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# MOLECULAR MIMICRY OF HOST SIALOME BY HUMAN PATHOGENS TO EXPLOIT IMMUNOREGULATORY SIGLECS

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**Summary**

This thesis focuses on the characterization of two human-specific pathogens that mimic host sialic acid surface structures to engage human immunoregulatory Siglec receptors. Furthermore, it explores the use of sialic acid and sialic acid-like molecules as tools and therapeutics.

The first chapter introduces the different types of sialic acids and their diverse functions in prokaryotes and eukaryotes. It reviews different viral, bacterial, fungal and parasitic pathogens that use sialic acid, in particular as molecular mimicry, to engage the host and subvert the host immune system.

The second chapter characterizes the interactions between *Escherichia coli* K1, which mimics host polysialic acid, and the paired receptors Siglec-11 and Siglec-16. *E. coli* K1 engages the inhibiting receptor Siglec-11 to escapes killing. In contrast, binding to Siglec-16, an activating immunoregulatory receptor, increases inflammatory responses and phagocytosis. Chapter two also introduces a murine model to study the interaction of pathogens with paired receptors.

The third chapter describes interactions between *Neisseria gonorrhoeae*, the causative agent of gonorrhea, and human Siglecs as well as their potential impact on pathogenesis. The interactions are mediated by sialylated lipooligosaccharide (LOS) structures that mimic human glycosphingolipids. Additionally, gonococcal porins engage Siglecs in a sialic acid-independent manner. In many individuals, a fusion event between *SIGLEC5* and *SIGLEC14* genes and a polymorphism in the *SIGLEC16* allele lead to the loss of functional Siglec-14 and Siglec-16, respectively. The impact of the loss of these activating receptors on the infection rate is explored in a remote population of Namibian pastoralists, which have a high burden of gonorrhea.

The increasing number of infections and antibiotic resistance of *N. gonorrhoeae* pose a risk to the future of gonorrhea treatment. Chapter 3 introduces a novel therapeutic strategy to that may treat and prevent gonococcal infections. *N. gonorrhoeae* incorporates host sialic acid into its LOS to inhibit the classical and alternative pathway of the host complement system. *N. gonorrhoeae* also

incorporates sialic acid analogs (e.g. legionaminic acid) into its LOS, which are usually not available to the pathogen. Consequently, it is not able to inhibit the classical pathway anymore. Administration of legionaminic acid to a gonorrhea mouse model leads to a reduced clearance time and infection burden.

The 9-O-acetyl modification of sialic acid is very common in humans and other animals. However, it is very difficult to study since this modification is very unstable. The addendum chapter suggests a chemical solution to this problem. The oxygen atom in the 9-O-acetyl group is substituted by a nitrogen atom, which leads to a chemically and biologically stable 9-N-acetyl group. This simple approach opens up many new opportunities to study 9-O-acetylated sialic acid, for example its role in host-pathogen interactions.

**Zusammenfassung**

Der Schwerpunkt dieser Doktorarbeit ist die Charakterisierung von zwei menschenspezifischen Krankheitserregern, die die Sialinsäure-Oberflächenstrukturen des Wirts nachahmen können. Dadurch sind sie in der Lage an immunregulatorische Siglec-Rezeptoren des Menschen zu binden. Darüber hinaus wird die Nutzung von Sialinsäuren und sialinsäure-artigen Molekülen als biochemische Werkzeuge und Therapeutika untersucht.

Das erste Kapitel stellt Sialinsäure als einen Zucker mit vielen Funktionen in Prokaryoten und Eukaryoten vor. Dabei werden verschiedene virale, bakterielle, mykotische und parasitäre Krankheitserreger inspiziert, die Sialinsäure nutzen um an den Wirt zu binden und dessen Immunsystem zu unterwandern.

Im zweiten Kapitel wird die Interaktion zwischen *Escherichia coli* K1 welches die Polysialinsäure des Wirts nachahmt und den gepaarten Rezeptoren Siglec-11 und Siglec-16 charakterisiert. *E. coli* K1 bindet den inhibierenden Siglec-11-Rezeptor und entkommt dadurch der Immunerkennung. Im Gegensatz dazu ist der immunregulatorische aktivierende Siglec-16-Rezeptor der entzündliche Reaktionen und Phagozytose erhöht. Darüber hinaus stellt das zweite Kapitel ein Maus-Modell vor, mit welchem man die Interaktionen zwischen Krankheitserregern und gepaarten Rezeptoren untersuchen kann.

Im dritten Kapitel werden die Interaktionen zwischen dem Gonorrhoe-Erreger *Neisseria gonorrhoeae* und menschlichen Siglec-Rezeptoren sowie deren potentieller Einfluss auf die Pathogenese beschrieben. Die Interaktionen werden durch die mit Sialinsäure modifizierten Lipooligosaccharide (LOS) vermittelt, welche menschliche Glykosphingolipide nachahmen. Zusätzlich binden Gonokokken-Porine, unabhängig von Sialinsäure, an Siglec-Rezeptoren.

In manchen Menschen führt eine Genfusion zwischen *SIGLEC5* und *SIGLEC14* sowie ein Polymorphismus in *SIGLEC16* zum Verlust von funktionellen Siglec-14- und Siglec-16-Rezeptoren. Der Einfluss dieses Verlusts von aktivierenden Rezeptoren auf die Infektionsrate wird in einer abgelegenen Population von namibischen Hirten untersucht, welche ein sehr hohes Gonorrhoe-Vorkommen haben.

Die zunehmende Anzahl von Infektionen und Antibiotikaresistenzen von *N. gonorrhoeae* gefährden die zukünftige Behandlung von Gonorrhoe. Das dritte Kapitel stellt eine neue, therapeutische Strategie vor, die Gonorrhoe möglicherweise behandeln und verhindern kann. *N. gonorrhoeae* inkorporiert Sialinsäure vom Wirt in sein LOS womit es den klassischen und alternativen Weg des Wirt-Komplementsystems umgehen kann. Ein Therapieansatz könnte die Verabreichung von sialinsäureartigen Zuckern darstellen, die der Erreger in sein LOS inkorporiert. Da ihm diese Zuckerstrukturen normalerweise nicht zur Verfügung stehen kann der Erreger den klassischen Weg des Komplementsystems nicht mehr umgehen und wird vom Immunsystem erkannt. Die Verabreichung von Legionaminsäure, als sialinsäureartigen Zucker wurde an einem Gonorrhoe-Maus-Modell getestet und führte zu einer reduzierten Heilungszeit und einer geringeren Erregerbelastung.

Die 9-O-Acetylierung von Sialinsäure kommt in Menschen und anderen Tieren sehr häufig vor. Allerdings ist es sehr schwierig diese Modifizierung zu untersuchen, da sie sehr unstabil ist. Das Zusatzkapitel schlägt eine chemische Lösung für dieses Problem vor. Das Sauerstoffatom in der 9-O-acetyl-Gruppe wird mit einem Stickstoffatom ersetzt und wird dadurch zu einer chemisch und biologisch stabilen 9-N-acetyl-Gruppe modifiziert. Dieser einfache Ansatz eröffnet viele neue Möglichkeiten, die 9-O-Acetylierungen zu untersuchen, wie zum Beispiel ihre Rolle in Wirt-Erreger-Interaktionen.

Chapter 1:

# Introduction

**Sialic acid: The sweet and sour sugarcoat**

Sialic acids are a family of nine-carbon backbone monosaccharides with over 50 known members. These sugars are present on the cell surface of eukaryotic and some prokaryotic organisms, typically at the terminal end of N-glycans, O-glycans and glycosphingolipids. Given their prominent terminal position, they play important roles in a wide array of biological functions like cell-cell recognition and ligand-receptor interactions.

The vast diversity of sialic acid arises from different linkages, which attach them to other sialic acids or glycans, and a variety of modifications. One of the most common and best studied sialic acid is *N*-acetylneuraminic acid (Neu5Ac) (**Fig. 1**).

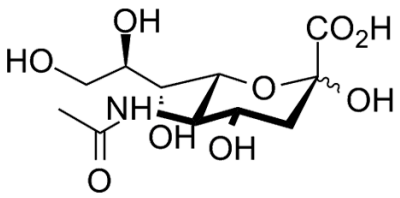


**Figure 1 | Chemical structure of *N*-acetylneuraminic acid (Neu5Ac)**

Hydroxylation of the *N*-acetyl group at the C-5 position leads to *N*-glycolyneuraminic acid (Neu5Gc). Interestingly, humans are not able to synthesize Neu5Gc because of a mutation in the gene that encodes for the cytidine 5'-monophospho-Neu5Ac hydroxylase enzyme (Cmah) that converts CMP-Neu5Ac into CMP-Neu5Gc. Another common modification is the O-acetylation of the different hydroxyl groups. The sugar 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$) is an example for this modification and one of the most abundant sialic acids found in mammals, beside Neu5Ac and Neu5Gc (*Varki and Schauer*, 2009). The total complement of different sialic acids, linkages and their modes of presentation on a particular organelle, cell, tissue, organ, or organism at a distinct time and under specific conditions is termed 'sialome' (*Varki and Angata*, 2006).

**Multiple roles of sialic acids in the immune system**

Mammalian cells typical decorate their surface with tens of millions of sialic acid molecules, leading to an estimated concentration of >100 mM of sialic acid in the glycocalyx of a B cell (*Collins et al.*, 2004). Given this high abundance, it is not

surprising that sialic acids have numerous roles in the immune system (*Varki and Gagneux*, 2012).

One important role is the regulation of fluid phase innate immunity. The major serum protein, factor H, recognizes sialic acid as 'self' and gets deposited onto native cell surfaces. Factor H protects cells from the alternative pathway of the complement system that is an innate component of the immune system's natural defense against infections (*Fearon*, 1979; *Pangburn and Muller-Eberhard*, 1978; *Pangburn et al.*, 1980; *Meri and Pangburn*, 1990). By competing with factor B for binding to C3b, the complement component that binds directly to microbial surfaces, factor H inhibits the formation of the C3 convertase. Factor H also accelerates the decay of the C3 convertase and together with Factor I mediates the cleavage of C3b. Mutations in the sialic acid-binding domains of factor H increase the risk of complement-mediated inflammatory processes, such as hemolytic-uremic syndrome, membranoproliferative glomerulonephritis and age-dependent macular degeneration (*Atkinson and Goodship*, 2007; *Morgan et al.*, 2011; *Donoso et al.*, 2010).

Factor H was the only vertebrate sialic acid-binding protein known until the 1980s. It was then found that treatment of endothelial venules in lymph node sections abolished the interaction with leukocytes (*Rosen et al.*, 1985). This finding contributed to the discovery of the cell adhesion molecule family of Selectins. These lectins, namely L-selectin (on leukocytes), P-selectin (on platelets and endothelium) and E-selectin (on endothelium), play a crucial role in leukocyte trafficking from the bloodstream into the surrounding tissues by using sialylated fucosylated glycans on their target cell surface as ligands (*McEver*, 1991; *Cummings and Smith*, 1992; *Rosen*, 1993; *Varki*, 1994; *Lasky*, 1995).

In the early 1990s, a family of immunoregulatory receptors that recognize sialic acid was discovered (*Kelm et al.*, 1994). The first receptor was Sialoadhesin, a very large protein expressed on macrophages that binds to sialic acid-containing ligands (*Crocker et al.*, 1991). At the same time it was found that the B cell 'adhesion molecule' called CD22 was a sialic acid-binding lectin as well (*Sgroi et al.*, 1993; *Powell et al.*, 1993; *Hanasaki et al.*, 1994). Subsequent cloning and comparison of Sialoadhesin and CD22 revealed that the two proteins share high homology in the

amino-terminal domains. Similar domains were found in CD33 and in the myelin-associated glycoprotein (MAG) (*Crocker et al.*, 1994). These findings suggested that Sialoadhesin, CD22, CD33 and MAG belonged to a single family of proteins that was named sialic acid-recognizing Ig-superfamily lectins (Siglecs) (*Kelm et al.*, 1994; *Powell and Varki*, 1995). In the following twenty years, other Siglec receptors were discovered and to date the Siglec family comprises at least 17 members in primates (*Angata et al.*, 2004; *Crocker et al.*, 2007; *Cao et al.*, 2009; *Pillai et al.*, 2012; *Macauley et al.*, 2014). The Siglec family can be divided into two subsets based on their sequence similarity and evolutionary conservation. Siglec-1, Siglec-2, Siglec-4 and Siglec-15 belong to the 'conserved' subset. Orthologs of this subset can be found in all mammals and sometimes even in fish, amphibians and birds (*Crocker et al.*, 2007). The other subset, called CD33-related Siglecs (CD33rSiglecs), has a high sequence identity with 50-99%. These Siglecs are found in one cluster, in humans on the chromosome 19, and are rapidly evolving though various events like gene conversion, exon loss, exon shuffling and gene duplication (*Angata et al.*, 2004; *Angata*, 2006). The selection pressure driving evolution of these receptors is different among mammalian species, resulting in different repertoires of CD33rSiglecs in each of them (*Angata et al.*, 2004). For example, Siglec-13 is present in chimpanzees but not in humans and Siglec-11 is present in primates but not in rodents (*Wang et al.*, 2012; *Hayakawa et al.*, 2005). **Figure 2** shows the repertoire of human Siglecs.

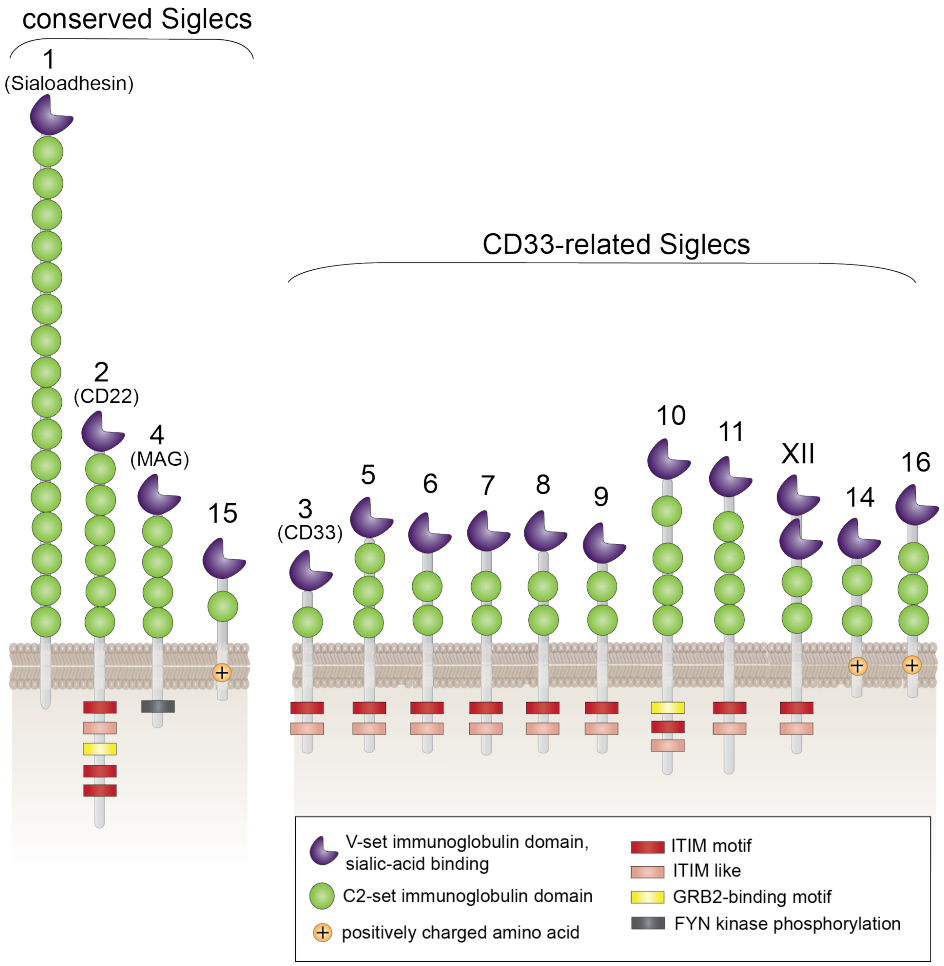


**Figure 2 | Human Siglec repertoire.** Siglecs are type I membrane proteins with amino-terminal V-set domains that bind to sialic acid and a varying number of C2-set immunoglobulin domains. Siglecs can be divided in into "conserved" and "CD33-related" subsets based on evolutionary conversation and sequence identity. Roman numerals indicate a mutation of the conserved arginine in the V-set domain, which is essential for sialic acid binding.

The extracellular domains of the Siglecs contain an amino-terminal sialic acid-binding V-set domain and different numbers of C2-set immunoglobulin like domains. However, their transmembrane and intracellular domains, which determine the signaling functions of these immunoregulatory receptors, are different among Siglecs. Based on these differences, another classification of the Siglec family can be made by dividing them into inhibitory and activating Siglecs. Inhibitory Siglecs have immunoreceptor tyrosine-based inhibitory motifs (ITIM) and ITIM-like motifs in their intracellular domain. These motifs recruit SHP-1 and SHP-2 phosphatases that inhibit pro-inflammatory signaling cascades (*Yu et al.*, 2001; *Whitney et al.*, 2001; *Angata et al.*, 2002; *Avril et al.*, 2004; *Nitschke*, 2009; *Ravetch and Lanier*, 2000). Activating Siglecs can recruit adapter proteins via their transmembrane domain that includes a positively charged amino acid (lysine or arginine). These adapter proteins are either DNAX activating protein of 10 kDa or 12 kDa (DAP10 or DAP12) and they

contain immunoreceptor tyrosine-based activating motifs (ITAMs) (*Angata et al.*, 2006; *Angata et al.*, 2007; *Cao et al.*, 2008). Kinases of the SRC family phosphorylate the tyrosine of ITAMs and generate binding sites for Syk/ZAP70 kinases. Activation of these kinases leads to the initiation of a number of intracellular signaling cascades like the MAPK/ERK or PI3K/AKT/mTOR pathway. These pathways activate transcription factors like nuclear factor kappa-B (NFκB), interferon regulatory factor 3 (IRF) and activator protein 1 (API), which upregulate expression of type I interferons, including interferon-α (IFNα), and pro-inflammatory cytokines like TNF-α or IL-6. Therefore, inhibiting Siglecs dampen pro-inflammatory responses like cytokine secretion and activating Siglecs induce pro-inflammatory responses.

Interestingly, it has been reported that interaction of some ligands with immunoregulatory receptors that associate with ITAM-bearing adaptors produce inhibitory effects, leading to the term inhibitory ITIM (ITAMi) (*Hamerman et al.*, 2009). However, it has not been reported so far that the ITAMs associated with Siglecs can be turned into ITAMis.

CD33-related Siglecs are so far reported mainly expressed on immune cells (*Lock et al.*, 2004). However specific Siglecs have also been found on other cell types. For instance, Siglec-5 and -14 are expressed on amniotic epithelium (*Ali et al.*, 2014), Siglec-6 is expressed on placental trophoblasts (*Brinkman-Van der Linden et al.*, 2007) and Siglec-XII on epithelial cells (*Mitra et al.*, 2011).

The above described functions of sialic acid as ‘self’ molecule, mediator of leukocyte trafficking through selectins and cell activation by Siglec receptors are only a few examples of the many different and important roles that sialic acid plays in the immune system (*Varki and Angata*, 2006; *Crocker et al.*, 2007; *Pillai et al.*, 2012; *Varki and Gagneux*, 2012; *Macauley et al.*, 2014).

**Pathogens bind to host sialic acids**

The abundance and terminal position of sialic acids at the cell surface make them a prominent target for exploitation by microbes. Numerous viruses, bacteria, fungi and parasites use sialic acid to recognize and engage host cells.

A very well studied example is influenza virus, which recognizes sialic acid with haemagglutinin lectins and cleaves it with neuraminidases. Binding of sialic acid is

often very specific and well adapted to the host species. For example, human influenza virus A strains bind preferentially to Neu5Acα2-6Gal in contrast to avian strains, which bind to Neu5Acα2-3Gal epitopes (*Lehmann et al.*, 2006). Besides influenza viruses, which belong to the family of orthomyxoviridae, many other viruses of the coronaviridae, adenoviridae, and papillomaviriae families, to name only a few, bind to sialic acid, often in a linkage-specific manner or through specific modifications like O-acetylation (*Lehmann et al.*, 2006).

Similar to viral infections, adhesion of bacteria to host cells represents an initial and essential step in pathogenesis. *Escherichia coli* K99 expresses adhesion S-fimbriae that bind preferentially to gangliosides carrying Neu5Gcα2-3Gal and Neu5Acα2-8Neu5Ac structures (*Hanisch et al.*, 1993). The pathogen *Helicobacter pylori*, which causes a variety of human gastric diseases, like gastritis, peptic ulcer and gastric cancer, produces two sialic acid-specific adhesins: SabA, which recognizes terminal α2-3 linkages, and the neutrophil activating protein, HPNAP, which binds Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNac (*Mahdavi et al.*, 2002) (*Miller-Podraza et al.*, 1997). Other than these two and many other Gram negative species, Gram positives like *Streptococcus gordonii* attach to sialic acid as first contact point with the host as well (*Yajima et al.*, 2005; *Bensing et al.*, 2004; *Lehmann et al.*, 2006).

Besides hemagglutinin and adhesins, bacterial toxins also engage sialic acid to target the host cells. The toxin of *Salmonella typhi*, which leads to typhoid fever, exclusively binds to Neu5Ac glycans. Since Neu5Ac is prominent on human cell surfaces, the specificity of the toxin may have contributed to the human specificity of typhoid disease (*Deng et al.*, 2014). Other examples of sialic acid-targeting toxins include the cholera toxin from *Vibrio cholera*, which binds to GM1 gangliosides of the intestine epithelia (*Lencer et al.*, 1999), toxins of *Clostridium botulinum* and *tetani* causing botulism and tetanus, respectively (*Kitamura et al.*, 2005), as well as *Bordetella pertussis*'s toxin the causative agent of whooping cough (*Kitamura et al.*, 2005).

In the kingdom of fungi, several sialic acid-specific lectins have been isolated and characterized; however in many cases, their natural functions are not understood yet. Some pathogenic fungi, especially airborne species, can cause severe infections

in immunocompromised patients. A sialic acid-dependent lectin that agglutinates human erythrocytes is secreted by the yeast *Histoplasma capsulatum*, the causative agent of histoplasmosis, a severe pulmonary infection that is commonly found in tropic areas (*Taylor et al.*, 2004). In developed countries, one of the most important airborne fungal human pathogens is *Aspergillus fumigatus.* Individuals inhale the conidia, which possess lectins that adhere to sialic acid on basal lamina proteins in lung tissue. This leads to allergic bronchopulmonary aspergillosis and usually to fatal invasive disease in immunocompromised individuals (*Bouchara et al.*, 1997; *Latgé*, 2001; *Tronchin et al.*, 2002).

Other than bacteria and fungi, some protozoa utilize sialic acid as first point of contact as well. *Plasmodium falciparum*, the causative agent of malignant Malaria, a disease that affects millions of people worldwide, uses sialic acid to enter human erythrocytes. The erythrocyte-binding proteins EBA-175, EBA-140 and EBA-181 of *P. falciparum* bind to sialic acid on erythrocyte receptors like glycophorin A and consequently invade the host cells (*Orlandi et al.*, 1992; *Gilberger et al.*, 2003; *Hadley et al.*, 1987; *Mayer et al.*, 2001).

**Sialic acid mimicry of bacterial pathogens**

Many microorganisms, especially pathogens, have evolved strategies to decorate their surfaces with sialic acid to mimic the host and hence avoid, subvert or inhibit the innate immunity. These strategies include *de novo* synthesis of sialic acid, scavenging host sialic acid, using *trans*-sialidases, scavenging host precursors, using sialic acid-like molecules and other strategies, which mechanisms have not been studied yet (**Table 1**).

**Table 1 I** Human pathogens that use different mechanism to mimic the host sialome.

| Name: | Causative agent of: | Reference: |
|---|---|---|
| ***De novo* synthesis** | | |
| *Neisseria meningitidis* group B, C, W, Y | Septicemia, meningitis | (*Virji*, 2009) |
| *Escherichia coli* K1 and K92 | Neonatal septicemia, meningitis (K1) | (*Robbins et al.*, 1974) (*Glode et al.*, 1977) |
| *Streptococcus agalactiae* | Neonatal pneumonia, septicemia, meningitis, premature delivery | (*Rubens et al.*, 1987) |
| *Mannheimia haemolytica* | Bovine respiratory disease zoonotic infections | (*Puente-Polledo et al.*, 1998) |
| *Campylobacter jejuni* | Enteritis, Guillain-Barre syndrom | (*Hughes and Cornblath*, 2005) (*Yuki and Odaka*, 2005) |
| **Uptake of free sialic acid** | | |
| *Hemophilus influenzae* | Bacteremia, pneumonia, epiglottitis, meningitis | (*Vimr et al.*, 2000) |
| *Hemophilus ducreyi* | Chancroid | (*Schilling et al.*, 2001) |
| *Pasteurella multocida* | Pasteurellosis, zoonotic infections | (*Steenbergen et al.*, 2005) |
| **Use of *trans*-sialidases** | | |
| *Trypanosoma cruzi* | Chagas's disease | (*Previato et al.*, 1985) |
| *Trypanosoma brucei* | Sleeping sickness | (*Pontes et al.*, 1993) |
| *Corynebacterium diphteriae* | Diphtheria | (*Mattos-Guaraldi et al.*, 1998) |
| **Scavenging of host CMP-Sialic acid** | | |
| *Neisseria gonorrhoeae* | Gonorrhea | (*Parsons et al.*, 1988) |
| *Neisseria meningitides group A* | Meningitis | (*Gilbert et al.*, 1996; *Shell et al.*, 2002) |
| **Mechanism unknown** | | |
| *Sporotrichium schenkii* | Sporothrichosis | (*Alviano et al.*, 1999) |
| *Cryptococcus neoformans* | Cryptococcois, fungal meningitis and encephalitis in immunocompromised patients | (*Rodrigues et al.*, 1997) |
| *Paracoccidioides brasiliensis* | Paracoccidioidomycosis | (*Soares et al.*, 1993) |
| *Candida albicans* | Candidiasis | (*Soares et al.*, 2000) |
| *Pseudomonas aeruginosa* | Associated with cystic fibrosis in immunocompromised patients | (*Khatua et al.*, 2013). |
| *Leishmania donovani* | Leishmaniasis | (*Khatua et al.*, 2013). |

### *De novo* synthesis of sialic acid by pathogens

Sialic acid was first detected in bacteria in the 1950s, when Barry et al. discovered a repeating subunit of a capsular polysaccharide in the supernatant of E. coli (*Barry*, 1958). Further studies elucidated that *E. coli* K1 contains a genetic locus that encodes for *de novo* synthesis of sialic acid starting from UDP-GlcNAc, a common cell wall precursor. Furthermore, the operon encodes for sialyltransferases that synthesize the final product α2-8-linked sialic acid polymer (PSA) that coats *E. coli* K1 (*Troy et al.*, 1982; *Vimr and Lichtensteiger*, 2002). The capsule protects *E. coli* K1, which is a causative agent septicemia and neonatal meningitides, from host immune attacks. Another pathogen that causes meningitides in children and young adults, *N. meninigitidis* group B, synthesizes PSA *de novo* and uses it to protect itself from complement-mediated killing (*Swartley et al.*, 1996) (*Vogel and Frosch*, 1999). Humans express PSA on certain proteins in the brain, where it plays crucial roles in morphogenesis and development (*Rutishauser*, 2008) (*Weinhold et al.*, 2005). The similarity of the human PSA to the bacterial one is suggested to cause the poor immunogenicity of the bacterial capsule, complicating the development of carbohydrate-based vaccines against these pathogens. Carbohydrate-based vaccines have been very successful for other capsulated pathogens. For example *N. meninigitidis* group C synthesizes and expresses a α2-9-linked sialic acid capsule (*Bhattacharjee et al.*, 1975), which is highly immunogenic. Based on this structure, a successful vaccines could be developed (*Frasch*, 1989; *Pöllabauer et al.*, 2005).
Many more pathogens are able to synthesize sialic acid *de novo* and incorporate it in their cell-surface structures. A few examples are *E. coli* K92, *C. jejuni* and *S. agalactiae* also known as group B *Streptococcus* (**Table 1**).

Interestingly, every known microorganism to date that has sialic acid biosynthesis pathways is synthesizing Neu5Ac or an acetylated form of Neu5Ac, which are prominent in humans, mustelids, pinnipeds and new world monkeys (*Ng et al.*, 2014; *Springer et al.*, 2014). It seems that microorganisms are not able to emulate the animal CMAH enzyme that hydroxylases the acetyl group of Neu5Ac to form Neu5Gc, which is mainly present in animals other than the previous mentioned.

**Uptake of free sialic acid by pathogens**

The human pathogen *Haemophilus influenzae* is able to take up free sialic acid from its environment and uses it either as a source of carbon and nitrogen or decorates it cell surface with it (*Vimr et al.*, 2000). Interestingly, *H. influenzea* does not possess the genes for the biosynthesis of sialic acid or a sialidase. Therefore *H. influenzae* is dependent on sialidases of other microorganisms in the same niche or the host itself to produce free sialic acid by cleaving sialic acid from sialoglycans. For the uptake *H. influenzea* relies on specific transporter, which bind sialic acid with high specificity and affinity (*Severi et al.*, 2005). Upon uptake of sialic acid the CMP-Neu5Ac synthetase converts Neu5Ac into its activated form, CMP-Neu5Ac, which is used by sialyltransferases to decorate the lipooligosaccharide (LOS) (*Hood et al.*, 2001; *Hood et al.*, 1999). Many variations of the *H. influenzea* LOS, sialylated and non-sialylated, mimic host structures, mainly glycolipids and glycoshingolipids, and thus help the pathogen to hide and avoid a host immune response (*Harvey et al.*, 2001b).

Other examples of pathogens that take up free sialic acid are *Haemophilus ducreyi* and *Pasteurella multocida* (*Schilling et al.*, 2001; *Steenbergen et al.*, 2005; *Li et al.*, 2012).

**Pathogens use *trans*-sialidases**

Usually in order to add sialic acid to a glycan, a sialyltransferase requires an activated donor, for example in form of CMP-sialic acid, from which it transfers the sialic acid to the acceptor glycan. However, the protozoan parasites *Tryponomonas cruzi* and *tryponomonas brucei*, causative agents of Chagas'disease and sleeping sickness, respectively, evolved a different and very elegant strategy to coat their surface with sialic acid (*Previato et al.*, 1985; *Pontes et al.*, 1993). They express surface *trans*-sialidase that transfers sialic acid from a donor sialoglycan to a terminal galactosyl or *N*-acetylgalactosamine residue. The *trans*-sialidase modifies endogenous as well as host cell surfaces. The sialylated endogenous acceptors are mucin-like molecules that protect the pathogens from innate immunity in the bloodstream. Whereas modifications of the host cell surface by the *trans*-sialidase facilitates adhesion and invasion. So far, there are no vaccines available to protect from Chaga's disease and sleeping sickness. The important functions of the *trans*-

sialidase in the pathogenesis of theses diseases make it a promising target for drug design and therapeutic intervention (*Freire-de-Lima et al.*, 2015; *Silva et al.*, 2009).

The first *trans*-sialidase in bacteria has been described in *Corynebacterium diphtheriae*, which causes diphtheria (*Mattos-Guaraldi et al.*, 1998; *Kim et al.*, 2010). The functional role and implication of *C. diphtheriae trans*-sialidase in pathogenesis might have similar features to *Tryponomonas* spp, however the *C. diphtheria* specific details have yet to be investigated.

Lastly, the CstII sialyltransferase of *Campylobacter jejuni,* besides having α2-3- and α2-8-sialyltransferase activity that uses CMP-Neu5Ac as donor substrates, also shows trans-sialidase activity *in vitro* (*Cheng et al.*, 2008).

**Scavenging of host CMP-sialic acid**

*Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease gonorrhea, is not able to synthesize or take up free sialic acid. However, it expresses an extracellular sialyltransferase (Lst) that uses CMP-Neu5Ac, which is present in small amounts in host bodily fluids, to decorate its LOS with sialic acid. This has been discovered when Ward et al. observed that many strains of *Neisseria gonorrhoeae* directly isolated from patients were resistant to complement-mediated killing of human serum, but lost resistance when subcultured (*Ward et al.*, 1970). Further studies showed that supplementing the subcultures *in vitro* with red blood cell lysates, human serum or urogenital secretions restored the resistance to complement-mediated killing, suggesting they contained a host factor that induced resistance (*Martin et al.*, 1982; *Patel et al.*, 1984). Parsons et al. discovered then that CMP-Neu5Ac is this host resistance-inducing factor that facilitates *N. gonorrhoeae* to resist the complement-mediated killing in human serum (*Parsons et al.*, 1988).

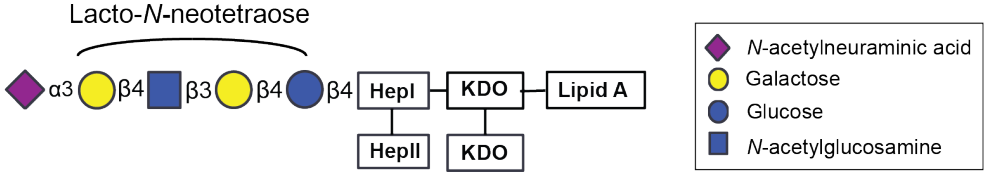


**Figure 2 I Common LOS structure of *N. gonorrhoeae*.** The lacto-*N*-neotetraose structure can be modified with Neu5Ac if CMP-Neu5Ac is present in the environment. (HepII has glycan extensions, which are not shown here)

Lacto-*N*-neotetraose (LNnT) is a Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-4 structure (**Fig. 2**) of the LOS in most *N. gonorrhoeae* strains (*Mandrell et al.*, 1988). This structure mimics human paragloboside, a precursor of human blood group antigens on erythrocytes (*Mandrell et al.*, 1988; *Harvey et al.*, 2001b). LNnT structure binds to the asialo-glycoprotein receptor, which is expressed on male urethral epithelium, and facilitates invasion (*Harvey et al.*, 2001a). Gonococci with the LNnT structure can also attach to sperm, which express the asialo-glycoprotein receptor, and use sperm as carrier to be transmitted to a new host (*Edwards and Apicella*, 2004). If CMP-Neu5Ac is present in the host environment, the LNnT is siaylated at the terminal Gal with a α2-3 linkage (*Gilbert et al.*, 1996). Sialylation enables gonococci to recruit factor H and hide from human complement, since factor H is a complement factor, which physiological role is to recognize and coat host cell surface to protect them from the host complement. (*Ram et al.*, 1998). Studies have shown that sialylated gonococci are less invasive (*van Putten*, 1993). To counteract this disadvantage, *N. gonorrhoeae* relies heavily on phase variation *in vivo*. Certain glycosyltransferases are switched 'off' resulting in a truncated LOS that cannot be sialylated, making the bacteria more invasive. Besides the phase variation of the LOS, gonococci possess many other mechanisms and surface antigens that are under phase variation. Therefore in any given host environment, gonococci has a population equipped with an adequate set of 'tools' that help it to survive and exhibit virulence (*van Putten*, 1993; *Edwards and Apicella*, 2004).

Another bacterium capable of scavenging host CMP-Neu5Ac is *N. meninigitidis* group A, a close relative to *N. gonorrhoeae*. It posses a similar extracellular sialyltransferase, which is involved in LOS sialylation and helps the pathogen to avoid complement-mediated killing (*Gilbert et al.*, 1996; *Shell et al.*, 2002).

**Unknown mechanisms of sialic acid surface decoration**

The opportunistic pathogen *Pseudomonas aeruginosa* preferentially colonizes immunocompromised individuals and is associated with diseases like cystic fibrosis (*Williams et al.*, 2010). Bioinformatics searches of the *P. aeruginosa* genome have indicated the absence of endogenous genes involved in sialic acid biosynthesis. Experiments with growth media have suggested that sialic acid is adsorbed from the

environment. Is still unclear if sialic acid is adsorbed as free sugar or in the nucleotide-activated form, incorporated by a *trans*-sialidase, or if there is a novel mechanism involved (*Khatua et al.*, 2013).

The mechanisms of sialic acid surface incorporation are still poorly studied in fungal and parasitic pathogens. It is often unclear if they use similar mechanism as mentioned above or convergently evolved mechanisms that have not been described so far. For example, it is unclear how the virulent parasite *Leishmania donovoni* coats its surface with 9-O-acetylated-sialic acids (*Khatua et al.*, 2013) and how pathogenic fungi like *Sporothrix schenckii*, *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* and *Candida albicans* incorporate sialic acid in their cell wall (*Alviano et al.*, 1999).

**Expression of sialic acid-like molecules**

Sialic acid belongs to a broader family of nine-carbon backbone acidic sugars called nonulosonic acids (NulO). Other members of this family are pseudaminic acid (Pse), legionaminic acid (Leg) and deaminoneuraminic acid (KDN), which are shown in **Figure 3**. Pse and Leg are expressed by many microorganisms, both commensal and pathogenic, but they have not been found in eukaryotes.

In 1995, Leg was discovered in the LOS of *Legionella pneumophila*, the causative agent of Legionnaires'disease (*Knirel et al.*, 1994). *Acinetobacter baumanii* and *C. jejuni* are further examples of pathogens that have legionaminic acid in their cell surface glycoconjugates (*Vinogradov et al.*, 2014; *Schoenhofen et al.*, 2009).

The flagellin proteins of *Campylobacter coli*, *C. jejuni*, and *H. pylori* are heavily glycosylated with pseudaminic acid. It is required for the development of the flagella and therefore plays an important role in their motility within host as well as the invasion of the host (*Chou et al.*, 2005).

The presence of legionaminic acid and pseudaminic acid on pathogen cell surfaces has been suggested to lead to subversion of the host immune system due to its similarity to Neu5Ac. However, detailed studies of the mechanisms are still lacking.

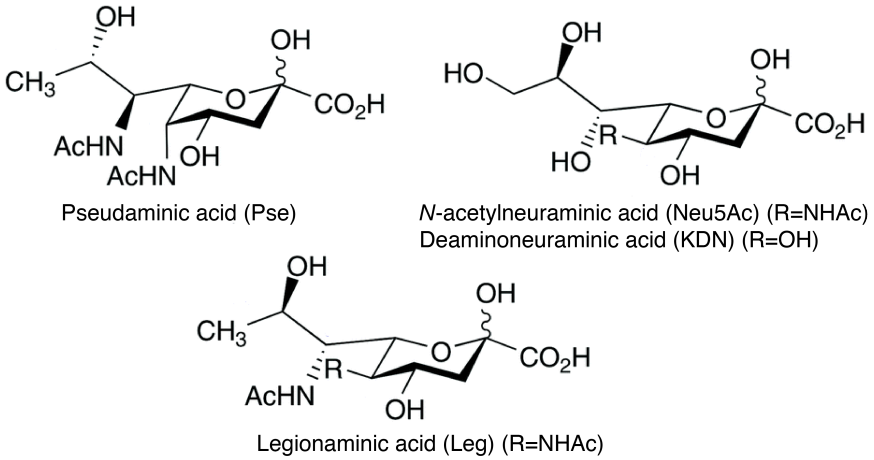


**Figure 3 | Chemical structures of the non-ulsonic acids pseudaminic acid, legionaminic acid, deaminoneuraminic acid and *N*-acetylneuraminic acid** (adapted from (*Zunk et al.*, 2014))

## Evolution of sialic acid molecular mimicry

A phylogenetic analysis of the nonulosonic acid family shed light on how the molecular mimicry in bacteria evolved (*Lewis et al.*, 2009). Analysis of the first thousand prokaryotic genomes sequenced, showed that around 20% have clusters of genes, called nonulosonic pathway biosynthesis genes (NAB), which show similarities to the clusters involved in bacterial sialic acid biosynthesis (*Lewis et al.*, 2009). Previously, it has been suggested that sialic acid was an animal innovation and later emerged in bacteria to mimic host sialic acid. However this analysis suggests that the bacterial biosynthesis of the sialic acid might have evolved from an ancient nonulosonic pathway that was present very early in cellular evolution going as far back as to a common ancestor of bacteria and archaea. (*Lewis et al.*, 2009) (*Kandiba and Eichler*, 2013). So it seems that when sialic acid emerged in animals, pathogens took advantage of their NABs and adapted them in order to mimic and eventually exploit the host.

## Host Siglecs respond to sialic acid molecular mimicry of pathogens

To counteract molecular mimicry of pathogens and their exploitation of the immune system, human have evolved different mechanisms to keep up in this ‘arms race’ between host and pathogen. Siglecs play an important role in recognizing ‘self’, mimicking pathogens and subsequently induce an inflammatory response. However, at the same time Siglecs are also a prominent target for exploitation by these pathogens since most of them have immune inhibiting functions.

Sialoadhesin regulates cell-cell interactions in the host, however it is an important host defense receptor as well. The α2-3 and α2-8-linked sialic acids on *C. jejuni* LOS are recognized by sialoadhesin expressed on macrophages (*Heikema et al.*, 2010). These interactions induce rapid pro-inflammatory cytokines and type I interferon responses and phagocytosis and therefore actively finding the infection (*Klaas et al.*, 2012). However, sialoadhesin is also targeted for exploitation by pathogens. For example, the human immunodeficiency virus (HIV) binds to sialoadhesin expressed on dendritic cells through its host cell derived α2-3-sialylated glycosphingolipids, which facilitate *trans*-infection of permissive cells (*Rempel et al.*, 2008; *Izquierdo-Useros et al.*, 2012).

Carlin et al. found that Group B *Streptococcus* can exploit the inhibitory function of multiple CD33rSiglecs via its sialylated capsule. The interaction inhibits the host immune response like pro-inflammatory cytokine secretion and phagocytosis and therefore increases the survival of the pathogen (*Carlin et al.*, 2007).
Siglec-10 is the only Siglec so far that has been described to bind sialic acid-like molecules. Pseudaminic acid on flagella of *C. jejuni* promotes anti-inflammatory signaling via Siglec-10 engagement (*Stephenson et al.*, 2014).

Interestingly, not only inhibiting Siglecs interact with GBS, but also activating Siglecs like Siglec-14. Siglec-5 and Siglec-14 are so-called ‘paired receptor’ because they have very similar extracellular domains, suggesting they recognize similar ligands, but they have opposite signaling properties. Siglec-5 inhibits and Siglec-14 activates the inflammatory response (*Angata et al.*, 2006). Siglec-14 counteracts the exploitation of Siglec-5 by GBS by activating MAPK and AKT signaling pathways (*Ali et al.*, 2014). However, not all individuals express Siglec-14 due to a deletion polymorphism. This can have implications for the course of diseases or infections that involve these receptors (*Yamanaka et al.*, 2009). For example, *SIGLEC14*-null allele is suggested to play a role in the risk of women colonized with GBS to give premature birth. The amniotic epithelium, the site of initial contact of invading GBS and the fetus, expresses Siglec-5 and Siglec-14 in a human-specific manner. If Siglec-14 is not expressed due to the null polymorphism, the risk of premature delivery is increased (*Ali et al.*, 2014). These results suggest that activating Siglecs play an important role to counteract the exploitation of the inhibiting Siglecs by

pathogens. Other activating Siglecs are Siglec-13, Siglec-16 and Siglec-17. High amount of polymorphisms in the genes of these activating receptors lead to the suggestion that the host selects against these receptors. Only some individuals in the human population are able to express Siglec-16, whereas Siglec-13 and Siglec-17 are completely missing in humans (*Wang et al.*, 2012). It has been suggested that activating receptors, in absence of an infection or presence of excessive infection, might be detrimental to the host because they cause increased inflammation. Therefore the host has to balance its selection for and against activating receptors.

It has been suggested that the fast evolution of genes involved in sialic acid biology help the host to escape pathogens by changing its surface epitopes and specificity of Siglecs, but at the same time maintain self-recognition by innate immune cells (*Varki and Angata*, 2006; *Padler-Karavani et al.*, 2014). This can be described with a classic evolutionary concept the so-called 'Red Queen effect' (**Fig. 4**), which is inspired by the novel of Lewis Carroll 'Through the Looking-Glass': "Now, here, you see, it takes all the running you can do, to keep in the same place," the queen says to Alice. Translated into the world of host-pathogen interactions it means that multicellular organisms with long lifecycles, like animals, must rapidly evolve to survive the constant attacks of microbial pathogens that replicate much faster. In **Figure 4** the primary 'Red Queen Effect' describes how the host must evolve new ways to display sialic acid to escape from pathogen recognition, which is also rapidly evolving to recognize host sialic acids. However this introduces a secondary 'Red Queen Effect' which suggest that then host Siglecs have to evolve to maintain self-recognition of the evolved host sialome. Siglecs pressured to evolve and escape sialylated pathogens that exploit them, which possess another primary 'Red Queen Effect' (*Varki and Angata*, 2006; *Padler-Karavani et al.*, 2014).

