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**Siglec Interactions with a Sialylated Bacterial Pathogen**

A dissertation submitted in partial satisfaction of the  
requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

**Aaron Foster Carlin**

Committee in charge:

Professor Victor Nizet, Chair  
Professor Ajit Varki, Co-Chair  
Professor Joshua Fierer  
Professor Christopher Glass  
Professor Stephen Hedrick

2007



**The dissertation of Aaron Foster Carlin is approved, and it is acceptable in quality and form for publication on microfilm:**

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**Co-Chair**

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**Chair**

**University of California, San Diego**

**2007**

**DEDICATION**

*To my family*

## EPIGRAPH

**“An individual has not started living until he can rise above the narrow confines of his individualistic concerns to the broader concerns of all humanity.”**

**Martin Luther King Jr.**

**“I want to stand as close to the edge as I can without going over. Out on the edge you see all the kinds of things you can’t see from the center.”**

**Kurt Vonnegut**

**“Men acquire a particular quality by constantly acting a particular way. . . you become just by performing just actions, temperate by performing temperate actions, brave by performing brave actions.”**

**Aristotle**

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## LIST OF COMMON ABBREVIATIONS

SIA, sialic acid

GBS, Group B *Streptococcus*

Neu5Ac, N- *acetyl* neuraminic acid

THB, Todd Hewitt Broth

Siglec, sialic acid recognizing immunoglobulin-like lectin

CPS, capsular polysaccharide

hCD33rSiglec, human CD33-related Siglec

Cm, Chloramphenicol

Erm, Erythromycin

MOI, multiplicity of infection

CHO, Chinese hamster ovary cell

CFU, colony forming units

U937, human monocytic cell line

SHP-1, Src homology domain 2-containing tyrosine phosphatase-1

SHP-2, Src homology domain 2-containing tyrosine phosphatase-2

ITIM, immunoreceptor tyrosine-based inhibitory motif

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## ABSTRACT OF THE DISSERTATION

# **Siglec Interactions with a Sialylated Bacterial Pathogen**

by

**Aaron Foster Carlin**

Doctor of Philosophy in Biomedical Sciences  
University of California, San Diego, 2007

Professor Victor Nizet, Chair  
Professor Ajit Varki, Co-chair

Group B *Streptococcus* (*Streptococcus agalactiae* or GBS) is the leading cause of bacterial sepsis and meningitis in human newborns. The GBS capsular polysaccharide (CPS) is a major virulence factor that contains terminally linked sialic acids (Sias) that are important for escaping detection by the innate immune system. The structure, position, and linkages of the Sia-capped oligosaccharide subunits that make up the CPS of GBS are identical to some of the most common Sia-capped glycans that coat the cell surface of all human cells.

The Sia-recognizing immunoglobulin (Ig) superfamily lectins (Siglecs) are a family of type I transmembrane surface proteins many of which are expressed on the surfaces of human leukocytes. The human CD33/Siglec-3 Related Siglecs (hCD33rSiglecs) are a rapidly evolving subgroup of the Siglecs that are composed of (a) an extracellular V-set domain that engages Sia-capped glycans, and (b) an intracellular domain that contains inhibitory motifs that are classically involved in reducing leukocyte activation. The CD33rSiglecs are postulated, but not proven, to

interact with “self” Sias present on the surface of human cells, thereby reducing leukocyte-mediated autoimmune responses.

We hypothesized that sialylated microorganisms producing mimics of host cell glycans would engage CD33rSiglecs, thus causing inhibition of leukocyte activation. Here we demonstrate that the sialylated pathogen, GBS, functionally engages human CD33rSiglecs by two distinct mechanisms. The first mechanism by which GBS binds CD33rSiglecs is dependent on CPS sialylation as we had hypothesized. The second mechanism involves a novel interaction between a GBS surface protein and human Siglec-5 that is Sia-independent. This latter finding opens up the possibility that the capacity of microorganisms to target CD33rSiglecs may not be restricted to sialylated pathogens, but instead may be a more universal mechanism of immune evasion.

Using a novel experimental system incorporating immobilized, synthetic sialylated glycans, we confirm that CD33rSiglec engagement of Sias *in trans* can cause inhibition of neutrophil activation. These findings further support the hypothesis that the natural function of CD33rSiglecs is to recognize endogenous glycans. but that certain microorganisms have evolved to target these cell surface receptors in order to subvert the immune system.

## **Chapter I**

# **Subversion of Vertebrate Sialic Acid Binding Lectins by Microorganisms**

## INTRODUCTION AND SUMMARY

One of the few seemingly universal biological findings is that all cells of every species studied to date are covered with a complex array of glycans (oligosaccharides or polysaccharides) (Rambourg and Leblond, 1967; Varki, 2007). These individual oligo/polysaccharides are often covalently attached to proteins or lipids on the cell surface and can be extremely diverse due to different monosaccharide compositions, modifications, linkage and branching patterns (Taylor and Drickamer, 2006; Varki, 1999). Glycan expression is often developmentally regulated and can change rapidly in response to variations in the extracellular environment or due to intracellular events. Despite the abundant possibilities for glycan diversity, most individual organisms, tissues, and cells express a particular array of glycans under defined conditions. This distinct pattern of glycan expression is frequently conserved within an individual species suggesting strict regulatory control (Lowe and Marth, 2003).

Cell-surface glycans have been a major constituent of the outermost perimeter of all cells for billions of years; therefore it is not surprising that glycans and glycan-binding receptors (lectins) have a considerable impact on interactions between cells. Glycans and their receptors have been shown to be particularly important at the interface between microbial pathogens (broadly defined here as viruses, bacteria, parasites, or fungi that infect multicellular organisms) and their hosts (Hooper and Gordon, 2001; Olofsson and Bergstrom, 2005; Rostand and Esko, 1997). Exposed surface glycans that are expressed in a taxa-specific, species-specific, or cell-specific

fashion make convenient ligands for lectin receptors expressed by immune cells and microorganisms (Martin et al., 2005) (Wolf et al., 1998); (Suzuki et al., 2000).

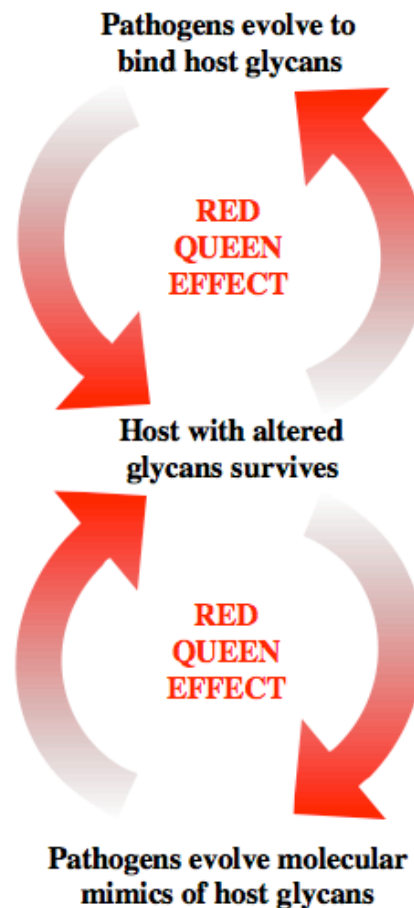
There are numerous examples of microbial surface receptors such as adhesins, pili, fimbriae, or hemagglutinins, as well as microbial toxins that recognize glycans with exquisite specificity (Bishop and Gagneux, 2007; Ilver et al., 2003; Sharon and Lis, 2004). In fact, pathogenic organisms synthesize the vast majority of known glycan-binding lectins (Angata and Varki, 2002; Ilver et al., 2003). Likewise, human leukocytes which are responsible for detecting and responding to “foreign” microorganisms within the environment of “self” host tissues express numerous glycan binding receptors (Sharon and Lis, 2001). These carbohydrate-binding receptors appear to recognize exogenous microbial glycosylation patterns and promote inflammation, but conversely recognize endogenous oligosaccharide ligands with an opposite effect to inhibit the inflammatory response. One example of an exogenous glycan ligand, peptidoglycan, is highly conserved across bacterial species and is exploited as a pathogen-associated molecular pattern (PAMP) recognized by pattern recognition receptors (PRRs) expressed on host leukocytes (Akira, 2006; Bishop and Gagneux, 2007; Remer et al., 2005; Weis et al., 1998). Recognition of peptidoglycan by these lectin receptors increases the activation state of individual leukocytes and promotes the release of inflammatory cytokines that further amplify the immune response (Kirschning and Schumann, 2002; O'Neill, 2002). Conversely, lectins expressed by the immune system, including factor H and members of the I-type lectin families, recognize common endogenous oligosaccharides that are rarely expressed on

microorganisms (Crocker and Varki, 2001; Geijtenbeek et al., 2004) (Sonnenburg et al., 2004). In the case of Factor H, recognition of endogenous ligands is necessary in order to maintain immune homeostasis in the presence of "self" (Pangburn, 2000). This finding suggests that leukocyte lectin receptors may play dual roles, both activating inflammatory responses upon recognition of specific "foreign" glycans common to invading microbes while simultaneously maintaining immune homeostasis upon recognition of "self" oligosaccharide ligands (Gordon, 2002; Linehan et al., 2000).

One likely evolutionary consequence of this array of lectins produced on both sides of the host-pathogen interface is the concomitant rapid evolution of glycans covering both host and pathogen surfaces (Gagneux and Varki, 1999). Since the great majority of microorganisms replicate at a much faster rate than their hosts, potential parasitic microorganisms can quickly evolve multiple mechanisms of subverting host glycans or lectins to gain a selective advantage. In an evolutionary process known as a "Red Queen" effect, continual compensatory evolutionary changes in glycan structure in the host organism are required in order to maintain the original status quo (**Figure 1.1**) (Van Valen, 1974; Varki, 2006) (Angata and Varki, 2002).

One effective mechanism by which pathogens escape immune detection is by coating their surfaces in glycans that closely resemble host surface glycosylation (molecular mimicry) (Moran et al., 1996; Tsai, 2001; Vimr and Lichtensteiger, 2002). By mimicking host cell glycans, a pathogen can avoid detection by leukocyte lectins





**Figure 1.1 Evolutionary Diversification of Glycans.** Each arrowed circle represents a potential vicious cycle driven by a Red Queen effect, in which hosts are constantly trying to evade the more rapidly evolving pathogens that infect them. The host requires glycans for critical functions but must constantly change them to evade glycan binding pathogens without impairing their own survival. In addition pathogens can mimic host glycans thereby disguising themselves as the host. Again the host is forced to modify their own glycans in order to allow differential recognition of exogenous microorganisms. There are also possible secondary Red Queen effects involving glycan binding proteins such as the CD33 Related Siglecs that recognize “self” (Varki and Angata, 2006). Modified from (Varki A., Cell, 2006)

that strictly recognize exogenous microbial glycans while simultaneously engaging host lectins that recognize endogenous host glycosylation patterns.

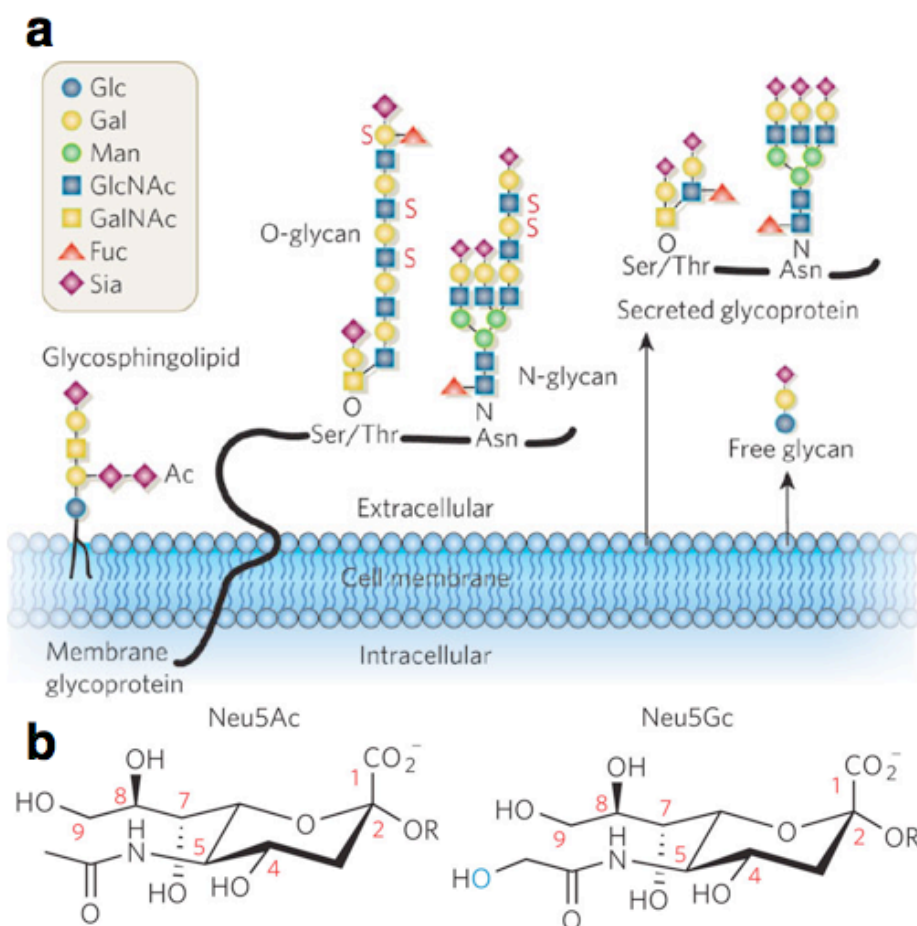
Although microbial lectins recognize many glycan targets, a disproportionate number specifically target sialic acid (Sia) or Sia-containing epitopes (Lehmann et al., 2006). Likewise, a large number of animal lectins bind Sias possibly to function as negative regulators of the immune system (Varki, 2007). Animal and microbial lectins likely evolved to target Sias for similar reasons such as their accessibility (terminal positions on glycans), diversity (provides selectivity), and expression patterns (abundant in vertebrates but rare in microorganisms). Although pathogens utilize Sias as targets for attachment and cellular invasion, and the host itself uses Sias for immune homeostasis and cellular trafficking, in both cases Sias are distinguishing markers of host cells. Given the species distribution of Sias this 'rule' is generally true, but in the case of Sia-expressing pathogens, the typically useful molecular assumption that "Sia=self" may lead to disastrous consequences.

This review discusses the basic features of sialic acid and its importance as a target for endogenous lectins of the vertebrate immune system. Focus is also placed on how Sia-expression allows microorganisms to commandeer some of these host lectins and the consequences of those interactions. Particular attention is paid to factor H, a regulator of the non-cellular arm of innate immunity that has been demonstrated to interact with various pathogens, and the sialic acid-recognizing immunoglobulin-like lectins (Siglecs), a family of Sia-binding lectins found on cells of the innate

immune system that only recently have been shown to interact with Sia-expressing pathogens.

## SIALIC ACIDS

*Structure and Diversity of Sialic Acids.* Sias are  $\alpha$ -keto acids, have a common nine-carbon backbone structure, and can be modified in numerous ways producing a diverse family of more than 50 distinct monosaccharides that are typically found at the outermost ends of N-glycans, O-glycans, and glycosphingolipids (Figure 1.2) (Angata and Varki, 2002). The two most common Sias found in mammals are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) (Figure 1.2). Humans cannot synthesize Neu5Gc due to a human-specific mutation in the CMP-N-acetylneuraminic acid hydroxylase (CMAH) gene that catalyzes the production of CMP-Neu5Gc via enzymatic addition of one oxygen atom to the N-acetyl group of the substrate CMP-Neu5Ac (Varki, 2001). Thus, although the total quantity of Sias on the surface of humans cells are similar to other vertebrates, humans have a relative abundance of Neu5Ac on their cell surfaces (Brinkman-Van der Linden et al., 2000; Muchmore et al., 1998). Due to the human specific mutation, Neu5Gc is a foreign molecule and consequently most humans produce Neu5Gc specific IgM, IgG, and IgA antibodies (Tangvoranuntakul et al., 2003).



**Figure 1.2 Sialic acids (Sias) on cell surfaces and secreted glycans.** (a) Sias are typically found at the terminal position of cell associated and secreted N- and O-linked glycoproteins and cell surface glycosphingolipids. (b) The two most common Sias found in mammals are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) which differ by a single oxygen atom. N Ac, *O*-acetyl ester; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; S, sulphate ester. Figures taken with permission from (Varki, A., Nature, 2007)

*Species Distribution of Sialic Acids.* The evolutionary distribution of glycans in living organisms fall into four general patterns: glycans conserved across taxa, glycans specific to a particular lineage, glycans similar across distant taxa, and glycans conspicuously absent from very restricted taxa (Bishop and Gagneux, 2007). Sias are most likely evolutionarily ancient but are predominantly expressed in the deuterostome lineage of metazoans where their relative expression level and complexity have greatly increased (Angata and Varki, 2002). In addition to these higher multicellular eukaryotes, there are small numbers of microorganisms that utilize multiple mechanisms to attach Sia to their surface glycans (Mandrell and Apicella, 1993; Vimr et al., 2004) (Cross and Takle, 1993). Thus, Sias belong to the third category of glycan distribution in which similar glycans are produced by evolutionarily distant taxa. Interestingly, virtually all microorganisms that express sialylated glycans infect vertebrate hosts (Moran et al., 1996; Tsai, 2001; Vimr and Lichtensteiger, 2002). Furthermore, all sialylated pathogens infecting humans incorporate Neu5Ac, a common human endogenous monosaccharide, while none utilize Neu5Gc, a “foreign” sugar in humans, apparently because Neu5Ac expression is a successful form of molecular mimicry. This pattern of Sia expression in certain pathogenic microorganisms could have come about because of maintenance of evolutionarily old Sia synthesis machinery, convergent evolution of bacterial genes, or lateral transfer of Sia-related genes from other bacteria or multicellular organisms. Due to the complexity and number of proteins necessary to synthesize Sias, it is less likely that microorganisms completely reinvented the Sia-synthetic machinery.

**Biological Importance of Sialic Acids.** Until the 1980's it was thought that Sias functioned merely to provide negative charge and hydrophilicity to the cell surface, mask subterminal galactose residues from their receptors, and act as ligands for receptors on pathogens and toxins (Varki, 1999; Varki, 2007). In mammals, the biophysical properties of Sia alone are known to be involved in diverse biological processes such as neuronal plasticity and learning, glomerular filtration, and electrostatic repulsion of circulating erythrocytes (Fujimoto et al., 2001; Johnson et al., 2005; Kerjaschki et al., 1985; Ronn et al., 2000; Suzuki et al., 1998). Subsequently it was shown that Sias were essential for the early embryonic development of mice but are not required for embryogenesis of other organisms such as *Drosophila melanogaster* or *Caenorhabditis elegans*, suggesting that Sias have additional intrinsic functions in vertebrates beyond providing negative charge, hydrophilicity, and masking of underlying oligosaccharide epitopes (Schwarzkopf et al., 2002). Over the past three decades it was discovered that Sias are essential components of numerous motifs recognized by secreted and cell-surface Sia-recognizing vertebrate lectins involved in many biological systems, including the immune system.

**Sia-decoration by pathogenic microorganisms.** *Escherichia coli* (K1 and K92), *Neisseria meningitidis* (groups B, C, Y, and W135), and group B *Streptococcus* (GBS) all express capsular Sias, and represent three of the most common causes of bacterial sepsis and meningitis, particularly in infants and young children. Sialylated bacterial glycans include polysaccharide capsules (K-antigens), that can be produced by Gram-positive or Gram-negative bacteria, as well as glycoproteins, or glycolipids

(Brooks et al., 2004; Fischetti and American Society for Microbiology, 2006). Bacterial glycolipids are commonly referred to as lipopolysaccharides (LPS) or lipooligosaccharides (LOS) and are found exclusively in the outer membrane of Gram-negative bacteria where they can be modified with Sias. In addition to sialylated bacteria, the presence of sialylated glycans have been discovered on viral, parasitic, and fungal pathogens (Vimr and Lichtensteiger, 2002)<sup>33</sup>, (Rodrigues et al., 1997); (Chava et al., 2004). In fact, the list of verified Sia-expressing pathogens continues to expand, and with the addition of recently available genomic data, the presence of Sia-synthetic gene clusters has been shown to be more widespread than previously anticipated (Lewis et al., 2006). The selective advantage gained by Sia-decoration is evidently so strong that microorganisms have evolved at least four mechanisms of cell-surface sialylation. *E. coli* K1 and GBS are two examples of organisms that utilize de novo biosynthesis of Sias to produce a sialylated capsular polysaccharide (CPS) (Chaffin et al., 2005; Vimr et al., 1995; Wessels et al., 1992). *Haemophilus influenzae* lacks the genes necessary for Sia synthesis but can scavenge Sia from its surroundings, activate it with CMP-Sia synthetase, and attach it to its surface (Vimr et al., 2000). *Neisseria gonorrhoeae* has an even greater truncation of the Sia synthesis pathway and is able to scavenge minute amounts of CMP-Sia from its environment and transfer the Sia from this activated sugar donor nucleotide to its cell-surface LOS (Parsons et al., 1988). The final mechanism of pathogen sialylation is employed by *Trypanosoma cruzi* and *Corynebacterium diphtherium*, which express a cell surface trans-sialidase that first cleaves Sias off cell surfaces then functions as a Sia-

transferase that attaches the free Sia to the pathogen surface (Cross and Takle, 1993; Mattos-Guaraldi et al., 1998). Although mechanisms of microbial Sia acquisition and expression the benefits of a sialylated surface are the same. Sias provide a negative surface charge for electrostatic repulsion, mask underlying glycan structures that can serve as easily recognizable PAMPs, and we hypothesize that they act by directly engaging host sialic acid binding lectins in addition to factor H that are involved in immune regulation.

### **SIA-BINDING LECTINS AS REGULATORS OF IMMUNITY**

*Factor H.* The complement system is composed of the classical, alternative, and lectin branches that together form the major non-cellular arm of the innate immune system. The alternative complement system is evolutionarily ancient and its activation is a continuous, spontaneous, and self-amplifying process that is designed in such a way that it will attack any cell surface unless it is recognized as a host cell (Pangburn, 2000; Smith et al., 1999). One key recognition protein in this system is factor H, which binds host cell surfaces and downregulates alternative pathway C3/C5 convertase activity, and serves as a cofactor for factor I, a serine protease that inactivates C3b (Alsenz et al., 1984; Kuhn et al., 1995). Factor H recognition of host cell surfaces requires the presence of Sias and/or certain polyanions such as sulphated glycosaminoglycans (GAGs). For example, the enzymatic removal of Sias from sheep erythrocytes is sufficient to convert them from nonactivators to activators of alternative complement (Fearon, 1978; Pangburn and Muller-Eberhard, 1978). Both



endogenous targets of factor H, Sias and sulfated glycosaminoglycans, are cell surface glycans abundantly found in vertebrates while rare in microorganisms.

***Sialylated microorganisms that engage factor H.*** In 1981 Smith and coworkers published a paper describing the discovery that *N. gonorrhoeae* grown in human serum and secretions converted from a serum-sensitive phenotype to serum resistance (Veale et al., 1981). A low molecular weight dialyzable substance isolated from human serum and cells, which was later identified as the nucleotide sugar CMP-Neu5Ac, was responsible for rendering gonococci resistant to killing by normal human serum (Parsons et al., 1988); (Patel et al., 1984). Subsequently, it was shown that incubation of the bacteria with purified CMP-Neu5Ac resulted in the addition of Sia to the surface LOS acceptor substrate Gal $\beta$ 1-4GlcNAc. This terminal Sia was sufficient to engage human factor H, inactivate alternative complement deposition on the bacterial surface, and thereby protect the bacteria from serum killing (Smith et al., 1992). Further studies on the interactions of *N. gonorrhoeae* with factor H lead to the discovery of a new Sia-binding site located in the final four (16-20) short consensus repeats (SCRs) of factor H (Ram et al., 1998).

Sialylation of the LOS of other Gram-negative bacteria such as *H. influenzae* and *N. meningitides* were shown to be important for resistance to serum killing (Bouchet et al., 2003; Hood et al., 1999; Moran et al., 1996). Furthermore, the sialylated capsular polysaccharides of GBS, *N. meningitidis*, and *E. coli* K1 are proven virulence factors that decrease the activation of the alternative complement cascade (Edwards et al., 1982; Jarvis and Vedros, 1987; Kahler et al., 1998; Van Dijk et al.,

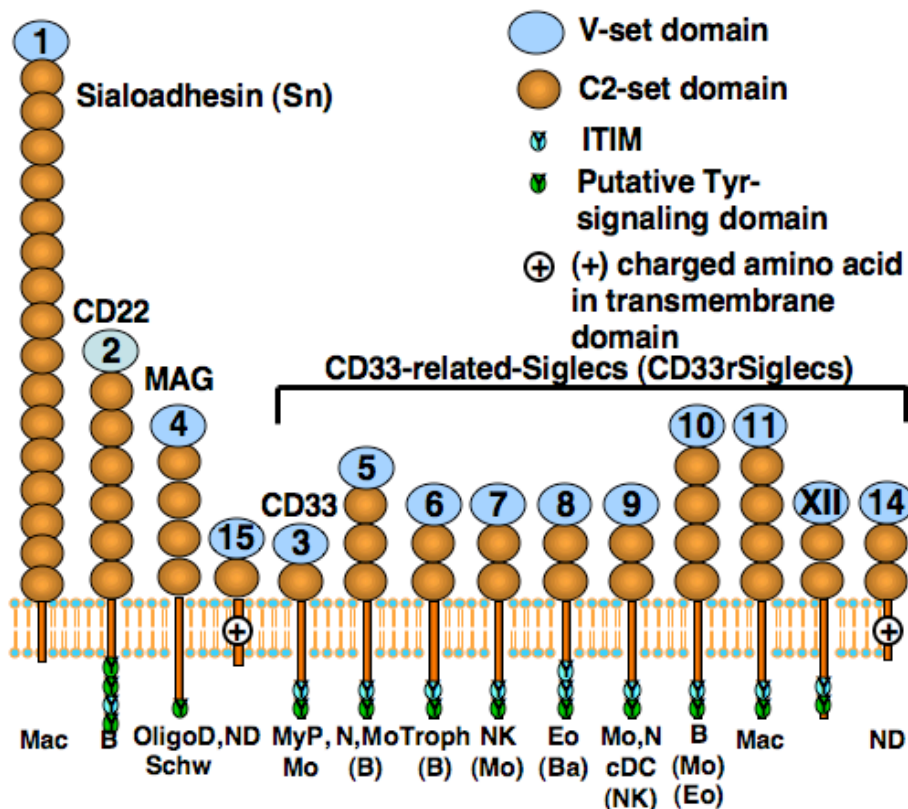
1979). Desialylation of the trypomastigote stage of the protozoan parasite *Trypanosoma cruzi* causes the accumulation of a greater proportion of active complement C3b to inactive iC3b resulting in increased lysis of the organism (Tomlinson et al., 1994). Sindbis virus, an enveloped virus that buds from cells, is surrounded by a host cell membrane that contains varying amounts of sialylated oligosaccharides depending on the origin of that cell (Hirsch et al., 1983). It was shown that the ability of Sindbis virus to activate alternative complement is inversely correlated with the Sia content of the host cell from which its membrane is derived (Hirsch et al., 1981).

In addition to Sia-mediated interactions numerous pathogens express specific proteins that are able to functionally engage factor H including the M protein of *Streptococcus pyogenes*, Yad A protein of *Yersinia enterocolitica*, and GP120 and GP41 of human immunodeficiency virus (HIV).

**Siglecs.** The Siglecs are a large family of type-I transmembrane proteins with an amino terminal V-set immunoglobulin domain that binds Sias, followed by a variable number of constant-region type 2 (C2-set) immunoglobulin domains (Varki and Angata, 2006). The Siglecs can be divided into two groups based on sequence similarity and evolutionary conservation (Figure 1.3). Sialoadhesin (Siglec-1), CD22 (siglec-2), myelin associated glycoprotein (MAG or Siglec-4), and Siglec-15 are only distantly related based on sequence identity but have clear orthologues in all mammalian species examined (Crocker et al., 2007). In contrast, the CD33/Siglec-3-related subgroup (Siglecs -3 and -5 through -14 in primates) share between 50-99%

identity but are rapidly evolving by multiple mechanisms that include gene duplication, exon shuffling, gene conversion, and gene loss (Angata et al., 2004). One consequence of this rapid evolutionary change is that CD33rSiglecs vary greatly between species. While Humans express nine CD33rSiglecs, mice only express five making it difficult to assign true orthologues.

Siglecs function as regulators of cell adhesion, signaling, and endocytosis. There are a wide variety of sialylated ligands that Siglecs can potentially recognize, although each individual Siglec appears to have a characteristic specificity for particular sialylated ligands (Alphey et al., 2003; Blixt et al., 2003) (Bochner et al., 2005). While the negative charge of Sia is broadly sufficient to explain the binding of factor H and the selectins, Siglecs often interact specifically with particular Sia-linkages, and in some cases such as Siglec-4 (MAG), to the extended oligosaccharide chain (Varki, 2007) (Pan et al., 2005; Vyas et al., 2005). Siglec-1 (Sialoadhesin) is expressed on the surface of a subset of macrophages and is one of the largest immunoglobulin super-family proteins known. Recent data from sialoadhesin deficient mice suggest that it plays a role in positively modulating T-cell activation in response to particular inflammatory stimuli (Jiang et al., 2006) (Ip et al., 2007; Oetke et al., 2006). Siglec-1 has also been implicated in direct recognition and internalization of sialylated pathogens (Jones et al., 2003; Monteiro et al., 2005; Vanderheijden et al., 2003). Siglec-2 (CD22) is a B-cell specific molecule that is a negative modulator of B-cell receptor activation and an important regulator of B-cell



**Figure 1.3 Siglec-family proteins in humans.** Siglecs are type I membrane proteins with an amino-terminal V-set domain (binds Sias) and varying numbers of C2-set domains. Siglecs are divided into two subfamilies based on sequence similarity and evolutionary conservation. The CD33rSiglecs share 50-99% sequence similarity, differ in composition between species, and their intracellular domains frequently contain conserved tyrosine based signaling motifs. Siglec-1, -2, -4, -15 have orthologues in all mammals examined but have lower sequence similarity. Siglec-14 and -15 have been shown to interact with DAP-12 through the positive charge located in their transmembrane domains. Cellular expression pattern is listed below each Siglec. B, B cells; Ba, basophils; cDCs, conventional dendritic cells; Eo, eosinophils; ITIM, immunoreceptor tyrosine-based inhibitory motif; Mac, macrophages; Mo, monocytes; MyP, myeloid progenitors; N, neutrophils; ND, not determined; NK, natural killer cells; OligoD, oligodendrocytes; pDCs, plasmacytoid dendritic cells; Schw, Schwann cells; Troph, trophoblasts.

homeostasis and survival whose signaling in particular circumstances may be dependent or independent of recognition of  $\alpha$ 2-6-linked Sias (Poe et al., 2004) (Grewal et al., 2006; Tedder et al., 2005). Siglec-4 (MAG) is specifically expressed in glial cells and recognizes gangliosides containing Sias or sulfate esters. MAG's ligand recognition is required for myelin stability and may play a role in inhibiting neuronal sprouting and outgrowth following injury. The newly discovered Siglec-15 has been highly conserved throughout evolution and may play a role in regulating immunity through recruitment of DAP-10 and DAP-12 adaptor proteins (Angata et al., 2007).

With the exception of Siglec-5 (unpublished observation) and Siglec-6, which have recently been found in the human placenta, the human CD33rSiglecs (Siglec-3, -5 through -11, -XII, and -14) are expressed in cells of haemopoietic origin (Crocker et al., 2007). Each CD33rSiglec is expressed in a cell specific fashion and has specificity for different sialylated ligands, suggesting that each protein mediates a distinct and partially overlapping function. The CD33rSiglecs contain cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) as well as an additional tyrosine based motif that in certain proteins is an immunoreceptor tyrosine-switch motif (ITSM). Receptors with ITIM motifs generally function as inhibitory receptors that suppress activation signals produced by immunoreceptor tyrosine-based activation motifs (ITAMs). ITIM-bearing CD33rSiglecs are hypothesized, but never proven, to be involved in recognizing Sias as self and in turn send inhibitory signals that downregulate possible autoimmune responses (Crocker and Varki, 2001) (Avril et al., 2004). Recently, it has been shown that Siglec-F, a murine CD33rSiglec expressed on

eosinophils, plays an important role in inhibiting the eosinophil induced inflammatory response in a lung model of allergic response (Zhang et al., 2007). This supports the hypothesis that CD33rSiglecs are important in down-modulating the cellular immune response.

