*Campylobacter jejuni* lipo-oligosaccharides modulate DC-mediated T cell polarization in a sialic acid linkage dependent manner

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Running title

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Key words

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Abbreviations used in this paper: DC, Dendritic cell; GBS, Guillain-Barré syndrome; C. jejuni, Campylobacter jejuni; LOS, lipo-oligosaccharide; Siglec, Sialic acid-binding Ig-like lectin; Sn, Sialoadhesin; Th, T helper cell; PAA, polyacrylamide
Abstract
Carbohydrate mimicry between Campylobacter jejuni lipo-oligosaccharides (LOS) and host neural gangliosides plays a crucial role in the pathogenesis of Guillain-Barré syndrome (GBS). Campylobacter jejuni LOS may mimic various gangliosides, which affects the immunogenicity and the type of neurological deficits in GBS patients. Previous studies have shown the interaction of LOS with sialic acid-specific siglec receptors, although the functional consequences remain unknown. Cells that express high levels of siglecs include dendritic cells (DCs) that are crucial for initiation and differentiation of immune responses. We confirm that α2,3-sialylated GD1a/GM1a mimics and α2,8-sialylated GD1c mimic LOS structures interact with recombinant Sn and siglec-7 respectively. Although the linkage of the terminal sialic acid of LOS did not regulate expression of DC maturation markers, it displayed clear opposite expression levels of IL-12 and OX40L, molecules involved in DC-mediated Th differentiation. Accordingly, targeting DC-expressed siglec-7 with α2,8-linked sialylated LOS resulted in Th1 responses, whereas Th2 responses were induced by targeting with LOS containing α2,3-linked sialic acid. Thus, our data demonstrate for the first time that, depending on the sialylated composition of Campylobacter jejuni LOS, specific Th differentiation programs are instructed, possibly through targeting of distinct DC-expressed siglecs.
Introduction

Infection with *Campylobacter jejuni* usually causes uncomplicated gastro-enteritis, however in rare cases this infection can lead to the Guillain-Barré syndrome (GBS). GBS is a post-infectious immune-mediated disorder of the peripheral nerves and nerve roots. Molecular mimicry between lipo-oligosaccharides (LOS) present on the cell wall of *C. jejuni* and gangliosides found in the human nervous system are thought to play a critical role in the pathogenesis of *C. jejuni*-related GBS (2). The terminal glycans of both gangliosides and LOS are composed of α2,3-linked or α2,8-linked sialic acids. In patients with *C. jejuni*-related GBS cross-reactive antibodies are induced during infection against these bacterial structures. The specificity of the antibodies is highly associated with the type of neurological deficits.

Sialic acids are a wide family of carbohydrates, consisting of a nine-carbon backbone (4). They are distinguished from other monosaccharides, which are composed of 5 or 6 carbon sugars. Sialic acids are often found at the outer ends of surface exposed oligosaccharide chains attached to proteins and lipids to provide the oligosaccharide with a negative charge. In addition, in this terminal position they serve as ligands for lectins to mediate cell-cell interactions (27). Cst-II has been identified as the sialyltransferase responsible for the sialylation pattern of *C. jejuni* expressed LOS, acting bifunctional by catalyzing the formation of both α2,3- and α2,8-linked sialic acids (16). Loss of sialic acid expression on LOS by *cst-II* mutant strains resulted in lower DC activation and subsequent DC-mediated B cell responses (30). The ganglioside mimics of GD1a/GM1a and GD1c expressed by *C. jejuni* LOS are both sialylated. Interestingly, the GD1a/GM1a mimics of *C. jejuni* LOS that express terminal α2,3-linked sialic acids are associated with...
pure motor forms of GBS (25), whereas GD1c mimics of LOS exposing terminal α2,8-linked sialic acids are associated with GBS with ophthalmoplegia (18). Thus the sialylation pattern of *C. jejuni* LOS could be an important pathogen-related factor for the induction of GBS.

Sialic acid-binding Ig-like lectins (siglecs) are the best characterized I-type lectins involved in the recognition of sialic acids (3). Siglecs are predominantly expressed on cells of the immune system (12), whereby different leukocyte subsets express their own variety of siglecs. Siglecs are categorized into two subsets, the evolutionary well-conserved group consisting of sialoadhesin (Sn, or siglec-1), CD22 (or siglec-2), MAG (or siglec-4) and siglec-15 and the rapidly evolving CD33-related siglecs (siglecs-3,-5,-7,-8,-9,-10,-11,-14 and -16) (13). All siglecs have an unique glycan binding specificity, depending mainly on the linkage of the sialic acid and the underlying glycan (48). Siglecs are thought to play a role in both positive and negative regulation of immune responses (12,33). CD33-related siglecs mainly act as negative immunoregulators via their ITIM motifs. In contrast, siglec-14, -15 and -16 lack ITIMs and interact with DAP-12, an ITAM containing receptor. Sn on the other hand, lacks known signalling motifs (13).