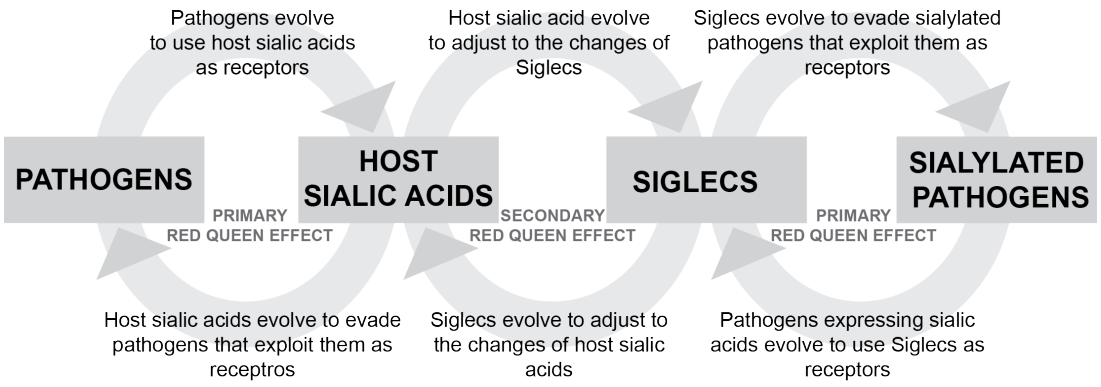


**Figure 4 | Red queen effects involving sialic acids, Siglecs and pathogens** adapted from (*Varki and Angata*, 2006; *Padler-Karavani et al.*, 2014)

## Conclusions

As described in this introduction, sialic acids are important molecules that mediate multiple important functions in the maintenance of vertebrate host physiology. Many pathogens, in all kingdoms of life, from bacteria to fungi, take advantage of the important role sialic acid plays in the host. They invented many different strategies to decorate their surface with sialic acid and sialic acid like molecules and subvert the host. The host responds with different mechanisms as well like using activating receptors that recognize the decorated pathogen. In this ongoing 'arms race' between host and pathogen many battles, which have been described above or elsewhere, are fought. However many more are still unknown. This thesis aims to characterize two of these so far unknown battles.

## Aims of this thesis

This research project explores and characterizes the interactions between Siglec receptors and two human-specific pathogens, *E. coli* K1 and *N. gonorrhoeae*, as well as their impact on the host immune system, which have not been studied before. Furthermore it explores the use of legionaminic acid, as a treatment for gonorrhea.

1. Characterization of the interaction between paired receptors Siglec-11 and Siglec-16 and the human pathogen *E. coli* K1.
2. Description of the interactions between multiple Siglecs and the causative agent of gonorrhea, *N. gonorrhoeae*.
3. Analysis of the incorporation of legionaminic acid into the gonococcal LOS from CMP-Leg precursors, which are suggested to have antimicrobial effects on *N. gonorrhoeae*.
4. Addendum: Measuring the incorporation of a synthetic sialic acid analog, which is used as tool for studying unstable 9-O-acetyl-sialic acid, into mammalian cells.

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# Chapter 2:

# Paired Siglec receptors generate opposite inflammatory responses to a human-specific pathogen

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Contribution: Cloning of Siglec-16-Fc construct
Purification of Siglec-Fc constructs
Bacteria binding assays
Analysis of cytokine expression
Mouse infection studies

**Abstract**

Paired immune receptors display near-identical extracellular ligand-binding regions but have intracellular sequences with opposing signaling functions. While inhibitory receptors dampen cellular activation by recognizing self-associated molecules, the functions of activating counterparts are less clear. Here we studied the inhibitory receptor Siglec-11 that shows uniquely human expression in brain microglia and engages endogenous polysialic acid to suppress inflammation. We demonstrated that the human-specific pathogen *Escherichia coli* K1 uses its polysialic acid capsule as a molecular mimic to engage Siglec-11 and escape killing. In contrast, engagement of the activating counterpart Siglec-16 increases elimination of bacteria. Since mice do not have paired Siglec receptors, we generated a model by replacing the inhibitory domain of mouse Siglec-E with the activating module of Siglec-16. Siglec-E16 enhanced pro-inflammatory cytokine expression and bacterial killing in macrophages, and boosted protection against intravenous bacterial challenge. These data elucidate uniquely human interactions of a pathogen with Siglecs, and support the long-standing hypothesis that activating counterparts of paired immune receptors evolved as a response to pathogen molecular mimicry of host ligands for inhibitory receptors.

**Introduction**

Paired receptors are membrane proteins that are mainly expressed on immune cells. They share significantly conserved amino acid sequences within their extracellular domains, but have both activating and inhibitory members (*Barrow and Trowsdale*, 2006; *Lanier*, 2001; *Kuroki et al.*, 2012). The inhibitory receptors possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within their cytoplasmic region. Phosphorylation of ITIM tyrosine residues by Src kinases generates specific binding sites for Src homology 2 (SH2) domains of phosphatases SHP-1 and SHP-2 (*Tourdot et al.*, 2013). In contrast, the activating receptors have short cytoplasmic regions and transmembrane domains with a positively charged amino acid that associate with adaptor proteins like DAP12 (*Kameda et al.*, 2013). DAP12 contains cytosolic immunoreceptor tyrosine-activating motifs (ITAMs) that can be phosphorylated at tyrosine residues to activate MAP kinase signaling cascades, leading to production of pro-inflammatory cytokines (*Lanier*, 2009; *Hirayasu and Arase*, 2015).

Although many of the inhibitory receptors have been shown to bind to endogenous ligands, it has been more difficult to identify ligands of the activating receptors, and their functions have hence not been clearly elucidated (*Yamada and McVicar*, 2008). When the activating and inhibitory receptors recognize the same ligand, the inhibitory receptors usually have higher affinity. This supports the notion that the inhibitory receptors function in the maintenance of immunological quiescence by the recognition of self-associated molecular patterns (SAMPs) (*Varki*, 2011). Interestingly, many pathogens have evolved successful molecular mimicry mechanisms to bind directly to inhibitory repressors in order to suppress the immune response of the host. It has been suggested that binding of pathogens to inhibitory receptors might have driven the evolutionary emergence of activating counterparts (*Barclay and Hatherley*, 2008). This is consistent with the fact that many of the paired receptor families are evolving rapidly, indicative of pressure from pathogens (*Vilches and Parham*, 2002). However the presumed ligands on pathogens have been difficult to identify, and are currently limited to a few viral glycoproteins (*Kuroki et al.*, 2012).

Paired receptors have been identified among immunoglobulin (Ig)-like receptors that include the killer cell Ig-like receptors (KIRs), leukocyte Ig-like receptors (LILRs), murine paired Ig-like receptors (PIRs), Fc receptor, leukocyte-associated inhibitory receptors (LAIRs), NKp46 and Siglecs (*Yamada and McVicar*, 2008). Siglecs (sialic acid-binding immunoglobulin-like lectins) are a subset of I-type lectins (*Varki and Crocker*, 2009; *Macauley et al.*, 2014). The extracellular portion of each Siglec family member includes a distinct number of Ig-like domains that allows these receptors to reach sialylated ligands extending from the same cell membrane, from other cells, or bind to soluble ligands (*Fong et al.*, 2015; *Crocker et al.*, 2007; *Linnartz et al.*, 2010). The cytosolic segment of some Siglecs contains ITIMs, and interactions with SAMPs prevent unwanted inflammatory responses under homeostatic conditions. Conversely, their disengagement releases these brakes and results in cellular activation (*Chen et al.*, 2014). Other members of the Siglec family (Siglec-14, -15, and -16) do not possess ITIMs and recruit DAP12. Siglecs that recruit DAP12 are thus classified as “activating”. Interestingly, the outermost extracellular segments of Siglec-5 and Siglec-14 are kept nearly identical through ongoing gene conversion events between the *SIGLEC5* and *SIGLEC14* loci, while the intracellular parts drive opposite responses (*Angata et al.*, 2006). The two

proteins can thus work as paired receptors in the modulation of responses to group B *Streptococci* (GBS): bacteria inhibit phagocytosis by targeting the inhibitory Siglec-5; conversely, recognition of GBS by Siglec-14 leads to activation of MAP kinase pathway, and to more efficient clearance of the pathogen (*Ali et al.*, 2014). Similarly, the sialic acid-binding properties of human Siglec-11 and Siglec-16 are indistinguishable due to the nearly identical extracellular domains that are the result of gene conversion events (*Cao et al.*, 2008; *Wang et al.*, 2012). However, the two proteins possess intracellular domains capable of inducing opposing signals. Moreover, while the *SIGLEC11* gene is fixed in the human population, the overall *SIGLEC16* allele frequency is 0.22 and the majority of the population carry an inactive *SIGLEC16P* variant containing a 4 nucleotide deletion that disrupts the open reading frame(*Cao et al.*, 2008; *Wang et al.*, 2012). Lastly, previous comparative analysis has detected Siglec-11 expression in brain microglia of humans, but not in the closely related hominids, like chimpanzees (*Hayakawa et al.*, 2005).

In this study, we investigated the relevance of Siglec-11 and Siglec-16 in the regulation of the innate immune response to the pathogen *Escherichia coli* K1, an important cause of meningitis in neonates and infection in the urinary tract (*Croxen and Finlay*, 2010; *Wiles et al.*, 2008). We found it intriguing that *E. coli* K1 produces a capsular polysaccharide made of α2-8-linked sialic acids, which is a perfect mimic of the preferred ligand of Siglec-11. It was also remarkable that *E. coli* K1 is a human-specific pathogen, and it seems to exploit a receptor (Siglec-11) that is expressed in the brain only in humans. We show here that Siglec-11 and -16 were indeed capable of modulating responses to *E. coli* K1 in opposite directions, demonstrating that they can act as paired receptors. To demonstrate that activating Siglecs confer better protection to bacterial infection *in vivo*, we therefore generated a novel mouse model of human-type paired Siglec receptors by replacing Siglec-E with a chimeric receptor that includes the extracellular part of Siglec-E and the transmembrane segment of human Siglec-16. In contrast to the to the native ITIM-bearing inhibitory Siglec-E, the chimeric receptor Siglec-E16 was able to produce protective inflammatory responses to bacterial infection.

## Results

*Siglec-11 and -16 are paired receptors expressed on tissue macrophages*

Siglec-11 and -16 share a high degree of amino acid sequence identity in their extracellular region, with the two outermost Ig-like domains being 99% identical (**Figure 1A,B**). The underlying two domains are separated from the first two domains by a short linker domain in Siglec-11. *SIGLEC11* includes an additional exon encoding a polypeptide that separates the four Ig domains from the cell membrane.

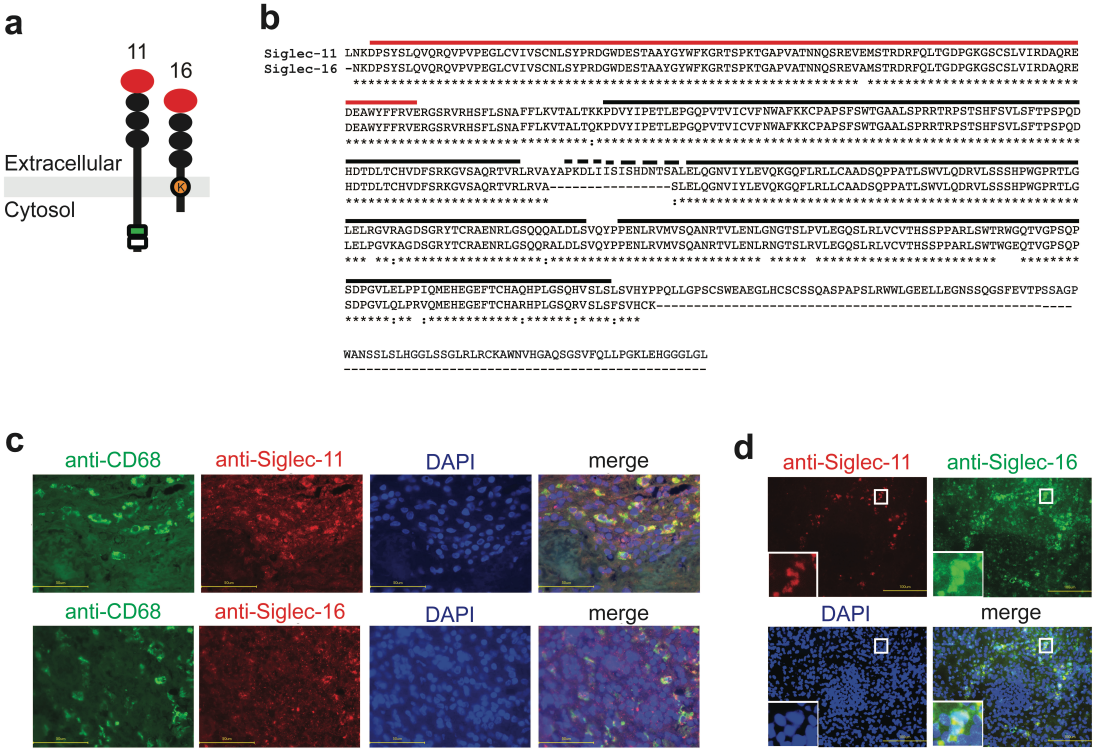


**Figure 1 I Siglec-11 and Siglec-16 are paired receptors expressed on macrophages in humans**
(A) Schematic representation of the two proteins. Filled circles represent Ig domains of the V-set (red) or CH2-type (black). Siglec-11 contains an ITIM (green box) and an ITIM-like (white box) domain in the intracellular tail. Siglec-16 has a positively charged amino acid (K, lysine) in the transmembrane span for interaction with DAP12. (B) Alignment of the amino acid sequences corresponding to the extracellular parts of the two receptors. Lines indicate domains. (C) Co-immunofluorescence of macrophage marker CD68 and Siglec-11 or Siglec-16 in spleen samples. Cells were stained with fluorescently labeled antibodies and DAPI. The scale bar is 50 micron. (D) Immunofluorescence reveals that Siglec-11 and Siglec-16 can be expressed on the same cell in spleen samples. Cells were stained with fluorescently labeled antibodies and DAPI. The insets are from higher magnification images representing the region included in the white box. The scale bar in yellow is 100 micron.

To understand the contribution of Siglec-11 and Siglec-16 to the modulation of immune responses, we studied expression of these two receptors in human tissues. First, we developed antibodies that could specifically discriminate the two proteins (**Figure S1**). In agreement with previous studies (*Wang et al.*, 2012; *Angata et al.*, 2002; *Wang and Neumann*, 2010), Siglec-11 was detected in spleen, lung, liver,

bladder and in brain (**Figure S2**). Siglec-16 was expressed at generally lower levels, but detectable in spleen and the other organs, particularly in association with inflammatory states. Co-staining with CD68 indicated that these Siglecs were present on macrophages (**Figure 1C**). In an independent study, Siglec-11 and -16 mRNA expression was reported on human microglia, resident macrophages in the brain (**Figure S3**) (*Bennett et al.*, 2016; *Sierra et al.*, 2013). Notably, Siglec-11 and Siglec-16 could be detected on the same splenic cells (**Figure 1D**), indicating that inhibitory and activating receptors can be expressed in the same cells at the same time.

*Pathogenic E. coli K1 engages Siglec-11 and Siglec-16 via its surface sialic acid capsule, generating opposite immune responses*

Siglecs are found on innate immune cells that provide a first line in defense against foreign agents. To escape elimination, pathogens continuously evolve strategies to abolish recognition or rewire inflammatory responses of the host. The preferred ligands of Siglec-11 are α2-8-linked polysialic acids, which are common structures in the human brain (*Angata et al.*, 2002; *Shahraz et al.*, 2015; *Wang and Neumann*, 2010). Interestingly, *E. coli* K1, a prominent cause of bacterial meningitis in neonates, produces a capsule made of the identical α2-8-linked polysialic acids (*Troy*, 1979; *Croxen and Finlay*, 2010). The K1 capsule confers serum resistance and anti-phagocytic properties (*Hoffman et al.*, 1999; *Xie et al.*, 2004). Moreover, although both capsular and acapsular *E. coli* K1 strains can traverse brain microvascular endothelial cells *in vitro* and enter the central nervous system *in vivo*, only capsulated bacteria are found in positive CSF cultures and cause *E. coli* meningitis (*Xie et al.*, 2004).

We investigated if *E. coli* uses its capsule to hijack Siglec-11 function during bacterial infection. First, we tested whether *E. coli* K1 can engage inhibitory Siglecs. Among the protein tested, *E. coli* K1 exhibited the strongest binding to Siglec-11 (**Figure 2A**). A similar binding profile was observed for the activating counterpart Siglec-16, suggesting that the region responsible for bacterial binding is located within the first two near-identical extracellular domains of the two Siglecs. To understand if the capsular sialic acid mediated Siglec binding, we compared a wild-type parent K1 strain to a mutant, deficient in sialic acid biosynthesis (D*neu*DB). Only the wild-type strain bound Siglec-11-Fc. Further confirmation was achieved by gain

of function analysis, as introduction of the encoding biosynthesis of the K1 capsule into an unrelated non-encapsulated *E. coli* K12 stain conferred binding to the Siglec-11-Fc (**Figure 2B** and **Figure S4**). Thus the polysialic acid capsule was necessary and sufficient for Siglec-11 binding and appeared to represent the key determinant for binding of *E. coli* K1 to Siglec-11-Fc.

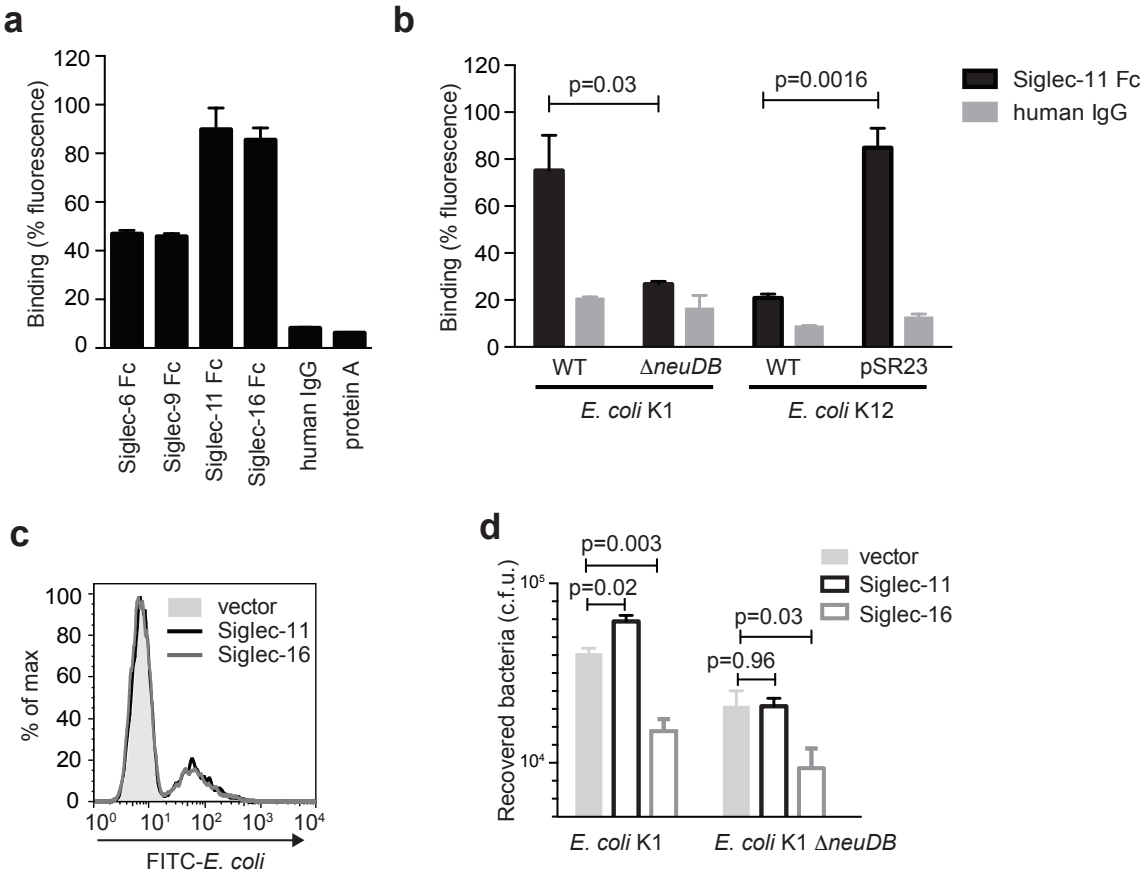


**Figure 2 I Human Siglec-11 and Siglec-16 bind similarly to *E. coli* K1, but drive opposite responses.**
(A) *E. coli* K1 binding to Siglec-11 and Siglec-16. Data are represented as mean ± SEM, n=3. (B) The polysialic acid capsule of *E. coli* K1 is necessary for binding to Siglec-11. *E. coli* K1 and an acapsular mutant (D*neu*DB) were tested for binding to Siglec-11. Introduction of the locus for capsule biosynthesis in *E. coli* K12 results in bacterial binding to Siglec-11. Data are represented as mean ± SEM, n=3. (C) Expression of Siglec-11 or Siglec-16 on microglia CHME-5 cells results in increased bacterial adherence. The low overall extent of binding was likely due to the low efficiency of transfection of these cells. (D) Bacterial killing by microglia CHME-5 cells expressing Siglecs. Recovered bacteria are indicated. Data are represented as mean ± SEM, n=3.

To determine if *E. coli* K1 capsule could bind Siglec-11 and Siglec-16 on a cell surface, we transfected microglial CHME-5 cells with Siglec-11 and Siglec-16, and evaluated binding of fluorescein-labeled *E. coli* K1 by flow cytometry. This analysis revealed a subpopulation of cells expressing Siglec-11 or Siglec-16 that were bound by bacteria (**Figure 2C**). To evaluate the contribution of Siglec-11 and -16 in innate immune response against bacteria, microglial cells were infected with *E. coli* K1 and

bacterial survival was determined. Remarkably, more bacteria were recovered from cells expressing Siglec-11, while expression of Siglec-16 resulted in higher bacterial killing (**Figure 2D**). Siglec-11-dependent suppression of bacterial killing was not observed for the isogenic *E. coli* D*neu*DB mutant deficient in capsule biosynthesis. The presence of Siglec-16 reduced survival of acapsular bacteria, suggesting that additional sialic acid-independent interactions might occur between Siglec-16 and *E. coli*, or that Siglec-16 altered overall cellular reactivity.

*Engineered expression of activating Siglec receptors in mice*

Studies of paired human Siglec-11/16 receptors are complicated by the weak expression on accessible primary cells, such as blood monocytes or monocyte-derived macrophages, the low population frequency of the functional *SIGLEC16* gene, and the high degree of outbreeding in the human population. Meanwhile, due to rapid evolution in the *SIGLEC* gene family in mammals, the repertoire of Siglecs differs substantially in humans and rodents, and human-type paired Siglec receptors are not found in mice. To address the relevance of paired Siglec receptors in a genetically defined *in vivo* model, we envisioned the generation of a mouse line expressing an activating receptor exhibiting ligand specificity identical to the native inhibitory murine Siglec-E (Figure 3A). First, to demonstrate that such a chimeric receptor Siglec-E16 could be expressed and signal properly, we tested its ability to recruit the DAP12. Immunoprecipitation of Siglec-E16 from lysates of cells co-transfected with Siglecs and DAP12 constructs resulted in the co-precipitation of DAP12, whereas no DAP12 was detected on immunoprecipitation of native Siglec-E (Figure 3B). We then monitored cytokine modulation by Siglec-E16. Compared to Siglec-E expressing wild-type cells, murine macrophages with Siglec-E16 produced higher levels of pro-inflammatory IL-6 and lower levels of anti-inflammatory IL-10 (Figure 3C). These two experiments demonstrated that Siglec-E16 productively interacts with signaling pathways to alter inflammatory responses in cell lines.

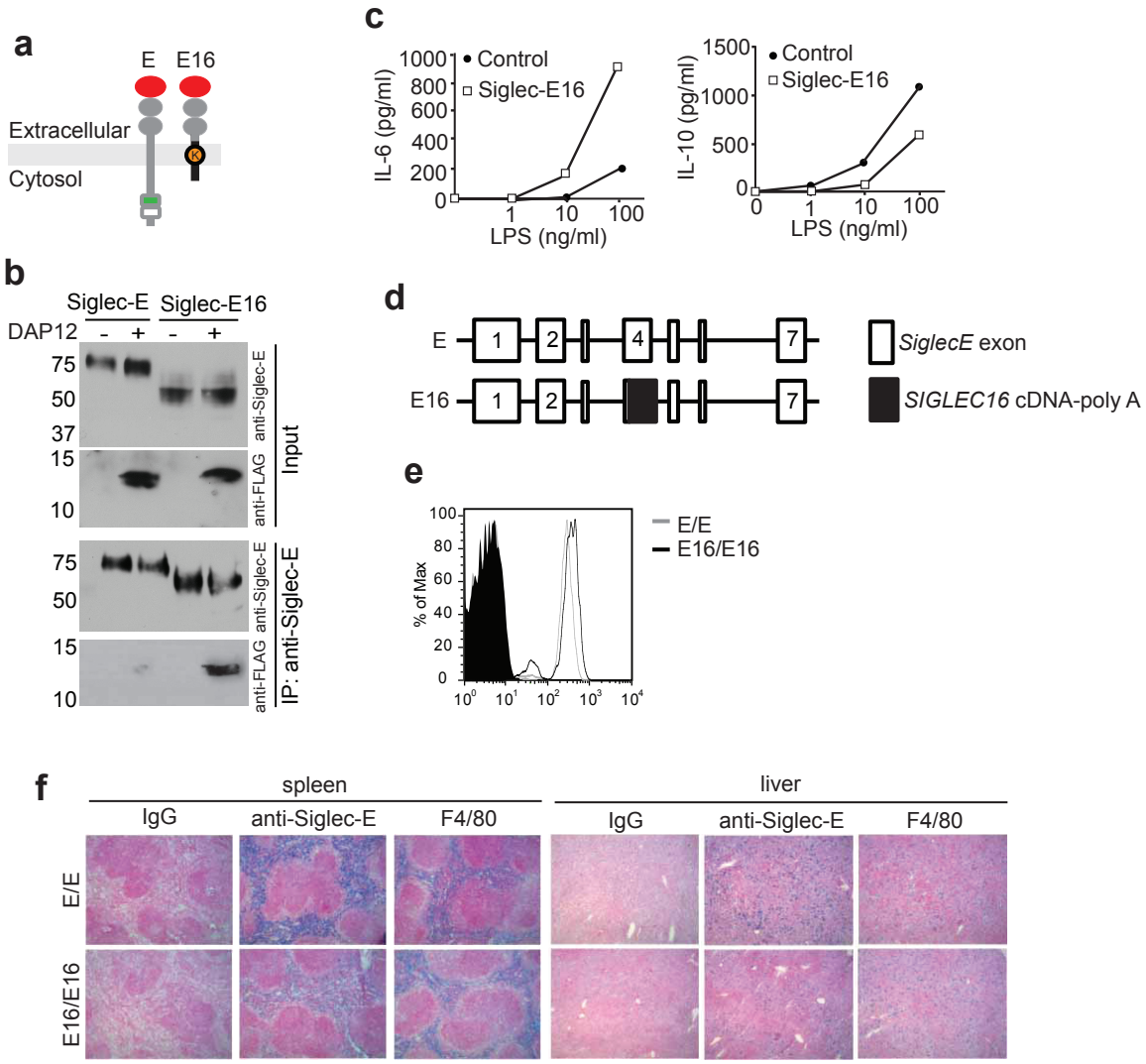


**Figure 3 I Engineered expression of activating Siglec-E16 in mice**
(A) Schematic representation of Siglec-E and Siglec-E16 receptors. The parts of Siglec-E16 derived from mouse Siglec-E or human Siglec-16 are drawn in gray and black, respectively. (B) Immunoprecipitation of Siglec-E16 results in co-precipitation of DAP12. HEK293A cells were transfected with constructs for Siglecs and FLAG-tagged DAP12. Proteins were detected with anti-Siglec-E and anti-FLAG antibodies. (C) Modulation of cytokine production by activating Siglec-E16. Stable RAW264.7 macrophage cell lines were stimulated with increasing doses of LPS. IL-6 and IL-10 were quantified in cell supernatants. Data are represented as mean ± SEM, n=3. (D) Schematic representation of the *Siglec-e* and *Siglec-e16* loci. (E) Expression of Siglec-E and Siglec-E16 in blood neutrophils. Ly6G-positive cells were stained with Siglec-E (lines) or isotype control antibodies (solid). (F) Expression of Siglec-E and Siglec-E16 in spleen and liver. Siglec expression is marked with blue color. Nuclei were stained with a red dye.

We then generated the mouse line E16 by genomic insertion of a cDNA encoding the transmembrane part of Siglec-16 at exon 4 of *Siglece* (**Figure 3D**). As transcription of Siglec-E and chimeric receptor Siglec-E16 are driven by the same promoter, we expected a similar expression pattern. Indeed, Siglec-E16 was found on blood neutrophils at levels comparable to Siglec-E (**Figure 3E**). We did not detect Siglec-E or Siglec-E16 on other blood cells (data not shown). We then analyzed Siglec expression in organs, and detected Siglec-E16 in spleen and liver

macrophages (**Figure 3F** and **Figure S5**). Minor variations in expression were expected, as the two receptors have differences in their transmembrane domain. Siglec-E replacement did not altered basal values of hematology and serum chemistry (**Tables S1** and **S2**). Together, these data indicated that the chimeric Siglec-E16 exhibits an expression pattern similar to the endogenous, inhibitory Siglec-E, but drives cellular activation.

*Activating Siglec receptors produce a protective response against bacterial infection*

To evaluate the role of paired Siglec receptors in bacterial infection, we first tested if *E. coli* K1 could recognize the extracellular domain that is common in Siglec-E and Siglec-E16. Fluorescein-labeled bacteria bound to Siglec-E to a similar extent as Siglec-11 (**Figure 4A**). We then studied bacterial survival in blood from homozygous wild type (E/E) or homozygous E16 (E16/E16) mice, and found enhanced killing of *E. coli* K1 in blood from homozygous E16/E16 (**Figure 4B**). This effect was not present using *E. coli* deficient in sialylated capsule biosynthesis, suggesting that the effect was dependent on capsule interaction with the Siglecs. Furthermore, activation of MAP kinases, as measured by phosphorylation of p44/Erk1 and p38, was increased in bone marrow derived macrophages of E16/E16 mice compared to E/E controls upon *E. coli* K1 challenge (**Figure 4C**). Finally, we asked if activating Siglec-E16 conferred an advantage to the host during bacterial infection. We used an *in vivo* model of experimental hematogenous *E. coli* K1 meningitis, which mimics the pathogenesis of *E. coli* meningitis in humans and was used to study the role of the K1 capsule (*Huang et al.*, 1995; *Kim et al.*, 1992). In this model, bacteria are injected intravenously, resulting in bacteremia and subsequent entry of bacteria into the central nervous system. Compared to wild type E/E mice, we observed reduced bacterial counts in blood, spleen and liver of the E16/E16 animals (**Figure 4D**). We found no differences in the number of bacteria in organs with low Siglec expression, such as brain and kidneys (**Figure S6**). Further corroborating the immunoregulatory role of Siglec-E16 as an activating receptor, we detected higher levels of the pro-inflammatory cytokines IL-6, MCP-1 and IL-12 in the serum of E16/E16 animals (**Figure 4E**). Interestingly, a single *SigleceE16* allele could alter marginally *E. coli* K1 recovery in spleen (**Figure S7**). In summary, these data demonstrate that activating Siglec receptors do confer an advantage to the host during infection with a bacterial species that can interact with related inhibitory Siglecs to dampen the immune

responses of the host. Activating Siglec-E16 engages *E. coli* K1, leading to increased intracellular signaling and pro-inflammatory cytokine responses, which elicits a protective innate immune response against the pathogen.

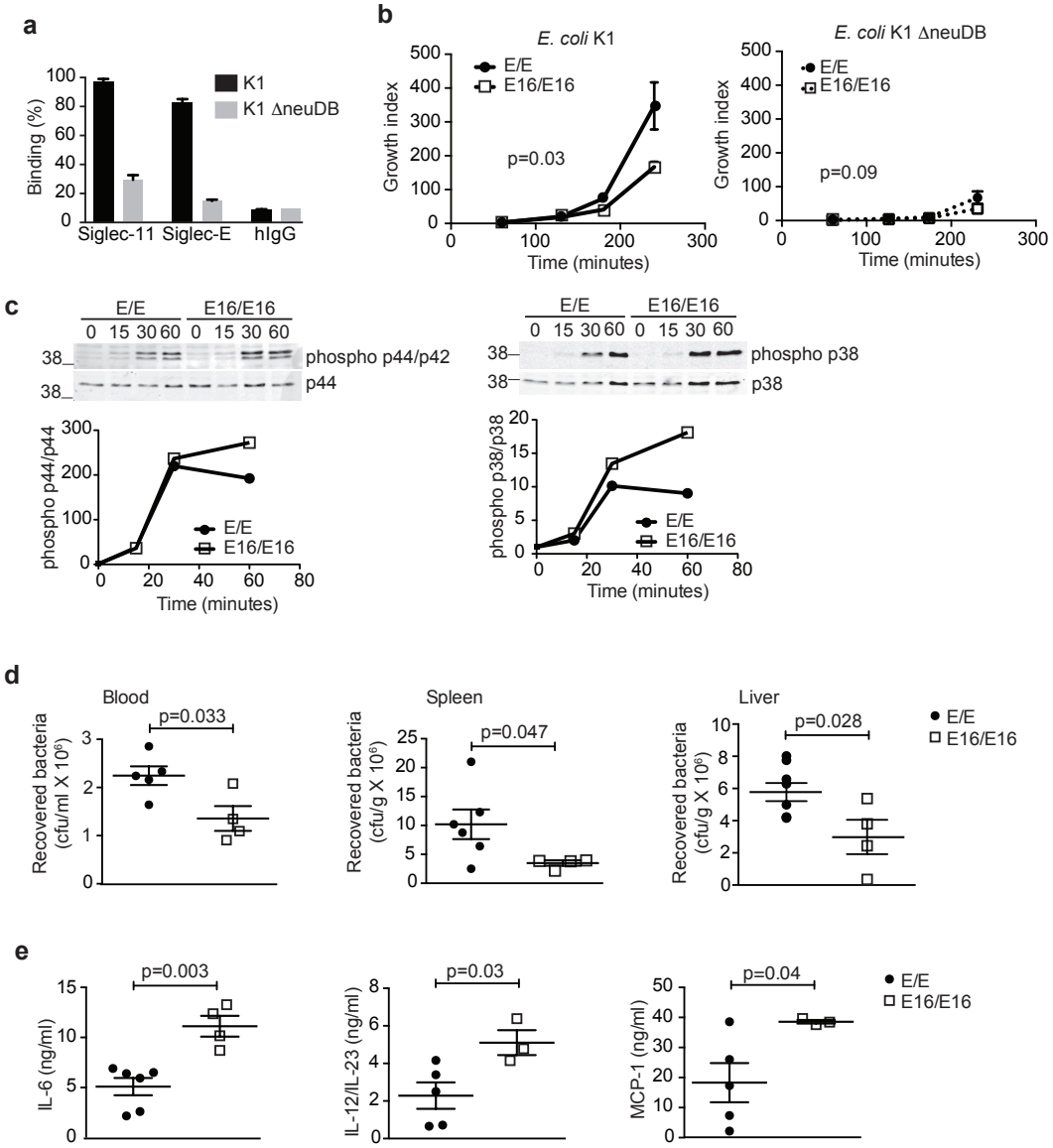


**Figure 4 | Activating Siglecs confer protection against *E. coli* K1 challenge**
(A) *E. coli* K1 binding to the extracellular part of Siglec-E. Data are represented as mean ± SEM, n=3. (B) *E. coli* survival in blood from E/E or E16/E16 mice, one hour after infection. Data are represented as mean ± SEM, n=4. (C) Activation of MAP kinase signaling cascade in bone marrow-derived macrophages. (D) Recovered bacteria from blood, spleen and liver. Mean ± SEM are indicated, n=4-6. (E) Cytokine levels in serum one hour after bacterial challenge. Mean ± SEM are indicated, n=4-6.

## Discussion

The innate immune system relies on receptors that distinguish molecules of the host from those of pathogens (*Janeway and Medzhitov*, 2002). Macrophages, neutrophils, and dendritic cells are strategically located in distinct anatomical compartments to sense conserved features of microbial pathogens via pattern-recognition proteins

(*Kawai and Akira*, 2010; *Iwasaki and Medzhitov*, 2015) and mount adequate innate immune defense functions. At the same time, systems are in place to balance cellular reactivity to provide maximal protection from infection with minimal immunopathology. The molecular features and signaling properties of the inhibitory Siglecs suggest important role in balancing inflammatory responses in resting cells via recognition of host sialic acids as SAMPs (*Varki*, 2011; *Macauley et al.*, 2014; *Linnartz-Gerlach et al.*, 2014). For instance, Siglecs in microglia alleviate neurotoxicity (*Claude et al.*, 2013; *Wang and Neumann*, 2010); CD33/Siglec-3 controls secretion of proinflammatory cytokines (*Lajaunias et al.*, 2005). Engagement of such inhibitory Siglecs results in tyrosine phosphorylation within the cytoplasmic ITIM domain and recruitment of downstream phosphatases. Dephosphorylating signaling intermediates causes them to act on their respective targets to dampen inflammatory signals relayed by activating receptors (*Crocker et al.*, 2007).

In this study, we investigated the role of the Siglec-11 and Siglec-16 in bacterial infection, and developed a novel mouse model to demonstrate the relevance of such putative paired Siglec receptors *in vivo*. Given that the extracellular domains of Siglec-11 and Siglec-16 are very similar, we suspected they could bind similar ligands. However, whereas Siglec-11 is an inhibitory receptor, Siglec-16 was shown to associate with DAP12, suggesting that it activates inflammatory responses (*Angata et al.*, 2002; *Cao et al.*, 2008). It is also interesting that the two *SIGLEC* genes underwent a very unusual sequence of gene conversion events during human evolution, and that *SIGLEC16* is often inactivated in the human population (*Wang et al.*, 2012). We speculated that *SIGLEC16* first emerged as a countermeasure to pathogens that exploit interaction with Siglec-11 to avoid immune responses by the host. We also suspected that activating Siglecs have the long term potential to be deleterious, perhaps by altering the inflammatory set point of cells, as their genes are frequently inactivated (*Angata and Varki*, 2014).

We studied Siglec-11 and Siglec-16 function in relation to a pathogen that causes meningitis and produces a capsular homopolymer made of a2,8-linked sialic acid – the same glycan structure identified as a potential ligand of Siglec-11 (*Hayakawa et al.*, 2005). In this regard it is intriguing that Siglec-11 was found in the brain of humans, but not of chimpanzee (*Hayakawa et al.*, 2005), and that *E. coli* K1 is a human-specific pathogen. We demonstrated that Siglec-11 and Siglec-16 are expressed on macrophages throughout the human body, including simultaneously on

the same cell type, indicating that could behave as paired receptors. We also showed that *E. coli* K1 uses molecular mimicry strategies to engage Siglec-11 to blunt innate immune responses responsible for bacterial killing. By contrast, cellular expression of activating Siglec-16 promotes bacterial elimination. It is interesting that we observed Siglec expression in brain and bladder, which are common sites of infection of *E. coli* K1 and other uropathogenic strains (*Croxen and Finlay*, 2010). Similar interaction might occur with other pathogens such as *Neisseria meningitis* serotype B that produces the same capsular saccharide (*Troy*, 1979; *Freiberger et al.*, 2007).

We then studied the relevance of human-type paired receptors in the response to bacterial infection using mice expressing engineered Siglec-E16 receptors. While the binding properties of Siglec-E16 are virtually undistinguishable from Siglec-E, it can engage DAP12 and drive pro-inflammatory responses, due to increased activation of MAP kinase signaling cascade. Mice expressing activating Siglec-E16 produced higher levels of proinflammatory cytokine upon intravenous administration of E. coli K1. Within an hour, animals expressing Siglec-E16 restricted bacterial dissemination, whereas *E. coli* K1 survived better when allowed to interact with the endogenous inhibitory Siglec-E.

Building on this and previous research, we suggest that some bacterial pathogens exploit molecular mimicry of sialylated SAMPs to bind to inhibitory Siglec receptors and escape immune responses of the host. Likely to counteract such pathogen subversion, the host has evolved receptors that combine the binding properties of the inhibitory receptors to intracellular elements that activate immune responses. Similar patterns of receptor evolution have been described for activating receptors of natural killer cells (*Abi-Rached and Parham*, 2005; *Akkaya and Barclay*, 2013; *Vilches and Parham*, 2002). The advantage of activating receptors in protecting against pathogens would be balanced by a greater risk for unwanted inflammation, which could select against them and drive a high frequency of the non-functional alleles.

## Materials and Methods

*Bacteria and cell lines*

*E. coli* RS218 is a spontaneous streptomycin-resistant mutant of *E. coli* RS218 (O18:K1:H7) that was isolated from the cerebrospinal fluid of a neonate with *E. coli* meningitis(*Silver et al.*, 1980). *E. coli* Δ*neu*DB strain SE1634 is a *neu*DB deficient strain that lack genes necessary for production of cytoplasmic precursors to the K1 exopolysaccharide capsule (*Kim et al.*, 2003). *E. coli* K12 strain DH5α and *E. coli* K12 strain carrying the plasmid pRS32 for K1 capsular polysaccharide (*Silver et al.*, 1981) were also used in this study. *E. coli* were propagated in Brain Heart Infusion broth, BHI (Difco, BD Diagnostics, Franklin Lakes, NJ) at 37 ˚C with shaking. For all binding and infection studies, bacteria were cultivated to an optical density at 600 nm equivalent to 0.6. HEK293 and RAW264.7 were obtained from ATCC. CHME-5 microglia was described in (*Janabi et al.*, 1995).

*Siglec-Fc/bacteria binding assay*

96-well plates were coated with 1 μg/well protein A (Thermo Scientific, Waltham, MA) in 50 mM carbonate buffer pH 9.5 overnight at 4 ˚C. Wells were washed with PBS-T (0.05% Tween-20 in PBS) and blocked with 1% BSA in PBS for 1 hour at room temperature. 2.5 μg/well Siglec-Fcs, produced as previously described (*Padler-Karavani et al.*, 2014), were incubated for 2 hours at room temperature. Afterwards, wells were washed with PBS-T. *E. coli* were pelleted, washed with PBS and then incubated with 0.1% fluorescein isothiocyanate (FITC, Sigma) in PBS for 1 hour at 37 ˚C with rotation. Bacteria were extensively washed with PBS to remove trace amounts of free FITC and then resuspended in PBS at an optical density of 1. A volume of 0.1 ml of FITC-labeled bacteria was added to each well. Plates were centrifuged at 500 g for 10 minutes and incubated for 1 hour at room temperature. After washing to remove unbound bacteria, the residual fluorescent intensity was measured using a SpectraMax M3 (Molecular Devices, Sunnyvale, CA).

*Adherence to microglia cells and killing assay*

CHME-5 cells were transfected with constructs for expression of Siglecs using Neon (Life Technologies, Grand Island, NY). Cells were infected with *E. coli* K1 strains at a multiplicity of infection of 0.1 bacteria per cell, followed by incubation for 30 minutes

at 37˚C. Cells were washed and lysed with 0.01% Triton X-100 and bacteria were counted by serial dilutions. For bacteria adherence assay, FITC-labeled bacteria were incubated with transfected cells. Cells were washed and analyzed by flow cytometry using a FACScalibur (BD Biosciences, San Jose, CA).

*MAP kinase activation*

Murine macrophages were derived from bone marrow cells cultured with conditional media obtained from culture supernatants of L929 cells for 6 days. Cells were incubated with bacteria ($10^5$ c.f.u./test) for different time points, washed with PBS and lysed in lysis buffer (1% NP-40, 20 mM Tris pH 8, 150 mM NaCl). Lysates were spun at 12,000 x g. Protein concentration of the supernatant was measured with a BCA kit (Pierce). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with anti-phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, Danvers, MA, cat 9216), anti-p38 MAPK (BioLegend, San Diego, CA, cat 620422), anti-phospho-ERK1/2 (pT202/pY204) (BD Bioscience, cat 612358), or anti-p44 MAP Kinase (Cell Signaling, cat 4372).

*Analysis of cytokine expression*

Supernatant were collected from stable RAW264.7 cell lines expressing Siglec-E16 stimulated with LPS (Sigma) for 24 hours. Serum was separated from whole blood by incubation in BD Microtainer tubes (cat 365956). IL-6 concentration was measured using an ELISA kit from R&D Systems (Minneapolis, MN). IL-10, IL-12, MCP-1, TNF-$\alpha$ concentrations were measured using ELISA kits from BioLegend.

*Generation of Siglec-E16 mouse line*

The E16 mouse line was generated by GenOway (France) by homologous recombination of a DNA cassette including human Siglec-16 cDNA encoding for the transmembrane and intracellular tail within the exon 4 of *SIGLECE* in embryonic stem cells in C57BL/6 background.

