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TLR4-Mediated Sensing of *Campylobacter jejuni* by Dendritic Cells Is Determined by Sialylation

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In Guillain-Barré syndrome (GBS), ganglioside mimicry of *Campylobacter jejuni* lipo-oligosaccharide (LOS) drives the production of cross-reactive Abs to peripheral nerve gangliosides. We determined whether sialic acid residues in *C. jejuni* LOS modulate dendritic cell (DC) activation and subsequent B cell proliferation as a possible mechanism for the aberrant humoral immune response in GBS. Highly purified sialylated LOS of *C. jejuni* isolates from three GBS patients induced human DC maturation and secretion of inflammatory cytokines that were inhibited by anti-TLR4 neutralizing Abs. The extent of TLR4 signaling and DC activation was greater with LOS of the wild type isolates than with nonsialylated LOS of the corresponding *sialyltransferase* gene knockout (cst-II mutant) strains, indicating that sialylation boosts the DC response to *C. jejuni* LOS. Supernatants of LOS-activated DCs induced B cell proliferation after cross-linking of surface IgGs in the absence of T cells. Lower B cell proliferation indices were found with DC supernatants after DC stimulation with cst-II mutant or neuraminidase desialylated LOS. This study showed that sialylation of *C. jejuni* LOS enhances human DC activation and subsequent B cell proliferation, which may contribute to the development of cross-reactive anti-ganglioside Abs found in GBS patients following *C. jejuni* infection. The Journal of Immunology, 2010, 185: 748–755.

The Guillain-Barré syndrome (GBS) is a postinfectious neuropathy characterized by rapidly progressive muscle weakness. In its most severe form, patients have a paralysis of all cranial, limb, and respiratory muscles for which they require mechanical ventilation for months. Various types of infection may precede GBS, but *Campylobacter jejuni* is the predominant cause, especially in patients with extensive weakness (1, 2). Patients with *C. jejuni*-related GBS frequently have serum Abs to human peripheral nerve gangliosides, which induce multiple neurotoxic effects after passive transfer to mice (3, 4). *C. jejuni* triggers the production of anti-ganglioside Abs in these patients by molecular mimicry. More specifically, some *C. jejuni* strains express lipo-oligosaccharides (LOSs) with similar sialic acid (N-acetyl-neuraminic acid) carbohydrate moieties present in gangliosides (5, 6), resulting in the production of cross-reactive Abs (7). Rabbits sensitized with ganglioside GM1-like *C. jejuni* LOS produce cross-reactive anti-ganglioside Abs and develop a neuropathy and flaccid paresis, similar to patients (8). The variation in ganglioside mimicry is controlled by the sialyltransferase cst-II gene, which is associated with *C. jejuni* from GBS patients (9, 10). Therefore, LOS sialylation is a key factor in the development of GBS after *C. jejuni* infection.

However, in cases with uncomplicated *C. jejuni* gastroenteritis not leading to GBS, the Ab response to LOS is usually very low or even undetectable (5). The mechanism responsible for the induction of a high Ab response to LOS in GBS is unknown. We hypothesized that the immunogenicity of LOS during *C. jejuni* infection, which is required to induce the high titer cross-reactive Abs to gangliosides in GBS, also depends on the sialylation of the LOS. Sialylated LOS is found more frequently in *C. jejuni* isolates from GBS patients than in isolates from enteritis controls (5), and sialic acids are known to modulate the immune response in other types of infection (11). This process may be controlled by dendritic cells (DCs), which constitute one of the first lines of mucosal immune defense and are pivotal in bridging innate and adaptive immunity (12). In the current study, the maturation and cytokine production of human DCs in response to sialylated LOS from three GBS-related *C. jejuni* isolates was compared with the DC response to nonsialylated LOS from the corresponding cst-II mutant strains. In addition, the proliferation of human tonsillar B cells in response to soluble factors from these activated DCs was determined. Our study showed that the sialylation of *C. jejuni* LOS modulated the DC activation that subsequently contributes to stronger proliferation of naive mucosal B cells.

**Materials and Methods**

**Patients and controls**

Pretreatment, acute-phase serum samples were obtained from 27 GBS patients who participated in a previous clinical trial (13). All of these...
patients fulfilled the diagnostic criteria for GBS (14) and had preceding diarrhea and a positive serology for a recent C. jejuni infection (15). Acute-phase serum samples from 20 patients with culture-proven C. jejuni gastroenteritis without GBS were used as controls, as well as serum samples from 30 healthy blood donors. All experiments were performed according to the guidelines of the medical ethical committee of the Erasmus Medical Center, and all patients provided written informed consent.

