Interaction of *Streptococcus pyogenes* with extracellular matrix components resulting in immunomodulation and bacterial eradication

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Abstract

Streptococcus pyogenes is a major human pathogen that causes a variety of diseases ranging from mild skin and throat infections to fatal septicemia. In severe invasive infections, *S. pyogenes* encounters and interacts with components of the extracellular matrix (ECM), including small leucine rich-proteoglycans (SLRPs). In this study, we report a novel antimicrobial role played by SLRPs biglycan, decorin, fibromodulin and osteoadherin, specifically in promoting the eradication of *S. pyogenes* in a human sepsis model of infection. SLRPs can be released from the ECM and de novo synthesized by a number of cell types. We reveal that infection of human monocytes by *S. pyogenes* induces the expression of decorin. Furthermore, we show that the majority of genetically distinct and clinically relevant *S. pyogenes* isolates interact with SLRPs resulting in decreased survival in blood killing assays. Biglycan and decorin induce TLR2 and TLR4 signaling cascades resulting in secretion of proinflammatory and chemotactic molecules and recruitment of professional phagocytes. Surprisingly, SLRP-mediated elimination of *S. pyogenes* occurs independently of TLR activation. Our results indicate that SLRPs act in concert with human serum, enhancing deposition of complement activation fragments and the classical activator C1q on the bacterial surface, facilitating efficient microbial eradication. Addition of the complement C3 inhibitor compstatin significantly reverses SLRP-induced blood killing, confirming active complement as a key mediator in SLRP-mediated bacterial destruction. Taken together our results add to the functional repertoire of SLRPs, expanding to encompass their role in controlling bacterial infection.
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Introduction

Streptococcus pyogenes is a major human pathogen responsible for significant morbidity and fatality globally. *S. pyogenes* primarily inhabits the epithelial surfaces of the nasopharynx and skin, colonizing asymptomatically in the majority of incidences. However, the bacterium possesses a vast array of virulence factors, promoting diseases ranging from superficial skin to life-threatening invasive infections, in addition to post-infection immune-mediated sequalae [1]. Invasive streptococcal diseases are associated with a high mortality rate, resulting in approximately 500,000 deaths worldwide annually, mostly in low income countries [2]. Currently, no vaccine is available to prevent *S. pyogenes* infections. Therefore, in order to tackle and prevent *S. pyogenes* disease, a greater understanding of the host-pathogen dynamic is required.

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Small leucine-rich proteoglycans (SLRPs) are multifactorial molecules, discernible by their central domain of tandem leucine-rich repeats (LRR) flanked by conserved cysteine rich domains at both termini [3]. As proteoglycans, these molecules consist of core protein domains and glycosaminoglycan (GAGs) side chains which are differentially processed, vary according to tissue site and play fundamental roles in extracellular matrix assembly and maintenance [3,4]. The view that SLRPs act solely as structural components of the extracellular matrix (ECM) has significantly changed in the last decade. The two archetypal class I SLRPs, decorin and biglycan, interact with multiple binding partners regulating a wide spectrum of cellular processes [5–9]. Increasing evidence indicates that these SLRPs act as damage-associated molecular patterns (DAMPs) when released from the ECM following tissue injury or upon secretion from activated macrophages and resident cells [7,10,11]. Interaction of soluble SLRPs with a number of germline-encoded pathogen recognition receptors (PRRs) modulates multiple signaling cascades triggering sterile inflammation and potentiating pathogen-mediated inflammation [7,12,13].

Soluble biglycan acts as an endogenous ligand of Toll-like receptors (TLR) 2 and TLR4 by activating p38, ERK and NF-κB signaling pathways [10,14]. In macrophages, CD14 is required for biglycan-mediated TLR2/4-dependent inflammation [15], inducing the expression of TNF-α and a suite of macrophage chemoattractants, which further facilitate the recruitment of multiple innate and adaptive immune cells to the site of injury [10,11,13,16,17]. In addition, biglycan crosslinks TLR2/4 and P2X receptors stimulating NLRP3 inflammasome activation, thus promoting the expression of pro-interleukin 1β [18].

The decorin interactome spans a myriad of binding partners, which participate in the regulation of a wide range of biological processes [5]. Importantly, decorin also binds TLR2 and TLR4 with an affinity similar to bacteria-derived ligands, promoting induction of p38, MAPK and NF-κB pathways and synthesis of pro-inflammatory cytokines IL-12 and TNF-α [19]. In addition, decorin enhances inflammation through stimulation of programmed cell death 4 (PDCD4) via inhibition of transforming growth factor-β1 and microRNA-21, both inhibitors of PDCD4 [19]. Increased PDCD4 results in suppression of the anti-inflammatory cytokine IL-10 thus contributing to a pro-inflammatory environment [19]. Biglycan and decorin act as danger signals, engaging multiple receptors resulting in a rapid inflammatory reaction. However, it is less known what roles SLRPs play during bacterial infection.

It is well established that bacterial interaction with ECM components is vital for adherence and colonization of specific anatomical sites and invasion of certain host cells [20]. *S. pyogenes* produces adhesins that interact with fibronectin, collagen and laminin allowing attachment to the ECM, which is a critical step in establishing infection [21]. These adhesion steps are crucial especially in the case of invasive *S. pyogenes* strains that infect deep tissues, where they come in contact with ECM components including SLRPs [22]. Recent studies have highlighted that SLRPs also display complement modifying functions [23,24]. The complement system is one of the first lines of defense against invading pathogens and plays an important role in combatting *S. pyogenes* infection. Invading pathogens activate complement through three pathways (classical, alternative and lectin pathways) leading to the generation of inflammatory anaphylatoxins and deposition of complement-derived opsonins on the surface of the bacteria [25]. Opsonization by C3b, C4b and C1q enables recognition of bacteria by professional phagocytes resulting in microbial destruction [25].

We have previously shown that both fibromodulin and osteoadherin activate the classical pathway of complement via interaction with C1q globular head domains, whereas biglycan and decorin interact with the collagenous stalk domain of C1q, leading to inhibition [23,26]. Alternatively, SLRPs alter complement function through the acquisition of soluble complement regulators, C4b-binding protein (C4BP) and factor H (FH), as exemplified by both fibromodulin and osteoadherin [23,27]. Recently we have observed that ECM proteins interact with bacteria and influence complement activation. Two opposing scenarios result from this dynamic interplay. In one instance, bacteria can use ECM proteins to prevent the deposition of complement components, resulting in decreased susceptibility to complement-mediated attack and eradication by phagocytes [28], affording a significant survival strategy for the bacteria. In contrast, bacterial interaction with ECM proteoglycans may actually be detrimental to the microbe. We have shown that several SLRPs can be bound by a number of pathogens, resulting in enhanced complement-mediated killing. Our previous work has provided evidence that the interaction of the emerging respiratory pathogen, *Moraxella catarrhalis*, with PRELP (proline/arginine-rich end leucine-rich repeat protein), fibromodulin, osteoadherin and biglycan act synergistically with complement, leading to enhanced complement activation and increased serum killing, highlighting their role as novel antibacterial components of innate immunity [29,30].

The interaction of *S. pyogenes* with SLRPs is not well documented and the outcome of such interplay is unknown. Since *S. pyogenes* inhabits anatomical sites rich in SLRPs, and given the multiple roles SLRPs play in modulating complement and innate immune signaling cascades we sought to define whether *S. pyogenes* interact with SLRPs and what consequence this may have on bacterial viability.

