Cell Chemical Biology

Synthetic Lipoteichoic Acid Glycans Are Potential Vaccine Candidates to Protect from Clostridium difficile Infections

Graphical Abstract

Highlights
- Lipoteichoic acid (LTA) glycan antigens of Clostridium difficile were synthesized
- The synthetic LTA glycans are recognized by antibodies of C. difficile patients
- Formulated as a glycoprotein, LTA glycans elicit strong IgG responses in mice
- Immunization with the LTA glycoprotein protects mice from challenge with C. difficile

Authors
Felix Broecker, Christopher E. Martin, Erik Wegner, ..., Claney L. Pereira, Chakkumkal Anish, Peter H. Seeberger

Correspondence
achakkum@its.jnj.com (C.A.), peter.seeberger@mpikg.mpg.de (P.H.S.)

In Brief
Broecker et al. show that synthetic lipoteichoic acid (LTA) glycan antigens derived from Clostridium difficile resemble epitopes of the natural polysaccharide. The synthetic LTA antigens are able to induce antibodies in mice that protect from infection with C. difficile.

Broecker et al., 2016, Cell Chemical Biology 23, 1–9
August 18, 2016 © 2016 Elsevier Ltd.
http://dx.doi.org/10.1016/j.chembiol.2016.07.009
INTRODUCTION

*Clostridium difficile* infections (CDIs) are a primary cause of nosocomial diarrhea and antibiotic-associated colitis (Kelly and LaMont, 2008). Infection and death rates have increased drastically in recent years, partially due to emerging hypervirulent, antibiotic-resistant strains (Zilberberg et al., 2008). Infection and death rates have increased significantly to morbidity and mortality (Kelly and LaMont, 2008). Vaccination with surface antigens may prevent colonization, reduce the reservoir, and thus limit recurrence more efficiently than toxin-neutralizing approaches. Due to the well-known role of glycans in mucosal bacterial adhesion (Moxon and Kroll, 1990), *C. difficile* surface glycans are potential targets for colonization-preventing vaccines.

Three *C. difficile* surface glycans, PS-I, PS-II, and lipoteichoic acid (LTA) (also called PS-III), are known (Ganeshapillai et al., 2008; Reid et al., 2012). PS-II and LTA glycans recovered from *C. difficile* grown in culture are immunogenic in animals and elicit Abs that recognize *C. difficile* bacteria (Bertolo et al., 2012; Adamo et al., 2012; Cox et al., 2013). However, isolated C. difficile glycans cannot be used for the structure-immunogenicity relationship studies required for rational vaccine development due to their heterogeneity and low expression levels in culture (Ganeshapillai et al., 2008; Reid et al., 2012). Therefore, chemical synthesis is the only feasible approach to obtain defined molecules for these studies. The potential of synthetic LTA glycans for vaccines against intestinal Gram-positive bacteria has recently been demonstrated. Immunization with a synthetic LTA fragment of *Enterococcus faecalis* mediated protection against infection in animal models (Laverde et al., 2014). Moreover, synthetic *C. difficile* PS-I and PS-II glycans are promising vaccine candidates as they are recognized by natural Abs from CDI patients and are immunogenic in mice (Martin et al., 2013a; Oberli et al., 2011; Adamo et al., 2012). We recently reported the first synthesis of *C. difficile* LTA glycans and their recognition by Abs from CDI patients (Martin et al., 2013b).

Here, we disclose the immunologic properties of synthetic *C. difficile* LTA glycans. The glycans did not induce innate immune responses in vitro, but elicited LTA-specific Abs that recognized *C. difficile* and limited bacterial colonization in mice in vivo. This study underlines the potential of synthetic LTA glycans as vaccine candidates to prevent CDI.

SUMMARY

Infections with *Clostridium difficile* increasingly cause morbidity and mortality worldwide. Bacterial surface glycans including lipoteichoic acid (LTA) were identified as auspicious vaccine antigens to prevent colonization. Here, we report on the potential of synthetic LTA glycans as vaccine candidates. We identified LTA-specific antibodies in the blood of *C. difficile* patients. Therefore, we evaluated the immunogenicity of a semi-synthetic LTA-CRM197 glycoconjugate. The conjugate elicited LTA-specific antibodies in mice that recognized natural LTA epitopes on the surface of *C. difficile* bacteria and inhibited intestinal colonization of *C. difficile* in mice in vivo. Our findings underscore the promise of synthetic LTA glycans as *C. difficile* vaccine candidates.