Previous studies have demonstrated that inhibitory siglecs interact with sialylated pathogens like *Neisseria meningitidis* and Group B Streptococci with the potential to subvert immune responses (6,10,26). LOS of *C. jejuni* strains mimicking GD1a/GM1a were shown to interact with Sn (21), whereas siglec-7 bound to LOS structures mimicking gangliosides with terminal α2,8-linked sialic acids such as GD1c (6). Sn has a preference for clustered oligosaccharides terminating in Neu5Acα2,3Gal (α2,3-sialylated glycans) (13). The CD33-related inhibitory receptor siglec-7 has an unusual preference...
for α2,8-disialylated structures over α2,3- and α2,6-sialylated glycans (5,49). These α2,8-
disialylated epitopes are mainly found on gangliosides (37).

DCs are professional antigen-presenting cells important for the initiation and
differentiation of immune responses. Like other immune cells, DCs express a variety of
siglecs on their cell surface, including siglec-7 (48). Sn can be induced on immature
monocyte-derived DCs following exposure to rhinovirus (28), most likely via IFN-α
production, which is a potent inducer of Sn expression on monocytes (50). Immature DCs
reside in the tissue where they sense pathogens. Upon pathogen recognition, DCs migrate
to the lymph node where they arrive as fully mature DCs to promote the polarization of
naïve T cells to T helper 1 (Th1) cells, T helper 2 (Th2) cells, Th17 cells or regulatory T
cells (7,14,42). Classically, Th1 cells are critical for immunity to intracellular organisms,
whereas Th2 cells convert immunity to extracellular pathogens and are the major
mediators of class switching in B cells (34,51).

Because of their siglec expression and important immune regulatory role, we
hypothesized that different LOS structures of C. jejuni isolates from GBS patients would
differentially influence human DC biology. We show here that the interaction of DCs
with GD1a/GM1a LOS mimics and GD1c LOS mimics both induced DC maturation to
similar levels. However, the GD1a/GM1a mimic induced a more pronounced Th2
skewing, whereas the GD1c mimic induced DC-mediated Th1 responses. Thus, our data
demonstrate for the first time that the sialylated composition of C. jejuni LOS structures
determines DC-mediated T helper cell polarization in a manner that would be consistent
with siglec-dependent modulation.
Material and Methods

Ethics statement

The study was approved by the VU university medical center (VUmc) Amsterdam and the Commissie Wetenschappelijk Onderzoek (CWO) by written consent. A written informed consent was obtained from the healthy donors for the use of blood samples.

Human dendritic cells

Monocytes were isolated from the blood of healthy donors (Sanquin, Amsterdam, The Netherlands) through Ficoll gradient centrifugation and positive selection of CD14<sup>+</sup> cells using MACS sorting (Miltenyi Biotec, CA, USA). Isolated monocytes (purity >99%) were cultured in RPMI 1640 (Invitrogen, Gibco, CA, USA) supplemented with 10% FCS (BioWhittaker, Walkersville, MD), 1,000 U/ml penicillin (BioWhittaker, Walkersville, MD), 1,000 U/ml streptomycin (BioWhittaker, Walkersville, MD), and 2 mM glutamine (BioWhittaker, Walkersville, MD) in the presence of IL-4 (500 U/ml; Biosource, CA, USA) and GM-CSF (800 U/ml; Biosource, CA, USA) for 7 days (43). Anti-DC-SIGN stainings confirmed that >99% of our cells constituted of DCs. Maturation was induced by the addition of 10 ng/ml LPS (E. Coli; Sigma-Aldrich, MO, USA) for 24 hours. In order to cleave off sialic acids from the cell surface, DCs (5x10<sup>6</sup>/ml) were pre-incubated with the Vibrio cholerae neuraminidase (Roche, 2.5x10<sup>2</sup> U/ml) for 1 hour at 37°C in TSM (20 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>) (40). Sialic acid-specific lectin stainings confirmed complete removal of sialic acids.
Bacterial strains and LOS purification

Four *C. jejuni* strains (GB2, GB11, GB16 and GB19) were isolated from different GBS patients as described previously (21). The strains were cultured as reported (21) and LOS was purified by hot phenol-water extraction as described (24). Mutagenesis of the *cst-II* target gene was described previously (17) and the corresponding *cst-II* mutants derived from GB11 (Cst-II KO) or derived from GB2 (GB2 Cst-II KO) express a truncated LOS outer core due to the absence of sialic acids thus lacking ganglioside mimicry (47). GB19 LOS was incubated with the *Vibrio cholerae* neuraminidase (Roche) o/n at 37°C to remove sialic acids, followed by inactivation of neuraminidase for 30 min. at 80°C (GB19 neuraminidase). Purified LOS yields were determined by silver staining and mass spectrometry (18,30).