**Human cell cultures**

PBMCs from healthy volunteers were isolated by density-gradient (Lymphoprep, Greiner Bio-one, Frickenheim, Germany) centrifugation. Monocytes were isolated by positive selection using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. To obtain monocyte-derived DCs, monocytes were cultured in six-well plates for 6 d in the presence of GM-CSF (600 U/ml; BioSource International, Camarillo, CA) and IL-4 (400 U/ml; R&D Systems, Minneapolis, MN). Immature monocyte-derived DCs were washed, seeded at 1 × 10^6 cells/ml in 96-well plates, and stimulated with heat-inactivated C. jejuni whole bacteria or purified C. jejuni LOS fractions for 18 h. Neutralizing mAb to TLR4 (clone HTA125) and isotype control mlgG2a (both used at 25 μg/ml) were purchased from eBioscience (San Diego, CA). Human tonsils were obtained from children who underwent routine tonsillectomy at Sophia Children’s Hospital (Erasmus Medical Center, Rotterdam, The Netherlands). Tonsils were crushed in a 100-m nylon mesh and washed extensively in PBS. After isolation of mononuclear cells by density gradient (Lymphoprep), B cells were isolated by depletion of CD43^+ cells with CD43-microbeads (Miltenyi Biotec). This procedure yielded a population with typically 15 ± 9% CD5^+ cells and <2% contaminating CD3^+ cells (denoted as resting B cells). FACS sorting using a FACSaria cell sorter was performed to further separate this cell population into IgD^+CD27^ and IgD^-CD27^ fractions. B cells isolated from tonsils were cultured in triplicate in a 96-well plate supplemented with or without DC supernatant (1:1) in culture medium and goat anti-human IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). After 48 h, cells were supplemented with 0.5 μCi [3H]thymidine per well and harvested with an automated harvester after an additional 18 h. [3H]Thymidine incorporation in the newly synthesized DNA from proliferating B cells was measured in a β-counter (Wallac). 

### Bacteria and LOS purification

Three C. jejuni strains were isolated from different GBS patients (GB2, GB11, and GB19). The carbohydrate structure of the C. jejuni LOS core Ag was determined previously by mass spectrometry (Table I) (6, 10).

### LOS core structure of C. jejuni LOS from GB2, GB11, and GB19

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Structure</th>
<th>Ganglioside Mimic</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB2-, GB11 WT</td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>GM1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Neu5Ac</td>
<td>Glc</td>
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<tr>
<td></td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>GD1α</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Neu5Ac</td>
<td>Glc</td>
</tr>
<tr>
<td>GB19 WT</td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>GD1c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neu5Ac</td>
<td>Glc</td>
</tr>
<tr>
<td>GB2-, GB11-cst-II mutant</td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>None</td>
</tr>
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<tr>
<td></td>
<td>Neu5Ac</td>
<td>GalNAc-Gal-Hep-Hep</td>
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</tr>
<tr>
<td></td>
<td>Neu5Ac</td>
<td>Gal-Hep-Gal-Hep-Hep</td>
</tr>
<tr>
<td>GB19-cst-II mutant</td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>None</td>
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<td></td>
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<td>Glc</td>
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**FACS analysis**

FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA). The following mAbs were used: CD86-PE, CD80-FITC, CD40-FITC, HLA-DR-PE, CD11c-Cy5, CD5-FITC, CD19-allophycocyanin, IgD-PE, CD3-FITC, CD27-allophycocyanin, and CD43-FITC. All mAbs were purchased from BD Pharmingen (Franklin Lakes, NJ), except for CD80-FITC (Beckman Coulter, Fullerton, CA) and CD40-FITC (Serotek, Oxford, U.K.).

### TLR4 cell lines

HEK293/TLR4 cells were grown in T75 flasks and harvested upon confluence, generally after 6 d of culture. Cells (10^6 cells/ml) were plated into 96-well plates at 100 μl/well and stimulated with LOS or control medium for 24 h. Additional studies on TLR4 were performed with a wild type (WT) macrophage cell line derived from the C3H/HeJ mouse, which has a mutated nonfunctional TLR4; a corresponding C3H/HeJmTLR4 cell line with wild-type TLR4 was used as a control. These cells were seeded in 96-well plates (5 × 10^4 cells/well) and stimulated with LOS or with the TLR3 agonist R848 (3M Pharmaceuticals, St. Paul, MN) as a positive control. IL-6 was measured in the supernatant after 18 h of stimulation.

