Supplementary Information

Synthetic Oligosaccharide-based Vaccines Protect Mice from *Clostridioides difficile* Infections

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Experimental Procedures

**Preparation of Microarrays.** Amine-functionalized oligosaccharides or proteins were immobilized on commercial N-hydroxysuccinimide (NHS) ester-activated microarray slides (CodeLink Activated Slides; SurModics) using a piezoelectric spotting device (S3; Scienion) such that 64 identical subarrays were contained on each slide. Samples for spotting were diluted in 50 mM sodium phosphate buffer, pH 8.5. Slides were incubated in a humid chamber for 24 h at room temperature to complete coupling reactions. Remaining NHS ester groups were deactivated with 50 mM ethanolamine in 50 mM sodium phosphate buffer, pH 9, for 1 h at 50 °C. Slides were rinsed three times with deionized water, dried by centrifugation (300 x g, 5 min) and stored desiccated until use.

**Preparation of Glycoconjugates with DNAP Crosslinker.** Glycoconjugate preparation with di-p-nitrophenyl adipate (DNAP) followed a modified procedure as described before.\(^{23}\) The amounts of oligosaccharides listed in Figure 1B were combined with a ~6-fold molar excess of DNAP crosslinker in 400 μL anhydrous dimethyl sulfoxide (DMSO)/pyridine (2:1) and 10 μL triethylamine (Et\(_3\)N). The respective reaction mixtures were incubated for 2 h at room temperature while stirring. Solvents were evaporated via lyophilization. Dried reaction products were successively washed with dichloromethane and chloroform (ten times 1 mL each) until thin-layer chromatography (TLC) revealed complete removal of non-reacted crosslinker. The washed reaction products were solubilized in DMSO, transferred to new reaction tubes and lyophilized. Dried products were reacted with CRM\(_{197}\) (Pfénex) in 100 mM sodium phosphate buffer, pH 8, for 24 h at room temperature while stirring. The amounts of CRM\(_{197}\) for each reaction are listed in Figure 1B. Resulting glycoconjugates were washed and concentrated with deionized water using 10 kDa centrifugal filter units (Merck Millipore). Protein concentrations were determined by measuring the absorbance at 280 nm in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), using the calculated extinction coefficient of CRM\(_{197}\), 54,320 M\(^{-1}\) cm\(^{-1}\).

**Preparation of Glycoconjugates with DSAP Crosslinker.** Glycoconjugates with di-N-succinimidyl adipate (DSAP) crosslinker were prepared as described (19). Oligosaccharide 3 was dissolved in 210 μL anhydrous DMSO with 10 μL Et\(_3\)N, oligosaccharide 5 in 190 μL anhydrous DMSO with 10 μL methanol and 10 μL Et\(_3\)N. The respective solutions were added drop-wise over a period of 30 min to stirred solutions containing a ~10-fold molar excess of DSAP solubilized in 115 μL (for 3) or 180 μL (for 5) anhydrous DMSO. Mixtures were incubated for additional 1.5 h at room temperature while stirring. After addition of 400 μL 100 mM sodium phosphate buffer, pH 7.4, non-reacted crosslinker was extracted twice with 10 mL chloroform. The upper aqueous phase was recovered and immediately reacted with CRM\(_{197}\) in 100 mM sodium phosphate buffer, pH 7.4, for 24 h at room temperature while stirring. The amounts of CRM\(_{197}\) for each reaction are shown in Figure 1B. Resulting glycoconjugates were washed and concentrated with deionized water using 10 kDa centrifugal filter units (Merck Millipore). Protein concentrations were determined with the Micro BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer’s protocol.

**Preparation of Formalin-inactivated TcdB Antigen.** Lyophilized recombinant TcdB (Pfénex) was solubilized in PBS containing 0.05% formaldehyde to a protein concentration of 1 mg mL\(^{-1}\). After incubation for 1 h at room temperature while stirring, the toxoid was recovered using PBS and 10 kDa centrifugal filter units (Merck Millipore). Protein concentration was determined by measuring absorbance at 280 nm in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), using the calculated extinction coefficient of TcdB, 287,660 M\(^{-1}\) cm\(^{-1}\).

