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An oral pH-stable and protease-responsive polypeptide nanogel vaccine against *Clostridioides difficile*

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Abstract

Background *Clostridioides difficile* infection remains a major healthcare burden with no licensed vaccine. Parenteral toxoid vaccines have failed in phase 3 trials, and the mucosal strategies that succeed in preclinical models still require rectal instillation or intranasal boosting after injection, each with an exogenous adjuvant. Oral delivery would be more deployable, but demands a carrier that protects antigen during gastric transit and releases it in the intestine.

Methods We encapsulated the recombinant TcdB receptor-binding domain in a genipin-cross-linked poly(L-lysine)-block-poly(L-threonine) nanogel. Antigen release was characterized at pH 3.0, 7.4 and 8.0 with and without protease. Mice received three weekly oral immunizations. Antibodies were measured by ELISA, splenocyte responses by ELISpot, and toxin neutralization in Vero cell against six strains spanning clades 1-5. Protection was assessed after lethal challenge.

Results Here we show that the nanogel retains antigen across pH 3.0-8.0 without protease and releases it upon proteolysis at neutral-to-alkaline pH. Three oral immunizations elicit serum and intestinal antibodies, every immunoglobulin G subclass measured, and concurrent interferon-γ-, interleukin-4- and interleukin-17-secreting splenocytes. Responses persist for six months without a booster. Vaccinated mice show improved survival, less weight loss, preserved cecal weight and colonic architecture, and lower colonic inflammation, although colonization persists. Immune

sera neutralize toxin from ribotype 027 strains and, at higher titers, from Asian ribotype 023 and 017 isolates.

Conclusions An adjuvant-free polypeptide nanogel enables needle-free oral delivery and confers durable, toxin-directed protection in mice. Protection reflects toxin neutralization rather than bacterial clearance, indicating that colonization-targeting antigens are needed for sterilizing immunity.

Plain Language Summary

Clostridioides difficile is a bacterium that causes severe diarrhea, usually after antibiotics, and no vaccine against it is licensed. Injected vaccines have failed in large trials, probably because they do not raise immunity inside the gut, where the infection happens. Vaccines that do raise gut immunity still need a nasal spray on top of an injection, or an enema, and an added immune stimulant. We made a vaccine that is simply swallowed. The toxin fragment sits inside a tiny protein gel that survives stomach acid and opens when gut enzymes break it down. Three oral doses, with no added stimulant, protected mice against lethal infection, and antibodies were still present six months later. The vaccine prevented illness but did not clear the bacterium from the gut.

Keywords: *Clostridioides difficile*, oral vaccine, mucosal immunity, TcdB, polypeptide nanogel, toxin-neutralizing antibody

Introduction

Clostridioides difficile is a spore-forming anaerobic bacterium and a leading cause of antibiotic-associated diarrhea, particularly in healthcare settings[1,2]. Despite advances in antimicrobial therapy and infection control, *C. difficile* infection (CDI) continues to cause substantial morbidity, mortality, and healthcare costs, driven in part by high recurrence rates[3]. CDI is mediated primarily by two exotoxins, toxin A (TcdA) and toxin B (TcdB), with accumulating evidence indicating that TcdB alone is sufficient to induce the full spectrum of clinical disease[4,5].

Although vaccination has long been pursued as a preventive strategy for CDI, no licensed vaccine is currently available. Recent phase 3 clinical trials of parenteral alum-adjuvanted toxoid vaccines failed to demonstrate protective efficacy, underscoring the limitations of systemic immunization against a mucosal pathogen [6,7]. The inability of parenteral vaccination to effectively engage the intestinal immune compartment has been proposed as a central contributing factor [8]. Recent work further demonstrated that mucosal vaccination can induce immune mechanisms distinct from those elicited by parenteral immunization, including fecal antibody responses and colonic TH17-skewed tissue-resident memory T cells that are associated with protection against *C. difficile* colonization [9]. Together, these

findings highlight the importance of mucosal immunity in effective CDI vaccine design. While a synthetic liposomal adjuvant system (GLA 3M-052 LS) delivered intramuscularly and intranasally improved immunogenicity and memory B-cell responses compared with alum [10], and rectal immunization with a multivalent formulation achieved colonization clearance in mice [9], both approaches still rely on parenteral or invasive mucosal delivery and complex exogenous adjuvant systems. Accordingly, there remains a need for a non-invasive mucosal vaccine platform capable of protecting antigens during gastrointestinal transit and inducing protective intestinal immunity without the use of exogenous adjuvants.

Oral vaccination represents a practical mucosal immunization strategy for CDI prevention: it directly targets gut-associated lymphoid tissue (GALT), stimulates secretory IgA and local immune responses at the intestinal site of infection, and enables entirely non-invasive, self-administered delivery amenable to mass vaccination campaign[11]. However, developing effective oral vaccines against CDI remains technically challenging, primarily due to antigen degradation in the acidic and enzymatic gastrointestinal environment and inefficient delivery to immune inductive sites such as Peyer's patches. An ideal oral vaccine platform must therefore protect antigen integrity throughout gastrointestinal transit while simultaneously

99 providing immunostimulatory signals, ideally without the need for exogenous
100 adjuvants[12].
101 Biodegradable polypeptide-based polymers represent a promising class of oral
102 vaccine delivery systems[13]. Composed of naturally occurring amino acids, these
103 materials combine biocompatibility with tunable physicochemical properties that can
104 protect antigens during gastric transit and enable controlled release in the
105 intestine[14]. In addition to their role as delivery vehicles, such polymers may also
106 function as intrinsic adjuvants by facilitating antigen uptake and presentation.
107 In this study, we developed an oral vaccine platform based on a pH-stable,
108 protease-responsive poly(L-lysine)-*block*-poly(L-threonine) (PLL_{20}-*b*-PLT_{40}) polymer
109 encapsulating a recombinant TcdB receptor-binding domain. We evaluated its ability
110 to induce systemic and mucosal immune responses, confer protection against lethal
111 challenge with a hypervirulent *C. difficile* strain, generate durable immunity following
112 oral immunization, and neutralize TcdB across multiple clinically relevant ribotypes.

Methods

Bacterial strains and plasmids

C. difficile strains R20291 (RT027), VPI 10463 (RT087), BI-1 (RT027), and clinical isolates NCKUH K16 (RT023), NCKUH A4 (RT017), and M120 (RT078) were cultured anaerobically in brain-heart infusion broth supplemented with yeast extract and L-cysteine (BHIS) at 37 °C. For molecular cloning, *E. coli* DH5α was used. For recombinant protein expression, ClearColi BL21(DE3) electrocompetent cells (Lucigen) were used to minimize endotoxin contamination. A pET-21B(+) plasmid encoding the C-terminal combined repetitive oligopeptides (CROPs) region of TcdB (residues 1852-2363), originally amplified from *C. difficile* R20291 genomic DNA, was described previously [15] and was used for expression of rTcdB here.

Recombinant protein expression and purification

ClearColi BL21(DE3) transformed with pET21b-tcdB was cultured in LB medium containing 100 μg/mL ampicillin at 37°C. Protein expression was induced with 0.5 mM IPTG at OD_{600} = 0.6, followed by incubation at 20°C for 16 h. Cells were harvested, lysed by sonication in binding buffer (50 mM NaH_2PO_4, 500 mM NaCl, 25 mM imidazole, pH 6.5), and clarified by centrifugation. His-tagged rTcdB was purified using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions.

Protein purity was assessed by SDS-PAGE and Coomassie blue staining. Endotoxin levels were confirmed to be <0.1 EU/μg by LAL assay (Associates of Cape Cod). Purified protein was dialyzed against PBS and stored at -80°C.

