

Group B *Streptococcus* suppression of phagocyte functions by protein-mediated engagement of human Siglec-5

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Group B *Streptococcus* (GBS) is a leading cause of invasive bacterial infections in human newborns. A key GBS virulence factor is its capsular polysaccharide (CPS), displaying terminal sialic acid (Sia) residues which block deposition and activation of complement on the bacterial surface. We recently demonstrated that GBS Sia can bind human CD33-related Sia-recognizing immunoglobulin (Ig) superfamily lectins (hCD33rSiglecs), a family of inhibitory receptors expressed on the surface of leukocytes. We report the unexpected discovery that certain GBS strains may bind one such receptor, hSiglec-5, in a Sia-independent manner, via the cell wall-anchored β protein, resulting in recruitment of SHP protein tyrosine phosphatases. Using a panel of WT and mutant GBS strains together with Siglec-expressing cells and soluble Siglec-Fc chimeras, we show that GBS β protein binding to Siglec-5 functions to impair human leukocyte phagocytosis, oxidative burst, and extracellular trap production, promoting bacterial survival. We conclude that protein-mediated functional engagement of an inhibitory host lectin receptor promotes bacterial innate immune evasion.

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Abbreviations used: AUS, *Arthrobacter ureafaciens* sialidase; CPS, capsular polysaccharide; GBS, group B *Streptococcus*; hCD33rSiglec, human CD33-related Siglec; HIHS, heat-inactivated human serum; NET, neutrophil extracellular trap; Sia, sialic acid; Siglec, Sia-recognizing Ig superfamily lectin.

Group B *Streptococcus* (GBS) is a common cause of sepsis and meningitis in human newborns (Dermer et al., 2004). The GBS capsular polysaccharide (CPS) is a critical virulence factor containing a terminal α 2-3-linked *N*-acetylneuraminic acid, identical to the most common sialic acid (Sia) capping many surface glycans on all human cells (Varki, 2007). To date, structures of nine antigenically distinct GBS CPS have been described (serotypes Ia, Ib, and II-VIII), each composed of unique arrangements of galactose, glucose, and/or *N*-acetylglucosamine but invariably possessing Sia on the branching terminus of the repeating unit (Paoletti and Kasper, 2003). By increasing affinity of serum factor H for C3b and accelerating intrinsic decay of the C3 convertase, host Sia counterregulates complement activation (Pangburn, 2000). Likewise, by decorating its own surface with Sia, GBS creates a nonactivating environment restricting alternative complement deposition (Marques et al., 1992), impairing opsonophagocytic clearance and promoting disease progression (Wessels et al., 1989).

The Sia-recognizing Ig superfamily lectins (Siglecs) are a type-I transmembrane protein family, consisting of an N-terminal Sia-binding V-set domain and a variable number of extracellular C2-set domains (Varki and Angata, 2006; Crocker et al., 2007; Varki, 2007). Human CD33-related Siglecs (hCD33rSiglecs), comprising Siglecs 3, 5-11, XII, 14, and 16, are a rapidly evolving subgroup of Siglecs predominantly expressed on leukocytes (Crocker et al., 2007). The most prominent Siglecs on human monocyte/macrophages, neutrophils, and dendritic cells are Siglec-5 (hSiglec-5) and Siglec-9 (hSiglec-9), each of which contains two intracellular tyrosine-based signaling motifs (Crocker et al., 2007). The membrane-proximal motif (LHYASL) conforms to the canonical immunoreceptor tyrosine-based inhibitory motif (I/L/V)xYxx(L/V),

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whereas the distal motif (TEYSEI) resembles the immunoreceptor tyrosine-based switch motif (TXYXX[V/I]) found in the CD150/SLAM subfamily (Ravetch and Lanier, 2000). The immunoreceptor tyrosine-based inhibitory motif is present in various inhibitory receptors that antagonize kinase-dependent activation cascades (Taylor et al., 2000) and function by recruiting Src homology 2 domain-containing tyrosine phosphatases SHP-1 (Zhang et al., 2000) and SHP-2 (Gergely et al., 1999). Both hSiglec-5 and hSiglec-9 can recruit SHP-1 and SHP-2 and function as inhibitory receptors of leukocytes (Avril et al., 2006a).

We recently demonstrated that GBS of different serotypes bind hCD33rSigelec (Carlin et al., 2007) and that GBS serotype III engages Siglec-9 on the surface of cells, including human neutrophils, in a Sia- and Siglec-specific manner to down-regulate their immune responsiveness (Carlin et al., 2009). In this paper, we report the unexpected discovery that certain GBS strains bind hSiglec-5 in a Sia-independent manner, via a specific protein anchored to the bacterial cell wall. Using a combination of bacterial and immunological reagents, we characterize the protein-mediated interaction of GBS with Siglec-5 on the surface of human leukocytes and present functional evidence that it represents a novel bacterial immune evasion mechanism.

RESULTS AND DISCUSSION

GBS interacts with hSiglec-5-Fc through a trypsin-sensitive bacterial protein

We tested WT GBS isolates expressing sialylated CPS of serotypes Ia (strain A909) and III (strain COH1), isogenic Sia-negative mutants of each strain generated by allelic replacement of the CMP-Neu5Ac-synthase gene (Δ NeuA), and GBS pretreated with trypsin to remove cell surface proteins, for binding to chimeras of hSiglec5 (hSiglec-5-Fc) and hSiglec-9 (hSiglec-9-Fc) by flow cytometry. Corroborating our previous ELISA-based findings (Carlin et al., 2007), serotype III GBS bound to hSiglec-9-Fc and serotype Ia bound to hSiglec-5 (Fig. 1, A and B). The GBS serotype III strain bound to hSiglec-9-Fc in a Sia-specific manner because this interaction was lost for the isogenic Sia-negative Δ NeuA mutant but unaltered by trypsin treatment (Fig. 1 A). In contrast, attachment of the GBS serotype Ia strain to hSiglec-5-Fc was maintained in the Sia-deficient mutant and eliminated by trypsin treatment (Fig. 1 B), linking binding to a trypsin-sensitive protein rather than the anticipated sugar-lectin interaction.