**SDS-PAGE.** Protein samples were dissolved in Laemmli buffer [40 % (v/v) glycerol, 0.25 M Tris-HCl, pH 6.8 with 4 % (w/v) SDS and 0.015 % (w/v) bromophenol blue] and heated at 95 °C for 5 min.
An amount of 2 μg protein (CRM or glycoconjugate) was loaded per lane. PageRuler Plus Prestained Protein Ladder 10 to 250 kDa (Thermo Scientific) was used as size marker (3 μL per lane). Samples were run on 10 % SDS-PAGE gels at 20 V cm⁻¹ and stained with 0.5 % (w/v) Coomassie Brilliant Blue R-250 in 50 % (v/v) methanol and 10 % (v/v) acetic acid for 30 min. Stained gels were destained with 50 % (v/v) methanol and 10 % (v/v) acetic acid.

MALDI-TOF MS. Mass spectra were acquired with an Autoflex Speed MALDI-TOF system (Bruker Daltonics). Samples were spotted using the dried droplet technique with 2,5-dihydroxyacetophenone (DHAP) as matrix on MTP 384 ground steel target plates (Bruker Daltonics). Samples were prepared by mixing 2 μL of desalted protein sample with 2 μL of DHAP matrix and 2 μL of 2 % (v/v) trifluoroacetic acid (TFA) prior to spotting. The mass spectrometer was operated in linear positive mode. Mass 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.

Pilot Immunization Studies with Glycoconjugate 14. Female, 6-8 weeks old C57BL/6 mice (Charles River) were immunized s.c. with an amount of 14 corresponding to 1 μg of glycan antigen per injection. The immunogen was diluted in sterile PBS to a final volume of 100 μL per injection. For immunizations with Alum adjuvant, 14 was pre-incubated with 1 μL per μg protein of Alum Alhydrogel (Brenntag) the day before, and the mixture was rotated for 24 h at 4 °C. For immunizations with Freund’s adjuvant (Sigma-Aldrich), a solution of 14 was combined with an equal volume of Complete Freund’s adjuvant (CFA) (priming immunization) or Incomplete Freund’s adjuvant (ICFA) (boosting immunizations) to a homogeneous emulsion immediately before injection. For immunizations with AddaVax (similar to MF59) (InvivoGen) a solution of 14 was combined with an equal volume of AddaVax to a homogeneous emulsion immediately before injection (priming and boosting immunizations). The 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 of Laboratory Animal Science Associations and were approved by the Landesamt für Gesundheit und Soziales of Berlin, Germany (protocol number G0135/14). All efforts were made to minimize suffering.

Challenge Studies. 6-8 weeks old female C57BL/6 mice (Charles River, Sulzfeld) were used for immunization experiments. Each injection comprised a volume of 100 μL using sterile PBS as diluent. Sham-immunizations with PBS contained either 17.3 μL Alum or 50 μL AddaVax. One dose of CRM contained 17.3 μg protein with either 17.3 μL Alum or 50 μL AddaVax. One dose of 12 contained 7 μg protein, corresponding to 1 μg of 1, and 7 μL Alum. Each dose of 13 contained 16.7 μg protein, corresponding to 1 μg of 2, and 16.7 μL Alum. Each dose of 14 contained 14.3 μg protein, corresponding to 1 μg of 3, and 50 μL AddaVax. Each dose of 15 contained 15 μg protein, corresponding to 1 μg of 4, and 15 μL Alum. Each dose of 16 contained 17.3 μg protein, corresponding to 1 μg of 5, and either 17.3 μL Alum or 50 μL AddaVax. Each dose of formalin-inactivated TcdB contained 75 μg protein and 75 μL Alum. 13 days after the last immunization, mice were rendered susceptible to C. difficile infection with intraperitoneal (i.p.) injections of clindamycin (20 mg per kg body weight) for one day. The next day, mice were challenged via oral gavage with 5x10⁷ CFUs of the C. difficile strains M68 or VPI 10463. M68 is a clindamycin-resistant ribotype 017 strain isolated from a hospital outbreak in Dublin, Ireland, expressing toxin B (TcdB) but not toxin A (TcdA). VPI 10463 is a highly virulent ribotype 087 strain expressing both toxins. Intestinal colonization was quantified 5 days after the infection by determining the C. difficile CFUs in fecal suspensions that were plated at limited dilutions on selective T.C.C.F.A. agar plates and cultivated for 48 h at 37 °C under anaerobic conditions. The degree of colonization is displayed as CFUs per gram feces. Enterococcus spp. CFUs grown on blood agar plates served as control. Characteristic colonies were counted and identified at random by MALDI-TOF MS analysis. Histopathological analysis of colon samples was performed to determine the degree of colitis.
For passive transfer experiments, mice received 200 µL of pooled sera i.p. on days 0 and 1 with the challenge performed on day 0 two hours after the first serum transfer, using $5 \times 10^7$ CFUs of strain VPI 10463. mAbs were applied i.p. on days 0, 1 and 2 (100 µg per injection) and mice were challenged on day 0 two hours after the first antibody transfer, using $5 \times 10^7$ CFUs of VPI 10463. 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 of Laboratory Animal Science Associations and were approved by the Regierung von Mittelfranken, Germany (AZ 54-2532.1-47/13).