*Sialylated microorganism that engage Siglecs.* Over 20 pathogenic microorganisms have evolved mechanisms of incorporating Sias into their glycoconjugates, and in many of these organisms Sias are proven virulence factors. It is widely assumed that Sias increase pathogenicity by providing a negative charge that reduces interactions with host cells via electrostatic repulsion and/or by avoiding alternative complement deposition by recruiting host factor H. In either case a Sia alone attached to the surface of a microorganism could provide a negative charge or serve as a binding site for factor H. However, microorganisms synthesize complex oligosaccharides that mimic not only Sia, but also the linkages and underlying sugars that are present abundantly in their mammalian hosts. One possible reason for mimicry of extended sialylated oligosaccharide structures would be to engage Siglecs which recognize not only the Sia monosaccharide but its linkage and in some cases underlying sugars. Recently, five pathogenic microorganism, including one virus, one parasite, and three bacteria, have been demonstrated to interact with members of the Siglec family.

Sialoadhesin (Siglec-1) due to its size and conformation is thought to extend its Sia-binding domain past the surface glycocalyx of macrophages and into the extracellular space. Sialoadhesin binds to  $\alpha$ 2-3-linked-Sias, and unlike many other

siglecs, lacks intracellular signaling domain suggesting that it is mainly involved in Sia-dependent cellular interactions. The first microorganism that was shown to interact with Siglecs was *N. meningitidis* which attaches Sias to its LOS to create an Neu5Ac $\alpha$ 2-3Gal epitope that is recognized by both Siglec-1 as well as Siglec-5. It was demonstrated using Sia-positive and -negative *N meningitidis* and Sialoadhesin-expressing and -deficient mice that the bacteria are engaged and phagocytosed in a Sia- and Sialoadhesin-specific manner (Jones et al., 2003). This study suggested that Sialoadhesin in conjunction with other receptors may play a role in immune defense against sialylated pathogens by allowing host macrophages to engage a mammalian epitope on the bacterial surface. Subsequent studies involving *T. cruzi* trypomastigotes and the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) demonstrated that Sialoadhesin is responsible for interactions with and phagocytosis of these sialylated microorganisms. However in this case both *T. cruzi* and the PRRSV both infect macrophages in their respective hosts. PRRSV has a very selective tropism for subpopulations of differentiated macrophages in vivo, and can only infect porcine alveolar macrophages (PAMs) and African Green Monkey kidney cells in vitro (Duan et al., 1997). It was demonstrated that PRRSV infection of PAMs requires the expression of Sialoadhesin (Siglec-1) on target macrophages as well as viral expression of Sia (Delputte and Nauwynck, 2004) (Vanderheijden et al., 2003). *T. cruzi* utilizes its trans-sialidase enzyme to sialylate itself during the infective bloodstream trypomastigote stage. The Sia on its surface has been shown to interact in a Sia-specific fashion with Sialoadhesin and promote pathogen entry into

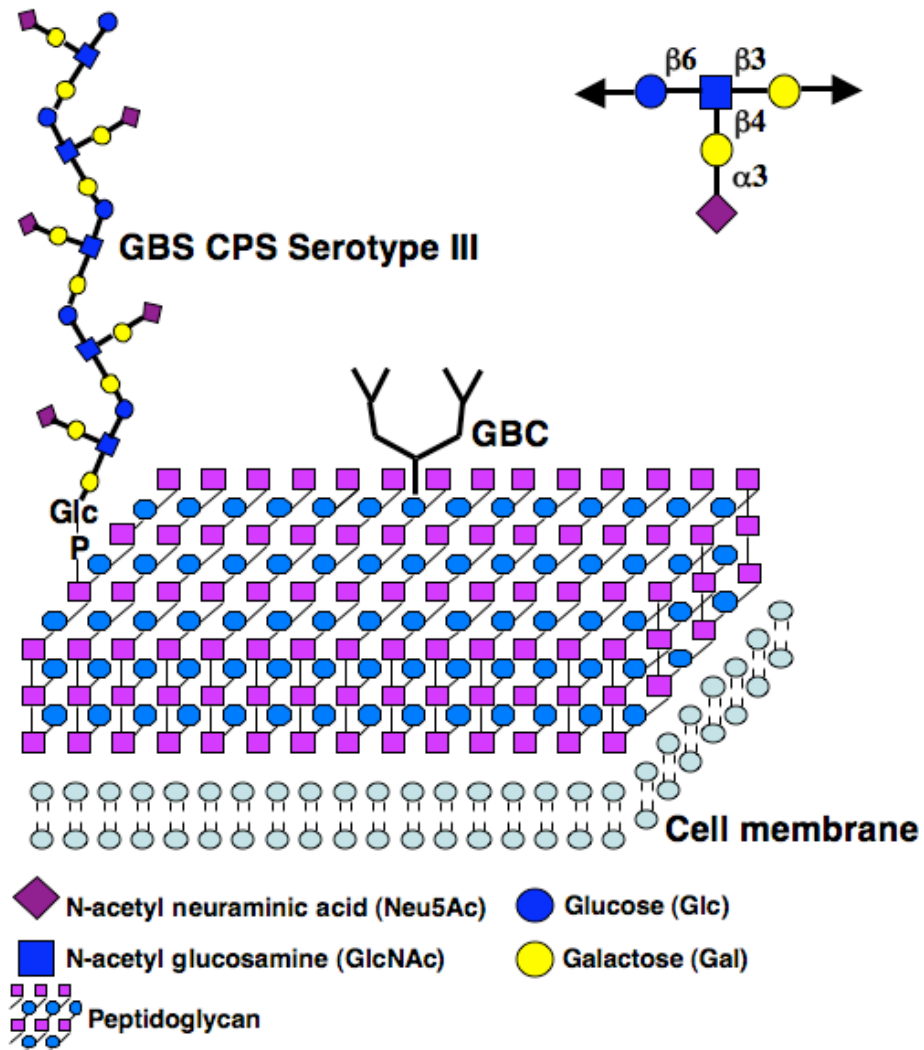
macrophages (Monteiro et al., 2005). Clearly Sialoadhesin can recognize and phagocytose microorganisms, however the ultimate consequences of this interaction for the host as well as the pathogen may differ depending on the microorganism and its tropism.

The CD33rSiglecs are predominantly expressed by cells of the innate immune system, utilize intracellular tyrosine signaling domains, and can recognize a variety of Sia-linkages and oligosaccharide structures in a Siglec-specific fashion. In addition to *N. gonorrhoeae*, another human pathogen shown to be capable of interacting with a hCD33rSiglec is *Campylobacter jejuni*. *C. jejuni* can express a specific disialo-LOS structure, Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-3Gal, and has been shown to interact with human Siglec-7 (hSiglec-7). Bacterial engagement of Siglec-7 increases was shown to increase the association of sialylated *C. jejuni* with NK cells and monocytes (Avril et al., 2006). Although the functional significance of Sia-expressing pathogens engaging CD33rSiglecs is unknown, it has been hypothesized that this interaction would recruit Siglecs and their ITIMs to sights of pathogen contact with immune cells. These ITIM domains would then recruit src-homology phosphatases (SHP-1 and SHP-2) that would act to down-modulate the inflammatory reaction of these cells. Alternatively, CD33rSiglecs are capable of acting as endocytic receptors when cross-linked by antibodies and therefore may act as endocytic receptors responsible for pathogen ingestion by phagocytes.

GBS synthesizes a capsular polysaccharide (CPS) that is covalently anchored to the peptidoglycan cell wall, forming the outermost layer of the bacterial surface and



is composed of repeating subunits of four monosaccharides – glucose, galactose, N-acetylglucosamine, and N-acetylneuraminic acid (Neu5Ac), polymerized in one of nine different serotype-specific configurations (Ia, Ib, II-VIII) based on antibody recognition (**Figure 1.4**) (Cieslewicz et al., 2005). Although the physical structure of the CPS of each GBS serotype is unique, all invariably share a terminal Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-(3/4)GlcNAc epitope. The structure, position, and linkage of these terminal oligosaccharides are identical to some of the most widely expressed Sia-containing glycans found on all cells in the human body. In the following Chapters, I will test the hypothesis that GBS cell surface sialylation is necessary and sufficient to engage human Siglecs. In addition, I will explore the functional consequences of hCD33rSiglec interactions with Sias presented in trans, which shares relevance both for human leukocyte interactions with endogenous cells and proteins as well as interactions with exogenous Sia-expressing pathogenic organisms.



**Figure 1.4 Group B *Streptococcus* surface structures.** The GBS surface consist of an inner membrane surrounded by a thick layer of peptidoglycan with covalently attached polysaccharides. The GBS serotype III capsular polysaccharide is depicted attached to the peptidoglycan cell wall along with the repeating unit structure that includes glycosidic linkages (top right). CPS, capsular polysaccharide; GBC, group b carbohydrate. (modified from Amanda Lewis)

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## **Chapter II**

# **Group B Streptococcal Capsular Sialic Acids Interact with Siglecs (Immunoglobulin-Like Lectins) on Human Leukocytes**

## ABSTRACT

Group B *Streptococcus* (GBS) is classified into nine serotypes that vary in capsular polysaccharide (CPS) architecture, but share in common the presence of a terminal sialic acid (Sia) residue. This position and linkage of GBS Sia closely resembles that of cell surface glycans found abundantly on human cells. CD33-related Siglecs (CD33rSiglecs) are a family of Sia-binding lectins expressed on host leukocytes that engage host Sia-capped glycans and send signals that dampen inflammatory gene activation. We hypothesized that GBS evolved to display CPS Sia as a form of molecular mimicry limiting the activation of an effective innate immune response. In this study, we apply a panel of immunologic and cell-based assays to demonstrate that GBS of several serotypes interact in a Sia- and serotype-specific manner with certain human CD33rSiglecs, including hSiglec-9 and hSiglec-5 expressed on neutrophils and monocytes. Modification of GBS CPS Sia by O-acetylation (OAc) has recently been recognized, and we further show that the degree of OAc can markedly impact the interaction between GBS and hSiglecs-5, -7, and -9. Thus, production of Sia-capped bacterial polysaccharide capsules that mimic human cell surface glycans in order to engage CD33rSiglecs may be an example of a previously unrecognized bacterial mechanism of leukocyte manipulation.

## INTRODUCTION

Group B *Streptococcus* (GBS) is the leading cause of bacterial pneumonia, sepsis and meningitis in human newborns and is increasingly recognized as a pathogen in adult populations including diabetics, pregnant women and the elderly. A critical factor contributing to GBS virulence is its surface capsular polysaccharide (CPS). The CPS forms the outermost layer of the bacterial surface and is typically composed of repeating subunits of four monosaccharides – glucose, galactose, N-acetylglucosamine, and N-acetylneuraminic acid, polymerized in serotype-specific configurations (Cieslewicz et al., 2005). GBS isolates are categorized as belonging to one of nine different serotypes (Ia, Ib, II-VIII) based on antibody recognition. Although the physical structure of the CPS of each GBS serotype is unique, all GBS serotypes invariably share a terminal  $\alpha$ 2-3-linked N-acetylneuraminic acid (Neu5Ac), identical to the predominant sialic acid (Sia) found on human cells. It has recently been discovered that GBS modify their CPS Sia by addition of a single O-acetyl group at the seventh carbon position that can subsequently migrate to the eighth or ninth carbon position (Lewis et al., 2006; Lewis et al., 2004). GBS synthesize and modify Sia using a *de novo* pathway in which four genes, *neuA-neuD*, are responsible for Sia synthesis and O-acetylation, while *cpsK* mediates transfer of Sia to the CPS subunit (Chaffin et al., 2005; Haft and Wessels, 1994; Lewis et al., 2006; Suryanti et al., 2003; Vann et al., 2004).

The Sia-recognizing immunoglobulin (Ig) superfamily lectins (Siglecs) are a family of structurally-related type I transmembrane proteins composed of one unique



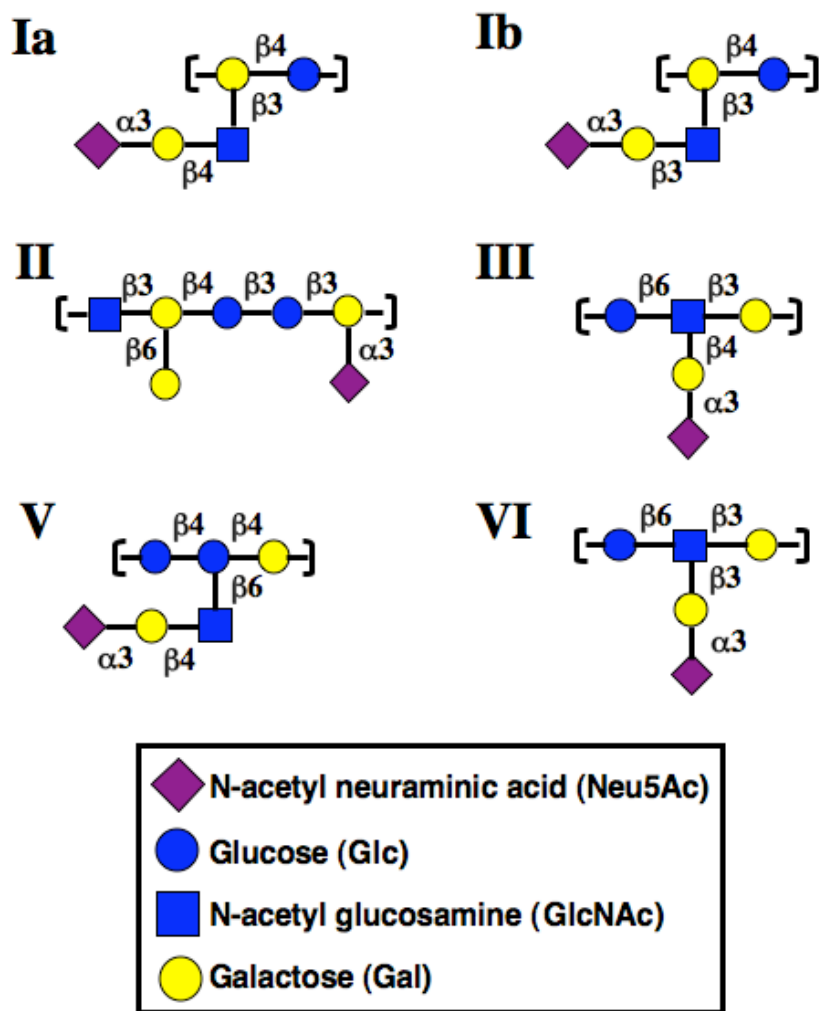
(or two in the case of Siglec-XII) V-set Ig domains responsible for recognizing sialylated glycoconjugates, followed by a variable numbers of structural C2-set Ig domains (Varki and Angata, 2006). The CD33/Siglec-3-related Siglecs (CD33rSiglecs) are a rapidly evolving subgroup of the Siglecs and in humans consists of Siglecs-3, -5-11, -XII. CD33rSiglecs, with the exception of Siglec-XII, which lacks a functional Sia binding domain, are expressed predominantly on the surfaces of leukocytes in a cell type-specific fashion suggesting distinct functional roles (Crocker, 2005). The CD33rSiglecs share 50-80% sequence similarity with the highest similarity in the V-set domain involved in Sia recognition and the two intracellular tyrosine signaling motifs. The membrane-proximal signaling domain is an immunoreceptor tyrosine-based inhibitory motif (ITIM) with the canonical sequence (I/L/V)xYxx(L/V) whereas the membrane-distal domain is an ITIM-like motif, whose role is less well defined (Ravetch and Lanier, 2000; Vely et al., 1997). Upon phosphorylation by a Src-family kinase, these motifs can recruit protein tyrosine phosphatases SHP-1, SHP-2, and SHIP. Many transmembrane proteins containing these motifs are involved in inhibitory signaling that dampens or counteracts activating signals sent by other immunoreceptors such as those containing immunotyrosine-activating motifs (ITAMs).

In this study we examine whether CPS Sia of various GBS serotypes can specifically engage specific human CD33rSiglecs present on human leukocytes involved in control of bacterial infections. Further, we examine how O-acetyl modification of CPS Sia can modify the interaction between GBS and these leukocyte

surface receptors. The implications of GBS surface expression of a human-like monosaccharide, Neu5Ac, are then discussed in the context of molecular mimicry and innate immune evasion.

## RESULTS

*Sialylated GBS bind to many different human Siglecs.* Well-characterized sialylated GBS strains from serotypes Ia, Ib, II, III, V, and VI (CPS structures shown in **(Figure 3.1)**) and an asialo- serotype III  $\square$ NeuA allelic exchange mutant were tested for their ability to bind to hSiglecs-3, -5, -6, -7, -8, -9, -10, and -11. Human Siglec-Fc chimeras were pre-bound to protein A-coated ELISA plates in a high avidity format and whole cell heat-killed FITC-labeled GBS added to the wells. Each of the GBS serotypes tested were capable of binding to at least one hCD33rSiglec, while the asialo- $\square$ NeuA mutant did not bind to any of the hSiglec constructs tested (**Figure 3.2**). In parallel experiments, similar interactions were observed between live (Calcein-AM labeled) GBS and hSiglec-Fc constructs (data not shown). The representative serotype Ia and Ib WT GBS strains were very effective at binding to many different hSiglecs in this high avidity assay format. Interestingly, the serotype III strain common in late onset GBS disease was only recognized effectively by Siglec-9. Furthermore, in contrast to hSiglec-9, where all GBS serotypes tested bound, not one of the GBS



**Figure 2.1** All GBS serotypes described to date contain a terminal  $\alpha$ 2-3 linked Sia on its CPS. The repetitive subunits of the CPS of GBS serotypes Ia, Ib, II, III, V, and VI.

Group B <i>Streptococcus</i> Isolate and Serotype							
	la	lb	II	III	V	VI	III asialo-
Siglec-3 (CD33)	2 ± 0.3	2 ± 0.2	4 ± 0.5	3 ± 0.1	3 ± 0.9	2 ± 0.2	3 ± 0.1
Siglec-5	76 ± 10	64 ± 14	3 ± 0.4	3 ± 0.8	14 ± 3	8 ± 0.5	5 ± 0.5
Siglec-6	56 ± 3	45 ± 3	1 ± 0.3	1 ± 0.2	13 ± 2	8 ± 2	3 ± 1
Siglec-7	73 ± 2	38 ± 1	5 ± 1	3 ± 1	3 ± 3	1 ± 0.3	4 ± 1
Siglec-8	48 ± 11	43 ± 10	2 ± 0.8	1 ± 0.1	17 ± 2	9 ± 3	4 ± 1
Siglec-9	63 ± 8	39 ± 12	11 ± 13	38 ± 3	10 ± 0.6	18 ± 0.4	3 ± 2
Siglec-10	2 ± 0.5	3 ± 3	3 ± 1	2 ± 0.6	2 ± 0.6	2 ± 0.3	1 ± 0.1
Siglec-11	39 ± 6	34 ± 6	1 ± 0.4	2 ± 1.2	17 ± 7	7 ± 1	5 ± 1

Binding (%)	0-5%	6-15%	16-30%	31-50%	>50%
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**Figure 2.2** All GBS serotypes bind to at least one hCD33rSiglec while several serotype interact with multiple hCD33rSigs. FITC-GBS were added to hSiglec-Fc chimera coated wells and the efficiency of binding was tested. The values represent the mean of at least three separate experiments ± standard deviation.

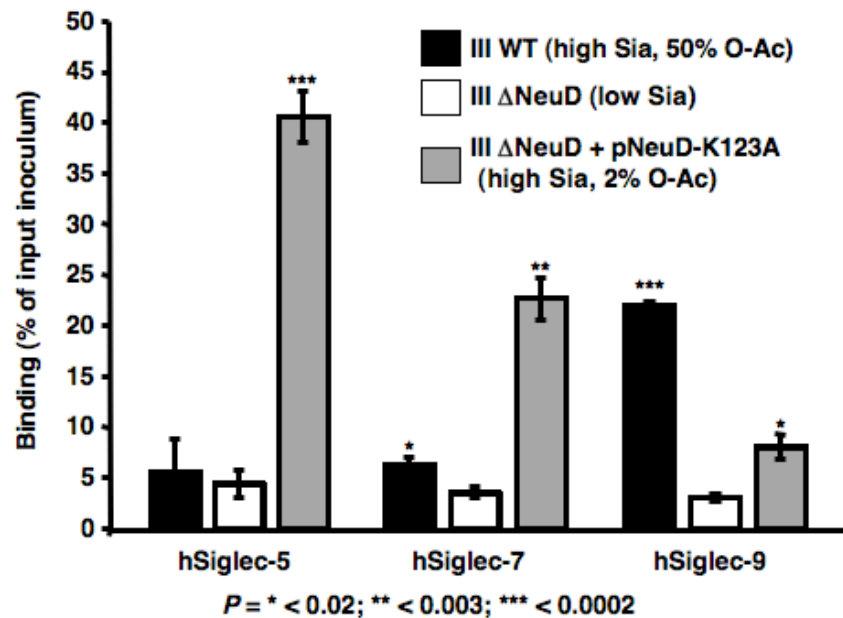
serotypes tested was able to bind to hSiglec-3 or hSiglec-10. Many of the hSiglecs to which GBS bound are located on the cell surfaces of leukocytes involved in innate immunity to bacterial infections. Human neutrophils express Siglec-5 and -9 on their surface, human monocytes express Siglec-5, -7 and -9, and human macrophages express Siglec-5 and 11 (Angata et al., 2002; Floyd et al., 2000; Lock et al., 2004).

***GBS serotype III binding to hSiglec-9 is Sia-specific.*** The significant loss of hSiglec-9 binding of the isogenic asialo- mutant compared to the WT serotype III parent strain (**Figure 3.2**) suggests a Sia-dependent interaction. Independent verification of Sia-dependence was achieved by treatment of WT serotype III FITC-GBS with either trypsin (to cleave bacterial cell surface proteins leaving the bacterial CPS intact), or an exogenous sialidase (to remove all Sias attached to the surface of the bacteria). Consistent with our previous results using the asialo- $\square$ NeuA mutant, the sialidase- treated (“enzymatically asialo-”) bacteria had significantly diminished binding to hSiglec-9 (**Figure 3.2**). In contrast, the trypsin-treated serotype III GBS largely retained its ability to bind to hSiglec-9. We thus conclude that serotype III GBS interacts with hSiglec-9 in a Sia-specific manner.

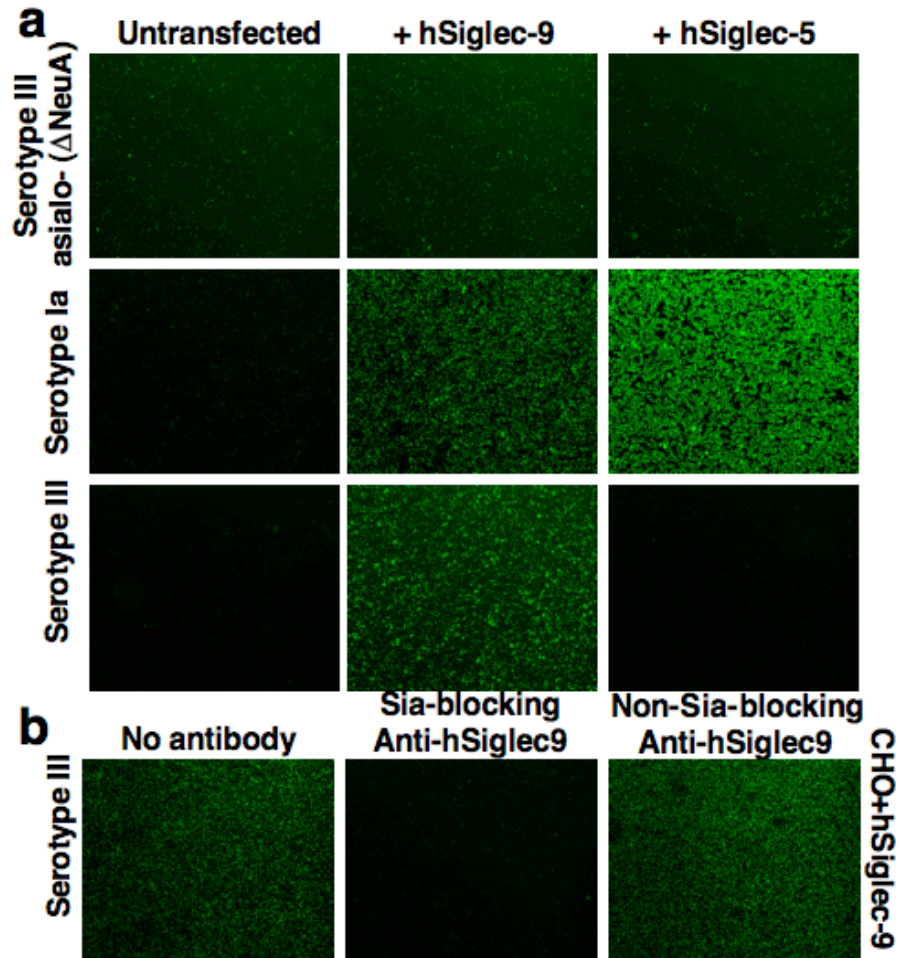
***O-acetylation of GBS CPS Sia alters binding to hSiglecs-5, -7, and -9.*** GBS modify CPS Sias with O-acetyl groups at the 7, 8, and 9 carbon positions. It was previously shown that O-acetylation of Sias present on synthetic or natural glycan probes affects the binding of some Siglecs (Brinkman-Van der Linden and Varki, 2000; Kelm et al., 1998; Sjoberg et al., 1994). To determine if O-acetylation of GBS Sias modifies interaction with hCD33rSiglecs we utilized a similar assay format as

used in **Figure 3.2** with three isogenic serotype III strains: (a) the WT parent strain that expresses O-acetylation on ~50% of total Sias, (b) an isogenic allelic exchange mutant of the O-acetyltransferase,  $\Delta$ NeuD, displaying very little Sia and no O-acetylation, and (c) the  $\Delta$ NeuD mutant transformed with a NeuD expression plasmid harboring a single amino acid mutation in the active site (pNeuD-K123A), which restores sialylation but reduces O-acetylation to ~2% (Lewis et al., 2006). Like the asialo- serotype III  $\Delta$ NeuA mutant, the serotype III  $\Delta$ NeuD mutant with low Sia expression was also unable to bind to hSiglecs-5, -7 and -9 (**Figure 3.3**) nor any of the other hCD33rSiglecs tested (data not shown). The restoration of CPS sialylation with greatly diminished O-acetylation (complemented strain  $\Delta$ NeuD + pNeuD-K123A) altered binding patterns to hSiglecs-5, -7, and -9 in comparison to the WT parent GBS. In the case of hSiglecs-5 and -7, removal of bacterial O-acetylation increased bacterial binding. However, in the case of hSiglec-9, the removal of O-acetylation from CPS Sias had the opposite effect, such that binding of the bacterium was inhibited. All other hCD33rSiglecs tested showed no significant change in binding affinity between the WT highly O-acetylated serotype III GBS parent strain and its low O-acetylated derivative  $\Delta$ NeuD + pNeuD-K123A (data not shown).

***GBS engage CHO cells expressing hSiglec-9 or hSiglec-5 in a Sia- and Siglec-specific manner.*** To determine if the previous binding patterns were representative of GBS interactions with cell surface expressed hCD33rSiglecs, we stably transfected CHO cells with vectors expressing hSiglecs-5 and -9, and compared adherence of sialylated and non-sialylated serotype III FITC-GBS by fluorescence



**Figure 2.3 GBS O-Acetylation alters binding to hCD33rSiglecs.** Binding of WT serotype III GBS, its isogenic  $\Delta$ NeuD mutant, and the  $\Delta$ NeuD mutant complemented with pNeuD-K123A to hSiglecs-5, -7, and -9. Values represent the mean of three separate experiments  $\pm$  SD. P values are two-tailed T-tests comparing each sialylated GBS group with the non-sialylated negative control.



**Figure 2.4 Sialylated GBS adhere to CHO cells expressing hSigslecs in a Sia- and Siglec-specific manner.** (a) FITC-serotype III asialo-  $\Delta$  NeuA (top row), sialylated WT serotype Ia (middle row), and sialylated WT serotype III (bottom row) GBS were allowed to adhere to untransfected CHO cells (first column) or CHO cells expressing either hSiglec-9 (second column) or hSiglec-5 (third column). (b) Adherence of sialylated serotype III FITC-GBS to CHO cells expressing hSiglec-9 +/- 10ug/ml hSiglec-9 Sia-blocking or Sia-non-blocking antibodies. Images were captured with an inverted microscope, appropriate fluorescent filter set, and CCD digital camera. Images are representative of three separate experiments with similar results.



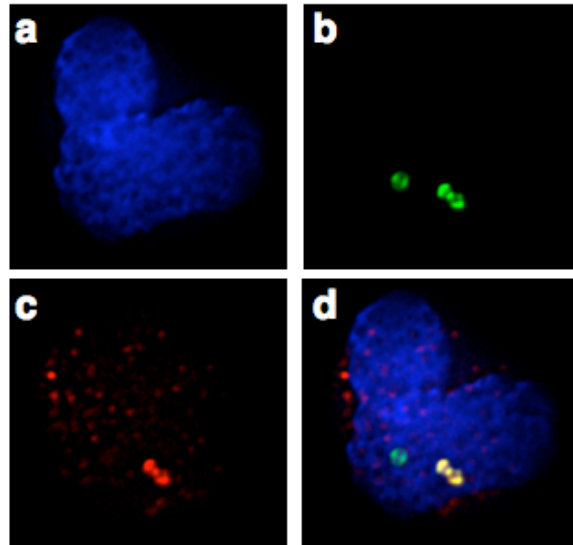
microscopy. A low background level of either sialylated or nonsialylated GBS adhered to the surface of untransfected CHO cells (**Figure 3.4a, first column**). Likewise, the non-sialylated  $\square$ NeuA mutant GBS had a relatively low level of adherence to CHO cells whether or not hSiglecs were expressed (**Figure 3.4a, first row**). In contrast, the WT sialylated serotype Ia and III strains had greatly increased interaction with CHO cells expressing specific hSiglecs compared to the untransfected CHO cells (**Figure 3.4a, second and third rows**). As observed using the *in vitro* binding assays, the serotype Ia GBS strain engaged CHO cells expressing either hSiglec-5 or -9, while the serotype III GBS strain bound only CHO cells expressing hSiglec-9. To verify that this interaction reflects an engagement of the bacterial Sia with the Sia-binding V-set domain of hSiglec-9, we performed additional studies with Sia-blocking and Sia-non-blocking monoclonal antibodies to hSiglec-9. Binding of WT serotype III GBS was inhibited by the addition of monoclonal antibodies that blocks the Sia-binding pocket of hSiglec-9. On the contrary, when Sia-non-blocking antibodies against hSiglec-9 were added, no inhibition of GBS binding was observed (**Figure 3.4b**). Similar results were found in assays using the serotype Ia GBS WT strain (data not shown). Therefore, serotype III GBS interact with cell surface expressed hSiglec-9 in a manner dependent on both bacterial Sia and the ability of hSiglec-9 to interact with Sia. The results obtained from our *in vitro* hSiglec-Fc binding studies closely parallel those obtained using full length hSiglecs expressed on the surface of cells, indicating that our *in vitro* system represents a useful technique for assessing bacterial binding to hCD33rSiglecs.

***Sialylated GBS interact with hSiglec-9 present on the neutrophil surface.***

Neutrophils play an important role in immune defense against GBS infections (Henneke and Berner, 2006). These specialized leukocytes also express relatively high levels of Siglec-9 and Siglec-5 on their surface. To test the ability of GBS to engage Siglecs on the surface of purified human neutrophils, PMN were incubated with sialylated FITC-GBS at 37°C for 5 min followed by washing with 4°C PBS + 1% BSA (to prevent internalization). Neutrophil-GBS interactions were visualized using fluorescent deconvolution microscopy using an hSiglec-9 specific Fab antibody fragment and fluorescently tagged secondary antibody to label Siglec-9. Punctate staining of hSiglec-9 on the surface of human neutrophils was observed in the presence or absence of bacteria (**Figure 3.5**). When sialylated GBS were allowed to interact with the neutrophil surface, clear foci of colocalization between hSiglec-9 staining (red) and GBS (green) were observed (**Figure 3.5**).