Siglec-Fc binding

Nunc maxisorp 96-wells ELISA plates (Nalge Nunc international, Rochester, NY) were coated overnight at 4°C with 20 µg/ml LOS. As negative control for siglec binding the plates where coated with the non-sialylated sugar GalNAc coupled to polyacrylamide (PAA), whereas as positive control the wells were coated with α2,3-sialyllactose, α2,6 sialyllactose, α2,8-linked di-, tri- or polysialic acid coupled to polyacrylamide (20 µg/ml, Lectinity, Moscow, Russia) in PBS. Wells were blocked for 30 min. at 37 °C with TSM with 1% BSA (Calbiochem). For soluble siglecs to bind their sialylated ligands a precomplexed conformation is required, therefore Sn-Fc and siglec-7-Fc (10 µg/ml) were
pre-complexed with goat-anti-human-Fc-PO (0.5 µg/ml) in TSM with 1% BSA for 60 min. at room temperature. This mixture consisting of siglec-Fc proteins complexed with anti-human-Fc antibodies was added to the wells with coated LOS or control glycans and incubated for 60 min. at room temperature. TMB substrate was added to the wells and binding of the pre-complexed siglec-Fc proteins to their ligands was analyzed by measuring the optical density at 450 nm.

Flow cytometry

For antibody stainings, DCs (5x10^4/well) were incubated with 10 µg/ml 7D2 (20) (anti-Sn), S7.5A (35) (anti-siglec-7) or PE-labeled anti-OX40L (BD Biosciences, CA, USA) for 45 min. at room temperature in PBS with 1% BSA. Cells were washed in PBS and for siglec stainings the cells were counter stained with a secondary FITC-labeled goat anti-mouse IgG (Zymed, CA, USA) for 30 min. at RT in PBS with 1% BSA. Stainings were analyzed by flow cytometry (FACS Calibur, BD Biosciences, CA, USA).

For maturation assays, DCs (5x10^4/well) were incubated with PE-labeled antibodies against human CD80 (BD Biosciences, CA, USA), CD83 (IOtest, Newcastleton, GB) CD86 and MCH II (both from BD Biosciences, CA, USA) in PBS for 45 min. at 4°C. After washing, binding was analyzed by flow cytometry (FACS Calibur, BD Biosciences, CA, USA).

Glycan binding assay
DCs (5x10^4/well) were pretreated for 45 minutes at 37°C with blocking antibodies to human siglec-7 (clone 7.7a) (35), followed by a 30 minute incubation at 37°C with 10 µg/ml biotinylated PAA attached to galactose, α2,3-sialyllactose, α2,6 sialyllactose and di- or tri-linked α2,8-sialic acid (Lectinity, Moscow, Russia) in TSM with 1% BSA. Cells were washed in TSM and stained with Alexa 488-labeled streptavidin (Invitrogen, CA, USA) for 30 min. at at 37°C in TSM with 1% BSA. After washing, binding was analyzed by flow cytometry (FACS Calibur, BD Biosciences, CA, USA).

**TLR4 activation**

HEK293-TLR4/MD2 co-transfectants (31) were grown in T75 flasks in RPMI 1640 (Invitrogen, Gibco, CA, USA) supplemented with 10% FCS (BioWhittaker, Walkersville, MD), 10,000 U/ml penicillin (BioWhittaker, Walkersville, MD), 10,000 U/ml streptomycin (BioWhittaker, Walkersville, MD), 10,000 U/ml glutamine (Sigma-Aldrich, MO, USA) and G418 (1 mg/ml). 10^5 cells in 100 µl RPMI were plated into 96-wells flat bottom plates and stimulated with LOS for 24 hours. *E. coli* LPS served as positive control for TLR4 activation. Subsequently, supernatants were analyzed for IL-8 production by ELISA according to the manufacturer’s guidelines (Biosource, CA, USA).

**Real-Time PCR**

Cells were lysed and mRNA was isolated using an mRNA Capture kit (Roche, Basel, Switzerland). cDNA was synthesized using the Reverse Transcription System kit (Promega, WI, USA) following manufacturer’s guidelines. Oligonucleotides were
designed by using the computer software Primer Express 2.0 (Applied Biosystems) and
synthesized by Invitrogen Life Technologies (Invitrogen, Breda, The Netherlands). Real-
Time PCR analysis was performed as previously described with the SYBR Green method
in an ABI 7900HT sequence detection system (Applied Biosystems), using GAPDH as
endogenous reference (15).

