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Discovery of Lipids from *B. longum* subsp. *infantis* using Whole Cell MALDI Analysis

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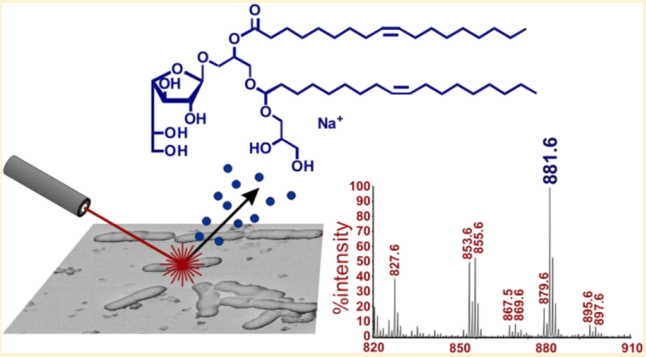
Supporting Information

ABSTRACT: Bifidobacteria are dominant members of the microbial community in the intestinal tract of infants, and studies have shown that glycolipids extracted from the cell surface of these bacteria elicit beneficial immune responses. Accordingly, the identification and structural characterization of glycolipids from the cell wall of bifidobacteria is the first step in correlating glycolipid structure with biological activity. Using whole cell MALDI as a screening tool, we herein present for the first time the identification and structural elucidation of the major polar lipids from *Bifidobacterium longum* subs. *infantis*. The lipids identified include an unprecedented plasmenyl cyclophosphatidic acid and a mixed acetal glycolipid, with the latter subsequently being isolated and found to suppress the innate immune response.



INTRODUCTION

Bifidobacteria are dominant members of the microbial community in the intestinal tract of infants and are considered to be beneficial to human health. Although much research in this area is still in its infancy, bifidobacteria are thought to exhibit anticancer properties, alleviate the symptoms of irritable bowel syndrome, and reduce atopic disease.[1,2] For these reasons, *B. longum* has been used as a probiotic to improve health,[3] and how the bacteria exert their biological effects is a growing field of research.[4] Despite this, there is little published literature on the composition and structure of bifidobacterial glycolipids in comparison to that on other bacterial species.[5] Veerkamp and co-workers identified the presence of mono-, di-, and trigalactosyldiglyceride derivatives as the major constituents of *Bifidobacterium bifidum* var. *pennsylvanicus*,[6] and more recently, Novik and co-workers[7] analyzed the principal glycolipids from several bifidobacterial species and strains using thin-layer chromatography (TLC) and found increased antiglycolipid antibody responses against glycolipid fractions. However, in both studies, only limited detail pertaining to glycolipid structure was presented.

Given the growing interest in the beneficial effects of bifidobacteria, we thus sought to analyze the glycolipid content of the bifidobacterial cell wall. One way to achieve this is to use a variety of NMR spectroscopy techniques and more traditional lipid and carbohydrate analyses; however, we also envisioned the use of whole cell matrix-assisted laser desorption/ionization (MALDI) analysis as a means to rapidly identify key molecular ions correlating to unprecedented glycolipids. While the screening of extracted bacterial lipids or glycolipids using MALDI is now commonplace,[8] whole bacterial cell MALDI analysis allows for efficient lipid fingerprinting.[9] Whole cell MALDI, however, has not been used for the identification of novel glycolipid structures. To this end, we explored the use of whole cell MALDI in combination with collision induced dissociation (CID) for the discovery of novel cell wall glycolipids from *B. longum* subs. *infantis* (ATCC 15702). Following identification, the major lipids were then isolated and subsequently probed for their immunomodulatory activities. Accordingly, this work represents a first step toward understanding the correlation between bifidobacterial cell wall composition and biological activity.

RESULTS AND DISCUSSION

To begin our studies into the glycolipid composition *B. longum* subsp. *infantis*, we first subjected the bacteria to whole cell MALDI analysis so as to rapidly determine the cell wall glycolipid profile and the presence of unique glycolipids. Heat-killed cells of *B. longum* subsp. *infantis* were thus suspended in water and were cocrystallized in a 2,5-dihydroxybenzoic acid (DHB) matrix for analysis by MALDI mass spectrometry (Figure 1). As illustrated

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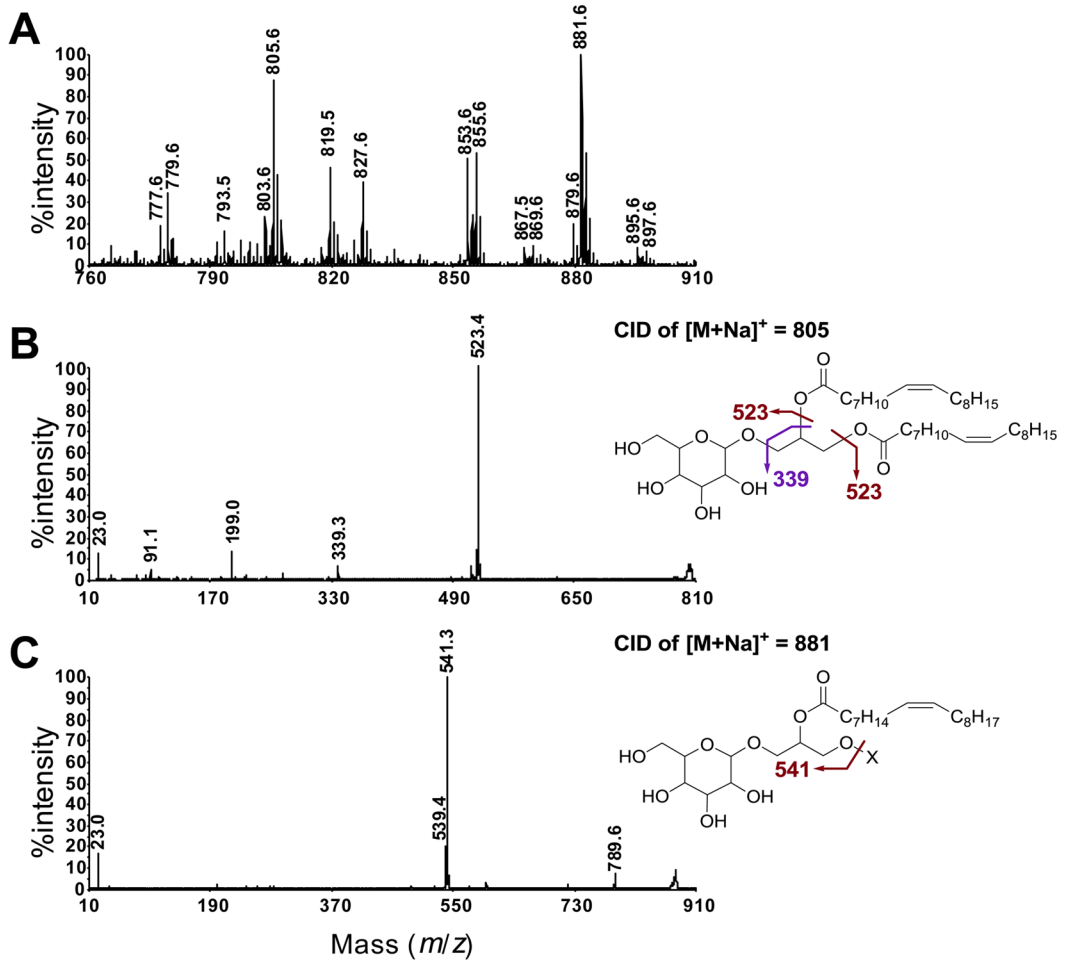

Figure 1. (A) MALDI spectrum of whole cells of heat-killed *B. longum* subsp. *infantis*. (B) CID of *m*/*z* 805. (C) CID of *m*/*z* 881.

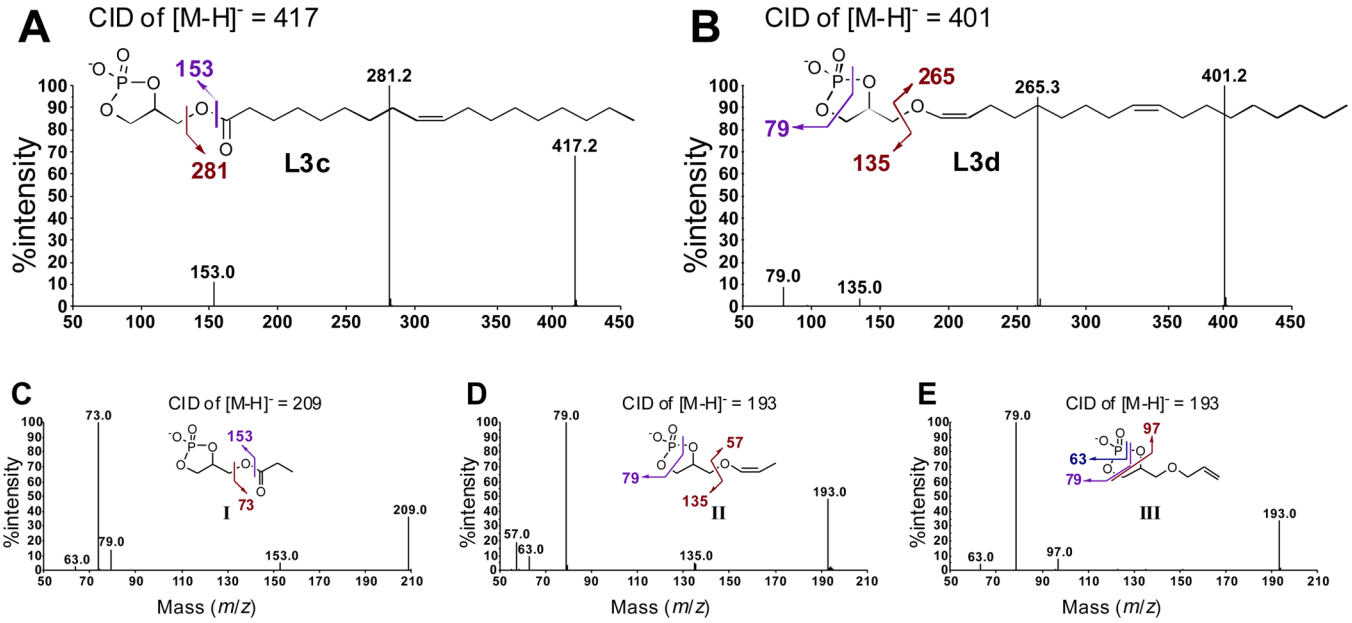

Figure 2. ESI-MS^2 spectra of the isolated products (A) cyclophosphatidic acid **L3c** and (B) plasmalogen **L3d** and synthetic samples of (C) propanoylcyclophosphatidic acid, (D) prop-1-enylcyclophosphatidic acid, and (E) prop-2-enylcyclophosphatidic acid.