### Cytokine detection by ELISA

Cytokines present in DC supernatants, including IL-6, IL-8, IL-10, IL-12p40, and TNF-α, were determined by a capture ELISA, according to the manufacturer’s guidelines (BioSource International). Detection of

**Table I. Carbohydrate outer core structure of LOS from C. jejuni strains isolated from GBS patients used in the current study**

<table>
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<tr>
<td></td>
<td>Neu5Ac</td>
<td>Glc</td>
</tr>
<tr>
<td>GB19 WT</td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>GD1cα</td>
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<td></td>
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<tr>
<td></td>
<td>Neu5Ac</td>
<td>Glc</td>
</tr>
<tr>
<td>GB2-, GB11-cst-II mutant</td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>None</td>
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<tr>
<td></td>
<td>Neu5Ac</td>
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<tr>
<td></td>
<td>Neu5Ac</td>
<td>Gal-Hep-Gal-Hep-Hep</td>
</tr>
<tr>
<td>GB19-cst-II mutant</td>
<td>Gal-GalNAc-Gal-Hep-Hep</td>
<td>None</td>
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<td></td>
<td>Neu5Ac</td>
<td>Glc</td>
</tr>
</tbody>
</table>

Molecular structures were determined by mass spectrometry and were published previously (6).

*Glc* is structurally similar to GDP.

Neu5Ac, N-acetylneuraminic acid, Gal, galactose; GalNAc, N-acetyl galactosamine; Hep, heptose; cst-II, sialyltransferase knockout mutant.
B cell activating factor (BAFF) in DC supernatants was performed by the quantikine ELISA kit from R&D Systems.

LOS and ganglioside ELISA

The LOS ELISA was performed as described previously (18). For determining the adsorption of IgG anti-LOS activity, LOS was incubated with serum (diluted 1:100) for 3 h at 4°C. The supernatants were centrifuged and tested for residual anti-LOS activity. Percentage of inhibition was defined as

\[
\text{OD (serum without LOS) } - \text{OD (serum with LOS)} \times 100.
\]

The antiganglioside ELISA was performed as previously described (7).

Statistics

Significant differences in DC activation, cytokine production, and B cell proliferation were evaluated by nonparametric Wilcoxon signed-rank tests or Mann–Whitney U tests (GraphPad Prism 5, GraphPad Software, San Diego, CA). A p value <0.05 was considered significant.

Results

High Ab titers to sialylated C. jejuni LOS in serum from GBS patients

To demonstrate that the production of Abs to sialylated LOS is related to the development of GBS, acute-phase serum samples were tested from GBS patients with a preceding C. jejuni infection (n = 27), enteritis controls with a C. jejuni infection but no GBS (n = 20), and healthy controls without a recent C. jejuni infection (n = 30). The LOS for this serological study was purified from the GB11 WT strain, a C. jejuni isolated from a patient with GBS, which is highly sialylated and mimics the gangliosides GM1 and GD1a (Table I).

High titers of IgM and IgG Abs to the LOS from the GB11 WT were found exclusively in the serum from patients with GBS (Fig. 1A). Based on the extinctions for anti-LOS Abs found in the healthy control group, cut-off values for positivity were defined (Fig. 1A). In the group of GBS patients, 18 (67%) were positive for anti-LOS IgG, and 13 (48%) were positive for anti-LOS IgM. These results showed that the C. jejuni infections resulted in significant Ab responses to sialylated LOS only in the GBS patients.

Next, we determined whether these Abs in the serum from GBS patients are directed to the sialic acid residues in C. jejuni LOS by performing adsorption studies with sialylated LOS from the WT strain and with nonsialylated LOS from the corresponding cst-II knockout mutant strain. cst-II is a sialyltransferase that is essential for the synthesis of α(2-3)– and α(2-8)–linked sialic acids to galactose in the outer core of LOS from C. jejuni (10). The GB11 cst-II knockout mutant strain expresses a truncated LOS outer core without sialic acids and lacks ganglioside mimicry (Table I) (6, 10). IgG Ab activity to GB11 WT LOS in serum from GBS patients was inhibited much more efficiently when preincubated with LOS from the same strain (median 96% inhibition) than with LOS from the GB11 cst-II mutant strain (median 37% inhibition; p = 0.002) (Fig. 1B). These results confirmed that the high Ab activity to C. jejuni LOS in patients with GBS is caused by an immune response mainly directed to the sialylated moieties in LOS.