Cytokine ELISA

IL-12p70 ELISA was performed as described (44). In short, mAb 20C2 was coated
overnight to Nunc maxisorp 96-wells ELISA plates (Nalge Nunc international, Rochester,
NY). The plates were blocked for 30 min. at 37 °C with PBS supplemented with 1% BSA
and the supernatants together with mAb C8.6-biotin (BD biosciences) were added to the
plates. IL-12p70 was detected with a streptavidin-PO conjugate and optical density was
measured at 450 nm.

T helper differentiation assay

The T helper assay was performed as described before (14). Briefly, monocytes were
isolated from healthy blood donors and DCs were generated as described before. At day
6, maturation was induced by culturing the cells with 10 ng/ml LOS for 2 days. At day 8,
DCs were harvested and washed. Maturation of the DC was tested by flow cytometric
analysis of CD86 expression. DCs (5x10^3) were incubated with 2x10^4 naïve CD4^+ T cells
that were purified from PBLs using MACS isolation (Miltenyi Biotec, CA, USA). At day
5 and day 9, human rIL-2 (10 U/ml, Cetus, Emeryville, CA) was added to the culture. At
day 14, resting T helper cells were restimulated with PMA (30 ng/ml, Sigma-Aldrich, Missouri, USA) and ionomycin (1 µg/ml, Sigma-Aldrich) for 6 hours. During the last 5 hours, Brefeldin A (10 µg/ml, Sigma-Aldrich) was present to enable detection of the intracellular production of IL-4 and IFN-γ (BD Biosciences) by flow cytometry (FACS Calibur, BD Biosciences, CA, USA).

Statistics

Significant differences in cytokine expression by DCs activated by GB11 or GB16 LOS were evaluated by Mann-Whitney U tests and differences in secretion of cytokines by DCs was evaluated by Unpaired t tests (GraphPad Prism 4.02, GraphPad Software, San Diego, CA). A p value <0.05 was considered significant.
Results

Differential binding of Sn and siglec-7 to sialylated LOS structures

Recent publications have demonstrated that LOS with terminal α2,3-linked sialic acid (GB11) could bind to Sn (21), whereas LOS with terminal α2,8-linked sialic acid interacted with siglec-7 (6), however, the functional consequences of siglec binding were not evaluated on a cellular level. In this study, we used GB11, the LOS structure that mimics the gangliosides GD1a/GM1a containing terminal α2,3-linked sialic acid (Fig. 1A), and GB16, the LOS structure that mimics the ganglioside GD1c, exposing a terminal α2,8-linked sialic acid (Fig. 1B) (18). As control, we used the same LOS glycan structure from a C. jejuni mutant derived from GB11, that lacks the terminal sialic acid through deletion of the cst-II gene (Cst-II KO), coding for the sialyltransferase (17) (Fig. 1C).

First, the sialic acid binding specificities of the Sn-Fc and siglec-7-Fc proteins were evaluated. Therefore, we analyzed binding of Sn-Fc and siglec-7-Fc to sialylated or N-acetylgalactosylated glycan PAA probes. Sn-Fc interacted with α2,3-linked sialic acids (Fig. 2A), whereas siglec-7-Fc bound with the highest avidity to α2,8-linked di- and tri-sialic acid (Fig. 2B) and no siglec-Fc binding was observed to the negative control N-acetylgalactosamine conjugated PAA (Fig. 2A and 2B), confirming reported Sn and siglec-7 carbohydrate specificities (36).

Next, we determined the potency of Sn and siglec-7 to interact with the sialylated GB11 and GB16 LOS structures. Sn-Fc only displayed binding to GB11 carrying α2,3-linked sialic acid. In contrast, siglec-7-Fc interacted both with GB16 carrying α2,8-linked sialic acid, as well as GB11, due to low affinity interactions with α2,3-linked sialic acids. To assess the sialic acid requirement for this siglec-glycan interaction, binding of siglec-Fc
to LOS structures without terminal sialic acids (Cst-II KO) was investigated (29). Both siglec-Fc proteins did not bind to the mutant without sialic acid (Fig. 2C). Thus, we demonstrate that GB11 and GB16 interact specifically with Sn-Fc or siglec-7-Fc, in a sialic acid and linkage-dependent manner.

Interaction of *C. jejuni* sialylated LOS structures with DC-expressed siglec

To evaluate whether the differential siglec binding of GB11 and GB16 could influence immune responses, we first analyzed the siglec expression pattern of human DCs, an important cell for induction and differentiation of immune responses. In concordance with the literature, human immature DCs expressed only siglec-7, whereas Sn was not expressed by immature DCs (Fig. 2D) (12,32).