Synthesis of polypeptide-based polymers

PLL_{20}-*b*-PLT_{40} block copolypeptides were synthesized by sequential ring-opening polymerization of N-carboxyanhydrides (NCAs) using hexylamine as the initiator, following previously reported procedures [16-18]. Briefly, hexylamine was dissolved in dichloromethane (6.6 mM) and added to Z-L-lysine (ZLL) NCA in dichloromethane (0.2-0.3 M) at a feed molar ratio of 1:20. The solution was stirred at room temperature for 2 days. O-benzyl-L-threonine (BnT) NCA in dichloromethane (0.2-0.3 M) was then added at a ZLL:BnT feed molar ratio of 1:2, and the mixture was stirred at room temperature for an additional 4 days, followed by precipitation with diethyl ether. Benzyl and Z protecting groups were subsequently removed to yield PLL_{20}-*b*-PLT_{40}. Molecular weight was characterized by size-exclusion chromatography (SEC; Shimadzu, two Shodex HFIP 806M columns, hexafluoro-2-propanol eluent, 35 °C, 0.8 mL min^{-1}, calibrated with poly(methyl methacrylate)), and composition was confirmed by ^{1}H NMR (AVANCE III HD 600; PZLL-*b*-PBnT in

trifluoroacetic acid-d_1, PLL-*b*-PLT in D_2O). PLL_{20} and PLL_{10}-*b*-PLT_{20} variants were synthesized similarly with adjusted monomer ratios.

Preparation of antigen-encapsulated polymer particles

rTcdB (25 or 50 μg per dose) was encapsulated within genipin-cross-linked gel particles using a water-in-oil emulsion process. The aqueous phase consisted of polymer and rTcdB dissolved in 0.5 mL deionized water. Genipin (Challenge Bioproducts) was added as the cross-linker at a genipin-to-amino-group molar ratio (C_Gen) of 0.25, and the emulsion was pulsed by probe sonication for 5 min on ice, followed by a further 5 min pulse and stirring at 350 rpm for 24 h. Gel particles were purified by centrifugation (7000 rpm, 1 min) and repeated n-hexane extraction until the oil phase was colorless. Protein-encapsulated particles were transferred to a Vivaspin 6 centrifugal concentrator (100 kDa MWCO; Sartorius) and free antigen was removed by centrifugation (2000 rpm, 10 min); encapsulation efficiency was determined from unbound protein in the filtrate by BCA assay. Particle size and zeta potential were measured by dynamic light scattering (Otsuka ELSZ-1000), in triplicate.

Polymer cytotoxicity assay

Vero cells (ATCC CCL-81) were seeded at 5×10^4 cells per well and exposed to ten-fold serial dilutions (10^{-1} to 10^{-3}) of PLL_{20}, PLL_{10}-*b*-PLT_{20} or PLL_{20}-*b*-PLT_{40}, prepared from stock solutions of 5.15 mg/mL (PLL_{20}) and 3.93 mg/mL (PLL_{10}-*b*-PLT_{20} and PLL_{20}-*b*-PLT_{40}). These concentrations refer to the complete genipin-cross-linked material. The polypeptide content was equivalent across formulations; the higher total concentration of PLL_{20} reflects the larger amount of genipin required to cross-link its higher density of lysine ε-amino groups. Cytotoxicity was quantified by lactate dehydrogenase release (Cytotoxicity Detection Kit, Roche) and expressed relative to Triton X-100-treated cells, which served as the positive control for maximal lysis (100%).

Serum biochemistry

Blood was collected by terminal cardiac puncture seven days after the third oral immunization. Samples were allowed to clot, centrifuged, and the serum was submitted to New South Ocean Diagnostic Laboratory (Tainan, Taiwan) for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine. Serum creatinine was below the laboratory's lower reporting limit (<1.0 mg/dL) in all groups.

pH stability and release studies

To simulate gastrointestinal conditions, antigen-encapsulated particles were incubated at 37 °C in buffers at pH 3.0 (gastric), pH 7.4, and pH 8.0 (intestinal). At the indicated time points, samples were centrifuged and rTcdB concentration in the supernatant was measured by Bradford assay. To assess protease-triggered release, bromelain (1 mg/mL), used as a model protease to evaluate protease-responsive degradation, was added to mimic the intestinal enzymatic environment.

Animals and immunizations

All animal experiments were conducted and reported in accordance with the ARRIVE 2.0 guidelines and approved by the Institutional Animal Care and Use Committee of National Cheng Kung University (IACUC approval #107056). Six-week-old male C57BL/6JNarl mice (BioLASCO Taiwan) were housed under specific-pathogen-free conditions with ad libitum access to food and water. Mice were randomly assigned to experimental groups, with group sizes specified in the respective figure legends. For immunization, mice received 100 μL of vaccine formulation by oral gavage weekly for three consecutive weeks. Control groups received normal saline or empty polymer. Blood was collected via submandibular

puncture weekly before immunization and monthly after the final dose for long-term studies.

ELISA for antigen-specific antibodies

The 96-well half-area plates (Corning) were coated with 2 μg/mL rTcdB in carbonate buffer (pH 9.6) overnight at 4°C. After blocking with 1% BSA in PBS-Tween 20 (PBST), serial dilutions of serum or fecal extracts were added and incubated for 1 h at 37°C. Fecal samples were processed by homogenizing feces (1:10 w/v) in PBS containing protease inhibitors, followed by centrifugation and collection of supernatants. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgG (1:5000), IgG1, IgG2b, IgG2c (each 1:1000), or IgA (1:1000) (Southern Biotech), followed by TMB substrate (Thermo Fisher). Reactions were stopped with 1 M H_2SO_4 and absorbance measured at 450 nm. Endpoint titers were defined as the highest dilution yielding $OD_{450} \geq 0.5$ (IgG) or ≥ 0.1 (IgA) above background.

Toxin neutralization assay

Vero cells (ATCC CCL-81) were seeded at 3×10^4 cells/well in 96-well plates and cultured overnight. For serum neutralization assays (Fig. 4c), purified TcdB (4 ng/mL) was pre-incubated with serial dilutions of immune sera for 1 h at 37°C, added to Vero cells, and incubated for 18 h. Cell viability was quantified by MTT assay

(absorbance at 490 nm) and expressed as a percentage of untreated controls. For cross-strain neutralization assays (Fig. 4d), culture supernatants from six *C. difficile* strains grown in TY medium for 16 h were centrifuged (13,000 rpm, 3 min) and incubated with serially diluted immune sera at 37°C for 18 h. Neutralizing activity was assessed microscopically by cell rounding; the neutralizing titer was defined as the reciprocal of the highest serum dilution preventing cell rounding.

Fecal and intestinal-wash cytotoxicity assay

Fecal pellets and intestinal washes collected at study termination were serially diluted (10^{-1} to 10^{-3}) and applied directly to Vero cells; no exogenous toxin was added. Cell viability was measured by MTT assay after 18 h and expressed as a percentage of untreated controls. Purified TcdB (4 ng/mL) was included as a separate positive cytotoxicity control. This assay therefore reports the net cytotoxic activity of the luminal contents rather than neutralizing titer.

Cytokine ELISpot

Spleens were harvested from immunized mice 4 weeks post-final immunization. Single-cell suspensions were prepared and stimulated with 10 μg/mL rTcdB for 48 h. IFN-γ, IL-4, and IL-17A secreting cells were quantified using mouse ELISpot kits (BD Biosciences) according to the manufacturer's instructions. Spots were developed,

counted using an automated ELISpot reader (CTL), and expressed as spot-forming units (SFU) per 10^4 splenocytes after subtracting background from unstimulated controls.

C. difficile spore preparation

C. difficile R20291 was cultured in BHIS medium for 7 days at 37°C under anaerobic conditions to promote sporulation. Cultures were washed five times with sterile water to remove vegetative cells, and spore preparations were stored at 4°C. Spore concentration was determined by plating serial dilutions on BHIS agar supplemented with 0.1% sodium taurocholate under anaerobic conditions.

