The Generation of Synthetic Antibody Reagents for *Clostridium difficile* Toxins

by

Sylvia Cien Man Wong

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Graduate Department of Molecular Genetics
University of Toronto

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Abstract

The symptoms of \textit{C. difficile} infection are primarily caused by two toxins, toxin A and toxin B. Some strains produce a third known toxin, \textit{C. difficile} transferase (CDT) toxin; however, its role in virulence remains unclear. I aimed to develop synthetic antibodies using phage display technology to block toxin entry by binding to the receptor-binding domain (RBD) of the toxins. I first described the generation of anti-toxin A and anti-toxin B Fabs. I presented Fab A3, which bound to the full-length toxin, but did not functionally inhibit toxin entry. In chapter 2, I described the generation of novel anti-CDTb antibodies. I further demonstrated that five of the anti-CDTb antibodies could functionally inhibit CDTb binding in an ELISA-based assay and on cultured cells. These antibodies can be used as tools to understand the toxins’ role in human disease and potentially be used as therapeutics.
Acknowledgments

I am very thankful for having the chance to work with many great scientists in Dr. Jason Moffat’s and Dr. Sachdev Sidhu’s labs. These people made the lab a very motivating, supportive, and intellectually stimulating environment, where I was able to learn and expand my critical thinking skills. Several people that I would like to thank personally are Dewald Van Dyk who was the first person that taught me everything I need to know about phage display, and Bryce Nelson, who was always there when I needed to talk, whether it was about experiments or life decisions. These people have made my graduate experience a wonderful one.

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I would like to thank the entire TRAC team for generating the IgGs and Nick Jarvik who assisted me with obtaining the SPR data on the Fabs and IgG. I would like to thank Sheng Xue for producing the recombinant extracellular domain of LSR. Most importantly, I would like to thank my collaborators, Dr. Panagiotis Papatheodorou and Dr. Klaus Aktories, for kindly providing me with the RBD-CDTb, full-length CDTb, CDTa, CDTb-Dylight 488, and the HeLa cell lines for the experiments. This project would not exist without their assistance.

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<th>Full name / definition</th>
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDI</td>
<td><em>C. difficile</em> infection</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CDT</td>
<td><em>C. difficile</em> transferase (binary toxin)</td>
</tr>
<tr>
<td>CDTa</td>
<td>Enzymatic component of CDT</td>
</tr>
<tr>
<td>CDTb</td>
<td>Receptor-binding component of CDT</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>EC\textsubscript{50} of antibody</td>
<td>Half-maximal effective concentration of an antibody, which can bind to the antigen</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment / fragment antigen binding</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable region</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HCT116</td>
<td>Human colorectal carcinoma cells</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>LCT</td>
<td>Large clostridial toxin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LSR</td>
<td>Lipolysis-stimulated lipoprotein receptor</td>
</tr>
<tr>
<td>NA</td>
<td>Neutravidin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>RBD</td>
<td>Receptor-binding domain</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>scFv</td>
<td>Single-chain variable fragment</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TcdA</td>
<td>Gene for toxin A</td>
</tr>
<tr>
<td>TcdB</td>
<td>Gene for toxin B</td>
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<tr>
<td>VH</td>
<td>Variable heavy chain</td>
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<tr>
<td>VH1, VH2, VH3</td>
<td>Complementarity determining regions of the variable heavy chain</td>
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<tr>
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<td>Variable light chain</td>
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<td>Complementarity determining regions of the variable light chain</td>
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Chapter 1
Introduction
1 Introduction

1.1 *C. difficile* infection (CDI)

*Clostridium difficile* is a gram-positive, spore-forming bacterium that infects the epithelial cells of the lower intestine and is usually transmitted via the fecal-oral route from patients[1], healthcare workers, and / or contaminated surfaces[2-4]. *C. difficile* infection (CDI) commonly occurs in the elderly, immunocompromised patients, and patients undergoing antibiotic therapy unrelated to CDI[5-7].

*C. difficile* is becoming one of the most problematic pathogens in hospital settings because antibiotics such as clindamycin, penicillins, cephalosporins and fluoroquinolones can deplete the intestinal microflora that normally prevents the invasion of certain pathogenic bacteria[5,8,9]. This imbalance allows *C. difficile* to overgrow and release toxins that cause the symptoms of the disease[7,10,11]. Overuse of antibiotics is also a problem, especially for patients who are prescribed broad-spectrum antibiotics as the first choice of antibiotics for treating unknown infections[10-12].

Approximately 5-10% of hospital patients infected with toxigenic strains of *C. difficile* are asymptomatic but the typical symptoms of CDI include watery diarrhea, fever, abdominal pains, and in severe cases life-threatening pseudomembranous colitis[13-18]. Since its discovery in 1935, hospital outbreaks of CDI have increased worldwide, and it was reported in 2007 that more than $3.2 billion per year was spent in healthcare for the diagnosis and treatment of CDI in the US alone[19,20]. Severity and recurrence of the disease is increasing in frequency, caused either by reinfection with a new strain of *C. difficile* or incomplete eradication of the bacterial spores from a previous infection[6]. The BI/NAP1/027 strain of *C. difficile* has been associated with majority of the outbreaks worldwide[9,21,22]. In a recent survey, BI/NAP1/027 comprised 53% of 155 *C. difficile* isolates in the data collected from 16 outbreaks in Ontario. This strain is also associated with the continual relapse of the disease[23].
1.2 *Clostridium difficile* toxins

The symptoms of the disease are caused by the release of toxins from the bacteria during the late log growth phase[10,24]. There are three known toxins produced by *C. difficile*: two large clostridial toxins, toxin A (TcdA), toxin B (TcdB), and some strains produce a binary toxin called the *C. difficile* transferase (CDT) toxin[13], with most disease-causing strains of *C. difficile* producing both TcdA and TcdB[10,13,25]. Infections from the BI/NAP1/027 strain are particularly severe due to a mutation in the loci that regulates the expression of toxins A and B, which effectively causes overexpression of the toxins[22]. TcdB is 1000 times more toxic than TcdA in toxicity assays on cultured cells[26,27]; however, it is unclear which toxin is more essential for causing symptoms associated with infection. It was once thought that the presence of both TcdA and TcdB cause synergistic damage to the colon but TcdB requires TcdA to initiate tissue damage [28,29]; however, upon closer examination of A\(^+\)B\(^+\) strains, it was found that A\(^+\)B\(^+\) strains caused disease with the same or increased severity compared to A\(^+\)B\(^-\) strains[26,30-32]. Furthermore, the A\(^+\)B\(^-\) strain has been associated with at least 4 outbreaks[24,31,33-35], but it is not clear whether or not CDT also contributed to these outbreaks.

