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# Biochemical and Immunological Characterizations of the Receptor Binding Domain of *C. difficile* Toxin B

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## Abstract

*Clostridium difficile* (Cd) is an emerging nosocomial pathogen responsible for antibiotic-associated pseudo-membranous colitis and diarrhea in hospital acquired infections. Clostridial toxins A (TcdA) and B (TcdB) which specifically bind to unknown glycoprotein(s) on the surface of epithelial cells disrupt the intestinal barrier and ultimately lead to acute inflammation and diarrhea. There is still debate as to whether the receptor binding domains (RBD) of toxins can individually elicit protection in the hamster challenge model. In this study, a TcdB RBD which was derived from *C. difficile* strain VPI10463 with >95% amino acid sequence identity to hyper-virulent strain BI/NAP1/027 was designed and expressed in *Escherichia coli*. Recombinant RBD (rRBD) was purified, characterized biologically and immunologically and found to have the following properties: (a) capable of binding to the cell surface of both Vero and Caco-2 cells and entering into the cytosol; (b) devoid of hemagglutinin activity (HA); (c) the ability to up-regulate cell surface markers expressions and cytokines secretions from dendritic cells; (d) eliciting anti-TcdB neutralizing antibody responses that could weakly cross-neutralize TcdA in the absence of adjuvant; (e) and inducing weak protection against a lethal dose of Cd spores in the hamster challenge model. Therefore, rRBD shows potential as an immunogen to be included in the development of vaccines against *Clostridium difficile*-associated diseases.

**Keywords:** *C. difficile* infection; Antibiotic-associated pseudo-membranous colitis; *C. difficile* toxins; Receptor binding domain; Toll-like receptor agonist; Vaccine

## Introduction

In the past two decades, because of an increase in multi-drug resistant strains, *Clostridium difficile*-associated diseases (CDAD), such as diarrhea, pseudomembranous colitis, and toxic megacolon have become serious emerging infectious diseases worldwide [1-3]. A hyper-virulent and antibiotic-resistant epidemic strain NAP1/027 recently found in developed countries poses a major challenge for CDAD prevention since strain VP110463 had been used as the standard in most current vaccine development [4,5]. More importantly, *C. difficile* relapse was found to be around 15-35% within a few weeks after the standard therapy using either vancomycin and/or metronidazole [6]. The pathogenicity of *C. difficile* infections (CDI) is largely correlated to clostridial toxins A and B (TcdA and TcdB) which are secreted in the gastrointestinal environment of infected hosts and disrupt the epithelial cell barriers in the small intestine [7]. The mechanism underlying TcdA and TcdB toxicity involves three steps: (a) binding to unidentified receptor protein(s) on the surface of intestinal epithelium and internalization through its C-terminal receptor binding domain, (b) auto-cleavage and translocation of the N-terminal glucosyltransferase domain from the endosomal membrane to the cytosol; and (c) the N-terminal enzymatic region that inactivates the Rho GTPase family by glycosylation [8].

TcdA-specific antibodies in patient sera were found to positively correlate with the prevention of CDAD recurrence [9-12], so TcdA-specific monoclonal antibodies are currently being tested in clinical trials [13-15]. In addition, different *C. difficile* vaccine strategies are being evaluated; the most advanced being vaccination with formalin-inactivated toxins [9,16-19]. Immunization with the receptor binding domain (RBD) of TcdA as antigens formulated with different adjuvant has been shown to elicit toxin-neutralizing antibody responses and protect mice from toxin challenges [20-26]. TcdB RBD has been less studied. A recent publication [27] has indicated that recombinant fragment of TcdB RBD in the presence of adjuvant could elicit neutralizing antibody responses against TcdB but not TcdA, only vaccine candidates containing both RBDs from TcdA and TcdB would confer protection in an *in vivo* hamster challenge model. Dingle et al. [28] also showed recombinant TcdB RBD to have poor biological functions such as not being able to agglutinate red blood cells, or to compete and directly block the cytotoxicity of TcdB and TcdA.

TcdB RBD is predicted to have a molecular size of approximately 63 kDa and is composed of 22-24 homologous repetitive peptides which may contain 4 potential lectin-like receptor-binding sites [8,22,28,29]. The specific roles and functions of these 4 putative binding regions are unclear, but they correlate with the lectin-like binding to the synthetic oligosaccharide, Galα1-3Galβ1-4GlcNAc [28-30]. In this study, a consensus sequence of TcdB RBD was identified from different *C. difficile* strains deposited in the NCBI protein database. A consensus recombinant TcdB RBD (rRBD) was designed and expressed in *Escherichia coli*; the purified rRBD was characterized biologically and

immunologically to test its merits as a potential vaccine candidate against CDAD.

## Materials and Methods

### Ethics statement

All experiments were conducted in accordance with the guidelines of the Laboratory Animal Center of National Health Research Institutes (NHRI). Animal use protocols have been reviewed and approved by the Institutional Animal Care and Use Committee of National Health Research Institutes (Approved protocol No. NHRI-IACUC-100053-A).

