



Dual actions of group B *Streptococcus* capsular sialic acid provide resistance to platelet-mediated antimicrobial killing

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Circulating platelets have important functions in thrombosis and in modulating immune and inflammatory responses. However, the role of platelets in innate immunity to bacterial infection is largely unexplored. While human platelets rapidly kill *Staphylococcus aureus*, we found the neonatal pathogen group B *Streptococcus* (GBS) to be remarkably resistant to platelet killing. GBS possesses a capsule polysaccharide (CPS) with terminal α 2,3-linked sialic acid (Sia) residues that mimic a common epitope present on the human cell surface glycoalkal. A GBS mutant deficient in CPS Sia was more efficiently killed by human platelets, thrombin-activated platelet releasate, and synthetic platelet-associated antimicrobial peptides. GBS Sia is known to bind inhibitory Sia-recognizing Ig superfamily lectins (Siglecs) to block neutrophil and macrophage activation. We show that human platelets also express high levels of inhibitory Siglec-9 on their surface, and that GBS can engage this receptor in a Sia-dependent manner to suppress platelet activation. In a mouse i.v. infection model, antibody-mediated platelet depletion increased susceptibility to platelet-sensitive *S. aureus* but did not alter susceptibility to platelet-resistant GBS. Elimination of murine inhibitory Siglec-E partially reversed platelet suppression in response to GBS infection. We conclude that GBS Sia has dual roles in counteracting platelet antimicrobial immunity: conferring intrinsic resistance to platelet-derived antimicrobial components and inhibiting platelet activation through engagement of inhibitory Siglecs. We report a bacterial virulence factor for evasion of platelet-mediated innate immunity.

platelets | group B *Streptococcus* | innate immunity | sialic acid | Siglec

Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) causes sepsis and meningitis in human newborns and infants (1). In most developed countries, public health interventions such as universal culture screening of pregnant women or risk factor-based decision algorithms guide intrapartum antibiotic prophylaxis to reduce neonatal GBS transmission (2). Nevertheless, the incidence of GBS infection in infants below 3 mo of age is ~0.53 cases per 1,000 births in European countries, 0.67 cases per 1,000 births in the Americas, and at least twice as high in less developed countries not practicing intrapartum antibiotic prophylaxis, with overall case fatality at ~7 to 10% (3). Invasive GBS infections are increasingly reported in nonpregnant adult populations, especially the elderly, diabetics, and immune-compromised individuals (4).

GBS systemic dissemination indicates intrinsic resistance to clearance by innate immune defenses operating in the bloodstream. The best-studied GBS virulence factor is its capsular polysaccharide (CPS), represented by 10 unique serotypes (Ia, Ib, II to IX) that differ antigenically, but which all share a critical terminal sialic acid (Sia) residue α 2,3- linked to an underlying galactose within the repeating structure. This GBS Sia motif is identical to a common epitope prominently displayed on surface

glycoproteins and glycolipids of all mammalian cells, an example of bacterial molecular mimicry of its human host. CPS Sia is a critical determinant for GBS survival in vivo (5), interfering with host complement pathways to block C3b deposition, impairing opsonophagocytosis (6), and reducing generation of chemotactic C5a, limiting phagocyte recruitment (7). Sia mimicry also allows GBS to functionally engage inhibitory Sia-binding Ig-like lectins (Siglecs) (8), key immunoregulatory receptors expressed by leukocytes including neutrophils and macrophages (9). Inhibitory Siglecs (e.g., human Siglec-5, Siglec-7, or Siglec-9; murine Siglec-E) recognize the ubiquitous Sia epitopes of host cells as “self-associated molecular patterns,” prompting recruitment of Src homology region 2 domain-containing phosphatases (SHP) to their intracellular immunotyrosine-based inhibitory motif (ITIM) domain(s), thus limiting cellular activation under homeostatic conditions (10). GBS Sia mimicry allows the pathogen to bind inhibitory Siglecs, suppressing oxidative burst, proinflammatory cytokine release, neutrophil extracellular trap (NET) production, and phagocytosis, cumulatively enhancing GBS survival in human blood and virulence in murine infection models (11, 12).

Significance

Platelets have an important role in blood clotting, but also function in immune responses, including the release of antimicrobial peptides that eliminate pathogens such as *Staphylococcus aureus*. Here we observe that group B *Streptococcus* (GBS), a leading cause of sepsis and meningitis in human newborns, is not killed by platelets. The key to GBS platelet resistance is a polysaccharide capsule that coats the bacterial surface and contains sialic acid (Sia), a common sugar present on all human cells. GBS Sia blocks platelet-derived antimicrobial factors, and also engages an inhibitory receptor (Siglec-9) on the platelet surface to block platelet activation. Platelet resistance is a novel consequence of GBS “molecular mimicry” of its host and contributes to its propensity to produce bloodstream infection.

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From the host perspective, bacterial clearance from the bloodstream is commonly attributed to neutrophils, the most abundant circulating leukocyte, or complement-mediated lysis. However, the complex outer membrane of Gram-negative bacteria, the target of complement attack, is absent in Gram-positive GBS, whose highly cross-linked peptidoglycan and thick cell wall are not permeabilized by the membrane attack complex. Recently, increased attention has focused on the innate immune functions of another abundant cell type in circulating blood, the platelet (13, 14). These tiny (2 μM to 3 μM) anucleated cells play a central role in coagulation but are also recruited and activated in response to microbial pathogens (15). Platelets express formyl peptide receptors, Toll-like receptors (TLRs), and chemokine receptors to sense pathogen-associated molecular patterns and display directed chemotaxis (16, 17), whereupon they can engage microbes and undergo degranulation to release small cationic platelet microbicidal proteins (PMPs) and kinocidins with direct antimicrobial activities (18, 19).

A direct contribution of platelets in defense against GBS infection has not been described. In stark contrast to another leading invasive Gram-positive bacterial pathogen of humans, *Staphylococcus aureus*, we find GBS to be highly resistant to platelet killing in vitro and in vivo. Our mechanistic analyses identify dual roles of GBS CPS Sia to suppress platelet activation and block killing by PMPs, revealing an unexpected function of Siglec receptors in regulating platelet immunity, and pinpointing a specific bacterial determinant that counteracts platelet-mediated host defense.

Results

GBS Displays Intrinsic Resistance to Platelet-Mediated Killing. A role for circulating platelets in antimicrobial defense, although not commonly studied, is increasingly appreciated (13, 15, 20). Consistent with recent studies (21, 22), induction of thrombocytopenia in mice with anti-CD41 antibody impaired bloodstream clearance of a USA300 strain of methicillin-resistant *S. aureus* (MRSA) following i.v. challenge (Fig. 1A). In contrast, anti-CD41 treatment did not affect bloodstream clearance of a wild-type (WT) strain of invasive serotype III GBS strain under identical challenge conditions (Fig. 1A). Strikingly, when coincubated with freshly isolated human platelets at a 1:10 ratio, MRSA were rapidly killed, with viable cfu reduced 80% within 2 h, while GBS proliferated to reach eightfold higher cfu levels than the input inoculum during the same time period (Fig. 1B). Likewise, resistance to human platelet killing was observed in a panel of five additional GBS strains of differing CPS serotype (SI Appendix, Fig. S1A). Similarly, thrombin-activated releasates from platelets (ranging from 0.8×10^7 to 5.0×10^8 cells) showed dose-dependent bactericidal activity against MRSA, in marked contrast to dose-dependent promotion of GBS growth from the same releasates (Fig. 1C). This finding likely reflects enriched nutritional content of releasates prepared from greater platelet numbers, yet simultaneously devoid of anti-GBS bactericidal/static activity. In sum, GBS appears intrinsically resistant to platelet killing.