Less than 15%[36-39] of clinical *C. difficile* isolates produce CDT with only 2-8%[40,41] producing CDT alone. The role of this toxin in virulence and pathogenicity remains unclear. However, there is some speculation that CDT may play a role in virulence, since 65% of the *C. difficile* clinical isolates identified during an outbreak in Pittsburgh produce CDT[42]. In an examination of strains that are A\(^-\)B\(^-\)CDT\(^+\), these strains caused enterotoxic effects and nonhemorrhagic fluid responses in the rabbit ileal loop test [43]. In contrast, A\(^-\)B\(^-\)CDT\(^+\) strains did not cause disease or death when the hamsters were challenged with the strain, even though there was normal colonization of the bacteria [43]. A recent study suggests that CDT aids and increases *C. difficile* adherence on intestinal cells by changing the morphology of the colon cells to form microtubule protrusions[44]. Additionally, the BI/NAP1/027 strain also produces the binary toxin. Since this strain produces all three toxins, it suggests that CDT may have some role in infection, and thus, preventing the toxin entry of CDT might decrease *C. difficile* adherence and colonization.
1.3 Mode of action of toxin A (TcdA) and toxin B (TcdB)

The two large clostridial toxins, TcdA and TcdB, share 47% sequence identity and 63% sequence similarity[13,28,45]. The two toxins have similar structure and four of the same domains: 1) a glucosyltransferase domain, which is the enzymatic component of the toxin and is located at the N-terminus, 2) a cysteine protease domain, 3) a hydrophobic insertion domain, and 4) a receptor-binding domain (RBD), which is located at the C-terminus (Figure 1.1A)[45-47]. Once toxins A and B are secreted from C. difficile, they enter epithelial cells by binding to a currently unknown receptor through their receptor-binding domain[48] and are consequently endocytosed to form an endosome[13]. It has been suggested that the low pH environment within the endosome induces the formation of a pore through the hydrophobic insertion domain while the catalytic component of the toxin, the glucosyltransferase domain, is cleaved by the cysteine protease and released through the pore into the cytosol of the cell (Figure 1.2)[49-53].

Once the glucosyltransferase domain enters the cytosol, it inhibits Rho GTPases (Rho, Rac, and Cdc42) via glucosylation of the Thr37 residue within the pocket of Rho-GDP and prevents it from becoming activated by guanine-nucleotide-exchange factors (GEF) (Figure 1.2)[54-56]. Rho GTPases regulate many cellular processes including cell polarity, cell adhesion, and the assembly of the cellular cytoskeleton [57]. Inhibition of Rho GTPases causes disruption of the cytoskeleton and in turn causes cell rounding and cell death[13]. The intestinal epithelium contains tight junctions that regulate the permeability of ions, water, and macromolecules and serves as a barrier for the diffusion of these molecules. The disruption of the cytoskeleton caused by the toxins compromises the integrity of the tight junctions, and the resulting imbalance of electrolytes causes symptoms typical of CDI including diarrhea[58,59].

Although the host receptor of toxins A and B remains unknown, several studies have found that the toxins bind to carbohydrate-containing receptors such as Gal-α-(1,3)-Gal-β-(1,4)-GlcNAc and N-acetylglucosamine glycoproteins[60-63]. These receptors, however, are present in various animal models but absent in human, and identification of the human receptor remains to be discovered.
Figure 1.1 Domain organization of the large clostridial toxins (LCT), TcdA and TcdB, and the binary toxin, CDT. A) TcdA and TcdB are 2710 amino acids and 2366 amino acids in length, respectively. They consist of four domains: glucosyltransferase (GT), cysteine protease (CP), hydrophobic insertion (HI), receptor binding domain (RBD). The colour scheme for the diagram is maintained for Figure 1.2. B) Unlike the LCT, CDTa and CDTb are produced independently and assemble together on the cell surface. Mature CDTa and CDTb are 415 amino acids and 662 amino acids long, respectively. CDTa has enzymatic activity at the C-terminus domain and associates with CDTb at the N-terminus domain. CDTb must be cleaved at the activation domain in order to become active and bind to the receptor via the putative receptor binding domain, which is predicted to be at the C-terminus [64]. Figures adapted from Davies, A.H. et al (2011).
**Figure 1.2 Mechanism of LCT entry.** The toxins bind to an unknown receptor via RBD to initiate endocytosis. In the endosome, the low pH induces the formation of a pore, which is presumably caused by the hydrophobic insertion domain. The glucosyltransferase domain is suggested to be cleaved by the cysteine protease domain and released into the cytosol of the intestinal epithelial cells through the pore. Thereafter, the toxins inhibit Rho-GTPase activity by transferring a UDP-glucose molecule (red hexagon) on Thr$^{37}$ of the GDP-bound conformation of Rho-GTPases and prevent the exchange into GTP-bound active form. Normally, the change between the active and inactive form is regulated by guanine nucleotide-dissociation inhibitor (GDI), guanine-nucleotide-exchange factor (GEF) and GTPase-activating protein (GAP). GDI prevents Rho-GTPases from associating with the membrane and therefore prevents the exchange from GDP to GTP-bound form at the membrane. Rho-GTPases are activated and inactivated by GEFs and GAPs, respectively.
Figure 1.3 Mechanism of CDT entry. It is proposed that the precursor of CDTb undergoes a proteolytic cleavage and forms a heptamer conformation, which then binds to the host receptor, lipolysis-stimulated lipoprotein receptor (LSR), or the heptamers are formed on the cell surface. CDTa associates with the heptamer form of CDTb and the complex is endocytosed. The low pH in the endosome induces the heptamer to form a pore for CDTa release into the cytosol. CDTa transfers an ADP-ribose on G-actin subunits and prevents the polymerization of F-actin. Consequently, the depolarization of F-actin causes destabilization of the cytoskeleton of the cell. Figure adapted from Aktories, K., et al (2012) with permission from Elsevier Limited.
1.4 Mode of action of *C. difficile* transferase toxin

*C. difficile* transferase toxin is a binary toxin produced as two independent components, CDTa and CDTb with a molecular weight of ~48 kDa and ~99 kDa, respectively[65,66]. CDTa is the catalytic component of the toxin and associates with the receptor-binding component, CDTb, through its N-terminal region[67]. The C-terminal region of CDTa contains the enzymatic activity of the toxin (Figure 1.1B[67].