Results

Biglycan, decorin, fibromodulin and osteoadherin bind to *S. pyogenes* in a SLRP- and strain dependent manner

To determine whether biglycan, decorin, fibromodulin and osteoadherin modulate innate immune responses directed against *S. pyogenes* we first wanted to ascertain whether SLRPs interact with *S. pyogenes*. Employing biotin-labelled biglycan, decorin, fibromodulin and osteoadherin and flow cytometry analyses, we highlighted that all four SLRPs bound the M-type 6 *S. pyogenes* strain BAA-946 (Fig. 1). Interestingly, the M1 *S. pyogenes* strain AP1 only bound biglycan, but not decorin, fibromodulin or osteoadherin (Fig. 1A). In contrast, all other tested Gram-positive bacterial species including *Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Staphylococcus aureus*, did not bind these SLRPs (Fig. 1). To determine the clinical relevance of these interactions and to exclude the possibility that *S. pyogene*s BAA-946 interaction with SLRPs was a unique event, we included a panel of clinical isolates (*n = 16*) and assessed their interaction to all four SLRPs. (Fig. 2). These clinical isolates were chosen on the basis of their M-type, a clinically important epidemiological marker which represents a key virulence factor expressed on the surface of *S. pyogenes* [31]. We observed that the vast majority of isolates bound biglycan, decorin, fibromodulin and osteoadherin, independently of their M-type. Importantly, the intensity of binding varied and was both strain and SLRP-dependent.

Decorin and fibromodulin mRNA expression are increased in *S. pyogenes*-infected monocytes

Although SLRPs are sequestered in the ECM under physiological conditions, they can be proteolytically cleaved from the ECM or newly synthesized by a number of cell types to become danger signals, binding bacteria and activating the immune system [10,19]. Biglycan and decorin are early response genes, synthesized by macrophages and resident cells during inflammation [7]. Decorin protein concentrations are strongly increased in the plasma of septic individuals, while plasma biglycan levels are enhanced in patients suffering from lupus nephritis [19,32]. We therefore asked whether SLRPs

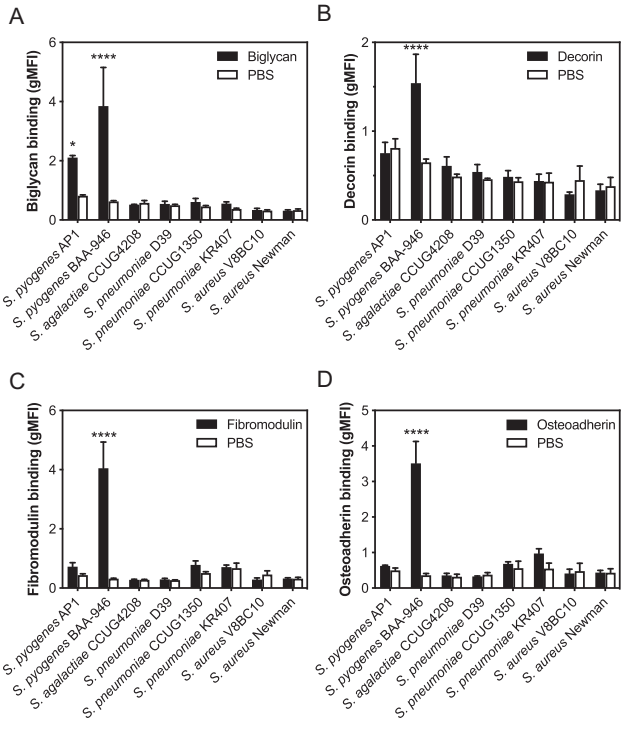

Fig. 1. *Streptococcus pyogenes*, but not other tested Gram-positive pathogenic bacteria, bind biglycan, decorin, fibromodulin and osteoadherin. Biotinylated (A) biglycan, (B) decorin, (C) fibromodulin, and (D) osteoadherin were incubated with Gram-positive pathogenic bacteria *S. pyogenes*, *S. agalactiae*, *S. pneumoniae* and *Staphylococcus aureus*. SLRPs binding was detected with fluorescently labelled streptavidin and analysed by flow cytometry. Data are shown as mean values and error bars indicate SD of three independent experiments. Two-way ANOVA with Sidak post hoc test in comparison with controls without SLRPs. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

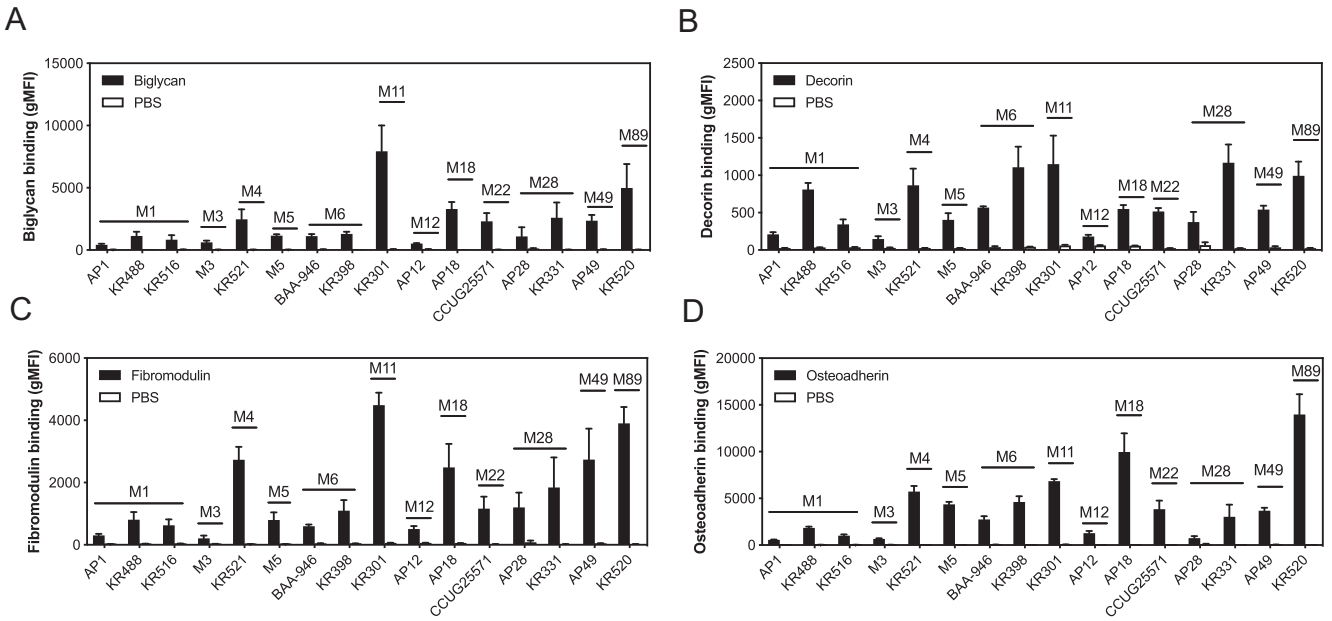

Fig. 2. The majority of tested *S. pyogenes* strains bind biglycan, decorin, fibromodulin and osteoadherin, independently of their M-type. Biotinylated (A) biglycan, (B) decorin, (C) fibromodulin, and (D) osteoadherin were incubated with *S. pyogenes* strains of various M-types, followed by fluorescently labelled streptavidin and analysis by flow cytometry. Data are shown as mean values and error bars indicate SD of three independent experiments.

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expression is increased in response to *S. pyogenes* infection of primary blood monocytes. Human CD14+ monocytes were purified from blood, infected with various *S. pyogenes* strains for 3 h, or treated with LPS as a control. The expression of biglycan, decorin, fibromodulin and osteoadherin was analysed by RT-qPCR, and represented as log2 of fold change. Osteoadherin mRNA was not detected in any sample, which was not surprising since osteoadherin is thought to be expressed only by odontoblasts and osteoblasts [33]. The expression of biglycan mRNA was increased in response to LPS, but not significantly changed by infection with any *S. pyogenes* strain (Fig. 3A). Fibromodulin mRNA expression was unchanged by LPS or *S. pyogenes* strains M4 and M11, but increased two-fold in response to strains M28 and M89 (Fig. 3B). The most striking effect was observed with decorin, where mRNA expression was strongly increased in response to all *S. pyogenes* strains: approximately 13-fold for M4, 20-fold for M28, 37-fold for M89 and 44-fold for M11 (Fig. 3C). Fibromodulin and decorin increase steadily overtime after infection (Fig. 3D–E), and their expression correlates with the MOI used – the more bacteria used to infected monocytes, the higher the expression of fibromodulin and decorin. (Fig. 3F–G).