**Synthetic Lipoteichoic Acid Glycans Are Potential Vaccine Candidates to Protect from Clostridium difficile Infections**

Felix Broecker,1,2 Christopher E. Martin,1,2,4 Erik Wegner,3 Jochen Mattner,3 Ju Yuel Baek,1,6 Claney L. Pereira,1,6 Chakkumkal Anish,1,6,* and Peter H. Seeberger1,2,7,*

1Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, 14424 Potsdam, Germany
2Department of Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany
3Mikrobiologisches Institut – Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen and Friedrich-Alexander Universität Erlangen-Nürnberg, 91054 Erlangen, Germany
4Present address: Bachem AG, 4416 Bubendorf, Switzerland
5Present address: Vaxxilon Deutschland GmbH, 12489 Berlin, Germany
6Present address: Bacterial Vaccines Discovery and Early Development, Janssen Pharmaceuticals (Johnson & Johnson), 2333 CK Leiden, The Netherlands
7Lead Contact
*Correspondence: achakkum@its.jnj.com (C.A.), peter.seeberger@mpikg.mpg.de (P.H.S.)
http://dx.doi.org/10.1016/j.chembiol.2016.07.009"
Other synthetic LTAs exert stimulating activity at comparable concentrations (Morath et al., 2002; Pedersen et al., 2010). Lipo-polsaccharide (LPS) served as our positive control. Stimulation with LPS, but not with 2, elevated surface expression of DC activation markers CD80 and CD86 (Figure 2B). In addition, cytokines were not induced by stimulation with 2, whereas LPS dose-dependently activated TNF-α and IL release (Figure 2C). Therefore, 2 did not activate innate immunity in DCs in vitro.

**Synthetic LTA Glycans Represent Natural Epitopes Recognized by Human Abs**

Next, we wondered if synthetic *C. difficile* LTA glycans can induce adaptive immunity by means of opsonizing Abs. Such Abs are the basis of protection against many bacterial infections (Astronomo and Burton, 2010). Isolated *C. difficile* LTA glycans elicit bacteria-opsonizing Abs (Cox et al., 2013) and Abs to 1–3, representing repeat units of the natural glycan, present in CDI patients indicates that natural and synthetic LTA glycans contain similar epitopes (Martin et al., 2013b). However, many factors drive the Ab response to an antigen, including size and charge (Martin et al., 2013a; Adamo et al., 2012). Therefore, we tested the antigenicity of synthetic LTA glycans by screening natural human Abs raised against the bacteria by glycan array.

Secretory immunoglobulin A (sgA) is involved in the defense against intestinal pathogens (Macpherson et al., 2001). The identification of sgA to PS-I and PS-II antigens suggests that *C. difficile* surface glycans are targets of sgA-mediated immunity (Martin et al., 2013b; Oberli et al., 2011). To identify sgA to synthetic LTA glycans, we screened fecal samples of CDI patients and control individuals using glycan arrays. In line with previous findings (Martin et al., 2013b; Oberli et al., 2011), we detected sgA to PS-I and PS-II antigens but did not identify sgA to 1–3. Natural *C. difficile* LTA glycan may therefore not induce sgA in detectable quantities.

In addition to sgA, mucosal tissues bear significant amounts of immunoglobulin G (IgG) capable of providing protection from pathogens (Ménard et al., 2010). High serum IgG levels to *C. difficile* toxin A are associated with protection from CDI (Kyne et al., 2000). Increased serum IgG levels to PS-I antigens in recovered CDI patients suggest that these Abs play a role in the defense against *C. difficile* (Martin et al., 2013a). To assess whether serum IgG to LTA glycans is increased during CDI, we screened serum samples from recovered CDI patients and control individuals with no history of CDI using glycan arrays (Figure 3A). Serum IgG levels to 1–3 were slightly higher in recovered patients, but without statistical significance (Figure 3B). The presence of IgG to LTA glycans in the control group may have been due to asymptomatic exposure to *C. difficile*. We detected significantly higher serum IgG responses to PS-I in recovered patients, while IgG levels to PS-II were almost similar in both groups, confirming previous findings (Martin et al., 2013a). The presence of serum IgG to 1–3 in human subjects showed that synthetic LTAs represent natural epitopes and are therefore promising antigens for further immunologic evaluation.

**Coupling of LTA Dimer 2 to CRM197 Yields a Glycoconjugate Vaccine Candidate that Efficiently Adsorbs to Alum Adjuvant**

To investigate the immunogenicity of synthetic LTA glycans we selected 2, as larger glycans are usually more immunogenic.
We intended to immunize mice with 4 either in the absence of adjuvant, co-administered with Freund’s adjuvant (FA), or alum. FA was selected due to its potency in promoting anti-glycan Abs in mice (Oberli et al., 2011; Martin et al., 2013a) and alum was chosen as it is a human-approved adjuvant (Astronomo and Burton, 2010). Adsorption of (glyco)protein antigens is necessary for alum to exert immunogenicity-enhancing effects (Clapp et al., 2011). Therefore, we quantitatively determined adsorption of 4 to alum with two in vitro methods. In a modified assay described by Skrastina et al. (2014), alum was mixed with 4 in PBS and incubated at 4°C for up to 24 hr. The concentration of non-adsorbed protein was used to calculate adsorption efficiencies in percent (Figure 5A). Native CRM197 served as control. Glycoconjugate 4 was dose-dependently adsorbed to alum more efficiently than CRM197. The efficiency did not majorly benefit from longer incubation times, indicating that the adsorption process was mostly completed within minutes. After 24 hr of incubation and a 1:1 ratio of 4 (3 μg) and alum (3 μL), 85.9% of the glycoconjugate was adsorbed to alum. These results were confirmed with a flow cytometric assay modified from Ugozzoli et al. (2011), in which adsorbed 4 was quantitatively detected with an anti-CRM197 antibody. Alum particles showed a distinct population in the scatterplot (Figure 5B). Again, we observed dose-dependent adsorption that was more efficient for 4 than for CRM197. The fraction of anti-CRM197-labeled particles reached 73.9% when 4 and alum were incubated at a 1:1 ratio (Figure 5C). Consequently, we chose these conditions to prepare alum-adsorbed 4 intended for immunizations.