The mechanism of CDT entry is similar to toxins A and B, where CDTb binds to a receptor to initiate toxin entry[13,65,68]. However, prior to CDTb binding, it must be activated by proteolytic cleavage at the N-terminus which subsequently produces a ~74 kDa protein. The activation of CDTb results in formation of a heptamer either in solution, which then interacts with the receptor, or forms a heptamer on the cell surface (Figure 1.3). After CDTb binds to the receptor, CDTa associates with CDTb and both components enter the cell via endosomes. The low pH in the endosome induces pore formation and only CDTa enters the cytosol of the host cell[65,68]. Thereafter, CDTa inhibits the polymerization of G-actin to F-actin by irreversibly transferring an ADP-ribose from NADP / NAD⁺ to the monomeric G-actin, presumably on Arg¹⁷⁷[13,69,70]. The imbalance of the ratio between G-actin and F-actin consequently causes disruption of the cytoskeleton leading to cell rounding and eventual cell death[69-71].

1.5 Lipolysis-stimulated lipoprotein receptor (LSR)

Using a haploid genetic screen developed by Dr. Thijn Brummelkamp[72], Papatheodorou *et al.* discovered that lipolysis-stimulated lipoprotein receptor (LSR) is the host receptor of CDT[73]. LSR is a single-pass membrane receptor that contains an Ig-like V-type domain located at the N-terminal region of the extracellular domain (Figure 1.4). It is a complex multimeric protein that consists of α or α¹ subunits that associate with two or three β subunits through disulfide bonds[74]. The receptor is present predominantly in the liver[74-76] but also moderately expressed throughout the gastrointestinal tract and in the pancreas.

In the presence of free fatty acids (FFA), particularly oleate, the receptor undergoes a reversible conformational change to enable binding to its natural ligands, lipoproteins ApoE and ApoB[77,78]. Lipoprotein binding induces receptor-mediated endocytosis, which delivers the
lipoprotein to the lysosome for degradation[77]. The specific site to which CDT binds to LSR and whether the binding site is similar to that of the lipoproteins are not clearly defined but it is likely that it associates with the Ig-like V type domain of the receptor that occupies the majority of the extracellular domain (Figure 1.4)[73]. Interestingly, it is suggested that LSR plays an important role in the formation of tricellular tight junctions[79,80]. Whether CDT binding causes the impairment of the receptor and further decreases the integrity of the tight junctions that leads to diarrhea in CDI is unknown and must be further investigated. It has also recently been shown that binary toxins produced by other clostridial species such as Clostridium spiroforme and Clostridium perfringens can bind to LSR[73,81].

![Figure 1.4 Domain organization of LSR](image)

**Figure 1.4 Domain organization of LSR.** LSR is a 71 kDa single-pass membrane receptor that contains an Ig-like V-type domain located in the extracellular domain, a transmembrane domain (TM) for membrane insertion, and an intracellular domain.

### 1.6 Difficulties in treatment and diagnosis

As of today, diagnosis and treatment of *C. difficile* infection remains difficult. The first line of treatment for non-severe CDI includes discontinuing use of *C. difficile*-associated antibiotics such as clindamycin and penicillins in preference for the administration of oral metronidazole and vancomycin[9,82]. However, patients are becoming less responsive to these antibiotics, especially those that are infected with the hypervirulent strain, BI/NAP1/027, with patients becoming more prone to relapse of the disease after initial improvement from the antibiotic treatments[6,9,20-22]. In severe cases, the colon must be removed, particularly in patients that have repeated relapses of the disease and develop pseudomembranous colitis[83,84].

Fecal transplantation is reported to be an effective treatment that has only recently been widely recognized and is slowly gaining acceptance in hospitals[85-90]. In this procedure, feces from a
healthy relative are transplanted to the colon of the infected patient via enema, gastroscope tube, or nasojejunal tube in order to replenish the microflora in the lower intestine. Various clinical studies have shown that the treatment has a high efficacy with 91% (n = 159) of reported cases of recurrent CDI cured when treated with this procedure[9,89,91], and patients that undergo the fecal transplantation procedure do not develop significant adverse side effects[92]. Although this is an effective treatment, there are limitations in these studies that need to be improved or addressed; a standard optimized protocol for fecal transplantation is currently unavailable, and factors such as the volume of fecal matter being transplanted, the route of donor-feces infusion, and the combined effects of other treatments in combination with fecal infusion in the procedures were not standardized between each study[91]. Further studies need to be performed to assess the risks of contracting additional infections.

The standard diagnostic tests used in hospitals for CDI diagnosis primarily rely on detection of toxins in patients’ stools; however several of these tests either have slow turnaround time such as the cell cytotoxic test, are expensive, insensitive, non-specific, and/or labor intensive[9,93]. Enzyme immunoassay (EIA), as an example, provides a fast test for detecting the toxins using antibodies but is insensitive and may provide a false-negative result[9,93]. Developing higher affinity antibodies directed towards both toxin A and B for EIA can provide a better detection system for the diagnosis of CDI.