The GBS β protein interacts with hSiglec-5 in a Sia-independent manner

To identify the proteins responsible for mediating attachment to hSiglec-5, we prepared whole cell lysates of the GBS serotype Ia strain that bound hSiglec-5-Fc and, as a control, the nonbinding GBS serotype III strain. To avoid hSiglec-Fc Sia-specific interactions, isogenic Δ NeuA mutants that were virtually devoid of detectable cell surface Sia (Fig. S1 A) were used. Proteins were separated under reducing conditions by SDS-PAGE, transferred to a PVDF filter,

and probed with hSiglec-Fc chimeras and secondary anti-human HRP-conjugated antibodies. The hSiglec-5-Fc chimera interacted with a GBS protein of an apparent molecular mass of 125 kD found in lysates of the GBS serotype Ia strain but not in the serotype III control (Fig. 1 C). The hSiglec-9-Fc chimera did not interact with this protein or any others, excluding nonspecific interactions with the human IgG-Fc portion of the chimeras. Based on size, surface location, sensitivity to trypsin, presence in the GBS serotype Ia strain, and absence in the GBS serotype III strain, we hypothesized that the protein mediating GBS interaction with hSiglec-5-Fc was the surface-expressed β protein (also known as Bac or β C; Lindahl et al., 2005). To produce a partially purified GBS β protein extract, we incubated the GBS serotype Ia and serotype III (negative control) Δ NeuA mutants at pH 9.7 for 4 h at 37°C, selectively releasing β protein (Lindahl et al., 1990), concentrated and separated supernatants by SDS-PAGE, and then blotted and probed with hSiglec-5-Fc. This sample was highly enriched for the 125-kD protein interacting with hSiglec-5-Fc (Fig. 1 D). Treatment of the protein extract with active or heat-inactivated (control) *Arthrobacter ureafaciens* sialidase (AUS) did not change hSiglec-5-Fc binding (Fig. 1 E). To determine the identity of the 125-kD GBS protein, the band reacting with hSiglec-5-Fc was excised, digested, and analyzed using MALDI-TOF MS peptide fingerprinting (Fig. S1 B). The monoisotopic masses of the peptide fragments listed were analyzed using the online database at Rockefeller University (<http://prowl.rockefeller.edu>) and the GBS β protein was identified with 100% certainty. Confirming the significance of the observed interaction, an isogenic β protein-deficient mutant (Δ Bac) of our parent GBS Ia strain lost the ability to bind hSiglec-5-Fc, but when it was complemented with the *bac* gene expressed on a plasmid vector (pBac), WT levels of hSiglec-5-Fc binding were restored (Fig. 1 F).

The β protein is required for GBS interactions with hSiglec-5 via its N-terminal domain

The GBS β protein N-terminal (cell wall distal) domain is known to bind human IgA-Fc, whereas its C-terminal domain can interact with human factor H (Areschoug et al., 2002). To map the domain for β protein-hSiglec-5 interaction, we preincubated GBS with or without polyclonal antibodies against full-length β protein (Beta Ab), its N-terminal domain (B6 Ab), or its C-terminal domain (75 kD antibody; Fig. 2 A). The Beta Ab and B6 Ab significantly blocked GBS binding to hSiglec-5-Fc, causing >75% ($P < 0.001$) and >95% ($P < 0.001$) inhibition, respectively (Fig. 2 B). In contrast, the 75-kD Ab did not interfere with GBS hSiglec-5-Fc binding (Fig. 2 B). Immunoblot confirmed that the N-terminal B6 domain, but not the 75-kD C-terminal fragment, bound hSiglec-5-Fc (Fig. 2 C). Note that recombinant B6 protein is size heterogeneous (Heden et al., 1991). Furthermore, GBS β protein bound hSiglec-5 and baboon Siglec-5, but not chimpanzee Siglec 5 (Fig. S2), mapping β protein binding to the hSiglec-5 V-set (lectin) domain because this domain contains

all amino acid residues in chimpanzee Siglec-5 that differ from the hSiglec5 sequence but are not shared by baboon Siglec-5.

GBS binding to cell surface-expressed hSiglec-5 is β protein dependent

To determine if GBS β protein could bind hSiglec-5 on a eukaryotic cell surface, we stably transfected CHO-K1 cells with an hSiglec-5 expression plasmid and applied FITC-labeled GBS to the monolayers. Nonadherent bacteria were washed away and fluorescent images of adherent GBS captured. WT GBS expressing β protein adhered to CHO cells expressing hSiglec-5 (Fig. 2 D) but not to nontransfected cells (not depicted). In contrast, the Δ Bac mutant did not adhere to CHO cells expressing hSiglec5, and binding was restored upon mutant

complementation with the pBac plasmid (Fig. 2 D). GBS attachment to transfected CHO cells was dependent on hSiglec-5, as anti-Siglec-5 antibody significantly blocked the binding (Fig. 2 D). Adherence was quantified by lifting the monolayers and analyzing single cells for adherent FITC-GBS by flow cytometry (Fig. 2 E). Adherent WT GBS or pBac-complemented Δ Bac mutant were present on the majority of cells, usually with more than one attached FITC-labeled bacterium per cell (greater shifts in fluorescence intensity). In contrast, very few Δ Bac mutant bacteria adhered to the CHO(hSiglec5) cells. Anti-Siglec-5 antibody reduced WT GBS binding to the level observed with the Δ Bac mutant (Fig. 2 E). We conclude that binding of GBS to the cell surface is a direct result of β protein-mediated binding to hSiglec-5.