*Mouse infection model*

All animal experiments were approved by the Committee on the Use and Care of Animals, UCSD and performed using accepted veterinary standards. 10–12 weeks old mice received *E. coli* RS218 ($10^7$ c.f.u.) in 100 ml PBS via the tail vein. One hour

later, mice were sacrificed and blood was collected by cardiac puncture. Animals were then perfused with Ringer solution as described previously(*Zhu et al.*, 2010). Organs were isolated from mice, homogenized with a magDNA (Roche, Basel, Switzerland) and plated for bacteria count.

*Immunohistochemistry*

Frozen human tissues were obtained from the National Cancer Institute funded Co-operative Human Tissue Network. Sections were blocked for endogenous peroxidases and endogenous biotin and overlaid either with control mouse IgGs (Abcam, Cambridge, MA, cat ab81032), mouse anti-CD68 (AbD Serotec, Raleigh, NC, cat MCA5709), mouse anti-Siglec-11 (R&D Systems, cat MAB3258), or mouse anti-Siglec-16 (R&D Systems, clones 706004, 706022 and 706032), followed by detection using appropriate secondary reagents, and developed using Vector Blue SK4200 (Vector Laboratories, Burlingame, CA), and Fast Red nuclear counterstain, following protocols of the UC San Diego Mouse phenotype Core http://mousepheno.ucsd.edu/. For immunofluorescence, mouse anti-Siglec-11 antibodies were biotinylated using Biotin-NHS (Thermo Scientific). Tissue sections were sequentially incubated with mouse anti-Siglec-16, Alexa Fuor488-conjugated anti-mouse IgG (Life Technologies), biotinylated anti-Siglec-11, and Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). Murine organs were isolated, snap-frozen in OCT and stored at -80 ˚C. Sections were blocked and stained with rat anti-F4/80 (AbD Serotec, cat MCA497), rat anti-Siglec-E (BioLegend, cat 677102) or rat IgG (Abcam, cat ab37361), followed by appropriate secondary reagents.

*Immunoprecipitation*

HEK293T expressing Siglec-E or Siglec-E16 and DAP12 were lysed and proteins were incubated with anti-Siglec-E antibodies (BioLegend) and protein G-Dynabeads (Life Technologies). Proteins were detected with goat anti-Siglec-E antibodies (R&D Systems, cat AF5806) or rabbit anti-FLAG antibodies (Sigma). Secondary antibodies were from LI-COR (Lincoln, NE). Signals were acquired with an Odyssey instrument (LI-COR) and analyzed by Image Studio software (LI-COR).

*Siglec-E16 expression on mouse cells*

Blood was obtained by cardiac puncture. Bone marrow neutrophils were isolated by Percol gradient. Splenocytes were obtained by mechanic disruption of spleen. Red blood cells were lysed by incubation in ACK buffer (Gibco). Cells were stained with anti-Siglec-E, anti-Ly6G clone 1A8 (BD Biosciences, cat 560599) or anti-F4/80 antibodies (BioLegend Inc, San Diego, cat 123115). Data were analyzed with FlowJo (FlowJo, LLC, Asland, OR).

*Statistical analysis*

For mouse experiments, no specific blinding method or exclusion criteria were used, but mice in each sample group were selected randomly. The sample size (n) of each experimental group is described in each corresponding figure legend, and all experiments were repeated at least with three biological replicates. Prism 6 software (GraphPad) was used for all statistical analyses. Quantitative data are expressed as means ± standard error of the mean (sem, represented as error bars). Results from each group were averaged and used to calculate descriptive statistics. Unpaired Student's t-test or ANOVA were used for comparisons involving two groups.

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Chapter 2b:

# Supplementary information: Paired Siglec receptors generate opposite inflammatory responses to a human-specific pathogen

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**Supplementary Table 1**

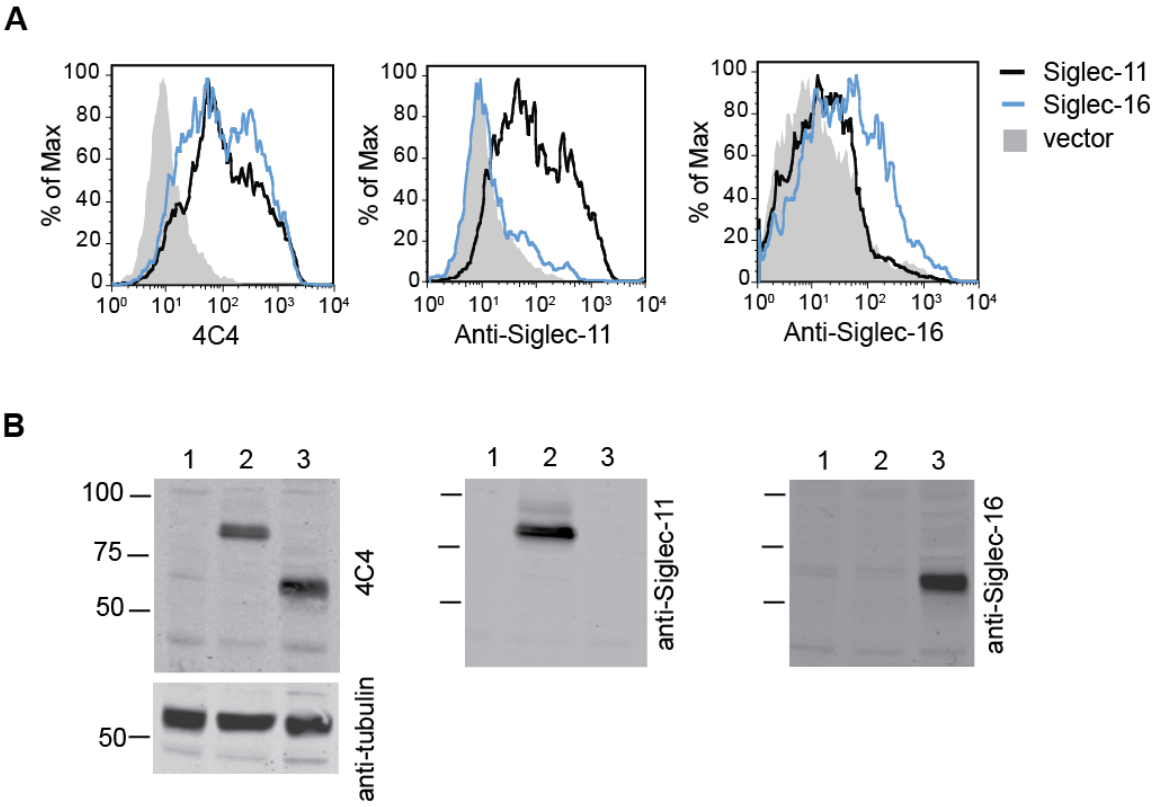
Hematology data for mice generated in this study

| | | E/E | | E16/E16 | |
|---|---|---|---|---|---|
| | | **average** | **st dev** | **average** | **st dev** |
| **WBC** | **K/uL** | 3.20 | 1.25 | 2.34 | 0.47 |
| **NE#** | **K/uL** | 0.41 | 0.25 | 0.21 | 0.07 |
| **LY#** | **K/uL** | 2.50 | 0.90 | 1.91 | 0.41 |
| **MO#** | **K/uL** | 0.20 | 0.11 | 0.16 | 0.05 |
| **EO#** | **K/uL** | 0.07 | 0.08 | 0.05 | 0.03 |
| **BA#** | **K/uL** | 0.01 | 0.02 | 0.01 | 0.01 |
| | | | | | |
| **NE%** | **%** | 12.60 | 5.04 | 9.17 | 3.25 |
| **LY%** | **%** | 78.59 | 7.43 | 81.57 | 5.45 |
| **MO%** | **%** | 6.19 | 1.46 | 6.53 | 1.25 |
| **EO%** | **%** | 2.24 | 2.22 | 2.33 | 1.61 |
| **BA%** | **%** | 0.38 | 0.38 | 0.41 | 0.37 |
| | | | | | |
| **RBC** | **M/uL** | 10.33 | 0.41 | 10.08 | 0.79 |
| **HB** | **g/dL** | 14.21 | 1.14 | 13.19 | 1.17 |
| **HCT** | **%** | 39.66 | 1.08 | 38.39 | 2.40 |
| **MCV** | **fL** | 38.43 | 1.11 | 38.14 | 1.51 |
| **MCH** | **Pg** | 13.77 | 1.04 | 13.09 | 0.33 |
| **MCHC** | **g/dL** | 35.81 | 2.35 | 34.34 | 1.31 |
| **RDW** | **%** | 17.58 | 0.57 | 17.66 | 1.00 |
| **PLT** | **K/uL** | 1147.93 | 105.29 | 975.79 | 78.59 |
| **MPV** | **fL** | 4.76 | 0.24 | 4.59 | 0.12 |

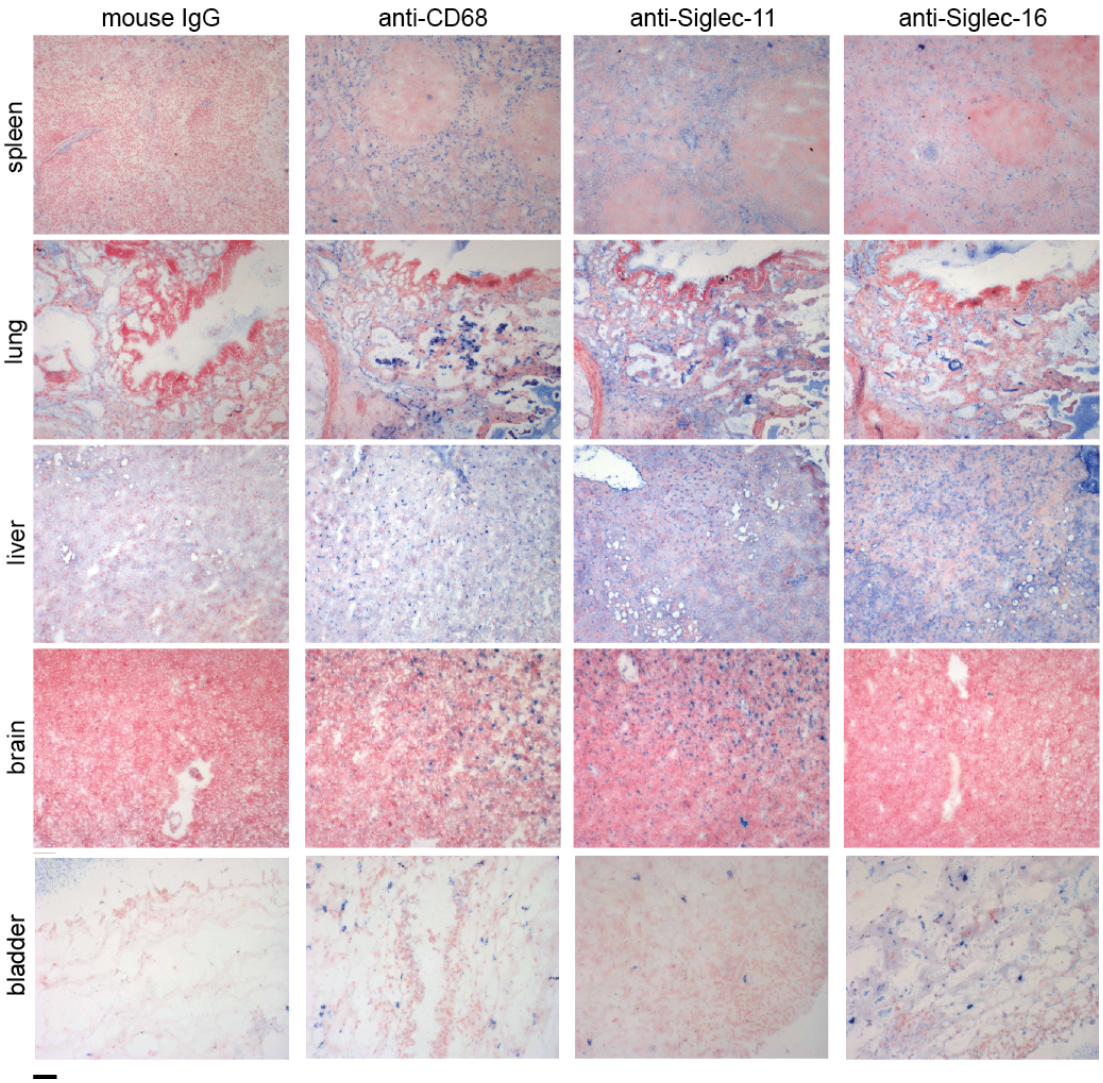
**Supplementary Table 2**

Serum chemistry data for mice generated in this study

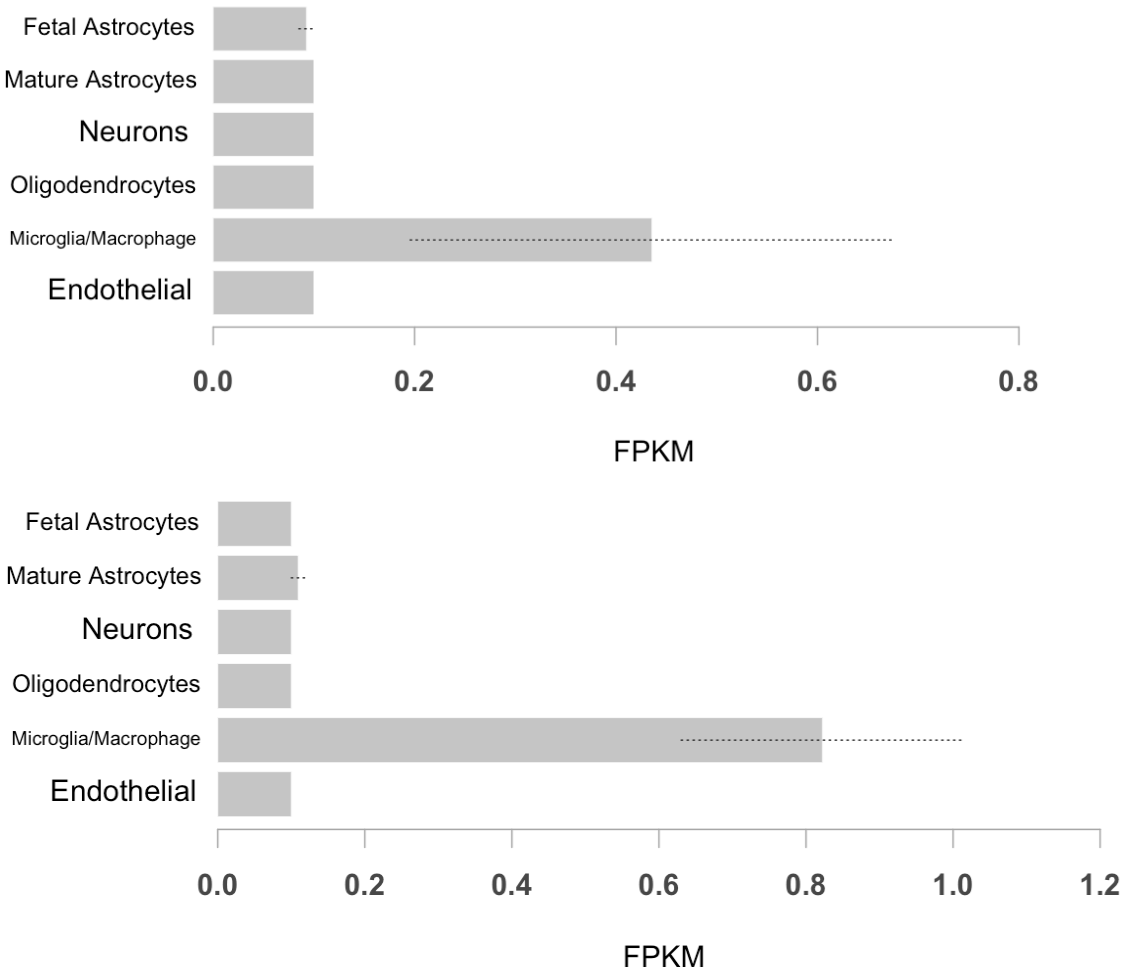
| | E/E | | E16/E16 | |
|---|---|---|---|---|
| | **mean** | **stdev** | **mean** | **stdev** |
| **Albumin** | 3.90 | 0.21 | 4.00 | 0.23 |
| **Alkaline Phosphatase** | 167.40 | 54.31 | 166.50 | 28.01 |
| **ALT** | 41.70 | 9.43 | 92.88 | 66.40 |
| **Anion Gap** | 44.85 | 2.29 | 43.50 | 1.73 |
| **AST** | 67.80 | 11.42 | 143.25 | 77.45 |
| **Bicarbonate** | 15.30 | 1.55 | 13.75 | 1.50 |
| **Bilirubin Total** | 0.03 | 0.02 | 0.05 | 0.01 |
| **BUN** | 24.30 | 3.73 | 24.88 | 3.17 |
| **Calcium** | 10.79 | 0.43 | 11.05 | 0.26 |
| **Chloride** | 100.35 | 2.49 | 96.50 | 3.54 |
| **Creatinine** | 0.12 | 0.02 | 0.13 | 0.04 |
| **Glucose** | 207.15 | 46.19 | 269.00 | 55.79 |
| **Potassium** | 6.63 | 1.43 | 9.21 | 2.95 |
| **Sodium** | 160.50 | 4.00 | 153.50 | 2.35 |
| **Total Protein** | 5.43 | 0.28 | 5.46 | 0.29 |



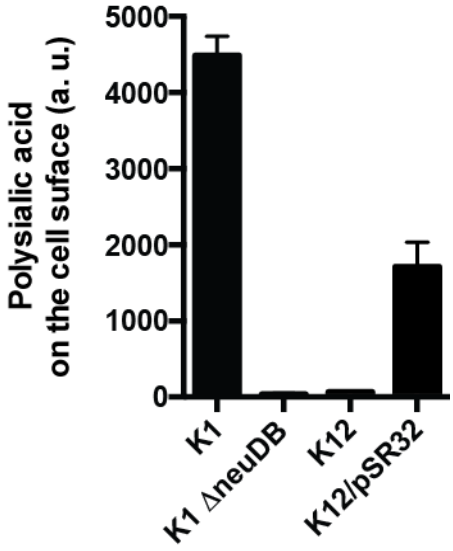

**Supplemental Figure 1 I Analysis of Siglec-11 and Siglec-16 specific antibodies**
(A) HEK293A cells expressing Siglec-11 or Siglec-16 were incubated with Siglec antibodies, and analyzed by flow cytometry. 4C4 monoclonal antibodies (which recognize both Siglecs) show expression levels of Siglec-11 and Siglec-16 proteins on the cell surface. Siglec-11 and Siglec-16 monoclonal antibodies were raised against peptides with amino acid sequences specific for Siglec-11 (PPARLELSWTRWGQTVGPSQPSDPGVLELPPIC) or Siglec-16 (PPARLSWTWGEQTVGPSQPSDPGVLQLPRVC). The affinity of Siglec-16 antibodies appears to be lower than the other antibodies. (B) Lysates from HEK293A cells expressing Siglecs were probed with 4C4 or Siglec-specific antibodies



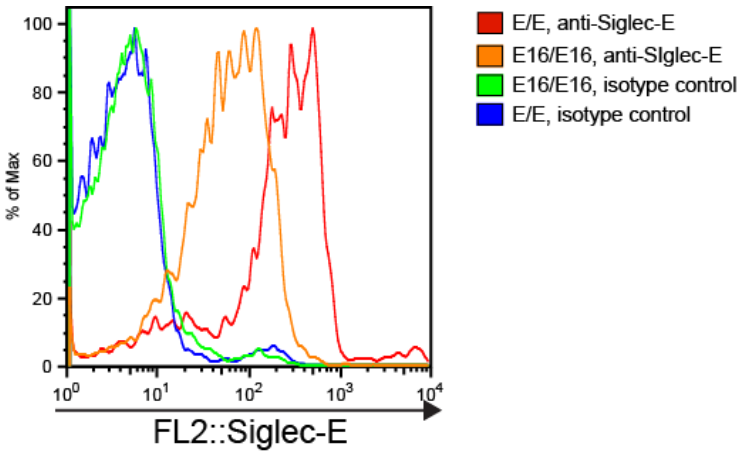

**Supplementary Figure 2**
Sections from human organs were stained with antibodies specific for the macrophage marker CD68, Siglec-11 or Siglec-16 or control IgG. Expression of receptors is marked with blue color. Nuclei were stained with a red dye.



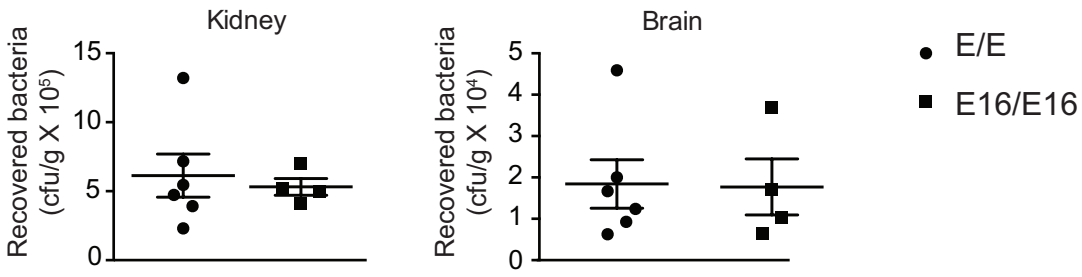

**Supplementary Figure 3 | Siglec-11 and Siglec-16 are expressed in human microglia**
Expression data of Siglec-11 and Siglec-16 in human cells in brain were retrieved from a recently updated database (www.BrainRNAseq.org) that includes cell type-specific gene expression profiles. Bennett and co-workers used anti-human TMEM119 rabbit polyclonal antiserum to identify and isolate microglia in postmortem and surgical human brain sections, and generated RNA sequencing profiles of pure microglia (*Bennett et al.*, 2016). Human samples were obtained from patients ranging in age from 8-63 years old, with the exception of the fetal astrocytes (18 gestation weeks). The human data are derived from temporal lobe cortexes with the exception of the fetal astrocytes (whole cortex). FPKM (Fragments Per Kilobase of transcript per Million mapped reads) is indicated for each cell type. Siglec-11 (top) and Siglec-16 (bottom) show specific expression in microglia.



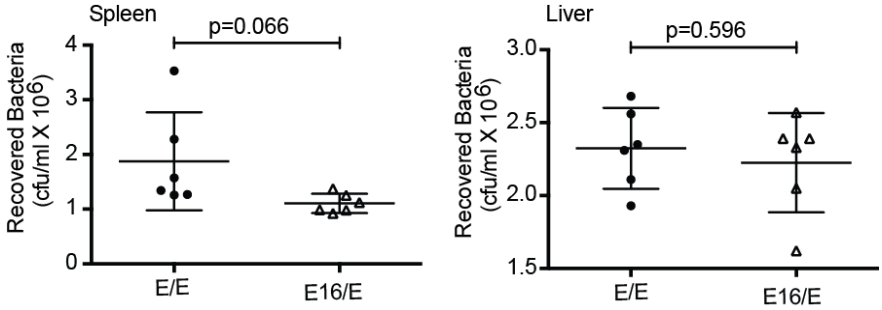

**Supplementary Figure 4 l Quantification of polysialic in bacterial strains used in this study.** Cells were incubated with 12E3 antibodies (binding to polysialic acid), washed and incubated with peroxidase-conjugated secondary antibodies to quantify capsular polysialic acid.




**Supplemental Figure 5 l** Expression of Siglec-E and Siglec-E16 on mouse spleens. Single cells suspension was obtained as indicated in the methods. Cells were stained with F4/80 and Siglec-E or isotype antibodies. F4/80-positive macrophages from E16/E16 mice show lower Siglec staining compared to E/E mice, as also seen in Figure 3f.




**Supplemental Figure 6 l** Recovery of bacteria in organs of mice infected with *E. coli* K1.




**Supplemental Figure 7 l** Recovery of bacteria in organs of mice infected with *E. coli* K1

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# Chapter 3:

# The human-specific pathogen *Neisseria gonorrhoeae* engages immunoregulatory Siglecs in a species-specific manner: Genetic implications for gonorrhea risk

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*Manuscript in preparation*

Contribution: Purification of Siglec-Fc constructs
Bacteria binding assays
Porin binding assays
Genotyping of Namibian cohort

**Abstract**

*Neisseria gonorrhoeae* causes the sexually transmitted disease gonorrhea in humans, and represents a major global health concern as it instigates serious sequelae including infertility in women. The recent increase in multidrug-resistant gonococcal strains may lead to an era of untreatable gonorrhea. This pathogen has evolved different mechanisms to evade the host immune system, including sialylation of its surface lipooligosaccharide (LOS), which results in resistance to complement-dependent killing and decreased antibody recognition. The CD33-related sialic acid binding immunoglobulin like lectins (CD33rSiglecs) expressed on innate immune cells recognize sialic acid-bearing glycans, leading either to anti-inflammatory or pro-inflammatory responses. We hypothesized that anti-inflammatory Siglecs are targeted by gonococci to limit host immune responses, and that pro-inflammatory Siglecs and *SIGLEC* gene polymorphisms represent a host evolutionary response to counteract this interaction. We now show that *N. gonorrhoeae* can engage multiple human CD33rSiglecs expressed in the genitourinary tract, including Siglec-11 and -16, which we detected on cervical epithelium. In addition to LOS sialic acid, we found that gonococcal porin (PorB) also mediated binding to Siglecs. PorB bound preferentially to human Siglecs compared to chimpanzee orthologs, which may provide another reason for the human restriction of gonorrhea. These novel interactions differentially modulate immune reactions such as cytokine secretions and phagocytosis. We also studied the distribution of null polymorphisms in *SIGLECS* in a Namibian cohort with a high prevalence of gonorrhea, and found that uninfected women preferentially harbor intact *SIGLEC16* alleles encoding an activating immune receptor. These results contribute to understanding of the mechanisms by *N. gonorrhoeae* have evolved to evade the human immune system, which could lead to the development of novel therapeutics to treat gonorrhea.

**Introduction**

Gonorrhea is a sexually transmitted disease that poses a major global health problem with about 78 million estimated infections worldwide in 2012 (*Newman et al.*, 2015). This disease is caused by *Neisseria gonorrhoeae*, a Gram negative bacterium that exclusively infects humans. Whereas urogenital epithelia are the main sites of infection, *N. gonorrhoeae* can also infect the conjunctiva, pharynx and rectal mucosa (*Edwards and Apicella*, 2004). Gonococci successfully proliferate in different host microenvironments and evade the human immune system by constantly modulating their surface antigenic makeup by phase variation and other mechanisms (*Edwards and Apicella*, 2004; *Virji*, 2009). The dynamic changes in the glycan extensions of gonococcal lipooligossacharide (LOS) is an excellent example of how *N. gonorrhoeae* evade the host immune response. The lacto-*N*-neotetraose (LNnT) structure of the LOS engages host surface receptors, like the asialoglycoprotein receptor (ASGP-R), and leads to the invasion of the urethral epithelia (*Harvey et al.*, 2001). Sialylation of LNnT by the gonococcal sialyltransferase (Lst), a surface-exposed outer membrane protein that uses host sialic acid in form of CMP-Neu5Ac (*Shell et al.*, 2002), makes the bacteria more resistant to complement-mediated killing by recruiting Factor H (*Ram et al.*, 1999). Sialic acid is present on mammalian cell surfaces where it is recognized as a 'self' molecule by receptors of the immune system (*Varki and Gagneux*, 2012). Interactions of sialic acid with sialic acid-binding Siglec lectins maintain immune cells in a quiescent state and prevent inflammation (*Crocker et al.*, 2007). Inhibitory effects are driven by ITIM motifs in their intracellular domain that recruits SHP kinases and quench pro-inflammatory signaling cascade. This property is targeted by pathogens trying to control host immune responses to escape elimination (*Chang and Nizet*, 2014). For example, group B Streptococci (GBS) evade host immune responses by engaging inhibitory Siglec-5 and Siglec-9 (*Carlin et al.*, 2007). Humans also express paired Siglec receptors – sets of receptors with similar ligand specificities but opposite signaling properties. For instance, Siglec-11 and Siglec-16 have a highly conserved extracellular domain and similar ligand specificities (*Schwarz et al.*, 2015). Activating Siglecs recruit the adaptor proteins DAP10 and DAP12 through a positively charged amino acid in the transmembrane domain (*Pillai et al.*, 2012; *Tourdot et al.*, 2013; *Angata et al.*, 2006; *Cao et al.*, 2008). The adaptor proteins contain ITAM motifs that recruit Syk kinase, which induces the pro-inflammatory signaling cascade (*Lanier*, 2009). It has been

suggested that activating Sigecs represent evolutionary responses to microbes exploiting inhibitory Siglecs. In fact, Siglec-14 has been shown to counteract the exploitation of Siglec-5 by GBS (*Ali et al.*, 2014), whereas Siglec-16 reduces survival of *E. coli* K1 during infection. Moreover, due to polymorphisms, not all humans are able to express these activating Siglec receptors, which can lead to different immune responses to infections (*Schwarz et al.*, 2015). In this study, we elucidated how *N. gonorrhoeae* interacts with human Siglecs to modulate their pathogenic potential and the host inflammatory response.

**Results & Discussion**

*N. gonorrhoeae engages human Siglec receptors*

Here we showed for the first time that *N. gonorrhoeae* bound to human Siglecs. These receptors are mainly expressed on the cell surface of innate immune cells such as macrophages and neutrophils, which produce key innate immune responses to gonococcal infections (*Virji*, 2009). Gonorrhea is a human specific disease. To determine whether gonococci interacted with Siglecs in a human-specific manner that may contribute to its human specificity, we compared recombinant soluble extracellular domains of human and chimpanzee homologues (fused with Ig-Fc) in a binding assay using *N. gonorrhoeae* strains F62 (**Fig. 1A**) and 15253 (**Fig. 1B**). While F62 can express the LNnT LOS epitope that can be terminally substituted with sialic acid via an α2-3 linkage (*Yamasaki et al.*, 1991) (**Suppl. Fig**.**1**), 15253 lacks the genes that encode LOS glycosyltransferases (Lgt) B, C and D (*Erwin et al.*, 1996) and thus can express only lactose extensions from HepI and HepII (*Mandrell et al.*, 1993) (**Suppl. Fig**.**6**). While human Siglec-3 (also known as CD33) bound strongly to strain F62 (grown in media containing CMP-Neu5Ac to sialylate LOS, **Suppl. Fig.1**), the chimpanzee homologue showed no binding (**Fig. 1a**). Differences in amino acid sequences between the extracellular V-set and/or C2 set domains of human and chimpanzee Siglec-3 that could contribute to differences in bacteria binding are shown in **Suppl. Fig. 2**.

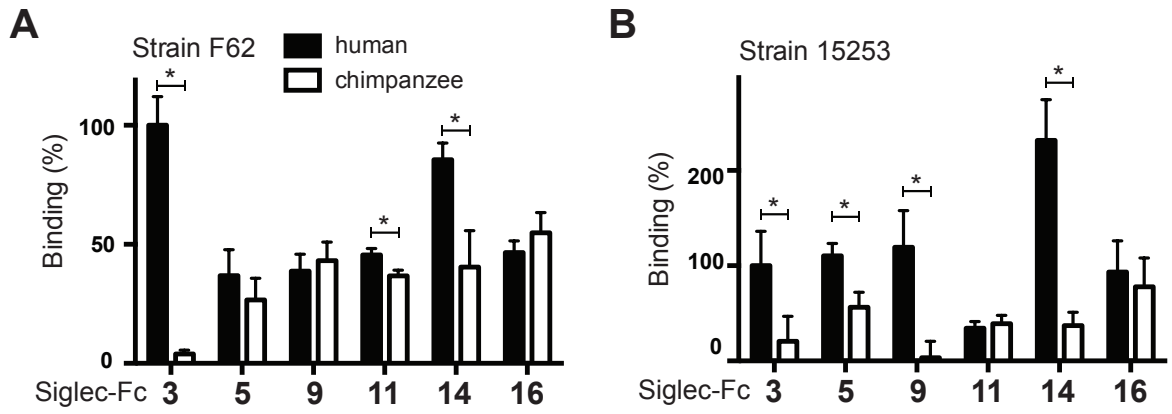


**Figure 1 I Human Siglecs bind to *Neisseria gonorrhoeae*** Recombinant human and chimpanzee Siglec-Fc fusion proteins are immobilized and binding of FITC-labeled, sialylated *N. gonorrhoeae* (A) strain F62 and (B) strain 15253 was measured. Binding is normalized to human Siglec-3 Fc and data are represented as mean ± SEM, n=3.

F62 bound well to all other human and chimpanzee Siglecs tested (Siglecs 5, 9, 11, 14 and 16), although the binding of human Siglec-11 and -14 was significantly

greater than the chimpanzee counterpart. Human Siglec-5, -11 and -14 have a significantly increased binding over the chimpanzee homologues but no difference was seen between the binding of the human and chimpanzee homologues of Siglec-9 and Siglec-16. The *N. gonorrhoeae* strain 15253, which was isolated from a disseminated infection, bound significantly better to human Siglec-3, -5, -9 and -14 than to its chimpanzee homolog (**Fig. 1b**). Amino acid sequence differences, as well as differences in post-translational modifications like N- and O-glycosylation between the human and chimpanzee homologs could be responsible for the difference in binding (**Suppl. Fig. 2-5**). Differences in binding between the strains could also be due to differences in LOS structure and their ability to sialylate (**Fig. 2a**) or other surface structures that could sterically hinder interactions.

*N. gonorrhoeae binding was only partially dependent on LOS sialic acid, and truncation of LOS increased binding*

Since Siglecs are lectins that usually bind to sialic acid, we hypothesized that the ligand sialylated LOS on *N. gonorrhoeae* (**Fig. 2a**) likely is the primary ligand for Siglecs. Growth of *N. gonorrhoeae* in media lacking CMP-Neu5Ac leads to expression of non-sialylated LOS because gonococci lack the ability to synthesize sialic acid *de novo;* the addition of CMP-Neu5Ac to media results in sialylation of LNnT LOS. The binding of sialylated and non-sialylated gonococci F62 differs between the Siglecs (**Fig. 2b**). Binding of Siglec-3 is solely dependent on sialic acid. Binding of Siglec-9 and Siglec-14 were enhanced by sialic acid, however residual binding suggested the presence of a sialic acid independent ligand. In contrast, the binding of Siglec-16 to F62 was significantly increased in the absence of sialic acid. Similar results are seen with sialylated and non-sialylated gonococci 15253 (**Suppl. Fig. 6**). These results lead to the hypothesis that an additional structure mediates the binding of Siglecs and gonococci. It is possible that the carbohydrate-binding pocket that confers lectin-like properties to Siglecs also binds to other glycan structures. To elucidate if Siglecs bind to non-sialylated LOS structures, we tested Siglec binding by flow cytometry to isogenic mutants of strain F62 that expressed truncated LOS glycans by (separately) inactivating the LOS glycosyltransferases *lgtD, lgtA, lgtE* and *lgtF* (**Fig. 2a**). Progressive truncation of LOS correlated with increased binding of Siglec-5, and as expected, to Siglec-14 to F62 (**Fig. 2c & f**). This inverse correlation between Siglec binding and LOS glycan length was not

significant with Siglec-9 (**Fig. 2d**). Binding of Siglec-11-Fc increases significantly with the truncation of the lactose epitope (**Fig. 2e**). Siglec-16-Fc binds very strongly to the LNnT structure (**Fig. 2g**). These data suggest the presence of an alternate ligand on *N. gonorrhoeae* for Siglecs. When present, sialylated LOS may be the primary binding site for LOS. Unsialylated LNnT LOS may hinder access of the alternate ligand to Siglecs. However, when LOS phase varies (e.g., lgtA 'off'), it may leave the alternate ligand more exposed and permit binding to Siglecs.

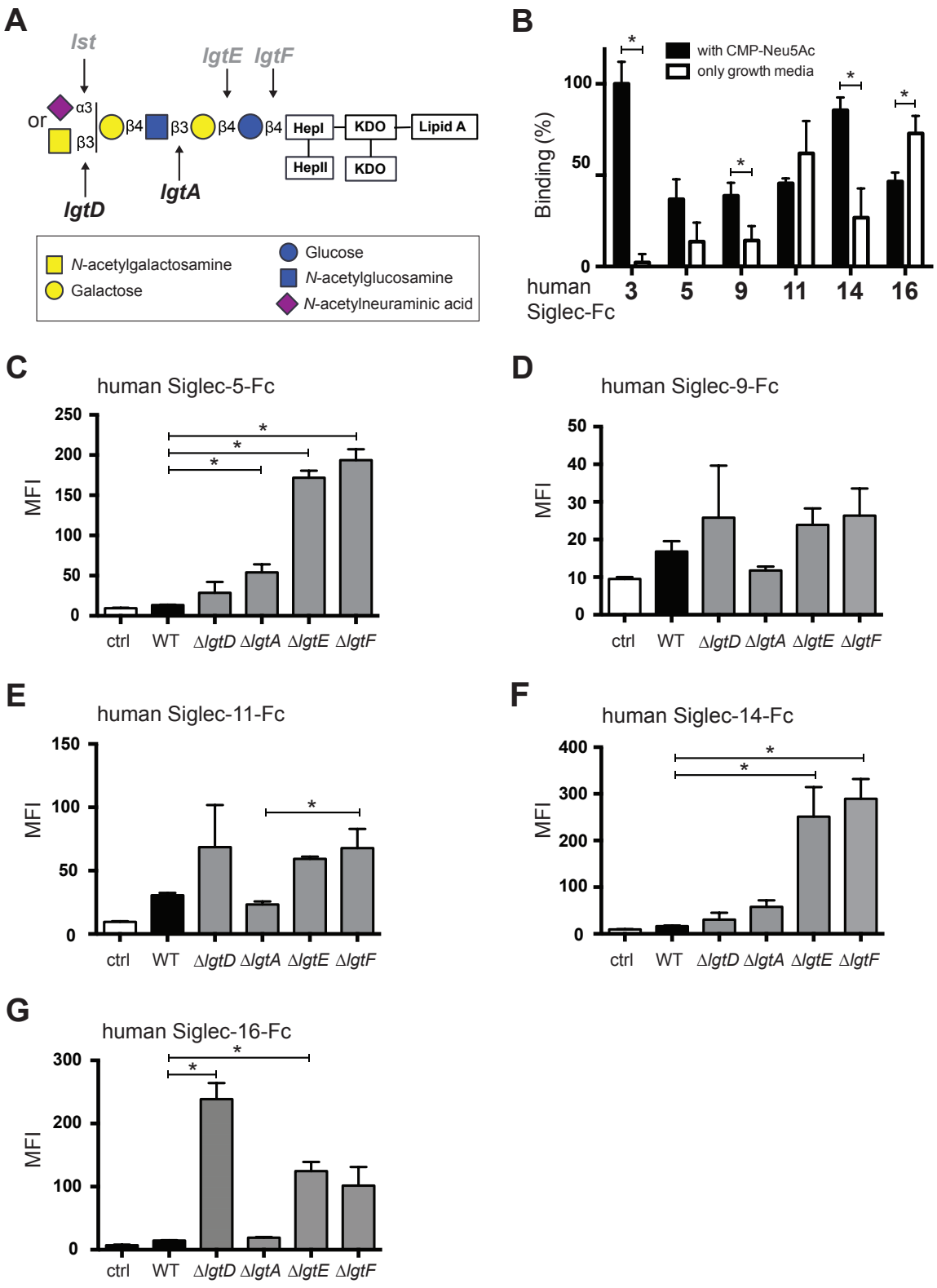


**Figure 2 l Siglec interactions with gonococci are sialic acid dependent and independent**
The LNnT LOS (A) of *N. gonorrhoeae* F62 strain can be sialylated by the sialyltransferase (Lst) if CMP-Neu5Ac is present. Phase variable genes are presented in grey. (B) Binding FITC-labeled F62 grown with and without CMP-Neu5Ac to immobilized human Siglec-Fcs. Binding is normalized to human Siglec-3 Fc. (C-F) Binding of human Siglec-Fcs to F62 with mutations in the glycosyltranferases lgtD, lgtA, lgtE and lgtF which lead to truncation of the LOS was measured by flow cytometry. Data are represented as mean ± SEM, n=3

*Gonococcal PorinB mediated binding to human Siglecs in a protein-dependent manner*

In addition to their main ligand, sialic acid, Siglecs have been reported to engage in protein-protein interactions (*Carlin et al.*, 2009; *Fong et al.*, 2015). Porin B (PorB) is the major outer membrane protein of *N. gonorrhoeae* and is essential for gonococcal survival; therefore we first explored the possibility that PorB mediated binding to the Siglecs. We tested two porins, PorB.1A (**Fig. 3a**) purified from the strain 15253 and PorinB.1B (**Fig. 3b**) purified from the strain F62. Both human and chimpanzee Siglec-3-Fc did not bind to either PorB molecules, which is consistent with the finding that binding of human Siglec-3-Fc to whole bacteria is solely dependent on LOS sialic acid. PorB.1A and PorB.1B both bound only to human, but not chimpanzee Siglec-Fcs -5, -9 and -14. Both PorB molecules bound to the human and chimpanzee Siglec-11 and -16, although binding to chimpanzee Siglecs was significantly diminished. The binding patterns of PorB.1A and PorB.1B were very similar, which could indicate a conserved binding region or motif between the two porins. The preference of porins to bind to human Siglecs over chimpanzee homologues strengthens the hypothesis that gonococcal interactions with Siglecs could contribute to the human specificity of gonorrhea.

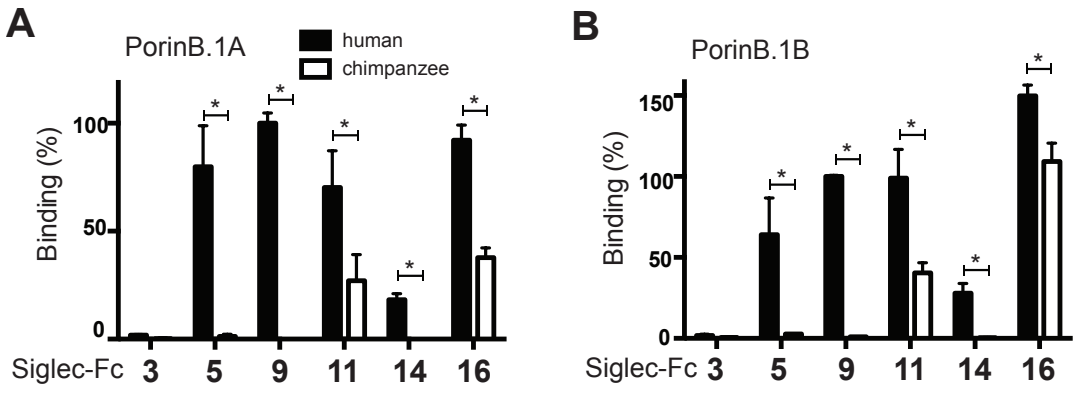


**Figure 3 | Siglecs bind to the gonococcal porin in a human specific manner**
Purified PorinB.1A from 15253 (A) and PorinB.1B from F62 (B) were immobilized and binding of human and chimpanzee Siglec-Fc proteins was measured. Binding is normalized to human Siglec-9-Fc and data are represented as mean ± SEM, n=3.

*N. gonorrhoeae modulated innate immune response via engagement of Siglecs*

The innate immune system plays an important role in the symptomatology of gonorrhea; the lack of a robust inflammatory response contributes to the lack of symptoms that is commonly seen, especially in women. To ask if Siglec interactions with *N. gonorrhoeae* modulates the immune response, we used a model system

where the monocytic cell line THP-1 was stably transfected with Siglec-5 or Siglec-14. The cytokine IL-6 is a major pro-inflammatory cytokine that is found in the secretions of infected patients (*Ramsey et al.*, 1995). THP-1 cells that express transfected Siglec-5 and were infected with *N. gonorrhoeae* expressed lower levels of IL-6 compared to an empty vector control (**Fig. 4a**). Expression of the activating Siglec-14 increased IL-6 secretion. These results indicate that the interaction between Siglecs and *N. gonorrhoeae* modulates the secretion of the cytokine, IL-6. In addition to cytokine secretion, phagocytosis is a common response to fight infections. We examined the phagocytic activity of THP-1 cells expressing Siglec-14 that were stimulated with purified PorB.1B. Increased phagocytic activity was seen when Siglec-14 expressing THP-1 cells were incubated with PorB.1B. The addition of a Siglec-14 blocking antibody reversed this effect, indicating that a functional interaction between Siglec-14 and PorB is a direct physical contact (**Fig. 4b**). These results indicate that gonococcal engagement of inhibitory Siglecs like Siglec-5 could contribute to the inhibition of pro-inflammatory responses as is seen in asymptomatic disease. On the other hand engagement of activating Siglecs like Siglec-14 can contribute to the pro-inflammatory response that may help clear gonococcal infection.