(Figure 1A), the mass spectrum showed several major molecular ions with three signals (*m*/*z* 779, 805, and 819) corresponding to a monoglycosyldiglyceride (MGDG) containing different fatty acyl chains (16:0/18:1, 18:1/18:1, and 18:1/19:1). Collision-induced dissociation (CID) of the ions with *m*/*z* 805 gave a major fragment ion peak at *m*/*z* 523, corresponding to loss of

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oleic acid, thus confirming the presence of MGDG (Figure 1B). The most abundant molecular ion in the mass spectrum (m/z 881), however, did not correlate to any known glycolipid structure, and the CID of these ions gave a major fragment with m/z 541, which suggested a monoacylated glycosyl glycerol (Figure 1C). The molecular ions at m/z 855 and 827 also showed this fragmentation pattern and thus appeared to belong to the same class of compounds. In this way, we were able to rapidly identify the two major classes of glycolipid in *B. longum* subs. *infantis*: the first being a MGDG and the second being an as yet unidentified, and potentially more complex, glycolipid.

To continue our investigations into the nature of the lipids found within *B. longum* subsp. *infantis*, the bacteria were cultured in 18 L of medium, isolated by centrifugation, and then freeze-dried. Extraction of the resulting cell mass (6 g) using chloroform/methanol/water yielded 480 mg of crude lipid, and analysis of the extracts by TLC ($CHCl_3$/MeOH (6/1); see the Supporting Information) revealed three lipid fractions (**L1**−**L3**) and two glycolipid fractions (**GL1** and **GL2**). The compounds were then separated by silica gel column chromatography and analyzed using ESI-MS and NMR, in addition to lipid, sugar, and linkage analysis. This allowed for the ready identification of **L1** (R_f = 0.72) as oleic acid (m/z 281.2484), and the slightly more polar lipid **L2** (R_f = 0.62) as 10-hydroxyoctadecanoate (m/z 299.2579). Both structures matched previously published data.[10] The much more polar fraction **L3** (R_f = 0.05) was found to contain a mixture of diacylglyceryl phosphates, including 1,2-dioleoylglycerol 3-phosphate (**L3a**) and the plasmalogen 1-(1Z,9Z-octadeca-1,9-dienyl)-2-oleoylglycerol 3-phosphate (**L3b**), which matched data reported in the literature.[11] In addition, the presence of the plasmalogen was not surprising, as enol ether containing lipids have been observed in bifidobacteria previously.[12] In addition there were two unusual minor lipids, **L3c** and **L3d**, with principal negative mode ESI-HRMS signals m/z 417.2421 for $[C_{21}H_{38}O_5P]^-$ and m/z 401.2466 for $[C_{21}H_{38}O_6P]^-$, respectively, which suggested that both lipids were phosphatidic acids (cPAs).

To establish the structures of the cPAs, CID of both the natural products and chemically synthesized MS standards were performed. CID of **L3c**, with parent ion m/z 417.2421, produced a readily identifiable fragmentation pattern (Figure 2A), with two main fragments m/z 281 (oleate) and m/z 153 (cyclo-glycerylphosphate) in accordance with that of oleoyl cyclo-phosphatidic acid (C18:1-cPA).[13] On the other hand, cPA **L3d** gave a parent ion with m/z 401.2466 (Figure 2B), which dissociated into four fragments: m/z 265 (C18:1 enolate), m/z 135 (methylenedioxaphospholane), m/z 97 ($H_2PO_4^-$), and m/z 79 (PO_3^-). This fragmentation pattern suggested the presence of an enol ether and enabled us to assign **L3d** as C18:1-plasmenyl-cPA. To corroborate our assignments, we prepared three synthetic cPAs to compare fragmentation patterns with those of the isolated natural products (Figures 2C−E). As illustrated, the CID fragmentation pattern of the ester derivative (**I**; Figure 2C) led to cleavage at either side of the ester linkage and the formation of the propionate (m/z 73) and cycloglycerylphosphate ions (m/z 153), as was observed in the isolated cPA ester **L3c**. In comparison, CID of the synthetic cPA enol ether **II** (Figure 2D) saw cleavage of the phosphate and the glyceryl ether linkage to produce ions with m/z 79 and 135, respectively. This fragmentation pattern was consistent with the CID of the isolated plasmenyl cPA **L3d**, thereby confirming the presence of the hitherto unprecedented cPA enol ether linkage. To further support our assignment, the synthetic cPA ether **III** was also prepared and subjected to CID (Figure 2E). Here fragmentation of the phosphate occurred to produce the $H_2PO_4^-$, PO_3^-, and PO_2^- fragments, while the ether linkage remained intact. Remarkably, plasmenyl cPA **L3d** has never been observed in nature before, and while acyclic enol ether containing lipids (plasmalogens) are commonly detected in animal tissues and anaerobic bacteria,[14] they have only recently been observed in bifidobacteria.[12] The presence of cPAs in *B. longum* is even more notable, as this is the first time they have been identified in bacteria.

Following the identification of the lipids **L1**−**L3** present in *B. longum* subsp. *infantis*, our attention then turned toward the structural elucidation of the purified glycolipids **GL1** and **GL2**. As mentioned previously, whole cell MALDI gave some indication about the two main classes of glycolipid present, and lipid, sugar, and linkage analysis in addition to R_f values, yields, HRMS, and NMR was used to provide a more thorough overview of the chemical characteristics of the individual glycolipids (Table 1). **GL1** and **GL2** were then fully characterized using ^{1}H and ^{13}C NMR, and assignments were made on the basis of COSY, HSQC, and HMBC experiments (Tables 2 and 3). From these data, the assignment of **GL1** as an MGDG proved to be correct. The mobility of the glycolipid on TLC supported an MGDG structure, and sugar analysis allowed for the sugar residue to be identified as a terminal galactopyranose (t-Gal*p*). Lipid analysis confirmed that oleic acid accounted for most of the lipid content, and HRMS of **GL1** (m/z 805.5770 for $[C_{45}H_{82}O_{10} + Na]^+$) was consistent with the presence of a monoglycosyldiglyceride. Finally, NMR analysis confirmed the presence of a β-linked galactopyranose ($\delta_{H\text{-}1}$ 4.24 ppm, $J_{1,2}$ = 7.8 Hz and $\delta_{C\text{-}1}$ 104.7 ppm and the small $J_{3,4}$ = 3.3 Hz).[15] Accordingly, **GL1** was assigned as a β-D-galactopyranosyl-diglyceride (Figure 3). All proton and carbon NMR signals were

Table 1. Chemical Characteristics of Isolated Compounds

	GL1	GL2
R_f	0.56	0.41
yield[a]	1.0	1.8
neutral sugar[a]		
D-Gal	10.6	15.6
D-Glc	0.8	
total	11.4	15.6
linkage analysis[b]		
t-Gal*f*		1.0
t-Gal*p*	0.8	
t-Glc*p*	0.2	
fatty acid content[a]	32.0	54.7
fatty acids[a]		
14:0		1.7
DMA 16:1n-7		1.0
16:0	6.5	13.3
16:1n-7	2.3	4.7
DMA 18:1n-9		36.4
18.00	4.3	3.1
18:1n-9	41.6	23.4
18:1n-7	38.9	9.7
19:0br		3.1
9,10-Mt 18:0	2.9	
HRMS	805.5770	881.6349

[a]In wt %. [b]Molar ratios. [c]Fatty acids present under 1% are not shown.

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Table 2. Selected NMR Data (ppm) and Peak Assignments for GL1

GL1	*β*-t-Gal*p*	4.24 (H1)	3.55 (H2)	3.50 (H3)	3.89 (H4)	3.51 (H5)	3.81 (H6a)	3.76 (H6b)
		104.7 (C1)	71.8 (C2)	73.7 (C3)	69.3 (C4)	75.6 (C5)	61.8 (C6)	

Table 3. Selected NMR Data and Peak Assignments for GL2

no.	δ_H (ppm)[a]	δ_C (ppm)	COSY (H–H)	HMBC (H–C)
1	4.94 (s)	108.6	2	3,4,7
2	3.99 (dd, 1.0, 2.4)	80.9	1,3	
3	4.01 (dd, 2.4, 4.7)	78.2	2	
4	4.03 (dd, 3.0, 4.7)	85.1	5	3
5	3.79 (m)	71.5	4,6	
6	3.67 (m)	63.7	5	4
7a	3.65 (m)	66.0	7b,8	1,8,9
7b	3.82 (m)		7a,8	1,8,9
8	5.14 (p, 5.2)	71.8	7a,7b,9a,9b	7,9,31
9a	3.62 (m)	64.2	8,9b	7,8,13
9b	3.79 (m)		8,9a	7,8,13
10a	3.48 (dd, 10.0, 6.2)	67.0	10b	11,12,13
10b	3.63 (m)		10a	11,12,13
11	3.81 (m)	71.2	10a,10b,12a,12b	
12a	3.58 (m, 11.4, 6.1)	63.8	11,12b	10,11
12b	3.68 (m)		11,12a	
13	4.54 (t, 5.9)	104.2	14	9,10,14
14	1.62 (m)	25.2	13	15

[a]Peak multiplicities and coupling constants (in Hz) are provided in parentheses.

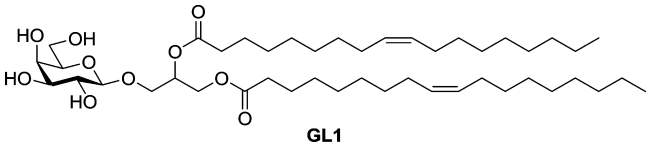

Figure 3. Structure of **GL1**.

also found to be in accordance with literature data for this compound.[8d] The presence of *β*-D-galactopyranosyl-diglyceride is not surprising, as it is common in both plants and bacteria[16] and has been previously observed in *B. bifidum* var. *pennsylvanicus*.[6]

As suggested by the whole cell MALDI results, glycolipid **GL2** appeared to be of novel composition, and its unique mobility on TLC, intermediate to that of mono- and diglycosyldiglycerides, further piqued our interest in this compound. Constituent sugar and linkage analyses of **GL2** revealed the presence of terminal galactofuranose (t-Gal*f*), which is a configuration previously observed in bifidobacterial glycolipids isolated by Veerkamp in the 1970s,[6] and the presence of this sugar residue was readily confirmed by ^{1}H NMR, which showed a singlet at δ 4.94 ppm with a small vicinal coupling constant (<2 Hz) corresponding to H-1 of a *β*-Gal*f* moiety.[17] The assignment was confirmed by ^{13}C chemical shift and direct spin–spin coupling constant ($^1J_{C,H}$) of the anomeric carbon (108.6 ppm, 172 Hz)[18] and by the HMBC between the anomeric proton and C-4 (δ 85.1 ppm). All other sugar resonances (^{1}H and ^{13}C), assigned on the basis of the COSY, HSQC, and HMBC 2D NMR spectra, were also consistent with a *β*-t-Gal*f* moiety.[18]

A unique characteristic of **GL2**, however, was the positive ion HRMS (*m*/*z* 881.6349), corresponding to $[C_{48}H_{90}O_{12} + Na]^+$, a molecular formula too high in oxygen for a monoglycosyldiglyceride. This proposed formula, however, fits an assumed condensation product of a hexose, two glycerols, a fatty acid, and a lipid ether with an additional degree of unsaturation. The latter substructure was confirmed by lipid analysis (Table 1), which in addition to the anticipated palmitic and oleic acids revealed high amounts of dimethyl acetals (DMAs). DMAs typically arise from enol ether type lipids (e.g., plasmalogens) following methanol/acid treatment.[10a,19] Furthermore, NMR spectroscopy of **GL2** showed an unusual triplet at δ_H 4.54 ppm (J = 5.9 Hz) with an associated δ_C 104 ppm. COSY and HMBC experiments then revealed that this proton was part of a long-chain acetal, thus explaining the observed DMAs in the lipid analysis.