1.7 Antibodies

Antibodies, also termed immunoglobulins (Ig), consist of a fragment antigen-binding (Fab) region that comprises the paratope for binding to proteins called antigens, together with the fragment crystallizable (Fc) region that initiates biological effects such as recruiting immune cells to destroy foreign antigens[94,95]. The paratope of an antibody binds to a particular binding site on the antigen called an epitope. The Fab consists of two heavy chains and two light chains, each containing a variable region and a constant region (Figure 1.5)[95,96]. Within each variable region are three areas called the complementarity determining region (CDR) that dictate an antibody’s variability in binding to different antigens[97] and are denoted as variable light 1 (VL1), variable light 2 (VL2), variable light 3 (VL3), variable heavy 1 (VH1), variable heavy 2 (VH2), and variable heavy 3 (VH3) in the thesis. Studies have shown that Fabs, autonomous
single VH domains, and single-chain fragment variables (scFvs) of an antibody (Figure 1.5) are sufficient for antigen binding[98-101].

In the human immune system, there are five main immunoglobulin isotypes, IgA, IgD, IgE, IgG, and IgM, each differing in their response to a particular antigen, site of action, and biological properties[102]. Immunoglobulin G is the primary antibody isotype found in human serum, and the heavy and light chains are arranged such that each bivalent molecule contains two antigen binding fragments. Within IgGs, there are four subclasses that also differ in their ability to elicit effector responses such as complement activation for the removal of invading pathogens[94]. IgG1 is used commonly to generate therapeutic antibodies, as this subclass is found at higher levels in the serum and can activate effector molecules more potently than IgG2, IgG3, and IgG4[94,95].

**Figure 1.5 Schematic of IgG structure.** IgGs are complex multimeric proteins composed of four polypeptides with two heavy (blue) and two light chains (green) that are folded in a Y shape. The heavy and light chains are joined by disulfide bonds (-S-S-) and the variable regions of each chain are located at the ends of the antibody (VL in light green and VH in light blue). Each variable region has three complementarity regions (CDR) shown as black circles. An IgG has two fragment antigen-binding (Fab) regions and a fragment crystallizable (Fc) region. A Fab is composed of a single variable heavy and light chain, and a single constant heavy and light chain. A single-chain variable fragment (scFv) can be synthesized as a single variable heavy and light chain, joined by a short peptide linker (orange line). The Fab and the Fc region are separated by a hinge (red) that adds flexibility to the antibody.
1.8 Benefits of antibody treatment against bacterial toxins

Bacteria has a natural tendency to mutate in order to become better suited to a hostile environment (such as becoming resistant to antibiotics), and therefore targeting secreted virulence factors such as toxins might be a better therapeutic alternative. This is particularly true when considering that the pathogenicity of a bacterial infection is usually caused either by the bacterial surface protein, the toxins released by them, or other virulence factors[103]; by neutralizing the bacterial toxins, the pathogenicity of some bacteria can be reduced and allow the immune system to clear the bacterial infection[103]. One example already approved and used effectively as a treatment against *C. botulinum* poisoning uses human immune serum to neutralize the botulinum toxin[104]. Monoclonal antibodies against *C. difficile* toxins, toxin A and toxin B, called CDA1 and MDX1388, respectively, are currently in phase II clinical trials and have been shown to decrease the recurrence of CDI when treated as a cocktail[105].

Engineered antibodies can bind very specifically and with very high affinity to their targets and can be used to block toxin entry by binding to the receptor-binding domain of the toxins and prevent it from interacting with the receptor[48]. With this approach, the antibodies can act upon all variants of the bacteria, including those that may be missed by some antibiotics due to changes in the bacteria mode of infection or colonization. Additionally, the likelihood of mutations in the RBD of the toxins to prevent antibody binding is low because it may also prevent binding of the toxin to the host receptor. Furthermore, in contrast to chemical inhibitors, the Fc portion of antibodies also allows the recruitment of immune cells[94,103] and can prime the immune system to produce natural antibodies against the toxins[106].

1.9 Exploiting the filamentous phage lifecycle for phage display

High-affinity antibodies can be developed in a selection process called phage display[97,107-109]. To understand the principle of phage display, we must first understand the lifecycle of a filamentous phage. The lifecycle begins with binding of the N2 domain of the pIII phage coat protein to the pili of *E. coli* (Figure 1.6)[110,111]. This initial step allows binding of the N1 domain of the coat protein to a co-receptor, TolA, found on the surface of the bacteria[112,113]. Thereafter, the phage penetrates the outer membrane of the bacteria and translocates its ssDNA
into the bacterial cytoplasm. The host’s DNA polymerase and RNA polymerase converts the viral ssDNA into dsDNA, and express the viral proteins for ssDNA replication through the rolling circle method[114]. The viral ssDNA is packaged into the phage particles followed by their assembly into phage and subsequent secretion from E. coli without cell lyses[111]. Using the M13 filamentous phage, we can construct a specialized vector called a phagemid and fuse the protein of interest to the phage coat protein[97,111,115,116]. The protein can then be expressed on the surface of phage particle and the phage particle can be amplified in E. coli for phage display (Figure 1.7)[115].

**Figure 1.6 The lifecycle of filamentous phage.** Phage particles initiate contact with bacteria bearing the F-pilus. Additional interactions between pIII domains and bacterial receptors occur with pilus retraction, followed by injection of the phage genome into the cell interior. ssDNA is converted to the dsDNA replicative form, which enables the synthesis of viral proteins and the generation of new ssDNA required for replication. Nascent viral coat proteins take up residence in the bacterial inner membrane in preparation for viral assembly, and pV pre-packages the ssDNA viral genome for assembly and extrusion. Viral assembly and export occurs through a membrane pore without cell lysis. Figure and caption was taken from Miersch, S. and Sidhu, S.S. (2012) with permission from Elsevier Limited.
1.10 Principles of phage display

Phage display was first documented by George P. Smith in 1985[115]. He proposed that various proteins could be fused to the coat protein such as pIII of a filamentous bacteriophage and be expressed on the surface of the bacteriophage along with the coat protein without affecting the infectivity of the phage particle. This concept allows the exploration and determination of novel protein-protein interactions by incubating a protein-displayed phage library with a particular immobilized target protein and carrying out multiple rounds of selection to enrich for protein-phage particles that bind specifically to the target protein (Figure 1.8). Since the genetic code of the protein of interest is linked directly to the DNA that is coding the coat protein being expressed on the surface of the phage particle, the DNA sequence of the protein can be determined easily by Sanger sequencing thereby allowing elucidation of the protein that binds to the target protein.