C. jejuni LOS induces DC activation through TLR4

To further explore the human immune response to C. jejuni LOS, DCs derived from peripheral blood monocytes from healthy blood donors were cultured in the presence of heat-inactivated C. jejuni whole bacteria. The three C. jejuni WT strains isolated from GBS patients were cultured with DCs in multiplicities of infection (MOIs) ranging from 1:2 to 1:100. MOIs of 1:2 already induced upregulation of the DC surface-expressed costimulatory molecules CD80, CD86, and CD40, and HLA-DR, indicating cellular activation (Fig. 2A). In agreement with these observations, highly purified LOS from these C. jejuni WT strains also induced a dose-dependent upregulation of CD80 on these DCs from a starting concentration of 0.1 ng/ml (Fig. 2B). Activation of DCs was also reflected by secretion of inflammatory cytokines in the supernatant (Fig. 3A).

After stimulation of DCs with C. jejuni LOS, there was a dose-dependent increase in the supernatant levels of IL-6, IL-8, IL-10, IL-12p40, and TNF-α (Fig. 4B).

DC activation by microbial patterns is mediated through TLR4 expressed on the cell surface or intracellularly (19). Interactions between microbial Ags and TLR4 lead to downstream activation of NF-kB that regulates the transcription of proinflammatory cytokines, such as TNF-α, IL-8, and IL-12p40 (20). TLR4 is a member of the pattern recognition receptor family and binds to endotoxin or lipid A present in LPS from Gram-negative bacteria. The C. jejuni LOS is devoid of the repetitive oligosaccharides present in the O-specific chain of LPS, but it does contain the evolutionarily conserved lipid A structure. Therefore, it is presumed that LOS may

\[\text{FIGURE 1. High serum reactivity to C. jejuni LOS in GBS patients due to sialylation of LOS. A, Serum IgG and IgM Ab activity to sialylated LOS from GB11 WT, a C. jejuni isolate from a GBS patient, was significantly greater in acute-phase serum from C. jejuni-related GBS patients than in acute-phase serum from C. jejuni enteritis patients without GBS or healthy controls. B, Absorption of IgG Ab activity, expressed as percentage of inhibition, to LOS from GB11 WT in serum samples from GBS patients by preincubation with 100 μg/ml LOS was significantly lower with LOS from the GB11 cst-II mutant strain, indicating that the Abs recognize the sialic acids in LOS from GB11 WT.}\]
also induce DC activation through TLR4. To test this, DCs were cultured with a neutralizing mouse anti-human TLR4 mAb during LOS stimulation. Neutralization of TLR4 inhibited C. jejuni LOS-induced secretion of IL-12p40 by DCs (Fig. 3B). In three independent experiments, each with a different blood donor, the mean inhibition of IL-12p40 secretion by anti-human TLR4 mAb, compared with the isotype control for all LOS concentrations tested, was 20.2 ± 11.9%. The inhibition by this mAb of the secretion of IL-10 in these three experiments was 39.8 ± 14.1%. In addition, DC activation and secretion of IL-6, IL-8, and TNF-α were attenuated by the anti-human TLR4 mAb. To demonstrate that C. jejuni LOS specifically induces cell activation through TLR4, a macrophage cell line from the C3H/HeJ mouse lacking functional TLR4 and the knock-in C3H/HeJmTLR4 cell line with a functional TLR4 were incubated with C. jejuni GB11 WT LOS. Overnight stimulation resulted in no secretion of IL-6 in the WT cell line, but there was a dose-dependent secretion of IL-6 in the C3H/HeJmTLR4 cell line (Fig. 3C). In sum, human DC activation occurs at low concentrations of C. jejuni LOS and is mediated, in part, through TLR4, leading to induction of cytokine secretion.