Endotoxin contamination of SLRPs enhances killing of *S. pyogenes*

SLRP expression was achieved using eukaryotic Freestyle HEK 293 cells, devoid of endotoxin. In our original approach we did not screen for LPS contamination based on this factor. However, following preliminary experiments we carried out an extensive analysis for endotoxin on all SLRPs and found variable levels of contamination. Working on the estimation that one unit of LPS/ml (EU/ml) is equal to 100 pg of LPS/ml [10], we found that LPS contamination per µg of SLRPs were as follows: biglycan (18 pg/ml), decorin (38 pg/ml), fibromodulin (26 pg/ml), and osteoadherin (6.8 pg/ml). We therefore aimed at understanding whether LPS itself would affect bacterial survival due to enhanced immune response against *S. pyogenes* using an ex

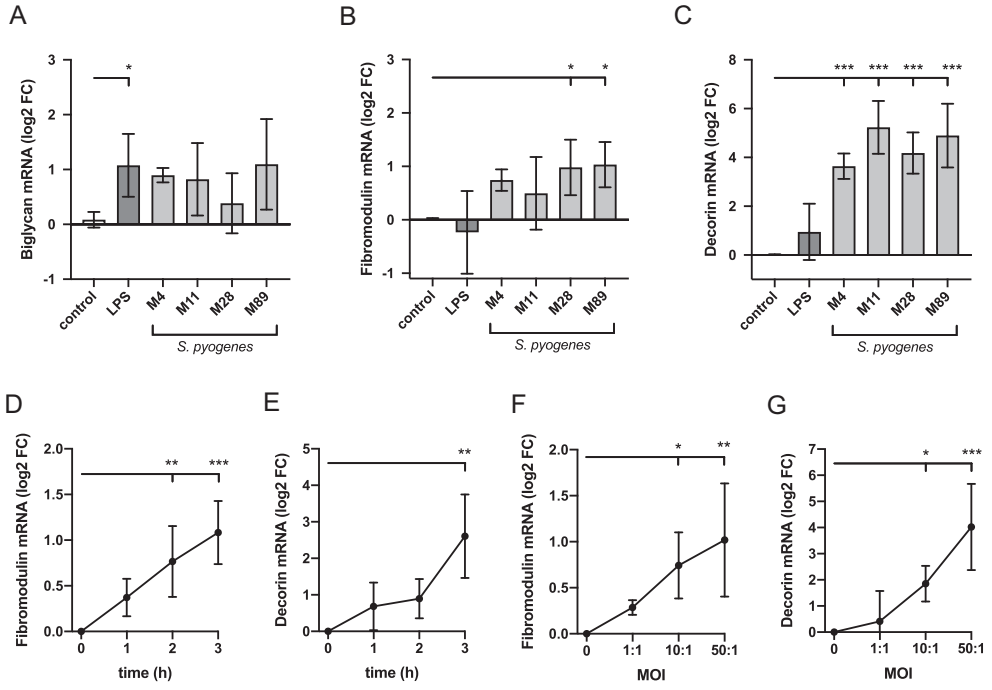

Fig. 3. *S. pyogenes* infection induces decorin and fibromodulin mRNA expression by primary monocytes. CD14^{+} monocytes were isolated from blood and infected with *S. pyogenes.* RNA was extracted, reverse transcribed and qPCR analysis was performed. (A-C) Monocytes were infected with *S. pyogenes* M4, M11, M28 or M89 (multiplicity of infection, MOI 10:1) or treated with LPS for 3 h, and analysed for biglycan (A), fibromodulin (B) and decorin (C) mRNAs. Of note, osteoadherin mRNA was not detected in any sample. (D, E) Monocytes were infected with *S. pyogenes* carrying M28 (MOI 10:1) for 0 (initial uninfected control), 1, 2, and 3 h, and the levels of fibromodulin (D) and biglycan (E) mRNA were assessed. (F, G) Monocytes were infected with *S. pyogenes* carrying M28 for 3 h, with MOIs 1:1, 10:1, 50:1 and the levels of fibromodulin (F) and biglycan (G) mRNA were assessed. The mRNA expression is represented as log2 of fold change in relation to the housekeeping genes Cyclophilin and HPRT. Data are shown as mean values and error bars indicate SD of three independent experiments. One-way ANOVA with Dunnett post hoc test in comparison with controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

vivo whole human blood model. The *S. pyogenes* strain carrying M28 was picked as a representative strain to assess the potential effect of LPS contamination in our model, instead of testing all *S. pyogenes* strains as it is not the main focus of this study. Our results showed that LPS as low as 0.1 ng/ml significantly enhanced killing of *S. pyogenes* and therefore a potential impact of SLRP mediated killing would be masked (Fig. 4A). The final concentration of LPS when using contaminated SLRPs was in the range of 0.6–2.6 ng/ml. In an effort to inhibit LPS toxicity we preincubated SLRPs with polymyxin B (PMB) which has a high neutralizing affinity for LPS [34]. In order to assess whether PMB could neutralize LPS and prevent enhanced killing of *S. pyogenes* in our blood killing model, we incubated LPS either with PBS or 100 µg/ml PMB for 1 h and then spiked blood with either LPS cocktail and determined survival of *S. pyogenes*. Treatment of highly purified LPS with PMB neutralized LPS endotoxin activity and reduced *S. pyogenes* killing to that observed in human blood alone (Fig. 4A). This confirmed that pre-incubation of SLRPs with PMB can neutralize LPS contamination and illustrate true SLRP-dependent enhanced blood killing.

Interestingly, LPS-induced blood killing seemed to be donor-dependent: although a majority of donors displayed a significant reduction in *S. pyogenes* survival when their blood was treated with LPS (Fig. 4A compiles the results of 4 donors), a small number of donors showed much less sensitivity to LPS (Fig. 4B). Donors D6 and D8 showed no reduction in survival of *S. pyogenes* when their blood was treated with LPS, unlike D4. Moreover, LPS-induced blood killing seemed to be dependent on the *S. pyogenes* strain as well (data not shown).

Decorin enhances blood killing of *S. pyogenes* in a strain-dependent fashion

We then questioned whether SLRPs could also influence *S. pyogenes* survival in an ex vivo human sepsis model. Decorin was investigated first because it is the most strongly upregulated SLRP in response to *S. pyogenes* infection (Fig. 3). Proteins were pre-treated with 100 µg/ml of PMB for 30 min in order to neutralize LPS. First, *S. pyogenes* M89 was pre-incubated with 100 µg/ml of either bovine serum albumin (BSA, as negative control), decorin, or decorin treated with PMB prior to addition to whole