### Construction of TcdB RBD

The identification and design of the consensus sequence for TcdB RBD were performed according to a methodology described previously [31]. Briefly, TcdB RBD sequences from different *Clostridium difficile* strains deposited in the NCBI database were aligned for sequence analysis using the alignment tools from Vector NTI Advance 11.5 (Life technologies, Carlsbad, CA). This consensus sequence was analyzed by online software (http://www.ebi.ac.uk/Tools/pfa/radar/) for the detection of repetitive protein sequences and potential ligand binding sites. The nucleotide sequence of tcdB rRBD coding for the C-terminal 537 amino acids of TcdB was optimized for E. coli codon usage (Figure 1), chemically synthesized (GeneArt; Life technologies) for cloning and expressed in E. coli. Gene encoding for TcdB RBD was inserted into a pET-22b vector (Novagen, Darmstadt, Germany) containing a polyhistidine tag coding sequence at the 3'-end before the NdeI and XhoI restriction sites. The resulting pET-22b_tcdB RBD construct was transformed into *E. coli* JM109 (DE3) (Promega, Madison, WI) for TcdB RBD (rRBD) expression.

MMVSGLIYIN DSLYYFKPPV NNLITGFVTV GDDKYYFNPI NGGAA
QSGVLQTGVF STEDGFKYFA PANTLDENLE GEAIDFTGK
ELDGEMHYFS PETGKAFKGL NQIGDDKYYF NSDGVMQKGF VSINDNKHYF DDSGVMKVGY
TEIDGKHFYF AENGEMQIGV FNTEDGFKYF AHHNEDLGNE EGEEISYSGI
DDSFTAVVGW KDLEDGSKYY FDEDTAEAYI GLSLINDGQY YFNDDGIMQV GFVTINDKVF
YFSDSGIIES GVQNIDDNYF YIDDNGIVQI GVFDTSDGYK YFAPANTVND NIYGQAVE
GLVRVGEDVY YFGETYTIET GWIYDMENES DKYYFVPETK KACKGINLID DIKYYFDEKG
IMRTGLISFE NNNYYFNENG EIQFGYINIE DKMFYFGEDG VMQIGVFNTP DGFKYFAHQN
TLDENFEGES INYTGWLGLD EKRYYFTDEY IAATGSVIID GEEYYFDPDT AQLVISE

**Figure 1:** Amino acid sequence of the C-terminal repetitive and putative receptor binding domain from *C. difficile* toxin B (TcdB RBD). (A) The amino acid sequence (537 residues) of TcdB RBD was identified using the online software (http://www.ebi.ac.uk / Tools/pfa/radar/). The localization of the 4 long-repeat (LR) regions of oligossacharide-binding sites are highlighted in red color.

## Production of rRBD

rRBD was expressed in *E. coli* JM109 (DE3) (Promega) grown in LB medium at 20 oC for 16 hours following induction with 1 mM isopropyl-β-D- thiogalacto-pyranoside (IPTG). The purification process for rRBD is briefly described below. Cells from 2 liters of culture medium were harvested by centrifugation and stored at -20°C before re-suspension in lysis buffer (50 mM sodium phosphate buffer, pH 7.2 containing 250 mM NaCl and 5 mM imidazole). The cells were physically disrupted using a French Press (Constant System, Daventry, UK) at 27 Kpsi and the supernatant was collected by filtration through a 0.22 μm filter. The crude extract was directly applied onto a nickel affinity chromatography column (GE Healthcare, Uppsala, Sweden) for purification of rRBD at 4°C. After sequential washes with low concentrations of imidazole buffer, rRBD was eluted using a lysis buffer containing 500 mM imidazole. The eluent was then dialyzed in a 30 kDa cut-off dialysis bag against phosphate buffered saline (PBS), pH 7.2 containing 10% glycerol. To remove bacterial endotoxin, the rRBD solution was passed through an E membrane (Pall Corporation, Ann Arbor, MI). All purification steps were analyzed by 8% SDS-PAGE. The residual endotoxin was determined using the Limulus amoebocyte lysate (LAL) assay (Associates of Cape Cod, Inc., Cape Cod, MA).

### Circular dichroism analysis

The purified rRBD was diluted in 10 mM phosphate buffer (pH 7.0) at a concentration of 50 μg/mL. Spectra were obtained using a J-185 spectropolarimeter (Jasco, Easton, MD) with temperature control, and data were acquired in continuous scanning mode using a path length of 0.5 mm, at a 0.2 nm interval and an accumulation time of 10-15 s/min. The far UV scan range was set between 190-250 nm with a scan speed of 50 nm/min. All data were processed using Jascot software, and a background spectrum obtained from PBS buffer was subtracted from the acquired sample spectrum. For the thermal stability test, the temperature gradient was set between 30 and 95°C. CD spectra were recorded at an interval of 5°C. The percent of the protein structure that was helical was calculated from mean residue ellipticity ([θ]) at 208 nm according to Freenfield and Fasman [32].

### Immuno-fluorescence staining and confocal microscopy for cell-binding and entry analysis

Vero cells were seeded in 75T flasks with VP-SFM containing 4mM glutamine at 37°C, 5% $CO_2$, and allowed to grow to 80% confluency. Cells were isolated and suspended with VP-SFM culture media at $2 \times 10^5$ cells/mL inside the flow tube. Resuspended cells were mixed with final 80 μg/mL of rRBD at 4°C for 5, 15, and 30 min. After washing three times with cold PBS buffer, cells were sequentially fixed and then permeabilized with buffers specific for flow analysis (eBioscience, San Diego, CA). Anti-Fc receptor antibody (BD science) was added for 10 min before stepwise incubation with the specific anti-TcdB antibody (GeneTex) and a secondary antibody conjugated with fluorescein isothiocynate (FITC) (Sigma-Aldrich) on ice for 30 min. At every step, cells were washed with cold PBS three times. After the last PBS washing step, cells were further washed with double distilled $H_2O$ to remove salt. Cells were simultaneously mounted onto glass slides, and nuclei stained with DAPI (Invitrogen, Carlsbad, CA.) and preparations subjected to -20°C before confocal microscopy analysis (Leica TCS SP5 II; Leitz, Heidelberg, Germany).