**Microarray-assisted Antibody Binding Analyses.** Spotted and quenched microarray slides were blocked using PBS with 1 % (w/v) BSA (PBS-BSA) for 1 h at room temperature, washed three times with PBS and dried by centrifugation. FlexWell 64 grids (Grace Bio-Labs) were applied to yield 64 wells for individual experiments. Slides were incubated with serum samples diluted 1:100 (unless mentioned otherwise) or with processed stool samples diluted 1:100 in PBS with 0.01 % (v/v) Tween-20 and 1 % (w/v) BSA (PBS-T-BSA) for 1 h at room temperature in a humid chamber. Wells were washed three times using PBS with 0.1 % Tween-20 (PBS-T0.1). Grids were removed and slides were dried by centrifugation (300 x g, 5 min). Dried microarrays were incubated with fluorescence-labeled detection antibodies diluted in PBS-T-BSA for 1 h at room temperature in a humid chamber. Following dilutions were used: α-mouse IgA (cat.no. F9384), 1:200; α-mouse IgG (cat.no. A-31574), 1:400; α-mouse IgG1 (cat.no. A-21125), 1:400; anti-mouse IgG2a (cat.no. A-21241), 1:400; α-mouse IgG3 (cat.no. A-21151), 1:200; α-mouse IgM (cat.no. 715-585-140), 1:200. All detection antibodies were from Life Technologies, except α-mouse IgA (Sigma-Aldrich) and α-mouse IgM (Dianova). After incubation with detection antibody, slides were washed three times with PBS-T0.1, rinsed once with H2O and dried by centrifugation. Microarray slides were scanned with a GenePix 4300A scanner (Molecular Devices; Sunnyvale, CA, USA). The photomultiplier tube voltage was adjusted to reveal scans free of saturated signals. Image analysis was carried out with the GenePix Pro 7 software. Background-subtracted mean fluorescence intensity (MFI) values were exported for further analysis.

**Processing of Stool Samples for Microarray and ELISA.** To fresh stool samples two volumes of PBS supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich) were added. After vigorous vortexing samples were incubated in ice for 1 h and then centrifuged (10,000 x g for 20 min). The supernatant was recovered and used for further analyses.

**Flow Cytometry.** Formalin-inactivated *C. difficile* bacteria (strain M68 and strain VPI10463) were incubated with murine antisera diluted 1:20 in PBS for 1 h, washed three times with PBS, and stained with anti-mouse IgG FITC produced in goat (cat. no. F0257; BD Biosciences) diluted 1:100 in PBS for 1 h. After three washing steps with PBS, the bacterial cells were subjected to flow cytometry using a FACS Canto II instrument (BD Biosciences). About 2,000 events (strain M68) or 10,000 events (strain VPI 10463) were counted for each individual measurement.