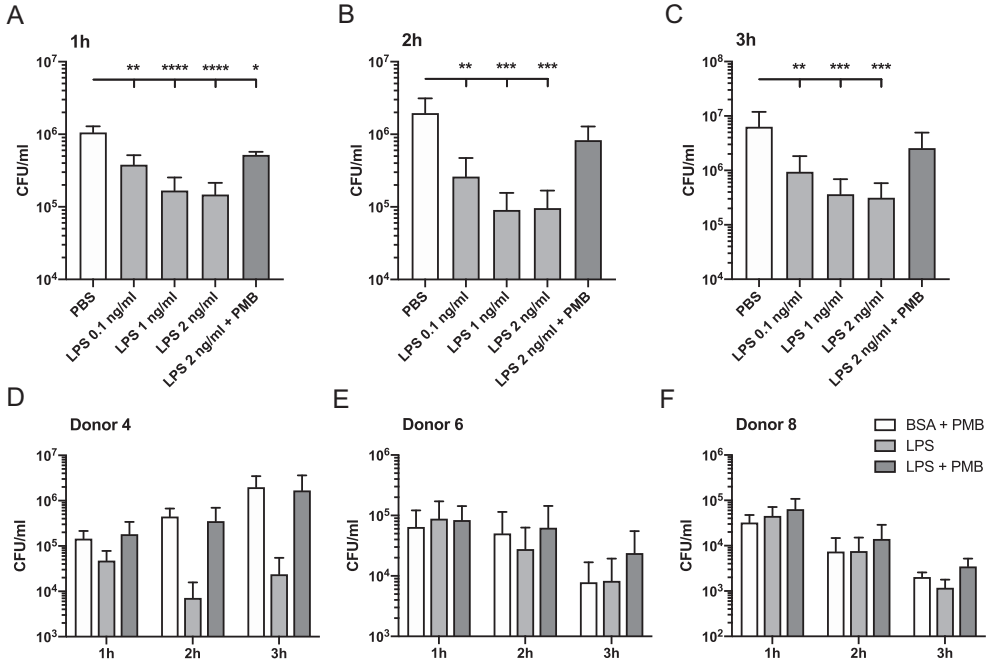

Fig. 4. LPS alone stimulates blood killing of *S. pyogenes*, in a donor specific manner, and its effect is reversed by polymyxin B (PMB). (A–C) Blood was treated with either ultrapure LPS (0.1, 1 and 2 ng/ml) or LPS (2 ng/ml) pre-treated with PMB (100 µg/ml) to assess the effect of LPS on *S. pyogenes* killing (strain M28), after 1 h (A), 2 h (B), and 3 h (C). Data are shown as mean values and error bars indicate SD of four independent experiments (each from a separate blood donor). Statistical analysis was performed on log10-transformed bacteria counts using one-way ANOVA with Dunnett post hoc test in comparison with PBS as a negative control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (D–F) Blood was treated with 200 µg/ml BSA, or 1 ng/ml of LPS, both pre-treated with 100 µg/ml polymyxin B prior to addition to whole blood, or 1 ng/ml LPS only. Data are shown as mean values and error bars indicate SD of two independent experiments for each donor D4 (D), D6 (E) and D8 (F).

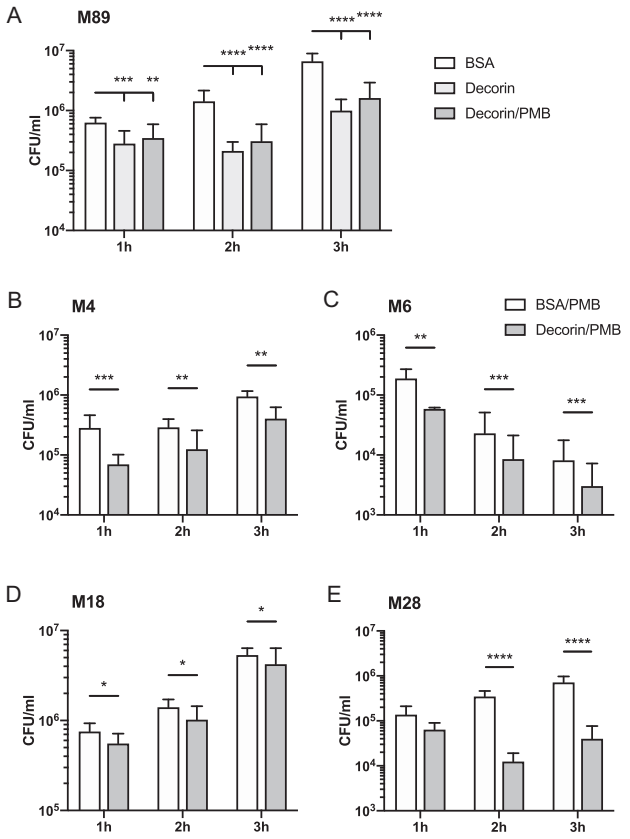

Fig. 5. Decorin enhances killing of several *S. pyogenes* strains in a whole blood model. (A) *S. pyogenes* carrying M89 was incubated for 30 min with BSA, decorin or decorin pre-treated with PMB (100 μg/ml for 30 min). Bacteria were then added to whole blood and their survival was assessed after 1, 2 and 3 h. (B, C, D, E) Several *S. pyogenes* strains of different M-types were incubated with BSA or decorin, both pre-treated with PMB (100 μg/ml for 30 min) prior to infection of whole blood: *S. pyogenes* carrying (B) M4, (C) M6, (D) M18, or (E) M28. Data are shown as mean values and error bars indicate SD of at least three independent experiments. Statistical analysis was performed on log10-transformed bacteria counts using two-way ANOVA with Sidak post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

blood. The survival of *S. pyogenes* M89 was decreased by decorin, either pre-treated or not with PMB, showing that decorin increased blood killing of *S. pyogenes* independently of LPS contamination (Fig. 5A).

Next, we examined a number of clinically relevant M-type strains; M4, M6, M18 and M28. These isolates were incubated with BSA or decorin (both pre-treated with PMB) for 30 min prior to inoculation in human blood. Survival was assessed at 1, 2 and 3 h post-infection where a general trend of decreased survival when incubated with decorin was observed (Fig. 5A–E). Decorin significantly enhanced blood killing of M4, M6 and M28, as well as M18 to a lesser extent, suggesting that different M-types exhibit different susceptibilities to decorin-mediated blood killing (Fig. 5B–E).

M28 is the second most abundant *emm* type in Europe [35], third worldwide [36], and one of the most prevalent *emm* types associated with invasive infections in Europe and North America [37]. M28 is especially associated with gyneco-obstetrical infections and overrepresented among cases of puerperal sepsis and neonatal *S. pyogenes* infections [38]. Due to the clinical importance of M28 and practical reasons, the downstream experiments were performed using this strain.

TLR2 and TLR4 signaling is not involved in decorin or biglycan-induced killing of *S. pyogenes*

Decorin and biglycan are structurally related, distinguished from each other by the number of chondroitin or dermatan sulphate side chains at their N-terminal ends: one for decorin or two for biglycan [39]. Similar to decorin, biglycan enhanced killing of *S. pyogenes* strain M28 in whole blood (Fig. 6E–G). Biglycan and decorin have been shown to act as endogenous ligands for TLR2 and TLR4, triggering their signaling and leading to a rapid sterile inflammatory response [10,19]. We therefore asked whether the increase in *S. pyogenes* killing in whole blood when treated with biglycan and decorin could be due

to increased TLR2- and TLR4-mediated proinflammatory secretion leading to increased phagocyte activation and bacterial engulfment. Two TLR chemical inhibitors were used to pre-treat the blood prior to infection with *S. pyogenes*. The oxidized phospholipid oxPAPC inhibits TLR2 and TLR4 signaling by competitive interaction with their accessory proteins [40], while CLI-095 (also called TAK-242) inhibits TLR4 signaling by blocking the intracellular domain of TLR4 [41]. We tested whether these chemical inhibitors function in our model of whole human blood. The secretion of several cytokines known to be induced by TLR2 and TLR4 signaling was assessed in blood treated with biglycan and decorin by ELISA. IL-8 was significantly increased in response to biglycan and decorin (Fig. 6A–B), while TNF-α was induced by biglycan (Fig. 6C–D). Finally, CLI-095 and oxPAPC successfully inhibited biglycan and decorin-induced cytokine secretion, making it similar to untreated blood (Fig. 6A–D).