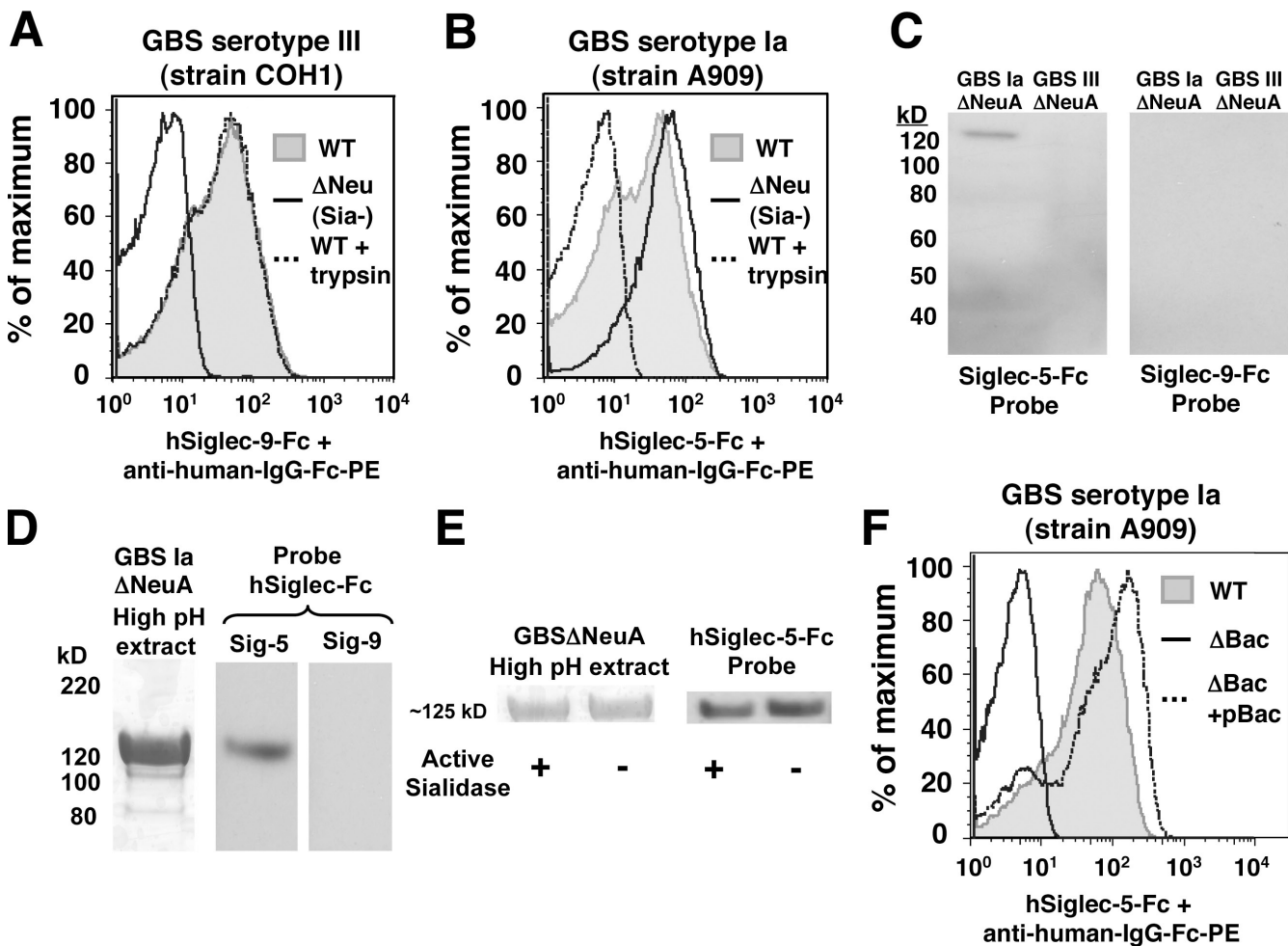


Figure 1. The GBS β protein mediates attachment to hSiglec-5-Fc in a Sia-independent manner. (A and B) WT GBS serotype III (A) and Ia (B) and their isogenic Sia-deficient Δ NeuA mutants were incubated with hSiglec-9-Fc and hSiglec-5-Fc, respectively, followed by PE-conjugated secondary antibodies, and analyzed by flow cytometry for Sia-dependent binding of hSigslecs. Similar analysis was performed with WT GBS serotype III (A) and Ia (B) pretreated with or without trypsin to determine protein-dependent binding of hSigslecs. (C) Western blot analysis of GBS whole cell lysates from GBS serotype Ia and III Δ NeuA mutants with hSiglec-Fc chimeras as probes. The analysis identified an ~125-kD specific hSiglec-5 binding protein in the Ia strain. (D) Analysis of GBS extracts prepared under basic conditions (pH 9.7) also identified an ~125-kD protein that binds hSiglec5. (E) AUS treatment (50 mU/ml for 2 h) does not affect binding of the 125-kD protein to hSiglec5. (F) WT GBS serotype Ia and isogenic mutant Δ Bac (lacking the β protein), or the plasmid complemented mutant Δ Bac + pBac, were incubated with hSiglec-5-Fc and PE-conjugated secondary antibody and analyzed by flow cytometry. All experiments were performed in duplicate and repeated three (A and B) or four (C-F) times with similar results. Representative experiments are shown.

GBSs expressing β protein colocalize with hSiglec-5 on the surface of human monocytes

GBS–U937 monocyte interactions were visualized using fluorescent deconvolution microscopy with a hSiglec-5–specific antibody, a fluorescently tagged (red) secondary antibody to label hSiglec-5, and Hoechst DNA stain (blue) to visualize cell nuclei. WT GBS expressing β protein colocalized with clear aggregations of hSiglec-5 present on the U937 cell surface (Fig. 3 A). Increased quantities of hSiglec-5 at the interface of the Δ Bac mutant and the U937 cell surface were not observed; consequently, the Manders coefficient for colocalization of bacteria and hSiglec-5 was significantly less in the Δ Bac mutant compared with the WT GBS strain (Fig. 3 A).