GBS Capsular Sia Increases Resistance to Platelets and Platelet-Derived Antimicrobial Peptides. The surface CPS of GBS is a major virulence factor that promotes resistance to bloodstream clearance (23), with terminally exposed Sia residues that interfere with complement C3b deposition and opsonophagocytosis (6). We noted that a serotype 14 strain of *Streptococcus pneumoniae*, which shares the same capsule structure as serotype III GBS except its terminal Sia, was sensitive to killing by human platelets (SI Appendix, Fig. S1B). To determine its contribution to platelet resistance, we employed an isogenic mutant GBS strain lacking Sia (ΔSia) secondary to a mutation in the *neuA* gene encoding CMP-Neu5Ac synthase (24). In contrast to the resistant WT GBS strain, bloodstream survival of the platelet-sensitive ΔSia GBS mutant

in vivo was increased upon CD41-induced thrombocytopenia (Fig. 1D). Compared with the WT parent GBS strain, the ΔSia mutant had reduced proliferation on exposure to human platelets (Fig. 1E) or platelet releasate (Fig. 1F), while plasmid complementation of *neuA* in the ΔSia mutant restored growth to WT GBS levels (Fig. 1E and F). Similarly, treatment of WT GBS with purified sialidase from *Arthrobacter ureafaciens* (AUS) increased susceptibility to killing by human platelets (Fig. 1G) and platelet releasate (SI Appendix, Fig. S2A), and increased platelet killing of an isogenic ΔSia mutant in the GBS serotype Ia background compared with its WT parent strain was also noted (SI Appendix, Fig. S3A). The releasate of thrombin-activated platelets contains several cationic antimicrobial peptides (18) including platelet factor-4 (PF-4) (25) and human β -defensin 1 (HBD-1) (19). Loss of capsular Sia expression by genetic mutation or AUS treatment increased GBS sensitivity to killing by both PF-4 (Fig. 1H and SI Appendix, Fig. S2B) and HBD-1 (Fig. 1I and J). Together, these results indicate that the Sia component of the GBS polysaccharide augments resistance to antimicrobial factors released by activated platelets, but cannot in and of itself fully explain the dramatic overall resistance of GBS to platelet killing (Fig. 1B), nor how platelet numbers fail to influence on GBS clearance in vivo (Fig. 1A).

GBS Capsular Sia Subverts Platelet Antimicrobial Activity by Inhibiting Platelet Activation. Platelet-mediated antimicrobial defense requires not only molecular effectors of bacterial killing (e.g., PF-4, HBD-1, and others) but also activation processes by which the platelets respond to bacterial encounter by receptor-linked signaling (e.g., TLR2, adenosine receptors), leading to degranulation and release of these effectors at the focus of infection (14, 17). We hypothesized that GBS Sia may further subvert platelet defense by blocking normal activation kinetics. P-selectin is an α -granule component exposed on the platelet surface during granule fusion with the outer membrane, whereupon it modulates activated platelet interactions with leukocytes and endothelium. The glycoprotein IIb/IIIa complex, recognized by monoclonal antibody PAC-1, is a platelet activation marker (~80,000 copies per platelet) that binds fibrinogen, von Willebrand factor, and other matrix and clotting system components. By flow cytometry, platelet expression of P-selectin or activated glycoprotein IIb/IIIa was significantly greater upon challenge with the GBS ΔSia mutant compared with the WT parent strain in both the serotype III (Fig. 2A) and serotype Ia (SI Appendix, Fig. S3B) backgrounds. Corroborating the genetic deletion study, enzymatic removal of Sia from WT GBS capsule using AUS restored platelet activation to the level seen with the ΔSia mutant (Fig. 2B). Representative transmission electron microscopy of human platelets incubated for 3 h with WT GBS showed that most platelets retained their discoid shape with normal features of a resting platelet, including microtubular coils, glycogen store, and an open canalicular system (Fig. 2C). In contrast, pronounced evidence of processing degranulation, degeneration, and necrosis is seen in platelets incubated with the isogenic ΔSia mutant (Fig. 2C).

GBS Capsular Sia Inhibits Platelet Activation by Interacting with Siglec-9 Expressed on the Platelet Surface. We previously reported a mechanism whereby GBS capsular Sia suppresses the activation of myeloid cells, that is, neutrophils and macrophages, by engagement of inhibitory CD33-related Siglec receptors on the leukocyte cell surface (reviewed in ref. 26). Specifically, GBS binds the most-abundant Siglec on human neutrophils, Siglec-9, in a Sia-dependent manner to suppress neutrophil innate immune phenotypes including oxidative burst, phagocytosis, extracellular trap formation, and bacterial killing (11). Further genetic studies in a knockout mouse lacking Siglec-E, a murine paralog of human Siglec-9, confirmed that GBS blunts macrophage immune responses in a Sia- and Siglec-dependent manner to promote its own survival (12). Recently, it was reported that Siglec-7 was present

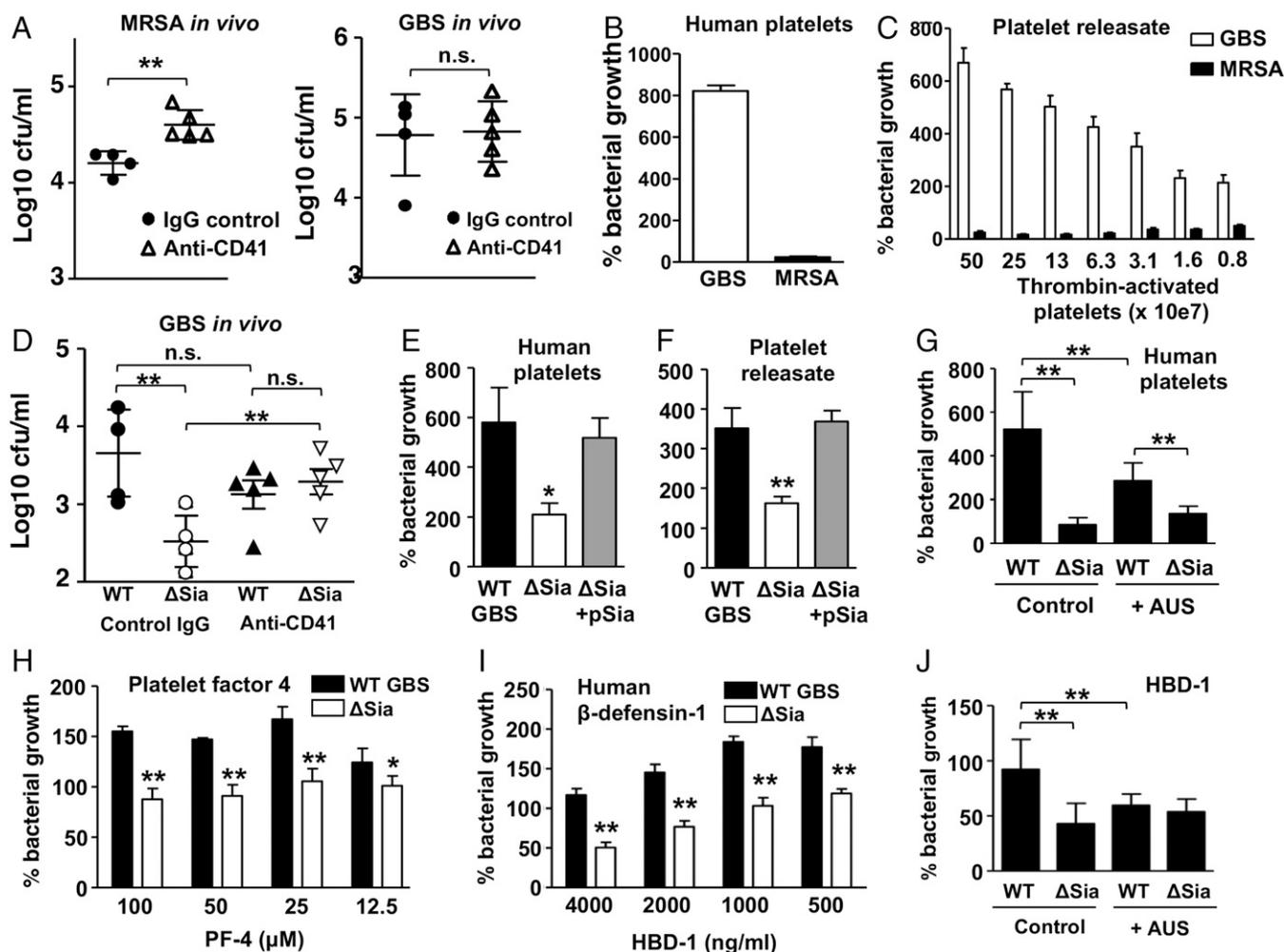


Fig. 1. Sialylated bacterial pathogen GBS displays intrinsic resistance to platelet-mediated killing. (A) Impaired clearance of MRSA from the bloodstream of mice rendered thrombocytopenic by anti-CD41 antibody treatment compared with IgG isotype control; similar treatment did not influence bloodstream counts of GBS. (B) GBS proliferates when coincubated in the presence of freshly isolated human platelets, whereas MRSA is killed. (C) GBS proliferates in a dose-dependent manner when exposed to thrombin-activated platelet releasate, whereas the same releasates have antimicrobial activity against MRSA. (D) In contrast to the resistant WT parent GBS strain, in vivo bloodstream survival of an isogenic Sia capsule-deficient (Δ Sia) mutant is increased upon CD41-induced thrombocytopenia. (E and F) Compared with WT GBS, the Δ Sia mutant has reduced proliferation on exposure to (E) human platelets or (F) platelet releasate; complementation of Sia capsule expression to the mutant restores WT growth levels. (G) Treatment of WT GBS with AUS sensitizes it to human platelet killing. (H and I) Increased susceptibility of Δ Sia mutant GBS to killing by the platelet-derived antimicrobial peptides (H) PF-4 and (I) HBD-1. (J) AUS treatment of WT GBS renders it more susceptible to HBD-1. For in vivo experiments, A and D show means \pm SD ($n = 4$ or 5 per group); n.s., nonsignificant; $^{***}P < 0.01$. For ex vivo and in vitro experiments, C and E through J, means \pm SD ($n = 3$ per experiment, mean of at least three independent experiments) are shown; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the GBS WT-infected group.