To assess the role of TLR signaling in biglycan- and decorin-induced bacterial killing, whole blood was treated with oxPAPC or CLI-095 for 1 h prior to infection with bacteria previously pre-incubated with BSA, biglycan or decorin, and bacteria were counted after 1, 2 and 3 h. The two TLR inhibitors did not rescue bacteria from killing triggered by biglycan or decorin, suggesting that biglycan and decorin-induced killing of *S. pyogenes* is not the result of SLRPs interfering with TLR signaling (Fig. 6E–G).

Biglycan, decorin, fibromodulin and osteoadherin enhance killing of *S. pyogenes* in whole blood through increased complement activation

In our initial *S. pyogenes* - SLRP binding experiments we observed that the majority of *S. pyogenes* isolates bound fibromodulin and osteoadherin to a similar degree as biglycan and decorin (Fig. 2). In addition, previous work by our group showed that both fibromodulin and osteoadherin accelerated serum killing of *M. catarrhalis*, by enhancing complement deposition and subsequent membrane attack complex (MAC) formation [30]. Therefore, based on these observations, we investigated whether fibromodulin and/or osteoadherin also augmented killing of *S. pyogenes* in whole blood. Interestingly, both fibromodulin and osteoadherin significantly increased killing of the *S. pyogenes* M28 strain over a three-hour time course, in a similar manner to both biglycan and decorin (Fig. 7A–C).

As SLRPs can regulate complement activity, we hypothesized that SLRPs-induced killing in whole blood was due to increased complement deposition on the surface of *S. pyogenes* followed by enhanced elimination of bacteria by phagocytes. The C3-targeted complement inhibitor Compstatin Cp40 [42] was used to treat whole blood before infection with *S. pyogenes* pre-treated with biglycan, decorin, fibromodulin, or osteoadherin. As expected, Cp40 treatment resulted in an increase in survival of *S. pyogenes* in the absence of SLRPs, as Cp40 blocked complement-mediated killing of the bacteria (Fig. 7A–C). More importantly, Cp40 reversed the SLRP-induced increase in *S. pyogenes* killing, indicating that SLRPs enhance blood killing of bacteria through increased complement deposition and subsequent enhanced opsonophagocytosis (Fig. 7A–C).

To confirm the role of complement in SLRPs-induced blood killing, the deposition of complement components on the bacterial surface was assessed using flow cytometry. In agreement with the blood killing assays, we observed that biglycan, decorin, fibromodulin, and osteoadherin significantly enhanced the deposition of C3b (Fig. 8A). We then asked whether SLRPs induce the classical pathway by assessing the deposition of C1q and C4b. Deposition of C1q was significantly increased in bacteria treated with biglycan, decorin, fibromodulin, and osteoadherin compared to BSA (Fig. 8B), while deposition of C4b was increased with biglycan, decorin and fibromodulin, but not osteoadherin (Fig. 8C). This suggests that biglycan, decorin and fibromodulin activate the classical pathway through binding of C1q at the bacterial surface followed by activation of C4, while osteoadherin increases the binding of C1q, but without activating the classical pathway.

SLRPs have been shown to inhibit binding of the complement inhibitor C4BP at the surface of the respiratory pathogen *M. catarrhalis*, leading to increased complement deposition and serum-mediated killing of the bacteria [30]. A number of highly virulent *S. pyogenes* strains are also known to bind C4BP as a complement evasion strategy [43]. C4BP was bound by our M28 *S. pyogenes* strain, but there was no difference in binding between BSA- or SLRPs-treated bacteria (Fig. 8D), which shows that the increased complement deposition when bacteria were treated with SLRPs in not due to an inhibition of C4BP binding.

Discussion

Streptococcus pyogenes ranks in the top ten list of pathogens causing the highest global fatalities [2], and is responsible for infections in both immune-competent and compromised patients [1,2]. *S. pyogenes* causes skin and soft tissue infection, promoting substantial tissue inflammation, ECM degradation and potential release of SLRPs [44]. In soluble form, class I SLRPs, biglycan and decorin can engage with PRRs, activating proinflammatory cascades inducing the expression of numerous chemoattractants resulting in phagocyte recruitment [10,19]. In this study we add to the functional