## **DISCUSSION**

Group B *Streptococcus* is the leading bacterial pathogen associated with neonatal sepsis and meningitis (Doran and Nizet, 2004). The adaptive immune system of neonates, especially preterm neonates, is impaired in its ability to produce a significant immunoglobulin response to an infection with an encapsulated bacterium such as GBS (Bauer et al., 2002; Henneke and Berner, 2006). The neonate is thus very dependent on its innate immune system to provide protection from GBS infection



**Figure 2.5 GBS attached the surface of human neutrophils colocalize with hSiglec-9.** FITC-GBS were added to isolated purified human neutrophils and allowed to interact for 5 min after which cells were fixed and hSiglec-9 was labeled using fluorescent antibodies. Cells were mounted onto slides and images were acquired using DeltaVision Deconvolution Microscopy and CCD camera. Three images representing the nucleus (a, blue Hoechst stain), bacteria (b, green FITC), and hSiglec-9 (c, red PE) are shown separately and then combined in one merged image (d). The areas of colocalization between FITC-GBS (green) and hSiglec-9 (red) appear yellow in the merged image. The experiment was repeated three times and this image is representative of many GBS-neutrophil interactions.

and disease in concert with passive antibody acquired from the mother. In humans, neutrophils and monocytes/macrophages are important elements of the innate immune defense against invasive bacterial pathogens. These cells express different members of a family of cell surface Sia-recognizing receptors called the CD33rSiglecs (neutrophils siglec-5 and siglec-9; monocytes siglec-5, siglec-7, siglec-9; and macrophages siglec-5, siglec-11) that are capable of sending inhibitory signals to the cells to modulate inflammatory responses.

In this study, we asked whether sialylated GBS engage hCD33rSiglecs that are present on human neutrophils and monocyte/macrophages. We show that all GBS serotypes interact with at least one hCD33rSiglec and that some serotypes engage multiple hCD33rSiglecs. Serotypes Ia and Ib were the most promiscuous, engaging the largest number of hCD33rSiglecs with the greatest strength, whereas serotype II appears to be the least effective binder. We further demonstrate that GBS CPS Sia can engage hSiglec-9 as expressed on the surface of CHO cells and human neutrophils. Each serotype tested displays the same terminal a2,3 linkage of Sia; however, due to the way in which the repetitive subunit is polymerized into the CPS, types Ia and Ib will contain the highest density of Sias over a given length of CPS and serotype II will have the fewest (one Sia per two monosaccharides for Ia and Ib vs. one Sia per five monosaccharides for II, see **Figure 3.1**). Most glycan-protein interactions have low binding affinities when compared to protein-protein interactions and thus the capacity of type Ia and Ib to form dense arrays of Sias, in addition to having the proper binding epitope, may be essential for creating sufficient avidity for strong binding.

Careful analysis of the specificity of the serotype III GBS interaction with hSiglecs revealed that biochemical and genetic perturbation of bacterial sialylation or Sia O-acetylation altered Siglec binding. Cell-based assays confirmed and extended the *in vitro* studies, showing that hSiglec-9-transfected CHO cells-GBS interactions required both bacterial sialylation and accessibility of the hSiglec-9 Sia-binding domain for interaction with type III GBS. Furthermore, the presence of O-acetylation of CPS Sias altered the binding of GBS to hSiglecs-5, -7, and -9. In the context of the GBS CPS, O-acetylation blocks binding to hSiglecs-5 and -7 while promoting binding to hSiglec-9. Further studies are needed to determine the precise mechanism(s) by which O-acetylation modifies hSiglec-GBS binding. It may be that O-acetyl modification of GBS CPS Sias directly changes the binding affinity of the V-set domains for the Sias, or alternatively, alters the three dimensional architecture of the CPS (Kadirvelraj et al., 2006), with secondary effects on the GBS-hSiglec binding interaction.

CD33rSiglecs contain an ITIM and ITIM-like motif that can be phosphorylated and recruit Src homology phosphatases SHP-1 and SHP-2 that are thought to play a role in downregulation of cell activation (Avril et al., 2004; Ikehara et al., 2004). Numerous *in vitro* studies have shown the capacity of CD33rSiglecs to act in an inhibitory fashion including negative regulation of T-cell signaling (Ikehara et al., 2004) and inhibition of NK cell toxicity (Nicoll et al., 2003). Therefore, it is possible that the GBS CPS serotypes have evolved to engage hCD33rSiglecs on neutrophils and monocyte/macrophages in order to exert suppressive effects upon the innate

immune response. Furthermore, hSiglec-9 can activate accelerated apoptosis in human neutrophils (von Gunten et al., 2005), an additional mechanism for avoidance of phagocytic killing. Conversely, it has also recently been shown that many CD33rSiglecs, including hSiglec-9, can act as endocytic receptors (Biedermann et al., 2006) that could theoretically aid in the internalization of bound GBS. Thus evolutionary forces modulating hSiglec-GBS interactions could be operative from both the pathogen and host perspectives.

Sia expression by GBS and other pathogens plays an important role in resistance to host complement mediated killing, perhaps through binding of host factor H to impair complement deposition on the bacterial surface (Aoyagi et al., 2005; Marques et al., 1992; Wessels et al., 1995). In addition to the data we present here supporting the interaction between GBS CPS Sias and multiple CD33rSiglecs, it was recently shown that hSiglec-5 expressed on CHO cells can bind to sialylated *Neisseria meningitides* (Jones et al., 2003) and hSiglec-7 present on dendritic cells can bind to an isolate of sialylated *Campylobacter jejuni* (Avril et al., 2006). Therefore, it may be that GBS and other bacteria have evolved a shared virulence mechanism of decorating their surfaces with a human-like monosaccharide, the Sia Neu5Ac, which acts as a ligand for both factor H and hCD33rSiglecs. In this fashion, the Sia-expressing bacterial pathogens could simultaneously interfere with complement and cellular components of innate immunity. In the particular case of GBS, clinical disease is seen in the neonate and other special populations including pregnant women, the elderly and diabetics (Edwards et al., 2005; Palazzi et al., 2004). Future studies could

examine both the patterns of hCD33rSiglec expression and the cellular responses mediated by these receptors in these populations for correlations with increased susceptibility to GBS infection.

## METHODS

***Bacteria and growth conditions.*** GBS wild-type (WT) strains of serotypes Ia (A909), Ib (M709), II (DK23), III (COH1), V (NCTC10/84) and VI (NT-6) are well-characterized isolates from human neonates with invasive infections. Generation of the  $\Delta$ NeuA and  $\Delta$ NeuD mutants in serotype III strain COH1 was previously reported (Lewis et al., 2006; Lewis et al., 2004). GBS were grown in Todd-Hewitt broth (THB), pH 7.5, or on THB agar plates (THA). For antibiotic selection, 10  $\mu$ g/ml erythromycin (Em) or 2.5  $\mu$ g/ml chloramphenicol (Cm) were used. *Escherichia coli* strains were grown in Luria-Bertani broth (LB); antibiotic selection employed 500  $\mu$ g/ml Em or 50  $\mu$ g/ml Cm. For functional assays, until otherwise noted, bacteria were grown to early-exponential phase in THB, washed three times with pyrogen-free phosphate-buffered saline (PBS), resuspended in appropriate buffers, and adjusted to the desired concentrations using a spectrophotometric method confirmed by pour-plate colony counts. For enzymatic removal of CPS Sias, GBS were incubated in sterile PBS with 100mU/ml *Arthrobacter ureaficiens* sialidase (AUS) for 1 h at 37°C and then washed 3 times with PBS. Trypsin-treated bacteria were incubated with 0.5% trypsin + EDTA in PBS for 30 min at 37°C and washed 5 times with PBS. Prior to Chinese hamster ovary (CHO) cell binding experiments using Sia-blocking and non-

Sia-blocking antibodies, GBS were preincubated for 10 min in PBS + 5% normal goat serum and then washed twice.

***GBS FITC labeling.*** GBS were grown overnight and diluted 1:100 in 50 ml of THB, grown to early exponential phase, pelleted and washed three times with sterile PBS pH 7.4, and heat-killed by incubation at 56°C for 40 min. GBS were then washed in 50 mM carbonate buffer pH 8.0, resuspended in 5 ml carbonate buffer pH 8.0 + 0.1% FITC, and incubated for 1 h at 37°C. Bacteria were extensively washed in PBS to remove trace amounts of free FITC and then resuspended in assay buffer (20 mM HEPES, 150 mM NaCl, 1% BSA, pH 7.4). GBS-FITC were then enumerated using a bacterial cytometer and a fluorescent microscope.  $1 \times 10^6$  GBS-FITC were resuspended in PBS + 1% BSA and analyzed by flow cytometry (BD FACS caliber) to verify that bacterial staining was uniform throughout each sample.

***Siglec-Fc binding assay.*** Immulon 4 ELISA plates were coated with 0.5 mg/ml Protein A in coating buffer (50 mM carbonate buffer pH 9.5) overnight at 4°C. Wells were washed three times with assay buffer (20 mM HEPES, 150 mM NaCl, 1% BSA) then blocked with assay buffer for 1 h at room temperature. Human CD33rSiglec-Fc chimeras diluted in assay buffer were added to individual wells at 0.5 mg/ml and allowed to adhere for at least 3 h at room temperature. Wells were washed three times with assay buffer and  $1 \times 10^7$  FITC-GBS suspended in assay buffer were added to each well and centrifuged. Bacteria were allowed to adhere for 10 minutes at



37°C. Initial fluorescent intensity was verified, wells washed to remove unbound bacteria, and the residual fluorescent intensity (ex. 488 / em. 530) measured using a CytoFluor<sup>II</sup> fluorescent plate reader.

***Soluble receptors and stable cell expression.*** Soluble hSiglec-Fc chimeras were produced as previously described (Angata and Varki, 2000). CHO-K1 cells stably expressing either full length hSiglec-5 or hSiglec-9 were produced by transfecting WT CHO-K1 cells with either pcDNA3.1(+)-Neo-hSiglec-5 or pcDNA3.1(+)-Hygro-hSiglec-9 using Fugene-6 transfection reagent per manufacturers directions. The expression plasmid pcDNA3.1(+)-Hygro-hSiglec-9 was produced by subcloning human SIGLEC-9 from pcDNA3.1(+)-Neo into pcDNA3.1(+)-Hygro. Stable transfectants were selected by growth in F12 (Ham's) medium + 1 mg/ml G418 or 800 mg/ml hygromycin. Stably expressing CHO+ hSiglec-9 were further selected using Flow Activated Cell Sorting at the UCSD/Veteran's Administration San Diego Flow Cytometry Core.

***CHO cell adhesion assay.*** CHO-K1 cells +/- hSiglec expression were grown to 80% confluency in 100 mm cell culture dishes, lifted using PBS + 2 mM EDTA, and  $2.5 \times 10^5$  cells added to each well of 24 well plates and allowed to grow overnight. CHO cells were washed once with 37°C serum free F12 (Ham's) medium and resuspended in F12 (Ham's) medium +/- 10 mg/ml antibodies for 10 min at 37°C and 5% CO<sub>2</sub>. FITC-GBS were added at an multiplicity of infection (MOI) of 100:1, spun

down onto the cell monolayer, and allowed to adhere for 15 min at 37°C and 5% CO<sub>2</sub>. Nonadherent bacteria were removed by repeated washing and fluorescent images were acquired with a 5X objective lens using a Zeiss Axiovert 40 inverted microscope with appropriate fluorescent filters and CCD camera.

***Isolation of human neutrophils.*** Venous blood was drawn from healthy volunteers under institutional IRB approval using vacutainers containing EDTA. Neutrophils were isolated by density gradient centrifugation using Polymorphprep™ solution (Axis Shield PoC AS, Oslo, Norway) according to the manufacturer's instructions. The neutrophil layer was washed with pyrogen-free PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> at 4°C; contaminating erythrocytes were hypotonically lysed. Subsequently, neutrophils were washed twice and finally resuspended in Hank's Balanced Salt Solution without Ca<sup>2+</sup> and Mg<sup>2+</sup>. Viability of cells exceeded 95% as assessed by trypan blue exclusion.

***Human neutrophil and GBS fluorescent microscopy.*** FITC-GBS were added to isolated human neutrophils in RPMI 1640 without serum at an MOI of 10:1, centrifuged to initiate contact, incubated at 37°C in 5% CO<sub>2</sub> for 5 min, then washed with ice cold PBS + 1% BSA and spun down at 100 x g for 5 min at 4°C. Neutrophils were fixed using 2% paraformaldehyde in PBS for 10 min at 4°C. Human neutrophil Siglec-9 was labelled using mouse anti-hSiglec-9 antibody Fab fragments at 5 mg/ml

in PBS + 1% BSA for 30 min on ice. Anti-hSiglec-9 Fab fragments were prepared using BD Pharmingen mouse anti-hSiglec-9 clone E10-286 and Pierce ImmunoPure IgG1 Fab and F(ab)<sub>2</sub> preparation kit according to the manufacturer's instructions for producing Fab antibody fragments. Neutrophils were washed, goat anti-mouse PE antibody was added in PBS + 1% BSA, and the cells incubated at 4°C for 30 min. The cells were washed again, Hoechst stain added at 1:1000 dilution in PBS for 2 min on ice, then cells rewashed and resuspended in 50 ml of PBS + 1% BSA. Cells were added to glass slides and allowed to dry in the dark at 4°C and coverslips were mounted using Gelvatol. Images were captured with a DeltaVision Restoration microscope system (Applied Precision Inc., Issaquah, WA, USA) using a Photometrics Sony Coolsnap HQ charged-coupled device (CCD) camera system attached to an inverted, wide-field fluorescent microscope (Nikon TE-200). Optical sections were acquired using a 100x (NA 1.4) oil immersion objective in 0.2µm steps in the z-axis using the attached Applied Precision Inc. motorized stage. The fluorescent markers were excited with a standard mercury arc lamp and fluorescence was detected using a standard DAPI, FITC, Rhodamine filter set.

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## **Chapter III**

# **CD33-related Siglec Ligation by Sialic Acids Dampens Innate Responses: Implications for the Pathogenicity of Sialylated Microorganisms**

## INTRODUCTION

Cellular recognition and balanced regulation are essential components of the vertebrate innate immune system that allow it to respond appropriately when challenged with microorganisms, while minimizing collateral damage to host cells and tissues. Though much is known about antagonistic interactions of host innate immune cells with microbes, less is understood about mechanisms that dampen excessive innate immune responses. One family of proteins postulated to play a role in intrinsic host cell recognition and negative regulation of the inflammatory response are the CD33-related Siglecs (CD33rSiglecs).

The sialic acid (Sia)-recognizing immunoglobulin (Ig) superfamily lectins (Siglecs) are a family of structurally-related type I transmembrane proteins mostly expressed on cells of the immune system (Varki, 2007) (Crocker et al., 2007). The CD33rSiglecs are a rapidly evolving subgroup of the Siglecs, which are expressed in a cell type-specific fashion, predominantly on mature cells of the innate immune system, such as neutrophils, eosinophils, macrophages, monocytes, dendritic cells, natural killer cells, and mast cells. The extracellular regions of CD33rSiglecs consist of an N-terminal Ig-like V-set domain responsible for binding sialylated glycoconjugates, and a variable number of Ig-like C2-set domains. The CD33rSiglecs recognize common Sia-capped glycans that are attached to proteins and lipids present on the surface of all mammalian cells. Individual CD33rSiglecs have characteristic Sia-binding specificity profiles, but have relatively low binding affinities (typically in the millimolar range), requiring clustering of both ligands and receptors to mediate functional interactions.

Given the frequent expression of Sias on many successful human pathogens such as *Neisseria meningitidis* (Hackenthal, 1969) (Bhattacharjee et al., 1975) and Group B *Streptococcus* (GBS) (Wessels et al., 1987); (Schifferle et al., 1985), coupled with the preference of human CD33rSiglecs for *N*-acetylneuraminic acid (Neu5Ac), the type of sialic acid found on such pathogens, it was originally thought that the purpose of CD33rSiglecs on innate immune cells was to recognize and respond to such pathogens (Jones et al., 2003). However, preferential recognition of Neu5Ac by human CD33rSiglecs appears to be the recent outcome of rapid evolution of Sia-binding specificities following the human-specific loss of expression of N-glycolylneuraminic Acid (Neu5Gc) (Chou et al., 1998) (Irie et al., 1998) (Muchmore et al., 1998) (Angata et al., 2001; Chou et al., 2002)), the other major cell surface Sia in non-human primates. As CD33rSiglecs of innate immune cells in chimpanzees and gorillas (our closest evolutionary cousins) strongly prefer binding to Neu5Gc, and Neu5Gc is never synthesized by bacterial pathogens, we concluded that that the natural ligands of CD33rSiglecs are likely to be endogenous Sias, and not those expressed on microbes (Sonnenburg, Altheide, Varki 2004).

All CD33rSiglecs except Siglec-14 have two conserved cytoplasmic tyrosine-based motifs, comprising a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) with the canonical sequence (I/L/V)<sub>x</sub>Yxx(L/V) and a membrane-distal ITIM-like motif. Upon phosphorylation by Src-family kinases, these motifs can recruit protein tyrosine phosphatases SHP-1 and SHP-2. Classically, receptors with ITIMs act as inhibitory receptors and suppress activation signals that

originate from tyrosine-based activation motifs (ITAMs) through recruitment of tyrosine and inositol phosphatases (Gergely et al., 1999; Ravetch and Lanier, 2000). This combination of extracellular Sia-binding domains that recognize ubiquitously expressed endogenous host Sias with intracellular inhibitory signaling motifs lead us to propose that CD33rSiglecs function primarily as molecules that recognize “self”, in order to dampen innate immune responses (Sonnenburg, Altheide, Varki 2004, Angata and Varki 2006). Further, we have suggested that pathogens that express sialic acids may simply be taking advantage of this mechanism, to masquerade as “self” (Angata and Varki 2006). Consistent with this general hypothesis, studies of the interaction of Siglec-7 on natural killer cells with GD3 on malignant melanoma cells indicated that Sia recognition could be a mechanism for tumors to evade this type of innate immune response (Nicoll et al., 2003).

Many *in vitro* studies have demonstrated the importance of the ITIM and ITIM-like motif of CD33rSiglecs in inhibiting cellular activation and proliferation (Avril et al., 2004; Avril et al., 2005; Balaian et al., 2003; Ikehara et al., 2004; Lajaunias et al., 2005; Paul et al., 2000; Ulyanova et al., 2001; Vitale et al., 1999), Siglec-dependent adhesion (Biedermann et al., 2007; Taylor et al., 1999; Walter et al., 2005), and induction of apoptosis (Nutku et al., 2003; von Gunten et al., 2005). However, the vast majority of these “functional” studies on CD33rSiglecs were carried out using cross-linking antibodies as ligands, and/or on Siglec-transfected cell lines. Such antibodies are likely not the natural ligands of CD33rSiglecs, and they have the additional confounding effect of inducing rapid Siglec endocytosis in many

circumstances (Bernstein, 2002; Nguyen et al., 2006). We recently showed that mice deficient in Siglec-F (a CD33rSiglec expressed on mouse eosinophils and activated T-cells), showed increased bone-marrow, blood, and tissue eosinophilia in a model of induced lung allergy (Zhang et al., 2007). Although this result is again consistent with the role of CD33rSiglecs as negative regulators of innate immunity, it still remains to be proven that recognition of Sias by CD33rSiglecs is actually required for down-regulation of the host leukocyte response.

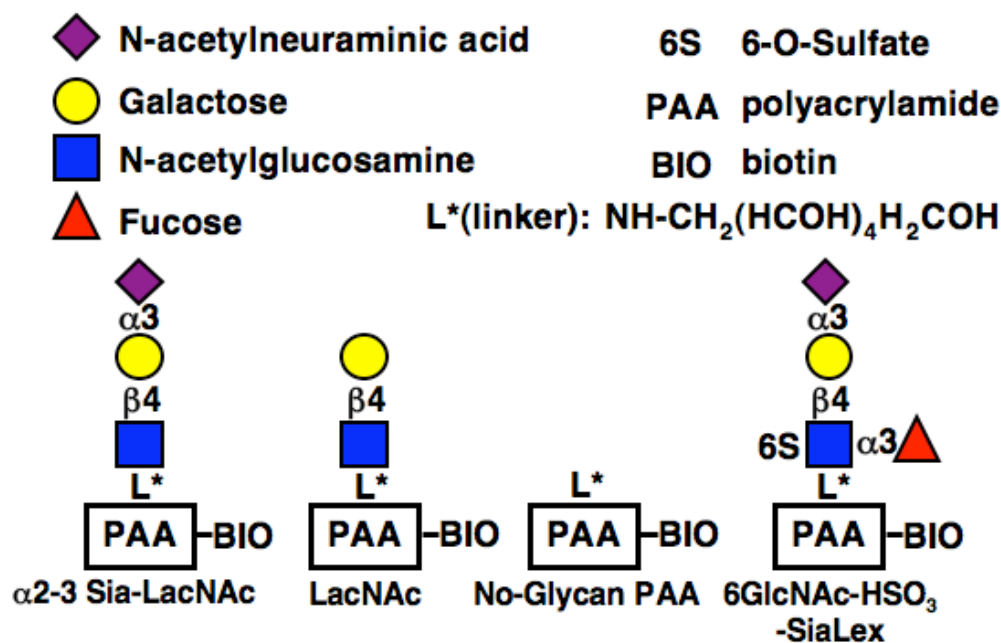
One reason behind the paucity of experimental evidence of signaling mediated by CD33rSiglec binding of sialylated ligands is that these receptors are also involved in interactions with Sias on the autologous cell surface (so-called “*cis*”-interactions) (Collins et al., 2004; Freeman et al., 1995; Hanasaki et al., 1995; Razi and Varki, 1998). As local concentrations of Sias on immune cell surfaces are high, these data initially suggested that interactions with Sias on opposing cell surfaces (*trans*-interactions) were not possible without removal of the *cis* Sias. However, recent studies have shown that high densities of Sias presented as highly multimerized polyvalent probes (Collins et al., 2006), adjacent cell surfaces (Collins et al., 2004), or bacterial capsular polysaccharides (Carlin et al., 2007), can indeed engage Siglecs *in trans*, by competing out the *cis*-Sia ligands at sites of contact.

Here we directly test for the first time the hypothesis that ligation of CD33rSiglecs by Sias *in trans* is responsible for the negative regulatory signals transmitted to Siglec-expressing innate immune leukocytes. We show that the functional outcome of such Sia engagement *in trans* has implications not only for

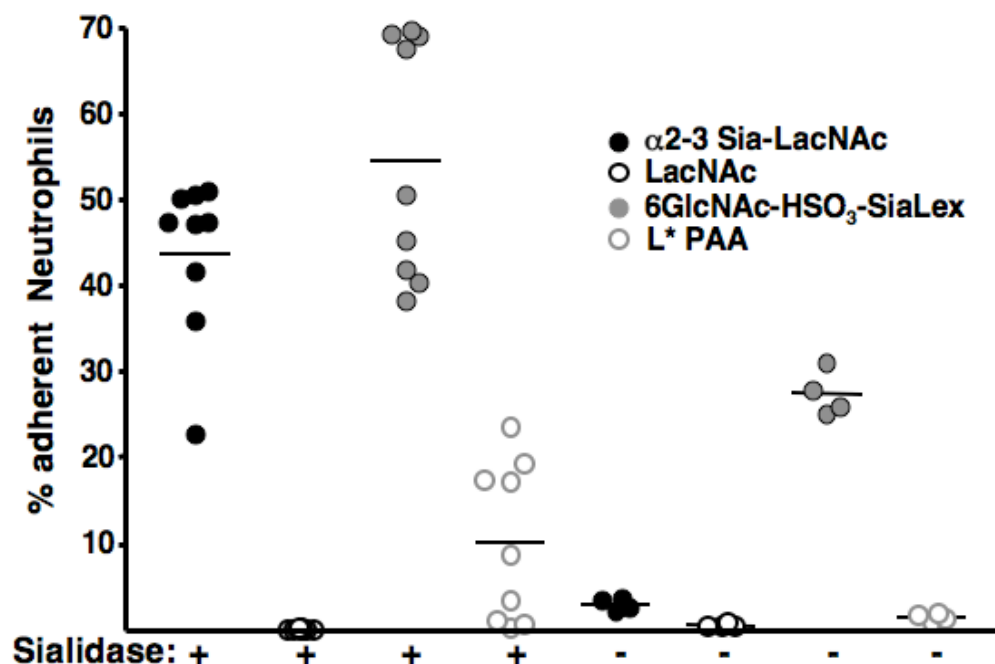
dampening cellular innate immune responses against host cells, but can also be subverted by pathogenic sialylated microorganisms such as GBS. We begin by studying interactions of native neutrophils with immobilized Sias, and then focus on the specific interactions involving the major human neutrophil CD33rSiglec, Siglec-9, including its interactions with serotype III GBS.

## RESULTS AND DISCUSSION

*Human neutrophils bind multimerized Sias when presented in trans on ELISA plates.* In order to overcome *cis*-interactions of CD33rSigelecs with Sias on neutrophil surfaces, we generate a highly multimerized two-dimensional glycan-coated surface by binding polyvalent glycan-containing polyacrylamide probes with or without Sias (**Figure 3.1**) to ELISA plates. In order to establish that the CD33rSigelecs expressed by human neutrophils (predominantly Siglec-5 and Siglec-9), could engage sialylated glycans presented in this manner, we first pre-treated the neutrophils with sialidase to remove cell surface Sias and eliminate the potential for *cis*-interactions. When such sialidase-treated primary human neutrophils were added to polyacrylamide probe coated wells, more than 40% of cells adhered to wells containing either of two sialylated glycans (Sia $\alpha$ 2-3LacNAc or GlcNAc6SO<sub>3</sub>SialylLex) but did not bind to wells coated with LacNAc, a glycan lacking Sias, or to the control polyacrylamide backbone without any glycans (**Figure 3.2**). Even in the absence of sialidase pretreatment almost 30% of neutrophils continued to adhere to the wells coated with



**Figure 3.1 Multivalent glycan polyacrylamide probes.** Cartoon representation of the multivalent glycan-bearing polyacrylamide probes. Two probes containing Sias and two probes without Sias were utilized in the assays. Each polyacrylamide probe contains approximately 8 oligosaccharides. Probes (purchased from GlycoTech) were immobilized on ELISA plates by overnight incubation at 4°C in 50mM carbonate buffer pH 9.4.

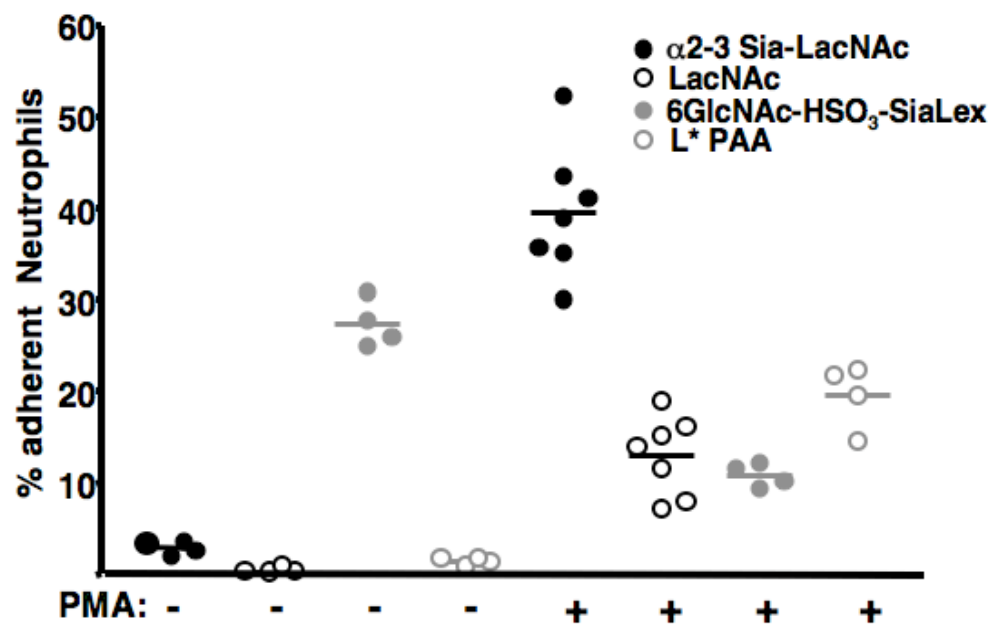


**Figure 3.2 Human neutrophils bind multimerized Sias when presented in trans on ELISA plates.** Human neutrophils fluorescently labeled with Calcein AM were pretreated with or without *Arthrobacter ureafaciens* sialidase and added to ELISA wells coated with glycans conjugated to polyacrylamide in a multivalent fashion. The fluorescent intensity (FI) was measured in each well before and after washing away non-adherent neutrophils. The percent adherent neutrophils was determined by dividing the (final FI) by (initial FI) and multiplying by 100. Graph represents two experiments for sialidase-treated neutrophils and one experiment for non-sialidase treated neutrophils performed in quadruplicate. The line in each group represents the mean.



GlcNAc6SO<sub>3</sub>SialylLex, a preferred ligand of Siglec-9 (Consortium for Functional Glycomics, <http://www.functionalglycomics.org>) (**Figure 3.1**). In contrast, we observed a reduction of neutrophil adherence to Sia $\alpha$ 2-3LacNAc, a general ligand for both Siglec-5 and Siglec-9, though this modest binding was still significantly greater than background binding to probes that lacked Sia. Thus, the adherence of neutrophils to a Sia-glycan coated ELISA plate in the absence of sialidase treatment is contingent both upon a high density of Sia-containing ligands and upon the affinity for each individual ligand. Though antibody inhibition studies are needed to confirm the Siglecs involved, this is the first direct evidence that CD33rSiglecs can mediate adhesive interactions with Sias *in trans*, even without removal of competing *cis* Sias.

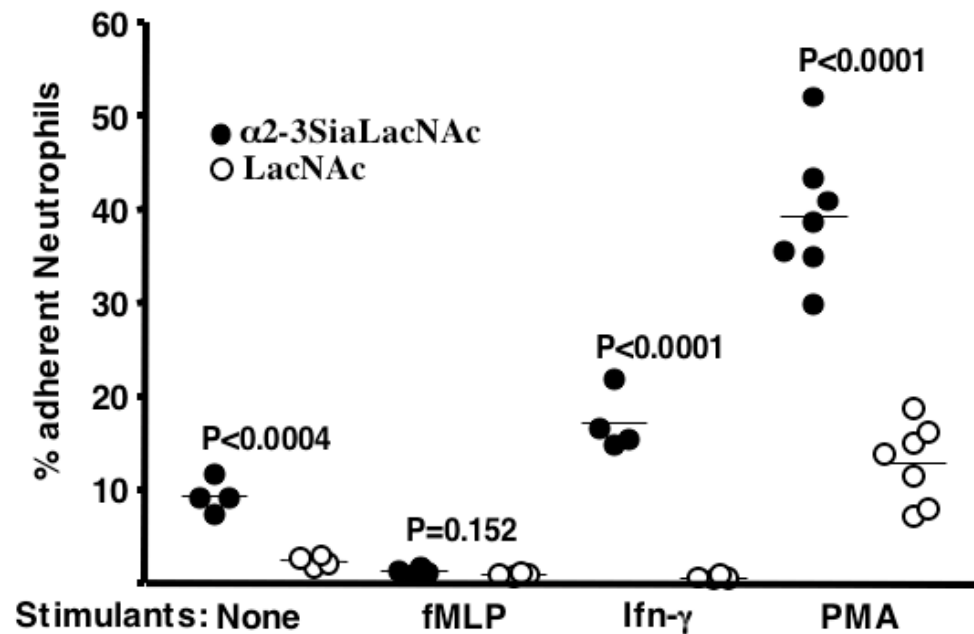
***Activated neutrophils have increased binding to Sia<sub>2-3</sub>LacNAc coated surfaces.*** Previous studies have shown that activation of neutrophils by stimulants such as phorbol 12-myristate 13-acetate (PMA), which acts on protein kinase C, results in proteolytic cleavage of the highly sialylated CD43 glycoprotein from the neutrophil cell surface (Campanero et al., 1991; Lopez et al., 1998; Remold-O'Donnell and Parent, 1994; Rieu et al., 1992). PMA also causes mobilization of endogenous sialidases to the neutrophil cell surface, cleaving Sias and perhaps contributing to neutrophil spreading, deformability, and adhesion (Cross and Wright, 1991). We hypothesized that neutrophil activation would thus decrease the local concentration of Sias on the cell surfaces, allowing for increased CD33rSiglec interactions *in trans*. In order to test this hypothesis, we pretreated primary human neutrophils with PMA prior to studying interactions with the immobilized glycans. Following PMA treatment



**Figure 3.3 PMA activated neutrophils have increased binding to Sia $\alpha$ 2-3LacNAc coated surfaces.** (a) Human neutrophils with or without pretreatment with PMA were added to probe coated ELISA wells and allowed to adhere and percent adherent neutrophils was calculated as described in Figure 3.2. PMA, phorbol 12-myristate 13-acetate.

there was a general non-specific increase in neutrophil binding to coated ELISA wells that lacked Sias (**Figure 3.3**). However, PMA treatment significantly increased binding to the Sia $\alpha$ 2-3LacNAc coated wells from < 10% of cells to ~40% of cells (**Figure 3.3**). In contrast, binding of human neutrophils to GlcNAc6SO<sub>3</sub>SialylLex coated wells decreased when the cells were treated with PMA, such that mean binding did not differ from that observed in LacNAc-coated wells (**Figure 3.3**). Treatment of human neutrophils with immunostimulants has been shown to cause increased surface expression of Siglec-5 (Erickson-Miller et al., 2003) and its effect on Siglec-9 is unknown. Thus, divergent results for neutrophil binding to the Sia-glycans following PMA pretreatment could be the result of changes of cell surface Siglec expression, changes in membrane mobility, and/or altered surface expression of other receptors.