### Fluorescence-activated cell sorting flow cytometry (FASC)

Vero cells were seeded in 75T flasks containing VP-SFM/4mM glutamine and allowed to grow to 80% confluentcy at 37°C. An aliquot of resuspended cells ($5 \times 10^5$ cells) were mixed with 50 μg/mL of rRBD at 4°C for 5 minutes. After washing with cold PBST, 1 μg of either anti-TcdA antibody PCG-4 or anti-his tag antibody (AbD Serotec, Oxfordshire, UK) was added to the cells and the mixture was incubated on ice for 30 min. After washing twice with cold PBS, an FITC-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO.) was added and mixed for 30 min for surface staining. Before flow

cytometry analysis, propidium iodide (Sigma-Aldrich) was added to assess cell viability.

### Hemagglutinin activity assay

Hemagglutinin (HA) activity assay was performed as described by Wren et al. [33]. In brief, 1 nmole of rRBD or 50 pmole of TcdA RBD (positive control) in 25 μL used as the starting samples, were serially diluted two-fold with PBS buffer in 96-well round-bottom plates. A 25 μL of a 2% of rabbit erythrocyte (pre-washed with PBS) suspension was added into the wells. The mixtures were incubated at 4°C overnight. HA activity was calculated by visual scoring.

### Mouse immunogenicity study

BALB/c mice were purchased from the National Animal Center in Taiwan and held in the Animal Center of the NHRI. The general immunization protocol is briefly described below. Three groups of mice (6 BALB/c mice per group) were vaccinated with three intramuscular injections of 0, 3 or 10 μg, respectively, of rRBD every two weeks. Before each immunization, mice were bled to collect sera that were stored at -20°C before being used for anti-RBD antibody titer determination using RBD-specific ELISA and anti-TcdB neutralization assay as described below.

### Antigen-specific ELISA

ELISA plate wells were coated with 100 ng of rRBD overnight and blocked with 5% nonfat dry milk (w/v) in PBS. Mouse antisera serially diluted 2-fold with PBS containing 1% BSA (Calbiochem, Darmstadt, Germany) were added to the wells followed by incubation at room temperature (RT) for 2 hours. After washing with 3 × PBST, either anti-IgG isotypes (Invitrogen, Carlsbad, CA.) or HRP-conjugated IgG (KPL, Gaithersburg, MD) specific antibodies diluted in PBS containing 1% BSA were added to the wells and incubated at RT for 1 hour. After washing with 3 × PBST, the plates were treated with TMB peroxidase substrate (KPL) at room temperature in the dark for 20 min. To determine anti-RBD titers, $OD_{450}$ nm absorbance was measured using a spectrophotometer. End-point serum titers were defined as maximum dilution representing 0.2 cut-off value of $OD_{450}$ nm measurement. This cut-off value was determined as the mean plus two standard deviations of the mean optical density value from six pre-immunized mouse sera diluted at a 1:20. The maximum dilution of each serum sample was finally represented as geometric mean titer (GMT).

### Anti-TcdB neutralization assay

The anti-TcdB neutralization assay was performed according to the protocol previously described by Huang et al. [31]. Briefly, Vero cells ($2 \times 10^4$ per well) were seeded into 96-well plates containing VP-SFM culture medium and 4mM glutamine at 37°C, and allowed to grow to confluentcy Sera either from mice or hamsters immunized with rRBD were serially diluted two-fold with fresh VP-SFM and mixed with an equal volume of 40 pg/mL toxin B (The Native Antigen Company Ltd, Oxfordshire, UK) and incubated at room temperature for 1 hour. The mixture was added to the 96-well plates containing Vero cells and incubated at 37°C for 24 hours. Anti-TcdB neutralization titers were calculated as the highest serum dilution which could prevent 50% of cell from rounding due to TcdB cytotoxicity. Cellular toxicity was recorded using a microscope equipped with a camera.

### Surface markers and cytokines analyses for DC maturation

Analysis of DC maturation was performed *in vitro* as previously described [34]. C57BL/6 mice were purchased from National Animal Center in Taiwan and held in the Animal Center of the NHRI. In brief, bone marrow-derived DCs (BMDCs) were collected from the tibiae of 6 to 8-week old C57BL/6 females. Bone marrow cells were isolated by vigorously washing with LCM (RPMI 1640 containing 1% antibiotics with penicillin and streptomycin, 10% heat-inactivated FBS, 50 μM β-mercaptoethanol, and 50 mM HEPES) and treated with lysis buffer to remove erythrocytes. BMDC were re-suspended at $2 \times 10^6$ cells per mL in LCM and treated with 20 ng/mL of recombinant granulocyte macrophage colony stimulating factor (MoGM-CSF) (Peprotech, Rocky Hill, NJ) on days 0 and 3. An aliquot of suspended BMDCs equivalent to $2 \times 10^6$ cells/mL was seeded into 24-well plates on day 6. Varying concentrations of rRBD with or without 10 ng of polymyxin B were added to the wells. LPS (1000 EU, Sigma-Aldrich) served as control. After 16 to 18 hr incubation at 37°C, BMDCs were analyzed by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ) to evaluate the up-regulation of cell surface markers. In order to exclude immature DCs, representing 50% of the total cell population, the $CD11c^+$ cell population was gated for surface marker staining with specific monoclonal antibodies to CD40, CD80, CD86, and MHC-II. In addition, cell culture supernatants were collected for cytokine expression. Cytokines such as IL-6, IL-12p40 and TNF-α were determined using specific cytokine kits purchased from eBioscience (San Diego, CA ).