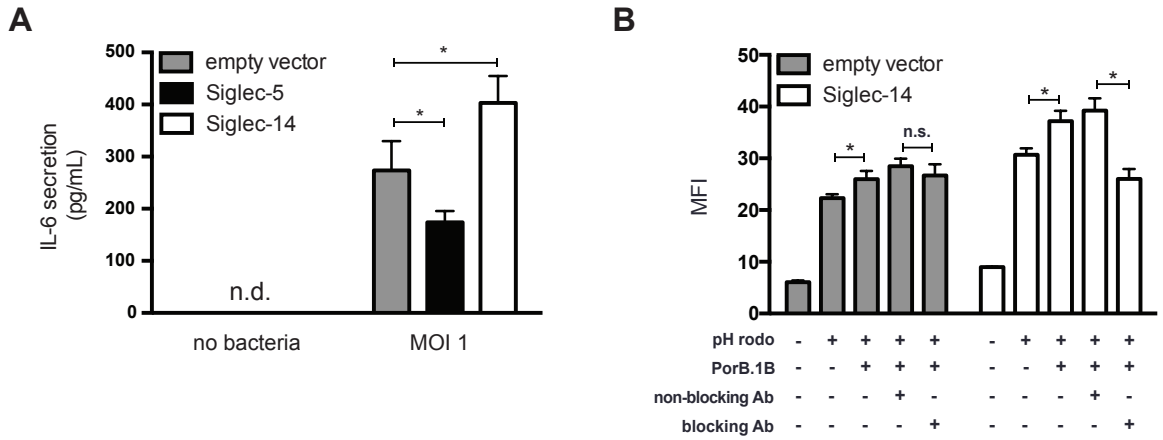


**Figure 4 l Siglec-5 and Siglec-14 modulate pro-inflammatory response in opposite directions**
(A) The secretion of pro-inflammatory cytokine IL-6 was measured in THP-1 expressing either Siglec-5 or Siglec-14 after incubation with F62 for 24 hours. (B) Phagocytic activity of THP-1 cells expressing Siglec-14 was measured by adding PorB.1B and monitoring the uptake of fluorescent particles (pH rodo). A blocking anti-Siglec-14 antibody could reverse the uptake. Data are represented as mean ± SEM, n=3.

*Siglec-11 and Siglec-16 are expressed on human cervical epithelium*

Siglecs are mainly found on cells of the innate immune system: monocytes express Siglec-3 and Siglec-9; neutrophils express Siglec-5, Siglec-9 and Siglec-14 (*Crocker*

*et al.*, 2007) and macrophages express Siglec-3, Siglec-11 and Siglec-16. Some Siglecs also have been found on other cell types. For example, Siglec-11 is found on ovarian fibroblasts (*Wang et al.*, 2011) and Siglec-5 is seen on amniotic epithelia (*Ali et al.*, 2014). We detected Siglec-11 and Siglec-16 on human cervical columnar epithelium **(Fig. 5)**, but not Siglec-3, Siglec-5, Siglec-9 and Siglec-14 (data not shown). The presence of these Siglec-11 and Siglec-16 on the cervical epithelium, the main site of gonococcal infection in females, may play a crucial role in host defense. We hypothesize that *N. gonorrhoeae* engages the inhibitory Siglec-11, which downregulates the pro-inflammatory response and enables the bacteria to escape immune detection establish asymptomatic infection. Siglec-11 is expressed in all humans, however due to a polymorphism not all humans are able to express Siglec-16 (*Wang et al.*, 2012). In the individuals that are able to express this activating receptor, Siglec-16 could enable the host to detect *N. gonorrhoeae* and mount a pro-inflammatory immune response that results in symptoms and facilitate clearance of bacteria.

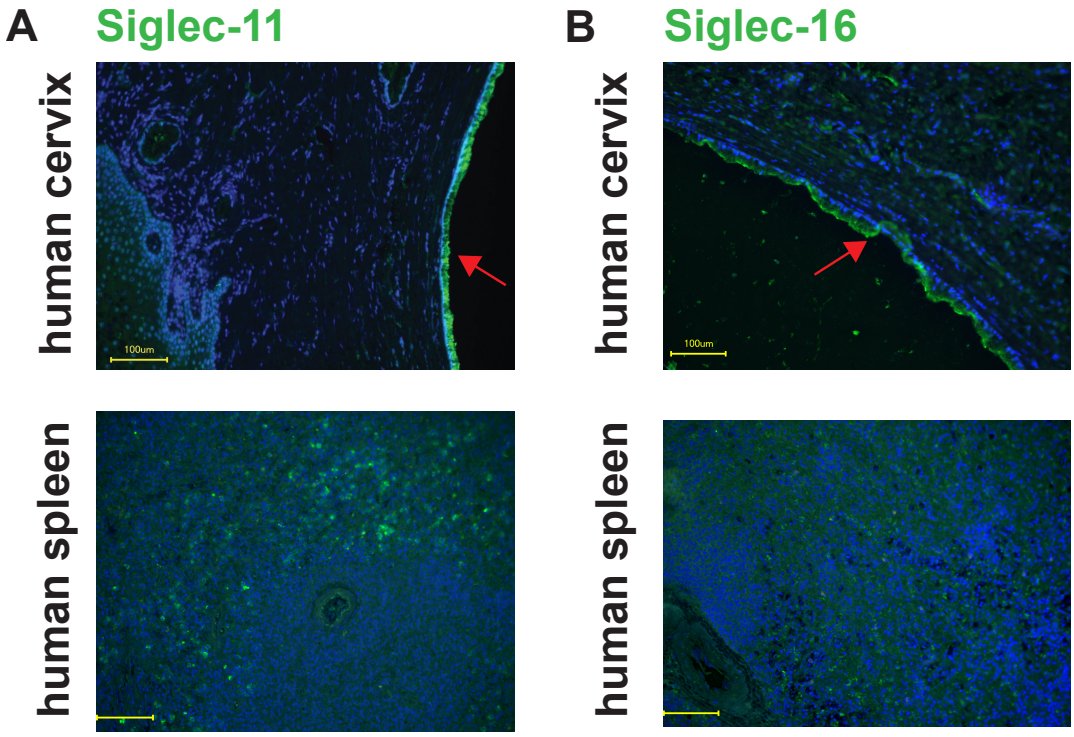


**Figure 5 I Siglec-11 and Siglec-16 are expressed on human cervical epithelium** Immunofluorescent staining of human cervixes and spleen as positive control with (A) anti-Siglec-11 and (B) anti-Siglec-16 antibodies (green). Nuclei are stained blue (DAPI), red arrows indicate columnar cervical epithelium and yellow bar indicates 100 μm.

*Genetic differences in human Siglecs influence burden of N. gonorrhoeae*

Similar to Siglec-16, Siglec-14 is also not expressed in every individual (*Yamanaka et al.*, 2009). The absence of these activating receptors could contribute to

asymptomatic disease and prolonged carriage of gonococci. The rs3865444 polymorphism in Siglec-3 could potentially also contribute, as it leads to differential expression of two Siglec-3 isoforms (*Malik et al.*, 2013). The C allele leads to a higher expression of the major isoform with a V-set sialic acid-binding domain, which can bind to sialylated gonococci. The A allele decreases the expression of the major isoform. The C allele therefore potentially poses a higher risk for dampening the pro-inflammatory response by providing gonococci with more sialic acid binding exploitable inhibitory Siglec-3 receptors.

To test if these genetic variations in human Siglecs are associated with gonococcal infections, we genotyped a human cohort with a high burden of gonorrhea. A cross-sectional study of Namibian pastoralists has shown that over 64% of the tested sexually active adult population is infected with *N. gonorrhoeae* with a higher prevalence in females (72%) compared to males (57%) (*Hazel et al.*, 2014).

We first used a non-parametric approach by performing a chi square test (**Tab. 1**). We found that and found that infected females express the Siglec-16 receptor significantly less frequently with an allele frequency of the SIGLEC16 with 0.155 compared to uninfected women with 0.213. However this approach was not sufficiently constrained to find evidence of association for the other genotypes, we only found trends that showed higher expression of activating Siglec-14 receptor expressed in the uninfected population with an allele frequency of 0.729 compared to 0.693 in the infected population**.** Although not statistically significant, this trend supported our hypothesis that activating Siglec-14 is protective. We saw a similar trend when females and males were analyzed separately. The pathology of the gonococcal infection is very different between males and females and therefore it is important to look at the two populations separately as well. There were no significant differences between the A and C alleles in Siglec-3 among infected and uninfected

persons.

| | | | | | | Allele Frequency | | Chi-square test | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Siglec-5/Siglec-14** | | **wt** | **het** | **null** | **total** | **wt** | **null** | **Chi-square** | **df** | **P value** |
| **all subjects** | **uninfected** | 81 | 48 | 15 | **144** | 0.729 | 0.271 | 1.360 | 2 | 0.507 |
| | **infected** | 129 | 98 | 30 | **257** | 0.693 | 0.307 | | | |
| **female** | | | | | | | | | | |
| | **uninfected** | 30 | 16 | 7 | **53** | 0.717 | 0.283 | 2.748 | 2 | 0.253 |
| | **infected** | 65 | 59 | 13 | **137** | 0.690 | 0.310 | | | |
| **male** | | | | | | | | | | |
| | **uninfected** | 51 | 32 | 8 | **91** | 0.736 | 0.264 | 1.441 | 2 | 0.487 |
| | **infected** | 64 | 39 | 17 | **120** | 0.696 | 0.304 | | | |
| **Siglec-16/Siglec-16P** | | **Siglec-16P** | **het** | **Siglec-16** | **total** | **Siglec-16P** | **Siglec-16** | | | |
| **all subjects** | **uninfected** | 98 | 39 | 6 | **143** | 0.822 | 0.178 | 5.236 | 2 | 0.073 |
| | **infected** | 164 | 74 | 2 | **240** | 0.838 | 0.163 | | | |
| **female** | | | | | | | | | | |
| | **uninfected** | 35 | 15 | 4 | **54** | 0.787 | 0.213 | 6.479 | 2 | 0.039 |
| | **infected** | 92 | 39 | 1 | **132** | 0.845 | 0.155 | | | |
| **male** | | | | | | | | | | |
| | **uninfected** | 63 | 24 | 2 | **89** | 0.843 | 0.157 | 1.163 | 2 | 0.559 |
| | **infected** | 72 | 35 | 1 | **108** | 0.829 | 0.171 | | | |
| **Siglec-3 rs3865444 (C/A)** | | **C** | **het** | **A** | **total** | **C** | **A** | (analysis without A/A) | | |
| **all subjects** | **uninfected** | 140 | 8 | 1 | **149** | 0.966 | 0.034 | 0.868 | 1 | 0.351 |
| | **infected** | 247 | 21 | 1 | **269** | 0.957 | 0.043 | | | |
| **female** | | | | | | | | | | |
| | **uninfected** | 54 | 3 | 0 | **57** | 0.974 | 0.026 | 0.709 | 1 | 0.400 |
| | **infected** | 134 | 13 | 0 | **148** | 0.956 | 0.044 | | | |
| **male** | | | | | | | | | | |
| | **uninfected** | 86 | 5 | 1 | **92** | 0.962 | 0.038 | 0.113 | 1 | 0.737 |
| | **infected** | 113 | 8 | 1 | **122** | 0.959 | 0.041 | | | |

**Table 1 | Siglec genotypes in a Himba cohort with high burden of gonorrhea**.
The Himba cohort with a high burden of gonorrhea infections was genotyped for three Siglec loci. The Siglec-5/Siglec-14 locus has the wildtype allele (wt), which leads to the expression of Siglec-5 and Siglec-14 and the null allele that only expresses Siglec-5. The Siglec-16/Siglec-16P locus has the Siglec-16 allele that expresses the Siglec-16 receptor and the loci of the pseudogene *SIGLEC16P* that cannot express Siglec-16 due to a base pair deletion. The polymorphism rs3865444 influences the expression level of two isoforms of Siglec-3 (CD33) the major form with the V-set domain (sialic acid binding domain) and the minor form without the V-set domain. C leads to an increased expression of the major form and A decreases it. The allele frequency is calculated according to the Hardy-Weinberg equation and Chi-square test was performed.

There is the possibility that the sample size was too low to see a significant difference in the allele frequency in most of the comparisons with this method. Another explanation could be that the effect of Siglec-5/-14 and Siglec-3 (confined to innate immune cells) are less relevant than Siglec-16 that is associated with the cervical epithelium, and/or their net effects do not drive selection of the protective genotype. On the other hand, Siglec-16 is not only expressed on macrophages but also on the cervical epithelium, which is the main site of infection. This additional site of expression could enhance contact with gonococci and thus drive selection for the protective Siglec-16 genotype. It is worth noting that immortalized cervical epithelial

cells also release pro-inflammatory cytokines following stimulation with *N. gonorrhoeae* (*Fichorova et al.*, 2001).

We tried then a parametric approach using logistic regressions, the parameterization helped us achieve sufficient power to make significant observations.

The univariate logistic regression screen of women with the homozygous genotype for Siglec-16 indeed showed a significant protective association with an odds ratio of 0.092 (P=0.034; 95% confidence interval (CI)=0.005-0.636) (**Fig. 6a, Suppl. Tab. 1**). The same analysis showed no significant protection or risk for the different genotypes in males (**Fig. 6b**). In an additive model of homozygous Siglec-14 wt and Siglec-16 wt, Siglec-16 showed again significant protection with an odds ratio of 0.089 (P=0.032; CI =0.004-0.620) and Siglec-14 with 0.713 (P=0.280; CI =0.384-1.318), which was not significant (**Fig. 6c, Suppl. Tab. 1**).

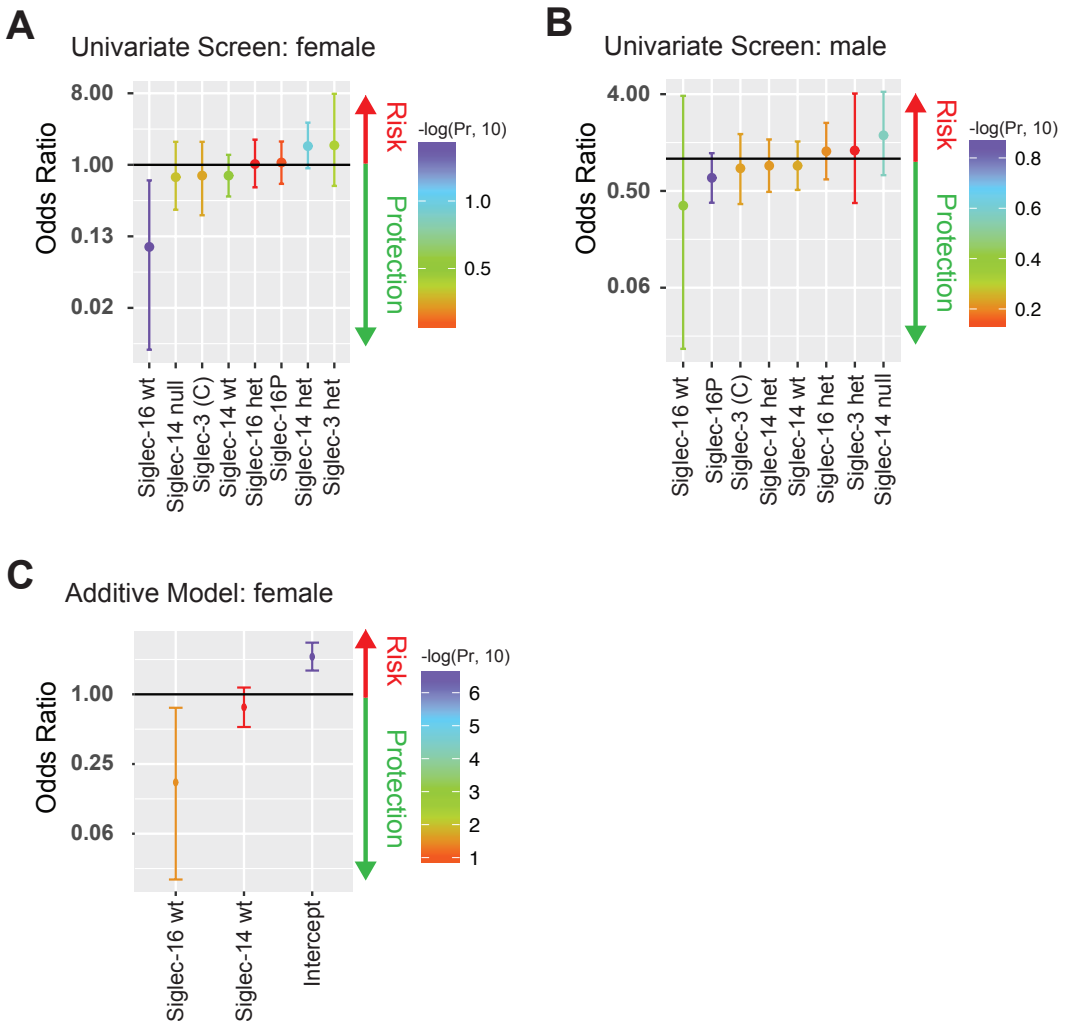


**Figure 6 I Activating Siglecs are protective**
Univariate Screen of females (A) and males (B). Additive Model of females, which are homozygous for SIGLEC16 and Siglec-14 wt (C).

The data presented in this work suggest that interaction of *N. gonorrhoeae* with Siglecs play a role in the innate immune response during infection (**Fig. 7**). Upon binding, the inhibitory receptors Siglec-3, -5, -9 and -11 suppress the pro-inflammatory response, which could contribute to decreased clearance of infection and overall appearance of an asymptomatic infection. The engagement of activating Siglec receptors, like -14 and -16, leads to an activation of the pro-inflammatory signaling cascade. This can contribute to a better clearance of a symptomatic infection. However these activating receptors are only expressed on certain individuals. The presence of these polymorphisms could contribute to different ability of clearing a gonococcal infection among individuals that face a high burden of gonorrhea in the community.

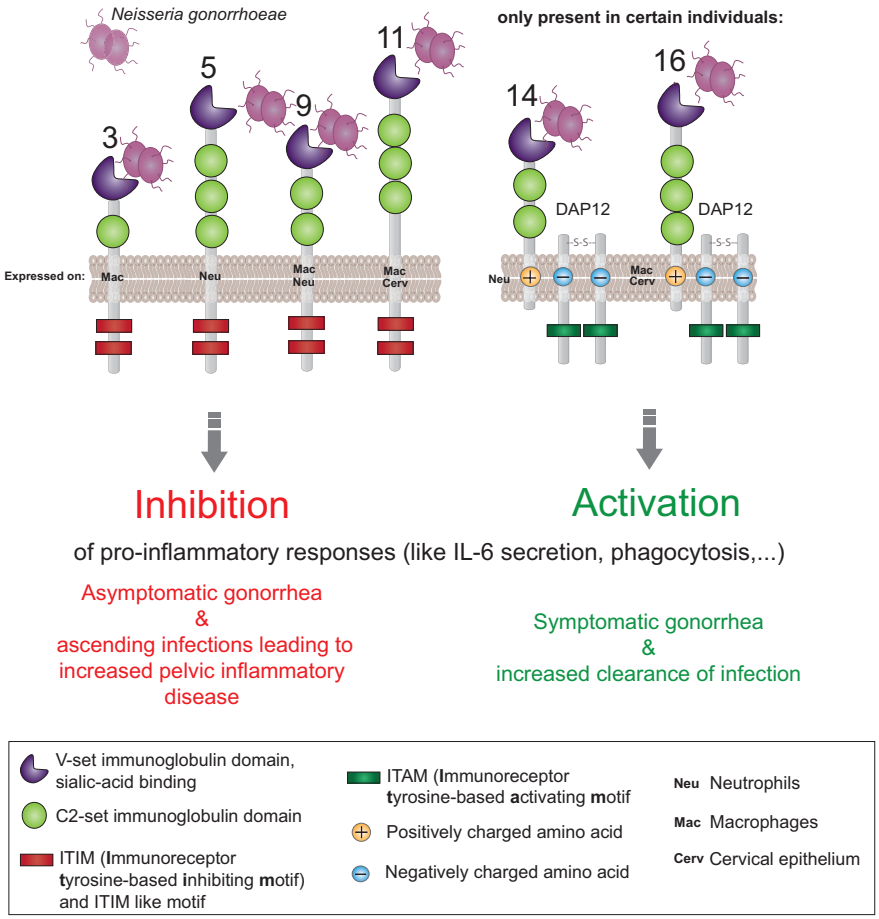


**Figure 7 I Possible mechanism of Siglec gonococci interactions**
Neisseria gonorrhoeae engages human immunoregulatory Siglec receptors. Upon binding, the inhibitory receptors Siglec-3, -5, -9 and -11 suppress the proinflammatory response, which could contribute to decreased clearance of infection and overall appearance of an asymptomatic infection. The engagement of activating Siglec receptors, like -14 and -16, leads to an activation of the pro-inflammatory signaling cascade. This can contribute to a better clearance of a symptomatic infection. However these activating receptors are only expressed on certain individuals. The presence of these polymorphisms could contribute to different ability of clearing a gonococcal infection among individuals, which face a high burden of gonorrhea in the community.

**Materials and Methods**

*Bacteria and cell lines*

*Neisseria gonorrhoeae* F62 was isolated from an uncomplicated infection (*Schneider et al.*, 1982). *Neisseria gonorrhoeae* 15253 was isolated from a disseminated infection (*O'Brien et al.*, 1983). *N. gonorrhoeae* were grown overnight on chocolate agar plates with IsoVitaleX (BD Bioscience) or in GC broth supplemented with IsoVitaleX at 37 ˚C and 5% $CO_2$. When indicated, growth media was supplemented with 30 $\mu$M CMP-Neu5Ac (Nacalai USA, Inc.). Incorporation of Neu5Ac has been confirmed by loss of Erythrina Cristagalli lectin (ECA, Vector Laboratories), which binds to the lactosamine epitope (**Suppl. Fig. 1**). For all binding and infection studies, bacteria were cultivated to an optical density at 600 nm equivalent to 0.4-0.6. THP-1 cells were grown in RPMI-1640 (Gibco) with 10% fetal calf serum (Gemini Bio-Products) at 37 ˚C and 5% $CO_2$.

*Siglec-Fc production*

Siglec-Fcs have been produced as described in (*Padler-Karavani et al.*, 2014). Siglec-Fc vectors were transfected into HEK293A cells in serum-free media with Nutridoma-SP (Roche). Culture supernatant was collected, and Siglec-Fc's were purified on a Sepharose Protein A column. After washing with Tris-buffered saline (20 mM Tris–HCl, 150 mM NaCl, pH 8.0; TBS), the Siglec-Fc's were desialylated by neuraminidase derived from Arthrobacter ureafaciens (Sigma- Aldrich) for 1 h at room temperature. After extensive washing with TBS, the Siglec-Fc's were eluted with 0.1 M glycine–HCl pH 3.0 and concentrated by Amicon centrifugation.

*Bacteria binding assay*

96-well plates were coated with 1 µg/well protein A (Thermo Scientific, Waltham, MA) in 50 mM carbonate buffer pH 9.5 overnight at 4 ˚C. Wells were washed with PBS-T (0.05% Tween-20 in PBS) and blocked with 1% BSA in PBS for 1 hour at room temperature. 1 µg/well Siglec-Fcs were incubated for 2 hours at room temperature. Afterwards, wells were washed with PBS-T. *N. gonorrhoaea* were pelleted, washed with HBSS and then incubated with 0.1% fluorescein isothiocyanate (FITC, Sigma) in PBS for 1 hour at 37 ˚C with rotation. Bacteria were extensively washed with HBSS to remove trace amounts of free FITC and then resuspended in HBSS at an optical density of 1. A volume of 0.1 ml of FITC-labeled

bacteria was added to each well. Plates were centrifuged at 500 g for 10 minutes and incubated for 1 hour at room temperature. After washing to remove unbound bacteria, the residual fluorescent intensity was measured using a SpectraMax M3 (Molecular Devices, Sunnyvale, CA).

*Porin binding assays*

96-well plates were coated with 1 µg/well PorinB.1A, PorinB.1B, purified as mentioned in (*Massari and Wetzler*, 2012) or purified IgA-Br as negative control (*Fong et al.*, 2015) in 50 mM carbonate buffer pH 9.5 overnight at 4 ˚C. Wells were washed with PBS-T (0.05% Tween-20 in PBS) and blocked with 1% BSA in PBS for 1 hour at room temperature. Wells were washed and incubated with 5 *µ*g/mL Siglec-Fc in 1% BSA PBS-T for 2 hours at room temperature. Wells were washed and incubated with goat HRP-conjugated anti-human IgG (1:5000, 109-035-003 Jackson ImmunoResearch Laboratories Inc.) for 1 hour at room temperature. Wells were washed and incubated with TMB substrate (BD OptEIA) and absorbance was measured using a SpectraMax M3 (Molecular Devices, Sunnyvale, CA).

*Cytokine secretion analysis*

THP-1 cells expressing Siglec-5 or Siglec-14 (*Yamanaka et al.*, 2009) were differentiated with 10 ng/mL PMA in a 24 well plate for 24 hours and infected with MOI=1 of *N. gonorrhoeae* F62. Supernatant was collected after 24 hours. IL-6 concentration was measured with ELISA standard kit from BioLegend.

*Phagocytosis assay*

THP1 cells transduced with an expression plasmid for Siglec-5, Siglec-14, or empty vector were differentiated with 12.5 ng/ml of phorbol myristate acetate (PMA) for 24 hours. Next, the cells were washed with sterile culture media, and then exposed to 10 ug/ml of PIB for 10 minutes before the addition of pHrodo Red *S. aureus* BioParticles (prepared as per manufacturer's instructions). After incubation at 37 degrees Celsius for 2 hours, the cells were washed with PBS, and then detached with 5mM EDTA in PBS. The detached cells were centrifuged for 5 minutes at 500 g, and washed with PBS. After another centrifugation, the cells were resuspended in PBS and assayed for phagocytosis of the pHrodo BioParticles by flow cytometry. All samples were performed in triplicate.

*Immunohistochemistry*

Deparaffinized sections were blocked for endogenous peroxidases and endogenous biotin and subjected to heat induced antigen retrieval using pH 9.0 buffer and pressure cooker de-cloaking chamber (Biocare Medical) for 15 minutes at 110 degrees. After the slides cooled to 37 C, the sections were overlaid with primary antibodies at the appropriate dilutions and incubated in a humid chamber overnight at 4 C. Following washing after each incubation, the sections were sequentially overlaid with biotinylated anti-mouse, HRP-Streptavidin, biotinyl tyramide to amplify, either HRP streptavidin again or fluorescently labeled Streptavidin. If HRP streptavidin was the final step, the substrate used for color development was AEC (Vector laboratories, Burlingame CA) and nuclei were counterstained with Mayer's hematoxylin and the slides were aqueous mounted for viewing and photography using a Olympus Magnafire digital photomicrography on an Olympus BH2 light microscope. If fluorescence tags were used, the nuclei were counterstained using Hoechst and coverslipped and viewed with photography using the Keyence BZ9000 with the appropriate filters.

*Genotyping of Siglecs in Namibian cohort*

Genomic DNA was isolated from FDA cards of Namibian cohort (*Hazel et al.*, 2014) with E.Z.N.A. MicroElute Genomic DNA Kit (OMEGA). Genotyping of the Siglec-3 polymorphism rs3865444 was performed as described in (*Schwarz et al.*, 2016). Genotyping of Siglec-5 and Siglec-14 was performed as described in (*Yamanaka et al.*, 2009). Genotyping of Siglec-16 was performed by PCR with primers (Forward: GCATGTCTGATCACCTCAGTTGGGAAAG; Reverse: CCCTGACTCTCCTGTACTGATAAACC) and OneTaq MasterMix (New England Biolabs). Followed by restriction digest with TspRI (New England Biolabs), which cuts a polymorphism in disequlibrium with the SIGLEC16P (*Wang et al.*, 2012).

*Statistical analysis*

Gene Association Analysis: Gene-Disease association was assessed between binary variables representing each genotype (homozygous dominant, heterozygous, homozygous recessive) of the three loci (Siglec-3, Siglec-16, Siglec 5/14), and we used models considering the presence or absence of each genotype.

Models were learned from gender-segregated data to account for the unique mechanisms of virulence across genders. Univariate logistic regressions were constructed to predict the magnitude and significance of association between each genotype and the disease. The univariate models provided assessments of the single genotype contributions to the disease. Multivariate additive and interaction models were constructed using the R::MASS::stepAIC function. Multivariate additive models were constructed using backward selection from the complete additive model including all genotypes. Multivariate interactions models were constructed using bidirectional model selection from the complete additive model. Code is deposited at github.com/bkellman/Gh_genotypes/.

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Chapter 3b:

# Supplementary information: The human-specific pathogen *Neisseria gonorrhoeae* engages immunoregulatory Siglecs in a species-specific manner: Genetic implications for gonorrhea risk

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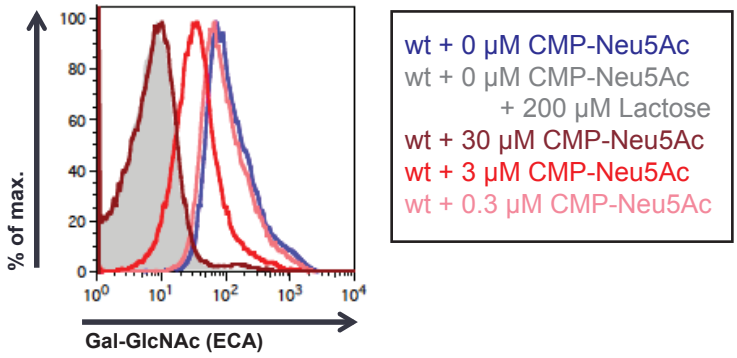
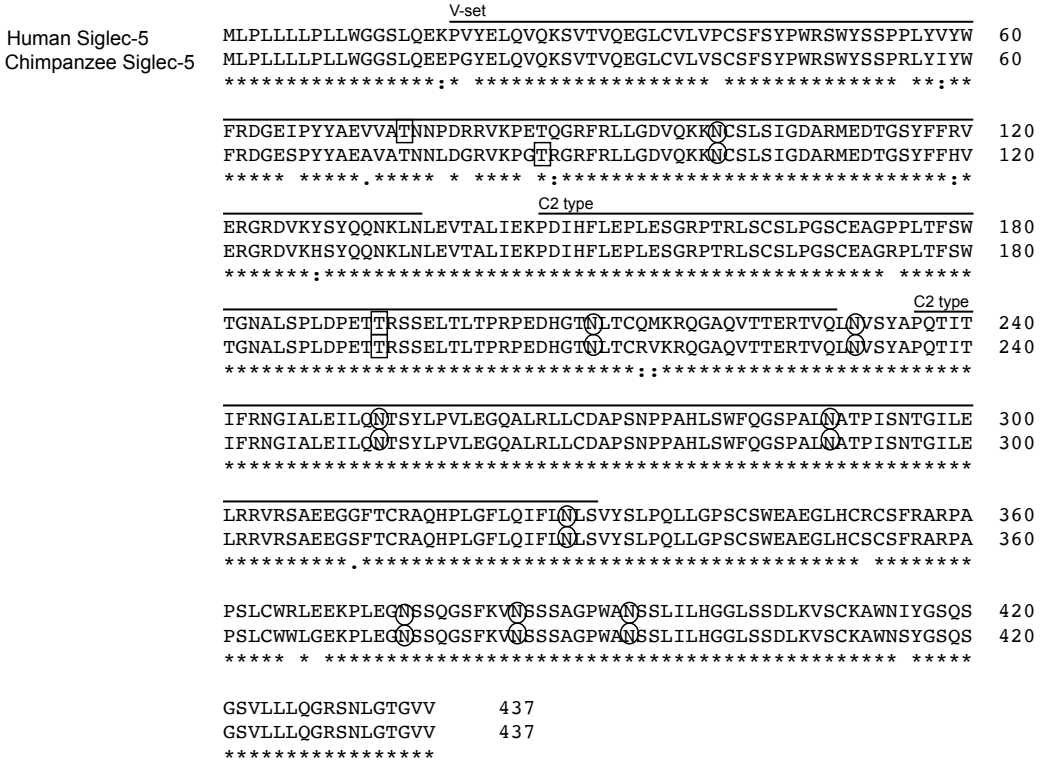



**Supplement Figure 1 I Monitoring Neu5Ac incorporation into F62 LOS with ECA lectin**
The Erythrina Cristagalli lectin (ECA) recognizes the lactosamine epitope and with increasing concentration of supplemented CMP-Neu5Ac the binding to F62 decreases. Free lactose is used as inhibitor of ECA.

```
                                  V-set
Human Siglec-3      MPLLLLLPLLWAGALAMDPNFWLQVQESVTVQEGLCVLVPCTFFHPIPYYDKNSPVHGYW  60
Chimpanzee Siglec-3 MPLLLLLPLLWAGALAMDPKIRLQVQESVTVQEGLCVLVPCTFFHPIPYYDKNSPVHGYW  60
                    ********************:: ************************************

                    FREGAIISRDSPVATNKLDQEVQEETQGRFRLLGDPSRNNCSLSIVDARRRDNGSYFFRM 120
                    FREGPIVSGDSPVATNKPDQEVQEETQGRFRLLGDLSRNNCSLSIVDARRRDNGSYFFRM 120
                    **** *:* ******** ***************** ***********************
                                       C2 type
                    ERGSTKYSYKSPQLSVHVTDLTHRPKILIPGTLEPGHSKNLTCSVSWACEQGTPPIFSWL 180
                    ERGSTKYSYKSPQLSVHVTDLTHRPKILIPGALDPGHSKNLTCSVSWACEQGTPPIFSWL 180
                    ********************************:*:*************************

                    SAAPTSLGPRTTHSSVLIITPRPQDHGTNLTCQVKFAGAGVTTERTIQ       228
                    SAAPTSLGPRTTHSSVLIITPRPQDHGTNLTCQVKFAGAGVTTERTIQ       228
                    **********************************************
```

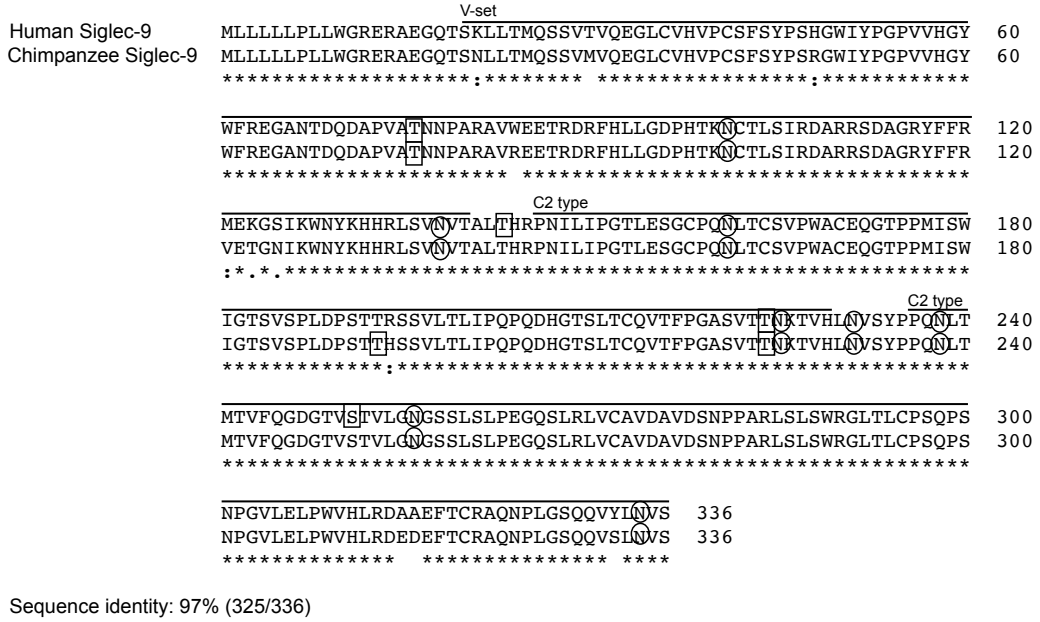
Sequence identity: 96% (218/228)

**Supplement Figure 2 I Alignment of extracellular domain of human and chimpanzee Siglec-3**
The extracellular domains of Siglec-3 are aligned and possible O-glycosylation sites are indicated with a rectangle and N-glycosylation sites with a circle (predicted with NetOGlyc 4.0 Server and NetNGlyc 1.0 Server (*Steentoft et al.*, 2013).




Sequence identity: 97% (426/437)

**Supplement Figure 3 | Alignment of extracellular domain of human and chimpanzee Siglec-5**
The extracellular domains of Siglec-5 are aligned and possible O-glycosylation sites are indicated with a rectangle and N-glycosylation sites with a circle (predicted with NetOGlyc 4.0 Server and NetNGlyc 1.0 Server (*Steentoft et al.*, 2013)).



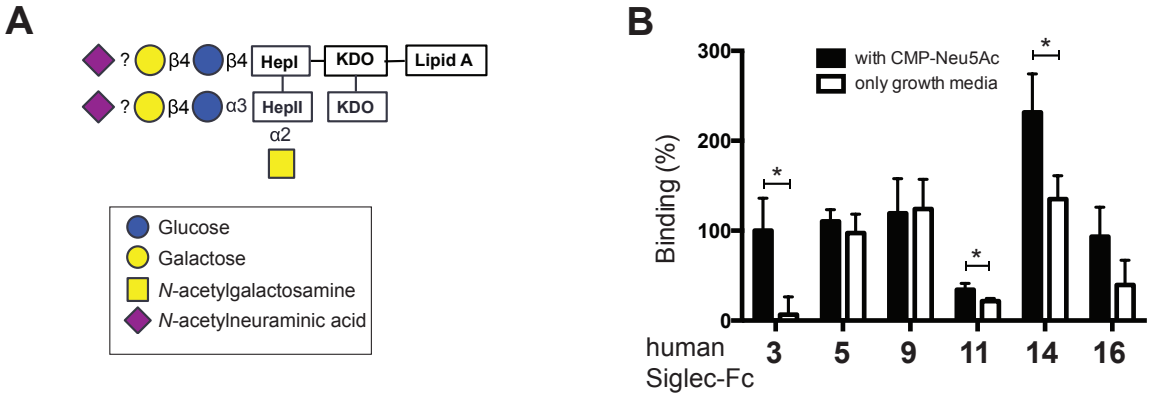

Sequence identity: 97% (325/336)

**Supplement Figure 4 | Alignment of extracellular domain of human and chimpanzee Siglec-9**
The extracellular domains of Siglec-9 are aligned and possible O-glycosylation sites are indicated with a rectangle and N-glycosylation sites with a circle (predicted with NetOGlyc 4.0 Server and NetNGlyc 1.0 Server (*Steentoft et al.*, 2013)).

```
                                  V-set
Human Siglec-14          MLPLLLLPLLWGGSLQEKPVYELQVQKSVTVQEGLCVLVPCSFSYPWRSWYSSPPLYVYW 60
Chimpanzee Siglec-14     MLPLLLLPLLWGGSLQEEPGYELQVQKSVTVQEGLCVLVSCSFSYPWRSWYSSPPLYVYW 60
                         ****************:* ****************** ******************

                         FRDGEIPYYAEVVATNNPDRRVKPETQGRFRLLGDVQKKNCSLSIGDARMEDTGSYFFRV 120
                         FRDGESPYYAEAVATNNLDGRVKPGTRGRFRLLGDVQKKNCSLSIGDARMEDTGSYFFHV 120
                         ***** *****.***** * **** *:*********************************:*

                                                  C2 type
                         ERGRDVKYSYQQNKLNLEVTALIEKPDIHFLEPLESGRPTRLSCSLPGSCEAGPPLTFSW 180
                         ERGRDVKHSYQQNKLNLEVTALIEKPDIHFLEPLESGHPTRLSCSLPGSCEAGRPLTFSW 180
                         *******:****************************:*************** ******

                                                                        C2 type
                         TGNALSPLDPETTRSSELTLTPRPEDHGTNLTCQVKRQGAQVTTERTVQLNVSYAPQNLA 240
                         TGNALSPLDPETTRSSELTLTPRPEDHGTNLTCQVKRQGAQVTTERTVQLNVSYAPQNLA 240
                         ************************************************************

                         ISIFFRNGTGTALRILSNGMSVPIQEGQSLFLACTVDSNPPASLSWFREGKALNPSQTSM 300
                         ISIFFRNGTGTALRILSNGMSVPIQEGQSLFLACTVDSNPPASLSWFREGKALNPSQTSM 300
                         ************************************************************

                         SGTLELPNIGAREGGEFTCRVQHPLGSQHLS      331
                         SGTLELPNIGAREGGEFTCRVQHPLGSQHLS      331
                         ********************************
```

Sequence identity: 96% (318/331)

**Supplement Figure 5 I Alignment of extracellular domain of human and chimpanzee Siglec-14**
The extracellular domains of Siglec-14 are aligned and possible O-glycosylation sites are indicated with a rectangle and N-glycosylation sites with a circle (predicted with NetOGlyc 4.0 Server and NetNGlyc 1.0 Server (*Steentoft et al.*, 2013)).




**Supplement Figure 6 I Siglec interactions with gonococci are sialic acid dependent and independent** (A) The lipooligosaccharid of N. gonorrhoaea 15253 can be sialylated (B) Binding FITC-labeled 15253 grown with and without CMP-Neu5Ac to immobilized human Siglec-Fcs. Binding is normalized to human Siglec-3 Fc. Data are represented as mean ± SEM, n=3.

| Univeriate Screen | | | Odds Ratio | P-value | 95% CI |
|---|---|---|---|---|---|
| | **female** | | | | |
| | | **Siglec-16 wt** | 0.092 | 0.034 | (0.005-0.636) |
| | | **Siglec-14 null** | 0.700 | 0.472 | (0.271-1.951) |
| | | **Siglec-3 (C)** | 0.730 | 0.555 | (0.231-1.951) |
| | | **Siglec-14 wt** | 0.731 | 0.308 | (0.398-1.337)) |
| | | **Siglec-16 het** | 1.021 | 0.952 | (0.519-2.081) |
| | | **Siglec-16P** | 1.069 | 0.831 | (0.575-1.969) |
| | | **Siglec-14 het** | 1.724 | 0.106 | (0.904-3.409) |
| | | **Siglec-3 het** | 1.758 | 0.392 | (0.541-7.881) |
| | **male** | | | | |
| | | **Siglec-16 wt** | 0.365 | 0.413 | (0.017-3.859) |
| | | **Siglec-16P** | 0.664 | 0.131 | (0.388-1.126) |
| | | **Siglec-3 (C)** | 0.813 | 0.588 | (0.377-1.698) |
| | | **Siglec-14 het** | 0.860 | 0.598 | (0.490-1.513) |
| | | **Siglec-14 wt** | 0.861 | 0.571 | (0.511-1.447) |
| | | **Siglec-16 het** | 1.168 | 0.615 | (0.017-3.859) |
| | | **Siglec-3 het** | 1.194 | 0.763 | (0.386-4.058) |
| | | **Siglec-14 null** | 1.653 | 0.265 | (0.702-4.208) |
| **Additive Model** | | | | | |
| | **female** | | | | |
| | | **Siglec-16 wt** | 0.089 | 0.032 | (0.004-0.620) |
| | | **Siglec-14 wt** | 0.713 | 0.280 | (0.384-1.318) |
| | | **Intercept** | 3.201 | 1.39E-07 | (2.106-5.025) |
| | **male** | | | | |
| | | **Intercept** | 1.722 | 0.009 | (1.149-2.620) |
| | | **Siglec-16P** | 0.664 | 0.131 | (0.388-1.126) |

**Supplementary Table 1 l** Values of univariate Screen and additive Model for females and males.

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Chapter 4:

# Utilizing CMP-sialic acid analogs to unravel Neisseria gonorrhoeae lipooligosaccharide-mediated complement resistance and design novel therapeutics

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Contribution: HPLC analysis of nonulosonate sugar incorporation into lipooligosaccharide

**Abstract**

*Neisseria gonorrhoeae* deploys a novel immune evasion strategy wherein the lacto-N-neotetraose (LNnT) structure of lipooligosaccharide (LOS) is capped by the bacterial sialyltransferase, using host cytidine-5'-monophosphate (CMP)-activated forms of the nine-carbon nonulosonate (NulO) sugar *N*-acetyl-neuraminic acid (Neu5Ac), a sialic acid (Sia) abundant in humans. This allows evasion of complement-mediated killing by recruiting factor H (FH), an inhibitor of the alternative complement pathway, and by limiting classical pathway activation ("serum-resistance"). We utilized CMP salts of six additional natural or synthetic NulOs, Neu5Gc, Neu5Gc8Me, Neu5Ac9Ac, Neu5Ac9Az, legionaminic acid (Leg5Ac7Ac) and pseudaminic acid (Pse5Ac7Ac), to define structural requirements of Sia-mediated serum-resistance. While all NulOs except Pse5Ac7Ac were incorporated into the LNnT-LOS, only Neu5Gc incorporation yielded high-level serum-resistance and FH binding that was comparable to Neu5Ac, whereas Neu5Ac9Az and Leg5Ac7Ac incorporation left bacteria fully serum-sensitive and did not enhance FH binding. Neu5Ac9Ac and Neu5Gc8Me rendered bacteria resistant only to low serum concentrations. While serum-resistance mediated by Neu5Ac was associated with classical pathway inhibition (decreased IgG binding and C4 deposition), Leg5Ac7Ac and Neu5Ac9Az incorporation did not inhibit the classical pathway. Remarkably, CMP-Neu5Ac9Az and CMP-Leg5Ac7Ac each prevented serum-resistance despite a 100-fold molar excess of CMP-Neu5Ac in growth media. The concomitant presence of Leg5Ac7Ac and Neu5Ac on LOS resulted in uninhibited classical pathway activation. Surprisingly, despite near-maximal FH binding in this instance, the alternative pathway was not regulated and factor Bb remained associated with bacteria. Intravaginal administration of CMP-Leg5Ac7Ac to BALB/c mice infected with gonorrhea (including a multidrug-resistant isolate) reduced clearance times and infection burden. Bacteria recovered from CMP-Leg5Ac7Ac-treated mice were sensitive to human complement *ex vivo*, simulating *in vitro* findings. These data reveal critical roles for the Sia exocyclic side-chain in gonococcal serum-resistance. Such CMP-NulO analogs may provide a novel therapeutic strategy against the global threat of multidrug-resistant gonorrhea.