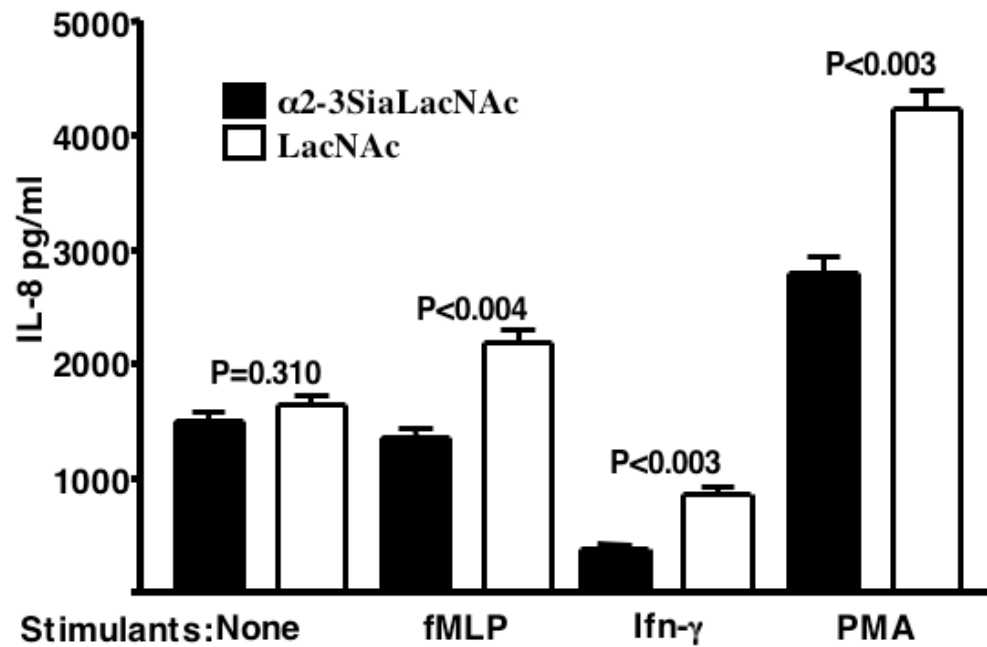
We next tested the capacity of other stimulants to increase human neutrophil binding to sialylated glycans. Neutrophils were incubated with or without fMLP, interferon-gamma (IFN- $\gamma$ ) or PMA and adherence measured as described above. Although < 10% of neutrophils adhered to wells when no additional stimuli were added, a significant difference in binding between the sialylated glycan and the LacNAc control remained (**Figure 3.4**). In addition, treatment with either IFN- $\gamma$  or PMA increased binding to the Sia $\alpha$ 2-3LacNAc glycans. In contrast, addition of fMLP abolished neutrophil adhesion to the glycan-coated plates (**Figure 3.4**). Overall, while much of the capacity of primary human neutrophils to engage Sias *in trans* in this assay remains masked at baseline, activation using common proinflammatory stimuli



**Figure 3.4 Activated neutrophils have increased binding to Sia $\alpha 2-3\text{LacNAc}$  coated surfaces.** Human neutrophils pretreated with or without fMLP, Ifn- $\gamma$ , or PMA were analyzed for adherence to probe coated wells as described above. Graphs represent one experiment performed in quadruplicate, lines are equal to means neutrophil adherence, statistical analysis was by Student's t-test. PMA, phorbol 12-myristate 13-acetate; fMLP, formylated methionine leucine proline tripeptide; Ifn- $\gamma$ , interferon-gamma.

generally increases these interactions, possibly by decreasing neutrophil cell surface sialylation and/or by increasing CD33rSiglec expression. Further studies are needed to examine if activation of neutrophils prior to presentation to sialylated glycans will further enhance these interactions. For the moment, our data are consistent with the hypothesis that CD33rSiglecs on neutrophils will have increased capacity to bind to Sias *in trans* during inflammatory activation, allowing improved recognition of other host cells as “self”.

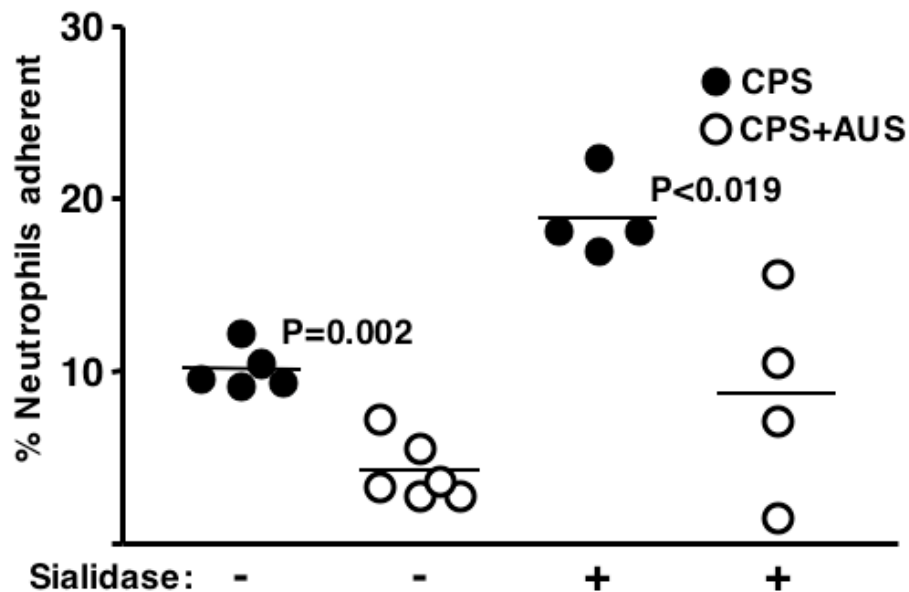
***Sia binding in trans decreases neutrophil secretion of the proinflammatory cytokine interleukin-8.*** In order to measure the functional consequences of human neutrophil engagement of Sias presented *in trans*, we measured the release of the proinflammatory cytokine interleukin-8 (IL-8) by neutrophils interacting with either Sia $\alpha$ 2-3LacNAc or LacNAc glycans. Human neutrophils that were in contact with either of the probes were treated with or without fMLP, IFN- $\gamma$  or PMA as described above, and incubated at 37°C for 6 h. Supernatants were collected and the concentration of IL-8 measured by ELISA assay. Cells that had been in contact with the sialylated glycan Sia $\alpha$ 2-3LacNAc secreted significantly less IL-8 than neutrophils contacting the non-sialylated LacNAc glycans (Figure 3.5). No significant difference was observed in the (lower) levels of IL-8 secretion by untreated neutrophils interacting with glycans  $\pm$  Sia (Figure 3.5). IL-8 is released by activated neutrophils and macrophages during inflammatory responses, and is responsible for recruiting additional leukocytes to areas of inflammation (Baggiolini and Clark-Lewis, 1992). As activated neutrophils secrete less IL-8 when they are contacting Sias *in trans*, this



**Figure 3.5** Sia binding *in trans* decreases neutrophil secretion of the proinflammatory cytokine IL-8. Human neutrophils were allowed to adhere to ELISA wells coated with Sia $\alpha$ 2-3LacNAc or LacNAc oligosaccharides and subsequently treated with or without fMLP, Ifn- $\gamma$ , or PMA and incubated at 37°C for 6 hours. Supernatants were collected, and IL-8 was measured by ELISA. Graph contains data from one experiment performed in triplicate, error bars represent the standard error of means, statistical analysis was by Student's t-tests. PMA, phorbol 12-myristate 13-acetate; fMLP, formylated methionine leucine proline tripeptide; Ifn- $\gamma$ , interferon-gamma.

effect is consistent with Sia engagement of CD33rSiglecs sending inhibitory signals. Further studies with blocking and non-blocking antibodies will help to define which CD33rSiglecs are involved in these responses (the most likely candidates being Siglecs -5 and -9).

***Neutrophils adhere to the capsular polysaccharide of GBS in a Sia-specific manner.*** Another way that Sias can be presented to neutrophils in a high-density format is on the surface of sialylated bacteria. The capsular polysaccharide (CPS) of serotype III GBS is a dense collection of glycans that covers the bacterial surface and contains a high concentration of terminal Sia $\alpha$ 2-3LacNAc oligosaccharides, which interact highly selectively with Siglec-9 (Carlin et al., 2007). To determine if human neutrophils adhere to the GBS CPS in a Sia-dependent manner, we isolated purified CPS from serotype III GBS, deposited it on wells of ELISA plates, and then treated some of the wells with sialidase to remove terminal Sias. Neutrophils ( $\pm$  sialidase pretreatment) were then allowed to adhere to these GBS CPS-coated wells. Neutrophils, whether or not pretreated with sialidase, adhered significantly more to native GBS CPS than to GBS CPS from which Sias had been removed (Figure 3.6). The observation that neutrophils bound sialylated CPS even without prior sialidase treatment indicates that Sias on the surface of GBS are densely arrayed enough to overcome cis-Sia interactions and allow Siglec-9 binding in trans. It is also possible that other components of GBS cause stimulation of neutrophils, altering the concentration of neutrophil surface Sia and/or CD33rSiglec expression, thereby further enhancing in trans interactions.

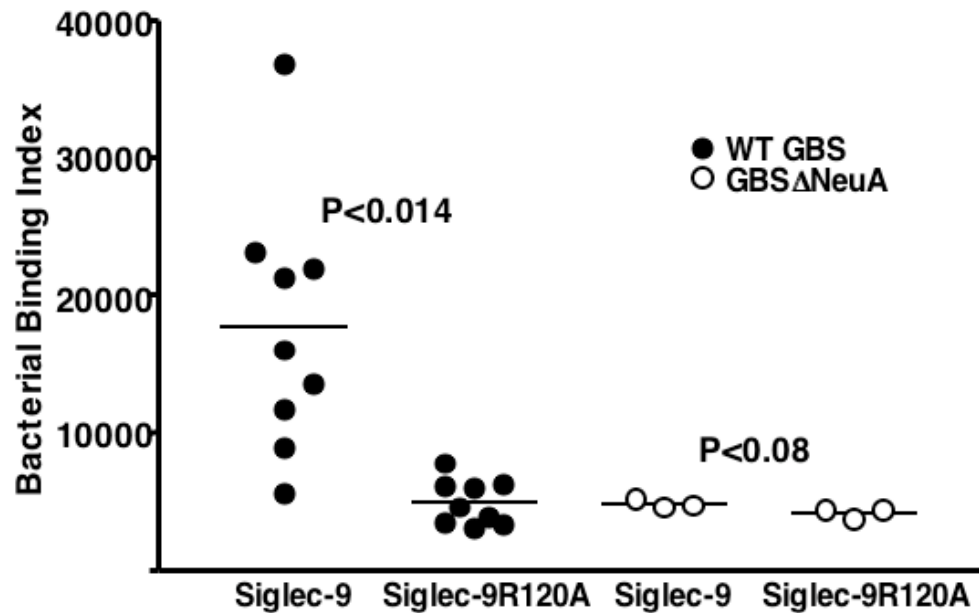


**Figure 3.6 Neutrophils adhere to the capsular polysaccharide of *group B Streptococcus* in a Sia-specific manner.** The capsular polysaccharide (CPS) of serotype III GBS strain COH1 was purified and immobilized in ELISA wells. The CPS was then treated with or without bacterial sialidase. Neutrophils with or without sialidase pretreatment were added to the wells and allowed to adhere before washing away non-adherent neutrophils. Graph represents data from one experiment, with statistical analysis by Student's t-test.



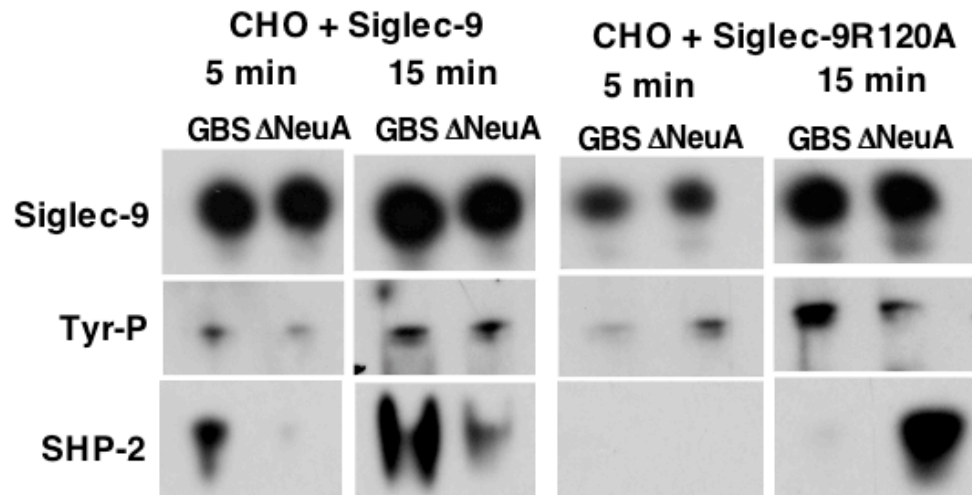
***CHO cells expressing Siglec-9 but not Siglec-9R120A functionally engage sialylated GBS.*** Further studies in isolated neutrophils were limited by complexities of the many simultaneous events that occur when these cells interact with GBS as well as the existence of multiple CD33rSiglecs. To better isolate the role of Siglec-9 interactions with GBS serotype III CPS, CHO cells were transfected with constructs expressing hSiglec-9 (CHO-Siglec-9) or hSiglec-9 possessing a single arginine mutation (R120A) in the Sia-binding pocket that abolishes Sia-binding (CHO-Siglec-9R120A). The stably transfected CHO cells were then infected with FITC-labeled sialylated WT GBS, or its isogenic mutant strain ( $\square$ NeuA) lacking a key enzyme in the Sia biosynthesis pathway ( $\square$ NeuA) required for Sia expression on the CPS. Bacteria were allowed to adhere to the cell monolayer and non-adherent bacteria were removed by repeated washing. CHO cells were then lifted using PBS + 5mM EDTA, and analyzed by flow cytometry for the presence of adherent FITC-GBS. GBS adhered to CHO cells only when the bacteria expressed Sias and the CHO cells expressed the wild-type version of Siglec-9 that could bind to Sias (**Figure 3.7**). As with neutrophils, the surface concentration of Sias on the bacteria was sufficient to engage a CD33rSiglec (Siglec-9) *in trans*, without sialidase pretreatment of the CHO cell surface.

Next, we analyzed the effect of Sia-GBS engagement of Siglec-9 on tyrosine-phosphorylation and recruitment of the protein tyrosine phosphatase SHP-2. Wild-type (WT) sialylated GBS and the Sia-deficient  $\square$ NeuA mutant were centrifuged down onto CHO-Siglec-9 or CHO-Siglec-9R120A cells to initiate bacterial contact with the



**Figure 3.7 CHO cells expressing Siglec-9 but not Siglec-9R120A bind Sia-GBS.** (a) CHO cells expressing hSiglec-9 (CHO-Siglec-9) or an arginine mutant (R120A) hSiglec-9R120A (CHO-Siglec-9R120A) that cannot bind Sias were incubated with FITC-labeled WT GBS or the Sia-deficient GBSΔNeuA. CHO cells were lifted and analyzed by flow cytometry for adherence of FITC-GBS. Bacterial binding index (BBI) is the (% CHO cells that are FITC positive) multiplied by (mean fluorescent intensity of the positive cell population). Binding experiments were performed three times with WT GBS and once with GBSΔNeuA in triplicate, and lines represent the mean BBI. Statistical analysis was by Student's t-test.

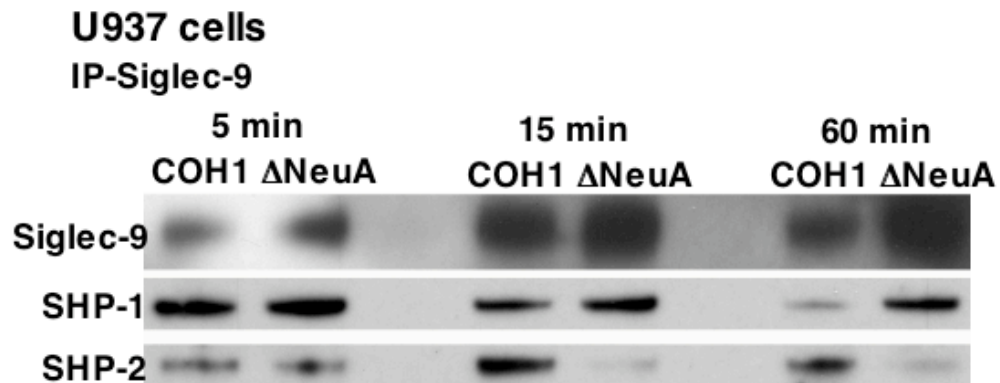
cell surface at 37°C. At the indicated times points, cells were washed with ice cold wash buffer, lysed at 4°C, Siglec-9 immunoprecipitated, and tyrosine-phosphorylation and the co-immunoprecipitation of SHP-2 was measured by Western blots. Interactions of CHO-Siglec-9 with WT GBS caused rapid tyrosine phosphorylation and recruitment of SHP-2 first detected within 5 min of bacterial contact and increased by 15 min. Infection of the same cells with the GBS  $\Delta$ NeuA mutant also triggered some tyrosine phosphorylation of the receptor and recruitment of SHP-2, but the response was both decreased and delayed compared to infection with the WT GBS (**Figure 3.8**). When WT GBS were allowed to interact with CHO-Siglec-9R120A and CHO-Siglec-9 cells, similar levels of Siglec-9 tyrosine phosphorylation were observed, but SHP-2 co-immunoprecipitation was not observed (**Figure 3.8**). In contrast, when the Sia-deficient GBS  $\Delta$ NeuA mutant was added to CHO-Siglec-9R120A the levels and rate of tyrosine phosphorylation and SHP-2 association were similar or increased compared to CHO-Siglec-9 (**Figure 3.8**). Overall, we can conclude that, the increased rate and levels of Siglec-9 tyrosine phosphorylation and SHP-2 recruitment observed when WT GBS interacted with CHO-Siglec-9 was dependent on Siglec-9 binding the CPS Sias of GBS *in trans*. Taken together, these data support the hypothesis that interactions of GBS serotype III CPS with Siglec-9 on innate immune cells is likely to be beneficial to the pathogen, by dampening the reactivity of the immune cells. Further studies are needed to elucidate the relevant signaling pathways downstream of SHP-2 recruitment to hCD33rSiglecs.



**Figure 3.8 CHO cells expressing Siglec-9 but not Siglec-9R120A functionally engage Sia-GBS.** CHO-hSiglec-9 or CHO-hSiglec-9R120A were incubated with WT GBS or GBS $\Delta$ NeuA for 5 and 15 minutes. CHO cells washed in ice-cold wash buffer and lysed at 4°C before immunoprecipitating Siglec-9. Proteins were separated by reducing SDS-PAGE and transferred to PVDF and probed with antibodies against Siglec-9, phosphotyrosine, and SHP-2 followed by HRP-conjugated secondary and ECL reagent.

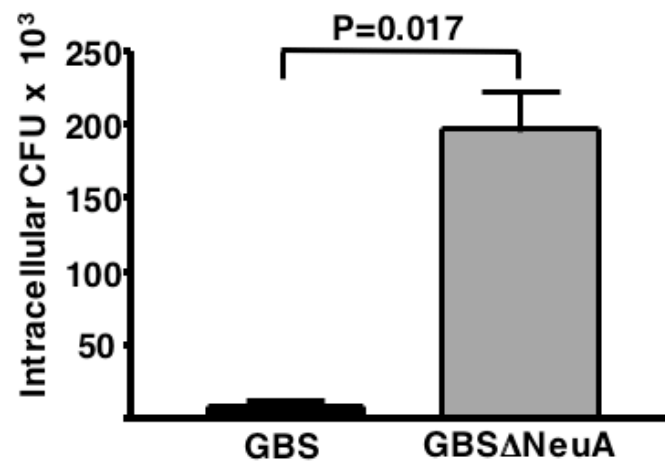
***Human monocyte interactions with sialylated GBS leads to increased recruitment of SHP-2 to the cytosolic tail of Siglec-9.*** To further study GBS serotype III CPS Sia interactions with hSiglec-9 in the context of leukocytes that normally express this CD33rSiglec, we incubated these bacteria with U937 cells (a human monocyte cell line), lysed the cells, and immunoprecipitated Siglec-9 to determine if the protein tyrosine phosphatases SHP-1 and SHP-2 were recruited to the receptor. Interaction with WT sialylated GBS lead to a clear increase in SHP-2 recruitment to Siglec-9 by 15 min that continued for at least 60 min, effects absent in parallel studies with the Sia-deficient  $\square$ NeuA mutant (**Figure 3.9**), although it appeared that the  $\square$ NeuA mutant caused a slight increase in the quantity of SHP-1 that was co-immunoprecipitated with Siglec-9 at 60 min (**Figure 3.9**). In this regard, we note that there was substantial baseline interaction of SHP-1 with the cytosolic tail of Siglec-9 that may have affected the capacity of GBS to alter this interaction. Regardless, the increase in recruitment of SHP-2 to the cytosolic tail of Siglec-9 occurred in both CHO-Siglec-9 cells and human monocytes, and is dependent on *in trans* interaction of Sias on the GBS CPS with cell surface Siglec-9.

***Human monocytes phagocytose less bacteria and release less IL-8 in response to sialylated GBS.*** Since the interaction of sialylated GBS with human monocytes alters the interaction of Siglec-9 with protein tyrosine phosphatases known to regulate immune activation, we tested the capacity of Sia on the surface of GBS CPS to alter the functional response of leukocytes to infection. WT or  $\square$ NeuA GBS



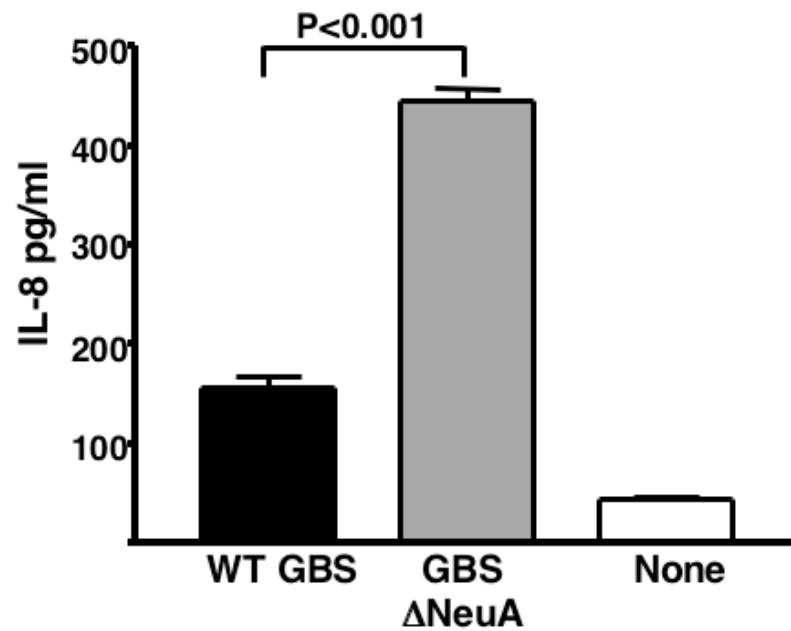
**Figure 3.9** *Human monocyte interactions with sialylated GBS leads to increased recruitment of SHP-2 to the cytosolic tail of Siglec-9.* U937 cells (human monocyte cell line) were incubated with WT GBS or GBS $\Delta$ NeuA for 5, 15 minutes, and 60 minutes before washing in ice cold wash buffer and lysing at 4°C followed by immunoprecipitation of Siglec-9. Proteins were separated by reducing SDS-PAGE and transferred to PVDF and probed with antibodies against Siglec-9, SHP-1, and SHP-2 followed by HRP-conjugated secondary and ECL reagent.

were added to human U937 monocytes for 30 min at 37°C. All extracellular bacteria were then killed using antibiotics, internalized bacteria recovered by monocyte lysis, and plated by serial dilutions for enumeration of intracellular colony forming units (cfu). The WT GBS-expressing Sias were phagocytosed approximately 10-fold less than the non-sialylated mutant GBS $\Delta$ NeuA, consistent with the role of Sias as inhibiting the phagocyte response (**Figure 3.10**). Secretion of IL-8 by human monocytes in response to WT sialylated-GBS was also diminished in comparison to the non-sialylated  $\Delta$ NeuA mutant (**Figure 3.11**). Thus, interactions of human monocytes with Sia-GBS leads to decreased bacterial phagocytosis and dampened release of proinflammatory cytokines. Taken together, these data further support the hypothesis that Sias on the surface of bacteria decrease leukocyte responses via direct interaction with CD33rSiglecs on the immune cell surface.



**Figure 3.10 Human monocytes phagocytose less sialylated GBS.** U937 cells were incubated with WT GBS or GBSΔNeuA for 30 minutes before washing and adding media + antibiotics to kill all extracellular bacteria. After 2 hours cells were washed, lysed, lysates serially diluted, and plated on Todd Hewitt Agar (THA) to enumerate colony-forming units (CFU) corresponding to intracellular bacteria. GBS phagocytosis assays were performed twice in triplicate. Graph is from one representative experiment. Statistical analysis was performed using Student's t-test. Error bars represent standard deviation.





**Figure 3.11 u937 cells secrete less IL-8 in response to sialylated GBS.** U937 cells were incubated with WT GBS or GBS $\Delta$ NeuA for 30 minutes before washing and adding media + antibiotics to kill all extracellular bacteria. After 8 hours supernatants were collected and the concentration of IL-8 was determined by ELISA. IL-8 assays were performed twice in triplicate. Graph is from one representative experiment. Statistical analysis was performed using Student's t-test. Error bars represent standard error of the mean.

## METHODS

***Bacteria and growth conditions.*** GBS WT serotype III strain COH1 is a well-characterized isolates from human neonates with invasive infections. Generation of the  $\Delta$ NeuA mutants in serotype III strain COH1 were previously reported (Lewis et al., 2007). GBS were grown in Todd-Hewitt broth (THB), pH 7.5, or on THB agar plates (THA). For functional assays, unless otherwise noted, bacteria were grown to early-exponential phase in THB, washed three times with pyrogen-free phosphate-buffered saline (PBS), resuspended in appropriate buffers, and adjusted to the desired concentrations using a spectrophotometric method confirmed by pour-plate colony counts.

***Soluble receptors and stable CHO cell expression.*** Soluble hSiglec-Fc chimeras were produced as previously described (Angata and Varki, 2000). CHO-K1 cells stably expressing full length hSiglec-9 or hSiglec-9R120A were produced by transfecting WT CHO-K1 cells with pcDNA3.1(+)-Hygro-hSiglec-9 or hSiglec9-R120A using Fugene-6 transfection reagent per manufacturers directions as previously described (Carlin et al., 2007).

***Cell growth conditions and isolation of human neutrophils.*** CHO-K1 cells were grown in Ham's F10 media supplemented with 10% FBS at 37C and 5% CO<sub>2</sub>. The human monocyte cell line U937 was grown in RPMI supplemented with 10mM HEPES, 1mM Na pyruvate, 10% FCS, and glucose at 37°C and 5% CO<sub>2</sub>. Human

neutrophils were prepared as previously described using Polymorphprep<sup>TM</sup> solution (Axis Shield PoC AS, Oslo, Norway) according to the manufacturer's instructions (Carlin et al., 2007).

***Neutrophil adhesion assays.*** ELISA plates (Immulon 4) were coated with polyacrylamide probes (Glycotech) at 1 $\mu$ g/well in 100 $\mu$ l 50mM carbonate buffer pH 9.4 overnight at 4°C. Wells were washed once with assay buffer (HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup> + 1% BSA) and blocked in assay buffer for at least one hour. Purified human neutrophils were incubated with Calcein AM for 30 minutes as directed (Molecular Probes) at 37°C with or without *Arthrobacter ureaficiens* sialidase 50mU/ml. Neutrophils were washed 3 times in HBSS without Ca<sup>2+</sup> or Mg<sup>2+</sup> and resuspended in HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup> + 1% BSA and added to ELISA plates 5x10<sup>5</sup> cells/well. and allowed to adhere for 30 minutes at 37°C. After 30 minutes the initial fluorescence intensity in each well was determined using a fluorescent plate reader (Cytofluor II) and non-adherent cells were removed by gentle washing with HBSS w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>. Final fluorescent intensity was measured and used to calculate % Neutrophils adherent to individual ELISA wells. For IL-8 assays neutrophils were allowed to adhere for 10 minutes prior to stimulation with fMLP, LPS, PMA, or Interferon- $\gamma$  and incubated for 6 hours before collecting supernatants. IL-8 concentration was determined by ELISA assay as previously described (Eckmann et al., 1993).

***Mammalian protein isolation and Western blots.*** GBS were added to mammalian cells at an MOI of 20:1 and centrifuged to initiate contact. At specified times cells were washed with ice cold wash buffer (TBS pH 7.4, 1mM EDTA, 1mM NaF, sodium pervanadate), lysed with ice cold lysis buffer (TBS pH 7.4, 1mM EDTA, 1mM NaF, 1% Triton-X, complete EDTA-free protease inhibitor cocktail, sodium pervanadate) for 30 min rotating at 4°C, and centrifuged at 15,000 G for 20 min at 4°C. Protein concentration were measured by BCA protein quantitation kit (Pierce) and protein concentrations were normalized prior to immunoprecipitation overnight with mouse anti-human Siglec-9 antibody (BD Pharmingen) and 4 fast flow protein-G sepharose beads (Amersham Biosciences) as instructed by manufacturer. Proteins were separated by SDS-PAGE under reducing conditions, transferred to PVDF, probed with biotinylated goat anti-human Siglec-9 antibody (R&D systems), mouse anti-phosphotyrosine 4G10 (Upstate), rabbit anti-SHP-1 (santa cruz biotechnology), or rabbit anti-SHP-2 (Upstate) along with either streptavidin-HRP (Amersham) or HRP conjugated goat anti-rabbit antibody (Biorad) and ECL reagent (Pierce). For assays using CHO cells, N-glycans were removed using PNGase F. After the immunoprecipitation reaction, protein (20ul total volume) was resuspended in 31.6  $\mu$ l H<sub>2</sub>O + 2.4  $\mu$ l 0.5M beta mercaptoethanol (2-mE) + 6  $\mu$ l 10% SDS in 200 mM Tris pH 7.3 and incubated at 100°C for 5 min. Reaction was cooled and 5  $\mu$ l 20% NP 40, 0.8  $\mu$ l 0.5M 2-mE, and 5  $\mu$ l PNGase F were added and reaction was incubated overnight at 37°C. Samples were then resuspended in SDS-PAGE loading buffer and processed as described above.

***GBS adherence assays.*** CHO cell adherence assays were performed as described previously (Carlin et al., 2007). FITC-GBS adherence to individual CHO-K1 cells was analyzed by flow cytometry (BD FACS caliber) after lifting CHO cell monolayers in PBS + 5mM EDTA. Results were expressed as a bacterial binding index, calculated by multiplying the percentage of FITC-positive cells in the cell gate by the mean fluorescence intensity of FITC-positive cells within the cell gate. For CHO cell flow cytometry experiments side- and forward-scatter properties were used to distinguish cells (cell gate) from free bacteria, and 20,000 events were collected from the cell gate.