### Preparation of *C. difficile* spores and hamster challenge model

The protocol for preparation of *C. difficile* spores was modified from Lyras et al., [35]. Briefly, *C. difficile* strains VPI10463 were streaked on 10 anaerobic blood agar plates and grown anaerobically at 37°C to induce sporulation at around 5 to 6 days. The cells were harvested with disposable loops and washed in 10 mL PBS, and heat-shocked at 56°C for 30 min to kill surviving vegetative cells. The spores were collected by low-speed centrifugation and resuspended in DMEM, aliquoted and frozen at -80°C. The frozen spores were then quantified before use by plating ten-fold serial dilutions of the spores onto Taurocholatefructose-agar (TFA) plates which were prepared with agar plus taurocholate-cefoxitin- cycloserinefructose-agar (TCCFA) without cycloserine and cefoxitin. The hamster challenge model was performed as follows. Six hamsters per group (6 weeks old and weighing 100-130 g) purchased from National Animal Center in Taiwan and held in the Animal Center of the NHRI were given clindamycin orogastrically (30 mg/kg) to render them susceptible to *C. difficile* infection (day 0). On day-5 post clindamycin treatment hamsters in each group were gastrically inoculated with 100 c.f.u. of *C. difficile*, and monitored twice daily for 5 days and daily thereafter. Animal bedding was changed and faecal pellets were collected daily. Specimens were inoculated onto selective TCCFA plates and incubated anaerobically at 37°C to determine if they were colonized with *C. difficile*. Faecal pellets were collected daily for 12 days, then weekly until the study terminated (at least 14 days). Each hamster group was assessed for *C. difficile* colonization and survival rate.

### Statistical analysis

Data were expressed using Prism 5 version 5.01 (GraphPad Software, Inc.). Antibody titer was displayed as means ± SEM from the experiments. Statistical difference was analyzed using two-tailed

students' t-test by comparison of the means obtained in each treatment with the control group. The p-value <0.05 is considered to be significant.

## Results

### Design of TcdB RBD

According to previous studies [22,27-28,30,35], the receptor binding domain of TcdB is located between the C-terminal residues 1834 to 2366 and has a molecular weight of approximately 63 kDa. Sequences of TcdB RBD based on different *C. difficile* isolates deposited in the NCBI protein database including VPI10463 and ATCC9689 which are the reference strains for *C difficile* toxin studies and BI/NAP1/027 hyper-virulent strains were aligned and examined. The results indicated that the TcdB RBD amino acid sequences are conserved between these strains with 90-97% identity that is consistent with those previously reported by Stabler et al. [36]. The selected amino acid sequence of TcdB RBD is identical to that of *C. difficile* strains VPI10463 and composed of 537 residues (Figure 1). Based on the crystal structure reported by Ho et al. [29], this sequence comprises four potential oligosaccharide receptor-binding sites with core sequences QxGVFxTEDGFKYFA xxN as shown in Figure 1.

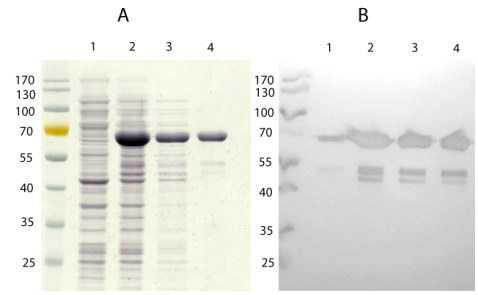


**Figure 2:** The expression and the purity of rRBD were confirmed by SDS-PAGE analysis (A) and Western blotting using toxin B-specific monoclonal antibody. (B). Purity of rRBD is shown in panel. A: lanes 1 to 4 correspond to cell lysate before IPTG induction, cell lysate after IPTG induction, supernatant of IPTG-induced cell lyseis, and eluent of 500 mM imidazole, respectively. The first lane contains molecular markers.

### Production of recombinant TcdB RBD (rRBD)

The coding sequence of rRBD was designed using codon usage optimization, chemically synthesized, inserted into pET-22b vector and successfully expressed in E. coli as shown in Figure 2. After single-step purification using Ni-affinity chromatography, highly purified rRBD (>95% purity) was obtained and its purity confirmed by SDS-PAGE (Figure 2A), and the Western blot analysis using anti-TcdB specific monoclonal antibody (Figure 2B). The degradation products are likely the result of proteolytic digestion during the purification process. In any event, at least 20 mg of highly enriched rRBD (Figure 2B lane 3) could be easily obtained from 0.5 liter of bacterial culture. Most of the *E. coli* endotoxin (LPS) was successfully removed by passing the rRBD preparation through an E membrane. Residual LPS in the purified rRBD was found to be below 0.01 EU per μg of protein based on the Limulus assay. We found that the best condition for preserving rRBD integrity was to store the protein at 3 mg/mL in PBS containing 10% (v/v) of glycerol at -80°C.

### Secondary structure analysis by circular dichroism (CD)

The secondary protein structure of rRBD was investigated and analyzed using circular dichroism. Figure 3A shows the CD spectra of rRBD at room temperature. According to the method developed by Freenfield and Fasman [32], the major secondary protein structure of rRBD is estimated to contain 47% of β-sheet structure; this is consistent with other reports that the fragments of TcdA RBD form stable β-solenoid secondary structures [29,31]. The CD secondary structure analysis supports that rRBD was correctly folded to form a stable β-solenoid structure that melts at 49°C (Figure 3A).