C. difficile infection model

Four weeks (or 6 months for long-term studies) after the final immunization, mice were treated with an antibiotic cocktail (kanamycin 400 mg/L, gentamicin 35 mg/L, colistin 57 mg/L, metronidazole 215 mg/L, vancomycin 45 mg/L) in drinking water for 3 days, followed by regular water for 1 day. One day before infection, mice received intraperitoneal clindamycin (10 mg/kg) and omeprazole (29 mg/kg, proton pump inhibitor) to increase susceptibility. Mice were challenged with 1×10^8 R20291 spores by oral gavage. In the dose-finding experiment shown in Supplementary Fig. 2, mice immunized with the 25 μg formulation were challenged with 1×10^6 R20291

spores. Clinical scoring, body weight, and survival were monitored twice daily for 6 days. Mice reaching predetermined humane endpoints (>25% weight loss or clinical score >14) were euthanized. At study termination, cecum weight, colon length, bacterial burden, and toxin levels were assessed.

Fecal bacterial burden and toxin quantification

Fecal pellets were collected, weighed, and homogenized in PBS. Serial dilutions were plated on BHIS agar with cycloserine-cefoxitin-fructose agar (CCFA) under anaerobic conditions. Colonies were counted after 48 h incubation. *C. difficile* glutamate dehydrogenase (GDH) was measured using C. DIFF CHEK-60 kit (TechLab). Toxin A/B levels were quantified using C. DIFFICILE TOX A/B II ELISA kit (TechLab) according to manufacturer's instructions.

Quantitative RT-PCR

Colonic tissue was excised, snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed with SuperScript III (Invitrogen). qRT-PCR was performed using SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 3 system. Gene expression was normalized to β-actin and calculated using the 2^(-ΔΔCt) method. Primer sequences are provided in Supplementary Table 1.

Histological analysis

Cecal and colonic tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin (H&E). Blinded histological scoring was performed by a veterinary pathologist assessing epithelial damage, inflammatory cell infiltration, submucosal edema, and crypt architecture on a scale of 0-3 for each parameter (total score 0-12).

Statistics and Reproducibility

Data are presented as mean ± standard deviation (SD). Statistical comparisons between two groups were performed using unpaired Student's t-test or Mann-Whitney U test for non-parametric data. Multiple group comparisons used one-way or two-way ANOVA with Tukey's or Dunnett's post-hoc test as appropriate. Survival curves were compared using log-rank (Mantel-Cox) test. All statistical analyses were performed using GraphPad Prism 9.0. Group sizes are stated in each figure legend; the challenge experiments used 10 mice per group. Particle size and zeta potential were measured in triplicate. No data were excluded, and no statistics were derived from fewer than three independent values. *P*-values <0.05 were considered statistically significant.

Results

Design and characterization of the rTcdB-encapsulated polypeptide nanogel

To develop an oral vaccine against *C. difficile* infection, we encapsulated the recombinant TcdB C-terminal receptor-binding domain (rTcdB) within a genipin-cross-linked polypeptide nanogel. Two polymer architectures were initially evaluated: a graft copolymer (PLL_{20}-*g*-$Indo_{0.3}$) and a block copolymer (PLL_{20}-*b*-PLT_{40}) (Fig. 1a). In a screening immunization, only the rTcdB-loaded block copolymer elicited an antibody response significantly above the saline control, whereas responses to the graft configuration did not differ from control (Supplementary Fig. 1a). Among the polymer variants tested, PLL_{20}-*b*-PLT_{40} elicited the highest IgG response (Supplementary Fig. 1b); IgA responses did not differ significantly among variants. PLL_{20}-*b*-PLT_{40} also showed the lowest cytotoxicity towards Vero cells, consistent with charge shielding by the longer PLT segment, although all three polymers were cytotoxic at the lowest dilution tested (65-77%) and the differences between variants emerged only at 10^{-2} and 10^{-3} dilutions (Fig. 1c). PLL_{20}-*b*-PLT_{40} was therefore selected for all subsequent studies. The selected nanogel had a hydrodynamic diameter of 348.5 nm and a zeta potential of −18.09 mV, whereas the corresponding empty nanogel carried a net positive surface charge (+23.43 mV); this charge reversal upon loading is consistent with incorporation of the antigen (Fig. 1b). Serum markers of hepatic (AST, ALT) and renal (blood urea nitrogen, creatinine) function did not

differ between polymer-immunized mice and saline-treated controls (Fig. 1d), indicating no detectable organ toxicity attributable to the formulation.

Dose optimization and durable antibody responses

We next determined the rTcdB dose required for effective immunization. After three weekly oral immunizations, PLL_{20}-*b*-PLT_{40} loaded with 50 μg rTcdB elicited significantly higher serum IgG and IgA than the 25 μg formulation, while empty polymer and saline controls remained at baseline (Fig. 2a). Consistent with this, mice immunized with the 25 μg formulation were not significantly protected from lethal challenge, showing no significant differences in survival or body-weight change relative to infected controls (Supplementary Fig. 2). The 50 μg dose was therefore selected for subsequent challenge studies. To obtain an initial assessment of long-term humoral durability, rTcdB-specific antibodies were monitored for six months after the final immunization. Both serum IgG and IgA remained significantly elevated above control levels throughout this period without any booster dose (Fig. 2b), indicating that the polypeptide platform supports durable antibody responses.

pH-stable, protease-triggered antigen release

To characterize the antigen-release behavior of the nanogel, we measured rTcdB release under conditions mimicking different gastrointestinal pH environments. In the absence of protease, minimal rTcdB release was observed over 30 h at pH 3, 7.4, or 8, indicating that the genipin-cross-linked network remains largely stable across this pH

range (Fig. 3b). Addition of bromelain, used here as a model protease to assess protease-responsive degradation, increased rTcdB release, particularly under neutral-to-alkaline conditions (pH 7.4-8), whereas release remained limited under acidic conditions (pH 3) (Fig. 3b). These findings suggest that antigen release from the nanogel is driven primarily by protease-mediated matrix degradation rather than pH alone, supporting a model in which rTcdB is retained under acidic gastric conditions and released more efficiently in a protease-containing intestinal environment (Fig. 3a).

Systemic immunity and cross-ribotype TcdB neutralization

We next characterized the systemic immune response elicited by the vaccine. Analysis of rTcdB-specific IgG subclasses revealed significant induction of IgG1, IgG2a, IgG2b, IgG2c and IgG3 after the third immunization, with IgG2b showing the largest increase, indicating engagement of both Th1- and Th2-associated isotypes (Fig. 4a). Consistent with engagement of cellular immunity, ELISpot analysis of splenocytes one month after the final immunization showed significantly increased frequencies of IFN-γ-, IL-4-, and IL-17-secreting cells upon rTcdB restimulation, reflecting concurrent Th1, Th2, and Th17 responses (Fig. 4b). To assess the functional capacity of the antibodies, sera were tested for their ability to neutralize purified

TcdB; vaccinated sera protected Vero cells from TcdB-induced cytopathic effects in a dose-dependent manner (Fig. 4c). We further examined cross-strain reactivity against TcdB present in culture supernatants from six clinically relevant *C. difficile* strains spanning major ribotypes. Vaccinated sera neutralized TcdB from the RT027 strains (BI-1 and R20291; titer 625), with markedly higher titers against the Asian clinical isolates RT023 (NCKUH K16; titer 3125) and RT017 (NCKUH A4; titer 3125) (Fig. 4d). Neutralization of the toxin A-negative RT017 strain confirmed that protective activity was directed specifically against TcdB. In contrast, no neutralizing activity above the control serum threshold was detected against VPI 10463 (RT087) or M120 (RT078), both of which yielded titers of 5, equivalent to the unimmunized control. These strain-dependent differences suggest that the receptor-binding domain used as immunogen shares antigenic determinants with several clinically important ribotypes, but does not elicit detectable cross-neutralization against all TcdB variants. The findings further indicate that sequence variation within toxin B may influence the breadth of neutralizing antibody responses elicited by this vaccine platform.