The connectivity of the acetal center was readily established by HMBC analysis, which showed correlations between the acetal center and two CH_2– groups of two distinct glycerols. One glycerol was acylated at the 2-position (δ_H 5.20 ppm), as evidenced by an HMBC with a lipid carbonyl (δ_C 174.2), while the 1-position showed an HMBC with the H-1 of the galactofuranose. The second glycerol did not show any further HMBCs and thus appeared to contain two free hydroxyl groups. Taken as a whole, this analysis allowed us to propose the structure of **GL2** to be a 1-*β*-D-galactofuranosyl-3-glyceroplasmalomonoglyceride (Figure 4).

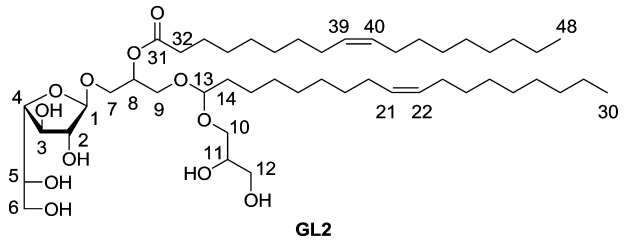

Figure 4. Structure of **GL2**.

To confirm the structure of **GL2**, ESI-MS^2 was used. The major molecular ion peak in positive ion mode (*m*/*z* 881.6) was fragmented using CID, and the most abundant fragment ion (*m*/*z* 541.3) was attributed to the loss of glycerol and acetal (Figure 5A). Additional fragments of *m*/*z* 789.6, 719.6, 259.1, and 203.1 were also detected and could be explained by the loss of glycerol, galactose, fatty acetal, and fatty acid, respectively. In addition, MS^2 in negative ion mode (Figure 5B) showed the quasi molecular ion of *m*/*z* 893.6 $[M + Cl]^-$ and loss of HCl (*m*/*z* 857.6 $[M - H]^-$). CID fragmentation of the *m*/*z* 893.6 $[M + Cl]^-$ ion provided a major fragment at *m*/*z* 281.2 corresponding to the oleate anion. Higher mass fragments consisted of *m*/*z* 431.3 (loss of galactose and oleic acid), *m*/*z* 575.4 (loss of oleic acid), and *m*/*z* 593.4 (loss of oleoyl). The loss of these fragments confirmed the connectivity of the individual components and unequivocally corroborated the unusual structure of **GL2**.

Although acetal-containing glycolipids have been previously isolated from natural sources, thus far, these have been restricted to structures carrying the acetal on the C-6 position of a galactose residue, such as plasmalogalactosylceramides (isolated from human brain),[20] plasmalogalactosylalkylglycerol from equine brain,[21] and glyceroplasmalopsychosine from bovine brain.[22] Glycerol acetals have been observed before: for example, in

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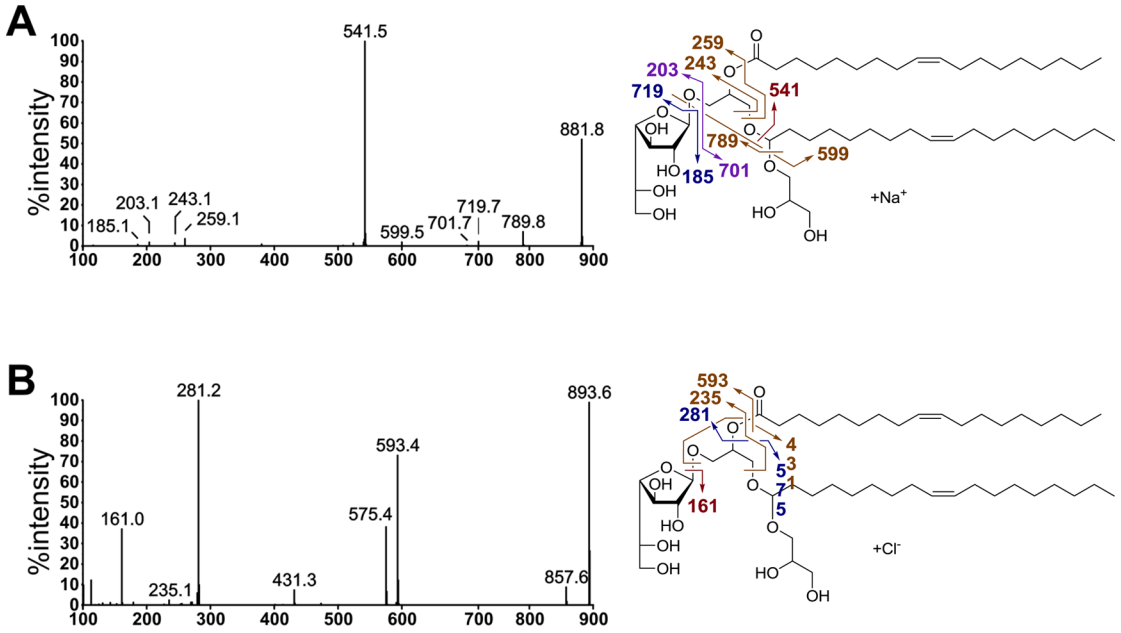

Figure 5. MS^2 of **GL2**: (A) positive ion mode ($[M + Na]^+$ m/z 881.8); (B) negative ion mode ($[M + Cl]^-$ m/z 893.6).

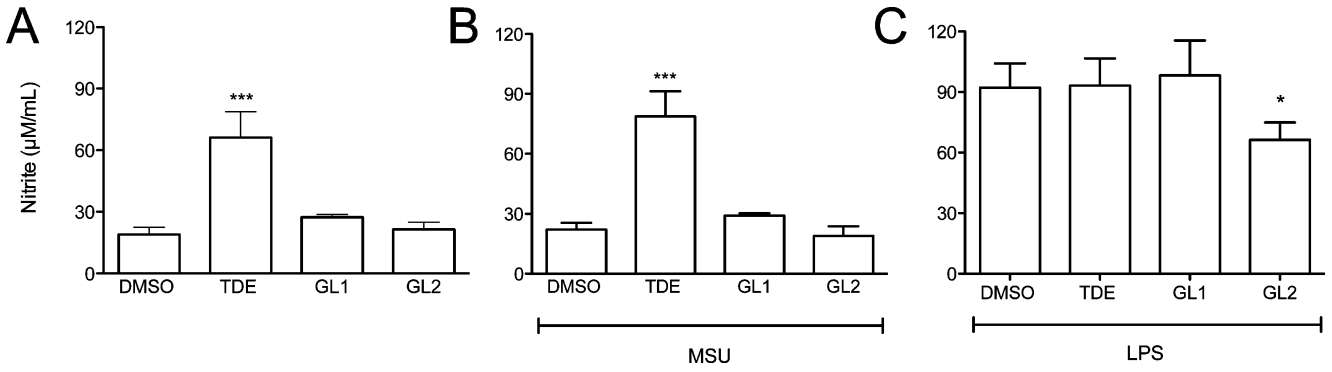

Figure 6. BMMs were stimulated by MSU crystals (200 mg/mL) or LPS (100 ng/mL) in the presence of 40 μM TDE, **GL1**, or **GL2** for 96 h. Nitric oxide levels in culture supernatants were measured using the Greiss assay: (A) glycolipids alone; (B) glycolipids + MSU; (C) glycolipids + LPS. Values are the mean ± SEM of combined data from three independent experiments. Legend: (***) $p < 0.001$ (one-way ANOVA); (*) $p < 0.05$ (Mann–Whitney).

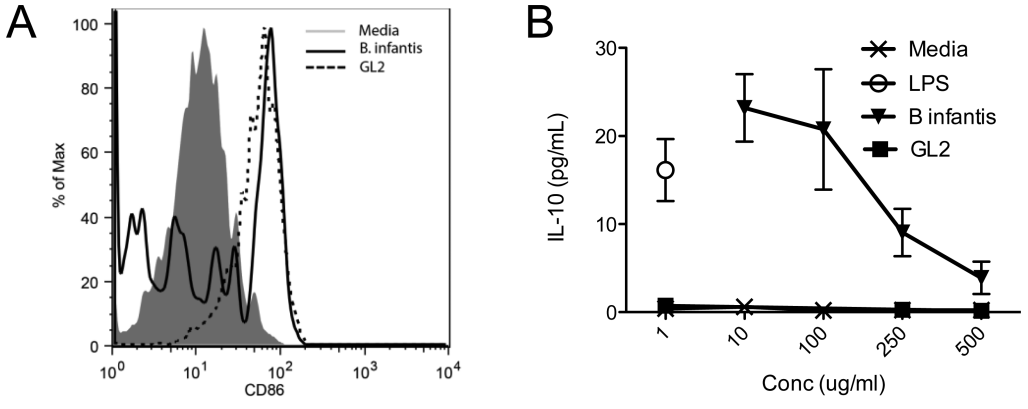

Figure 7. Human DCs treated with LPS, *B. infantis*, or **GL2** for 24 h: (A) CD86 expression by DC analyzed by flow cytometry; (B) IL-10 levels in culture supernatants measured by ELISA.

aldehydogenic ethanolamine phospholipids (isolated from *Clostridium butyricum*)[23] and other phosphatidyl plasmalogens from clostridia.[24] The occurrence of a glycerol acetal of a galactosylglyceride, however, is unprecedented.