***GBS phagocytosis assay.*** Bacterial cultures were inoculated from a single colony and grown overnight in THB and cultures were diluted 1:10 in fresh THB and GBS were grown to an OD<sub>600</sub> of 0.4 washed once in PBS + 1% BSA and preopsonized with RPMI + 80% heat inactivated (56C for 40 minutes) human serum for 15 minutes at 37C. GBS were then added to u937 cells at an MOI of 10:1 to produce a final concentration of RPMI + 10% heat inactivated human serum. Bacteria and cells were spun together to initiate contact, incubated at 37°C for 30 minutes, washed once with serum free RPMI, and resuspended in RPMI + penicillin 5 µg/ml and gentamicin 100 µg/ml for 2 hours at 37°C to kill extracellular bacteria. The cells were washed in PBS + 1% BSA and lysed using PBS + 0.025% Triton X-100 to liberate intracellular bacteria. Dilutions were plated on THA plates, placed at 37°C overnight, and GBS

CFU were counted. For interleukin-8 assays, after 30 minutes of contact with GBS antibiotics were added to u937 cells in order to kill all extracellular bacteria and cells were incubated for 6 hours at 37°C. After 6 hours supernatants were removed and IL-8 was measured as previously described (Eckmann et al., 1993).

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## **Chapter IV**

# **Group B Streptococcal $\alpha$ Protein Binds Human Siglec-5 to Impair Phagocytosis and Promote Placental Attachment**

## INTRODUCTION

*Streptococcus agalactiae* or Group B *Streptococcus* (GBS) is the most common cause of bacterial sepsis, pneumonia, and meningitis in newborns (Dermer et al., 2004). The majority of GBS neonatal disease results from ascending spread of bacteria colonizing the maternal genital tract to access the amniotic fluid and fetus; alternatively the infant can aspirate infected vaginal fluids during passage through the birth canal (Johri et al., 2006; Schuchat, 1998). GBS is also an important cause of morbidity in pregnant women resulting in chorioamnionitis, spontaneous mid-gestation abortion, and premature rupture of placental membranes (Berkowitz and Papiernik, 1993; Moller et al., 1984; Muller et al., 2006).

A critical factor for GBS virulence is its capsular polysaccharide (CPS) containing a terminal  $\alpha$ 2-3-linked *N*-acetylneuraminic acid, identical to the most common sialic acid (Sia) that caps many surface glycans on all human cells (Varki, 1997; Wessels et al., 1989). To date, the structures of nine antigenically distinct GBS CPS have been described (serotypes Ia, Ib, II-VIII), each composed of a unique arrangements of galactose, glucose, and/or *N*-acetylglucosamine, but invariantly possessing the above mentioned Sia residue on the branching terminus of the repeating unit (Paoletti and Kasper, 2003). Through increasing affinity of serum factor H for C3b and accelerating intrinsic decay of the C3 convertase, host Sia plays an important counter-regulatory role limiting alternative complement pathway activation (Fearon, 1978; Whaley and Ruddy, 1976). By decorating its own surface with Sia, GBS creates a nonactivating environment for alternative complement deposition (Edwards et al.,

1980; Marques et al., 1992), consequently impairing opsonophagocytic clearance and promoting disease progression (Rubens et al., 1990; Wessels et al., 1989).

The Sia-recognizing immunoglobulin (Ig) superfamily lectins (Siglecs) are a family of structurally related type I transmembrane proteins consisting of an N-terminal Sia-binding V-set domain and a variable number of extracellular C2-set domains (Crocker et al., 2007; Varki, 2007; Varki and Angata, 2006). Human CD33rSiglecs (hCD33rSiglecs), comprising Siglecs 3, 5 to 11, XII, and 14, are a rapidly evolving subgroup of Siglecs that are predominantly expressed on leukocytes in a cell type specific fashion (Angata et al., 2006; Crocker et al., 2007). Human Siglec-5 (hSiglec-5) is expressed on human monocytes, macrophages, neutrophils, and dendritic cells and contains two intracellular tyrosine-based signaling motifs (Cornish et al., 1998; Lock et al., 2004; Munday et al., 1999). The membrane proximal motif (LHYASL) conforms to the canonical immunoreceptor tyrosine-based inhibitory motif (ITIM) (I/L/V)<sub>x</sub>Y<sub>xx</sub>(L/V), while the distal motif (TEYSEI) is similar to the immunoreceptor tyrosine-based switch motif (TXYXX(V/I)) found in the CD150/SLAM subfamily (Avril et al., 2004; Bruhns et al., 1999; Ravetch and Lanier, 2000). The ITIM motif is present in various inhibitory receptors that antagonize kinase-dependent activation cascades (Daeron et al., 1995; Shultz et al., 1997; Taylor et al., 2000) and function by recruiting Src homology 2 (SH2) domain-containing tyrosine phosphatases SHP-1 (Yusa et al., 2002; Zhang et al., 2000) and SHP-2 (Gergely et al., 1999). Human Siglec-5 has been shown to recruit both SHP-1 and SHP-2 and function as an inhibitory receptor of leukocytes (Avril et al., 2005).

GBS serotype III engages hCD33rSiglecs on the surface of cells including human neutrophils in a Sia- and Siglec-specific manner (Carlin et al., 2007). Here we report the unexpected discovery that certain other GBS strains can also 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 analyze the protein-mediated interaction of GBS with Siglec-5 on the surface of human leukocytes and its functional consequences. In addition, we identify human-specific expression of Siglec-5 on primary human amniotic epithelial membranes, and discuss its potential implications for the pathogenesis of ascending GBS infection.

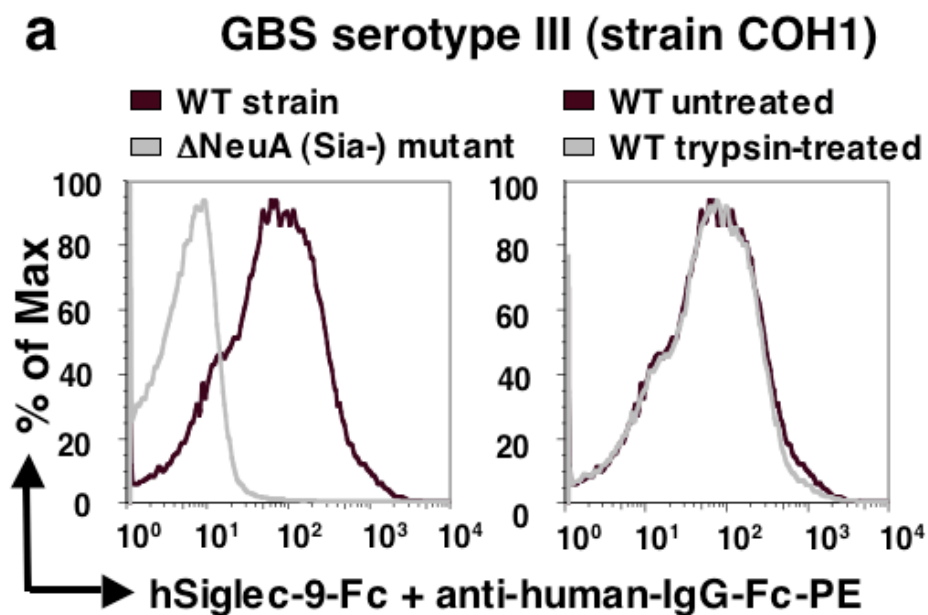
## RESULTS

*GBS interacts with hSiglec-5-Fc through a trypsin-sensitive bacterial protein.* We tested the well-characterized WT GBS isolates expressing sialylated CPS of serotypes Ia (strain A909) and III (strain COH1), isogenic Sia-negative mutants of each strain generated by precise allelic replacement of the CMP-Neu5Ac-synthase gene ( $\square$ NeuA), and GBS pretreated with trypsin to remove cell surface proteins, for their abilities to bind to hSiglec-5-Fc and human Siglec-9-Fc (hSiglec-9-Fc) chimeras by flow cytometry. In accordance with our previous results using an ELISA-based binding assay (Carlin et al., 2007), serotype III GBS bound to hSiglec-9-Fc and serotype Ia bound to hSiglec-5 (**Figure 4.1A-B**, black lines). Furthermore the GBS serotype III strain bound to hSiglec-9-Fc in a Sia-specific manner, since this interaction was lost when the isogenic Sia-negative  $\square$ NeuA mutant was tested in the

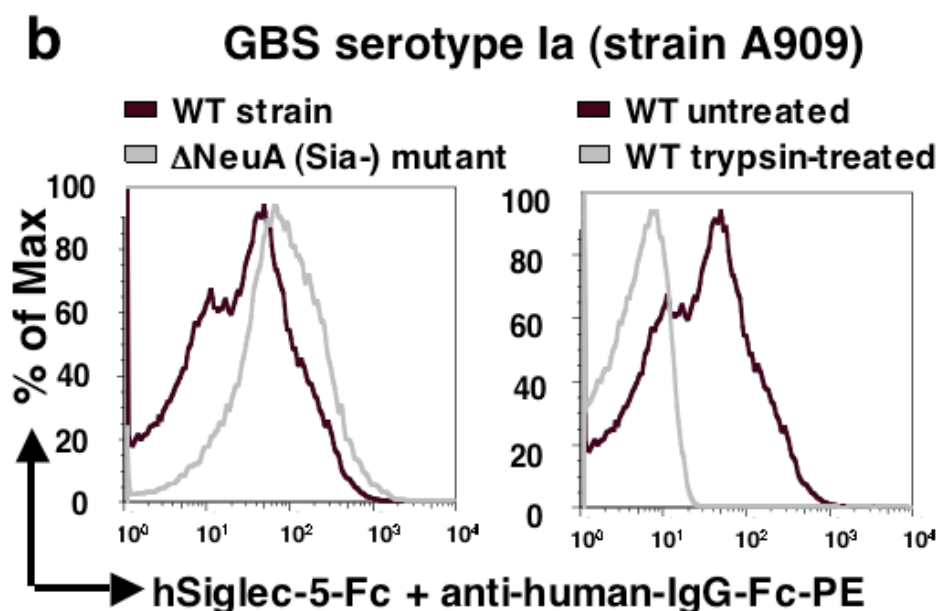
same assay (**Figure 1A**). Trypsin treatment of the GBS serotype III strain did not alter binding of the bacterium to hSiglec-9-Fc (**Figure 4.1a**). In contrast, the isogenic Sia-deficient  $\Delta$ NeuA serotype Ia mutant showed increased interaction with hSiglec-5-Fc compared to the Sia-expressing WT parent strain (**Figure 4.1b**). Furthermore, when the GBS serotype Ia strain was treated with trypsin, all capacity to bind hSiglec-5-Fc was lost (**Figure 4.1b**). Thus, attachment of GBS serotype Ia strain to hSiglec-5-Fc is dependent on a trypsin-sensitive protein and not the anticipated sugar/lectin interaction.

*The GBS  $\Delta$ protein interacts with hSiglec-5 in a Sia-independent manner.* In order to identify the protein(s) 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 GBS serotype III strain that did not interact with hSiglec-5-Fc. To reduce or avoid hSiglec-Fc Sia-specific interactions, isogenic  $\Delta$ NeuA mutants were utilized that are virtually devoid of detectable cell surface Sia as measured by DMB-HPLC (**Figure 4.2a**). The 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 was found to interact with a GBS protein of apparent M.W. 125 kDa found only in lysates of the GBS serotype Ia strain and not in the serotype III strain (**Figure 4.2b**). Furthermore, the hSiglec-9-Fc chimera did not interact with this protein or any others ruling out non-specific protein interactions with the human IgG-Fc portion of the

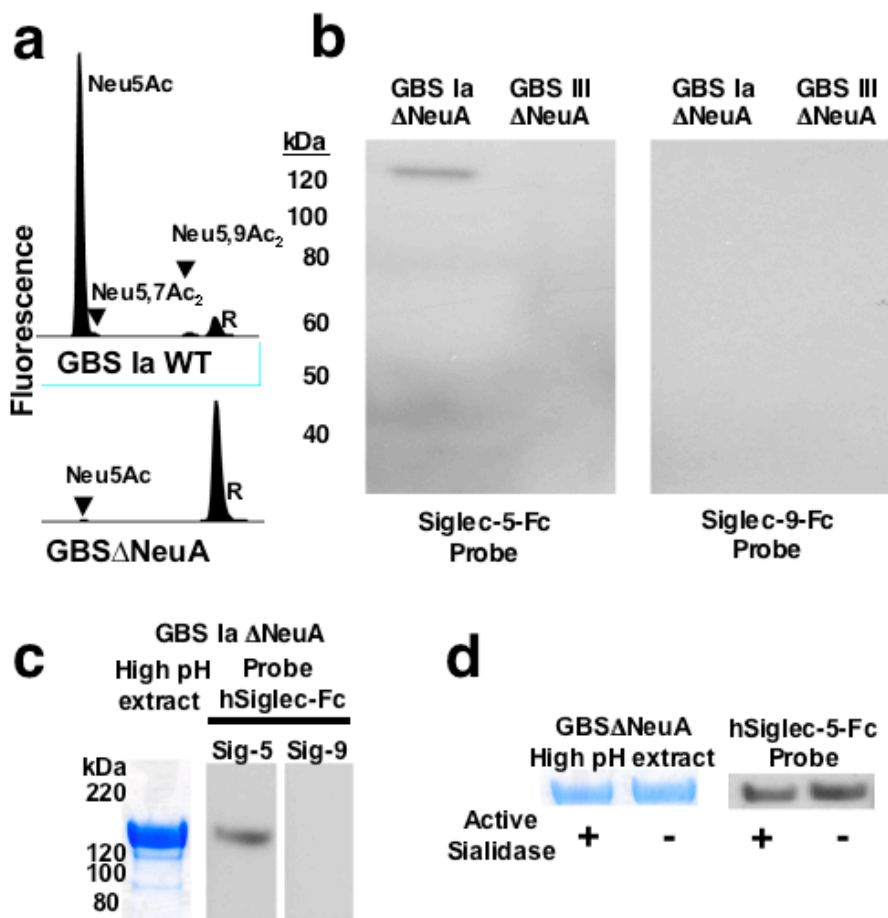




**Figure 4.1a** GBS serotype III binds hSiglec-9-Fc in a Sia-specific manner (a) WT GBS serotype III strain COH1, its isogenic allelic exchange mutant  $\Delta$ NeuA (deficient in extracellular Sia), and trypsin treated WT GBS were incubated on ice with hSiglec-9-Fc, then PE-conjugated secondary antibody, and analyzed by flow cytometry.

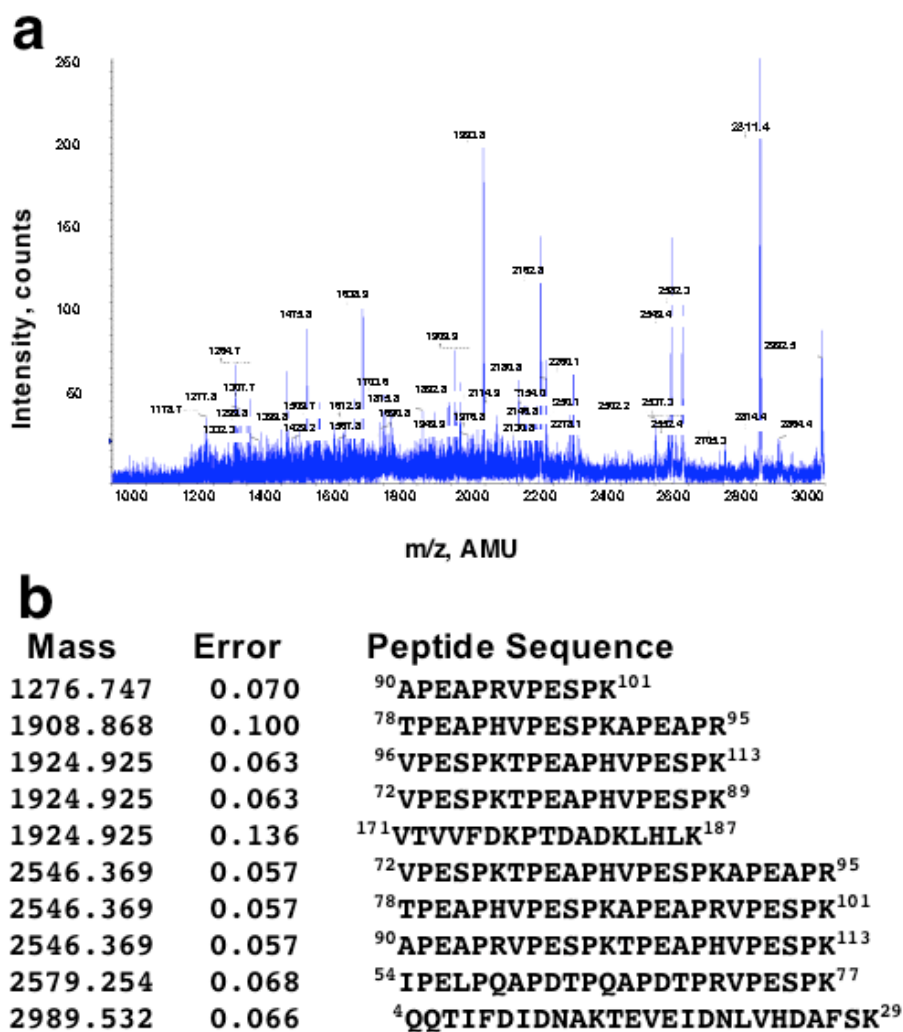


**Figure 4.1b** GBS serotype Ia binds hSiglec-5-Fc in a trypsin sensitive protein-dependent and Sia-independent manner (b) WT GBS serotype Ia strain A909, its isogenic allelic exchange mutant  $\Delta$ NeuA (deficient in extracellular Sia), and trypsin treated WT GBS were incubated on ice with hSiglec-5-Fc, then PE-conjugated secondary antibody, and analysed by flow cytometry.



**Figure 4.2 A 125kDa GBS protein interacts with hSiglec-5-Fc in a Sia-independent manner.** (A) HPLC resolution of fluorescently labeled Sias isolated from WT GBS serotype Ia strain A909 and  $\Delta$ NeuA allelic replacement mutant. Elimination of the neuA gene depletes virtually all cell surface Sia. (B) GBS whole cell lysates from GBS serotype Ia and III  $\Delta$ NeuA mutants probed with hSiglec-Fc chimeras identifies ~125 kD specific hSiglec-5 binding protein in the Ia strain. (C) GBS supernatants extracted under basic conditions (pH 9.7) and analyzed by SDS-PAGE identify similar ~125 KD protein that binds hSiglec5. (D) *Arthrobacter ureafaciens* sialidase treatment (50 mU/ml x 2 h) does not affect binding of the 125 kD protein to hSiglec5.

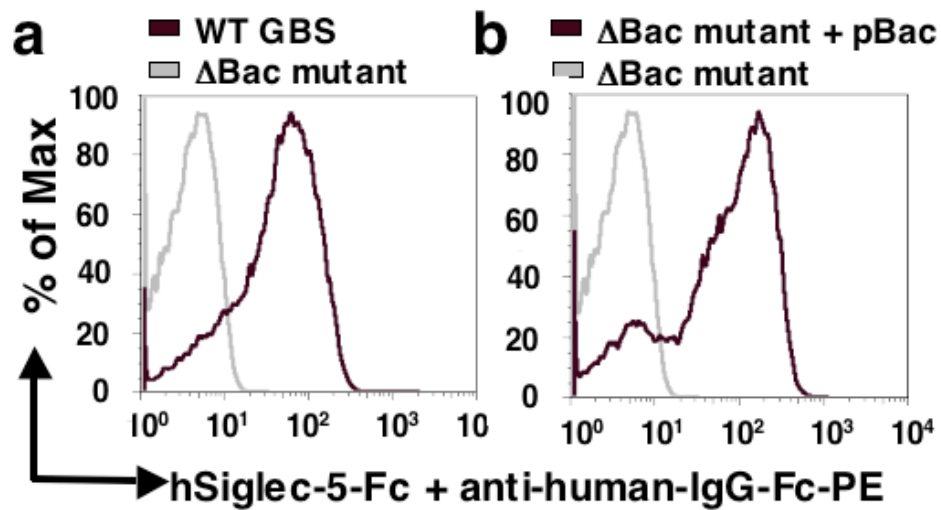
Siglec chimeras. Based on the size, surface location, sensitivity to trypsin, presence in the GBS serotype Ia strain and absence in the GBS serotype III strain, we hypothesized that the protein mediating GBS interaction with hSiglec-5-Fc was the surface-expressed  $\alpha$  protein (also known as C-reactive  $\alpha$  antigen, Bac or  $\alpha$ C) (Areschoug et al., 2002a). In order to produce a partially purified extract of GBS  $\alpha$  protein, we incubated the GBS serotype Ia and serotype III (negative control)  $\alpha$ NeuA mutants at pH 9.7 for 4 h at 37°C (Stalhammar-Carlemalm et al., 1993), concentrated and separated the supernatants by SDS-PAGE, then probed as described above. This procedure produced a sample that was highly enriched for the 125 kDa protein interacting with hSiglec-5-Fc (**Figure 4.2c**). To prove that interaction of this 125 kDa protein with hSiglec-5-Fc was not dependent on residual Sia, we treated the protein extract with active or heat-inactivated (control) *Arthrobacter ureafaciens* sialidase and found no changes in hSiglec-5-Fc binding (**Figure 4.2d**). To determine the identity of the 125 kDa GBS protein, the band reacting with hSiglec-5-Fc was excised, digested, and analyzed using MALDI-TOF MS peptide fingerprinting (**Figure 4.3a,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  $\alpha$  protein was identified with a p value = 0.005. In accordance with our data, the GBS  $\alpha$  protein is a trypsin-sensitive, cell wall anchored protein of ~125 kDa that is present in the serotype Ia strain used in these experiments but is absent from virtually all serotype III GBS strains (Lindahl et al., 2005).



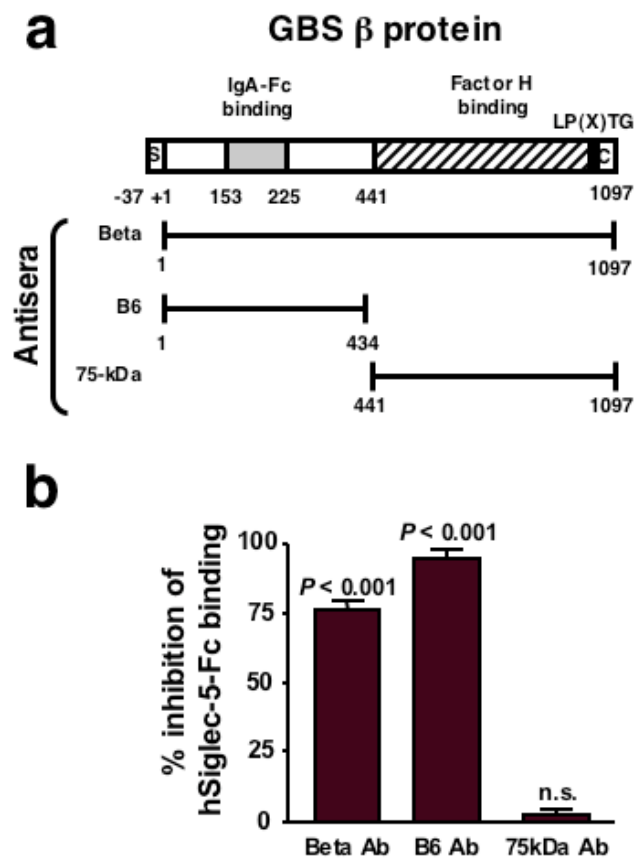
**Figure 4.3 MALDI-TOF analysis of Siglec-5 binding protein identifies the  $\beta$  protein of GBS** (a) The protein of interest was destained, concentrated, and analyzed by MALDI-TOF mass spectrometry. (b) Fingerprint data was analyzed with the online database at Rockefeller University (<http://prowl.rockefeller.edu>). The  $\beta$  protein of GBS was identified with 100% certainty.

*The  $\beta$  protein is required for GBS interactions with hSiglec-5-Fc.* To determine if the  $\beta$  protein was necessary for GBS to bind to hSiglec-5-Fc, we compared the capacity of GBS serotype Ia strain A909 and its isogenic  $\beta$  protein deficient mutant ( $\beta$ Bac) to bind to hSiglec-5-Fc using flow cytometry as described in **Figure 1**. The GBS  $\beta$ Bac mutant lost the ability to bind hSiglec-5-Fc (**Figure 4.4a**), and when the  $\beta$ Bac mutant was complemented with the *bac* gene expressed on a plasmid vector (pBac), WT levels of hSiglec-5-Fc binding were restored (**Figure 4.4b**).

*The N-terminal domain of the  $\beta$  protein interacts with the V-set domain of hSiglec-5.* The GBS  $\beta$  protein has two characterized host protein-binding domains located at opposite ends of the protein. The N-terminal, cell wall distal domain, binds human IgA-Fc while the C-terminal domain interacts with human Factor H (Areschoug et al., 2002b). In order to map the  $\beta$  protein domain responsible for interacting with hSiglec-5-Fc, we pre-incubated GBS with or without polyclonal antibodies directed against the full length  $\beta$  protein (Beta Ab), the N-terminal domain (B6 Ab), or the C-terminal domain (75 kDa antibody) (**Figure 4.5a**), then added hSiglec-5-Fc chimera to measure the hSiglec-5-Fc interactions by flow cytometry. The Beta Ab and B6 Ab significantly inhibited > 75% ( $P < 0.001$ ) and > 95% ( $P < 0.001$ ) of GBS binding to hSiglec-5-Fc, respectively (**Figure 4.5b**). In contrast, the 75 kDa Ab did not significantly interfere with GBS binding to hSiglec-5-Fc (**Figure 4.5b**). To determine



**Figure 4.4 The  $\beta$  protein of GBS is required for interaction with hSiglec-5-Fc** (a) WT GBS Ia and its isogenic allelic exchange mutant GBS $\Delta$ Bac (deficient in the  $\beta$  protein) were incubated on ice with hSiglec-5-Fc, PE-conjugated secondary, and analyzed by flow cytometry. (b) GBS $\Delta$ Bac and GBS $\Delta$ Bac complemented with a plasmid expressing the  $\beta$  protein (GBS  $\Delta$ Bac + pBac) were treated and analyzed as described above.

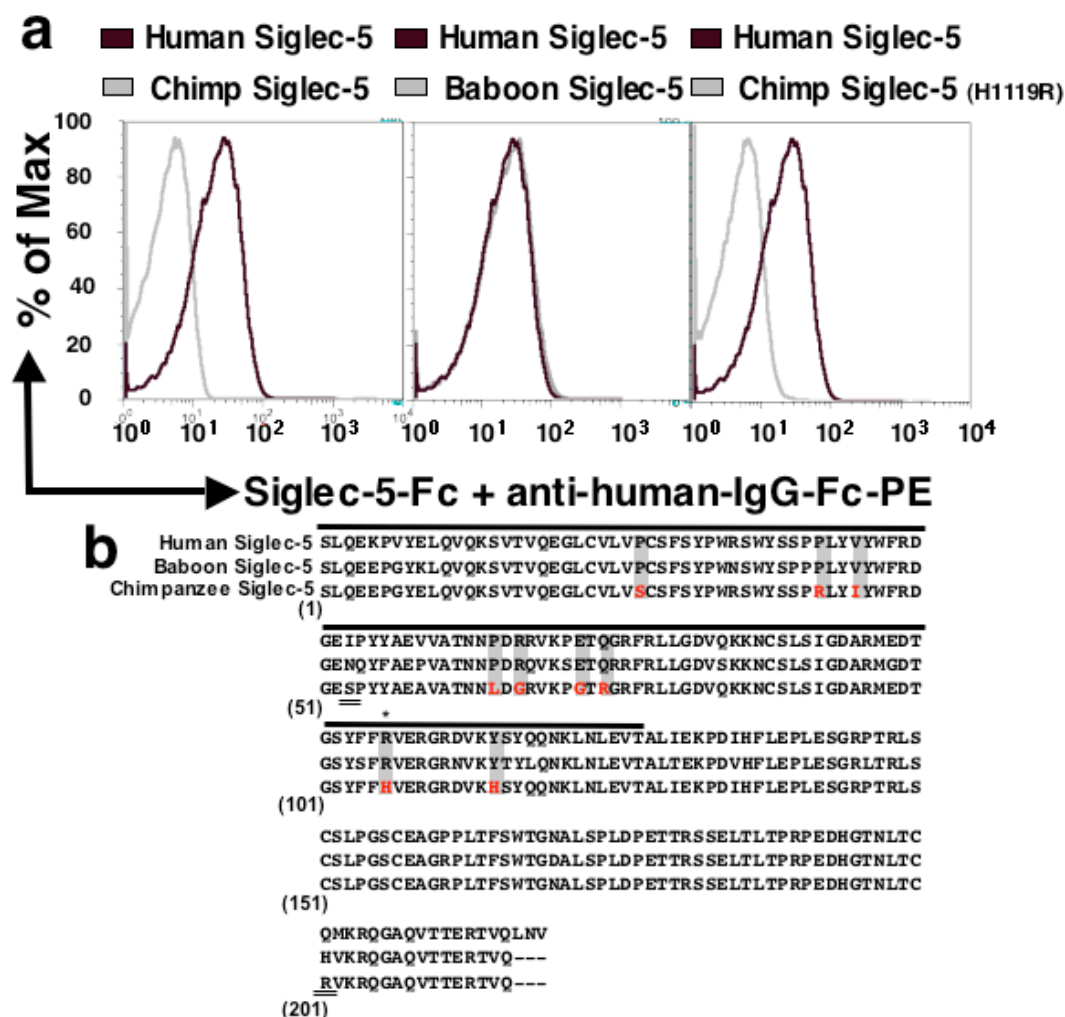


**Figure 4.5 The N-terminal domain of the  $\beta$  protein is responsible for binding hSiglec-5-Fc** (a) Schematic of GBS  $\beta$  protein the domains of the protein utilized to generate Beta, B6, and 75-kDa rabbit antisera. (b) GBS Ia were incubated with or without rabbit antisera against the  $\beta$  protein and the percent of hSiglec-5-Fc binding as measured by flow cytometry was determined.

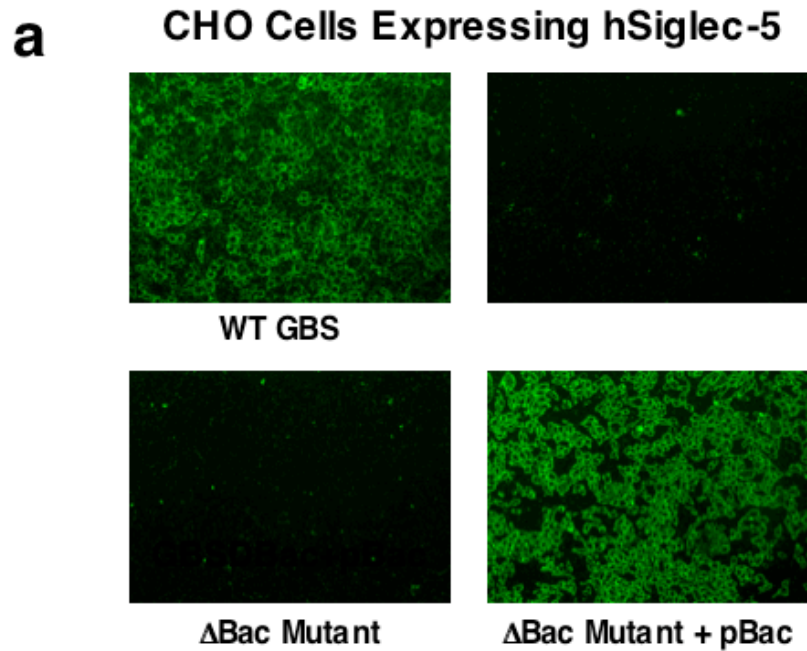


the region of Siglec-5 responsible for  $\square$  protein interactions, we compared the binding of hSiglec-5-Fc, chimpanzee Siglec-5-Fc (cSiglec-5-Fc), and baboon Siglec-5-Fc (bSiglec-5-Fc) to WT GBS. The bacterium bound both hSiglec-5-Fc and bSiglec-5-Fc, but was unable to bind to cSiglec-5-Fc (**Figure 4.6a**). One major difference between Siglec-5 in chimpanzees vs. humans and baboons is that cSiglec-5 harbors a mutation in the binding pocket arginine residue (R119H) essential for engaging Sia (Angata et al., 2004). To ascertain whether the interaction between GBS and cSiglec-5-Fc could be rescued by reintroduction of this arginine residue, we tested binding of GBS to cSiglec-5-Fc with a site-directed introduction of the reverse mutation – cSiglec-5-Fc(H119R). This change allows cSiglec-5-Fc(H119R) to bind Sia but still does not induce binding to GBS (**Figure 4.6a**). The human, chimpanzee, and baboon Siglec-5-Fc chimeras are composed of the V-set and first C2-set domains of Siglec-5 fused to the Fc portion of human IgG. We aligned the protein sequences of the V-set and first C2-set domain of hSiglec-5, cSiglec-5, and bSiglec-5 to examine the possible amino acids important for Siglec-5 Beta protein interactions. There are nine amino acids that are identical in humans and baboons but different in chimpanzees, including H119 that we restored to arginine (**Figure 4.6b**). Seven of these amino acid differences involve substitution of proline residues or charged amino acids that could significantly alter protein structure. In addition, all of these amino acid differences occur in the V-set domain, indicating that this is where the specific interaction of GBS  $\square$  protein with hSiglec-5 is likely mediated.