Oral vaccination protects against lethal *C. difficile* challenge

We evaluated protective efficacy in a mouse model of lethal CDI. Mice were orally immunized with PLL_{20}-*b*-PLT_{40} (50 μg rTcdB), treated with an antibiotic cocktail, and

challenged with *C. difficile* R20291 spores (Fig. 5a). Vaccinated mice were substantially protected, with markedly improved survival compared with the infection and empty-polymer groups (Fig. 5e). This protection was accompanied by attenuated disease: vaccinated mice lost significantly less body weight and recovered to baseline, whereas infected controls exhibited sustained weight loss (Fig. 5c, d). Vaccinated animals were also protected from the cecal inflammation characteristic of CDI, retaining significantly higher normalized cecum weight than infected controls, while colon length did not differ significantly among groups (Fig. 5f, g). Gross examination confirmed preserved cecal and colonic architecture in vaccinated mice (Fig. 5b). Although fecal bacterial burden in vaccinated mice was lower than in the unvaccinated infection group, it did not differ significantly from the empty-polymer group and remained markedly above uninfected levels, indicating that vaccination did not confer bacterial clearance (Fig. 5h).

Vaccination elicits mucosal immunity and limits intestinal inflammation

To investigate the basis of mucosal protection, we examined antibody and inflammatory responses in the intestine after challenge. Vaccinated mice had significantly elevated rTcdB-specific IgG and IgA in intestinal washes compared with controls, demonstrating that oral immunization established antigen-specific antibodies

384 at the mucosal surface (Fig. 6a, b). Luminal samples from vaccinated mice were also
385 markedly less cytotoxic to Vero cells than those from infected or empty-polymer
386 controls, at every dilution tested (Fig. 6c, d). Because exogenous toxin was not added
387 to these samples, this assay reports the net cytotoxic activity of the luminal contents
388 and does not by itself distinguish antibody-mediated neutralization from a lower
389 luminal toxin burden. Consistent with reduced toxin-mediated damage, vaccinated
390 mice showed markedly lower pro-inflammatory cytokine and chemokine responses, at
391 both the protein and transcript levels, than infected and empty-polymer controls,
392 comparable to uninfected mice (Supplementary Fig. 3). Histological examination of
393 the colon confirmed this protection: .vaccinated mice exhibited preserved mucosal
394 architecture with minimal leukocyte infiltration and significantly lower
395 histopathology scores than the infection and empty-polymer groups, although scores
396 remained slightly above those of uninfected controls (Fig. 6e, f).

397 **Discussion**

398 In this study, we developed an oral vaccine platform using a biodegradable, pH-
399 stable poly(L-lysine)-*block*-poly(L-threonine) polypeptide polymer. Oral
400 immunization with the recombinant TcdB receptor-binding domain encapsulated in
401 this polymer induced systemic and mucosal antibody responses, cellular immune

activation, and protection against lethal CDI in a mouse model, without the need for parenteral administration or a booster dose.

The protective performance of this platform is enabled by its antigen-release mechanism. The polypeptide network is cross-linked by genipin through covalent bonds between lysine residues, rendering it stable across the gastrointestinal pH range; in the absence of protease, no antigen was released at gastric or intestinal pH. Release instead required proteolytic cleavage and occurred preferentially under neutral-to-alkaline conditions. This protease-triggered, rather than pH-dependent, behavior supports a model in which the nanogel remains largely intact during gastric transit and releases antigen more efficiently in a protease-containing intestinal environment where mucosal uptake may occur. The poly(L-threonine) block further contributes to this design: its β-sheet conformation shields the cationic charge of the poly(L-lysine) backbone, which both stabilizes antigen encapsulation and reduces the cytotoxicity associated with cationic polymers, consistent with the favorable biocompatibility we observed.

The failure of recent phase 3 trials of parenteral alum-adjuvanted CDI vaccines highlights that systemic immunity alone may be insufficient for effective CDI prevention [6,7]. A liposomal adjuvant platform incorporating TLR4 and TLR7/8

agonists improved antitoxin IgG avidity, IgG2c class switching, and mucosal IgA responses compared with alum; however, both adjuvants conferred complete survival, and the mucosal component was associated with better preservation of gut barrier integrity rather than with survival itself, and the regimen still combined intramuscular priming with an intranasal boost [10]. More recently, a multivalent rectal vaccine combining inactivated toxins and non-toxin surface antigens achieved complete bacterial clearance in mice, while the same formulation given intraperitoneally failed to reduce colonization burden, demonstrating that mucosal delivery route, not antigen composition alone, determines whether sterilizing immunity can be achieved [9]. Our findings extend this concept by showing oral polymer-encapsulated antigen delivery can engage gut-associated lymphoid tissue and induce intestinal IgA besides systemic immunity [19].

We selected the TcdB receptor-binding domain (residues 1852-2363) as the immunogen because TcdB is sufficient to cause the full spectrum of CDI, and anti-TcdB antibodies represent the primary correlate of protection in both human and animal models [5]. Using recombinant protein rather than chemically inactivated toxoid avoids potential disruption of conformationally sensitive neutralization epitopes, a concern that similarly motivated the use of point-mutated toxin constructs

in recent mucosal vaccine designs [9]. The neutralizing activity confirmed in Vero cell assays demonstrates that antigen immunogenicity is preserved through the oral delivery process.

The cross-strain neutralization profile showed both the breadth and limitations of immunity elicited by the RT027-derived immunogen. Vaccinated sera exhibited high neutralizing titers against RT023 and RT017 clinical isolates and moderate titers against RT027 strains, confirming conservation of neutralizing epitopes across these clades. In contrast, no activity above the unimmunized control threshold was detected against VPI 10463 (RT087, clade 1) or M120 (RT078, clade 5), likely reflecting clade-specific divergence in the receptor-binding domain. Notably, neutralization of the toxin A-negative RT017 strain confirmed TcdB-specific activity. It should be noted that supernatants were normalized by total protein rather than TcdB concentration, so titers partly reflect inter-strain differences in toxin secretion; formal cross-strain efficacy *in vivo* remains to be established. This cross-strain analysis extends beyond either the liposomal adjuvant platform [10] or the rectal mucosal vaccine [9], both of which were evaluated against a single challenge strain, and addresses cross-subtype protection as an outstanding question for CDI vaccines [10]. The absence of neutralizing activity against RT087 and RT078 likely reflects clade-

456 specific sequence divergence in TcdB, as subtypes across major clades differ by 3-
457 15% in amino acid sequence [20].

458 Oral vaccination induced antigen-specific cellular immune responses characterized
459 by IFN-γ, IL-4, and IL-17 secretion. Although IL-17-associated pathways have been
460 linked to intestinal immunopathology in other contexts, vaccinated animals showed
461 reduced pro-inflammatory cytokine expression and preserved intestinal architecture
462 after challenge, indicating that this response reflects productive mucosal immunity
463 rather than pathological inflammation [21]. This is consistent with the finding that
464 TH17-skewed tissue-resident memory T cell responses in the colonic lamina propria
465 correlate with *C. difficile* clearance following rectal vaccination, suggesting that
466 TH17-associated mucosal responses may be a common protective mechanism across
467 different mucosal vaccine platforms [9].

468 Oral vaccination did not achieve antigen-specific clearance of colonization:
469 although fecal bacterial burden was lower than in untreated infected mice, it did not
470 differ from the empty-polymer group. Nevertheless, robust protection against disease
471 was observed, as evidenced by reduced toxin activity, attenuated clinical
472 manifestations, and improved survival. CDI pathology is driven by toxin-mediated
473 epithelial injury rather than bacterial burden per se [22]. This is consistent with

474 bezlotoxumab, a clinically approved anti-TcdB monoclonal antibody that reduces CDI
475 recurrence through toxin neutralization alone without directly affecting bacterial
476 colonization [23]. Incorporating colonization-targeting antigens such as flagellar
477 components or cell-wall enzymes, as validated in recent mucosal vaccine work, may
478 be a logical next step toward achieving sterilizing immunity with this platform [9].
479 Durability was assessed with the same 50 μg formulation used in the challenge
480 studies, so the six-month antibody persistence observed here applies directly to the
481 protective dose. That antigen-specific reactivity remained detectable for six months
482 without a booster suggests that the polymer platform contributes to prolonged antigen
483 availability. This observation is consistent with previous reports that biodegradable
484 polymer-based delivery systems can promote sustained antigen exposure and
485 prolonged immune stimulation [24]. The biodegradable and pH-stable nature of the
486 polymer, together with its compatibility with oral delivery and scalable synthesis,
487 supports its potential for clinical translation. Although antibody responses persisted
488 for six months without booster administration, whether this humoral durability
489 translates into sustained functional protection remains to be determined in dedicated
490 long-term challenge studies.