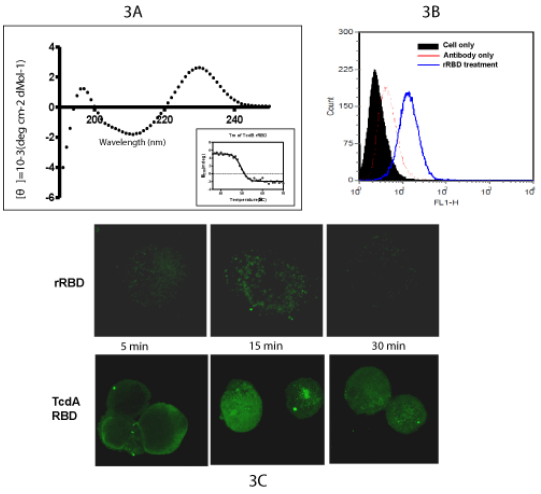


**Figure 3:** (A) CD spectra of rRBD. (B) Vero cell binding ability of rRBD was characterized by flow-cytometry. (C) Immuno-fluoresence signals of rRBD and TcdA RBD binding to Vero cell were analyzed by confocal micrscopy after 5, 15 and 30 minutes rRBD inoculation.

### rRBD binding to Vero cells

To further confirm that purified rRBD was correctly refolded, a Vero cell-binding assay was performed using flow cytometry. The results shown in Figure 3B indicate that within 5 minutes rRBD at a concentration of 0.3 μM (20 μg/mL) unambiguously binds to the Vero cell surface. rRBD also strongly binds to Caco-2 cells in a dose-dependent manner (data not shown). As seen in Figure 3C, binding of TcdA RBD to Vero cell surfaces promotes its cellular internalization through receptor-mediated/clathrin-dependent pathway endocytosis [31]. To verify whether or not rRBD exhibited this biological activity, a Vero cell binding assay was performed and results were analyzed by immuno-fluorescence and confocal microscopy [31]. After 5 minutes, rRBD binding was detected as speckled immunofluorescent spots on the surface of Vero cells. This was different from what was observed with TcdA RBD where immuno-fluorescence signals were uniformly distributed over the cell surface (Figure 3C). This difference in cell surface binding patterns suggests that TcdA RBD and TcdB RBD may have different receptor-binding specificities. After 15 minutes of rRBD incubation, the immuno-fluorescence signals did not decrease but rather increased over time (Figure 3C). This suggests that rRBD most

likely had been internalized from the cell surface to the cytosol as shown by confocal microscopy (Figure 3C, 15 min picture). The results support rapid cell-binding of rRBD as illustrated by strong anti-rRBD fluorescence signals on the cell surface within 5 minutes and entry into cytosol after 15 min (Figure 3C, 15 min picture). In addition, the majority of rRBD inside the cytosol was degraded within 30 minutes (Figure 3C, 30 min picture). These experiments indicate that rRBD specifically binds to cell surfaces, is internalized into the cytosol, and then degraded within a short period of time. In contrast, the anti-TcdA RBD fluorescence signals were decreased, but not disappeared in 30 minutes (Figure 3C). This suggests that rRBD and TcdA RBD may internalize into different compartments, so the rate of degradation is different.

The hemagglutinin activity of rRBD was evaluated using rabbit erythrocytes. It was observed that rRBD could not agglutinate rabbit erythrocytes at concentrations as high as 2 nmoles. TcdA RBD used as the positive control easily agglutinated rabbit erythrocyte at a concentration of 2 pmoles [31]. Our result is consistent with the previous report [28] that recombinant TcdB RBD does not have HA activity.

## TcdB RBD can activate mouse dendritic cell maturation

A and TcdA RBD have been reported to have the ability to up-regulate cell surface marker expression and cytokine secretion from dendritic cells [31,38]. To further investigate whether rRBD could play a role in regulating the immune system, rRBD was tested for its ability to promote the maturation of mouse Dendritic Cells (DC). Bone marrow-derived DCs (BMDCs) from C57BL/6 mice were treated with increasing amounts (0.6 to 60 μg) of rRBD. Cell surface biomarkers associated with DC maturation (CD40, CD80, CD86, and MHC-II) and the secretion of pro-inflammatory cytokines (IL-6, IL12, and TNF-α) were examined using FACS and cytokine-specific ELISA, respectively. In order to preclude the interference of LPS contamination, rRBD samples used in the studies contained very low amounts of residual LPS (0.03 EU/μg of protein). In addition, polymyxin B was added to DC samples to prevent activation by LPS through the Toll-like receptor 4 pathway. It was found that surface biomarkers of dendritic cell maturation were up-regulated and that the production of pro-inflammatory cytokines (IL-6, IL-12, and TNF-α) was significantly increased in a dose-dependent manner, from 0.2 to 2 μM (data not shown). Subsequent analyses and repeats were performed using a concentration of 1 μM of rRBD in the final assay solution. DC maturation biomarkers were up-regulated (Figure 4A). The results were not influenced by LPS contamination since there was no significant difference between polymyxin B-treated and non-treated samples. A significant increase in the production of pro-inflammatory cytokines (IL-6, IL12, and TNF-α) was detected in rRBD-treated BMDCs culture supernatant (Figure 4B). Interestingly, polymyxin B reduced at least 50% of the production of the pro-inflammatory cytokines (Figure 4B). To further confirm that the production of the pro-inflammatory cytokines was the result of treatment with rRBD, both rRBD and LPS were boiled for 10 minutes to destroy their biological functions. The boiling treatment did not affect LPS-induced DC activation but fully abolished rRBD DC-activation ability (data not shown). Combined these data clearly demonstrate that DC activation is mediated by rRBD. These results confirm that rRBD has intrinsic adjuvant functions that would be useful for its formulation as a candidate vaccine against CDAD.