**Author summary**

*Neisseria gonorrhoeae*, the causative agent of the sexually transmitted infection, gonorrhea, has developed widespread resistance to almost every conventional antibiotic currently in clinical use. Novel therapeutics are urgently needed against this pathogen. Gonococci have the capacity to scavenge CMP-*N*-acetyl-neuraminic acid (CMP-Neu5Ac, a CMP-activated 9-carbon sugar that is a member of the 'sialic acid family') from the host to 'cap' its lipooligosaccharide with Neu5Ac, which renders gonococci resistant to complement, a key arm of innate immune defenses. Here, we show that gonococci also utilize derivatives (or analogs) of CMP-Neu5Ac, which not only fail to render the bacteria resistant to complement, but also prevent complement inhibition mediated by the 'physiologic' human sialic acid donor, CMP-Neu5Ac. When administered intravaginally to mice, a representative analog significantly shortened the duration and burden of gonococcal infection. Thus, CMP-sialic acid analogs may represent promising preventive or therapeutic agents against multidrug-resistant gonorrhea that poses a global threat to public health.

**Introduction**

Sialic acids (Sias) are a family of 9-carbon sugars (nonoses, or nonulosonates (NulOs)) expressed in the tissues of every vertebrate and some "higher" invertebrates (*Chen and Varki*, 2010). Sias serve a wide variety of biological roles, including modulation of several aspects of immune function (*Varki and Gagneux*, 2012). For example, cell surface-associated Sia regulates the immune system by inhibiting complement activation. Sheep erythrocytes are resistant to lysis by the human alternative pathway because surface Sias increase the affinity of factor H (FH; inhibitor of the alternative complement pathway) for C3b, that is deposited on these or other host cells or microbial surfaces upon activation of complement (*Fearon*, 1978). Recent work has shown that FH C-terminal domains 19 and 20 bound simultaneously to C3b and glycosaminoglycans or Sias on host cells, which served to inhibit the alternative pathway (*Kajander et al.*, 2011). This was further confirmed by structural data that suggest a trimolecular complex of the two C-terminal FH domains, Sia and C3b (*Blaum et al.*, 2015). Neuraminidase treatment of sheep erythrocytes removes cell surface Sias and reduces the affinity of FH for C3b, which permits complement activation and promotes hemolysis. Typically, FH binds

vertebrate cell surfaces via Sias to allow preferential protection of host cells (i.e., reduce complement-mediated damage).

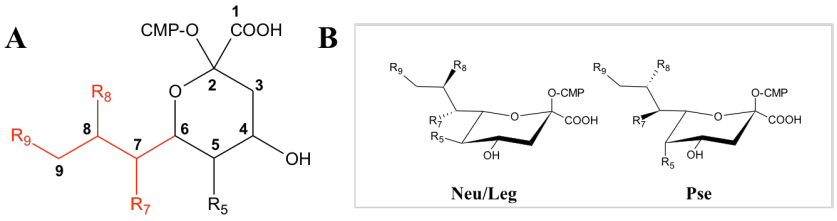
Many microbes express Sias and other unique microbial NulOs (e.g., legionaminic (Leg) and pseudaminic (Pse) acid) on their surfaces that contribute to pathogenesis in numerous ways including subverting complement activation, promoting biofilm formation and facilitating colonization (*Severi et al.*, 2007). Some pathogens such as *Neisseria gonorrhoeae (Ng), Haemophilus influenzae, Histophilus somni* (*Haemophilus somnus*) and serogroup A *N. meningitidis* lack the ability to synthesize Sias, but scavenge these molecules (such as Neu5Ac or Neu5Gc, or the cytidine-monophospho (CMP)-activated form CMP-Neu5Ac) from the host. Other pathogens, for example, *Escherichia coli* K1, *Streptococcus agalactiae*, *N. meningitidis*, groups B, C, W and Y*, Campylobacter jejuni* and certain Leptospira, can synthesize their own nonulosonic acids such as Neu5Ac, Leg5Ac7Ac or Pse5Ac7Ac *de novo*. Sialylation of gonococcal lacto-N-neotetraose (LNnT; Galβ1-4GlcNAcβ1-3Gal-Glcβ1-4HepI) lipooligosaccharide (LOS) using CMP-Neu5Ac enhances resistance of *Ng* to complement-dependent killing by decreasing binding of IgG against select bacterial targets such as porin B (PorB) protein (*Elkins et al.*, 1992), which attenuates the classical pathway. LNnT LOS sialylation with Neu5Ac also enhances FH binding, which results in inhibition of the alternative pathway (*Ram et al.*, 1998).

The purpose of this study was to use CMP-NulOs to define the structural basis of Neu5Ac-mediated complement inhibition by gonococci. CMP-NulO analogs that serve as substrates for gonococcal LOS sialyltransferase (Lst) and result in NulO modified LOS, may prevent Neu5Ac-mediated serum resistance. This could translate into a novel therapeutic approach to combat infections caused by *Ng*, a microorganism that has developed resistance to almost every conventional antibiotic.

## Results

*Substrate specificity of gonococcal lipooligosaccharide sialyltransferase*

Humans, unlike other old-world primates, lack a functional CMP-Neu5Ac hydroxylase enzyme (CMAH) and cannot convert CMP-Neu5Ac to the C5 glycolyl (Gc) derivative, CMP-Neu5Gc. In addition to CMP-Neu5Gc (relevant for the mouse model of gonorrhea), we also included other analogs with additional structural variations at carbons 7, 8 and 9, the exocyclic moiety of NulOs. **Fig. 1** illustrates the CMP-NulOs selected and prepared for this study. In summary, the analogs synthesized include two C9 modified CMP-Neu5Ac analogs (CMP-Neu5Ac9Ac, CMP-Neu5Ac9Az), a C8 modified CMP-Neu5Gc analog (CMP-Neu5Gc8Me), and two C7/C9 modified CMP-Neu5Ac analogs (CMP-Leg5Ac7Ac, CMP-Pse5Ac7Ac) were synthesized. See Methods and Materials and also Supplemental **Tables S1** and **S2** and Supplemental **Figure S1** for further details of synthesis/characterization. All of the CMP-NulO analogs except CMP-Pse5Ac7Ac exhibit the same absolute configuration as CMP-Neu5Ac, making them candidate substrates for gonococcal LOS sialyltransferase (Lst).




| CMP-NulO | R5 | R7 | R8 | R9 |
|---|---|---|---|---|
| CMP-Neu5Ac | NH-Acetyl | OH | OH | OH |
| CMP-Neu5Ac9Ac | NH-Acetyl | OH | OH | O-Acetyl |
| CMP-Neu5Ac9Az | NH-Acetyl | OH | OH | $N_3$ |
| | | | | |
| CMP-Neu5Gc | NH-Glycolyl | OH | OH | OH |
| CMP-Neu5Gc8Me | NH-Glycolyl | OH | O-Methyl | OH |
| | | | | |
| CMP-Leg5Ac7Ac | NH-Acetyl | NH-Acetyl | OH | H |
| | | | | |
| CMP-Pse5Ac7Ac | NH-Acetyl | NH-Acetyl | OH | H |

**Figure 1 I** Structures of the CMP-nonulosonate (CMP-NulO) analogs used in this study. (A) General chemical structure, and (B) $^2C_5$ chair chemical structure of CMP-nonulosonates used. All NulO sugars except for pseudaminic acid (Pse5Ac7Ac) have the same stereochemistry (D-*glycero*-D-*galacto* configuration), where Pse has an L-*glycero*-L-*manno* configuration, differing stereochemically at carbons 5, 7 and 8. For reference, the nine carbon atoms of the NulOs are numbered in (A), where the NulO exocyclic moiety is highlighted in red.

Gonococcal Lst adds a terminal Neu5Ac residue to LNnT LOS when CMP-Neu5Ac is supplied exogenously (gonococci are unable to synthesize CMP-Neu5Ac) (*Mandrell et al.*, 1990). To define the substrate specificity of gonococcal Lst, we used an isogenic LOS glycosyltransferase D (*lgtD*) deletion mutant of gonococcal strain F62, called *Ng* F62 ΔlgtD in subsequent *in vitro* experiments. *LgtD* is a phase-variable gene product that adds a terminal GalNAc residue to HepI LNnT (*Yang and Gotschlich*, 1996); 'capping' LNnT with GalNAc will prevent LOS sialylation. Thus, deleting *lgtD* permits more homogenous expression of LNnT and uniform sialylation. *Ng* F62 ΔlgtD was grown in media alone (unsialylated) or in media containing either CMP-Neu5Ac or one of the other six CMP-NulOs (listed in the **Table 1** and **Fig. 1**), each at a concentration of ~30 $\mu$M (20 $\mu$g/ml). Following incubation for 2 h at 37 °C, bacterial LOS was examined by western blotting using monoclonal antibody (mAb) 3F11, which binds to the terminal lactosamine residue of LNnT; any extension beyond the terminal Gal (for example with a NulO) will abrogate mAb 3F11 binding.

| NulO | Incorporation into LOS | Serum resistance | FH binding | IgG binding | C3 / C4 deposition |
|---|---|---|---|---|---|
| **None (unsialylated)** | − | none | − | +++ | +++ |
| **Neu5Ac** | Yes | high [A] | +++ | + / − | + / − |
| **Neu5Gc** | Yes | high | +++ | ND [B] | ND |
| **Neu5Ac9Ac** | Yes | low [C] | + | + | ++ |
| **Neu5Ac9Az** | Yes | none | − | ++ | +++ |
| **Neu5Gc8Me** | Yes | low [C] | − | + | ++ |
| **Leg5Ac7Ac** | Yes | none | − | +++ | +++ |
| **Pse5Ac7Ac** | No | none | − | ND | ND |

**Table 1 I** Summary of nonulosonate (NulO) incorporation by *N. gonorrhoeae* lipooligosaccharide and key functional consequences
[A] >100% survival in 10% serum
[B] ND, not done
[C] >100% survival in 3.3% serum and <10% survival in 10% serum

As shown in **Fig. 2A**, growth in media containing CMP salts of Neu5Ac, Neu5Gc, Neu5Ac9Ac, Neu5Ac9Az, Neu5Gc8Me and Leg5Ac7Ac resulted in decreased binding of mAb 3F11. This suggests that these CMP-NulOs served as substrates for gonococcal Lst in the context of live bacteria and the respective NulOs are incorporated into LNnT. Only Pse5Ac7Ac was not incorporated into LNnT LOS. CMP-Pse5Ac7Ac, differs from the other CMP-NulOs stereochemically at C5, C7 and C8, and was not anticipated to be utilized by gonococcal Lst. Consistent with decreased mAb 3F11 binding and addition of a NulO residue, silver staining of LOS showed the appearance of a second, slower migrating band in the 6 inside lanes (**Fig. 2A**). Whole cell ELISA with mAb 3F11 confirmed results of western blotting

(**Fig. 2B**). Direct measurement of NulO incorporation into wild-type *Ng* F62 was shown for Neu5Gc using chicken polyclonal IgY Ab that specifically recognizes Neu5Gc (**Fig. 2C**). This method directly demonstrates the presence of Neu5Gc on the bacterial surface. Finally, mass spectrometric analysis of LOS from bacteria grown in CMP-NulOs confirmed addition of the respective NulO onto LOS (Supplemental **Table S3**).

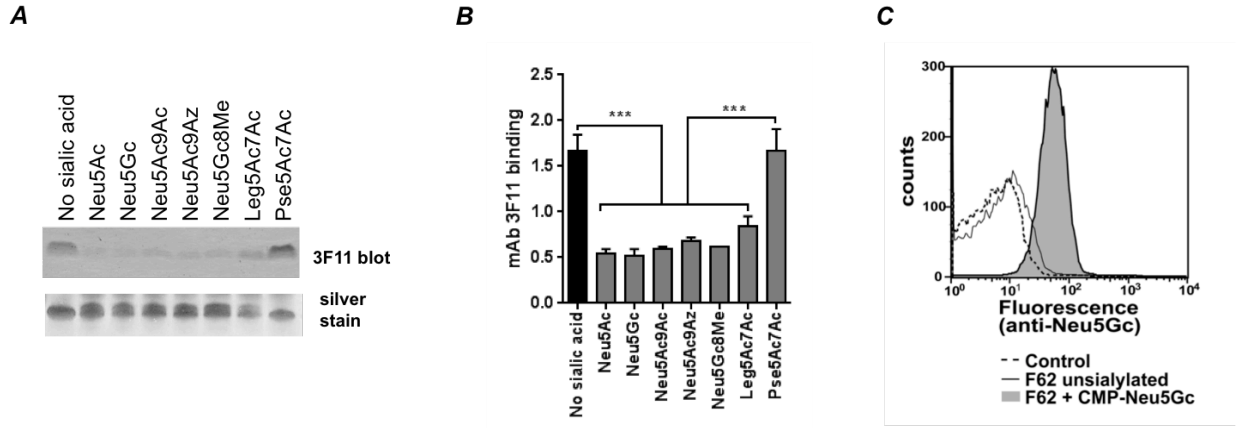



**Figure 2 I** Substrate specificity of gonococcal LOS sialyltransferase (Lst). (A) *N. gonorrhoeae (Ng)* strain F62 ΔlgtD was grown in gonococcal media alone ('No sialic acid') or in media supplemented with CMP salts of the indicated sialic acid (Sia), each at a concentration of 20 μg/ml (30 μM), for 2 h at 37 ºC. Bacteria were washed, pellets treated with protease K, lysed in 4× LDS buffer and lysates were separated on 12% Bis-Tris gels using MES running buffer. LOS was transferred to a PVDF membrane and probed with monoclonal antibody (mAb) 3F11 that recognizes the lacto-*N*-neotetraose only in the non-sialylated state; the addition of a Sia residue abrogates mAb 3F11 binding (upper panel). LOS was also visualized by silver staining following electrophoresis on 16.5% tricine gels (lower panel). (B) Incorporation of NulO by LNnT LOS was assessed using whole cell ELISA with mAb 3F11, *** P<0.001. (C) Wild-type *Ng* F62 was grown with (shaded) or without (solid line) 30 μM CMP-Neu5Gc for 3 hours and analyzed by flow cytometry using a polyclonal Neu5Gc-specific chicken IgY Ab followed by a FITC conjugated donkey anti-chicken IgY secondary Ab. As a negative control, wild-type *Ng* F62 was grown in CMP-Neu5Gc as above and incubated with the secondary Ab only (dashed line).

*Serum resistance mediated by incorporation of NulOs*

The addition of a terminal Neu5Ac residue to the LNnT LOS of *Ng,* as occurs *in vivo* or following the addition of CMP-Neu5Ac to growth media, results in resistance to complement-dependent killing (*Smith et al.*, 1992). We next determined the effects of incorporation of the five structural analogs of Neu5Ac on the ability of *Ng* F62 ΔlgtD ability to resist complement-dependent killing by normal human serum at concentrations of 10%, 6.7% or 3.3%. Bacteria were grown either in media alone, or media supplemented with 30 μM (~20 μg/ml) of each of the CMP-NulOs. As shown in **Fig. 3**, only CMP-Neu5Ac (serum-resistant control) and CMP-Neu5Gc conferred

full (>100%) survival at serum concentrations of 10%. Neu5Ac9Ac and Neu5Gc8Me incorporation conferred >100% survival only in 3.3% serum, but did not protect bacteria (<10% survival) when serum concentrations were raised to 6.7%. The addition of Neu5Ac9Az and Leg5Ac7Ac to LOS did not increase bacterial survival at any serum concentration tested. As expected, Pse5Ac7Ac, which does not incorporate into LOS, did not affect serum resistance.

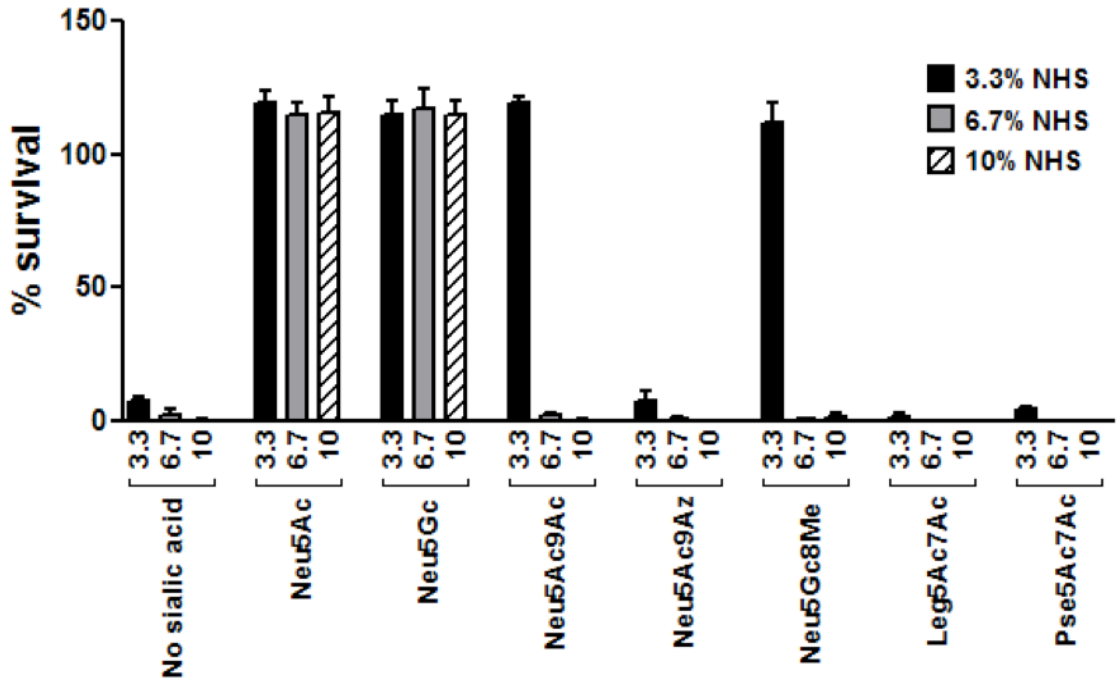



**Figure 3 I** Select sialic acid (Sia) analogs enhance gonococcal serum resistance. *N. gonorrhoeae* (*Ng)* F62 ΔlgtD was grown in media alone (no CMP-Sia added), or media that contained 20 μg/ml (~30 μM) of each of the indicated CMP-Sias (NulOs). Resistance of bacteria to complement-dependent killing in the presence of 3.3%, 6.7% or 10% normal human serum (NHS) was measured in serum bactericidal assays. The mean (SD) of two independent experiments is shown. A survival greater than 100% indicates growth of bacteria.

*FH binding and complement activation mediated by incorporation of NulOs*

A schematic of the complement cascade is provided in Supplemental **Figure S2**. The addition of a terminal Neu5Ac residue to LNnT LOS enhances binding of the alternative pathway inhibitor, FH, and this contributes to the ability of sialylated gonococci to resist killing by complement (*Ram et al.*, 1998). We next examined FH binding to *Ng* F62 ΔlgtD grown in the presence of each of the CMP-NulOs (20 μg/ml each) (**Fig. 4**; see Supplemental **Figure S3** for representative histograms). Maximal FH binding was seen with Neu5Ac and Neu5Gc, a modest level of FH binding was seen with Neu5Ac9Ac; the remaining NulOs did not enhance FH binding significantly above levels seen with the unsialylated strain. Further, modification of LOS with

Neu5Ac or Neu5Gc yielded similar amounts of FH binding, even with low CMP-Sia concentrations ranging from 0.5 to 4 μg/ml (~0.75 - ~4 μM), with near maximal FH binding observed at the lowest concentration tested (**Fig. 4B**).

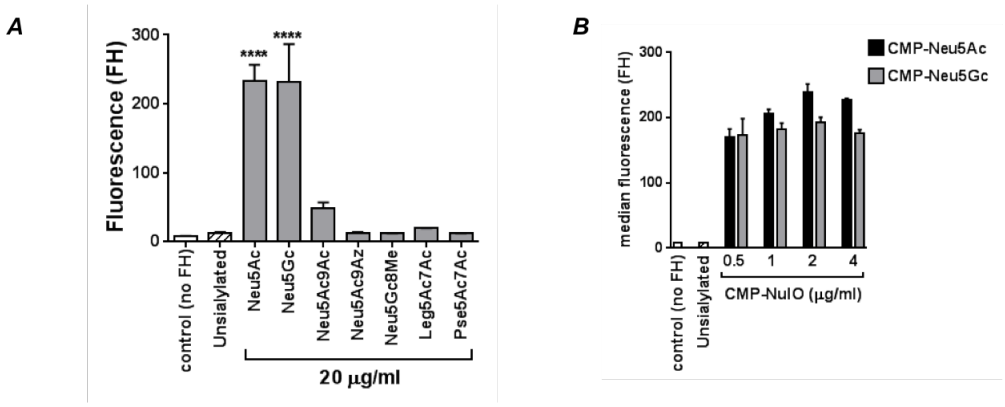



**Figure 4** | Factor H (FH) binding when sialic acid (Sia) analogs (NulOs) were substituted into *N. gonorrhoeae* (*Ng)* lipooligosaccharide (LOS). (A) *Ng* F62 ΔlgtD was grown for 2 h in media alone, or media supplemented with 20 μg/ml (~30 μM) of each of the indicated CMP-sialic acids (NulOs). Binding of bacteria to FH (10 μg/ml) was measured by flow cytometry using anti-FH mAb 90X. The control (open bar) indicates reaction mixtures that lacked FH. The median fluorescence was recorded for each reaction and the Y-axis shows the mean fluorescence (SD) of FH binding of 2 independent experiments. The mean (SD) of 2 independent repeat experiments is shown. (B) FH binding to *Ng* F62 ΔlgtD grown in CMP-Neu5Ac and CMP-Neu5Gc at concentrations ranging from 0.5 μg/ml to 4 μg/ml. Bacteria were incubated with FH (1 μg/ml) and bound FH was detected as indicated above.

We next examined binding of *Ng*-specific IgG and IgM present in normal human serum (NHS), and deposition of complement components C3, C4 and factor B (FB) on *Ng* F62 ΔlgtD grown in the presence of the following representative CMP-NulOs: Neu5Ac represented a NulO that bound high levels of FH and conferred high level serum resistance; Neu5Gc8Me did not bind FH and conferred low level serum resistance; Neu5Ac9Ac bound modest amounts of FH and conferred low levels of serum resistance and Neu5Ac9Az and Leg5Ac7Ac neither bound FH nor conferred serum resistance. *Ng* F62 ΔlgtD with an unmodified (unsialylated) LNnT LOS was used as the control serum sensitive strain. Experiments were carried out using serum concentrations of 3.3% and 10%; the 3.3% serum concentration discriminated serum-killing of the Neu5Ac9Az phenotype from serum–resistance of the Neu5Gc8Me and Neu5Ac9Ac phenotypes.

Consistent with previous studies (*Elkins et al.*, 1992; *Ram et al.*, 1998; *McQuillen et al.*, 1999), modification of LOS with Neu5Ac decreased IgG binding and resulted in maximal inhibition of complement (lowest C3, C4 and FB deposition) at both serum concentrations tested, while high levels of complement activation products were deposited on *Ng* F62 ΔlgtD (unsialylated) (**Fig. 5**).

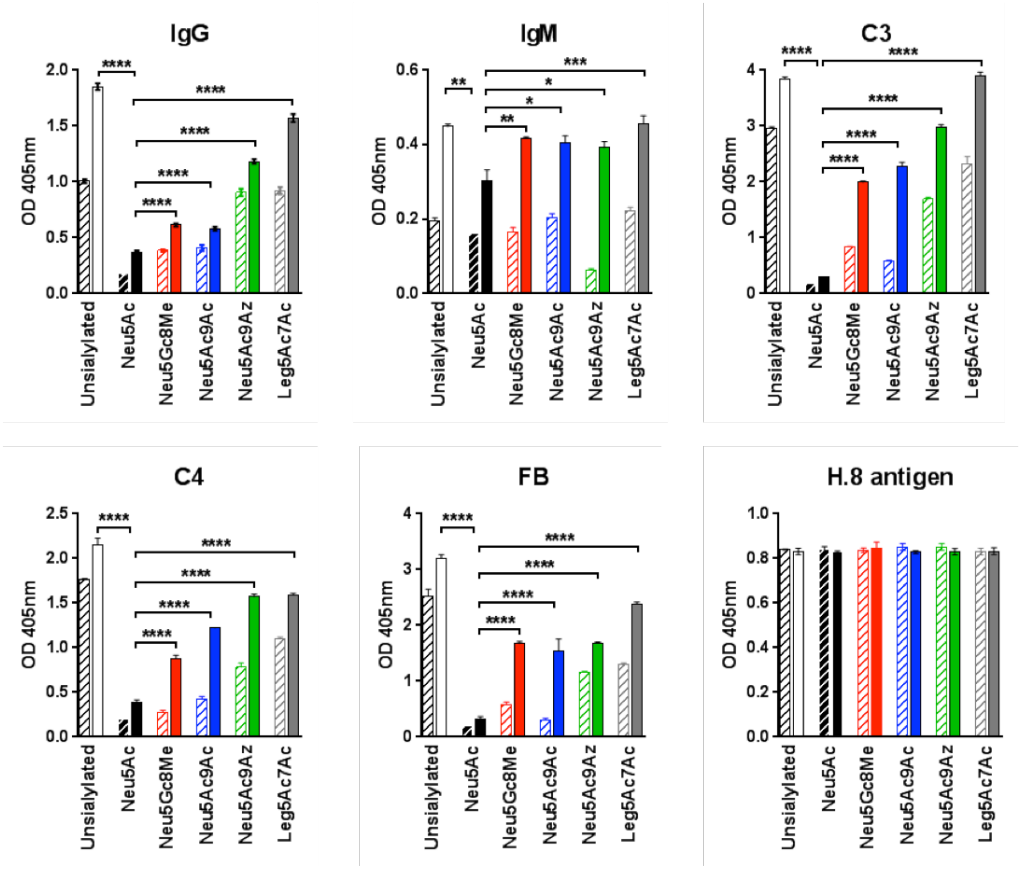



**Figure 5** | IgG, IgM, and complement components C3, C4 and FB deposition on *N. gonorrhoeae* (*Ng)* F62 that have incorporated each indicated sialic acid analog into lipooligosaccharide (LOS). *Ng* F62 ΔlgtD was grown in media alone (open bars), or media supplemented with 20 $\mu$g/ml (~30 $\mu$M) of each of the indicated CMP-Sias (NulOs). Bacteria were incubated with either 3.3% or 10% normal human serum (NHS) at 37 ºC for 10 min and IgG and IgM binding, and C3, C4 and factor B (FB) deposited on bacteria were measured by whole cell ELISA. Data with 3.3% and 10% NHS is shown using hatched and solid bars, respectively. mAb 2-8C-4-1 [62] that detects the Neisserial lipoprotein H.8 was similar across all groups and confirmed similar capture of bacteria in all wells (bottom right graph). Measurement of gonococcal H.8 lipoprotein antigen was performed to assess similarity of bacterial capture across microtiter wells. The mean (SD) of three observations is shown. Significance of differences in Ig or complement component binding/deposition using each analog vs, Neu5Ac is indicated for results that used 10% NHS only (for simplicity): *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 (ANOVA). Controls with bacteria alone yielded $OD_{405nm}$ readings <0.1 (negative).

LOS substitution with Neu5Ac significantly decreased IgG and IgM binding compared to all other CMP-NulOs, which could at least in part, contribute to decreased C4 deposition. While LOS modification with Neu5Ac9Ac and Neu5Gc8Me (both resistant only to 3.3%, but not 10% serum) yielded low levels of C3, C4 and FB deposition in 3.3% serum, only a modest decrease in deposition of these components was noted in 10% serum when compared with *Ng* F62 ΔlgtD (unsialylated) that was not likely to be sufficient to subvert killing by complement. Neu5Gc8Me and Neu5Ac9Ac substitution also reduced IgG binding, but not to the extent seen with Neu5Ac. The two analogs that left *Ng* F62 ΔlgtD fully serum

sensitive, Neu5Ac9Az and Leg5Ac7Ac, yielded the highest levels of Ig binding and complement deposition among the analogs tested. A summary of CMP-NulOs utilized by Lst, Ab and complement component binding, and serum resistance associated with each of the tested CMP-NulOs is found in **Table 1**.

*Select NulO derivatives inhibit Neu5Ac-mediated serum resistance*

We next asked whether the two NulOs, Neu5Ac9Az and Leg5Ac7Ac, that were incorporated into gonococcal LOS but did not enhance serum resistance, could prevent the ability of CMP-Neu5Ac to enhance serum resistance of *N. gonorrhoeae*. These CMP-activated analogs were added at concentrations of 20, 2 or 0.2 μg/ml to growth media 15 min after the addition of CMP-Neu5Ac (20 μg/ml) to *Ng* F62 ΔlgtD (**Fig. 6A**), and bacteria were allowed to grow for 2 h, followed by bactericidal assays using 10% NHS. As shown in **Fig. 6A**, both CMP-Neu5Ac9Az and CMP-Leg5Ac7Ac prevented serum resistance mediated by CMP-Neu5Ac, even when the latter was present in a 100-fold molar excess. Adding CMP-Leg5Ac7Ac and CMP-Neu5Ac to the growth media simultaneously, each a concentration of 20 *μ*g/ml, also yielded similar results – the mean survival of *Ng* F62 ΔlgtD was only 7.6%. Similarly, CMP-Leg5Ac7Ac prevented CMP-Neu5Gc-mediated serum resistance (**Fig. 6B**). CMP-Neu5Ac9Az and CMP-Leg5Ac7Ac also prevented Neu5Ac-mediated serum resistance of a ceftriaxone-resistant (CRO-R) isolate, H041 (**Fig. 6C**), however prevention was less than with *Ng* F62 ΔlgtD (minimal prevention seen with CMP-NulO concentrations of 0.2 *μ*g/ml). In the unsialylated state, H041 is intrinsically more serum resistant than F62 ΔlgtD (H041 shows >100% survival in 3.3% NHS, while F62 shows <10% survival in 3.3% NHS).

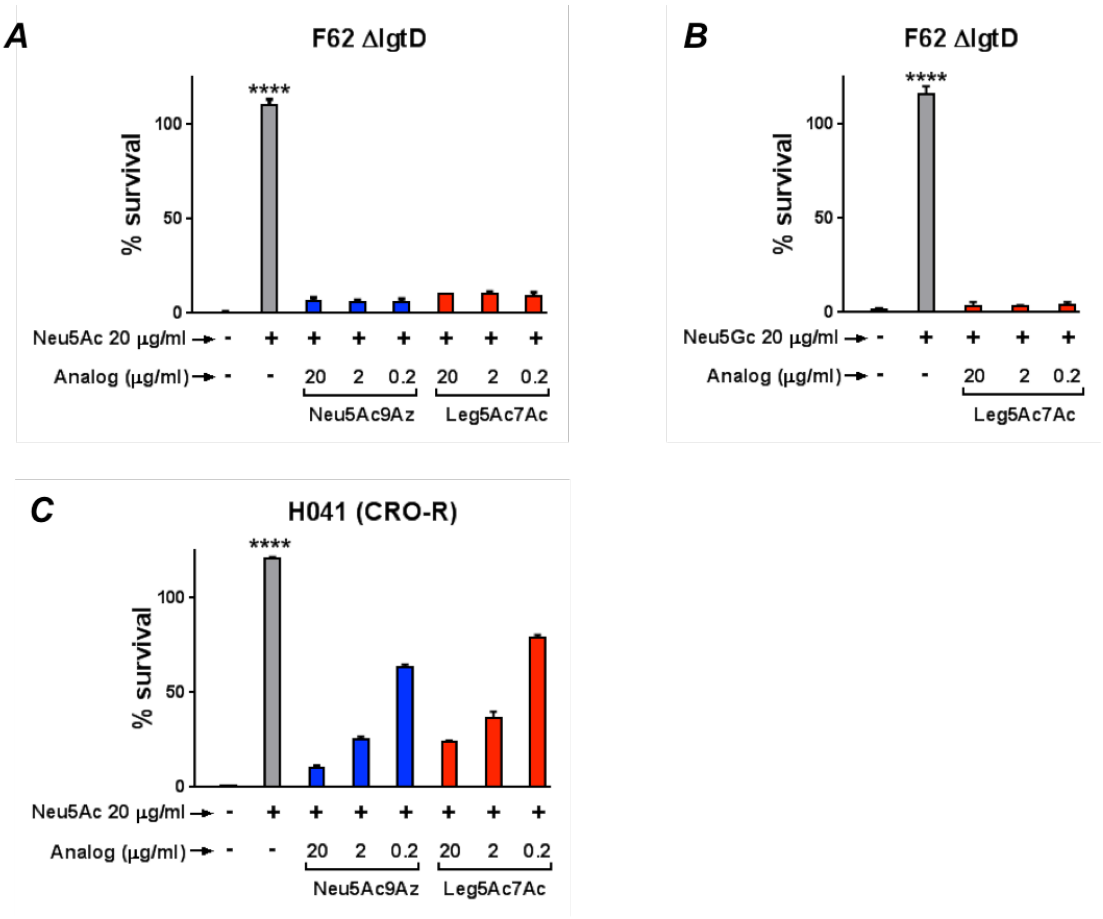



**Figure 6** | Survival of *N. gonorrhoeae* (*Ng)* first incubated in CMP-Neu5Ac or CMP-Neu5Gc followed by incubation with CMP-NulOs. *Ng* F62 ΔlgtD (A and B) and ceftriaxone-resistant (CRO-R) *Ng* H041 (C) were suspended in media containing 20 *μ*g/ml CMP-Neu5Ac or CMP-Neu5Gc as indicated for 15 min at 37 ºC. Following incubation, CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac (at concentrations of 20, 2 or 0.2 *μ*g/ml) were added to bacterial suspensions and then incubated for 2 h. Bacteria were serially diluted and serum bactericidal assays were performed using 2000 CFU and 10% normal human serum (NHS). Controls included bacteria in media that lacked any CMP-Sia altogether or bacteria that were incubated in CMP-Neu5Ac or CMP-Neu5Gc only. Y-axis, % survival (mean (SD) of two independent experiments is shown). ****, P<0.0001 compared to all other reactions (ANOVA).

*Competing NulOs block Neu5Ac-mediated complement inhibition*

To define the mechanism whereby Neu5Ac9Az and Leg5Ac7Ac blocked complement resistance by Neu5Ac, we studied Ab binding to, and complement component deposition on *Ng* F62 ΔlgtD that was incubated with CMP-Neu5Ac (20 *μ*g/ml), followed 15 min later with either CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac at concentrations of 20, 2 or 0.2 *μ*g/ml. Controls included bacteria grown in the absence of any Sia, or bacteria grown in CMP-Neu5Ac alone.

As shown in **Fig. 7** (and above in **Fig. 5**), incorporation of Neu5Ac into LOS decreased IgG binding and also diminished deposition of complement components C4 and C3. However, addition of CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac at all

concentrations tested resulted in lesser reduction in IgG binding in 10% NHS and also in the corresponding deposition of C4 and C3. Similar results were also seen when a lower concentration of serum, 3.3% NHS was used (Supplemental **Figure S4**).

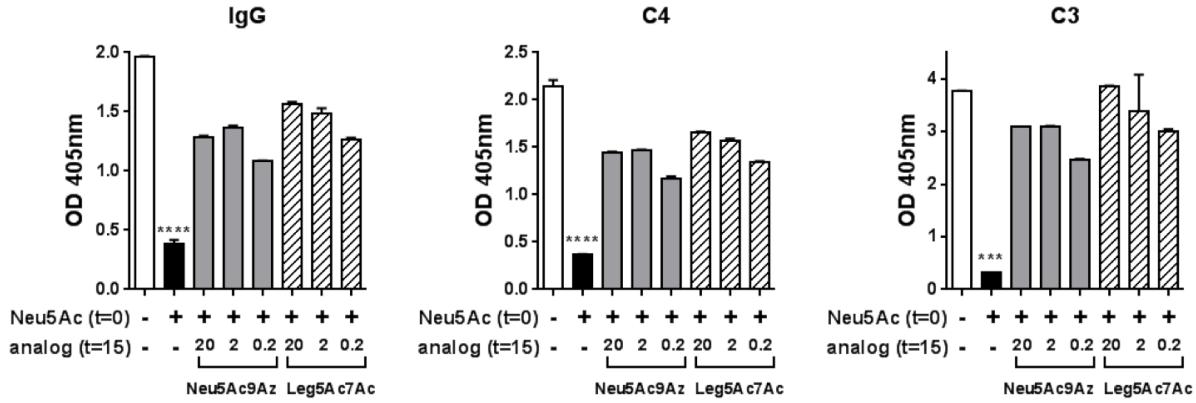



**Figure 7** | CMP-Neu5Ac9Az and CMP-Leg5Ac7Ac interfere with inhibition of the classical and alternative pathways of complement mediated by CMP-Neu5Ac. *N. gonorrhoeae (Ng)* F62 ΔlgtD was incubated with CMP-Neu5Ac for 15 min followed by addition of CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac (at concentrations of 20, 2 or 0.2 μg/ml) for 2 h as described in Fig. 6. Bacteria were incubated in 10% normal human serum (NHS) and IgG binding and complement C3 and C4 fragment deposition was measured by ELISA as described in Fig. 5. Mean (±SD) of two independent experiments is shown. Controls with heat-inactivated serum were carried out as described in Fig. 5. MAb 2-8C-4-1 that detects the Neisserial lipoprotein H.8 was similar across all groups and confirmed similar capture of bacteria in all wells ($OD405_{nm}$ values ranged from 0.964 to 1.039). ***, P<0.001; ****, P<0.0001 compared to other bars in the same graph (ANOVA).

CMP-Neu5Ac9Az and CMP-Leg5Ac7Ac also resulted in lesser reduction in FB deposition (**Fig. 8A** and Supplemental **Figure S4**); surprisingly these NulOs did not interfere with FH binding to *Ng* F62 ΔlgtD (**Fig. 8B**). These data seem paradoxical because under physiological conditions, FH functions to irreversibly dissociate factor Bb from C3bBb and also prevents factor B from binding C3; thus, FH and FB/FBb binding usually bear inverse proportions (reviewed in (*Ferreira et al.*, 2010)). The relatively unimpeded activation of the alternative pathway, evidenced by high FB deposition seen in the presence of the competing CMP-NulOs that occurs in the face of FH binding, may suggest impaired FH function when Leg5Ac7Ac or Neu5Ac9Az are expressed on the bacterial surface concomitantly with Neu5Ac.

Ab-dependent classical pathway activation is essential for killing of *N. gonorrhoeae* (*Ingwer et al.*, 1978; *Lewis et al.*, 2009b); disabling the classical pathway abrogates killing of otherwise fully serum sensitive gonococci (*Lewis et al.*, 2009b). To define the role of the alternative pathway in mediating killing by CMP-Leg5Ac7Ac, the function of FB was blocked with an anti-factor Bb mAb (*Gulati et al.*, 2012). Blocking

the alternative pathway enabled unsialylated *Ng* F62 ΔlgtD to survive 56% in the pooled NHS used in these assays (**Fig. 8C**). As expected growth of bacteria in CMP-Neu5Ac abrogated killing (CFU at $t_{30}$ was 115% of the CFUs at $t_0$) (**Fig. 8C**). Addition of CMP-Leg5Ac7Ac (20 $\mu$g/ml) alone to media, or 15 min after the addition of CMP-Neu5Ac (20 $\mu$g/ml), resulted in 76% and 112% survival, respectively, when FB function was blocked (**Fig. 8C**). By contrast (and consistent with the data in **Fig. 6A**), the parallel control bactericidal reactions with all pathways intact (i.e. no anti-Bb) showed 0% survival when bacteria were grown in CMP-Leg5Ac7Ac alone, increasing only to 8% when this NulO was added to media 15 min after CMP-Neu5Ac. Thus, interfering with the function of LOS Neu5Ac by CMP-Leg5Ac7Ac on *Ng* F62 ΔlgtD appears to require a functioning alternative pathway.

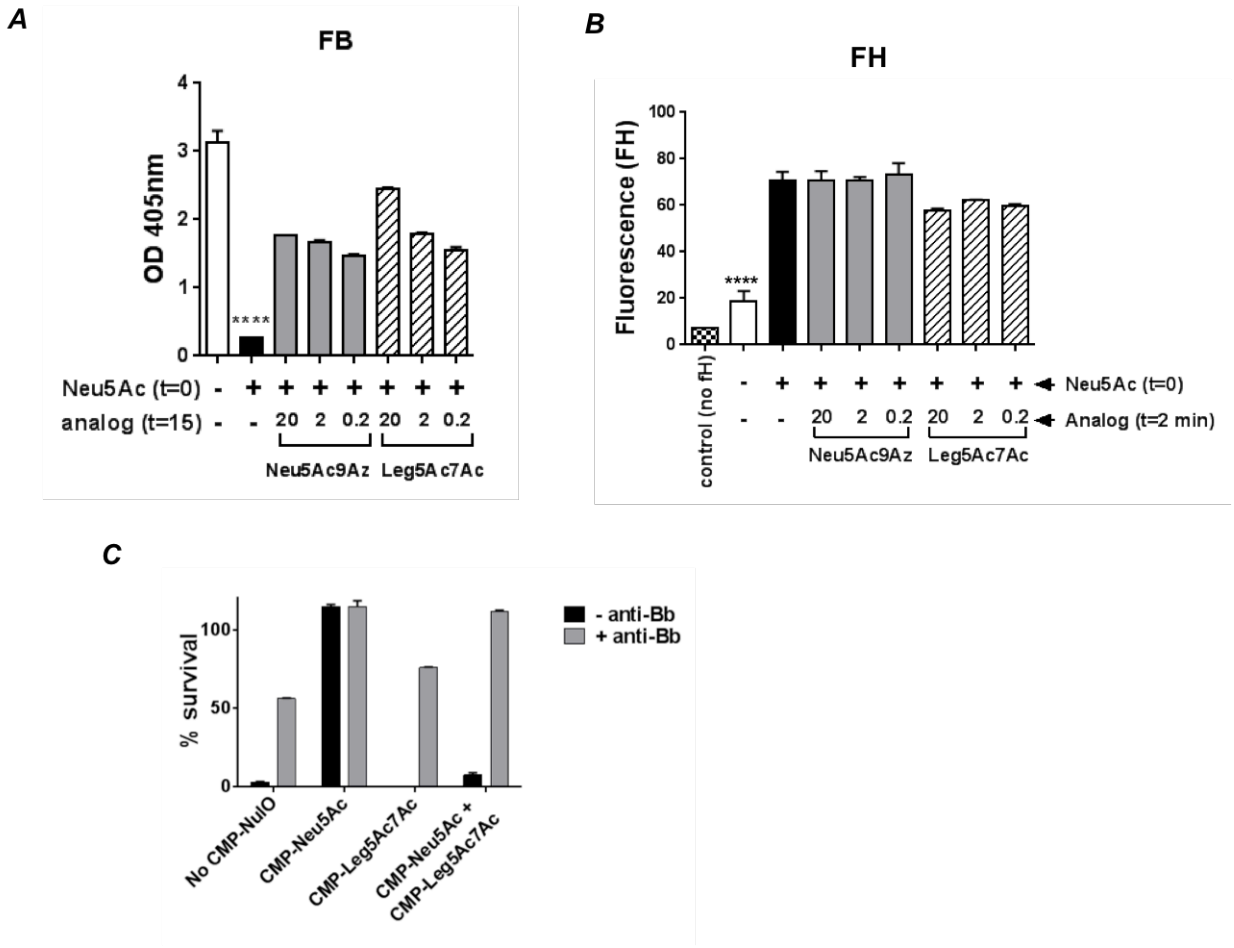



**Figure 8** | CMP-Neu5Ac9Az and CMP-Leg5Ac7Ac block CMP-Neu5Ac-mediated alternative pathway inhibition despite continued factor H (FH) binding. *N. gonorrhoeae (Ng)* F62 ΔlgtD was incubated in media that contained CMP-Neu5Ac (20 μg/ml), followed by addition of either CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac (20, 2 or 0.2 μg/ml). Bacteria were grown for 2 h and factor B (FB) binding (A) to bacteria following incubation with 10% normal human serum (NHS) was measured by whole cell ELISA as described in Fig. 7. Binding to purified FH (B) was measured by flow cytometry using polyclonal goat anti-human FH. The Y-axis represents $OD_{405nm}$ for FB binding and median fluorescence of FH binding. Mean (SD) of two independent observations is shown for each measurement. ****, P<0.0001 (ANOVA) compared to other reactions. (C) Alternative pathway activation is required for maximal killing of *Ng* F62 ΔlgtD grown in CMP-Leg5Ac7Ac-containing media. Bacteria were grown in media alone, or media containing CMP-Neu5Ac (20 μg/ml) or CMP-Leg5Ac7Ac (20 μg/ml) or both CMP-NulOs together (each at 20 μg/ml) as indicated on the X-axis. Serum bactericidal assays were performed using normal human serum (all complement pathways intact; black bars) or NHS treated with an anti-factor Bb mAb that blocks FB function (AP inactivated, but classical and lectin pathways intact; grey bars). Y-axis, percent survival. The mean (range) of two independent experiments is shown.