In summary, oral polymer-encapsulated rTcdB vaccination induced durable antibody responses, cross-strain toxin neutralization in vitro, and protection against lethal CDI *in vivo*. Because all experiments were conducted in young adult mice whereas CDI predominantly affects elderly populations, future studies should evaluate this platform in aged mouse models, explore the addition of colonization-targeting antigens, and examine whether combination approaches integrating toxin-specific and surface antigen-specific immunity, as recently demonstrated with rectal vaccination, can further enhance protective efficacy.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. Additional raw data are available from the corresponding author on reasonable request.

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Author contributions

Lin YS and Huang IH designed the study. Jan JS and Chen YF synthesized the polypeptide polymers and developed the nanogel platform. Tsai PJ provided the clinical *C. difficile* isolates and performed PCR ribotyping. Lin YS performed the experiments. Lin YS and Tsai CS analyzed the data, prepared the figures and wrote the manuscript. JM reviewed the manuscript. Huang IH supervised the project, provided resources, wrote and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Figure legends

Figure 1. Architectural design, physicochemical characterization, and biocompatibility of the rTcdB-encapsulated polypeptide nanogel. (a) Schematic representation of the two polymer configurations evaluated for antigen encapsulation. The graft copolymer PLL_{20}-*g*-$Indo_{0.3}$ and the block copolymer PLL_{20}-*b*-PLT_{40} were cross-linked with genipin to entrap recombinant TcdB C-terminal receptor-binding domain (rTcdB); PLL_{10}-*b*-PLT_{20} shares the same block architecture with shorter chain lengths. **(b)** Hydrodynamic diameter and zeta potential of empty PLL_{20}-*b*-PLT_{40} and of the candidate formulations loaded with 50 μg rTcdB, determined by dynamic light scattering. **(c)** *In vitro* cytotoxicity of serially diluted PLL_{20}, PLL_{10}-*b*-PLT_{20}, and PLL_{20}-*b*-PLT_{40} measured in Vero cells by LDH assay. Cytotoxicity is expressed relative to Triton X-100, which served as the positive control for maximal cell lysis (100%). No LDH release above background was detected for PLL_{20}-*b*-PLT_{40} at the 10^{-2} and 10^{-3} dilutions (0% cytotoxicity). Undiluted stocks were 5.15 mg/mL (PLL_{20}) and 3.93 mg/mL (PLL_{10}-*b*-PLT_{20} and PLL_{20}-*b*-PLT_{40}); these refer to the cross-linked material, with equivalent polypeptide content across formulations. **(d)** Serum biochemical markers of hepatic (AST, ALT) and renal (BUN, creatinine) function in mice after three oral immunizations, compared with reference physiological ranges. Data are presented as mean ± standard deviation. Statistical comparisons were performed by one-way ANOVA (n.s., not significant; $*P < 0.05$; $****P < 0.0001$).

Figure 2. Dose optimization and long-term antibody durability of the oral polypeptide vaccine. (a) rTcdB-specific serum IgG (1:200 dilution) and IgA (1:50

dilution) measured by ELISA after each of three oral immunizations, comparing the control group, empty PLL$_{20}$-*b*-PLT$_{40}$, and PLL$_{20}$-*b*-PLT$_{40}$ loaded with 25 μg or 50 μg rTcdB. The 50 μg formulation elicited significantly higher IgG and IgA than the 25 μg formulation, supporting selection of the higher dose for subsequent challenge studies. **(b)** Longitudinal rTcdB-specific serum IgG and IgA in mice immunized with PLL$_{20}$-*b*-PLT$_{40}$ (50 μg rTcdB), monitored from before immunization through six months after the final immunization. Antibody titers remained elevated above the control group for six months without booster, indicating durable humoral responses. Data are presented as mean ± standard deviation. Statistical comparisons were performed by two-way ANOVA (***$P < 0.001$; ****$P < 0.0001$). IgA y-axes in (a) and (b) contain axis breaks.

Figure 3. Design rationale and in vitro release behavior of the genipin-cross-linked PLL$_{20}$-*b*-PLT$_{40}$ nanogel. (a) Proposed gastrointestinal transit and antigen-release mechanism. The genipin-cross-linked network remains intact in the stomach (pH 1.5-3.5) despite exposure to pepsin, whereas cleavage of the polypeptide matrix by intestinal proteases in the colon (pH 7.0-8.0) releases encapsulated rTcdB for mucosal uptake. Lower panels: proposed structural basis for nanogel stability, and genipin-mediated cross-linking of PLL ε-amino groups (schematic; not drawn to mechanistic detail). **(b)** Cumulative in vitro release of rTcdB at pH 3.0, 7.4 or 8.0 over

30 h, with or without bromelain (1 mg/mL), used as a model protease to mimic intestinal proteolysis. Data are mean ± SD (n=3 per time point).

Figure 4. Systemic humoral and cellular immunity and cross-ribotype TcdB neutralization. (a) rTcdB-specific IgG subclass distribution (IgG1, IgG2a, IgG2b, IgG2c, IgG3) in serum measured by ELISA after each of three oral immunizations, indicating a mixed Th1/Th2 response. **(b)** Frequencies of rTcdB-specific cytokine-secreting splenocytes (IFN-γ, IL-4, IL-17) enumerated by ELISpot one month after the third immunization, comparing rTcdB-stimulated and non-stimulated splenocytes. **(c)** Serum-mediated neutralization of purified TcdB: serially diluted sera were incubated with TcdB and applied to Vero cells, with cell viability measured by MTT assay. **(d)** Cross-ribotype neutralization of TcdB in culture supernatants from six clinically relevant *C. difficile* strains spanning major ribotypes, expressed as reciprocal neutralizing titers. Culture supernatants were normalized by total protein content prior to analysis. Data are presented as mean ± standard deviation (n = 4 for a-c). Statistical comparisons were performed by one-way ANOVA (a, c) or two-way ANOVA (b) (n.s., not significant; *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

Figure 5. Vaccination protects mice against lethal *C. difficile* challenge. (a) Experimental design. C57BL/6 mice (n = 10 per group) were orally immunized with PLL_{20}-*b*-PLT_{40} (50 μg rTcdB) weekly for three weeks, treated with an antibiotic cocktail, and challenged with 1×10^8 *C. difficile* R20291 spores following

clindamycin and proton-pump-inhibitor administration. Mice were monitored for six days post-infection. **(b)** Representative gross morphology of cecum and colon from each group; scale bar, 1 cm. **(c)** Body weight kinetics from five days before to six days after infection. **(d)** Net change in body weight following infection. **(e)** Kaplan-Meier survival curves. **(f)** Cecum weight normalized to body weight, an index of cecal inflammation. **(g)** Colon length. **(h)** Fecal bacterial burden (log CFU/g) one day after infection. Groups comprised mock (uninfected, untreated), infection (infected, untreated), empty polymer (infected, immunized with empty PLL_{20}-*b*-PLT_{40}), and vaccination (infected, immunized with 50 µg rTcdB formulation). Data are presented as mean ± standard deviation. Statistical comparisons were performed by one-way ANOVA (n.s., not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Survival curves were compared by the log-rank (Mantel-Cox) test; PLL_{20}-*b*-PLT_{40} vs. infection group, **$P < 0.01$; PLL_{20}-*b*-PLT_{40} vs. empty group, *$P < 0.05$.