FIGURE 2. Human DCs are dose dependently activated by C. jejuni whole bacteria and C. jejuni LOS. A, Immature DCs (10^5 cells/well) were incubated for 18 h with heat-inactivated C. jejuni GB11 WT whole bacteria at an MOI of 1:10 (upper panels) or 100 ng/ml of purified LOS from the same strain (lower panels). Cells were stained with mAbs to the costimulatory molecules CD86, CD80, and CD40, as well as to HLA-DR to determine DC activation. Open graphs represent unstimulated DCs, and shaded graphs represent DCs stimulated with C. jejuni whole bacteria or LOS. Representative results of 1 of 15 experiments. B, DCs were stimulated with increasing concentrations of LOS from C. jejuni GB19 WT. Upregulation of CD80 indicating DC activation was observed at 0.1 ng/ml and increased further at higher concentrations of LOS. Open graphs represent unstimulated cells, and shaded graphs represent LOS-stimulated cells. Representative results of 1 of 15 experiments.

FIGURE 3. C. jejuni LOS stimulates DCs to secrete inflammatory cytokines partially through TLR4. A, DCs stimulated with 100 ng/ml LOS from C. jejuni GB11 WT or medium as control were harvested after overnight incubation. Cytokine levels in DC supernatants measured in 10 experiments are expressed as mean ± SD. B, DCs were stimulated with LOS from C. jejuni GB11 WT (control) and incubated concomitantly with a neutralizing mouse anti-human TLR4 mAb or an isotype control mAb. IL12p40 levels in DC supernatants of one experiment, tested in duplo in two dilutions, are presented as mean ± SD. Similar results were observed in two separate experiments from different donors having a variable background IL-12p40 production (described in Results). *p = 0.03. C, C3H/HeJ WT and TLR4 knock-in C3H/HeJmTLR4 cell lines were stimulated with different concentrations of C. jejuni GB11 WT LOS, R848 (positive control), or medium in duplo. Cells were stimulated overnight, and levels of secreted IL-6 in supernatant from stimulated cells are presented as mean ± SD.
**FIGURE 4.** Sialylation of *C. jejuni* LOS increases DC activation and cytokine secretion. A, DCs were stimulated with 1 ng/ml LOS from *C. jejuni* GB11 WT (filled graph), GB11 cst-II mutant strain (graph outlined by black line) or not stimulated (graph outlined by gray line). After overnight incubation, DCs were harvested, and surface expression of CD80 and CD86 was determined by flow cytometry. B, DCs were incubated overnight with 0.1–100 ng/ml of LOS from GB11 WT (●) and GB11 cst-II mutant strains (○). Cytokine levels in supernatant were determined by ELISA. In the lower right panel, 1 ng/ml of LOS from GB2 WT and GB19 WT (black bars) were compared with their corresponding cst-II mutant strains (open bars). *p < 0.05; **p < 0.005, Wilcoxon signed-rank test.

*C. jejuni* LOS carbohydrate moiety modulates DC activation

To assess whether the carbohydrate moiety of *C. jejuni* LOS influences the DC response, LOS from the three WT strains and corresponding cst-II mutant strains were compared for their ability to induce DC activation and secretion of cytokines. The expression of surface costimulatory molecules on DCs was greater after incubation with sialylated LOS from the GB11 WT strain compared with nonsialylated LOS from the GB11 cst-II mutant strain (Fig. 4A). A similar greater expression of these markers on DCs was found after stimulation with LOS from GB2 and GB19 WT strains compared with LOS from the GB2 and GB19 mutant strains. In addition, the secretion of pro- and anti-inflammatory cytokines was significantly greater when DCs were stimulated with LOS from WT strains compared with their cst-II mutant strains (Fig. 4B). An ~10-fold greater concentration of LOS from the GB11 cst-II mutant compared with the GB11 WT was needed to obtain similar levels of induced cytokine secretion. The cst-II mutants of two other GBS-associated tested *C. jejuni* isolates showed similar results (Fig. 4B, lower right panel). These data indicated that the carbohydrate moiety of *C. jejuni* LOS modulates human DC activation and cytokine secretion.