on activated human platelets and, when cross-linked by Sia ligands, accelerated platelet senescence through apoptosis (27). By flow cytometry, we corroborated the presence of Siglec-7 on activated human platelets yet observed even higher expression of Siglec-9 (Fig. 3A), the same Siglec exploited by GBS to suppress neutrophil immune functions. In addition, an anti-Siglec-9 blocking antibody significantly reduced GBS binding to human platelets in a plate-based assay, an effect not seen with Siglec-7 or Siglec-5 blocking antibodies (Fig. 3B). The Sia-dependent and Siglec-9-dependent binding of GBS to human platelets was confirmed by flow cytometry using the Δ Sia mutant and the anti-Siglec-9 blocking antibody (Fig. 3C). Engagement of Sia ligands by inhibitory Sigs leads to tyrosine phosphorylation of their cytoplasmic tail, whereupon ITIMs recruit SHP phosphatases, important negative regulators of immune and inflammatory receptor signaling (9). In line with this, platelets exposed to Sia-expressing WT GBS exhibited more Siglec-9 phosphorylation compared with those challenged with the Δ Sia mutant (Fig. 3D

and E). To confirm the inhibitory effect of platelet activation, we employed a recent technique to selectively modify GBS Sia through mild sodium metaperiodate oxidation, followed by aldehyde quenching using 4-methyl-3-thiosemicarbazide (28). Recapitulating effects seen with the Δ Sia mutant, chemical modification of Sia blocked Siglec-9 binding to WT GBS (Fig. 3F) and increased platelet activation in response to WT GBS challenge (Fig. 3G). We conclude that the regulatory role of Siglec-9 to control immune cell responsiveness is not restricted to myeloid cells, but rather extends to platelets. Molecular mimicry of host Sia epitopes by the pathogen GBS allows it to suppress platelet activation and release of bactericidal effectors.

Mice Lacking Inhibitory Siglec-E Show Increased Platelet Activation upon GBS Bloodstream Infection. GBS interacts with murine Siglec-E, the inhibitory receptor most closely related to human Siglec-9 (26). We confirmed expression of Siglec-E on murine platelets (Fig. 4A) and determined that efficient GBS binding to murine

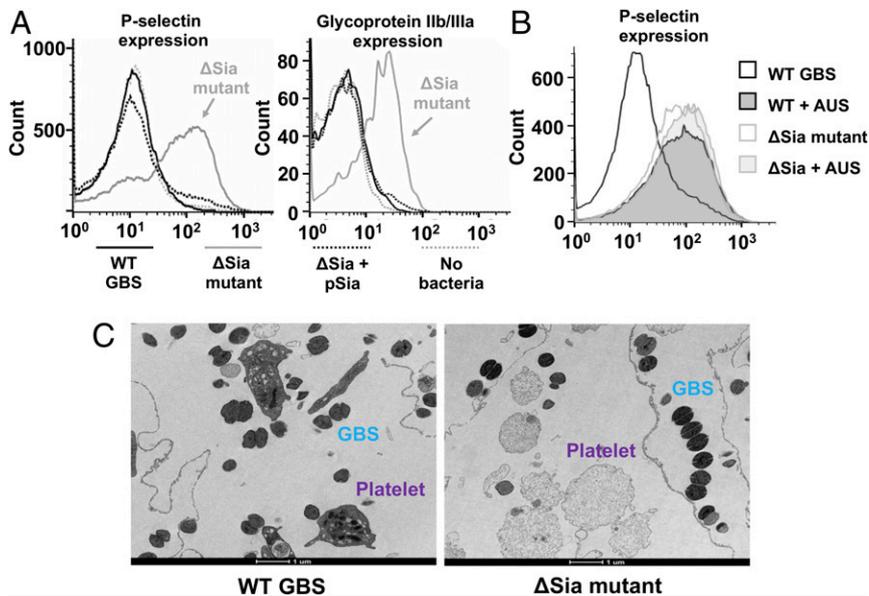


Fig. 2. GBS capsular Sia subverts platelet antimicrobial activity by inhibiting platelet activation. (A) Shown by flow cytometry, platelet expression of P-selectin or PAC-1 (activated GP IIb/IIIa) is greater upon challenge with the Δ Sia mutant compared with the WT parent GBS strain. (B) Enzymatic removal of the Sia from the WT GBS capsule by AUS increases platelet activation to the level seen in the Δ Sia mutant. (C) Representative transmission electron microscopy of human platelets incubated for 3 h with WT GBS shows most platelets retaining their unactivated discoid shape, whereas pronounced evidence of processing degranulation, degeneration, and necrosis is seen in platelets incubated with the isogenic Δ Sia mutant. Plots and images are representative of at least three independent experiments.

platelets requires both bacterial Sia and the presence of Siglec-E (Fig. 4B). Capsular Sia is a critical GBS survival factor in vivo, with capsule-deficient strains so rapidly cleared that the calculated LD50 is up to 10⁵-fold greater than corresponding WT parent strains (6). With that confounding factor in mind, we focused our analysis on short-term effects of Siglec-E expression on platelet activation following WT i.v. GBS infection. Siglec-E knockout ($SigE^{-/-}$) mice were generated on a C57BL/6 background by gene targeting (SI Appendix, Fig. S4); the $SigE^{-/-}$ animals exhibited normal platelet counts and thrombin-mediated platelet activation (SI Appendix, Fig. S5 and Table S1) similar to WT controls. Consistent with prior findings (12), bacterial counts were significantly reduced in $SigE^{-/-}$ mice compared with the parent strain 4 h after infection (Fig. 4C). However, despite the lower number of bloodstream bacteria, P-selectin expression on platelets of $SigE^{-/-}$ mice was significantly increased vs. WT controls (Fig. 4D). These results strongly suggest that GBS expression can blunt platelet activation in vivo through Siglec-E engagement. Also, whereas

bloodstream clearance of WT GBS was increased in $SigE^{-/-}$ mice compared with WT mice, no difference was seen between WT and $SigE^{-/-}$ mice in clearance of the Δ Sia GBS mutant (Fig. 4E), further supporting a specific role of GBS Sia engagement of Siglec-E in modulating platelet immunity.