*N. gonorrhoeae LOS does not preferentially incorporate Leg5Ac7Ac*

A possible explanation for the 'dominant-suppressive' effect of Leg5Ac7Ac and Neu5Ac9Az over Neu5Ac in the bactericidal assays in **Fig. 6** is preferential incorporation of these competing NulOs into gonococcal LOS. However, characterization of Lst enzyme function has revealed that conversion efficiency for *N. meningitidis* and *N. gonorrhoeae* F62 a2,3-sialyltransferases with CMP-Leg5Ac7Ac as a donor is comparable to that with CMP-Neu5Ac (*Watson et al.*, 2015). Further, binding of mAb 3F11 (binds only to unsialylated LNnT; extensions beyond the terminal Gal of LNnT abrogates 3F11 binding) to *Ng* F62 ΔlgtD grown in concentrations ranging from 0.125 to 25 μg/ml of CMP-Neu5Ac or CMP-Leg5Ac7Ac were similar, providing additional evidence that Leg5Ac7Ac (when used alone) is not preferentially added to the *Ng* LNnT LOS (Supplemental **Figure S5**). Finally, the ratio of Leg5Ac7Ac:Neu5Ac incorporated into *Ng* F62 ΔlgtD LOS,after CMP-Leg5Ac7Ac was added to growth media 15 min after CMP-Neu5Ac had been added, was approximately 1:2 (Supplemental **Figure S6B**). Collectively, these data provide strong evidence that the effects of Leg5Ac7Ac in preventing the complement-inhibiting effects of Neu5Ac were not because of the preferential addition of Leg5Ac7Ac on to LOS.

*CMP-Leg5Ac7Ac attenuates gonococcal infection in mice*

Given the urgent need for novel therapies against multidrug-resistant gonorrhea that has spread globally, we next tested the ability of CMP-Leg5Ac7Ac to attenuate gonococcal infection in mice. CMP-Leg5Ac7Ac was chosen because it most effectively blocked Neu5Ac-mediated complement inhibition (**Figs. 5, 7** and **8**) and because Leg5Ac7Ac-α2,3-lactose-FCHASE substrates are more resistant to microbial and human sialidases (that *Ng* may encounter in the genital tract) than Neu5Ac-α2,3-lactose-FCHASE substrates (*Watson et al.*, 2011). Two groups of 17β-estradiol-treated BALB/c mice (10 mice per group) were infected with wild-type strain F62. We used wild-type *Ng* for the experiments *in vivo* because phase variation of LOS (i.e., 'GalNAc capping' via LgtD) could constitute a possible mechanism of 'drug resistance'. One group was treated with 10 *μ*g CMP-Leg5Ac7Ac intravaginally daily; the control group was untreated. A third group of mice (n=10) was infected with F62 Δ*lst* (unable to sialylate LOS) and served as a control. As shown in **Fig. 9A**, mice treated with CMP-Leg5Ac7Ac cleared infection faster than

untreated mice (median clearance time was 6 days in treated mice compared to 10 days for untreated mice; P<0.0001, Kaplan-Meier survival curve; clearance times were compared between groups using a log-rank test). Mixed model analysis indicated significant differences in colonization trends of F62 wild-type over time between the two groups comparing CMP-Leg5Ac7Ac versus untreated mice (P=0.0005) (**Fig. 9B**). A significant difference in the Mean Areas Under the Curve (mean AUCs) ($log_{10}$ CFU versus time) between the treated and untreated groups challenged with F62 WT was also observed (**Fig. 9C**). To address the possibility that non-specific stimulation of the immune system by CMP-Leg5Ac7Ac may have accounted for its activity, we examined the effects of CMP-Leg5Ac7Ac treatment on the burden of infection caused by an Lst deletion mutant of F62 (F62 *Δlst)* that is unable to add NulO to its LOS. As shown in Supplemental **Fig. S7**, CMP-Leg5Ac7Ac treatment did not affect the course of infection (measured as time to clearance or AUC) of F62 *Δlst*.

To confirm that CMP-Leg5Ac7Ac treatment reduced serum resistance of *Ng in vivo*, we incubated bacteria recovered directly from mice (without sub-passage on media) with normal human serum and measured their survival after 30 min. As shown in **Fig. 9D**, the wild-type bacteria from untreated mice remained resistant to complement over all 5 days; bacteria recovered from the CMP-Leg5Ac7Ac treatment group became progressively more serum sensitive over time. As expected, the *Δlst* mutant was uniformly serum sensitive on all days. This experiment was limited to 5 days, because the numbers of bacteria recovered from the treatment and *Δlst* groups thereafter were too low to perform the assay.

Similar to findings with F62, CMP-Leg5Ac7Ac significantly attenuated the duration and burden of infection caused by CRO-R isolate H041 in mice (**Fig 10**.). Of note, the H041 isolate is naturally more serum resistant than F62, resulting in minimal clearance at day 10 of saline-treated mice (**Figs. 10A** and **10B**).

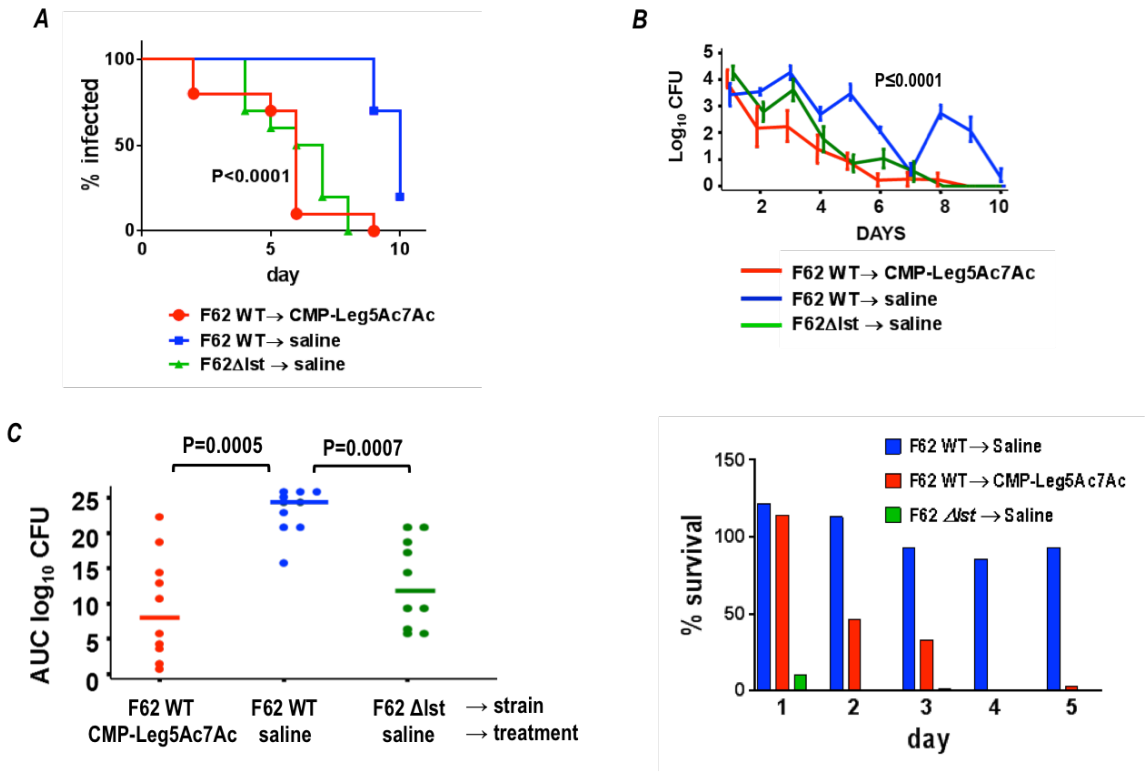



**Figure 9 I** CMP-Leg5Ac7Ac treatment reduces the duration of infection and the burden of *N. gonorrhoeae (Ng)* F62 in the BALB/c mouse vaginal colonization model. Three groups of 17β-estradiol treated BALB/c mice (n=10/group) were inoculated and treated as follows: i) wild-type F62; saline (vehicle control), ii) wild-type *Ng* F62; treated with CMP-Leg5Ac7Ac 10 μg/ml intravaginally daily and iii) *Ng* F62 ΔIst; saline. Vaginal swabs were obtained daily to quantify *Ng* CFUs. (A) Kaplan Meier analysis of time to clearance. (B) Colonization of bacteria ($log_{10}$ CFU) measured daily. (C) Bacterial burdens consolidated over time (Area Under the Curve [$log_{10}$ CFU] analysis) for the three groups. There were no statistically significant differences noted between the group treated with CMP-Leg5Ac7Ac and the *Ng* F62 ΔIst group. (D) Complement resistance of *Ng* recovered directly from the genital tract of mice simulates findings *in vitro*. Equal volumes of vaginal secretions from all mice within a group on each day were pooled, serially diluted and incubated with 10% NHS. Survival of bacteria at 30 min relative to 0 min was measured and the percentage survival expressed on the Y-axis.

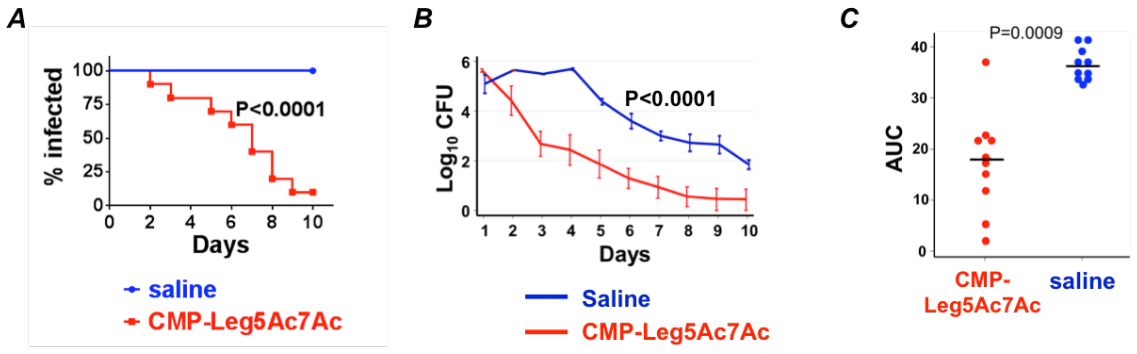



**Figure 10** I CMP-Leg5Ac7Ac treatment reduces the duration and burden of ceftriaxone-resistant (CRO-R) *N. gonorrhoeae (Ng)* H041 in the BALB/c mouse vaginal colonization model. Two groups of 17β-estradiol treated BALB/c mice (n=10/group) were challenged with 9 x $10^5$ CFU *Ng* H041. One group received CMP-Leg5Ac7Ac 10 μg/ml intravaginally daily; the other group received saline (vehicle control). Vaginal swabs were obtained daily to quantify *Ng* CFUs. (A) Kaplan Meier analysis of time to clearance. (B) Colonization of bacteria ($log_{10}$ CFU) measured daily. (C) Bacterial burdens consolidated over time (Area Under the Curve [$log_{10}$ CFU] analysis) for the two groups.

## Discussion

Over 40 years ago, Ward and colleagues showed that gonococci recovered directly from male urethral secretions fully resisted killing by human complement PMID: 4987874. However, sub-passage of gonococci on routine culture media abrogated the property of serum resistance and thus was termed 'unstable' serum resistance. Almost two decades later, seminal work performed by Smith and co-workers (*Smith et al.*, 1992; *Nairn et al.*, 1988; *Parsons et al.*, 1993) identified CMP-Neu5Ac as the molecule scavenged by *Ng in vivo* that was responsible for conferring unstable serum resistance. The only molecule modified by Neu5Ac on *Ng* is LOS (*Mandrell et al.*, 1990). Sialylation of LOS is mediated by Lst, an enzyme located on the *Ng* outer membrane (*Shell et al.*, 2002). The two gonococcal HepI LOS extensions that can be modified by Neu5Ac are lacto-N-neotetraose (LNnT) (Neu5Acα2–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–4HepI) and the $P^K$ LOS (Neu5Acα2–6Galα1–4Galβ1–4Glcβ1–4HepI) (*Gulati et al.*, 2005). Here we show that substrate specificity of gonococcal Lst in the context of intact bacteria extends to five additional CMP-Sia analogs listed in **Table 1**.

Several previous studies have elucidated mechanisms of gonococcal LNnT LOS Neu5Ac-mediated serum resistance and point to regulation of both the classical and alternative pathways of complement (*Elkins et al.*, 1992; *Ram et al.*, 1998; *Jarvis*, 1994)[25]. The presence of LOS Neu5Ac on gonococci decreases binding of select mAbs directed against gonococcal PorB (*Elkins et al.*, 1992). Another report suggested that LOS Neu5Ac did not affect the quantity of anti-LOS IgM binding to bacteria, but perhaps masked epitopes usually recognized by pentameric IgM molecules that resulted in limited engagement of C1q and subsequent activation of the classical pathway (*Zaleski and Densen*, 1996). The addition of Neu5Ac via an α2–3 linkage to the terminal Gal of LNnT also enhances binding of human FH, the principal inhibitor of the alternative pathway of complement. Enhancement of FH binding to sialylated gonococci also requires the presence of gonococcal PorB specifically; specifically replacement of gonococcal PorB with meningococcal PorB3 does not augment FH binding (*Madico et al.*, 2004). Neu5Ac that enhances FH binding and results in high-level serum resistance of gonococci is also specific for the LNnT LOS structure. Previously we reported that addition of α2–6-linked Neu5Ac to the terminal Gal of the $P^K$-like (or L1) LOS did not enhance FH binding and conferred resistance only to lower concentrations of serum relative to serum resistance seen with Neu5Ac α2–3-linked to LNnT LOS [23]. Binding of FH hastens

dissociation of alternative pathway C3/C5 convertases (C3b,Bb and C3bC3b,Bb, respectively) (*Ferreira et al.*, 2010) and likely contributes to the barely detectable levels of FB on the Neu5Ac and Neu5Gc-bearing strains even in 10% serum.

In contrast to the high-level serum resistance seen with LOS bearing Neu5Ac and Neu5Gc, NulOs with further modifications at C7, C8 and C9 were equally well incorporated and yet conferred only low level serum resistance (Neu5Ac9Ac or Neu5Gc8Me) or no serum resistance (Leg5Ac7Ac and Neu5Ac9Az). These data also suggest that the negative charge of Sias alone is not sufficient for FH binding or complement evasion – Neu5Gc8Me, Leg5Ac7Ac and Neu5Ac9Az are all negatively charged, yet do not enhance FH binding or classical pathway inhibition. In contrast, N-glycolyl substitution at the 5-carbon position of Neu5Ac (to Neu5Gc) facilitates FH binding and increases serum resistance. The importance of the C9 substitutions reported here are in accordance with several previous studies as discussed below. Removal of the 9-carbon was associated with loss of 90% of the complement inhibition by Sia (*Michalek et al.*, 1988). The extent of 9-O-acetylation of Sias on erythrocytes varies widely across mouse strains and correlates directly with the susceptibility of erythrocytes to lysis by the alternative pathway (*Varki and Kornfeld*, 1980). Similarly, increased expression of 9-O-acetylated sialoglycans on the red cells of individuals with visceral leishmaniasis is associated with greater alternative pathway activation (*Chava et al.*, 2004). Fearon showed that removal of C8 and C9 substitutions of Sia by $NaIO_4$ treatment rendered sheep erythrocytes susceptible to lysis by the alternative pathway (*Fearon*, 1978). Using ligand-based nuclear magnetic resonance (NMR), Blaum et al recently showed that the O8 and O9 hydroxyl groups of the glycerol chain of Neu5Ac that was α2–3-linked to lactose formed hydrogen bonds with the amide and carbonyl groups, respectively, of the W1198 residue in FH domain 20 (*Blaum et al.*, 2015). In addition the O9 also formed a hydrogen bond with the W1183 backbone (*Blaum et al.*, 2015). Effective and unimpeded alternative pathway inhibition when the 5Ac group is replaced with 5Gc is also consistent with a prior study (*Michalek et al.*, 1988).

Previously we reported that the addition of Neu5Ac to gonococcal LOS diminished classical pathway activation (less C4 fragment deposition) (*McQuillen et al.*, 1999). In the present study, using relatively low numbers of bacteria in Ab binding and complement deposition assays to more closely simulate conditions in serum bactericidal assays, we observed a substantial reduction in binding of IgG to

sialylated (Neu5Ac and Neu5Gc) bacteria. Reduced IgG binding could account, at least in part, for classical pathway inhibition mediated by Neu5Ac. Downstream events, including C4, C3 and FBb deposition (the latter a measure of alternative pathway recruitment), were effectively regulated only in low serum concentrations when LOS expressed Neu5Gc8Me and Neu5Ac9Ac, but were not likely to reach a threshold that permitted bacterial survival in 10% complement. Of note, the intermediate phenotype exhibited by CMP-Neu5Ac9Ac treated bacteria, compared to CMP-Neu5Ac9Az treatment, is likely due to the instability of the C9 O-acetyl group where some of the Neu5Ac9Ac is converted to Neu5Ac, as this instability was observed during preparation and storage of CMP-Neu5Ac9Ac.

Sialic acids (Sias) are present on all vertebrate cell types and play diverse roles in immune function (*Varki and Gagneux*, 2012). Humans (and birds and reptiles) lack a functional CMP-Neu5Ac hydroxylase enzyme (CMAH) and therefore cannot convert Neu5Ac to Neu5Gc (*Chou et al.*, 1998). In contrast to humans, most other mammals studied (with the exception of ferrets (*Ng et al.*, 2014) and new world monkeys (*Springer et al.*, 2014)) including mice, possess a functional CMAH and therefore express both Neu5Ac and Neu5Gc glycoconjugates. Thus, the LOS of *Ng* recovered from mice during experimental studies of gonococcal colonization may be modified with either Neu5Ac and/or Neu5Gc. The ability of Neu5Gc on *Ng* LOS to also inhibit complement validates the use of the mouse model to study complement interactions with gonococci and evaluate vaccines and therapeutics that may rely on complement activation for their efficacy. The observation that Neu5Gc also facilitates binding of human FH and results in high-level serum resistance that simulates the findings with Neu5Ac would validate the use of human FH transgenic mice to elucidate the role of complement in pathogenesis of sialylated gonococci.

Binding of purified FH (independent of C3 fragment deposition as occurs in context of normal serum) to sialylated gonococci is human specific, with weak binding observed to chimpanzee FH (*Ngampasutadol et al.*, 2008). This raises the question of the utility of wild-type mice, whose endogenous FH is not known to bind directly to sialylated gonococci, for studies related to sialic acid. Distinct from the mechanism that involves direct FH binding described above, gonococcal LOS Neu5Ac also regulates non-human (e.g., rhesus macaque) C3 deposition on gonococci, albeit to a lesser extent than seen in species (chimpanzee and human) whose FH directly bind (i.e., in a C3 independent manner) to sialylated bacteria (*Ngampasutadol et al.*,

2008). Although not formally demonstrated on sialylated gonococci, we have shown that Neu5Ac substitution of meningococcal LNnT LOS limits non-human (in this instance, rat) C3 deposition (*Lewis et al.*, 2012). This mechanism represents a cooperative interaction of the C-terminal FH domains with C3 fragments that bind to bacteria and Neu5Ac-LNnT (*Lewis et al.*, 2012) that is concordant with the prior model proposed by Kajander and colleagues (*Kajander et al.*, 2011). A key role for sialic acid in bacterial survival *in vivo* in wild-type mice has been shown in **Fig. 10** (lower 'virulence' of the Lst mutant), and replicates prior findings (*Lewis et al.*, 2015; *Wu and Jerse*, 2006).

We and others previously have proposed a role for bacterial Neu5Ac (reviewed in (*Severi et al.*, 2007)), including Leg5Ac7Ac in immune evasion (*Schoenhofen et al.*, 2006; *Schoenhofen et al.*, 2009), possibly through increased FH binding. However, in the context of *Ng*, the current data (**Fig. 4B** and Supplemental **Figure S3**) refute this notion for Leg5Ac7Ac. Paradoxically, incorporation of Leg5Ac7Ac by *Ng* LOS appears to confer heightened susceptibility of *Ng* to the immune system.

*Ng* has developed widespread resistance to almost every conventional antibiotic currently in clinical use (*Unemo and Shafer*, 2014). High-level resistance to ceftriaxone was first reported in 2011 (*Ohnishi et al.*, 2011a; *Ohnishi et al.*, 2011b) and has ushered in an era of potentially untreatable gonorrhea. There is an urgent need for novel therapeutics and vaccines against this disease (*CDC*, 2012; *WHO*, 2012). LOS sialylation is an important component of gonococcal pathogenesis; isogenic mutants that cannot sialylate their LOS are at a disadvantage *in vivo* compared to their wild-type counterparts (*Wu and Jerse*, 2002). Manipulation of gonococcal LOS 'sialylation' could represent a novel preventive or treatment strategy. We acknowledge that further work is needed to elucidate the mechanistic details of the 'dominant negative' effect of Leg5Ac7Ac/ Neu5Ac9Az over Neu5Ac substitution of gonococcal LOS, however given the dire need for alternative therapeutics against drug-resistant gonorrhea, we decided to focus first on the practical utility of these findings. Here we have shown that select derivatives of Sia, such as Neu5Ac9Az and Leg5Ac7Ac block Neu5Ac-mediated serum resistance (**Fig. 6**). *In vitro*, the effects of these CMP-Sia derivatives are also evident when added to bacteria shortly after adding CMP-Neu5Ac. *In vivo*, however, bacteria actively replicate for several generations and are likely to incorporate the competing Sia analog over time. Of note, the effects of CMP-Neu5Ac were negated even when

CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac were provided at ~100-fold lower concentrations. These competing CMP-NulOs diminished inhibition of the classical pathway (determined by measuring both increased IgG binding and C4 deposition) and serum resistance elicited by CMP-Neu5Ac. Surprisingly, FBb continued to bind to *Ng* in an uninhibited manner (**Fig. 8A**) in the face of high FH binding (**Fig. 8B**). This raises the possibility that FH is rendered 'non-functional' (i.e., does not display decay accelerating activity and liberate FBb from C3/C5 convertases) when Leg5Ac7Ac- or Neu5Ac9Az-substituted LOS and Neu5Ac-subsituted LOS are concomitantly expressed on the bacterial surface. The reasons for the 'dominant-suppressive' effect of the competing NulOs over complement inhibition by Neu5Ac remain to be elucidated. Based on the phylogeny of NulOs (*Lewis et al.*, 2009a) it is likely that *Ng* Lst may have evolved from an ancestral transferase that originally used CMP-bacterial NulOs such as CMP- Leg5Ac7Ac as substrates.

In addition to its ability to counteract Neu5Ac-mediated complement inhibition effectively, CMP-Leg5Ac7Ac was chosen to study therapeutic efficacy *in vivo* because of the sialidase resistance exhibited by Leg5Ac7Ac, as evidenced by the resistance of Leg5Ac7Ac-α(2,3)-lactose-FCHASE to mammalian and microbial sialidases [18]. Sialidases are present in the female genital tract (*Briselden et al.*, 1992; *McGregor et al.*, 1994; *Paulesu and Pessina*, 1982; *von Nicolai et al.*, 1984; *Wiggins et al.*, 2001); resistance of Leg5Ac7Ac-substituted LNnT on *Ng* to sialidases would be a desired attribute for a therapeutic CMP-NulO. Indeed, mice treated with CMP-Leg5Ac7Ac cleared wild-type *Ng* F62 more rapidly (**Fig. 9A**) and harbored lower bacterial burdens over the course of infection (**Fig. 9B and 9C**) analogous to clearance and colonization levels using a Lst knockout mutant of strain F62. Furthermore, as daily treatment of mice with CMP-Leg5Ac7Ac took hold, surviving bacteria isolated from mice during the course of infection became progressively more sensitive to killing by complement (**Fig. 9D**), consistent with organisms having incorporated Leg5Ac7Ac (**Figs. 2 and 3**). Under the influence of CMP-Leg5Ac7Ac, conversion of the infecting strain into a phenotype unable to sialylate only with Neu5Ac (and/or Neu5Gc in the mouse (*Chou et al.*, 2002)) resulted in an attenuation of bacterial burden similar to that seen with the Lst mutant, which manifests incomplete virulence in the mouse model of gonococcal infection [36]. In contrast to strain F62, gonococcal strain H041 is fully resistant to ceftriaxone (CRO-R), resistant to 3.3% serum, and more resistant to the bactericidal properties conferred by NuIOs

Neu5Ac9Az and Leg5Ac7Ac than strain F62 Δ*lgtD* (Figs. 6A and 6C). Nevertheless, mice treated with CMP-Leg5Ac7Ac also cleared strain H041 more rapidly and harbored lower bacterial burdens than saline-treated infected animals (**Fig. 10**).

In conclusion, these findings have shed new light on the molecular basis of gonococcal LOS sialylation and how this translates to complement regulation on the bacterial surface. CMP-Sia analogs that interfere with serum resistance of gonococci mediated by CMP-Neu5Ac may predict their efficacy as topical anti-gonococcal therapeutics in vivo. The identification of such Sia analogs that can interfere with a key virulence mechanism of *Ng* paves the way to develop novel therapeutic strategies against rapidly spreading multi-drug resistant gonococcal infections.

**Materials and Methods**

*Synthesis of CMP-Nonulosonate (CMP-NulO) compounds*

Neu5Ac, Neu5Gc and Neu5Ac9Az nonulosonates were purchased from commercial sources (Inalco Pharmaceuticals, Sigma, Sussex Research Laboratories Inc.). Neu5Ac9Ac and Neu5Gc8Me were synthesized using published methods (*Ogura et al.*, 1987; *Yu et al.*, 2011). Leg5Ac7Ac and Pse5Ac7Ac were prepared enzymatically using methods in (*Schoenhofen et al.*, 2009) and (*Schoenhofen et al.*, 2006), respectively. CMP-activation of nonulosonate sugars was performed enzymatically using appropriate CMP-Sia, CMP-legionaminic acid and CMP-pseudaminic acid synthetases from either *N. meningitidis*, *C. jejuni* or *Helicobacter pylori* (*Schoenhofen et al.*, 2006; *Schoenhofen et al.*, 2009; *Gilbert et al.*, 1997; *Guerry et al.*, 2000; *Li et al.*, 2012). Reactions contained 50 mM Tris pH 8-9, 50 mM $MgCl_2$, 15.7 mM CTP, 15 mM nonulosonate, 4 units pyrophosphatase per mM of CTP, and sufficient quantities of CMP-NulO synthetase enzyme to obtain optimal conversion at 5-6 hours. CMP-NulO enzymatic reaction mixtures were then passed through an Amicon Ultra-15 (10,000 molecular weight cut-off) or Ultra-4 (5,000 molecular weight cut-off) filter membrane before purification. Filtered CMP-NulO samples were then lyophilized and desalted/purified using a Superdex Peptide 10/300 GL (GE Healthcare) column in ammonium bicarbonate or NaCl solutions. To achieve additional purity, elution fractions containing individual CMP-NulOs were subjected to anion-exchange chromatography (Mono Q 4.6/100 PE, GE Healthcare) using either an ammonium bicarbonate or NaCl gradient. When NaCl gradients were used, CMP-NulOs were 'desalted' by gel filtration (Superdex Peptide 10/300 GL) using 1 mM NaCl.

Quantification of CMP-NulO preparations was determined using the molar extinction coefficient of CMP (ε260=7,400). Prior to lyophilization, CMP-NulOs were suspended in sodium hydroxide or NaCl solutions at molar ratios of 1:2 (NulO: salt).

Purified CMP-nonulosonates were dissolved in >99% $D_2O$ and structural analysis performed by mass spectroscopy (MS) using either a Varian Inova 500 MHz ($^1H$) spectrometer with a Varian Z-gradient 3 mm probe or a Varian 600 MHz ($^1H$) spectrometer with a Varian 5 mm Z-gradient probe. All spectra were referenced to an internal acetone standard ($\delta_H$ 2.225 ppm and $\delta_C$ 31.07 ppm). Results that are shown in Supplemental Table S1 and Supplemental Figure S1 verify the production and purity of each CMP-nonulosonate compound made for this study; CMP-LegAc7Ac and CMP-Pse5Ac7Ac, which were confirmed based on NMR data presented in (*Schoenhofen et al.*, 2009) and (*Schoenhofen et al.*, 2006).

CMP-NulOs were also characterized using capillary electrophoresis (CE)-MS analysis. Separation of ions was achieved by CE (Prince CE system [Prince Technologies, Netherlands]) in a 90 cm long bare fused-silica capillary (365 μm OD x 50 μm ID). The 30 mM morpholine aqueous running CE buffer (adjusted to pH9 with formic acid) was coupled with the capillary sheath fluid (isopropanol: methanol [2:1]) at their interface prior to mass spectrometry (API3000 mass spectrometer [Applied Biosystems/Sciex, Concord, ON, Canada]). Supplemental Table S2 indicates measured m/z ions of each CMP-nonulosonate compound using CE-MS. Measurements correspond to calculated masses.

*Bacterial strains and growth conditions*

A mutant of *Ng* strain F62 (*Schneider et al.*, 1982) that lacked expression of lipooligosaccharide glycosyltransferase D *(lgtD),* called F62 ΔlgtD (*Song et al.*, 2000), was provided by Dr. Daniel C. Stein (University of Maryland). LgtD adds a GalNAc residue to the terminal Gal of the HepI lacto-N-neotetraose species (*Yang and Gotschlich*, 1996). Therefore, any extension of the HepI of F62 ΔlgtD is limited to the addition of a NulO residue that is transferred from the CMP-NulO added to growth media.

Bacteria (F62 ΔlgtD) grown overnight on chocolate agar plates were suspended in gonococcal liquid media supplemented with IsoVitaleX (*McQuillen et al.*, 1994) that contained specified concentrations of the CMP-NulO. Bacteria were then incubated at 37 ºC for the period specified in each experiment.

A spontaneous streptomycin-resistant mutant of *Ng* F62 (*Exley et al.*, 2007), kindly provided by Dr. Ann E. Jerse, was used in mouse infection studies. An isogenic *lst* deletion mutant, derived from this strain, was constructed using plasmid pUC18-lst-Kan (*Gulati et al.*, 2005).

Strain H041 (sequence type (ST) 7363; NG-MAST ST 4220) was isolated from a female commercial sex-worker in Kyoto, Japan (*Ohnishi et al.*, 2011a). This isolate is highly resistant to ceftriaxone (MIC 2–4 μg/ml) and to several other antibiotics (*Ohnishi et al.*, 2011a). H041 was rendered resistant to streptomycin by transformation with *rpsL* derived from streptomycin resistant *Ng* strain FA1090. Both wildtype H041 and streptomycin resistant H041 expressed predominantly the sialylatable HepI lacto-*N*-neotetraose LOS, confirmed by silver stain and western blot with mAb 3F11.

*Antibodies*

Anti-FH mAb (Quidel, catalog no. A254 (mAb 90X)) or goat anti-human FH were used in flow cytometry assays to detect human FH binding to bacteria. Goat polyclonal antibodies against C3, C4 and factor B (FB) were obtained from Complement Technology, Inc (Tyler, TX). Anti-factor Bb (Quidel) added to serum at 100 μg/ml was used to block factor B function (*Gulati et al.*, 2012), thereby disabling the alternative pathway in serum bactericidal assays. Alkaline phosphatase conjugated anti-human IgG and IgM were purchased from Sigma (St. Louis, MO). mAb 3F11 (mouse IgM, kindly provided by Dr. Michael A. Apicella, University of Iowa) binds to the unsialylated HepI lacto-*N*-neotetraose structure; sialylation of LOS results in decreased binding of mAb 3F11 (*Yamasaki et al.*, 1991). MAb 2-8C-4-1 (*Ram et al.*, 2007) recognizes Neisserial H.8 lipoprotein and was used in whole cell ELISA assays to measure capture of bacteria on microtiter wells. FITC conjugated anti-mouse IgG and anti-goat IgG, and alkaline phosphatase conjugated anti-mouse IgM, anti-mouse IgG and anti-goat IgG were all obtained from Sigma. Neu5Gc incorporation into LNnT LOS was detected using a Neu5Gc-specific chicken polyclonal IgY Ab (1:2,000) (*Diaz et al.*, 2009) followed by FITC conjugated donkey anti-chicken IgY secondary Ab (1:200; Jackson ImmunoResearch).

*SDS-PAGE and Western blotting for LOS analysis*

Gonococcal lysates treated with protease K (100 μg/ml) and NuPAGE® LDS Sample Buffer (4X) (Invitrogen) were separated on NuPAGE® 12% Bis-Tris (Invitrogen) gels using Novex® MES running buffer (Invitrogen) followed by transfer to an Immobilon PVDF membrane (Millipore) by western blotting. Membranes were blocked with PBS/1% milk and probed with tissue culture supernatants that contained mAb 3F11. mAb 3F11-reactive LOS bands were disclosed with anti-mouse IgM conjugated to alkaline phosphatase followed by the addition of BCIP®/NBT-Purple Liquid Substrate (Sigma). Silver staining for LOS was performed following electrophoresis on 16.5% Criterion™ Tris-Tricine gels (Bio-Rad) using the Bio-Rad Silver Stain kit.

*Mass spectrometry of gonococcal LOS*

*Ng* F62 ΔlgtD was grown in media containing the CMP salts of Neu5Ac, Neu5Gc, Neu5Ac9Ac, Neu5Gc8Me, Neu5Ac9Az and Leg5Ac7Ac and LOS was extracted, de-O-acylated and analyzed by MS as described previously (*Bouchet et al.*, 2003).

*Flow cytometry for factor H binding*

Factor H (FH) binding to bacteria was performed using flow cytometry as described previously (*Gulati et al.*, 2012). Briefly, bacteria (*Ng* F62 ΔlgtD) were harvested from chocolate agar plates and grown in liquid media that contained the specified concentration of the CMP-NulO as described above. Bacteria were then washed with Hanks Balanced Salt Solution (HBSS) containing 1mM $Ca^{2+}$ and 1 mM $Mg^{2+}$ ($HBSS^{++}$) and incubated with FH purified from human plasma (Complement Technology, Inc.; concentration specified for each experiment). Bound FH was detected using either anti-FH mAb (Quidel, catalog no. A254 (mAb 90X)) or affinity-isolated polyclonal goat anti-human FH, followed by FITC conjugated anti-mouse IgG or anti-goat IgG, respectively (Sigma); both Abs had similar performance characteristics. All reaction mixtures were carried out in $HBSS^{++}$/1% BSA in a final volume of 50 μl. Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson) and data were analyzed using FlowJo (version 7.2.5; Tree Star, Inc.).

*Whole cell ELISA for complement component deposition*

C3 and C4 fragment deposition on, and FB binding to bacteria were measured by whole cell ELISA as described previously (*McQuillen et al.*, 1999; *Gulati et al.*, 2012). Briefly, ~$10^6$ organisms in $HBSS^{++}$ were incubated with NHS (at concentrations of 3.3% or 10%) in a reaction volume of 100 µl for 10 min at 37°C. This time point was chosen based on the kinetics of complement deposition on gonococci in previously published data (*McQuillen et al.*, 1999). Reactions were stopped after 10 min by washing three times with ice-cold HBSS containing 5 mM phenylmethylsulfonyl fluoride at 4 ºC. Organisms were resuspended in 200 µl of the same buffer, and 50 µl of each sample applied per well of a 96-well U-bottomed polystyrene microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA) and incubated for 3 h at 37°C. Plates were washed with PBS containing 0.05% Tween 20. Primary antibodies (polyclonal goat anti-human C3, C4 and FB) were diluted in PBS, and secondary antibodies diluted in PBS-0.05% Tween 20 prior to use. To ensure similar capture of bacteria incubated under different conditions, we measured the amount of gonococcal H.8 antigen (*Cannon*, 1989) expression using mAb 2-8C-4-1 (*Ram et al.*, 2007), followed by anti-mouse IgG-alkaline phosphatase conjugate.

*Serum bactericidal assay*

Serum bactericidal assays were performed as described previously (*McQuillen et al.*, 1994). Bacteria harvested from an overnight culture on chocolate agar plates and ~$10^5$ CFU of *Ng* were grown in liquid media containing the specified concentration of CMP-Sia as specified for each experiment. Bacteria were diluted in Morse A and ~2000 CFU of *Ng* F62 ΔlgtD were incubated with NHS (concentration specified for each experiment). The final reaction volumes were maintained at 150 µl. Aliquots of 25 µl of reaction mixtures were plated onto chocolate agar in duplicate at the beginning of the assay ($t_0$) and again after incubation at 37 °C for 30 min ($t_{30}$). Survival was calculated as the number of viable colonies at $t_{30}$ relative to $t_0$.

To measure the sensitivity of bacteria recovered directly from mouse secretions to human complement, an equal aliquot of secretions from each mouse within each group were pooled (pools were made to ensure adequate number of bacteria for the assay over the first 5 days). Secretions from each pool were serially diluted, each dilution was incubated with 10% NHS and survival at $t_{30}$ relative to $t_0$ was carried out

as described above. Only those dilutions that yielded ~100–300 CFU at $t_0$ were considered in order to ensure similar bacteria-to-complement ratio across groups.

*Mouse vaginal colonization model*

Female BALB/c mice 5–6 weeks of age (Jackson Laboratories) in the diestrus phase of the estrous cycle were started on treatment (that day) with 0.5 mg of water soluble 17β-estradiol (Sigma) in 200 μl of water given subcutaneously on each of three days; –2, 0 and +2 days (before, the day of and after inoculation) to prolong the estrus phase of the cycle and promote susceptibility to *Ng* infection. Antibiotics (vancomycin, colistin, neomycin, trimethoprim and streptomycin) ineffective against *Ng* were also used to reduce competitive microflora (*Jerse et al.*, 2011). Mice (n=20) were then infected with 3.4 x $10^5$ CFU of strain F62. One group of mice (n=10) was treated with 10 μg CMP-Leg5Ac7Ac (1 mg/ml in sterile $H_2O$) daily intravaginally while the remaining 10 mice (controls) were given saline (vehicle control). A third group (n=10) was infected with 2.5 x $10^5$ CFU of *Ng* F62 Δlst (unable to sialylate) and received no treatment. In a separate control experiment, *Ng* F62 Δlst infection was compared with *Ng* F62Δ*lst* infection 'treated' with CMP-Leg5Ac7Ac.

*Statistics*

Experiments that compared clearance of *N. gonorrhoeae* in independent groups of mice estimated and tested three characteristics of the data (*Gulati et al.*, 2013): Time to clearance, longitudinal trends in mean $\log_{10}$ CFU and the cumulative CFU as area under the curve (AUC). Statistical analyses were performed using mice that initially yielded bacterial colonies on Days 1 and/or 2. Median time to clearance was estimated using Kaplan-Meier survival curves; the times to clearance were compared between groups using a log-rank test. Mean $\log_{10}$ CFU trends over time were compared between groups using a linear mixed model with mouse as the random effect using both a random intercept and a random slope. A quadratic function in time was determined to provide the best fit; random slopes were also quadratic in time. A likelihood ratio test was used to compare nested models (with and without the interaction term of group and time) to test whether the trend differed over time between the two groups. The mean AUC ($\log_{10}$CFU vs. time) was computed for each mouse to estimate the bacterial burden over time (cumulative

infection); the means under the curves were compared between groups using the nonparametric rank sum test because distributions were skewed or kurtotic.

*Ethics statement*

Collection of human sera and its use were approved by the University of Massachusetts Medical School Institutional Review Board (IRB). Informed, written consent was obtained from all serum donors (Docket # H00005614). Use of animals in this study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School (Docket # A-1930).