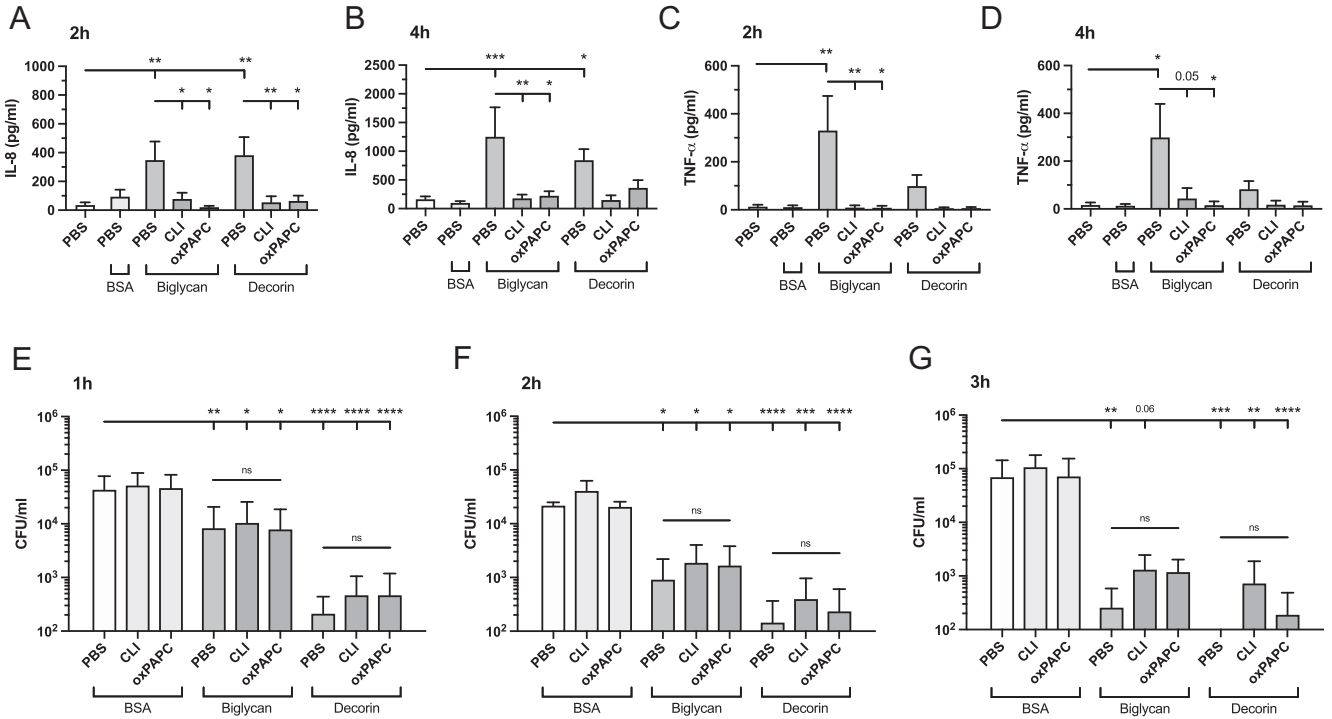

Fig. 6. Biglycan and decorin-induced killing of *S. pyogenes* is not mediated through TLR signaling. (A–D) Biglycan and decorin-induced secretion of TNF-α and IL-8 is blocked by TLR inhibitors CLI-095 and oxPAPC. Whole blood was treated for 1 h with the TLR4 inhibitor CLI-095 (5 μg/ml), the TLR2/TLR4 inhibitor oxPAPC (50 μg/ml) or left untreated (LPS), prior to addition of 8 μg/ml of biglycan, decorin or BSA. After 2 h and 4 h, plasma was collected and an ELISA was performed to assess the concentration of TNF-α (A, B) and IL-8 (C, D). BSA, biglycan and decorin had previously been incubated with polymyxin B to block any effect due to LPS contamination. Data are shown as mean values and error bars indicate SD of four independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett post hoc test in comparison to PBS-treated blood. (E–G) Whole blood was incubated for 1 h with the TLR4 inhibitor CLI-095 (5 μg/ml) or the TLR2/TLR4 inhibitor oxPAPC (50 μg/ml), prior to infection with M28 *S. pyogenes*. Bacteria had been incubated with BSA, biglycan or decorin for 30 min before infection (all pre-treated with PMB). The number of remaining live bacteria in blood was enumerated after 1 h (E), 2 h (F) and 3 h (G). Data are shown as mean values and error bars indicate SD of three independent experiments. Statistical analysis was performed on log10-transformed bacteria counts using one-way ANOVA with Dunnett post hoc test in comparison to BSA-treated bacteria in PBS-treated blood. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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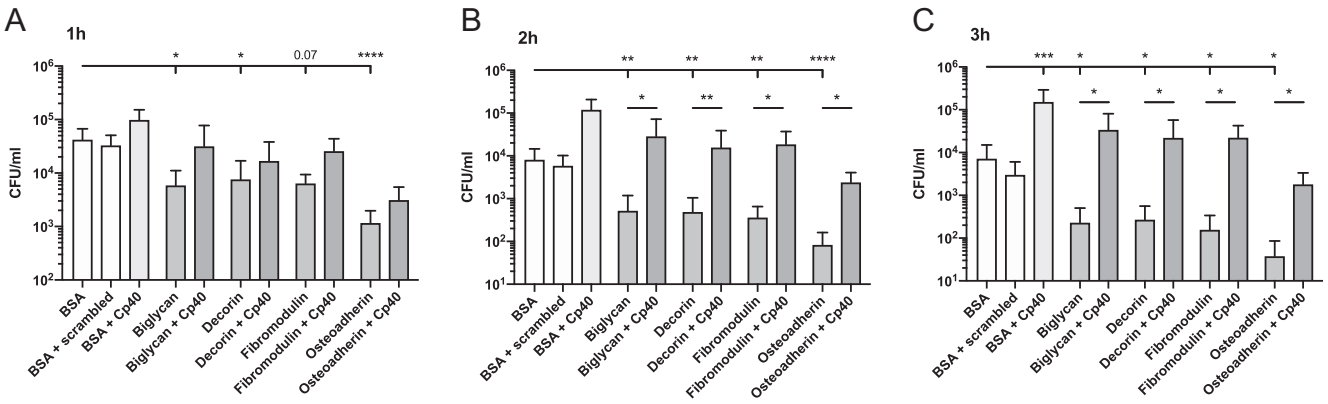

Fig. 7. All SLRPs enhanced killing of *S. pyogenes* in whole blood through increased complement deposition. Whole blood was treated for 30 min with the complement inhibitor compstatin Cp40 (20 μM), prior to infection with M28 *S. pyogenes*. Scrambled peptides were used as negative control. Bacteria were incubated with 100 μg/ml of BSA, biglycan, decorin, fibromodulin, or osteoadherin (all pre-treated with PMB) for 30 min before infection. The number of remaining bacteria in blood was enumerated after 1 h (A), 2 h (B) and 3 h (C). Data are shown as mean values and error bars indicate SD of four independent experiments. Statistical analysis was performed on log10-transformed bacteria counts using one-way ANOVA with Sidak post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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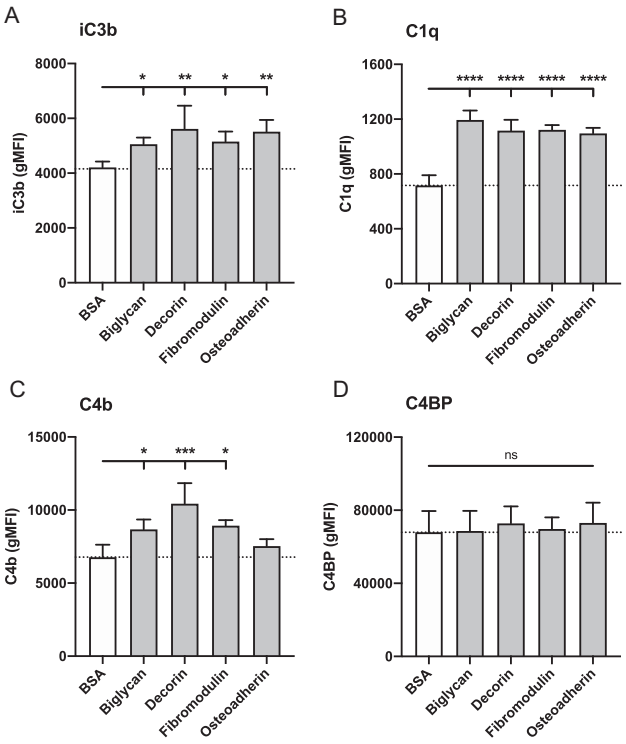

Fig. 8. All SLRPs increase complement deposition at the surface of *S. pyogenes*. M28 *S. pyogenes* was incubated with 100 µg/ml of BSA, biglycan, decorin, fibromodulin, or osteoadherin for 45 min followed by addition of NHS to a final concentration of 1% for another 15 min (A, B, C) or 5% of NHS for 30 min (D). Flow cytometry was used to analyse the deposition of complement components (A) iC3b, (B) C1q, (C) C4b and (D) C4BP on bacteria. Data are shown as mean values and error bars indicate SD of at least three independent experiments. One-way ANOVA with Dunnett post hoc test in comparison to BSA-treated bacteria. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, ns: non significant.

repertoire of SLRPs, characterizing their role as antimicrobial mediators facilitating enhanced *S. pyogenes* killing in an ex vivo human sepsis model. Noteworthy, this novel function was not restricted to biglycan and decorin, with class II SLRPs, fibromodulin and osteoadherin also exhibiting this activity.

Interestingly, we observed that the antibacterial activity of biglycan, decorin, fibromodulin and osteoadherin was not due to enhanced proinflammatory responses as a result of SLRPs docking with their multiple innate immune receptors, but rather via increased C3b/iC3b deposition, boosting serum opsonization on the streptococcal surface thus promoting phagocytosis and efficient eradication. All tested SLRPs enhanced C1q and C3b/iC3b deposition while all but osteoadherin increased C4b binding on *S. pyogenes*. This suggests that SLRP biglycan, decorin and fibromodulin primarily enhanced complement deposition via the classical pathway. Previous work by our group showed that biglycan, decorin, fibromodulin and osteoadherin bind serum C1q. However, distinct domains where preferentially bound by specific SLPRs; while fibromodulin and osteoadherin bound the globular head domain and activated the classical pathway, biglycan and decorin inhibited activation through interaction with the collagenous tail domain [23,26]. We hypothesize that because of the different conditions at the surface of bacteria, biglycan and decorin initially bind C1q by the tail region, but it is then transferred and turned around to bind bacterial surface molecules or antibodies deposited at the surface, where it can act as an opsonin and activate the classical pathway, as shown by the increase in C4b deposition. Biglycan and decorin would then act by increasing the recruitment of C1q to the bacteria surface, leading to increased opsonization and complement-mediated phagocytosis.

Biglycan and decorin are structurally highly similar, distinguished from each other by the presence of one (decorin) or two (biglycan) chondroitin/dermatan sulfate GAG side chains at their N-terminal ends [39]. Fibromodulin and osteoadherin carry one to four keratan sulfate GAG chains, between their leucine-rich repeats [45]. Glycosaminoglycans on cell surfaces are known to play a role in bacterial adhesion to host cells [46], in particular functioning as receptors in M protein-mediated adhesion of *S. pyogenes* [47]. SLRPs might therefore bind the bacterial surface through their GAG side chains. The binding of GAGs would

depend on the nature and number of GAG chains, as well as the M protein carried by *S. pyogenes*, which can be reflected in the variability in *S. pyogenes* strains binding (Fig. 2).