To test the ability of immature DC-expressed siglec-7 to bind sialylated glycans, the interaction of PAA-conjugated with α2,3-linked sialyllactose, α2,6-linked sialyllactose and α2,8-linked sialic acids (di- and trisialic acid) with DCs was analyzed by flow cytometry. N-acetylgalatosamine (Fig. 2A and 2B) was not suitable as a negative control in these cellular assays because of its ability to bind to the DC-expressed C-type lectin MGL. Instead we used Galactose as a negative control since both Galactose and N-acetylgalactosamine are known substrates of sialyltransferases. It is well accepted that siglecbs expressed on immune cells often have endogenous cis-ligands resulting in masking of siglecbs by recognition of sialic acids present on the same cell membrane. Masking of siglecbs by endogenous sialic acid ligands generally results in low binding to sialylated trans-ligands (40). Siglecbs can be unmasked and released from their cis-ligands by neuraminidase treatment. To release DC-expressed siglecbs from potential cis-ligands,
DCs were pre-treated with neuraminidase before measuring sialic acid binding. α2,3-linked sialic acid and α2,6-linked sialic acid displayed low binding to DCs, whereas α2,8-linked di- and tri-sialic acid bound with high affinity to DCs, suggesting interaction with siglec-7. The binding of sialylated glycans was clearly augmented when siglecs were unmasked by neuraminidase treatment. To determine the contribution of siglec-7 in binding of α2,8-sialylated glycans, as also present on GB16, siglec-7 on DCs was blocked with a monoclonal antibody prior to PAA-conjugated glycan incubation. As expected, binding of α2,8-sialylated glycans was exclusively mediated by DC-expressed siglec-7 (Fig. 2E). In addition, neuramindase treatment or maturation of DCs with LPS could not improve the low binding of α2,3-linked sialylated structures (data not shown).

**TLR4 activation and DC maturation by LOS**

DC activation following pathogen recognition is mediated by TLRs (41). TLR4 recognizes the lipid A moiety of LPS, a glycolipid expressed on the cell surface of Gram-negative bacteria (19) (45). To investigate the potency of LOS to trigger TLR4, HEK cells transfected with TLR4 and MD2 were used. As siglecs are exclusively expressed on leukocytes and in the nervous system, it is generally accepted that HEK cells do not express siglecs. Furthermore, HEK cells are not able to bind sialylated glycans (23). After overnight incubation with LOS, IL-8 levels, corresponding to TLR4 activation, were analyzed. Both GB11 and GB16 showed high TLR4 activation, comparable to the control LPS of *E. coli*. Compared to GB11, the lowered capacity of GB16 to induce IL-8 production after TLR4 engagement may indicate that the sialic acid linkage affects the ability to trigger TLR4 activation. The sialic acid mutant Cst-II KO, lacking sialic acids,
clearly showed a reduced capacity to trigger TLR4 (Fig. 3A). This corresponds with our previous results showing that TLR4-mediated sensing of *C. jejuni* by DCs is determined by sialylation (30). Differential effects on TLR4 triggering could not be due to variations in lipid A moiety since no apparent differences were found between in the lipid A composition from the *C. jejuni* strains used in this study by mass spectrometry. TLR4 activation can be modulated by glycan binding to lectins expressed by immature DCs (46). To test if maturation induction of immature DCs is influenced by differently linked sialic acids expressed on LOS, we measured the expression levels of maturation markers CD80, CD83, CD86 and MHCII after overnight incubation of immature DCs with LOS. *E. coli* LPS was used as a positive control. For all LOS concentrations, GB11 and GB16 induced similar levels of expression of markers associated with DC maturation, indicating that the type of linkage of sialic acid, being α2,3- or α2,8-linked, did not influence DC maturation (Fig. 3B-E). This data suggest that the presence of sialic acid as well as the linkage could modulate TLR4 activation; however both sialylated strains induced DC maturation to a similar extent. These results indicate that sialylated LOS induced maturation is partly independent of TLR4.

**Sialic acid linkage of LOS influences the cytokine profile of DCs**

To obtain more information about the functional consequences of DC interactions with differentially sialylated LOS structures, we analyzed expression levels of IL-12p40/70 and OX40L, which are molecules linked to functional T helper 1 and T helper 2 cell polarization respectively (14). DCs were incubated with *E. coli* LPS or LOS structures GB11, the *Cst-II* mutant and GB16 for 6 hours and mRNA levels of IL-12p40 were
analyzed. The pro-inflammatory Th1 skewing cytokine IL-12p40 was strongly upregulated in DCs incubated with GB16 (Fig. 4A). Interestingly, the strong TLR4 activating GB11 displayed the lowest IL-12p40 response. These results were confirmed by ELISA, in which DCs incubated with GB16 secreted higher amounts of the Th1 skewing cytokine IL-12p70 compared to DCs incubated with GB11 (Fig. 4B). In contrast to this, the Th2 polarizing molecule OX40L was expressed at the highest level on DCs incubated with GB11 and low expressed on DCs incubated with GB16 as shown by RT-PCR (Fig. 4C) and flow cytometry (Fig. 4D). Together, these results predict that sialic acids present on GB11 and GB16 have differential effects on key molecules involved in DC-mediated T helper cell differentiation.