Antibodies to Synthetic LTA Glycans Raised in Mice Bind to C. difficile and Limit Bacterial Colonization In Vivo

Three groups of mice received three doses of 4 containing 3 μg glycan antigen administered either without adjuvant, with alum, or with FA (Figure 6A). Ab responses were followed by microarray-assisted serum IgG analysis. Microarrays covalently functionalized with LTAs 1–3 and control oligosaccharides allowed for multiplexed detection of glycan-specific IgG without requiring a protein carrier as in conventional ELISA (Geissner et al., 2014). Glycoconjugate 4 elicited LTA-specific Abs (Figure 6B). Identification of IgG1, IgG2a, and IgG3 subtypes indicated that class switch, a hallmark of T cell-dependent immunity, occurred (Figure 6C). IgG levels to LT antigen increased over time. Post-immune (week 5) IgG1 and IgG2a levels were highest in mice immunized with 4 and FA, while alum promoted highest levels of IgG3. The effect of adjuvants on anti-glycan Ab levels was less pronounced than observed previously (Martin et al., 2013a) and post-immune IgG levels to the CRM197 carrier protein were almost identical in the three groups of mice (Figure 6D).

The low adjuvant activity of FA and alum may have been due to degradation of 4 in the presence of the adjuvants. We therefore compared the integrity of 4 either in soluble or adjuvant-formulated form. After 7 days of incubation at 37°C, 4 was extracted from the adjuvants using methods described previously (Miles et al., 2013a) and non-conjugated 4 was purified by size exclusion chromatography. MALDI-TOF mass spectrometry analysis revealed that 4 was mostly (>85%) intact in both formulations (Figure 4C). The mass shift was quantified by MALDI-TOF mass spectrometry (MS) analysis to ~5,300 Da (Figure 4C), corresponding to about four molecules of C24 linked per molecule of CRM197 on average.

Figure 2. Synthetic LTA Glycans Do Not Induce Innate Immunity In Vitro

(A) Murine bone marrow cells were differentiated to BMDCs with granulocyte macrophage colony-stimulating factor (GM-CSF) (see Figure S1 for details) and stimulated with LTA 2 or LPS in vitro. Then, surface levels of activation markers (B) and cytokine release (C) were measured.

(B) Flow cytometric analysis of CD80 and CD86 surface levels. Representative results for LTA 2 or LPS treated BMDCs, showing mean ± SEM of 2 experiments. LTA 2 concentrations: 0.1, 0.5, 1, and 10 μM; LPS concentrations: 0.01, 0.05, 0.1, 1, and 10 μg/mL. Control bars represent unstimulated cells. n.d., not detectable.

(C) Quantitation of cytokine release by ELISA. Bars show mean ± SEM of two experiments. LTA 2 concentrations: 0.1, 0.5, 1, and 10 μM; LPS concentrations: 0.1, 0.05, 0.1, 1, and 10 μg/mL. Control bars represent unstimulated cells. n.d., not detectable.
SDS-PAGE analysis showed no signs of degradation (Figure S4). The presence of glycan antigens on 4 was further verified by a competition microarray experiment using pooled week 5 post-immune serum from the alum group (Figure 6E). IgG binding to 2 was efficiently competed by pre-incubation with adjuvant-extracted 4 similar to non-formulated glycoconjugate. Thus, there was no evidence that alum or FA influenced the stability of 4.

Next we assessed whether the external adjuvants influenced glycan epitope recognition, as observed previously for the C. difficile glycan antigen PS-I where alum guided IgGs mainly to the entire repeating unit, whereas with FA smaller oligosaccharides were also recognized (Martin et al., 2013b; Broecker et al., 2016). Epitope recognition may influence Ab reactivity to the natural LTA polysaccharide. Therefore, we compared the reactivity of IgG1 to 1–3 before and after the second boost immunization (weeks 3 and 5, respectively) among the three groups of mice (Figure 6F). The boost increased IgG1 to 1–3 when no adjuvant or alum was used. At week 5, Ab levels to 1 and 3 were comparable with 2, indicating that with these two formulations the minimal glycan epitope recognized by the Abs was monomer 1 or a substructure thereof. In contrast, boosting in the presence of FA elevated IgG1 levels to 2 and 3 but not to 1, which was only weakly recognized at week 5. Here, the minimal glycan epitope seemed to be the monomer with two phosphodiesters 3. Differential epitope recognition may be explained by the nature of immune responses promoted by the adjuvants. FA induces a Th1- and alum a Th2-type response (Brewer and Alexander, 1997). The IgG1 to IgG2a ratio helps to differentiate between Th1- and Th2-type responses (Coffman et al., 1988). We found no significant differences in the IgG2a to IgG1 ratios among the three groups of mice (Figure 6G), indicating that Th skewing did not play a major role in differential epitope recognition.