Having successfully characterized the major glycolipids from *B. longum* subsp. *infantis*, we then set out to determine the ability of these glycolipids to modulate the innate immune response. To this end, the ability of the isolated compounds to induce NO

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production in bone marrow derived macrophages (BMMs) was compared to NO production induced by the positive control trehalose diester (TDE).[25] As illustrated (Figure 6A), TDE induced NO production by BMMs; however, the purified glycolipids **GL1** and **GL2** showed no such activity. Although **GL1** and **GL2** failed to induce NO production in BMMs, it was proposed that these glycolipids could modify NO responses in the presence of other stimuli. To investigate this, BMMs were stimulated with either the autoinflammatory agent monosodium urate (MSU) crystals or lipopolysaccharide (LPS) in the presence of **GL1**, **GL2**, or the control glycolipid TDE. Here, cotreatment with MSU crystals and glycolipids had no additional effect on NO production by BMMs (Figure 6B); however, **GL2** treatment resulted in a decrease in NO production by LPS-stimulated BMMs (Figure 6C). On the basis of these findings it appears that **GL2** partially suppresses LPS-induced nitric oxide production by macrophages.

Next we determined whether **GL2** affected dendritic cell (DC) maturation. The ability of DCs to present antigen and induce T-cell responses is enhanced by exposure to bacteria and bacterial products such as LPS. DC maturation is associated with the upregulation of surface activation markers such as CD86 and cytokine production. Human dendritic cells treated with either heat inactivated *B. longum* subsp. *infantis* or **GL2** showed upregulation of CD86 expression (Figure 7A) indicating DC maturation. Although the majority of dendritic cells expressed high CD86 levels, *B. longum* subsp. *infantis* induced more variation in low CD86 expression in comparison to **GL2**. This likely reflects the heterogeneity of the glycolipid composition of the whole bacterium ,where different components induce different levels of dendritic cell activation. Interestingly, low concentrations of *B. longum* subsp. *infantis* induced IL-10 production by DC that decreased at higher concentrations, whereas IL-10 production was not induced by **GL2** treatment (Figure 7B).

These data indicated that both **GL2** or high concentrations of *B. longum* subsp. *infantis* may induce a tolerogenic or suppressed DC phenotype, as illustrated by surface marker activation in the absence of cytokine production. Different species of bifidobacteria have been previously found to induce CD86 expression and the production of varying levels of IL-10 when using DCs harvested from cord blood, although the molecular causes of these responses have not been investigated.[4d] The ability of **GL2** to suppress NO production by LPS-stimulated macrophages and the development of tolerogenic DC phenotypes would be beneficial for improved control of inflammation in IBD and allergy, and in light of our observations it would be useful to determine the presence of this glycolipid in different strains of bifidobacteria.

Unfortunately, sufficient quantities of acyl-cPA **L3c** and plasmenyl-cPA **L3d** could not be obtained for biological assessment; however, it is important to note that acyl-cPAs have been observed to exhibit a number of biological properties,[26] such as DNA polymerase α inhibition and the inhibition of cancer cell invasion and metastasis.[27] Accordingly, it seems likely that plasmenyl-cPAs have interesting biological activities, which we will explore in due course.

CONCLUSION

Herein, we characterized the major polar lipids from *Bifidobacterium longum* subsp. *infantis* using MALDI as a starting point for the discovery of novel structures. While several known compounds were identified, such as glyceryl phosphates **L3a−c** and β-galactopyranosyl diglyceride **GL1**, the detection of the plasmenyl cyclophosphatidic acid **L3d** is unprecedented and the biological activity of this species will be worth reviewing. In addition, a 1-(β-D-Gal*f*)-3-glyceroplasmalomonoglyceride (**GL2**) is reported herein for the first time and represents an entirely new class of glycolipids. Neither **GL1** or **GL2** induced innate inflammatory responses, yet the potential for **GL2** to suppress LPS-induced macrophage activation and induce a tolerogenic DC phenotype is worthy of further investigation as a contributing factor in the beneficial properties of bifidobacteria in IBD and allergy.

EXPERIMENTAL SECTION

Bacterial Strains. *B. longum* subs. *infantis* (ATCC 15702) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Bacterial Growth. The *B. longum* culture was plated onto refined *Clostridium* media (RCM) agar and incubated anaerobically at 37 °C for 24 h. Glycolipid production was conducted anaerobically at 37 °C for 24 h in 18 L using carbohydrate-free Man−Rogosa−Sharp (MRS) media in a 20 L Braun bioreactors. The culture was then centrifuged at 3000*g* for 10 min to yield a watery paste, and the cell mass was freeze-dried. Next, the dried cell mass was washed three times with PBS buffer (pH 7.3) and centrifuged (15 min, 20000 rpm, 4 °C) or until the amount of carbohydrate detected in the washes by colorimetric assay was less than 1% (w/w). To remove the PBS buffer, the cells were washed with deionized water and centrifuged and then freeze-dried and weighed.

Whole Cell MALDI. Matrix solution was prepared by dissolving 2,5-dihydroxybenzoic acid (DHB) at 30 mg/mL in acetonitrile and water (7/3 v/v). Samples were prepared by suspending bacterial cells at 10 mg/mL in Milli-Q water (1 mL). The cell suspension (1 μL) and MALDI matrix (1 μL) were deposited using the dry droplet method on a stainless steel plate. The MS/MS experiments were conducted operating in reflectron positive ion mode. Mass calibration was performed externally using a peptide mixture containing des-arg^1-bradykinin, angiotensin 1, neurotensin and glu^1-fibrinopeptide B.

Thin-Layer Chromatography (TLC). Bifidobacterial glycolipid samples were dissolved in $CHCl_3$/MeOH (2/1) and applied to dried silica precoated TLC plates. The bifidobacterial glycolipids were eluted with $CHCl_3$/MeOH (6/1, v/v) and the plates were sprayed and developed with a cerium molybdate (Hanessian's) stain and molybdate stain to detect phosphorus.[28]

Extraction and Purification. Bifidobacterial dried cell mass (6.0 g) was extracted twice with $CHCl_3$/MeOH/water (400 mL, 10/5/1, v/v/v), allowing the layers to settle for 18 h at 37 °C. After filtration, the combined organic extracts were then evaporated to dryness to yield a residue (0.8 g), which was suspended in $CHCl_3$/MeOH/water (100 mL, 5/5/4.5, v/v/v) and rested overnight (16 h, 4 °C) to remove the polar material. The organic layer was collected, evaporated to dryness, and analyzed by TLC. The crude lipid extracts were preabsorbed onto silica gel and fractionated by silica column chromatography. Glycolipids were eluted successively with a stepwise gradient of MeOH in $CHCl_3$ and, if needed, further purified using preparative TLC.

NMR Spectroscopy. Purified glycolipids were dissolved in 0.7 mL of $CDCl_3$/MeOD (2/1, v/v). Samples were analyzed at 30 °C and the chemical shift (δ) measured relative to TMS (0 ppm). NMR spectra were recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C.

ESI-TOF MS. All high-resolution mass spectra and MS-MS experiments were performed using electrospray ionization mass spectrometry in positive or negative ion mode on a Q-Tof mass spectrometer.

Alditol Acetate Method. The constituent sugar neutral composition was determined by GC-MS following TFA mediated acid hydrolysis and derivatization to alditol acetates.[29] Briefly, samples were hydrolyzed with 2 M TFA (400 μL, 1 h, 120 °C), neutralized with 2 M NH_4OH (0.4 mL), using 50 μg of *myo*-inositol as an internal standard, reduced with 2% (w/v) $NaBH_4$ in water, and then acetylated with acetic anhydride (200 μL) in pyridine. Alditol acetates were quantified by GC on an SGE

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BPX90 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) with the GC oven programmed from 80 °C (held for 1 min) to 150 °C at a rate of 25 °C min^{-1} and then to 250 °C at a rate of 4 °C min^{-1} and analyzed by MS. Identifications were based on peak retention times and by comparison of electron impact spectra with standard spectra.

Glycosyl Linkage Analysis. Samples (~0.5 mg) were methylated as described by Ciucanu and Kerek,[30] with the exception that samples were dispersed in DMSO (0.5 mL) and heated (50 °C) overnight under argon. After extraction into chloroform, the methylated polysaccharides were hydrolyzed with TFA, reduced, and then acetylated before analysis by GC-MS as described above. Identifications were based on peak retention times and by comparison of electron impact spectra with standard spectra. Here, the partially methylated alditol acetate standards were prepared following the method of Doares et al.[31]

Sugar Absolute Configuration. Crude glycolipid extracts (1 mg) were hydrolyzed with 2 M TFA (1 h, 120 °C) and then dried. To this were added a solution of *R*-(−)-1-amino-2-propanol in methanol (20 μL, 1.6 M), acetic acid (17 μL, 3.5 M), and sodium cyanoborohydride in methanol (13 μL, 3% w/v) (1 h, 65 °C). After the residues were dried, the samples were acetylated, as mentioned above, dissolved in acetone, and then injected onto a GC-MS using a nonpolar column (HP-5 ms, 30 m, 0.32 mm i.d.). Here, the temperature program started at 180 °C (held for 1 min), rose to 220 °C at a rate of 2 °C/min (held for 2 min), and then rose again to 250 °C at a rate of 1 °C/min (held 5 min). Crude samples were compared with derivatives of D- and L-glucose and galactose references.

Fatty Acid Analysis. Fatty acid methyl esters (FAME) were prepared by direct transesterification from samples of biomass (ca. 0.5 mg) without extraction, as described by Svetashev et al.[32] In brief, samples were dissolved in 1% sodium methoxide in dry methanol (30 min, 80 °C) and then treated with 5% anhydrous HCl in methanol (30 min, 80 °C). The resulting FAMEs and DMA were taken up in hexane and analyzed by GC and GC-MS.[33] The position of the double bond in the monounsaturated fatty acids and DMAs was located by GC-MS of dimethyl disulfide adducts.[34] The hydroxyl fatty acids were converted into their TMS derivatives.[35] The position of the cyclopropane group was elucidated after separation of saturated fatty acids fraction by means of silver ion SPE.[41] This separation step was followed by hydrogenation in acetic acid over Adams' catalyst to open the cyclopropane ring.[36] The resulting fatty acids were converted to pyrrolidides[34] and analyzed by GC-MS analyses. The analysis was performed on a gas chromatograph equipped with a flame ionization detector (FID) and polar (SolgelWax, 30 m, 0.32 mm i.d.) column. Hydrogen was used as the carrier gas with a split ratio of 1:30 and a separation temperature of 195 °C. GC-MS analyses were performed on a gas chromatograph equipped with an inert mass selective detector and nonpolar (HP-5 ms, 30 m, 0.32 mm i.d.) column.[37] Helium was used as the carrier gas. The program started at 100 °C, with a temperature gradient of 5 °C/min to 160 °C and then 1 °C/min to 240 °C before being left at 240 °C for 25 min.

