Host-Pathogen Interactions in Guillain-Barré Syndrome:

The role of *Campylobacter jejuni* lipooligosaccharide sialylation

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Host-Pathogen Interactions in Guillain-Barré Syndrome:

The role of *Campylobacter jejuni* lipooligosaccharide sialylation

Gastheer-pathogeen interacties in Guillain-Barré syndroom:
De rol van *Campylobacter jejuni* lipooligosaccharide sialylering

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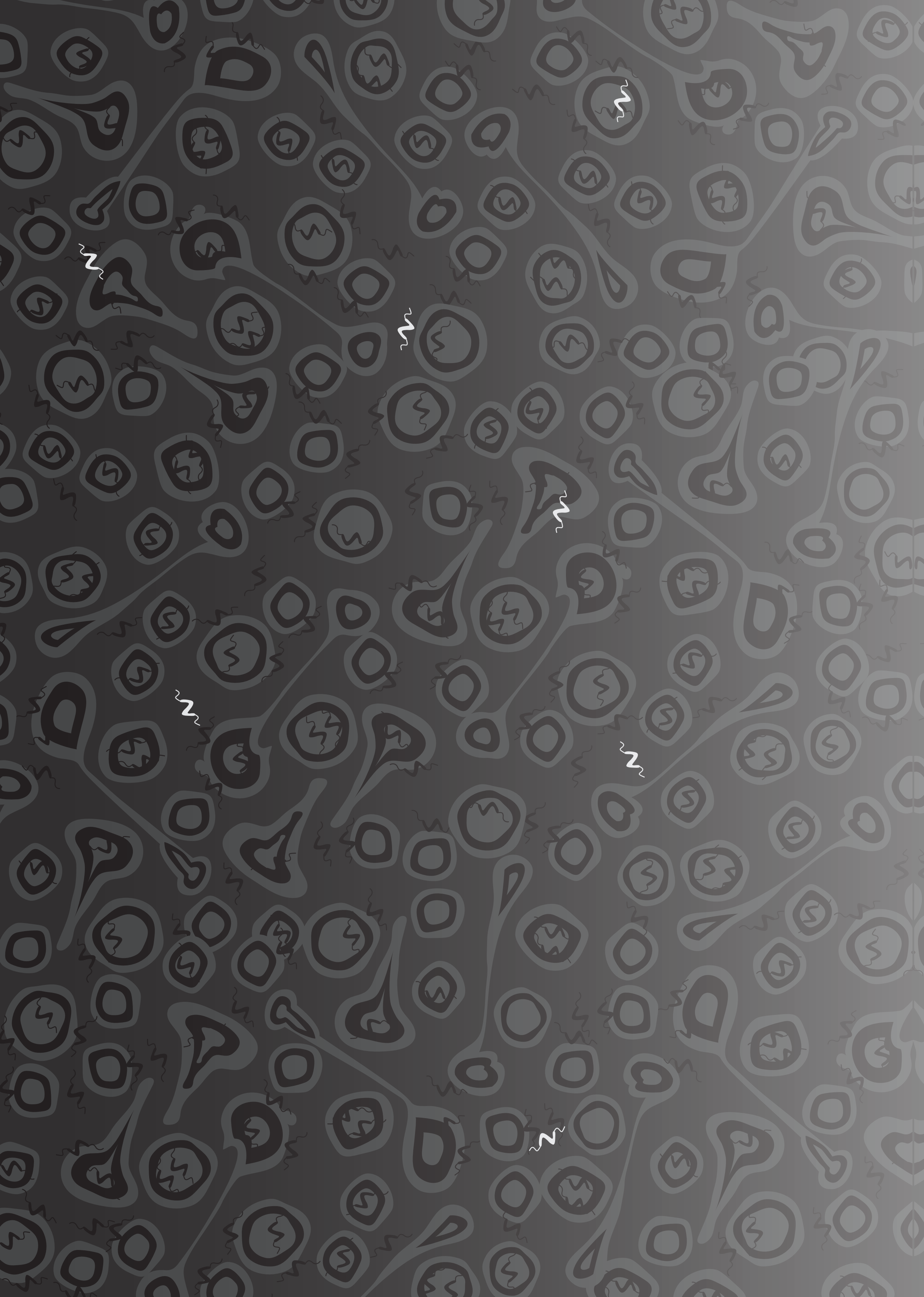
Contents

Chapter 1	General introduction	7
Chapter 2	Particular lipooligosaccharide loci and capsule types co-occur in Guillain-Barré syndrome associated *Campylobacter jejuni*	29
Chapter 3	The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion	49
Chapter 4	Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides	69
Chapter 5	Enhanced, sialoadhesin-dependent uptake of Guillain-Barré syndrome-associated *Campylobacter jejuni* strains by human macrophages	91
Chapter 6	Siglec-7 specifically recognizes *Campylobacter jejuni* strains associated with oculomotor weakness in Guillain-Barré syndrome and Miller Fisher syndrome	111
Chapter 7	Selective depletion of neuropathy-related antibodies from human serum by monolithic affinity columns containing synthetic gangliosides	129
Chapter 8	General discussion	145
Chapter 9	Summary	163
Chapter 10	Nederlandse samenvatting	171
	Curriculum vitae	181
	PhD Portfolio	183
	List of publications	187
	Dankwoord	191



Chapter 1

General introduction

Introduction

Pathogenesis of *Campylobacter jejuni* infection

Campylobacter jejuni (*C. jejuni*) is a spiral, comma-shaped Gram-negative bacterium which is motile due to bipolar flagella. *C. jejuni* is frequently present in the intestines of poultry and birds, where it is considered to be part of the normal intestinal flora (1). During slaughter procedures, poultry meat products often become contaminated with fecal content containing *C. jejuni* (2, 3). As a consequence of the extensive consumption of chicken worldwide, the handling of raw chicken and ingestion of undercooked chicken meat are the main causes of *C. jejuni* infection in humans (3). Apart from poultry, other sources of *C. jejuni* infection include raw milk, (swimming) water and pets (4).

Upon ingestion, *C. jejuni* can pass through the human gastrointestinal tract without clinical symptoms; however, infection with *C. jejuni* will often lead to a diarrheal illness (5). In the Netherlands, approximately 80,000 people per year (range, 30,000 – 160,000) are estimated to experience acute gastroenteritis caused by *Campylobacter* (6). The symptoms include fever, abdominal pain, and slimy or bloody diarrhea that lasts for several days (7). *C. jejuni* diarrhea is self-limiting, though complications such as bacteraemia, post-infectious reactive arthritis or Guillain-Barré syndrome (GBS) occasionally occur. In view of the broad spectrum of clinical disease presentations associated with *C. jejuni* infection, microbial as well as host factors are likely to contribute to *C. jejuni* pathogenesis.

In order to cause disease, *C. jejuni* must be able to move away from the peristaltic force in the intestinal lumen and penetrate the intestinal mucus to reach the underlying intestinal epithelium (Fig.1). Several components in the mucus layer aid in defense against

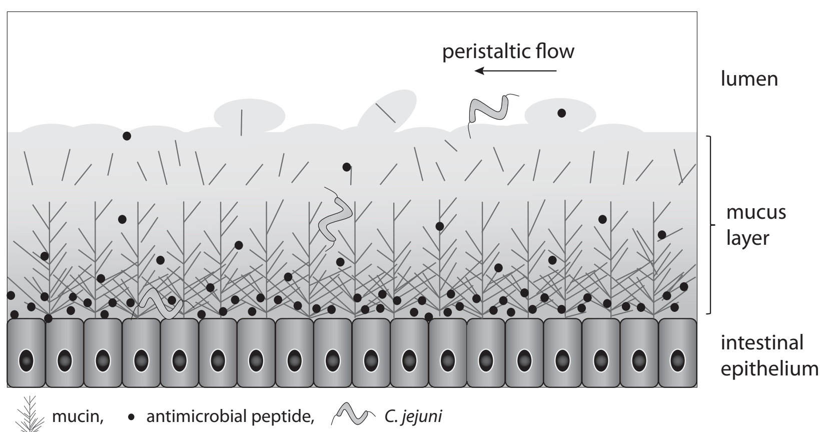


Figure 1. The luminal environment. Schematic representation of the luminal environment showing the intestinal lumen, and the mucins and antimicrobial peptides in the mucus layer which overlies the intestinal epithelium.

pathogens. To be able to cross the mucus, *C. jejuni* has to evade or combat these defense systems. For example, *C. jejuni* needs to avoid getting trapped in mucins, large net-like polymers able to physically block, stick- or bind to bacteria that penetrate the mucus layer (8). The broad range of bactericidal antimicrobial peptides, produced by epithelial cells, are another important component of the intestinal mucosal defense system which *C. jejuni* needs to combat next (9).

The small size and spiral (corkscrew) appearance of *C. jejuni* (Fig. 2A) are morphological characteristics of the bacterium that facilitate penetration of the mucus layer. Bipolar flagella facilitate motility and enable *C. jejuni* to move towards the mucus, which is an attractive, nutrition-rich environment for *C. jejuni*. A slimy polysaccharide capsule is present on the outer membrane of *C. jejuni* (Fig. 2B). The capsule prevents entrapment of bacteria in lumenal mucins and protects against environmental stress, such as osmotic changes (10, 11). Lipooligosaccharides (LOS) expressed on the surface of *C. jejuni* play a role in defense against mucosal antimicrobial peptides (12).

The ability of *C. jejuni* to adhere to and invade intestinal epithelial cells contributes to the pathogenicity of this bacterium. Contact with the epithelium can provoke inflammatory responses and may lead to damage of this cellular barrier. Several *C. jejuni* virulence factors play a part in epithelial adhesion and invasion. The current view of the *C. jejuni* adhesion/invasion process is that the binding of particular bacterial surface adhesins to their cognate receptors on intestinal epithelial cells is required to initiate bacterial invasion (13). Recently, it was demonstrated that the *C. jejuni* proteins CadF (Campylobacter adhesion to fibronectin) and FlpA (fibronectin-like protein A) act in a coordinated manner to bind fibronectin on the extracellular matrix of intestinal epithelial cells (14). The cooperative action of fibronectin binding and flagella-mediated secretion of Cia proteins (Campylobacter invasion antigens) stimulates host cell membrane ruffling (14). Subsequently, *C. jejuni*-induced intracellular signaling events promote bacterial uptake through invoking rearrangement of the epithelial cell cytoskeleton (14). Cytolethal distending toxin (CDT) is a major virulence factor that triggers cell cycle arrest and apoptosis in epithelial cells through DNase activity (15). *C. jejuni* starts to shed outer membrane vesicles containing CDT upon sensing of epithelial cells (16). CDT also induces interleukin-8 secretion from epithelial cells, which could contribute to inflammatory diarrhea (17). Other *C. jejuni* proteins reported to be involved in epithelial adhesion and/or invasion include PorA (porin), cj0091 (putative lipoprotein), JlpA (a surface-exposed lipoprotein), pldA (phospholipase A), CapA (putative autotransporter), DNAJ (transcriptional regulatory), racR (reduced ability to colonize) and FlaA (flagellin subunit A) (18-22). The capsule and LOS also play a role in epithelial invasion (23, 24). The regions associated with the biosynthesis of the capsule and the LOS are two of the most variable regions in the genome of *C. jejuni* (25). Gene variability in these regions has resulted in the display of a great variety of both capsule and LOS structures by

different *C. jejuni* strains (26, 27). It is unclear whether particular capsule structures or LOS structures are associated with a higher invasion potential.

Clearly, *C. jejuni* is equipped with many virulence factors (Fig. 2C). Although *C. jejuni* related disease is limited to gastroenteritis in the majority of individuals, severe post-infectious complications such as GBS can develop in rare cases.

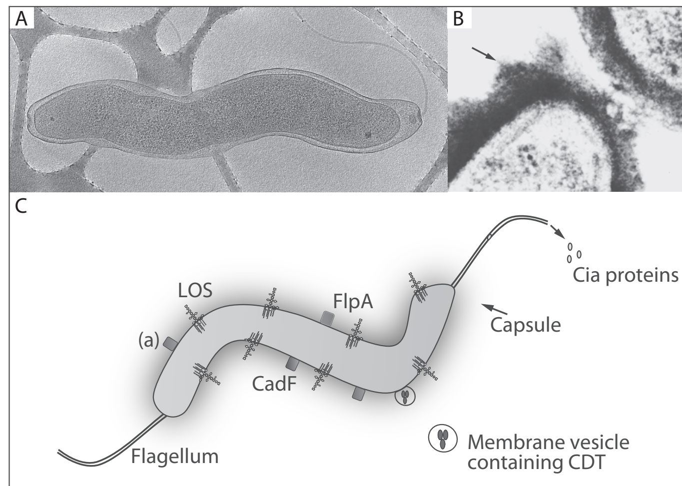


Figure 2. *Campylobacter jejuni*. **A.** Cryo-electron tomographic visualization of *C. jejuni* supported by a lacey carbon film (kindly provided by R. I. Koning). **B.** Electron microscopy visualization of a *C. jejuni* fragment. Alcian Blue dye was used to stain the polysaccharide capsule (indicated by the black arrow; copyright A. V. Karlyshev ©). **C.** Schematic presentation of *C. jejuni* showing the capsule, bipolar flagella, surface proteins CadF and FlpA, flagellum secreted Cia proteins, membrane vesicle secreted CDT and membrane-anchored LOS. CadF, Campylobacter adhesion to fibronectin; FlpA, fibronectin-like protein A; Cia, Campylobacter invasion antigen; LOS, lipooligosaccharides; (a) other surface proteins or lipoproteins.

Guillain-Barré syndrome

With a global incidence of 1-2 cases per 100,000 population annually, GBS is the most common cause of acute neuromuscular paralysis worldwide (28). GBS is a post-infectious, immune-mediated disease of the peripheral motor and sensory nerves, which is characterized by symmetric pain, sensory deficits and weakness of the limbs (29-32). The disease is monophasic, with an acute progressive phase, a plateau phase and a recovery phase (33). About one-third of patients with GBS require artificial respiration; in US hospitals, mortality is reported in 2-12% of the cases (34, 35). The rate of GBS-mediated mortality may be higher in developing countries, due to the unavailability of proper treatment (36). After the symptoms have stabilized, most patients gradually improve within a period of weeks to months, depending on the severity of neurological damage. After six months, 20% of patients with GBS are still unable to walk without support

(37). Severe fatigue is a common sequelae, which has been reported in 60-80% of GBS patients, and can last for years (38, 39).

In particular, the peripheral motor and sensory nerves and the peripheral nerve roots are damaged in patients with GBS. Peripheral nerves carry the signal impulses from the central nervous system to the limbs and organs, through cable-like bundles of axons. The axons are surrounded by myelin-producing Schwann cells which protect the axon and increase the speed of nerve conduction. Gangliosides are enriched in the membranes of both axons and Schwann cells (40, 41) (Fig. 3).

Based on nerve conduction studies and pathological criteria, GBS has been classified into different subtypes. Notable differences in geographic distribution between these subtypes were identified. Two axonal subtypes of GBS, acute motor axonal neuropathy (AMAN) and acute motor-sensory axonal neuropathy (AMSAN), occur more frequently in China, Japan, India and Central America (36, 42-44). A demyelinating subtype, acute inflammatory demyelinating polyneuropathy (AIDP), is the most common form of GBS in Europe and Northern America (45). Miller Fisher syndrome (MFS) is a restricted variant of GBS characterized by a lack of coordination (ataxia), loss of tendon reflexes (areflexia) and paralysis of the eye muscles (oculomotor weakness, ophthalmoplegia), without limb weakness (46). MFS affects around 5% of GBS patients in Western countries, but is more common in Eastern Asia (47)

During the acute phase of GBS, auto-antibodies with specificity for gangliosides are frequently detected in patient serum (48, 49). These antibodies can bind to ganglioside structures present on human peripheral nerves (45, 50). Electrophysiological and histological studies have revealed demyelination or axonal degradation of the peripheral nerves in patients (51-53). The mechanism by which auto-antibodies elicit nerve damage is presumably as follows: antibody binding to gangliosides on the peripheral nerves leads to activation of the complement system. For example, in fatal cases of GBS and in animal models, C3b complement deposition was detected on myelin membranes (in case of AIDP) and nodes of Ranvier (in case of AMAN) (Fig. 4) (54-56). As a consequence

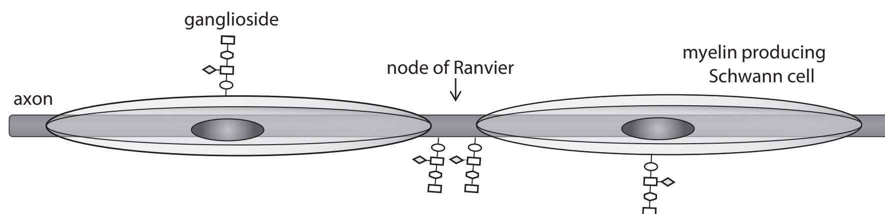


Figure 3. Schematic representation of a peripheral nerve. A peripheral nerve is composed of an axon which is surrounded by myelin producing Schwann cells. Gangliosides are imbedded in the outer membrane of both, the axon and the Schwann cells. Nodes of Ranvier are periodic gaps between myelin sheaths.

of C3b deposition, further activation of the complement cascade occurs, resulting in generation of the complement membrane attack complex (MAC, C5b-9). MAC deposits can be detected on Schwann cell membranes in nerve biopsies of patients with acute AIDP (56, 57), and the formation of these deposits has been reported to precede myelin degradation (58). Additionally, studies using *ex vivo* animal models demonstrated that accumulation of MAC on axons results in nerve disruption and nerve injury, by disturbance of the membrane potential which is crucial for fluent nerve conduction (59). Macrophages also seem to be involved. Histopathological characterization of nerve tissue from patients with GBS has revealed the presence of macrophages (52), which appear within days after the onset of nerve dysfunction (56). However, it is unclear what the role of these macrophages is. Possibly, macrophages recruited upon complement activation may cause further damage to the nerves. Alternatively, the macrophages may

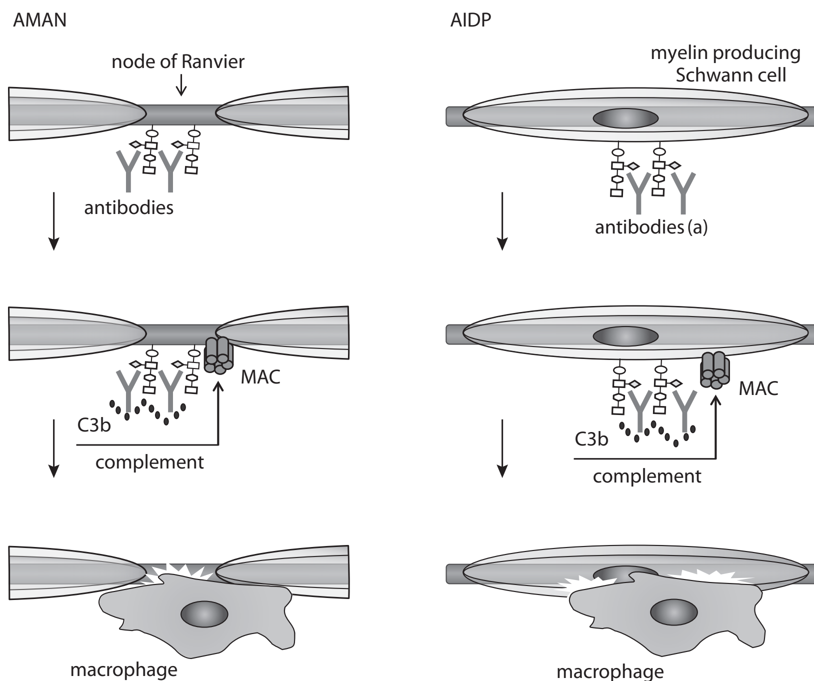


Figure 4. Mechanisms of nerve damage in AMAN and AIDP. In AMAN (left panel), cross-reactive anti-ganglioside antibodies bind to gangliosides in the nodes of Ranvier; whereas in AIDP (right panel), gangliosides in the membrane of Schwann cells are targeted. Antibody binding leads to activation of the classical complement pathway, resulting in insertion of the membrane attack complex (MAC). This causes axonal damage in AMAN patients and disruption of myelin in patients with AIDP. Macrophages either cause further damage or are involved in the clearance of damaged tissue fragments. (a) The role of anti-ganglioside antibodies in AIDP is less firmly established than in AMAN.

be involved in the scavenging and clearance of neural tissue fragments which have been disrupted by the complement MAC.

The role of anti-ganglioside antibodies in AIDP, the demyelinating form of GBS, is currently debated. A recent study proposes that anti-ganglioside antibodies only play a role in AMAN and that AMAN patients may have been erroneously classified as AIDP (60).

Antecedent infection and molecular mimicry

GBS is frequently preceded by a microbial infection, one to three weeks prior to the onset of disease. Serological studies have demonstrated that 30-40% of patients with GBS had been recently infected with *C. jejuni* (61). Although less frequent, other pathogens such as cytomegalovirus, Epstein-Barr virus, *Mycoplasma pneumoniae* and *Haemophilus influenzae* are also associated with GBS (61, 62). In about half of the patients, no prior infection is detected. Unidentified triggers, such as asymptomatic bacterial of viral infections, may be involved in the onset of disease in these patients.

The structural similarity (molecular mimicry) between sialylated carbohydrate structures expressed by microbes and ganglioside structures present in human peripheral nerves plays an important role in the development of GBS (63-65). During infection, an antibody response directed to microbial antigens is induced. For *C. jejuni* and *Haemophilus influenzae* in particular, it has been established that due to the structural similarity of bacterial antigens and self-antigens (gangliosides), cross-reactive auto-antibodies can be formed (66, 67). These antibodies not only target the microbe but, in rare cases, also the neural tissue of the host. Predominantly, the gangliosides GM1, GM1b, GD1a and GalNAc-GD1a and ganglioside complexes are targeted (68-70). This leads to complement-mediated tissue destruction, as mentioned above. In *C. jejuni* and *Haemophilus influenzae*, ganglioside mimicry can be found within the LOS expressed on the bacterial surface (71, 72). A clear causal relationship between other GBS-associated microbes and the onset of GBS has not yet been established but molecular mimicry may play a role. Cytomegalovirus does not express ganglioside mimics, but induces ganglioside GM2 expression on cytomegalovirus-infected fibroblasts (73). Anti-GM2 antibodies found in CMV-infected patients with GBS are hypothesized to be induced by the CMV infection (73). Anti-ganglioside antibodies have also been detected in patients with GBS who had a recent infection with Epstein-Barr virus or *Mycoplasma pneumoniae*. It has been speculated that these microbes can also express ganglioside mimics, or that they induce the expression of ganglioside-like structures on host cells (62, 74).

Campylobacter jejuni lipooligosaccharide and molecular mimicry

C. jejuni LOS is composed of two covalently linked domains: lipid A, a hydrophobic membrane anchor; and a surface exposed, non-repeating core oligosaccharide, consisting

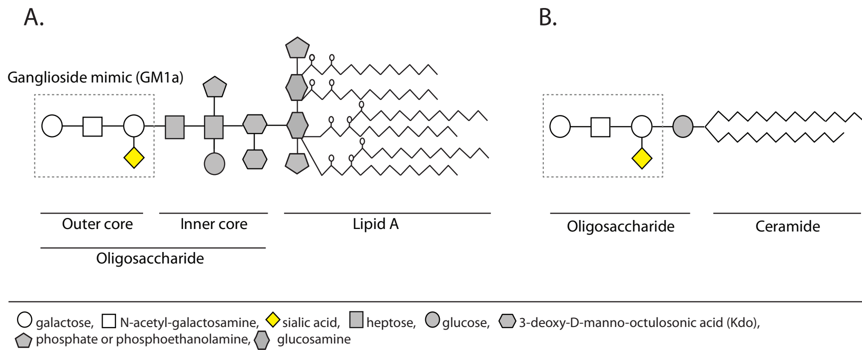


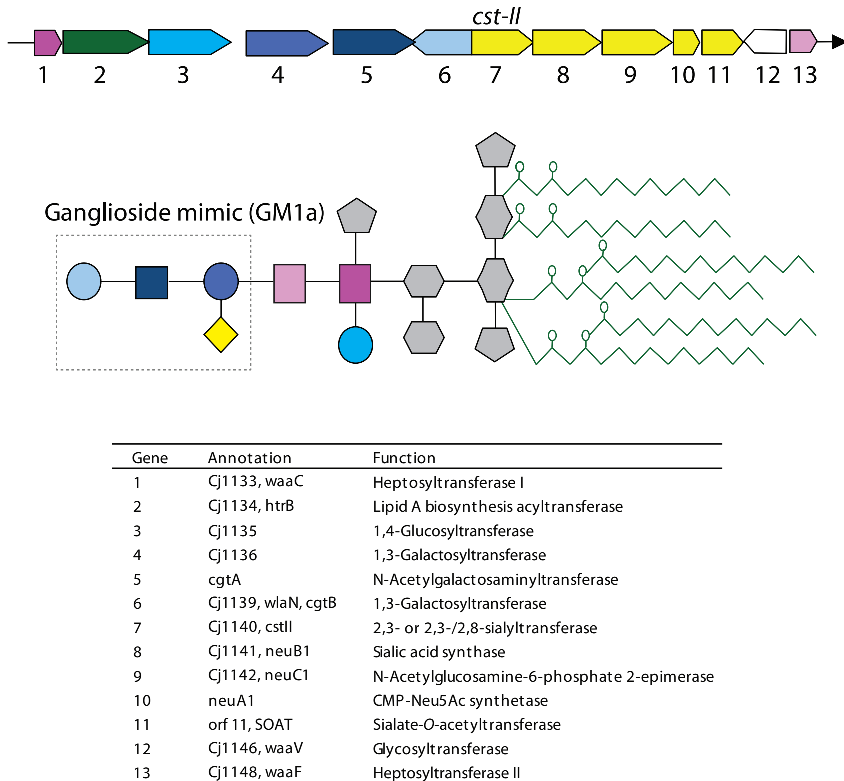
Figure 5. Ganglioside mimicry. A subset of *C. jejuni* strains express LOS that contain epitopes which mimic gangliosides. **A.** Schematic representation of an LOS structure that contains a ganglioside mimic. Ganglioside mimicry occurs in the outer oligosaccharide core and is indicated here as GM1a. The inner and outer cores are surface-exposed. Lipid A is embedded in the outer cell wall layer of *C. jejuni*. **B**. Schematic representation of a ganglioside (GM1a). Gangliosides are glycosphingolipids with an extracellular sialylated oligosaccharide chain and a ceramide tail that is embedded in the outer leaflet of the plasma membrane. Gangliosides are predominantly found in nerve cell membranes but are present in membranes of other cell types as well.

of an inner and outer core region. Only the outer core region of the LOS can contain epitopes that mimic nerve gangliosides (Fig. 5).

The *C. jejuni* genes involved in ganglioside mimicry are located within the LOS biosynthesis locus, a genetically highly-diverse gene cluster that is interchangeable between strains (75, 76). Genetic diversity has led to the formation of several LOS genotypes (LOS locus classes). Thus far, 19 distinct LOS locus classes, A through S, have been identified (27). Genes involved in the production of ganglioside mimics are only present in LOS classes A, B, C, M and R (27). In particular, the genes responsible for the synthesis, modification and transfer of sialic acid, found in the A, B, C, M and R LOS classes, are necessary for the production of ganglioside mimics, and are crucial for the induction of anti-ganglioside antibodies and hence GBS (77). Gene alterations, mutations and other mechanisms such as phase variation within these LOS classes (A, B, C, M and R) all contribute to structural variations in the ganglioside mimics produced (76).

Comparative genotyping has demonstrated that *C. jejuni* strains with LOS class A (Fig. 6) are associated with the development of GBS (77, 78), and strains with LOS class B are associated with the development of MFS (77, 78).

The *C. jejuni* sialyltransferase Cst-II, sialylates the LOS, and consequently determines the expression of ganglioside-mimicking LOS structures. Intriguingly, variability in *cst-II* can be detected in the *C. jejuni* strains isolated from GBS and MFS patients, and is highly associated with the clinical phenotype of the subsequent disease. Due to polymorphisms in the gene sequence, *C. jejuni* Cst-II can either be mono-functional (Thr51),



Gene	Annotation	Function
1	Cj1133, waaC	Heptosyltransferase I
2	Cj1134, htrB	Lipid A biosynthesis acyltransferase
3	Cj1135	1,4-Glucosyltransferase
4	Cj1136	1,3-Galactosyltransferase
5	cgtA	N-Acetylgalactosaminyltransferase
6	Cj1139, wlaN, cgtB	1,3-Galactosyltransferase
7	Cj1140, cstII	2,3- or 2,3-/2,8-sialyltransferase
8	Cj1141, neuB1	Sialic acid synthase
9	Cj1142, neuC1	N-Acetylglucosamine-6-phosphate 2-epimerase
10	neuA1	CMP-Neu5Ac synthetase
11	orf 11, SOAT	Sialate-*O*-acetyltransferase
12	Cj1146, waaV	Glycosyltransferase
13	Cj1148, waaF	Heptosyltransferase II

Figure 6. *C. jejuni* genes involved in the production of ganglioside mimics. Schematic representation of genes present in an LOS class A biosynthesis locus, together with an LOS structure and gene annotation and gene function of genes present in a class A biosynthesis locus. LOS class A is associated with GBS and contains the genes necessary for the production of the ganglioside mimic GM1a. Other ganglioside mimics including GM3-, GM2-, GD3-, GD1a- and GD1c- mimics can also be produced with a class A LOS biosynthesis locus. Color coding is used to represent gene functionality for LOS production. Genes 7-11 (yellow) are involved in sialic acid biosynthesis and transfer. A ganglioside mimic is only produced when sialic acid is present in the LOS outer core. Mutation of gene 7 (*cst-II*), which encodes a sialic acid transferase, leads to an LOS structure without sialic acid. *Cst-II* knockout mutants were used in chapters 3-6.

with α(2,3)-sialyltransferase activity, or bi-functional (Asn51) with both α(2,3)- and α(2,8)-sialyltransferase activities (76). There is a connection between the functionality of Cst-II and the syndrome that develops after *C. jejuni* infection (79). Strains with mono-functional Cst-II express monosialylated LOS structures that mimic, for example, the gangliosides GM1a, GM1b, GM2 and GD1a present on peripheral nerves (Fig. 7). Mono-sialylated *C. jejuni* strains are predominantly isolated from the stools of GBS patients, and in agreement with this observation, antibodies against monosialylated structures are frequently detected in the serum of patients with GBS (77, 80). Bi-functional Cst-II leads

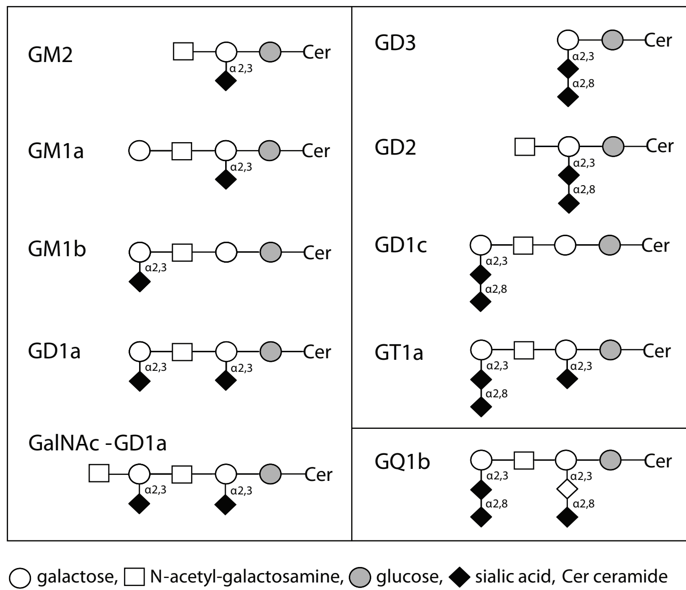


Figure 7. Mono- and disialylated ganglioside structures. Schematic illustration of the ganglioside structures relevant for this thesis. These ganglioside structures can be mimicked by the *C. jejuni* outer core LOS, except for GQ1b. However, instead of a ceramide-bound glucose which is present in gangliosides, the *C. jejuni* LOS contains a heptose, followed by an inner sugar core. *C. jejuni* LOS also has a lipid A transmembrane tail instead of a ceramide tail. Monosialylated structures with α(2,3)-linked sialic acid residues are represented in the left panel, Disialylated structures with α(2,3/2,8)-linked sialic acid residues are represented in the right panel.

to the production of disialylated gangliosides-mimicking structures, including GD2-, GD3-, GD1c- and GT1a-mimics, which share a structural resemblance with epitopes on ganglioside GQ1b (Fig. 7). The presence of anti-GQ1b antibodies is associated with GBS variants characterized by oculomotor weakness, including MFS (79, 81, 82). Interestingly, ganglioside GQ1b is highly enriched in the human cranial nerves innervating the eye muscles which are affected in MFS, explaining the vulnerability of these structures to anti-GQ1b antibodies (83).

Immune activation leading to GBS

The induction of an antibody response to sialylated LOS is a crucial step in the pathogenesis of GBS. It is largely unknown how these carbohydrate structures are recognized and presented by the host immune system, or how the immune system is activated to produce cross-reactive anti-ganglioside antibodies. In order to determine which cells and receptors might play a role in immune activation, the primary focus lies on immune cells which *C. jejuni* initially encounters during its passage through the intestinal mucosa.

Bacteria can cross the intestinal epithelial barrier in at least three ways (Fig. 8). First, some pathogens have the capacity to invade the epithelium and gain access to the sub-epithelial lamina propria. Second, bacteria can invade CX3CR1+ antigen presenting cells that can extend their dendrites in between epithelial cells after dislodging tight junctions (84, 85). Third, bacteria can be taken up via specialized epithelial microfold cells (M-cells) (86). M-cells are equipped with a large variety in pattern recognition receptors (PRRs) and are located on top of Peyer's patches, a mucosa associated lymphoid tissue in which adaptive immune responses are mounted.

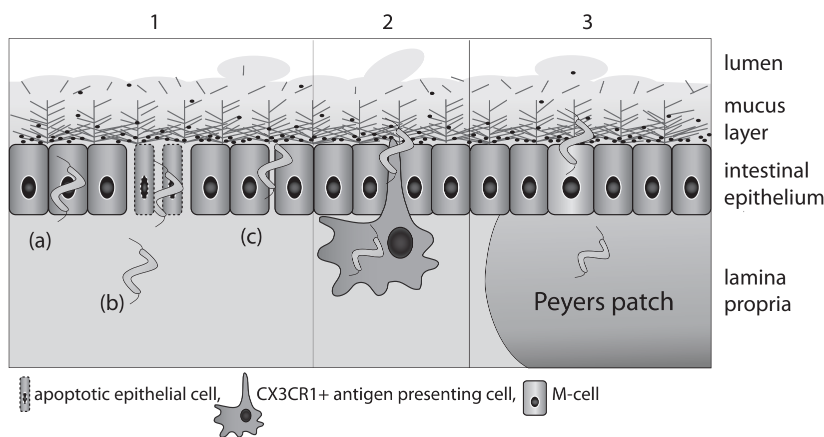


Figure 8. Potential mechanisms by which *C. jejuni* crosses the intestinal epithelium.
C. jejuni could cross the intestinal epithelium; (1) by invasion of intestinal epithelium cells through (a) cell-invasion, (b) cell-damaging or (c) disruption of tight junctions between epithelial cells; (2) by invasion of CX3CR1+ antigen presenting cells; (3) by invasion of specialized epithelial M-cells (microfold cells)

Residential macrophages and dendritic cells, situated beneath the intestinal epithelium, come into contact with *C. jejuni* once the bacterium has passed through the intestinal epithelium. Macrophages sense and kill invading bacteria, whereas sensing of bacterial fragments by dendritic cells induces activation of signaling pathways resulting in cytokine and chemokine production. $CD103^{+}$ dendritic cells have the capacity to

migrate to the intestine draining lymph nodes where they can present antigens to naïve T cells and induce T cell differentiation (87).

The immune receptors involved in the binding of sialylated *C. jejuni* LOS are likely to contribute to the immune-events leading to anti-ganglioside antibody formation. In this regard, the sialic acid-binding immunoglobulin-like (Siglec) family is an interesting family of receptors. Siglecs are expressed on the surface of immune cells and are involved in cell-to-cell communication, immune signaling and the binding of pathogens (88). Each Siglec contains an N-terminal 'V-set' Ig domain which binds sialic acid-containing ligands (Fig. 9). A variable number (1-16) of 'C-set' Ig domains extend the ligand binding

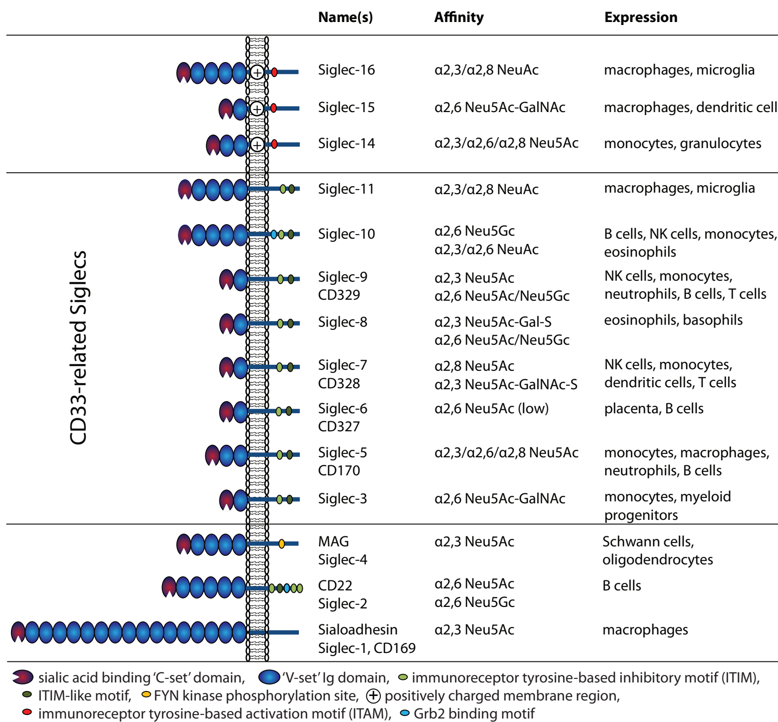


Figure 9. Human Siglecs. Sialic acid-binding immunoglobulin like-lectins (Siglecs) contain a sialic acid binding 'C-set' Ig domain and a variable number of 'V-set'Ig domains. Each Siglec has a distinct specificity for binding to sialylated ligands. Siglecs are predominantly expressed on immune cells but have also been detected on other cell types including Schwann cells and oligodendrocytes. Sialoadhesin, MAG (myelin-associated glycoprotein, Siglec-4) and Siglec-15 do not contain intracellular signaling motifs. CD22 and most CD33-related Siglecs have immunoreceptor tyrosine-based inhibitor motifs (ITIM) and ITIM-like motifs in their cytoplasmic tail. Siglec-14, -15 and -16 contain a positively-charged trans-membrane spanning region. This positive charge mediates the association with DAP12, an adaptor protein which bears an immunoreceptor tyrosine-based activation motif (ITAM). A FYN kinase phosphorylation site is present on MAG. CD22 and Siglec-10 contain Grb2 binding motifs. Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; Gal, Galactose, GlcNAc, N-acetylglucosamine; S, Sulphate.

site away from the membrane surface (89). A subgroup of the Siglec family, serve as regulators of the immune system through tyrosine-based signaling motifs located in their cytoplasmic tail (90, 91). The cytoplasmic tails of sialoadhesin (Siglec-1, Sn) and MAG (myelin-associated glycoprotein, Siglec-4) lack tyrosine-based signaling motifs.

Each Siglec displays distinct specificity for recognition of particular sialylated ligands. Siglec-7, expressed on NK cells, monocytes, dendritic cells and T cells, binds to sialylated *C. jejuni* strains (88). Specific recognition of sialylated LOS versus sialylated LOS by the host immune system can be considered as a crucial step in anti-ganglioside antibody formation. Binding of Siglec-7 to *C. jejuni* may therefore be an initial immune event that contributes to the eventual development of GBS. Hence, therefore it is important to determine whether GBS-associated *C. jejuni* strains specifically bind to Siglec-7 or other members of the Siglec family, and what the consequences of this binding are for the infection and pathogenicity of *C. jejuni*.

Besides immune recognition, immune activation is another pivotal step in antibody formation. Pattern recognition receptors expressed on dendritic cells and macrophages are committed to sense pathogens in their immediate environment. Toll-like receptors (TLRs) are responsible for the induction of cytokine production upon sensing of microbial structures. A recent study demonstrated that sialylation of LOS elicits increased cytokine production by dendritic cells via activation of TLR4, leading to enhanced B cell proliferation (92). TLR4 recognizes the lipid A part of LOS, suggesting that co-receptors, including Siglecs, may be involved in sialic acid-mediated enhancement of the TLR4 response.

It is unclear how sialylated LOS are presented to B cells, in which anatomical compartments B cells are activated and whether T cell help is involved. Anti-ganglioside antibodies detected in patients with *C. jejuni*-associated GBS are mainly of the IgA and IgG isotypes (93, 94). IgA antibodies are probably produced by plasma B cells in Peyer's patches whereas IgG antibodies either originate from activated B cells in mesenteric lymph nodes or, in case bacteria or bacterial epitopes reach the bloodstream, activated B cells in the marginal zone of the spleen. The IgG anti-ganglioside antibodies predominantly belong to the IgG1 and IgG3 subclasses, which is suggestive of T cell dependent responses (95, 96). Currently, it is not established whether a T cell response contributes to the development of anti-ganglioside antibodies.

CD1 glycoproteins, expressed on antigen-presenting cells, are involved in the presentation of microbial glycolipids to T cells (97) and might play a role in the induction of anti-glycolipid antibodies, including anti-ganglioside antibodies. GM1-like LOS specifically bind to purified mouse and human CD1d (98). Immunization studies in mice have demonstrated, however, that anti-ganglioside antibodies of IgG1 and IgG3 subclasses are produced in the absence of CD1d (in $GalNAcT^{-/-}$ $CD1d^{-/-}$ mice), indicating a CD1d-independent pathway for anti-ganglioside antibody production (98). Further studies are required to determine whether T cells are involved in the anti-ganglioside antibody response.

Treatment of GBS

Treatment of patients with GBS either consists of administration of high concentrations of immunoglobulins (IVIg) or plasma exchange (plasmapheresis). These treatment modalities are combined with supportive care, including mechanical ventilation when required. In both treatment modalities, the immune response of the patient is targeted. The precise mechanism of action of IVIg has not been definitively established yet, but IVIg is proposed to have pleitropic immune modulatory effects. It is thought that the IVIg interacts with the Fc receptors on immune cells, leading to anti-inflammatory effects that reduce the severity of the immune response. Anti-cytokine antibodies in IVIg inhibit cytokine function whereas complexes of IVIg inhibit components of the complement system (99). *In vitro*, pre-incubation of complement containing serum with IVIg resulted in reduced C3b deposition on ELISA plate wells coated with GM1, compared to non-pretreated complement containing serum (100). Another hypothesis is that the high concentration of IgG in IVIg accelerates the clearance of IgG, including the clearance of the pathogenic anti-ganglioside antibodies (101).

During plasmapheresis, plasma, including auto-antibodies, complement components and cytokines is removed from the patient's blood. Only the blood cells are returned to the blood circulation, diluted with either fresh donor plasma or a plasma substitute. The outcome of treatment with plasmapheresis is equivalent to IVIg; however, IVIg is the preferred method as it is better tolerated, associated with fewer complications and easy to administer (102). In many developing countries, IVIg is not a treatment option due to the high costs. As access to a plasmapheresis centers is not always available, many patients in developing countries only receive supportive care.

Alternative, preferably low-cost and more specific therapeutic interventions for GBS are highly desirable. Novel strategies to block GBS-specific immune responses, or treatments that result in faster clearance of pathogenic antibodies could lead to less severe neurological damage and faster patient recovery.

Aims and outline of this thesis

The aim of the work described in this thesis was to identify interactions between *C. jejuni* and the human host which contribute to the development of GBS. In particular, we focused on the role of sialylated LOS of *C. jejuni*.

In **Chapter 2**, LOS class typing and LOS allele typing was performed on a large collection of GBS/MFS-associated *C. jejuni* strains, in order to further investigate the role of LOS sialylation in the development of GBS. Furthermore, PCR-based genotyping was performed to assess whether other genes involved in virulence of C. *jejuni* are associated with the development of GBS. In **Chapter 3**, the role of LOS sialylation in *C. jejuni* adhesion to and invasion of the intestinal epithelial cell line Caco-2 was determined. Invasion of the intestinal epithelium may lead to increased exposure of *C. jejuni* to the subepithe-

lial immune system, and therefore may contribute to post-infectious GBS. In **Chapter 4**, the interaction of *C. jejuni* with mouse sialoadhesin (Siglec-1) was characterized. Sialoadhesin is expressed by a subset of macrophages, which are present in the intestine, mesenteric lymph nodes and the spleen. Sialoadhesin binds to gangliosides with α(2,3)-linked sialic acid residues. Similar residues are present on LOS of GBS-associated *C. jejuni* strains. In **Chapter 5,** the binding of human sialoadhesin to *C. jejuni* was assessed. Using human macrophages, the functional consequences of sialoadhesin binding on bacterial uptake, bacterial survival and macrophage activation were determined. **In Chapter 6,** we determined the epitope specificity of *C. jejuni* for Siglec-7 binding. The Siglec-7 binding property was correlated with serological and diagnostic records from patients with GBS and MFS. In **Chapter 7,** we assessed whether synthetic gangliosides coupled to a monomeric matrix can be used to selectively deplete anti-ganglioside antibodies from patient serum. Fast and specific depletion of pathogenic anti-ganglioside antibodies may reduce the severity of neurological damage inflicted by these antibodies in patients with GBS. **Chapter 8** contains a general discussion including future perspectives.

References

1. Hermans D, Van Deun K, Martel A*, et al.* (2011) Colonization factors of *Campylobacter jejuni* in the chicken gut. *Vet Res* 42(1):82.
2. Berrang ME, Buhr RJ, Cason JA, & Dickens JA (2001) Broiler carcass contamination with *Campylobacter* from feces during defeathering. *J Food Prot* 64(12):2063-2066.
3. Luber P, Brynestad S, Topsch D, Scherer K, & Bartelt E (2006) Quantification of *campylobacter* species cross-contamination during handling of contaminated fresh chicken parts in kitchens. *Appl Environ Microbiol* 72(1):66-70.
4. Young KT, Davis LM, & Dirita VJ (2007) *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol* 5(9):665-679.
5. Butzler JP (2004) *Campylobacter*, from obscurity to celebrity. *Clin Microbiol Infect* 10(10):868-876.
6. Mangen MJJ, Havelaar AH, & de Wit GA (2004) Campylobacteriosis and sequelae in the Netherlands. *RIVM report 250911004*.
7. Skirrow MBB, M. J. (2000) Clinical aspects of *Campylobacter* infection,. *In I. Nachamkin and M. J. Blaser (ed.), Campylobacter, 2nd ed. ASM Press* 2nd ed. :p. 69-88.
8. Hansson GC (2012) Role of mucus layers in gut infection and inflammation. *Curr Opin Microbiol* 15(1):57-62.
9. Lievin-Le Moal V & Servin AL (2006) The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. *Clin Microbiol Rev* 19(2):315-337.
10. Cameron A, Frirdich E, Huynh S, Parker CT, & Gaynor EC (2012) The hyperosmotic stress response of *Campylobacter jejuni*. *J Bacteriol* 194(22):6116-6130.
11. Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, & Weiser JN (2007) Capsule enhances *pneumococcal* colonization by limiting mucus-mediated clearance. *Infect Immun* 75(1):83-90.
12. Keo T, Collins J, Kunwar P, Blaser MJ, & Iovine NM (2011) *Campylobacter* capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials. *Virulence* 2(1):30-40.

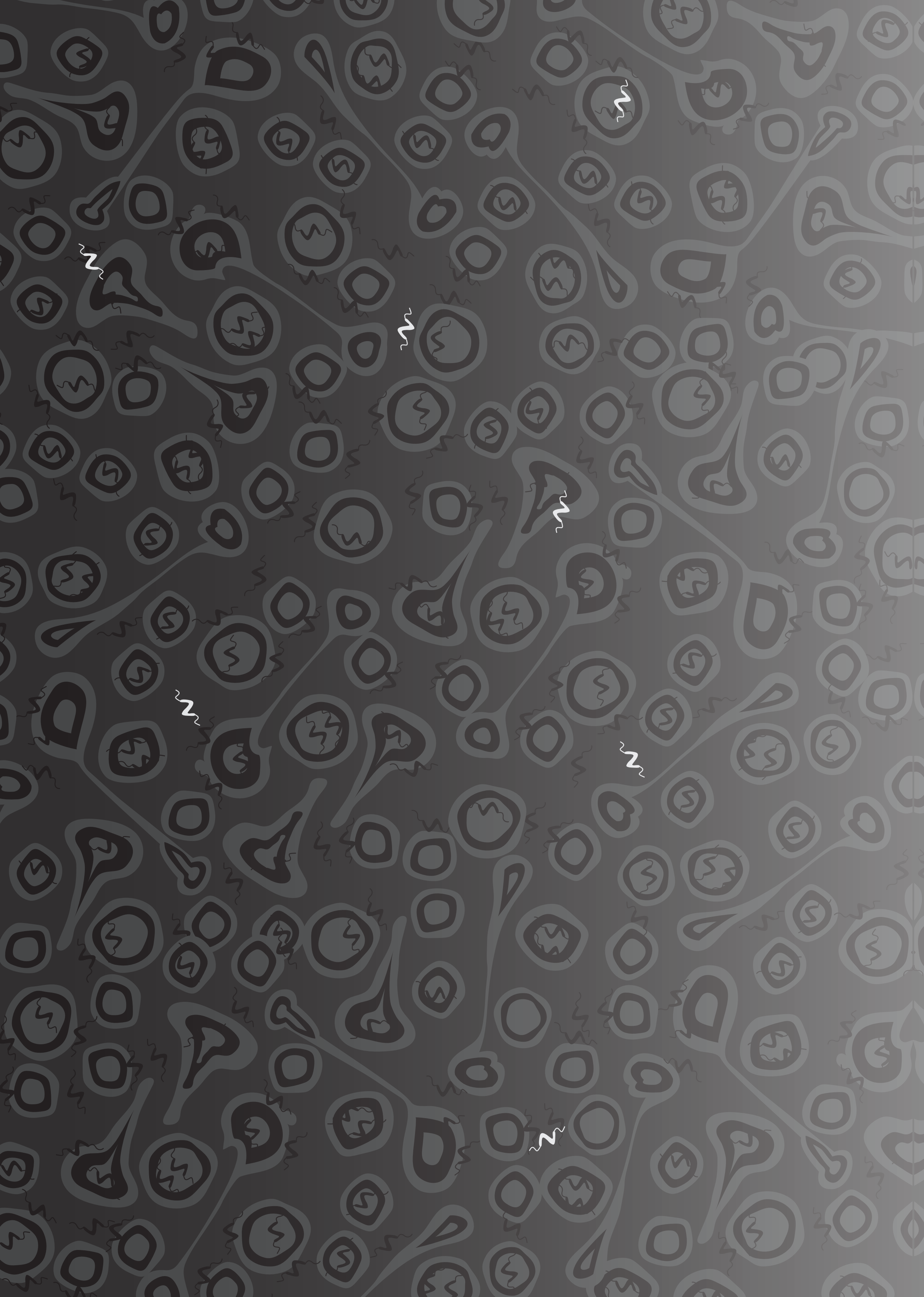
13. O Croinin T & Backert S (2012) Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism? *Front Cell Infect Microbiol* 2:25.
14. Eucker TP & Konkel ME (2012) The cooperative action of bacterial fibronectin-binding proteins and secreted proteins promote maximal *Campylobacter jejuni* invasion of host cells by stimulating membrane ruffling. *Cell Microbiol* 14(2):226-238.
15. Jain D, Prasad KN, Sinha S, & Vishwakarma AL (2009) Cell cycle arrest & apoptosis of epithelial cell line by cytolethal distending toxin positive *Campylobacter jejuni. Indian J Med Res* 129(4):418-423.
16. Lindmark B, Rompikuntal PK, Vaitkevicius K*, et al.* (2009) Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*. *BMC Microbiol* 9:220.
17. Hickey TE, McVeigh AL, Scott DA*, et al.* (2000) *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infect Immun* 68(12):6535-6541.
18. Ziprin RL, Young CR, Byrd JA*, et al.* (2001) Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. *Avian Dis* 45(3):549-557.
19. Bras AM, Chatterjee S, Wren BW, Newell DG, & Ketley JM (1999) A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. *J Bacteriol* 181(10):3298-3302.
20. Nuijten PJ, van den Berg AJ, Formentini I, van der Zeijst BA, & Jacobs AA (2000) DNA rearrangements in the flagellin locus of an flaA mutant of *Campylobacter jejuni* during colonization of chicken ceca. *Infect Immun* 68(12):7137-7140.
21. Oakland M, Jeon B, Sahin O, Shen Z, & Zhang Q (2011) Functional characterization of a lipoprotein-encoding operon in *Campylobacter jejuni*. *PLoS One* 6(5):e20084.
22. Ashgar SS, Oldfield NJ, Wooldridge KG*, et al.* (2007) CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. *J Bacteriol* 189(5):1856-1865.
23. Bacon DJ, Szymanski CM, Burr DH, Silver RP, Alm RA, & Guerry P (2001) A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol Microbiol* 40(3):769-777.
24. Kanipes MI, Holder LC, Corcoran AT, Moran AP, & Guerry P (2004) A deep-rough mutant of *Campylobacter jejuni* 81-176 is noninvasive for intestinal epithelial cells. *Infect Immun* 72(4):2452-2455.
25. Parkhill J, Wren BW, Mungall K*, et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403(6770):665-668.
26. Penner JL & Aspinall GO (1997) Diversity of lipopolysaccharide structures in *Campylobacter jejuni*. *J Infect Dis* 176(Suppl 2):S135-138.
27. Parker CT, Gilbert M, Yuki N, Endtz HP, & Mandrell RE (2008) Characterization of lipooligosaccharide-biosynthetic loci of *Campylobacter jejuni* reveals new lipooligosaccharide classes: evidence of mosaic organizations. *J Bacteriol* 190(16):5681-5689.
28. Hughes RA & Rees JH (1997) Clinical and epidemiologic features of Guillain-Barré syndrome. *J Infect Dis* 176 Suppl 2:S92-98.
29. Ruts L, Drenthen J, Jongen JL*, et al.* (2010) Pain in Guillain-Barré syndrome: a long-term follow-up study. *Neurology* 75(16):1439-1447.
30. Hahn AF (1998) Guillain-Barré syndrome. *Lancet* 352(9128):635-641.
31. Hughes RAC & Rees JH (1997) Clinical and epidemiologic features of Guillain-Barré syndrome. *J Infect Dis* 176(Suppl 2):S92-98.
32. Hughes RA & Cornblath DR (2005) Guillain-Barré syndrome. *Lancet* 366(9497):1653-1666.
33. van Doorn PA, Ruts L, & Jacobs BC (2008) Clinical features, pathogenesis, and treatment of Guillain-Barré syndrome. *Lancet Neurol* 7(10):939-950.