β protein binding to Siglec-5 promotes GBS leukocyte attachment but impairs phagocytosis

In a binding experiment at 4°C to prevent phagocytosis, the WT GBS strain bound to a greater degree than did the isogenic

Δ Bac mutant to U937 monocytes (Fig. 3 B, $P = 0.01$) and human neutrophils (Fig. 3 C, $P < 0.001$). To correlate binding to phagocytic uptake, we incubated bacteria with U937 monocytes for 30 min at 37°C, killed extracellular bacteria using antibiotics, and lysed monocytes to recover internalized bacteria for enumeration. U937 cells phagocytosed more Δ Bac mutant bacteria than either the WT GBS parent strain or the complemented mutant (Fig. 3 D, $P < 0.001$). Antibody cross-linking of Siglec-5 causes rapid endocytosis of the receptor so that only ~5–10% remains on the cell surface after 45 min (unpublished data). Upon depletion of hSiglec-5 from the U937 cell surface using an anti-hSiglec-5 antibody, phagocytosis of WT GBS approached the levels seen with the isogenic Δ Bac mutant (Fig. 3 D). Thus, although GBSs expressing β protein bind to human leukocytes more avidly, they are phagocytosed less efficiently, and this reduction depends on cell surface expression of hSiglec-5. Additional differential fluorescence studies to quantify phagocytosed versus extracellular

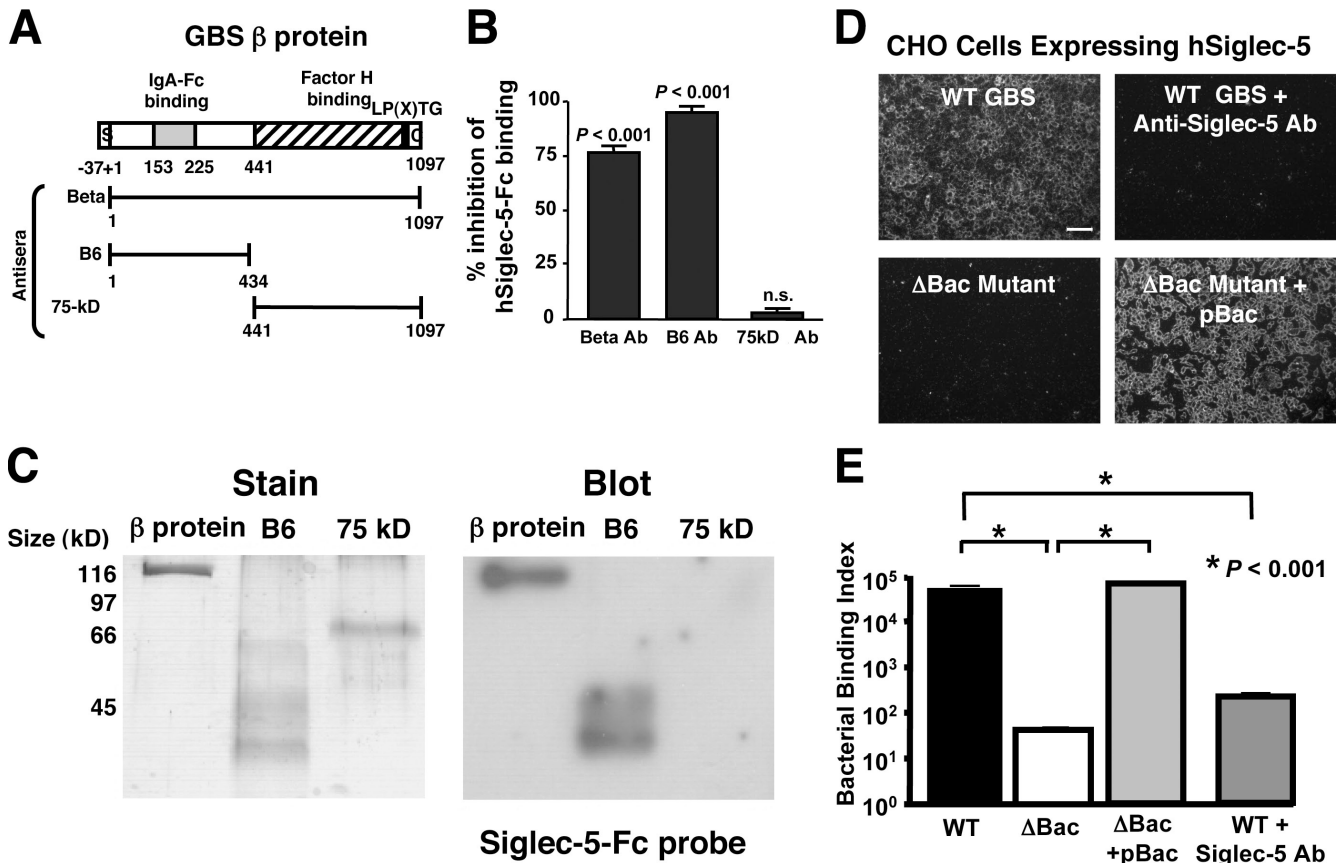


Figure 2. The N-terminal domain of the β protein mediates hSiglec-5–Fc interactions and can promote GBS binding to CHO cells expressing hSiglec-5. (A) Schematic of the GBS β protein, including the peptide fragments previously used to generate rabbit polyclonal antisera Beta, B6, and 75 kD. (B) WT GBS were preincubated with rabbit polyclonal antisera targeting various domains of the β protein before addition of hSiglec-5-Fc, and analysis of hSiglec-5-Fc binding by flow cytometry. (C) Western blot analysis shows direct binding of Siglec-5 to the B6 region of the β protein. (D) FITC-labeled GBS were allowed to adhere to CHO cells transfected with hSiglec-5 for 20 min and nonadherent GBS were removed by repeated washing. In blocking experiments, CHO cells were preincubated with anti-Siglec-5 antibody for 10 min. Bar, 100 μ m. (E) To quantify GBS attachment to individual cells, CHO cell monolayers were lifted with PBS + 5 mM EDTA and analyzed by flow cytometry. All experiments repeated three (B, D, and E) or two (C) times with similar results. Images show pooled data (B and E) or a representative experiment (C and D). Error bars represent standard deviation. Statistical analysis was performed using one-way ANOVA with Tukey’s post-test.

adherent bacteria corroborate these observations (Fig. S3, A and B). A similar inhibitory effect of GBS β protein expression on U937 cell phagocytosis was observed in serum-free assays, excluding any theoretical confounding effect of the β protein binding to IgA (Fig. S3 C).