Characterization Data for L3a and L3b. *1,2-Dioleoylglycerol 3-phosphate (**L3a**):* R_f = 0.05 ($CHCl_3$/MeOH, 6/1, v/v); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 2/1, v/v) δ 5.31−5.29 (m, 4H, CH-9′ and CH-10′), 5.22−5.18 (m, 1H, H-2), 4.37 (dd, $J_{1a,2}$ = 2.1 Hz, $J_{1a,1b}$ = 11.8 Hz, 1H, H-1a), 4.09 (dd, $J_{1b,2}$ = 6.7 Hz, $J_{1a,1b}$ = 11.8 Hz, 1H, H-1b), 3.96−3.92 (m, 2H, CH_2-3), 2.36−2.31 (m, 4H, CH_2-2′), 2.00−1.96 (m, 8H, CH_2-8′, and CH_2-11′), 1.60−1.54 (m, 4H, CH_2-3′), 1.31−1.29 (m, 8H, CH_2-7′ and CH_2-12′), 1.28−1.27 (m, 4H, CH_2-4′), 1.25−1.22 (m, 28H, CH_2-5′−6′ and CH_2-13′−17′), 0.86 (t, $J_{17',18'}$ = 7.0 Hz, 6H, CH_3-18′); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 2/1, v/v) δ 174.1 (C═O), 130.3 (C-9′ and C-10′), 70.6 (C-2), 63.7 (C-3), 62.7 (C-1), 34.5 (C-2′), 32.1 (C-16′), 30.4−29.3 (C-4′−7′ and C-12′−15′), 27.3 (C-8′ and C-11′), 25.0 (C-3′), 22.9 (CH_2-17′), 14.1 (C-18′). HRMS (ESI) *m*/*z* calcd for $[C_{39}H_{72}O_8P]^-$ 699.4970, obsd 699.5009.

*1-(1Z,9Z-Octadeca-1,9-dienyl)-2-oleoylglycerol 3-phosphate (**L3b**):* R_f = 0.06 ($CHCl_3$/MeOH, 6/1, v/v); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 2/1, v/v) δ 5.88 (d, $J_{1'',2''}$ = 6.1 Hz, 1H, H-1″), 5.31−5.29 (m, 4H, CH-9″, CH-10″, CH-9′ and CH-10′), 5.16−5.12 (m, 1H, H-2), 4.32 (dt, $J_{1'',2''}$ = 6.1 Hz, $J_{2'',3''}$ = 7.2 Hz, 1H, H-2″), 3.92−3.83 (m, 4H, CH_2-1 and CH_2-3), 2.36−2.31 (m, 2H, CH_2-2′), 2.00−1.96 (m, 10H, CH_2-3″, CH_2-8″, CH_2-11″, CH_2-8′, and CH_2-11′), 1.60−1.54 (m, 2H, CH_2-3′), 1.31−1.29 (CH_2-4″, CH_2-7″, CH_2-12″, CH_2-7′, and CH_2-12′), 1.28−1.27 (m, 2H, CH_2-4′), 1.25−1.22 (m, 28H, CH_2-5″, CH_2-6″, CH_2-13″−17″, CH_2-5′, CH_2-6′, and CH_2-13′−17′), 0.86 (t, J = 7.0 Hz, 6H, CH_3-18″ and CH_3-18′); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 2/1, v/v) δ 174.1 (C═O), 144.9 (C-1″), 130.3 (C-9″, C-10″, C-9′ and C-10′), 107.9 (C-2″), 71.8 (C-2), 70.4 (C-1), 66.2 (C-3), 34.5 (C-2′), 32.1 (CH_2-16″ and CH_2-16′), 30.4−29.3 (C-4″−7″, C-12″−15″, C-4′−7′, and C-12′−15′), 27.3 (C-3″, C-8″, C-11″, C-8′, and C-11′), 25.0 (C-3′), 22.9 (CH_2-17″ and CH_2-17′), 23.3 (C-3″), 14.1 (C-18″ and C-18′); HRMS (ESI) *m*/*z* calcd for $[C_{39}H_{72}O_7P]^-$ 683.5021, obsd 683.5042.

Characterization Data for GL1 and GL2. *3-O-(β-D-Galactopyranosyl)-1,2-di-O-oleoylglycerol (**GL1**):* R_f = 0.56 ($CHCl_3$/MeOH, 6/1, v/v); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 2/1) δ 5.30−5.28 (m, 4H, CH-9″ and CH-10″), 5.30−5.28 (m, 1H, H-2), 4.40 (dd, $J_{1a,2}$ = 3.0 Hz, $J_{1a,1b}$ = 12.1 Hz, 1H, H-1a), 4.24 (d, $J_{1',2'}$ = 7.7 Hz, H-1′), 4.23 (dd, $J_{1b,2}$ = 6.1 Hz, $J_{1a,1b}$ = 12.3 Hz, 1H, H-1b), 3.97 (dd, $J_{2,3a}$ = 5.4 Hz, $J_{3a,3b}$ = 10.8, 1H, H-3a), 3.89 (dd, $J_{3',4'}$ = 3.3 Hz, $J_{4',5'}$ = 0.8, 1H, H-4′), 3.81 (dd, $J_{5,6a'}$ = 6.9 Hz, $J_{6a',6b'}$ = 11.6 Hz, 1H, H-6a′), 3.76 (dd, $J_{5,6b'}$ = 5.5 Hz, $J_{6a',6b'}$ = 11.4 Hz, 1H, H-6b′), 3.73 (dd, $J_{2,3b}$ = 6.2 Hz, $J_{3a,3b}$ = 10.7 Hz, 1H, H-3b), 3.55 (dd, $J_{1',2'}$ = 7.9 Hz, $J_{2',3'}$ = 9.8, 1H, H-2′), 3.51 (ddd, $J_{4',5'}$ = 0.7 Hz, $J_{5',6a'}$ = 6.9, $J_{5',6b'}$ = 5.6, 1H, H-5′), 3.50 (dd, $J_{2',3'}$ = 9.9 Hz, $J_{3',4'}$ = 3.3, 1H, H-3′), 2.33 (t, $J_{2'',3''}$ = 7.0, 4H, H-2″), 2.00−1.95 (m, 8H, CH_2-8″, and CH_2-11″), 1.59−1.54 (m, 4H, CH_2-3″), 1.31−1.29 (m, 8H, CH_2-7″ and CH_2-12″), 1.28−1.27 (m, 4H, CH_2-4″), 1.25−1.22 (m, 28H, CH_2-5″−6″ and CH_2-13″−17″), 0.86 (t, $J_{17'',18''}$ = 6.5 Hz, 6H, CH_3-18″); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 2/1) δ 173.4 (C═O), 129.0 (C-9″ and C-10″), 103.2 (C-1′), 74.5 (C-5′), 72.5 (C-3′), 70.3 (C-2′), 69.7 (C-2), 67.8 (C-4′), 66.9 (C-3), 62.0 (C-1), 60.6 (C-6′), 33.3 (C-2″), 30.9 (C-16″), 29.2−28.2 (C-4″−7″ and C-12″−15″), 26.1 (C-8″ and C-11″), 24.0 (C-3″), 21.7 (CH_2-17″), 13.0 (C-18″); HRMS (ESI) *m*/*z* calcd for $[C_{45}H_{82}O_{10} + Na]^+$ 805.5800, obsd 805.5770.

*3-O-(β-D-Galactofuranosyl)-1-O-(glycer-1-yloxyoleyl)-2-O-oleoylglycerol (**GL2**):* R_f = 0.41 ($CHCl_3$/MeOH, 6/1, v/v); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 2/1) δ 5.34−5.29 (m, 4H, CH-9″, CH-10″), 5.14 (p, $J_{1a,2} = J_{1b,2} = J_{2,3a} = J_{2,3b}$ = 5.2 Hz, 1H, H-2), 4.94 (bs, 1H, H-1′), 4.54 (t, $J_{1'',2'''}$ = 5.9 Hz, 1H, H-1‴), 4.03 (dd, $J_{3',4'}$ = 4.7 Hz, $J_{4',5'}$ = 3.0 Hz, 1H, H-4′), 4.01 (dd, $J_{2',3'}$ = 2.4 Hz, $J_{3',4'}$ = 4.7 Hz, 1H, H-3′), 3.99 (dd, $J_{1',2'}$ = 1.0 Hz, $J_{2',3'}$ = 2.4 Hz, 1H, H-2′), 3.82−3.78 (m, 4H, H-3b, H-2‴, H-1a and H-5′), 3.68−3.62 (m, 5H, H-3a‴, CH_2-6′, H-3a, H-1a‴, and H-1b), 3.58 (m, $J_{2''',3b'''}$ = 6.1 Hz, $J_{3a''',3b'''}$ = 11.4 Hz, 1H, H-1a‴), 3.48 (dd, $J_{1b''',2'''}$ = 6.2 Hz, $J_{1a''',1b'''}$ = 10.0 Hz, 1H, H-1b‴), 2.31−2.36 (t, $J_{2'',3''}$ = 7.5 Hz, 2H, CH_2-2″), 2.00−1.96 (m, 8H, CH_2-8″, CH_2-11″), 1.62 (m, 2H, H-2‴), 1.60−1.54 (m, 2H, CH_2-3″), 1.31−1.29 (CH_2-7″, CH_2-12″), 1.28−1.22 (m, 32H, CH_2-4″−6″, CH_2-13″−17″), 0.86 (t, J = 7.0 Hz, 6H, CH_3-18″); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 2/1) δ 174.2 (C═O), 130.0 (C-9″ and C-10″), 108.6 (C-1′), 104.2 (C-1‴), 85.1 (C-4′), 80.9 (C-2′), 78.2 (C-3′), 71.8 (C-2), 71.5 (C-5′), 71.2 (C-2‴), 67.0 (C-1‴), 66.0 (C-3), 64.2 (C-1), 63.8 (C-2‴), 63.7 (C-6′),34.5 (C-2″), 32.1 (CH_2-16″ and CH_2-16′), 30.4−29.3 (C-4″−7″, C-12″−15″, C-4′−7′, and C-12′−15′), 27.2 (C-8″ and C-11″), 25.2 (C-3″ and C-2‴), 22.9 (CH_2-17″), 25.2 (C-3″), 14.2 (C-18″); HRMS (ESI) *m*/*z* calcd for $[C_{48}H_{90}O_{12} + Na]^+$ 881.6324, obsd 881.6349.