To determine whether Abs raised with 4 recognized the natural LTA polysaccharide on C. difficile, whole-bacteria ELISA was performed. ELISA wells were coated with inactivated bacteria and incubated with pooled serum. The level of bacteria-binding IgG was determined as post-immune (week 5) to pre-immune (week 0) ratios (Figure 6H). IgG raised using 4 without adjuvant or alum significantly opsonized all investigated C. difficile strains, including type strain DSM 1296 and clinical isolates of ribotypes 001, 014, 027, 046, and 078. No binding was observed to Gram-negative and -positive control bacteria, Salmonella enterica, and Streptococcus pneumoniae, respectively. Clostridium bifermentans, but not Clostridium perfringens, was recognized by the Abs, similar to Abs raised with natural LTA polysaccharide (Cox et al., 2013). These observations indicated that C. difficile and C. bifermentans share similar LTA epitopes. IgG raised using 4 and FA weakly bound to C. difficile strains, but also to all other bacteria except C. perfringens.

Finally, we addressed whether immunization with 4 could limit C. difficile colonization in vivo. A group of four mice were immunized three times with 4 in the presence of alum (Figure 6I). This immunization regime was selected as it proved suitable to induce high levels of LTA-specific IgGs (Figure 6C) that recognized the surface of C. difficile (Figure 6H). Two control groups included mice immunized with an equal amount of alum-adjuvanted CRM197 as well as age-matched non-immunized animals. The latter group was included to account for possible effects of the immunization procedure on innate immunity that may have an impact on bacterial colonization. Mice were rendered susceptible to infection with clindamycin (Buffie et al., 2012) and orally challenged with live C. difficile bacteria 7 days after the final immunization. Five days post-challenge, the degree of bacterial colonization was determined by counting colony-forming units (CFUs) in the feces of the mice. Immunization with 4 significantly reduced colonization by C. difficile compared with the non-immunized and CRM197-immunized control groups, whereas no difference was observed for control bacteria, such as Enterococcus spp. (Figure 6J). The colonization-inhibiting effects were LTA specific, as immunization with CRM197 alone did not significantly affect C. difficile levels compared with non-immunized mice.

**DISCUSSION**

Here, we disclosed the immunologic properties of synthetic C. difficile LTA glycans. While LTAs are known stimulants of innate immunity by engaging TLR2 (Schwandner et al., 1999), we found no evidence that synthetic LTAs activated DCs in vitro. The lipid anchor of LTA (Reid et al., 2012) that was not included in the synthetic glycans may be required for stimulating activity. LTA lipid chains interact with a hydrophobic pocket of TLR2 for which, however, ligands without hydrophobic regions are also known (Kang et al., 2009). Another reason for the lack of innate immune stimulation may be the unusual structure of the C. difficile LTA.
The soluble glycoconjugate was highly immunogenic, suggesting that the attached glycan moieties of 2 provided intrinsic adjuvant activity. Adjuvant and immunomodulatory effects have been described before for isolated, including lipid-free, LTAs (Chorpenning et al., 1979; Himanen et al., 1993). Moreover, 2 is highly charged and the immunogenicity-promoting effect of charged moieties of glycans against C. difficile is established (Adam et al., 2012; Tzianabos et al., 2003; Müller-Loennies et al., 2000). The use of alum elevated the level of bacteria-binding Abs mainly of the IgG3 subclass. In contrast, FA proved not to be suitable as adjuvant since the elicited IgGs only weakly recognized C. difficile. This observation may be explained by the epitope recognition pattern of the IgGs that was different from Abs raised without adjuvant or with alum. Ab recognition of the monomeric LTA repeating unit 1 may be crucial for efficient binding to the natural polysaccharide on C. difficile.

Finally, we showed that immunization with the glycoconjugate and alum limited bacterial colonization in a mouse model of CDI.

**SIGNIFICANCE**

Despite the unmet medical need, no vaccine against the hospital-acquired pathogen C. difficile is yet available. Toxin-based vaccines are currently in clinical testing but cannot preclude colonization. C. difficile surface glycans are promising targets for colonization-preventing vaccines that may help to limit the reservoir for the bacteria and prevent recurrent disease episodes. Biologic studies with isolated C. difficile glycans are hampered by heterogeneity and weak polysaccharide expression in bacterial culture in vitro. We used synthetic, well-defined glycan fragments of the C. difficile lipoteichoic acid surface polysaccharide and studied their immunologic properties. Synthetic LTA glycans did not induce innate immune responses in vitro. However, the detection of anti-LTA serum antibodies in C. difficile patients provided a rationale to assess their immunogenic properties. A potential vaccine candidate composed of the immunogenic carrier protein CRM197 and a synthetic LTA glycan was immunogenic in mice. The antibodies opsonized C. difficile and limited intestinal colonization in mice in vivo, rendering synthetic LTA glycans promising vaccine candidates against C. difficile. Glycoconjugate vaccines would be complementary to toxin-neutralizing vaccination approaches that are currently under intense investigation.