**Sialylation of *C. jejuni* LOS modulates DC response**

The observations presented above demonstrated that the carbohydrate structure of LOS modulates the DC response and suggested that the sialylation of LOS mediated this effect. To further determine the modulating effects of sialylation, LOS from the *C. jejuni* GB19 WT was incubated with neuraminidase (NA) from *Arthrobacter ureafaciens*, cleaving off the α(2-8)-linked and terminal α(2-3)-linked sialic acid residues. The desialylation of LOS from the GB19 WT by this NA treatment (LOS from *C. jejuni* GB19 WT treated with neuraminidase [GB19 WT NA]) was confirmed by showing that the binding of a mAb to GD3 was lost, whereas the LOS gained reactivity to a serum with Abs to asialo-GM1 (Supplemental Fig. 3). DCs were incubated with this LOS overnight, and the effect was compared with that of LOS from GB19 WT and cst-II mutant strains. DC activation and TNF-α secretion induced by LOS from the GB19 WT NA was lower than that induced by LOS from the GB19 WT and just as low as that induced by LOS from the GB19 cst-II mutant (Fig. 5A, 5B). The secreted levels of IL-6, IL-8, IL-10, and IL-12p40 were also lower, similar to the levels seen after stimulation with LOS from the cst-II mutant. These data demonstrated that DC activation is enhanced by sialylation of the LOS from *C. jejuni*.

To assess whether the enhanced cell activation by sialylated LOS was specifically due to differences in TLR4 signaling, a TLR4-transfected cell line (HEK293/TLR4) was used that secretes IL-8 in response to TLR4 ligation (20). Nonsialylated LOS from the GB19 cst-II mutant and GB19 WT NA induced significantly lower IL-8 levels compared with LOS from the GB19 WT (Fig. 5C). These data suggested that the sialylation of *C. jejuni* LOS modulates activation of DCs through differences in LOS/TLR4 signaling.

**Sialylation of *C. jejuni* LOS influences proliferation of naive B cells through DC-derived soluble factors**

Supernatants from DCs stimulated with microbial products are known to enhance B cell responses (21). In light of the previous observations, we hypothesized that the DC activation by *C. jejuni* may also affect B cell responses. A proliferation assay was designed in which human tonsillar B cells were cultured in the presence of DC-derived supernatant. These mucosal B cells were stimulated with low concentrations of goat anti-human IgM to cross-link BCRs, mimicking Ag recognition as a first signal for B cell activation. Stimulation of B cells with *C. jejuni* LOS alone with concentrations ≤10 μg/ml had no effect, consistent with the absence or low TLR4 expression in human nonactivated B cells (22). Next, B cells were cultured in the presence of supernatant from DCs previously stimulated with *C. jejuni* LOS, which resulted in an enhancement of B cell proliferation (Fig. 6A). Because centroblasts previously activated in vivo may proliferate without further exogenous stimulation, flow cytometric sorting of naïve IgD⁺CD27⁺ and IgD⁺CD27⁻ centroblasts was performed. Comparison of the two subsets derived from CD43⁻ cells showed that the naive B cell population (CD43⁻ IgD⁺CD27⁻) proliferated in the presence of DC supernatant (Fig. 6A). The extent of B cell proliferation correlated with the cytokine response and activation of DCs by LOS (Fig. 6B), and it increased dose dependently with the LOS concentrations (Fig. 6C). Supernatants from DC cultures stimulated with desialylated LOS from GB19 WT NA and GB19 cst-II mutant were less effective in enhancing B cell proliferation (Fig. 6C). These results demonstrated that *C. jejuni* LOS influences mucosal B cell proliferation through DC activation, even without cell–cell interactions between DCs.
and B cells. Sialylated LOS from *C. jejuni* enhanced this proliferation of B cells through activation of DCs.

**Contributing factors for DC-induced B cell proliferation**

To determine which DC-derived soluble factor(s) enhance B cell proliferation, inhibition studies were performed for candidate molecules. Heating the supernatant to 80°C abrogated its stimulatory effect. This confirmed our previous observation that the thermolabile LOS does not interact directly with B cells. The observed thermo-instability suggests that temperature-sensitive molecules, such as proteins, may be involved. In addition, freeze-thawing had mild attenuating effects. Next, the role of candidate cytokines known to influence B cells was investigated. Supplementing culture media with neutralizing Abs to IL-6 and IL-12 did not inhibit or abrogate the effect (Supplemental Fig. 4). These results implied that the enhanced B cell proliferation orchestrated by *C. jejuni*-stimulated DCs is likely induced by heat unstable factors and does not seem to be the result of common B cell stimuli, such as IL-6 and IL-12 (21, 23). BAFF is a candidate factor produced by DCs that stimulates B cell proliferation, but supernatants from DCs stimulated overnight by *C. jejuni* LOS in our assays did not contain detectable levels of BAFF (detection limit: 35 pg/ml).