Synthesis of MS^2 Standards. *1-O-Propanoylglycerol.* 1,2-*O*-Isopropylideneglycerol (1.8 g, 13.6 mmol) was dissolved in a mixture of propionic anhydride (10 mL) and pyridine (20 mL), and this mixture was stirred for 18 h. The mixture was concentrated, and residual solvents were removed by coevaporation with acetic acid. The residue was dissolved in a mixture of acetic acid (20 mL) and water (5 mL) and stirred for 2 h at 60 °C, after which the reaction mixture was concentrated and coevaporated with toluene. Purification of the residue using silica gel column chromatography (EtOAc/PE, 1/1, v/v → EtOAc) gave the title compound as a colorless oil (1.4 g, 9.5 mmol, 70%): R_f = 0.38 (EtOAc); IR ν 3379, 2982, 2947, 2887, 1718, 1463, 1422, 1381, 1351, 1279, 1190, 1118, 1083, 1046, 980, 928, 807 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.47 (dd, $J_{1a,2}$ = 4.9 Hz, $J_{1a,1b}$ = 11.5 Hz, 1H, H-1a), 4.44 (dd, $J_{1a,2}$ = 5.8 Hz, $J_{1a,1b}$ = 11.5 Hz, 1H, H-1b), 4.22−4.25 (m, 1H, H-2), 3.99 (dd, $J_{2,3a}$ = 3.8 Hz, $J_{3a,3b}$ = 11.5 Hz, 1H, H-3a), 3.89 (dd, $J_{2,3b}$ = 7.0 Hz, $J_{3a,3b}$ = 11.5 Hz, 1H, H-3b), 2.68 (q, $J_{2',3'}$ = 7.6 Hz, 2H, CH_2-2′), 1.45 (t, $J_{2',3'}$ = 7.6 Hz, 3H, CH_3-3′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.0 (C═O), 70.2 (C-2), 65.1 (C-1), 63.4 (C-3), 27.4 (C-

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2′), 9.0 (C-3′); HRMS (ESI) m/z calcd for $[C_6H_{12}O_4 + H]^+$ 149.0808, obsd 149.0809.

1-O-Prop-2-enyl-2,3-O-isopropylideneglycerol. 1,2-*O*-Isopropylideneglycerol (5.35 mL, 5.0 g, 37.8 mmol) was coevaporated with DMF, dissolved in DMF (150 mL), and cooled to 0 °C using an ice bath. Allyl bromide (6.4 mL, 5.0 g, 41.6 mmol, 1.1 equiv) was added and, with continuous stirring, NaH (60% in mineral oil, 1.8 g, 45 mmol, 1.2 equiv) was added slowly, portionwise. After stirring was continued for 1 h at room temperature, the reaction mixture was quenched by the addition of ice−water (100 mL) and extracted with ether (2×). The combined ethereal extracts were washed with water and brine, dried ($MgSO_4$), filtered, and concentrated. Purification of the residue by silica gel column chromatography (PE) gave the title compound as a colorless oil (5.45 g, 31.6 mmol, 84%): R_f = 0.52 (PE/EtOAc, 9/1, v/v); IR ν 3081, 2986, 2933, 2864, 1456, 1422, 1380, 1371, 1257, 1213, 1150, 1076, 1052, 999, 926, 844, 793, 737 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 5.89 (ddt, $J_{2',3a'}$ = 17.3 Hz, $J_{2',3b'}$ = 10.5 Hz, $J_{2',1'}$ = 5.8 Hz, 1H, H-2′), 5.27 (d, $J_{2',3a'}$ = 17.4 Hz, 1H, H-3a′), 5.18 (d, $J_{2',3b'}$ = 10.5 Hz, 1H, H-3b′), 4.27 (p, $J_{1,2} = J_{2,3}$ = 6.0 Hz, 1H, H-2), 4.07−3.99 (m, 3H, H-3a and CH_2-1′), 3.73 (t, $J_{2,3b} = J_{3a,3b}$ = 6.8 Hz, 1H, H-3b), 3.52 (dd, $J_{1a,2}$ = 5.8 Hz, $J_{1a,1b}$ = 9.8 Hz, 1H, H-1a), 3.43 (dd, $J_{1b,2}$ = 5.3 Hz, $J_{1a,1b}$ = 9.8 Hz, 1H, H-1b), 1.42 (s, 3H, CH_3 iPr), 1.36 (s, 3H, CH_3 iPr); ^{13}C NMR (125 MHz, $CDCl_3$) δ 134.5 (C-2′), 117.3 (C-3′), 109.4 (C_q iPr), 74.7 (C-2), 72.5 (C-1′), 71.1 (C-1), 66.8 (C-3), 26.7, 25.4 (2 × CH_3 iPr); HRMS (ESI) m/z calcd for $[C_9H_{16}O_3 + H]^+$ 173.1172, obsd 173.1168.

1-O-Prop-2-enylglycerol. 1,2-*O*-Isopropylideneglycerol (1.72 g, 10 mmol) was dissolved in a mixture of acetic acid (20 mL) and water (5 mL) and stirred for 4 h at 50 °C, after which the reaction mixture was concentrated and coevaporated with toluene. Purification of the residue by Kugelrohr distillation gave the title compound as a colorless oil (1.15 g, 8.7 mmol, 87%): R_f = 0.62 (EtOAc/MeOH, 4/1, v/v); IR ν 3343, 2940, 2833, 1450, 1398, 1269, 1111, 928, 852, 735 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 5.81 (ddt, $J_{2',3a'}$ = 17.4 Hz, $J_{2',3b'}$ = 10.3 Hz, $J_{2',1'}$ = 5.9 Hz, 1H, H-2′), 5.20 (dd, $J_{2',3a'}$ = 17.4 Hz, $J_{3a',3b'}$ = 1.3 Hz, 1H, H-3a′), 5.13 (d, $J_{2',3b'}$ = 10.3 Hz, 1H, H-3b′), 3.93 (d, $J_{1',2'}$ = 6.0 Hz, 2H, CH_2-1′), 3.77−3.72 (m, 1H, H-2), 3.49 (dd, $J_{2,3a}$ = 4.5 Hz, $J_{3a,3b}$ = 11.8 Hz, 1H, H-3a), 3.46 (dd, $J_{1a,2}$ = 4.0 Hz, $J_{1a,1b}$ = 10.7 Hz, 1H, H-1a), 3.41 (dd, $J_{2,3b}$ = 6.2 Hz, $J_{3a,3b}$ = 11.8 Hz, 1H, H-3b), 3.36 (dd, $J_{1b,2}$ = 7.0 Hz, $J_{1a,1b}$ = 10.7 Hz, 1H, H-1b); ^{13}C NMR (125 MHz, $CDCl_3$) δ 133.7 (C-2′), 118.2 (C-3′), 72.0 (C-1′), 70.7 (C-1), 70.3 (C-2), 62.6 (C-3); HRMS (ESI) m/z calcd for $[C_6H_{12}O_3 + H]^+$ 133.0859, obsd 133.0858.

E-/Z-1-O-Prop-1-enylglycerol. A mixture of 1-*O*-prop-2-enylglycerol (132 mg, 1.0 mmol), tris(triphenylphosphine)rhodium(I) chloride (60 mg, 65 μmol, 6.5 mol %), and DBU (200 mg, 1.3 mmol, 1.3 equiv) in ethanol/H_2O (20 mL, 9/1, v/v) was refluxed for 4 h, after which TLC analysis (EtOAc) showed complete conversion of the starting material into a slightly higher running product. The mixture was concentrated in vacuo and coevaporated with ethanol to remove traces of water, and the residue was purified by silica gel column chromatography (PE → PE/EtOAc, 3/1, v/v) to give the title compound as a mixture of *E* and *Z* isomers in a 5/2 ratio (112 mg, 0.85 mmol, 85%): R_f = 0.31 (EtOAc); IR ν 3876, 3361, 2987, 2943, 2911, 1453, 1406, 1393, 1310, 1251, 1182, 1118, 1066, 1050, 999, 928, 900, 869, 723 cm^{-1}; HRMS (ESI) m/z calcd for $[C_6H_{12}O_3 + H]^+$ 133.0859, obsd 133.0858. Data for the *E*-isomer are as follows: ^1H NMR (500 MHz, $CDCl_3$) δ 6.15 (d, $J_{1,2}$ = 12.4 Hz, 1H, H-1′), 4.71 (dq, $J_{1,2}$ = 12.4 Hz, $J_{2,3}$ = 6.6 Hz, 1H, H-2′), 3.86−3.80 (m, 1H, H-2), 3.66−3.49 (m, 4H, CH_2-1 and CH_2-3), 1.46 (d, $J_{2',3'}$ = 6.6 Hz, 3H, CH_3-3′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 146.2 (C-1′), 99.0 (C-2′), 70.5, 69.9, 63.6 (C-1, C-2, and C-3), 12.4 (C-3′). Data for the *Z* isomer are as follows: ^1H NMR (500 MHz, $CDCl_3$) δ 5.89 (bd, $J_{1,2}$ = 6.1 Hz, 1H, H-1′), 4.33 (p, $J_{1,2} = J_{2,3}$ = 6.8 Hz, 1H, H-2′), 3.86−3.80 (m, 1H, H-2), 3.68−3.49 (m, 4H, CH_2-1 and CH_2-3), 1.49 (d, $J_{2',3'}$ = 6.9 Hz, 3H, CH_3-3′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.6 (C-1′), 101.5 (C-2′), 72.7, 70.8, 63.5 (C-1, C-2 and C-3), 9.1 (C-3′).

General Procedure for the Synthesis of Cyclophosphatidic Acids I−III. To a solution of $POCl_3$ (0.93 mL, 1.5 g, 10 mmol, 10 equiv) in dry THF (5 mL) at −78 °C, under an argon atmosphere, was slowly added pyridine (0.81 mL, 0.79 g, 10 mmol, 10 equiv). After the mixture was stirred for 5 min at −78 °C, a solution of the diol (1 mmol) in dry THF (5 mL) was added dropwise over 10 min, while the temperature was maintained at −78 °C. After 30 min, the reaction mixture was warmed to room temperature over 2 h, and the mixture was poured into an ice−water solution (50 mL) containing $KHCO_3$ (5 g, 50 mmol). The aqueous layer was washed with ethyl acetate and concentrated to form a white semisolid residue. The solids were extracted with methanol and filtered. Concentration of the mother liquor yielded the crude potassium cyclophosphate, which was purified using silica gel column chromatography (EtOAc → EtOAc/MeOH, 3/1, v/v) to yield the pure cyclophosphatidic acids as white solids.