34. Winer JB, Hughes RAC, Anderson MJ, Jones DM, Kangro H, & Watkins RP (1988) A prospective study of acute idiopathic neuropathy. II. Antecedent events. *J Neurol Neurosurg Psychiatry* 51(5):613-618.
35. Alshekhlee A, Hussain Z, Sultan B, & Katirji B (2008) Guillain-Barré syndrome: incidence and mortality rates in US hospitals. *Neurology* 70(18):1608-1613.
36. Islam Z, Jacobs BC, van Belkum A*, et al.* (2010) Axonal variant of Guillain-Barré syndrome associated with *Campylobacter* infection in Bangladesh. *Neurology* 74(7):581-587.
37. Hughes RA, Swan AV, Raphael JC, Annane D, van Koningsveld R, & van Doorn PA (2007) Immunotherapy for Guillain-Barré syndrome: a systematic review. *Brain* 130(Pt 9):2245-2257.
38. Merkies IS, Schmitz PI, Samijn JP, van der Meché FG, & van Doorn PA (1999) Fatigue in immune-mediated polyneuropathies. European Inflammatory Neuropathy Cause and Treatment (INCAT) Group. *Neurology* 53(8):1648-1654.
39. Garssen MP, Van Koningsveld R, & Van Doorn PA (2006) Residual fatigue is independent of antecedent events and disease severity in Guillain-Barré syndrome. *J Neurol* 253(9):1143-1146.
40. Ganser AL & Kirschner DA (1984) Differential expression of gangliosides on the surfaces of myelinated nerve fibers. *J Neurosci Res* 12(2-3):245-255.
41. Sheikh KA, Deerinck TJ, Ellisman MH, & Griffin JW (1999) The distribution of ganglioside-like moieties in peripheral nerves. *Brain* 122 (Pt 3):449-460.
42. McKhann GM, Cornblath DR, Griffin JW*, et al.* (1993) Acute motor axonal neuropathy: a frequent cause of acute flaccid paralysis in China. *Ann Neurol* 33(4):333-342.
43. Ramos-Alvarez M, Bessudo L, & Sabin AB (1969) Paralytic syndromes associated with noninflammatory cytoplasmic or nuclear neuronopathy. Acute paralytic disease in Mexican children, neuropathologically distinguishable from Landry-Guillain-Barré syndrome. *Jama* 207(8):1481-1492.
44. Sobue G, Li M, Terao S*, et al.* (1997) Axonal pathology in Japanese Guillain-Barré syndrome: a study of 15 autopsied cases. *Neurology* 48(6):1694-1700.
45. Hughes RAC, Hadden RD, Gregson NA, & Smith KJ (1999) Pathogenesis of Guillain-Barré syndrome. *J Neuroimmunol* 100(1-2):74-97.
46. Fisher M (1956) An unusual variant of acute idiopathic polyneuritis: syndrome of ophthalmoplegia, ataxia and areflexia. *N. Engl. J. Med.* 255:57-65.
47. Mori M, Kuwabara S, Fukutake T, Yuki N, & Hattori T (2001) Clinical features and prognosis of Miller Fisher syndrome. *Neurology* 56(8):1104-1106.
48. Jacobs BC, Hazenberg MP, van Doorn PA, Endtz HP, & van der Meché FGA (1997) Cross-reactive antibodies against gangliosides and *Campylobacter jejuni* lipopolysaccharides in patients with Guillain-Barré or Miller Fisher syndrome. *J Infect Dis* 175:729-733.
49. Yuki N, Taki T, & Handa S (1996) Antibody to GalNAc-GD1a and GalNAc-GM1b in Guillain-Barré syndrome subsequent to *Campylobacter jejuni* enteritis. *J Neuroimmunol* 71(1-2):155-161.
50. Yuki N, Yamada M, Koga M*, et al.* (2001) Animal model of axonal Guillain-Barré syndrome induced by sensitization with GM1 ganglioside. *Ann. Neurol.* 49(6):712-720.
51. Illa I, Ortiz N, Gallard E, Juarez C, Grau JM, & Dalakas MC (1995) Acute axonal Guillain-Barré syndrome with IgG antibodies against motor axons following parenteral gangliosides. *Ann Neurol* 38(2):218-224.
52. Hughes R, Atkinson P, Coates P, Hall S, & Leibowitz S (1992) Sural nerve biopsies in Guillain-Barré syndrome: axonal degeneration and macrophage-associated demyelination and absence of cytomegalovirus genome. *Muscle Nerve* 15(5):568-575.
53. Hall SM, Hughes RA, Atkinson PF, McColl I, & Gale A (1992) Motor nerve biopsy in severe Guillain-Barré syndrome. *Ann Neurol* 31(4):441-444.
54. Susuki K, Yuki N, Schafer DP*, et al.* (2012) Dysfunction of nodes of Ranvier: a mechanism for anti-ganglioside antibody-mediated neuropathies. *Exp Neurol* 233(1):534-542.

55. Hafer-Macko C, Hsieh ST, Li CY, *et al.* (1996) Acute motor axonal neuropathy: an antibody-mediated attack on axolemma. *Ann Neurol* 40(4):635-644.
56. Hafer-Macko CE, Sheikh KA, Li CY, *et al.* (1996) Immune attack on the Schwann cell surface in acute inflammatory demyelinating polyneuropathy. *Ann Neurol* 39(5):625-635.
57. Putzu GA, Figarella-Branger D, Bouvier-Labit C, Liprandi A, Bianco N, & Pellissier JF (2000) Immunohistochemical localization of cytokines, C5b-9 and ICAM-1 in peripheral nerve of Guillain-Barré syndrome. *J Neurol Sci* 174(1):16-21.
58. Stoll G, Schmidt B, Jander S, Toyka KV, & Hartung HP (1991) Presence of the terminal complement complex (C5b-9) precedes myelin degradation in immune-mediated demyelination of the rat peripheral nervous system. *Ann Neurol* 30(2):147-155.
59. McGonigal R, Rowan EG, Greenshields KN, *et al.* (2010) Anti-GD1a antibodies activate complement and calpain to injure distal motor nodes of Ranvier in mice. *Brain* 133(Pt 7):1944-1960.
60. Sekiguchi Y, Uncini A, Yuki N, *et al.* (2012) Antiganglioside antibodies are associated with axonal Guillain-Barré syndrome: a Japanese-Italian collaborative study. *J Neurol Neurosurg Psychiatry* 83(1):23-28.
61. Jacobs BC, Rothbarth PH, van der Meché FGA, *et al.* (1998) The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. *Neurology* 51(4):1110-1115.
62. Yu RK, Usuki S, & Ariga T (2006) Ganglioside molecular mimicry and its pathological roles in Guillain-Barré syndrome and related diseases. *Infect Immun* 74(12):6517-6527.
63. Yuki N (1997) Molecular mimicry between gangliosides and lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Guillain-Barré syndrome and Miller Fisher syndrome. *J Infect Dis* 176(Suppl 2):S150-153.
64. Koga M, Gilbert M, Li J, *et al.* (2005) Antecedent infections in Fisher syndrome: a common pathogenesis of molecular mimicry. *Neurology* 64(9):1605-1611.
65. Moran AP, Prendergast MM, & Appelmelk BJ (1996) Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol Med Microbiol* 16(2):105-115.
66. Yuki N, Susuki K, Koga M, *et al.* (2004) Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barré syndrome. *PNAS* 101(31):11404-11409.
67. Mori M, Kuwabara S, Miyake M, *et al.* (2000) *Haemophilus influenzae* infection and Guillain-Barré syndrome. *Brain* 123(Pt 10):2171-2178.
68. Willison HJ & Yuki N (2002) Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125(12):2591-2625.
69. Hiraga A, Kuwabara S, Ogawara K, *et al.* (2005) Patterns and serial changes in electrodiagnostic abnormalities of axonal Guillain-Barré syndrome. *Neurology* 64(5):856-860.
70. Kuijf ML, Godschalk PC, Gilbert M, *et al.* (2007) Origin of ganglioside complex antibodies in Guillain-Barré syndrome. *J Neuroimmunol* 188(1-2):69-73.
71. Godschalk PC, Kuijf ML, Li J, *et al.* (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndromes. *Infect Immun* 75(3):1245-1254.
72. Severi E, Randle G, Kivlin P, *et al.* (2005) Sialic acid transport in *Haemophilus influenzae* is essential for lipopolysaccharide sialylation and serum resistance and is dependent on a novel tripartite ATP-independent periplasmic transporter. *Mol Microbiol* 58(4):1173-1185.
73. Ang CW, Jacobs BC, Brandenburg AH, *et al.* (2000) Cross-reactive antibodies against GM2 and CMV-infected fibroblasts in Guillain-Barré syndrome. *Neurology* 54(7):1453-1458.
74. Sharma NS, Wallenstein EJ, Novik E, Maguire T, Schloss R, & Yarmush ML (2009) Enrichment of hepatocyte-like cells with upregulated metabolic and differentiated function derived from embryonic stem cells using S-NitrosoAcetylPenicillamine. *Tissue Eng Part C Methods* 15(2):297-306.
75. Gilbert M, Godschalk PCR, Karwaski MF, *et al.* (2004) Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain-Barré syndrome, by horizontal exchange. *Infect. Immun.* 72(2):1162-1165.

76. Gilbert M, Karwaski M-F, Bernatchez S, *et al.* (2002) The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J. Biol. Chem.* 277(1):327-337.
77. Godschalk PCR, Heikema AP, Gilbert M, *et al.* (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J Clin Invest* 114(11):1659-1665.
78. Islam Z, van Belkum A, Wagenaar JA, *et al.* (2009) Comparative genotyping of *Campylobacter jejuni* strains from patients with Guillain-Barré syndrome in Bangladesh. *PLoS ONE* 4(9):e7257.
79. Yuki N (2007) *Campylobacter* sialyltransferase gene polymorphism directs clinical features of Guillain-Barré syndrome. *J Neurochem* 103 Suppl 1:150-158.
80. Yuki N, Yoshino H, Sato S, Shinozawa K, & Miyatake T (1992) Severe acute axonal form of Guillain-Barré syndrome associated with IgG anti-GD1a antibodies. *Muscle Nerve* 15(8):899-903.
81. Ohtsuka K, Nakamura Y, Hashimoto M, *et al.* (1998) Fisher syndrome associated with IgG anti-GQ1b antibody following infection by a specific serotype of *Campylobacter jejuni*. *Ophthalmology* 105(7):1281-1285.
82. Aranyi Z, Kovacs T, Sipos I, & Bereczki D (2012) Miller Fisher syndrome: brief overview and update with a focus on electrophysiological findings. *Eur J Neurol* 19(1):15-e13.
83. Chiba A, Kusunoki S, Obata H, Machinami R, & Kanazawa I (1993) Serum anti-GQ1b IgG antibody is associated with ophthalmoplegia in Miller Fisher syndrome and Guillain-Barré syndrome: clinical and immunohistochemical studies. *Neurology* 43(10):1911-1917.
84. Chieppa M, Rescigno M, Huang AY, & Germain RN (2006) Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* 203(13):2841-2852.
85. Mowat AM & Bain CC (2011) Mucosal macrophages in intestinal homeostasis and inflammation. *J Innate Immun* 3(6):550-564.
86. Brandtzaeg P (2010) Function of mucosa-associated lymphoid tissue in antibody formation. *Immunol Invest* 39(4-5):303-355.
87. Schulz O, Jaensson E, Persson EK, *et al.* (2009) Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* 206(13):3101-3114.
88. Avril T, Wagner ER, Willison HJ, & Crocker PR (2006) Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 74(7):4133-4141.
89. Crocker PR, Paulson JC, & Varki A (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* 7(4):255-266.
90. Avril T, Floyd H, Lopez F, Vivier E, & Crocker PR (2004) The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells. *J Immunol* 173(11):6841-6849.
91. Crocker PR (2005) Siglecs in innate immunity. *Curr Opin Pharmacol* 5(4):431-437.
92. Kuijf ML, Samsom JN, van Rijs W, *et al.* (2010) TLR4-mediated sensing of *Campylobacter jejuni* by dendritic cells is determined by sialylation. *J Immunol* 185(1):748-755.
93. Koga M, Yuki N, Takahashi M, Saito K, & Hirata K (1998) Close association of IgA anti-ganglioside antibodies with antecedent *Campylobacter jejuni* infection in Guillain-Barré and Fisher's syndromes. *J Neuroimmunol* 81(1-2):138-143.
94. Shahrizaila N & Yuki N (2012) Antiganglioside antibodies in Guillain-Barré syndrome and its related conditions. *Expert Rev Neurother* 11(9):1305-1313.
95. Koga M, Yuki N, Hirata K, Morimatsu M, Mori M, & Kuwabara S (2003) Anti-GM1 antibody IgG subclass: a clinical recovery predictor in Guillain-Barré syndrome. *Neurology* 60(9):1514-1518.
96. Jacobs BC, Koga M, van Rijs W, *et al.* (2008) Subclass IgG to motor gangliosides related to infection and clinical course in Guillain-Barré syndrome. *J Neuroimmunol* 194(1-2):181-190.

97. Porcelli SA & Modlin RL (1999) The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu Rev Immunol* 17:297-329.
98. Matsumoto Y, Yuki N, Van Kaer L, Furukawa K, Hirata K, & Sugita M (2008) Cutting edge: Guillain-Barré syndrome-associated IgG responses to gangliosides are generated independently of CD1 function in mice. *J Immunol* 180(1):39-43.
99. Hughes RA, Swan AV, & van Doorn PA (2012) Intravenous immunoglobulin for Guillain-Barré syndrome. *Cochrane Database Syst Rev* 7:CD002063.
100. Yuki N, Watanabe H, Nakajima T, & Spath PJ (2011) IVIG blocks complement deposition mediated by anti-GM1 antibodies in multifocal motor neuropathy. *J Neurol Neurosurg Psychiatry* 82(1):87-91.
101. Yu Z & Lennon VA (1999) Mechanism of intravenous immune globulin therapy in antibody-mediated autoimmune diseases. *N Engl J Med* 340(3):227-228.
102. van der Meché FG & Schmitz PI (1992) A randomized trial comparing intravenous immune globulin and plasma exchange in Guillain-Barré syndrome. Dutch Guillain-Barré Study Group. *N Engl J Med* 326(17):1123-1129.



Chapter 2

Particular lipooligosaccharide loci and capsule types co-occur in Guillain-Barré syndrome associated *Campylobacter jejuni* strains

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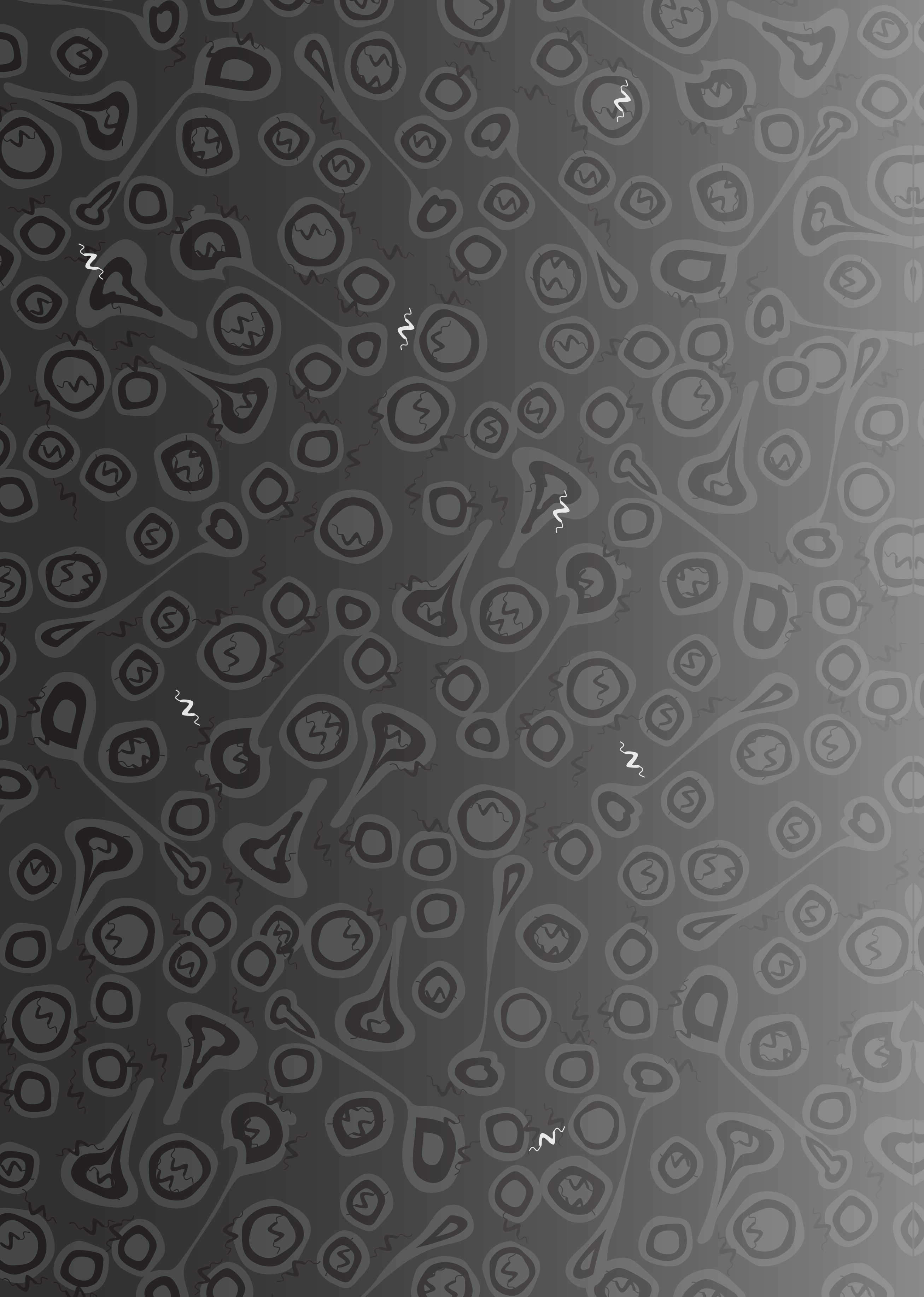

Chapter 3

The sialylated lipooligosaccharide outer core of *Campylobacter jejuni* is an important determinant for epithelial cell invasion

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Abstract

Campylobacter jejuni is a frequent cause of bacterial gastroenteritis worldwide. Lipooligosaccharide (LOS) has been identified as an important virulence factor that may play a role in microbial adhesion and invasion. Here we specifically address the question of whether LOS sialylation affects the interaction of *C. jejuni* with human epithelial cells. For this purpose, 14 strains associated with Guillain-Barré syndrome (GBS), 34 enteritis-associated strains, the 81-176 reference strain, and 6 Penner serotype strains were tested for invasion of two epithelial cell lines. *C. jejuni* strains expressing sialylated LOS (classes A, B, and C) invaded cells significantly more frequently than strains expressing nonsialylated LOS (classes D and E) ($P < 0.0001$). To further explore this observation, we inactivated the LOS sialyltransferase (Cst-II) via knockout mutagenesis in three GBS-associated *C. jejuni* strains expressing sialylated LOS (GB2, GB11, and GB19). All knockout strains displayed significantly lower levels of invasion than the respective wild-types. Complementation of a Δ*cst-II* mutant strain restored LOS sialylation and reset the invasiveness to wild-type levels. Finally, formalin-fixed wild-type strains GB2, GB11 and GB19, but not the isogenic Δ*cst-II* mutants that lack sialic acid, were able to inhibit epithelial invasion by viable GB2, GB11, and GB19 strains. We conclude that sialylation of the LOS outer core contributes significantly to epithelial invasion by *C. jejuni* and may thus play a role in subsequent postinfectious pathologies.

Introduction

Campylobacter jejuni is recognized as a leading cause of bacterial gastroenteritis worldwide. Poorly handled or improperly cooked poultry meat, raw milk, pets and untreated water are thought to be sources of infection (1). The disease spectrum caused by *C. jejuni* ranges from asymptomatic infection to severe inflammatory bloody diarrhea (2). Furthermore, *C. jejuni* infection has been associated with the development of post-infectious complications such as the Guillain Barré Syndrome (GBS) (3). The apparent variation in gastro-intestinal disease outcome is likely to be affected by the expression of virulence factors that are associated with specific pathogenic mechanisms, e.g. *C. jejuni* motility (4), attachment (5) and invasion (6-8). Motility and chemotaxis appear to be necessary for epithelial adherence of *C. jejuni*, whereas the expression of functional flagella may determine the capacities for *C. jejuni* to invade the epithelium and to effectively colonize the mouse intestine (7, 9-11).

Next to the role of flagella in the regulation of *C. jejuni* invasiveness, lipooligosaccharide (LOS) structures have been generally implicated in microbial invasion (12-18). To date, eight major and distinctive LOS biosynthesis gene clusters, here referred to as LOS classes, have been described for *C. jejuni* (19), and this number continues to increase (*Parker et. al*., submitted). Sequencing and microarray analysis of the LOS biosynthesis gene locus of the *C. jejuni* genomes have also revealed this locus to be highly variable (20, 21), which may contribute to the variation in *C. jejuni* associated pathologies.

A subgroup of *C. jejuni* strains that express the LOS class A, B or C gene locus, harbor genes involved in sialic acid biosynthesis and are therefore able to synthesize sialylated LOS (20, 22-24). The *cst-II* gene encodes for a sialyltransferase (25), that is necessary for the transfer of sialic acid onto the LOS core in *C. jejuni* class A and B strains. Class C *C. jejuni* strains depend on the gene *cst-III* for LOS sialylation. Hence, only *C. jejuni* strains expressing LOS classes A, B or C are capable of LOS sialylation. Previously, we have shown that the presence and expression of the *cst-II* gene is specifically associated with GBS and required for the induction of anti-ganglioside antibody responses which is the hall-mark of this post-infectious complication (22, 26). Based on this prior work, we hypothesized that LOS sialylation (and consequently *C. jejuni* LOS subclasses) may be involved in *C. jejuni* invasiveness.

Therefore, a panel of 48 human isolates and 7 human control strains were assessed for invasiveness into two human epithelial carcinoma cell lines (Caco-2 and T84). To specifically explore the role of sialylation, we generated three GBS-associated sialyltransferase (Cst-II) knock-out *C. jejuni* strains (GB2Δ*cst-II*, GB11Δ*cst-II* and GB19Δ*cst-II*). These GB2Δ*cst-II*, GB11Δ*cst-II* and GB19Δ*cst-II* mutants were tested for their ability to adhere and invade Caco-2 cells. Finally, we tested if complementation of the Δ*cst-II* mutant would restore the invasion associated function of this gene-product.

Materials and Methods

Bacterial strains

Fourteen GBS- and 34 enteritis-associated *C. jejuni* strains, isolated from Dutch patients, 6 Penner serotype strains and the 81-176 enteritis reference strain, were used in this study (see Table 1). To minimize in vitro passages, *C. jejuni* strains were recovered from the original patient-isolated glycerol stock by culturing on Butzler agar plates (Becton Dickinson, Breda, The Netherlands). A second passage was allowed for optimal vitality before using these strains in experiments. After recovery cells were harvested in Hanks Balanced Salt Solution (HBSS) (Life Technology, Breda, The Netherlands) and densities were adjusted according to the optical density at 600 nm (OD_{600}).

Typing of the LOS biosynthesis gene cluster

To determine the class of LOS locus present in each *C. jejuni* strain, genomic DNA was isolated using the DNeasy Tissue kit (Qiagen, Venlo, The Netherlands). PCR analysis was done with primer sets specific for the classes A, B, C, D and E as previously described (22). PCR assays were performed in a Perkin Elmer GeneAmp PCR System 9700 (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), applying 35 cycles of 1 min 94°C, 1 min 52°C, 2 min 72°C.

Knockout mutagenesis

Strains GB2, GB11 and their Δ*cst-II* mutants, GB2Δ*cst-II* and GB11Δ*cst-II*, respectively, have been described before (22). A Δ*cst-II* mutant of a third GBS-related strain that is described here, GB19, was generated using the same procedure used for the knock-out mutagenesis in strains GB2 and GB11 (22). Briefly, the target gene (*cst-II*) and approximately 700 bp of upstream and downstream flanking sequences were amplified and cloned into the pGem-Teasy vector (Promega Corp, Leiden, The Netherlands). Inverse PCR was used to introduce a *BamHI* restriction site and a deletion of approximately 800bp in the target gene. Inverse PCR products were digested with *BamHI* (Fermentas, St. Leon-Rot, Germany) and ligated to the *BamHI* digested chloramphenicol resistance (Cm^r) cassette. Constructs were electroporated into electrocompetent GB19 *C. jejuni* cells and recombinants were selected on Mueller-Hinton plates (Becton Dickinson, Breda, The Netherlands) containing 20μg/ml chloramphenicol (Difco, Alphen aan den Rijn, The Netherlands).

Mass spectrometry

Samples were prepared for LOS mass-spectrometric analysis by overnight growth of *C. jejuni* strains at 37°C on Butzler agar plates in a micro-aerobic atmosphere. Material from one confluent agar plate in a micro-aerobic atmosphere was harvested and treated with

proteinase K at 60 µg/ml, RNase A at 200 µg/ml, and DNase I at 100 µg/ml (Promega, Leiden, The Netherlands). *O*-deacylated LOS samples were prepared and analyzed by capillary electrophoresis coupled to electro-spray ionization mass spectrometry (CE-ESI-MS) (27).

Complementation of the *cst-II* gene

We used site specific homologous recombination to restore the wild-type phenotype of the GB11Δ*cst-II* mutant strain (manuscript in preparation). In short, a construct containing the *cst-II* gene together with its promoter region and a gene encoding erythromycin resistance were cloned in the same orientation and were transformed by electroporation into electrocompetent GB11Δ*cst-II* mutant cells. The electroporated cells were plated on selective blood agar plates containing 10µg/ml erythromycin (Sigma Aldrich, Zwijndrecht, The Netherlands) and incubated at 42°C in a micro-aerobic environment. Colonies formed were sub-cultured to purity and stored at -80°C until further use.

SDS-PAGE and Western blot assay

To analyze *C. jejuni* LOS sialylation, a 10% SDS-PAGE gel was run. Strains were harvested from an overnight Butzler agar plate, whereafter concentrations were equalized by OD 600 nm measurement. Bacterial cell suspensions were lysed using glass beads (MP Biomedicals, Solon, OH, USA). Lysates were digested with proteinase K at 60 µg/ml for 4 h at 56 °C and equal amounts were run on a 10% SDS-PAGE Tris-HCl gel for 2 h. As a marker the pre-stained SDS-PAGE standards broad range molecular weighted was used (Bio-Rad, Nazareth Eke, Belgium). After electrophoresis, the LOS was transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) for a Western-blot assay. The nitrocellulose membrane was blocked overnight with 0.05% (v/v) Tween-20 (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 5% (W/V) nonfat milk (Bio-Rad, Nazareth Eke, Belgium). The next day the membranes were washed three times for 10 minutes with PBS and incubated with horse radish peroxidase (HRP) labeled cholera toxin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 1% blocking buffer as a detection agent. Presence or absence of sialylated LOS was visualized with an ECL detection kit (Biocompare, San Francisco, USA) and a Kodak photo film (Roche-Diagnostics, Almere, The Netherlands) according to the manufacturer's protocol.

Bacterial growth assay

Bacterial growth characteristics of the clinical isolates and their corresponding mutants were determined in Mueller-Hinton broth (Becton Dickinson, Breda, The Netherlands) and in a specific antibiotic-free cell culture medium, which is used in the gentamicin exclusion assay. Bacterial strains were inoculated at equal OD at 600 nm, equivalent to 5.0 x 10^4 CFU/ml, and incubated at 37°C, while gently shaking in a micro-aerobic envi-

ronment. Bacterial cell counts and OD 600 nm were determined at 4, 8, 18, 24, 36 and 42 h post-inoculation, respectively.

Intestinal epithelial cell line

Human intestinal epithelial Caco-2 and T84 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Breda, The Netherlands) and 1% non-essential amino acids (NEAA) (Invitrogen, Breda, The Netherlands). The cells were routinely grown in a 75-cm^2 flask (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) at 37°C in a humidified 5% CO_2-95% air incubator. Confluent stock cultures were washed with PBS (Invitrogen, Breda, The Netherlands), trypsinized with trypsin-EDTA (Lonza, Verviers, Belgium) and 5.0 x10^5 cells were seeded in a new 75-cm^2 flask.

Adhesion and invasion

Adherence and invasion of *C. jejuni* was determined by growing the intestinal epithelial cells (Caco-2 or T84) to confluence for 48 h at a final approximate density of 5.0 x10^6 cells per well (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), without allowing them to differentiate in the case of Caco-2 cells. The adherence and invasion assays were performed by incubating the epithelial cells with *C. jejuni* at a ratio of 1:100. Bacteria and epithelial cells were coincubated for 2 h at 37 °C in a 5% CO_2 and 95% air atmosphere to assess adherence. For invasion, a subsequent 2 h of incubation of the epithelial cells was allowed. After incubation, monolayers were washed 3 times with pre-warmed PBS. To kill extra-cellular bacteria, monolayers were treated for 3 h with a bactericidal concentration of gentamicin (480 µg/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) in DMEM medium containing 10% FBS and 1% NEAA as described previously (7). For all strains, sensitivity to this concentration of gentamicin was confirmed. After washing, epithelial cells were lysed with 0.1% Triton X-100 (Cornell, Philadelphia, PA, USA) in PBS for 15 minutes at room temperature. The number of invaded *C. jejuni* was determined by plating serial dilutions of the lysis mix onto freshly prepared blood agar plates. After incubation for 24 to 36 h at 37 °C in a micro-aerobic environment, colonies were counted. The percentage of bacteria that invaded was calculated by dividing the number of *C. jejuni* that invaded the cells by the number of *C. jejuni* inoculated onto the cells times 100%. For determination of adherence, cells were washed three times extensively with PBS and the cell monolayer was lysed with 0.1% Triton X-100 after which serial dilutions were plated onto blood agar plates (Becton Dickinson, Breda, The Netherlands).

Inhibition of invasion

Formalin fixed, wild-type *C. jejuni* and their Δ*cst-II* mutants were used to inhibit invasion of viable *C. jejuni* GB2, GB11 and GB19. Briefly, GB2, GB11, GB19 and their Δ*cst-II* mutants

at a starting concentration of 5.0 x10^9 CFU/ml, determined at OD 600 nm, were fixed in 3.6% formalin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS for 10 minutes. By washing the fixed cells 3 times in PBS the excess of formalin was removed. The sterility of the control cultures confirmed fixation to be complete. Caco-2 cells at a density of 5.0 x10^4 cells per well were pre-incubated for 30 minutes with formalin-killed wild-type or Δ*cst-II* mutant *C. jejuni* at a multiplicity of infection (MOI) ranging from 100 to 5000. Subsequently, the Caco-2 cells were washed to remove excess dead *C. jejuni* bacteria whereafter fresh medium was added. Viable wild-type cells were added at a MOI of 100 and invasion was assessed by the gentamicin exclusion protocol as described earlier.

Statistical analysis

Statistical analysis was performed using Instat software (version 2.05a; Graphpad Software, San Diego, CA). Because the invasiveness of strains differed widely, log transformation was used to equalize variances. Invasiveness was expressed as the geometric mean number of CFU per milliliter retrieved from the infected cell line in all three to six invasion experiments per *C. jejuni* strain performed. Differences in invasiveness between LOS class A, B, and C strains and LOS class D and E strains, and between GBS-associated and enteritis-associated strains, were tested for significance with a Mann-Whitney U test, since column statistics showed that the Gaussian distribution was unequal for the strains. A two-tailed value with $P < 0.05$ indicated statistical significance. Statistical analysis for differences in adherence and invasion between wild-type and knockout mutant strains was performed, and differences were tested for significance with a paired *t* test.

Results

LOS sialylation is associated with increased epithelial cell invasion

We observed a wide range of invasion capacities among the *C. jejuni* strains (Table 1). Categorization of *C. jejuni* strains into those carrying sialylated ($n = 30$) and nonsialylated ($n = 18$) LOS established that the sialylated-LOS producers, classes A, B and C, were more invasive than the nonsialylated producers, classes D and E (median CFU per milliliter, 408,300 for classes A, B, and C and 11,190 for classes D and E; $P < 0.0001$) (Fig. 1A). Notably, on average, the GBS-associated strains ($n = 14$) invaded significantly better than the enteritis-associated strains ($n = 34$) (median CFU per milliliter, 632,700 versus 49,630, respectively; $P = 0.0046$) (Fig. 1B). The invasiveness of the *C. jejuni* Penner serotype strains corresponded with LOS class expression of sialylated or nonsialylated LOS, with the exception of the Penner serotype strain O:4. Thus, Penner serotype strain O:4 and also an enteritis-associated strain RIVM 15, invaded poorly, despite the presumed expression of sialylated LOS due to the presence of a class A or C LOS biosynthesis gene cluster,

Table 1. *C. jejuni* strains and their invasiveness for Caco-2 cells

Strains [a]	LOS locus	Invasion % [b]	No. of invading *C. jejuni* per 100 cells	Ganglioside mimic [c]	Illness
GB2	A	3.4 ± 0.55	285 - 395	GM1a, GD1a	GBS
GB11	A	2.2 ± 0.7	150 - 290	GM1a, GD1a	GBS
GB19	A	0.8 ± 0.29	51 - 109	GD1c	GBS
GB3	A	0.12 ± 0.046	7 - 16	GM1a, GD1a	GBS
GB22	A	0.05 ± 0.026	3 - 7	GM1a, GD1a	GBS
GB23	A	1.17 ± 0.14	103 - 131	GM2	GBS
GB29	A	0.73 ± 0.06	67 – 79		GBS
E990521	A	3.0 ± 1.15	185 - 415		Enteritis
E991095	A	1.9 ± 0.81	110 - 271		Enteritis
E9126	A	1.2 ± 0.58	70 - 178		Enteritis
P19	A	4.7 ± 1.4	330 - 610	GM1a, GD1a	Enteritis
P10	A	4.23 ± 1.86	237 - 609	GD3	Enteritis
P4	A	0.0054 ± 0.00092	0.44 – 0.63	GM1a, GD1a	Enteritis
GB17	B	3.05 ± 1.75	130 – 480	GM1b, GD1c	GBS
GB25	B	0.27 ± 0.13	14 - 40	GM1b, GD1c	GBS
GB31	B	0.97 ± 0.15	82 – 112	GM1a, GD1a	GBS
GB37	B	0.16 ± 0.03	13 – 19		GBS
Rivm 16	B	1.98 ± 0.7	192 – 205		Enteritis
Rivm 38	B	0.037 ± 0.023	1.0 – 6.0		Enteritis
Rivm 129	B	0.084 ± 0.026	5.0 – 11		Enteritis
E989123	B	0.29 ± 0.011	18 – 40		Enteritis
E981033	B	0.26± 0.075	18 - 33	GM1a	Enteritis
E98652	B	0.028 ± 0.006	2 - 4	GM1a, GQ1b	Enteritis
81176	B	0.26 ± 0.06	20 – 32	GM2, GM3	Enteritis
GB13	C	0.2 ± 0.017	18 - 22	GM1a	GBS
GB38	C	1.8 ± 0.77	103 – 257		GBS
Rivm 15	C	0.00075 ± 0.00014	0.061 – 0.089		Enteritis
Rivm 83	C	2.75 ± 1.28	147 – 403		Enteritis
Rivm 93	C	3.5 ± 1.15	235 – 465		Enteritis
Rivm 109	C	1.22 ± 0.44	78 – 166		Enteritis
Rivm 116	C	0.25 ± 0.13	12 – 38	GM1a. GQ1b	Enteritis
E98682	C	0.010 ± 0.0036	0.6 – 1.4	GM1a	Enteritis
E981087	C	0.13 ± 0.031	10 - 16	GM2	Enteritis
P1	C	0.01 ± 0.001	0.9 – 1.1	GM1b	Enteritis
P2	C	0.005 ± 0.0017	0.33 – 0.67		Enteritis
Rivm 3	D	0.005± 0.0012	0.38 – 0.62		Enteritis
Rivm 33	D	0.017 ± 0.0045	1 – 2		Enteritis
Rivm 65	D	0.018 ± 0.0026	1 – 2		Enteritis
Rivm 67	D	0.0097 ± 0.0013	0.5 – 1		Enteritis
Rivm 95	D	0.019 ± 0.003	1 – 2		Enteritis
Rivm 104	D	0.0082 ± 0.0014	0.68 – 0.96	none	Enteritis
E98706	D	0.014 ± 0.0025	1.15 – 1.65		Enteritis

E970873	D	0.14 ± 0.02	12 – 16	none	Enteritis
GB4	E	0.009 ± 0.003	0.5 – 1		GBS
Rivm 37	E	0.081 ± 0.029	5 – 11		Enteritis
Rivm 46	E	0.0065 ± 0.0027	0.38 – 0.92		Enteritis
Rivm 47	E	0.097 ± 0.028	6 – 12		Enteritis
Rivm 50	E	0.0065 ± 0.00096	0.56 – 0.74		Enteritis
Rivm 61	E	0.011 ± 0.0066	1 – 2		Enteritis
E9141	E	0.074 ± 0.013	5 - 9		Enteritis
E9144	E	0.14 ± 0.03	11 - 17		Enteritis
E9146	E	0.08 ± 0.015	6 - 10	none	Enteritis
E98623	E	0.004 ± 0.0015	0.2 – 0.5	none	Enteritis
E98624	E	0.003 ± 0.00075	0.23 – 0.4	none	Enteritis
P3	E	0.0045 ± 0.0013	0.32 – 0.58		Enteritis

a GB, GBS-associated strain; E, enteritis-related strain; P, Penner serotype strain. Strain 81-176 was used as a positive control.
b Data are means ± standard deviations for at least three independent experiments and are calculated as the percentage of bacteria that survived the gentamicin treatment.
c The LOS structures showing the ganglioside mimics of 18 strains were elucidated by mass spectrometry and immunological methods; for 7 strains, LOS structures were elucidated by immunological methods only. Data were not available for the other strains.

respectively. Strain 81-176 invaded the Caco-2 cell line as well as it did in previous studies, although most of these invasion studies were performed using a different cell line and a shorter incubation period (see Table 1). All Dutch clinical strains that contain LOS genes of class A, B or C are thought to express sialylated LOS (22). Characterization of the LOS ganglioside mimic structures and determination of the presence or absence of sialylation for the GBS strains (GB2, GB3, GB4, GB11, GB13, GB17, GB19, GB22, GB23, GB25 and GB31) and enteritis strains (E98-623, 624, 652, 682, 706, 1033 and 1087) were

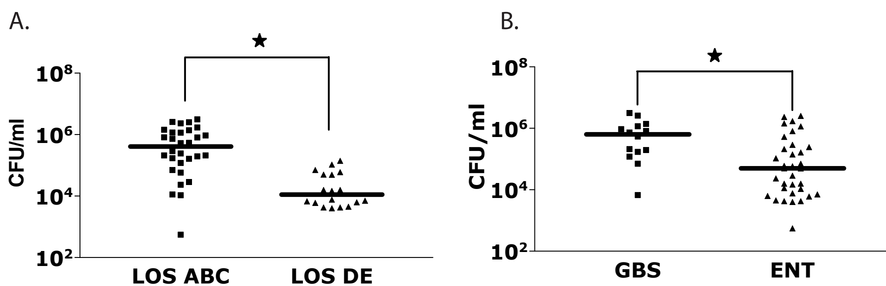

Figure 1. The invasiveness of *C. jejuni* is dependent on sialylation of the LOS. Scattergrams show the invasion of Caco-2 cells by Dutch *C. jejuni* strains, categorized with respect to the type of LOS that is expressed (sialylated LOS of classes A, B, and C [*n* = 30] versus non-sialylated LOS of classes D and E [*n* = 18]) **A.** or the clinical outcome of infection, i.e., GBS (*n* = 14) versus uncomplicated gastroenteritis (ENT) (*n* = 34) **B.** Experiments were performed in triplicate and repeated at least three times. For each strain, a geometric mean outcome (number of CFU per millilitre) was calculated. The differences between the geometric means of groups of strains were tested with the Mann-Whitney U statistic. The median for each group of strains is shown.

carried out previously by immunological methods (28, 29). These results are shown in Table 1.

LOS phenotype characteristics of different *C. jejuni* strains and Δ*cst-II* mutants

As determined by mass spectrometry analysis, GB19 expressed sialylated LOS in the form of ganglioside mimic GD1c (also referred to as GD3, due to the structural similarity to human GD3). GD1c contains disialic acid bound to the terminal galactose residue. All three Δ*cst-II* mutants were chemically defined and found not to express of sialylated LOS. The LOS structures of *C. jejuni* strains GB2, GB11, GB19 and their associated Δ*cst-II* mutants are shown in Fig. 2. For a subset of the strains, comprising GB3, GB4, GB13, GB17, GB22, GB23, GB25 and GB31, ganglioside mimic structures were determined previously by mass spectrometry (Table 1) (30). LOS structures of the Penner serotype strains O:1, O:2, O:3, O:4, O:10, O:19 and 81-176 (Table 1) have been characterized previously by other researchers (15, 31-35). As can be seen by the absence of data for some strains in Table 1, mass spectrometry data on LOS structures were not available for all bacteria.

Strain	Structure	Ganglioside mimic
GB2/GB11	Gal-GalNac-Gal-Hep-Hep- NeuAc Glc	GM1
	Gal-GalNac-Gal-Hep-Hep- NeuAc NeuAc Glc	GD1a
GB2/GB11Δ*cst-II*	Gal-GalNac-Gal-Hep-Hep- Glc	No
	GalNac-Gal-Hep-Hep- Glc	No
	Gal-Hep-Hep- Glc	No
GB19	Gal-GalNac-Gal-Hep- NeuAc Glc NeuAc	GD1c
GB19Δ*cst-II*	Gal-GalNac-Gal-Hep- Glc	No

Figure 2. Proposed LOS outer core structures as determined by mass spectrometry analysis. Note that GB2 and GB11 express a mixture of the sialylated LOS ganglioside mimics GM1 and GD1a, whereas GB19 expresses sialylated LOS only in the form of GD1c. In all three strains, knockout mutagenesis of *cst-II* resulted in loss of expression of sialylated LOS.

Knockout mutagenesis of *cst-II* does not affect bacterial growth rate significantly

To exclude the possibility that differences in viability and growth rate would influence the results of our invasion assays, we assessed the growth rates of wild-type strains GB2, GB11 and GB19 and their Δ*cst-II* mutants in Mueller Hinton medium and in the cell culture medium used in the Caco-2 cell invasion assays. No significant differences in growth rates were observed between the wild-type GB2, GB11 and GB19 and Δ*cst-II* strains and their Δ*cst-II* mutants during the time span of our invasion experiments (results not shown).

Disruption of *cst-II* significantly affects the invasiveness of *C. jejuni* into intestinal epithelial cells

We compared the capacity of the *C. jejuni* wild-type strains GB2, GB11 and GB19 to adhere to and invade into Caco-2 cells with those of their respective Δ*cst-II* mutants. At an MOI of 100, wild-type and mutant strains adhered equally well to the human Caco-2 cell line (Fig. 3A). The only exception was the GB11Δ*cst-II* strain, which displayed a lower

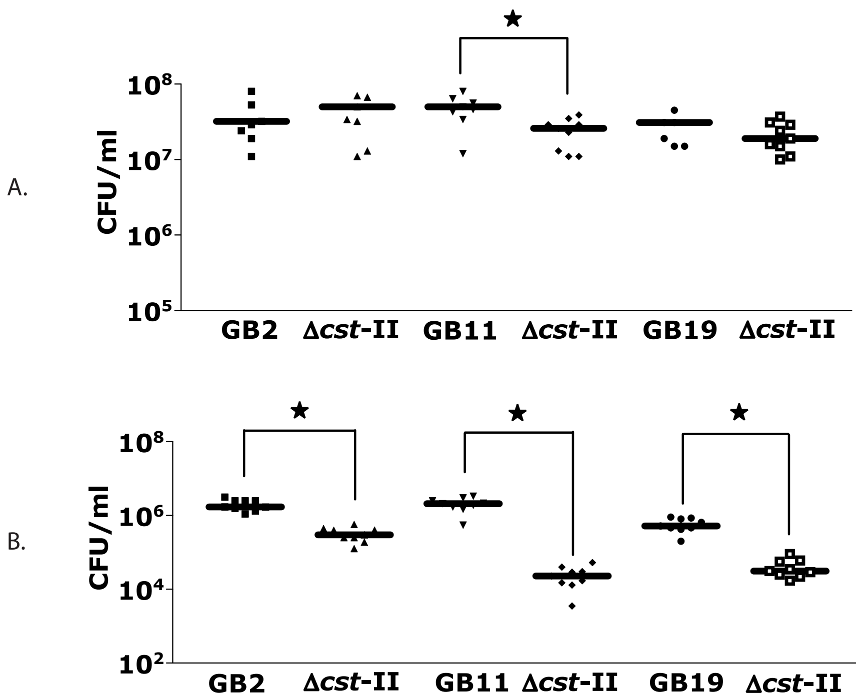

Figure 3. LOS sialylation plays an important role in invasion. *C. jejuni* wild-type strains GB2, GB11, and GB19 and their respective *cst-II* mutants were studied for adherence to **A.** and invasion of **B.** human enterocyte-like Caco-2 cells. Differences in adhesion and invasion were tested for significance by using the standard *t* test. Data are expressed as geometric means for 3 experiments each performed in triplicate. An astriks (★) indicates that a significant differences was detected.

level of adherence than wild-type GB11 a factor (*P* = 0.031). GB2Δ*cst-II*, GB11Δ*cst-II* and GB19Δ*cst-II* all showed a significant reduction in invasiveness as compared to their wild-type parent strain (*P* = 0.005, *P* = 0.002 and *P* = 0.008, respectively) (Fig. 3B).

In order to study whether the role of sialic acid in *C. jejuni* invasion is restricted to interactions with Caco-2 cells, a small selection of *C. jejuni* strains (P3, GB2, GB11 and GB13) and Δ*cst-II* mutants (GB2Δ*cst-II* and GB11Δ*cst-II*) were tested for invasiveness for the T84 human intestinal epithelial cell line (data not shown). The levels of invasiveness of all wild-type strains was similar in both cell types. Again, Δ*cst-II* mutants displayed reduced (by log 1 to 1.5) invasion of T84 cells. Together, these data establish that LOS sialylation contributes significantly to the invasion intestinal epithelial cells by *C. jejuni* into. We excluded variation in microbial motility as the mechanism underlying the reduced invasion of the Δ*cst-II* mutant strains by performing quantitative swarming assays (data not shown).

Complementation of the GB11Δ*cst-II* mutant restores expression of sialylated LOS

Site-specific homologous recombination was used to reinstall the *cst-II* gene, together with its promoter region, in the GB11Δ*cst-II* strain. Using HRP-labeled cholera toxin as a detection agent, we confirmed the expression of sialylated LOS of the wild-type GB11 strain and of three selected clones of the complemented GB11Δ*cst-II* mutant by a Western blot assay (Fig. 4, lane 1, 3, 4 and 5 respectively). The GB11Δ*cst-II* mutant did not

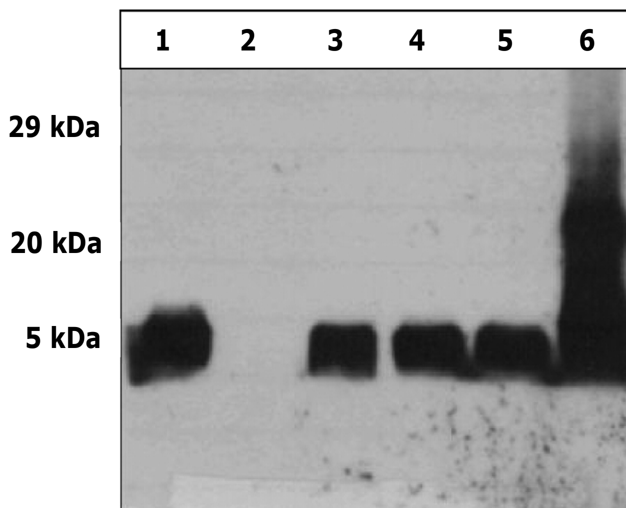

Figure 4. Western blot assay for analysis of cholera toxin binding at the LOS of wild-type GB11, its Δ*cst*-II mutant, and the complemented GB11Δ*cst*-II mutant strain. Lane 1, LOS of the GB11 wild-type strain; lane 2, LOS of the GB11Δ*cst*-II mutant strain; lanes 3, 4, and 5, LOS from three selected clones of the complemented GB11Δ*cst-II* mutant; lane 6, LOS of the 11168 genome strain, used as a positive control. The LOS band is present at around 7 kDa.