β protein expression can increase SHP recruitment to hSiglec-5

CD33rSiglecs mediate inhibitory signaling in immune cells via recruitment of SHP protein tyrosine phosphatases. We incubated the WT GBS strain and Δ Bac mutant with CHO cells expressing hSiglec-5, performed immunoprecipitation for hSiglec-5, and then probed for coimmunoprecipitation of tyrosine phosphatase SHP-2. SHP-2 was not recruited to hSiglec-5 in the absence of bacteria (Fig. 4 A). However, when

CHO(hSiglec-5) cells were exposed to WT GBS, SHP-2 was recruited to hSiglec-5 beginning at \sim 5 min and reached a maximum at 15–20 min (Fig. 4 A). In contrast, no recruitment of SHP-2 to hSiglec-5 on CHO(hSiglec-5) was detected after exposure to the GBS Δ Bac mutant. Similarly, U937 monocytes, which had no detectable SHP-2 associated with hSiglec-5 at baseline, showed recruitment of SHP-2 in response to WT GBS but not the Δ Bac mutant (Fig. 4 B). Recruitment of SHP-1 to Siglec 5 in U937 monocytes did not differ appreciably after exposure to the WT and Δ Bac strain (Fig. 4 B); however, when β protein was expressed heterologously in a Sia-deficient acapsular strain of *Lactococcus lactis*, a contribution of the protein to recruitment of both SHP-2 and SHP-1 to hSiglec-5 could be detected (Fig. 4 B). The observed GBS β protein-dependent SHP-2 recruitment to Siglec-5 resembles Sia-dependent

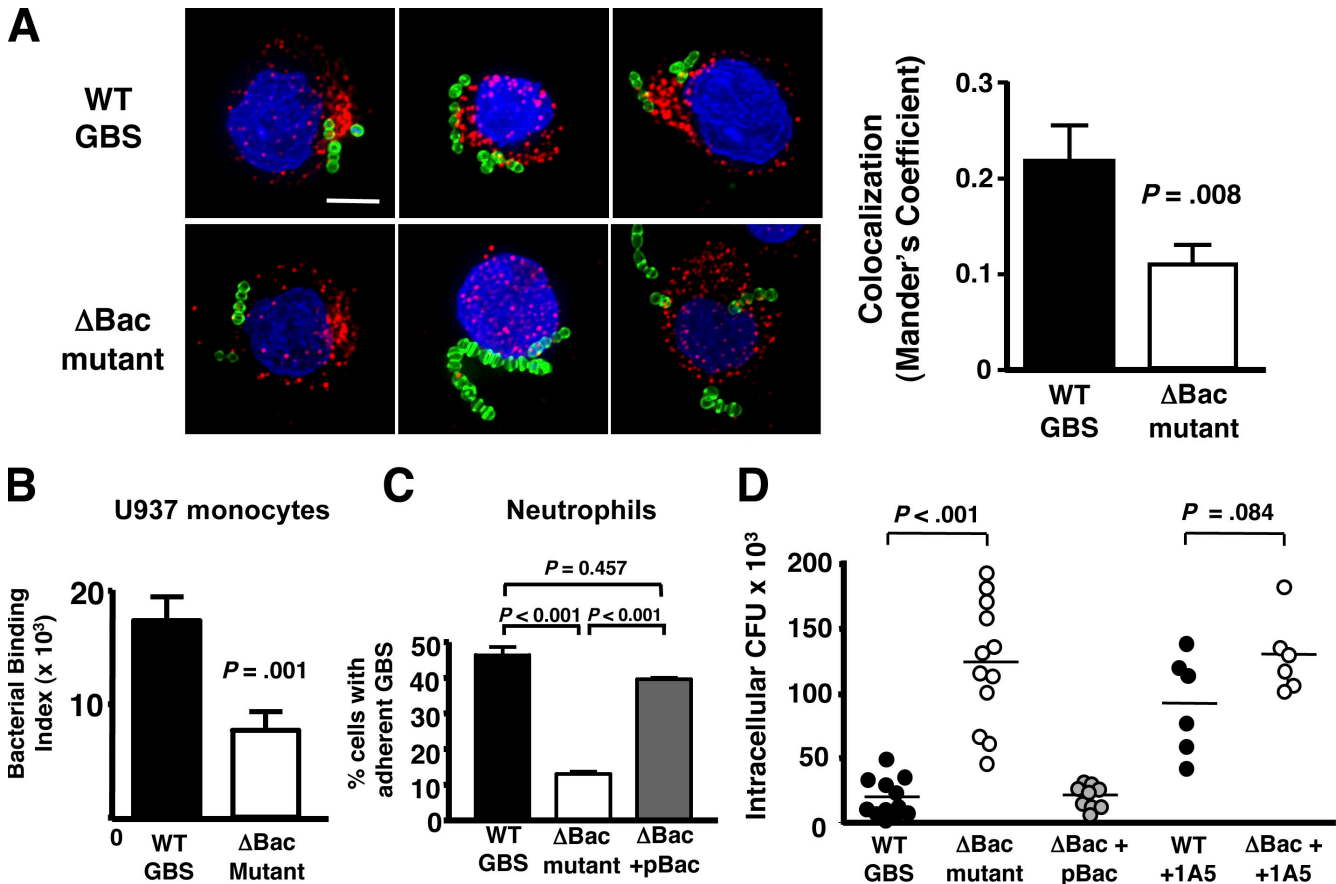


Figure 3. GBS expressing β protein colocalize with hSiglec-5 on U937 leukocytes and neutrophils, increasing bacterial attachment but impairing their phagocytosis (A) Colocalization of FITC-WT GBS (green) with hSiglec 5 (red) on the cell surface of U937 cells (blue nuclei) not observed in parallel studies with the isogenic FITC-labeled β protein-deficient mutant, as visualized by deconvolution microscopy. Bar, 5 μ m. (B) FITC-labeled GBS were allowed to interact with U937 for 60 min at 4°C (to prevent phagocytosis). Adherence was measured by flow cytometry. (C) Primary human neutrophils were incubated with live GBS-FITC on ice to allow cell-cell interactions but block phagocytosis. The percentage of neutrophils with adherent bacteria was measured using flow cytometry. (D) To calculate phagocytic uptake of GBS, bacteria were added to U937 at MOI 10:1 for 30 min, washed, antibiotics added for 2 h to kill extracellular bacteria, and then cells lysed and dilutions plated to enumerate intracellular CFU. Where indicated, U937 cells were preincubated with anti-Siglec-5 Ab (1-A5) for 50 min, which results in endocytosis of the receptor, leaving \sim 10% of original levels of Siglec-5 on the surface before addition of GBS. Horizontal bars represent the mean. Each experiment was performed in triplicate and repeated three times with similar results. Representative images (A) or pooled data (Manders coefficient; B–D) are shown. Error bars represent standard deviation. Statistical analysis was performed using one-way ANOVA with Tukey's post-test.

recruitment of SHP-2 to hSiglec9 in U937 cells exposed to serotype III GBS (Fig. S4).