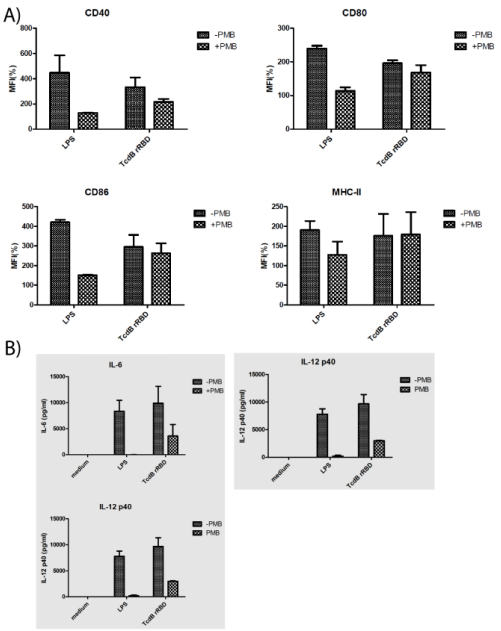


**Figure 4:** (A) Up-regulation of surface biomarkers on bone marrow-derived DC (BMDCs) by rRBD. BMDCs from C57BL/6 were collected and treated with GM-CSF at day 0 and 3. At day 6, rRBD (1 μM final concentration) was added, and 18 hours later DCs were collected for surface biomarkers expression (including CD40, CD80, CD86, and MHC II) by flowcytometry. Samples were divided into groups treated or not treated with polymyxin B (PMB) to assess the influence of residual LPS contamination. Surface marker signaling was normalized by calculating the ratio of mean of fluorescence intensity (MFI) between medium control and treatments. (B) Cytokines secretion from BMDC treated with tcdA rRBD. After BMDCs were treated with rRBD at day 6 for 18 hours, culture supernatants were collected and analyzed for their cytokine profiles using specific cytokine ELISA: IL-6, IL12-p40, and TNF- α.

## Immunogenicity of rRBD in mice

To assess the immunogenicity of rRBD, three groups of mice (n=6) were vaccinated with different amounts of rRBD alone (0, 3 and 10 μg). The results shown in Table 1 indicate that rRBD is a poor immunogen since mice vaccinated with 3 × 3 μg of rRBD alone produced poor anti-RBD IgG antibodies (titer=800) as measured by RBD-specific ELISA. Only antisera from mice immunized with 3 doses of 10 μg rRBD alone induced significant antibody responses (titer=3200; $p<0.05$). Although antisera from mice vaccinated with 3 × 10 μg of rRBD showed both IgG1 and IgG2 isotypes antibody responses, systemic IgA antibody responses were not elicited by rRBD vaccination (data not shown).

To test whether mouse anti-RBD antibodies elicited by rRBD functionally neutralize the cytotoxicity of toxins (TcdA and TcdB), antisera were tested in a Vero cell cytotoxicity assay as described in Materials and Methods. As shown in Table 1, antisera from mice immunized with 3 × 3 μg of rRBD were found to have 1/16 neutralization titer preventing 50% of cell death as a result of either toxin A or toxin B cytotoxicity. The neutralization titers obtained from

mice immunized with 3 × 10 μg of rRBD were found to be significantly high against toxin B (titer=64; p<0.01), but insignificant against toxin A as compared to those obtained from mice vaccinated with 3 × 3 μg dose (Tables 1). The current results indicate that 3 × 10 μg of rRBD alone was capable of inducing functional neutralizing antibodies against both toxins A (titer=16) and B (titer=64). Interestingly, the anti-RBD IgG antibody responses elicited by immunization with freeze-thaw or heat-treated rRBD were found to be significantly lower and have no neutralizing activity (data not shown). This shows the importance of preserving a functionally active conformation of rRBD.

| Mice immunized with various amount of rRBD (μg) | | Anti-rRBD IgG titersa | Neutralization Titerb | |
|---|---|---|---|---|
| | | | TcdB | TcdA |
| 0 | Pre-immune | <100 | <4 | <4 |
| | 2 doses | <100 | NP | NP |
| | 3 doses | <100 | <4 | NP |
| 3 | Pre-immune | <100 | <4 | <4 |
| | 2 doses | 400 | NP | NP |
| | 3 doses | 800 | 16 | 16 |
| 10 | Pre-immune | <100 | <4 | <4 |
| | 2 doses | 400 | NP | NP |
| | 3 doses | 3200 | 64 | 16 |
| TcdA RBDc | 3 × 10 μg | 800 | <4 | 256 |

aThe titers were obtained with sera pooled from 6 mice

bThe neutralization titer against toxins was defined as the highest sample dilution which could prevent 50% cell rounding induced by toxins

cThe immunogenicity studies of TcdA RBD will be published elsewhere

*NP means not performed

**Table 1:** *C. difficile* toxin B neutralization titers of antisera from groups of 6 mice immunized 3 times with varying amounts of rRBD.

