Supplemental Information

Synthetic Lipoteichoic Acid Glycans

Are Potential Vaccine Candidates to Protect

from Clostridium difficile Infections

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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, pH8.5. 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).
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. and washed 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