1-O-Propanoylcyclophosphatidic Acid Potassium Salt (I). 1-*O*-Propanoylglycerol (163 mg, 1.1 mmol) was subjected to the general procedure for the synthesis of cyclophosphatidic acids to produce the title compound **I** as a white solid (234 mg, 0.94 mmol, 86%): R_f = 0.29 (MeOH/EtOAc, 1/1, v/v); IR ν 3358, 2976, 2941, 1552, 1465, 1407, 1371, 1297, 1221, 1105, 1073, 1008, 971, 811 cm^{-1}; ^1H NMR (600 MHz, D_2O) δ 4.64 (dp, $J_{1a,2}$ = 3.5 Hz, $J_{1b,2} = J_{2,3a} = J_{2,3b} = J_{2,P}$ = 6.5 Hz, 1H, H-2), 4.27 (ddd, $J_{2,3a}$ = 6.5 Hz, $J_{3a,3b}$ = 9.7 Hz, $J_{3a,P}$ = 12.0 Hz, 1H, H-3a), 4.26 (dd, $J_{1a,1b}$ = 12.2 Hz, $J_{1a,2}$ = 3.5 Hz, 1H, H-1a), 4.13 (dd, $J_{1b,2b}$ = 12.2 Hz, $J_{1b,2}$ = 6.0 Hz, 1H, H-1b), 4.00 (dt, $J_{2,3b}$ = 7.0 Hz, $J_{3a,3b} = J_{3a,P}$ = 9.5 Hz, 1H, H-3b), 2.38 (q, $J_{2',3'}$ = 7.6 Hz, 2H, CH_2-2′), 1.03 (t, $J_{2',3'}$ = 7.6 Hz, 3H, CH_3-3′); ^{13}C NMR (150 MHz, D_2O) δ 174.6 (s, C═O), 71.4 (d, J_P = 2.6 Hz, C-2), 63.1 (d, J_P = 1.1 Hz, C-3), 61.6 (d, J_P = 5.4 Hz, C-1), 24.5 (s, C-2′), 5.7 (d, J_P = 6.4 Hz, C-3′); ^{31}P NMR (120 MHz, D_2O) δ 18.0 (ddd, $^3J_{H\text{-}2,P}$ = 3.5 Hz, $^2J_{H\text{-}3a,P}$ = 11.9 Hz, $^2J_{H\text{-}3b}$ = 9.5 Hz); HRMS (ESI) m/z calcd for $[C_6H_{10}O_6P]^-$ 209.0220, obsd 209.0221.

1-O-Prop-1-enylcyclophosphatidic Acid Potassium Salt (II). 1-*O*-Prop-2-enylglycerol (132 mg, 1.0 mmol) was subjected to the general procedure for the synthesis of cyclophosphatidic acids to produce the title compound **II** as a white solid with an *E*/*Z* ratio of 1.8/1 (178 mg, 0.77 mmol, 77%): R_f = 0.21 (MeOH/EtOAc, 1/1, v/v); IR ν 3336, 2947, 2835, 1450, 1411, 1213, 1103, 1071, 1019, 928, 884, 813 cm^{-1}; HRMS (ESI) m/z calcd for $[C_6H_{10}O_5P]^-$ 193.0271, obsd 193.0273. Data for the *E* isomer are as follows: ^1H NMR (500 MHz, $CDCl_3$) δ 6.17 (dq, $J_{1',2'}$ = 12.5 Hz, $^4J_{1',3'}$ = 1.6 Hz, 1H, H-1′), 4.87 (dq, $J_{1',2'}$ = 12.5 Hz, $J_{2,3}$ = 6.8 Hz, 1H, H-2′), 4.59−4.53 (m, 1H, H-2), 4.20 (ddd, $J_{2,3a}$ = 6.5 Hz, $J_{3a,3b}$ = 9.3 Hz, $J_{3a,P}$ = 12.3 Hz, 1H, H-3a), 3.90 (ddd, $J_{2,3b}$ = 7.1 Hz, $J_{3a,3b}$ = 9.2 Hz, $J_{3b,P}$ = 18.3 Hz, 1H, H-3b), 3.76−3.71 (m, 2H, CH_2-1), 1.41 (dd, $J_{2',3'}$ = 6.8 Hz, $^4J_{1',3'}$ = 1.5 Hz, 3H, CH_3-3′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.3 (s, C-1′), 103.2 (s, C-2′), 74.7 (d, J_P = 1.9 Hz, C-1), 69.4 (d, J_P = 5.8 Hz, C-2), 65.7 (s, C-3), 11.4 (C-3′). Data for the *Z* isomer are as follows: ^1H NMR (500 MHz, $CDCl_3$) δ 5.97 (dq, $J_{1',2'}$ = 6.2 Hz, $^4J_{1',3'}$ = 1.7 Hz, 1H, H-1′), 4.59−4.53 (m, 1H, H-2), 4.87 (app p, $J_{1',2'} = J_{2,3}$ = 6.9 Hz, 1H, H-2′), 4.21 (ddd, $J_{2,3a}$ = 6.5 Hz, $J_{3a,3b}$ = 9.3 Hz, $J_{3a,P}$ = 12.3 Hz, 1H, H-3a), 3.90 (ddd, $J_{2,3b}$ = 7.1 Hz, $J_{3a,3b}$ = 9.2 Hz, $J_{3b,P}$ = 18.3 Hz, 1H, H-3b), 3.82 (d, $J_{1,2}$ = 5.2 Hz, 1H, CH_2-1), 1.43 (dd, $J_{2',3'}$ = 7.0 Hz, $^4J_{1',3'}$ = 1.7 Hz, 3H, CH_3-3′); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.0 (s, C-1′), 101.7 (s, C-2′), 75.0 (d, J_P = 1.9 Hz, C-1), 71.5 (d, J_P = 5.7 Hz, C-2), 65.6 (s, C-3), 8.4 (C-3′).

1-O-Propen-2-ylcyclophosphatidic Acid Potassium Salt (III). 1-*O*-Allylglycerol (132 mg, 1 mmol) was subjected to the general procedure for the synthesis of cyclophosphatidic acids to produce the title compound **III** as a white solid (187 mg, 0.81 mmol, 81%): R_f = 0.21 (MeOH/EtOAc, 1/1, v/v); IR ν 3364, 2934, 1567, 1494, 1406, 1349, 1289, 1225, 1077, 1031, 974, 803, 769 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 5.82 (ddt, $J_{2',3a'}$ = 17.3 Hz, $J_{2',3b'}$ = 10.5 Hz, $J_{2',1'}$ = 5.9 Hz, 1H, H-2′), 5.22 (d, $J_{2',3a'}$ = 17.3 Hz, 1H, H-3a′), 5.15 (d, $J_{2',3b'}$ = 10.5 Hz, 1H, H-3b′), 4.52 (h, $J_{1,2} = J_{2,3} = J_{2,P}$ = 6.0 Hz, 1H, H-2), 4.19 (ddd, $J_{2,3a}$ = 6.4 Hz, $J_{3a,3b}$ = 9.3 Hz, $J_{3a,P}$ = 12.8 Hz, 1H, H-3a), 3.98 (d, $J_{1',2'}$ = 6.1 Hz, 1H, CH_2-1′), 3.84 (q, $J_{2,3b} = J_{3a,3b} = J_{3b,P}$ = 8.5 Hz, 1H, H-3b), 3.55 (d, $J_{1,2}$ = 5.3 Hz, 1H, CH_2-1); ^{13}C NMR (125 MHz, $CDCl_3$) δ 133.4 (s, C-2′), 118.6 (s, C-3′), 75.1 (d, J_P = 2.4 Hz, C-1), 72.1 (s, C-1′), 70.0 (d, J_P = 5.8 Hz, C-2), 65.8 (d, J_P = 1.4 Hz, C-3); ^{31}P NMR (120 MHz, D_2O) δ 18.3; HRMS (ESI) m/z calcd for $[C_6H_{10}O_5P]^-$ 193.0271, obsd 193.0275.

Immunological Methods. All extracted compounds were confirmed to be free of endotoxin, as determined by the Limulus amebocyte lysate (LAL) assay.[38]

Macrophage Assay. Bone marrow derived macrophages were generated as previously described.[25] Briefly, bone marrow cells from C57Bl/6 male mice were cultured in complete IMDM (2.5 × 10^5 cells/mL) supplemented with 10 ng/mL of GM-CSF. On days 2, 5, and 7 half

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the culture media in each well was removed and supplemented with fresh cIMDM containing 10 ng/mL GM-CSF. On day 10 the cells were primed with 10 ng/mL of IFNγ for 3 h. Cells were then treated with 100 ng/mL of LPS or 200 μg/mL of MSU crystals and the test compounds. Aliquots of supernatant were removed every 24 h for analysis. NO production was measured using the Greiss reaction.[39] Cytokine production was measured by ELISA.

DC Assay. Dendritic cells were generated as previously described.[40] Briefly, PBMCs were isolated from whole human blood and cultured in complete RPMI (1% autologous human serum, 2 mM glutamax, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) for 1 h and the nonadherent cells discarded. The adherent monocyte layer was cultured for 7 days in cRPMI supplemented with 200 IU/mL of GMCSF and 500 U/mL of IL-4; the medium was changed every 2 days. On day 7 the nonadherent cells were collected, counted, and plated out at 5×10^5/mL in a 96-well plate with the test compounds for 18 h. The culture supernatant was removed for cytokine analysis by ELISA, and the cells were stained with fluorescently labeled antibodies against the cell surface markers CD80, CD86, HLA-DR, and CD11c before being analyzed by flow cytometry.

■ ASSOCIATED CONTENT

Supporting Information

Figures giving characterization data, including NMR spectra and mass spectrometry data for all isolated and synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Picard, C.; Fioramonti, J.; Francois, A.; Robinson, T.; Neant, F.; Matuchansky, C. *Aliment Pharmacol. Ther.* **2005**, *22*, 495−512.

(2) Reddy, B. S.; Rivenson, A. *Cancer Res.* **1993**, *53*, 3914−18.

(3) Lee, J.-H.; O'Sullivan, D. J. *Microbiol. Mol. Biol. Rev.* **2010**, *74*, 378−416.

(4) (a) Isolauri, E. *Am. J. Clin. Nutr.* **2001**, *73*, 1142S−1146S. (b) Gareau, M. G.; Sherman, P. M.; Walker, W. A. *Nat. Rev. Gastroenterol. Hepatol.* **2010**, *7*, 503−514. (c) Sanders, M. E.; Guarner, F.; Guerrant, R.; Holt, P. R.; Quigley, E. M. M.; Sartor, B.; Sherman, P. M.; Mayer, E. A. *Gut* **2013**, *62*, 787−796. (d) Young, S. L.; Simon, M. A.; Baird, M. A.; Tannock, G. W.; Bibiloni, R.; Spencely, K.; Lane, J. M.; Fitzharris, P.; Crane, J.; Town, I.; Addo-Yobo, E.; Murray, C. S.; Woodcock, A. *Clin. Diagn. Lab. Immunol.* **2004**, *11*, 686−690.