For our purposes, the sequence of the Fab component of an antibody is fused to pIII in a phagemid. Since a phagemid does not encode all the necessary genes for phage packaging and assembly, it requires co-infection with a helper phage such as M13K07 that has all the genes for phage packaging in addition to the mutation, Met40Ile, in gII. This mutation reduces the activity of single-stranded DNA replication of the helper phage and preferentially packages the phagemid DNA into the phage particles[111]. The sequences of the CDRs of a Fab in the library are randomized via oligonucleotide-directed mutagenesis to provide at least $10^{10}$ unique phage-Fabs in the library[97]. Since the Fab fragments are fully humanized and modular, reformatting to fully bivalent IgGs, that include Fc functions, can be performed easily.

In order to isolate Fabs that bind to the target protein, the library is pre-incubated with a protein that is either the fusion tag of the target protein or an unrelated protein such as neutravidin before the library is incubated with the immobilized target protein. The unbound phage from the library is removed during the washing step and the bound phage is eluted. The eluted phage is amplified in *E. coli* with the help of M13K07 for the next round. This process is repeated for 3 to 4 rounds with the phage-infected *E. coli* plated on selective media agar plates for the growth of single colonies after rounds 3 and 4. Each colony is infected by a single phagemid, and thus each
colony can be screened for binding specificity towards the target protein using ELISAs and subsequently be sequenced to determine unique phage-Fab sequences[97].

Figure 1.7 Phagemid vector for phage display. A phagemid vector contains a site for replication of single-stranded DNA and phage packaging with the aid of M13K07 helper phage (f1 ori). The vector also contains a double-stranded DNA origin of replication (322 ori) and an ampicillin resistant gene (Amp') for propagation of the plasmid in E. coli. In this case, a protein of interest is fused to the coat protein P8, and it can be expressed along with P8 under the control of a promoter. The four minor coat proteins, P3, P6, P7, and P9 have also been used previously for phage display. The coat protein of the newly packaged phage contains mainly wild-type coat proteins from the helper phage but also coat proteins displaying the protein of interest. The phagemid is preferentially packaged into the phage due to a mutation in the genome of the helper phage which results in an inefficient packaging of wild-type genome. Figure was adapted from Sidhu, S.S. (2001) with permission from Elservier Limited.
Figure 1.8 Antibody selection from M13 bacteriophage libraries. Libraries of antibodies (in this example, Fabs) are displayed on the surfaces of phage particles as fusions to a coat protein. Each phage particle displays a unique antibody and also encapsulates a vector that contains the encoding DNA. Highly diverse libraries (>10^10) can be constructed and represented as phage pools, which can be used in selections for binding to immobilized antigen. Antigen-binding phage are retained by the immobilized antigen, and the nonbinding phage are removed by washing. The retained phage pool can be amplified by infection of an *Escherichia coli* host, and the amplified pool can be used for additional rounds of selection to eventually obtain a population that is dominated by antigen-binding clones. At this point, individual phage clones can be isolated and subjected to DNA sequencing to decode the sequences of the displayed antibodies. Figure was modified and caption was taken from F.A. Fellouse and S.S. Sidhu (2006)[109] with permission from Dr. S.S. Sidhu and Nature Publishing Group.

The advantage of phage display over the hybridoma technique, which is the traditional method for antibody generation, is that much more control and manipulation can be applied to the selection environment in order to develop high affinity and specific antibodies in a short period of time. Furthermore, since these libraries are fully humanized, these antibodies can also be developed as therapeutics without the cumbersome process of humanizing the antibodies [108,109,116]. In the hybridoma approach, an antigen of interest is injected into an animal in order to induce an immune response and produce B-cells. These cells, which produce antibodies against the antigen, are immortalized by fusion with cancer cells such as myeloma cells. The disadvantage of this approach is that there is no control over the serum environment during the selection process, takes much longer, and is much more expensive[117].
1.11 Thesis rationale and objectives

In spite of many functional studies, the mechanism of the pathogenicity of *C. difficile* toxins has not been clear and the importance of the role of each toxin for infectivity has been debated. Currently, there are no known CDT-specific antibodies that can inhibit the toxin’s entry, and current antibodies in clinical trials against toxins A and B that are treated as a mixture of monoclonal antibodies do not provide complete and long-lasting protection against the effects of the toxins in patients[105].

In order to understand the role of these toxins, effective tools must be developed to inhibit toxin entry in order to determine if there is additive protection through inhibition of all three toxins. These tools can also have applications for understanding the mechanism of *C. difficile* toxin entry such as determining important toxin-receptor epitopes. The goal of my project was to develop high-affinity reagents that can block cellular entry of all three known *C. difficile* toxins by targeting the receptor-binding domains of the toxins. In chapter 2, I describe the development of high-affinity synthetic antibodies against toxins A and B using the phage display method instead of the traditional hybridoma method with the aim of generating more effective or cross-reactive anti-toxin A and anti-toxin B antibodies to be used as research tools for studying these toxins, and possibly as diagnostic purposes. In chapter 3, I describe development of high-affinity synthetic antibodies against CDT using the same method. These antibodies were characterized for their binding properties using various affinity assays, such as ELISA and surface plasmon resonance. The antibodies were also tested for their ability to inhibit toxin entry and prevent toxicity to cultured cells.

20-30% of infected patients experience relapse after *C. difficile*-directed antibiotic treatment[6,9,20,22,118]; so by developing antibodies against all three toxins that have neutralization properties, the antibodies can potentially be used as an additional and more effective therapy to relieve the symptoms of CDI. Additionally, obtaining CDT antibodies can be used to potentially inhibit the effects of binary toxins produced from other bacteria, such as *C. spiroforme* and *C. perfringens*, as there is high sequence homology between CDT and these toxins.
Chapter 2
Developing high-affinity synthetic human antibodies for toxin A and toxin B
2 Developing high-affinity synthetic human antibodies for toxin A and toxin B

Despite many efforts of generating inhibitors against toxins A and B[106,119,120], including antibodies, there are few examples of reagents/drugs that can provide durable protection against the toxins and no reagents/drugs that can detect or effectively prevent intoxication of both toxins in an animal as a single agent. High-affinity antibodies that are cross-reactive for both toxins could be beneficial for efficient and accurate toxin detection as diagnostics for clinical surveillance studies. A single neutralizing antibody treatment could also limit unknown effects that may result from administration of multiple therapeutic agents in an animal model system.