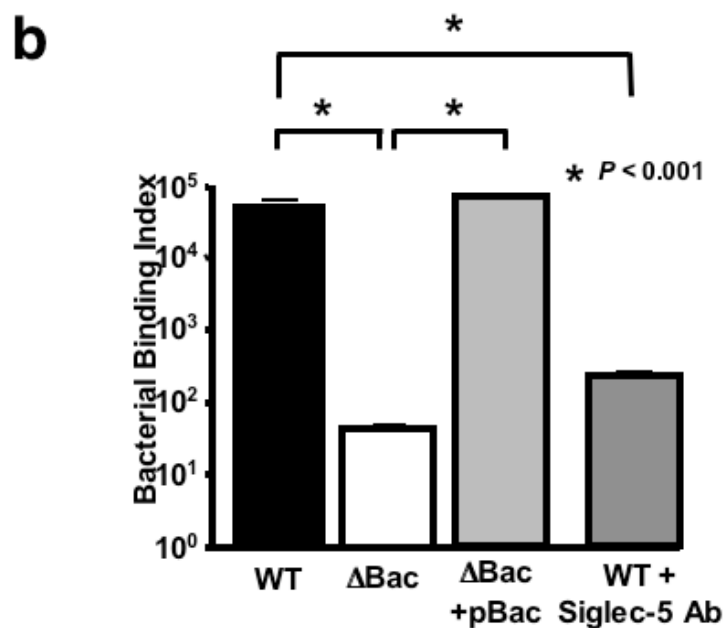
***GBS binding to CHO cells expressing hSiglec-5 is  $\alpha$  protein-dependent.*** To determine if the GBS  $\alpha$  protein was capable of binding to hSiglec-5 in the context of the host cell surface, we stably transfected CHO-K1 cells with a plasmid expressing hSiglec-5 and allowed FITC-labeled GBS to interact with the CHO cell monolayers. Nonadherent bacteria were removed by washing and fluorescent images of GBS adhering to the monolayer were captured using an inverted fluorescent microscope and FITC filter set. The WT GBS strain expressing  $\alpha$  protein adhered to CHO cells expressing hSiglec-5 (**Figure 4.7a**) but not to nontransfected cells (data not shown). In contrast, the  $\alpha$ Bac mutant did not adhere to CHO cells expressing hSiglec5, and this binding could be restored by complementation of the  $\alpha$ Bac mutant with the pBac plasmid expressing  $\alpha$  protein (**Figure 4.7a**). GBS attachment to the transfected CHO cells was dependent on binding to hSiglec-5, since an anti-Siglec-5 antibody significantly reduced the FITC-GBS cell adherence (**Figure 4.7a**). To quantify GBS adherence to CHO cells expressing hSiglec-5, we lifted the monolayers using 5 mM EDTA in PBS and analyzed single cells for adherent FITC-GBS by flow cytometry (**Figure 4.7b**). Adherent bacteria of the WT GBS strain or pBac-complemented  $\alpha$ Bac mutant were present on > 64% of CHO cells and the majority of cells had more than one attached FITC-labeled bacterium (based on greater shifts in fluorescence intensity). In contrast, < 4% of CHO(hSiglec5) cells had adherent  $\alpha$ Bac mutant bacteria and antibody blocking reduced to < 7% the proportion of cells binding the WT GBS strain (**Figure 4.7b**). We conclude that binding of GBS the cell surface is a direct result of  $\alpha$  protein mediated binding to hSiglec-5.



**Figure 4.6** The V-set domain of hSiglec-5 is likely involved in interactions with the GBS  $\beta$  protein (a) WT GBS Ia were incubated on ice with human, chimpanzee, and baboon Siglec-5-Fc, PE-conjugated secondary, and analyzed by flow cytometry. A chimpanzee Siglec-5-Fc with a His to Arg mutation at amino acid 119 that restores Sia binding activity was used as described above (b) Protein sequence alignment of human, chimpanzee, and baboon V-set (black over-line) and first C2-set domains. There are nine amino acids that are the same in humans and baboons but are different in chimpanzees (gray boxes with chimpanzee amino acid in red) including the chimpanzee H119R mutation (star) in the active site. All are located in the V-set domain of Siglec-5. Double underlines represent locations where all three species have different amino acids.



**Figure 4.7a** GBS interacts with CHO cells in a  $\beta$  protein and Siglec-5 dependent manner (a) WT GBS,  $\Delta$ Bac mutant, and  $\Delta$ Bac + pBac were labeled with FITC and allowed to adhere to CHO cells expressing Siglec-5. Non-adherent GBS were removed by washing and fluorescent images were captured using an inverted microscope with appropriate filter set and CCD digital camera. Images are representative of three separate experiments with similar results.

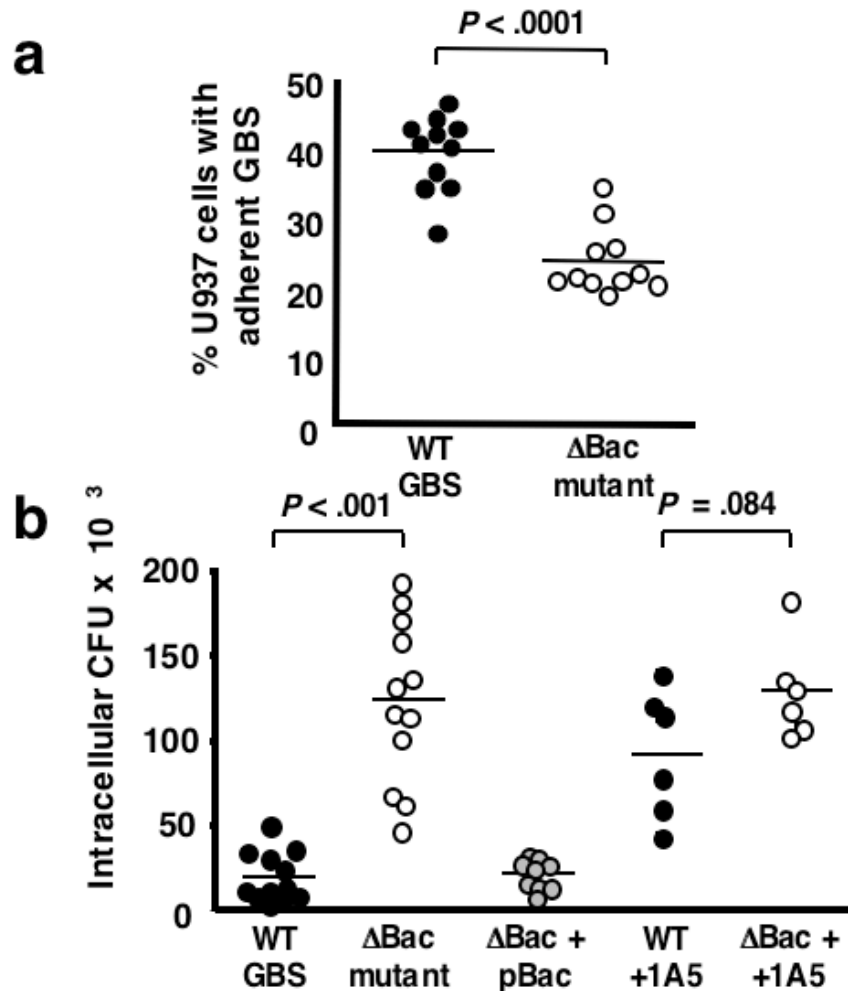


**Figure 4.7b** GBS interacts with CHO cells in a  $\beta$  protein and Siglec-5 dependent manner (b) After non-adherent FITC-GBS were washed away CHO cell monolayers were lifted with PBS + 5mM EDTA and analyzed by flow cytometry to determine the percentage of cells with FITC-GBS attached. Bacterial Binding Index = (% of cells positive for adherent GBS-FITC) x (mean fluorescent intensity). Flow cytometry experiments were performed three times, graph shows cumulative data from all experiments, error bar represents standard deviation, statistical analysis by one-way ANOVA with Tukey's post-test.

*□* protein increases GBS attachment to human leukocytes but impairs phagocytosis. To study GBS *□* protein interactions with hSiglec-5 in the context of leukocytes that normally express this CD33rSiglec, we incubated the bacteria with U937 cells (a human monocyte cell line) and purified human neutrophils from healthy volunteers. First we sought to quantify simple adherence of GBS to these cells, and thus conducted a binding experiment at 4°C in order to prevent phagocytosis of the bacteria. As before, the percentage of cells with bound FITC-labeled bacteria was determined by flow cytometry. The WT strain bound to a significantly higher percentage of U937 human monocytes (**Figure 4.8a**,  $P < 0.001$ ) and human neutrophils (**Figure 4.9**) than did the isogenic *□*Bac mutant. Next, in order to determine whether increased adhesion of *□* protein-expressing GBS to human leukocytes correlated with increased phagocytic uptake, we incubated WT GBS, the isogenic *□*Bac mutant, and the complemented *□*Bac mutant + pBac with human U937 monocytes for 30 min at 37°C. All extracellular bacteria were then killed using antibiotics, and internalized bacteria recovered by monocyte lysis and plating serial dilutions for enumeration of intracellular colony forming units (cfu). In contrast to the leukocyte adherence results, *□*Bac mutant bacteria were phagocytosed by the U937 cells to a significantly greater extent than either the WT GBS parent strain or the pBAC complemented mutant (**Figure 4.8b**,  $P < 0.001$ ). To ascertain whether this effect was due to interaction between the GBS *□* protein of GBS and hSiglec-5 on the leukocyte surface, we preincubated U937 cells with an anti-hSiglec-5 antibody for 50 min at 37°C. Antibody crosslinking of Siglec-5 causes rapid endocytosis of the

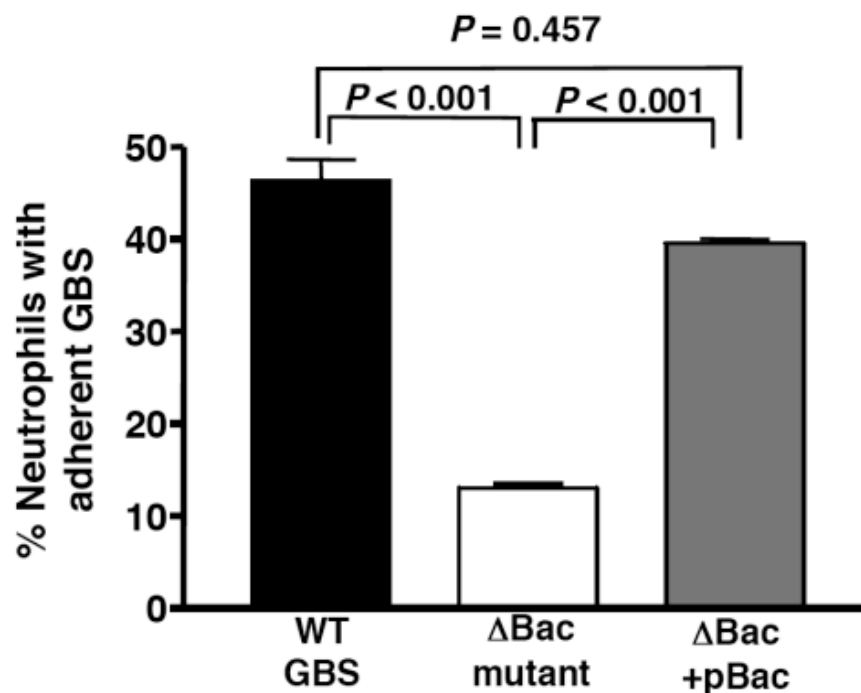
receptor so that only ~5-10% remains on the cell surface after 45 min (data not shown). Upon depletion of hSiglec-5 from the surface of the U937 monocytes, WT GBS expressing  $\square$  protein were phagocytosed more efficiently such that intracellular bacterial counts were not statistically different from the isogenic  $\square$ Bac mutant (**Figure 4.8b**). Thus, although GBS expressing  $\square$  protein bind to human leukocytes more avidly, they are phagocytosed less efficiently, and this reduction is dependent on cell surface expression of hSiglec-5. Additional differential fluorescence studies to quantify phagocytosed vs. extracellular adherent bacteria corroborate these observations and show the results of the antibiotic protection assay are not secondary to differences in intracellular survival of the GBS WT vs.  $\square$ Bac mutant strains (**Figure 4.10a,b**).

*GBS expressing  $\square$  protein increase recruitment of protein tyrosine phosphatases to Siglec-5.* CD33rSiglecs mediate inhibitory signaling in immune cells via recruitment of protein tyrosine phosphatases such as SHP-1 and SHP-2. To determine if interaction between GBS expressing  $\square$  protein and hSiglec-5 would alter protein tyrosine phosphatase recruitment, we incubated the WT GBS strain and  $\square$ Bac mutant with CHO cells expressing hSiglec-5 for varying durations, performed immunoprecipitation for hSiglec-5, then probed for coimmunoprecipitation of tyrosine phosphatase SHP-2 (SHP-1 is predominantly expressed in hematopoietic cells). SHP-2 was not recruited to hSiglec-5 in the absence of bacteria (**Figure 4.11a**). However, when CHO(hSiglec-5) cells were exposed to WT GBS, SHP-2 was recruited to

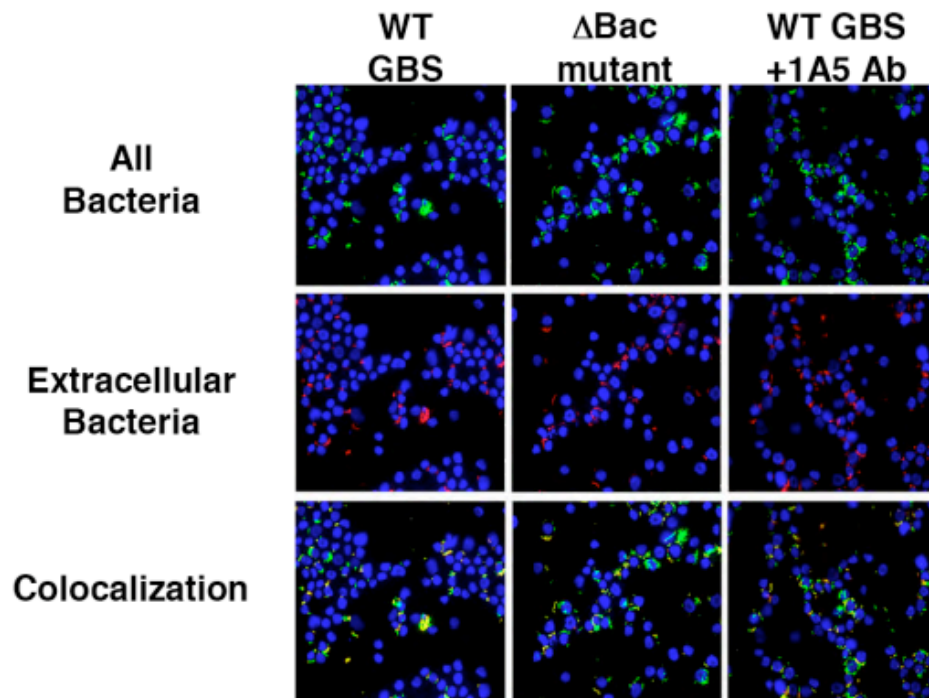


**Figure 4.8  $\beta$  protein expression by GBS increases attachment to leukocytes but impairs phagocytosis** (A) FITC labeled GBS were allowed to interact with U937 for 60 min at 4°C (to prevent phagocytosis); adherence was measured by flow cytometry. (B) To calculate phagocytic uptake of GBS, bacteria were added to U937 at MOI 10:1 for 30 min, washed, antibiotics added for 2 h to kill extracellular bacteria, then cells lysed and dilutions plated to enumerate intracellular colony forming units (cfu). Where indicated, U937 cells were pre-incubated with anti-Siglec-5 Ab (1-A5) for 50 min which results in endocytosis of the receptor leaving ~10% of original levels of Siglec-5 on the surface prior to addition of GBS.

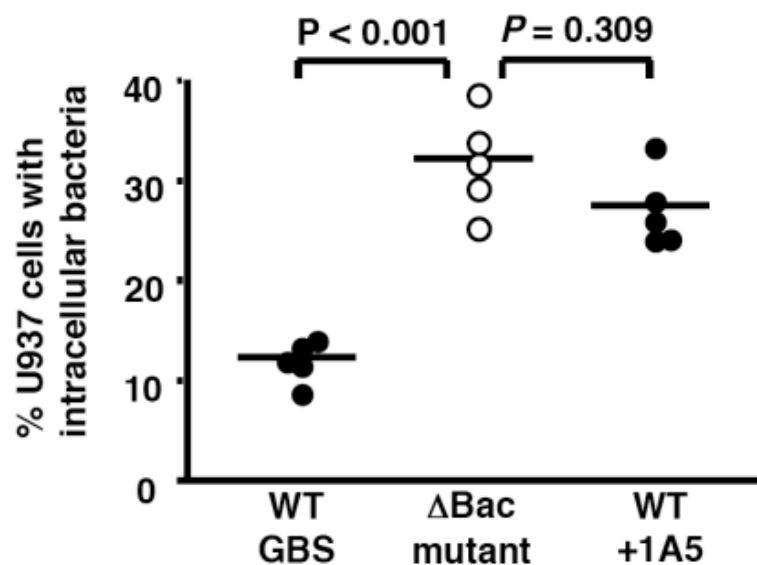




**Figure 4.9  $\beta$  protein expression by GBS increases adherence to the human neutrophils.** Primary human neutrophils were incubated with live GBS-FITC on ice to allow cell-cell interactions but impair phagocytosis. Percent neutrophils with adherent bacteria was measured using flow cytometry. Flow cytometry experiments were performed three times in triplicate, graph shows data from one representative experiment, error bar represents standard deviation, statistical analysis by one-way ANOVA with Tukey's post-test.



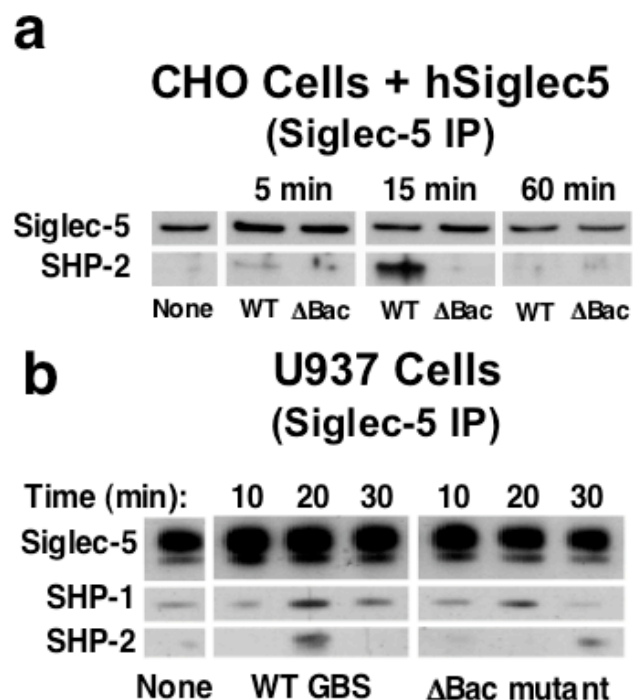
**Figure 4.10a GBS  $\beta$  protein impairs phagocytosis.** u937 with or without preincubation with anti-Siglec-5 Ab (1A5) were incubated with FITC-GBS for 60 minutes at 37 C to allow phagocytosis of bacteria. Cells were washed with ice cold wash buffer and extracellular bacteria were labeled on ice with anti-group B carbohydrate antibody and PE-conjugated secondary to stain extracellular bacteria only. Cells were fixed, nuclei stained (Hoechst), and images were acquired using deconvolution microscopy. Images are representative of five different randomly selected fields.



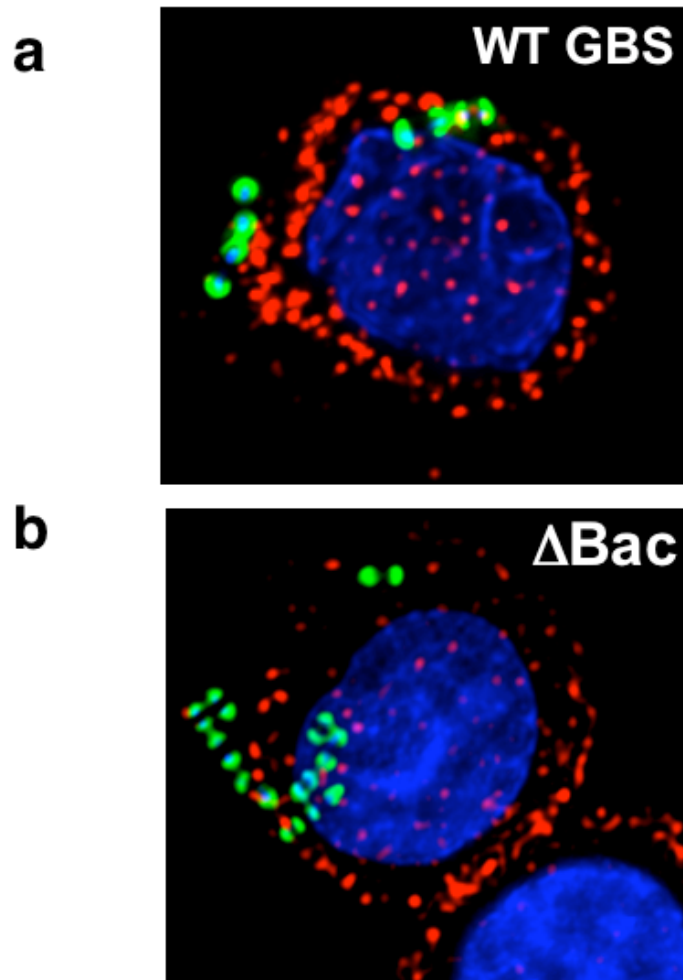
**Figure 4.10b GBS  $\beta$  protein impairs phagocytosis.** Total number of u937 cells with at least one phagocytosed bacteria (green on merge) were divided by the total number of u937 cells with at least one adherent bacteria (yellow or green on merge) and multiplied by 100 to obtain % u937 cells with intracellular bacteria. More than 400 cells total from each group were counted. Graph represents the % of positive cells from 5 randomly selected fields per group. Lines represent the means with statistical analysis by one way ANOVA with Tukey's posttest.

hSiglec-5 beginning at approximately 5 min and reaching a maximum at 15-20 min (**Figure 4.11a**). In contrast, when CHO(hSiglec-5) cells were exposed to the GBS  $\Delta$ Bac mutant, no recruitment of SHP-2 to hSiglec-5 was detected. To determine if GBS expressing  $\Delta$  protein induces phosphatase recruitment in leukocytes, we performed similar assays in U937 monocytes. Unstimulated U937 cells had a baseline level of SHP-1 recruitment but no detectable SHP-2 associated with hSiglec-5. When WT GBS type Ia were added to the U937 cells, an enhanced and sustained recruitment of SHP-1 was observed that was absent in parallel assays with the  $\Delta$ Bac mutant (**Figure 4.11b**). In addition, WT bacteria expressing  $\Delta$  protein induced a larger amount and faster rate of SHP-2 recruitment (**Figure 4.11b**).

*GBS expressing  $\Delta$  protein colocalize with Siglec-5 on the surface of human monocytes.* To visualize the interaction between GBS expressing  $\Delta$  protein and hSiglec-5 on the U937 cell surface, we incubated live FITC-GBS (green) with the monocytes for 1 h at 37°C, then washed away nonadherent bacteria. GBS-U937 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  $\Delta$  protein colocalized with clear aggregations of Siglec-5 present on the U937 cell surface (**Figure 4.12a**); increased quantities of hSiglec-5 at the interface of the  $\Delta$ Bac mutant and the U937 cell surface were not observed (**Figure 4.12b**). The recently discovered hSiglec-14 was found to be >99% similar to hSiglec-5 in its extracellular



**Figure 4.11** GBS expressing  $\beta$  protein increase recruitment of protein tyrosine phosphatases to hSiglec-5 and co-localize with the receptor on the cell surface. (A) Immunoprecipitation for hSiglec-5 followed by detection of SHP-2 coimmunoprecipitation by Western blot analysis in CHO cells expressing hSiglec-5 exposed to WT GBS and the isogenic  $\beta$  protein-deficient mutant. (B) SHP-1 and SHP-2 recruitment to hSiglec-5 by coimmunoprecipitation studies in U937 cells exposed to WT and  $\beta$  protein-deficient GBS.



**Figure 4.12  $\beta$  protein expressing GBS colocalize with Siglec-5 on the surface of human monocytes.** (a) FITC labeled live WT GBS or (b) GBS $\Delta$ Bac were incubated with u937 cells at 37 C for 60 minutes. GBS-u937 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.

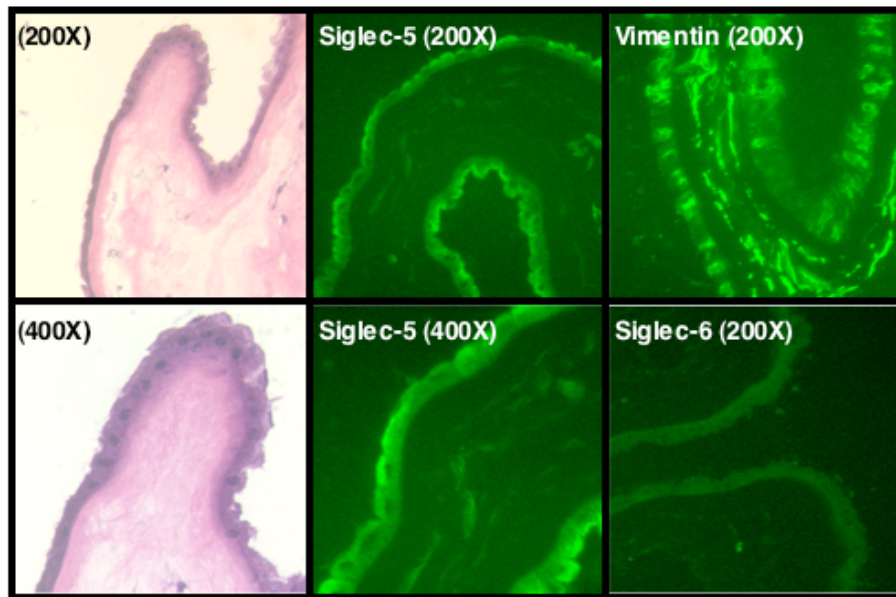
domain, to share similar glycan binding properties, but to possess an intracellular domain that interacts with the activating receptor DAP12. Based on Western blot analysis probed with a polyclonal antibody recognizing both hSiglec-5 and hSiglec-14 and a monoclonal antibody specific for hSiglec5, U937 cells express little or no hSiglec-14 (data not shown).

*Human-specific expression of Siglec-5 on the amniotic epithelium.*

CD33rSiglecs are known to be expressed primarily on leukocytes. One known exception is the expression of Siglec-6 in the trophoblast of the human placenta (Patel et al., 1999), an expression pattern that we have recently found to be human-specific, i.e., not found in our closest living evolutionary relatives, the great apes (Brinkman-Van der Linden et al., 2007). In pursuing further immunohistological studies we made the surprising observation that Siglec-5 is strongly expressed on the amniotic epithelium of human placental sections (**Figure 4.13**), though not in other areas of the placenta. Again, comparisons with chimpanzee placentae revealed that this expression is human-specific (**Figure 4.14**). Thus, humans appear to have specifically upregulated expression of multiple CD33rSiglecs in the placenta and embryonic membranes for as yet undetermined reasons.

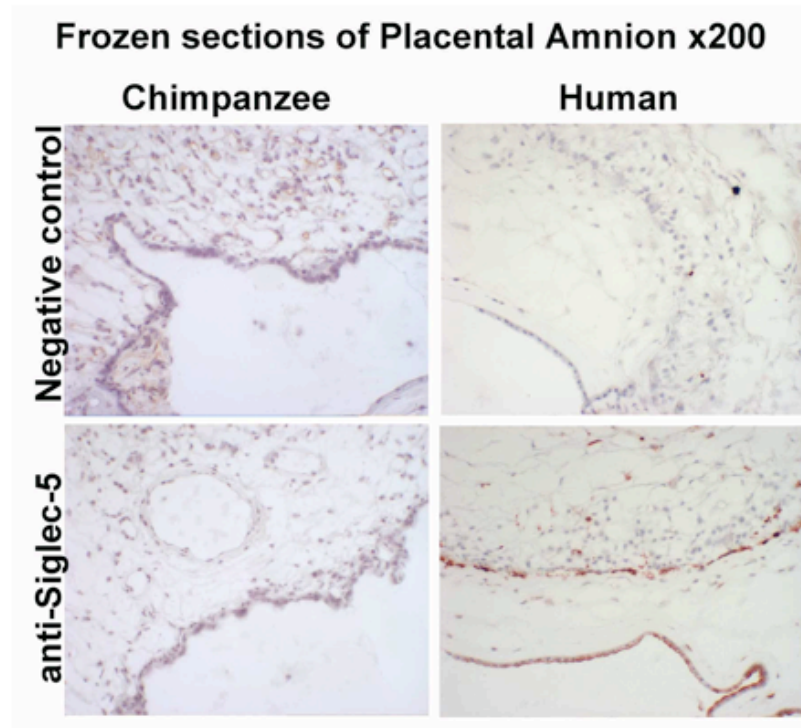
*□ protein expression by GBS increases adherence to the amniotic epithelium.*

During ascending infection, GBS contacts the amniotic epithelium after invasion of the placental membranes (Winram et al., 1998). To study the role of □ protein expression in GBS placental interactions, we added FITC-labeled WT GBS and the

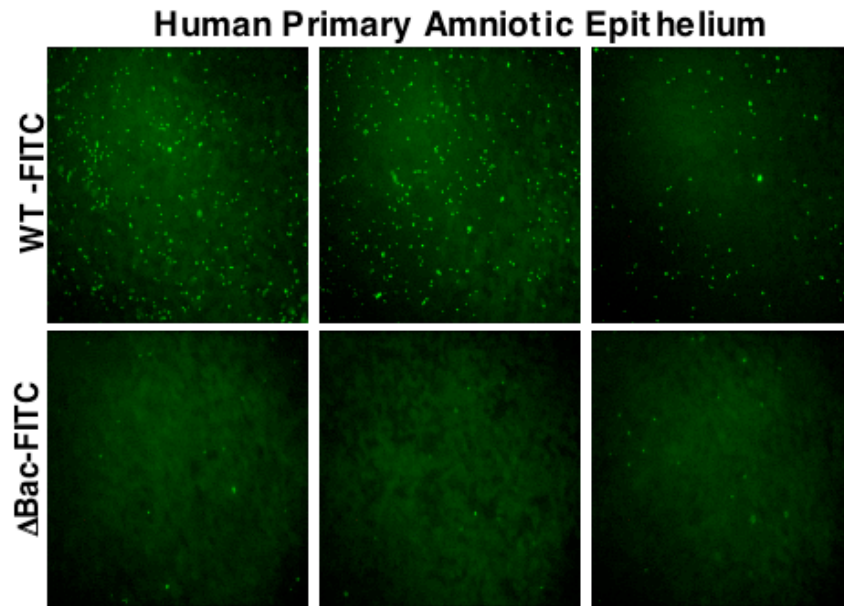


**Figure 4.13 Siglec-5 is expressed on the luminal surface of human amniotic epithelial cells.** Human amniotic membranes were separated from placental membranes, fixed, and stained with hematoxylin and eosin or with monoclonal antibodies recognizing Siglec-5, Siglec-6 (negative control), or Vimentin (positive control), followed by secondary anti-mouse antibodies, mounted, and pictures were captured using a fluorescent microscope with appropriate filter set and CCD digital camera. Primary human amnions from two different individuals were analyzed and images are representative of both tissues.