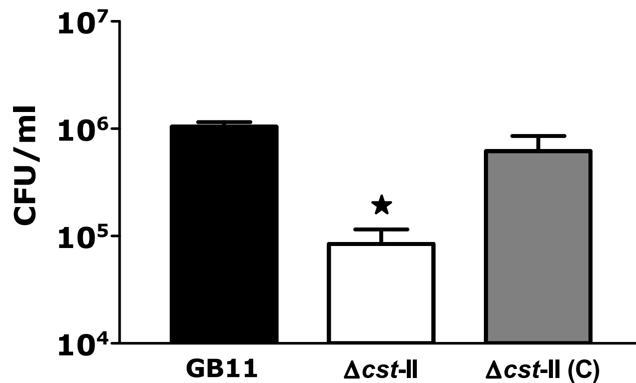

Figure 5. Complementation of the GB11 Δ*cst*-II mutant restores the wild-type phenotype for invasion observed with GB11. The *C. jejuni* wild-type strain GB11, the GB11Δ*cst*-II mutant, and the complemented GB11Δ*cst*-II (C) mutant were studied for invasion of human enterocyte-like Caco-2 cells. Data are geometric means from at least three independent experiments, each performed in duplo. Error bars, standard deviations

express sialylated LOS (Fig. 4, lane 2). LOS isolated from the 11168 genome strain was used as a positive control for binding of the HRP-labeled cholera toxin (Fig. 4, lane 6).

Complementation of the GB11Δ*cst-II* mutant restores invasiveness

The Western blot assay provided evidence that the complemented mutant was now capable of LOS sialylation. With the gentamicin exclusion assay, we were able to show that this complementation also restored invasiveness to wild-type levels (Fig. 5). These results reiterate the importance of LOS sialylation in invasion

Fixed, sialylated LOS-containing strains inhibit invasion of their viable counterparts

The decreased invasiveness of GB2Δ*cst-II*, GB11Δ*cst-II* and GB19Δ*cst-II* and the restored wild-type invasion phenotype of the complemented GB11Δ*cst-II* mutant clearly indicate a role for *C. jejuni* LOS sialylation in invasion. In order to further address the involvement of LOS sialylation in invasion, we designed an inhibition assay. We preincubated the Caco-2 cells with formalin-fixed, nonviable sialylated wild-type strains (GB2, GB11 and GB19) before incubating with viable sialylated wild-type strains (GB2, GB11 and GB19). We found reductions of as much as 1 to 2 log units in invasion by viable wild-type strains. When Caco-2 cells were preincubated with an excess of formalin-fixed nonsialylated LOS Δ*cst-II* mutants, no differences in invasion were found relative to the invasion control (Fig. 6). The invasion control groups consisted of Caco-2 cells that were incubated only with the viable wild-type strains GB2, GB11 or GB19. These results corroborate that LOS sialylation is an important determinant of epithelial cell invasiveness.

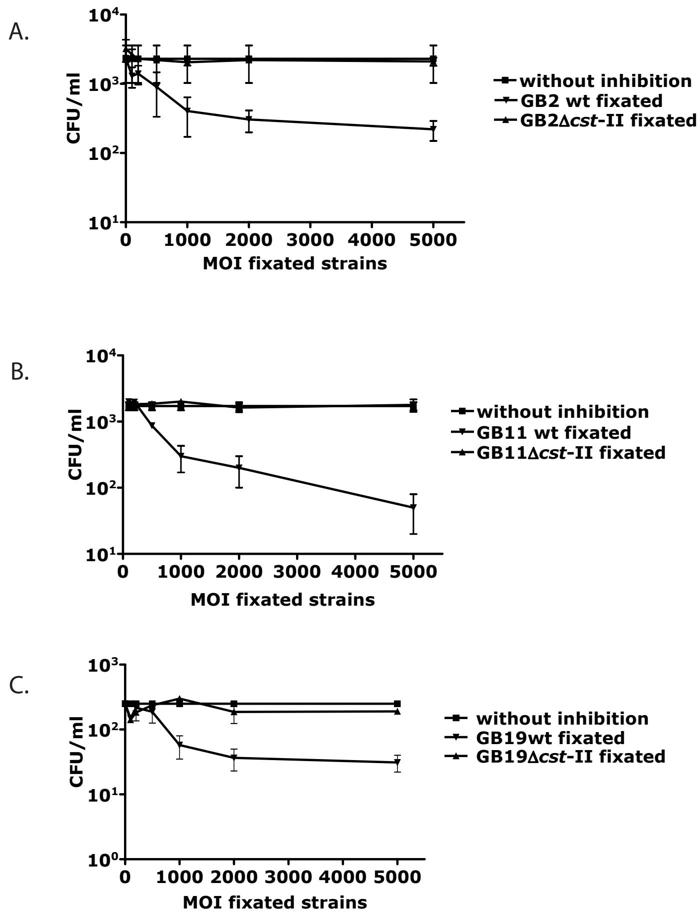

Figure 6. *C. jejuni* strains GB2, GB11, and GB19 invade Caco-2 cells via a sialylated-LOS-dependent mechanism(s). The levels of invasion by viable wild-type strains GB2 (**A**), GB11 (**B**), and GB19 (**C**) were assessed in the presence of either formalin-fixated GB2, GB11, or GB19 wild-type (wt) bacteria (sialylated LOS) or the respective fixated Δ*cst*-II mutants (truncated LOS, non-sialylated). Data are means from at least three independent experiments; error bars, standard deviations.

Discussion

The mucosal epithelial cells are the first to interact with enteric pathogens such as *C. jejuni*. This microorganism may temporarily colonize the intestines in the absence of any clinical symptom. On the other hand, *C. jejuni* has been implicated in the pathogenesis of immune-mediated pathologies, e.g., GBS. Because *C. jejuni* infection can present with

such a wide range of symptoms, it is crucial to further identify factors andmechanisms that control *C. jejuni* epithelial invasion and persistence (36). We hypothesized that the factors that regulate *C. jejuni* epithelial invasion may contribute to post-infectious sequelae, e.g., GBS.

Several *C. jejuni* outer membrane proteins, e.g., CadF, JlpA and PEB1 play a role in epithelial adhesion and invasion (37-39). Recently, PEB1 has also been identified as an amino acid transport system, which is essential for microbial growth. Previous studies that identified microbial LOS as a generally important factor for invasion have been confirmed for *C. jejuni* (12, 14-16). Here we specifically addressed if and to what extent sialylation of *C. jejuni* LOS contributes to microbial invasion. Therefore, we performed a large-scale survey by testing a heterogenic panel of 48 human-isolated *C. jejuni* strains, 7 human control strains, and 3 sialyltransferase (*cst-II*) knockout strains. The knockout strains were previously shown to lack the capacity of LOS sialylation (22).

Our studies indicate that LOS sialylation facilitates epithelial invasion (Table 1), since *C. jejuni* strains expressing sialylated LOS invaded significantly more frequently than nonsialylated LOS strains ($P<0.0001$). Two strains with presumed LOS sialylation displayed low invasiveness. These results show that LOS sialylation must be regarded as an important contributor for *C. jejuni* invasiveness but not the single determinant. Earlier reports support the hypothesis that several factors determine invasiveness (12, 14-16). Similar contributions of sialic acid to invasiveness have been established for other pathogens (40, 41). In contrast, one study reports on inhibition of invasion by sialic acid (42).

Our experiments with the GB2, GB11 and GB19 sialyltransferase (*cst-II*) knockout strains further established the importance of LOS sialylation, since these mutated strains expressing nonsialylated LOS displayed significantly lower invasiveness than their respective wild-type controls. The methods for generation of such knockout strains may be accompanied by various technical side effects, e.g., mutation of genes other than the target gene. Furthermore, insertion of an antibiotic resistance cassette may induce expression or silencing of adjacent genes and gene products. Therefore, we set up experiments using a complemented Δ*cst-II* mutant strain. We show that this procedure indeed restored sialylation of the LOS (Fig. 4) and subsequent invasiveness to wild-type-levels (Fig. 5).

In our studies, only the GB11Δ*cst-II* mutant strain showed diminished adherence relative to that of its wild-type parent strain, indicating a less important role for LOS sialylation in epithelial adhesion than in invasion. These findings indicate that adhesion and invasion are regulated by different sets of factors. Adhesion is likely established by proteins such as CadF, JlpA and PEB1 (37-39), whereas invasion is more influenced by LOS sialylation in the strains we tested.

To support that invasion is facilitated by LOS sialylation, we established that formalin-fixed wild-type strains GB2, GB11 and GB19, but not the isogenic Δ*cst-II* mutants, were

able to inhibit epithelial invasion by viable GB2, GB11 and GB19 strains. These findings may have two implications; first, these data may help to identify novel epithelial invasion receptors. Secondly, these experiments may lead to the discovery of specific agents that can be used to block microbial invasion.

Previously, sialylation of *C. jejuni* LOS was associated with GBS (22, 43, 44). Isolates from GBS patients mainly synthesize sialylated LOS of classes A and B (± 80%) (30). Strains isolated from enteritis patients show a more mixed LOS composition, with a tendency towards nonsialylated LOS expressed by the classes D and E. Notably, the presence of strains expressing LOS classes A and B in enteritis patients is around 20-25%. Therefore, the enhanced invasiveness of GBS-associated strains seems to result from the frequent presence of LOS class A and B strains in this patient group (28). We hypothesize that among other risk-factors, enhanced invasiveness (e.g., through LOS class A expression) contributes to the development of postinfectious complications such as GBS.

In conclusion, we demonstrate that *C. jejuni* strains expressing sialylated LOS have an overall increased capacity to invade intestinal epithelial cells. Knockout mutagenesis of the *cst-II* gene and complementation and blocking experiments provide additional evidence on the role of LOS sialylation in invasion of the intestinal epithelium. Understanding the function of LOS sialylation in epithelial cell invasion may provide us with potential target structures for future therapeutic interventions in *C. jejuni*-mediated diarrheal disease and its postinfectious complications.

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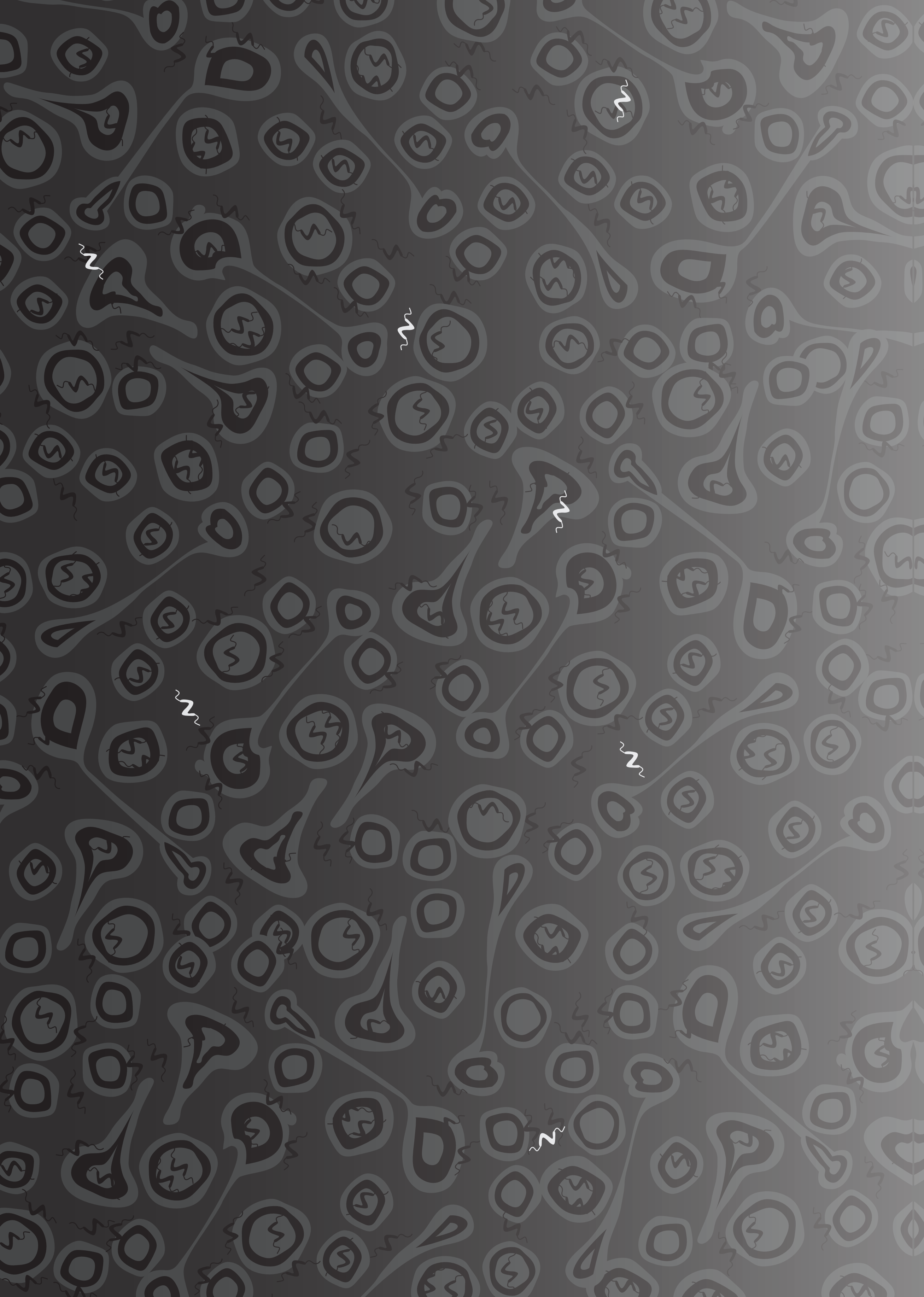
References

1. Nachamkin IB, M. J. (1992) *Campylobacter* (ASM Pres, Washington, DC 20036-2804) 2nd Ed.
2. Ketley JM (1997) Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* 143 (Pt 1):5-21.
3. Nachamkin I, Mishu Allos B, & Ho T (1998) *Campylobacter* species and Guillain-Barré syndrome. *Clin Microbiol Rev* 11(3):555-567.

4. Morooka T, Umeda A, & Amako K (1985) Motility as an intestinal colonization factor for *Campylobacter jejuni*. *J Gen Microbiol* 131 (Pt 8):1973-1980.
5. Karlyshev AV, Everest P, Linton D, Cawthraw S, Newell DG, & Wren BW (2004) The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks. *Microbiology* 150(Pt 6):1957-1964.
6. Hanel I, Muller J, Muller W, & Schulze F (2004) Correlation between invasion of Caco-2 eukaryotic cells and colonization ability in the chick gut in *Campylobacter jejuni*. *Vet Microbiol* 101(2):75-82.
7. Szymanski CM, King M, Haardt M, & Armstrong GD (1995) *Campylobacter jejuni* motility and invasion of Caco-2 cells. *Infect Immun* 63(11):4295-4300.
8. Byrne CM, Clyne M, & Bourke B (2007) *Campylobacter jejuni* adhere to and invade chicken intestinal epithelial cells in vitro. *Microbiology* 153(Pt 2):561-569.
9. Yao R, Burr DH, Doig P, Trust TJ, Niu H, & Guerry P (1994) Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. *Mol Microbiol* 14(5):883-893.
10. Wassenaar TM, Bleumink-Pluym NM, & van der Zeijst BA (1991) Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. *Embo J* 10(8):2055-2061.
11. Yao R, Burr DH, & Guerry P (1997) CheY-mediated modulation of *Campylobacter jejuni* virulence. *Mol Microbiol* 23(5):1021-1031.
12. Kanipes MI, Holder LC, Corcoran AT, Moran AP, & Guerry P (2004) A deep-rough mutant of *Campylobacter jejuni* 81-176 is noninvasive for intestinal epithelial cells. *Infect Immun* 72(4):2452-2455.
13. Preston A, Mandrell RE, Gibson BW, & Apicella MA (1996) The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit Rev Microbiol* 22(3):139-180.
14. Muller J, Meyer B, Hanel I, & Hotzel H (2007) Comparison of lipooligosaccharide biosynthesis genes of *Campylobacter jejuni* strains with varying abilities to colonize the chicken gut and to invade Caco-2 cells. *J Med Microbiol* 56(Pt 12):1589-1594.
15. Guerry P, Szymanski CM, Prendergast MM*, et al.* (2002) Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. *Infect. Immun.* 70(2):787-793.
16. Perera VN, Nachamkin I, Ung H*, et al.* (2007) Molecular mimicry in *Campylobacter jejuni*: role of the lipo-oligosaccharide core oligosaccharide in inducing anti-ganglioside antibodies. *FEMS Immunol Med Microbiol* 50(1):27-36.
17. Lambotin M, Hoffmann I, Laran-Chich MP, Nassif X, Couraud PO, & Bourdoulous S (2005) Invasion of endothelial cells by Neisseria meningitidis requires cortactin recruitment by a phosphoinositide-3-kinase/Rac1 signalling pathway triggered by the lipo-oligosaccharide. *J Cell Sci* 118(Pt 16):3805-3816.
18. Swords WE, Buscher BA, Ver Steeg Ii K*, et al.* (2000) Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol Microbiol* 37(1):13-27.
19. Parker CT, Horn ST, Gilbert M, Miller WG, Woodward DL, & Mandrell RE (2005) Comparison of *Campylobacter jejuni* lipooligosaccharide biosynthesis loci from a variety of sources. *J Clin Microbiol* 43(6):2771-2781.
20. Gilbert M, Karwaski M-F, Bernatchez S*, et al.* (2002) The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J. Biol. Chem.* 277(1):327-337.
21. Phongsisay V, Perera VN, & Fry BN (2006) Exchange of lipooligosaccharide synthesis genes creates potential Guillain-Barré syndrome-inducible strains of *Campylobacter jejuni*. *Infect Immun* 74(2):1368-1372.
22. Godschalk PC, Heikema AP, Gilbert M*, et al.* (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J Clin Invest* 114(11):1659-1665.

23. Gilbert M, Brisson J-R, Karwaski M-F, *et al.* (2000) Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384. Identification of the glycosyltransferase genes, enzymatic synthesis of model compounds, and characterization of nanomole amounts by 600-MHz ^{1}H and ^{13}C NMR analysis. *J. Biol. Chem.* 275(6):3896-3906.
24. Guerry P, Ewing CP, Hickey TE, Prendergast MM, & Moran AP (2000) Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. *Infect. Immun.* 68(12):6656-6662.
25. Chiu CP, Watts AG, Lairson LL, *et al.* (2004) Structural analysis of the sialyltransferase Cst-II from *Campylobacter jejuni* in complex with a substrate analog. *Nat. Struct. Mol. Biol.* 11(2):163-170.
26. van Belkum A, van den Braak N, Godschalk P, *et al.* (2001) A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. *Nat. Med.* 7(7):752-753.
27. Li J, Koga M, Brochu D, Yuki N, Chan K, & Gilbert M (2005) Electrophoresis-assisted open-tubular liquid chromatography/mass spectrometry for the analysis of lipooligosaccharide expressed by *Campylobacter jejuni*. *Electrophoresis* 26(17):3360-3368.
28. Ang CW, Laman JD, Willison HJ, *et al.* (2002) Structure of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity and clinical features of Guillain-Barré and Miller Fisher patients. *Infect. Immun.* 70(3):1202-1208.
29. Godschalk PC, van Belkum A, van den Braak N, *et al.* (2007) PCR-restriction fragment length polymorphism analysis of *Campylobacter jejuni* genes involved in lipooligosaccharide biosynthesis identifies putative molecular markers for Guillain-Barré syndrome. *J Clin Microbiol* 45(7):2316-2320.
30. Godschalk PC, Kuijf ML, Li J, *et al.* (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndromes. *Infect Immun* 75(3):1245-1254.
31. Aspinall GO, McDonald AG, Raju TS, Pang H, Moran AP, & Penner JL (1993) Chemical structures of the core regions of *Campylobacter jejuni* serotypes O:1, O:4, O:23, and O:36 lipopolysaccharides. *Eur J Biochem* 213(3):1017-1027.
32. Aspinall GO, McDonald AG, Raju TS, *et al.* (1993) Chemical structure of the core region of *Campylobacter jejuni* serotype O:2 lipopolysaccharide. *Eur J Biochem* 213(3):1029-1037.
33. Aspinall GO, Lynch CM, Pang H, Shaver RT, & Moran AP (1995) Chemical structures of the core region of *Campylobacter jejuni* O:3 lipopolysaccharide and an associated polysaccharide. *Eur J Biochem* 231(3):570-578.
34. Aspinall GO, McDonald AG, & Pang H (1994) Lipopolysaccharides of *Campylobacter jejuni* serotype O:19: structures of O antigen chains from the serostrain and two bacterial isolates from patients with the Guillain-Barré syndrome. *Biochemistry* 33(1):250-255.
35. Nam Shin JE, Ackloo S, Mainkar AS, *et al.* (1998) Lipo-oligosaccharides of *Campylobacter jejuni* serotype O:10. Structures of core oligosaccharide regions from a bacterial isolate from a patient with the Miller-Fisher syndrome patient and from the serotype reference strain. *Carbohydrate Res* 305:223-232.
36. Watson RO & Galan JE (2008) *Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes. *PLoS Pathog* 4(1):e14.
37. de Melo MA & Pechere JC (1990) Identification of *Campylobacter jejuni* surface proteins that bind to Eucaryotic cells in vitro. *Infect Immun* 58(6):1749-1756.
38. Konkel ME, Garvis SG, Tipton SL, Anderson DE, Jr., & Cieplak W, Jr. (1997) Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol* 24(5):953-963.
39. Pei Z, Burucoa C, Grignon B, *et al.* (1998) Mutation in the peb1A locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. *Infect Immun* 66(3):938-943.
40. Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ, & Wiley DC (1988) Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* 333(6172):426-431.
41. Schenkman RP, Vandekerckhove F, & Schenkman S (1993) Mammalian cell sialic acid enhances invasion by Trypanosoma cruzi. *Infect Immun* 61(3):898-902.
42. van Putten JP, Grassme HU, Robertson BD, & Schwan ET (1995) Function of lipopolysaccharide in the invasion of Neisseria gonorrhoeae into human mucosal cells. *Prog Clin Biol Res* 392:49-58.

43. Nachamkin I, Ung H, Moran AP, *et al.* (1999) Ganglioside GM1 mimicry in *Campylobacter* strains from sporadic infections in the United States. *J Infect Dis* 179(5):1183-1189.
44. Yuki N, Ichihashi Y, & Taki T (1995) Subclass of IgG antibody to GM1 epitope-bearing lipopolysaccharide of *Campylobacter jejuni* in patients with Guillain-Barré syndrome. *J Neuroimmunol* 60(1-2):161-164.



Chapter 4

Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides

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Abstract

In *Campylobacter jejuni* (*C. jejuni*) induced Guillain-Barré syndrome (GBS), molecular mimicry between *C. jejuni* lipooligosaccharide (LOS) and host gangliosides leads to the production of cross-reactive antibodies directed against the peripheral nerves of the host. Currently, the presence of surface exposed sialylated LOS in *C. jejuni* is the single known bacterial pathogenesis factor associated with the development of GBS.

Using a unique, well-characterized strain collection, we demonstrate that GBS-associated *C. jejuni* strains bind preferentially to sialoadhesin (Sn, Siglec-1, or CD169), a sialic acid receptor found on a subset of macrophages. In addition, using a whole-cell enzyme-linked immunosorbent assay (ELISA), *C. jejuni* strains with sialylated LOS bound exclusively to soluble Sn. Mass spectrometry revealed that binding was sialic acid-linkage specific with a preference for α(2,3)-linked sialic acid attached to the terminal galactose of the LOS chain as seen in the gangliosides GD1a, GM1b and GM3. This molecular interaction was also related to functional consequences as a GBS-associated *C. jejuni* strain that bound Sn in a whole-cell ELISA adhered to surface-expressed Sn of Sn-transfected CHO cells but was unable to adhere to wild type CHO cells. Moreover, a sialic acid negative mutant of the same *C. jejuni* strain was unable to bind Sn transfected CHO cells.

This is the first report of the preferential binding of GBS-associated *C. jejuni* strains to the Sn immune receptor ($P = 0.014$). Moreover, because this binding is dependent on sialylated LOS, the main pathogenic factor in GBS progression, the current findings bring us closer to unraveling the mechanisms that lead to formation of cross-reactive antibodies in GBS disease.

Introduction

Campylobacter jejuni (*C. jejuni*), a food-borne gram negative bacterium, is the major cause of bacterial gastroenteritis worldwide. In addition to enteritis, infection with *C. jejuni* may also lead to a neurological complication called the Guillain-Barré Syndrome (GBS). GBS is an autoimmune disease affecting the peripheral nerves. Antibodies raised by the host during an infection with *C. jejuni* possess the capacity to cross-react with structures on human nerve tissue, resulting in neurological complications for the host (1). Further, high titers of anti-ganglioside antibodies are frequently found in the sera of GBS patients (2, 3). Gangliosides are glycosphingolipids with an extracellular sialylated oligosaccharide chain and a ceramide tail that is embedded in the outer leaflet of the plasma membrane. Although predominantly found in the nervous system, gangliosides are present on other cell surfaces as well.

C. jejuni has lipooligosaccharide (LOS) structures on its outer membrane. Biochemical and structural analysis of LOS outer core oligosaccharides has identified sialylated moieties that are structurally similar to several gangliosides (4-6). During infection, the structural similarity between *C. jejuni* LOS and human gangliosides, also known as molecular mimicry, facilitates the induction of anti-ganglioside antibodies and the development of GBS (1, 7-9). The *C. jejuni* genes involved in ganglioside mimicry are located within the LOS biosynthesis locus, a gene cluster that is interchangeable between strains and is genetically highly diverse (10, 11). Therefore, several LOS classes (A through S) have been identified (12). LOS class, gene alterations, mutations and mechanisms such as phase variation in the LOS locus, contribute to structural variations in the ganglioside mimics produced (11). The presence of LOS biosynthesis locus-encoded genes responsible for synthesis, modification and transfer of sialic acid, found in LOS classes A, B and C, is crucial in the induction of anti-ganglioside antibodies and hence GBS (13, 14). Sialylated LOS is also involved in other aspects of *C. jejuni* pathogenesis. *C. jejuni* strains expressing sialylated LOS invade human epithelial intestinal cells significantly more frequently than strains expressing nonsialylated LOS (15). However, the receptor for *C. jejuni* attachment to human epithelial intestinal cells is unknown.

Certain *C. jejuni* strains are known to bind to Siglec-7, a member of the sialic acid binding immunoglobulin like lectin (Siglec) family (16). Siglecs are present on the cell surface of a range of immune-associated cells and are involved in cell to cell interactions and signaling. A subset of the Siglec family, the CD33-related Siglecs, can serve as regulators of the immune system through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail (17, 18). In addition, several recently described human Siglecs, Siglecs-14, -15 and -16 can interact with the immunoreceptor tyrosine-based activation motif (ITAM) adaptor, DAP12, and therefore potentially mediate the activation of intracellular signaling (19, 20).

Sialoadhesin (Sn, Siglec-1, or CD169) is a macrophage-restricted Siglec that has been associated with inflammatory and autoimmune diseases. For example, Sn levels are elevated on activated macrophages within the inflamed organs of several inflammatory disorders, including rheumatoid arthritis, experimental autoimmune encephalomyelitis (EAE), and experimental autoimmune uveoretinitis (EAU) (21-23). This elevated expression may have functional consequences since Sn-deficient mice show a reduced severity of EAE and EAU (23, 24). With a poorly conserved cytoplasmic tail and the absence of tyrosine-based signaling motifs, Sn seems to be more involved in cell-to-cell communication and ligand binding than intracellular immunoregulation. It has been shown that macrophages expressing Sn can bind and internalize sialylated *Neisseria meningitidis* in a Sn- and sialic acid-dependent manner (25). Further, HIV-1 can interact with Sn, probably via a sialic acid residue on gp120, with binding resulting in enhanced infectivity and facilitates transinfections in alpha interferon (IFN-α)-stimulated CD14+ monocytes. Furthermore, Sn gene expression is elevated in $CD14^{+}$ monocytes from patients infected with HIV-1 (26).

Each Siglec has a unique specificity for certain sialylated glycans, with Sn preferring sialic acid conjugates with an α(2,3)galactose (gal) linkage (27). This α(2,3)Gal linkage is often found on the LOS of GBS-associated *C. jejuni* strains.

Because of the connection of *C. jejuni* infection with autoimmune disease such as GBS and its clinical variant Miller Fisher Syndrome (MFS), we investigated whether α(2,3)-linked sialic acid residues on the surface of *C. jejuni* strains could interact with Sn and whether this interaction was characteristic for GBS-associated strains.

Material & Methods

Bacterial strains and culture conditions

Bacterial strains utilized in this study comprise (i) a group of eight Penner serotype reference strains (28, 29) (Supplemental Table 1A), (ii) 29 well-characterized GBS- or MFS-associated *C. jejuni* strains isolated from GBS and MFS patient stool samples (GB13, GB14 and GB26, GB27 were cultured from diarrheal stools of family members of two GBS patients after a family outbreak of *C. jejuni* enteritis) (30-32) (Supplemental Table 1B), (iii) 54 age and sex matched enteritis-associated *C. jejuni* strains isolated from Dutch patients with active diarrhea, and (iv) a sialic acid transferase (*cst-II*) knockout mutant of a GBS-associated strain (GB11Δ*cst-II*) (13).

C. jejuni strains were cultured from -80°C stocks and maintained on Colombia blood agar (BA) plates (Becton Dickinson BV, Alphen aan den Rijn, The Netherlands) supplemented with 10 µg vancomycin/ml in a microaerophilic atmosphere at 37°C. For culture of the GB11Δ*cst-II* mutant strain, chloramphenicol (20 µg/ml) was added to the growth

medium. Prior to experimentation, all strains were cultured overnight on BA-plates containing vancomycin only. The LOS outer core structures of the GBS-associated strains utilized in this study have previously been reported (31). LOS was purified by hot phenol-water extraction as previously described (2). For the enteritis-only strains, we used mass spectrometry data as reported (33) to determine whether sialic acid was present in the LOS outer core. PCR screening to verify the LOS class was performed as previously described (13).

Cell culture and preparation of Fc-conjugates

Wild-type Chinese Hamster Ovary cells (CHO-wt), CHO cells expressing Sn domain 1-17 (CHO-Sn), and CHO cells expressing a Sn mutant with an amino acid substitution in the sialic acid binding pocket at amino acid position 97 (arginine to alanine; CHO-SnR97A) were generated as previously described (34). Cells were maintained in Ham/F-12 medium (PPA Laboratories, Cölbe, Germany), containing penicillin/streptomycin, 2 mM L-glutamine and 10% fetal calf serum (FCS) and were routinely grown in plastic 75-cm^2 flasks (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) at 37°C in a humidified 5% CO_2-95% air incubator. With respect to Sn-Fc production, CHO cells expressing murine Sn recombinant Fc fusion protein domain 1-3 (Sn-Fc) or its mutant form (SnR97A-Fc) were generated as previously described (35). Cells were cultured in 225 cm^2 flasks and expanded into roller bottles in glutamine free Glasgow Minimal Essential Medium (GMEM) (Sigma-Aldrich, Zwijndrecht, The Netherlands) containing 100 µM L-methionine sulfoximine (Sigma-Aldrich), glutamine synthetase (GS) supplement (Sigma-Aldrich), penicillin/streptomycin and 10% dialyzed FCS (Invitrogen, Leek, The Netherlands). The FCS concentration was adjusted to 2% once cells covered ca. 80% of the surface of the bottle. When 100% confluence was reached, cells were put on X-VIVO-10 serum-free media (Lonza, Verviers, Belgium), and medium containing Sn-Fc or SnR97A-Fc was harvested weekly. The concentration of Sn-Fc and SnR97A-Fc produced was determined using an Fc-specific enzyme-linked immunosorbent assay ELISA.

Quantification of Fc-conjugates

A 96-well Maxisorb ELISA plate (NUNC Inc. Uden, The Netherlands) was coated with 0.01 mg goat anti-human IgG (Sigma-Aldrich)/ml, followed by incubation for 2 h at room temperature. After washing, wells were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Series of 5-fold dilutions of the Sn-Fc- or SnR97A-Fc-conjugates were added to the plates, followed by incubation for 2 h at room temperature. Five-fold dilutions of a sample containing a known concentration of Siglec-Fc were used as a reference. After washing, wells were incubated with 1/2,000-diluted peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich). Plates were washed four times with 0.05% Tween 20 in PBS and developed with 100 µl TMB substrate (3',3',5',5'-tetramethyl-

benzidine; Sigma-Aldrich)/well. After an appropriate incubation time (5 to 10 minutes), the reaction was stopped by adding 100 µl 2 M H_2SO_4/well. The intensity of the signal was measured spectrophotometrically at 450 nm by using a 96-well microplate reader (Bio-Rad, Veenendaal, The Netherlands), and the concentrations were determined.

Sialoadhesin-Fc ELISA

With respect to the ganglioside/SnFc ELISA, equal amounts (300ng/well) of purified bovine brain ganglioside (GA1, GM1, GM2, GM3, GD1a, GD1b, GD3 and GT1b (Sigma-Aldrich)) diluted in ethanol, were applied to 96-well Maxisorp ELISA plates (NUNC Inc.). Plates were then incubated for 2 h at room temperature, allowing the ethanol to evaporate. For the LOS/Sn-Fc ELISA, plates were coated overnight at 37 ºC using 2 µg of LOS in PBS plus 0.2% trichloroacetic acid per well. After a washing step, the wells were blocked for 2 h at room temperature and 2 h at 4ºC with 1% BSA in PBS (pH 7.8). In the meantime, 1.25 µg Sn-Fc conjugate/ml was precomplexed with 1,4000-diluted peroxidase-conjugated anti-human IgG (IgG-PO) (Sigma-Aldrich) in PBS with 0.05 % normal goat serum for 1 h at room temperature with shaking. After a washing step, 100 µl precomplexed Sn-Fc was added/well, and plates were incubated for 2 hat room temperature. Plates were washed four times with PBS and developed using TMB as previously mentioned.

Finally, for the *C. jejuni*-Sn-Fc ELISA, fresh overnight *C. jejuni* cultures grown on BA plates were harvested and washed, and optical density at 600 nm (OD_{600}) was adjusted to 0.2 in +/++PBS (i.e., containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$. After heat inactivation at 56ºC for 45 min, 100 µl of each sample/well was added in duplicate to 96-well Maxisorp ELISA plates (NUNC Inc.), and the open plates were incubated overnight at 37ºC in order to allow the fluid to evaporate. After overnight incubation and washing, wells were blocked for 1 h with 1% BSA in PBS at 37ºC. In some cases, the bacteria were pre-treated with neuraminidase for 16 *h* using 0.05 U of *Arthrobacter ureafaciens* α2-3,6,8,9-neuraminidase (Calbiochem, Breda, The Netherlands)/ml in PBS-1 % BSA. Thereafter, the ELISA protocol previously described for the ganglioside Sn-Fc ELISA was followed with the exception that the *C. jejuni*/Sn-Fc ELISA plates were washed with PBS containing 0.05 % Tween 20 to minimize nonspecific binding.

Statistical procedure

For statistical analysis, strains were divided into either positive or negative groups depending on their Sn binding properties. Strains exhibiting an OD_{450} value higher than 2 times the background signal plus 2 times the standard deviation were classified as positive for Sn binding and strains with a lower OD_{450} signal as negative. The chi-square test was used to indicate whether a significant difference existed in Sn binding between

GBS-associated and enteritis-only strains. We consider $P \leq 0.05$ to be statistically significant.

FITC-labeling of *C. jejuni*

Fresh overnight cultured *C. jejuni* were harvested in +/+ PBS and incubated for 1 h with 5 µl of fluorescein isothiocyanate (FITC; 100mg/ml in dimethyl sulfoxide)/ml with shaking. Bacteria were washed in PBS and heat inactivated for 45 min at 56°C, and OD_{600} was adjusted to 1.0 in +/+ PBS.

Binding of *C. jejuni* to Sn-expressing and wild-type CHO cells

CHO-wt, CHO-Sn and CHO-SnR97A were grown to ~ 80% confluence on glass cover slips and, after being washed with serum-free medium, were incubated for 2 h at 37°C along with various FITC-labeled *C. jejuni* strains in media containing 1% FCS. For this procedure, a bacterium/cell ratio of 100:1 was used. After a washing step, the cells were fixed for 20 min in methanol at -20°C and counterstained using 3 µM propidium iodide (PI). For flow cytometry analysis, semi-confluent CHO cells were harvested from 75-cm^2 flasks using PBS containing 2mM EDTA. Cells were incubated for 45 min with FITC-labeled bacteria (bacteria/cell ratio of 100:1) in a 37°C incubator with shaking. After being washed, the cells were analyzed by using a FACSCalibur (Becton Dickinson BV). In control experiments, the cells were incubated for 15 min with 1/10 diluted 3D6, a monoclonal rat anti-mouse antibody raised against Sn, prior to incubation with the bacteria. To confirm Sn expression on CHO-Sn and CHO-SnR97A, 1/100-diluted 3D6 and 1/1,000 diluted Alexa Fluor 633-conjugated goat anti-rat IgG (H+L) secondary antibody (Invitrogen) was used.

Results

Binding of Sn-Fc to purified gangliosides

To validate our batch of Sn-Fc and to extend our knowledge of Sn/ganglioside interactions and affinities using ELISA, we determined the ability of Sn-Fc to bind to a panel of purified bovine brain gangliosides. Gangliosides were coated on ELISA plates and incubated with Sn-Fc precomplexed with anti-human IgG-PO. It has been shown that Sn preferentially binds to α(2,3)-linked sialic acid glycoconjugates with strong affinity for sialic acid residues in the terminal position of a Gal-GalNAc-Gal backbone (27). We confirmed these findings with Sn binding properties in a ranking order of GD1a > GT1b > GM3. No Sn binding was observed either when sialic acid was absent (GA1), sialic acid was linked to the inner galactose of (Gal)-GalNAc-Gal (GM1a, GM2), or there was an α(2,8)-linkage (GD1b, GD3) (Fig. 1, Fig. 2). In conclusion, the binding efficiency of the Sn variant used here is in agreement with literature data.

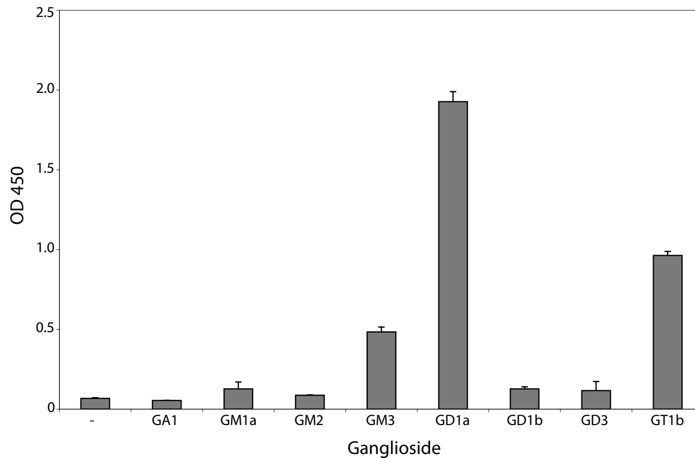

Figure 1. Sialoadhesin-Fc binding to purified gangliosides. Purified bovine gangliosides dissolved in ethanol were coated on ELISA plates, incubated with Sn-Fc precomplexed with anti-human IgG-PO, and visualized by using TMB. As a control for nonspecific binding of the precomplexed Sn-Fc, ethanol-coated wells were used (-). The data are depicted as means and standard deviations of quadruple measurements. Schematic structures of the gangliosides that were tested are depicted in Fig. 2.

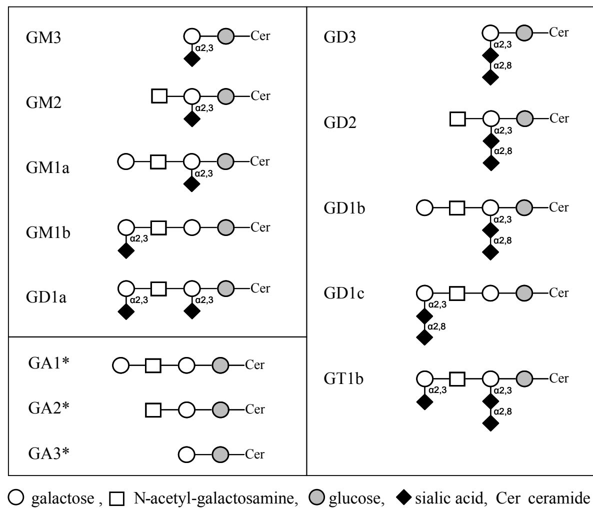

Figure 2. Schematic representation of the human ganglioside structures relevant to the present study. The galactose-sialic acid linkages are indicated. These structures are mimicked by *C. jejuni* in the outer core LOS. However, instead of the ceramide bound glucose, the *C. jejuni* LOS has a heptose, followed by an inner sugar core, and *C. jejuni* LOS has a lipid A transmembrane tail instead of a ceramide tail. * GA1, GA2 and GA3 or asialo-GM1, -GM2 and -GM3, are considered not to be gangliosides.

Sialic acid dependent interaction of Sn-Fc with heat inactivated *C. jejuni* strains and purified LOS

Similar to the ganglioside ELISA described above, we set up a whole cell bacterial ELISA using eight heat-inactivated *C. jejuni* Penner strains with known ganglioside structures. These included not only Penner strains harboring ganglioside mimics that are the most important in GBS pathology (GM1, GM2, GM3, GD1a and GD3) but also control strains possessing no ganglioside mimic (none or GA3). Consistent with the ganglioside binding pattern, we found strong Sn binding for strains P2 (GM3), P4 (GM1, GD1a) and P19 (GM1a, GD1a) all having terminal α(2,3)-linked sialic acid residues in the LOS. Sn binding was also

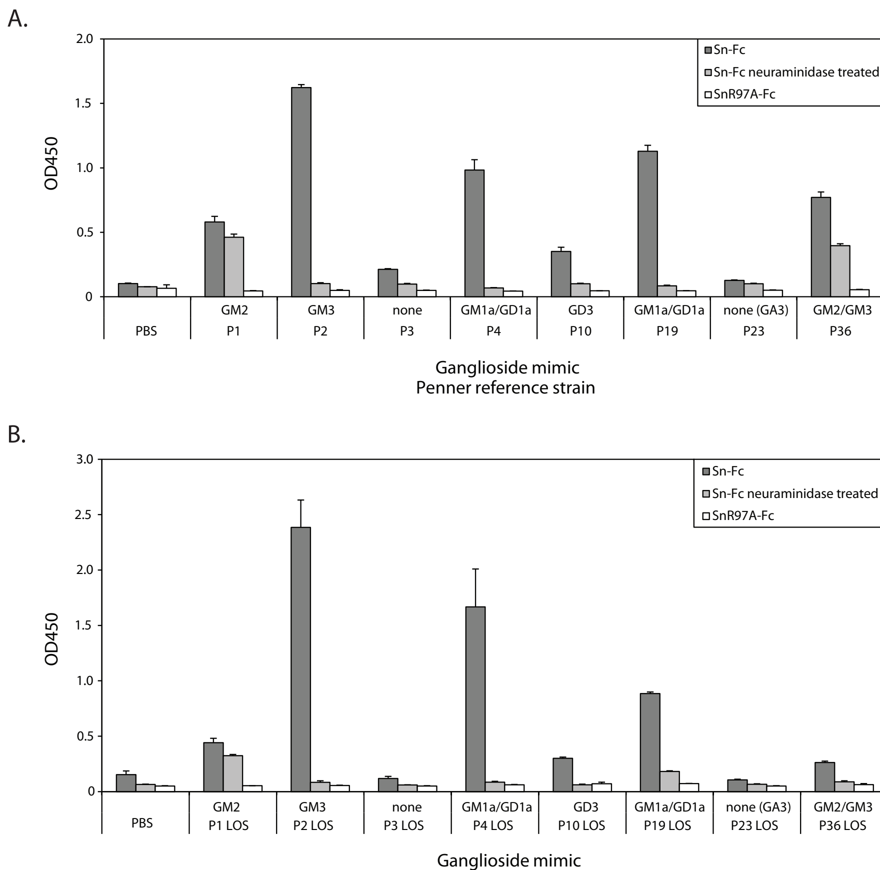

Figure 3. Heat-inactivated *C. jejuni* Penner strains (**A**) or purified LOS of the same Penner strains (**B**) were coated on ELISA plates, and incubated with precomplexed Sn conjugates Sn-Fc or SnR97A-Fc, and visualized using TMB. For binding assays, Penner strains and LOS were either left untreated or were treated with neuraminidase. The bars represent a single experiment which was repeated at least three times, with means and standard deviations of three measurements. Schematic structures of the ganglioside mimics that are expressed by the Penner strains are depicted in Fig 2.

observed for P36 (GM2, GM3). Surprisingly, P1 (GM2) and P10 (GD3) also showed binding to Sn. It is possible that the presence of some undetected GM3 is responsible for this phenomenon. Binding of Sn to strains P3 (none) and P23 (GA3), which lack ganglioside mimics, was found to be low or zero (Fig. 3A). To confirm that these interactions were sialic acid dependent, we treated the Penner strains with neuraminidase before incubation with the Sn-Fc conjugate. Neuraminidase treatment completely abolished Sn recognition of P2 (GM3), P4 (GM1, GD1a), P10 (GD3), and P19 (GM1, GD1a), showing that Sn binding to these strains is sialic acid specific. Sn binding to P1 (GM2) and P36 (GM2, GM3) was reduced, although not to background levels. Reduction in binding is probably due to the loss of the GM3 mimic. GM2, with an internal sialic acid, is less sensitive to neuraminidase treatment, so residual binding might be because of the presence of this structure.

The ELISA was also performed using the Sn mutant SnR97A-Fc. This mutant has an amino acid substitution (R97A) in the sialic acid binding pocket and lacks the ability to bind sialic acid conjugates. None of the strains showed binding to SnR97A, ruling out nonspecific binding to other parts of the protein (Fig. 3A).

To date, no ganglioside-like or sialylated structures other than the sialylated LOS have been found on the surface of *C. jejuni* strains. Therefore, Sn binding to *C. jejuni* strains as observed in the whole cell Sn-Fc ELISA is almost certainly due to the presence of sialylated LOS structures on the bacterial surface. To show that LOS is the ligand for Sn, we also performed Sn-Fc and SnR97A-Fc ELISAs on purified LOS (Fig. 3B), using LOS from the same Penner strains as used in Fig. 3A. Other than some relative differences in signal intensity for P19 and P36, the Sn binding pattern for purified LOS was very similar to the Sn binding pattern for intact bacteria, indicating that the interaction is really LOS-specific.

Binding of *C. jejuni* to Sn-expressing CHO cells

When attached to ELISA plate wells, *C. jejuni* and purified LOS from *C. jejuni* were both able to bind to precomplexed soluble Sn. *In vivo*, however, Sn is exposed on cell surfaces. Therefore, to test whether sialic acid-dependent binding to soluble Sn could be reproduced using cell surface-expressed Sn, we used CHO cells stably transfected with mouse Sn cDNA (CHO-Sn). Binding of *C. jejuni* to CHO-Sn cells was compared to that of parental CHO cells (CHO-wt), as well as CHO cells transfected with the Sn mutant R97A (CHO-SnR97A). GB11, a GBS associated strain, and its Campylobacter sialic acid transferase knockout mutant (GB11Δ*cst-II*) were used. These strains were first tested for Sn binding using the Sn-Fc ELISA, and as expected, GB11 (possessing a mix of GM1a- and GD1a-like structures) bound to Sn, whereas GB11Δ*cst-II* (possessing GA1, GA2 and GA3) did not (Fig. 2 and 4A).

Immunofluorescent staining (Fig. 4B) showed a clear association between GB11 and Sn-expressing CHO cells but no binding of GB11 to CHO-wt or CHO-SnR97A cells. GB11Δ*cst-II*

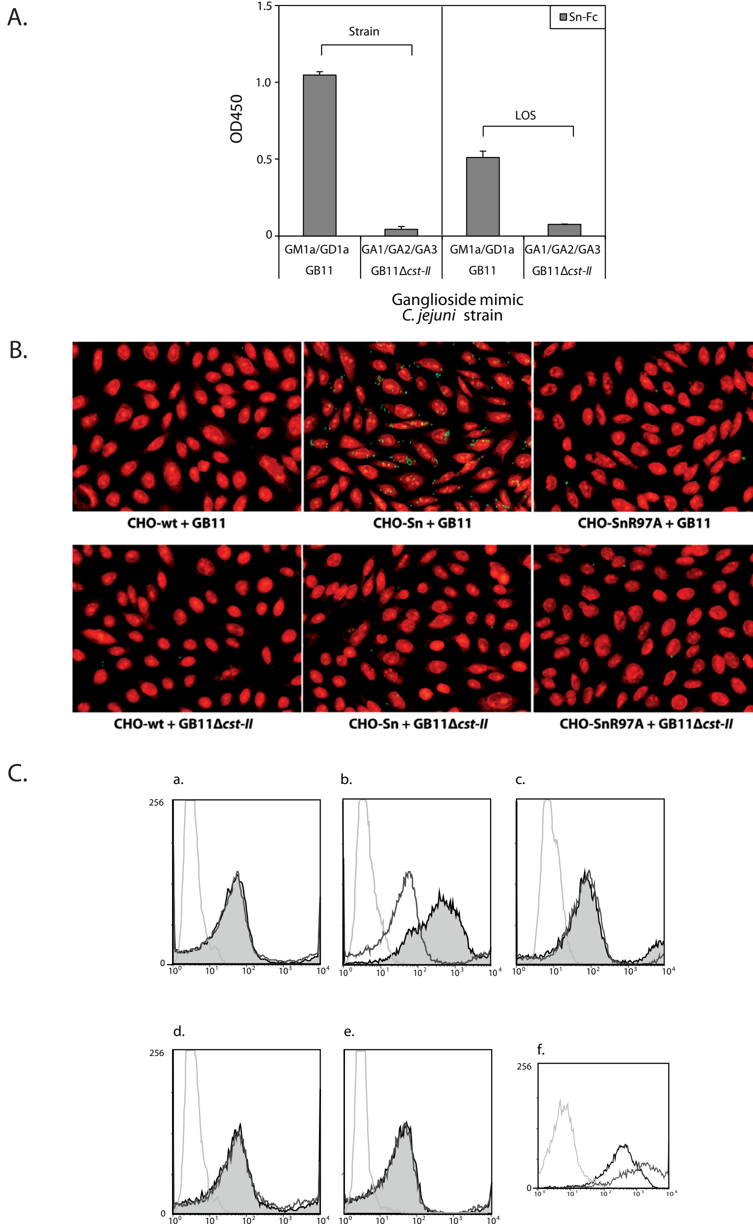

Figure 4. A. Sn binding to GBS-associated strain GB11 and the sialic acid negative mutant GB11Δ*cst-II*. Whole bacteria and purified LOS was tested for Sn binding using ELISA. A schematic representation of the ganglioside mimicking structures expressed by GB11 and GB11Δ*cst-II* can be found in Fig. 2. The structures were determined by mass spectrometry and previously published (20). **B.** Immunostaining of wild-type-, Sn- and SnR97A-expressing CHO cells, incubated with FITC-labeled GB11 or the sialic acid-negative mutant GB11Δ*cst-II*. Cells were counter stained using PI. **C.** Flow cytometric analysis of binding

between CHO cells and *C. jejuni* strains. Wild type- and Sn-expressing CHO cells were incubated with FITC-labeled GB11 or GB11Δ*cst-II*, with or without preincubation using 3D6 (a monoclonal rat anti-mouse antibody against Sn). Expression of Sn on the CHO cells was confirmed using 3D6 and an Alexa Fluor 633-labeled secondary antibody. (a) CHO-wt, (b) CHO-Sn, (c) CHO-SnR97A, incubated with GB11 and GB11Δ*cst-II*. (d) CHO-wt, (e) CHO-Sn cells, preincubated with 3D6 before the addition GB11 or GB11Δ*cst-II*. GB11 is depicted as filled grey curves; GB11Δ*cst-II* as dark grey open curves. Light grey open curves indicate CHO cells without the addition of bacteria. (f) Expression of Sn (or Sn-R97A) on CHO cells. The light grey line is CHO-wt, the back line is CHO-Sn, and the dark grey line is CHO-SnR97A.

did not bind to any of the CHO cell-lines, indicating that the binding of GB11 to CHO-Sn was actually sialic acid dependent. Flow cytometric analysis of CHO cells incubated with FITC-labeled *C. jejuni* (Fig. 4C) confirmed these findings with a shift in the fluorescent signal observed when GB11 incubated with CHO-Sn was compared to GB11Δ*cst-II* incubated with CHO-Sn. This effect was not observed when GB11 and GB11Δ*cst-II* were incubated with CHO-wt or CHO-SnR97A. Further, upon preincubated of CHO-Sn with 3D6, no difference in binding was observed between GB11 and GB11Δ*cst-II*, indicating that the binding was strictly Sn dependent. Together, these results show that *C. jejuni* with ganglioside mimics in their LOS bind to cell-exposed Sn in a sialic acid-dependent manner.