**Microbiota Analyses.** Fecal specimens were obtained after vaccination, but before infection and analyzed for the composition of the intestinal microbiota by 16S rRNA sequencing. Bacterial genomic DNA from stools was isolated using a ZR Fecal DNA MicroPrep™ kit (Zymo research). The variable V3 and V4 regions of the 16S rRNA genes were amplified with Illumina-Nextera compatible, degenerate region-specific primers: Illumina341F: TCGTCGGCAGCGTCAGATGTATAAGAGACAGCTACGCGGTAA; Illumina806R: GTCTCAGGGGCTCG GAGATGTATAAGAGACAGGACTACHVGGGTATCTAATCC (Integrated DNA Technologies). Amplicons were gel purified, then dual-indexed barcodes and Illumina flow cell adaptor sequences were attached by eight cycles of PCR, amplicons purified by AMPureXP magnetic beads and quantified using a Qbit device (Invitrogen); normalized, pooled libraries were sequenced on an Illumina MiSeq device using a 600-cycle v3 paired-end kit. Quality-controlled, demultiplexed paired-end reads
were joined, classified using the RDP Classifier 2.10 (16S rRNA training set 10) database and results were analyzed using MEGAN5 software⁴⁹ and Metagenassist. Numbers of total reads generated per sample are shown in Supplementary Table 1.

Quantitative PCR. Bacterial DNA was isolated from mouse feces using the ZR Fecal DNA MicroPrep kit (Zymo Research) according to the manufacturer’s recommendations. Previously reported⁵⁰-⁵² primer and probe sequences for the detection of *C. difficile* were: *TcdA*-for, CAGTCCGATTGCAAGTATTGACAA; *TcdA*-rev, AGTAGTATCTACTACCATTAAAGCTCG; *TcdA*-probe, FAM-TTGAGATGATAGCAGTGTCAGGATT-TAMRA; *TcdB*-for, GAAAGTCAAATTTGACCTCAAT; *TcdB*-rev, GCTGCACCTAAACTTACACCA; *TcdB*-probe, FAM-ACAGATGCAGCAGAAGTGGTGAATT-TAMRA; *Cdff16SrRNA1*-for, TTGAGCGATTACTTCCGTTAAAGA; *Cdff16SrRNA1*-rev, TGACTGAGCCTACCCGTATATCA; *Cdff16SrRNA2*-for, CCACGCGTTACTCACTCCG-TAMRA; *Cdff16SrRNA2*-rev, GTACTGCACCTTCTTGATTATYAGAG; *Cdff16SrRNA2*-rev, TGCCTCTCAAATATATTATCCCGATTAG (*C. difficile* 16S rRNA gene GenBank accession number: AB548672); *Cdff16SrRNA2*-for, GCAAGTGGACGGATTACTTCCGTT; *Cdff16SrRNA2*-rev, GTACTGCACCTTCTTGATTATYAGAG; *Cdff16SrRNA2*-rev, TGCCTCTCAAATATATTATCCCGATTAG (*C. difficile* 16S rRNA gene GenBank accession number: NR074454). Quantitative PCRs were run on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) using the TaqMan Universal Master Mix II (Thermo Fisher Scientific) according to the manufacturer’s recommendations and the following cycling conditions: 2 min 50 °C, 10 min 95 °C, (15 sec 95 °C, 1 min 60 °C)x40. Cycle threshold (Ct) values were correlated with *C. difficile* CFUs to generate standard curves that were used to calculate CFU equivalents.

ELISA. High binding 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA) were coated overnight at 4 °C with 10 µg/mL (50 µL/well) of CRM₁₉₇ (Pfénex) in PBS. The plates were washed once with PBS-T0.1 and blocked with PBS-BSA for 1h at room temperature. After washing three times with PBS-T0.1, the plates were incubated with serum or processed stool samples diluted 1:100 in PBS-BSA (50 µL per well) for 1h at 37 °C. The plates were washed three times with PBS-T0.1 and incubated for 1h at room temperature with HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich) diluted 1:10,000 in PBS-BSA (50 µL per well). Plates were washed three times with PBS-T0.1 and developed using 3,3',5,5'-tetramethylbenzidine (BD Biosciences). The reaction was stopped by adding 2% H₂SO₄ and absorbance at 450 nm was recorded.