It is worth noting that treatment of blood with Cp40 did not entirely block SLRPs-induced killing in whole blood, as the survival of SLRPs-treated *S. pyogenes* did not reach the level of BSA-treated when Cp40 was used (Fig. 7). Cp40 inhibits complement at the C3 level, and therefore does not inhibit C1q, which can act as an opsonin and promote phagocytosis, independently of complement activation [48]. C1q binding is strongly enhanced in all SLRPs-treated *S. pyogenes* (Fig. 8). Furthermore, Cp40 treatment was reported to increase C4b opsonization on porcine endothelial cells [49]. Therefore the difference between BSA + Cp40 and SLRPs+Cp40 most likely reflects C3b/iC3b-independent C4b- or C1q-induced opsonophagocytosis. Cp40 was least effective at inhibiting osteoadherin-induced killing, compared to the other SLRPs (Fig. 7), which is consistent with the fact that osteoadherin increased C1q binding but not C4b activation (Fig. 8): osteoadherin-induced killing seems to primarily happen through enhanced C1q opsonization without activation of complement. Altogether, this suggests that SLRPs increase C1q deposition, which then enhances phagocytosis of *S. pyogenes* through opsonization by C1q, independently of active complement, and – in the case of biglycan, decorin, and fibromodulin - complement classical pathway activation involving opsonization by C3b and C4b.

One major question is how *S. pyogenes* becomes coated with SLRPs during infection. One potential mechanism is via secretion by monocytes/macrophages and resident cells upon activation. We have shown that monocyte infection with *S. pyogenes* of different M-types strongly stimulated decorin mRNA expression at a higher level than stimulation of monocytes with LPS alone, suggesting that decorin may serve as both a warning signal and complement enhancing factor following infection with live bacteria. Another potential mechanism involves tissue destruction and proteolytic release of SLRPs during *S. pyogenes* invasive infections. Degradation of the ECM permits bacterial dissemination and is orchestrated by the section of bacterial enzymes and exploitation of host proteases [44]. *S. pyogenes* secretes streptokinase, which catalyzes the conversion of inactive plasminogen to plasmin resulting in fibrinolysis and degradation of ECM components [50]. The vast majority of invasive *S. pyogenes* strains also secrete a cysteine protease, pyrogenic exotoxin type B (SpeB), with broad substrate specificity, responsible for cleaving a suite of both host and bacterial proteins [51]. Among others, SpeB targets several actors of the human ECM; it cleaves fibronectin and degrades vitronectin [52]. SpeB also cleaves pro-matrix metalloproteinases (MMPs) into active MMPs, which catalyze ECM degradation [53–55]. It is tempting to speculate that during *S. pyogenes* infection, SpeB may enhance proteolysis of the ECM, resulting in SLRP liberation facilitating increased complement deposition and decrease *S. pyogenes* survival.

Among the four Gram-positive pathogenic bacteria that were screened, only *S. pyogenes* was positive for binding of biglycan, decorin, fibromodulin and osteoadherin. *Streptococcus agalactiae*, *Streptococcus pneumoniae* and *S. pyogenes* are closely related, but each species has evolved its own virulence factors, including surface proteins that are critical for interaction with host cells and innate immune resistance [56,57]. The M protein is the major virulence determinant for *S. pyogenes*, providing antiphagocytic functions required for survival in human tissues and blood [31]. M proteins are highly variable, and these variations may explain fluctuations in SLRPs binding to different M-types and concomitant survival in human blood that we have observed. Interestingly, the highly invasive M1 and M3 isolates which are associated with life-threatening infections bound the lowest to all tested SLRPs, potentially indicating the presence of an effective immune evasive strategy. In contrast M89 isolates are associated with non-invasive infections and were bound highest by SLRPs and effectively neutralized in whole blood killing assays. More work is required to evaluate the exact binding ligands on the streptococcal surface and to evaluate whether certain *S. pyogenes* strains have evolved to prevent SLRPs interaction.

Our observation that minute concentrations of LPS decreased the survival of *S. pyogenes* in a blood infection model is new and unexpected. LPS is a potent immunologic stimulant but the amount needed to alter the immune response is not clear and depends on the experimental conditions [58]. In our model, the use of contaminated proteins led to range of 0.6–2.6 ng/ml of LPS, which is comparable to commercially produced recombinant proteins [59]. LPS contamination in protein expression is indeed very widespread, even when using a eukaryotic cell system as we do here. This notion that even the smallest contamination with LPS can lead to a striking difference in survival of *S. pyogenes* in a blood model should be considered when testing the effect of various molecules, and the use of PMB is very much needed.

Our data highlight an interaction of *S. pyogenes* with biglycan, decorin, fibromodulin and osteoadherin, which have a complement modulating activity resulting in enhanced killing of the bacteria. This SLRP-mediated enhanced killing of *S. pyogenes* may be a novel effective immune mechanism helping to control bloodstream infections.

Materials and methods

Bacterial strains and culture conditions

The M-types of clinical and laboratory *S. pyogenes* strains used in this study are listed: M1 (AP1, KR488, KR516), M3, M4 (KR521), M5, M6 (BAA-946, KR398), M11 (KR301), M12 (AP12), M18 (AP18), M22 (CCUG25571), M28 (AP28, KR331), M49 (AP49), and M89 (KR520). M protein gene typing was determined according to the protocol available from the Centers for Disease Control and Prevention (CDC), United States [60]. *S. pyogenes* strains, *Streptococcus agalactiae* CCUG4208, and *Streptococcus pneumoniae* isolates (CCUG1350, D39 and KR407) were grown overnight in 25 ml Todd-Hewitt broth at 37 °C and 5% CO_2 without shaking. *Staphylococcus aureus* strains (Newman and V8 BC10) were grown in tryptic soy broth at 37 °C with shaking (180 rpm). Overnight cultures were normalized to an OD_{600nm} = 0.1 in fresh medium and grown under the same conditions until an OD_{600nm} = 0.3–0.4, corresponding to mid-exponential phase of growth. Bacteria were centrifuged and washed once in sterile PBS and normalized to an OD_{600nm} = 1 which equates to 1–2 × 10^8 CFU/ml.

Proteins, antibodies and sera

Human recombinant SLRPs, biglycan, decorin, fibromodulin and osteoadherin, were expressed with a hexa-histidine tag sequence using a pCEP4 vector in FreeStyle 293-F cells (Invitrogen) as described in detail [30]. SLRPs were labelled with biotin using the EZ-Link Sulfo-NHS-LC-Biotinylation kit (ThermoFisher Scientific) according to the manufacturer's guidelines.

The following primary antibodies were used for the analysis of complement deposition on *S. pyogenes* surface by flow cytometry; monoclonal mouse anti-human C4BP MK104 (homemade [61]), monoclonal mouse anti-human C1q (Quidel A201), monoclonal mouse anti-human C4c (Quidel A211), monoclonal mouse anti-human iC3b (Quidel A209), all used at a 1:1000 dilution. Fluorescently labelled goat anti-rabbit Alexa Fluor 647 (AF647) (A21246, Invitrogen) or goat anti-mouse AF647 (A21235, Invitrogen) secondary antibodies (1:1000 dilution) were used to detect primary antibodies. Detection of biotinylated proteins was achieved using streptavidin-AF647 conjugate (S21374; ThermoFisher Scientific).

Normal human serum (NHS) was prepared from freshly drawn blood from at least 8 healthy volunteers as described previously [30]. All volunteers provided written consent according to the recommendations of the local ethical committee in Lund, Sweden (permit 2017/582). Heat-inactivated (HI) serum was achieved by heating NHS at 56 °C for 30 min.