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Chapter 4b:

# Supplementary information:

# Utilizing CMP-sialic acid analogs to unravel Neisseria gonorrhoeae lipooligosaccharide-mediated complement resistance and design novel therapeutics

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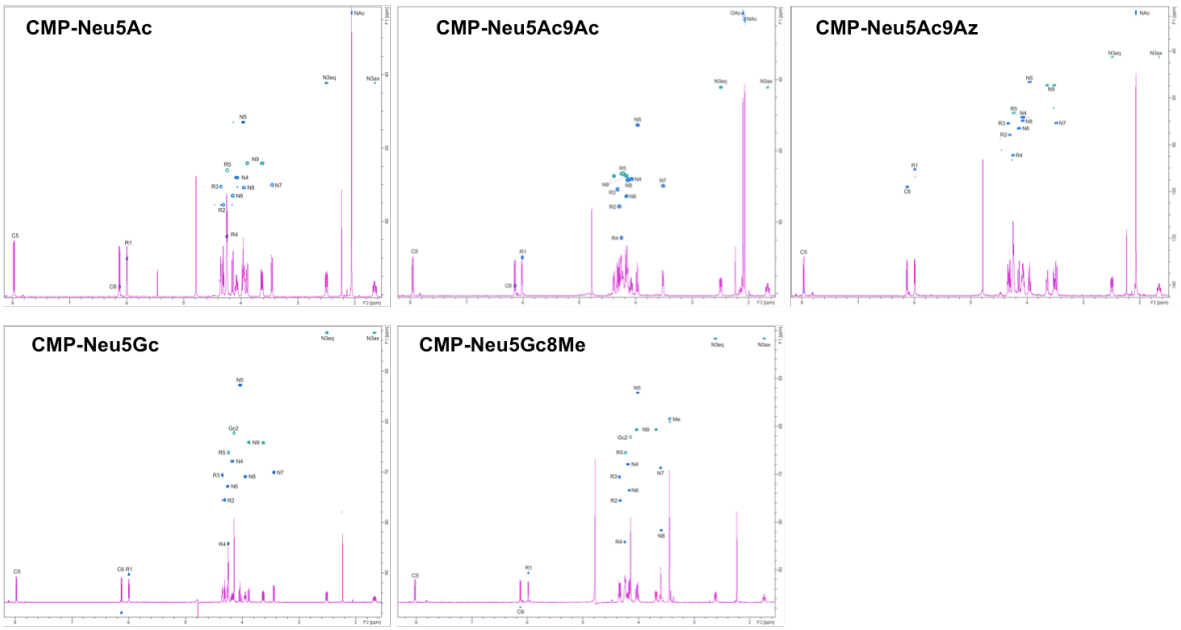
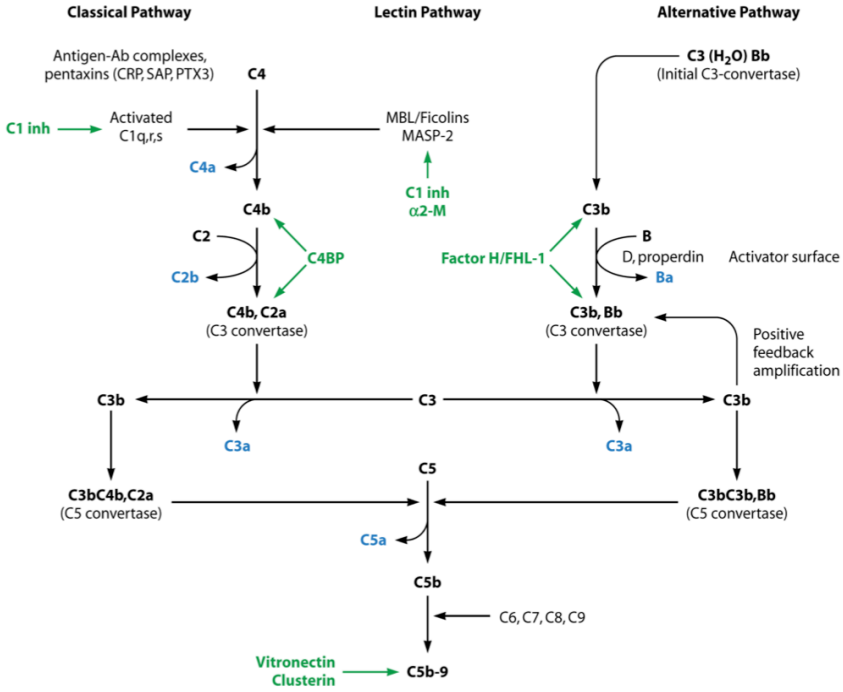
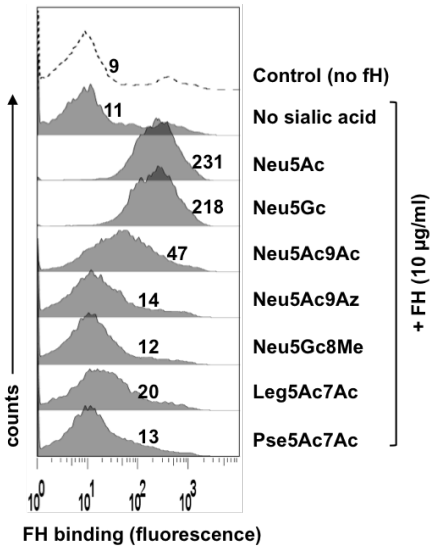


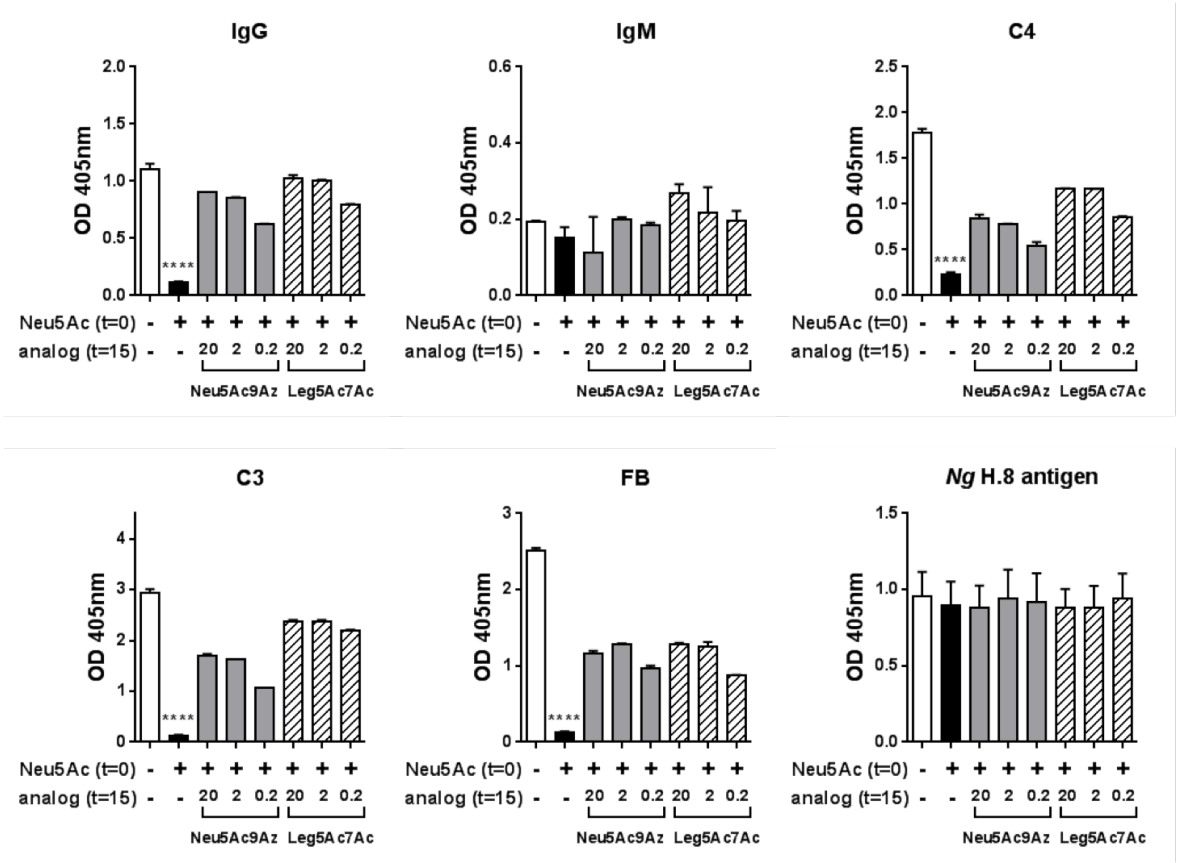

**Supplemental Figure S1 I** The $^{1}H$ spectrum and $^{1}H$-$^{13}C$ HSQC correlation spectrum of different CMP-nonulosonic acids used in this study. Spectra for CMP-Pse5Ac7Ac and CMP-Leg5Ac7Ac have been described previously (*Schoenhofen et al.*, 2006; *Schoenhofen et al.*, 2009). Spectra were recorded on a Varian Inova Unity 500 MHz spectrometer with standard Varian pulse sequences in $D_2O$ at 25°C, with 16 scans for the $^{1}H$ spectrum and 64 scans for HSQC. C, cytosine; R, ribose; N, Nonulosonic acid; NAc, 5-NHAc CH3 regions of either Neu5Ac, Neu5Ac9Ac, or Neu5Ac9Az; OAc, 9-OAc CH3 region of Neu5Ac9Ac; Me, 8-OMe region of Neu5Gc8Me; Gc, glycolyl region of Neu5Gc or Neu5Gc8Me. Acetone was included as an internal reference.



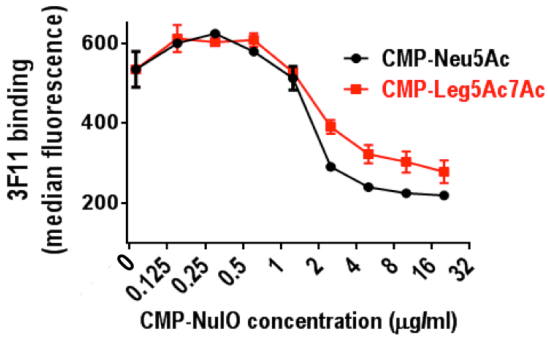

**Supplemental Figure S2 I** Schematic representing activation/regulation of the complement cascade. The fragments released into solution are indicated in blue font. The key fluid-phase regulators are indicated in green font. KEY: CRP, C-reactive protein; SAP, serum amyloid P component; PTX3, pentraxin 3; C1 inh, C1 inhibitor; α2-M, α2-macroglobulin; C4BP, C4b-binding protein; FHL-1, factor H like protein-1. From *Ram S, Lewis LA, Rice PA. Clin Microbiol Rev. 2010. 23(4):740-780.*



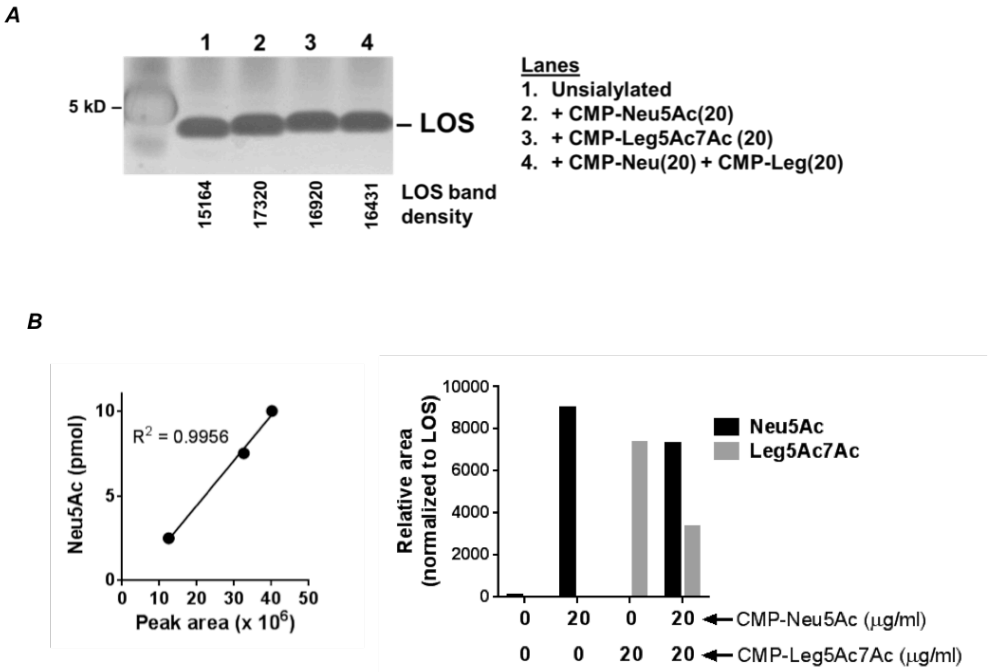

**Supplemental Figure S3** I Factor H (FH) binding to *N. gonorrhoeae* (*Ng)* grown in media containing CMP-Sia analogs. Representative histogram tracings of an experiment depicted in Figure 3. X-axis, fluorescence ($log_{10}$ scale); Y-axis, counts (linear scale). Numbers alongside each histogram represent the median fluorescence. Control represents bacteria that were incubated in buffer alone (no added FH).



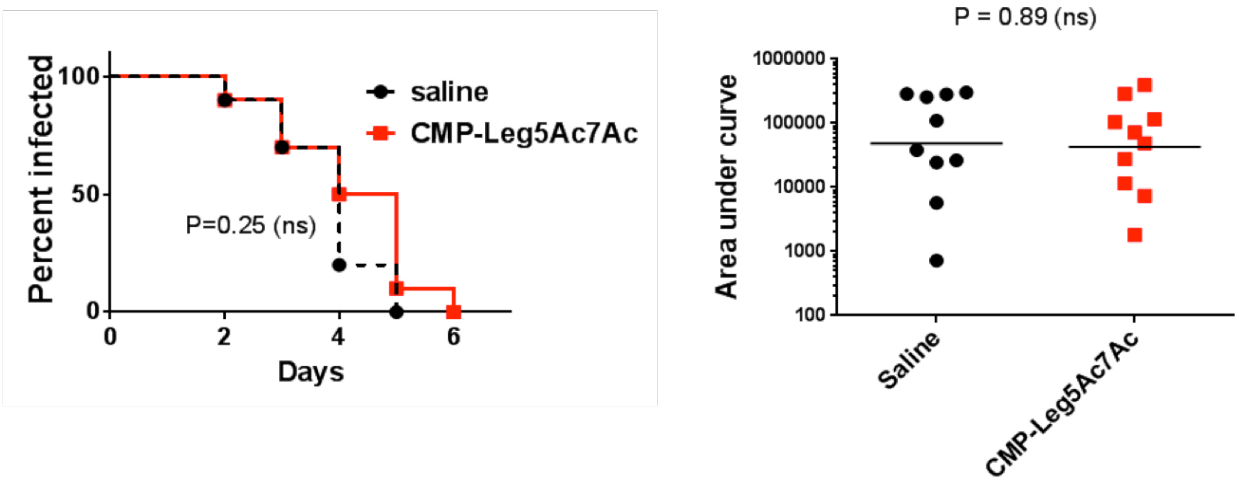

**Supplemental Figure S4 I** CMP-Neu5Ac9Az and CMP-Leg5Ac7Ac interfere with inhibition of the classical and alternative pathways of complement mediated by CMP-Neu5Ac. *N. gonorrhoeae(Ng)* F62 ΔlgtD was incubated with 20 μg/ml Neu5Ac for 15 min followed by addition of CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac (at concentrations of 20, 2 or 0.2 μg/ml) for 2 h as described in Fig. 5. Bacteria were incubated in 3.3% NHS and IgG and IgM binding and deposition of complement components C3, C4 and FB was measured by ELISA. *Ng* H.8 lipoprotein was performed to measure bacterial capture to microtiter wells. Mean (±SD) of two independent experiments is shown.




**Supplemental Figure S5 I** Neu5Ac and Leg5Ac7Ac are incorporated into Ng LNnT LOS with similar efficiency. *N. gonorrhoeae* (*Ng*) F62 ΔlgtD was grown in media alone, or media containing decreasing concentrations (2-fold dilutions ranging from 25 μg/ml to 0.125 μg/ml) of CMP-Neu5Ac or CMP-Leg5Ac7Ac. Binding of mAb 3F11 was measured by flow cytometry. mAb 3F11 binds only to unsubstituted LNnT LOS; extensions beyond the terminal Gal of LNnT abrogates 3F11 binding. Y-axis, median fluorescence (mean (SD) of duplicate samples of one experiment); X-axis, CMP-NulO concentration.




**Supplemental Figure S6** | Quantification of the relative amounts of Neu5Ac and Leg5Ac7Ac in the LOS of *N. gonorrhoeae (Ng)* strain F62 lgtD grown in media containing no CMP-NulO, or in media containing CMP-Neu5Ac alone (20 μg/ml), CMP-Leg5Ac7Ac alone (20 μg/ml), or in media where CMP-Leg5Ac7Ac was added 15 min after CMP-Neu5Ac (both CMP-NulOs at 20 μg/ml). LOS was extracted on a small-scale from a 12 ml culture volume using a modification of the phenol-chloroform method (*Apicella et al.*, 1994). (A) Estimation of the relative amounts of LOS in the samples. Equal volumes of each preparation taken just prior to the final lyophilization step were loaded on a 4-12% Bis-Tris gel (Invitrogen/Life Technologies) with MES running buffer and LOS was revealed by silver staining. The relative intensities of the LOS bands were estimated using ImageJ software (NIH) and are indicated below each lane. (B) Estimation of the relative amounts of Neu5Ac and Leg5Ac incorporated by Ng F62 lgtD lacto-N-neotetraose LOS. Lyophilized LOS extracts samples were dissolved in H2O. Acid hydrolysis of the NulOs was performed with 0.1 M hydrochloric acid at 80 °C for 1 h to release them from the underlying LOS backbone Samples were cooled to room temperature, neutralized with NaOH and then derivatized with 1,2-diamino-4,5-methylene-dioxybenzene (DMB) and analyzed by high performance liquid chromatography (HPLC), as described below. The DMB derivatization reagent was made by mixing 14 mM DMB (Sigma), 18 mM sodium hydrosulfite, 0.75 M 2-mercaptoethanol, and 1.6 M acetic acid, followed by incubation at 50 °C for 2.5 h (*Hara et al.*, 1989) (*Padler-Karavani et al.*, 2013). DMB-derivatized samples were analyzed on a LaChrom Elite HPLC System (Hitachi) using a Phenomenex Gemini 5μ C18 250 × 4.6-mm HPLC column at room temperature. Fluorescence was detected at 448 nm using excitation at 373 nm. To separate NulOs, an isocratic solvent composition of 88% water, 7% methanol and 5% acetonitrile was used at a flow rate of 0.9 mL/min; the data collection time was expanded to 90 min. A Neu5Ac standard curve is shown on the left. Relative amounts of each of the NulOs (normalized to the respective LOS band densities) are indicated on the graph on the right as the normalized peak area.




**Supplemental Figure S7**. CMP-Leg5Ac7Ac treatment does not alter the course of infection with *Ng* F62 *Δ*lst. BALB/c mice were infected with a LOS sialyltransferase deletion mutant of *Ng* F62 (F62 *Δ*lst) and given either saline (vehicle control; black lines/circles; n=10 mice) or CMP-Leg5Ac7Ac (red line/squares; n=10 mice) and bacterial burdens monitored daily. The left graph shows time to clearance of infection and the right graph compares the Areas Under Curves (AUCs) across the two groups.

NMR chemical shifts δ (ppm) for CMP-5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid (CMP-Neu5Ac).

| | | | |
|---|---|---|---|
| H3ax | 1.65 | | |
| H3eq | 2.49 | C3 | 42.2 |
| H4 | 4.07 | C4 | 68.0 |
| H5 | 3.95 | C5 | 52.8 |
| H6 | 4.14 | C6 | 72.9 |
| H7 | 3.45 | C7 | 70.0 |
| H8 | 3.94 | C8 | 70.7 |
| H9 | 3.62; 3.89 | C9 | 64.0 |

NMR chemical shifts δ (ppm) for CMP-5-hydroxyacetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid (CMP-Neu5Gc).

| | | | |
|---|---|---|---|
| H3ax | 1.67 | | |
| H3eq | 2.51 | C3 | 42.4 |
| H4 | 4.18 | C4 | 67.8 |
| H5 | 4.04 | C5 | 52.7 |
| H6 | 4.25 | C6 | 72.8 |
| H7 | 3.44 | C7 | 70.0 |
| H8 | 3.95 | C8 | 70.9 |
| H9 | 3.63; 3.88 | C9 | 64.2 |

NMR chemical shifts δ (ppm) for CMP-5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-nonulosonic acid (CMP-Neu5Ac9Az).

| | | | |
|---|---|---|---|
| H3ax | 1.65 | | |
| H3eq | 2.49 | C3 | 42.4 |
| H4 | 4.07 | C4 | 68.2 |
| H5 | 3.95 | C5 | 53.1 |
| H6 | 4.15 | C6 | 72.9 |
| H7 | 3.48 | C7 | 70.5 |
| H8 | 4.07 | C8 | 69.7 |
| H9 | 3.51; 3.64 | C9 | 54.5 |

NMR chemical shifts δ (ppm) for CMP-5-acetamido-9-O-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid (CMP-Neu5Ac9Ac).

| | | | |
|---|---|---|---|
| H3ax | 1.66 | | |
| H3eq | 2.49 | C3 | 42.1 |
| H4 | 4.08 | C4 | 67.9 |
| H5 | 3.97 | C5 | 52.8 |
| H6 | 4.17 | C6 | 72.7 |
| H7 | 3.51 | C7 | 69.7 |
| H8 | 4.15 | C8 | 68.1 |
| H9 | 4.16; 4.39 | C9 | 67.1 |

NMR chemical shifts δ (ppm) for CMP-5-hydroxyacetamido-8-O-methyl-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid (CMP-Neu5Gc8Me).

| | | | |
|---|---|---|---|
| H3ax | 1.74 | | |
| H3eq | 2.62 | C3 | 41.6 |
| H4 | 4.20 | C4 | 67.9 |
| H5 | 4.01 | C5 | 52.9 |
| H6 | 4.16 | C6 | 73.3 |
| H7 | 3.60 | C7 | 68.7 |
| H8 | 3.59 | C8 | 81.7 |
| H9 | 3.68; 4.03 | C9 | 60.7 |

**Table S1** shows NMR chemical shifts for CMP-NulOs prepared in this study. All spectra were referenced to an internal acetone standard (δH 2.225 ppm and δC 31.07 ppm)

| Compound | Observed m/z | Calculated mass | Formula (M) | Comments |
|---|---|---|---|---|
| CMP-Pse5Ac7Ac | 638.5 | 639.5 | C22H34O15N5P | $[M-H]^-$ |
| CMP-Leg5Ac7Ac | 638.4 | 639.5 | C22H34O15N5P | $[M-H]^-$ |
| CMP-Neu5Ac | 613.5 | 614.4 | C20H31N4O16P | $[M-H]^-$ |
| CMP-Neu5Gc | 629.3 | 630.5 | C20H31N4O17P | $[M-H]^-$ |
| CMP-Neu5Gc8Me | 643.3 | 644.5 | C21H33N4O17P | $[M-H]^-$ |
| CMP-Neu5Ac9Ac | 655.5 | 656.5 | C22H33N4O17P | $[M-H]^-$ |
| CMP-Neu5Ac9Az | 638.3 | 639.5 | C20H30N7O15P | $[M-H]^-$ |

**Table S2** shows CE-MS data of purified CMP-NulO sugars prepared in this study**.** The detected ions indicated here were not present in negative control samples (data not shown).

| | Detected ions | Det. Mass (Da) | Theoretical mass (Da) | Compositions |
|---|---|---|---|---|
| **Neu5Ac** | 1027.5 | 3085.5 | 3084.8 | 1Neu5Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 1PE, LipidA-OH |
| | 1068.5 | 3208.5 | 3207.9 | 1Neu5Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH |
| | 1076 | 3231 | 3229.9 | 1Neu5Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH + Na[A] |
| **Neu5Ac9Ac[B]** | 1068.5 | 3208.5 | 3207.9 | 1Neu5Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH |
| | 1076 | 3231 | 3229.9 | 1Neu5Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH + Na |
| **Neu5Gc** | 1033 | 3102 | 3100.8 | 1Neu5Gc 3Hex, 2HexNAc, 2Hep, 2Kdo, 1PE, LipidA-OH |
| | 1073.5 | 3223.5 | 3223.9 | 1Neu5Gc 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH |
| | 1081.5 | 3247.5 | 3245.9 | 1Neu5Gc 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH + Na |
| **Neu5Gc8Me** | 1037.5 | 3115.5 | 3114.8 | 1Neu5Gc8Me 3Hex, 2HexNAc, 2Hep, 2Kdo, 1PE, LipidA-OH |
| | 1078.5 | 3238.5 | 3237.9 | 1Neu5Gc8Me 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH |
| | 1086 | 3261 | 3259.9 | 1Neu5Gc8Me 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH + Na |
| **Leg5Ac7Ac** | 1036 | 3111 | 3109.8 | 1Leg5Ac7Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 1PE, LipidA-OH |
| | 1077 | 3234 | 3232.9 | 1Leg5Ac7Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH |
| | 1084 | 3255 | 3254.9 | 1Leg5Ac7Ac 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH + Na |
| **Neu5Ac9Az** | 1036 | 3111 | 3109.8 | 1Neu5Ac9Az 3Hex, 2HexNAc, 2Hep, 2Kdo, 1PE, LipidA-OH |
| | 1076.5 | 3232.5 | 3232.9 | 1Neu5Ac9Az 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH |
| | 1084 | 3255 | 3254.9 | 1Neu5Ac9Az 3Hex, 2HexNAc, 2Hep, 2Kdo, 2PE, LipidA-OH + Na |

[A] 9Ac will be lost in the de-O-acylation step
[B] Na adducts in solvent

**Table S3** shows mass spectrometric analysis of Ng F62 ΔlgtD LOS sialylated with different NuIOs**.**

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Chapter 5:

# Conclusion and perspectives

The objective of this research was to characterize specific interactions between the two human pathogens *Escherichia coli* K1 and *Neisseria gonorrhoeae*, and immunoregulatory Siglecs, and explain how these bacteria might use molecular mimicry to exploit host immunity. Moreover, this thesis explores new treatments for these infectious diseases.

*E. coli* K1 interacts with the paired receptors Siglec-11 and Siglec-16 by mimicking human neuronal polysialic acid. The increased killing of *E. coli* K1 mediated by Siglec-16 supports the long-standing hypothesis that activating receptors evolved to counteract the exploitation of inhibiting immunoregulatory receptors by pathogens (*Barclay and Hatherley*, 2008).

The interactions between *E. coli* K1 and Siglec-11/-16 might have an impact on the pathogenesis of bacterial meningitis caused by *E. coli* K1. Since Siglec-11 and Siglec-16 are expressed in the brain uniquely in human and *E. coli* K1 is a solely human pathogen, these interactions are difficult to study in an animal model. Further advancements in models that closely resemble human physiology might help to elucidate the role of these receptors in bacterial meningitis.

Interestingly, *N. meningitidis* group B, the causative agent of epidemic bacterial meningitis, expresses α2-8-linked sialic acid capsule as *E. coli* K1 (*Troy*, 1979; *Freiberger et al.*, 2007) and might engage Siglec-11 and -16 in a similar way. It has been shown before that different strains of *N. meningitides* bind Siglec receptors; however, Siglec-11 and Siglec-16 were not included (*Jones et al.*, 2003).

It might be interesting to further study not only the interaction between these pathogens and Siglec-11 and-16 during the infection, but also the polymorphism in the *SIGLEC16* gene. Part of the human population is not able to express a functional Siglec-16 receptor due to a 4 base pair deletion in the *SIGLEC16 gene (Wang et al., 2012)*. This polymorphism might indicate there is a selection against the expression of Siglec-16. Although the current hypothesis and the work presented here describe that Siglec-16 helps to clear pathogens by increasing pro-inflammatory signaling, it remains to be studied if the presence of activating receptors results in a disadvantage for the host. This might depend on the level of Siglec-16 expression and its capability to mediate pro-inflammatory signaling. Whereas pro-inflammatory signaling might help to clear an infection, too much signaling might contribute to

tissue damage during an infection or to the systemic dysregulation of pro-inflammatory responses, which cause sepsis.
Furthermore, the SIGLEC16 polymorphism may contribute to the fact that the *N. meningitides* only causes systemic infections in very few individuals although 10% of adults carry *N. meningitides* as commensals in their nasopharnynx (*Virji*, 2009).

The discovery of *N. gonorrhoeae* engaging human Siglecs may be yet another mechanism that the pathogen uses to subvert the human immune system (*Virji*, 2009). It seems that activating receptors like Siglec-14 and Siglec-16 contribute to an increased pro-inflammatory response upon infection and might help to clear it. However, the role of pro-inflammatory responses in the gonococcal pathogenesis is ambivalent (*Virji*, 2009; *Criss and Seifert*, 2012). For example, *N. gonorrhoeae* has been reported to exploit and survive within neutrophils that are infiltrating the site of infection upon pro-inflammatory stimuli (*Criss and Seifert*, 2012). Multiple factors, like sex and site of infection, play a role in the effectiveness of a pro-inflammatory stimulus. Therefore, similar as describe above in the case of *E. coli* K1 and *N. meningitides*, it might be possible that in some circumstances activating Siglecs are beneficial for the host to clear the gonococcal infection, but in other circumstances they are disadvantageous. To answer this question more studies in different tissue cell lines as well as animals models have to be conducted.
Ascending gonococcal infections can also cause pelvic inflammatory disease, a major cause of infertility (*Gradison*, 2012). It might be important to study if interactions between *N. gonorrhoeae* and Siglecs contribute to pelvic inflammatory disease. If this is true, it could be a driver of selection for certain polymorphisms in *SIGLEC* genes, given the impact on fertility.

Gonorrhea might become untreatable due to resistance to available antibiotics. The supplementation strategy of CMP-legionaminic acid introduced described in Chapter 4 might be a promising novel strategy for prevention and treatment strategy.
It will be crucial to determine if human cells are able to utilize CMP-legionaminic acid and to incorporate it into their glycoconjugates. These would be disadvantageous because humans possess anti-legionaminic acid antibodies (Matthies et al., 2015) and therefore the treatment could lead to autoimmune reactions. Furthermore the level of CMP-sialic acids and therefore the degradation of are crucial for the success

of the treatment strategy. Over 40 years ago an enzyme that hydrolyzes CMP-sialic acids has been described, but so far the gene has not been identified (Kean and Bighouse, 1974).

The role of this enzyme is still unclear. It might be a measure to control the level of CMP-sialic acid. A certain serum level of CMP-sialic acid is needed sialylate certain proteins extracellular, however too much might be detrimental because pathogens like *N. gonorrhoeae* might take advantage of it. It needs to be explored if the hydrolase, which might be able to inactivate CMP-legionaminic acid as well, is expressed in the vaginal tract. This would be a disadvantage for the treatment strategy that utilizes CMP-legionaminic acid. It could be interesting to expand this strategy to other sialic acid analogs and nonulosonic acids as well as other pathogens. For example in the case of an infection by *H. influenzae*, a major cause of lower respiratory tract infections in infants and children in developing countries. *H. influenzae* uses free sialic acid to decorate its cell surface (*Vimr et al.*, 2000). If the pathogen can utilize other nonulosonic acid like legionaminic acid, the administration of it could help to clear the infection.

Many pathogens mimic sialic acid to subvert the host immune responses and many of these pathogens might interact with Siglecs. Siglecs already have been suggested for therapeutic interventions in cancer and autoimmune disease (*O'Reilly and Paulson*, 2009; *Jandus et al.*, 2011). With an increasing number of pathogens becoming resistant to antibiotic treatments, it might be interesting to explore Siglecs as potential targets for treatment of infections as well. To implement a Siglec based treatment strategy it will crucial to study the level of expression of Siglecs and the amount of inflammatory signaling they are able to mediate in respective tissues and the environment of the infection.

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Addendum 1:

# A Chemical Biology Solution to Problems with Studying Biologically Important but Unstable 9-O-Acetyl Sialic Acids

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*Manuscript submitted*

Contribution: Glycan incorporation in mammalian cells
*In vivo* glycan turn over studies

**Abstract**

9-O-Acetylation is a common natural modification on sialic acids (Sias) that terminate many vertebrate glycan chains. This ester group has striking effects on many biological phenomena, including microbe-host interactions, complement action, regulation of immune responses, sialidase action, cellular apoptosis, and tumor immunology. Despite such findings, 9-O-acetyl sialoglycoconjugates have remained largely understudied, primarily because of marked lability of the 9-O-acetyl group to even small pH variations, and/or the action of mammalian or microbial esterases. Our current studies involving 9-O-acetylated sialoglycans on glycan microarrays revealed that even the most careful precautions cannot assure complete stability of the 9-O-acetyl group. We now demonstrate a simple chemical biology solution to many of these problems by substituting the oxygen atom in the ester with a nitrogen atom, resulting in sialic acids with chemically and biologically stable 9-N-acetyl group. We present an efficient one-pot multienzyme method to synthesize a sialoglycan containing 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc) and compare it to the one with naturally occurring 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9$Ac_2$). Conformational resemblance of the two molecules was confirmed by computational molecular dynamics simulations. Microarray studies showed that a Neu5Ac9NAc-sialoglycan is a ligand for viruses naturally recognizing Neu5,9$Ac_2$, with a similar affinity but with much improved stability in handling and study. Feeding of Neu5Ac9NAc or Neu5,9$Ac_2$ to mammalian cells resulted in comparable incorporation and surface expression as well as binding to 9-O-acetyl-Sia-specific viruses. However, cells fed with Neu5Ac9NAc remained resistant to viral esterases and showed a slower turnover. This simple approach opens numerous research opportunities that have heretofore proved intractable.

**Introduction**

The first sialic acid discovered (crystallized by Gunner Blix from a hot mild acid extract of bovine submaxillary mucin in 1936) contained two acetyl groups, only one possibly attached to nitrogen (*Lundblad*, 2015). In retrospect, Blix likely isolated a 9-O-acetyl variant of the common sialic acid *N*-acetylneuraminic acid (Neu5Ac), 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9$Ac_2$) (**1, Figure 1**). Neu5,9$Ac_2$ along with *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are the three most frequently occurring sialic acid forms in mammals (*Schauer*, 1982b). It has long been known that 9-O-acetylation of sialic acids masks the recognition by influenza A virus hemagglutinin (*Rogers et al.*, 1986) and by other lectins such as Factor H (*Varki and Kornfeld*, 1980; *Shi et al.*, 1996), CD22/Siglec-2 (*Shi et al.*, 1996; *Sjoberg et al.*, 1994; *Kelm et al.*, 1994), and Sialoadhesin/Siglec-1 (*Kelm et al.*, 1994), while being required for the binding of influenza C virus hemagglutinin (*Rogers et al.*, 1986; *Herrler et al.*, 1985; *Herrler et al.*, 1987; *Muchmore and Varki*, 1987; *Enard et al.*, 2002). Despite such early recognition and understanding of their importance, studies of the biological significance of sialoglycans presenting this common O-acetylated form of sialic acid have lagged far behind those of the parent molecules. The reasons are many, and have been detailed elsewhere, including their lability to both acidic and basic conditions (often used in standard purification methods for glycans and glycoconjugates), their propensity to migrate from one position to another, and their relative or absolute effects in blocking sialidase action (*Schauer*, 1982a; *Rogers et al.*, 1986; *Schauer*, 1978). However, when it has been further studied, such O-acetyl group modification turns out to be a key determinant modulating recognition by viruses (*Rogers et al.*, 1986; *Langereis et al.*, 2015; *Song et al.*, 2016), antibodies (*Cheresh et al.*, 1984; *Padler-Karavani et al.*, 2013; *Padler-Karavani et al.*, 2012; *Lu et al.*, 2012), and mammalian lectins (*Sjoberg et al.*, 1994; *Kelm et al.*, 1994; *Padler-Karavani et al.*, 2012; *Padler-Karavani et al.*, 2014), as well as modulating sialidase action (*Schauer*, 1982b; *Rogers et al.*, 1986; *Schauer*, 1978) and cellular apoptosis (*Birks et al.*, 2011; *Malisan et al.*, 2002; *Malisan and Testi*, 2002; *Bhunia et al.*, 2002; *Chen and Varki*, 2002).

Overall, exploration of the functions of Neu5,9$Ac_2$ and its biological and pathological interactions has been greatly hampered by the chemical instability of the O-acetyl group and/or the esterase cleavage of such a group (*Herrler et al.*, 1985). Taken together with the fact that it is eliminated by basic conditions during conventional

approaches to glycomic analysis such as beta-elimination and permethylation, it has come to the point where 9-O-acetylation tends to be simply ignored in many studies. Synthetic analogs of Neu5,9Ac$_2$ with chemical modifications of the 9-position of sialic acids generated included a 9-N-acetyl analog, 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc) (**2**, **Figure 1**), which was shown to mimic Neu5,9Ac$_2$ in binding to influenza C virus without being destroyed by esterase activity of the hemagglutinin-esterase (*Herrler et al.*, 1992). This observation was not however further explored for chemical and biological studies of Neu5,9Ac$_2$. Herein, we report a more efficient chemoenzymatic method for synthesizing Neu5Ac9NAc and a sialoside containing this Neu5,9Ac$_2$ analog. Applications in glycan microarray and cell feeding studies are presented, laying the foundation for a new approach to elucidate the important roles of 9-O-acetylation of sialic acids.

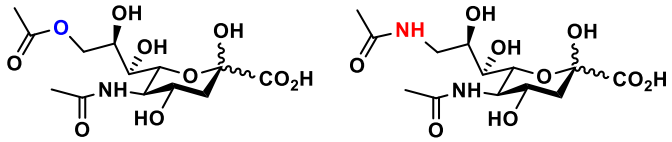



**Figure 1 I** Structures of 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$) (1) and its *N*-acetyl analog Neu5Ac9NAc (2).

## Results

*Problems with Instability of O-Acetyl Sialic Acids in Preparing and Using Glycan Microarrays*

The early described glycan microarrays did not have much representation of O-acetyl sialic acids (*Feizi et al.*, 2003). In an attempt to address this deficiency, our groups have been collaborating to synthesize, print, and study matched pairs of sialoglycans that either have or do not have O-acetyl esters. Due to their relative ease of synthesis and handling compared to 7-O- or 8-O-acetyl analogs, sialosides with 9-O-acetylated sialic acids were the ones studied first. Over the last few years we have been collaborating with several other groups to study the binding of various proteins to such glycans using glycan microarray technology (*Padler-Karavani et al.*, 2013; *Padler-Karavani et al.*, 2012; *Lu et al.*, 2012; *Padler-Karavani et al.*, 2014; *Feng et al.*, 2015; *Deng et al.*, 2014a; *Laubli et al.*, 2014; *Deng et al.*, 2014b; *Scobie et al.*, 2013; *Padler-Karavani et al.*, 2011). In revisiting some of these studies, we

noticed that some of the 9-OAc-glycans used for microarray printing were partially de-O-acetylated.

*Investigating and Minimizing 9-O-Acetyl Group Loss at Various Steps in Sample Preparation, Preparing and Using of Glycan Microarrays*

Based on the above observations, we investigated the extent of de-O-acetylation at the final steps of the synthesis and entire glycan arraying process, for the model 9-OAc-glycan Neu5,9$Ac_2$α3Galβ4GlcβPro$N_3$ and its reduced form Neu5,9$Ac_2$α3Galβ4GlcβPro$NH_2$. The azide-containing glycan was analyzed using nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS). It was found to be pure, without any loss of OAc group. The compound was then subjected to hydrogenolysis using $H_2$ and Pd-C in water with a drop of glacial acetic acid. The reaction was completed in one hour as detected using MS. To minimize de-O-acetylation during the celite filtration step, which uses $Na_2CO_3$ as a mild base (*Chokhawala et al.*, 2008), filtration after catalytic hydrogenation was replaced by passing the reaction solution through a nylon syringe filter (0.2 mm). The product was then purified using a short C-18 cartridge (water as an eluant) instead of the commonly and previously used Bio-Gel purification, as it is faster. The fractions containing the pure product were collected and lyophilized. No noticeable loss of the O-acetyl group was observed using the improved procedures described above.

Then, the reduced glycan was quantified for the OAc loss at each step of glycan microarray process. Our results revealed that ~45% of the OAc group was either lost from the 9-position of Neu5Ac or migrated to 7- and 8-positions of the sialic acid upon conventional analysis procedures including release of sialic acids with acetic acid, derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB), followed by high performance liquid chromatography (HPLC) analysis of fluorescent adducts (**Figure 2A**). In comparison, *Arthrobacter ureafaciens* sialidase (AUS) (*Chokhawala et al.*, 2007) treatment followed by DMB derivatization at low temperature (*Samraj et al.*, 2014) showed only 1.5% loss of the OAc group (**Figure 2B**). This means there is no significant de-O-acetylation during initial preparation of stock solution in water (it should be noted that the final synthetic product is a neutral sodium salt - earlier studies in which the acid form was prepared was associated with some de-O-acetylation, data not shown) and AUS treatment with low temperature DMB derivatization. However, about 3% loss of OAc was observed during storage in

phosphate buffer (pH 8.4) at room temperature, a condition popular for current microarray printing. Furthermore, about 5% of the sialic acid is de-O-acetylated under the standard blocking condition used for glycan arrays (ethanolamine in Tris-HCl buffer, pH 9.0). The glycan stock solution was also analyzed again after three months of storage at -20 °C and a few freeze-thaw cycles. It was noticed that another 4% of the OAc group was released over three months. Therefore, a small amount of de-O-acetylation occurs under the storage conditions and under conditions used for microarray printing and slide blocking. Any further loss of O-acetylation after printing during the binding and analysis, however, cannot be directly monitored.

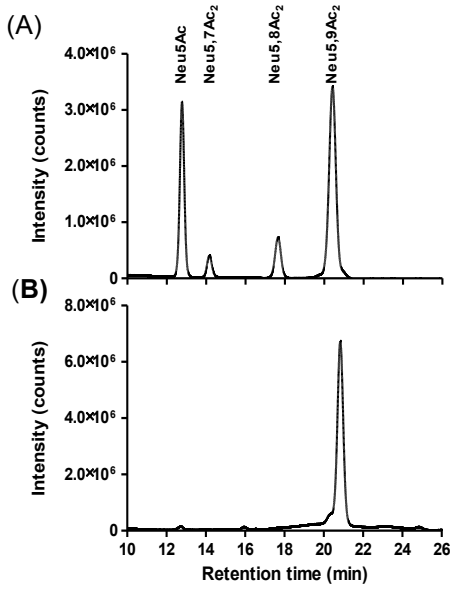



**Figure 2 I** DMB derivatization and HLPC analysis of sialic acids released from $Neu5,9Ac_2\alpha3Gal\beta4Glc\beta ProNH_2$ using acetic acid (A) and AUS (B).

Overall, definitive conclusions about binding of probes can therefore only be made where binding is exclusively to the O-acetylated sialoglycan spots and not to the corresponding non-O-acetylated ones, as discussed later. A more general conclusion is that, even under the most carefully managed handling by skilled experimentalists, some loss of 9-O-acetylation appears inevitable. Taken together with the knowledge that esterases of bacterial (*Corfield et al.*, 1988; *Corfield et al.*, 1993; *Steenbergen et al.*, 2009; *Rangarajan et al.*, 2011; *Phansopa et al.*, 2015) and vertebrate (*Hayes and Varki*, 1989; *Takematsu et al.*, 1999; *Schauer and Shukla*, 2008) origin are very common in biological systems, there is an intractable problem facing the systematic study of the chemistry and biology of sialic acid 9-O-acetylation.

*Chemical Synthesis of Neu5Ac9NAc Precursor 6-Acetamido-6-deoxy-N-acetylmannosamine (ManNAc6NAc) and Enzymatic Synthesis of Neu5Ac9NAc*

A new efficient chemoenzymatic synthetic strategy was developed for the synthesis of Neu5Ac9NAc. The design was to chemically synthesize ManNAc6NAc, a precursor of Neu5Ac9NAc, followed by a sialic acid aldolase-catalyzed reaction (*Yu et al.*, 2004; *Li et al.*, 2008) for the synthesis of desired Neu5Ac9NAc. ManNAc6NAc was synthesized in a 82% yield from previously obtained 6-azido-6-deoxy-*N*-acetylmannosamine ($ManNAc6N_3$) (*Yu et al.*, 2005) using a simple one step reduction and simultaneous acetylation process achieved by adding thioacetic acid to a pyridine solution containing $ManNAc6N_3$ (*Shangguan et al.*, 2003) Neu5Ac9NAc was readily obtained from ManNAc6NAc and pyruvate with a 78% yield using a reaction catalyzed by a sialic acid aldolase from *Pasteurella multocida* (PmAldolase) (*Li et al.*, 2008).

*One-pot Multienzyme (OPME) Synthetic Approach for Facile Production of Neu5Ac9NAcα3Galβ4GlcβProNH$_2$ for Glycan Microarray Studies*

ManNAc6NAc was also used directly for synthesizing a sialoside analog Neu5Ac9NAcα3Galβ4GlcβPro$N_3$ via an efficient one-pot multienzyme (OPME) sialylation system (*Yu et al.*, 2005; *Yu et al.*, 2006a; *Yu et al.*, 2006b) containing PmAldolase, (*Li et al.*, 2008) a CMP-sialic acid synthetase from *Neisseria meningitidis* (NmCSS) (*Yu et al.*, 2004), and *Pasteurella multocida* sialyltransferase 1 M144D mutant (PmST1 M144D) (*Sugiarto et al.*, 2012). Neu5Ac9NAcα3Galβ4GlcβPro$N_3$ was obtained in 84% yield and was readily converted in a quantitative yield to Neu5Ac9NAcα3Galβ4GlcβProNH$_2$ by catalytic hydrogenation using $H_2$ in the presence of Pd/C (*Yu et al.*, 2007).

*Glycan Microarray Studies of Viral Proteins and Human Siglec-9 Binding to Sialosides Containing Neu5,9Ac$_2$ and Neu5Ac9NAc*

The synthesized glycan Neu5Ac9NAca3Galb4GlcbProNH$_2$ was added to our previously described glycan library for glycan microarray studies (*Deng et al.*, 2014a). Proteins known to recognize Neu5,9$Ac_2$ were tested including immunoglobulin Fc-fused human Siglec-9-Fc (hSiglec-9) (*Zhang et al.*, 2000), as well as viral proteins (hemagglutinins in an esterase inactivated form) of porcine torovirus

(PToV-P4-Fc, PToV) and bovine coronavirus (BCoV-Mebus-Fc, BCoV) (*Langereis et al.*, 2015). The results were compared to those obtained using Neu5,9Ac$_2$α3Galβ4GlcβProNH$_2$, and Neu5Acα3Galβ4GlcβProNH$_2$ lacking 9-modification at sialic acid as probes in the same slides (**Figure 3**). Results from glycan microarray studies (**Figure 3A**) showed that hSiglec-9 prefers binding to Neu5Ac-glycan without 9-modification at sialic acid (white column), but with observable binding to both Neu5Ac9NAc (black column) and Neu5,9Ac$_2$ (grey column)-containing glycans. In comparison, both PToV and BCoV prefer binding to both sialoglycans with 9-modifications such as Neu5Ac9NAc and Neu5,9Ac$_2$. BCoV is a known viral protein probe for 9OAc sialic acids (both Neu5,9Ac$_2$ and Neu5Gc9Ac) and especially 7,9-di-OAc sialic acids (*Langereis et al.*, 2015). The more specific 9OAc-sialic acid viral probe PToV (prefers Neu5,9Ac$_2$ over Neu5Gc9Ac) (*Langereis et al.*, 2015) binds to both 9OAc and 9NAc glycans although it shows slightly stronger binding to the 9OAc derivative. The specific recognition of 9-modified sialic acid by esterase-inactive PToV and BCoV, the stability of 9NAc, and the esterase lability of 9OAc were evident by comparing the binding study results of esterase active PToV-treated samples (**Figure 3B**) with those non-treated ones (**Figure 3A**).

*Feeding of Free Neu5Ac9NAc and Neu5,9Ac$_2$ to Human Cells Followed by Measuring Cell Surface Incorporation with PToV Probes*

Human Burkitt lymphoma B cells (BJA-B K20) cells are hypo-sialylated due to the lack of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (*Hinderlich et al.*, 2001), therefore maximizing exogenous sialic acid incorporation. After three days of feeding with either 1 mM of free Neu5Ac9NAc or Neu5,9Ac$_2$, cells were probed with PToV-P4-Fc (PToV) with or without active esterase to measure cell surface incorporation of the sugars. Both Neu5Ac9NAc and Neu5,9Ac$_2$ were detected on the cell surface, but only Neu5Ac9NAc-fed ones were resistant to virus-hemagglutinin-esterase activity (**Figure 4**).