Discussion

Prominent roles of platelets in driving or influencing immune and inflammatory responses are increasingly recognized (13, 14). However, direct in vivo experimental evidence for an essential platelet function in innate host defense against bloodstream infection remains limited. During the clotting process, human and rabbit platelets release antibacterial substances from their granules that show activities predominantly against Gram-positive bacterial strains such as *S. aureus* (29, 30). The laboratories of Yeaman and coworkers (18, 25, 31, 32) achieved careful biochemical identification and activity testing of several such peptide classes and variants under the rubric of thrombocidins, PMPs, or

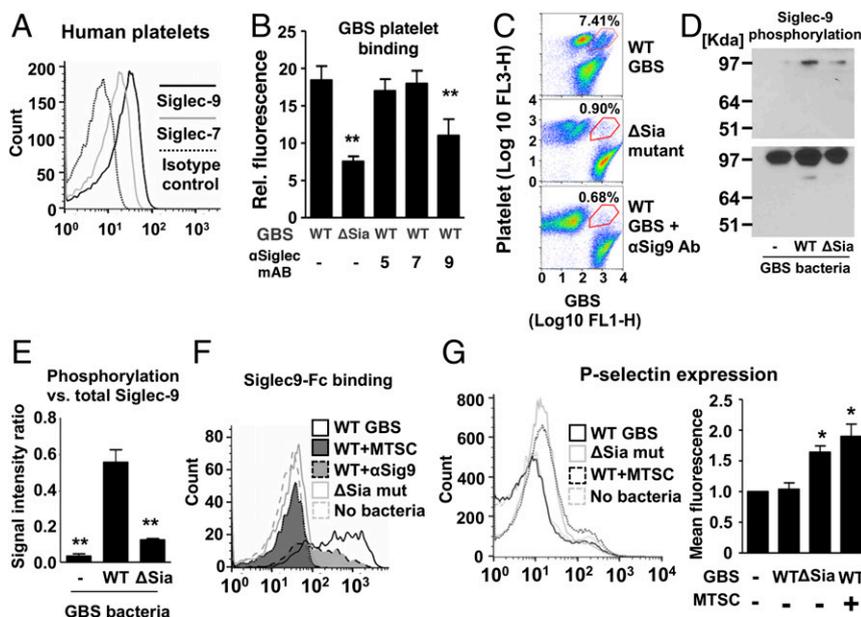


Fig. 3. GBS capsular Sia inhibits platelet activation by interacting with Siglec-9 expressed on the platelet surface. (A) Flow cytometry detects expression of Siglec-9 and Siglec-7 on human platelets. (B and C) Siglec-9 blocking antibody significantly reduces GBS Sia-dependent binding to human platelets as assessed in (B) a plate binding assay and (C) by flow cytometry. (D) Platelets were challenged with GBS strains, then lysed, and protein samples were investigated for phosphorylation (activation) of inhibitory Siglec-9 by immunoprecipitation and Western blot, with densitometric quantification shown in E. (F) Flow cytometry shows that elimination (Δ Sia mutant) or chemical modification (MTSC) of Sia reduces Siglec-9 binding to GBS. (G) Flow cytometry shows that elimination (Δ Sia mutant) or chemical modification (MTSC) of Sia increases platelet activation marker P-selectin upon exposure to GBS. Means \pm SD ($n = 3$) are shown; * $P < 0.05$ and ** $P < 0.01$ compared with GBS WT control. Bar graphs are the total of at least three independent experiments. Flow cytometry histograms or Western blot images are representative of at least three independent experiments.

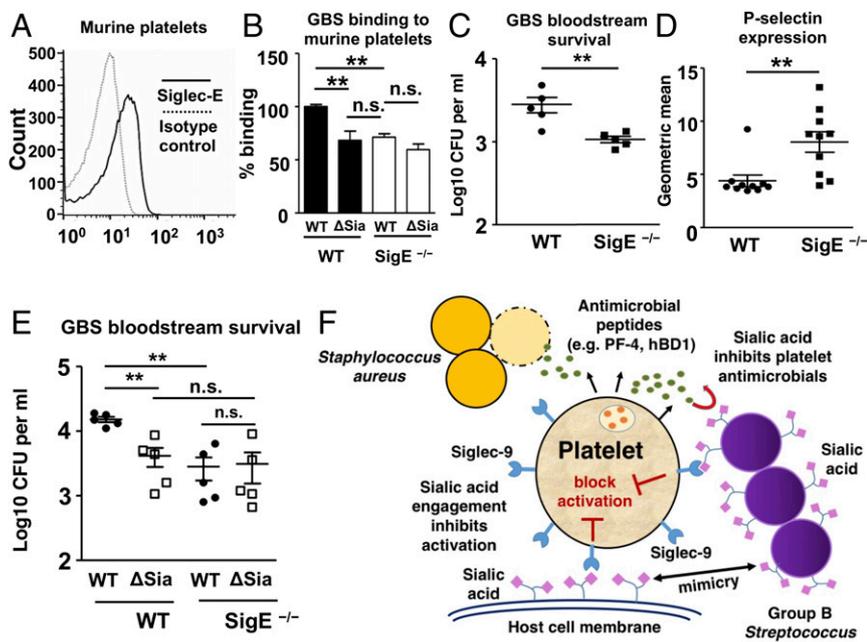


Fig. 4. Mice lacking inhibitory Siglec-E show increased platelet activation upon GBS bloodstream infection. (A) Flow cytometry detects expression of Siglec-E on murine platelets. (B) GBS binds to murine platelets in a Sia-dependent and Siglec-E-dependent manner. (C) Increased bacterial clearance from the blood following GBS i.v. infection in SigE^{-/-} mice compared with WT mice. (D) Increased platelet P-selectin expression following GBS i.v. infection in SigE^{-/-} mice compared with WT mice. (E) The enhanced bloodstream clearance of GBS in SigE^{-/-} mice vs. WT mice is abrogated in studies with a GBS Sia-deficient mutant (Δ Sia). (F) Schematic representation of the dual roles of GBS capsular Sia in counteracting platelet antimicrobial immunity: conferring intrinsic resistance to platelet-derived antimicrobial components and inhibiting platelet activation through engagement of inhibitory Siglecs. Means \pm SD ($n = 3$) are shown; $**P < 0.01$. Figures are representative of at least three independent experiments (for A and B) or a total of two independent experiments (for C–E). (F) Schematic depicting GBS Sia dual roles to counteract platelet immunity: conferring intrinsic resistance to platelet-derived antimicrobial components and inhibiting platelet activation through engagement of inhibitory Siglecs.

platelet kinocidins. Activated platelets further augment *S. aureus* clearance through release of interleukin-1 β , which boosts macrophage phagocytosis, and by a dual action of HBD-1 to stimulate NETs (19). Consistent with a multifaceted role in host defense, antibody-mediated platelet depletion renders mice significantly more susceptible to systemic *S. aureus* infection (21, 22).

Here we report that the prominent human pathogen GBS is remarkably resistant to human platelet killing and that platelet depletion does not influence GBS bloodstream clearance in a murine infection model. Thrombocytopenia and pathological clotting (e.g., disseminated intravascular coagulation), hallmarks of advanced bacterial sepsis, can likewise be observed in severe neonatal GBS infection (33). GBS strains isolated from septic patients, which have an enhanced ability to bind fibrinogen on their surface, preferentially stimulate the aggregation of platelets (34), although this phenomenon appears to be independent of the Sia epitope on serotype III GBS CPS (35). Here we find that terminal Sia in the GBS CPS provides protection against killing by human platelets, their releasates, and two well-characterized platelet-derived antimicrobial peptides, HBD-1 and PF-4. Other groups have used isogenic mutants to demonstrate that terminal Sias present in the lipooligosaccharide of *Campylobacter jejuni* or *Neisseria gonorrhoeae* contribute to resistance against neutrophil-derived cationic host defense peptides (36, 37).

GBS suppressed platelet activation and release of antimicrobial factors through Sia-dependent engagement of the inhibitory receptor Siglec-9. Immunoreceptor-mediated activation of platelets in response to bacterial components has been studied in detail, especially among TLRs, with documentation of TLR-1, TLR-2, TLR-3, TLR-4, TLR-6, TLR-7, and TLR-9 expression on platelets and demonstration of functional signaling outcomes including regulation by MyD88 (16, 38). A role of Siglec-9 and other inhibitory Siglecs (e.g., human Siglec-5, murine Siglec-E) in blunting TLR and MAPK activation pathways has been reported in neutrophils and macrophages (reviewed in ref. 26), and here we observe that their immunoregulatory function extends to the anucleate but granule rich platelets. During bone marrow hematopoiesis, a common myeloid progenitor cell gives rise to neutrophils, macrophages, and megakaryocytes—the producer of platelets. We suspect, as the role of platelets in host defense is increasingly appreciated, further parallels with phagocytic cell signaling and effector pathways are likely to be uncovered.

Platelets contain many potent immunologically active molecules that contribute directly and indirectly to innate and adaptive host defenses (39). Given their abundance in blood, platelets can serve as sentinels for detecting invading microorganisms through TLRs and other receptor pathways (38). Activated platelets release antimicrobial peptides (studied here) but also produce both proinflammatory [e.g., interleukin-1 β (40)] and antiinflammatory [e.g., transforming growth factor-1 (41)] cytokines that can modulate the broader inflammatory/immune response through “cross-talk” with leukocytes (e.g., neutrophils and macrophages) and vascular endothelium (22, 39). To the extent that platelet inhibitory Siglecs counterregulate these platelet activation phenotypes, the ability of GBS Sia to blunt platelet activation via Siglec engagement may propagate a broader immune perturbation that influences the progression of systemic GBS infection. Reciprocally, GBS Sia-mediated inhibition of neutrophils and macrophages (via Siglec-9 and Siglec-5) (11, 12) can suppress release of cytokines that normally promote platelet activation (e.g., IL-1 β , IL-6, and IL-8), magnifying the functional deficit in platelet immunity.