**EXPERIMENTAL PROCEDURES**

**Ethics Statement**

The study protocol (protocol no. 4,439) for the analysis of stool and serum specimens of patients with CDI and control individuals was approved by the Ethics Committee of the University of Erlangen. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science and the European Health Law of the Federation.
In Vitro Differentiation of Murine Bone Marrow Cells and Stimulation Assays

Bone marrow cells were isolated from femurs and tibias of 6 week old female BALB/c mice. Single-cell suspensions were obtained by gently passing cell clumps through a cell strainer. Suspensions were adjusted to 10^6 cells per mL and seeded into 6-well cell culture plates (3 mL per well) in RPMI-1640 medium with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 20 ng/mL recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF; ProTech) for 8 days at 37°C and 5% CO2. Every 2–3 days, cells were fed with 1 mg CRM197 (Pfenex) in 1 mL of 100 mM Na-phosphate buffer (pH 7.4), unreacted spacer extracted with chloroform. The aqueous phase was reacted with serum or fecal samples diluted 1:100 in 1% BSA and 0.01% Tween 20 in PBS for 1 hr and washed three times with 0.1% Tween 20 in PBS. Slides were blocked with 1% BSA in PBS for 1 hr and washed three times with 0.1% Tween 20 in PBS for 1 hr, washed three times with 0.1% Tween 20 in PBS and once with deionized water. After scanning with a GenePix 4300A microarray scanner (Molecular Devices), images were analyzed with GenePix Pro 7.

Alum Adsorption Studies

Two assays were used to quantify the adsorption of glycoconjugate 4 or CRM197 to alum. First, with a modified assay described by Skrastina et al. (2014), alum particles (3, 1.5, 0.3, or 0.03 μg) were mixed with 3 μg of 4 or CRM197 in PBS (final volume 20 μL) and vortexed briefly. Samples were incubated at 4°C for 0.5, 1, 4, or 24 hr while rotating or used directly after mixing. Samples were centrifuged at 3,000 × g for 10 min. The protein concentration in the supernatant was determined with the Micro BCA Protein Assay Kit (Thermo Scientific) modified such that it allowed for measuring small volume samples (5 μL) in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Protein concentrations were referenced to samples with alum particles but without protein set to 100% and samples with protein but without alum set to 0%. The second assay was a flow cytometric method described by Ugozzoli et al. (2011) with minor modifications. Different concentrations of 4 or CRM197 (0.1, 1, 5, or 10 μg) were incubated with 10 μL of alum in PBS (final volume 70 μL) at 4°C while rotating for 24 hr and subjected to flow cytometry as described above.

Flow Cytometry

Stimulated BMDCs were analyzed in a BD FACSCanto II flow cytometer (BD Biosciences), counting at least 10,000 events per sample. Detection Abs: goat anti-human IgA FITC (Sigma-Aldrich; diluted 1:100), goat anti-human IgG Alexa Fluor 647 (1:400), goat anti-mouse IgG1 Fluor 594 (1:400), goat anti-mouse IgG2α Alexa Fluor 647 (1:200), goat anti-mouse IgG3 Alexa Fluor 488 (1:200) (all by Life Technologies). All incubation steps were at room temperature (RT).
Immunizations

Female C57BL/6 mice (6–8 weeks old) purchased from Charles River, Germany, were kept in individually ventilated cages in the animal facility of the Federal Institute for Risk Assessment, Berlin, Germany, under specific-pathogen-free conditions. Mice were immunized subcutaneously (s.c.) with 4 without adjuvant, with alum Alhydrogel adjuvant (Brenntag), or with FA. The initial immunizations with FA were performed with Complete Freund’s adjuvant and consecutive immunizations with incomplete Freund’s adjuvant (both by Sigma-Aldrich). For immunizations with alum, 4 was mixed with alum 1:1 (w/v) and incubated for 24 hr at 4°C before immunization.

ELISA

Commercial ELISA kits were used to determine IL-1β, -10, -12, and -6 (PeproTech) and TNF-α (R&D Systems) levels in BMDC culture supernatants. To determine the serum IgG levels binding to CRM197 or to the surface of bacteria, ELISA wells were coated with 10 ng/mL CRM197 or formalin-inactivated

Figure 6. Glycoconjugate 4 Elicits Anti-LTA IgGs in Mice that Bind to C. difficile and Limit Bacterial Colonization In Vivo

(A) Mice were immunized with 4 in different adjuvant formulations. Serum IgG responses were followed at indicated time points.