Histopathology. Intestinal tissue obtained from the ascending, transverse and descending colon was fixed in 10% buffered formalin, embedded in paraffin, and cut into 2 µm thick sections. Colonic sections were deparaffinized, stained with H&E by the Department of Pathology of the FAU Erlangen-Nürnberg, and evaluated microscopically in a double-blinded manner. Briefly, the intestinal damage shown in the presented figures was evaluated in at least 5 colonic tissue sections per mouse using the following parameters: (a) inflammatory infiltrate, (b) submucosal edema, (c) epithelial damage and the number of intraepithelial lymphocytes (IELs) (d), lymph follicles (LF) (e) and crypt abscesses (CA) (f). A histologic grading of the severity of the tissue damage was performed for each parameter a-c (score between 0 and 3) and the number of IELs (score of 0: 0 IELs, score of 1: < 5 IELs, score of 2: < 10 IELs and score of 3: > 10 IELs) or lymph follicles (LF) and crypt abscesses (CA) (score of 0: 0 LF or CA, score of 1: 1 LF or CA, score of 2: 2 LF or CA and score of 3: 3 LF or CA), leading to a cumulative score between 0 (no signs of inflammation) and 18 (very severe inflammation and epithelial damage). For the initial challenge studies with strain M68, inflammation scores reflect the average of the three tissue sections with most prominent pathology obtained from at least five colon sections per mouse. For the repeat challenge studies, inflammation scores reflect the average of the three tissue sections with most prominent pathology obtained from at least 15 (M68) or 10 (VPI 10463) colon sections per mouse.
Additional References

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Supplementary Table 1. Number of total reads generated per sample and numbers of reads assigned to the order *Clostridioides* and the family *Peptostreptococcaceae*.

| Vaccine          | Mouse no. | Total reads | Reads assigned to *Clostridioides* | Reads assigned to *Peptostreptococcaceae* |
|------------------|-----------|-------------|-----------------------------------|------------------------------------------|
| No (naïve mice)  | 1         | 21,225      | 0                                 | 1                                        |
|                  | 2         | 21,225      | 0                                 | 5                                        |
|                  | 3         | 25,739      | 0                                 | 39                                       |
|                  | 4         | 10,725      | 0                                 | 1                                        |
|                  | 5         | 23,556      | 0                                 | 17                                       |
| PBS + Alum       | 1         | 12,114      | 0                                 | 0                                        |
|                  | 2         | 16,714      | 0                                 | 0                                        |
|                  | 4         | 17,070      | 0                                 | 0                                        |
|                  | 5         | 12,969      | 0                                 | 0                                        |
| CRM₁₉₇ + Alum    | 1         | 23,309      | 0                                 | 0                                        |
|                  | 2         | 13,287      | 0                                 | 0                                        |
|                  | 3         | 13,282      | 0                                 | 0                                        |
|                  | 4         | 19,139      | 0                                 | 0                                        |
|                  | 5         | 16,151      | 0                                 | 0                                        |
| **16** + Alum    | 1         | 25,584      | 0                                 | 0                                        |
|                  | 2         | 25,280      | 0                                 | 0                                        |
|                  | 3         | 22,038      | 0                                 | 0                                        |
|                  | 4         | 22,812      | 0                                 | 0                                        |
|                  | 5         | 20,846      | 0                                 | 0                                        |
| **12** + Alum    | 1         | 19,636      | 0                                 | 0                                        |
|                  | 2         | 18,048      | 0                                 | 0                                        |
|                  | 4         | 24,340      | 0                                 | 0                                        |
|                  | 5         | 19,460      | 0                                 | 0                                        |
| **15** + Alum    | 1         | 20,024      | 0                                 | 0                                        |
|                  | 2         | 21,383      | 0                                 | 0                                        |
|                  | 3         | 27,729      | 0                                 | 0                                        |
|                  | 4         | 21,251      | 0                                 | 0                                        |
|                  | 5         | 24,183      | 0                                 | 0                                        |
| TcdB + Alum      | 1         | 19,329      | 0                                 | 0                                        |
|                  | 2         | 17,717      | 0                                 | 0                                        |
|                  | 4         | 25,076      | 0                                 | 0                                        |
| PBS + AddaVax    | 1         | 35,943      | 0                                 | 0                                        |
|                  | 2         | 24,213      | 0                                 | 0                                        |
|                  | 3         | 29,083      | 0                                 | 0                                        |
|                  | 5         | 27,577      | 0                                 | 0                                        |
| CRM₁₉₇ + AddaVax | 1         | 22,610      | 0                                 | 0                                        |
|                  | 2         | 23,245      | 0                                 | 0                                        |
|                  | 3         | 29,052      | 0                                 | 0                                        |
|                  | 5         | 33,637      | 0                                 | 0                                        |
| **16** + AddaVax | 1         | 23,491      | 0                                 | 0                                        |
|                  | 2         | 23,294      | 0                                 | 0                                        |
|                  | 3         | 18,572      | 0                                 | 0                                        |
|                  | 4         | 19,787      | 0                                 | 0                                        |
|                  | 5         | 18,856      | 0                                 | 0                                        |
| **13** + AddaVax | 1         | 25,431      | 0                                 | 0                                        |
|                  | 2         | 17,287      | 0                                 | 0                                        |
|                  | 3         | 21,914      | 0                                 | 0                                        |
|                  | 4         | 23,358      | 0                                 | 0                                        |
|                  | 5         | 19,061      | 0                                 | 0                                        |
| **14** + AddaVax | 1         | 26,341      | 0                                 | 0                                        |
|                  | 2         | 17,296      | 0                                 | 0                                        |
|                  | 3         | 21,914      | 0                                 | 0                                        |
|                  | 4         | 23,358      | 0                                 | 0                                        |
|                  | 5         | 19,051      | 0                                 | 0                                        |
Supplementary Figure 1. Structures of synthetic oligosaccharides and of an exemplary glycoconjugate.