Sn binding properties of GBS-associated and enteritis-only *C. jejuni* strains

Based on the findings that *C. jejuni* was able to bind Sn in a sialic acid linkage-specific manner, with a preference for terminal α(2,3)-linked sialic acid residues, and the knowledge that this sialic acid linkage is frequently found in the LOS of GBS associated strains, we screened a well-characterized GBS-associated strain collection (n = 29), and an age and sex-matched enteritis control group (n = 54) for Sn binding. For this process, a whole-cell Sn-ELISA was used with heat-inactivated *C. jejuni* strains coated onto the ELISA plate. A total of 20 (69%) of the 29 GBS-associated strains tested were found to be positive for Sn binding in the ELISA (Fig. 5A), including all strains containing GD1a-like LOS (GB2, GB3, GB11, GB18, GB21, GB22, GB28, and GB31). The latter strains always expressed a combination of a GD1a and a GM1a ganglioside mimic. Strains GB13 and GB14, expressing a GM1a like structure only, produced negative Sn binding results in the ELISA. Therefore, and because the bovine ganglioside GM1a did not show binding in the ganglioside ELISA, the GD1a part of the GD1a/GM1a harboring strains is most likely responsible for Sn binding. Other strains that were positive for Sn binding at least had GM1b- or GM2-like LOS present. Strains MF7 and GB23, also with GM2-like LOS, showed little binding. The difference in binding affinity that was observed for strains with similar LOS structures was probably due to differences in expression rates of the mimics.

Strain GB27 showed very strong Sn binding which is surprising since we previously reported that its LOS outer core is a GA1-like structure (31). GB27 LOS genes are identical to LOS genes of GB26 but earlier sequence analysis had shown that the sialyltransferase gene (*cst-II*) was variable in these two strains due to phase variation (i.e., alterations in

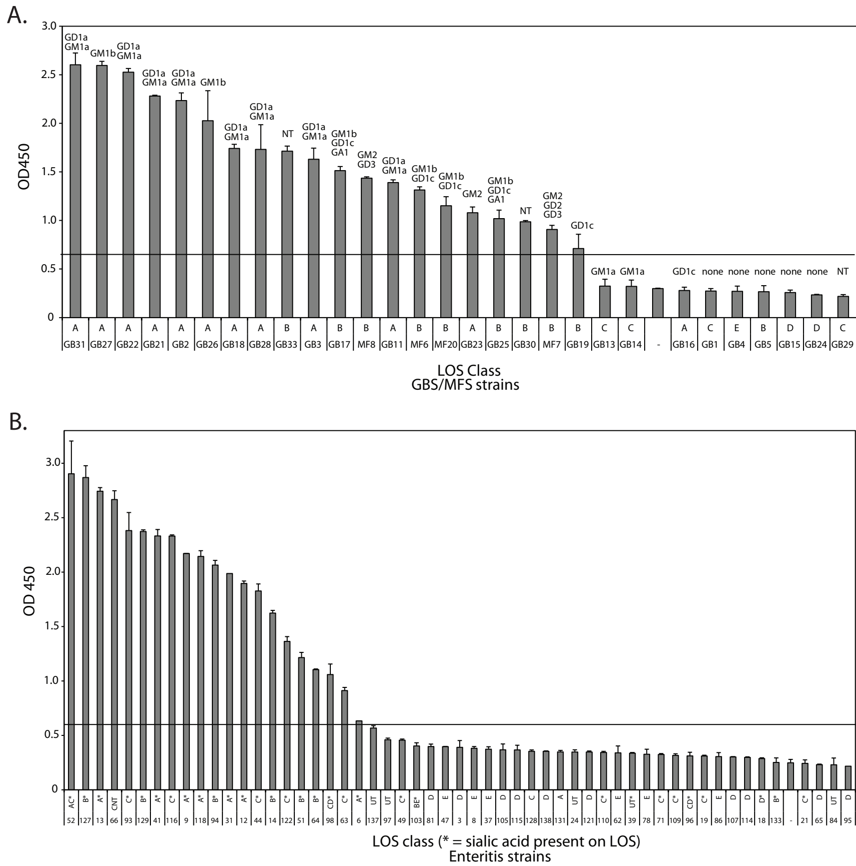

Figure 5. A. Sialoadhesin binding to GBS-associated strains. **B.** Sialoadhesin binding to enteritis only strains. Binding of Sn-Fc to GBS-associated strains and enteritis-only strains was evaluated by ELISA. Heat-inactivated bacteria were coated onto plates, incubated with Sn-Fc precomplexed with IgG-PO, and visualized using TMB. The bars represent a single experiment which was repeated at least two times, with means and standard deviations of two measurements per experiment. -, PBS control for background staining. The black line represents 2 times (signal plus the standard deviation) the negative control. Strains with OD_{450} values above this line were considered to be positive for Sn binding. UT, untypeable; NT, not tested. Schematic structures of the gangliosides mimics that are expressed by the GBS- and MFS-associated strains are depicted in Fig. 2.

a hypervariable homopolymeric G tract). GB26 had a 9-G tract in *cst-II* that predicted a complete translation product consistent with a sialylated outer core, while GB27 had a 10-G tract that predicted a premature translation stop resulting in no sialyltransferase activity in that strain. Because of the heterogeneity of homopolymeric G tracts, we suggest that the *cst-II* gene had a 9-G tract and was turned on in the GB27 sample that was tested for Sn binding. We sequenced the *cst-II* gene of 4 samples of minimally passaged GB27 cultures and indeed found that they contained 9-G tracts (turned on) in the *cst-II* gene. We also confirmed the presence of a GM1b mimic in the LOS outer core

Table 1. Binding of Sn to GBS- or enteritis-related *C. jejuni* strains [a]

Strain	Count (%) within strains	
	Negative	Positive
GBS	9 (31.0 %)	20 (69 %) [b]
Enteritis	32 (59.3 %)	22 (40.7 %)

[a] Strains were divided into positive or negative for Sn binding characteristics. Strains with OD values higher than 2 times the background signal plus 2 times the standard deviation were classified as positive for Sn binding, and strains with a lower signal as negative for Sn binding.
[b] A chi-square test analysis showed that the GBS group of strains exhibited significantly more Sn binding than the enteritis group of strains ($P = 0.01$)

of these samples by electrophoresis-assisted open-tubular liquid chromatography mass spectrometry (Supplemental Table 2). Clearly, GB27 is a strain that has the capacity to synthesize a GM1b mimic and to bind strongly to Sn.

The GBS-associated strains were significantly more often positive for Sn binding when compared to the enteritis strains ($P = 0.014$) (Table 1). For the enteritis strains, 22 (41%) of 54 strains tested, were positive (Fig 5B). All strains that were positive for Sn binding possessed either a class A, a class B or a class C LOS gene locus. Strains with such an LOS class contain genes involved in sialic acid synthesis, modification and transfer. The LOS outer core of these enteritis-only strains (except for strain 66) have previously been analyzed by electrophoresis-assisted open-tubular liquid chromatography–electrospray mass spectrometry (Supplemental data Table 1 in reference (33)). This analysis confirmed that sialic acid was present in the LOS outer core of the enteritis-only strains that were positive for Sn binding. The mass spectrometry analysis of the enteritis-only strains did not allow determination of the complete LOS outer core structures. However, correlations can be made for the strains that had mass species identical to GBS and MFS strains for which the LOS outer core structures are known (see reference (31)). For example, strains 19, 49, 71, 109 and 110 all had the same mass species as GB13 and GB14 which were previously shown to display GM1a mimicry. Therefore, it is reasonable to expect that these strains also possess sialic acid in their LOS outer cores but still are negative for Sn binding. Strains 9, 12, 13, 31 and 41 had mass species identical to GB11, which has GM1a/GD1a mimicry. Similar to GB11, these five strains were positive for Sn binding.

Discussion

It has been well established that LOS structures, expressed on the surface of *C. jejuni,* play an important role in development of the postinfectious autoimmune disorders GBS and MFS. Cross-reactive, nerve damaging antibodies are produced during infection due to molecular mimicry between *C. jejuni* outer core LOS and gangliosides on peripheral

nerves. The expression of genes involved in LOS sialylation are a prerequisite for mimicry, since truncated LOS structures without sialic acid show a reduced reactivity with GBS patient serum and fail to induce an anti-ganglioside antibody response in mice (13).

Here we report that the sialylated structures on the surface of *C. jejuni* are able to specifically bind to Sn, an immune receptor of the Siglec family that has been linked to various autoimmune diseases. We show that the ligand for Sn is the outer core LOS. Moreover, binding to Sn is sialic acid dependent as neuraminidase treated strains, as well as a sialic acid transferase knock-out strain, failed to bind to Sn. Sn specifically recognizes LOS structures with a terminal α(2,3) linked sialic acid conjugate as seen in GD1a, GM3 and GM1b. Crucially, upon screening a large panel of pathogenic GBS-associated and non-pathogenic enteritis strains, significantly more GBS-associated strains bound Sn compared to enteritis-only strains ($P = 0.014$).

What is the consequence of Sn binding? It is unclear whether binding to Sn on host cells affects the fate of *C. jejuni*. Sn is expressed on a subset of macrophages, predominantly on macrophages in the subcapsular sinus (SCS) and medulla of the lymph nodes and on metallophilic macrophages of the spleen (36). Lymph node macrophages are involved in capturing and processing of trafficking antigens from the lymph. A recent study (37) showed that SCS lymph node macrophages have limited phagocytic activity; therefore, Sn binding may not be pivotal for intracellular infection by and survival of the bacterium. In contrast, SCS macrophages possess the ability to retain antigens on their cellular surface, which may lead to antigen presentation to follicular dendritic cells (FDC) (38). As such, Sn expression on SCS macrophages may allow capture of soluble ganglioside-mimicking LOS fragments and FDC presentation, leading to B cell maturation and subsequent antibody production.

Another feature suggesting that Sn is not primarily involved in the initial events of *C. jejuni* invasion comes from our own observations. Although we have shown that *C. jejuni* strains with sialylated LOS invade intestinal epithelial cells better than nonsialylated strains (15), this effect was found not to be sialic acid linkage dependent. Moreover, intestinal epithelial cells are not known to express Sn. Therefore, factors and/or pattern recognition receptors other than Sn must play a role in *C. jejuni* invasion of the intestinal epithelium.

In our study we used murine Sn which has a high degree of protein sequence similarity, up to 79% in the sialic acid binding domain, to human Sn as well as similar α(2,3)-linkage and sialic acid-dependent binding properties (21). Extrapolation to the human situation is therefore plausible.

Our results show that especially strains with a GD1a, GM3 or GM1b ganglioside epitope and only strains with a class A, B or C LOS were positive for binding to Sn. Only these LOS classes harbor genes involved in LOS sialylation and ganglioside mimicry. Not just GBS-associated strains but also 40.7 % of enteritis-only strains were positive for Sn binding. Although these strains possess the Sn binding epitope, infection was not associated with the development of neurological dysfunctions. Therefore, other factors

that play a role in macrophage-mediated Sn binding and subsequent processes must contribute to the development of GBS. Single nucleotide polymorphisms (SNPs) that contribute to genetic variations between hosts might alter macrophage function and behavior. Genetic polymorphisms in genes encoding the macrophage-mediators tumor necrosis factor–alpha and matrix metalloproteinase-9 have been associated with the more severe forms of GBS (39). Furthermore, coinfection with another bacterial species or virus might direct the immune system towards Sn-mediated autoimmunity. For example, viral infections can induce release of interferons (IFNs), factors that have been shown to upregulate Sn on the surface of monocyte-derived macrophages (40).

Not all GBS associated strains tested were positive for Sn binding. Strains GB13 (GM1a) and GB14 (GM1a) lack a terminal α(2,3)-linked sialic acid residue that, most likely, is necessary for Sn binding. In fact, these strains were not isolated from a GBS patient but from two family members of a GBS patient during a family related *C. jejuni* enteritis outbreak. Although we classified these strains as GBS-associated because the patient serum reacted with LOS fractions from the *C. jejuni* strains isolated from the family members, a separate uncultured strain might have triggered development of GBS. Strain GB1 and GB5 with LOS classes C and B harbor genes required for ganglioside mimicry, but do not express ganglioside-like epitopes. Sequence analysis of these strains revealed one or more base deletions in LOS-associated genes, resulting in a truncated LOS outer core without sialic acid (31). Anti-GM1 antibodies were detected in the acute phase serum of the GBS patient from whom strain GB1 was isolated (41). It might be that the base deletions in the LOS of GB1 occurred later in the course of the infection or during laboratory procedures. Strains GB4, GB15 and GB24 lack the genes essential for ganglioside mimicry. Although these strains were cultured from the stools of GBS-patients, no anti-ganglioside antibodies were detected in the acute-phase sera of these patients (31, 41), suggesting that another pathogenic mechanism than molecular mimicry was involved.

Binding of *C. jejuni* strains to Siglec-7, which has a preference for α(2,8)-linked sialic acid glycans, has already been demonstrated in an earlier study (16). In the same study, however, no binding was found for Sn, not even using LOS purified from strain HS:19 (GM1, GD1a). Although variation in the signals between strains was observed in our experiments, all strains possessing GD1a-like LOS we tested were positive for Sn binding. It is unclear, therefore, why no Sn binding was observed using strain HS:19 in this publication (16). We also tested our strains for binding to Siglec-7, and preliminary experiments revealed that especially the MFS associated strains MF6, MF7, MF8 and MF20 had high affinity for Siglec-7 (data not shown). Although these strains were also positive for Sn binding, the Siglec-7 pathway might generate additional immune responses leading to the specific oculomotor nerve dysfunction seen in MFS. Future studies will focus on this interesting aspect.

In this publication, using various test systems, we demonstrate that *C. jejuni* strains expressing α(2,3)-linked sialylated surface structures are able to bind to Sn. Our finding

that GBS-associated strains preferentially bind Sn when compared to enteritis strains brings us nearer to an understanding of the mechanisms involved in the formation of cross-reactive antibodies in *C. jejuni*-mediated GBS.

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References

1. Willison HJ & Yuki N (2002) Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125(12):2591-2625.
2. Jacobs BC, Hazenberg MP, van Doorn PA, Endtz HP, & van der Meché FGA (1997) Cross-reactive antibodies against gangliosides and *Campylobacter jejuni* lipopolysaccharides in patients with Guillain-Barré or Miller Fisher syndrome. *J Infect Dis* 175:729-733.
3. Yuki N, Ho TW, Tagawa Y, *et al.* (1999) Autoantibodies to GM1b and GalNAc-GD1a: relationship to *Campylobacter jejuni* infection and acute motor axonal neuropathy in China. *J Neurol Sci* 164(2):134-138.
4. Moran AP, Rietschel ET, Kosunen TU, & Zahringer U (1991) Chemical characterization of *Campylobacter jejuni* lipopolysaccharides containing N-acetylneuraminic acid and 2,3-diamino-2,3-dideoxy-D-glucose. *J. Bacteriol.* 173(2):618-626.
5. Aspinall GO, McDonald AG, Raju TS, Pang H, Moran AP, & Penner JL (1993) Chemical structures of the core regions of *Campylobacter jejuni* serotypes O:1, O:4, O:23, and O:36 lipopolysaccharides. *Eur J Biochem* 213(3):1017-1027.
6. Bowes T, Wagner ER, Boffey J, *et al.* (2002) Tolerance to self gangliosides is the major factor restricting the antibody response to lipopolysaccharide core oligosaccharides in *Campylobacter jejuni* strains associated with Guillain-Barré syndrome. *Infect. Immun.* 70(9):5008-5018.
7. Moran AP & Prendergast MM (1998) Molecular mimicry in *Campylobacter jejuni* lipopolysaccharides and the development of Guillain-Barré syndrome [letter]. *J Infect Dis* 178(5):1549-1551.
8. Yuki N (1997) Molecular mimicry between gangliosides and lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Guillain-Barré syndrome and Miller Fisher syndrome. *J Infect Dis* 176(Suppl 2):S150-153.
9. Ang CW, Jacobs BC, & Laman JD (2004) The Guillain-Barré syndrome: a true case of molecular mimicry. *Trends Immunol* 25(2):61-66.
10. Gilbert M, Godschalk PCR, Karwaski MF, *et al.* (2004) Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain-Barré syndrome, by horizontal exchange. *Infect. Immun.* 72(2):1162-1165.
11. Gilbert M, Karwaski M-F, Bernatchez S, *et al.* (2002) The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J. Biol. Chem.* 277(1):327-337.

12. Parker CT, Gilbert M, Yuki N, Endtz HP, & Mandrell RE (2008) Characterization of lipooligosaccharide-biosynthetic loci of *Campylobacter jejuni* reveals new lipooligosaccharide classes: evidence of mosaic organizations. *J Bacteriol* 190(16):5681-5689.
13. Godschalk PCR, Heikema AP, Gilbert M*, et al.* (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J Clin Invest* 114(11):1659-1665.
14. van Belkum A, van den Braak N, Godschalk P*, et al.* (2001) A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. *Nat. Med.* 7(7):752-753.
15. Louwen R, Heikema A, van Belkum A*, et al.* (2008) The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. *Infect Immun* 76(10):4431-4438.
16. Avril T, Wagner ER, Willison HJ, & Crocker PR (2006) Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 74(7):4133-4141.
17. Avril T, Floyd H, Lopez F, Vivier E, & Crocker PR (2004) The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells. *J Immunol* 173(11):6841-6849.
18. Crocker PR (2005) Siglecs in innate immunity. *Curr Opin Pharmacol* 5(4):431-437.
19. Cao H, Lakner U, de Bono B, Traherne JA, Trowsdale J, & Barrow AD (2008) SIGLEC16 encodes a DAP12-associated receptor expressed in macrophages that evolved from its inhibitory counterpart SIGLEC11 and has functional and non-functional alleles in humans. *Eur J Immunol* 38(8):2303-2315.
20. Angata T, Tabuchi Y, Nakamura K, & Nakamura M (2007) Siglec-15: an immune system Siglec conserved throughout vertebrate evolution. *Glycobiology* 17(8):838-846.
21. Hartnell A, Steel J, Turley H, Jones M, Jackson DG, & Crocker PR (2001) Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. *Blood* 97(1):288-296.
22. Perry VH, Crocker PR, & Gordon S (1992) The blood-brain barrier regulates the expression of a macrophage sialic acid-binding receptor on microglia. *J Cell Sci* 101 (Pt 1):201-207.
23. Wu C, Rauch U, Korpos E*, et al.* (2009) Sialoadhesin-positive macrophages bind regulatory T cells, negatively controlling their expansion and autoimmune disease progression. *J Immunol* 182(10):6508-6516.
24. Jiang HR, Hwenda L, Makinen K, Oetke C, Crocker PR, & Forrester JV (2006) Sialoadhesin promotes the inflammatory response in experimental autoimmune uveoretinitis. *J Immunol* 177(4):2258-2264.
25. Jones C, Virji M, & Crocker PR (2003) Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol Microbiol* 49(5):1213-1225.
26. Rempel H, Calosing C, Sun B, & Pulliam L (2008) Sialoadhesin expressed on IFN-induced monocytes binds HIV-1 and enhances infectivity. *PLoS ONE* 3(4):e1967.
27. Crocker PR, Kelm S, Dubois C*, et al.* (1991) Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. *Embo J* 10(7):1661-1669.
28. Ang CW, Noordzij PG, de Klerk MA, Endtz HP, van Doorn PA, & Laman JD (2002) Ganglioside mimicry of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity in rabbits. *Infect Immun* 70(9):5081-5085.
29. Szymanski CM, Michael FS, Jarrell HC*, et al.* (2003) Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from *Campylobacter* cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. *J. Biol. Chem.* 278(27):24509-24520.
30. Ang CW, van Doorn PA, Endtz HP*, et al.* (2000) A case of Guillain-Barré syndrome following a family outbreak of *Campylobacter jejuni* enteritis. *J. Neuroimmunol.* 111:229-233.
31. Godschalk PC, Kuijf ML, Li J*, et al.* (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndromes. *Infect Immun* 75(3):1245-1254.

32. Kuijf ML, Godschalk PC, Gilbert M, *et al.* (2007) Origin of ganglioside complex antibodies in Guillain-Barré syndrome. *J Neuroimmunol* 188(1-2):69-73.
33. Dzieciatkowska M, Liu X, Heikema AP, *et al.* (2008) Rapid method for sensitive screening of oligosaccharide epitopes in the lipooligosaccharide from *Campylobacter jejuni* strains isolated from Guillain-Barré syndrome and Miller Fisher syndrome patients. *J Clin Microbiol* 46(10):3429-3436.
34. Crocker PR, Mucklow S, Bouckson V, *et al.* (1994) Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. *Embo J* 13(19):4490-4503.
35. Vinson M, van der Merwe PA, Kelm S, May A, Jones EY, & Crocker PR (1996) Characterization of the sialic acid-binding site in sialoadhesin by site-directed mutagenesis. *J Biol Chem* 271(16):9267-9272.
36. Crocker PR, Paulson JC, & Varki A (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* 7(4):255-266.
37. Phan TG, Grigorova I, Okada T, & Cyster JG (2007) Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol* 8(9):992-1000.
38. Heinen E, Braun M, Coulie PG, *et al.* (1986) Transfer of immune complexes from lymphocytes to follicular dendritic cells. *Eur J Immunol* 16(2):167-172.
39. Geleijns K, Emonts M, Laman JD, *et al.* (2007) Genetic polymorphisms of macrophage-mediators in Guillain-Barré syndrome. *J Neuroimmunol* 190(1-2):127-130.
40. Delputte PL, Van Breedam W, Barbe F, Van Reeth K, & Nauwynck HJ (2007) IFN-alpha treatment enhances porcine Arterivirus infection of monocytes via upregulation of the porcine Arterivirus receptor sialoadhesin. *J Interferon Cytokine Res* 27(9):757-766.
41. Ang CW, Laman JD, Willison HJ, *et al.* (2002) Structure of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity and clinical features of Guillain-Barré and Miller Fisher patients. *Infect. Immun.* 70(3):1202-1208.

Supplemental Table 1A. Penner serotype reference strains

Penner strain	HS(a) serotype	Origin	Strain type	Ganglioside-mimic	LOS class
P1	1	CCUG(b) 10935	Serostrain	GM2	C
P2	2	CCUG 10936	Serostrain	GM3	C
P3	3	CCUG 10937	Serostrain	none	E
P4	4	CCUG 10938	Serostrain	GD1a, GM1a	A
P10	10	CCUG 10943	Serostrain	GD3	A
P19	19	CCUG 10950	Serostrain	GD1a, GM1a	A
P23	23	CCUG 10954	Serostrain	none (GA3)	B
P36	36	CCUG 10966	Serostrain	GM2, GM3	B

Supplemental Table 1B. GBS/MFS-associated *C. jejuni* strains

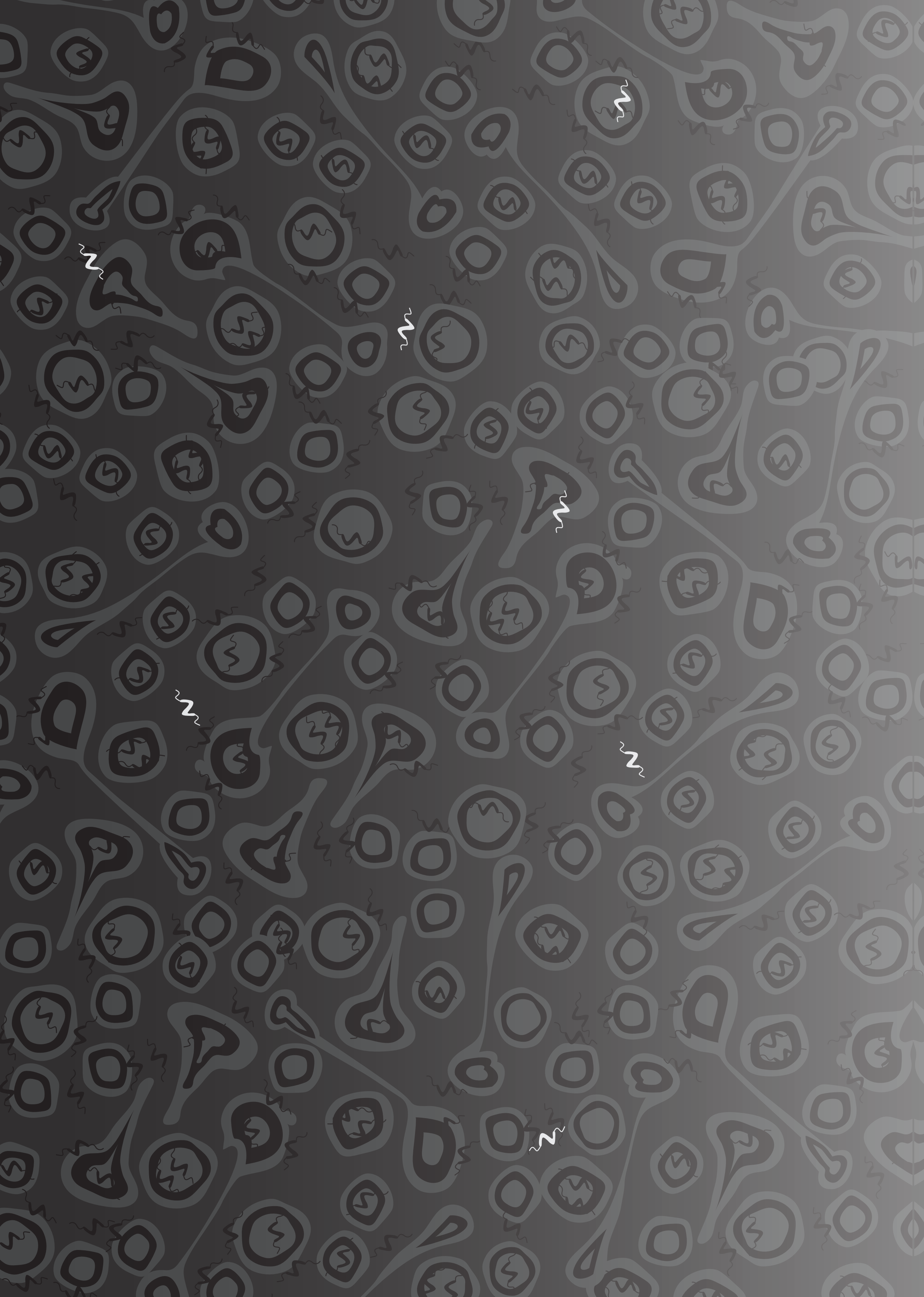
GBS/MFS strain	HS serotype	Origin	Patient diagnose	Ganglioside-mimic	LOS class
GB1	1	The Netherlands	GBS	none (GA1)	C
GB2	UT(c)	The Netherlands	GBS	GD1a, GM1a	A
GB3	19	The Netherlands	GBS	GD1a, GM1a	A
GB4	37	The Netherlands	GBS	none	E
GB5	4, 64	The Netherlands	GBS	none (GA2)	B
MF6	4, 64	The Netherlands	MFS	GM1b, GD1c	B
MF7	35	The Netherlands	MFS	GM2, GD2, GD3	B
MF8	23, 36	The Netherlands	MFS	GM2, GD3	B
GB11	2	The Netherlands	GBS	GD1a, GM1a	A
GB13	2	The Netherlands	enteritis, family GBS	GM1a	C
GB14	2	The Netherlands	enteritis, family GBS	GM1a	C
GB15	5, 34	The Netherlands	GBS	none	D
GB16	13, 66	Belgium	GBS with ophthalmoplegia	GD1c	A
GB17	4, 13, 64	The Netherlands	GBS	GM1b, GD1c, GA1	B
GB18	19	The Netherlands	GBS	GD1a, GM1a	A
GB19	4, 50	The Netherlands	GBS with ophthalmoplegia	GD1c	B
MF20	2	The Netherlands	MFS	GM1b, GD1c	B
GB21	13, 65	The Netherlands	GBS	GD1a, GM1a	A
GB22	13, 64	Netherlands Antilles	GBS	GD1a, GM1a	A
GB23	4, 13, 43	The Netherlands	GBS	GM2	A
GB24	31	The Netherlands	GBS	none	D
GB25	2	The Netherlands	GBS with ophthalmoplegia	GM1b, GD1c, GA1	B
GB26	1, 44	The Netherlands	enteritis, family GBS	GM1b	A
GB27	1, 44	The Netherlands	enteritis, family GBS	GM1b	A
GB28	19, 38	Netherlands Antilles	GBS	GD1a, GM1a	A
GB29	NT(d)	The Netherlands	GBS	not yet determined	C
GB30	NT	The Netherlands	GBS	not yet determined	B
GB31	13, 50	Netherlands Antilles	GBS	GD1a, GM1a	A
GB33	NT	The Netherlands	GBS	not yet determined	B

Penner (A) and GBS/MFS-associated (B) *C. jejuni* strains used in this study. The ganglioside mimicking structures on the surface of each strain was identified using mass spectrometry. The LOS class was determined using PCR. [(a)]HS, Heat stable (Penner serotyping system), [(b)]CCUG, Culture Collection University of Göteborg, [(c)]UT, Untypable , [(d)]NT, Not tested.

Supplemental Table 2.

	Observed Ions (*m/z*)		Molecular mass (Da)		Proposed composition
Strain	[M-4H]4-	[M-3H]3-	Observed	Calculated1	
GB27	1016.0	1354.0	4066.5	4067.3	Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A*
	1023.0	1364.0	4095.5	4095.4	Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A
	1030.0	1373.0	4123.0	4124.4	Gly1•Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A*
	1037.0	1383.0	4152.0	4152.4	Gly1•Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A
	1047.0	1396.0	4191.5	4190.4	Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A*
	1054.0	1405.0	4219.0	4218.4	Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A
	1061.0	1414.0	4246.5	4247.4	Gly1•Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A*
	1068.0	1424.0	4275.5	4275.5	Gly1•Neu5Ac1•HexNAc1•Hex4•Hep2•PEtn1•Kdo2•lipid A

Negative ion ESI-MS data and proposed compositions of intact LOS from *C. jejuni* GB27.
Electrophoresis-assisted open-tubular liquid chromatography mass spectrometry (EA-OTLC-MS) was performed as described by Dzieciatkowska et al. (*Biochemistry* 2007, 46:14704-14714). The presence of NeuAc was also confirmed by the fragment ion at *m/z* 290 when tandem mass spectrometry was carried out on the triply charged ions at *m/z* 1365 and 1405 (data not shown).
Isotope-average mass units were used for calculation of molecular mass values based on proposed compositions as follows: Hex,162.14; HexNAc, 203.20; Hep, 192.17; Kdo, 220.18; *P*, 79.98; *P*Etn, 123.05; Neu5Ac, 291.26; Gly, 57.05; $C_{14:0}$, 210.36; $C_{16:0}$, 238.41; HexN, 161.16; HexN3N, 160.17; 3-OH-$C_{14:0}$, 226.36; H_2O, 18.01. * Lipid A consists of 1 HexN, 1 HexN3N, 3 *N*-(3-OH-$C_{14:0}$), 1 *O*-(3-OH-$C_{14:0}$), 1*O*-($C_{14:0}$) and 1*O*-($C_{16:0}$). The others contain 1 HexN, 1 HexN3N, 3 *N*-(3-OH-$C_{14:0}$), 1 *O*-(3-OH-$C_{14:0}$) and 2 *O*-($C_{16:0}$).



Enhanced, sialoadhesin-dependent uptake of Guillain-Barré syndrome-associated *Campylobacter jejuni* strains by human macrophages

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Abstract

Molecular mimicry between *Campylobacter jejuni* sialylated lipooligosaccharides (LOS) and human nerve gangliosides can trigger the production of cross-reactive antibodies which induce Guillain-Barré syndrome (GBS). To better understand the immune events leading to GBS, it is essential to know how sialylated LOS are recognized by the immune system. Here, we show that GBS-associated *C. jejuni* strains bind to human sialoadhesin (hSn), a conserved, mainly macrophage-restricted I-type lectin. Using hSn-transduced THP-1 cells, we observed that *C. jejuni* strains with α(2,3)sialylated LOS, including strains expressing GM1a- and GD1a-like epitopes, bind to hSn. This observation is of importance, as these epitopes are frequently the targets of the cross-reactive antibodies detected in GBS patients. Interestingly, the Sn binding domains were not constitutively exposed on the surface of *C. jejuni*. Heat inactivation and the environmental conditions which foodborne *C. jejuni* encounters during its passage through the intestinal tract, such as low pH and contact with bile constituents, exposed LOS and facilitated Sn binding. Sn binding enhanced bacterial uptake and increased the production of interleukin-6 (IL-6) by primary human Sn-expressing monocyte-derived macrophages, compared to control conditions where Sn was blocked using neutralizing antibodies or when nonsialylated *C. jejuni* was used. Sn-mediated uptake has been reported to enhance humoral immune responses. As *C. jejuni* strains expressing ganglioside mimics GD1a and GM1a are closely associated with GBS, Sn binding might be a determining event in the production of cross-reactive antibodies and the development of GBS.

Introduction

Guillain-Barré syndrome (GBS) is an acute, rapidly progressing, post-infectious neuropathy which results in severe muscle paresis. In the acute phase of the development of GBS, auto-antibodies with specificity for gangliosides are frequently detected in patient serum (1, 2). These antibodies bind to ganglioside structures which are enriched on the peripheral nerves, resulting in immune-mediated damage and subsequent paralysis (3). Auto-antibodies against α(2,3)sialylated carbohydrate epitopes, present in gangliosides GM1a and GD1a, are especially detected in GBS patients (3, 4). Although it is accepted that antecedent infection by microorganisms carrying surface-exposed ganglioside-like structures can lead to production of anti-ganglioside antibodies (5-7), the precise immune-processes leading to anti-ganglioside antibody production are unclear. Infection with *Campylobacter jejuni* (*C. jejuni*), an intestinal pathogen, most commonly precedes the production of anti-ganglioside antibodies and the development of GBS (7). Lipooligosaccharides (LOS) are a major *C. jejuni* surface antigen that might contain sialylated carbohydrate moieties which are structurally identical to the carbohydrate moieties on human gangliosides (8, 9). Depending on gene content, phase variation and mutations in the LOS biosynthesis loci, *C. jejuni* can express various ganglioside-like structures (10). The presence of genes involved in sialic acid biosynthesis and transfer is essential for the production of these ganglioside mimics (11).

Recent studies have demonstrated that sialylation of LOS enhances the infectivity of bacteria, elicits enhanced immune responses and induces the production of anti-ganglioside antibodies leading to GBS (12-15). In particular, sialylated *C. jejuni* strains are more invasive in intestinal epithelial cells than nonsialylated strains (12) and in patients, sialylated strains are associated with an increased severity of gastro-enteritis (13). In addition, sialylation induces a stronger IgM antibody response in the human host (12, 13). By generating a Campylobacter sialyltransferase (*cst-II*) knockout mutant, we were able to demonstrate that sialylation of LOS modulates dendritic cell (DC)-mediated T helper cell differentiation and enhances DC-driven B-cell proliferation (14, 15). Most importantly, the presence of *cst-II* in *C. jejuni* is crucial for the induction of anti-ganglioside antibodies (16), which have the capacity to induce peripheral nerve destruction and paralysis in rabbits and mice (17, 18).

Specific recognition of sialylated LOS versus nonsialylated LOS by the host immune system can be considered as a crucial step in anti-ganglioside antibody formation.

Toll-like receptor 4 (TLR-4) interacts with the lipid A component of LOS; however, sialylation of the LOS outer core appears to influence TLR-4 signalling, as neuraminidase desialylated LOS and *cst-II* mutant-LOS activate DCs less efficiently leading to reduced B-cell proliferation, compared to wild-type strains (15). We hypothesize that other receptors which specifically bind to sialylated carbohydrates determine sialylated *C. jejuni* LOS recognition. Two members of the sialic acid-binding immunoglobulin-like lectins

(siglecs) family have been demonstrated to specifically recognize sialylated *C. jejuni* LOS. A sialic acid-specific interaction with siglec-7 was demonstrated previously (19), and we have recently shown that sialoadhesin (Sn, Siglec-1, CD-169) from mice is able to bind to *C. jejuni* LOS in a sialic acid-dependent manner. Interestingly, especially GBS-associated *C. jejuni* strains bound murine Sn (mSn) (20). Sn is a conserved siglec found in both rodents and humans, which is mainly expressed on macrophages (21). Therefore, in the current study, we aimed to identify whether *C. jejuni* binds to human Sn (hSn) expressed on macrophages, and assessed the consequences of hSn binding on bacterial uptake, bacterial survival and macrophage activation.

Results

C. jejuni binds to human Sn expressed on Sn-transduced THP-1 cells

THP-1 cells transduced with full length hSn cDNA (THP-1-Sn) (21) were used to identify whether *C. jejuni* interacts with hSn. Flow cytometric analysis confirmed high levels of hSn membrane expression on THP-1-Sn cells (MFI = 1526.3 ± 24.7; $n = 3$); whereas hSn expression was low on untransduced THP-1 cells (MFI = 6.7 ± 0.2; $n = 3$; Fig. 1A). To assess whether *C. jejuni* binds to hSn, THP-1-Sn cells and control THP-1 cells were incubated with heat inactivated FITC-labelled *C. jejuni* strain GB11, which was previously shown to bind mSn (20). Flow cytometric analysis revealed a strong association of GB11-FITC with THP-1-Sn cells (MFI = 81.2 ± 7.3; $n = 3$) while virtually no FITC signal was detected on THP-1 cells (MFI = 3.5 ± 0.3; $n = 3$; Fig. 1B). To visualize the binding of *C. jejuni* strain GB11 to hSn, THP-1-Sn and control THP-1 cells incubated with *C. jejuni*-FITC were cytospun onto glass slides, fixed and stained with anti-hSn-PE. Fluorescence microscopy revealed a clear co-localization of *C. jejuni* strain GB11 with hSn on THP-1-Sn cells (Fig. 1C, upper panel), whereas almost no interaction between GB11 and control THP-1 cells was observed (Fig. 1C, lower panel).

Binding of *C. jejuni* to THP-1-Sn cells is sialic acid- and Sn-dependent

To determine whether the binding of *C. jejuni* to hSn was sialic acid-specific, we incubated THP-1-Sn cells with heat-inactivated, FITC-labelled wild type *C. jejuni* strain GB11 or FITC-labelled bacteria from a previously generated sialic acid GB11 knockout mutant (denoted GB11Δ*cst-II*) (16). Flow cytometric analysis demonstrated that 82.2% of THP-1-Sn cells bound GB11, whereas only 0.4% bound GB11Δ*cst-II*, indicating that the presence of sialic acid was crucial for hSn binding (Fig. 1D). To confirm that GB11 bound to Sn and not to other cell-surface proteins, THP-1-Sn cells were treated for 15 min with an antibody against hSn before addition of the bacteria. Pretreatment with the anti-hSn antibody almost completely neutralized the binding of GB11 to THP-1-Sn cells, reducing

the number of positive cells to 2.9% (Fig. 1D). Pre-incubation with an isotype control antibody had no effect on Sn-binding (Fig. 1D).

Ganglioside mimic-specific interaction of *C. jejuni* with human Sn

Using an ELISA we previously demonstrated that mSn binds to *C. jejuni* strains which express terminal α(2,3)-linked sialic acid residues, as found in gangliosides such as GM3, GD1a, GM1b and GT1b (20, 22). To identify whether hSn has a similar binding profile

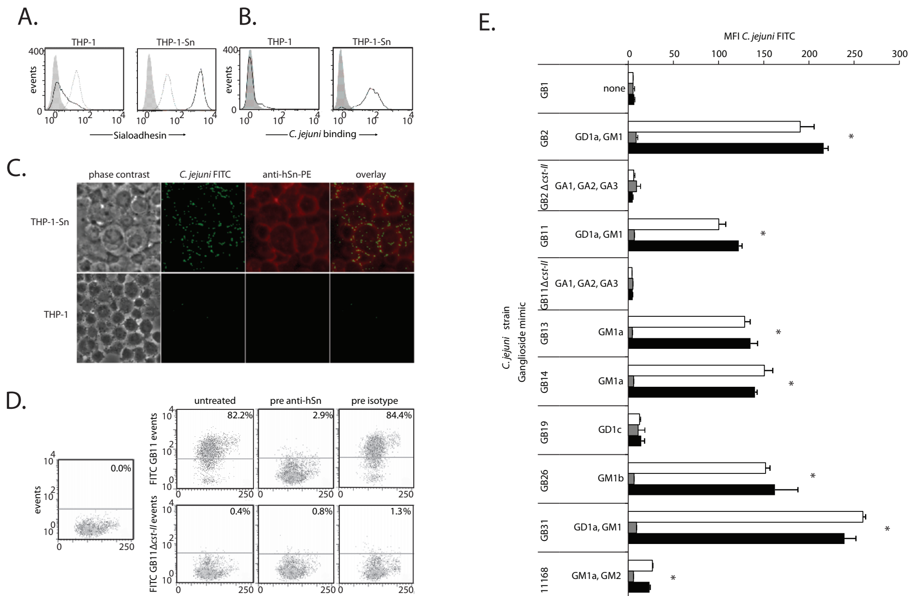

Figure 1. *C. jejuni* interacts with human Sn expressed on THP-1-Sn cells in a sialic acid- and Sn-dependent manner. **A.** Confirmation of Sn expression on THP-1-Sn cells. Flow cytometric analysis of Sn expression on THP-1 and THP-1-Sn cells, using an anti-hSn-PE antibody or isotype control antibody. Events indicate the FL-2 signal within the live cell population of unstained cells (filled grey curves), isotype control stained cells (dotted line curve) and anti-hSn-PE stained cells (black line curve). **B.** Flow cytometric analysis of the interaction of heat inactivated FITC-labelled *C. jejuni* strain GB11 incubated with THP-1 or THP-1-Sn cells for 2 h. Events indicate the FL-1 signal within the living cell population in the absence (filled grey curves) or presence of FITC-labelled GB11 (black line curve). **C.** Immunofluorescent staining of hSn on THP-1 and THP-1-Sn cells incubated with heat inactivated FITC-labelled *C. jejuni* strain GB11 for 2 h, using an anti-hSn-PE antibody. *C. jejuni* is shown in green, hSn in red. **D.** Flow cytometric analysis of the interaction of FITC-labelled *C. jejuni* strains GB11 and GB11Δ*cstII* with THP-1-Sn cells, in the presence or absence of hSn blocking using an anti-hSn antibody or isotype control antibody, respectively. The percentages of cells binding *C. jejuni* within the living cell populations are indicated. **E.** Binding of a panel of FITC-labelled, heat inactivated *C. jejuni* strains with known ganglioside mimicking structures to THP-1-Sn cells. The cells were either untreated (white bars), or treated with an antibody against hSn (grey bars) or an isotype control antibody (black bars). Living cells were gated and used for analysis. Results are the mean values from one representative experiment ± the SD of triplicate experiments. Results represent the data from one experiment that was repeated at least two times, with means ± SD of triplicate measurements; * $p < 0.05$ (one-way ANOVA).

to mSn, the binding of *C. jejuni* strains with known ganglioside mimicking structures to hSn was tested using THP-1-Sn cells. In agreement with the mSn binding capacities, *C. jejuni* strains GB2, GB11, GB26 and GB31 positively bound hSn, and *C. jejuni* strains GB1, GB11Δ*cst-II* and GB19 did not bind hSn (Fig. 1E) (20). Unexpectedly, hSn bound the *C. jejuni* strains GB13 and GB14 which were not recognized by mSn (Fig. 1E). GB13 and GB14 both express GM1a-like LOS (11). The GM1a structure does not contain a terminally-linked sialic acid residue, but instead contains an α(2,3)-linked sialic acid attached to an internal galactose of the LOS outer core, and hSn can apparently interact with this internal sialic acid. The enteritis-associated *C. jejuni* reference strain 11168 also has GM1a-ganglioside mimicry. Investigation of the hSn binding capacity of this strain indicated that 11168 could bind to THP-1-Sn cells; however, at a relatively low level compared to strains GM13 and GM14 (Fig. 1E). Similarly to GB11Δ*cst-II*, GB2Δ*cst-II* did not bind to hSn, again demonstrating that an absence of sialic acid in the LOS prevents hSn binding (Fig. 1E). It should be noted that THP-1-Sn cells were also incubated with an antibody against human Sn for 15 min prior to addition of the bacteria, as a control for Sn-specific binding. Pre-incubation with anti-hSn antibody significantly reduced the Sn binding capacity of all strains, as illustrated by a reduction in cell-associated fluorescence intensity to background levels ($p \leq 0.05$; one-way ANOVA); however, an isotype control antibody did not affect cell-associated fluorescence (Fig. 1E). To determine if the strains were adequately labelled, the FITC-labelled bacteria were analysed by flow cytometry. Although there was slight variation in the labelling intensities, the MFI was generally high (MFI = 1053 ± 170; Supplemental Fig. 1)

Growth conditions determine ganglioside mimic exposure and Sn binding

In the previous experiments, heat inactivated bacteria grown on BA-plates were used to determine Sn-dependent binding. The binding of untreated bacteria grown on BA-plates to THP-1-Sn cells was also assessed and, surprisingly, no binding was observed. Cholera toxin (CT) strongly binds to ganglioside GM1 (23). To determine whether the ganglioside mimics were adequately exposed on the bacterial surface, living and heat inactivated bacteria from the GB11 (GM1 positive) and GB11Δ*cst-II* (GM1 negative) strains were incubated with CT-biotin and streptavidin-FITC, and analysed by flow cytometry. Indeed, CT-biotin was able to bind to strain GB11 when the bacteria were heat inactivated; however, the binding was severely reduced when the strain was left untreated (Fig. 2A). As expected, CT-biotin did not bind to GB11Δ*cst-II,* which lacks a GM1-like structure.

We investigated whether the exposure of bacteria to similar environmental conditions as they would encounter after ingestion, such as low pH or bile salts in the stomach and intestine respectively, would influence the exposure of ganglioside mimics on the bacterial cell surface. Therefore, *C. jejuni* strain GB11 was cultured on BA-plates, FITC-labelled, incubated for one *h* in either PBS pH 3.0 or PBS pH 7.0 containing 0.1 % DOC, then the

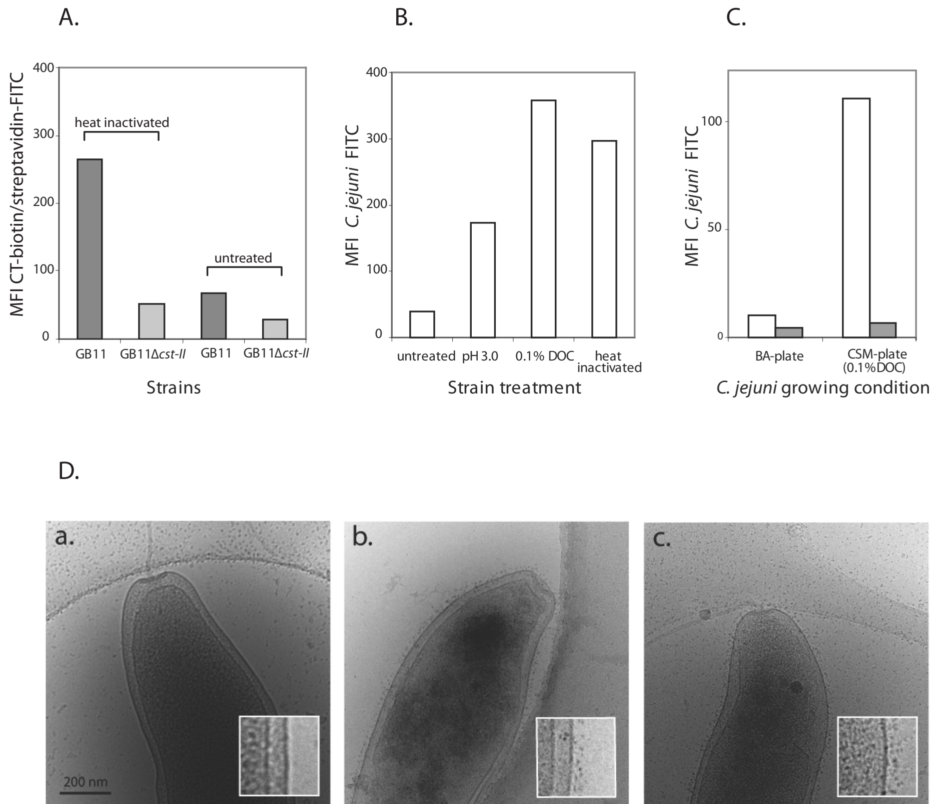

Figure 2. Exposure of ganglioside mimics on the surface of *C. jejuni*. **A**. Flow cytometric analysis of the expression of ganglioside mimics on the surface of *C. jejuni*. Bacteria were either heat inactivated or left untreated, then subsequently incubated with CT-biotin and streptavidin-FITC. **B.** Flow cytometric analysis of the binding of FITC-labelled *C. jejuni* strain GB11 to THP-1-Sn cells. Bacteria grown on BA-plates were untreated, incubated for 1 h in PBS pH 3.0 or PBS pH 7.0 containing 0.1% DOC, or heat inactivated. **C.** Flow cytometric analysis of the binding of FITC-labelled *C. jejuni* strain GB11 to THP-1-Sn cells. Bacteria were grown on either BA-plates or CSM-plates. THP-1-Sn cells were left untreated (white bars) or pretreated to block Sn binding using an anti-hSn antibody (grey bars). **D**. Cryo-EM visualization of *C. jejuni* strain GB11 grown on BA-plates and untreated (a), grown on BA-plates and heat inactivated (b), or grown on CSM-plates and untreated. The bacteria were incubated with CT-biotin followed by streptavidin-conjugated quantum dots.

binding to THP-1-Sn cells was analyzed using FACS. Compared to untreated bacteria, incubation at pH 3.0 and, to even greater extent, incubation in 0.1% DOC enhanced the bacterial ability to bind THP-1-Sn cells (Fig. 2B). Furthermore, when the bacteria were freshly cultured on commercial CSM-plates which contain 0.1% DOC, Sn-specific binding of the bacteria to THP-1-Sn cells was also observed (Fig. 2C). In order to localize the exposure of ganglioside mimics on the bacterial cell surface, untreated GB11 cultured on BA-plates, GB11 cultured on BA-plates and heat-inactivated, and CSM-plate cultured GB11 were incubated with CT-biotin, then subsequently with streptavidin-conjugated

quantum dots and visualized by cryo-electron microscopy (cryo-EM). Quantum dots were visible on the heat inactivated and CSM-plate grown bacteria, and covered the surface of the bacteria (Fig. 2D). Interestingly, no quantum dots were present on the surface of most of the BA-plate grown, non-heat inactivated bacteria.