Figure 6. Vaccination elicits mucosal antibodies, neutralizes luminal toxin, and protects colonic tissue. (a) rTcdB-specific IgG and **(b)** rTcdB-specific IgA in intestinal washes measured by ELISA after challenge, comparing mock, infection, empty polymer, and vaccination groups. **(c)** Neutralization of toxin in fecal samples and **(d)** in intestinal washes: serially diluted samples were applied to Vero cells and cell viability was measured, with purified TcdB (4 ng/mL) as a positive cytotoxicity control. Higher viability indicates greater luminal toxin-neutralizing activity. **(e)**

Representative hematoxylin and eosin-stained colonic sections; arrows indicate lymphocyte infiltration; scale bar, 100 µm. **(f)** Histopathology scores of colonic tissue. Mock animals scored 0 in all cases; the absence of an error bar reflects zero variance rather than missing data. Data are presented as mean ± standard deviation. Statistical comparisons were performed by one-way ANOVA (n.s., not significant; $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$).

Supplementary Figure 1. Polymer selection screening by antibody response. (a) rTcdB-specific serum IgG and IgA measured by ELISA after three oral immunizations, comparing the graft copolymer (PLL_{20}-*g*-$Indo_{0.3}$) and block copolymer (PLL_{20}-*b*-PLT_{40}) configurations, each as empty or rTcdB-loaded formulations, against a saline control. Only the rTcdB-loaded block copolymer elicited a robust antibody response, whereas responses to the graft configuration did not differ from the saline control. **(b)** rTcdB-specific serum IgG and IgA across the polymer variants tested (PLL_{20}, PLL_{10}-*b*-PLT_{20}, PLL_{20}-*b*-PLT_{40}). PLL_{20}-*b*-PLT_{40} elicited the highest IgG response; IgA responses did not differ significantly among the variants. Data are presented as mean ± standard deviation. Statistical comparisons were performed by two-way ANOVA (n.s., not significant; $***P < 0.001$; $****P < 0.0001$). The IgA y-axes in (a) and (b) contain axis breaks.

Supplementary Figure 2. The 25 µg formulation confers incomplete protection against lethal challenge. (a) Kaplan-Meier survival curves and **(b)** net change in

body weight following 1×10^6 *C. difficile* R20291 spore challenge in mice immunized with PLL_{20}-*b*-PLT_{40} (25 μg rTcdB), compared with mock and infection groups (n = 5 per group). Neither survival nor body-weight change differed significantly between the vaccinated and infection groups, indicating that the 25 μg dose was insufficient for protection and motivating dose escalation to 50 μg. Data are presented as mean ± standard deviation. Statistical comparisons were performed by one-way ANOVA, and survival was analyzed by the log-rank test (n.s., not significant).

Supplementary Figure 3. Vaccination suppresses colonic pro-inflammatory cytokine responses. (a) Pro-inflammatory cytokine and chemokine concentrations (TNF-α, IL-6, IL-1β, MIP-2, MCP-1) in intestinal washes measured by ELISA after challenge. **(b)** Corresponding cytokine and chemokine transcript levels in colonic tissue measured by quantitative RT-PCR, normalized to β-actin. In both compartments, the vaccination group showed inflammatory responses comparable to the mock group and significantly lower than the infection and empty-polymer groups. Groups comprised mock, infection, empty polymer, and vaccination (50 μg rTcdB). Data are presented as mean ± standard deviation. Statistical comparisons were performed by one-way ANOVA (n.s., not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Both y-axes contain axis breaks.

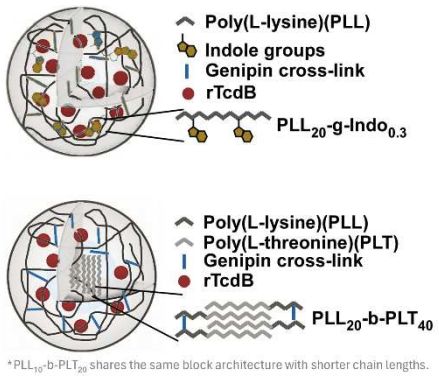
Supplementary information

Supplementary Table 1. Oligonucleotide primers used for quantitative reverse transcription PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
TNF-α (*Tnf*)	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-6 (*Il6*)	AGGATACCACTCCCAACAGAC	GTGCATCATCGTTGTTCATAC
IL-1β (*Il1b*)	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAAT
MIP-2 (*Cxcl2*)	TGTCAATGCCTGAAGACCCTGCC	AACTTTTTGACCGCCCTTGAGAGTGG
MCP-1 (*Ccl2*)	CCCACTCACCTGCTGCTACT	TCTGGACCCATTCCTTCTTG
β-actin (*Actb*)	ACTGCCGCATCCTCCTCCTC	TGCCACAGGATTCCATACCC

All primers were designed against *Mus musculus* sequences; official gene symbols are given in parentheses. β-actin (*Actb*) served as the endogenous reference gene for normalization using the $2^{-\Delta\Delta Ct}$ method.

a

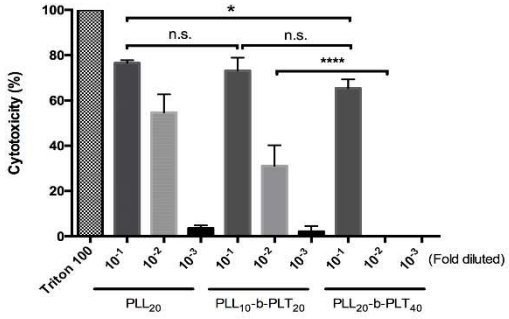


*PLL_{10}-b-PLT_{20} shares the same block architecture with shorter chain lengths.

b

Formulation	Particle size (Mean)	Zeta potential (Mean)
PLL_{20}-b-PLT_{40} (empty)	244.6 nm	+23.43 mV
PLL_{20} (50 μg)	275.6 nm	-6.93 mV
PLL_{10}-b-PLT_{20} (50 μg)	318.1 nm	-12.02 mV
PLL_{20}-b-PLT_{40} (50 μg)	348.5 nm	-18.09 mV

c



d

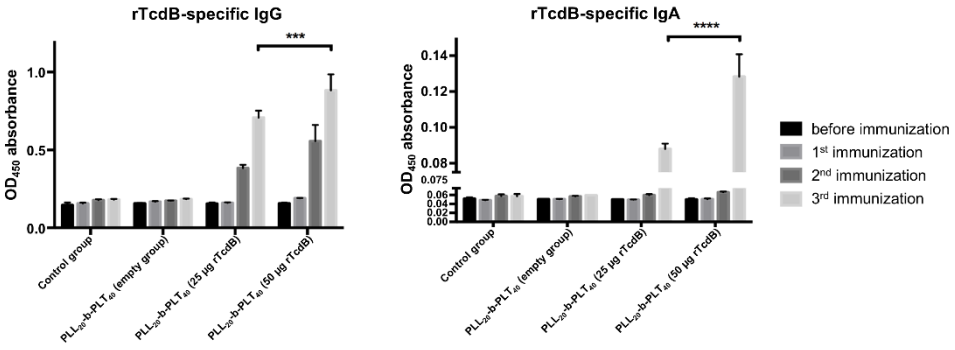
	AST (U/L)	ALT (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
Normal values	85.09 ± 22.78	30.80 ± 4.34	36.52 ± 3.20	0.39 ± 0.03
Control	80	57	30	<1.0
PLL_{20} (rTcdB)	67	50	30	<1.0
PLL_{10}-b-PLT_{20} (rTcdB)	73	50	23	<1.0
PLL_{20}-b-PLT_{40} (rTcdB)	63	53	30	<1.0