We conclude that GBS Sia has dual roles in counteracting platelet antimicrobial immunity: conferring intrinsic resistance to platelet-derived antimicrobial components and inhibiting platelet activation through engagement of inhibitory Siglecs (schematic in Fig. 4E). We note that, while *S. aureus* was readily killed in vitro by platelets and their releasate, the GBS Δ Sia mutant exhibits only growth inhibition in parallel assays. With the caveat that in vitro assays cannot model the full complexity and/or effectiveness of platelet-mediated immunity in vivo, there are likely to be additional bacterial factors contributing to GBS platelet resistance and host factors contributing to the partial effectiveness of platelets as a bactericidal cell, with Sia a highly significant component, as shown by the multiple complementary approaches of this study. Currently, the Sia epitopes of the GBS capsule are the principal target of GBS vaccines now proposed to enter phase III clinical trials for prevention of early- and late-onset infections in human newborn infants (42). GBS suppression of platelet activation and its intrinsic resistance platelet antimicrobial factors further explain the central role of CPS Sia in bloodstream survival and virulence of this foremost human neonatal pathogen.

Materials and Methods

Mouse studies were conducted in accord with protocols approved by the University of California, San Diego (UCSD) Institutional Animal Care and Use Committee. Blood collection and platelet isolation from healthy adult volunteers under informed consent and subsequent experimentation was approved by the UCSD Human Research Protection Program IRB. Well-characterized human serotype III GBS isolate (COH-1) from a case of human neonatal sepsis and isogenic mutant (Δ Sia) generated by allelic replacement of the *neuA* gene were analyzed in detail. Platelets were isolated by centrifugation from human or murine blood anticoagulated with acid citrate-dextrose buffer (ACD; Sigma Aldrich). Platelet releasates were collected after activation with 0.1 U/mL of bovine thrombin (Sigma-Aldrich) for 30 min. Recombinant PF-4 and HBD-1 were obtained from Bachem Americas and reconstituted in 96-well plates in serial dilutions. To remove or modify GBS

Sia for binding or activation assays, strains were exposed to either sialidase treatment or mild periodate oxidation. Flow cytometry was used for platelet surface P-selectin, PAC-1, and Siglec expression using specific antibodies. The 10- to 12-wk-old WT C57BL/6 and SigE^{-/-} mice were challenged 1×10^8 CFU of bacteria by tail vein injection, then killed 4 h later for blood collection by cardiac puncture. Platelet depletion was achieved with 1 mg/kg of anti-CD41 antibody. Detailed procedures for all studies are described in *SI Appendix, Materials and Methods*.

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Supplementary Information for

Dual Actions of Group B Streptococcus Capsular Sialic Acid Provide Resistance to Platelet-Mediated Antimicrobial Killing

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Figs. S1 to S6
Tables S1 to S2
References for SI reference citations

Supplementary Information Text

Materials and Methods

Bacterial Strains. Well-characterized human serotype III GBS isolate (COH-1) from a case of human neonatal sepsis was used in this study. An isogenic mutant (Δ Sia) lacking the terminally linked sialic acid in the capsule polysaccharide repeat unit generated by allelic replacement of the *neuA* gene was previously described (1) and has normal growth characteristics compared to its WT parent strain (see SI Appendix **Fig. S6**). Allelic replacement of the *neuA* to create a Δ Sia derivative in GBS serotype Ia isolate A909 was reported in (2). Community-acquired MRSA strain USA300/TCH1516 (3) was also employed. Bacterial strains were propagated in Todd-Hewitt broth (THB, Difco, BD Diagnostics) at 37°C without shaking. For *in vitro* and *in vivo* experiments, bacteria were grown to mid-exponential phase and re-suspended to an optimal density at 600nm (OD_{600}) = 0.4, followed by serial dilution and enumeration of colony forming units (CFU) to verify each experimental inoculum.

Platelet Isolation. Human platelets were isolated from fresh blood obtained from healthy donors using acid-citrate-dextrose buffer (ACD; Sigma Aldrich) as an anticoagulant following an established method (4) with a few modifications. Blood was centrifuged at 200 x g for 15 min without brake to obtain platelet rich plasma (PRP); PRP was centrifuged at 260 x g for 10 min to isolate platelets. Platelets were resuspended in serum-free Roswell Park Memorial Institute (RPMI) 1640 media (Fisher Scientific) at room temperature. Murine platelets were isolated from fresh blood collected by cardiac puncture with a 25 g

needle attached to a syringe containing 100 μ l acid citrate dextrose (ACD) buffer. Collected blood was centrifuged at 100 x *g* for 10 min without brake. PRP was removed into a separate tube and centrifuged at 400 x *g* for 10 min with low brake, and the platelet pellet resuspended in RPMI 1640.

Platelet Releasate Preparation. Platelets were activated with 0.1 U/ml bovine thrombin (Sigma-Aldrich) for 30 min, then centrifuged at 1000 x *g* for 10 min to obtain cell-free releasate. For control releasates, platelets without thrombin stimulation were treated in the same way, and 0.1U/ml thrombin was added to the cell-free control releasate.

Platelet Killing (Growth Inhibition) and Releasate Killing (Growth Inhibition). Isolated human platelets were inoculated with the bacterial strain(s) stated in each experiment at a multiplicity of infection (MOI) = 0.01. Platelet releasate was inoculated with 1×10^5 CFU of the bacterial for 2 h at 37°C + 5% CO₂. After incubation, infected platelets were sonicated (Fisher Sonic Dismembrator 550) 2 times for 3 sec, diluted, and plated on THB agar plates. Percent killing was determined as the number of surviving CFU divided by the bacterial inoculum x 100%.

Platelet Depletion Model. For platelet depletion studies, 1 mg/kg endotoxin and azide-free anti-CD41 antibody (clone MWReg30, Biolegend, San Diego, CA, USA) or 1 mg/kg isotype control rat IgG1 (clone RTK2071, Biolegend, San Diego, CA, USA) was injected intraperitoneally (i.p.). Then 16 h after antibody injection, GBS or MRSA (5×10^7 CFU/mouse) was injected intravenously (i.v.) via tail vein. Four h after bacteria injection,

mice were euthanized by CO₂, blood collected by cardiac puncture, and serial dilutions plated on THB agar to determine surviving bacterial CFUs.

Recombinant Antimicrobial Peptide Killing. Recombinant platelet factor 4 (PF4) and human β -defensin-1 (hBD1) were obtained from Bachem Americas (Torrance, CA, USA) and reconstituted in 96-well plates in serial dilutions. All wells were inoculated with 1×10^4 CFU of the stated bacterial strain for 2 h at 37°C. After incubation, wells were sonicated (Fisher Sonic Dismembrator 550) 2 times for 3 secs, diluted, and plated on THB agar plates. Percent killing was determined as the number of surviving CFU divided by the bacterial inoculum x 100%.

Siglec9-Fc Binding Assay. Chimeric recombinant human Siglec9-Fc fusion protein was generated as described previously (5). GBS strains were resuspended in RPMI at 4°C with 10 μ g/ml human Siglec-9 Fc. After 1 h, PE-anti human IgG Fc (Biolegend, San Diego, CA, USA) was added and the sample analyzed by flow cytometry. For blocking studies, anti-Siglec-9 antibody (Clone 191240, R&D Systems, Minneapolis, MN, USA) was used at 1:200 dilution.

GBS Surface Sialic Acid Removal and Modification. To remove or modify the sialic acid on GBS for binding assays, strains were exposed to either sialidase treatment or mild periodate oxidation. For sialidase treatment, GBS was incubated with 100 mU/ml *Arthrobacter ureafaciens* sialidase (AUS, Sigma Aldrich, St Louis, MO, USA) for 2 h at 37°C, then washed twice with PBS. Selective periodate oxidation of GBS sialic acid

containing glycans was accomplished by incubating heat killed GBS with fresh 2 mM NaIO₄ (Sigma Aldrich) on ice x 20 min to generate aldehydes at the C7 or C8 position of sialic acid. To stop the reaction, NaIO₄ was centrifuged (10,000 x g for 10 min at 4°C) then washed 3 times with ice-cold PBS. Sialic acid aldehydes were subsequently quenched with MTSC (Sigma Aldrich) as performed previously (6).