(B) Representative microarray scans showing IgG1 of one mouse before (week 0) and after (week 5) immunization (see Figure S3 for details).

(C) Microarray-inferred serum IgG levels. Data points are mean ± SEM of three mice.

(D) ELISA-inferred anti-CRM197 IgG levels at weeks 0 and 5. Bars represent mean ± SEM of two experiments.

(E) Competition microarray studies with adjuvant-extracted 4. Week 5 pooled serum of the alum group diluted 1:1,500 was pre-incubated with the indicated competitors before IgG binding signals to 2 were determined. Bars show mean ± SD of three microarray spots (see Figure S4 for details).

(F) Comparison of serum IgG1 levels to 1–3 at weeks 3 and 5. Bars show mean ± SD of three mice. n.d., not detectable.

(G) Comparison of IgG2a to IgG1 ratios. Bars represent mean ± SEM of three mice, except for the alum group (two mice, as one mouse did not respond to 4).

(H) Serum IgG binding to bacteria assessed by ELISA. Fold change was calculated by dividing binding signals of week 5 to week 0 serum. Bars show mean ± SEM of two experiments in duplicate. *p < 0.05, two-sided unpaired Student’s t test against C. perfringens.

(I) Mice were immunized with either 4 or CRM197 as control, both in the presence of alum, and orally challenged with C. difficile. Five days later, bacterial colonization was quantified by determining fecal CFUs. Age-matched non-immunized mice otherwise treated identically served as additional controls.

(J) Fecal CFUs of C. difficile or Enterococcus spp. 5 days post-challenge. Each data point represents one mouse, horizontal lines are mean values. *p < 0.05; n.s., not significant, Mann-Whitney U test.
bacteria (OD$_{600}$ = 0.2) in PBS overnight at 4°C, blocked with 1% BSA in PBS for 1 hr at RT, incubated with pooled sera diluted 1:500 in 1% BSA and 0.01% Tween 20 in PBS for 1 hr at RT. IgG levels were quantified with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Dianova; 1:1,000,000) and 1-Step Ultra TMB-ELISA Substrate (Thermo Scientific).

**Stability Studies of Glycoconjugate 4**

To assess its long-term and temperature stability, a solution of 4 was first filter sterilized (0.22 μm pore size). The glycoconjugate was adsorbed to alum at a ratio of 1:1 (w/v) or formulated as 50% (v/v) emulsion with incomplete Freund’s adjuvant (Sigma-Aldrich). The samples contained 15 μg of 4 in a final volume of 100 μL in sterile PBS. A solution of 4 in PBS served as control. The samples were incubated for 1 week at different temperature regimes (7 days at 4°C, 6 days at 4°C, and 1 day at 37°C, 4 days at 4°C and 3 days at 37°C, or 7 days at 37°C). 4 was extracted from alum particles with an extraction buffer composed of 0.6 M sodium citrate dihydrate, 0.55 M sodium phosphate dibasic, 30 mM SDS (pH 8.5) (Zhu et al., 2012). To 100 μL of alum-adsorbed 4, 200 μL of extraction buffer was added, mixed ten times by inversion and incubated at 60°C for 2.5 hr with gentle inversion of the samples every 20 min. Samples were centrifuged at 425 × g for 2 min. The supernatant was directly used for SDS-PAGE or, after washing three times with deionized water using 10 kDa centrifugal filter units (Merck Millipore) to remove potential detached glycans, for competition microarray studies. 4 was extracted from incomplete Freund’s adjuvant emulsions with a procedure described previously (Miles and Saul, 2009) with minor modifications. To the emulsions (100 μL), 50 μL of benzyl alcohol was added and the mixture was vortexed for 20 min at maximum speed on a benchtop vortex. Samples were centrifuged at 16,100 × g for 10 min. 4 was recovered from the middle aqueous phase that was used directly for SDS-PAGE or, after washing as above, for competition microarray studies.

**Competition Microarray Studies**

Pooled serum of mice immunized with 4 in the presence of alum (week 5, diluted 1:50) was pre-incubated with adjuvant-extracted 4 at 0.4 μg/mL or CRM197 (50 μg/mL) as control for 5 min in 1% BSA and 0.01% Tween 20 in PBS, before applying to blocked microarray slides. The remaining procedure followed the microarray binding assay method described above. Goat anti-mouse IgG Alexa Fluor 635 (Life Technologies) diluted 1:400 was used for detection.

**Challenge Studies**

Female, 6- to 8-week-old C57BL/6 mice received three s.c. doses of glycoconjugate 4, or an equal amount of CRM197, in the presence of alum at 2-week intervals. Six days after the third immunization, mice were injected intraperitoneally with 20 mg of clindamycin per kg body weight (Buffie et al., 2012). The following day, mice were challenged via oral gavage with 10$^7$ CFUs of the *C. difficile* in Dublin, Ireland. Clin. Microbiol. Infect. 13, 298–304. Ganeshappai, J., Vinogradov, E., Rousseau, J., Weese, J.S., and Monteiro, M.A. (2008). Clostridium difficile cell-surface polysaccharides composed of pentaglycosyl and hexaglycosyl phosphate repeating units. Carbohydr. Res. 343, 703–710.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.07.009.