IFN-α and *C. jejuni* LOS induce the expression of Sn by monocyte-derived macrophages

To study Sn-dependent binding and internalization, and the functional consequences of Sn binding in a more biologically relevant setting, we used Sn-expressing primary human macrophages. As it has been demonstrated that Sn expression can be induced on $CD14^+$ monocytes by stimulation with IFN-α (21), we investigated whether IFN-α induced expression of Sn on human monocyte-derived macrophages (MDM). After two days of culture with IFN-α, MDMs exhibited expression of Sn (MFI = 68.4 ± 4.3; $n = 3$); whereas non-IFN-α stimulated MDMs remained negative for Sn (MFI = 3.9 ± 0.5; $n = 3$; Fig. 3A). We also tested if *C. jejuni* LOS could directly induce expression of Sn by MDMs, and therefore whether LOS could potentially modulate its own recognition. Flow cytometric analysis revealed that GB11 LOS potently induced the expression of Sn by MDMs, at a similar level as IFN-α treatment (Fig. 3B). The induction of Sn by *C. jejuni* LOS was not sialic acid-dependent, as treatment with nonsialylated LOS isolated from strain GB11Δ*cst-II* or treatment with purified *E. coli* LPS resulted in similar levels of Sn expression in MDMs (Fig. 3B)

Binding to Sn enhances the uptake of *C. jejuni* by MDMs

As primary monocyte-derived macrophages express many different pattern recognition molecules, which may not all be present on THP-1 cells, we assessed whether the binding of *C. jejuni* to hSn on IFN-α-induced primary MDMs (Sn^+MDMs) was similar to THP-1-Sn cells. The Sn-specific binding of a selection of *C. jejuni* strains to Sn^+MDMs was determined. In particular, three strains (GB11, GB26 and GB31) that positively bound THP-1-Sn cells and two strains (GB1 and GB11Δ*cst-II*) that did not bind THP-1-Sn cells were included in the flow-cytometric binding assay. All of the *C. jejuni* strains tested showed an interaction with Sn^+MDMs. However, the fluorescence intensities of Sn^+MDMs incubated with strains GB11, GB26 and GB31 were higher than strains GB1 and GB11Δ*cst-II* (Fig. 3C), indicating that increased numbers of GB11, GB26 and GB31 were associated with each individual Sn^+MDM cell. Pretreatment of Sn^+MDMs with an antibody against hSn significantly reduced the binding of strains GB11, GB26 and GB31 ($p \leq 0.05$; one-way ANOVA), to similar levels as Sn^+MDMs incubated with strains GB1 and GB11Δ*cst-II*. Pretreatment with the hSn antibody had no significant effect on the interaction of Sn^+MDMs with strains GB1 and GB11Δ*cst-II*. Additionally, pretreatment with an isotype control antibody did not influence the interaction of Sn^+MDMs with any *C. jejuni*

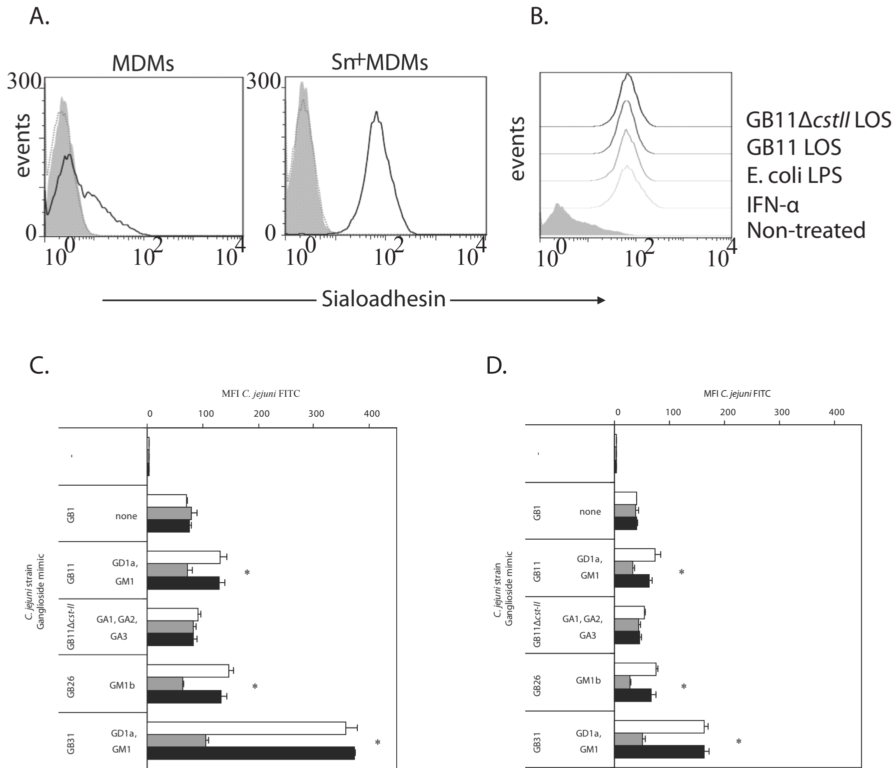

Figure 3. Sn-specific binding of sialylated *C. jejuni* strains results in enhanced uptake by Sn⁺MDMs. Human monocyte-derived macrophages were either untreated (MDMs) or treated with IFN-α to induce expression of Sn (Sn⁺MDMs), then Sn expression and the capacity to bind and internalize *C. jejuni* were quantified. FITC-labelled, heat inactivated *C. jejuni* stains were incubated with the cells for 2 h prior to flow cytometric analysis. Living cells were gated and used for analysis. **A.** Flow cytometric analysis of the expression of Sn on human MDMs and Sn⁺MDMs in unstained cells (filled grey curve), and cells stained with anti-hSn-PE (black line curve) or a PE-labelled isotype control antibody (dotted line curve). **B.** Flow cytometric analysis of the expression of Sn on untreated human MDMs or MDMs incubated for 48 h with either IFN-α (500 U/ml), *E. coli* LPS (10 ng/ml), *C. jejuni* GB11 or GB11Δ*cstII* LOS (10 ng/ml), and subsequently stained with anti-hSn-PE. **C.** Interaction of FITC-labelled *C. jejuni* strains containing known ganglioside mimicking structures with Sn⁺MDMs which had been untreated (white bars), stained with anti-hSn-PE (grey bars) or stained with an isotype control antibody (black bars). Results represent data from one experiment that was repeated at least one time. Means ± SD of triplicate measurements are shown; * $p < 0.05$ (one-way ANOVA). **D.** Internalization of *C. jejuni* strains by Sn⁺MDMs. Sn⁺MDMs were incubated with FITC-labelled bacteria as described in **C.**, then treated with trypan blue prior to flow cytometric analysis to discriminate between external and internalised bacteria; * $p < 0.05$ (one-way ANOVA).

strain, indicating that the enhanced interactions of strains GB11, GB26 and GB31 with Sn⁺MDMs, compared to strains GB1 and GB11Δ*cst-II,* were hSn-specific.

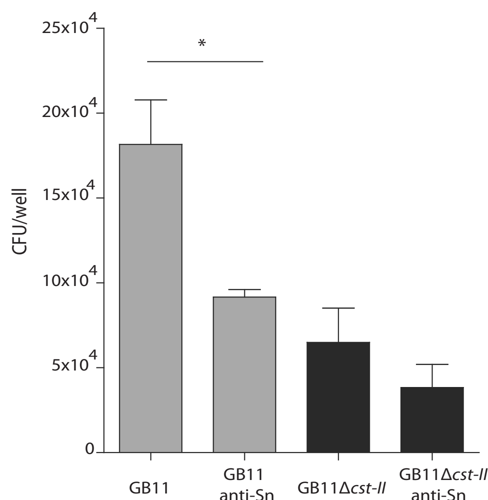

Figure 4. Sn-mediates the phagocytosis *C. jejuni*. Sn+MDMs were untreated or pretreated with an antibody against hSn, then incubated with *C. jejuni* strains GB11 or GB11Δ*cstII* for 3 h. The gentamicin exclusion assay was used to quantify the number of internalized bacteria. Results represent data from one experiment that was repeated at least two times. Means ± SEM of triplicate measurements are shown; * $p < 0.05$ (*t*-test).

To determine whether Sn-binding is a prerequisite to bacterial endocytosis, the extracellular bacteria in the experiment described above were quenched using trypan blue, and the cells were reanalysed using flow cytometry. The MFI of Sn+MDMs incubated with hSn binding strains GB11, GB26 and GB31 was higher than Sn+MDMs incubated with the non-hSn-binding strains GB1 and GB11Δ*cst-II* (Fig. 3D), indicating that Sn binding leads to an increased uptake of bacteria. In agreement with this hypothesis, preincubation with anti-Sn significantly decreased bacterial uptake ($p \leq 0.05$; one-way ANOVA). There was no difference in the interaction of untreated and isotype control antibody-treated cells, confirming that the increased internalization of strains GB11, GB26 and GB31 was Sn-dependent. To assess the quenching efficiency of trypan blue, FITC-labelled *C. jejuni* strains were analysed by flow cytometry before and after incubation with trypan blue. Trypan blue severely reduced the fluorescent signal of all strains (Supplemental Fig. 2).

Sn-specific binding enhances phagocytosis of living *C. jejuni* by Sn+MDMs

To further assess phagocytosis using live, unstained bacteria, we performed gentamicin exclusion assays using Sn+MDMs which had been incubated for 3 h with fresh, CSM-plate grown GB11 or GB11Δ*cst-II*. Sn-specific binding significantly increased the uptake of *C. jejuni* by untreated cells, compared to cells pretreated with anti-Sn antibody ($p \leq 0.05$; two-tailed *t*-test; Fig. 4). The survival of *C. jejuni* in Sn+MDMs was also assessed, and we observed that all of the bacteria were dead after 24 h, indicating that Sn binding does not result in altered intracellular trafficking or escape of *C. jejuni* from lysosomal degradation.

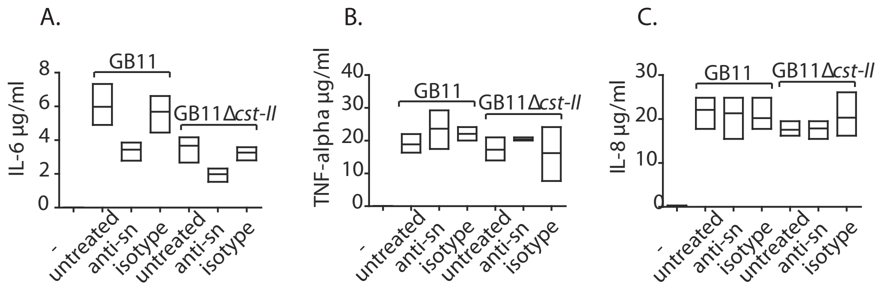

Figure 5. Production of the cytokine IL-6 is elevated in *C. jejuni*-treated Sn+MDMs. Sn+MDMs were untreated or pretreated with a hSn antibody or isotype control antibody, incubated for 6 h with *C. jejuni* strains GB11 or GB11Δ*cstII*, then IL-6 (**A**), IL-8 (**B**) and TNF-alpha (**C**) production were measured. The cytokine levels in the cell supernatants were quantified using a cytometric bead array human inflammatory cytokine kit. The plots display the mean value of triplicate measurements (indicated by the line) and the range values in one representative experiment that was repeated at least two times.

Sn binding elevates production of the cytokine IL-6 by Sn+MDMs

To assess if the interaction of *C. jejuni* with Sn affected cytokine production, Sn+MDMs were incubated for 6 h with wild type GB11 or GB11Δ*cst-II*, then the levels of six cytokines were measured in the cell culture supernatants. The cytokine IL-6 was specifically elevated in a Sn- and sialic acid-dependent manner (Fig. 5A). High levels of IL -8 and TNF-α were also induced by *C. jejuni* (19867 ± 3683 and 19704 ± 4709 pg/ml, respectively); however, the induction of these cytokines was not Sn-dependent (Fig. 5B and C). Other cytokines, such as IL-1b, IL10 and IL-12p70 were also induced in a Sn-independent manner to levels between ~100-400 pg/ml (data not shown).

Discussion

In this study, we demonstrated that *C. jejuni* strains isolated from patients with GBS can bind to Sn expressed on primary human macrophages, via the presence of sialic acid residues in the bacterial LOS. Using Sn-transduced THP-1 cells and primary human macrophages, we showed that *C. jejuni* strains, in particular those containing α(2,3)-linked sialic acid residues, as present in the gangliosides GM1a, GD1a, GM1b and GM3, bind to hSn. Ganglioside-like structures were not constitutively exposed on the bacterial surface, but required particular growth conditions or treatments. Direct growth of the bacteria on media containing DOC, or treatment of *C. jejuni* initially grown on BA-plates by heat-inactivation, incubation at pH 3.0 or exposure to DOC was necessary for Sn binding. In primary human macrophages, Sn binding resulted in enhanced bacterial uptake and increased cytokine release. Regardless of Sn binding, internalized bacteria

were killed within 24 h, suggesting that Sn binding does not enable *C. jejuni* to escape lysosomal degradation.

It has previously been reported that mSn has a preference for binding terminal α(2,3)-linked sialic acid, which is present on purified gangliosides such as GT1b, GD1a and GM3. (22). We recently demonstrated that the ganglioside-like structures present in the LOS on the surface of *C. jejuni* have similar binding properties to purified gangliosides (20). Using hSn in this study, we showed that internal α(2,3)-linked sialic acids, as present in GM1a-like LOS, as well as terminal α(2,3)-linked sialic acids can bind to hSn. This is of interest as *C. jejuni* strains carrying α(2,3)-sialylated LOS structures, especially the strains expressing GM1a- and GD1a-like LOS, are associated with GBS. It should be noted that the enteritis-associated *C. jejuni* reference strain 11168 could also bind Sn; however, at relatively low levels, despite the presence of GM1a-ganglioside mimicry. These data imply that non-GBS *C. jejuni* strains with the GM1a mimic carry additional structures that hinder binding to Sn. In agreement with this observation, it has been demonstrated that ganglioside complexes can attenuate siglec binding. (24).

Our finding that specific microenvironments are crucial for the binding of *C. jejuni* to Sn is of particular importance. In this study, both heat inactivated and living bacteria were used to assess Sn-dependent bacterial binding and uptake. An association with Sn could not be detected when living bacteria grown on standard BA-plates were tested, whereas heat inactivation resulted in Sn binding. This discrepancy was explained by exploring LOS exposure on living and heat inactivated *C. jejuni* using biotinylated-CT, which has a high binding affinity for surface-exposed GM1. In agreement with the observed Sn binding capacity, FACS analysis and cryo-EM microscopy revealed that GM1 was extensively exposed when *C. jejuni* were heat inactivated. The fact that GBS patients often have antibodies against *C. jejuni* LOS indicates that these structures are exposed during the course of infection. Therefore, we reasoned that the outer surface of *C. jejuni* is modified during the passage of food borne *C. jejuni* through the stomach and intestine, for example by losing the capsular layer or due to conformational changes in surface structures. Indeed, when living bacteria were incubated at low pH or in buffer containing the bile constituent DOC, or when the bacteria were grown on culture plates containing DOC, the LOS were exposed and able to bind Sn. Culture of *C. jejuni* in the presence of DOC may also enhance LOS expression; however, microarray based analysis has indicated that the expression of genes involved in LOS biosynthesis or modification are not upregulated in the presence of DOC (25).

We further explored the possible role of Sn in GBS by studying primary human macrophages. Using CHO cells transfected with mSn, we previously demonstrated that Sn is sufficient for the binding of ganglioside-like structures, without the need for additional co-receptors" (20). However, unlike most siglecs, Sn lacks intracellular signaling motifs thus most likely cooperates with other surface molecules. Our data clearly indicates that

Sn-binding leads to enhanced phagocytosis of *C. jejuni.* This is in line with a previously described role for Sn in microbial uptake. *Neisseria meningitides,* HIV and porcine reproductive and respiratory syndrome virus (PRRSV) are internalized upon Sn binding in a Sn-specific manner (21, 26, 27). These observations raise questions regarding the consequences of Sn-mediated phagocytosis. Sn binding may play a role in redirecting *C. jejuni* or the LOS to specific intracellular compartments. For example, Sn accompanies PRRSV from the cell surface to the inside of the cell just beneath the plasma membrane, after which the virus can be detected in early endosomes (27). Regardless of Sn-dependency, the uptake of *C. jejuni* by macrophages resulted in bacterial death within 24 h, indicating that Sn-mediated uptake of *C. jejuni* does not enable the bacteria to escape lysosomal degradation in macrophages.

Alternatively, Sn-mediated uptake of *C. jejuni* may affect the macrophage activation state and cytokine release, leading to altered innate immunity. Indeed, Sn-mediated *C. jejuni* uptake enhanced the release of IL-6 by primary macrophages. Moreover, in collaboration we have recently shown that sialylation of LOS increased the production of type I interferons in mice (28). These data imply that Sn-mediated uptake may determine the quality of the innate immune response to *C. jejuni*.

Sn may also indirectly affect antigen presentation to other cells of the immune system, via its selective expression pattern on specific immune cells. Sn is mainly expressed on tissue-resident macrophages found in the intestine, marginal zone of the spleen and in the sub-capsular sinus of lymph nodes (29). One function of these macrophages is the presentation of antigenic debris to follicular DCs and B-cells (30). As such, it can be envisaged that increased Sn-mediated binding and/or phagocytosis may lead to the presentation of more *C. jejuni* fragments, resulting in increased immune activation. Evidence for a direct role of Sn-mediated uptake in antibody production is provided by a recent immunization study in pigs, which demonstrated that direct targeting of the immunizing protein to Sn resulted in a more rapid and robust induction of specific IgM and IgG immune responses, compared to immunization with the protein alone (31). Further study is required to confirm whether Sn-dependent uptake leads to *C. jejuni* antigen trapping and presentation, resulting in cross-reactive anti-ganglioside antibody production.

In conclusion, this study demonstrates that GBS-associated sialylated *C. jejuni* strains are able to bind to hSn, which results in enhanced cytokine production and increased uptake of the bacteria by monocyte-derived macrophages. Sn-mediated differentiation between *C. jejuni* strains on the basis of ganglioside mimic expression may be an important initial event in the production of anti-ganglioside antibodies and the development of GBS.

Acknowledgements

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Experimental procedures

Bacterial strains

A panel of 11 well-characterized *C. jejuni* strains with known ganglioside-like structures was used in this study (Supplemental Table I) (11, 16). Eight strains isolated from GBS patients were selected, based on their ganglioside mimic-specific binding properties to mSn, as previously demonstrated using an ELISA (20). To verify sialic acid-specific binding, two sialic acid transferase (*cst-II*) knockout mutants, GB2Δ*cst-II* and GB11Δ*cst-II* (16), and the reference *C. jejuni* strain NCTC 11186 (32) were included. *C. jejuni* strains were routinely grown from -80ºC stocks and cultured on Colombia blood agar (BA) plates (BD Biosciences, Alphen aan den Rijn, The Netherlands), as previously described (20). For the cryo-EM experiments and gentamicin exclusion assays, bacteria were grown for one day on Campylobacter blood-free, charcoal based, selective medium agar (CSM) plates (BD Biosciences) which contain 0.1% deoxycholate (DOC).

FITC labelling of *C. jejuni* strains

C. jejuni cultures were grown for two days, harvested, washed with PBS and incubated for 1 h with 5 µl/ml FITC (100 mg/ml stock solution in DMSO) with shaking. Unbound FITC was removed by washing extensively with PBS, the bacteria were heat inactivated for 45 min at 56ºC in PBS containing 2 mM $MgCl_2$, then the bacteria were stored in 10% glycerol broth at -80ºC. Before use in binding experiments, the bacteria were thawed, washed and the optical density at 600 nm (OD_{600}) was adjusted to 1 in PBS (for *C. jejuni*, OD 1 ~ 2.5 x 10^9 CFU/ml). In some experiments, the bacteria were used directly after FITC-labelling, or incubated for 1 h in either PBS pH 3.0 or PBS pH 7.0 containing 0.1% DOC (Sigma). The fluorescence intensities of FITC-labelled *C. jejuni* were assessed using a FACSCalibur flow cytometer (BD Biosciences).

Culture of THP-1 cells and preparation of human Sn-expressing monocyte-derived macrophages

THP-1 (a human monocytic leukaemia cell line) and THP-1 cells transduced with full length human Sn cDNA (TSn or THP-1-Sn), were maintained as previously described (21). To prepare Sn-expressing monocyte-derived macrophages (Sn^+MDMs), human peripheral blood mononuclear cells were isolated from buffy coats by density gradient

centrifugation using Lymphoprep (Axis shield, Oslo, Norway), and CD14+ monocytes were isolated by positive selection using CD14-microbeads (Miltenyi Biotec, Utrecht, The Netherlands), according to the manufacturer's protocol. To obtain monocyte-derived macrophages (MDMs), the cells were grown on low attachment flasks in RPMI-1640 containing 10% human AB serum (HS), penicillin/streptomycin, 2 mM L-glutamine (growing medium), and 25 ng/ml human macrophage colony-stimulating factor (M-CSF; Invitrogen, Breda, The Netherlands) for 5 days. To obtain Sn^+MDMs, the media was refreshed with growing medium containing 500 U/ml IFN-α2a (PBL, Piscataway, USA) and the cells were cultured for 2 days. Sn was also induced by culturing MDMs for 2 days in media containing either *E. coli* LPS (10 ng/ml; Sigma-Aldrich, Zwijndrecht, Netherlands), or LOS isolated from *C. jejuni* GB11 or GB11Δ*cst-II* LOS (10 ng/ml), which was purified as previously described (15).

Expression of Sn on THP-1 cells and MDMs

To determine the expression of Sn on THP-1, THP-1-Sn, MDMs or Sn^+MDMs, the cells were incubated with PE-labelled mouse IgG1 κ anti-human CD169 monoclonal antibody (anti-hSn-PE; BioLegend, Uithoorn, The Netherlands) for 45 min at 4ºC, fixed in paraformaldehyde (PFA) and analysed using the FACSCalibur flow cytometer. A PE-labelled mouse IgG1 κ isotype antibody (BioLegend) was used as a control for background staining.

Binding of *C. jejuni* to Sn-transfected and wt THP-1 cells

THP-1 and THP-1-Sn cells were harvested, washed and resuspended in RPMI-1640 media containing 1% FCS. FITC-labelled *C. jejuni* were added at a cell:bacteria ratio of 1:100 and incubated for 2 h at 37°C in 5% CO_2. The cells were washed to remove unbound bacteria, fixed in 2% PFA and analysed using a FACSCalibur flow cytometer. To discriminate between living and dead cells, the nuclear stain 7-amino-actinomycin D (7-AAD; BD Biosciences) was added to the cells immediately before FACS analysis.

For the Sn-blocking experiments, the cells were incubated with anti-hSn-PE antibody for 15 min prior to the addition of bacteria; PE-labelled mouse IgG1, κ isotype control antibody (BioLegend) was used as a control for Sn-specific blocking. For immunofluorescent microscopy, the cells were incubated with FITC-labelled bacteria, stained with anti-hSn-PE, cytospin preparations were made and the expression of Sn was evaluated using an Olympus IX51 microscope and $Cell^F$ imaging software (Olympus, Zoeterwoude, Netherlands).

Ganglioside mimic exposure on *C. jejuni*

C. jejuni strains GB11 and GB11Δ*cst-II* were grown on BA plates either left untreated or heat inactivated and incubated with biotinylated cholera toxin (CT-biotin)(Sigma-

Aldrich) diluted 1:100. The cells were subsequently incubated with streptavidin-FITC diluted 1:100, washed, fixed using 2% PFA and analysed with a FACSCalibur flow cytometer.

Cryo-electron microscopy

C. jejuni strain GB11 was either grown on BA plates or on DOC containing CSM-plates. Bacteria were left untreated or heat inactivated and incubated CT-biotin diluted 1:100; then subsequently labeled with streptavidin-conjugated quantum dots 525 (1:50 vol/vol; (Invitrogen). The samples were vitrified using a Vitrobot Mark IV (FEI) at RT and 100% humidity, blotted for 2 sec at blot force 10 using filter paper and then plunged into a liquid ~2:1 mixture of liquid ethane and propane, which was cooled using liquid nitrogen. The vitrified samples were mounted in a Gatan 626 high tilt cryo holder and imaged using a Tecnai F20 TEM at 200 kV (FEI). The images were recorded with post-column energy filter 2k x 2k CCD cameras (GIF 2002; Gatan GmbH) in zero-loss mode using a slit width of 20 eV.

Binding and uptake of *C. jejuni* by Sn-expressing MDMs

Sn^{+}MDMs were harvested from low attachment culture plates using Cell Dissociation Buffer (Invitrogen), washed, resuspended in RPMI-1640 containing 1% HS and the binding of *C. jejuni* to Sn^{+}MDMs was determined as described for THP-1 cells. To distinguish between internalized and external bacteria, the external bacteria were quenched with 0.2 % trypan blue (MP Biomedicals, Illkirch, France) 20 min prior flow cytometric analysis.

Gentamicin exclusion assay

Human $CD14^{+}$ monocytes were grown on 24-wells in growing medium containing 25 ng/ml M-CSF. After 5 days, media was changed to media lacking penicillin/streptomycin, containing 500 U/ml IFN-α2a (PBL) and cells were grown for another 2 days. Cells were either left untreated or preincubated for 15 min with anti-hSn-PE antibody. *C. jejuni* harvested from overnight cultures on CSM-plates were added at an MOI of 50, incubated for 3 h, where after the cells were washed and the media was replaced with growth media containing 200 μg/ml gentamicin. After 2 h, cells were washed and lysed in HBSS containing 0.2% Triton X-100. Serial dilutions were prepared and plated on BA-plates. After overnight culture under microaerophilic conditions, the number of colonies were counted.

Cytokine measurements

Sn^{+}MDMs cultured in 96-well plates were either untreated, or pretreated with anti-hSn-PE or an isotype control antibody for 15 min, then incubated with heat inactivated *C. jejuni* strains at a cell:bacteria ratio of 1:100 in growing medium lacking penicillin/strep-

tomycin. The supernatants were harvested after 6 h and cytokine levels were measured using a cytometric bead array (CBA) Human Inflammatory Cytokine Kit (BD Biosciences), according to the manufacturer's instructions.

Statistical analysis

One-way ANOVA (SPSS software) and two-tailed t-tests (GraphPad Prism software) were used for statistical analysis, as indicated.

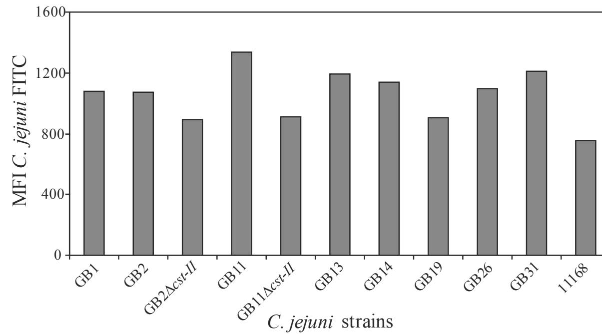
References

1. Jacobs BC, Hazenberg MP, van Doorn PA, Endtz HP, & van der Meché FGA (1997) Cross-reactive antibodies against gangliosides and *Campylobacter jejuni* lipopolysaccharides in patients with Guillain-Barré or Miller Fisher syndrome. *J Infect Dis* 175:729-733.
2. Yuki N, Taki T, & Handa S (1996) Antibody to GalNAc-GD1a and GalNAc-GM1b in Guillain-Barré syndrome subsequent to *Campylobacter jejuni* enteritis. *J Neuroimmunol* 71(1-2):155-161.
3. Hughes RAC, Hadden RD, Gregson NA, & Smith KJ (1999) Pathogenesis of Guillain-Barré syndrome. *J Neuroimmunol* 100(1-2):74-97.
4. Carpo M, Pedotti R, Allaria S, *et al.* (1999) Clinical presentation and outcome of Guillain-Barré and related syndromes in relation to anti-ganglioside antibodies. *J Neurol Sci* 168(2):78-84.
5. Jacobs BC, Van Doorn PA, Schmitz PIM, *et al.* (1996) *Campylobacter jejuni* infections and anti-GM1 antibodies in Guillain-Barré syndrome. *Ann. Neurol.* 40(2):181-187.
6. Yuki N (1997) Molecular mimicry between gangliosides and lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Guillain-Barré syndrome and Miller Fisher syndrome. *J Infect Dis* 176(Suppl 2):S150-153.
7. Jacobs BC, Rothbarth PH, van der Meché FGA, *et al.* (1998) The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. *Neurology* 51(4):1110-1115.
8. Aspinall GO, McDonald AG, Raju TS, *et al.* (1993) Chemical structure of the core region of *Campylobacter jejuni* serotype O:2 lipopolysaccharide. *Eur J Biochem* 213(3):1029-1037.
9. Moran AP, Rietschel ET, Kosunen TU, & Zahringer U (1991) Chemical characterization of *Campylobacter jejuni* lipopolysaccharides containing N-acetylneuraminic acid and 2,3-diamino-2,3-dideoxy-D-glucose. *J. Bacteriol.* 173(2):618-626.
10. Gilbert M, Karwaski M-F, Bernatchez S, *et al.* (2002) The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J. Biol. Chem.* 277(1):327-337.
11. Godschalk PC, Kuijf ML, Li J, *et al.* (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndromes. *Infect Immun* 75(3):1245-1254.
12. Louwen R, Heikema A, van Belkum A, *et al.* (2008) The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. *Infect Immun* 76(10):4431-4438.
13. Mortensen NP, Kuijf ML, Ang CW, *et al.* (2009) Sialylation of *Campylobacter jejuni* lipooligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect* 11(12):988-994.
14. Bax M, Kuijf ML, Heikema AP, *et al.* (2011) *Campylobacter jejuni* lipo-oligosaccharides modulate DC-mediated T cell polarization in a sialic acid linkage dependent manner. *Infect Immun* 79(7):2681-9.
15. Kuijf ML, Samsom JN, van Rijs W, *et al.* (2010) TLR4-mediated sensing of *Campylobacter jejuni* by dendritic cells is determined by sialylation. *J Immunol* 185(1):748-755.

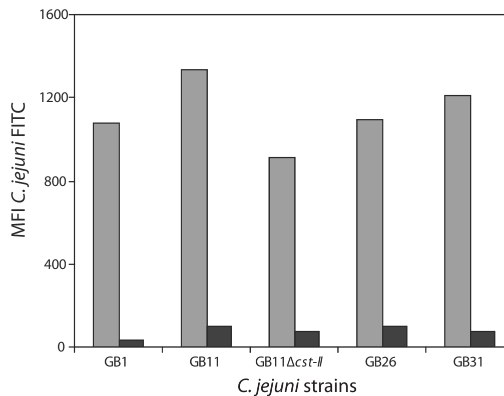
16. Godschalk PCR, Heikema AP, Gilbert M, *et al.* (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J Clin Invest* 114(11):1659-1665.
17. Yuki N, Susuki K, Koga M, *et al.* (2004) Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barré syndrome. *PNAS* 101(31):11404-11409.
18. Halstead SK, Zitman FM, Humphreys PD, *et al.* (2008) Eculizumab prevents anti-ganglioside antibody-mediated neuropathy in a murine model. *Brain* 131(Pt 5):1197-1208.
19. Avril T, Wagner ER, Willison HJ, & Crocker PR (2006) Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 74(7):4133-4141.
20. Heikema AP, Bergman MP, Richards H, *et al.* (2010) Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 78(7):3237-3246.
21. Rempel H, Calosing C, Sun B, & Pulliam L (2008) Sialoadhesin expressed on IFN-induced monocytes binds HIV-1 and enhances infectivity. *PLoS ONE* 3(4):e1967.
22. Crocker PR, Kelm S, Dubois C, *et al.* (1991) Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. *Embo J* 10(7):1661-1669.
23. Heyningen SV (1974) Cholera toxin: interaction of subunits with ganglioside GM1. *Science* 183(4125):656-657.
24. Rinaldi S, Brennan KM, Goodyear CS, *et al.* (2009) Analysis of lectin binding to glycolipid complexes using combinatorial glycoarrays. *Glycobiology* 19(7):789-796.
25. Malik-Kale P, Parker CT, & Konkel ME (2008) Culture of *Campylobacter jejuni* with sodium deoxycholate induces virulence gene expression. *J Bacteriol* 190(7):2286-2297.
26. Jones C, Virji M, & Crocker PR (2003) Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol Microbiol* 49(5):1213-1225.
27. Delputte PL, Van Breedam W, Barbe F, Van Reeth K, & Nauwynck HJ (2007) IFN-alpha treatment enhances porcine Arterivirus infection of monocytes via upregulation of the porcine Arterivirus receptor sialoadhesin. *J Interferon Cytokine Res* 27(9):757-766.
28. Huizinga R, Easton AS, Donachie AM, *et al.* (2012) Sialylation of *Campylobacter jejuni* Lipo-Oligosaccharides: Impact on Phagocytosis and Cytokine Production in Mice. *PLoS ONE* 7(3):e34416.
29. Crocker PR, Paulson JC, & Varki A (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* 7(4):255-266.
30. You Y, Myers RC, Freeberg L, *et al.* (2011) Marginal zone B cells regulate antigen capture by marginal zone macrophages. *J Immunol* 186(4):2172-2181.
31. Delputte PL, Van Gorp H, Favoreel HW, *et al.* (2011) Porcine sialoadhesin (CD169/Siglec-1) is an endocytic receptor that allows targeted delivery of toxins and antigens to macrophages. *PLoS ONE* 6(2):e16827.
32. Parkhill J, Wren BW, Mungall K, *et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403(6770):665-668.

C. jejuni strain	Disease association	Ganglioside mimic
GB1	GBS	none (GA1)
GB2	GBS	GD1a, GM1a
*GB2Δ*cstII*	-	GA1, GA2, GA3
GB11	GBS	GD1a, GM1a
*GB11Δ*cstII*	-	GA1, GA2, GA3
GB13	GBS	GM1a
GB14	GBS	GM1a
GB19	GBS	GD1c
GB26	GBS	GM1b
GB31	GBS	GD1a, GM1a
11168	enteritis	GM1, GM2

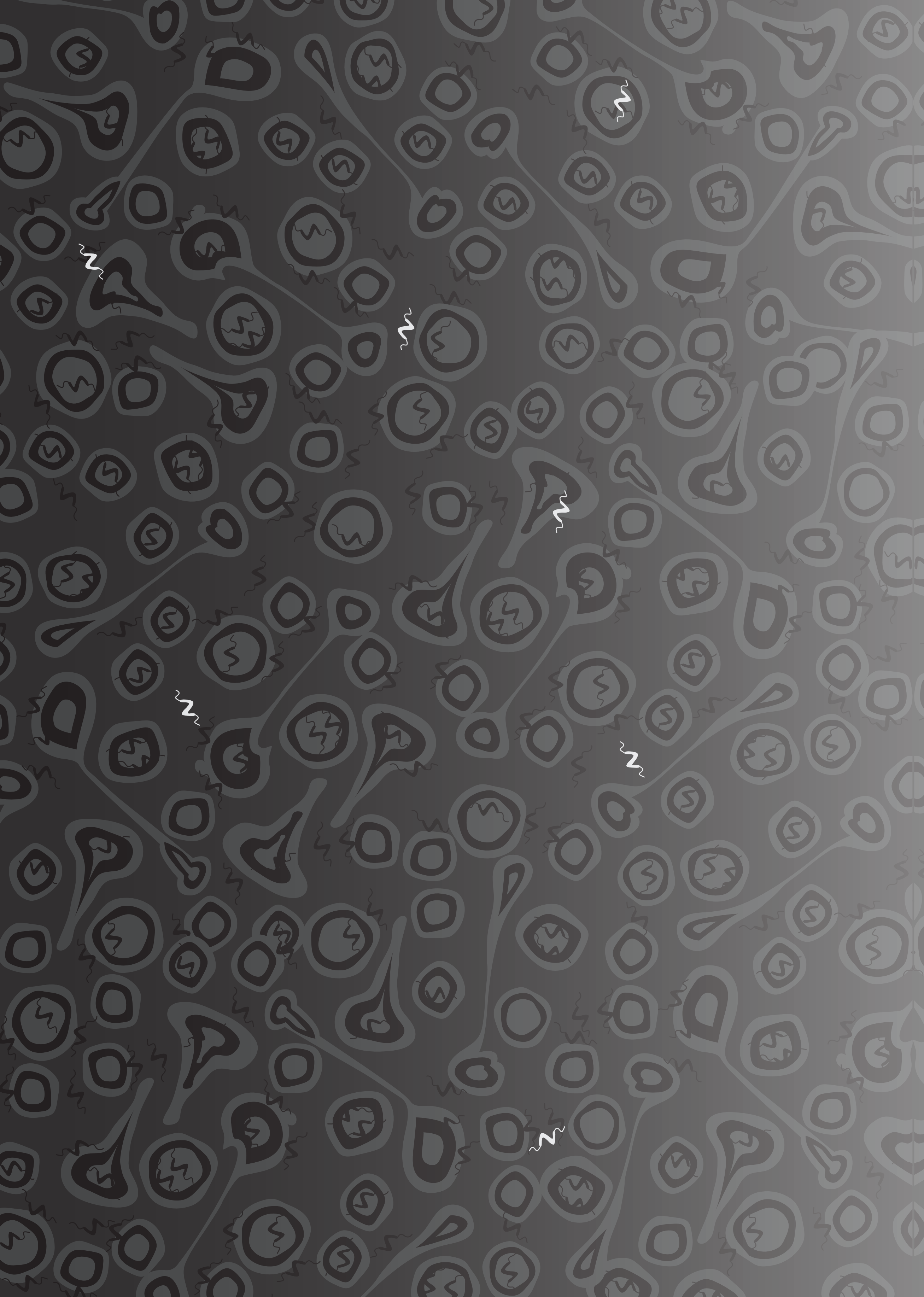
Supplemental Table 1. *C. jejuni* strains used in this study. The ganglioside mimicking structures on the surface of each *C. jejuni* strain were identified by mass spectrometry and have been previously described (11, 16). * Campylobacter sialic acid transferase (*cstII*) knockout mutants of strains GB2 and GB11.



Supplemental Figure 1. Flow cytometric analysis of FITC-labeled *C. jejuni* strains. In order to determine if *C. jejuni* strains were labeled adequately, FITC-labeled *C. jejuni* strains were analyzed using flow cytometry.



Supplemental Figure 2. Quenching efficiency of trypan blue. To assess the quenching efficiency of trypan blue, FITC-labeled *C. jejuni* strains were analyzed by flow cytometry before (grey bars) and after (black bars) incubation with trypan blue.



Chapter 6

Siglec-7 specifically recognizes *Campylobacter jejuni* strains associated with oculomotor weakness in Guillain-Barré syndrome and Miller Fisher syndrome

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Abstract

Due to molecular mimicry, *Campylobacter jejuni* lipooligosaccharides can induce a cross-reactive antibody response to nerve gangliosides, which leads to Guillain-Barré syndrome (GBS). Cross-reactive antibodies to ganglioside GQ1b are strongly associated with oculomotor weakness in GBS and its variant, Miller Fisher syndrome (MFS). Antigen recognition is a crucial first step in the induction of a cross-reactive antibody response, and it has been shown that GQ1b-like epitopes expressed on the surface of *C. jejuni* are recognized by sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7). We aimed to determine the epitope specificity of *C. jejuni* binding to Siglec-7, and correlate the outcome to disease symptoms in GBS and MFS patients. Using a well-defined GBS/MFS-associated *C. jejuni* strain collection, which included three sialic acid knockout strains, we found that Siglec-7 exclusively binds to *C. jejuni* strains that express terminal disialylated ganglioside mimics. When serological and diagnostic patient records were correlated with the Siglec-7 binding properties, we observed an association between Siglec-7 binding and the presence of anti-GQ1b antibodies in patient serum. In addition, Siglec-7 binding was associated with oculomotor weakness in GBS and MFS patients. Lipooligosaccharides-specific binding of *C. jejuni* to Siglec-7 may be an initiating event in immune recognition and presentation, and lead to anti-GQ1b antibody production and the development ocular weakness in GBS or MFS.

Introduction

Guillain-Barré syndrome (GBS) is an antibody-mediated autoimmune disease of the peripheral nerves, which mainly arises after gastrointestinal infection (1-3). GBS is characterized by rapidly progressing acute ascending paralysis, which can result in complete systemic paralysis and the need for artificial respiration (4). Miller Fisher syndrome (MFS) is a restricted variant of GBS, characterized by paralysis of the eye muscles (oculomotor weakness), lack of coordination and loss of tendon reflexes, without limb weakness (5). GBS-MFS overlap syndrome may also occur in patients who display a combination of limb and oculomotor weakness (6, 7).

Campylobacter jejuni is the predominant infection preceding the onset of weakness and paralysis in GBS and MFS (8, 9). *C. jejuni* strains isolated from GBS patients frequently express lipooligosaccharide (LOS) structures that contain glycan moieties which mimic gangliosides from the human peripheral nervous system (10). In these patients and in animal models, the antibodies raised during the immune response to *C. jejuni* LOS can cross-react with various gangliosides and lead to complement-dependent nerve destruction and paralysis (11, 12).

Guillain-Barré is a syndromic disease entity with a heterogeneous presentation of symptoms (2). Ganglioside mimicry in *C. jejuni* is strongly associated with the specificity of the cross-reactive antibody response and the clinical neurological phenotype. *C. jejuni* can express monosialylated and disialylated LOS with α(2,3)- or α(2,3/2,8)-linked sialic acid residues, respectively. Monosialylated *C. jejuni* strains are predominantly isolated from the stools of patients with GBS. In agreement with this observation, antibodies against monosialylated structures, including GM1a, GM1b, GD1a and GalNAc-GD1a are frequently detected in the serum of GBS patients (13, 14). In contrast, *C. jejuni* strains with disialylated LOS that mimic GQ1b-like epitopes including GD1c and GD3 are closely associated with MFS patients, who often have cross-reactive antibodies directed against GQ1b (15, 16). Iinterestingly, the human oculomotor nerves, which innervate the eye muscles and are affected in MFS, have a relatively high content of GQ1b, which could explain their vulnerability to damage mediated by anti-GQ1b antibodies (15).

Antigen recognition is a determining initial step in the development of immune responses leading to GBS or MFS. Sialylation of *C. jejuni* LOS is an important determinant for the development of GBS and MFS (17). Therefore, sialic acid-binding immunoglobulin-like lectins (Siglecs) expressed on immune-related cells may play a decisive role in immune recognition. Siglecs comprise a family of surface exposed receptors that are involved in sialic acid dependent cell-to-cell interactions and ligand binding (18). Additionally, Siglecs function as endocytic receptors in immune recognition of both bacteria and viruses (19-21).

We recently demonstrated that GBS-related *C. jejuni* strains specifically bind to sialoadhesin (Siglec-1) (22). Furthermore, other researchers have shown that *C. jejuni* strains

expressing disialylated LOS structures can bind to Siglec-7 (23); however, a limited number (*n* = 4) of strains were examined and no correlation was made with the clinical phenotype.

In this study, we determined the epitope specificity of *C. jejuni* for Siglec-7 binding. We examined a large and unique collection of *C. jejuni* strains (*n* = 29) derived from GBS and MFS patients, for which detailed information was available on the ganglioside mimicking structures expressed. In particular, we investigated the relationship between Siglec-7 binding and the presence of anti-ganglioside antibodies in patient serum and the specific clinical phenotypes. This study demonstrates that Siglec-7 specifically recognizes the *C. jejuni* strains associated with oculomotor weakness in GBS or MFS.

Materials and methods

Bacterial strains and culture conditions

A group of 29 successive and well-characterized *C. jejuni* strains isolated from the stools of either GBS or MFS patients (Supplemental Table 1), three previously described sialic acid transferase (*cst-II*) knockout mutants of GBS-associated strains (GB2Δ*cst-II*, GB11Δ*cst-II* and GB19Δ*cst-II*) (24, 25) and the reference strain NCTC 11168 were used in this study (22, 24, 26). Strains GB13, GB14, GB26 and GB27 were cultured from the diarrheal stools of the family members of two GBS patients after a family outbreak of *C. jejuni* enteritis (27) (Supplemental Table 1). The GBS-related and MFS-related strains predominantly originate from Dutch patients. Two strains from the Netherlands Antilles and one Belgian strain were included. *C. jejuni* strains were cultured from stocks held at -80°C stocks and maintained on Colombia blood agar plates (Becton Dickinson BV, Alphen aan den Rijn, The Netherlands) supplemented with 10 µg/ml vancomycin in a microaerobic atmosphere at 37°C. Chloramphenicol (20 mg/L) was added to the Δ*cst-II* mutant strain culture plates. For each experiment, all strains were freshly cultured for two days on blood agar plates containing only vancomycin. The LOS outer core structures of most GBS/MFS associated strains used in this study have been described previously (10). The LOS structures of *C. jejuni* GB29, GB30 and GB33 were determined using mass spectrometry analysis, as previously described (10). Genotyping by PCR was performed to verify the LOS classes, as previously described (24).

Serology and diagnosis

Serum samples obtained within 2 weeks of the onset of weakness and before treatment, were tested for the presence of IgM and IgG antibodies to the ganglioside GQ1b using a validated ELISA with predefined cut-off values, as previously described (28). The diagnosis of GBS or MFS was made by specialized neurologists, based on previously described criteria (29, 30).

Preparation of Siglec-7-Fc-conjugate

Chinese hamster ovary (CHO) cells expressing the full extracellular region of human Siglec-7 fused to recombinant Fc protein (CHO-Siglec-7-Fc) were generated (31) and Siglec-7-Fc was produced as previously described (22). Briefly, CHO-Siglec-7-Fc cells were cultured in glutamine-free Glasgow Minimal Essential Medium (Sigma-Aldrich, Zwijndrecht, The Netherlands) containing 100 μM L-methionine sulfoximine (Sigma-Aldrich), GS supplement (Sigma-Aldrich), penicillin/streptomycin and 10% dialysed foetal calf serum (Invitrogen, Leek, The Netherlands). Once the cells reached 80% confluency, the foetal calf serum concentration was adjusted to 2% and eventually, the cells were cultured in X-VIVO-10 serum free media (Lonza, Verviers, Belgium) and the media was harvested weekly. The concentration of Siglec-7-Fc was determined using an Fc-specific ELISA, as previously described (22).

Siglec-7-Fc ELISA

Two-day *C. jejuni* cultures grown on blood agar plates were harvested, washed and the optical density at 600 nm (OD_{600}) was adjusted to 0.2 in phosphate buffered saline (PBS) containing 2 mM $MgCl_2$ (PBS-Mg). After heat inactivation at 56ºC for 45 min, 100 μL of each sample was plated in triplicate in 96-well Maxisorp ELISA plates (NUNC Inc. Uden, The Netherlands). The plates were kept open overnight at 37ºC to allow the fluid to evaporate. After washing, the wells were blocked for 1 h using 1% bovine serum albumin in PBS at 37ºC. Simultaneously, 1 ml/L Siglec-7-Fc conjugate was precomplexed with peroxidase-conjugated anti-human IgG (IgG-PO; Sigma-Aldrich) diluted 1/3000 in PBS containing 0.05 % normal goat serum for 1 h at room temperature with shaking. After washing, 100 μL precomplexed Siglec-7-Fc was added per well, the plates were incubated for 2 h at room temperature, washed four times with PBS containing 0.05% Tween 20 and developed using 100 μL 3',3',5',5'-tetramethylbenzidine substrate (TMB; Sigma-Aldrich) per well. After an appropriate incubation time (5-10 min), the reaction was stopped by adding 100 μL of 2 M H_2SO_4 per well and signal intensity was measured spectrophotometrically at 450 nm using a 96-well microplate reader (Bio-Rad, Veenendaal, The Netherlands). With respect to the Siglec-7 inhibition experiment, equal amounts (300 ng/well) purified bovine brain GQ1b (Sigma-Aldrich) was coated on an ELISA plate and blocked to avoid non-specific binding. In parallel, precomplexed Siglec-7-Fc was incubated for 1 h with twice the number of bacteria we normally use in our Siglec-7 ELISA to coat the wells. Siglec-7 bound- or free bacteria were removed by centrifugation. The supernatant (i.e. non-adsorbed Siglec-7) was transferred to the GQ1b-coated plate and binding of Siglec-7 was determined as described above.

Statistical analysis

Two-tailed *t* tests and Mann-Whitney *U*-tests were performed using Prism software (GraphPad, La Jolla, CA, USA) as indicated; $P \leq 0.05$ was considered statistically significant.

Results

Recognition of *C. jejuni* by Siglec-7 is sialic acid-specific

Although it has been shown that disialylated ganglioside-like structures expressed on the surface of *C. jejuni* can bind to Siglec-7 in a sialic acid-dependent manner, the possibility of low-affinity Siglec-7 binding to monosialylated structures or complexes could not be excluded (23). Therefore, we aimed to determine the precise requirements of ganglioside-like structures for Siglec-7 binding.

Sialic acid-specific Siglec-7 binding was determined using three *C. jejuni* strains GB2, GB11 (both GM1a$^+$ GD1a$^+$) and GB19 (GD1c$^+$), and their sialic acid mutants GB2Δ*cst-II*, GB11Δ*cst-II* (both GA1$^+$ GA2$^+$ GA3$^+$) and GB19Δ*cst-II* (GA1$^+$; Fig. 1). Strain GB19 showed high Siglec-7 binding affinity in a whole cell Siglec-7-Fc ELISA (Fig. 2A). In agreement

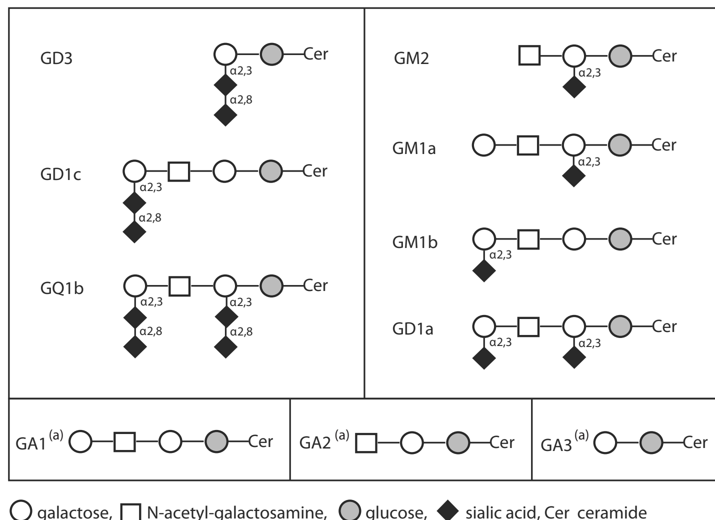

Figure 1. Schematic illustration of the ganglioside structures discussed in this study. These structures can be mimicked by the *C. jejuni* outer core lipooligosaccharides (LOS). However, instead of the ceramide-bound glucose, the *C. jejuni* LOS has a heptose, followed by an inner sugar core, and *C. jejuni* LOS has a lipid A transmembrane tail instead of a ceramide tail. Disialylated structures with α(2,3/2,8)-linked sialic acid residues are represented in the left panel; monosialylated structures with α(2,3)-linked sialic acid residues are represented in the right panel. (a) GA1, GA2 and GA3 (or asialo GM1, -GM2 and -GM3) contain no sialic acids and are considered not to be gangliosides.