Platelet Activation Measurement (P-selectin, PAC-1). Platelet surface P-selectin and PAC-1 expression were measured by flow cytometry. Briefly, 1 x 10⁷ platelets were infected with GBS with or without modification of surface sialic acid (described above) at MOI = 0.1 for 2 h with rotation at 37°C. Samples were incubated with PerCP/Cy5.5 anti-human CD41 and PE anti-human CD62p (P-selectin) antibody (Biolegend, San Diego CA, USA) or 1 µL of 25 µg/mL PAC1-FITC antibody (BD Bioscience) for 30 min at room temperature, diluted in 1 ml PBS, and expression of P-selectin measured by flow cytometry (FACSCalibur, BD Biosciences) and analyzed using FlowJo v10.2 software (FlowJo LLC, Ashland, OR). Mean fluorescence (PE and FITC) was calculated on cells gated on CD41.

Transmission Electron Microscopy. Isolated human platelets were inoculated with GBS strains at MOI = 0.1 in siliconized tubes, then incubated with rotation for 2 h at 37°C + 5% CO₂. The samples were then immediately fixed by adding modified Karnovsky's fixative (2.5% glutaraldehyde + 2% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.4) for at least 4 h, post-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 1 h, and stained in block in 2% uranyl acetate for 1 h. Samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50–60 nm on a Leica

UCT ultramicrotome, and picked up on Formvar and carbon-coated copper grids. Sections were stained with 2% uranyl acetate for 5 min and Sato's lead stain for 1 min. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and images obtained with an Eagle 4k HS digital camera (FEI). Images were taken from multiple random fields at magnifications ranging from 1400× to 30,000×.

Platelet Siglec Expression. Human and mouse platelet surface Siglec expression was measured by flow cytometry. Anti-Siglec-7 antibody (Clone 6-434, Biolegend, San Diego, CA, USA) or anti Siglec-9 antibody (Clone 191240, R&D Systems, Minneapolis, MN, USA) for human platelets, or anti Siglec-E antibody (clone M1304A01, Biolegend) for murine platelets, were added to platelets (1×10^6 platelets for human and 1×10^5 platelets for mice) in 100 μ l Tyrode's buffer. This was followed by incubation for 30 min at room temperature. The samples were diluted in 1 ml PBS and Siglec expression measured by flow cytometry (FACSCalibur, *BD* Biosciences) and analyzed using FlowJo v10.2 software (FlowJo LLC).

Platelet Binding Assays. GBS (1×10^7 CFU) resuspended in 100 μ l carbonate buffer were inoculated in each well of 96-well high binding plates (Corning, Corning, NY, USA) and incubated overnight at 4°C. The plate was washed x 3 and blocked at room temperature with phosphate-buffered saline (PBS) + 10% bovine serum albumin (BSA) for 1 h. Platelets were labeled with 2 μ g/ml calcein AM (AnaSpec, San Jose, CA, USA) and washed with PBS; labeled platelets were added to the assay and initial fluorescence intensity (FI) measured on a multi-plate reader (Enspire Alpha, Perkin Elmer, Waltham,

MA). After 30 min, non-adherent platelets were removed by washing x 5 with PBS using a multichannel pipetter and final FI measured. For antibody inhibition of Siglecs, platelets were incubated for 5 min in the presence or absence of anti-Siglec-7 antibody (Catalog # AF1138) or anti-Siglec-9 antibody (Clone #191240) from R&D Systems (Minneapolis, MD, USA) at 1:200 dilution before addition to the assays wells. For binding assessment by flow cytometry, WT serotype III GBS was labeled with 50 µg/ml fluorescein isothiocyanate (FITC) for 30 min at 37°C, washed and added to 1×10^7 human platelets at MOI = 1 in siliconized 2.0 ml tubes. Tubes were incubated at 37°C under rotation for 1h, then PerCP/Cy5.5 anti-human CD41 Antibody (Biolegend) added per manufacturer's protocol. Samples were analyzed for co-existence of FL-1 signal (bacteria) and FL-3 signal (platelets) using FlowJo software.

Immunoprecipitation for Siglec-9 Phosphorylation. Platelets were inoculated with GBS strains (MOI = 0.1) for 1 h at 37°C with rotation followed by lysis with RIPA buffer supplemented with PhosSTOP Phosphatase Inhibitor and cOmplete Protease Inhibitor (Roche). Lysates were incubated with anti-Siglec-9 antibodies (#624084, BD Pharmingen, San Diego, CA) conjugated to protein G-Dynabeads (Life Technologies) at 4°C for 1 h. Proteins were separated by reducing SDS-PAGE, transferred to PVDF and probed with anti-Siglec-9 (R&D Systems #BAF1139), followed by rabbit anti-SH-PTP1 (aka SHP; Santa Cruz Biotechnology #sc-287), HRP-conjugated secondary antibody and chemiluminescence substrate (Thermo Scientific).

Mouse Infection Studies. All use and procedures were reviewed and approved by the University of California, San Diego (UCSD) Institutional Animal Care and Use Committee. WT C57Bl/6 and SigE^{-/-} mice on the same genetic background (**Fig. S1, Tables S1, S2**) were studied. WT GBS cultures were washed x 1 in PBS, and 1 x 10⁸ CFU injected i.v. into 10 to 12-week-old WT or SigE^{-/-} mice. Four h after infection, mice were euthanized by CO₂ asphyxiation and blood collected by cardiac puncture with a 25g needle attached to a syringe containing 100 µl ACD buffer. Five µl each of PerCP/Cy5.5 anti-mouse CD41 antibody (Biolegend) and PE anti- mouse CD62p (P-selectin) antibody (Biolegend) were added to 100 µl of blood from each mouse. After 30 min incubation at room temperature, samples were diluted in 1 ml PBS, and expression of P-selectin measured by flow cytometry (FACSCalibur, BD Biosciences) with analysis by FlowJo (TreeStar) software. Mean fluorescence (PE) was calculated for cells gated on CD41. Part of the collected blood was dilution plated onto THB agar for enumeration of bacterial CFU.

Generation of *siglec-E* deficient mice. Siglec-E knockout (SigE^{-/-}) mice were generated on a C57BL/6 background by gene targeting as shown in **Fig. S4A**. Murine *Siglec-E* (cDNA sequence NM_031181) exon 4 and murine exon 5 was flanked by loxP sites enabling its conditional deletion. Floxed chimeras were bred with C57BL/6 Cre deleter mice to excise the loxP flanked sequence and to generate heterozygous mice carrying the knockout allele, and homozygous SigE^{-/-} mice were achieved through subsequent heterozygous crosses. Wild-type mice resulting from heterozygous crosses were maintained as controls. Siglec-E deficiency in SigE^{-/-} animals was confirmed via IHC in the liver and spleen (**Fig. S4B**). Siglec-E deficiency did not result in any gross

abnormalities. Animals bred normally with typical litter size ranges, and no gross pathologies were noted on hematology or serum chemistry panels (**Table S1** and **S2**).

Assessment of Mouse Platelet Activation. Platelets were isolated from 10-week-old female WT and SigE^{-/-} mice (n=3 each). Isolated platelets were stimulated with 0.1 or 1.0 U/ml thrombin for 30 min at 37°C. P-selectin expression was measured by flow cytometry and FL-2 signal intensity calculated as the geometric mean using FlowJo software.