Supplementary Figure 2. Immunogenicity of 14 with different adjuvants. (A) Immunization regime to assess immunogenicity of 14. Mice received 14 s.c. either with either Alum, Freund’s adjuvant (FA) or AddaVax. Sera of days 0 and 35 were subjected to microarray-assisted IgG analysis. (B) Microarray-inferred IgG levels to 3 expressed as mean fluorescence intensity (MFI) values. Diamonds represent individual mice (white: pre-immune, black: post-immune). Bars represent median + interquartile range. Microarray scans of one representative mouse (two spots of 3 printed at 1 mM and one spot at 0.5 mM, from top to bottom) are shown above the diagram.
Supplementary Figure 3. Vaccinated mice produce glycan-specific IgG. Mice were immunized three times with glycoconjugates, CRM197 or PBS as indicated in Figure 2A of the main manuscript. Pre- and post-immune sera were subjected to microarray-assisted IgG analysis. Serum IgG2a levels to indicated antigens are expressed as MFI values. Diamonds represent individual mice (white: pre-immune, black: post-immune). The displayed data is the average of two independent experiments in duplicates. Bars show median + interquartile range of five mice. Significance was inferred by two-tailed Mann-Whitney U tests (pre-immune vs. post-immune signals for each vaccine) with *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
**Supplementary Figure 4.** Antibody responses of immunized mice. (A-C) Serum antibody binding to inactivated *C. difficile* bacteria as determined by flow cytometry. Sera of vaccinated mice were exposed to *C. difficile* strains M68 and VPI10463 and antibody attachment to both strains was assessed by flow cytometry. (A) Representative histograms for strain M68 are shown, the geometric mean (GM) values of FITC-H intensity are listed below. (B and C) Fold change values were calculated by dividing the GM FITC-H values observed for post-immune sera by those observed for pre-immune sera of individual mice. Bars represent mean + SEM. Panel B shows data for strain M68, panel C data for strain VPI 10463. (D-E) Detection of glycan-specific immunoglobulin (Ig) by microarray (see immunization regime in Figure 2 A). (D) Ig to 2 in mice immunized with 13 adjuvanted with AddaVax. (E) Ig to 3 in mice immunized with 14 adjuvanted with AddaVax. Tables in panels A and B indicate if Ig to the respective oligosaccharide was detected, “+” meaning detectable, “(+)” weak/borderline detection, and “-“ undetectable.
Supplementary Figure 5. Intestinal microbiota are preserved in vaccinated mice. (A) Feces sampled from colons after the third immunization were subjected to 16S rRNA taxonomic analysis. (B) Species-level diversity (Shannon’s H'; left y-axis) and richness (Menhinick’s D; right y-axis). Individual mice are represented as circles, bars show mean + SEM of 3-5 mice. (C-F) Microbiota composition at phylum (C), order (D), family (E) and genus (F) levels. Stacked bars show averaged abundancies of the number of mice indicated above the bars. Only taxa with over 1% relative abundance in any sample are shown.
Supplementary Figure 6. Histopathology. (A) Histopathological analyses of colon tissue sections from mice infected with C. difficile M68. Subpanels 1-7: Histopathological analyses of sham-immunized (PBS) mice after C. difficile infection, showing diffuse infiltration of the lamina propria by inflammatory immune cells (subpanels 3 and 4), submucosal edema (subpanel 5; asterisk), crypt abscesses (subpanel 6) and epithelial damage (subpanel 7). Subpanel 8 shows small localized infiltrates after infection in a mouse immunized with 15, subpanel 9 lymph follicles with hyperplasia after infection in a mouse immunized with 14. Subpanel 10 shows the detection of intraepithelial lymphocytes of a sham-immunized mouse. Tissue sections without pathological signs from
uninfected and 15-vaccinated, infected mice are shown in subpanels 11 and 12, respectively. 