**AUTHOR CONTRIBUTIONS**

P.H.S. and C.A. initiated and designed the study. F.B. performed the experiments with assistance from C.A. C.E.M., J.Y.B., and C.L.P. synthesized compounds 1-3 and control oligosaccharides. J.M. and E.W. performed and evaluated mouse challenge studies. P.H.S., C.A., and F.B. wrote the manuscript. All authors have approved the final version of the manuscript.

**ACKNOWLEDGMENTS**

This work was funded by the Max Planck Society, the Körber Foundation (Körber Prize to P.H.S.), and the German Federal Ministry of Education and Research (grant nos. 0315447 and 03IS2201G), as well as by the German Research Foundation (DFG – CRC1181 (C04) and DFG - MA 2621/3-1 to J.M.). We express our gratitude to Dr. Bernd Lepenies for advice and support for animal ethics, Theresa Wagner for assistance with flow cytometry data analysis, Dr. Ulrich Nübel (Robert-Koch-Institut Wernigerode) and Dr. Jochen Klumpp (ETH Zürich) for providing formalin-inactivated bacteria. We thank Pfenix, Inc. for offering CRM197 and Toxin B proteins at a reduced price.

Received: December 30, 2014
Revised: June 28, 2016
Accepted: July 8, 2016
Published: August 11, 2016

**REFERENCES**


Supplemental Information

Synthetic Lipoteichoic Acid Glycans
Are Potential Vaccine Candidates to Protect from Clostridium difficile Infections

Felix Broecker, Christopher E. Martin, Erik Wegner, Jochen Mattner, Ju Yuel Baek, Claney L. Pereira, Chakkumkal Anish, and Peter H. Seeberger
Figure S1 Related to Figure 2. Flow Cytometric Analysis of Murine BM Cells and BMDCs. (A) Representative scatter plots showing expression of CD11c on BM cells stimulated for eight days with buffer control (left) or GM-CSF (right). (B) Representative scatter plots showing expression of CD80 and CD86 on BMDCs after stimulation with LTA or LPS. (C) Representative histograms showing expression of CD80 on stimulated BMDCs. (D) Representative histograms showing expression of CD86 on stimulated BMDCs.
Figure S2 Related to Figure 3. Detection of Natural Antibodies to Synthetic *C. difficile* LTA Glycans by Microarray. The spotting pattern is shown to the right. LTRT is the *Leishmania* lipophosphoglycan capping tetrasaccharide (Hewitt and Seeberger, 2001a and 2001b). PS-I disaccharide is the Rha-(1→3)-Glc minimal epitope and PS-I the pentasaccharide repeat unit of *C. difficile* PS-I (Martin et al., 2013a). Rha is mono-rhamnose (Martin et al., 2013a). PS-II is the *C. difficile* PS-II repeat unit hexasaccharide (Oberli et al., 2011). Toxin B is *C. difficile* toxin B protein purchased from Pfēnex, Inc. Buffer is 50 mM Na-phosphate, pH 8.5. Glycans were spotted at 0.5 and 1 mM (small and large spots, respectively) and Toxin B at 0.5 and 1 μM (small and large spots, respectively).

(A) Microarray scan representing sIgA in fecal samples of CDI patients and controls. Secondary Ab was goat anti-human IgA FITC (Sigma-Aldrich). Due to high background, binding signals of the ‘Patient E’ sample could not be determined. (B) Microarray scan representing serum IgG in serum samples of recovered CDI patients and control individuals with no history of CDI. Secondary Ab was goat anti-human IgG Alexa Fluor 647 (Life Technologies).
Table:

<table>
<thead>
<tr>
<th>No adjuvant Mouse 847 Week 0</th>
<th>No adjuvant Mouse 874 Week 3</th>
<th>No adjuvant Mouse 874 Week 5</th>
<th>No adjuvant Mouse 847 Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No adjuvant Mouse 872 Week 5</td>
<td>No adjuvant Mouse 820 Week 0</td>
<td>No adjuvant Mouse 877 Week 3</td>
<td>No adjuvant Mouse 820 Week 5</td>
</tr>
<tr>
<td>Alum Mouse 820 Week 0</td>
<td>Alum Mouse 787 Week 3</td>
<td>Alum Mouse 872 Week 3</td>
<td>Alum Mouse 620 Week 0</td>
</tr>
<tr>
<td>Alum Mouse 787 Week 5</td>
<td>Alum Mouse 672 Week 5</td>
<td>Freund’s Mouse 823 Week 0</td>
<td>Freund’s Mouse 823 Week 5</td>
</tr>
<tr>
<td>Freund’s Mouse 983 Week 0</td>
<td>Freund’s Mouse 978 Week 3</td>
<td>Freund’s Mouse 823 Week 3</td>
<td>Freund’s Mouse 983 Week 3</td>
</tr>
<tr>
<td>Freund’s Mouse 978 Week 5</td>
<td>Freund’s Mouse 823 Week 5</td>
<td>Freund’s Mouse 983 Week 5</td>
<td>PBS + BSA</td>
</tr>
</tbody>
</table>