β protein interferes with neutrophil activation and killing of GBS

Recently, we reported that GBS serotype III mimicry of host Sias allowed engagement of hSiglec-9, leading to reduced neutrophil phagocytosis, diminished oxidative burst, and impaired formation of neutrophil extracellular traps (NETs; Brinkmann et al., 2004), together promoting pathogen survival (Carlin et al., 2007). In this study, we performed additional neutrophil studies to ascertain how the newly discovered GBS β protein-mediated engagement of hSiglec-5 would affect these phagocyte functions. Compared with the isogenic Δ Bac mutant, the WT GBS strain elicited a weaker oxidative burst (Fig. 5 A), triggered the release of fewer NETs (Fig. 5 B), and stimulated less transcription of IL-8 (Fig. 5 C) upon co-incubation with human neutrophils. Moreover, the WT GBS strain showed increased survival versus the Δ Bac mutant in a neutrophil killing assay (Fig. 5 D). Inhibition of β protein

engagement of hSiglec-5 with an anti-hSiglec-5 monoclonal antibody increased neutrophil killing of GBS (Fig. 5 E). These findings indicated that β protein-expressing GBS strains can use this protein to suppress the innate immune function of phagocytic cells.

Protein-mediated Siglec engagement and GBS immune evasion

The β protein is the first example of a protein other than an antibody that directly engages the extracellular domains of an hCD33rSiglec in a Sia-independent manner. Previously, GBS and two other human pathogens, *Neisseria meningitidis* and *Campylobacter jejuni*, were shown to interact with hCD33r-Siglecs through cell surface-expressed Sia (Jones et al., 2003; Avril et al., 2006b; Carlin et al., 2007) and, in the case of GBS, these Sia-dependent interactions served to down-regulate neutrophil activation (Carlin et al., 2007). GBS association with hSiglec-5 in a protein-dependent manner reveals a novel mechanism by which pathogens may co-opt hCD33rSiglecs to their advantage. β protein-mediated engagement causes an accumulation of Siglec-5 at the sight of GBS contact and increases the recruitment of SHP protein tyrosine phosphatases to the receptor's cytosolic tail. Association of SHP family phosphatases with surface receptors can negatively regulate receptor-mediated phagocytosis (Oldenburg et al., 2001; Kant et al., 2002). Likewise, the antiphagocytic effect described in this paper is mediated by β protein-expressing GBS only when Siglec-5 is present on the leukocyte surface and, thus, likely depends on SHP recruitment to the surface receptor.

The GBS β protein is a large antigenic surface-anchored protein expressed in almost all GBS serotype Ib strains and some serotype Ia, II, and V strains but almost never in serotype III strains (Lindahl et al., 2005). Recently, it was shown that high levels of β protein expression are associated with increased virulence of GBS clinical isolates (Nagano et al., 2002). β protein is known to interact with two components of the human immune system, the Fc portion of serum IgA and factor H (Areschoug et al., 2002), and our present finding that β protein binds to hSiglec-5 identifies a third such interaction. Through nonimmune binding of IgA (Pleass et al., 2001), engagement of factor H (a major regulator of complement; Pangburn, 2000), and, now, attachment to hSiglec5, which is proposed to play an important role in regulating phagocyte function and the link to cellular immunity (Crocker et al., 2007), β protein appears to be a multifunctional virulence factor capable of interfering with several aspects of host defense to bacterial infection.

We have shown that GBS is able to engage different CD33rSiglecs using its sialylated CPS or the β protein. The Sia-binding sites of hCD33rSiglecs are rapidly evolving, a process that is hypothesized to be likely to facilitate continual host Sia self-recognition in the face of rapid changes in host Sia expression (Varki and Angata, 2006). In this scenario, rapid evolution of the host sialome, driven by Sia-binding pathogens such as malaria and influenza, requires that the CD33r-Siglecs also evolve rapidly to keep up with recognizing self.

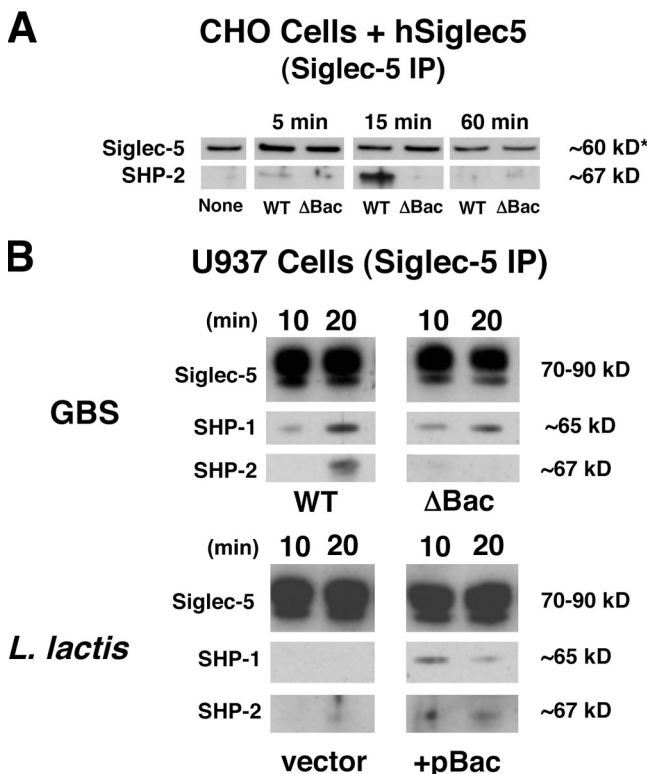


Figure 4. GBS β protein effects on recruitment of SHP family protein tyrosine phosphatases to hSiglec-5. (A) SHP-2 recruitment to hSiglec-5 by coimmunoprecipitation studies in CHO cells transfected with hSiglec-5 and exposed to WT and β protein-deficient GBS. (B) SHP-1 and SHP-2 recruitment to hSiglec-5 by coimmunoprecipitation studies in human U937 monocytes exposed to WT and β protein-deficient GBS or *L. lactis* expressing the GBS β protein or empty vector control. *N-glycans were removed using PNGase F before separation by SDS-PAGE in CHO cell experiments. All experiments performed three times with similar results. A representative experiment is shown.