**(B)** Histopathological analyses of colon tissue sections from mice infected with *C. difficile* VPI 10463. Subpanels 1-3: Signs of colitis in *C. difficile* VPI 10463 infected mice. Subpanels 4-5 and 10: Tissue sections of mice vaccinated with 15. Subpanels 6-9 show histopathological changes in sham-immunized, infected control mice, including: focal mucosal lesions (subpanel 6, red arrowhead), increased formation of apoptotic bodies in colon epithelial cells (subpanel 7, black arrowheads), granulocyte infiltration (subpanel 7, green arrowhead) and diffuse infiltration of the lamina propria by inflammatory immune cells (subpanels 8 and 9).

**Supplementary Figure 7.** Results of the repeat challenge studies with M68 and VPI 10463. The immunization and challenge regime was identical to that shown in Figure 3A in the main text. (A) Serum IgG1 levels expressed as MFI values. Only post-immune values (day 13 after third immunization) are shown, all pre-immune values were at baseline. (B) Results of CFU plating assays. (C) Histopathological scores. (D) *C. difficile* colonization levels measured by qPCR with the primers indicated in the Supplementary Methods section. Bars in all panels show median + interquartile range (n=5-7). Significance in B, C and D was inferred by Dunn-corrected Kruskal-Wallis tests [*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; n.s., not significant (P > 0.05)]. The CRM and TcdB groups for strain M68 in B, C and D only contain six data points, as one mouse each succumbed post-infection. Significance in A was inferred by two-tailed Mann-Whitney U tests (pre-immune vs. post-immune) with *P ≤ 0.05; **P ≤ 0.01.
Supplementary Figure 8. Binding signals to oligosaccharide 2. The images are representative scans of microarrays using post-immune sera of mice immunized with glycoconjugate 13 and mouse anti-IgG detection antibody. Oligosaccharide 2 was spotted in duplicate. The sera are from the same mice shown in Figure 4 F in the main manuscript. Sera from all mice except mouse #6 that showed no signal against 2 were pooled and used for passive serum transfer experiments.

Supplementary Figure 9. Detection of antigen-specific IgG in immunized mice. (A) Fecal specimens sampled at the indicated time point were subjected to microarray-assisted antibody analysis. (B) Fecal IgA and IgG levels to the indicated antigens expressed as MFI values. Diamonds represent individual mice (white, pre-immune; black, post-immune). (C) Fecal IgG levels to the CRM197 protein displayed as ELISA signal intensity (absorbance at 450nm). Statistical significance was inferred by two-tailed Mann-Whitney U tests (pre-immune vs. post-immune signals) with *P ≤ 0.05; **P ≤ 0.01.