In addition to developing novel synthetic antibodies against toxins A and B, my experiments were carried out to address two main questions: 1) can these antibodies from the phage-display screen bind to the toxins at a high-affinity and are these cross-reactive? 2) can these antibodies that bind to the toxin inhibit toxin entry? Results from the experiments would determine the potential of these antibodies for use as reagents for future research studies in the field.

My collaborator, John Tam from Dr. Roman Melnyk’s lab, assisted me in conducting the functional assay for determining the protective properties of my Fabs against toxins A and B on cultured cells. Dr. Roman Melnyk’s main research focus is on toxin B and routinely assesses the toxicity of toxin B on mammalian cells.

2.1 Results

2.1.1 Unique clones and binding specificity to recombinant His\textsubscript{6}-RBD\textsubscript{2304–2710}-toxin A and His\textsubscript{6}/GST-RBD\textsubscript{2286–2366}-toxin B

Phage display was performed on 6x histidine (His\textsubscript{6})-tagged RBD fragments of toxin A and toxin B (Figure 2.1A). The fragments were denoted as RBD\textsubscript{2304–2710}-toxin A and RBD\textsubscript{2286–2366}-toxin B (Figure 1.1A). These fragments were known to contain neutralizing epitopes upon llama immunization and produced a neutralizing single-VH domain of an antibody[121]. Since the fragment of RBD of toxin B was quite small, with an estimated molecular weight of 12.9 kDa, the protein may have been unstable. Therefore, I also cloned this fragment into a glutathione S-
transferase (GST)-tagged vector in order to improve the protein stability due to the additional size of the GST tag (Figure 2.1B). Phage display was also performed on the GST-tagged RBD_{2286-2366}-toxin B.

**Figure 2.1 Western blots on the RBD_{2304-2710}-toxin A and RBD_{2286-2366}-toxin B recombinant proteins.** A) Western blots of His_{6}-RBD_{2304-2710}-toxin A (2) and His_{6}-RBD_{2286-2366}-toxin B (1) recombinant proteins. The proteins were detected using anti-His_{6}-HRP (1:10000). His_{6}-RBD_{2286-2366}-toxin B and His_{6}-RBD_{2304-2710}-toxin A displayed a ~14 kDa band and ~35 kDa band, respectively with minimal background. B) Western blot of GST-RBD_{2286-2366}-toxin B recombinant protein. The protein was detected using anti-GST-HRP (1:10000). Two bands were detected in the western blot that corresponded to the GST-RBD_{2286-2366}-toxin B (~38 kDa) and GST (~27 kDa).

Following four rounds of phage-Fab selections, four unique phage-Fab clones that bound to RBD_{2304-2710}-toxin A and eleven unique phage-Fab clones that bound to RBD_{2286-2366}-toxin B with a specificity of > 5-fold higher than the background protein controls (based on neutravidin (NA), streptavidin (SA), bovine serum albumin (BSA), and GST) in ELISAs, were isolated (Figure 2.2). As a reference, our lab uses a five-fold difference as an arbitrary enrichment threshold to select Fabs for further characterization. From the eleven anti-toxin B phage-Fab clones, six were identified from the selections using His_{6}-tagged recombinant protein and the remaining five phage-Fab clones were identified from selections on GST-tagged toxin B. Upon conversion from phage-Fab to purified Fab protein, three of the Fabs lost their binding ability and specificity to the toxins (Figure 2.3B, C). Three purified anti-toxin A Fabs, A1, A3, and A4, were found to bind the protein with the specificity of > 5-fold greater than the NA control in ELISAs and at an absorbance of > 2.0 (Figure 2.3A) (Appendix C. Table 1). Fab A2 bound to
toxin A at a lower absorbance of 0.478 but still remained specific as its absorbance was 7.5-fold greater than the controls when 1 ug/ml of Fab was used in the assay. The ranking of the binding specificity of the Fabs against His$_6$-RBD-Toxin A was A3 > A1 > A4 > A2 (Appendix C. Table 1).

![Graph of the ELISA data of the binding specificity of phage-Fabs against RBD fragments of toxin A and toxin B recombinant proteins.](image)

**Figure 2.2** Graph of the ELISA data of the binding specificity of phage-Fabs against RBD fragments of toxin A and toxin B recombinant proteins. In the assay, the recombinant proteins were immobilized on protein binding plates and incubated with the phage-Fabs. After several washes, phage-Fab binding was detected using anti-M13-HRP (1:5000). All phage-Fabs bound to the RBD toxins with $\geq 5$ fold compared to the controls. A) ELISA data of four phage-Fabs from His$_6$-RBD$_{2304-2710}$-toxin A selection B) ELISA data of five phage-Fabs from His$_6$-RBD$_{2286-2366}$-toxin B selection. C) ELISA data of six phage-Fabs from GST-RBD$_{2286-2366}$-toxin B selection.

From the eleven purified Fabs that bound to RBD$_{2286-2366}$-toxin B, seven Fabs bound to His$_6$-RBD$_{2286-2366}$-toxin B while the remaining four Fabs, A5, A12, B1, and B2, lost their ability to bind to His$_6$-RBD$_{2286-2366}$-toxin B, with an absorbance of < 0.3 on ELISAs when 1 ug/ml of Fab was used (Figure 2.3B) (Appendix C. Table 2). When the concentration of the Fabs used in the ELISA was increased by 10 fold, the absorbance of Fab A5 increased by $\sim 9.7$ fold for His$_6$-
RBD\textsubscript{2286–2366}-toxin B but not in the control wells (Figure 2.3C). When increasing the concentration of Fabs A12, B1, and B2, there was no significant increase in the absorbance but there was a decrease in specificity of Fabs A12 and A9 (Figure 2.3C). The ranking of the binding specificity of the Fabs against His\textsubscript{6}-RBD\textsubscript{2286–2366}-toxin B was A10 \(>\) B3 \(>\) A6 \(>\) A8 \(>\) A11 \(>\) A9 \(>\) A7 \(>\) A5 \(>\) B1. These Fabs gave an absorbance fold difference of \(>\) 2 compared to the controls with 1 ug/ml of Fab was used (Appendix C. Table 2).