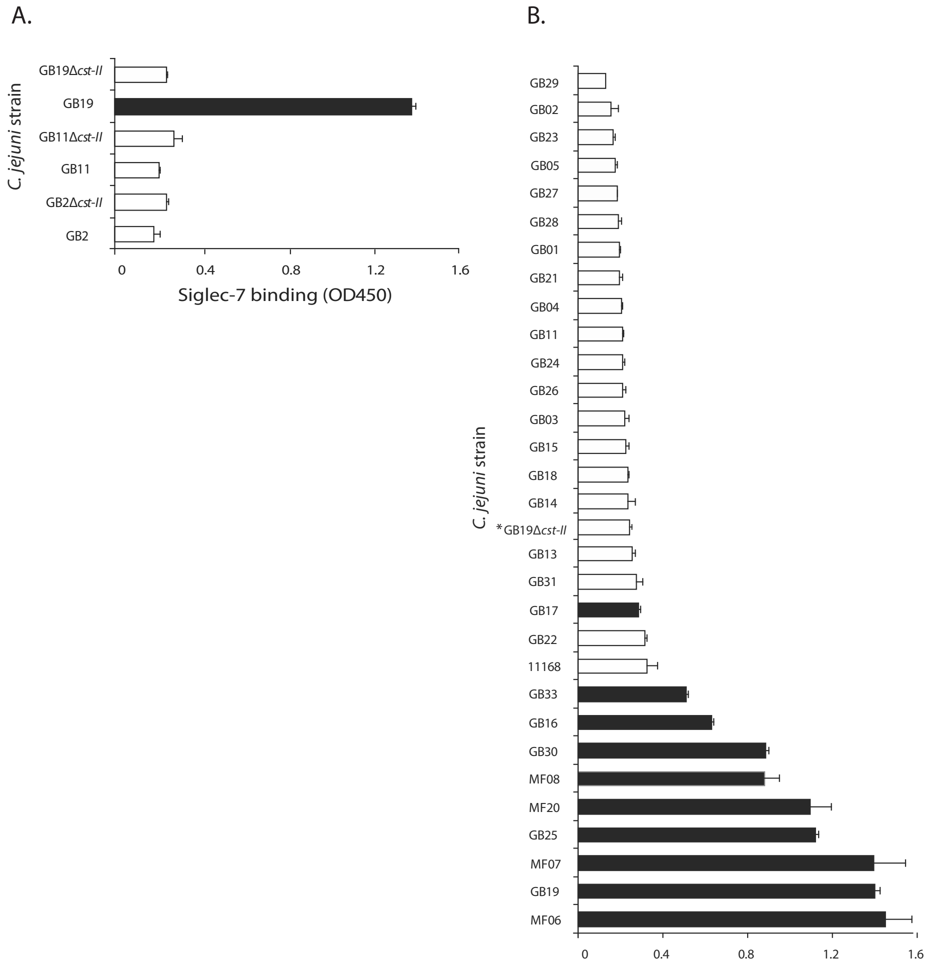

Figure 2. Evaluation of the binding of Siglec-7-Fc to *C. jejuni* strains using an ELISA. The strains were heat-inactivated, coated on ELISA plates, incubated with precomplexed Siglec-7 conjugate and visualized using 3′,3′,5′,5′-tetramethylbenzidine substrate. The bars represent a single experiment that was repeated at least three times, with means and standard deviations of triplicate measurements. Strains GB2Δ*cst-II*, GB11Δ*cst-II* and GB19Δ*cst-II* are the non-sialylated Campylobacter sialic acid transferase (*cst-II*) knockout mutants of the parental wild type strains GB2, GB11 and GB19, respectively. White bars, non-/monosialylated lipooligosaccharides (LOS); black bars, disialylated LOS. **A.** Siglec-7 binding to parental wild type and sialic acid transferase knockout *C. jejuni* strains. **B.** Siglec-7 binding to GBS- and MFS-associated *C. jejuni* strains. * Strain GB19Δ*cst-II* was included as a reference and it is considered to be a negative control for Siglec-7 binding.

with reports that disialylated carbohydrate structures specifically bind to Siglec-7 (23, 32), GB19 (GD1c^{+}) is disialylated at the terminal galactose of the oligosaccharide chain. GB19Δ*cst-II* demonstrated reduced Siglec-7 binding, indicating that the binding was sialic acid-specific. Comparable background levels of Siglec-7 binding were observed for the monosialylated strains GB2 and GB11, and their respective nonsialylated Δ*cst-II* mutants (Fig. 2A). In order to address whether Siglec-7 binds a similar epitope on ganglioside GQ1b, an inhibition ELISA was performed. Compared with GB19Δ*cst-II*, Siglec-7 binding to GQ1b was significantly ($P = 0.0005$; *t* test) reduced when strain GB19 was used for adsorption, demonstrating that strain GB19 inhibits Siglec-7 binding to GQ1b (Supplemental Fig.1). GB19Δ*cst-II* showed a reduction in the signal when compared to the non-blocking situation. This reduction was similar as observed with GB11 and GB11Δ*cst-II,* indicating that this effect was not dependent on sialic acid.

We concluded that Siglec-7 can bind to the disialylated GD1c-like structure present on *C. jejuni* LOS in a sialic acid-dependent manner; however, Siglec-7 cannot bind monosialylated GM1a- and GD1a-like structures.

In a large collection of GBS/MFS-associated *C. jejuni* strains, only disialylated *C. jejuni* strains bind Siglec-7

To further study ganglioside mimic-specific Siglec-7 binding, 25 GBS-related and 4 MFS-related *C. jejuni* strains with known LOS structures, and the reference strain NCTC 11168 were tested in the Siglec-7-Fc ELISA. A clear diversity in Siglec-7 binding was observed, with various strains showing high or low Siglec-7 binding capacity (Fig. 2B). In particular, strains MF06, GB19, MF07, GB25, MF20, MF08, GB30, GB16 and GB33 strongly bound Siglec-7. Strikingly, all of these strains have terminally disialylated LOS structures (Fig. 2B). The GD1c-like structure is disialylated in strains MF06, GB19, GB25, GB16 and GB33; whereas disialylation is present in the GD3-like structure of strains MF07 and MF08 (Fig. 1; Supplementary Table 1).

The exact structures of the ganglioside mimics present on strain GB30 could not be determined because mass spectrometry analysis yielded a complex profile. However, mass spectrometry analysis confirmed the presence of monosialic and disialic acids in the LOS outer core of strain GB30. Based on these results, it is probable that the GB30 LOS outer core contains GD3-like structures (Michel Gilbert, personal communication).

When the nonsialylated and monosialylated strains ($n = 20$) were compared to the disialylated strains ($n = 10$), we observed significantly higher Siglec-7 binding for the disialylated strains ($P < 0.0001$; Mann-Whitney *U* test; Fig. 3A). Binding of the nonsialylated and monosialylated strains was low and comparable to the binding of strain GB19Δ*cst-II*, which lacks sialic acid. Strain GB17 did not strongly bind Siglec-7, despite the presence of disialylated ganglioside-like structures. It is possible that this strain contains addi-

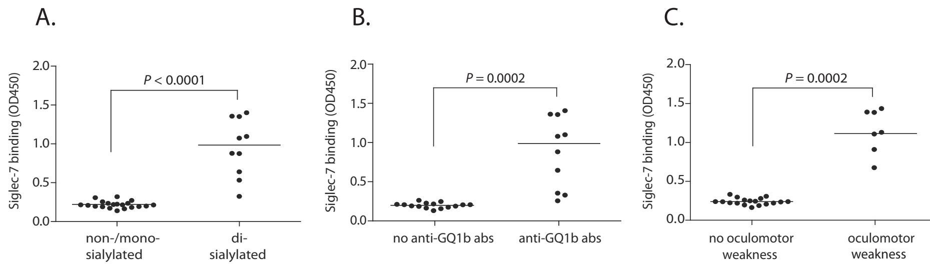

Figure 3. Relationship between Siglec-7 binding by *C. jejuni* and A, LOS sialylation; B, the presence of anti-GQ1b antibodies in GBS and MFS patient serum and C, oculomotor weakness in GBS and MFS patients. Siglec-7 binding was measured using an ELISA. Four individual bacterial strains were cultured from family members of patients with GBS, within two separate families (Supplementary Table 1); data from only one strain isolated from each family is included in B and C. The median values are indicated by the horizontal line; *P* values < 0.05 were considered statistically significant (Mann-Whitney *U*-test).

tional structures that hinder Siglec-7 binding. Therefore, we concluded that only *C. jejuni* equipped with terminally disialylated ganglioside-like structures can bind to Siglec-7.

Siglec-7 binding correlates with the presence of anti-GQ1b antibodies in the serum of patients with GBS

We determined whether Siglec-7 binding correlated with the presence of anti-GQ1b antibodies in the serum of GBS patients. The strains isolated from patients with a high anti-GQ1b antibody titre demonstrated significantly higher Siglec-7 binding than the strains isolated from patients who did not have anti-GQ1b antibodies ($P = 0.0002$; Mann-Whitney *U*-test; Fig. 3B; Supplemental Table 1). Seven of the nine strains that showed strong binding to Siglec-7 (78%) were isolated from GBS/MFS patients expressing anti-GQ1b antibodies; no patient serum was available for testing from the other two strains. Three strains (GB4, GB17 and GB22) that did not bind Siglec-7 were isolated from patients with anti-GQ1b antibodies. Strain GB4 expresses a class E LOS and therefore does not carry the genes necessary for sialylation, which is an essential determinant for ganglioside mimicry. We hypothesize that this strain (GB4) was not involved in triggering the patient's immune system and the subsequent development of GBS. Strain GB17 (GM1b$^+$, GD1c$^+$ GA1$^+$) contains disialylated LOS but did not bind to Siglec-7; however, the patient had (low) anti-GQ1b antibodies. Strain GB22 (GD1a$^+$ GM1a$^+$) does not express disialylated LOS; therefore, it probably does not bind to Siglec-7. In addition to anti-GQ1b antibodies, the patient from whom strain GB22 was isolated also had antibodies against GM1a (data not shown), suggesting that strain GB22 may contribute to induction of anti-GM1a antibodies but perhaps not anti-GQ1b antibodies.

C. jejuni Siglec-7 binding is associated with oculomotor weakness and MFS

As disialylated *C. jejuni* strains and anti-GQ1b antibodies are associated with oculomotor weakness (15, 33), we determined whether Siglec-7 binding also correlated with oculomotor weakness. Strikingly, all of the patients with oculomotor weakness (7/7; 100%) were infected with *C. jejuni* strains which showed a high binding affinity for Siglec-7 ($P = 0.0002$; Mann-Whitney *U*-test; Fig. 3C; Supplementary Table 1). Three of these strains were isolated from GBS patients and four were isolated from MFS patients. All of the patients with MFS had been infected with strains which had a high Siglec-7 binding affinity (4/4; 100 %). Two other strains which bound Siglec-7, GB30 and GB33, were isolated from GBS patients for whom no information on oculomotor weakness was available.

Discussion

In the present study, we report that sialylated structures on the surface of *C. jejuni* can bind to Siglec-7, a receptor of the Siglec family which is expressed on immune cells including dendritic cells. We demonstrated that the binding of *C. jejuni* to Siglec-7 is sialic acid-dependent, as a sialic acid transferase knockout strain could not bind Siglec-7 whereas the parental wild type strain could. Siglec-7 has a preference for binding disialylated sialic acid conjugates, such as those present in the ganglioside GQ1b (32). Indeed, only strains expressing disialylated ganglioside-like structures could bind Siglec-7; specifically, the GD1c- or GD3-like disialylated ganglioside-mimics present in the *C. jejuni* strain collection used in this study. Similarly to GQ1b, both GD1c and GD3 are disialylated at the terminal galactose of the carbohydrate chain. Infection with GD1c-positive or GD3-positive *C. jejuni* strains has been previously associated with the presence of cross-reactive anti-GQ1b antibodies in the serum of GBS or MFS patients (16, 33, 34). Upon screening a large panel of GBS-related and MFS-related *C. jejuni* strains, we observed an association between Siglec-7 binding and the presence of anti-GQ1b antibodies in patient serum. Furthermore, we found that Siglec-7 selectively recognized the *C. jejuni* strains that were isolated from GBS or MFS patients diagnosed with oculomotor weakness, strongly suggesting that the specific binding of *C. jejuni* to Siglec-7 is a marker for GBS and MFS with oculomotor weakness.

Our findings are in concordance with previous studies which reported that Siglec-7 can interact with terminally disialylated ganglioside structures, including GD3, GT1b and GQ1b (32, 35). An interaction of Siglec-7 with *C. jejuni* strains expressing disialylated LOS structures was also previously demonstrated using ELISA and CHO-cell adhesion assays (23); however, a limited number of strains were tested and the correlation with clinical phenotypes was not examined.

It has been suggested that the presence of other ganglioside mimics influences the interaction with Siglec-7 (23). Polyvinylidene difluoride glycoarray-based experiments revealed that a 1:1 complex of either GM1, GM2, GD1a, GD1b or GT1a with GD3 attenuated Siglec-7 binding (35). Therefore, the binding of Siglec-7 to the GD3-like structure of the strains MF07 ($GM2^{+}$ $GD2^{+}$ $GD3^{+}$) and MF08 ($GM2^{+}$ $GD3^{+}$) used in this study could potentially be affected by the presence of GM2. As Siglec-7 binding was clearly observed for these strains, complex attenuation is apparently not a major issue. However, complex attenuation may explain why strain GB17 ($GM1b^{+}$ $GD1c^{+}$ $GA1^{+}$) did not bind to Siglec-7. It should be noted that in serum of patient GB17, complex reactivity against GM1/GD1a, GD1a/GD1b and GD1a/GQ1b was observed (26). This suggests that the ganglioside-like epitopes on GB17 LOS form complexes. The formation of these complexes might prevent Siglec-7 binding. It is also possible that the GD1c-like structure was expressed in low levels on the surface of GB17 under the current culture conditions, resulting in low Siglec-7 binding.

The consequence of pathogen interactions with Siglec-7 is largely unknown. Siglec-7 is a member of the CD33-related Siglecs, which contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail. Pathogen interactions with Siglec-7 could therefore exert an inhibitory effect on immune activation, as ITIM signalling has been shown to restrain Siglec internalization via ITIM phosphorylation (36). However, Siglec-related pathogen uptake has also been reported (19). It is possible that *cis* interaction of Siglec-7 with self-ligands results in an inhibitory response; whereas pathogen interactions with Siglec-7 overrule this signal, possibly due to the activation of co-receptors and cytokine secretion, or a higher receptor affinity (37). Evidence for a Siglec-7-activated immune response was recently demonstrated, as Siglec-7 interactions resulted in the skewing of dendritic cells towards T helper 1 polarization, due to LOS-mediated OX40 ligand induction (38). However, the mechanisms by which this process could eventually lead to an anti-ganglioside antibody response and result in GBS or MFS with oculomotor weakness remain to be elucidated.

In conclusion, we demonstrate that oculomotor weakness in GBS and MFS is associated with *C. jejuni* strains that bind Siglec-7. Binding of *C. jejuni* to Siglec-7 may be an event that mediates anti-GQ1b antibody activation, leading to oculomotor weakness in patients with GBS or MFS. Identification of *C. jejuni* on the basis of Siglec-7 binding could be of diagnostic value for the detection of strains which have the potential to induce neurological symptoms. In cases of *C. jejuni* infection where a Siglec-7 binding strain is cultured from faecal samples, antibiotic treatment could be prescribed to prevent postinfectious neurological complications. Additional studies are necessary to identify the occurrence of Siglec-7 binding strains in uncomplicated enteritis.

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References

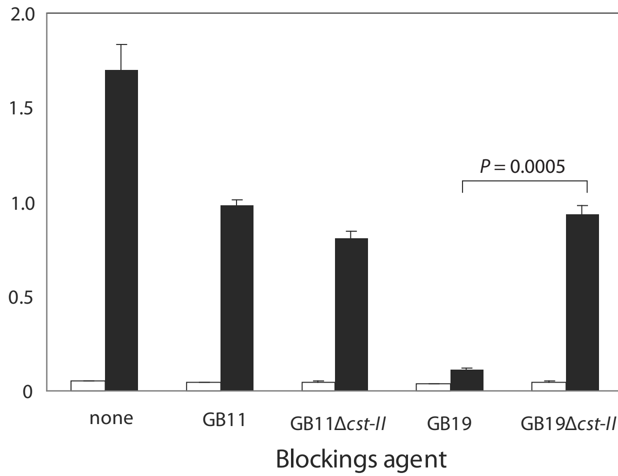
1. Hahn AF (1998) Guillain-Barré syndrome. *Lancet* 352(9128):635-641.
2. Hughes RA & Cornblath DR (2005) Guillain-Barré syndrome. *Lancet* 366(9497):1653-1666.
3. Tam CC, O'Brien SJ, Petersen I, Islam A, Hayward A, & Rodrigues LC (2007) Guillain-Barré syndrome and preceding infection with campylobacter, influenza and Epstein-Barr virus in the general practice research database. *PLoS ONE* 2(4):e344.
4. van Doorn PA, Ruts L, & Jacobs BC (2008) Clinical features, pathogenesis, and treatment of Guillain-Barré syndrome. *Lancet Neurol* 7(10):939-950.
5. Fisher M (1956) An unusual variant of acute idiopathic polyneuritis: syndrome of ophthalmoplegia, ataxia and areflexia. *N. Engl. J. Med.* 255:57-65.
6. Funakoshi K, Kuwabara S, Odaka M, Hirata K, & Yuki N (2009) Clinical predictors of mechanical ventilation in Fisher/Guillain-Barré overlap syndrome. *J Neurol Neurosurg Psychiatry* 80(1):60-64.
7. Rajabally YA, Hassan-Smith G, Notturno F, *et al.* (2011) Motor and sensory conduction failure in overlap of Guillain-Barré and Miller Fisher syndrome: two simultaneous cases. *J Neurol Sci* 303(1-2):35-38.
8. Jacobs BC, Rothbarth PH, van der Meché FGA, *et al.* (1998) The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. *Neurology* 51(4):1110-1115.
9. Koga M, Gilbert M, Li J, *et al.* (2005) Antecedent infections in Fisher syndrome: a common pathogenesis of molecular mimicry. *Neurology* 64(9):1605-1611.
10. Godschalk PC, Kuijf ML, Li J, *et al.* (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndromes. *Infect Immun* 75(3):1245-1254.
11. Hafer-Macko CE, Sheikh KA, Li CY, *et al.* (1996) Immune attack on the Schwann cell surface in acute inflammatory demyelinating polyneuropathy. *Ann Neurol* 39(5):625-635.
12. Yuki N, Susuki K, Koga M, *et al.* (2004) Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barré syndrome. *PNAS* 101(31):11404-11409.
13. Willison HJ & Yuki N (2002) Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125(12):2591-2625.
14. Hiraga A, Kuwabara S, Ogawara K, *et al.* (2005) Patterns and serial changes in electrodiagnostic abnormalities of axonal Guillain-Barré syndrome. *Neurology* 64(5):856-860.
15. Chiba A, Kusunoki S, Obata H, Machinami R, & Kanazawa I (1993) Serum anti-GQ1b IgG antibody is associated with ophthalmoplegia in Miller Fisher syndrome and Guillain-Barré syndrome: clinical and immunohistochemical studies. *Neurology* 43(10):1911-1917.
16. Ang CW, Laman JD, Willison HJ, *et al.* (2002) Structure of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity and clinical features of Guillain-Barré and Miller Fisher patients. *Infect. Immun.* 70(3):1202-1208.
17. van Belkum A, van den Braak N, Godschalk P, *et al.* (2001) A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. *Nat. Med.* 7(7):752-753.
18. Crocker PR, Paulson JC, & Varki A (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* 7(4):255-266.
19. Jones C, Virji M, & Crocker PR (2003) Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol Microbiol* 49(5):1213-1225.
20. Rempel H, Calosing C, Sun B, & Pulliam L (2008) Sialoadhesin expressed on IFN-induced monocytes binds HIV-1 and enhances infectivity. *PLoS ONE* 3(4):e1967.
21. Vanderheijden N, Delputte PL, Favoreel HW, *et al.* (2003) Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. *J Virol* 77(15):8207-8215.
22. Heikema AP, Bergman MP, Richards H, *et al.* (2010) Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 78(7):3237-3246.

23. Avril T, Wagner ER, Willison HJ, & Crocker PR (2006) Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 74(7):4133-4141.
24. Godschalk PCR, Heikema AP, Gilbert M*, et al.* (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J Clin Invest* 114(11):1659-1665.
25. Louwen R, Heikema A, van Belkum A*, et al.* (2008) The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. *Infect Immun* 76(10):4431-4438.
26. Kuijf ML, Godschalk PC, Gilbert M*, et al.* (2007) Origin of ganglioside complex antibodies in Guillain-Barré syndrome. *J Neuroimmunol* 188(1-2):69-73.
27. Ang CW, van Doorn PA, Endtz HP*, et al.* (2000) A case of Guillain-Barré syndrome following a family outbreak of *Campylobacter jejuni* enteritis. *J. Neuroimmunol.* 111:229-233.
28. Kuijf ML, van Doorn PA, Tio-Gillen AP*, et al.* (2005) Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. *J Neurol Sci* 239(1):37-44.
29. Asbury AK & Cornblath DR (1990) Assessment of current diagnostic criteria for Guillain-Barré syndrome. *Ann Neurol* 27(Suppl):S21-24.
30. Miller Fisher C (1956) An unusual variant of acute idiopathic polyneuritis (syndrome of ophthalmoplegia, ataxia and areflexia). *N Engl J Med* 225:57-65.
31. Vinson M, van der Merwe PA, Kelm S, May A, Jones EY, & Crocker PR (1996) Characterization of the sialic acid-binding site in sialoadhesin by site-directed mutagenesis. *J Biol Chem* 271(16):9267-9272.
32. Rapoport E, Mikhalyov I, Zhang J, Crocker P, & Bovin N (2003) Ganglioside binding pattern of CD33-related siglecs. *Bioorg Med Chem Lett* 13(4):675-678.
33. Yuki N (2007) Campylobacter sialyltransferase gene polymorphism directs clinical features of Guillain-Barré syndrome. *J Neurochem* 103 Suppl 1:150-158.
34. Yuki N (2001) Infectious origins of, and molecular mimicry in, Guillain-Barré and Fisher syndromes. *Lancet Infect. Dis.* 1:29-37.
35. Rinaldi S, Brennan KM, Goodyear CS*, et al.* (2009) Analysis of lectin binding to glycolipid complexes using combinatorial glycoarrays. *Glycobiology* 19(7):789-796.
36. Walter RB, Raden BW, Zeng R, Hausermann P, Bernstein ID, & Cooper JA (2008) ITIM-dependent endocytosis of CD33-related Siglecs: role of intracellular domain, tyrosine phosphorylation, and the tyrosine phosphatases, Shp1 and Shp2. *J Leukoc Biol* 83(1):200-211.
37. Paulson JC, Macauley MS, & Kawasaki N (2012) Siglecs as sensors of self in innate and adaptive immune responses. *Ann N Y Acad Sci* 1253(1):37-48.
38. Bax M, Kuijf ML, Heikema AP*, et al.* (2011) *Campylobacter jejuni* lipo-oligosaccharides modulate DC-mediated T cell polarization in a sialic acid linkage dependent manner. *Infect Immun*. 79(7):2681-9.

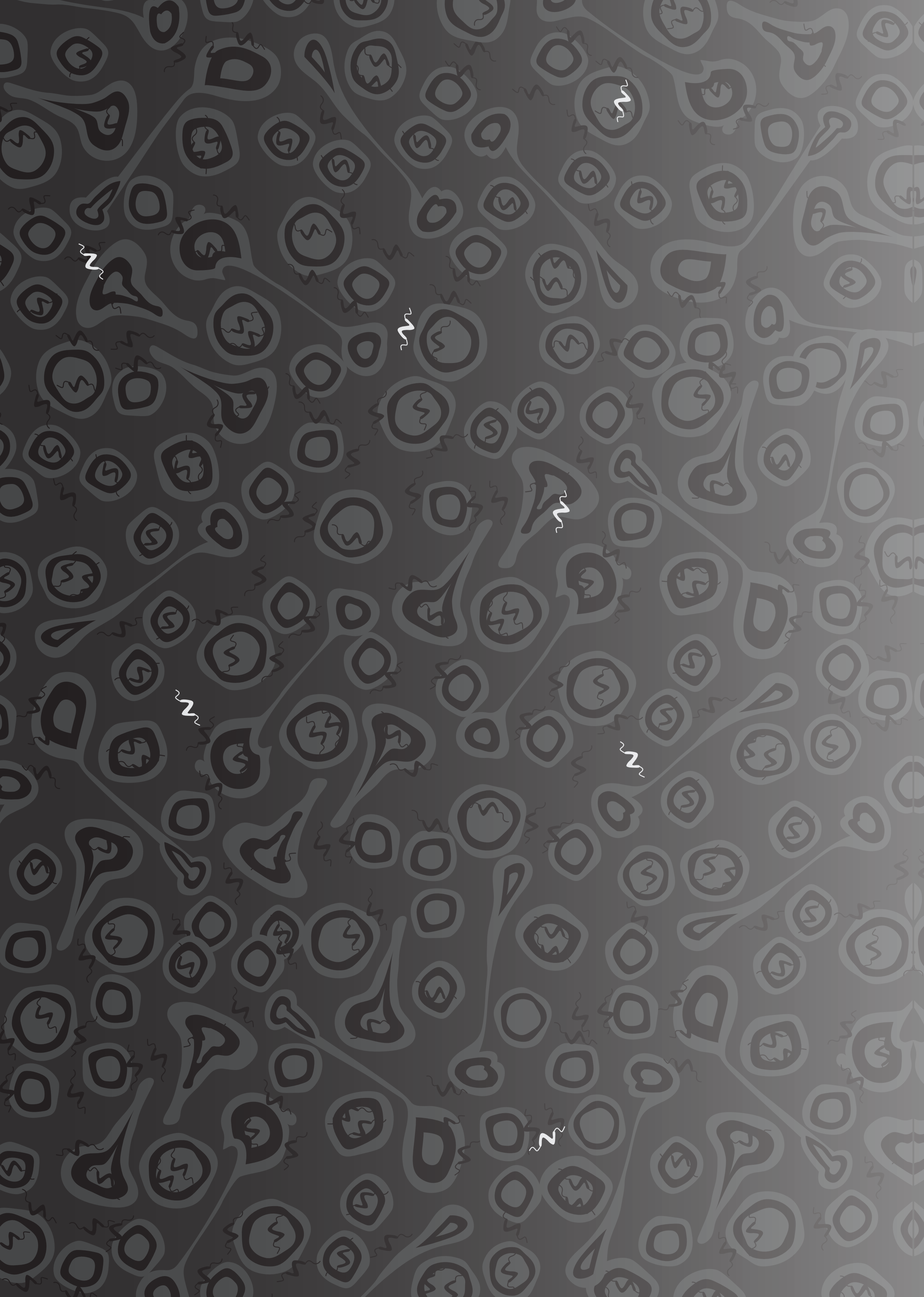
Supplemental Table 1. GBS- and MFS-associated *C. jejuni* strains used in this study. All strains were isolated from patient stools. The ganglioside mimicking structures on the surface of each strain were identified using mass spectrometry; serum antibodies to the ganglioside GQ1b were detected using an ELISA.

GBS/MFS strain	Patient diagnose	LOS class	Cst-II(a)	Ganglioside mimic	Siglec-7 binding	Anti-GQ1b antibodies	Oculomotor weakness
MF6	MFS	B	bifunctional	GM1b, GD1c	+	yes	yes
MF7	MFS	B	bifunctional	GM2, GD2, GD3	+	yes	yes
MF8	MFS	B	monofuctional	GM2, GD3	+	yes	yes
MF20	MFS	B	bifunctional	GM1b, GD1c	+	yes	yes
GB16	GBS	A	bifunctional	GD1c	+	yes	yes
GB19	GBS	A	bifunctional	GD1c	+	yes	yes
GB25	GBS	B	bifunctional	GM1b, GD1c, GA1	+	yes	yes
GB33	GBS	B	NT	GM1b, GD1c, GA1, GalNac-GMb1	+	NT(b)	unknown
GB30	GBS	B	NT	mono- and disialylated LOS(c)	+	NT(b)	unknown
GB4	GBS	E	absent	none	-	yes	no
GB17	GBS	B	bifunctional	GM1b, GD1c, GA1	-	yes(d)	no
GB22	GBS	A	monofuctional	GD1a, GM1a	-	yes	no
GB1	GBS	C	Cst-III	none (GA1)	-	no	no
GB2	GBS	A	monofuctional	GD1a, GM1a	-	no	no
GB3	GBS	A	monofuctional	GD1a, GM1a	-	no	no
GB5	GBS	B	off	none (GA2)	-	no	no
GB11	GBS	A	monofuctional	GD1a, GM1a	-	no	no
GB13	enteritis, family GBS	C	Cst-III	GM1a	-	no(e)	no
GB14	enteritis, family GBS	C	Cst-III	GM1a	-	no(e)	no
GB15	GBS	D	absent	none	-	no	no
GB18	GBS	A	monofuctional	GD1a, GM1a	-	no	no
GB21	GBS	A	monofuctional	GD1a, GM1a	-	no	no
GB23	GBS	A	monofuctional	GM2	-	no	no
GB24	GBS	D	absent	none	-	no	no
GB26	enteritis, family GBS	A	monofuctional	GM1b	-	no(e)	no
GB27	enteritis, family GBS	A	monofuctional	GM1b	-	no(e)	no
GB28	GBS	A	monofuctional	GD1a, GM1a	-	no	no
GB29	GBS	C	Cst-III	none	-	no	no
GB31	GBS	A	monofuctional	GD1a, GM1a	-	no	no

(a) Functionality of Cst-II based on a polymorphism (Asn51 or Thr 51) in the cstII gene, (b) = no serum available, (c) = mono-sialic acid and di-sialic acid were confirmed by mass spectrometry but the backbone could not be determined, (d) = multiple testing of the same serum sample demonstrated low serum reactivity against GQ1b, (e) = no GQ1b antibodies in the serum of the GBS patient



Supplemental Figure 1. *C. jejuni*-mediated inhibition of Siglec-7 binding to ganglioside GQ1b. Precomplexed Siglec-7-Fc was left untreated or preincubated with GB11, GB11Δ*cst-II*, GB19 or GB19Δ*cst-II*. Siglec-7 bound- or free bacteria were removed by centrifugation. The supernatant was transferred to a GQ1b-coated ELISA plate to detect the concentration of non-adsorbed Siglec-7. Siglec-7 binding was visualized using 3′,3′,5′,5′-tetramethylbenzidine substrate. The bars represent a single experiment with means and standard deviations of triplicate measurements. White bars, uncoated wells; black bars, GQ1b coated wells. *P* values < 0.05 were considered statistically significant (*t* test).



Chapter 7

Selective depletion of neuropathy-related antibodies from human serum by monolithic affinity columns containing ganglioside mimics

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Abstract

Monolithic columns containing ganglioside GM2 and GM3 mimics were prepared for selective removal of serum anti-ganglioside antibodies from patients with acute and chronic immune-mediated neuropathies. ELISA results demonstrated that anti-GM2 IgM antibodies in human sera and a mouse monoclonal anti-GM2 antibody were specifically and selectively adsorbed by monolithic GM2 mimic columns and not by blank monolithic columns or monolithic GM3 mimic columns. In control studies, serum antibodies against the ganglioside GQ1b from another neuropathy patient were not depleted by monolithic GM2 mimic columns. Fluorescence microscopy with FITC-conjugated anti-human immunoglobulin antibodies showed that the immobilized gangliosides were evenly distributed along the column. The columns were able to capture ~95% of the anti-GM2 antibodies of patients after only 2 min of incubation. A monolithic column of 4.4 μL can deplete 28.2 μL of undiluted serum. These columns are potential diagnostic and therapeutic tools for neuropathies related to anti-ganglioside antibodies.

Introduction

Antibodies against human peripheral nerve gangliosides are frequently encountered in various forms of immune-mediated neuropathies and may be directly involved in nerve damage (1). An example of such a neuropathy is the Guillain-Barré syndrome (GBS), in which half of the patients display significant levels of serum antibodies against various types of gangliosides. GBS is an acute post-infectious polyneuropathy, characterized by a rapidly progressive muscle weakness with a potentially devastating disease course. More than 20% of patients develop respiratory insufficiency requiring artificial ventilation at an intensive care unit. Overall the mortality is 5%, and at least 20% develop a sustained disability. *Campylobacter jejuni* is the most frequent cause of preceding infection, especially in the most severe forms of GBS (2). *C. jejuni* expresses lipooligosaccharides (LOS) on its surface, with carbohydrate moieties that are identical to gangliosides present in human neural cell membranes. This molecular mimicry can result in the production of antibodies against LOS during infection that cross-react with gangliosides. Antibodies to gangliosides are also found in patients with chronic forms of neuropathy, such as the paraproteinemic polyneuropathies. These forms of neuropathy are characterized by a progressive, incurable limb weakness usually leading to severe disability. At present no anti-ganglioside antibody specific treatment for these patients is available, although removal of the anti-ganglioside antibodies by selective immunoadsorption would be a rational approach.

Gangliosides are glycolipids containing a carbohydrate moiety with one or more sialic acid groups and a nonpolar ceramide unit by which gangliosides are anchored in cell membranes. Gangliosides are found abundantly in the human nervous system (3). The most widely known gangliosides are GM1, GM1b, GM2, GQ1b, GalNAc-GD1a, GT1a and GD1a (1, 2, 4) Antibodies in sera from patients with neuropathies bind to the extracellular exposed carbohydrate moieties of these gangliosides. Synthetic carbohydrate mimics of these gangliosides could be used to capture anti-ganglioside antibodies from the blood, as a specific treatment for these neuropathies. Synthetic ganglioside mimics possess various advantages over the natural gangliosides, which are usually purified from bovine brain. These advantages include a higher purity, tunable chemical properties, higher stability, possibly higher affinity, improved bioavailability, and no involvement of animals or risk of bovine-transmitted infections (5, 6). Recently, Pukin et al. synthesized analogs of various gangliosides (GM1, GM2 and GM3) in which alkyl spacers with different terminal functional groups (alkene, alkyne and azide) replace the natural nonpolar ceramide moiety (7), as well as di-, tetra- and octavalent derivatives of GM2 and GM1 gangliosides (8).

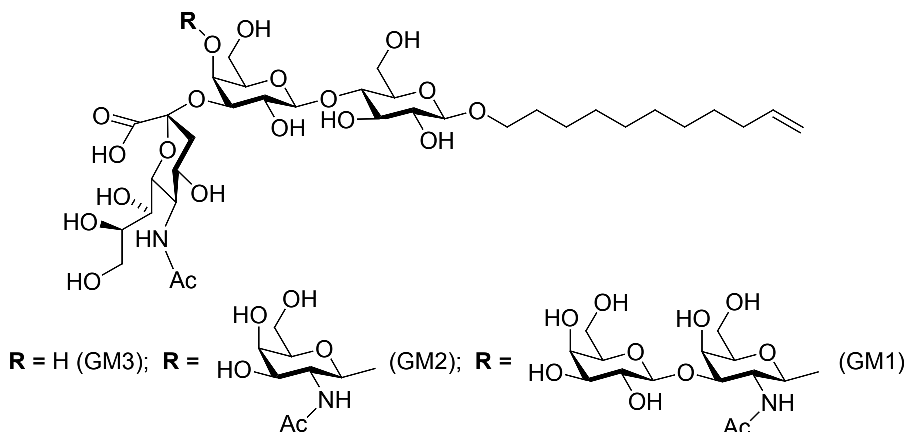

Figure 1. Structures of ganglioside mimics GM3, GM2 and GM1.

Figure 1 depicts the chemical structures of the ganglioside mimics GM1, GM2 and GM3. Serum antibodies from neuropathy patients showed a similar high affinity to these ganglioside mimics as to the bovine brain-derived gangliosides (7).

A range of synthetic GM1 mimics with a modified oligosaccharide part has been examined for binding the human anti-GM1 antibodies in solution inhibition and immunoadsorption (Sepharose columns) studies (9). It was found that the naturally derived GM1os (GM1 that lacks the ceramide aglycone) was superior to all investigated mimics. Thus, we focused on the genuine ganglioside analogues with an authentic carbohydrate structure and a functionalized aglycone part.

Carbohydrates with various functional groups have already been used as ligands, e.g., for microarrays on gold, silicon, glass surfaces (10-19), nanoparticles (20), and carbon nanotubes (21) and in monolithic columns (22-24). Monolithic supports have the advantage of a high surface density of reactive moieties that can bind oligosaccharides, which translates to a high loadability (25). Since this capacity is much higher than what could ever be obtained in e.g. wall-coated microchannels, this will increase the sensitivity in diagnostic applications. Flow-through applications would allow in principle the depletion of anti-ganglioside antibodies from blood as a rational treatment of GBS and other forms of immune-mediated neuropathies or more generally, diseases where binding between a receptor and a pathogenic molecule is involved.

We focused on obtaining a proof of principle that monolithic ganglioside mimic columns can efficiently capture antibodies from patients' serum samples and mouse monoclonal antibodies. This study is schematically summarized in Figure 2. Anti-GM2 antibodies were selected as the target, and thus, GM2 was chosen as ligand and attached via a 10-undecenyl spacer to a monolithic column. To determine the efficiency and selectivity of these affinity columns, we used sera obtained from a patient with

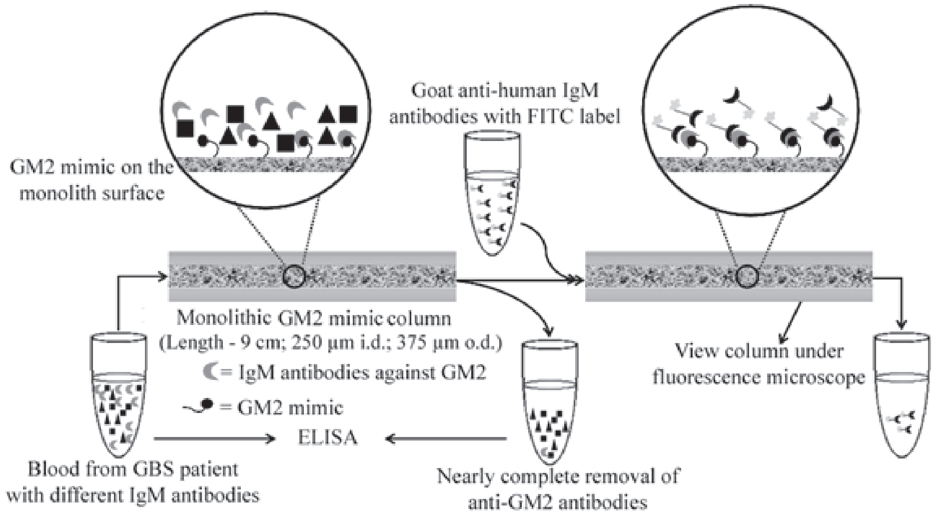

Figure 2. Schematic overview of the incubation studies with serum samples from patients with neuropathy and monolithic GM2 mimic columns to demonstrate selective depletion of serum anti-GM2 antibodies. The binding was measured by ELISA. The homogeneous distribution of captured antibodies along the column was measured by fluorescence microscopy and fluorescence conjugated anti-human IgM antibodies.

acute GBS (P1, patient 1) and a patient with chronic monoclonal gammopathy related polyneuropathy (P2, patient 2), both with high titers of IgM antibodies against GM2, and a mouse monoclonal antibody binding to GM2 (EM5). IgM antibodies against GM2 are found in various forms of immune-mediated neuropathy and in other disorders including patients with a human immunedeficiency virus 1 (HIV) infection, in which the anti-GM2 antibody level has prognostic relevance (26, 27).

Results and Discussion

Depletion of IgM antibodies using monolithic GM2 mimic columns

The monolithic GM2 mimic column was used to deplete anti-GM2 IgM antibodies from serum samples from the neuropathy patients P1 and P2. Figure 3A depicts the anti-GM2 IgM activity determined by ELISA in sera before and after exposure to this column, indicating that these antibodies were successfully depleted. The decrease of the IgM concentration was ~97.5 % in P1 (50 times diluted) and ~92 % in P2 (100 times diluted). Still, ~78 % of IgM antibodies in P1 were depleted when the serum dilution went from 1:50 to 1:5. This shows that a 4.4 µL monolithic column (250 µm i.d., 9 cm long) can deplete 28.2 µL undiluted serum, which corresponds to a capacity of 6.4 µL of undiluted serum per microliter of column volume. The kinetics of the adsorption are fast,

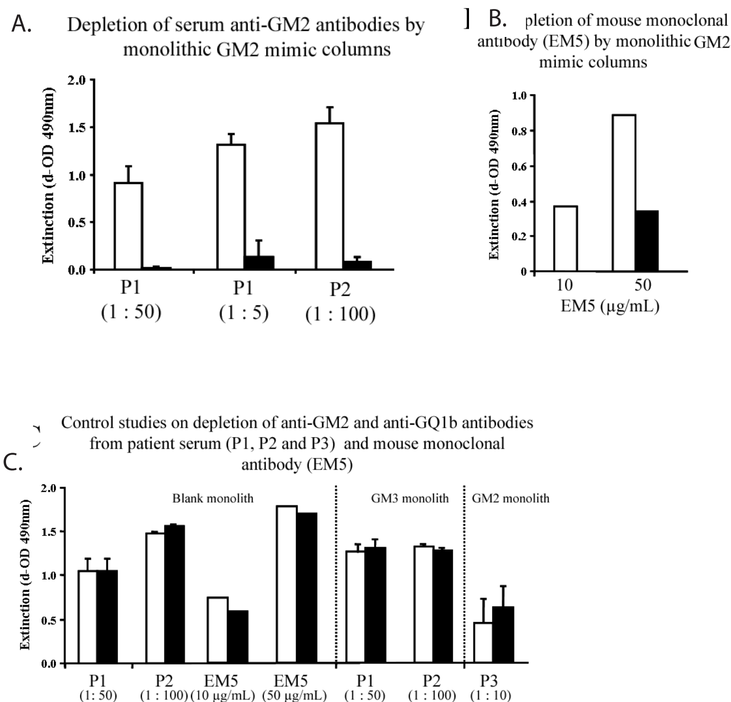

Figure 3. A. Depletion of serum anti-GM2 antibodies by monolithic GM2 mimic columns. Serum anti-GM2 IgM antibody concentration was determined by ELISAs in samples before (white bars) and after (black bars) exposure to the column. Serum from patient P1 with GBS was tested in two dilutions (1:50 and 1:5). Serum from patient P2 was tested in one dilution (1:100). **B.** Depletion of the mouse anti-GM2 monoclonal antibody EM5 (10 and 50 µg/mL) by monolithic GM2 mimic columns. The EM5 antibody concentration was determined by ELISAs in samples before (white bars) and after (black bars) exposure to the column. **C.** Control studies to confirm the specificity of interaction between anti-GM2 antibodies and GM2 mimics in the monolithic columns: (left panel) no depletion of anti-GM2 IgM antibodies from sera from patient P1 and P2 after infusion into blank monolithic columns; (middle panel) no depletion of anti-GM2 IgM antibodies in sera from patients P1 and P2 after infusion into monolithic GM3 mimic columns; (right panel) no depletion of anti-GQ1b IgM antibodies in serum from a neuropathy patient after infusion into monolithic GM2 mimic columns. Serum anti-ganglioside IgM antibody (GM2 + GQ1b) and mouse monoclonal antibody (EM5) concentrations were determined by ELISAs in samples before (white bars) and after (black bars) exposure to the column.

as the residence time of the serum in the columns was less than 2 min. Because of the strong binding of the serum anti-GM2 IgM to the GM2 mimic in the column, the bound antibodies could not be washed off the column with binding buffer solution.

To further demonstrate the specificity and capacity of these columns to capture anti-GM2 antibodies, mouse monoclonal IgM antibodies (EM5) binding to GM2 were infused

(see Figure 3B) (28). Studies with a dilution series of EM5 in concentrations up to 10 µg/mL showed that no residual activity to GM2 in the flow-through fraction was found after a single 2 min incubation with a monolithic GM2 mimic column. This column depleted more than 50% of the EM5 antibodies present at a concentration of 50 µg/mL of EM5 (Figure 3B). No depletion of EM5 (concentrations up to 50 µg/mL) was observed using blank monolithic columns (see Figure 3C).

Figure 3C depicts the ELISA data from three sets of control experiments to further confirm the specificity of the interaction between serum anti-GM2 antibodies and GM2 mimics in monolithic columns. First, sera from patients P1 and P2 were infused into blank monolithic columns (i.e., without GM2 mimics). No decrease in anti-GM2 IgM activity was observed confirming that these antibodies bind to GM2 mimics in the columns only (left panel, Figure 3C). Second, both sera were also infused into monolithic GM3 mimic columns. These act as a control antigen since the anti-GM2 antibodies in the patient sera did not bind to GM3 mimics in ELISA. No depletion of serum anti-GM2 antibodies was observed (middle panel, Figure 3C). Third, a serum sample from patient P3 with GBS with IgM antibodies to the ganglioside GQ1b (and no antibodies to GM2) was infused into the monolithic GM2 mimic column (right panel, Figure 3C). The anti-GQ1b antibody activity in serum from patient P3 was not reduced after incubation with the monolithic GM2 mimic column, indicating that no non-specific binding of antibodies occurs by the monolithic GM2 mimic column.

Fluorescence microscopy results

To define the homogeneity of the distribution of GM2 mimics in the monolithic columns and to visualize the IgM adsorption by these columns, staining studies were performed with FITC-conjugated anti-human IgM antibodies after infusion of patient serum samples into the columns. Both blank and monolithic GM2 mimic columns were first infused with serum P1 at two different dilutions (1:50 and 1:5). After prolonged washing and then staining with FITC-conjugated anti-human IgM antibodies, the columns were viewed under a fluorescence microscope. The recorded images are shown in Figure 4A-C.

Staining of the monolithic GM2 mimic columns with FITC-conjugated goat anti-human IgM, showed a significant and homogeneous labeling after infusion with serum from patient P1, and the fluorescence was visible over a greater length of the column after incubation with the 1:5 dilution than with the 1:50 dilution (Figure 4A and B, respectively). This indicates that most of the serum antibodies are immediately bound after introduction into the columns as long as free GM2 mimics remain available. The fluorescence images are in full agreement with the previously discussed results in ELISA showing depletion of serum anti-GM2 IgM activity after incubation with these columns (see Figure 3A). A monolithic GM2 mimic column not incubated with patient serum as a

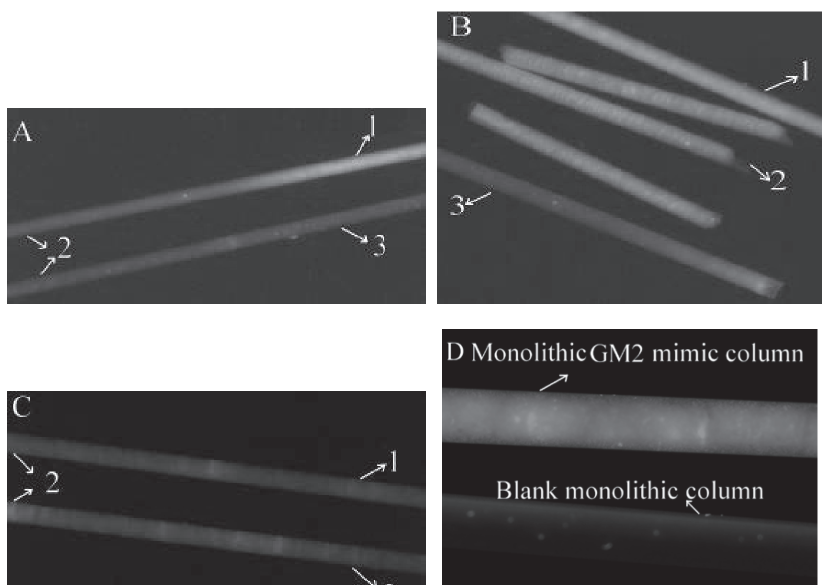

Figure 4. Fluorescence microscopy images of monolithic GM2 mimic columns infused with either serum or mouse monoclonal antibody and subsequently treated with relevant fluorescent labeled anti-human (FITC) or anti-mouse (Alexa Fluor 594) immunoglobulin: (A) Serum from patient P1 (1:50 dilution); (B) serum from patient P1 (1 : 5 dilution); (C) no serum treatment and; (D) mouse monoclonal antibody (EM5) incubated with blank monolithic columns and monolithic GM2 mimic columns; (1) initial part of column; (2) middle of column: (3) end of column.

control did not show any staining, confirming that the FITC-conjugated anti-human IgM antibodies do not bind to the column (Figure 4C).

Next a blank monolithic column (i.e. no GM2 present) and a monolithic GM2 mimic column were infused with mouse monoclonal antibodies (EM5), followed by treating both the columns with Alexa Fluor 594 conjugated anti-mouse IgM antibody. The obtained fluorescence images are depicted in Figure 4D. This clearly indicates that the mouse monoclonal antibodies are successfully captured only by the monolithic GM2 mimic column. This result is in full agreement with the ELISA data (see Figure 3B and 3C).

As additional controls, a blank monolithic column and a monolithic GM3 mimic column were infused with sera P1 (1:50) and P2 (1:100) and a monolithic GM2 mimic column was infused with serum P3 (1:10). Afterwards they were stained with FITC-conjugated goat anti-human IgM antibodies to detect bound serum antibodies in the column (see Supporting Information). In none of these cases significant staining was observed. These results prove that no IgM antibodies were bound to any of the columns (blank monolith, monolithic GM3 mimic and monolithic GM2 mimic), and they are in full

agreement with the ELISA data shown in Figure 3C. Thus, the columns are highly selective, as many other IgM antibodies must have been present in these serum samples. The overall results demonstrate both the efficiency and selectivity of monolithic GM2 mimic columns to capture anti-GM2 IgM antibodies from serum samples containing antibodies against gangliosides. The long-term stability of the columns was also checked, as this is of prime importance for any future diagnostic or therapeutic use. The antibody capturing efficiency remained intact even after two years of storage in a dry state (results not shown).

Gangliosides with an alkene-terminated spacer can be successfully incorporated in monolithic capillary affinity columns in a single step by *in situ* polymerization. Fluorescence microscopy images showed an even distribution of GM2 molecules along the entire column. Per microliter of GM2 column 6.4 µL of undiluted serum containing IgM antibodies (titer 1600) could be depleted after an incubation time of less than 2 min. GM2 columns specifically deplete IgM antibodies against GM2 and not IgM antibodies against the closely related ganglioside GQ1b or other IgM antibodies from a healthy volunteer. Both blank monolithic columns and monolithic GM3 mimic columns did not exhibit any non-specific adsorption of IgM antibodies. Overall, these affinity monolithic columns can be used to deplete IgM antibodies specifically from serum samples of patients suffering from GBS. This proof of principle with GM2 opens up interesting possibilities for the development of new forms of diagnostics and even treatments for GBS and related neuropathies with neurotoxic anti-ganglioside antibodies. For diagnostic purposes, one could envisage trapping, staining with fluorescent anti-human antibodies, followed by elution and detection to achieve rapid, selective and sensitive detection of various GBS-related antibodies.