If GBS CPS Sias have evolved to engage inhibitory CD33r-Siglecs, then these Sias would need to coevolve with the rapidly changing Sia-binding specificities of CD33rSiglecs to maintain functional interactions. The parallel evolution by GBS of a protein ligand that allows functional engagement of hSiglec-5 may have facilitated continued success of CPS serotypes (e.g., type Ia) that had lost their original binding affinity to host Siglec-5.

In sum, we demonstrate the presence of a microbial protein ligand that engages a member of the hCD33rSiglecs and find that this interaction increases bacterial resistance to phagocytosis and killing by human leukocytes. Recent studies suggest that Sia-expressing pathogenic microorganisms may use cell surface Sias to bind hCD33rSiglecs to dampen innate immune responses. Our present findings indicate that this pathogenic mechanism may not be limited to Sia-expressing

microorganisms and, thus, may represent a more widespread strategy of microbial immune evasion.

MATERIALS AND METHODS

Bacteria, growth conditions, and labeling. GBS WT strains of serotypes Ia (A909) and III (COH1) are well-characterized isolates from human neonates with invasive infections. Generation of the Δ NeuA and Δ Bac mutants and complemented mutants of A909 and COH1 have been previously reported (Areschoug et al., 2002; Lewis et al., 2004, 2007). GBS were grown and prepared for assays including sialidase and trypsin treatment as previously described (Carlin et al., 2007). Live GBS were incubated in PBS + 0.1% FITC (Sigma-Aldrich) at 37°C for 20 min; alternatively, heat-killed GBS were labeled as previously described (Carlin et al., 2007).

Siglec-Fc binding assays. hSiglec-Fc chimeras were produced as previously described (Angata and Varki, 2000). GBSs were resuspended in RPMI + 10% FCS at 4°C with 10 μ g/ml hSiglec-Fc, fluorescent secondary Ab, and analyzed by flow cytometry. In blocking experiments, GBS were preincubated