738

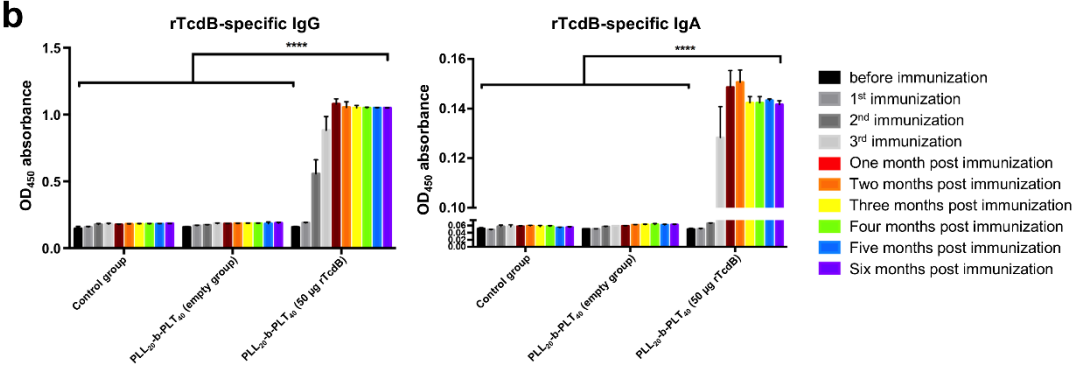
739

740

a

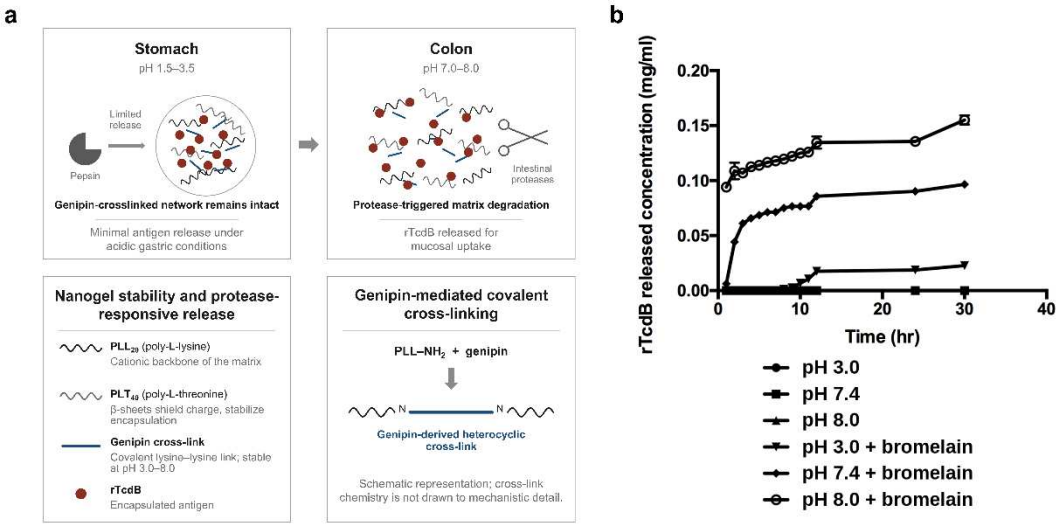


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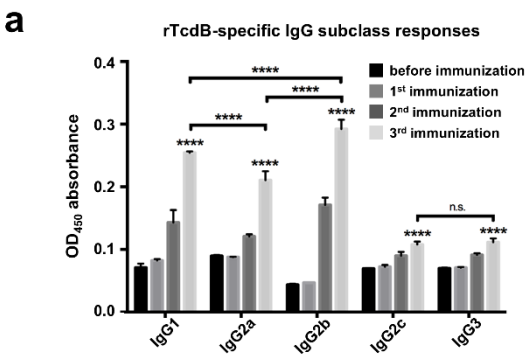
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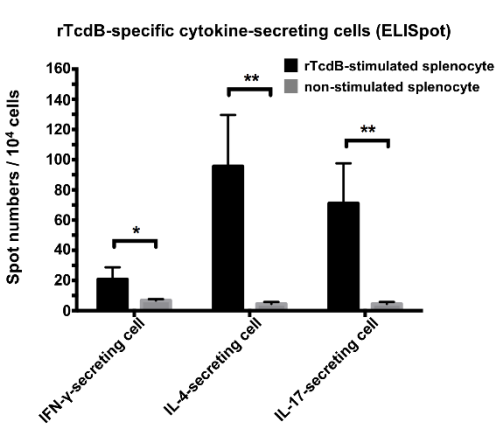
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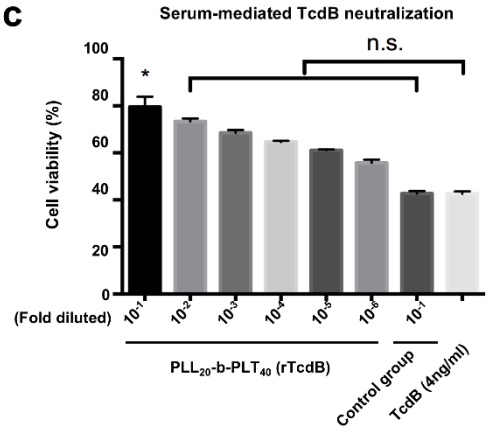

a
Stomach
pH 1.5–3.5
Limited release
Pepsin
Genipin-crosslinked network remains intact
Minimal antigen release under acidic gastric conditions
Colon
pH 7.0–8.0
Intestinal proteases
Protease-triggered matrix degradation
rTcdB released for mucosal uptake
Nanogel stability and protease-responsive release
PLL20 (poly-L-lysine)
Cationic backbone of the matrix
PLT40 (poly-L-threonine)
β-sheets shield charge, stabilize encapsulation
Genipin cross-link
Covalent lysine–lysine link; stable at pH 3.0–8.0
rTcdB
Encapsulated antigen
Genipin-mediated covalent cross-linking
PLL–NH2 + genipin
Genipin-derived heterocyclic cross-link
Schematic representation; cross-link chemistry is not drawn to mechanistic detail.
b
rTcdB released concentration (mg/ml)
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
Time (hr)
pH 3.0
pH 7.4
pH 8.0
pH 3.0 + bromelain
pH 7.4 + bromelain
pH 8.0 + bromelain