### Hamster challenge studies

To further evaluate the role of anti-toxin neutralizing antibodies *in vivo*, *C. difficile* spore challenge was performed in the hamster model as described in Materials and Methods. Three groups of hamsters (n=6) were vaccinated 3 times, 2 weeks apart either with PBS, 3 or 10 μg of rRBD intramuscularlly. A week after the third immunization, blood samples collected from immunized hamsters were assayed for anti-TcdB neutralizing antibody titers and found to be <4, 16 and 64 for PBS, 3 and 10 μg dose groups, respectively. Interestingly, hamster antisera had no neutralizing activity against TcdA. Two weeks after the third immunizations hamsters were gastrically inoculated with 100 c.f.u. of *C. difficile* (lethal dose). Three to 4 days post challenge 100%, 100% and 80% of hamsters died in the PBS, 3 and 10 μg groups, respectively (Figure 5). This 20% partial protection was insignificant since in the repeated experiment, we observed 20% of survival rate in both PBS and 10 μg of rRBD vaccinated hamster groups. In addition, significant amounts (>1 × $10^3$ pfu per μg of faecal) of *C. difficile* colonized the selective TCCFA plates when faecal pellets collected from the surviving hamsters were analyzed. To test whether rRBD formulated with alum could elicit better protection in the hamster challenge model, group of hamsters were immunized 3 × 10 μg of rRBD formulated with alum and challenged gastrically with 100 c.f.u. of C. difficile. The anti-rRBD IgG antibodies were found to increase 10 to 50 folds (Titer >5 × $10^4$) but the neutralization titer remained to be 64 and protected 1 out of 6 hamsters in the challenge studies. These results are very similar to previous reports that TcdB and TcdB RBD did not confer protection in the hamster challenge model [22,27,37].

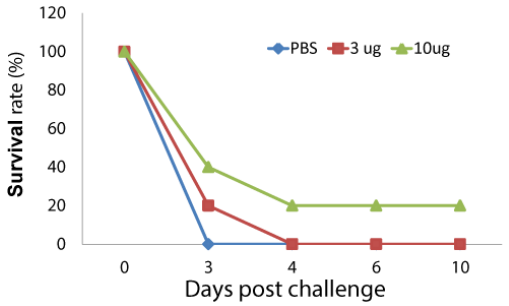


**Figure 5:** *C. difficile* spore challenge in hamster model studies. Four groups of hamsters (n=6) were gastrically inoculated with 100 c.f.u. of *C. difficile* (lethal dose) at 2 weeks after the third immunizations of rRBD (0, 3, and 10 μg). The challenge studies are described in the Materials and Methods. Survival rates are recorded.

## Discussion

Vaccine development against CDAD is urgently needed to control the rise of hospital-acquired Cd infections (CDI) which leads to excessive medical cost. TcdA toxoid-based vaccines against CDI are currently in phase III clinical trials [9,18]. The structural conformation of RBD undoubtedly correlates with its ability to elicit anti-toxin cross-neutralizing antibody responses. TcdA RBD is predicted to have >900 amino acids and 7 putative oligosaccharide (Galα1-3Galβ1-4GlcNAc) binding regions, whereas TcdB RBD has around 530 amino acids and 4 putative oligosaccharide binding sites [9,28-31,36]. Interestingly, among *C. difficile* strains deposited in the NCBI database, the amino acid sequences of the putative oligosaccharide binding sites of TcdA and TcdB shared about 50% identity or 70% similarity (Figure 6). To this end, we rationally designed two novel immunogens based on highly conserved protein sequences (90-97%) of TcdA RBD and TcdB RBD which would potentially be capable of inducing broadly neutralizing functional antibodies against both toxins. The biochemical and immunological functions of TcdA RBD have been characterized and submitted for publication [31]. In the present study, we have successfully engineered and expressed a codon usage optimized tcdB RBD synthetic gene in E. coli. More than 40 mg of highly purified rRBD (>95% purity) were consistently obtained from 1 liter of bacterial culture after purification using Ni-affinity chromatography. Downstream purification including E-membrane filtration essentially eliminated LPS contamination (<0.03 EU of LPS per μg of protein). This level of LPS is known not to influence animal immunogenicity studies.

Several functional assays were performed to assess rRBD biological activities. Crystal structure studies revealed that disorder in the C-terminal binding domain of TcdA affects its biological function [29]. As a result, the purified rRBD was first analyzed and found to consist of a stable β-solenoid secondary structure as shown by CD spectra

analysis (Figure 3A). This is consistent with previous reports [28-29,31] that the majority of the secondary structure of rRBD is formed by β-solenoids (>40%). The results obtained from Vero cell-binding assay as shown by FACS analysis and immuno-fluorescence assay (Figures 3B and 3C) provided direct evidence that rRBD is correctly folded to mediate its biological functions. Current immuno-fluorescence assays revealed that direct binding of rRBD to Vero cell surfaces occurs rapidly within 5 minutes. No loss of RBD-associated fluorescence signals in confocal microscopy indicated that RBD was transferred from the cell surface to the cytosol. The current result is consistent with a previous report [31] that TcdA RBD and its truncated fragments bind to Vero cell surfaces and enter the cytosol within 15 minutes of incubation. A point of interest is that TcdB RBD binds to specific receptor(s) on the surface of Vero cells as evidenced by the speckled pattern of the immuno-fluorescence signals. In contrast, TcdA RBD uniformly binds and covers the cell surface (Figure 3C, 5 min pictures). These different cell surface binding patterns indicate that TcdA RBD and TcdB RBD likely have different receptor-binding specificities as reported in previous studies [28-30,40]. In addition, a recent report [39] suggested that the C-terminal receptor binding domain of clostridial toxins mediates the first step of RBD-dependent endocytosis and that the translocation domain is required cell entry. To clarify the functions of translocation domain and putative carbohydrate-binding domains, the identification of natural cell-surface receptors are urgently needed