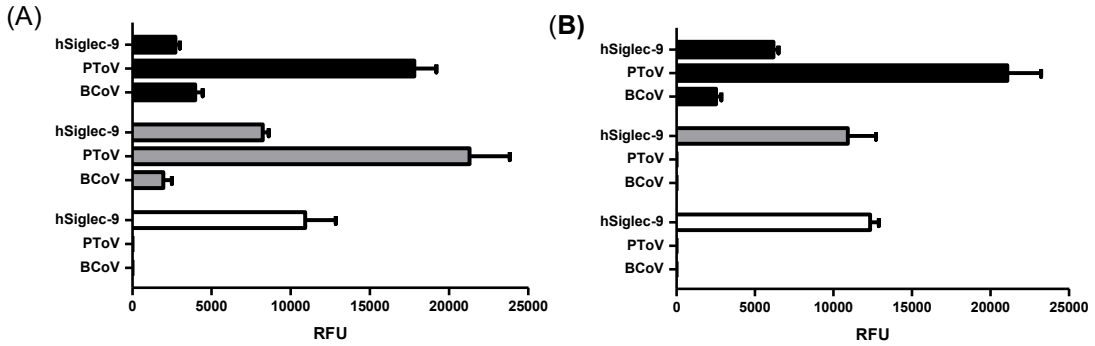



**Figure 3 I** Sialoglycan microarray binding specificity studies of human Siglec-9 (hSiglec-9-Fc, hSiglec-9), porcine torovirus hemagglutinin-esterase (PToV), and bovine coronavirus hemagglutinin-esterase (BCoV) (both PToV and BCoV were mutated to ablate their esterase activity) towards Neu5Ac9NAcα3Galβ4GlcβProNH$_2$ (black columns), Neu5,9Ac$_2$α3Galβ4GlcβProNH$_2$ (gray columns), and Neu5Acα3Galβ4GlcβProNH$_2$ (white columns) without esterase treatment (A) or treated with esterase active PToV (B).

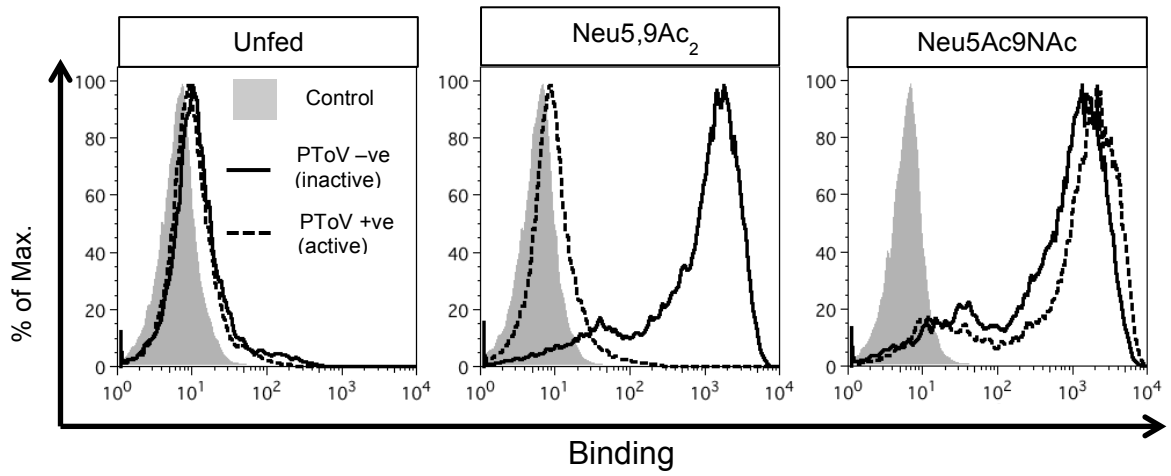



**Figure 4 I** Detection of incorporation of Neu5,9Ac$_2$ or Neu5Ac9NAc into hypo-sialylated human lymphoma BJA-B K20 cells. Cells were fed (or not) for 3 days with free Sias (1 mM) and then stained with PToV in inactive (mutated, solid black line) or active (dashed black line) O-acetylesterase forms. The binding was analyzed by flow cytometry.

*Feeding of Free Neu5Ac, Neu5,9Ac$_2$, Neu5Ac9NAc to Human BJA-B K20 Cells Followed by Measuring Cell Surface Incorporation of Sialic Acids with DMB Derivatization and HPLC Analysis*

BJA-B K20 cells were fed with 1 mM of Neu5Ac (**Figure 5A**), Neu5,9Ac$_2$ (**Figure 5B**), or Neu5Ac9NAc (**Figure 5C**) for 3 days. The cell membranes were recovered and sialic acids were released with *Vibrio cholerae* neuraminidase (*Chokhawala et al.*, 2007; *Varki and Diaz*, 1984). The released sialic acids were derivatized with DMB and subjected to HPLC analysis, which showed incorporation of each sugar on the cell membrane. Although BJA-B K20 cells are hypo-sialylated, they still express some Neu5Ac as can be observed on the cell membrane of Neu5,9Ac$_2$ or Neu5Ac9NAc-fed cells (**Figure 5B and 5C**).

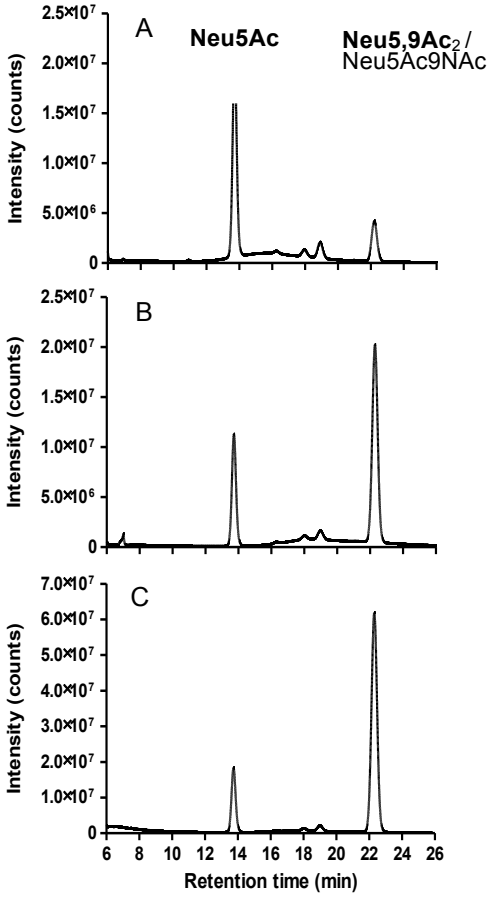



**Figure 5 I** DMB derivatization and HLPC analysis of sialic acids released from cell membranes of BJA-B K20 fed for 3 days with 1 mM of Neu5Ac (A), Neu5,9Ac$_2$ (B) or Neu5Ac9NAc (C).

*Monitoring Turnover of Neu5Ac9NAc and Neu5,9Ac$_2$ on the Cell Surface*

After feeding BJA-B K20 cells for two days with either 1 mM of free Neu5Ac9NAc or Neu5,9Ac$_2$, the sugars were removed from the cell culture medium (day 0) and the presence of the sialic acids on the cell surface was monitored for 4 days with the PToV (esterase inactive) probe (**Figure 6**). The turnover of Neu5,9Ac$_2$ on cell surface was shown to be faster than that of Neu5Ac9NAc.

*Feeding of Neu5Ac, Neu5,9Ac$_2$, and Neu5Ac9NAc to Human BJA-B K20 Cells Followed by Measuring Cell Surface Incorporation with Human CD22-Fc*

BJA-B K20 cells were fed with 3 mM of Neu5Ac, Neu5Ac9NAc, or Neu5,9Ac$_2$. After 3 days of feeding, BJA-B K20 cells were probed with human CD22-Fc/Siglec-2 to detect ligands (**Figure 7**). It was found that there was a high expression of CD22 ligand when the cells were fed with Neu5Ac (**Figure 7A**). However, when the cells were grown in the presence of Neu5Ac9NAc, the expression of the CD22 ligand was minimal (**Figure 7C**). The level of CD22 ligand expressed on the cells fed with

Neu5,9Ac2 (**Figure 7B**) was between the two which could be explained by possible partial de-O-acetylation of Neu5,9Ac2. This supports previous observations that human CD22-Fc recognizes α2–6-linked sialosides with Neu5Ac or N-glycolylneuraminic acid (Neu5Gc) as the preferred naturally existing sialic acid form and 9-O-acetyl modification on sialic acid blocks the binding (*Sjoberg et al.*, 1994; *Kelm et al.*, 1994). Furthermore, it demonstrates further that Neu5Ac9NAc can be a suitable substituent for natural Neu5,9Ac2 while has improved stability.

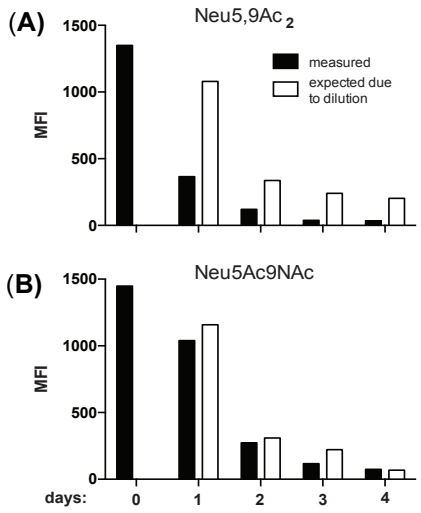



**Figure 6 I** Monitoring turnover of incorporated Neu5,9Ac$_2$ (A) and Neu5Ac9NAc (B) in BJA-B K20 cells. Cells were fed for two days with 1 mM of Neu5,9Ac$_2$ or Neu5Ac9NAc. After two days, the feeding with Neu5,9Ac$_2$ or Neu5Ac9NAc was stopped and the turnover of these sugars was measured with PToV (esterase inactive) probe by flow cytometry in the course of 4 days. The expected amount of sugar was calculated based on the initial MFI (Mean Fluorescence Intensity) at day 0 and the following cell doubling.

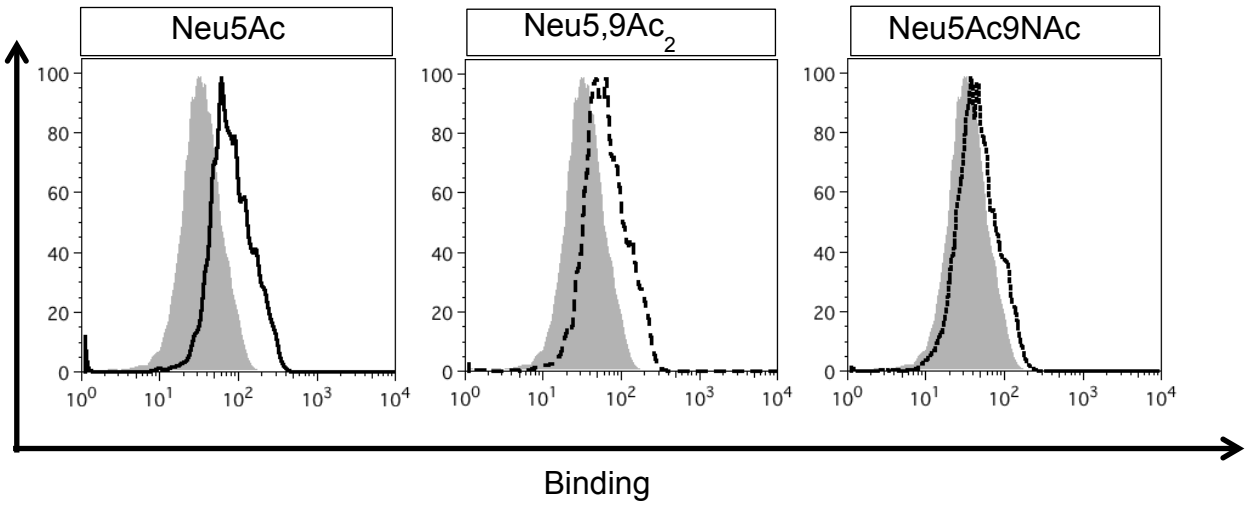



**Figure 7 I** Probing Neu5Ac, Neu5,9Ac$_2$ or Neu5Ac9NAc-fed human lymphoma BJA-B K20 cells with CD22-Fc to detect ligands. Cells were fed for 3 days with 3 mM of Neu5Ac (solid black line),

Neu5,9Ac$_2$ (dashed black line) or Neu5Ac9NAc (dotted line) and then stained with human CD22-Fc to detect ligands (and compared with non-fed cells, gray). The binding was analyzed by flow cytometry.

*Comparison of Sialosides Containing Neu5Ac9NAc and Neu5,9Ac$_2$ Using Molecular Dynamics*

Classical molecular dynamics (MD) simulations of Neu5,9Ac$_2$α3Galβ4GlcβProN$_3$ and Neu5Ac9NAcα3Galβ4GlcβProN$_3$ were carried out to investigate any differences that the chemical modification would introduce into the conformational ensemble in aqueous solution. The simulations were based on the GLYCAM carbohydrate force field (*Vanquelef et al.*, 2011) and TIP3P water model (*Jorgensen et al.*, 1983), with added electrostatic parameters for describing the N-acetylation. The total length of the simulation exceeded 2 ms for each of the two sialosides studied, which has previously been noted to provide good sampling of oligosaccharide conformational degrees of freedom (*Sattelle and Almond*, 2014).

**Figure 8** shows the results of our simulations projected onto the *(ϕ, ψ)* dihedral angles of the glycosidic linkage between Sia and Gal. The main observation is that the conformational ensembles of the N-acetylated and O-acetylated sialosides are highly equivalent. There are three free energy basins on the positive *ψ* half of the plot with the same shape and the relative free energies between the basins are similar to within 1 kcal/mol.

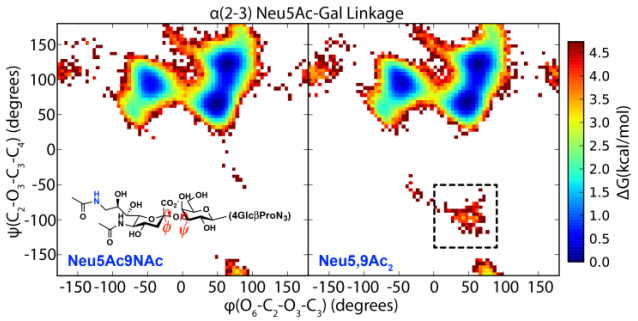



**Figure 8 I** Free energy diagram of glycosidic linkage in solution for Neu5Ac9NAcα3Galβ4GlcβProN$_3$ and the corresponding Neu5,9Ac$_2$α3Galβ4GlcβProN$_3$. The 9NAc and 9OAc analogs are compared in the left and right panels. The predominant states are almost identical for the two molecules, indicating that the 9NAc and 9OAc sialosides have similar structures in solution. The 9OAc sialoside has a sparsely populated state not seen in 9NAc (dotted box).

The classical MD simulation approximates the effect of the chemical modification as changing the force field parameters at the acetylation site. In particular, only the partial charges on the N-acetyl functional group are modified. To test this assumption, we used density functional theory to calculate the difference in electrostatic potential as a result of changing O-acetylation to N-acetylation

(*Harańczyk et al.*, 2007). The results are shown in **Figure 9** which confirm that the change in electrostatic potential is entirely localized to the acetylation site, which lends credence to the results of the classical simulations.

The O-acetylated sialoside appears to display a slightly higher amount of flexibility, as there exists a cluster of conformations at 3–4 kcal/mol higher in energy than the minimum (dotted box in **Figure 8**) where $\psi$ adopts negative values. These conformations represent far less than 1% of the whole ensemble, but are nonetheless interesting to investigate for understanding the effects of chemical modification. The overall conformational ensemble is shown in the top panel of **Figure 10**, and the negative-$\psi$ conformations are shown in the bottom panel, indicating that the position and orientation of Sia differ significantly between the full set vs. subset of conformations. The sialoside spends most of its time in an extended conformation, but occasionally it adopts a hairpin-like structure where the C7–C9 part of Sia bends back towards Glc. These hairpin conformations were not observed in the N-acetylated ensemble. The sialosides formed few intramolecular hydrogen bonds, which suggests that the chemical modification alters the conformational ensemble via the local hydrogen bonding network of the solvent (*Ren and Ponder*, 2003); a more detailed analysis of solvent degrees of freedom is needed to test this hypothesis.

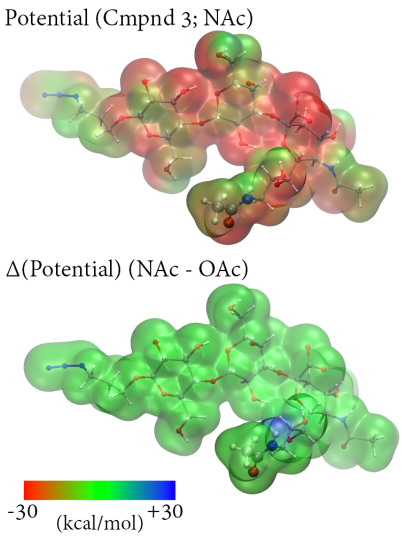



**Figure 9 I** Top: Calculated electrostatic potential for a representative sialoside structure extracted from simulations of Neu5Ac9NAcα3Galβ4GlcβProN$_3$ using the B3LYP/6-31G* level of theory (*Becke*, 1993; *Binkley et al.*, 1980) and plotted on the $r$=0.002 density isosurface. Bottom: Electrostatic potential difference computed between Neu5Ac9NAcα3Galβ4GlcβProN$_3$ and Neu5,9Ac$_2$α3Galβ4GlcβProN$_3$ plotted on the same isosurface; the O-acetylated potential was calculated by replacing *NH* with *O* in the N-acetylated structure. The difference between the potentials is very small, although *NH* group has a slightly positive potential relative to *O*.

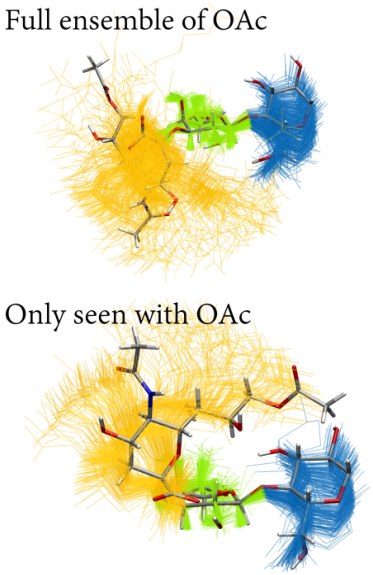



**Figure 10 l** Superimposed conformations of the 9-O-Ac sialoside simulations, showing only carbohydrate residues and aligned using galactose heavy atoms. One representative conformation is shown in licorice representation (*C*, gray; *O*, red; *N*, blue; *H*, white. *Top*: The full conformational ensemble of Neu5,9$Ac_2$α3Galβ4GlcβPro$N_3$ with snapshots taken 10 ns apart (Neu5,9$Ac_2$, yellow; Gal, green; Glc, blue). *Bottom*: The conformations corresponding to the dotted box in Figure 8 with snapshots taken 10 ps apart. These rarely seen conformations were only visited in the O-acetylated simulations but are only a small part of the ensemble, being higher in free energy.

## Discussion

This study began with the realization that even with the best of precautions, some de-O-acetylation occurs during preparation of sialoglycan arrays. In retrospect, definite conclusions to date by us and by others about 9-O-acetylation on such arrays can only be made in instances where binding is exclusively to the O-acetylated sialoglycan spot, and not to the corresponding non-O-acetylated one.[13] And even in this instance the ideal conclusive proof is to show loss of binding upon pretreatment with a Nidovirus 9-O-acetylesterase (such as PTOV and BCoV) (*Langereis et al.*, 2015). To be clear this does not overtly contradict our previous studies (*Padler-Karavani et al.*, 2013; *Padler-Karavani et al.*, 2012; *Lu et al.*, 2012; *Padler-Karavani et al.*, 2014; *Feng et al.*, 2015; *Deng et al.*, 2014a; *Laubli et al.*, 2014; *Deng et al.*, 2014b; *Scobie et al.*, 2013; *Padler-Karavani et al.*, 2011) as we rarely commented about the role of O-acetylation except when selective binding occurred (*Langereis et al.*, 2015). But it is important to prevent inadvertent misinterpretation of some of our published raw data such as binding patterns and heat maps, by readers who may not know the subtleties and to alert others who may

be studying these fascinating acetyl esters that modify important members of the vertebrate sialome.

Here we have presented a very practical solution to long-standing technical problems that have markedly inhibited progress in studies of the biology of 9-O-acetylated sialic acids. The strategy is replacing the labile OAc group with a more stable NAc group, which otherwise behaved functionally in a generally similar manner on microarrays, incorporation into cells after feeding, a subsequent expression on cell surfaces. The single obvious difference in both the array and cell feeding studies with or without binding studies using human CD22-Fc as a probe was the general resistance of the NAc group to both viral esterases and to endogenous cellular esterases. Of course in both instances we cannot rule out a very low level of esterase cleavage, and further studies are required.

The lack of cell lines that consistently express 9OAc sialic acids has also been a great limitation with cellular studies. The incorporation of the 9NAc molecules by feeding now allows one to ask if the cells become targets for infection by viruses that normally recognize the 9OAc group. Many other previously well-recognized but poorly studied biological or pathological roles of sialic acid *O*-acetylation may now become accessible. For example, while the disialoganglioside GD3 appears to protect cells from apoptosis, the unstable 9OAcGD3 is said to enhance apoptosis (*Birks et al.*, 2011; *Malisan et al.*, 2002; *Malisan and Testi*, 2002; *Bhunia et al.*, 2002; *Chen and Varki*, 2002). If feeding of cells expressing GD3 results in expression of 9NAc-GD3, this phenomenon can now be better studied. Likewise the potential role of O-acetylation in regulating Siglec functions could also now be better explored.

The rapid turnover of the *O*-acetyl groups in fed cells is very likely due to the action of a cytosolic sialic acid esterase we previously described (*Takematsu et al.*, 1999; *Varki et al.*, 1986; *Higa et al.*, 1987; *Butor et al.*, 1993). We predict the NAc-form to be resistant to this enzyme. The slower but continued turnover of the *N*-acetyl form is likely due to continued dilution by cell doubling. Assuming that the Neu5Ac9NAc is first degraded into ManNAc6NAc, the question also arises as to whether this molecule can be further metabolized. Biochemical analysis can be used to track possible outcomes.

In principle, the same approach could be tried to address difficulties in the studies of 4-O-acetyl sialic acid and the even more labile esters known to occur naturally at the

7- or 8- positions of sialic acids (*Schauer*, 1982b). A paper recently appeared showing an example of how this approach can work for 4-O-acetyl groups (*Bakkers et al.*, 2016). The approach can also potentially address the strong tendency for the 7-O-acetyl ester group to migrate to the 9-position under physiological conditions (*Schauer*, 1982b; *Varki and Diaz*, 1984; *Kamerling et al.*, 1987; *Diaz et al.*, 1989). While even more challenging to synthesize, N-acetyl versions of the 7,9-di-O-acetyl, 8,9-di-O-acetyl, 4,7-di-O-acetyl and 4,9-di-O-acetyl molecules that are known to occur in nature could be considered, as well as the 7,8,9-tri-O-acetyl entity. However, it remains to be seen if NAc analogs of such molecules recapitulate the conformation and biology of their natural counterparts, and also if they can all be efficiently incorporated by feeding the cells.

This approach could also potentially address the propensity of *O*-acetyl groups to migrate from one position to another (*Varki and Diaz*, 1984; *Kamerling et al.*, 1987). Even at near neutral/physiological pH values under which no significant de-*O*-acetylation occurred, the 7-*O*-acetyl group of Neu5,7$Ac_2$ is easily transferred to the 9-position, producing Neu5,9$Ac_2$ (possibly via an 8-position intermediate). Even more remarkably, the di-*O*-acetyl form Neu5,7,9$Ac_3$ quickly yields a dynamic equilibrium mixture of Neu5,7,9$Ac_3$ and Neu5,8,9$Ac_3$, in a molar ratio of ~1:1. Both the di-*O*-acetyl forms and the transient mono-8-*O*-acetyl form have been virtually impossible to study. Preparation of the NAc versions of these molecules could give the first hope of potentially understanding functions of the evanescent 8-*O*-acetyl group, and of differentiating between the recognition functions of 7,9 and 8,9 di-*O*-acetyl sialic acids, which are normally in a dynamic equilibrium state.

**Conclusions**

We present an efficient one-pot multienzyme method to synthesize a sialoglycan containing Neu5Ac9NAc and demonstrate that substituting the C9-oxygen atom in Neu5,9$Ac_2$ with a nitrogen atom is an effective approach to study sialosides containing unstable 9-O-acetyl sialic acid. Computational molecular dynamics simulations, mammalian cell feeding with or without binding studies using human CD22-Fc as a probe, and glycan microarray studies showed that Neu5Ac9NAc-sialoglycan structurally and conformationally resembles Neu5,9$Ac_2$-sialoglycan but with much improved stability in handling and study. This simple approach therefore

opens up numerous research opportunities to gain a new understanding of the biology that depends on the modification of sialic acids with O-acetyl group at various positions that have heretofore proved intractable.

**Methods**

*Materials*

Cy3 and PE affinipure goat anti-human IgG (H+L) antibodies were obtained from Jackson ImmunoResearch Laboratories. *Arthrobacter ureafaciens* sialidase (AUS) and *Vibrio cholerae neuraminidase* were purchased from EY Labs and Roche, respectfully. Chemical and biological material were purchased from commercial sources and used as received. Nuclear Magnetic Resonance (NMR) spectra were recorded in the NMR facility of the University of California, Davis on a Bruker Avance-400 NMR spectrometer (400 MHz for $^{1}$H, 100 MHz for $^{13}$C). Chemical shifts are reported in parts per million (ppm) on the d scale. High resolution electrospray ionization (ESI) mass spectra were obtained using a Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility at the University of California, Davis. Column chromatography was performed using Redi*Sep* Rf silica columns or an ODS-SM column (51 g, 50 mm, 120 Å, Yamazen) on the CombiFlash® Rf 200i system. Thin layer chromatography (TLC) was performed on silica gel plates (Sorbent Technologies) using anisaldehyde sugar stain for detection. Gel filtration chromatography was performed with a column (100 cm × 2.5 cm) packed with Bio-Gel P-2 Fine resins (Bio-Rad). *Pasteurella multocida* sialic acid aldolase (PmNanA), *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS), and *Pasteurella multocida* sialyltransferase 1 M144D mutant (PmST1 M144D) were expressed and purified as described previously.

*Preparation of Hemagglutinin-Esterase Probes for 9-OAc Sia*

The sequences for expressing the ectodomains of the hemagglutinin-esterase (HE) of two nidoviruses (PToV-P4 and BCoV-Mebus) were synthesized by GenScript (Piscataway, NJ), with varied degrees of insect codon optimization. To generate the probe molecules, the esterase domains were inactivated by changing the active site of the Ser residue to Ala by site-directed mutagenesis using Q5 mutagenesis (New England Biolabs). The HE proteins were linked to the baculovirus gp64 signal sequence peptide at their N-termini, and the C-terminus fused to a linker containing a

thrombin cleavage sequence, the Fc domain of Human IgG1, and a 6-His sequence. Constructs were cloned into pFastBac-1 (Life Technologies) to generate recombinant bacmids following the manufacturer's protocol. Recombinant baculoviruses were recovered by transfection of the bacmids into Sf9 insect cells using Cellfectin II (Life Technologies). Viruses were used to infect suspension High Five cells and the supernatant harvested 2–3 days post-infection. The proteins were purified by binding to a HiTrap ProteinG HP 5ml column (GE Healthcare Life Sciences, Piscataway, NJ) and eluted with 0.1M citrate, pH 3.0 (pH neutralization to 7.8 with 1 M of Tris-HCl, pH 9.0) using ÄKTA FPLC system (GE Healthcare Life Sciences). The HE-Fc containing fractions were dialyzed in PBS and concentrated using 30 kD Amicon Ultra-15 filters (EMD Millipore). Purified proteins were stored at -80 °C in aliquots.

*Cell Culture and Sugar Supplementation*

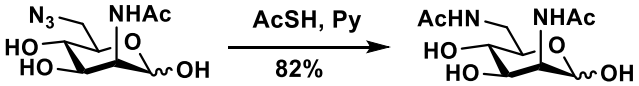
BJA-B K20 cells were propagated as suspension with RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per mL, 100 mg of streptomycin per mL in a humidified 5% $CO_2$, 37 °C atmosphere as described previously (*Hinderlich et al.*, 2001). For medium supplementation, Neu5Ac9NAc and Neu5,9$Ac_2$ were dissolved in PBS, titrated to a neutral pH and filter sterilized. The sugars were added at the indicated concentrations. For the sugar turnover experiments, the cells were fed as mentioned above and on day 0, the cell culture media was switched to RPMI 1640 medium, supplemented with 1% Nutridoma (Roche), 100 U of penicillin per mL and 100 mg of streptomycin without Neu5Ac9NAc and Neu5,9$Ac_2$.

*Flow Cytometry*

Cells were washed with PBS and incubated with 10 $\mu$g/mL of PToV probes in PBS with 1% BSA and 10 mM EDTA for 30 minutes on ice. Subsequently, cells were washed with PBS and incubated with PE goat anti-human IgG antibody for 30 minutes on ice. After an additional washing step, the cells were analyzed by FACSCalibur (BD).

*Synthesis of 2,6-Diacetamido-2,6-dideoxy-D-mannopyranose (ManNAc6NAc) from 2-acetamido-6-azido-2,6-dideoxy-D-mannopyranose (ManNAc6$N_3$)*

To a solution of ManNAc6$N_3$ (*Yu et al.*, 2005) (300 mg, 1.14 mmol) in pyridine (8 mL), thioacetic acid (2 mL) was added and the mixture was stirred at room temperature for 24 h. The solvent was concentrated in *vacuo*. The crude product was purified by column chromatography (ethyl acetate/methanol = 3:1) to produce ManNAc6NAc (262 mg, 82%) as a colorless amorphous solid. $^1$H NMR (400 MHz, $D_2O$) a-isomer: d 5.10 (d, *J* = 1.5 Hz, 1H), 4.31 (dd, *J* = 4.7, 1.5 Hz, 1H), 4.04 (dd, *J* = 9.8, 4.7 Hz, 1H), 3.96–3.86 (m, 1H), 3.68–3.33 (m, 3H), 2.07 (s, 3H), 2.03 (s, 3H); b-isomer: d 5.00 (d, *J* = 1.7 Hz, 1H), 4.46 (dd, *J* = 4.5, 1.7 Hz, 1H), 3.82 (dd, *J* = 9.0, 4.5 Hz, 1H), 3.68–3.33 (m, 4H), 2.11 (s, 3H), 2.04 (s, 3H); $^{13}$C NMR (100 MHz, $D_2O$) d 175.70, 174.77, 174.54, 174.53, 93.01, 74.43, 71.73, 70.24, 68.58, 68.49, 68.20, 53.98, 53.21, 40.27, 40.16, 22.04, 21.90, 21.77; HRMS (ESI) Anal. Calcd for $C_{10}H_{19}N_2O_2$ $[M+H]^+$: 263.1243, Found: 263.1241.




*Synthesis of 5,9-Diacetamido-3,5,9-Trideoxy-D-glycero-D-galacto-2-nonulopyranosylonic Acid (Neu5Ac9NAc)*

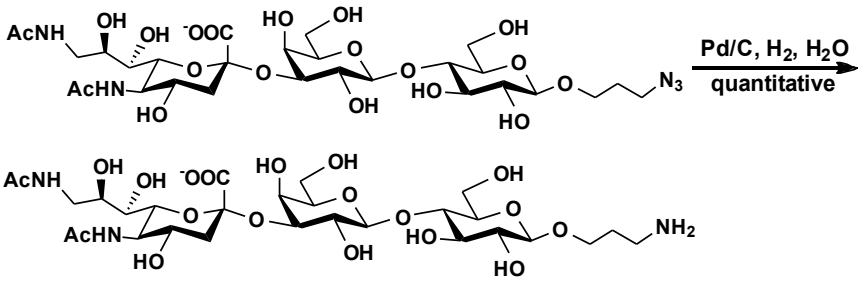
To a solution (10 mL) containing ManNAc6NAc (50 mg, 0.19 mmol), sodium pyruvate (210 mg, 1.9 mmol), and Tris-HCl buffer (100 mM, pH 7.5), PmAldolase (3.0 mg) was added and the reaction was incubated in an isotherm incubator for 48 h at 37 °C with agitation at 100 rpm. The reaction was quenched by adding the same volume of ice-cold ethanol and incubating at 4 °C for 1 h. The solvent was removed and the crude product was purified by column chromatography (ethyl acetate/methanol/water = 4:2:1) and followed by a short BioGel P-2 gel filtration column to give Neu5Ac9NAc (55.4 mg, 78%) as a colorless amorphous solid. $^1$H NMR (400 MHz, $D_2O$) d 4.06–3.92 (m, 3H, H-4, H-6, H-5), 3.80 (ddd, *J* = 9.0, 7.7, 3.1 Hz, 1H, H-8), 3.59 (dd, *J* = 14.1, 3.1 Hz, 1H, H-9), 3.44 (dd, *J* = 9.0, 1.1 Hz, 1H, H-7), 3.24 (dd, *J* = 14.1, 7.6 Hz, 1H, H-9), 2.22 (dd, *J* = 12.9, 4.8 Hz, 1H, H-3e), 2.06 (s, 3H, COC$H_3$), 2.02 (s, 3H, COC$H_3$), 1.83 (dd, *J* = 12.9, 11.4 Hz, 1H, H-3a); $^{13}$C NMR (100 MHz, $D_2O$) d 176.64, 174.68, 174.56, 96.36, 70.09, 69.77, 68.77, 67.25, 52.26, 42.76, 39.35, 22.08, 21.82; HRMS (ESI) Anal. Calcd for $C_{13}H_{21}N_2O_9$ $[M-H]^-$: 349.1247, Found: 349.1255.

*Synthesis of 3-Azidopropyl O-(5,9-Diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Ac9NAcα3Galβ4GlcβProN$_3$)*

Neu5Ac9NAcα3Galβ4GlcβProN$_3$ was synthesized following the general procedure for enzymatic synthesis of sialosides. Galb4GlcbProN$_3$ (*Yu et al.*, 2005) (46 mg, 0.11 mmol), ManNAc6NAc (43 mg, 0.16 mmol), sodium pyruvate (91 mg, 0.83 mmol) and CTP (87 mg, 0.16 mmol) were dissolved in Tris-HCl buffer (100 mM, pH 8.5, 10 mL) containing 20 mM of $MgCl_2$. The pH of the solution was further adjusted to 8.5 with 4 M NaOH. PmAldolase (1.5 mg), NmCSS (2.5 mg) and PmST1 M144D (2.5 mg) were added. The reaction was incubated in an isotherm incubator for 24 h at 37 °C with agitation at 100 rpm. The reaction was quenched by adding the same volume of ice-cold ethanol and incubating at 4 °C for 1 h. The formed precipitates were removed by centrifugation and the supernatant was concentrated. The residue was purified by passing through a BioGel P-2 gel filtration column followed by a C18 column ($H_2O/CH_3CN$ = 10:1) (70.9 mg, Yield 84%). $^1$H NMR (400 MHz, $D_2O$) d 4.52 (dd, *J* = 12.8, 7.9 Hz, 2H), 4.10 (dd, *J* = 9.9, 3.2 Hz, 1H), 4.06–3.86 (m, 4H), 3.88–3.53 (m, 13H), 3.54–3.43 (m, 3H), 3.37–3.23 (m, 2H), 2.76 (dd, *J* = 12.4, 4.6 Hz, 1H), 2.04 (s, 3H), 2.04 (s, 3H), 1.92 (p, *J* = 6.6 Hz, 2H), 1.81 (t, *J* = 12.1 Hz, 1H); $^{13}$C NMR (100 MHz, $D_2O$) d 174.92, 174.39, 173.81, 102.60, 102.11, 99.87, 78.17, 75.53, 75.14, 74.78, 74.33, 72.79, 72.73, 69.96, 69.58, 69.36, 68.30, 67.46, 67.34, 61.01, 60.01, 51.66, 47.86, 42.11, 39.61, 28.22, 22.02, 21.81; HRMS (ESI) Anal. Calcd for $C_{28}H_{46}N_5O_{19}$ [M-H]$^-$: 756.2787, Found: 756.2792.

*Synthesis of 3-Aminopropyl O-(5,9-Diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Ac9NAcα3Galβ4GlcβProNH$_2$)*

A catalytic amount of 10% palladium on charcoal (Pd/C) was added to the solution of Neu5Ac9NAcα3Galβ4GlcβProN$_3$ (5.6 mg) in $H_2O$ (1 mL). The mixture was stirred under hydrogen atmosphere for 3 h. The solution was diluted with MeOH (2 mL) and passed through a filter to remove the catalyst. The solvent was concentrated in *vacuo* to give Neu5Ac9NAcα3Galβ4GlcβProNH$_2$ (5.4 mg, quant.) as a colorless amorphous solid. HRMS (ESI) Anal. Calcd for $C_{28}H_{48}N_3O_{19}$ [M-H]$^-$: 730.2882, Found: 730.2914.




*Reduction of Neu5,9Ac$_2$α3Galβ4GlcβProN$_3$ to Neu5,9Ac$_2$α3Galβ4GlcβProNH$_2$*

Neu5,9Ac$_2$α3Galβ4GlcβProN$_3$ (20 mg) was dissolved in $H_2O$/MeOH (3 mL, 2:1 by volume) and a drop of glacial acetic acid was added. The mixture was stirred under hydrogen atmosphere in the presence of 10% palladium on charcoal for 1 h. The reaction mixture was then passed through a HyperSep C18 cartridge (1 g, 40–60 mm, Thermo) and eluted with water. The collection fraction containing the desired product was collected and lyophilized. The obtained white power was stored at -20 °C for long-term storage.

*HPLC Analysis of Neu5,9Ac$_2$a3Galb4GlcbProNH$_2$*

Neu5,9Ac$_2$α3Galβ4GlcβProNH$_2$ was first hydrolyzed in glacial acetic acid (2 M) for 3 h at 80 °C. AUS hydrolysis was performed based on the company protocol. DMB derivatization was performed as it is reported previously (*Hara et al.*, 1989). The DMB-derivatized samples were analyzed on a Dionex Ultra3000 HPLC System using a Phenomenex Gemini 5 mm C18 250 × 4.6 mm HPLC column at room temperature. The fluorescence was detected at 448 nm using excitation at 373 nm.

*Glycan Microarray Screening*

Glycan microarrays were fabricated using epoxide-derivatized slides (Corning by Thermo Fisher Scientific) and Arrayit SpotBot® Extreme Microarray Spotter as previously described (*Padler-Karavani et al.*, 2012; *Deng et al.*, 2014a; *Hinderlich et al.*, 2001). The arrays were printed with Stealth SMP3 microarray spotting pins from ArrayIt (Sunnyvale, CA, USA) generating 100 µm diameter spots. 4 pins were used; with each pin printing 4 replicate spots/well. The glycoconjugates were prepared at 100 *µ*M concentration in an optimized printing buffer (300 mM phosphate buffer, pH 8.4). They were then distributed into a 384-well source plate in 20 mL per well. To monitor printing quality, 4 replicate-wells of human IgG (Jackson ImmunoResearch) were used at 100 *µ*g/ml (in PBS) for each printing-pin. One complete array was

printed on each slide (within approx 12 hour/~28 slides). The humidity level in the arraying chamber was maintained at about 60–65% during printing. Printed slides were left on spotter deck overnight, allowing humidity to drop to ambient levels. Printed glycan microarray slides were blocked by 50 °C pre-warmed blocking solution (0.1 M Tris-HCl, 0.05 M ethanolamine, pH 9.0, 1 h). They were then washed twice with 50 °C pre-warmed water and dried. Slides were packed, vacuum-sealed and stored at RT until used.

To assure the quality of the printed slides, we used one or two slides for quality control using antibodies, lectins and serum samples with known glycan binding specificity. For glycan array binding, slides were fitted in a multi-well microarray hybridization cassette (AHC4X8S, ArrayIt, Sunnyvale, CA, USA) to divide into 8 subarrays. The subarrays were blocked with Ovalbumin (1% w/v) in PBS (pH 7.4) for 1 h at RT, with gentle shaking. For the esterase activity studies on the array, after removing the blocking solution, slides were first treated with 20 mg/mL PToV-P4-Fc (esterase active) in blocking buffer for 2 h at RT and then washed with PBS in 0.1% Tween 20 and then PBS (10 min/wash with shaking). Subsequently, diluted protein samples in blocking solution with various concentrations were added to each subarray (both esterase treated and non-treated slides). After incubating the protein samples for 2 h at RT with gentle shaking, the slides were washed with PBS in 0.1% Tween 20 and then PBS (10 min/wash with shaking). Cy3 affinipure goat anti-human IgG (H+L) antibody (Jackson ImmunoResearch) was used for the detection of BCoV-Mebus-Fc, PToV-P4-Fc and human Siglec-9-Fc. Diluted antibody in PBS was added to the subarrays and incubated for 1 h at RT. They were washed with PBS in 0.1% Tween 20, PBS and water (10 min/wash with shaking) and dried. The microarray slides were scanned using a Genepix 4000B microarray scanner (Molecular Devices Corp., Union City, CA, USA) at 100% laser power, PMT Gain 450 and 10 $\mu$M pixels. Data analysis was performed using Genepix Pro 7.0 analysis software (Molecular Devices Corp., Union City, CA) and the outputs were saved as gpr and jpg files. The gpr files then were saved as xls. The data were further analyzed with Excel. Local background subtraction was performed and data were plotted separately for each subarray. The binding specificity to glycoconjugates for each protein was plotted based on the RFU (Relative Fluorescence Units), average fluorescence value for 4 replicates, versus glycan IDs. The standard deviations were calculated and the error

bars were found for each glycan binding. The final graphs (Figure 3) were plotted based on the tested proteins versus RFU for each glycan in GraphPad Prism 5.

*Comparison of N-Acetylated and O-Acetylated Sialosides Using Molecular Dynamics*

The molecular dynamics simulations were carried out using the AMBER15 software suite, principally using *tleap* for setup and *pmemd.cuda* for dynamics (*Salomon-Ferrer and Case…*, 2013). The simulations employed the GLYCAM force field and TIP3P water model; the sialoside was solvated in a rectangular water box with roughly 1000 water molecules. The simulations used a 2.0 fs time step and a Langevin thermostat set to 298.15 K and a collision frequency of 1.0 $ps^{-1}$. The equilibration simulations used a Berendsen barostat set to 1.0 atm, a compressibility of $44.6 \times 10^{-6}$ $bar^{-1}$ and a time constant of 1.0 $ps^{-1}$; the production simulations were carried out at constant volume. The particle mesh Ewald method was used to treat long range electrostatics with a real-space cutoff of 8.0 Å. All input files and parameters are provided as part of the Supporting Information.

The parameters for the *N*-acetyl functional group were developed following the procedure outlined in Reference (*Kirschner et al.*, 2008). The initial guess force field for Neu5Ac9NAc was constructed by replacing the 9-O-acetyl group with a copy of the 5-N-acetyl group and the corresponding force field parameters. After 100 ps of equilibration in the NPT ensemble, we generated 50 ns of NVT dynamics, and saved 50 snapshots at 1 ns intervals. These snapshots were energy-minimized in the TeraChem software package with the dihedral angles constrained, employing the restricted Hartree-Fock method and 6-31G* basis set (*Wang and Song*, 2016; *Ufimtsev and Martínez*, 2008; *Ufimtsev and Martinez*, 2009b; *Ufimtsev and Martinez*, 2009a). The energy-minimized geometries were used as input to a restrained electrostatic potential calculation carried out using the R.E.D. server using the RESP-C2 charge model as appropriate for the GLYCAM force field (*Vanquelef et al.*, 2011). In the charge optimization, the charges for the terminal hydroxyl group were kept at the initial values and all hydrogen charges set to zero. The resulting final set of charges on the N-acetyl group has a slightly different total charge from the original O-acetyl group; the charge on the carbon atom that the N-acetyl group is bonded to is increased by +0.0014, instead of the +0.008 value used for O-acetyl. An analogous calculation was carried out for the $ProN_3$ functional group to obtain a complete set of charges for Neu5,9$Ac_2$α3Galβ4GlcβPro$N_3$. The valence force field

parameters for $ProN_3$ were copied over from the general AMBER force field (GAFF) (*Wang et al.*, 2004) by comparing GLYCAM atom types to GAFF atom types.

After the parameterization was finished, the production simulations of $Neu5,9Ac_2\alpha3Gal\beta4Glc\beta ProN_3$ and $Neu5Ac9NAc\alpha3Gal\beta4Glc\beta ProN_3$ were carried out to collect data for the free energy plots. The simulations were unbiased and each simulation ran for 2.5 $\mu$s, and configurations were saved every 10 ps. The free energy plot was constructed by projecting the ensemble of configurations onto the selected dihedral degrees of freedom.

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**Abbreviations**

AUS, *Arthrobacter ureafaciens* sialidase; DMB, 1,2-diamino-4,5-methylenedioxybenzene; ESI, electrospray ionization; HE, hemagglutinin-esterase; HPLC, high performance liquid chromatography; HRMS, hig resolution mass spectrometry; ManNAc6NAc, 6-acetamido-6-deoxy-*N*-acetylmannosamine; MD, molecular dynamics; MS, mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid; $Neu5,9Ac_2$, 9-*O*-acetyl-*N*-acetylneuraminic acid; Neu5Ac9NAc, 9-acetamido-9-deoxy-*N*-acetylneuraminic acid; NMR, nuclear magnetic resonance; OPME, one-pot multienzyme; Sia, sialic acid; TLC, thin layer chromatography.

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