SLRP-bacteria binding assays

To investigate binding of SLRPs to Gram-positive bacteria, strains were grown as described above. Bacteria were stained with 10 μM Carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich), washed in PBS and resuspended in 1% (w/v) BSA/PBS. Approximately 5 × 10^6 CFU of bacteria in 50 μl was mixed with 50 μl of 1% (w/v) BSA/PBS containing 100 μg/ml biotinylated fibromodulin (2.3 μM), osteoadherin (1.94 μM), biglycan (2.35 μM), or 200 μg/ml biotinylated decorin (4.94 μM). Bacteria-SLRP suspensions were incubated for 30 min at 37 °C following which bacteria were centrifuged at 5000 ×*g* for 10 min at room temperature (RT), washed once with 1% (w/v) BSA/PBS and incubated in 100 μl of streptavidin-AF647. Following incubation for 45 min at RT in the dark, bacteria were centrifuged and washed once in 1% (w/v) BSA/PBS and SLRPs bound to bacteria were detected using a CyFLOW Space flow cytometer (Partec). In examining SLRP interaction with clinical *S. pyogenes* strains of differing M-type, binding assays were performed in a V-bottom 96 well plate (Nunc) pre-coated with 10% (w/v) fetal calf serum (FCS)/PBS. Bound SLRPs were detected using a CytoFlex flow cytometer (Beckman Coulter). To determine background binding levels, bacteria incubated with streptavidin-AF647 in the absence of SLRPs was used. Bacteria were detected from the CFSE signal and a gating region was constructed to eliminate debris. To determine SLRP binding, geometric mean fluorescence signal was used.

Complement deposition assays

CFSE-labelled *S. pyogenes* strains (5 × 10^6 CFU) were incubated with either 100 μg/ml of 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, pooled NHS was added to a final concentration of 5% (C4BP), or 1% (C1q, C4b, iC3b) and incubated for a further 30 min (C4BP) or 15 min (C1q, C4b, iC3b) at 37 °C. After incubation bacteria were centrifuged at 5000 ×*g* for 10 min and washed once in 1% (w/v) BSA/PBS. Deposited complement products were detected using the outlined primary antibodies incubated at RT for 30 min. Following centrifugation and washing, fluorescently labelled secondary antibodies were used and incubated for 30 min at RT in the dark. Bacteria were centrifuged and washed once in 1% BSA/PBS and resuspended in 200 μl of PBS and complement components examined using a CytoFlex flow cytometer with geometric mean fluorescence intensity used to determine the amount of complement deposition. Isotype control, secondary antibody only staining and HI-serum was used as controls. CFSE stained and unstained bacteria were used for gating and a minimum of 10,000 events were examined.

SLRP contamination analysis

In order to perform *S. pyogenes* whole blood killing assays, lipopolysaccharide (LPS) contamination after each purification of SLRPs was tested with the LAL kinetic chromogenic assay (Endochrome-K; Charles River). Serial dilutions of known LPS was used to generate a standard curve according to manufacturers' instructions, permitting calculation of LPS contamination in SLRP samples. LPS contamination per µg of SLRPs were as follows; biglycan (18 pg/ml), decorin (38 pg/ml), fibromodulin (26 pg/ml), and osteoadherin (6.8 pg/ml). An optimal LPS neutralization method was to preincubate SLRPs with polymyxin B (PMB) (Sigma-Aldrich; 100 µg/ml) for 1 h prior to *S. pyogenes* blood killing assay.

Ex vivo blood killing assays

S. pyogenes (2×10^5 CFU) was incubated with 100 µg/ml PMB-treated SLRP for 30 min at 37 °C in a final volume of 50 µl. Human blood was taken from healthy volunteers and treated with lepirudin (Refludan 50 µg/ml; Celgene). Bacteria – SLRP suspension (25 µl) was added to 475 µl of blood in 1.5 ml sterile Eppendorf tubes. Blood-bacteria suspensions were incubated on an end-over-end shaker at 37 °C and 5% CO_2. At specific time points 50 µl of the suspension was serially diluted in PBS and plated onto blood agar plates. Agar plates were incubated overnight at 37 °C and 5% CO_2 and CFU/ml determined following enumeration of surviving *S. pyogenes*. Complement activation was blocked at the C3 level using compstatin Cp40 (20 µM), which was incubated with blood for 30 min at 37 °C and 5% CO_2 with rotation before the addition of bacteria-SLRP mixture. LPS from *Salmonella typhosa* (Sigma) was diluted in endotoxin-free water to indicated concentrations and was incubated in blood with bacteria simultaneously. TLR signaling was blocked in whole blood by treating for 1 h with the TLR inhibitors CLI-095 (5 µg/ml, Invivogen #tlrl-cli095) and oxPAPC (50 µg/ml, Invivogen #tlrl-oxp1).

Monocyte purification

Blood leukocyte concentrates (buffy coats) from anonymous blood donors were obtained through the Lund University Hospital Blood Center. The use of blood components for research use was approved by the local ethical review board in Lund (permit 2019/14). Peripheral blood mononuclear cells were isolated by density-gradient centrifugation. Briefly, one volume of buffy coat was diluted in 3 volumes of PBS-2 mM EDTA, and 1.5 volume of Lymphoprep (Stemcell technologies) was underlayed beneath the diluted buffy coat. The tubes were centrifuged 20 min at 800 ×*g* without breaks. The white interface layer containing the PBMCs was collected, and washed three times in PBS-2 mM EDTA. The PBMCs were counted and frozen until used. $CD14^+$ monocytes were isolated from previously frozen PBMCs by positive selection using CD14 MicroBeads (Miltenyi Biotec) and magnetic activated cell sorting (MACS), following the manufacturer's instructions.

RNA extraction, reverse transcription and qPCR

RNA was extracted with RNeasy Plus Minikit (Qiagen) and cDNA synthesis was performed using Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Quantitative PCR was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays for Biglycan (Assay ID Hs00959143_m1), Decorin (Hs00754870_s1), Fibromodulin (Hs00157619_m1) and Osteoadherin (Hs00192325_m1), while Cyclophilin (Hs99999904_m1) and hypoxanthine phosphoribosyltransferase 1 (HPRT) (Hs99999909_m1) were used as reference genes.

ELISA

Plasma was collected by adding 10 µl of EDTA to 500 µl of whole blood, then mixed and incubated on ice for 10 min. Tubes were centrifuged at 3000 *g* for 15 min at 4 °C. Aliquotes of plasma were stored at –20 °C until analysis. TNF-α and IL-8 were measured in plasma samples using human-specific Quantikine ELISA kits (R&D systems) following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. One- or two-way ANOVA analysis with Sidak or Dunnett post hoc test were used as indicated in each figure, $p < 0.05$ was considered significant.

For blood killing experiments (Figs. 4, 5, 6 and 7), statistical analyses were performed on bacteria counts after logarithmic transformation (log10). Unlike raw bacteria counts, log10-transformed bacteria counts were normally distributed which is a requirement for ANOVA tests.

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Writing - review & editing: Anna M. Blom, Guanghui Liu, Kristian Riesbeck, David Ermert, John D. Lambris.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: John Lambris is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for therapeutic purposes. John Lambris is the inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. John Lambris is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals [i.e., 4(1MeW)7 W/POT-4/APL-1 and PEGylated derivatives such as APL-2/pegcetacoplan].

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Abbreviations used:
AF647, Alexa Fluor 647; BSA, bovine serum albumin; CFSE, Carboxyfluorescein succinimidyl ester; C4BP, C4b-binding protein; Cp40, compstatin; ECM, extracellular matrix; GAG, glycosaminoglycan; HI, heat-inactivated; MAC, membrane attack complex; NHS, normal human serum; PMB, polymyxin B; SLRP, small leucine-rich proteoglycan; TLR, toll-like receptors.

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