**Figure S3 Related to Figure 6.** Detection of Anti-LTA Antibodies in Sera of Mice Immunized with Glycoconjugate 4 by Microarray. The spotting pattern is indicated to the right of the IgG3 microarray scan (see caption of Figure S2 for details). Microarray scans represent serum IgG1, IgG2a and IgG3. The four spots of immunogen 2 are highlighted. Results are representative of two experiments.
Figure S4 Related to Figure 6. Stability Studies of Glycoconjugate 4. (A) Experimental flowchart. (B) Denaturing SDS-PAGE analysis of adjuvant-extracted 4 after incubation at different temperature regimes shown in (A). CRM197 (100 μg) was loaded for comparison. Protein was visualized by silver staining. The total amount of 4 varies as fixed volumes of crude extracts were used. (C) Competition microarray experiment with adjuvant-extracted and washed 4 after incubation at the indicated temperature regimes. The IgG binding signals of 1:1500-diluted pooled post-immune serum (week 5, mice immunized with 4 in the presence of Alum) to 2 spotted at 0.1 mM with or without competitors are shown as bars (mean ± SD of three spots). Binding signals of serum without competitor was set to 100 % (white). As competitors, either CRM197 (grey) at 50 μg/mL or 4 (green, blue, black) at 0.4 μg/mL were pre-incubated for 5 min. with the serum before microarray analysis. Microarray scans of 2 spotted in triplicate are shown above the respective bars.
Supplemental Experimental Procedures

Patients
A positive Toxin ELISA and the growth of C. difficile identified CDI patients five to 25 days after the onset of clinical symptoms. The majority of CDI patients were hospitalized in intensive care units due to surgeries (abdominal, brain) or organ transplantation. The group of control patients reflects individuals whose stool samples were sent in for microbial analysis as well as control samples from individuals without diarrhea. All patient and control samples were age- and sex-matched and between 10 and 92 years old.

Preparation of Fecal Samples
Fresh fecal samples were weighed. Two volumes PBS supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) were added, samples vortexed vigorously and incubated on ice for 1 h. Samples were centrifuged for 20 min at 10,000 x g. Supernatants were used for microarray binding assays.

SDS-PAGE
Samples were dissolved in Lämmli buffer (0.125 M Tris, 20% (v/v) glycerol, 4% (w/v) SDS, 5% (v/v) beta-mercaptoethanol, bromophenol blue, pH 6.8) and boiled at 95°C for 5 min. Samples were run in a 10% polyacrylamide gel and stained with 0.025% Coomassie Brilliant Blue R-250 in an aqueous solution containing 40% (v/v) methanol and 7% (v/v) acetic acid. Gel was destained with an aqueous solution containing 50% (v/v) methanol and 10% (v/v) acetic acid. PageRuler Plus Prestained Protein Ladder (Thermo Scientific, Waltham, MA, USA) served as size marker. As alternative staining method, gels were stained by silver staining as follows. Gels were incubated in fixing solution (50 % (v/v) ethanol, 12 % (v/v) acetic acid with 0.05 % (v/v) aqueous 37 % formaldehyde) for 1 h. After washing twice with deionized water for 10 min., gels were incubated with 1 mM Na2S2O3 for 1 min. After washing three times with deionized water for 20 s. Gels were incubated in 10 mM AgNO3 with 0.08 % (v/v) aqueous 37 % formaldehyde for 12 min. After washing twice with deionized water for 20 s, gels were incubated in 5 M Na2CO3, 1 mM Na2S2O3 with 0.04 % (v/v) aqueous 37 % formaldehyde until protein bands became visible. The reaction was stopped by washing twice with deionized water for 2 min.

MALDI-TOF Mass Spectrometry
MALDI-TOF mass spectra of CRM197 and 4 were obtained with an Autoflex Speed instrument (Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in linear positive mode. Spectra were acquired over an m/z range from 30,000 to 210,000 and data was analyzed with the FlexAnalysis software provided with the instrument. 2',4'-dihydroxyacetophenone (DHAP) was used as matrix. Samples were spotted with the dried droplet technique.

Bacteria
Formalin-inactivated bacteria (Salmonella enterica, DSM 17058; Streptococcus pneumoniae, DSM 14377; C. difficile, DSM 1296 (similar to ATCC 9689) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Clostridium bifermentans was a kind gift of Dr. Jochen Klumpp (ETH Zurich, Switzerland). Clostridium perfringens and C. difficile clinical isolates RT 001, 014, 027, 046 and 078 were kindly provided by Dr. Ulrich Nübel (Robert-Koch-Institut, Wernigerode, Germany). They represent the most prevalent C. difficile strains in Germany (Zaiss et al., 2010).

Supplemental References