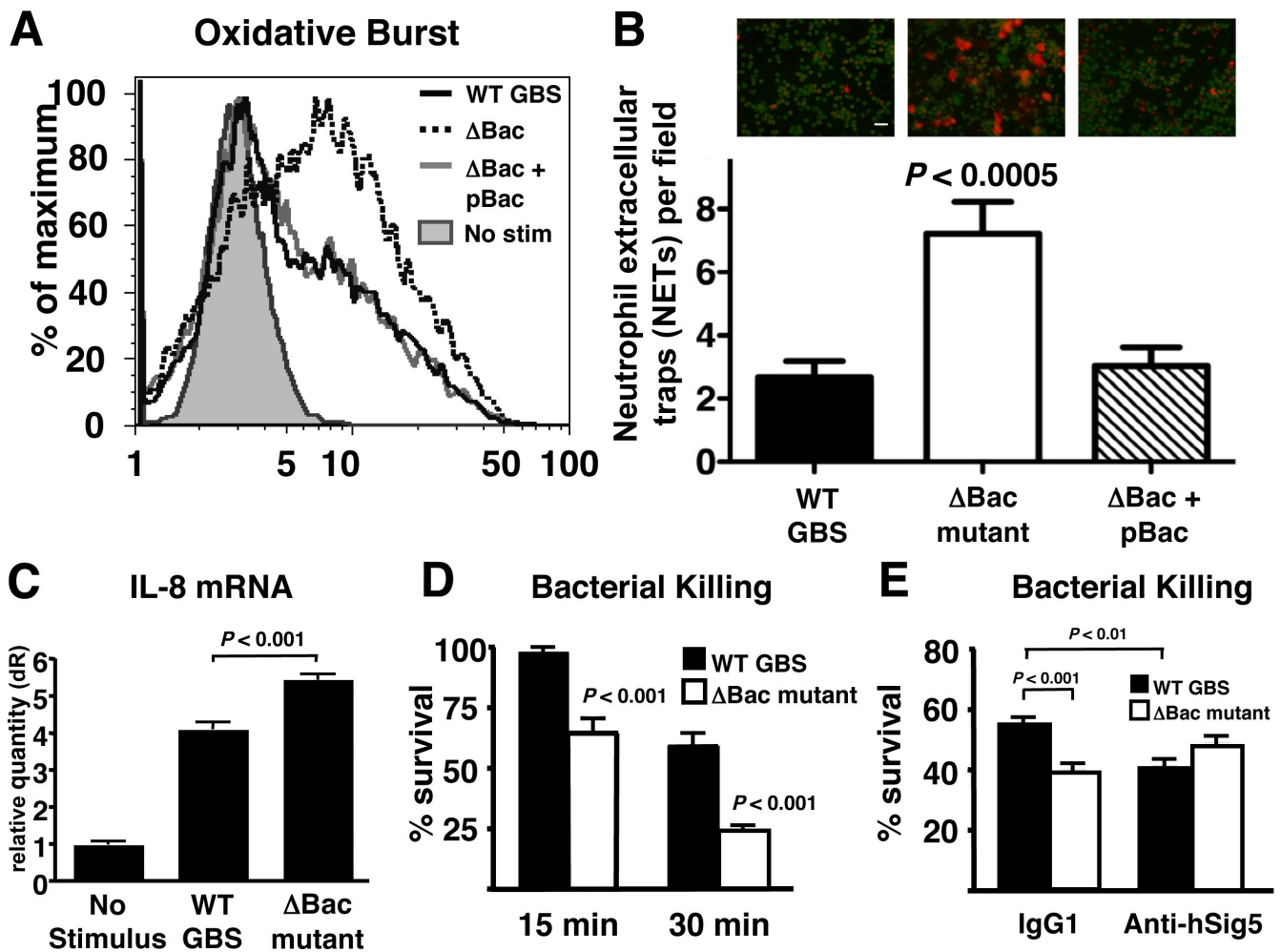


Figure 5. GBS β protein effects on human neutrophil activation and killing. (A) Oxidative burst activity of neutrophils exposed to GBS WT, Δ Bac mutant, and complemented mutant strains. (B) Production of NETs in response to GBS WT, Δ Bac mutant, and complemented mutant strains. Bar, 50 μ m. (C) Comparison of WT and Δ Bac mutant GBS strains in induction of IL-8 transcript from human neutrophils. (D and E) Survival of GBS WT and Δ Bac mutant strains upon coinubation with human neutrophils (D), with or without coinubation with anti-Siglec-5 Ab or control Ab (E). All experiments performed in triplicate and repeated three (A–C and E) or two (D) times with similar results. A representative experiment (A, C, and D) or pooled data (B and E) are shown. The error bars represent mean value \pm SD.

with polyclonal rabbit anti- β protein antibodies (Beta antibody, B6, or 75 kD; Heden et al., 1991; Areschoug et al., 2002) for 20 min at 4°C.

GBS protein isolation, purification, and Western blots. GBS were washed and disrupted in lysis buffer with multiple freeze-thaw cycles, and extracts were centrifuged at 4°C. Supernatants were concentrated with Amicon Ultra-10 (Millipore), separated by SDS-PAGE under reducing conditions, and either stained with GelCode blue (Thermo Fisher Scientific) or transferred to PVDF, blocked, probed with 5 μ g/ml Siglec-Fc chimeras and HRP-conjugated secondary Ab, and then detected with ECL reagent (Thermo Fisher Scientific). Partial purification of the β protein was performed with slight modifications as previously described (Stalhammar-Carlemalm et al., 1993). GBS were incubated in 50 mM Tris, pH 9.7, at 37°C and supernatants concentrated with Microcon 30 (Millipore), separated, and probed with Siglec-Fc chimeras.

Mammalian protein isolation and Western blots. Cells were infected with GBS, washed, and lysed with 4°C TBS lysis buffer. Protein concentrations were normalized and immunoprecipitation was performed with 1A5 anti-human Siglec-5 antibody (gift from P. Crocker, University of Dundee, Dundee, Scotland, UK) and protein G Sepharose beads (4 fast flow; BD). Proteins were separated by reducing SDS-PAGE, transferred to PVDF, and probed with anti-Siglec-5 antibody BAF1072 (R&D systems), rabbit anti-SHP-1 (Santa Cruz Biotechnology, Inc.), or rabbit anti-SHP-2 (Millipore) and appropriate HRP-conjugated secondary Abs (Bio-Rad Laboratories) and ECL reagent (Thermo Fisher Scientific). U937 cells were resuspended in RPMI + 0.5% FCS overnight before assays. For assays using CHO cells, N-glycans were removed using PNGase F before separation by SDS-PAGE.

GBS adherence and phagocytosis assays. CHO cell adherence assays were performed as described previously (Carlin et al., 2007). FITC-GBS were added to human leukocytes at 4°C in RPMI + 10% FCS, centrifuged to initiate contact, and rotated at 4°C for 30 min before analysis of adherence by flow cytometry. Phagocytosis assays were performed with or without preincubation in heat-inactivated human serum (HIHS). GBS MOI 10:1 were added to U937 cells and incubated at 37°C for 30 min before adding 5 μ g/ml of penicillin and 100 μ g/ml of gentamicin for 2 h to kill extracellular bacteria. Cells were washed and lysed and intracellular bacteria were enumerated using serial dilution plating. In specific assays, U937 cells were depleted of cell surface Siglec-5 by preincubation with 2 μ l/ml of anti-Siglec-5 1A5 Ab for 50 min at 37°C in RPMI before adding GBS.

GBS U937 cell deconvolution microscopy. Live FITC-GBS were added to U937 cells in HBSS MOI 5:1, incubated at 37°C for 10 min, washed, and resuspended in ice cold PBS + 1% BSA. Siglec-5 was labeled with 1A5 Ab and Alexa Fluor 647-conjugated secondary Ab (Invitrogen). Cells were fixed in 2% paraformaldehyde, spun onto poly-lysine-coated coverslips, and mounted in Prolong Gold with DAPI (Invitrogen). At least five images of GBS interactions were captured from three independent experiments as previously described (Carlin et al., 2007). Image Pro Plus software was used to determine Manders coefficients for green (GBS) colocalization with red (hSiglec-5) for every bacterial-cell interaction captured. The mean \pm SEM of WT and Δ Bac GBS were graphed and P-value was determined by a Student's *t* test.

Neutrophil functional assays. Human neutrophils were prepared as previously described (Carlin et al., 2007) using Polymorphprep solution (Axis-Shield PoC AS) according to the manufacturer's instructions. Experiments were performed as previously described (Carlin et al. 2009) with minor modifications. IL-8 transcript was assessed by quantitative real-time RT-PCR. GBS were incubated in HBSS + 10% HIHS for 20 min at 37°C and added to neutrophils at MOI of 10:1 in HBSS + 2% HIHS final concentration and incubated for 10 min (oxidative burst) or 30 min (NETs) before analysis. GBS were added and the bacteria and neutrophils were spun together at 500 *g* for 5 min to initiate the assay. For neutrophil killing assays performed in the presence of antibodies, 5 μ g/ml of anti-Siglec-5 Ab (clone 194128; R&D Systems) or

IgG1 control (Sigma-Aldrich) Ab was added to purified neutrophils 2 min before the assay. Protocols were approved by the University of California, San Diego Human Research Protection Program.

Online supplemental material. Fig. S1 shows analysis of GBS surface Sia expression and mass spectrometric identification of GBS β protein. Fig. S2 shows that GBS-expressing β protein binds to human and baboon Siglec-5 but not chimpanzee Siglec-5. Fig. S3 corroborates that GBS β protein impairs human monocyte phagocytosis in an hSiglec-5-dependent manner and under serum-free conditions. Fig. S4 shows that GBS serotype III Sia promotes recruitment of SHP-2 protein tyrosine phosphatases to hSiglec-9. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090691/DC1>.

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