T helper cell differentiation by LOS activated DCs is dependent on sialic acid linkage

The clearly regulated expression of OX40L and IL-12p40/70 by DCs incubated with LOS suggests that the LOS structures carrying different linkages of the sialic acids which target specific siglecs, would distinctly affect T helper differentiation. We therefore performed a DC-T cell co-culture assay and measured by flow cytometry at day 14 the production of IFNγ (Th1) and IL-4 (Th2) cytokines by CD4-positive T cells. As expected by the upregulation of OX40L, the α2,3-linked sialylated LOS structure GB11 induced a more pronounced Th2 response, which shifted towards Th1 differentiation when sialic acids were not present due to the mutation in the sialyltransferase gene (Cst-II KO). Surprisingly, not only the mutant but the siglec-7 binding α2,8-linked sialylated LOS structure GB16 induced a Th1 response as well, indicating that the differently linked sialic acids structures indeed induced opposite DC T helper cell differentiation, likely
mediated via DC-expressed siglecs (Fig. 5A). We observed identical T helper profiles with related LOS-structures from GB2 (similar to GB11 with terminal α2,3-linked sialic acid) and GB19 (similar to GB16 with terminal α2,8-linked sialic acid) (18) and their corresponding structures without sialic acids (GB2 Cst-II KO and GB19 neuraminidase (Fig. 5B). The T helper differentiation is in concordance with the cytokine expression data. In contrast, T helper polarization did not reflect the TLR4 activation, in which at the concentration of 10 ng/ml as used in the T helper differentiation assay, GB11 and GB16 showed higher TLR4 activation than LPS and the Cst-II KO. In conclusion, GB16 induced a distinct T helper differentiation program compared to GB11, which is displayed by the fact that these distinct LOS structures differentially affect OX40L upregulation and IL12p70 production by DCs.
Discussion

GBS is a post-infectious immune-mediated neuropathology, which is probably induced by carbohydrate mimicry between *C. jejuni* LOS and host gangliosides, resulting in a cross-reactivity antibody response to peripheral nerves. Although GB11 and GB16 were both isolated from patients with GBS, the clinical presentation is dissimilar. The specificity of the cross-reactive antibodies found in these patients is highly associated with the type of neurological deficits, probably explained by the distribution of the gangliosides and reachability by antibodies in the human peripheral nervous system.

A recent report was the first to describe the interaction between human monocyte-derived DCs and *C. jejuni* (22). In agreement with our data, this study showed that interaction with *C. jejuni* induced upregulation of DC cell surface maturation markers and triggering of NF-κB to stimulate the production of cytokines, including IL-12. Furthermore, this study showed that purified *C. jejuni* LOS was the major stimulant for the increased production of cytokines by DCs (22). Similarly, also murine DCs undergo activation and induce Th1-effector cell responses against *C. jejuni* (39). A subsequent study showed that *C. jejuni*-induced activation of DCs involved cooperative signaling through TLR4-MyD88 and TLR4-TRIF axis (38). We demonstrated that sialic acid expression by LOS is required for the induction of an efficient immune response by DCs (30). This correlates with earlier studies showing that sialic acids play an important role in DC biology (8,9).