Experimental Section

General Synthetic Methods

Fused-silica capillaries of 250 µm i.d. and 375 µm o.d. were obtained from Polymicro Technologies (Phoenix, AZ, USA). Anhydrous sodium hydrogen phosphate, bovine serum albumin (BSA) and O-phenylenediamine tablets were purchased from Sigma Aldrich, The Netherlands. Hydrogen peroxide, citric acid, and natural GM2 ganglioside derived from bovine brain were purchased from Merck, The Netherlands. Mouse monoclonal antibody (EM5) was kindly provided by Prof. Hugh Willison, University department of Neurology, Glasgow. Alexa Fluor 594 goat anti-mouse IgM was purchased from Invitrogen, The Netherlands. Natural GQ1b was purchased from Sanbio, The Netherlands. Serum samples (see Table 1) were provided by the Erasmus MC (Rotterdam, The Netherlands) with titers as indicated in the table. Gangliosides (GM2 and GM3) with a 1-undecenyl

Table 1. Patients' clinical diagnosis and serum antibody specificity, class and titer

Patient Code	Antibody		Antibody reactivity to gangliosides	Diagnosis
	Class	Titer		
P1	IgM	1600	GM2	GBS
P2	IgM	51200	GM2	Polyneuropathy and monoclonal gammopathy
P3	IgM	200	GQ1b	GBS
HV	IgM		None	Healthy control

spacer were synthesized as described before (7). Gas tight syringes (500 μL) were purchased from Alltech, The Netherlands. Syringe pumps (Harvard 11 PicoPlus, dual syringe) were purchased from VWR International, The Netherlands. (+)-N,N-diallyltartardiamide (DATD), 2-hydroxyethyl methacrylate (HEMA), piperazine diacrylamide (PDA) and 2,2′-azobis(2-methylpropion amidine) dihydrochloride (AMPA) were purchased from Sigma Aldrich, The Netherlands.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used to measure the IgM antibody binding to GM2 and GQ1b in serum samples. The wells of a 96-well plate (Nunc, Maxisorp) were treated either with ethanol or 300 pmol of ganglioside dissolved in ethanol. These solutions were subsequently allowed to evaporate to dryness. All the wells were then blocked with a solution of PBS (phosphate-buffered saline, pH = 7.8, 200 μL per well) containing 1% (w/v) of BSA for 2 h at room temperature and a further 2 h at 4 °C. The plates were then emptied by flicking and incubated overnight at 4 °C. The serum samples were diluted, initially 1:100, in PBS - 1% BSA, and added to 4 wells, 2 coated with GM2 or GQ1b and 2 with ethanol only. The following day, the plates were washed with an automated ELISA-washer (Elx50, Bio-Tek, UK) with PBS solution and filled with a solution of peroxidase-conjugated secondary antibodies (Jackson Immuno Research Labs) diluted to 1:2500 in PBS - 1% BSA for 1.5 h at room temperature. After washing, once again with the PBS solution, the plates were developed by adding 100 μL of substrate solution in citrate buffer (pH = 5.0-5.2). The substrate solution was prepared by dissolving one (5 mg) O-phenylenediamine tablet in 6.0 mL of 4 mM citric acid solution, 6.5 mL of 8 mM anhydrous sodium hydrogen phosphate solution and 12.5 mL of MilliQ water. Immediately prior to use 12.5 μL of 30% hydrogen peroxide solution was added to the above substrate solution. The reaction in the well was stopped by the addition of 100 μL of 2 M hydrochloric acid and the optical densities (OD) were read in an automated reader at 490 nm. The mean ODs of the blank (ethanol-coated) wells were subtracted from the mean ODs of the GM2-coated wells to obtain a specific OD (5, 6).

In situ preparation of ganglioside monolithic columns

A fused silica capillary of 100 cm total length was activated with 3-(trimethoxysilyl)propyl methacrylate. Subsequently an affinity monolithic column was prepared as described earlier.(24) In short: a solution of ganglioside mimics (GM2 or GM3, 5 mg) in methanol (30 µL) was added to a mixture of HEMA (30 µL), ammonium sulfate (8 mg), DATD (20 mg) and PDA (16 mg) in 250 µL of phosphate buffer, pH = 7.0 in an Eppendorf tube and mixed well, followed by de-aeration for a period of 5 min. Subsequently, the initiator AMPA (10 µL, 10% v/v in water) was added. The monolith solution was then sucked into the acrylate terminated capillary using vacuum and both ends of the capillary were sealed with a gas chromatography septum. The column was placed in an oven (T = 65 °C) for 12 h, which resulted in a monolithic ganglioside mimic column. The column was washed with water for 2 h at 2 µL/min.

Antibody depletion of serum samples

A three-step procedure to capture antibodies (IgM) from serum samples was followed. First the columns were washed with PBS buffer (pH = 7.8) for 1 h at 1.4 µL/min. Next various serum dilutions (P1 (1:50 and 1:5), P2 (1:100), P3 (1:10), HV (1:10)) in PBS buffer (pH = 7.8)) were prepared and in each case 180 µL were infused into the columns at 1 µL/min during 3 h. Subsequently the columns were washed again with PBS buffer for 1 h at 1.4 µL/min. All solutions were infused into the columns with a 500 µL syringe and a syringe pump. The collected samples were analyzed with ELISA.

Mouse monoclonal antibody (EM5) depletion

A three-step procedure to capture mouse monoclonal antibodies (EM5) was as follows: first the columns were washed with PBS buffer (pH = 7.8) for 1 h at 1.4 µL/min followed by infusion of 120 µL of EM5 (10 µg/mL or 50 µg/mL) in PBS buffer (pH = 7.8) for 2 h at 1 µL/min. Finally the columns were washed again with PBS buffer for 1 h at 1.4 µL/min.

Control experiments

Three sets of control experiments were performed to demonstrate that a) IgM antibodies against GM2 in sera P1 and P2 could only be depleted by monolithic GM2 mimic columns and not by either a blank monolithic column or a monolithic GM3 mimic column, b) monolithic GM2 mimic columns deplete IgM antibodies against GM2 only and not IgM antibodies against GQ1b nor various other species of antibodies and c) Mouse monoclonal antibody (EM5) could specifically be depleted by a monolithic GM2 mimic column and not by a blank monolithic column.

Fluorescence microscope (FM) measurements

A stereofluorescence microscope and Olympus IX 51 microscope were used to carry out FM measurements. The homogeneous distribution of IgM antibodies along the ganglioside monolithic columns was tested using FITC conjugated goat anti-human IgM. First, the column was washed with PBS buffer (pH = 7.8) for 30 min at 1 μL/min. Subsequently, the column was treated with FITC conjugated goat anti-human IgM solution for 1 h at 1 μL/min and finally washed with PBS buffer (pH = 7.8) for 45 min at 1 μL/min. The stained columns were evaluated using a fluorescence microscope. In case of a blank monolithic column and a monolithic GM2 mimic columns infused with mouse monoclonal antibody (EM5), the columns were treated with PBS buffer (pH = 7.8) for 1 h at 1.4 μL/min followed by treatment with 120 μL Alexa Fluor 594 goat anti-mouse IgM (1:100 dilution) for 2 h at 1 μL/min. The columns were immediately washed with PBS buffer (pH = 7.8) for 85 min at 1.4 μL/min and then viewed with a fluorescence microscope.

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References

1. Willison HJ & Yuki N (2002) Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125:2591-2625.
2. Ang CW, Jacobs BC, & Laman JD (2004) The Guillain-Barré syndrome: a true case of molecular mimicry. *Trends Immunol* 25(2):61-66.
3. Sonnino S, Mauri L, Chigorno V, & Prinetti A (2007) Gangliosides as components of lipid membrane domains. *Glycobiology* 17(1):1R-13R.
4. Hughes RA & Cornblath DR (2005) Guillain-Barré syndrome. *Lancet* 366(9497):1653-1666.
5. Kuijf ML, van Doorn PA, Tio-Gillen AP*, et al.* (2005) Diagnostic value of anti-GM1 ganglioside serology and validation of the INCAT-ELISA. *Journal of the Neurological Sciences* 239(1):37-44.
6. Sears P & Wong CH (1999) Carbohydrate mimetics: A new strategy for tackling the problem of carbohydrate-mediated biological recognition. *Angew. Chem. Int. Ed.* 38(16):2301-2324.
7. Pukin AV, Weijers C, van Lagen B*, et al.* (2008) GM3, GM2 and GM1 mimics designed for biosensing: chemoenzymatic synthesis, target affinities and 900 MHz NMR analysis. *Carbohydrate Research* 343(4):636-650.
8. Pukin AV, Branderhorst HM, Sisu C*, et al.* (2007) Strong inhibition of cholera toxin by multivalent GM1 derivatives. *Chembiochem* 8(13):1500-1503.
9. Townson K, Boffey J, Nicholl D*, et al.* (2007) Solid phase immunoadsorption for therapeutic and analytical studies on neuropathy-associated anti-GM1 antibodies. *Glycobiology* 17(3):294-303.
10. de Smet L, Pukin AV, Sun QY*, et al.* (2005) Visible-light attachment of Si-C linked functionalized organic monolayers on silicon surfaces. *Applied Surface Science* 252(1):24-30.
11. de Smet L, Stork GA, Hurenkamp GHF*, et al.* (2003) Covalently attached saccharides on silicon surfaces. *Journal of the American Chemical Society* 125:13916-13917.
12. Galanina OE, Mecklenburg M, Nifantiev NE, Pazynina GV, & Bovin NV (2003) GlycoChip: multiarray for the study of carbohydrate-binding proteins. *Lab on a Chip* 3(4):260-265.
13. Houseman BT, Gawalt ES, & Mrksich M (2003) Maleimide-functionalized self-assembled monolayers for the preparation of peptide and carbohydrate biochips. *Langmuir* 19(5):1522-1531.
14. Houseman BT & Mrksich M (2002) Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. *Chem Biol* 9(4):443-454.
15. Love KR & Seeberger PH (2002) Carbohydrate arrays as tools for glycomics. *Angew. Chem. Int. Ed.* 41(19):3583-3586.
16. Park S, Lee MR, Pyo SJ, & Shin I (2004) Carbohydrate chips for studying high-throughput carbohydrate-protein interactions. *J. Am. Chem. Soc.* 126(15):4812-4819.
17. Shin I, Park S, & Lee MR (2005) Carbohydrate microarrays: An advanced technology for functional studies of glycans. *Chemistry-a European Journal* 11(10):2894-2901.
18. Tetala KKR, Giesbers M, Visser GM, Sudholter EJR, & van Beek TA (2007) Carbohydrate microarray on glass: a tool for carbohydrate-lectin interactions. *Natural Product Communications* 2(4):391-394.
19. Wang DN (2003) Carbohydrate microarrays. *Proteomics* 3(11):2167-2175.
20. de la Fuente JM, Barrientos AG, Rojas TC*, et al.* (2001) Gold glyconanoparticles as water-soluble polyvalent models to study carbohydrate interactions. *Angewandte Chemie-International Edition* 40(12):2258-+.
21. Andersson CH, Lahmann M, Oscarson S, & Grennberg H (2009) Reversible non-covalent derivatisation of carbon nanotubes with glycosides. *Soft Matter* 5(14):2713-2716.
22. Bedair M & El Rassi Z (2004) Affinity chromatography with monolithic capillary columns I. Polymethacrylate monoliths with immobilized mannan for the separation of mannose-binding proteins by capillary electrochromatography and nano-scale liquid chromatography. *Journal of Chromatography A* 1044(1-2):177-186.

23. Tetala KKR, Chen B, Visser GM*, et al.* (2007) Preparation of a monolithic capillary column with immobilized alpha-mannose for affinity chromatography of lectins. *Journal of Biochemical and Biophysical Methods* 70(1):63-69.
24. Tetala KKR, Chen B, Visser GM, & van Beek TA (2007) Single step synthesis of carbohydrate monolithic capillary columns for affinity chromatography of lectins. *Journal of Separation Science* 30(17):2828-2835.
25. Tetala KKR & van Beek TA (2010) Bioaffinity chromatography on monolithic supports. *Journal of Separation Science* 33:1-17.
26. Wu XS, Okada N, Goto M, Iwamoto A, & Okada H (2000) The IgM antibody level against ganglioside GM2 correlates to the disease status of HIV-1-infected patients. *Microbiology and Immunology* 44(5):405-410.
27. Wu XS, Okada N, Momota H, Irie RF, & Okada H (1999) Complement-mediated anti-HIV-1 effect induced by human IgM monoclonal antibody against ganglioside GM2. *Journal of Immunology* 162(1):533-539.
28. Goodyear CS, O'Hanlon GM, Plomp JJ*, et al.* (1999) Monoclonal antibodies raised against Guillain-Barré syndrome-associated *Campylobacter jejuni* lipopolysaccharides react with neuronal gangliosides and paralyze muscle-nerve preparations. *Journal of Clinical Investigation* 104(6):697-708.

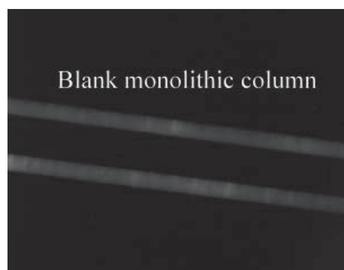


Fig. 1. Blank monolithic column treated with serum P1

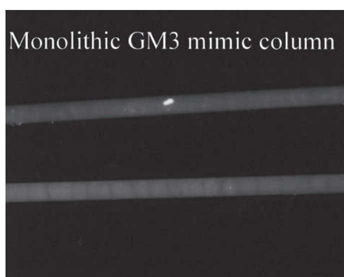


Fig. 2. Monolithic GM3 mimic column treated with serum P1

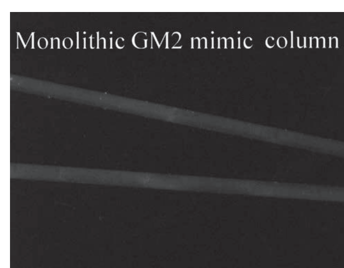


Fig. 3. Monolithic GM2 mimic column treated with serum P3.

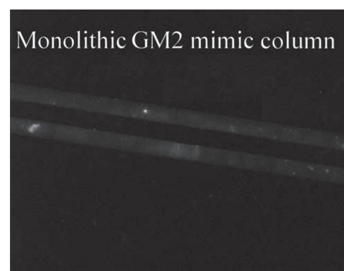
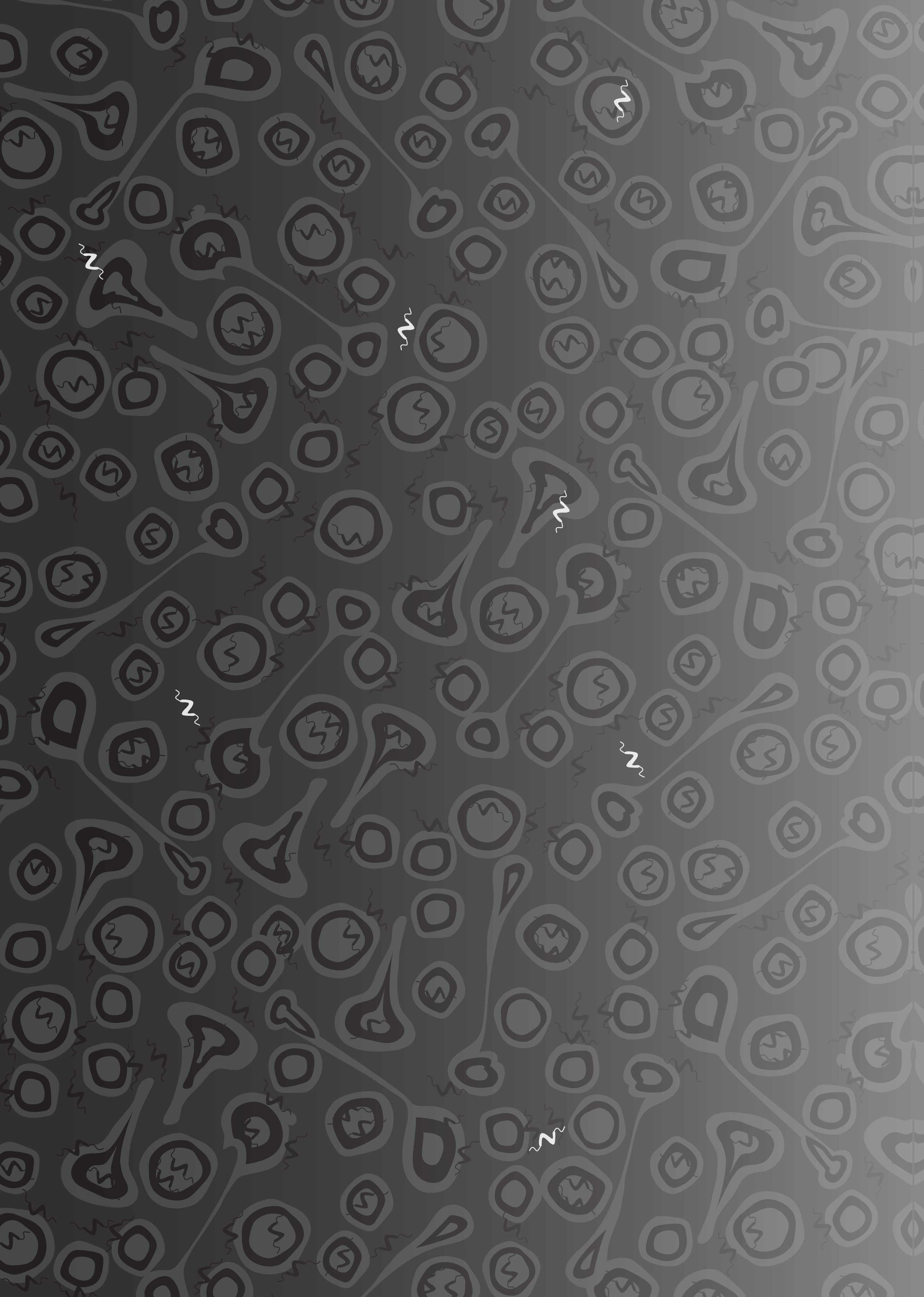


Fig. 4. Monolithic GM2 mimic column treated with serum of a healthy volunteer

Supporting Information. Four fluorescence microscopy images of various blank experiments. This information is available free of charge via the Internet at http://pubs.acs.org.



Chapter 8

General discussion

Sialylated epitopes on *C. jejuni* LOS are a major pathogenic factor in the development of GBS and MFS; however, the expression of sialylated epitopes alone is not sufficient to induce GBS. *C. jejuni* strains with sialylated LOS are frequently detected in the stools of patients with uncomplicated enteritis, whereas GBS is rare and develops in less than one in a thousand patients with an antecedent *C. jejuni* infection. The low incidence of GBS suggests that additional bacterial and/or host factors are involved in the development of this disease.

The aim of the work described in this thesis was to identify interactions between *C. jejuni* and the human host which contribute to the development of GBS. In particular, we focused on the role of sialylated LOS of *C. jejuni*. In most of the studies described in this thesis, a large, unique, mainly Dutch collection of GBS- and MFS-associated *C. jejuni* strains, isolated from human stool samples was examined. Detailed information was available on the ganglioside mimicking structures expressed. Additionally, three sialic acid knockout mutants of parental GBS-associated *C. jejuni* strains were generated and used to determine the role of sialic acid in host interactions. A large *C. jejuni* strain collection, isolated from stool samples of patients (all Dutch) with uncomplicated enteritis, served as a control group.

In this thesis, we report new GBS-associated bacterial virulence factors, we demonstrate that sialylation of *C. jejuni* LOS enhances intestinal epithelial cell-invasion, and we present two host immune receptors involved in the specific recognition of sialylated LOS. Additionally, we focused on the development of a novel treatment strategy for patients with GBS. In the following section, the results described in this thesis will be discussed in relation to the current literature. In addition, future perspectives will be addressed.

Bacterial factors associated with GBS

Comparative genotyping was performed on our GBS-, MFS- and uncomplicated enteritis-associated *C. jejuni* strains, with the aim to identify additional bacterial factors involved in the pathogenesis of GBS **(Chapter 2).** LOS genotyping confirmed and further established that *C. jejuni* strains with the sialylated LOS classes A, B and C are predominant within GBS- and MFS-associated strains, and also that strains with class A and B LOS loci are associated with GBS and MFS, respectively. These findings are in agreement with several studies performed globally (1-4). Despite the presence of genes involved in LOS sialylation, we did not find an association between the LOS class C and GBS development when GBS/MFS- and enteritis-associated *C. jejuni* strains were compared. An LOS class A or B was detected in 73% of our GBS- and MFS-associated strains compared to 36% of our enteritis-associated strains. Since there are allelic variants for both LOS class A (A1/A2) and LOS class B (B1/B2), we had hypothesized that there might be differences in allele distribution between GBS- and enteritis-associates strains with an A or B LOS

class. The allele A1 and B2 were, however, most prevalent (~80% each) amongst LOS class A and B strains, respectively, in both the GBS/MFS and the enteritis collection. The finding that the LOS A1 and B2 alleles are the most common allelic variants in enteritis-associated strains is in agreement with a Finnish study which shows that the majority of class A/B *C. jejuni* strains of human origin have an A1 or B2 allele (5). We observed that the presence of an A1 or B1 allele results in sialylation of both, the inner and the terminal galactose of the LOS outer core. In contrast, in strains with an A2 or B2 allele, only the terminal galactose of the LOS outer core is sialylated. *C. jejuni* strains with the A1 allele can produce GD1a/GM1a ganglioside mimics on their surface. These strains strongly associate with the development of GBS, as antibodies against GD1a and GM1a are the most frequently detected auto-antibodies in patients with GBS (6) and are likely more pathogenic as they are associated with severe GBS and poor prognosis (7, 8). The observation that the A1 allele was dominant in both the GBS- and enteritis-associated strains indicates that the capacity of *C. jejuni* to expresse GD1a/GM1a ganglioside mimics does, however, not necessarily lead to the induction of GBS. One of the reasons behind this could be the fact that the presence of genes involved in LOS sialylation does not guarantee the production of ganglioside mimics. For instance, within an LOS biosynthesis locus several distinct genetic mechanisms allow *C. jejuni* to vary the structure of the LOS outer core (9). These mechanisms include the presence of gene complements, phase variation because of homopolymeric C tracts, and gene inactivation by the deletion or insertion of a single base. The majority of the class A, B or C enteritis-associated strains that we tested (28/31; 90%), expressed sialylated LOS. It is therefore probably not the absence of sialic acid residues in the outer core LOS that explains why enteritis strains, which carry genes involved in LOS sialylation, did not trigger the development of GBS.

PCR-based genotyping of other bacterial genes that might be associated with the development of GBS led to the observation that certain capsule types (HS1, HS2, HS4, HS19 and HS23/36) are dominant in GBS- and MFS-associated *C. jejuni* strains. These capsule types were found in combination with LOS loci classes that contain genes involved in LOS sialylation (either LOS class A, B or C). High prevalence of capsule serotypes HS19 and HS23/36, in combination with sialylated LOS classes, has been observed by others in GBS-associated *C. jejuni* strains (3, 4). These and our observations raise the question whether there is a particular cause for having a combination of a certain capsule type and a sialylated LOS class in *C. jejuni*. The capsule and LOS are synthesized by enzymes located on distinct gene loci (10), and as such biosynthesis of the capsule and LOS can be considered to be independent. Multilocus sequence typing (MLST), a method based on the partial sequences of seven housekeeping genes, is used for studying the population structure and diversity of pathogens. MLST analysis showed that all *C. jejuni* strains with the capsule serotype HS19 belong to a similar genetic background, MLST clonal complex ST-22; these strains always appear to have a class A (A1) LOS locus (3, 4, 11).

A conserved genetic background, as demonstrated by others for HS19-positive strains, suggests that the co-occurrence of LOS classes and capsule types is clonally related. Based on MLST analysis, related, partially-related and unrelated genetic lineages were observed for the capsule/LOS class combinations found in our GBS/MFS strain collection. In several cases, horizontal transfer of LOS locus genes or capsule locus genes may have occurred between *C. jejuni* strains of genetically unrelated lineages. This is in agreement with results from other studies, which showed that *C. jejuni* is capable of gene exchange and horizontal transfer of both LOS and capsule gene clusters, and even complete LOS loci (9, 12, 13). In particular, the observation that genetically unrelated preservation of certain capsule types occurred in our GBS- and MFS strain collection argues for a causal relationship between capsule type and the development of GBS. This raises the question: what could be the role of the capsule in the pathogenesis of GBS? The *C. jejuni* capsule was shown to be involved in the invasion of epithelial cells and serum complement resistance (14, 15). Enhanced invasiveness and prolonged survival of *C. jejuni* in the intestinal mucosa may lead to increased exposure of bacterial epitopes to the immune system and eventually, the development of GBS. Moreover, the capsule is surface-exposed and is therefore likely to be involved in host-pathogen interactions.

Sialylated LOS is involved in invasion of intestinal epithelial cells

In **Chapter 3**, we assessed whether LOS sialylation contributes to intestinal epithelial cell adhesion and/or invasion. We observed that *C. jejuni* strains with sialylated LOS are more invasive in Caco-2 cells compared to nonsialylated strains. With the use of three Campylobacter sialic acid transferase (*cst-II*) knockout strains, all of which lack sialic acid on their LOS, we demonstrated that the invasion of Caco-2 cells is sialic acid-dependent. In agreement with this observation, Caco-2 invasion capacity was restored by complementation of the *cst-II* gene in a *cst-II* knockout strains. Furthermore, we recently demonstrated that sialylated *C. jejuni* strains translocate abundantly through polarized Caco-2 cells, without disrupting monolayer integrity as measured by the transepithelial electric resistance (16).

Many bacterial structures have been shown to play a role intestinal cell adhesion and invasion, including *C. jejuni* LOS (17). We are the first to show a role for LOS sialylation in *C. jejuni* cell-invasion. A more recent study, performed on *C. jejuni* strains isolated from chicken meat, confirmed our findings that strains with sialylated LOS exhibit a higher invasion potential compared to nonsialylated strains (18). However, the role of sialic acid in the invasion process may be strain and cell-type dependent, as the loss of sialic acid in the LOS outer core of *C. jejuni* strain 81-176 had no effect on the invasion of INT407 cells (19). Strain 81-176, which is highly transformable and has been studied widely, seems to have evolved in a somewhat different manner (20). Transposon mutagenesis in strain 81-176 revealed that CadF and Cia proteins, which are generally implied to be required

for *C. jejuni* cell-invasion (21), did not contribute to the invasiveness of this strain (22). Instead, genes on the pVir plasmid which are present in a subset (~10%) of *C. jejuni* strains are involved in 81-176 cell invasion (22-24). We detected pVir in 4 of 125 (3%) enteritis strains and in 1 of 21 (5%) GBS- and MFS-associated strains (25). Therefore, involvement of pVir is not likely to account for the enhanced invasive capacity of *C. jejuni* strains in our collection.

The precise contribution of sialylated LOS to invasion of the intestinal epithelium is unclear. Cell adhesion is an important first step in the invasion process. However, we did not observe a difference in *C. jejuni* adhesion to epithelial Caco-2 cells between sialylated and nonsialylated strains, suggesting that sialylated LOS may play a more important role in the internalization process. It is likely that *C. jejuni* uses host cell receptors for invasion. C-type lectins, involved in carbohydrate binding and lectin-glycoconjugate interactions, have been proposed to participate in initiation of the invasion process (26). As such, lectins expressed on the intestinal epithelium that specifically recognize sialylated structures may be responsible for the enhanced invasiveness of sialylated *C. jejuni* strains observed in Caco-2 cells. In particular, the identification of sialic acid binding receptors on intestinal epithelial cells would strongly support a direct role for sialylated LOS in intestinal epithelial invasion.

It should be noted that there is controversy concerning the role of sialylated LOS in the severity of diarrheal disease. Sialylation of *C. jejuni* LOS has been associated with an increased occurrence of bloody diarrhea and a longer duration of symptoms (27). In a recent study, however, no relation between the ability of *C. jejuni* to sialylate its LOS and either bloody diarrhea, hospitalization or campylobacteriosis was found (28). As mentioned earlier, we observed that sialylated *C. jejuni* strains translocate through polarized Caco-2 cells in high numbers, without disrupting the monolayer integrity (16). This questions whether *C. jejuni* might be able to cross the intestinal epithelial barrier without inducing cell lysis, and as a result provoke less severe diarrheal disease.

GBS-associated *C. jejuni* strains bind to sialoadhesin

Upon assessing the binding of GBS- and MFS-associated *C. jejuni* strains to members of the Siglec family, we observed that GBS-associated *C. jejuni* strains preferentially bind to Sn **(Chapter 4 & 5)**. Binding to mouse- and human-derived Sn (mSn and hSn, respectively) was assessed, and we observed that Sn from both species bound to *C. jejuni* strains which expressed terminal α(2,3)-linked sialic acids, as present in gangliosides GM1b, GD1a and GM3. Interestingly, hSn, but not mSn, was also able to bind to *C. jejuni* strains with internal α(2,3)-linked sialic acids, as present in GM1a-like structures. This is of importance as auto-antibodies directed to ganglioside GM1a are the most frequently detected antibodies in patients with GBS (6, 29). Using sialic acid mutant strains and purified LOS isolated from these sialic acid mutant strains, we demonstrated that the

α(2,3)-linked sialic acids present on the LOS outer core are involved in binding to both hSn and mSn. However, the binding of *C. jejuni* to Sn was not constitutive. We observed (**Chapter 5**) that *C. jejuni* must undergo particular treatments or growth conditions in order to expose the Sn binding domains. Heat inactivation, low pH or contact with a bile constituent during growth, exposed the LOS and facilitated Sn binding. Of interest in this observation is that no bacterial treatment was performed during the invasion studies described in **Chapter 3**, yet sialic acid-mediated invasion was demonstrated. This discrepancy may be related to the cascade of events that are necessary for optimal cell invasion (21). Adhesin/ligand mediated forces, together with rearrangement of bacterial cell surface structures during the adhesion process, could facilitate the exposure of bacterial LOS to cellular structures. Additionally, factors secreted by the intestinal epithelium may also contribute to LOS exposure.

Expression of Sn on human intestinal epithelial cells has not been reported; however, Sn is expressed by a subset of tissue macrophages, including macrophages in the intestine, lymph nodes and spleen (30). Sn is involved in the recognition of sialylated pathogens leading to endocytosis (31-33). With 16 repeating Ig-like domains, the extended length of Sn facilitates optimal interaction with pathogens in the environment. In the human intestine, Sn-positive macrophages populate the lamina propria (30). These sub-epithelial macrophages survey the environment for invading pathogens. Macrophage encounter of sialylated *C. jejuni* strains is likely to occur, as sialylated *C. jejuni* strains are highly invasive (**Chapter 3**) and able to translocate through epithelial cells in high numbers (16).

Using primary human macrophages, we demonstrated that *C. jejuni* binding to Sn leads to increased bacterial uptake and enhanced production of the cytokine IL-6. Our findings are supported by recent studies, which showed that sialic acid-mediated binding to Sn expressed on murine bone marrow-derived macrophages enhances phagocytosis and leads to enhanced secretion of proinflammatory cytokines including IL-6 (34, 35). Moreover, intravenously injected inactivated *C. jejuni* are readily captured by macrophages in the mouse spleen (34), and cytokines IL-6, TNF-α and IFN-β are induced in an Sn-dependent manner (35). In summary, sialic acid-specific binding of *C. jejuni* to Sn can result in macrophage-mediated immune activation in the intestine and the spleen.

Due to the absence of signaling motifs in its cytoplasmic tail, it is unlikely that Sn binding directly triggers cytokine production. Cooperation of Sn with other receptors such as TLRs, could explain Sn-mediated cytokine induction. Capture of LOS by Sn could, for example, enhance the contact between the LOS lipid A component and TLR4. Preliminary experiments using whole bacteria did not reveal enhanced Sn-mediated TLR4 activation, probably because lipid A, which is embedded in the outer membrane of *C. jejuni,* was not available for TLR4.

C. jejuni strains associated with oculomotor weakness bind to Siglec-7

In **Chapter 6** we demonstrated that disialylated *C. jejuni* strains isolated from GBS or MFS patients bind to Siglec-7 in a sialic acid-dependent manner. Previously, it was shown that Siglec-7 can bind to disialylated carbohydrates, including the disialylated epitopes present on *C. jejuni* LOS (36). We confirmed these findings and additionally correlated the property of Siglec-7 binding with patient serological and diagnostic records. Upon screening a large panel of GBS- and MFS-related *C. jejuni* strains, we observed an association between Siglec-7 binding and, (1) the expression of terminally disialylated LOS structures, (2) the presence of anti-GQ1b antibodies in patient serum, and (3) the diagnosis of oculomotor weakness in patients with GBS and MFS.

The functional consequences of *C. jejuni* binding to Siglec-7 remain to be established. In humans, Siglec-7 is expressed on monocytes, dendritic cells, natural killer (NK) cells and T lymphocytes (37). The trans-membrane immunoreceptor tyrosine based inhibitory motif (ITIM) is suggestive for inhibitory signaling by Siglec-7. In agreement, Siglec-7 binding inhibits NK cell cytotoxicity and has a negative effect on T cell receptor signaling (38, 39). The function of Siglec-7 may, however, be cell type dependent as it recently was demonstrated that antibody ligation and zymosan engagement of Siglec-7 leads to pro-inflammatory responses in monocytes but not in NK cells and T lymphocytes (40).

A role for Siglecs in antigen presentation leading to GBS?

Could Sn or Siglec-7 binding promote production of cross-reactive anti-ganglioside antibodies, as detected in GBS and MFS patients? In order to answer this question, we first have to consider the immune-events that lead to antibody production. These events comprise: exposure of bacterial epitopes to immune cells, recognition by immune cells, phagocytosis, immune activation through cytokine production, and antigen presentation to cells of the adaptive immune system.

In this thesis we demonstrate that sialylated *C. jejuni* strains can invade intestinal epithelial Caco-2 cells in high numbers. Epithelial invasion leads to abundant translocation of *C. jejuni* through epithelial cells (16). *In vivo*, such an event would expose *C. jejuni* to intestinal-tissue macrophages and dendritic cells which are situated beneath the intestinal epithelium. Sn and Siglec-7 bind to sialylated epitopes that are associated with the induction of GBS or MFS. Sn and Siglec-7 are expressed on macrophages and dendritic cells, respectively (30, 41). We show that Sn binding results in enhanced phagocytosis and cytokine production in primary human macrophages and others showed that binding to Siglec-7 can induce pro-inflammatory responses (40). Thus, by binding of sialylated LOS epitopes to Sn or Siglec-7, several of the necessary prerequisites for antibody production are met.

It remains unclear how sialylated ganglioside-like epitopes are presented to adaptive immune cells. Dendritic cells are well known for their ability to present antigenic

peptides, via MHC-I- and MHC-II-mediated presentation. However, the antigens which trigger GBS are not peptides but carbohydrates, and the structures involved in carbohydrate presentation to B cells are largely unknown. We hypothesize that Siglecs expressed on either dendritic cells or macrophages play a role in antigen presentation. Sn is expressed on marginal zone (MZ) macrophages in the spleen and subcapsular sinus macrophages in the lymph nodes (42). B cells involved in IgM and IgG antibody production are present in both of these organs. In the spleen, MZ macrophages capture and present antigenic debris to follicular dendritic cells and B cells (43). Whole bacteria or *C. jejuni* debris which enter the bloodstream during infection of the intestine will enter the spleen, where sialylated epitopes could be captured by Sn-positive macrophages (35). It can be envisaged that increased Sn-mediated binding and/or phagocytosis may lead to the presentation of more *C. jejuni* fragments, resulting in increased immune activation. In agreement, a recent immunization study in pigs demonstrates that direct targeting of the immunizing protein to Sn results in a more rapid and robust induction of specific IgM and IgG immune responses, compared to immunization with the protein alone (44). These latter findings suggest that Sn-mediated binding of sialylated LOS epitopes derived from *C. jejuni* also leads to enhanced humoral immune responses, resulting in the production of cross-reactive anti-ganglioside antibodies. Whether Sn directly presents epitopes to B cells or whether additional cells including dendritic cells are involved remains unclear.

Next to a role for macrophages, dendritic cells may play a role in antigen capture and presentation to B cells leading to the development of GBS. Dendritic cells express many PPRs which recognize pathogen-associated molecular patterns. Epitope binding by PRRs can lead to immune activation, internalization, and processing of the epitope or prolonged retention of the epitope on the cell surface (45). The sensing of sialylated LOS by TLR4 expressed on dendritic cells results in enhanced expression of inflammatory cytokines, compared to nonsialylated LOS (46). In the intestine, such cytokines can directly or indirectly activate the recruitment of circulating monocytes, which differentiate into pro-inflammatory macrophages upon sensing chemokine stimuli from the intestinal epithelium (47). Additionally, cellular activation may stimulate the migration of dendritic cells to the mesenteric lymph nodes. Mucosal dendritic cells loaded with microbial epitopes traffic towards these lymph nodes, where they interact with and activate B cells (48). Specific capture of *C. jejuni* disialylated carbohydrate epitopes by Siglec-7 expressed on the surface of dendritic cells may result in enhanced dendritic cell-mediated TLR4 activation in the intestine. The disialylated *C. jejuni* epitopes bound to Siglec-7 can, additionally, be presented to B cells in the mesenteric lymph nodes by dendritic cells that have migrated from the lamina propria.

In summary, we propose a model for sialylated LOS-mediated host-pathogen interactions that lead to GBS in Fig. 1.

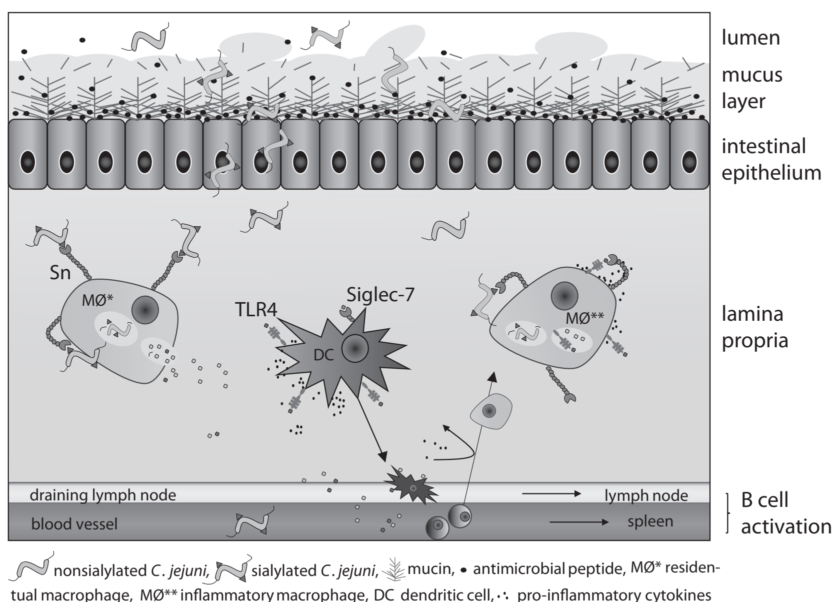


Figure 1. Model for the sialylated LOS-mediated host-pathogen interactions leading to the production of cross-reactive anti-ganglioside antibodies.
In the intestinal lumen, ingested *C. jejuni* penetrate the mucus layer by the use of bipolar flagella. The polysaccharide capsule prevents entrapment of *C. jejuni* in mucins and the LOS provides protection against mucosal antimicrobial peptides (15, 49). Several conserved bacterial surface structures contribute to the adherence to intestinal epithelial cells. Sialylated LOS expressed on a subset of *C. jejuni* strains facilitates enhanced invasion and translocation through the intestinal epithelium. Sn expressed on the surface of subepithelial tissue macrophages preferentially binds sialylated *C. jejuni* strains which are phagocytosed, killed and digested in lysosomal compartments. The fate of degraded particles is currently a topic of debate. Degraded bacterial particles may be released by the macrophage and shed into the environment or actively transferred from one phagocyte to another. TLR4 expressed on dendritic cells interacts with bacterial lipid A, leading to the production of pro-inflammatory cytokines. These cytokines may directly or indirectly activate the recruitment of infiltrating monocytes, which subsequently can differentiate into inflammatory macrophages and initiate additional immune activation. Other PRRs expressed on the surface of dendritic cells, including Siglec-7, sense the environment and capture microbial epitopes. Bacterial particles and dendritic cells coated with bacterial epitopes drain to the lymph nodes. Bacterial particles and whole bacteria that reach the bloodstream move towards the spleen. Specific recognition of sialylated epitopes by Sn expressed on subcapsular sinus and/or marginal zone macrophages, present in the lymph nodes and spleen, respectively, leads to presentation of sialylated epitopes to B cells.

A novel treatment strategy?

IVIg and plasmapheresis are proven, effective treatments for GBS (50). In both of these treatment modalities, the immune response of the patient is reduced. However, these treatments do not specifically target the pathogenic anti-ganglioside antibodies. Fast and specific depletion of anti-ganglioside antibodies from the plasma of patients with GBS may reduce neurological damage during the acute phase of GBS and possibly also

lead to faster patient recovery. In **Chapter 6** we provide proof of principle that synthetic gangliosides covalently bound to a polymeric structure (monolith) are able to capture anti-ganglioside antibodies from human serum. We have successfully depleted IgM and IgG anti-GM2 antibodies from serum in our experiments. Anti-GM2 antibodies have been related to cytomegalovirus-induced GBS (51). Unfortunately, we were not able to deplete anti-GM1 or anti-GD1a antibodies from serum using synthetic gangliosides coupled to monolithic columns. One explanation may be that the synthetic epitope is not recognized by anti-GM1 antibodies. However, when coupled to ELISA plates, synthetic GM1 showed a strong binding affinity for anti-GM1 antibodies from the serum of neuropathy patients (52), demonstrating that the epitope is adequately recognized. Perhaps the density of synthetic GM1 in the monolithic columns is insufficient, resulting in poor binding of the antibodies. In humans, the density of gangliosides on peripheral nerves is high (53). These densely-packed structures are specifically targeted by anti-ganglioside antibodies, despite the prevalence of gangliosides on many other cell surfaces. Additional experiments are needed to address whether a higher concentration of synthetic gangliosides in the monolithic structure may improve antibody binding.

Main conclusions

The work presented in this thesis has led to the following conclusions:

1. Most GBS- and MFS-associated *C. jejuni* strains express sialylated LOS structures.
2. *C. jejuni* LOS locus class A and B are associated with GBS and MFS, respectively.
3. The *C. jejuni* LOS allele A1 results in the production of GM1a- and GD1a-like ganglioside mimics.
4. *C. jejuni* capsule types HS1, HS2, HS4, HS19 and HS23/36 are the dominant types among GBS/MFS-associated strains.
5. Capsule type HS4 is associated with the development of GBS/MFS.
6. LOS sialylation is an important determinant of Caco-2 cell invasiveness.
7. GBS-associated *C. jejuni* strains bind to Sn in a sialic acid-dependent manner.
8. Both human and mouse Sn bind to terminal α(2,3)-linked sialic acid on *C. jejuni* LOS.
9. Human Sn, unlike mouse Sn, binds to internal α(2,3)-linked sialic acid of GM1a-like LOS.
10. Particular bacterial treatments or growth conditions are necessary to expose the Sn binding epitopes on *C. jejuni*.
11. Binding to Sn results in increased uptake of *C. jejuni* in primary human macrophages.
12. *C. jejuni* binding to Sn results in enhanced cytokine production in primary human macrophages.

13. Oculomotor weakness-associated *C. jejuni* strains bind to Siglec-7 in a sialic acid-dependent manner.
14. Synthetic GM2 coupled to a monolithic carrier is able to deplete anti-ganglioside antibodies from human serum.

Future perspectives

The role of the capsule in *C. jejuni*-host interactions

In our search for novel bacterial factors that may be involved in the development of GBS, we identified five main capsule types HS1, HS2, HS4, HS19 and HS23/36 among GBS- and MFS-associated strains. In order to establish whether these capsule types are indeed associated with the development of GBS, additional *C. jejuni* strains isolated from GBS and MFS patients from various geographical areas need to be characterized. Upon confirming of our results, the role of these capsule types in the pathogenesis of GBS and MFS could be further explored. In this regard, the role of the capsule in epithelial cell-invasion and in complement resistance deserves special attention.

The identification of pathogenic capsules opens new avenues for novel preventive strategies in the future. GBS-related capsule types could be considered for inclusion in capsule-based vaccines, which are currently under development (54). Additionally, screening of environmental sources, food products and human stool samples for the presence of specific *C. jejuni* LOS classes and capsule types may create an opportunity for preventive eradication of these virulent strains. However, it should be realized that the costs of such measures may be prohibitively high; therefore, cheap and rapid assays for the detection of pathogenic strains are desirable.

Epithelial invasion by *C. jejuni*

To support a direct role for sialylated LOS in intestinal epithelial invasion, our future research should focus on the identification of the epithelial cell-receptors that specifically bind to sialylated LOS. Additionally, we suggest that human organoids, derived from *in vitro* expansion of intestinal stem cells (55), could be used to study intestinal epithelial invasion in a more physiological setting.

The role of Siglecs in *C. jejuni*-host interactions

The identification of two receptors involved in the specific recognition of GBS- and MFS-associated strains has improved our understanding of the immune events that may lead to the development of GBS and MFS. To gain more insight into the role of Sn and Siglec-7 in immune activation, we suggest that it should be investigated whether Sn-mediated binding of sialylated *C. jejuni* LOS can enhance TLR4 activation, and the

functional consequences of *C. jejuni* binding to dendritic cell-expressed Siglec-7 should be assessed. Additionally, it seems important to determine whether binding to Sn affects the intracellular trafficking of *C. jejuni*, as it has been demonstrated that binding to lectins can lead to differential routing of antigens (56).

An *in vivo* mouse model would be suitable to address the question of whether Sn-dependent uptake leads to *C. jejuni* antigen trapping and presentation, resulting in cross-reactive anti-ganglioside antibody production. Transgenic *GalNAcT*$^{-/-}$ mice, which lack complex gangliosides (57), are used as immune-naive hosts to raise anti-ganglioside antibodies. Anti-ganglioside antibodies from the IgG1 and IgG3 subclasses are produced upon immunization of these mice with GM1-/GD1a-like LOS (58). Crossing of *GalNAcT*$^{-/-}$ mice with *Sn*$^{-/-}$ mice (59) would generate *GalNAcT*$^{-/-}$ *Sn*$^{-/-}$ double knockout mice. Such mice would be highly valuable to study the impact of Sn on the production of anti-ganglioside antibodies.

Identification of the host receptors involved in the production of anti-ganglioside antibodies will assist in the discovery of host genetic susceptibility factors. In this regard, we propose investigating whether GBS patients have specific alterations in the genes that encode Sn or Siglec-7, resulting in altered expression of these receptors. Sequencing of the genes that encode Sn and Siglec-7, or the genes that encode the structures involved in the downstream signaling pathways may lead to the identification of particular nuclear polymorphisms associated with the development of GBS or MFS.

Synthetic gangliosides in GBS serology

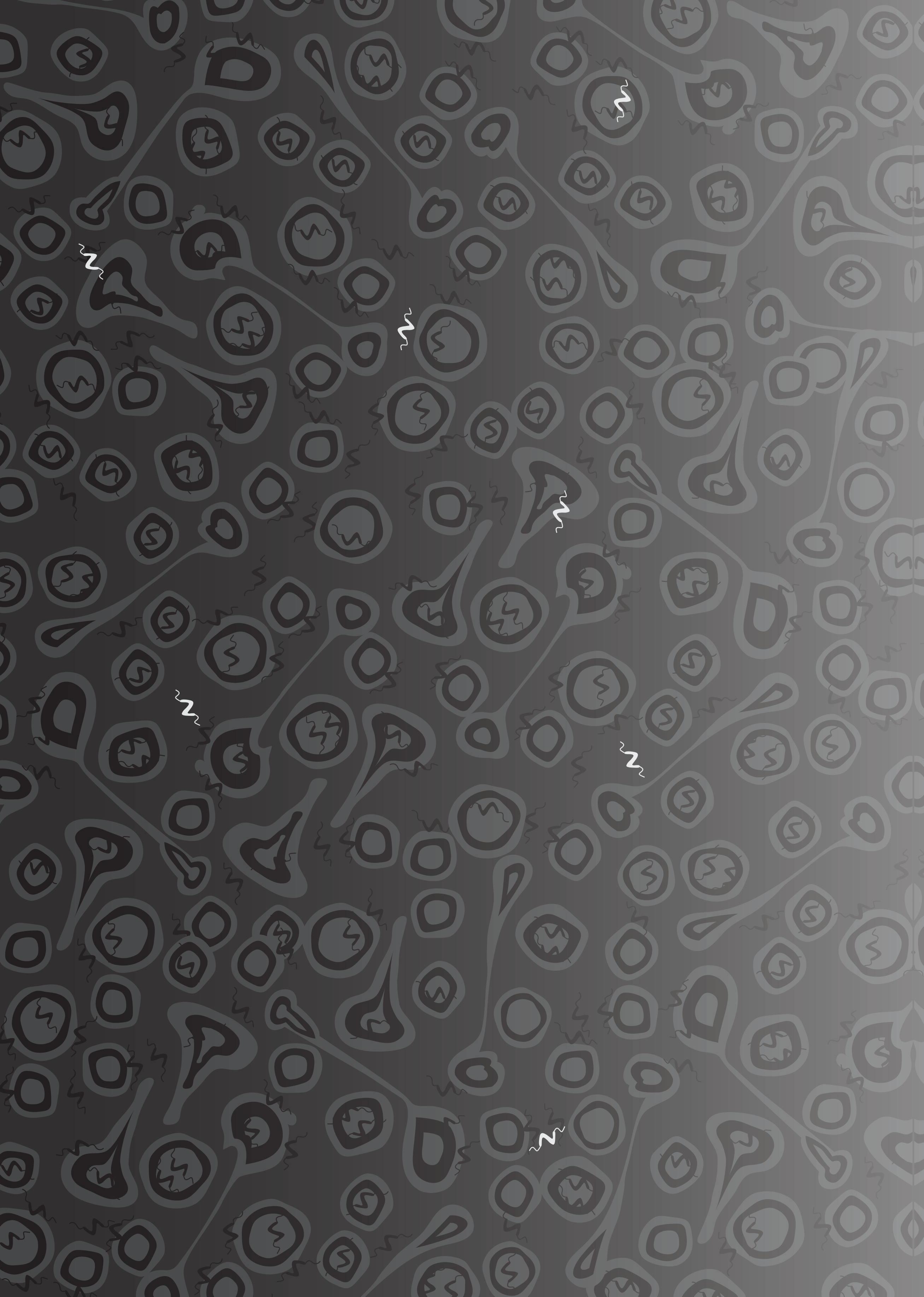
Unfortunately, synthetic gangliosides covalently bound to a monolithic matrix were not successful for depleting anti-GM1 antibodies from human serum. However, serum-derived IgM and IgG anti-GM1 antibodies did bind strongly to synthetic GM1 coupled to ELISA plates and Luminex® beads. GM1-coupled ELISA plates or Luminex® beads are unsuited for antibody depletion but instead can be used for the detection of anti-ganglioside antibodies in patient serum. Therefore, the use of synthetic gangliosides in GBS serology should be further explored. The fine specificity of various anti-ganglioside antibodies in GBS and MFS patients can be further investigated using synthetic gangliosides. This research will eventually lead to a better understanding of the heterogeneous clinical phenotype of GBS.

References

1. Godschalk PCR, Heikema AP, Gilbert M, *et al.* (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J Clin Invest* 114(11):1659-1665.
2. Koga M, Gilbert M, Takahashi M, *et al.* (2006) Comprehensive analysis of bacterial risk factors for the development of Guillain-Barré syndrome after *Campylobacter jejuni* enteritis. *J Infect Dis* 193(4):547-555.
3. Parker CT, Horn ST, Gilbert M, Miller WG, Woodward DL, & Mandrell RE (2005) Comparison of *Campylobacter jejuni* lipooligosaccharide biosynthesis loci from a variety of sources. *J Clin Microbiol* 43(6):2771-2781.
4. Islam Z, van Belkum A, Wagenaar JA, *et al.* (2009) Comparative genotyping of *Campylobacter jejuni* strains from patients with Guillain-Barré syndrome in Bangladesh. *PLoS ONE* 4(9):e7257.
5. Revez J & Hanninen ML (2012) Lipooligosaccharide locus classes are associated with certain *Campylobacter jejuni* multilocus sequence types. *Eur J Clin Microbiol Infect Dis*.
6. Willison HJ & Yuki N (2002) Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125(12):2591-2625.
7. Carpo M, Pedotti R, Allaria S, *et al.* (1999) Clinical presentation and outcome of Guillain-Barré and related syndromes in relation to anti-ganglioside antibodies. *J Neurol Sci* 168(2):78-84.
8. van den Berg LH, Marrink J, de Jager AE, *et al.* (1992) Anti-GM1 antibodies in patients with Guillain-Barré syndrome. *J Neurol Neurosurg Psychiatry* 55(1):8-11.
9. Gilbert M, Karwaski M-F, Bernatchez S, *et al.* (2002) The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J. Biol. Chem.* 277(1):327-337.
10. Parkhill J, Wren BW, Mungall K, *et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403(6770):665-668.
11. Dingle KE, Colles FM, Ure R, *et al.* (2002) Molecular characterization of *Campylobacter jejuni* clones: a basis for epidemiologic investigation. *Emerg Infect Dis* 8(9):949-955.
12. Karlyshev AV & Wren BW (2005) Development and application of an insertional system for gene delivery and expression in *Campylobacter jejuni*. *Appl Environ Microbiol* 71(7):4004-4013.
13. Gilbert M, Godschalk PCR, Karwaski MF, *et al.* (2004) Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain-Barré syndrome, by horizontal exchange. *Infect. Immun.* 72(2):1162-1165.
14. Bacon DJ, Szymanski CM, Burr DH, Silver RP, Alm RA, & Guerry P (2001) A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol Microbiol* 40(3):769-777.
15. Keo T, Collins J, Kunwar P, Blaser MJ, & Iovine NM (2011) *Campylobacter* capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials. *Virulence* 2(1):30-40.
16. Louwen R, Nieuwenhuis EE, van Marrewijk L, *et al.* (2012) *Campylobacter jejuni* translocation across intestinal epithelial cells is facilitated by ganglioside-like lipooligosaccharide structures. *Infect Immun* 80(9):3307-3318.
17. Kanipes MI, Holder LC, Corcoran AT, Moran AP, & Guerry P (2004) A deep-rough mutant of *Campylobacter jejuni* 81-176 is noninvasive for intestinal epithelial cells. *Infect Immun* 72(4):2452-2455.
18. Habib I, Louwen R, Uyttendaele M, *et al.* (2009) Correlation between genotypic diversity, lipooligosaccharide gene locus class variation, and caco-2 cell invasion potential of *Campylobacter jejuni* isolates from chicken meat and humans: contribution to virulotyping. *Appl Environ Microbiol* 75(13):4277-4288.
19. Guerry P, Szymanski CM, Prendergast MM, *et al.* (2002) Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. *Infect Immun* 70(2):787-793.
20. Wilson DL, Bell JA, Young VB, Wilder SR, Mansfield LS, & Linz JE (2003) Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. *Microbiology* 149(Pt 12):3603-3615.
21. O Croinin T & Backert S (2012) Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism? *Front Cell Infect Microbiol* 2:25.