(5) (a) Moran, A. P.; Prendergast, M. M.; Appelmelk, B. J. *FEMS Immunol Med. Microbiol.* **1996**, *16*, 105−115. (b) Weintraub, A. *Carbohydr. Res.* **2003**, *338*, 2539−2547. (c) Tupin, E.; Kinjo, Y.; Kronenberg, M. *Nat. Rev. Microbiol.* **2007**, *5*, 405−417. (d) Khan, A. A.; Stocker, B. L.; Timmer, M. S. M. *Carbohydr. Res.* **2012**, *356*, 25−36.

(6) Veerkamp, J. H. *Biochim. Biophys. Acta, Gen. Subj.* **1972**, *273*, 359−367.

(7) (a) Novik, G. I.; Astapovich, N. I.; Pastsyak, M.; Gam'yan, A. *Microbiology* **2005**, *74*, 678−683. (b) Novik, G.; Gamian, A.; Francisco, J. D. C.; Dey, E. S. *J. Biotechnol.* **2006**, *121*, 555−562. (c) Novik, G. I.; Astapovich, N. I.; Grzegorzewicz, A.; Gam'yan, A. *Microbiology* **2005**, *74*, 670−677. (d) Izhyk, A.; Novik, G.; Szwajcer Dey, E. *J. Supercrit. Fluids* **2012**, *62*, 149−154.

(8) (a) Gidden, J.; Denson, J.; Liyanage, R.; Ivey, D. M.; Lay, J. O., Jr *Int. J. Mass Spectrom.* **2009**, *283*, 178−184. (b) Fuchs, B.; Schiller, J. *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 83−98. (c) Shu, X.; Li, Y.; Liang, M.; Yang, B.; Liu, C.; Wang, Y.; Shu, J. *Int. J. Mass Spectrom.* **2012**, *321−322*, 71−76. (d) Paściak, M.; Sanchez-Carballo, P.; Duda-Madej, A.; Lindner, B.; Gamian, A.; Holst, O. *Carbohydr. Res.* **2010**, *345*, 1497−1503. (e) Paściak, M.; Kaczyński, Z.; Lindner, B.; Holst, O.; Gamian, A. *Carbohydr. Res.* **2010**, *345*, 1570−1574.

(9) (a) Ishida, Y.; Kitagawa, K.; Nakayama, A.; Ohtani, H. *Appl. Environ. Microbiol.* **2005**, *71*, 7539−7541. (b) Ishida, Y.; Madonna, A. J.; Rees, J. C.; Meetani, M. A.; Voorhees, K. J. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1877−1882. (c) Jones, J.; Stump, M.; Fleming, R.; Lay, J.; Wilkins, C. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1665−1674. (d) Jones, J. J.; Stump, M. J.; Fleming, R. C.; Lay, J. O.; Wilkins, C. L. *Anal. Chem.* **2003**, *75*, 1340−1347. (e) Schumaker, S.; Borror, C. M.; Sandrin, T. R. *Rapid Commun. Mass Spectrom.* **2011**, *26*, 243−253. (f) Calvano, C. D.; Zambonin, C. G.; Palmisano, F. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1757−1764.

(10) (a) Gunstone, F. D.; Harwood, J. L.; Dijkstra, A. J. *The lipid handbook with CD-ROM*; CRC Press: Boca Raton, FL, 2009; pp 1−791. (b) Kerwin, J. L.; Torvik, J. J. *Anal. Biochem.* **1996**, *237*, 56−64. (c) Kerwin, J. L.; Wiens, A. M.; Ericsson, L. H. *J. Mass Spectrom.* **1996**, *31*, 184−192.

(11) (a) Garigapati, V. R.; Bian, J.; Roberts, M. F. *J. Colloid Interface Sci.* **1995**, *169*, 486−492. (b) Tallman, K. A.; Kim, H.-Y. H.; Ji, J.-X.; Szapacs, M. E.; Yin, H.; McIntosh, T. J.; Liebler, D. C.; Porter, N. A. *Chem. Res. Toxicol.* **2007**, *20*, 227−234.

(12) Oberg, T. S.; Ward, R. E.; Steele, J. L.; Broadbent, J. R. *Appl. Environ. Microbiol.* **2012**, *78*, 880−884.

(13) Kobayashi, T.; Tanaka-Ishii, R.; Taguchi, R.; Ikezawa, H.; Murakami-Murofushi, K. *Life Sci.* **1999**, *65*, 2185−2191.

(14) Goldfine, H. *Prog. Lipid Res.* **2010**, *49*, 493−498.

(15) Gheysen, K.; Mihai, C.; Conrath, K.; Martins, J. C. *Chem. Eur. J.* **2008**, *14*, 8869−8878.

(16) (a) Wiegandt, H. *Glycolipids*; Elsevier: London, 1985; pp 1−314. (b) Hoelzl, G.; Doermann, P. *Prog. Lipid Res.* **2007**, *46*, 225−243.

(17) (a) Gerwig, G. J.; De Waard, P.; Kamerling, J. P.; Vliegenthart, J. F. G.; Morgenstern, E.; Lamed, R.; Bayer, E. A. *J. Biol. Chem.* **1989**, *264*, 1027−1035. (b) McConville, M. J.; Thomas-Oates, J. E.; Ferguson, M. A. J.; Homans, S. W. *J. Biol. Chem.* **1990**, *265*, 19611−19623.

(18) (a) Gorin, P. A. J.; Mazurek, M. *Can. J. Chem.* **1975**, *53*, 1212−1223. (b) Sassaki, G. L.; Iacomini, M.; Gorin, P. A. J. *An. Acad. Bras. Cienc.* **2005**, *77*, 223−234. (c) Wieneke, R.; Klein, S.; Geyer, A.; Loos, E. *Carbohydr. Res.* **2007**, *342*, 2757−2765.

(19) Caruso, U. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1283−1285.

(20) (a) Levery, S. B.; Nudelman, E. D.; Hakomori, S. *Biochemistry* **1992**, *31*, 5335−5340. (b) Nudelman, E. D.; Levery, S. B.; Igarashi, Y.; Hakomori, S. *J. Biol. Chem.* **1992**, *267*, 11007−11016.

(21) Yachida, Y.; Kashiwagi, M.; Mikami, T.; Tsuchihashi, K.; Daino, T.; Akino, T.; Gasa, S. *J. Lipid Res.* **1999**, *40*, 2271−2278.

(22) Hikita, T.; Tadano-Aritomi, K.; Iida-Tanaka, N.; Anand, J. K.; Ishizuka, I.; Hakomori, S. I. *J. Biol. Chem.* **2001**, *276*, 23084−23091.

(23) Matsumoto, M.; Tamiya, K.; Koizumi, K. *J. Biochem.* **1971**, *69*, 617−620.

(24) (a) Johnston, N. C.; Goldfine, H. *Eur. J. Biochem.* **1994**, *223*, 957−963. (b) Johnston, N. C.; Goldfine, H. *Biochim. Biophys. Acta, Lipids Lipid Metab.* **1988**, *961*, 1−12.

(25) (a) Khan, A. A.; Chee, S. H.; McLaughlin, R. J.; Harper, J. L.; Kamena, F.; Timmer, M. S. M.; Stocker, B. L. *ChemBioChem* **2011**, *12*, 2572−2576. (b) Stocker, B. L.; Khan, A. A.; Chee, S. H.; Kamena, F.; Timmer, M. S. M. *ChemBioChem* **2014**, *15*, 382−388.

(26) Fujiwara, Y. *Biochim. Biophys. Acta* **2008**, *1781*, 519−524.

(27) (a) Murakami-Murofushi, K.; Kaji, K.; Kano, K.; Fukuda, M.; Shioda, M.; Murofushi, H. *Cell Struct. Funct.* **1993**, *18*, 363−370. (b) Murakami-Murofushi, K.; Kobayashi, S.; Onimura, K.; Matsumoto, M.; Shioda, M.; Yoshida, S.; Shoji, M.; Murofushi, H. *Biochim. Biophys. Acta* **1995**, *1258*, 57−60. (c) Mukai, M.; Imamura, F.; Ayaki, M.; Shinkai, K.; Iwasaki, T.; Murakami-Murofushi, K.; Murofushi, H.;

Kobayashi, S.; Yamamoto, T.; Nakamura, H.; Akedo, H. *Int. J. Cancer* **1999**, *81*, 918−922.
(28) (a) Dittmer, J. C.; Lester, R. L. *J. Lipid Res.* **1964**, *5*, 126−127. (b) Vaskovsky, V. E.; Kostetsky, E. Y.; Vasendin, I. M. *J. Chromatogr.* **1975**, *114*, 129−141.
(29) Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. *Carbohydr. Res.* **1983**, *113*, 291−299.
(30) Ciukanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 8.
(31) Doares, S. H.; Albersheim, P.; Darvill, A. G. *Carbohydr. Res.* **1991**, *210*, 311−317.
(32) Svetashev, V. I.; Vysotskii, M. V.; Ivanova, E. P.; Mikhailov, V. V. *Syst. Appl. Microbiol.* **1995**, *18*, 37−43.
(33) Stránský, K.; Jursík, T.; Vítek, A.; Skořepa, J. *J. High Resolut. Chromatogr.* **1992**, *15*, 730−740.
(34) Carballeira, N. M.; Shalabi, F. *J. Nat. Prod.* **1994**, *57*, 1152−1159.
(35) Jiang, H.; McGiff, J. C.; Quilley, J.; Sacerdoti, D.; Reddy, L. M.; Falck, J. R.; Zhang, F.; Lerea, K. M.; Wong, P. Y.-K. *J. Biol. Chem.* **2004**, *279*, 36412−36418.
(36) McCloskey, J. A.; Law, J. H. *Lipids* **1967**, *2*, 225−230.
(37) Andersson, B. A.; Holman, R. T. *Lipids* **1974**, *9*, 185−190.
(38) Purified glycolipids were tested to be endotoxin-free at a sensitivity of 0.125 EU/mL with an endotoxin kit (Pyrotell, Limulus Amebocyte Lysate).
(39) (a) Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265−9269. (b) Stuehr, D. J.; Nathan, C. F. *J. Exp. Med.* **1989**, *169*, 1543−1555.
(40) (a) Romani, N.; Gruner, S.; Brang, D.; Kämpgen, E.; Lenz, A.; Trockenbacher, B.; et al. *J. Exp. Med.* **1994**, *180*, 83−93. (b) Sallusto, F.; Lanzavecchia, A. *J. Exp. Med.* **1994**, *179*, 1109−1118.
(41) Discovery Ag-Ion SPE for FAME fractionation and cis/trans separation (technical report); Supelco, 2006; http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/Bulletin/t406062.Par.0001.File.tmp/t406062.pdf.