**Discussion**

The current study demonstrated that the sialylation of *C. jejuni* LOS enhanced human DC activation via TLR4 signaling and that supernatants from activated DCs induced proliferation of mucosal B cells. *C. jejuni* strains isolated from GBS patients expressed sialylated LOS more frequently than did *C. jejuni* strains from patients with uncomplicated gastroenteritis (5). The sialylated outer core of *C. jejuni* LOS determines the specificity of the cross-reactive Abs to gangliosides and, thereby, the site of nerve damage and clinical phenotype in GBS. The current study showed that sialic acids in *C. jejuni* LOS boost DC activation and stimulate the subsequent B cell response. This enhancement may explain the high Ab activity to sialylated LOS in serum from patients who develop GBS after a *C. jejuni* infection. Breaking of the natural tolerance to host gangliosides may result in a cross-reactive Ab response to peripheral nerves and subsequent neuropathy.

*C. jejuni* infections are usually resolved before an adaptive immune response is mounted (24), indicating a key role of the innate immune response in clearing the infection. *C. jejuni* strains expressing sialylated LOS were found to invade intestinal epithelial cells significantly better compared with strains with nonsialylated LOS (16). DCs reside directly under the epithelial cell layer; therefore, they are well positioned to interact with invasive and noninvasive pathogens (25). Previous studies showed that *C. jejuni* strains induce an upregulation of costimulatory cell surface markers, including CD40, CD80, CD86, and HLA-DR, and secretion of inflammatory cytokines, including IL-6, IL-8, IL-10, IL-12p40, and TNF-α. Interestingly, high levels of these cytokines are also found in serum from patients in the acute stage of GBS (27, 28).

For most bacteria, the surface sialic acids may provide a protective mechanism against the immune response from the host. In *Neisseria meningitidis*, sialylation of LOS results in a reduced phagocytosis by DCs, without influencing cytokine secretion (29). Sialylation of LOS may also inhibit complement activation, as was demonstrated for *Neisseria gonorrhoeae* (30). Sialylation of LOS helps *C. jejuni* to invade intestinal epithelial cells (16). Despite these effects that may increase the survival or invasiveness of bacteria, proinflammatory effects of sialylation have also been described. For example, the sialylation of LOS from *N. meningitidis* resulted in enhanced siglec-dependent macrophage-mediated phagocytosis (31). In addition, *Haemophilus influenzae* WT strains expressing sialylated LOS induced a more extensive immune response than did the nonsialylated mutant strains in an in vivo model of otitis media in chinchillas (11). Therefore, sialic acids seem to have a dual role in microbial–host interactions and do not always function as anti-recognition molecules. The current study showed that sialylated LOS from *C. jejuni* WT strains induced stronger DC activation and subsequent B cell proliferation than did desialylated LOS from enzyme-treated or sialyltransferase cst-II mutant strains. These proinflammatory effects may contribute to the exaggerated Ab response to sialylated LOS in patients with GBS compared with the low Ab response to nonsialylated LOS after uncomplicated...
C. jejuni gastroenteritis. This enhanced Ab response may be disadvantageous for C. jejuni, although the possible additional anti-inflammatory effects of sialylation may result in an overall advantage for bacteria survival.

TLR4 engages LOS via lipid A, which leads to secretion of several inflammatory cytokines through activation of NF-kB transcription (20). Previous studies demonstrated NF-kB transcription in human DCs upon stimulation with C. jejuni LOS (26). Accordingly, the observed secretion of IL-12p40 and other cytokines induced by GBS-associated C. jejuni LOS in the current study was reduced when binding to TLR4 was prevented. HEK/TLR4 cells stimulated with C. jejuni LOS secreted high levels of IL-8, further indicating that cellular activation is mediated by direct interaction of C. jejuni LOS with TLR4. In agreement with these findings, C3H/HeJ mice lacking functional TLR4 show a diminished production of anti-ganglioside Abs after immunization with ganglioside-mimicking LOS from C. jejuni (32). Interestingly, IL-8 production by HEK/TLR4 cells was significantly reduced after stimulation with non-sialylated LOS from GB19 WT NA or the corresponding cst-II mutants) at concentrations ranging from 0.1–100 ng/ml. DC supernatant was used to stimulate B cells. B cell proliferation was assessed by [3H] thymidine incorporation. Linear regression lines with 95% confidence intervals (dotted) are shown. Spearman correlations were significant at p < 0.0001 for TNF-α and IL-6 and at p = 0.002 for IL-12p40. Results are from one of three representative experiments. C. DCs were stimulated with sialylated LOS from GB19 WT or with nonsialylated LOS from GB19 cst-II mutant strains or from GB19 WT treated with NA (GB19 WT NA). A range of LOS concentrations was used to demonstrate a dose-dependent effect. Supernatant from DCs stimulated with GB19 WT LOS induced greater B cell proliferation compared with GB19 cst-II mutant strain and GB19 WT NA LOS. Representative results from one of two experiments.