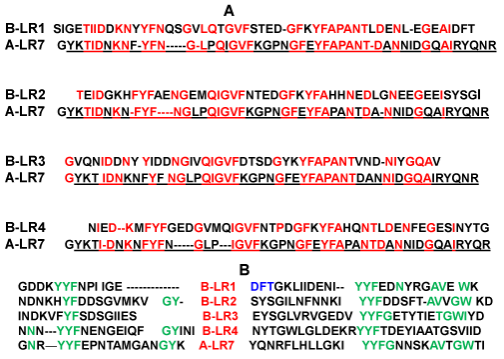


**Figure 6:** (A) Analysis of the putative oligosaccharide binding sites. A-LR7 (residues 795-844) and B-LR are the putative oligosaccharide binding sites of TcdA and TcdB, respectively. Since the crystal structure of A-LR7 was previously published [29], it was used for comparison with the putative oligosaccharide binding sites of TcdB. Identical amino acids are highlighted in red. (B) Amino acid sequence analysis of the regions in between the putative oligosaccharide binding sites of TcdB and LR7 of TcdA. Identical amino acids are highlighted in green.

Another point of interest from the current study is that rRBD shows comparable binding affinity for Vero cells (100 nM) to TcdA RBD (50 nM), but it does not agglutinate rabbit erythrocyte even at a 2 nmolar concentration, whereas TcdA RBD could easily agglutinate rabbit erythrocytes at 2 pmoles [31]. Since amino acid sequences of the putative oligosaccharide binding sites of TcdA and TcdB share no more than 70% similarity (Figure 6), differences in the cell-surface receptor-binding specificities of TcdA and TcdB reported in the present and previous studies [28-30,40] may account for the lack of HA activity. Differences in amino acid sequences between the long-repeats are clearly seen when TcdA and TcdB sequences are compared (Figure 6B). It will be of interest to study the specific role(s) and function(s) of each putative oligosaccharide binding region and to test whether rRBD and/or its fragments have the same cell-surface receptor-binding specificities. There is a need to identify the natural cell-surface receptors.

Both TcdA and cholera toxin had been shown to stimulate endothelial cells and DC activation [38,41,43], such activation has been confirmed to be the result of TcdA RBD [31,41]. In our current study, we have clearly shown that 1 μM of rRBD promoted the maturation of BMDC and an increased secretion of pro-inflammatory cytokines (Figure 4). rRBD functions as a toll-like receptor (TLR) agonist that was capable of enhancing immune responses. This was supported by the fact that rRBD in the absence of adjuvant elicits neutralizing antibody responses. It will be of interest to determine whether rRBD at 10 μg doses can effectively enhance specific IgG antibody responses against poor immunogens as there is strong evidence that rRBD has intrinsic immuno-stimulatory properties.

To obtain maximum protective efficacy, TcdA RBD and/or its fragments were formulated with different types of adjuvant such as alum, MF59, CFA/IFA, flagellin, heat-labile enterotoxin from *Eschelichia coli* (LT) and cholera toxin from *Vibrio cholerae* (CT), and/or vaccine delivery systems including recombinant vectors based on adenovirus, *Vibrio cholerae* and bacillus spores [21-26,44-47]. In these studies, high dosages of recombinant TcdA RBD and/or RBD fragments (100 μg) were intraperitoneally injected into animals to generate neutralizing antibodies against CDI. In contrast to these previous studies, results in Table 1 have shown that 10 μg of rRBD alone elicited antibody responses that inhibited the cytotoxicity of TcdA and TcdB *in vitro*. This result unambiguously demonstrates that rRBD can be a component of potential vaccine candidates against CDAD.

| | Biological Properties[a] | | | Immunological Properties[b] | | | |
|---|---|---|---|---|---|---|---|
| **Antigen** | | | | **Elicit neutralizing antibodies** | | **Elicit protection in hamster model** | **Activate dendritic cell** |
| | **Cell-binding** | **HA activity** | **Cell entry** | **TcdA** | **TcdB** | | |
| TcdA RBD | 50 nM | 0.4 pmoles | Fast | 256 | <4 | No | 0.4 μM |
| TcdB RBD | 100 nM | No | Fast | 16 | 64 | No | 1 μM |
| TcdB | Yes** | NP | Yes | NP | Yes | NP | NP |
| [a]Results are from a previous [31] and the current studies | | | | | | | |
| [b]The immunogenicity studies of TcdA RBD will be published elsewhere | | | | | | | |
| *NP means not performed | | | | | | | |
| **Yes refers to information from the literature [7-23,31]. | | | | | | | |

**Table 2:** Summary of biochemical and immunological properties of TcdA RBD and TcdB RBD.

In summary, the recombinant TcdB RBD based on *C. difficile* strain VPI10463 with >95% amino acid sequence identity to hyper-virulent

strain BI/NAP1/027 was successfully expressed in *Escherichia coli*, purified, characterized biologically and immunologically and found to have the following properties (Table 2): (a) capable of binding to the cell surface of both Vero and Caco-2 cells and entry into the cytosol; (b) devoid of hemagglutinin activity (HA); (c) functioning as a toll-like receptor agonist activating dendritic cell maturation; and (d) in the absence of adjuvant eliciting anti-TcdB neutralizing antibody responses that weakly cross-neutralize TcdA. As rRBD could induced weak protection in hamster challenge studies, rRBD will be formulated with TcdA RBD and/or other domains of TcdB in the present of adjuvant (alum) and tested whether it is potentially a good immunogen candidate to be included in future vaccines against *Clostridium difficile*-associated diseases.

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