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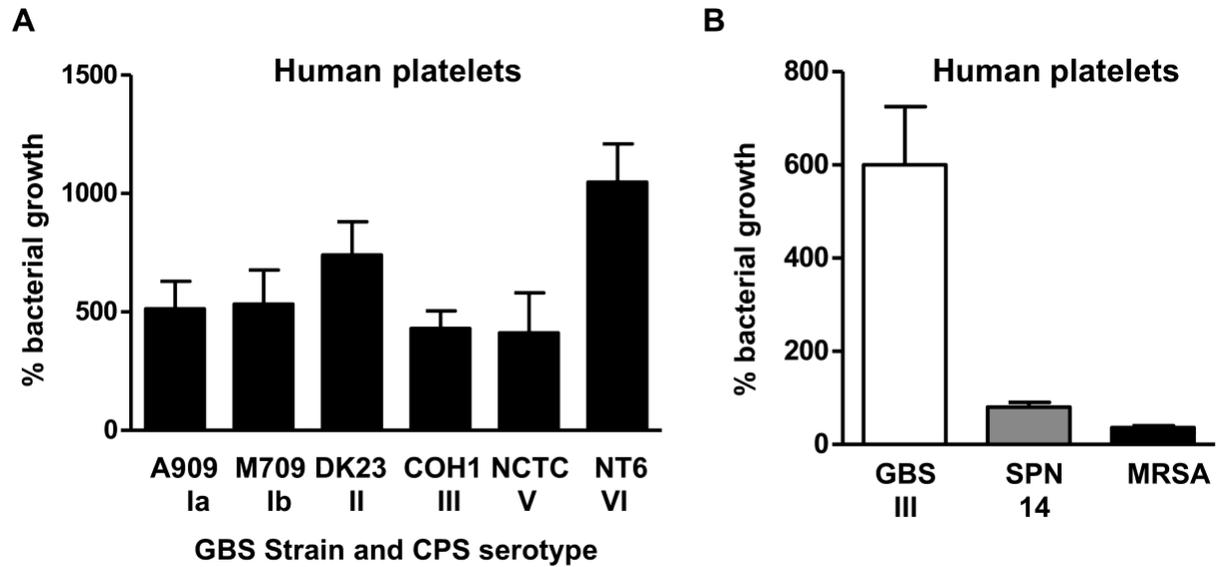


Fig. S1. (A) Group B *Streptococcus* (GBS) strains of various capsular serotype display intrinsic resistance to platelet-mediated killing. Representative GBS strains of capsular serotype Ia, Ib, II, III, V and VI proliferate when co-incubated in the presence of freshly isolated human platelets. (B) Serotype 14 *Streptococcus pneumoniae* (SPN), which shares the exact capsule structure of serotype III GBS except the terminal sialic acid, is sensitive to human platelet killing.

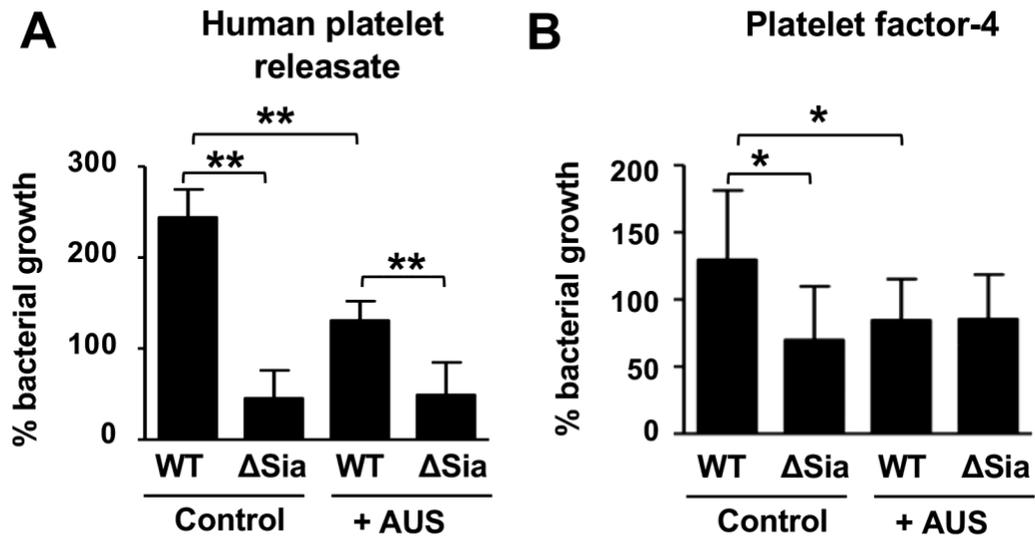


Fig. S2. Treatment of WT Group B *Streptococcus* (GBS) with *Arthrobacter ureafasciens* sialidase increases susceptibility to killing by (A) human platelet releasate and (B) the platelet-associated antimicrobial peptide, platelet factor-4 (PF-4). An isogenic sialic acid-deficient GBS mutant (Δ Sia) serves as a control. *, $P \leq 0.05$; **, $P \leq 0.01$

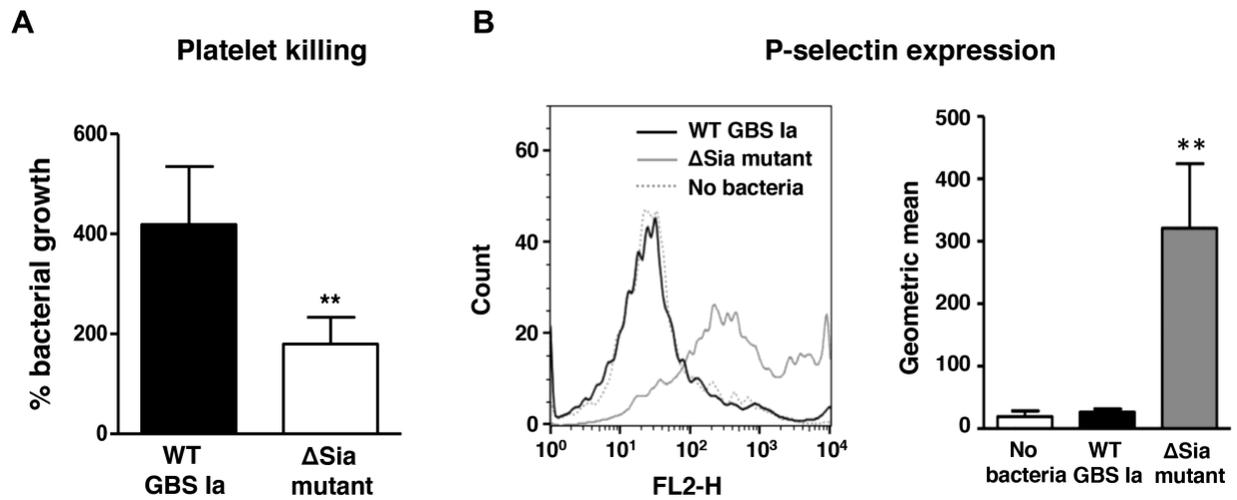


Fig. S3. (A) An isogenic sialic acid-deficient mutant (Δ Sia) in a WT GBS strain Ia background has reduced proliferation on exposure to human platelets. (B) Flow cytometry shows that elimination of the GBS Ia Δ Sia mutant is associated with increased activation of P-selectin on human platelets. Bar graphs and histogram images are total of at least three independent experiments. Flow cytometry histogram images are representative of at least three independent experiments. **, $P \leq 0.01$

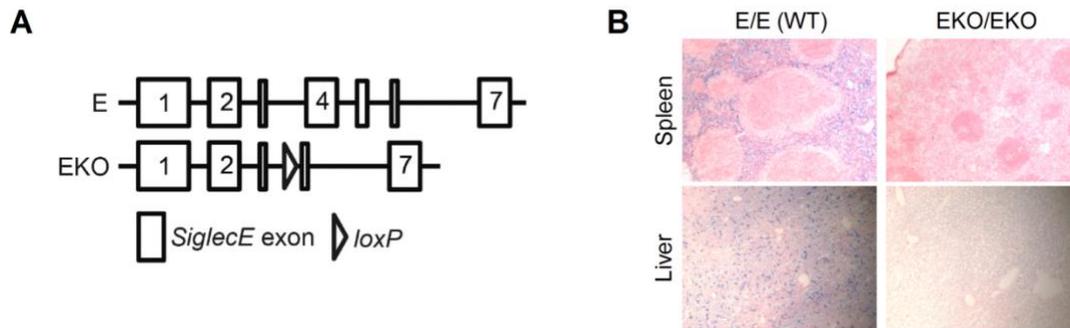


Fig. S4. Generation of Siglec-E deficient mice. (A) Schematic view of the *Siglec-E* locus in wild-type, and Siglec-E KO (EKO) mice. (B) Siglec-E staining in spleen and liver from wild-type or EKO male mice 8 weeks of age. Organs were isolated and snap-frozen in OCT. Sections were blocked and stained with rat anti-Siglec-E (BioLegend, cat: 677102), and secondary anti-rat IgG conjugated to alkaline phosphatase. Antibody binding (Blue) was detected using the substrate Vector Blu SK4200 (Vector Laboratories, Burlingame, CA). Nuclear Fast Red was used as a counterstain.