Activation of B cells is required to produce a cross-reactive Ab response to gangliosides and is an essential step in the pathogenesis of C. jejuni–related GBS. The relatively low affinity of the anti-ganglioside Abs and the absence of sustained serum titers in GBS (3) may be compatible with a T cell-independent immune response. Recently, a mechanism was described in which activated DCs provide direct B cell help in the absence of T cells (21, 23, 35–37). Our observation that C. jejuni LOS-activated DCs induced the proliferation of human tonsillar B cells via soluble factors, in which sialylation of LOS further enhances this effect, suggests a comparable mechanism for the Ab response to infection with C. jejuni. Coincubation with blocking mAbs to IL-6, IL-12, or a combination of both did not inhibit this B cell proliferation. In a previous study in which DCs were stimulated with Escherichia coli LPS, neutralizing mAbs to IL-6 and IL-12 abolished a similar effect on B cells (21). However, in this study, T cell help was also added to DC supernatants. DCs can directly induce T cell-independent proliferation and class-switching in B cells via expression of BAFF and a proliferation-inducing ligand (APRIL) and skew adaptive-immune responses by TLR-dependent mechanisms (36, 37). Recently published data demonstrated that BAFF is not a crucial factor for anti-ganglioside Ab production in a mouse model (38). In accordance, we were unable to detect BAFF production by human DCs in the current study. Further studies are required to demonstrate which soluble factors are

**FIGURE 6.** B cell proliferation is modulated by LOS sialylation and DC-derived solubles and correlates with cytokine levels. A. Human tonsils were depleted of CD43+ cells by magnetic labeling. The cells were further sorted by flow cytometry into IgD+CD27+ and IgD−CD27+ fractions. B cells (2 × 10^5 cells/well) were cultured on plates coated with polyclonal anti-IgM in the presence or absence of 1:1 supernatant from DCs. Supernatant used to stimulate B cells was from various experiments in which DCs were stimulated or not with C. jejuni LOS in different concentrations. After 48 h of coculture, proliferation was determined by [3H] thymidine incorporation. B. DCs were stimulated with LOS from different C. jejuni strains (GB2, GB11, GB19 WT, and cst-II mutants) at concentrations ranging from 0.1–100 ng/ml. DC supernatant was used to stimulate B cells. B cell proliferation was assessed by [3H] thymidine incorporation. Linear regression lines with 95% confidence intervals (dotted) are shown. Spearman correlations were significant at p < 0.0001 for TNF-α and IL-6 and at p = 0.002 for IL-12p40. Results are from one of three representative experiments. C. DCs were stimulated with sialylated LOS from GB19 WT or with nonsialylated LOS from GB19 cst-II mutant strains or from GB19 WT treated with NA (GB19 WT NA). A range of LOS concentrations was used to demonstrate a dose-dependent effect. Supernatant from DCs stimulated with GB19 WT LOS induced greater B cell proliferation compared with GB19 cst-II mutant strain and GB19 WT NA LOS. Representative results from one of two experiments.
successful for the proliferation of B cells by supernatants from \textit{C. jejuni}-activated DCs and whether B cells show gene rearrangements and mature into Ab-producing plasma cells in this particular context. In addition, future studies should account for the various enteric and tonsillar B cell subsets, which may differ in the expression of homing receptors (39).

In conclusion, our study showed that sialylation of LOS in \textit{C. jejuni} is a key determinant in the extent of human DC activation and subsequent B cell proliferation. This feature of \textit{C. jejuni} LOS may explain the high titers of serum Abs to LOS found in patients with GBS that cross-react with peripheral nerve gangliosides.

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Disclosures
The authors have no financial conflicts of interest.

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