Figure 2.3 Graph of the ELISA data of the binding specificity of the purified Fabs against His\textsubscript{6}-tagged RBD fragments of toxin A and toxin B recombinant proteins. Binding of purified Fabs was detected using anti-Flag-HRP (1:5000). A) ELISA data of purified Fabs from the toxin A selection against toxin A. B) ELISA data of 1 ug/ml of purified Fabs used in the ELISA against toxin B. C) ELISA data of 10 ug/ml of purified Fabs used in the ELISA against toxin B.

I also examined the specificity of the toxin B Fabs when incubated with immobilized GST-RBD\textsubscript{2286–2366}-toxin B by ELISAs. Surprisingly, the binding properties of some Fabs against the GST-tagged protein were different from the His-tagged toxin B. In the ELISAs, when 1 ug/ml of Fab was used, Fabs A11, A12, B1, B2, and B3 were unable to bind to GST-RBD\textsubscript{2286–2366}-toxin B (Figure 2.4A) (Appendix C. Table 3). Fab A9 specificity to the protein was decreased by 6 fold
compared to the ELISAs with immobilized His\textsubscript{6}-RBD\textsubscript{2286–2366}-toxin B. When the concentration of the Fabs used in the ELISA was increased by 10 fold, the absorbance of Fab A5 increased by \textasciitilde 2 fold for GST-RBD\textsubscript{2286–2366}-toxin B but not in the control wells (Figure 2.4B). When increasing the concentration of Fab A7, the specificity decreased by 4.5 fold. Overall, Fab A11 and B3 bound to His\textsubscript{6}-RBD\textsubscript{2286–2366}-toxin B in ELISAs, but did not bind to GST-RBD\textsubscript{2286–2366}-toxin B. The ranking of the binding specificity of the Fabs against GST-RBD\textsubscript{2286–2366}-toxin B was A10 > A8 > A6 > A5 > A7 > A9. These Fabs gave an absorbance fold difference of > 2 compared to the controls when 1 ug/ml of Fab was used (Appendix C. Table 3). Since Fabs A12, B1, and B2 did not bind to toxin B on two different tagged recombinant proteins, they were excluded from further experiments.

Figure 2.4 Graph of the ELISA data of the binding specificity of the purified toxin B Fabs against GST-tagged RBD fragments of toxin B recombinant protein. Binding of purified Fabs was detected using anti-Flag-HRP (1:5000). A) ELISA data of 1 ug/ml of purified Fabs used in the ELISA against the antigen B) ELISA data of 10 ug/ml of purified Fabs used in the ELISA against the antigen.
Interestingly, when determining the binding specificity of the Fabs, I discovered that Fabs A1, A2, and B3 were cross-reactive and bound to both His$_6$-RBD$_{2304-2710}$-toxin A and His$_6$-RBD$_{2286-2366}$-toxin B with high binding specificity at 1 ug/ml of Fab in ELISAs (Figure 2.5) (Appendix C Table 4, 5). The ranking of the binding specificity of the cross-reactive Fabs for His$_6$-RBD$_{2304-2710}$-toxin A was A1 > B3 > A2 and the ranking of the binding specificity of the cross-reactive Fabs for His$_6$-RBD$_{2286-2366}$-toxin B was B3 > A1 > A2.

\[ \text{Figure 2.5 ELISA data on cross-reactive Fabs. A) ELISA against RBD-toxin A. Fab B3 came from the toxin B selection. B) ELISA against RBD-toxin B. Fabs A1 and A2 came from the toxin A selection.} \]

\[ \text{2.1.2 Affinity measurements of anti-toxin A and anti-toxin B Fabs on RBD of the toxins by ELISA} \]

The relative affinities of the binders were estimated by performing competitive ELISAs. In this assay, different concentrations of antigen were used to bind to Fabs in solution and then incubated with immobilized antigen. The concept of the assay is that the binding site of a high affinity binder with a slow $k_{\text{off}}$ will be occupied during the incubation with the antigen in solution and prevent binding to immobilized antigen. Thus, detection of Fab binding on immobilized antigen would be low due to the competition with the antigen in solution. A low concentration of antigen that can inhibit Fab binding to immobilized antigen indicates a high affinity Fab. For the competitive ELISAs, the concentration of Fabs used in the assay was first established by measuring the half-maximal effective concentration of the antibody which can bind to the antigen (EC$_{50}$). The EC$_{50}$ values of the Fabs were determined by generating a binding curve for each Fab. The Fabs were serial diluted and incubated with immobilized recombinant RBD$_{2304-2710}$-toxin A and RBD$_{2286-2366}$-toxin B in the ELISAs. The absorbance from the ELISA was
plotted (Figure 2.6). The EC$_{50}$ values of each Fab from the binding curves are listed in Table 2.1 and the ranking of the EC$_{50}$ values for anti-RBD-toxin A Fabs was A4 > B3 > A1 > A3. The EC$_{50}$ for Fab A2 could not be determined but was estimated to be > 80 nM. The ranking of the EC$_{50}$ values for anti-RBD$_{2286-2366}$-toxin B Fabs was A2 > A7 > A5 > A6 > (A9 = A11) > A8 > A10 > A1 > B3.