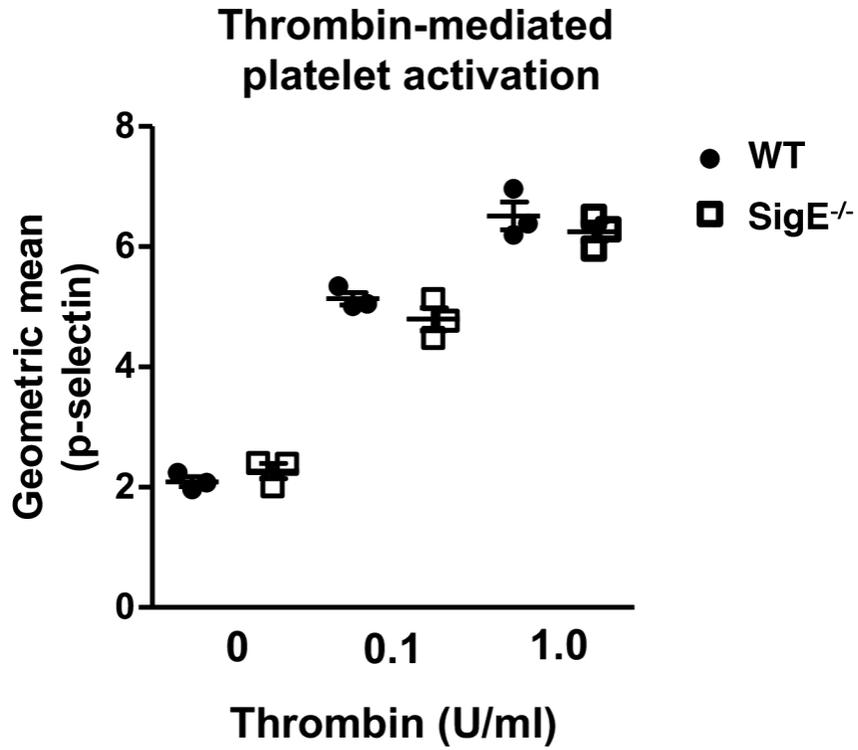


Fig. S5. Platelets isolated from WT and Siglec-E knockout (SigE^{-/-}) mice were stimulated with two different doses of thrombin. Flow cytometric analysis of platelet activation marker P-selectin is shown as the geometric mean.

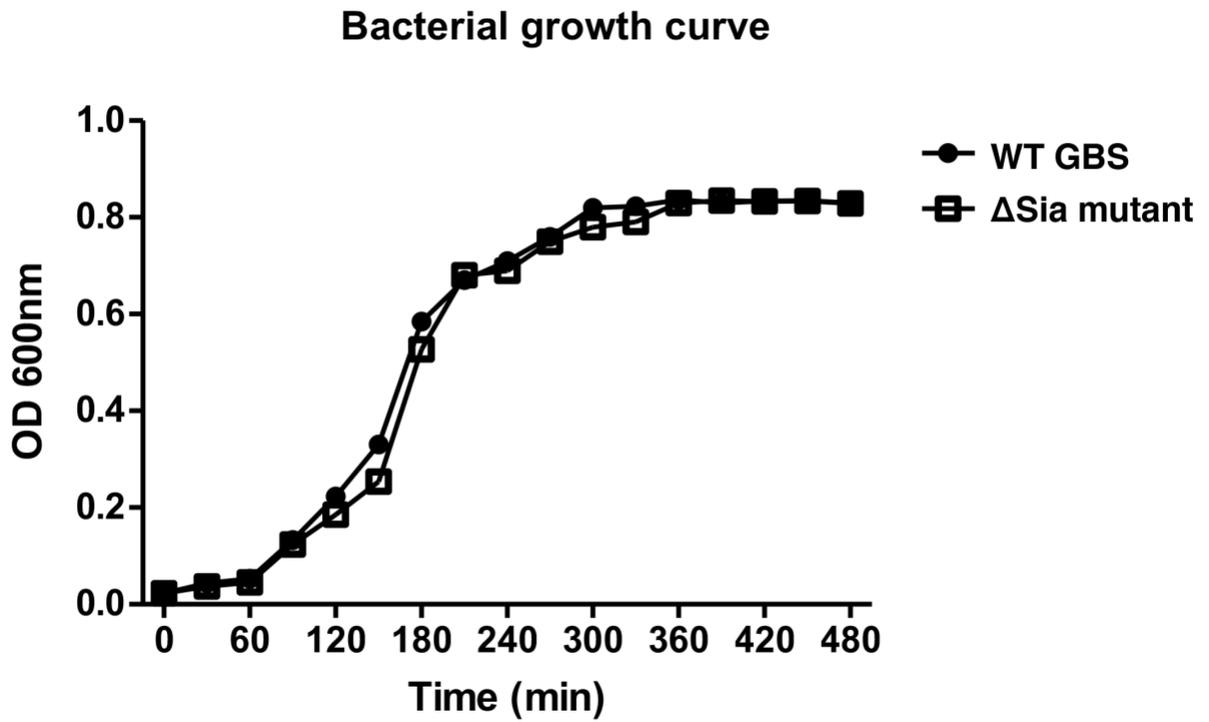


Fig. S6. Growth curve of WT serotype III GBS stain and the isogenic Δ Sia mutant in Todd-Hewitt Broth media used in preparing bacterial inocula. The graph shown is a representative of three independent experiments.

		E/E		EKO/EKO		<i>p</i> value (unpaired <i>t</i> test)
		Average	SD	Average	SD	
WBC	K/uL	2.62	0.46	2.31	1.01	0.51
NE#	K/uL	0.35	0.14	0.40	0.18	0.63
LY#	K/uL	2.08	0.47	1.67	0.77	0.29
MO#	K/uL	0.13	0.03	0.16	0.10	0.58
EO#	K/uL	0.04	0.02	0.06	0.07	0.40
BA#	K/uL	0.01	0.00	0.01	0.02	0.59
NE%	%	13.80	6.66	17.13	5.17	0.36
LY%	%	79.36	8.06	72.89	6.50	0.16
MO%	%	5.10	0.72	6.43	1.85	0.13
EO%	%	1.42	0.99	2.79	3.16	0.34
BA%	%	0.32	0.18	0.75	0.95	0.30
RBC	M/uL	10.47	0.36	9.39	1.32	0.08
HB	g/dL	14.19	0.37	12.85	2.02	0.14
HCT	%	39.85	0.86	36.79	5.66	0.22
MCV	fL	38.08	1.12	39.14	0.91	0.10
MCH	Pg	13.57	0.30	13.66	0.35	0.63
MCHC	g/dL	35.64	0.93	34.90	0.68	0.14
RDW	%	17.52	0.63	18.34	1.37	0.21
PLT	K/uL	1181.33	82.11	954.67	404.88	0.21
MPV	fL	4.74	0.33	4.97	0.56	0.42

Table S1. Hematology analysis of wild-type (E/E) and Siglec-E KO (EKO) mice. Blood was collected from male mice, 2 months of age. Average, standard deviation and *p* value are indicated, n=6/group.

	E/E		EKO/EKO		<i>p</i> value (unpaired t test)
	average	SD	average	SD	
Albumin (g/dL)	3.9	0.2	3.7	0.2	0.14
Alkaline Phosphatase (U/L)	167.4	54.3	159.8	43.6	0.77
ALT (U/L)	41.7	9.4	69.0	24.5	0.01
Anion Gap (mmol/L)	44.9	2.3	45.8	3.3	0.52
Bicarbonate (mmol/L)	67.8	11.4	129.8	68.6	0.64
Bilirubin Total (mg/dL)	15.3	1.5	14.8	3.1	0.07
BUN (mg/dL)	0.0	0.0	0.1	0.0	0.33
Calcium (mg/dL)	24.3	3.7	26.3	3.8	0.15
Chloride (mmol/L)	10.8	0.4	11.2	0.5	0.28
Creatinine (mg/dL)	100.4	2.5	98.5	4.2	0.18
Glucose (mg/dL)	0.1	0.0	0.1	0.0	0.94
Potassium (mmol/L)	207.2	46.2	209.5	75.9	0.17
Sodium (mmol/L)	6.6	1.4	8.0	2.4	0.49
Total Protein (g/dL)	160.5	4.0	159.0	4.2	0.84

Table S2. Serum chemistry analysis of wild-type (E/E) and Siglec-E KO (EKO) mice. Blood was collected from male mice, 2 months of age. Average, standard deviation and *p* value are indicated, n=5/group.