In addition, it has been shown that LOS with terminal α2,3-linked sialic acids bind to Sn (21), whereas LOS with terminal α2,8-linked sialic acid binds siglec-7 (6). In this study we have extended these results by investigating whether the sialylated LOS structures differentially affect DC maturation and subsequent T cell polarization. Dendritic cells
express various siglecs on their cell surface, including siglec-3, siglec-7, siglec-9, siglec-10 and siglec-15 (36). Because we were interested in siglec-mediated DC activation by α2,3- and α2,8-sialylated LOS structures that have been demonstrated before to bind to Sn and siglec-7 respectively (6,21), we measured Sn and siglec-7 expression on our human monocyte-derived DCs. On immature DCs we detected siglec-7 expression, whereas Sn was detected on mature DCs only, as demonstrated previously (28). With anti-siglec-7 antibodies we could completely block the binding of α2,8-linked sialylated structures to DCs (Fig. 2E). We could not detect α2,3-linked sialylated PAA binding to DCs, even not after neuraminidase treatment or LPS maturation followed by neuraminidase treatment. The effect of GB11 on DCs could be explained by higher affinity of Sn to GB11 due to composition of the underlying glycan structure, which is different in GB11 compared to the PAA probe. Furthermore, the lipid A tail of GB11 is likely required for DC modulation. In addition to our data (Fig. 2A and 2C), a recent study confirmed Sn binding to α2,3-sialylated LOS structures (21). However, we were not able to block GB11 induced DC maturation with anti-Sn antibodies (data not shown) suggesting that GB11 in addition to Sn may bind other DC expressed lectins. Since DCs express more lectins with a binding preference for α2,3-linked sialylated structures, including siglec-9 (36), we can not exclude that other DC-expressed lectins are responsible for the GB11 induced DC activation. Siglec-7 carries an inhibitory ITIM motif in its cytoplasmic tail. The possibility that LOS binds to siglec-7 and modulates subsequent cellular signalling needs to be further determined in future experiments in which phosphorylation of the ITIM motif as well as activation of signalling proteins downstream siglec-7 could be analyzed. Sn has no internalisation motif, albeit Sn can mediate endocytosis of bacteria possibly in synergy with other receptors (26).
As described previously, the sialic acid binding sites of siglecs expressed on several cell types are masked by endogenous ligands (40). Likewise, we found low binding of sialylated PAA structures to untreated DCs, whereas upon neuraminidase treatment, high binding of sialylated PAA structures was observed. When neuraminidase untreated DCs were incubated with GB16, we did find a Th1-skewed phenotype of DCs and a subsequent Th1 response, suggesting abolishment of the cis-interactions of siglec-7 with its ligand. The incubation of α2,8-sialylated PAA glycans could not abolish this cis-interaction (Fig. 2E), indicating that the conformation of the sialic acid with its underlying glycan and protein is not strong enough to compete for sialylated cis-ligands.

In contrast, the underlying glycan structure in combination with the lipid A part of GB16 LOS could be a better competitor and thus induce trans-interactions with siglec-7. In vivo, unmasking of DC-expressed siglecs can occur when sialic acids on bacteria outcompete cis interactions to mediate trans recognition, depending on avidity effects. In addition, other mechanisms could be involved to disrupt the cis-interactions of siglec-7, like binding of the LOS lipid A moiety to TLR4 to serve as a scaffold to recruit siglec-7. In a wide range of bacteria, intrinsic sialidase activity has been described, especially in strains isolated from various infections (11). Likewise, C. jejuni might possess sialidase activity, and hereby unmask DC-expressed siglecs to create their own binding sites and thus modulate DC function.

Although C. jejuni binding to siglecs has been observed in previous reports, the effects on immune responses of these interactions are poorly understood. As DCs are siglec expressing cells which can be programmed to promote Th1 responses or Th2 responses, we investigated the interaction of C. jejuni expressed LOS with DCs for subsequent T cell polarization. Interestingly, we found that activation of DCs with GB11, targeting
lectins that bind \( \alpha_{2,3} \)-linked sialic acids, induced Th2 skewing. In contrast, targeting
\( \alpha_{2,8} \)-linked sialic acid binding siglecs or lectins capable of binding galactose with
unsialylated LOS induced Th1 skewing. Unfortunately, we were not able to inhibit
cytokine and T helper responses by adding siglec-7 blocking antibody in our assays. The
antibody was only suitable for blocking binding assays (Fig. 2E), as it worked agonistic
in cellular assays causing siglec-7 signaling. The T helper skewing could help to explain
the differences in pathological conditions observed between patients after infection with
LOS structures mimicking GD1a/GM1a gangliosides (GB11) or LOS structures
mimicking GD1c gangliosides (GB16), as the nature of the T helper response determines
the class switching in mature B cells and thus the isotype of the antibodies produced. We
used highly purified LOS, so it is unlikely that our LOS preparations contain
contaminants that could trigger other PRRs on DCs. In addition, we found that TLR2
(data not shown) and TLR4 activation (Fig. 3A) by the sialylated LOS structures did not
reflect the T cell polarization data. This finding was in agreement with the study of Al-
Sayeqh et al. in which they showed that \textit{C. jejuni} activates NF-\( \kappa \)B independently of TLR4
(1).