**Figure 4.14 Siglec-5 expression on the amniotic membrane occurs in humans but not in chimpanzees.** Human and chimpanzee placentas were frozen in OCT, sectioned, mounted, and probed with an anti-hSiglec-5 antibody that crossreacts with chimpanzee Siglec-5, followed by alkaline phosphatase secondary antibody. Seven human placentas and two chimpanzee placentas were analyzed and images are representative of all tissues.



**Figure 4.15  $\beta$  protein expression by GBS increases adherence to the amniotic epithelium.** FITC labeled WT GBS or GBS $\Delta$ Bac were added to primary human amniotic membranes and allowed to adhere for 30 minutes. Non-adherent bacteria were removed by washing and fluorescent images were acquired using a microscope with appropriate fluorescent filter set and CCD digital camera. Binding assays were done in triplicate using amniotic membranes from two separate donors. Images are representative of two separate experiments.

isogenic  $\Delta$ Bac mutant to primary human amniotic epithelial membranes, washed to remove non-adherent bacteria, then visualized the membranes with fluorescent microscopy. WT GBS expressing  $\Delta$  protein showed greater adherence to the amniotic membranes than the GBS $\Delta$ Bac mutant (**Figure 4.15**).

## DISCUSSION

In this study we utilize WT GBS strains and isogenic mutants together with a panel of Siglec expressing cells and soluble Siglec-Fc chimeras to demonstrate a direct and functional interaction of the GBS  $\Delta$  protein with hSiglec-5. The  $\Delta$  protein is the first example of a protein other than an antibody that directly engages the extracellular domains of a hCD33rSiglec in a Sia-independent manner. Previously, GBS and two other human pathogens, *Neisseria meningitides* and *Campylobacter jejuni*, were shown to interact with hCD33rSigeles in a manner dependent on cell surface Sia expression and it was postulated that these interactions could serve to downregulate the innate immune response (Avril et al., 2006a; Avril et al., 2006b; Carlin et al., 2007; Jones et al., 2003). The capacity of GBS to associate with hSiglec-5 in a protein dependent manner represents a novel mechanism by which pathogens may co-opt hCD33rSigeles to their advantage.  $\Delta$  protein-mediated engagement causes an accumulation of Siglec-5 at the sight of GBS contact and increases the recruitment of the protein tyrosine phosphatases SHP-1 and SHP-2 to the receptor's cytosolic tail. Reduced phagocytosis of GBS expressing  $\Delta$  protein is likely linked to this phosphatase recruitment, since the antiphagocytic effect requires Siglec-5 expression on the

leukocyte cell surface. It is known that association of SHP-1 with surface receptors such as SHPS-1 (SIRP $\alpha$ , BIT, p84) negatively regulates Fc gamma and complement receptor-mediated phagocytosis (Kant et al., 2002; Okazawa et al., 2005; Oldenborg et al., 2001).

SHP-1 is an important regulator of signaling cascades that promote myeloid cell growth, survival, and activation including the production of TNF and iNOS in response to cellular activation by LPS (Hardin et al., 2006; Zhang et al., 2000). Targeting of phosphotyrosine phosphatases in order to subvert host cell signaling pathways is thought to play an important role in the pathogenicity of *Leishmania donovani*, *Neisseria gonorrhoeae*, *Mycobacterium tuberculosis*, and *Helicobacter pylori* (Higashi et al., 2002; Knutson et al., 1998; Nandan et al., 2000; Olivier et al., 2005). Activation of SHP-1 by *L. donovani* is important for parasite survival within macrophages and disease progression *in vivo* (Olivier et al., 2005). The Opa52 protein of *N. gonorrhoeae* interacts with CEACAM1 on T lymphocytes, increasing association with SHP-1 and SHP-2 and suppressing lymphocyte activation and proliferation (Boulton and Gray-Owen, 2002). Conversely, the Opa52 protein of *N. gonorrhoeae* interacts with CD66 on human phagocytes causing strong downregulation of SHP-1 and increased bacterial phagocytosis (Hauck et al., 1999).

The GBS  $\alpha$  protein is a large, antigenic surface anchored protein expressed in almost all GBS serotype Ib strains, 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  $\alpha$  protein expression are associated with increased virulence of GBS clinical

isolates (Nagano et al., 2002).  $\alpha$  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., 2002b); and our present finding that  $\alpha$  protein binds to hSiglec-5 identifies a third such interaction. Through nonopsonic binding of immunoglobulin, engagement of factor H (a major regulator of complement) (Pangburn, 2000), and attachment to hSiglec5 (proposed to play an important role in regulating cellular innate immunity) (Crocker and Varki, 2001; Zhang et al., 2007),  $\alpha$  protein appears to be a multifunctional virulence factor capable of interfering with several branches of immunity important in resistance to bacteria infection.

Ascending GBS infection of placental membranes is the cause of significant morbidity in pregnant women and mortality in neonates. We demonstrate that Siglec-5 is expressed on the luminal surface of human amniotic epithelial cells which, like Siglec-6 expression by placental trophoblasts, constitutes an example of human specific protein expression since these proteins are not expressed in the chimpanzee placenta. Regardless of the original reason for the human-specific up-regulation of CD33rSiglecs in the placenta and embryonic membranes, it is interesting that GBS infections of fetuses and neonates is a human-specific phenomenon, and has not been found in any other primate species, including chimpanzees (Elizabeth Strobert, Yerkes Primate Center, personal communication). GBS evolution to express  $\alpha$  protein may contribute to this extended tissue tropism, coupled with the altered preference of human Siglecs for Neu5Ac (the sialic acid found on pathogens like GBS).

GBS is able to engage different CD33rSiglecs using its sialylated CPS or the  $\alpha$  protein. It is possible that during GBS interactions with leukocytes, the close localization of CD33rSiglecs with the sialylated CPS may have facilitated selection for bacterial proteins that were able to directly engage CD33rSiglecs in a Sia-independent manner. It is known that the Sia-binding site of hCD33rSiglecs are rapidly evolving, most likely to facilitate continual host Sia binding in the face of rapid changes in host Sia expression (Brinkman-Van der Linden et al., 2000; Nagano et al., 2002; Sonnenburg et al., 2004; Varki, 2007). If GBS evolved to display Sias on its capsular polysaccharide (CPS) in order to engage CD33rSiglecs, then these Sias would be required to coevolve with the Sia-binding specificities associated with CD33rSiglec evolution in order to maintain functional interactions whereas protein mediated interactions would not. It is interesting to note that large scale sequencing of mammalian CD33rSiglec gene clusters revealed rapid mutation of the entire V-set domain not just amino acids that appear to be involved in formation of the Sia binding site. This could signify counter-adaptation where host Siglecs are now forced to evolve in order to avoid protein specific interactions that engage other parts of the V-set domain. In this context, it is apparent chimpanzees have acquired a mutation in the Sia-binding site of Siglec-5 that abolish Sia-binding as well as amino acid changes that eliminate  $\alpha$  protein binding; together these mutations may provide additional protection against GBS immune evasion and systemic infection.

GBS bacteremia in humans elicits specific IgM and IgG responses directed against the  $\alpha$  protein (Pannaraj et al., 2007). In the mouse model,  $\alpha$  protein can serve

as an effective maternal immunogen affording passive protection to the infant against lethal infection with  $\alpha$ -positive GBS strains (Madoff et al., 1992). Our studies suggest that the potential effectiveness of a future  $\alpha$  protein based vaccine strategy in humans could derive not only from increased antibody levels for opsonization, but also through interference with  $\alpha$  protein-mediated downregulation of human phagocyte function via hSiglec-5.

## METHODS

***Bacteria and growth conditions.*** GBS WT strains of serotypes Ia (A909) and III (COH1) are well-characterized isolates from human neonates with invasive infections. Generation of the  $\alpha$ NeuA mutants in serotype Ia strain A909 and serotype III strain COH1 were previously reported (Lewis et al., 2004) (Lewis et al., 2007). The  $\alpha$ Bac mutant of GBS strain A909 and its complemented mutant were described previously (Areschoug et al., 2002b). GBS were grown in Todd-Hewitt broth (THB), pH 7.5, or on THB agar plates (THA). For functional assays, unless otherwise noted, bacteria were grown to early-exponential phase in THB, washed three times with pyrogen-free phosphate-buffered saline (PBS), resuspended in appropriate buffers, and adjusted to the desired concentrations using a spectrophotometric method confirmed by pour-plate colony counts. For enzymatic removal of CPS Sias, GBS were incubated in sterile PBS with 100mU/ml *A. ureafaciens* sialidase (AUS) for 1 h at 37°C and then washed 3 times with PBS. Trypsin-treated bacteria were incubated in sterile 0.5% trypsin + EDTA in PBS for 30 min at 37°C and washed 5 times with PBS.

***GBS FITC labeling.*** To prepare heat-killed FITC-GBS, bacteria were grown overnight, diluted 1:100 in 50 ml of THB, grown to early exponential phase, pelleted, washed three times with sterile PBS pH 7.4, and heat-killed by incubation at 56°C for 40 min. GBS were washed in 50 mM carbonate buffer pH 8.0, resuspended in 5 ml carbonate buffer pH 8.0 + 0.1% FITC, and incubated for 1 h at 37°C. Bacteria were extensively washed in PBS and resuspended in the appropriate assay buffer. GBS-FITC were enumerated using a bacterial cytometer and fluorescent microscope.  $1 \times 10^6$  GBS-FITC were resuspended in PBS + 1% BSA and analyzed by flow cytometry (BD FACS caliber) to verify that bacterial staining was uniform throughout each sample. Heat killed FITC-GBS were used for CHO cell adherence assays. For live FITC-GBS, GBS were grown to optical density (OD) of 0.4, washed in PBS, incubated in PBS + 0.1% FITC for 30-60 min at 37°C, washed thoroughly, resuspended and analyzed as described above. Live FITC-GBS were used for all binding assays other than CHO cell binding assays.

***Analysis of GBS Sias using 1,2-Diamino-4,5-methylene dioxybenzene (DMB)-HPLC.*** GBS were washed and lysed by repeated hypotonic freeze-thawing to remove any intracellular Sias. Surface associated Sias were released from the remaining washed pellet using AUS. After filtration of the supernatant over a 10K molecular weight cutoff spin-column, Sias were derivatized with DMB (1,2-diamino-4,5-methylene dioxybenzene) and resolved by reverse phase HPLC as described previously (Lewis et al., 2004). Neu5,7Ac<sub>2</sub> and Neu5,9Ac<sub>2</sub> refer to O-acetyl-modified



Neu5Ac at the 7 and 9-carbon positions respectively. “R” refers to a reagent peak of unknown identity. Note that the A909 $\Delta$ NeuA sample was injected at 5X compared to the WT sample.

***Soluble receptors and stable CHO cell expression.*** Soluble hSiglec-Fc chimeras were produced as previously described (Angata and Varki, 2000). CHO-K1 cells stably expressing full length hSiglec-5 were produced by transfecting WT CHO-K1 cells with pcDNA3.1(+) $\Delta$ Neo-hSiglec-5 using Fugene-6 transfection reagent per manufacturers directions as previously described (Carlin et al., 2007).

***Cell growth conditions and isolation of human neutrophils.*** CHO-K1 cells were grown in Ham’s F10 media supplemented with 10% FBS at 37C and 5% CO<sub>2</sub>. The human monocyte cell line U937 was grown in RPMI supplemented with 10mM HEPES, 1mM Na pyruvate, 10% FCS, and glucose at 37°C and 5% CO<sub>2</sub>. Human neutrophils were prepared as previously described using Polymorphprep<sup>TM</sup> solution (Axis Shield PoC AS, Oslo, Norway) according to the manufacturer’s instructions (Carlin et al., 2007).

***Siglec-Fc binding assays.*** Bacteria were grown to OD 0.4, resuspended in RPMI + 10% FCS, incubated on ice with 10  $\mu$ g/ml Siglec-Fc, followed by PE-conjugated F(ab’)<sub>2</sub> fragment goat anti-human IgG Fc specific (Caltag) and analyzed by flow cytometry. For blocking antibody studies GBS were preincubated with polyclonal rabbit anti- $\square$  protein antibodies (Beta antibody, B6, or 75kDa) (Areschoug et al., 2002b; Heden et al., 1991) for 20 min on ice prior to incubation with Siglec-5-Fc chimeras. Bacteria were washed, incubated with FITC-conjugated F(ab’)<sub>2</sub>

fragment donkey anti-human IgG (H+L) (minimal cross-reaction to Rabbit) (Jackson Immunoresearch), and analyzed by flow cytometry.

***GBS protein isolation, purification, and Western blotting.*** GBS proteins were isolated by growing bacteria overnight in 500 ml THB, washing twice in 50mM Tris pH 7.0, and incubating in TBS pH 7.0 lysis buffer (TBS with mutanolysin 100 U/ml, 1mM EDTA, complete EDTA-free protease inhibitor cocktail (Roche), 1% Triton X-100) for 4 h rotating at 37°C. Bacteria were then subjected to multiple freeze-thaw cycles and the extract was centrifuged at 15,000 G for 20 minutes at 4°C. The supernatant was concentrated and washed with PBS using an Amicon Ultra-10 (10,000 molecular weight cut-off) (Millipore). GBS proteins were separated by SDS-PAGE under reducing conditions, stained with GelCode blue stain reagent (Pierce) or transferred to PVDF, blocked, and probed with Siglec-Fc chimeras (5 µg/ml) and HRP-conjugated goat anti-human IgG (H+L) followed by addition of ECL reagent (SuperSignal chemiluminescent reagent) (Pierce). A partial purification of the □ protein was performed with slight modifications as previously described (Stalhammar-Carlemalm et al., 1993). GBS were grown overnight in THB 500 ml, washed twice with 50mM Tris pH 7.0, and bacteria (~1g) resuspended in 10 ml of 50mM Tris pH 11.0 (10% v/v) to produce a final pH of 9.7. Bacteria were incubated 4 h at 37°C, centrifuged, the supernatant was removed, concentrated and washed in PBS using a microcon 30 (Millipore). Partially purified proteins containing the GBS □ protein were separated and probed as described above.

**Mass spectrometry.** Partially purified GBS proteins were separated by SDS-PAGE as described above. Proteins of interest were excised using an automated EXQuest Spot Cutter (BioRad). Selected proteins were destained in a freshly prepared 1:1 solution (v/v) of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, then washed three times in 50% acetonitrile/10 mM  $\text{NH}_4\text{HCO}_3$ , followed by a brief dehydration in 100% acetonitrile. Proteins were incubated overnight in 50% acetonitrile/10 mM  $\text{NH}_4\text{HCO}_3$  with 0.05  $\mu\text{g}$  trypsin (Roche). Isolated peptides were washed and concentrated in  $\text{C}_{18}$  ZipTips (Millipore) according to the manufacturer's protocol. Samples were directly eluted onto a 100 spot platform using a buffer containing 4- $\beta$ -hydroxycinnamic acid (Agilent), 50% acetonitrile, 10 mM diammonium citrate, and 0.1% trifluoroacetic acid. MALDI-TOF analysis was performed on an Applied Biosystems Q-Star XL hybrid mass spectrometer. MALDI-TOF fingerprint data was analyzed with the online database at Rockefeller University (<http://prowl.rockefeller.edu>).

**Mammalian protein isolation and Western blots.** GBS were added to mammalian cells at an MOI of 20:1 or 10:1 and centrifuged to initiate contact. At specified times cells were washed with ice cold wash buffer (TBS pH 7.4, 1mM EDTA, 1mM NaF, sodium pervanadate), lysed with ice cold lysis buffer (TBS pH 7.4, 1mM EDTA, 1mM NaF, 1% Triton-X, complete EDTA-free protease inhibitor cocktail, sodium pervanadate) for 30 min rotating at 4°C, and centrifuged at 15,000 G for 20 min at 4°C. Protein concentration were measured by BCA protein quantitation kit (Pierce) and protein concentrations were normalized prior to immunoprecipitation

overnight with mouse anti-human Siglec-5 antibody 1A5 and protein-G sepharose beads (4 fast flow BD) as instructed by manufacturer. Proteins were separated by SDS-PAGE under reducing conditions, transferred to PVDF, probed with biotinylated goat anti-human Siglec-5 antibody (R&D systems), rabbit anti-SHP-1 (santa cruz biotechnology), or rabbit anti-SHP-2 (Upstate) along with either streptavidin-HRP (Amersham) or HRP conjugated goat anti-rabbit antibody (Biorad) and ECL reagent (Pierce). For assays involving u937 cells, cells were washed 14 hours prior to addition of GBS and resuspended in RPMI + 0.5% FCS overnight. For assays using CHO cells, N-glycans were removed using PNGase F. After the immunoprecipitation reaction, protein (20  $\mu$ l total volume) was resuspended in 31.6  $\mu$ l H<sub>2</sub>O + 2.4  $\mu$ l 0.5M beta mercaptoethanol (2-mE) + 6  $\mu$ l 10% SDS in 200 mM Tris pH 7.3 and incubated at 100°C for 5 min. Reaction was cooled and 5  $\mu$ l 20% NP 40, 0.8  $\mu$ l 0.5M 2-mE, and 5  $\mu$ l PNGase F were added and reaction was incubated overnight at 37°C. Samples were then resuspended in SDS-PAGE loading buffer and processed as described above.

***GBS adherence assays.*** CHO cell adherence assays were performed as described previously (Carlin et al., 2007). FITC-GBS adherence to individual CHO-K1 cells was analyzed by flow cytometry (BD FACS caliber) after lifting CHO cell monolayers in PBS + 5mM EDTA. Results were expressed as a bacterial binding index, calculated by multiplying the percentage of FITC-positive cells in the cell gate by the mean fluorescence intensity of FITC-positive cells within the cell gate. For leukocyte adherence assays 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 prior to analysis by flow cytometry. For both CHO cell and leukocyte flow cytometry experiments side- and forward-scatter properties were used to distinguish cells (cell gate) from free bacteria, and 20,000 events were collected from the cell gate. For human amnion adherence assays primary human amniotic membranes were separated from fresh placentas and cut into 1 cm squares. FITC-GBS diluted in Hank's Balanced Salt Solution (HBSS) pH 7.4 were added to the amniotic membranes and spun down to initiate contact, incubated for 30 min at room temperature, and washed to remove non-adherent bacteria. Amniotic membranes were mounted onto slides and images were acquired using a fluorescent microscope with appropriate fluorescent filters and CCD camera.

***GBS phagocytosis assay.*** Bacterial cultures were inoculated from a single colony and grown overnight in THB and cultures were diluted 1:10 in fresh THB and GBS were grown to an OD<sub>600</sub> of 0.4 washed once in PBS + 1% BSA and preopsonized with RPMI + 80% heat inactivated (56°C for 40 minutes) human serum for 15 minutes at 37°C. GBS were then added to u937 cells at an MOI of 10:1 to produce a final concentration of RPMI + 10% heat inactivated human serum. Bacteria and cells were spun together to initiate contact, incubated at 37°C for 30 minutes, washed once with serum free RPMI, and resuspended in RPMI + penicillin 5  $\mu$ g/ml and gentamicin 100  $\mu$ g/ml for 2 hours at 37°C to kill extracellular bacteria. The cells were washed in PBS + 1% BSA and lysed using PBS + 0.025% Triton X-100 to liberate intracellular bacteria. Dilutions were plated on THA plates, placed at 37°C overnight, and GBS

CFU were counted. For phagocytosis assay carried out following antibody mediated depletion of Siglec-5 from the surface of U937 cells, the assays were carried out as described except U937 were pre-incubated with 2  $\mu$ l/ml anti-Siglec-5 antiserum (1A5) for 50 minutes at 37°C in RPMI prior to adding GBS. Addition of anti-Siglec-5 antibody causes rapid endocytosis of Siglec-5 from the surface of cells so that after 50 minutes ~10% of original Siglec-5 quantities remain on the surface. All assays were carried out in triplicate. GBS phagocytosis assays were carried out 4 times in the absence of Siglec-5 antibodies and twice with addition of Siglec-5 antibodies.

***GBS U937 cell deconvolution microscopy.*** FITC-GBS were added to U937 cells in RPMI 1640 + 10% heat inactivated human serum at a multiplicity of infection of 10:1, centrifuged to initiate contact, incubated at 37°C in 5% CO<sub>2</sub> for 60 min, washed with ice cold PBS plus 1% BSA, spun down, and resuspended in ice cold PBS + 1%BSA +5mM EDTA. hSiglec-5 was labeled on ice using mouse anti-hSiglec-5 specific antibody (1A5) and secondary anti-mouse IgG-Fc conjugated to AlexaFluor 647 (Invitrogen). Cells were fixed using 2% paraformaldehyde in PBS for 10 min at 4°C. Hoechst stain was added at a 1:2,000 dilution in PBS for 2 min on ice, and cells were washed and resuspended in 10  $\mu$ l of PBS plus 1% BSA and cells were added to glass coverslips and allowed to dry in the dark. Coverslips were mounted in Gelvatol. Images were captured with a DeltaVision Restoration microscope system (Applied Precision Inc., Issaquah, WA) using a Photometrics Sony Coolsnap HQ CCD camera system attached to an inverted, wide-field fluorescence microscope (Nikon TE-200). Optical sections were acquired using a 100x (numerical aperture, 1.4) oil immersion

objective in 0.2- $\mu$ m steps in the z axis, using the attached Applied Precision Inc. motorized stage. The fluorescent markers were excited with a standard mercury arc lamp, and fluorescence was detected using a standard DAPI (4',6'-diamidino-2-phenylindole)-FITC-Cy-5 filter set.

***Histology and immunohistochemistry.*** Human or chimpanzee placentas were frozen in OCT cut in 5  $\mu$ M slices, air dried, fixed in acetone, and endogenous peroxidases blocked with 0.03% H<sub>2</sub>O<sub>2</sub>. Tissue sections were coated with PBS + 1% BSA, blocked with avidin and biotin (Vector Avidin/Biotin kit), coated with PBS + 1% BSA, and primary anti-Siglec-5 antibody (R & D clone 194128) 5  $\mu$ g/ml added followed by an LSAB biotinylated link, and LSAB HRP streptavidin (DAKO). Next AEC (3-amino, 9-ethyl-carbazole) (Vector) was added followed by Mayer's hematoxylin. For amnion immunohistochemistry, primary human amniotic membranes were separated from placental sections and treated as described above except that streptavidin-FITC was added and sections visualized with a fluorescent microscope and appropriate filter set. All images were acquired using an upright microscope and CCD digital camera. For immunohistochemistry, two human placentas were examined and stained similarly. For histology seven human placentas and two chimpanzee placentas were analyzed and stained similarly.

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## **Chapter V**

# **The Surface-Anchored NanA Protein Promotes Pneumococcal Blood-Brain Barrier Invasion**



## INTRODUCTION

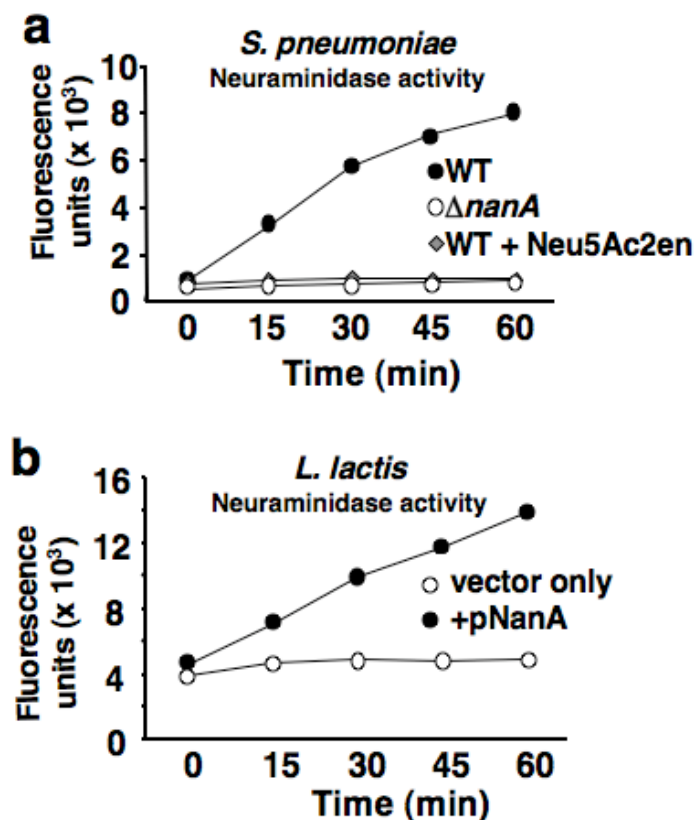
*Streptococcus pneumoniae* (SPN, pneumococcus) accounts for 50% of bacterial meningitis in humans. This often devastating infection carries a 30% mortality rate and up to half of survivors experience neurological sequelae, reflecting a wide spectrum of brain injuries including cortical neuronal necrosis and hippocampal neuronal apoptosis (Koedel et al., 2002; van de Beek et al., 2004; Weber and Tuomanen, 2007; Weisfelt et al., 2006). To cause meningitis, bloodborne bacteria must first attach to and penetrate human brain microvascular endothelial cells (hBMEC), the single cell layer comprising the majority of the blood-brain barrier (BBB) (Kim, 2003; Tuomanen, 1996). The molecular mechanisms underlying the distinct central nervous system (CNS) tropism of SPN are incompletely understood. All SPN clinical isolates express the surface anchored neuraminidase (sialidase) NanA (Camara et al., 1994; Pettigrew et al., 2006) that targets sialic acid residues on host cells and bacterial flora to promote SPN mucosal colonization (Shakhnovich et al., 2002; Tong et al., 2002). Here we use an isogenic SPN NanA-deficient mutant and heterologous expression of the protein to show that NanA is necessary and sufficient for adherence and invasion to hBMEC, in a manner independent of its neuraminidase activity. NanA promotes SPN-BBB interaction in a murine infection model, identifying the protein as proximal mediator of CNS entry by the pathogen.

## RESULTS

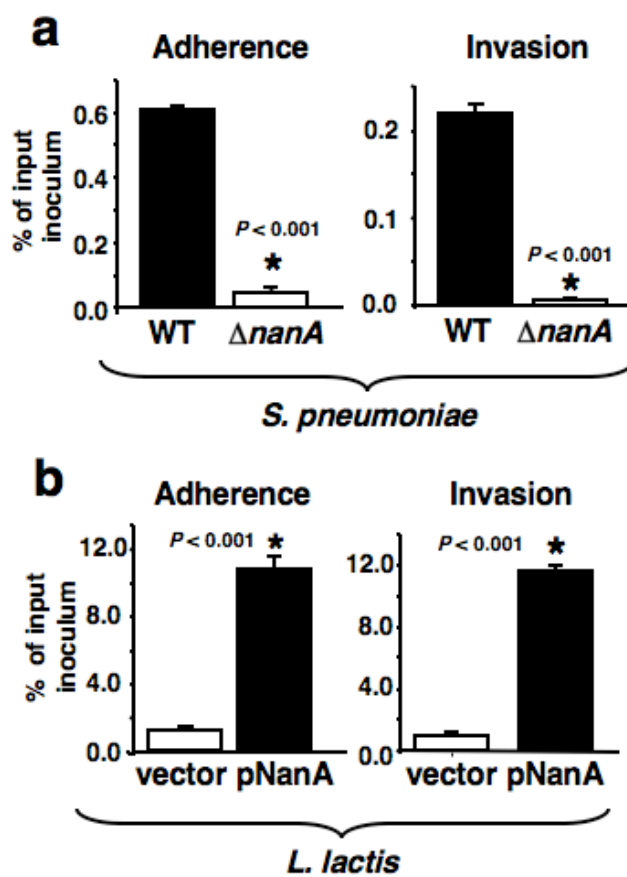
NanA is a molecule of 1,035 amino acids and ~107 kDa molecular mass

containing a C-terminal LP(X)TG anchor motif for sortase-mediated anchoring to the SPN cell wall (Camara et al., 1994; Lock et al., 1988). NanA neuraminidase has enzymatic activity on three linkage classes of substrates,  $\alpha$ 2-6 and  $\alpha$ 2-3 linkages of N-acetylneuraminic acid to galactose, and  $\alpha$ 2-6 linkages of N-acetylneuraminic acid to N-acetylgalactosamine (Scanlon et al., 1989). To investigate the potential contributions of NanA to SPN interactions with the BBB endothelium, we compared the well-characterized serotype 2 strain clinical isolate D39, originally isolated by Avery in 1916 (Avery et al., 1944) and highly virulent in animal infection models (Lanie et al., 2007), to its isogenic mutant  $\Delta$ NanA, confirmed to possess a targeted nonpolar mutation that eliminates NanA expression (Winter et al., 1997). Using a fluorescent assay, we found the  $\Delta$ NanA mutant to lack neuraminidase activity present in the WT D39 parent strain (**Figure 5.1a**). For gain of function analysis, we amplified the *nanA* gene from the D39 chromosome, cloned it into an expression vector to generate the recombinant plasmid pNanA, and used this to transform the nonpathogenic Gram-positive bacterium *Lactococcus lactis*. Compared to the empty vector control, pNanA conferred significant neuraminidase activity to *L. lactis* (**Figure 5.1b**).

Studies of microbial interactions with the human BBB have been greatly facilitated by the development of an immortalized hBMEC line retaining the morphological and functional characteristics of primary endothelium.(Kim, 2006) Adherence and intracellular invasion of hBMEC within a membrane-bound vacuole appears to be a common phenotypic property of several CNS bacterial pathogens



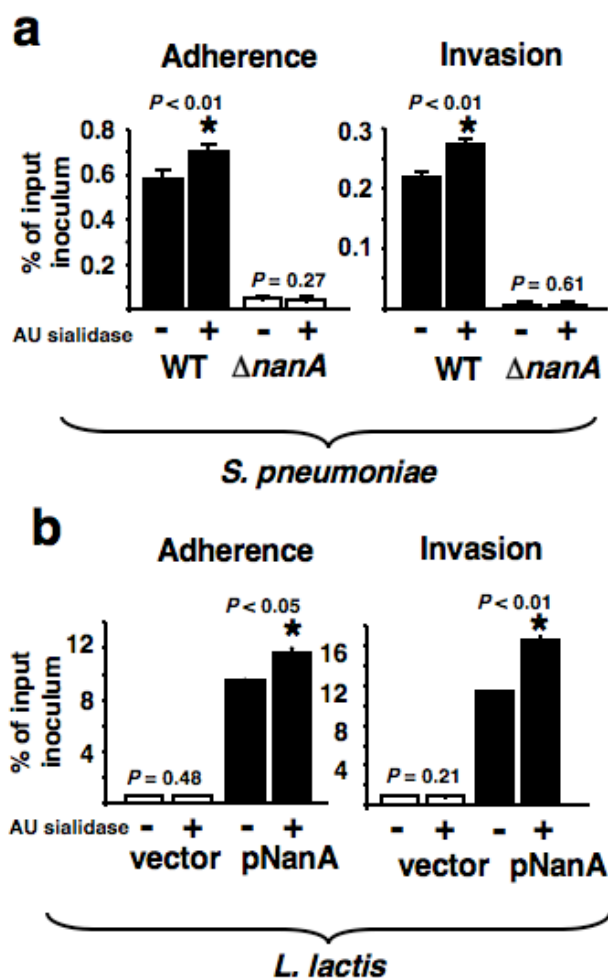
**Figure 5.1 NanA necessary for neuraminidase activity.** Bacterial lysates were incubated with 4MU-Neu5Ac to measure cleavage of Neu5Ac from 4-MU (fluorescent product) (a) Loss of neuraminidase activity in isogenic SPN  $\Delta NanA$  mutant and effectiveness of neuraminidase inhibitor Neu5Ac2en. (b) Gain of neuraminidase activity in *L. lactis* transformed with plasmid containing SPN NanA. Neuraminidase assay performed three times with similar results; representative experiment shown.