22. Novik V, Hofreuter D, & Galan JE (2010) Identification of *Campylobacter jejuni* genes involved in its interaction with epithelial cells. *Infect Immun* 78(8):3540-3553.
23. Bacon DJ, Alm RA, Burr DH, *et al.* (2000) Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infect Immun* 68(8):4384-4390.
24. Bacon DJ, Alm RA, Hu L, *et al.* (2002) DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81-176. *Infect Immun* 70(11):6242-6250.
25. Louwen RP, van Belkum A, Wagenaar JA, Doorduyn Y, Achterberg R, & Endtz HP (2006) Lack of association between the presence of the pVir plasmid and bloody diarrhea in *Campylobacter jejuni* enteritis. *J Clin Microbiol* 44(5):1867-1868.
26. Kieda C (1998) Role of lectin-glycoconjugate recognitions in cell-cell interactions leading to tissue invasion. *Adv Exp Med Biol* 435:75-82.
27. Mortensen NP, Kuijf ML, Ang CW, *et al.* (2009) Sialylation of *Campylobacter jejuni* lipooligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect* 11(12):988-994.
28. Zautner AE, Herrmann S, Corso J, Tareen AM, Alter T, & Gross U (2012) Epidemiological association of different *Campylobacter jejuni* groups with metabolism-associated genetic markers. *Appl Environ Microbiol* 77(7):2359-2365.
29. Hiraga A, Kuwabara S, Ogawara K, *et al.* (2005) Patterns and serial changes in electrodiagnostic abnormalities of axonal Guillain-Barré syndrome. *Neurology* 64(5):856-860.
30. Hartnell A, Steel J, Turley H, Jones M, Jackson DG, & Crocker PR (2001) Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. *Blood* 97(1):288-296.
31. Rempel H, Calosing C, Sun B, & Pulliam L (2008) Sialoadhesin expressed on IFN-induced monocytes binds HIV-1 and enhances infectivity. *PLoS ONE* 3(4):e1967.
32. Jones C, Virji M, & Crocker PR (2003) Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol Microbiol* 49(5):1213-1225.
33. Vanderheijden N, Delputte PL, Favoreel HW, *et al.* (2003) Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. *J Virol* 77(15):8207-8215.
34. Huizinga R, Easton AS, Donachie AM, *et al.* (2012) Sialylation of *Campylobacter jejuni* Lipo-Oligosaccharides: Impact on Phagocytosis and Cytokine Production in Mice. *PLoS ONE* 7(3):e34416.
35. Klaas M, Oetke C, Lewis LE, *et al.* (2012) Sialoadhesin promotes rapid proinflammatory and type I IFN responses to a sialylated pathogen, *Campylobacter jejuni*. *J Immunol* 189(5):2414-2422.
36. Avril T, Wagner ER, Willison HJ, & Crocker PR (2006) Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 74(7):4133-4141.
37. Nicoll G, Ni J, Liu D, *et al.* (1999) Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes. *J Biol Chem* 274(48):34089-34095.
38. Falco M, Biassoni R, Bottino C, *et al.* (1999) Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells. *J Exp Med* 190(6):793-802.
39. Ikehara Y, Ikehara SK, & Paulson JC (2004) Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. *J Biol Chem* 279(41):43117-43125.
40. Varchetta S, Brunetta E, Roberto A, *et al.* (2012) Engagement of siglec-7 receptor induces a pro-inflammatory response selectively in monocytes. *PLoS ONE* 7(9):e45821.
41. Lock K, Zhang J, Lu J, Lee SH, & Crocker PR (2004) Expression of CD33-related siglecs on human mononuclear phagocytes, monocyte-derived dendritic cells and plasmacytoid dendritic cells. *Immunobiology* 209(1-2):199-207.

42. Crocker PR, Paulson JC, & Varki A (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* 7(4):255-266.
43. You Y, Myers RC, Freeberg L, *et al.* (2011) Marginal zone B cells regulate antigen capture by marginal zone macrophages. *J Immunol* 186(4):2172-2181.
44. Delputte PL, Van Gorp H, Favoreel HW, *et al.* (2011) Porcine sialoadhesin (CD169/Siglec-1) is an endocytic receptor that allows targeted delivery of toxins and antigens to macrophages. *PLoS ONE* 6(2):e16827.
45. Khalili-Shirazi A, Hughes RAC, Brostoff SW, Linington C, & Gregson NA (1992) T cell responses to myelin proteins in Guillain-Barré syndrome. *J Neurol Sci* 111(2):200-203.
46. Kuijf ML, Samsom JN, van Rijs W, *et al.* (2010) TLR4-mediated sensing of *Campylobacter jejuni* by dendritic cells is determined by sialylation. *J Immunol* 185(1):748-755.
47. Spottl T, Hausmann M, Kreutz M, *et al.* (2001) Monocyte differentiation in intestine-like macrophage phenotype induced by epithelial cells. *J Leukoc Biol* 70(2):241-251.
48. Hooper LV, Littman DR, & Macpherson AJ (2012) Interactions between the microbiota and the immune system. *Science* 336(6086):1268-1273.
49. Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, & Weiser JN (2007) Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect Immun* 75(1):83-90.
50. Shahrizaila N & Yuki N (2011) The role of immunotherapy in Guillain-Barré syndrome: understanding the mechanism of action. *Expert Opin Pharmacother* 12(10):1551-1560.
51. Ang CW, Jacobs BC, Brandenburg AH, *et al.* (2000) Cross-reactive antibodies against GM2 and CMV-infected fibroblasts in Guillain-Barré syndrome. *Neurology* 54(7):1453-1458.
52. Pukin AV, Jacobs BC, Tio-Gillen AP, *et al.* (2011) Detection of antibodies in neuropathy patients by synthetic GM1 mimics. *Glycobiology* 21(12):1642-1650.
53. Ganser AL & Kirschner DA (1984) Differential expression of gangliosides on the surfaces of myelinated nerve fibers. *J Neurosci Res* 12(2-3):245-255.
54. Monteiro MA, Baqar S, Hall ER, *et al.* (2009) Capsule polysaccharide conjugate vaccine against diarrheal disease caused by *Campylobacter jejuni*. *Infect Immun* 77(3):1128-1136.
55. Sato T, Stange DE, Ferrante M, *et al.* (2011) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 141(5):1762-1772.
56. Herre J, Marshall AS, Caron E, *et al.* (2004) Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104(13):4038-4045.
57. Takamiya K, Yamamoto A, Furukawa K, *et al.* (1996) Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system. *Proc Natl Acad Sci U S A* 93(20):10662-10667.
58. Matsumoto Y, Yuki N, Van Kaer L, Furukawa K, Hirata K, & Sugita M (2008) Cutting edge: Guillain-Barré syndrome-associated IgG responses to gangliosides are generated independently of CD1 function in mice. *J Immunol* 180(1):39-43.
59. Oetke C, Vinson MC, Jones C, & Crocker PR (2006) Sialoadhesin-deficient mice exhibit subtle changes in B- and T-cell populations and reduced immunoglobulin M levels. *Mol Cell Biol* 26(4):1549-1557.



Chapter 9

Summary

Summary

Infection with the intestinal bacterium *Campylobacter jejuni* (*C. jejuni*) may lead to the development of Guillain-Barré syndrome (GBS) or Miller Fisher syndrome (MFS). GBS is a severe and potentially life-threatening neurological disease which is characterized by demyelination or axonal degradation of peripheral nerves, resulting in acute, progressive and fairly symmetrical paralysis. MFS is a milder variant of GBS, characterized by a lack of coordination, loss of tendon reflexes and paralysis of the eye muscles (oculomotor weakness). GBS and MFS are immune-mediated diseases that can develop following a microbial infection. Several bacteria and viruses have been associated with the induction of GBS. *C. jejuni* is the most frequently identified causative agent, and is associated with more severe forms of the disease. *C. jejuni* can have sialylated lipooligosaccharides (LOS) structures which are highly similar to peripheral nerve gangliosides. It is established that these sialylated LOS structures can induce the production of cross-reactive anti-ganglioside antibodies; these antibodies trigger nerve damage which may lead to paralysis in patients with GBS.

In industrialized countries, *C. jejuni* is predominantly transmitted through contaminated food. Once ingested, *C. jejuni* may penetrate the intestinal mucosa. The bacterial factors that contribute to mucosal penetration by *C. jejuni* include: the bipolar flagella, the surface adhesins involved in the adhesion to intestinal epithelial cells, and the bacterial structures involved in the invasion of intestinal epithelial cells. As such, these bacterial factors contribute to enhanced immune exposure which may lead to immune activation. In combination with the presence of sialylated LOS, these bacterial factors may contribute to the development of GBS. The identification of novel GBS-associated bacterial factors that contribute to enhanced immune exposure would be a pivotal step forward in the understanding of how *C. jejuni*-mediated GBS develops.

Besides the identification of bacterial factors, identification of the human factors involved in the development of GBS is also of great importance. It is clear that cross-reactive anti-ganglioside antibodies are induced in patients with GBS. However, it is largely unknown which immune events lead to the induction of these antibodies. Immune recognition of bacterial epitopes is a crucial first step in the induction of an antibody response, yet the specific receptors involved in the recognition of sialylated LOS are largely unknown.

The aim of this thesis was to identify interactions between *C. jejuni* and the human host which contribute to the development of GBS. In particular, we focused on the role of sialylated LOS of *C. jejuni*. We assessed the role of sialylated LOS of *C. jejuni* in adhesion and invasion of human intestinal epithelial cells and we studied whether sialylated *C. jejuni* strains can bind to members of the sialic acid-binding immunoglobulin-like lectin

(Siglec) family. Additionally, we search for novel GBS-associated *C. jejuni* virulence factors and we focused on the development of a new putative treatment strategy for GBS.

In **Chapter 2,** we further established that the sialylated LOS loci classes A and B of *C. jejuni* are associated with GBS and MFS, respectively. In search for novel virulence factors that may be involved in the development of GBS or MFS, we screened for the prevalence of genes encoding proteins known to be involved in *C. jejuni* virulence. However, this approach did not lead to the identification of new GBS- or MFS-associated genes. PCR-based capsule genotyping revealed that five capsule types (HS1, HS2, HS4, HS19 and HS23/36) were dominant among GBS/MFS-associated *C. jejuni* strains. Of these five capsule types, HS4 was significantly associated *C. jejuni* strains isolated from GBS or MFS patients. In order to establish whether these capsule types indeed are associated with GBS and MFS development, capsular genotyping on additional GBS- and MFS-associated strain from various geographical areas is necessary.

In **Chapter 3**, we observed that sialylated *C. jejuni* strains were able to invade human intestinal epithelial Caco-2 cells in significantly higher numbers compared to nonsialylated strains. Three previously constructed sialic acid knockout strains showed a significantly reduced ability to invade Caco-2 cells, compared to the parental wild type strains. No difference was observed in the ability of sialylated and nonsialylated *C. jejuni* strains to adhere to Caco-2 cells. Our findings suggest that sialic acids on *C. jejuni* LOS play a role in the cell invasion process. As such, sialylated LOS could contribute to increased exposure of bacterial components, including sialylated LOS, to the host immune system.

In **Chapter 4**, the interaction of *C. jejuni* strains with murine sialoadhesin (Sn) was assessed. Sn is a member of the Siglec family (Siglec-1) and is expressed on a subset of macrophages. We observed that *C. jejuni* strains that have monosialylated LOS with terminal α(2,3)-linked sialic acids on the LOS outer core were able to bind to murine Sn (mSn). We provided evidence that Sn binding is sialic acid-specific, as two sialic acid knockout strains which lacked α(2,3)-linked sialic acids on their LOS were unable to bind to mSn, whereas the parental wild type strains could bind mSn. Upon screening a large panel of *C. jejuni* strains, we demonstrated that significantly more GBS-associated *C. jejuni* strains bond to mSn, compared to uncomplicated enteritis-associated strains. Sn is expressed on residentual macrophages in the lamina propria and on splenic marginal zone and mesenteric lymph node subcapsular sinus macrophages. *C. jejuni* may encounter these macrophages once the bacterium has crossed the intestinal epithelium.

To determine whether binding to Sn could play a role in the pathogenesis of GBS in human, we assessed (**Chapter 5)** whether sialylated LOS on *C. jejuni* also interacted with human Sn (hSn) and what the functional consequences of binding to Sn were. We showed that *C. jejuni* can bind to hSn expressed on a monocytic cell line and primary human macrophages, in a sialic acid-dependent manner. Not only strains with terminally

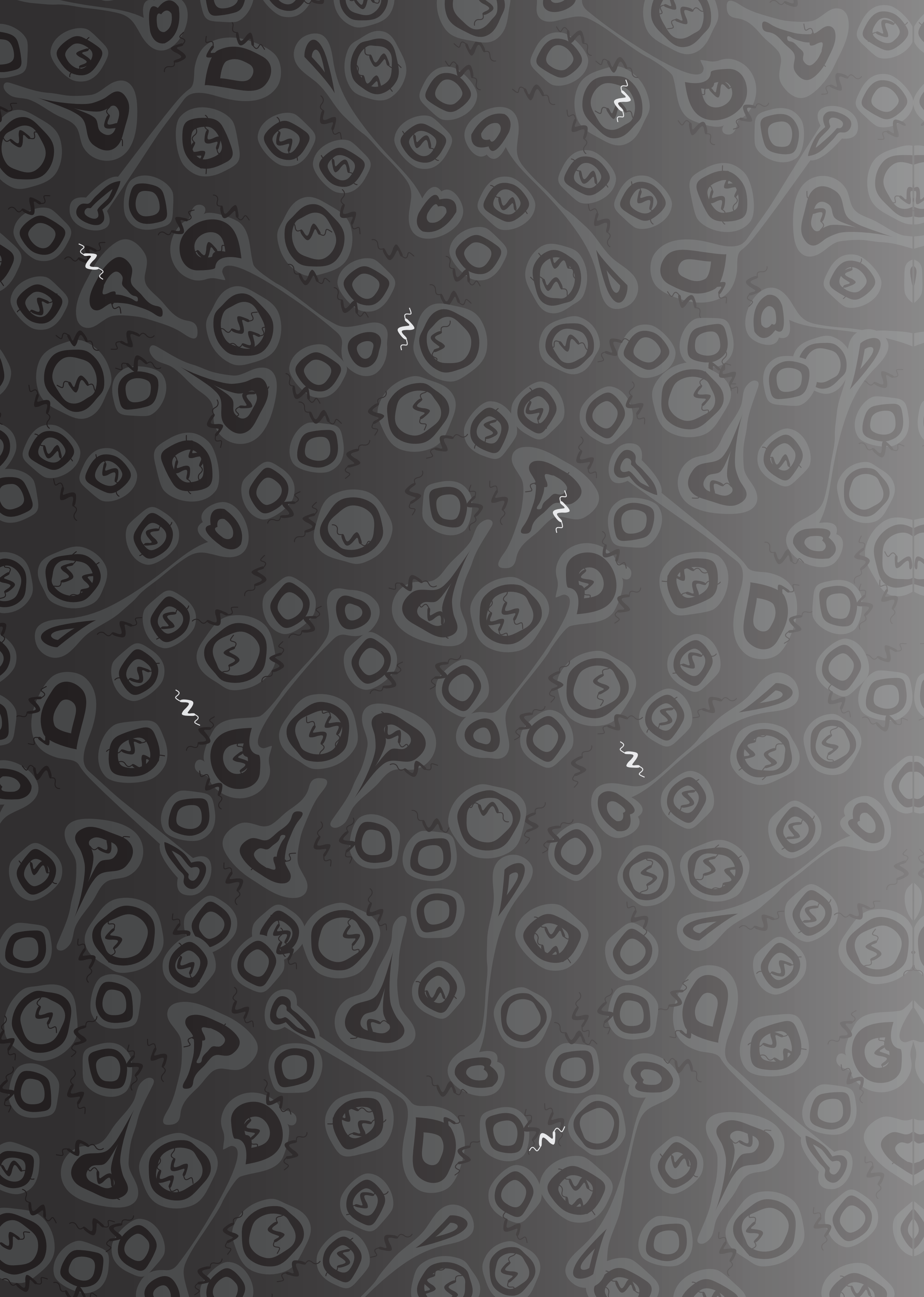
α(2,3)-linked sialic acids, but also strains with internal α(2,3)-linked sialic acids (as present in the ganglioside GM1a) bond to hSn. This observation is of importance as antibodies against GM1a are the most frequently detected anti-ganglioside antibodies in patients with GBS. Intriguingly, the binding of *C. jejuni* LOS to hSn was not constitutive, but required specific bacterial treatments or growth conditions. Heat inactivation, treatment with low pH or a bile constituent, or bacterial growth on culture plates which contained bile facilitated the binding of *C. jejuni* LOS to hSn, in a sialic acid dependent manner. Cryo-electron microscopy and labeling of *C. jejuni* LOS with cholera toxin revealed that these conditions were necessary in order to expose the LOS on the bacterial surface. The functional consequences of hSn binding were assessed using human monocyte-derived macrophages. Sn binding enhanced bacterial uptake and increased release of the cytokine IL-6, compared to control conditions when hSn was blocked using neutralizing antibodies or when nonsialylated *C. jejuni* was used. Sn-mediated differentiation of *C. jejuni* strains on the basis of their ganglioside mimic expression may be an important initial event in the induction of GBS-related immune responses.

In **Chapter 6** we studied the interaction between *C. jejuni* and human Siglec-7, another member of the Siglec family which is expressed on NK cells, monocytes, dendritic cells and T cells. We showed that Siglec-7 exclusively bond to strains which had terminal disialylated LOS, with α(2,8)-linked sialic acids. Additionally, we observed a correlation between Siglec-7 binding, the presence of anti-GQ1b antibodies in patient serum and the diagnosis of oculomotor weakness in either GBS or MFS. For future research we suggest that the functional consequences of *C. jejuni* binding to Siglec-7 on human dendritic cells should be assessed. Dendritic cells play a pivotal role in antigen presentation and immune activation and are therefore of interest in the pathogenesis of GBS.

In **Chapter 7**, we focused on a novel putative treatment strategy for GBS. Currently, patients with GBS are treated with either a high doses of purified immunoglobulins which are injected intravenously (IVIg) or plasma exchange (plasmapheresis). In both of these treatment modalities, the immune response of the patient is reduced, yet these treatments do not specifically target the anti-ganglioside antibodies. In **Chapter 7** we provided proof of principle that synthetic gangliosides covalently bound to a polymeric structure (monolith) were able to capture anti-ganglioside antibodies from human serum. We demonstrated successful antibody capture and depletion of class IgM or IgG anti-GM2 antibodies. Rapid, specific depletion of anti-ganglioside antibodies from GBS patient plasma could lead to less severe neurological damage in the acute phase of GBS and faster patient recovery.

The most important conclusions of the work described in this thesis are that sialylated *C. jejuni* strains are more invasive in Caco-2 cells compared to nonsialylated strains. GBS-associated *C. jejuni* strains preferentially bind to Sn in a sialic acid dependent manner. Binding to Sn might activate GBS-related immune responses, as we showed that

in primary human macrophages, sialic acid specific binding of *C. jejuni* to Sn resulted in increased bacterial uptake and enhanced cytokine production. MFS-related immune responses might be induced via the initial binding of disialylated LOS to Siglec-7, as we demonstrated that disialylated MFS- and oculomotor weakness-associated *C. jejuni* strains specifically bond to this Siglec, in a sialic acid dependent manner. The identification of receptors involved in the development of GBS and MFS is of importance to dissect the immune events that contribute to the production of anti-ganglioside antibodies and may lead to the identification of host susceptibility factors for GBS and MFS.



Nederlandse samenvatting

Nederlandse samenvatting

De darmbacterie *Campylobacter jejuni (C. jejuni)* is wereldwijd een van de belangrijkste veroorzakers van bacteriële diarree. Naast diarree kan een infectie met *C. jejuni* ook leiden tot de ontwikkeling van het Guillain-Barré syndroom (GBS). In Nederland worden jaarlijks 150 tot 300 getroffen door GBS. Hoewel GBS dus niet vaak voorkomt, is het een zeer ernstige en levensbedreigende ziekte.

In patiënten met GBS zijn de zenuwen die het centrale zenuwstelsel met de spieren verbinden beschadigd. Deze zenuwschade leidt tot gevoelsstoornissen en spierzwakte en heeft in ernstige gevallen volledige verlamming tot gevolg. Een mildere variant van GBS is het Miller Fisher syndroom (MFS), wat gekenmerkt wordt door een oogspierverlamming en evenwichtsstoornissen. GBS en MFS zijn immuun-gemedieerde ziektes die kunnen ontstaan na een microbiële infectie. Verschillende bacteriën en virussen worden in verband gebracht met GBS. Een voorafgaande infectie met de bacterie *C. jejuni* wordt het vaakst aangetoond en is geassocieerd met ernstige vormen van de ziekte.

GBS volgend op een *C. jejuni*-infectie wordt waarschijnlijk veroorzaakt door een gelijkenis tussen structuren op bacteriën en structuren op zenuwcellen van de mens. Aan het oppervlak van *C. jejuni* kunnen gesialyleerde suikerketens (lipooligosacchariden, LOS) voorkomen, die een grote overeenkomst vertonen met de ganglioside structuren die aanwezig zijn op zenuwcellen. Het is bewezen dat deze gesialyleerde suikerketens de productie van kruisreactieve anti-ganglioside antilichamen kunnen stimuleren. Als deze antilichamen binden aan gangliosiden op zenuwcellen, kan dit een immuunrespons op gang brengen die resulteert in zenuwbeschadiging en de klinische symptomen van GBS en MFS.

C. jejuni komt door het eten van besmet voedsel in het maag-darmkanaal terecht en is dan in staat om de mucuslaag (slijmlaag) van de darm te penetreren. Het daaronder liggende darmepitheel vormt een barrière voor de meeste bacteriën. *C. jejuni* is echter in staat om het darmepitheel te passeren, waarna het in contact komt met immuuncellen. Bacteriële factoren die het binnendringen van het darmepitheel faciliteren dragen waarschijnlijk ook bij aan een verhoogde blootstelling van bacteriële componenten aan het immuunsysteem. In combinatie met de aanwezigheid van gesialyleerde LOS op de bacterie, zouden deze factoren een rol kunnen spelen bij het ontstaan van GBS. De identificatie van GBS-geassocieerde bacteriële structuren die bijdragen aan verhoogde immuunblootstelling zou een belangrijke stap voorwaarts zijn in ons begrip van het ontstaan van *C. jejuni*-gemedieerd GBS.

Naast het identificeren van bacteriële factoren is de identificatie van humane factoren die bijdragen aan het ontstaan van GBS van groot belang. Het is duidelijk dat er kruisreactieve anti-ganglioside antilichamen voorkomen in het bloed van patiënten met GBS. Het is echter grotendeels onbekend welke immunologische gebeurtenissen

bijdragen aan het ontstaan van deze kruisreactieve antilichamen. Immuunherkenning van lichaamsvreemde componenten is een noodzakelijke stap voor het ontwikkelen van een antilichaamrespons, maar receptoren die betrokken zijn bij de specifieke herkenning van gesialyleerde LOS zijn nagenoeg onbekend. Het identificeren van dergelijke receptoren zal leiden tot meer inzicht in het ontstaan van GBS.

Het doel van het onderzoek dat beschreven staat in dit proefschrift was om de interacties tussen *C. jejuni* en de mens die bijdragen aan het ontstaan van het GBS te identificeren. We hebben ons hierbij vooral gericht op de rol van gesialyleerde LOS. We hebben de rol van gesialyleerde LOS bij het binnendringen van humaan darmepitheel bestudeerd en bekeken of gesialyleerde *C. jejuni*-stammen kunnen binden aan Siglecs. Siglecs zijn een familie van siaalzuurbindende receptoren die voorkomen op immuuncellen en die onder andere betrokken zijn bij het herkennen van pathogenen. Verder hebben we met behulp van genotypering gekeken of bepaalde genen die betrokken zijn bij virulentie (vermogen om schade te veroorzaken in een gastheer) vaker voorkomen bij GBS-geassocieerde C. *jejuni* stammen vergeleken met stammen die geassocieerd zijn met ongecompliceerde diarree. Tenslotte hebben we ons gericht op het ontwikkelen van een nieuwe behandelingsmethode voor patiënten met GBS.

In **Hoofdstuk 2** konden we bevestigen we dat *C. jejuni*-stammen met gesialyleerde LOS geassocieerd zijn met GBS en MFS. Onderzoek naar het voorkomen van genen die eiwitten coderen waarvan bekend is dat ze bijdragen aan de virulentie van *C. jejuni* hebben niet geleid tot de identificatie van GBS- of MFS-geassocieerde bacteriële genen. Genotypering van het kapsel liet echter zien dat vijf kapseltypes (HS1, HS2, HS4, HS 19 en HS23/36) dominant aanwezig zijn in GBS/MFS-geassocieerde *C. jejuni*-stammen, terwijl de kapseltypes in *C. jejuni*-stammen die geassocieerd zijn met ongecompliceerde diarree meer variatie vertoonden. Van de vijf gevonden kapseltypes in de GBS/MFS-gerelateerde stammen was kapseltype HS4 significant geassocieerd met GBS/MFS. Om met meer zekerheid vast te kunnen stellen of het kapsel inderdaad een rol zou kunnen spelen bij het ontstaan van GBS of MFS, zijn aanvullende studies nodig.

De rol van gesialyleerde LOS in het binden aan en binnendringen van darmepitheelcellen werd onderzocht in **Hoofdstuk 3**. We vonden dat gesialyleerde *C. jejuni*-stammen significant beter in staat waren om Caco-2 darmepitheelcellen binnen te dringen dan niet-gesialyleerde stammen. Drie siaalzuurmutanten waren significant minder goed in staat om darmepitheelcellen binnen te dringen in vergelijking met ongemuteerde stammen. Er werden echter geen verschillen gevonden in de capaciteit van *C. jejuni*-stammen om aan Caco-2-cellen te binden. Dit suggereert dat de siaalzuren die aanwezig zijn op de LOS van *C. jejuni* voornamelijk een rol spelen bij het binnendringen van de cel en niet bij de hechting aan de cel. Onze bevindingen wijzen daarom op een dubbele rol voor gesialyleerd LOS in de pathogenese van GBS. Naast het feit dat gesialyleerde LOS betrokken zijn bij immuunactivering, dragen deze structuren door een verhoogde

capaciteit om darmepitheel binnen te dringen waarschijnlijk ook bij aan verhoogde blootstelling van bacteriële componenten aan immuuncellen (die zich onder het darmepitheel bevinden).

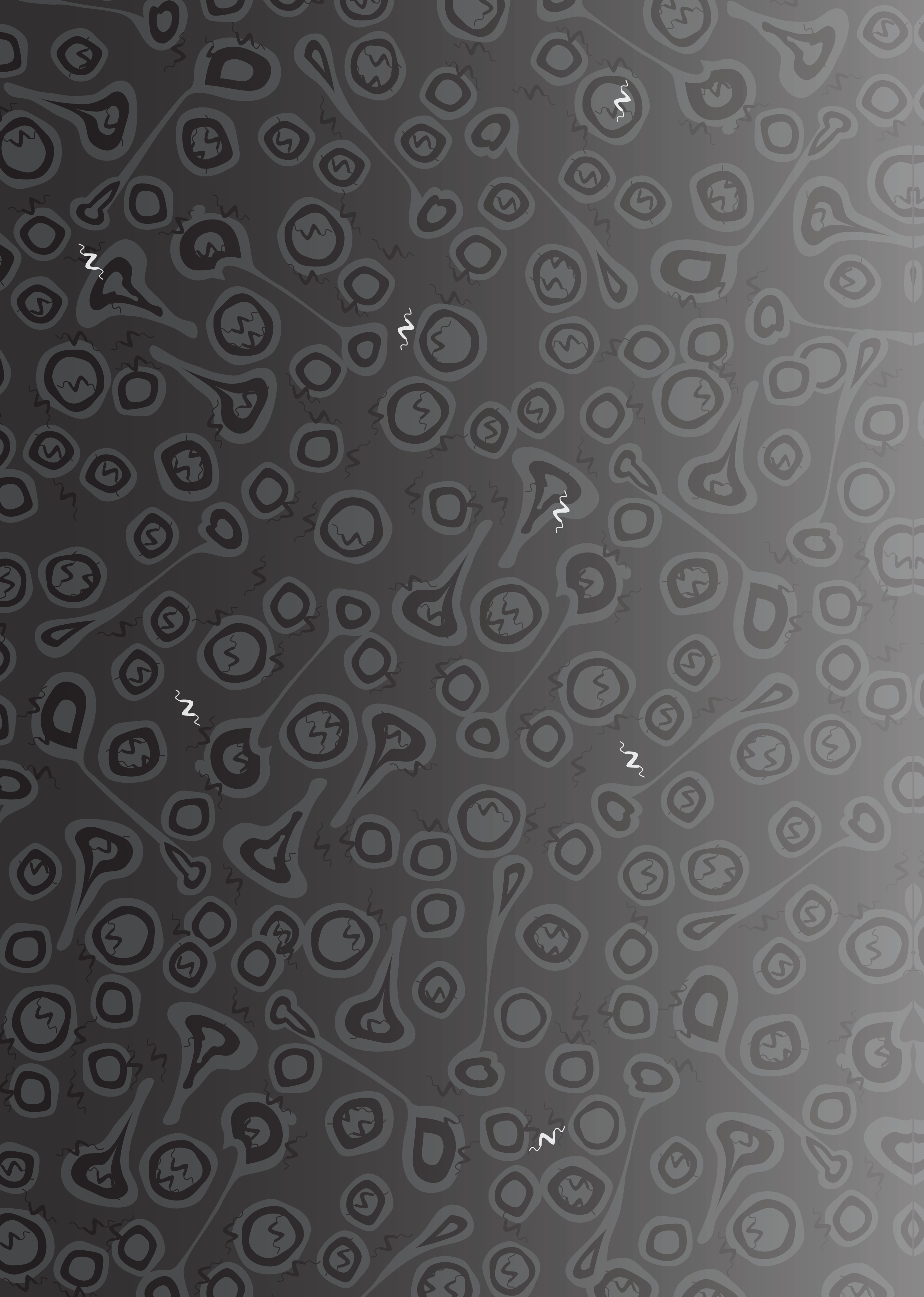
Hoofdstuk 4 beschrijft een studie over de binding van *C. jejuni* aan muis-sialoadhesine (Sn, Siglec-1). Sn is een siaalzuurbindende receptor die voorkomt op bepaalde macrofagen. In bindingsstudies laten we zien dat *C. jejuni*-stammen die α(2,3)-gekoppelde siaalzuren aan het uiteinde van de suikerketens van hun LOS hebben, in staat zijn om aan Sn te binden. De binding aan Sn was siaalzuurspecifiek. Twee siaalzuurmutanten waren namelijk niet in straat om aan Sn te binden, terwijl de representatieve niet-gemuteerde stammen wel aan Sn bonden. Het vergelijken van een groot aantal *C. jejuni*-stammen op basis van binding aan Sn leidde tot de observatie dat GBS-geassocieerde stammen significant vaker aan Sn bonden dan *C. jejuni*-stammen die geassocieerd zijn met ongecompliceerde diarree. Sn is aanwezig op macrofagen die voorkomen in de darm, de lymfeklieren van de darm en de milt. Het is aannemelijk dat *C. jejuni* bij het passeren van het darmepitheel in contact komt met deze macrofagen.

Om te bepalen of binding aan Sn ook een rol zou kunnen spelen in de pathogenese van GBS in de mens, onderzochten we of de gesialyleerde LOS van *C. jejuni* ook kunnen binden aan humaan Sn. In **Hoofdstuk 5** laten we zien dat dit inderdaad het geval is. Dit toonden we aan met behulp van bindingsexperimenten waarbij we gebruikmaakten van twee celtypen die Sn aan hun oppervlak tot expressie brengen. Naast stammen met eindstandige α(2,3)-gekoppelde siaalzuren konden ook stammen met interne α(2,3)-gekoppelde siaalzuren, onder andere aanwezig op ganglioside GM1a en GD1a, aan humaan Sn binden. Deze ontdekking is van belang, omdat antilichamen tegen GM1a en GD1a vaak gedetecteerd worden in het bloed van patiënten met GBS. Er waren specifieke condities noodzakelijk voor de binding van *C. jejuni* aan Sn. Alleen als *C. jejuni* op een bepaalde manier werd behandeld of gekweekt, bond het aan de receptor. Kortstondige verwarming, behandeling met een lage pH of met een galzout, of bacteriële groei in aanwezigheid van galzout waren condities die Sn-binding faciliteerden. Elektronenmicroscopie wees uit dat bovengenoemde condities nodig waren om de gesialyleerde suikerstructuren bloot te stellen aan de oppervlakte van *C. jejuni*. Met behulp van primaire humane macrofagen hebben we de gevolgen van *C. jejuni*-binding aan Sn bestudeerd. Onze experimenten lieten zien dat Sn-binding leidde tot verhoogde bacteriële opname en verhoogde productie van cytokine IL-6, vergeleken met controlecondities waarbij Sn werd geblokkeerd met antilichamen of wanneer niet-gesialyleerde *C. jejuni*-stammen werden gebruikt. Cytokine IL-6 is betrokken bij immuunactivering. Door binding van gesialyleerde LOS aan Sn kan het immuunsysteem dus worden geactiveerd. Deze activatie zou een belangrijke eerste stap kunnen zijn in de productie van anti-ganglioside antilichamen en de ontwikkeling van GBS.

Hoofdstuk 6 handelt over de interactie tussen *C. jejuni* en Siglec-7. Siglec-7 is een ander lid van de Siglec-familie, dat onder andere aanwezig is op het oppervlak van NK-cellen, monocyten, dendritische cellen en T-cellen. Dendritische cellen spelen een belangrijke rol bij interacties die kunnen leiden tot de productie van antilichamen, waardoor deze cellen interessant zijn voor onderzoek naar het ontstaan van GBS. We vonden dat Siglec-7 exclusief bond aan *C. jejuni*-stammen die dubbel-gesialyleerde LOS hadden. Verder is in hoofdstuk 7 te zien dat Siglec-7-binding correleerde met de diagnose oogspierverlamming in GBS en MFS. In toekomstig onderzoek zouden de functionele consequenties van *C. jejuni*-binding aan Siglec-7 op dendritische cellen onderzocht kunnen worden.

In **Hoofdstuk 7** beschrijven we een innovatieve en mogelijke toekomstige behandelingsmethode voor patiënten met GBS. Huidige behandelingen bestaan ofwel uit het toedienen van een hoge dosis gezuiverde immunoglobulinen, ofwel uit plasmavervanging. Ondanks dat beide behandelmethoden succesvol zijn in het onderdrukken van de immuunreactie in patiënten met GBS, zijn deze behandelingen niet specifiek gericht tegen de anti-ganglioside antilichamen. Deze anti-ganglioside antilichamen zijn nu juist waarschijnlijk de oorzaak van zenuwschade in GBS patiënten. We demonstreerden dat synthetische gangliosiden gekoppeld aan een polymere matrix in staat zijn om anti-ganglioside antilichamen te binden en te verwijderen uit humaan plasma. Met deze methode hebben we succesvolle verwijdering van anti-GM2 antilichamen uit serum kunnen bewerkstelligen. Snelle, specifieke verwijdering van anti-ganglioside antilichamen uit bloed van patiënten met GBS kan leiden tot minder neurologische schade in de acute fase van GBS, waardoor de patiënten mogelijk sneller herstellen en minder restschade hebben.

Sn en Siglec-7 zijn betrokken bij de specifieke herkenning van gesialyleerde LOS, aanwezig op GBS- en MFS-geassocieerde stammen. Dit is de belangrijkste conclusie van het werk beschreven in dit proefschrift. Deze bevinding heeft geleid tot de identificatie van immuuncellen en immuunresponsen die mogelijk indirect bijdragen aan de productie van anti-ganglioside antilichamen en de ontwikkeling van GBS en MFS.



Curriculum vitae

PhD portfolio

List of publications

Dankwoord

Curriculum vitae

Astrid Petra Heikema was born on April 25th, 1970 in Stedum, the Netherlands. In 1986 she finished her secondary school education at the Alberda MAVO in Uithuizen and received further education at the MLO (Middelbaar Laboratorium Onderwijs) in Groningen. She continued her education at the Hanze Hogeschool (HLO; Hoger Laboratorium Onderwijs) in Groningen where she specialized in Biotechnology. From 1993 till 1998, she worked as a research technician at the department of Physiological Chemistry of the Groningen University, under supervision of Dr. A. Huckriede, Dr. E. Agsteribbe and Prof. J. Wilschut. During this period she studied mitochondrial protein import in cells derived from mitochondrial myopathy patients. She also contributed to the development of a novel treatment method for patients with neuroblastoma. In 1998, Astrid continued her scientific carrier at the California Institute of Technology (Caltech), Pasadena, CA, United States. There she worked as a research technician III in the laboratory of Professor P. Bjorkman where she studied the interaction of cytomegalovirus encoded MHC class I homolog UL18 with leukocyte immunoglobulin-like receptor 1. Additionally, she contributed to the purification of Zn-α_2-glycoprotein, an MHC class I homolog which has a role in lipid storage homeostasis. Upon returning to the Netherlands in 2002, Astrid joined the department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam. Under supervision of Professor A. van Belkum, Astrid got introduced to *Campylobacter jejuni* induced Guillain-Barré syndrome. After being a research technician for several years, Astrid started her PhD study in 2008, supervised by Dr. J. Samsom, Dr. W. van Wamel, Prof. A. van Belkum and Prof. H. Endtz. The results of the research performed by Astrid were presented at several national and international scientific symposia and conferences. For one of her publications, Astrid received an award, "de Kiemprijs", from the Dutch society of Medical Microbiology (NVMM) for best first authorship of junior microbiologists. In three occasions, a conference travel award was granted to Astrid. Astrid participated in several courses provided by the Molecular Medicine Postgraduate School. She is a lecturer in the Research Master Infection & Immunity of the Erasmus University Rotterdam. Additionally, Astrid is a teaching assistant in the course Infectious Diseases, which is provided to second year medicine students annually by the department of Medical Microbiology and Infectious Diseases. After her graduation, Astrid will continue to work at the department of Medical Microbiology and Infectious Diseases and carry on her work on *Campylobacter jejuni* induced Guillain-Barré syndrome.

PhD portfolio

Name: Astrid P. Heikema
Institute: Erasmus University Rotterdam
Department: Medical Microbiology and Infectious Diseases
Promotor: Prof.dr. H.P. Endtz
Copromotors: Dr. J.N. Samsom and Dr. W.J.B. van Wamel

PhD education

- The basic introduction course on SPSS, Molecular Medicine, Erasmus Postgraduate school, 2009
- Introduction course confocal microscopy, Erasmus MC Optical Imaging Centre, 2010
- Workshop on Adobe Photoshop & Illustrator CS5 for PhD-students and other researchers, Molecular Medicine, Erasmus Postgraduate school, 2011
- Adaptive immune response in the mucosa: B cells and beyond, Molecular Medicine, Erasmus Postgraduate school, 2011

Conferences and Seminars

2007

- The 14th International Workshop on Campylobacter, Helicobacter and Related Organisms (CHRO), Rotterdam, the Netherlands

2008

- Research day, Medical Microbiology and Infectious Diseases (MMIZ), Rotterdam, the Netherlands

2009

- Keystone symposium, Innate, Adaptive and Regulatory Immune Responses to Intestinal Microbiota, Taos, New Mexico, USA
- Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands
- CampylobacterUK 2009 conference, Norwich, United Kingdom
- Guillain-Barré syndrome symposium, Rotterdam, the Netherlands
- Scientific fall meeting of the Dutch society for Gastro Enterology (NVGE), Veldhoven, the Netherlands
- Research day, Medical Microbiology and Infectious Diseases (MMIZ), Rotterdam, the Netherlands

2010

- Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands
- Research day, Medical Microbiology and Infectious Diseases (MMIZ), Rotterdam, the Netherlands

2011

- The 16th International Workshop on Campylobacter, Helicobacter and Related Organisms (CHRO), Vancouver, British Columbia, Canada
- CampyGerm conference, Gottingen, Germany

2012

- Scientific spring meeting of the Dutch society for Immunology (NVVI), Lunteren, the Netherlands
- Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands
- Peripheral Nerve Society / Inflammatory Neuropathy Consortium (PNS/INC) conference, Rotterdam, the Netherlands
- Research day, Medical Microbiology and Infectious Diseases (MMIZ), Rotterdam, the Netherlands

Research abroad

- Department of Cell Biology and Immunology, College of Life Sciences, University of Dundee, United Kingdom. Supervision: Prof. P. Crocker and Dr. H. Richards. April 2008 (one week)

Teaching

2008-2012

- Teaching assistant in the practical course Infectious Diseases, provided annually by the MMIZ to second year medicine students of the Erasmus University Rotterdam

2008-2012

- Supervisor of Bachelor and Master students

2010-2012

- Lecturer in the summer and winter course of the Research Master in Infection and Immunity of the Erasmus University Rotterdam

Grants

- Erasmus Trustfonds travel grant (2011)
- CHRO travel grant (2011)
- Rabobank travel grant PNS/INC (2012)

Presentations

Campylobacter-Siglec interactions in host-cell defence, CHRO, Rotterdam, 2007 (oral presentation).

Role of *Campylobacter jejuni* sialic acid in Guillain-Barré syndrome, Research day MMIZ, Rotterdam, 2008 (oral presentation).

Role of Siglec/sialic acid interaction in phagocytosis of *Campylobacter jejuni* by monocytes, Keystone symposium, Taos, New Mexico, USA, 2009 (poster presentation).

Role of Siglec/sialic acid interaction in phagocytosis of *Campylobacter jejuni* in monocytes, NVMM, Papendal, 2009 (oral presentation).

The specific interaction between sialoadhesin (Siglec-1) and sialylated *Campylobacter jejuni* lipooligosaccharides, CampylobacterUK, Norwich, UK, and Guillain-Barré syndrome symposium, Rotterdam, 2009 (oral presentation).

Affinity capturing of auto-antibodies using synthetic ganglioside epitopes for the treatment of induced Guillain-Barré Syndrome, NVGE, Veldhoven, 2009 (oral presentation).

Bacterial factors that contribute to Siglec binding and consequences for the host, Research day MMIZ, Rotterdam, 2009 (oral presentation).

The specific interaction between sialoadhesin (Siglec-1) and sialylated *Campylobacter jejuni* lipooligosaccharides, NVMM, Papendal, 2010 (oral presentation).

Antibody responses in Guillain-Barré syndrome pathology, Research day MMIZ, Rotterdam, 2010 (oral presentation).

Enhanced, sialoadhesin dependent uptake of GBS-associated *C. jejuni* strains in human macrophages, CHRO, Vancouver, Canada, 2011 (oral and poster presentation).

Interaction of *Campylobacter jejuni* with human macrophages, CampyGerm conference, Gottingen, Germany, 2011 (oral presentation).

In *C. jejuni* isolates causing Guillain-Barré syndrome, LOS class and capsule type are correlated, NVMM, Papendal, 2012 (poster presentation).

GBS- and MFS-associated *C. jejuni* strains specifically interact with sialic acid-binding immunoglobulin-like lectins (Siglecs), PNS/INC conference, Rotterdam, 2012 (oral and poster presentation).

A potential role for Siglecs in recognition of Guillain-Barré syndrome-related *Campylobacter jejuni* strains, Research day MMIZ, Rotterdam, 2012 (oral and poster presentation).

List of publications

Huckriede A, **Heikema A**, Sjollema K, Briones P, & Agsteribbe E (1995) Morphology of the mitochondria in heat shock protein 60 deficient fibroblasts from mitochondrial myopathy patients. Effects of stress conditions. *Virchows Arch* 427:159-165.

Huckriede A, **Heikema A**, Wilschut J, & Agsteribbe E (1996) Transient expression of a mitochondrial precursor protein. A new approach to study mitochondrial protein import in cells of higher eukaryotes. *Eur J Biochem* 237:288-294.

Heikema A, Agsteribbe E, Wilschut J, & Huckriede A (1997) Generation of heat shock protein-based vaccines by intracellular loading of gp96 with antigenic peptides. *Immunol Lett* 57:69-74.

Chapman TL, **Heikema AP**, West AP, Jr., & Bjorkman PJ (2000) Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). *Immunity* 13:727-736.

Kennedy MW, **Heikema AP**, Cooper A, Bjorkman PJ, & Sanchez LM (2001) Hydrophobic ligand binding by Zn-alpha 2-glycoprotein, a soluble fat-depleting factor related to major histocompatibility complex proteins. *J Biol Chem* 276:35008-35013.

Willcox BE, Thomas LM, Chapman TL, **Heikema AP**, West AP, & Bjorkman PJ (2002) Crystal structure of LIR-2 (ILT4) at 1.8 A: differences from LIR-1 (ILT2) in regions implicated in the binding of the Human Cytomegalovirus class I MHC homolog UL18. *BMC Struct Biol* 2:6.

Godschalk PCR, **Heikema AP**, Gilbert M, Komagamine T, Ang CW, Glerum J, Brochu D, Li J, Yuki N, Jacobs BC, van Belkum A, & Endtz HP (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J Clin Invest* 114:1659-1665.

Dzieciatkowska M, Brochu D, van Belkum A, **Heikema AP**, Yuki N, Houliston RS, Richards JC, Gilbert M, & Li J (2007) Mass spectrometric analysis of intact lipooligosaccharide: direct evidence for O-acetylated sialic acids and discovery of O-linked glycine expressed by *Campylobacter jejuni*. *Biochemistry* 46:14704-14714.

Dzieciatkowska M, Liu X, **Heikema AP**, Houliston RS, van Belkum A, Schweda EK, Gilbert M, Richards JC, & Li J (2008) Rapid method for sensitive screening of oligosaccharide epitopes in the lipooligosaccharide from *Campylobacter jejuni* strains isolated from

Guillain-Barré syndrome and Miller Fisher syndrome patients. *J Clin Microbiol* 46:3429-3436.

Louwen R, **Heikema AP**, van Belkum A, Ott A, Gilbert M, Ang W, Endtz HP, Bergman MP, & Nieuwenhuis EE (2008) The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. *Infect Immun* 76:4431-4438.

Heikema AP, Bergman MP, Richards H, Crocker PR, Gilbert M, Samsom JN, van Wamel WJ, Endtz HP, & van Belkum A (2010) Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides. *Infect Immun* 78:3237-3246.

Kuijf ML, Samsom JN, van Rijs W, Bax M, Huizinga R, **Heikema AP**, van Doorn PA, van Belkum A, van Kooyk Y, Burgers PC, Luider TM, Endtz HP, Nieuwenhuis EE, & Jacobs BC (2010) TLR4-mediated sensing of *Campylobacter jejuni* by dendritic cells is determined by sialylation. *J Immunol* 185:748-755.

Bax M, Kuijf ML, **Heikema AP**, van Rijs W, Bruijns SC, Garcia-Vallejo JJ, Crocker PR, Jacobs BC, van Vliet SJ, & van Kooyk Y (2011) *Campylobacter jejuni* lipooligosaccharides modulate dendritic cell-mediated T cell polarization in a sialic acid linkage-dependent manner. *Infect Immun* 79:2681-2689.

Tetala KK, **Heikema AP**, Pukin AV, Weijers CA, Tio-Gillen AP, Gilbert M, Endtz HP, van Belkum A, Zuilhof H, Visser GM, Jacobs BC, & van Beek TA (2011) Selective depletion of neuropathy-related antibodies from human serum by monolithic affinity columns containing ganglioside mimics. *J Med Chem* 54:3500-3505.

Huizinga R, Easton AS, Donachie AM, Guthrie J, van Rijs W, **Heikema AP**, Boon L, Samsom JN, Jacobs BC, Willison HJ, & Goodyear CS (2012) Sialylation of *Campylobacter jejuni* Lipo-Oligosaccharides: Impact on phagocytosis and cytokine production in mice. *PLoS ONE* 7:e34416.

Klaas M, Oetke C, Lewis LE, Erwig LP, **Heikema AP**, Easton A, Willison HJ, & Crocker PR (2012) Sialoadhesin promotes rapid proinflammatory and type I IFN responses to a sialylated pathogen, *Campylobacter jejuni*. *J Immunol* 189:2414-2422.

Louwen R, Horst-Kreft D, de Boer AG, van der Graaf L, de Knegt G, Hamersma M, **Heikema AP**, Timms AR, Jacobs BC, Wagenaar JA, Endtz HP, van der Oost J, Wells JM, Nieuwenhuis EE, van Vliet AH, Willemsen PT, van Baarlen P, & van Belkum A (2012) A novel link between

Campylobacter jejuni bacteriophage defence, virulence and Guillain-Barré syndrome. *Eur J Clin Microbiol Infect Dis* 80:3007-3318.

Louwen R, Nieuwenhuis EE, van Marrewijk L, Horst-Kreft D, de Ruiter L, **Heikema AP**, van Wamel WJ, Wagenaar JA, Endtz HP, Samsom J, van Baarlen P, Akhmanova A, & van Belkum A (2012) *Campylobacter jejuni* translocation across intestinal epithelial cells is facilitated by ganglioside-like lipooligosaccharide structures. *Infect Immun* 80:3307-3318.

Heikema AP, Jacobs BC, Horst-Kreft D, Huizinga R, Kuijf ML, Endtz HP, Samsom JN, & Van Wamel WJ (2012) *Clinical Microbiology and Infection* DOI: 10.1111/1469-0691.12073

Heikema AP, Koning RI, Duarte dos Santos Rico S, Rempel H, Jacobs BC, Endtz HP, Wamel WJB, & Samsom JN Enhanced, sialoadhesin-dependent uptake of Guillain-Barré syndrome-associated *Campylobacter jejuni* strains by human macrophages. *Submitted*.

Heikema AP, Horst-Kreft D, Poly F, Guerry P, Gilbert M, Li J, Eadie K, Wagenaar JA, van Belkum A, Samsom JN, Wamel WJB, & Endtz HP Particular lipooligosaccharide loci and capsule types co-occur in Guillain-Barré syndrome associated *Campylobacter jejuni* strains. *In preparation*.

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