In conclusion, we found that the molecules IL-12 and OX40L which are involved in DC-
mediated Th differentiation, displayed clear opposite expression levels when comparing
\( \alpha_{2,3} \)- and \( \alpha_{2,8} \)-linked sialylated LOS structures. Accordingly, GB11 with \( \alpha_{2,3} \)-linked
sialic acid promoted Th2 responses, whereas the LOS structure GB16, with \( \alpha_{2,8} \)-linked
terminal sialic acid induced a Th1 response. Together, these data indicate that besides the
presence of cross-reactive antibodies, the glycan structure of LOS and especially the
linkage of the terminal sialic acid, may affect the functional properties of DCs in T cell
polarization and could thus contribute to the different mechanisms of disease involved in GBS.
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References


Legends

Figure 1. Schematic representation of glycan structures from isolates of GBS patients and the cst-II KO mutant. (A) GB11 and GB2, containing terminal $\alpha$2,3-linked sialic acid and the corresponding ganglioside GD1a mimic. (B) GB16 and GB19, containing terminal $\alpha$2,8-linked sialic acid and the corresponding ganglioside GD1c mimic. (C) Cst-II KO, GB2 Cst-II KO and GB19 neuraminidase, lacking sialic acid.

Figure 2. Sn and siglec-7 have different binding specificites towards sialylated LOS structures. (A) Sn-Fc and (B) siglec-7-Fc proteins were pre-complexed with anti-human-Fc PO and added to coated PAA-conjugated glycan structures. As control for the sialic acid specificity of the siglec-Fc proteins, uncoated wells or GalNAc-PAA coated wells were used. Depicted are the relative bindings of pre-complexed siglec-Fc proteins compared to the binding of secondary antibody alone. (C) Sn-Fc (white bars) and siglec-7-Fc (black bars) pre-complexed with anti-human-Fc PO binding to coated GB11 ($\alpha$2,3-linked sialic acid), the Cst-II mutant (without sialic acid) and GB16 ($\alpha$2,8-linked sialic acid) LOS. (D) FACS analysis of immature DCs (thin line) and mature DCs (bold line) stained with Mab 7D2 (anti-Sn, left) or Mab S7.5A (anti-siglec-7, right). Dotted lines are the corresponding negative controls. (E) Binding of biotinylated glycosylated PAA-conjugated probes to immature DCs as measured by flow cytometry. Binding to non-treated DCs (white bar), neuraminidase pre-treated DCs (grey bar) or neuraminidase and anti-siglec-7 pre-treated DCs (black bar) of one representative donor is depicted. The
percentage of cells binding the PAA-glycoconjugates is depicted. For all experiments, one out of 5 representative experiments is shown.

Figure 3. LOS induces DC maturation and TLR4 activation. (A) TLR-4 and MD-2 transfected HEK-cells were triggered with 1, 10 or 100 ng/ml LOS or E. coli LPS for 16 hours. TLR4 activation was determined by analyzing IL-8 concentration in the supernatant by ELISA. DCs were incubated overnight with indicated concentrations of LOS or E. coli LPS. The levels of the co-stimulatory molecules (B) CD80, (C) CD83, (D) CD86 and (E) MHCII expressed on DCs were measured by FACS analysis. The average mean fluorescence intensity of 4 donors is shown.

Figure 4. IL-12 and OX-40L expression levels of DCs are dependent on sialic acid linkage of LOS. To analyze mRNA expression levels, DCs were incubated with 100 ng/ml LPS, GB11, the Cst-II mutant or GB16 for 6 hours. Cells were lysed and by real-time PCR the expression of (A) the Th1 related cytokine IL-12p40 or (C) the Th2 related molecule OX40L were analyzed. *p<0.05. To measure IL-12p70 and OX40L expression at the protein level, DCs were incubated for 24 hours with LPS, GB11, the Cst-II mutant or GB16. (B) The secretion of Th1 related cytokine IL-12p70 was analyzed by ELISA, *p<0.05, and (D) the expression of the Th2 related molecule OX40L on DCs was analysed by flow cytometry. (N=4).

Figure 5. T cell polarization by LOS-triggered DCs. LOS matured DCs with 10 ng/ml of (A) LPS, GB11, the Cst-II mutant and GB16 or (B) DCs matured with 10 ng/ml LPS, GB2, GB2 Cst-II KO, GB19 and GB19 sialidase were incubated with naïve T cells. After
14 days, the intracellular IL-4 and IFNγ production of T cells was measured by FACS analysis. Average percentages of positive cells of 2 donors are shown.
Figure 3

A  TLR-4 activation

- LPS
- GB11
- CstII KO
- GB16
- Medium

B  CD80

Concentration (ng/ml)

CD80 expression (MFI)

LPS
GB11
CstII KO
GB16

C  CD83

Concentration (ng/ml)

CD83 expression (MFI)

LPS
GB11
CstII KO
GB16

D  CD86

Concentration (ng/ml)

CD86 expression (MFI)

LPS
GB11
CstII KO
GB16

E  HLA-DR

Concentration (ng/ml)

HLA-DR expression (MFI)

LPS
GB11
CstII KO
GB16