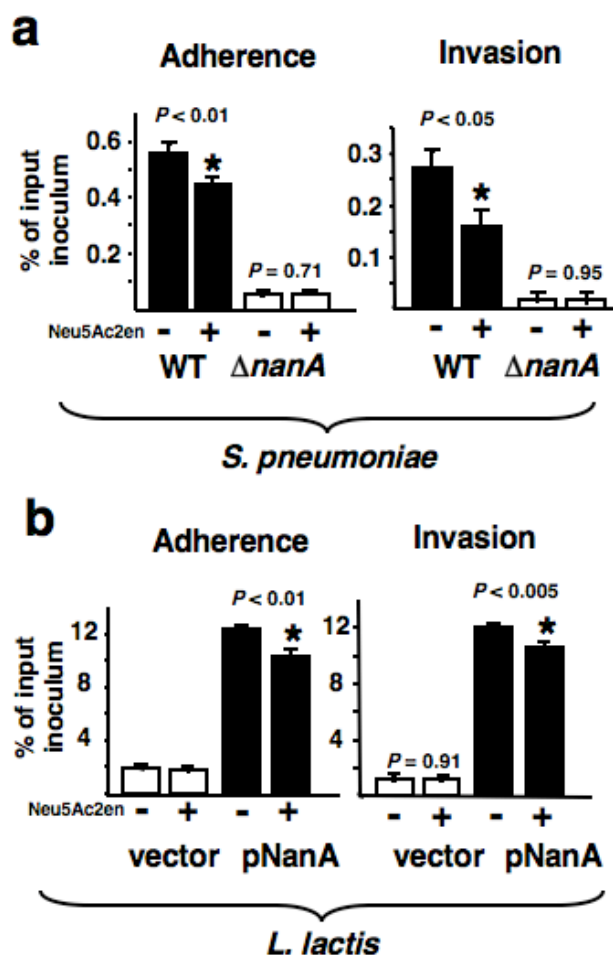
**Figure 5.2 SPN NanA contributes to hBMEC invasion and adherence.** Bacteria were allowed to interact with Human Brain Microvascular Endothelial Cells (hBMEC) to determine bacterial adherence or extracellular bacteria were killed with antibiotics to measure intracellular bacteria. (a) Decreased hBMEC adherence and invasion by  $\Delta$ NanA mutant compared to WT parent SPN strain. (b) Increased hBMEC adherence and invasion by *L. lactis* expressing SPN NanA. Adherence and invasion assays performed in triplicate and repeated three times; graphs show cumulative data from all experiments. Error bars represent standard deviation, statistical analysis by Student's T-test.

including SPN, group B *Streptococcus* (GBS), *Escherichia coli* K1, and *Haemophilus influenzae*. Using an *in vitro* assay, we found the SPN  $\Delta$ NanA mutant to exhibit a > 90% decrease in hBMEC adherence and invasion compared to the WT D39 parent strain (**Figure 5.2a**,  $P < 0.001$ ). Conversely, heterologous expression of SPN NanA in *L. lactis* conferred a > 10-fold increase in the bacterium's ability to adhere to and invade the cultured hBMEC (**Figure 5.2b**,  $P < 0.001$ ). The addition of exogenous neuraminidase (from *Arthrobacter ureafaciens*) failed to rescue the adherence and invasion defects of the SPN  $\Delta$ NanA mutant nor did it stimulate *L. lactis* hBMEC interactions in the absence of NanA (**Figure 5.3a,b**). However, for SPN or *L. lactis* expressing NanA, exogenous neuraminidase stimulated a modest (20-30%) but statistically significant increase in hBMEC adherence and invasion (**Figure 5.3a,b**). These results indicate that SPN NanA is both necessary and sufficient to promote efficient bacterial hBMEC adherence and invasion, and suggest this phenotype is enhanced but not dependent on the neuraminidase activity of the surface bound protein.

To further dissect the roles of SPN NanA and its enzymatic activity in mediating interactions with hBMEC, we utilized the broad-spectrum neuraminidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) (Holzer et al., 1993) (**Figure 5.1a**). When used at a concentration effective at inhibiting SPN neuraminidase activity (**Figure 5.1a**), Neu5Ac2en reduced but did not eliminate NanA-dependent adherence and invasion of hBMEC in both the SPN and *L. lactis* backgrounds (**Figures 5.4a,b**); the neuraminidase inhibitor had no effect on the low



**Figure 5.3 Exogenous sialidase cannot rescue the decrease in adherence and invasion caused by lack of NanaA** (a) Exogenous neuraminidase (*A. ureafaciens* sialidase) does not rescue hBMEC adherence and invasion defects of SPN  $\Delta$ NanA mutant. (b) Exogenous neuraminidase is not sufficient to promote increased hBMEC adherence and invasion by *L. lactis* lacking NanaA. Adherence and invasion assays performed in triplicate and repeated three times; graphs show cumulative data from all experiments. Error bars represent standard deviation, statistical analysis by Student's T-test.

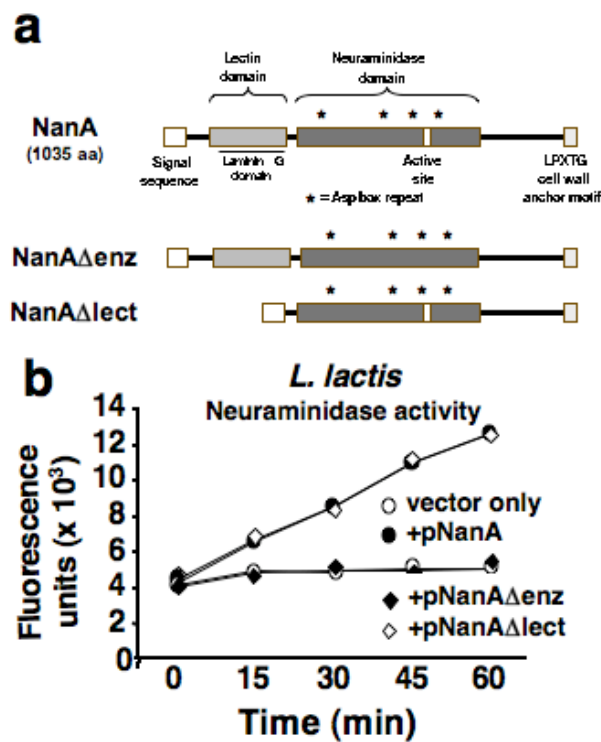


**Figure 5.4 NanA promotion of hBMEC invasion and adherence is only partially dependent upon neuraminidase activity.** (a) Addition of the neuraminidase inhibitor Neu5Ac2en reduces but does not eliminate hBMEC adherence and invasion by WT SPN. (b) Neuraminidase inhibition reduces but does not eliminate the ability of NanA to confer an hBMEC adherence and invasion phenotype to *L. lactis*. Adherence and invasion assays performed in triplicate and repeated three times; graphs show cumulative data from all experiments. Error bars represent standard deviation, statistical analysis by Student's T-test.

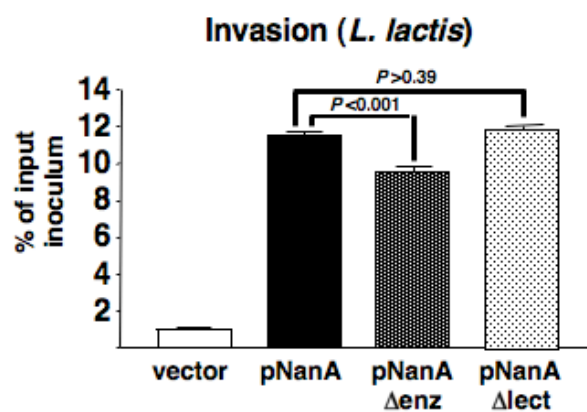
level baseline hBMEC interactions present in bacteria lacking NanA. These findings support a significant contribution of NanA to hBMEC invasion independent of its enzymatic activity.

Two targeted deletions were constructed in the recombinant NanA protein to determine their potential effects on NanA-mediated hBMEC interactions. Like other nonviral neuraminidases, SPN NanA shows a recurring motif (Ser/Thr-X-Asp-[X]-Gly-X-Thr-Trp/Phe) known as the “Asp box” that repeats four times along its length (Roggentin et al., 1989) (**Fig. 5.5a**). Amid the Asp box repeat domain, active site residues essential for the neuraminidase activity of SPN NanA have recently been identified (Yesilkaya et al., 2006), and we found that deletion of a region containing two of these residues (Glu647 and Arg663) from pNanA yielded a derivative plasmid (pNanA $\square$ enz) that did not confer neuraminidase activity when expressed in *L. lactis* (**Fig. 5.5a,b**). Similar to observations with chemical inhibition of NanA enzymatic activity, genetic deletion of the active site residues reduced but did not eliminate the ability of the recombinant protein to confer a phenotype of enhanced hBMEC adherence and invasion to *L. lactis* (**Fig. 5.6**). SPN NanA belongs to the superfamily of concavalinA-like glucanases/lectins, containing a sugar cleavage domain and a second “lectin domain” that binds sugars or resembles a domain that binds sugars; two such proteins have solved crystal structures, the *Vibrio cholerae* sialidase (Crennell et al., 1994) and the *Trypanosoma cruzi* trans-sialidase (Buschiazzo et al., 2002). Since the neuraminidase activity of NanA is hypothesized to promote SPN colonization by modulating carbohydrate moieties on epithelial cell surfaces and exposing higher





**Figure 5.5 Domain deletions of NanA** (a) Scheme for deletion of active site residues and lectin domain from recombinant SPN NanA protein. (b) Deletion of active site region eliminates NanA-induced neuraminidase activity in *L. lactis* while deletion of the NanA lectin domain has no effect. Neuraminidase assay performed three times with similar results; representative experiment shown.



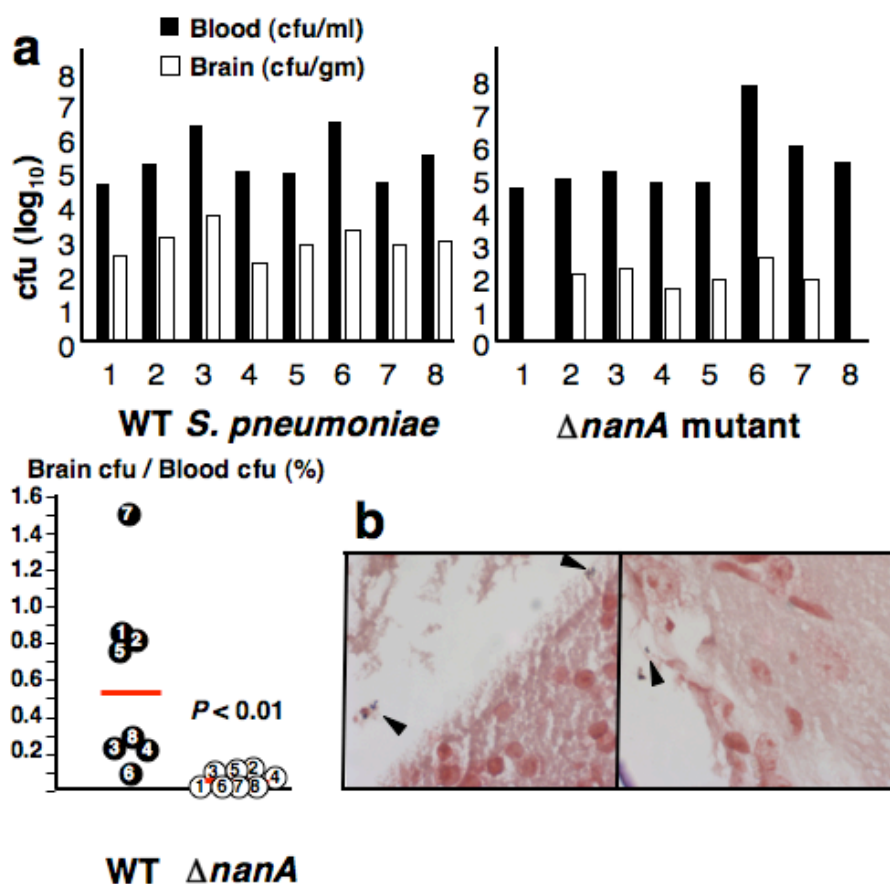
**Figure 5.6 Nan A neuraminidase activity and lectin binding domain are not required for adherence and invasion of hBMEC.** Deletion of active site residues reduces but does not eliminate the ability of NanA to confer an hBMEC adherence and invasion phenotype to *L. lactis*; deletion of NanA lectin domain has no effect. Adherence and invasion assays performed in triplicate and repeated three times; graphs show cumulative data from all experiments. Error bars represent standard deviation, statistical analysis by one-way ANOVA with Tukey's post test.

affinity receptors (King et al., 2006; Tong et al., 2002), it is conceivable that a NanA lectin function could complement its enzymatic activity in mediating host cell interactions. The laminin G-like candidate lectin domain of SPN NanA is situated at its N-terminus, and we generated a deletion derivative of pNanA lacking this domain, pNanA $\Delta$ lect (Fig. 5.5a). Elimination of the lectin domain of NanA did not affect neuraminidase enzymatic activity (Fig. 5.5b), nor did it reduce the ability of the recombinant protein to confer hBMEC invasion or adherence when expressed in *L. lactis* (Fig. 5.6).

Lastly, we sought to determine whether the marked contribution of SPN NanA to hBMEC adherence and invasion *in vitro* translated to increased capacity of SPN to penetrate the BBB *in vivo*. Our choice of animal model had to exclude the confounding contribution of NanA to sustained bacteremia, since a NanA-deficient SPN mutant was recently found to be cleared from the mouse circulation within 24 h of intravenous challenge while the WT parent strain proliferated (Manco et al., 2006). To focus specifically on SPN-BBB interactions, we developed a short-term (4 h) murine intravenous challenge model that incorporated terminal exsanguination by saline perfusion (to eliminate blood contamination), allowing calculation of the ratio of SPN colony forming units (cfu) in brain vs. blood at the experimental endpoint. We found that at 4 h the levels of WT D39 and isogenic  $\Delta$ NanA mutant SPN in blood were similar, but the median ratio of brain/blood cfu in the WT strain (0.512%) was 16-fold greater than (0.031%) ( $P < 0.01$ ) (Fig. 5.7a). Histopathologic analysis and tissue Gram stain could identify cocci associated with brain endothelium in sections from

WT-infected mice (**Fig. 5.7b**); CNS-associated bacteria were scant or absent in those animals infected with the isogenic  $\Delta$ NanA mutant. No acute inflammatory changes were seen in the brains of animals from either group in this short term experiment.

We conclude that NanA plays an important role in SPN invasion of BBB endothelial cells and bacterial entry into the CNS, the critical first step in the pathogenesis of meningitis. After SPN breaches the BBB endothelium, subsequent brain injury is more likely the byproduct of different SPN virulence determinants (e.g. the pore-forming toxin pneumolysin (Stringaris et al., 2002; Wellmer et al., 2002)) or host factors, since previous studies using direct intracerebral installation of SPN in mice or guinea pigs identified no differences between WT and  $\Delta$ NanA mutant in meningeal inflammation, hearing loss or neuronal injury scores (Wellmer et al., 2002; Winter et al., 1997). Nevertheless, a clinical correlation between the magnitude of free sialic acid levels in cerebrospinal fluid during pneumococcal meningitis and risk of adverse neurological sequelae including coma has been reported (O'Toole et al., 1971).



**Figure 5.7 NanA contributes to SPN blood-brain barrier penetration *in vivo*.** (a) Comparison of bacterial counts (cfu) recovered from the blood and brain of mice 4 h after intraperitoneal challenge with  $5 \times 10^7$  cfu of WT SPN strain D39 or its isogenic  $\Delta$  NanA mutant; while blood cfu did not differ between the two groups ( $P = 0.37$ ), the ratio of brain cfu: blood cfu in individual mice was significantly higher in mice infected with the WT SPN strain than the  $\Delta$  NanA mutant ( $P < 0.01$ ). Statistics by Student's T-test; red line shows median. (b) Representative histopathologic examination (100 x) of brain from WT SPN-infected mice showed Gram-positive cocci in association with brain microvascular endothelium or within the brain parenchyma (arrows); such findings were extremely rare or absent in animals infected with the  $\Delta$ NanA mutant.

## DISCUSSION

Loss of two additional SPN surface proteins has been previously associated with diminished invasion and transcytosis of cultured hBMEC cells – the fibronectin-binding protein PavA and choline-binding protein A (CbpA) (Pracht et al., 2005; Ring et al., 1998). Operating in the context of the low capsule, “transparent” phase variant phenotype of SPN, CbpA can present choline that in turn attaches to the platelet activating factor receptor on the hBMEC surface (Ring et al., 1998). Although the high-capsule, “opaque” phenotype of SPN predominates in the bloodstream (transparent forms are readily phagocytosed), the high-frequency switch between the two phase variants is sufficient to reveal a contribution of CbpA to BBB penetration in vivo (Orihuela et al., 2004). The studies of NanA reported in the present manuscript were all performed with inocula of SPN expressing the opaque phenotype. We propose that NanA and CbpA may function synergistically to promote passage of the phase-variant SPN bacterium across the BBB.

The conservation of NanA expression in all clinical isolates of SPN prompted interest in the recombinant protein as a universal vaccine antigen against this leading pathogen (Tong et al., 2005; Yesilkaya et al., 2006), since current conjugate polysaccharide vaccines only target a subset of the most common SPN serotypes. Our identification of NanA function in hBMEC adherence and invasion suggests such immunization strategies could have an added benefit in reducing the risk of meningitis as a secondary complication of bloodstream infection.

## METHODS

**Bacterial strains and preparation of inocula.** *Streptococcus pneumoniae* (SPN) serotype 23 strain D39 (NCTC 7466) and its isogenic D39 $\Delta$ NanA were used for these experiments. D39 $\Delta$ NanA is deficient in neuraminidase A and was constructed by nonpolar insertion-duplication mutagenesis of the *nanA* gene as previously described (Winter et al., 1997); identity was verified by erythromycin (2 $\mu$ g/ml) resistance. SPN cultures were grown in Todd-Hewitt broth (THB) + 0.5 yeast extract (THY media). *Lactococcus lactis* strain NZ9000 is derived from MG1363 and lacks the nisin operon (Kuipers et al., 1997); *L. lactis* transformed with pNanA, deletion plasmids pNanA $\Delta$ lect and pNanA $\Delta$ enz, or empty vector control were grown in THB + erythromycin (5 $\mu$ g/ml). After overnight incubation at 37°C, 5%CO<sub>2</sub>, bacteria were reinoculated into fresh media, grown to mid-logarithmic growth phase (OD<sub>600n</sub> = 0.4), centrifuged at 4,000 rpm x 5min, washed once with PBS, then resuspended in the appropriate buffer for use in the assays described below.

**Generation of NanA expression plasmids.** The NanA gene was amplified by PCR from the D39 chromosome using primers NanAUpF (5'-GATTGGAGAAAGGAGAGGGG-3') and NanADownR (5'-CGTATCAGCCTGAATGTCATC-3'), and the resulting amplicon directionally cloned into E. coli/Gram-positive shuttle plasmid pDCerm to yield the expression plasmid pNanA. Elimination of the critical active site residues of NanA from pNanA was accomplished by inverse PCR using primers NanAenzUp (5'-TGTATTTTGCACGTCTATTGTTC-3'), a reverse strand primer located upstream of the key residues, and NanAenzDown (5'-

GGTTGACTGGAGATCTTCAGGTTG-3'), a forward strand primer located just downstream of these residues. Blunt end ligation of the amplicon yielded pNanA $\Delta$ enz, an expression plasmid containing a version of NanA harboring deletion of residues 645 to 662 including two amino acids critical for neuraminidase activity. To eliminate the N-terminal lectin domain of NanA, a similar process of inverse PCR followed by blunt end ligation was performed using the primers NanAlecUp (5'-GGTTGAGCTCTCCCCGAAAG-3') and NanAlecDown (5'-ATGCCAGATGTAACGCATGTGCA-3'), yielding pNanA $\Delta$ lec, an expression plasmid containing NanA with a deletion of residues 74 to 229. The desired deletions in pNanA $\Delta$ enz and pNanA $\Delta$ lec were confirmed by direct sequencing of purified plasmids. All expression plasmids were used to transform *L. lactis* and transformants identified by erythromycin selection for functional analyses.

**Neuraminidase activity assay.** A quantitative assay utilizing 4-Methylumbelliferyl-N-acetyl- $\beta$ -D-neuraminic acid sodium salt hydrate (4-MU; Fluka USA) was used to assay neuraminidase activity. SPN (WT D39 and isogenic  $\Delta$ NanA mutant) and *L. lactis* expressing NanA constructs were centrifuged, washed once, resuspended in 100  $\mu$ l 0.1M phosphate citrate buffer (pH 6.5) to  $10^9$  colony forming units (cfu)/ml. Bacterial suspensions were sonicated (Fisher Sonic Dismembrator 550) on ice for 5 sec followed by a 15-sec rest intervals x 5 cycles. Sample aliquotes of 50  $\mu$ l were added to individual wells of a 96-well microtiter plate and mixed with 50  $\mu$ l of 4-MU diluted to 0.35% in phosphate citrate buffer. The plate was incubated at 37°C and fluorescence (excitation 360 nm, emission 460 nm) recorded every 15 minutes



from time 0 to 1 h; with reported values corrected for the reaction blank containing phosphate citrate buffer alone.

***Human brain microvascular endothelial cell assays.*** Immortalized human brain microvascular endothelial cell line hBMEC, a product of SV-40 large T antigen transformation maintaining the morphological and functional characteristics of primary endothelium, was obtained from Kwang Sik Kim (Johns Hopkins University, Baltimore, Maryland, USA). hBMEC were maintained in RPMI 1640 medium (Life Tech Technologies Inc., Grand Island, New York, USA) supplemented with 10% FBS, 10% NuSerum (Becton, Dickinson and Co., Bedford, Massachusetts, USA), 1% MEM nonessential amino acids, and were incubated at 37°C in 5% CO<sub>2</sub>. Bacterial adherence and invasion assays were performed essentially as previously described (Doran et al., 2005), and briefly summarized as follows. With 24 h of established confluence (~10<sup>5</sup> cells/well), hBMEC monolayers were washed twice with PBS, and fresh RPMI 1640 + 10% FBS added. SPN or *L. lactis* strains described above were resuspended in RPMI + 10% FBS and added to monolayers at multiplicity of infection = 10 bacteria/cell. Plates were centrifuged at 800 x g for 5 min to place bacteria on the monolayer surface, then incubated at 37°C in 5% CO<sub>2</sub> for 30 min (adherence assays) or 2 h (invasion assays). For evaluating adherence (total cell-associated bacteria), the wells were washed six times with PBS, 100  $\mu$ l trypsin added, cells lysed by repeated trituration, and dilutions plated on THY agar for enumeration of surviving cfu. For invasion assays, the wells were washed x 3 with PBS and antibiotics (5  $\mu$ g/ml penicillin + 100  $\mu$ g/ml gentamicin) added for 1 h (SPN) or 2 h (*L. lactis*) to kill

extracellular bacteria. At that point, wells were washed again x 3 with PBS, trypsin added, cells were lysed, and dilutions of the lysate plated on. Bacterial invasion was calculated as (recovered cfu/inoculum cfu) x 100%.

***Murine model of SPN blood-brain barrier invasion.*** Eight to 10 week old male BALB/c mice were injected intravenously with  $5 \times 10^7$  cfu of SPN D39 or its isogenic D39  $\Delta$ NanA mutant. Four hours after injection, blood was collected and serial dilutions plated to enumerate bacterial cfu in the circulation. Mice were then deeply anesthetized using an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg), chest opened, and blood cleared by inserting a 23 gauge needle into the left ventricle, perfusing with sterile PBS for 7 min using a peristaltic perfusion pump (Amersham Biosciences P-1), and allowing fluid to exit through the right atrium. After perfusion, brains were removed and the hemispheres were separated. One half of the brain was homogenized in PBS, diluted, and plated on THY agar plates to enumerate bacteria tightly adherent to the BBB; while the other half was fixed in PBS + 4% PFA for histopathological analysis.

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## **Chapter VI.**

### **Conclusions and Perspectives**

Numerous human pathogens that are the causative agents of serious disease express sialic acids on the terminal ends of their cell surface glycans or lipopoligosaccharides, including all serotypes of group B *Streptococcus*, *Neisseria meningitidis* groups B and C, *Escherichia coli* strain K1, *Campylobacter jejuni*, *Trypanasoma cruzi*, and *Cryptococcus neoformans*. These organisms produce complex sialylated oligosaccharide structures that also closely mimic some of the common glycans present on the surface of all human cells. The previously known benefits of sialylation to the pathogen (restriction of complement activation, inhibition of phagocytosis and negative charge repulsion) fail to explain the reasons for this remarkable mimicry. In this thesis, I report on a plausible explanation for this phenomenon.

The CD33rSiglecs are a subfamily of Siglec proteins that are expressed prominently on the surface of all cells of the human innate immune system. They recognize sialylated oligosaccharides using an extracellular V-set immunoglobulin domain and contain intracellular ITIM and ITIM-like motifs that typically play a role in negative regulation of immune cell function. The human CD33rSiglecs have been postulated, but never proven, to function by recognizing sialylated oligosaccharides with their extracellular domains, causing recruitment of protein tyrosine phosphatases to the cytoplasmic tail, and thereby inhibiting signaling cascades involved in cell activation.

Based on the binding specificities and negative signaling potential of the CD33rSiglecs we hypothesized that the sialylated oligosaccharides of the GBS



attached to the capsular polysaccharide of GBS would engage these receptors on leukocytes leading to a diminished inflammatory response. The capacity of GBS Sias to directly downregulate human cellular innate immunity could potentially contribute to GBS pathogenesis and reveal a paradigm shared by other sialylated infectious disease agents.

During the course of this dissertation work we discovered that all serotypes of GBS were capable of binding to at least one human CD33rSiglec. We demonstrated that serotype III and serotype Ia GBS, together responsible for a majority of GBS disease, engaged human Siglec-9 and Siglec-5 respectively. Serotype III GBS engaged Siglec-9 in a Sia-dependent mechanism as we had hypothesized but the serotype Ia GBS engaged Siglec-5 in a novel Sia-independent mechanism mediated by the  $\alpha$  protein of the microorganism. GBS CPS Sias and the  $\alpha$  protein functionally engaged human leukocyte CD33rSigeles causing changes in intracellular recruitment of protein tyrosine phosphatases. GBS expression of the  $\alpha$  protein or CPS Sias decreased bacterial phagocytosis by human leukocytes, and in the case of the  $\alpha$  protein this effect was demonstrated to occur directly through Siglec-5 engagement. Therefore, GBS has evolved two distinct mechanisms of interacting with CD33rSigeles that both result in inhibition of host leukocyte responses.

Our studies using GBS Sias to engage Siglec-9 are the first to show effects of Sia-binding *in trans* on intracellular signaling by CD33rSigeles. However, based on evolutionary comparisons between CD33rSigeles from humans and great apes we believe the intrinsic function of these receptors is to recognize Sias on “self” cell

surfaces and not on microorganisms. In order to further understand the relationship between Sia-binding and negative signaling by CD33rSiglecs on leukocytes we successfully developed an assay where human neutrophils bound to multivalent synthetic sialylated glycans presented *in trans* attached to ELISA plates. The Sia-dependent binding increases with neutrophil activation and likely involves the CD33rSiglecs, Siglec-5 and Siglec-9, although specific antibody blocking studies will be required to confirm. Neutrophils interacting with Sias *in trans* released less interleukin-8 (IL-8) upon stimulation, consistent with the hypothesis that engagement of CD33rSiglecs by Sias causes inhibition of the leukocyte inflammatory response. In addition, these results directly mimic the decreased IL-8 response observed when Siglec-9 on human monocytes interacts with sialylated GBS.

Since we began studying Siglec interactions with GBS it has been demonstrated that *N. meningitidis*, *C. jejuni*, *T. cruzi*, and porcine reproductive and respiratory syndrome virus (PRRSV), interact with various Siglecs present on cells of the innate immune system in a Sia-dependent manner (Avril et al., 2006; Delputte and Nauwynck, 2004; Jones et al., 2003; Monteiro et al., 2005; Vanderheijden et al., 2003). Until now research involving Siglecs has focused on sialylated pathogens and Sia-dependent interactions with these receptors. Our work on GBS Sia-independent interactions with Siglecs suggests that microbial subversion of Siglecs is not restricted to Sia-expressing pathogens, but may be a common mechanism of immune evasion. Future studies can aim at identifying new Sia-independent interactions between microorganisms and human Siglecs. For example, microorganisms could be

incubated with fluorescently labeled hSiglec-Fc chimeras in the presence of high quantities of blocking Sias and sorted using flow activated cell sorting (FACS) to identify potential Sia-independent interactions.

We have also shown that engagement of CD33rSiglecs by GBS leads to changes in recruitment of protein tyrosine phosphatases to Siglec intracellular domains. Furthermore, we demonstrated that GBS engagement of Siglec-5 and Siglec-9 leads to decreased bacterial phagocytosis and diminished release of pro-inflammatory cytokines such as IL-8. Based on previous data supporting the role of protein tyrosine phosphatases in downregulation of immune cell function, we believe that the increased recruitment of SHP-1 and SHP-2 to Siglecs is the cause of the decreased phagocytosis and IL-8 release (Kant et al., 2002; Salmond and Alexander, 2006; Shultz et al., 1997; Zhang et al., 2000). However, future studies should confirm this connection as well as determine the exact mechanisms by which SHP-1 and SHP-2 recruitment to Siglec-5 and Siglec-9 alter the activation of human leukocytes. Our preliminary results have revealed that there is rapid dephosphorylation of the MAP kinase Erk42/44 following leukocyte interaction with  $\square$  protein expressing GBS that is not observed in the GBS $\square$ Bac mutants. It is possible that Erk dephosphorylation is the direct result of phosphatase recruitment to Siglec-5, and that downregulation of this arm of the MAP kinase cascade may result in the observed leukocyte phenotypes.

Sialoadhesin or Siglec-1 is a large surface receptor with 17 extracellular Ig domains and no cytoplasmic signaling motifs, which is expressed on the surface of tissue macrophages. In contrast to the CD33rSiglecs that are thought to be involved in

negative regulation of innate immunity, Sialoadhesin mediates cell-cell interactions through recognition of cell surface Sias. Sialoadhesin has been implicated in the binding and phagocytosis of *N. meningitides* (Jones et al., 2003) and our studies using Sialoadhesin-Fc chimeras suggest that this receptor engages GBS in a sialic acid-specific manner. Sialoadhesin recognizes  $\alpha$ 2-3 linked Neu5Ac, which is the most common Sia and Sia-linkage found on the surface of pathogenic organisms. This receptor may have evolved to recognize and bind sialylated microorganisms that are capable of avoiding other arms of the immune system through molecular mimicry of host sialylated oligosaccharides. Future studies will focus on the role of Sialoadhesin in protection against sialylated GBS infection by comparing GBS infections in wild-type and Sialoadhesin knockout mice (Oetke et al., 2006).

*Streptococcus pneumoniae* (SPN) produces a cell-surface anchored neuraminidase A (NanA) that cleaves Sias from cell surfaces (Camara et al., 1994). We initially hypothesized that the neuraminidase of SPN would cleave Sias from the surface of host leukocytes, thereby leading to unmasking of CD33rSiglecs, decreased negative signaling, and heightened immune stimulation. Indeed, using SPN and the neuraminidase deficient mutant SPN $\Delta$ NanA, we observed a specific defect in the ability of the NanA mutant to adhere to and invade human brain microvascular endothelial cells (hBMEC). SPN is the leading cause of bacterial meningitis in humans and causes death in about 30% of cases. To cause meningitis, bacteria must first adhere to and invade the hBMEC, the single cell layer that comprises the majority of the blood brain barrier (BBB). We next demonstrated that SPN NanA was both

necessary and sufficient to cause bacterial adherence and invasion of hBMEC cells,. However, contrary to our original hypothesis, this activity was independent of its enzymatic function or lectin binding domain. Moreover, NanA contributed to SPN-BBB adherence in vivo in a mouse model of infection. Although the experiments contained in this dissertation do not address the effect of the SPN neuraminidase on CD33rSiglec function, this question remains relevant and should be explored.

Our ultimate goal is to understand the contribution of bacterial interactions with human Siglecs to the pathogenesis of bacterial infection. The studies described here using GBS show that this leading pathogen of human neonates does in fact functionally engage human CD33rSiglecs by multiple mechanisms, and that these interactions have the potential to subvert host immunity to the advantage of the bacterial pathogen. Of course, as GBS is also a common commensal on certain mucosal surfaces, the same mechanisms could be involved in maintaining the commensal state, becoming virulence factors only when other circumstances provide the opportunity. In future studies, we hope to better assess the ability of GBS interactions with hCD33rSiglecs to contribute to human disease. However, human CD33rSiglecs are rapidly evolving and are very different from murine CD33rSiglecs, making knockout mice a poor in vivo model for understanding these effects. Thus, we are producing multiple transgenic mice that are capable of expressing human CD33rSiglecs in a tissue specific manner using Cre-LoxP technology. As these CD33rSiglec humanized mice become available, we will test the specific contributions of CPS Sias and the  $\alpha$  protein to GBS pathogenesis in vivo.

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