743

744





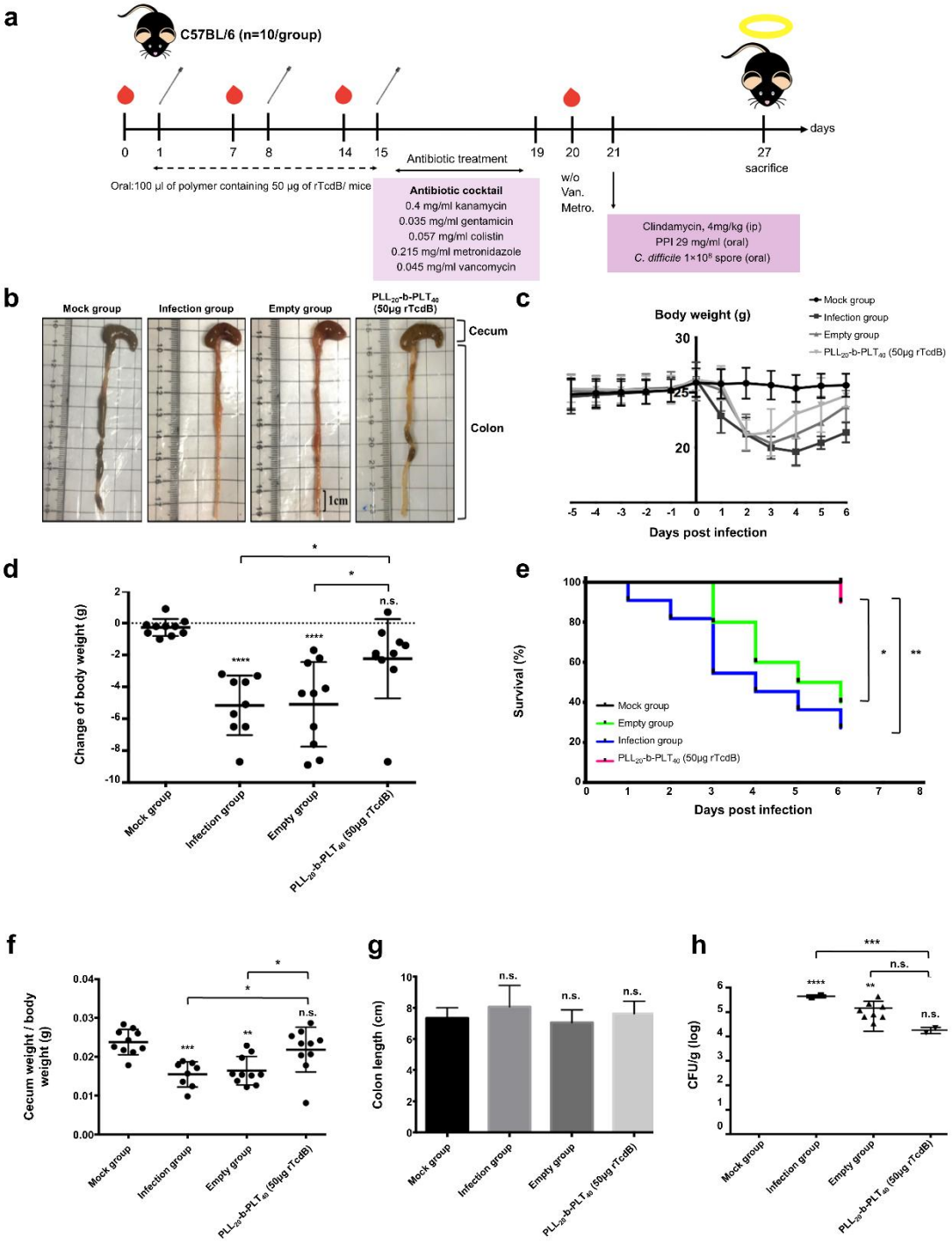


d

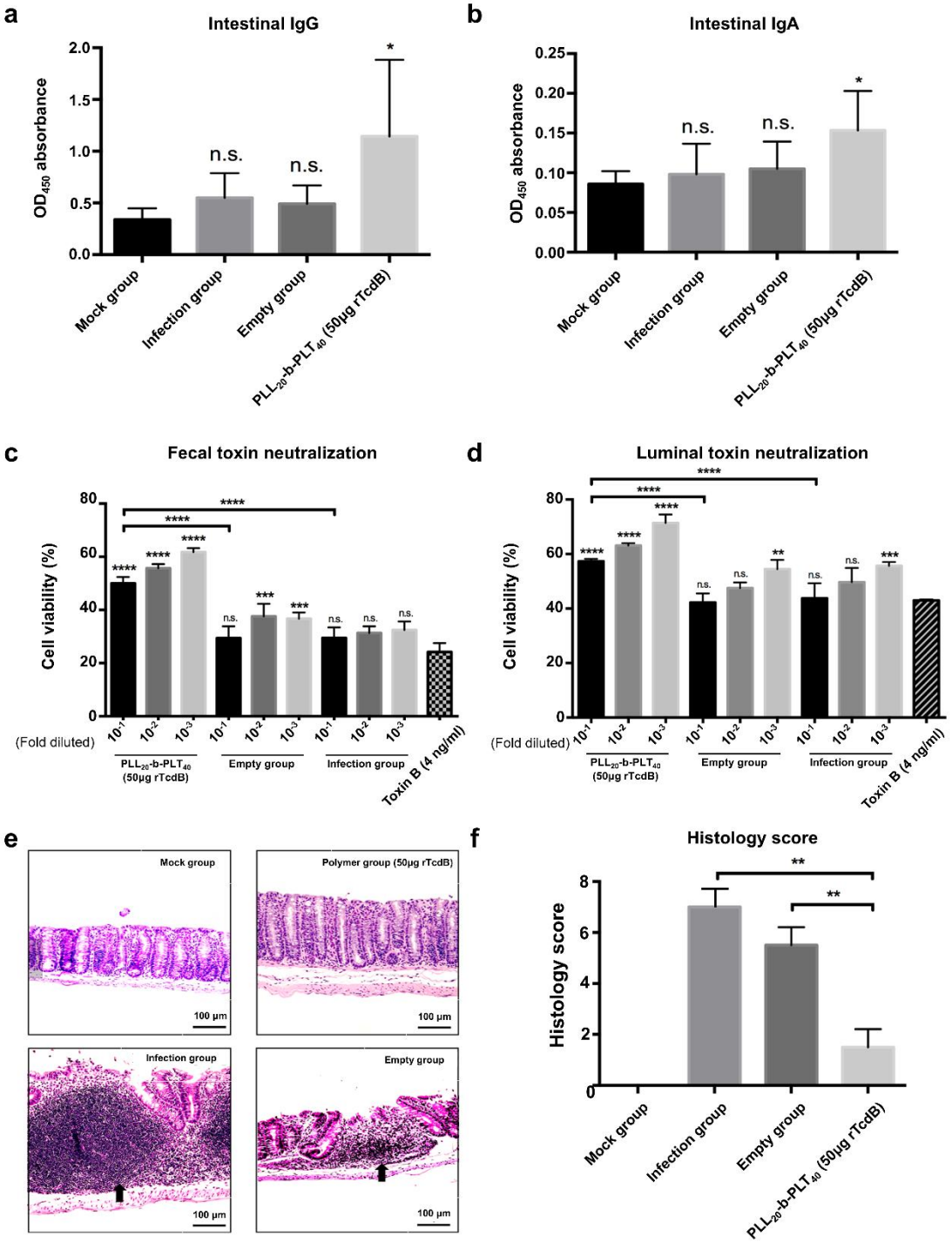
Cross-ribotype TcdB neutralization

Clade	Strain name	Ribotype	Toxin genotype	Sera titer (fold diluted)
1	VPI 10463	RT087	A+B+CDT-	5
2	BI-1	RT027	A+B+CDT+	625
2	R20291	RT027	A+B+CDT+	625
3	NCKUH K16	RT023	A+B+CDT+	3125
4	NCKUH A4	RT017	A-B+CDT-	3125
5	M120	RT078	A+B+CDT+	5
Control sera added in all different strains				5

745


a
C57BL/6 (n=10/group)
days
0 1 7 8 14 15 19 20 21 27
sacrifice
Antibiotic treatment
Oral:100 μl of polymer containing 50 μg of rTcdB/ mice
Antibiotic cocktail
0.4 mg/ml kanamycin
0.035 mg/ml gentamicin
0.057 mg/ml colistin
0.215 mg/ml metronidazole
0.045 mg/ml vancomycin
w/o Van. Metro.
Clindamycin, 4mg/kg (ip)
PPI 29 mg/ml (oral)
C. difficile 1×10⁸ spore (oral)
b
Mock group
Infection group
Empty group
PLL20-b-PLT40 (50μg rTcdB)
Cecum
Colon
1cm
c
Body weight (g)
Mock group
Infection group
Empty group
PLL20-b-PLT40 (50μg rTcdB)
Days post infection
d
Change of body weight (g)
n.s.
e
Survival (%)
Days post infection
f
Cecum weight / body weight (g)
g
Colon length (cm)
h
CFU/g (log)

746


a
Intestinal IgG
OD450 absorbance
2.0
1.5
1.0
0.5
0.0
n.s.
n.s.
*
Mock group
Infection group
Empty group
PLL20-b-PLT40 (50μg rTcdB)
b
Intestinal IgA
OD450 absorbance
0.25
0.20
0.15
0.10
0.05
0.00
n.s.
n.s.
*
Mock group
Infection group
Empty group
PLL20-b-PLT40 (50μg rTcdB)
c
Fecal toxin neutralization
Cell viability (%)
80
60
40
20
0
****
****
****
n.s.
***
***
n.s.
n.s.
n.s.
(Fold diluted)
10-1
10-2
10-3
PLL20-b-PLT40 (50μg rTcdB)
Empty group
Infection group
Toxin B (4 ng/ml)
d
Luminal toxin neutralization
Cell viability (%)
80
60
40
20
0
****
****
****
****
****
n.s.
n.s.
**
n.s.
n.s.
***
(Fold diluted)
10-1
10-2
10-3
PLL20-b-PLT40 (50μg rTcdB)
Empty group
Infection group
Toxin B (4 ng/ml)
e
Mock group
Polymer group (50μg rTcdB)
Infection group
Empty group
100 μm
f
Histology score
Histology score
8
6
4
2
0
**
**
Mock group
Infection group
Empty group
PLL20-b-PLT40 (50μg rTcdB)

747

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NewFigureS1.tif
- NewFigureS2.tif
- NewFigureS3.tif
- SupplementaryInformationCommsMed.docx