Table 2.1 EC$_{50}$ values from the binding curves of toxin A and B Fabs. These values were extrapolated from the binding curves in Figure 2.6 using Prism. A) Estimated EC$_{50}$ values of Fabs that bound to RBD of toxin A. B) Estimated EC$_{50}$ values of Fabs that bound to RBD of toxin B.
In the competitive ELISA, 10 nM and 100 nM of antigen (His$_6$-RBD$_{2304-2710}$-toxin A / His$_6$-RBD$_{2286-2366}$-toxin B) were used to pre-incubate with the Fab in solution. The Fab / antigen mixture was then incubated with immobilized antigen and binding on immobilized antigen was detected. The competitive ELISA showed that Fabs A1 and A3 binding was inhibited by 100 nM of His$_6$-RBD$_{2304-2710}$-toxin A with > 2-fold decrease in binding relative to the no competition control (Figure 2.7A) (Table 2.2A). Inhibition of Fabs A1 and A10 binding by 100 nM of His$_6$-RBD$_{2286-2366}$-toxin B was evident relative to the no competition control; however, they showed < 2-fold decrease in binding (Figure 2.7B) (Table 2.2B). The remaining Fabs were not significantly inhibited by either 10 nM or 100 nM of antigen in solution suggesting their affinity to be > 100 nM. This data suggested that the affinity of Fabs A1 and A3 for His$_6$-RBD$_{2304-2710}$-toxin A were the highest with an estimated affinity of between 10-100 nM, and Fabs A1 and A10 had an estimated affinity of ~100 nM for His$_6$-RBD$_{2286-2366}$-toxin B.

**Figure 2.7** Graph of the competitive ELISA data of RBD-toxin A and RBD-toxin B Fabs. Purified Fabs were pre-incubated with 0 nM, 10 nM, or 100 nM of antigen in solution and the mixture was then incubated with immobilized antigen. Purified Fab binding on immobilized antigen was detected using anti-Flag-HRP (1:5000). Inhibition of Fab binding to immobilized antigen indicated that the binding site of the Fab to the antigen was occupied during the pre-incubation step. The affinity of the Fabs was estimated from this assay. A) Competitive ELISA against RBD fragment of toxin A. Fabs A1 and A3 binding was inhibited at 100 nM of antigen in solution with > 2-fold decrease in binding to the immobilized antigen (dotted box) (mean % inhibition of Fab binding when competed with antigen in solution ± SD with technical replicates of n = 3). B) Competitive ELISA against RBD fragment of toxin B. Inhibition of Fabs A1 and A10 binding to the immobilized antigen at 100 nM of antigen in solution was less than 2-fold but was still evident (dotted box) (mean % inhibition of Fab binding when competed with antigen in solution ± SD with technical replicates of n = 3).
Table 2.2 Estimated affinities from competitive ELISAs of toxin A and B Fabs. Fabs that had an estimated affinities of \( \leq 100 \) nM are in yellow. The values were extrapolated from the graph in Figure 2.7. A) Estimated affinities of Fabs that bound to RBD of toxin A. B) Estimated affinities of Fabs that bound to RBD of toxin B.

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2.1.3 Binding affinity validation of anti-toxin A and anti-toxin B Fabs using SPR

The binding kinetics of the Fabs for their corresponding proteins were determined using surface plasmon resonance (SPR). The equilibrium dissociation constant (\( K_D \)) is the ratio of \( k_{off} \) to \( k_{on} \) and corresponds to the concentration of ligand at which it can saturate 50% of the receptor (antibody). The lower the \( K_D \) values of a protein, the higher the affinity. Typical \( K_D \) values for high-affinity antibodies are within the single-digit nanomolar range. Only Fabs, A1 and A3, showed binding to His\(_6\)-RBD\(_{2304-2710}\)-toxin A with \( K_D \) values of 8.98nM and 10.5nM, respectively (Table 2.3) (Figure 2.8A, C). Since I found that Fab A1 also bound to His\(_6\)-RBD\(_{2286-2366}\)-toxin B from our previous ELISA data, SPR was also performed on Fab A1 against His\(_6\)-RBD\(_{2286-2366}\)-toxin B. In agreement with ELISA data, the SPR data showed that Fab A1 also bound to His\(_6\)-RBD\(_{2286-2366}\)-toxin B with a \( K_D \) value of 5.73nM (Table 2.3) (Figure 2.8B). From the SPR data, I found two Fabs with affinities of < 10 nM towards their proteins. Fab A3 had a better fit than Fab A1 to the curve with a \( \chi^2 \) value of less than 10% of \( R_{max} \). The remaining Fabs did not bind to their protein in the SPR assay and considering their low affinities, based on the competitive ELISAs, they were not explored further.
Table 2.3 Surface plasmon resonance data for toxin A and B Fabs. The association rate constant \( (k_{\text{on}}) \) was determined by the rate at which the Fabs bound to the antigen when injected into the flow cell. The dissociation rate constant \( (k_{\text{off}}) \) was determined by the rate at which the Fabs were released from the ligand surface when PBS was injected. The \( K_D \) is the ratio of \( k_{\text{off}}/k_{\text{on}} \). For kinetic analysis, a 1:1 Langmuir model of global fittings of \( k_{\text{on}} \) and \( k_{\text{off}} \) was used. \( R_{\text{max}} \) and \( \text{Chi}^2 \) are displayed to show the confidence of the fit. For a good fit to the model, \( \text{Chi}^2 \) should be less than 20% of \( R_{\text{max}} \). TcdA showed a high \( \text{Chi}^2 \% \) of \( R_{\text{max}} (> 30\%) \).

<table>
<thead>
<tr>
<th>Fab</th>
<th>Antigen</th>
<th>( k_{\text{on}} ) (1/Ms)</th>
<th>( k_{\text{off}} ) (1/s)</th>
<th>( K_D )</th>
<th>( R_{\text{max}} )</th>
<th>( \text{Chi}^2 )</th>
<th>% ( \text{Chi}^2 ) of ( R_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>TcdA</td>
<td>2.61E+05</td>
<td>2.35E-03</td>
<td>8.98nM</td>
<td>11.54</td>
<td>6.14</td>
<td>53.21</td>
</tr>
<tr>
<td>A1</td>
<td>TcdB</td>
<td>3.37E+05</td>
<td>1.93E-03</td>
<td>5.73nM</td>
<td>21.88</td>
<td>9.46</td>
<td>43.2</td>
</tr>
<tr>
<td>A3</td>
<td>TcdA</td>
<td>2.51E+05</td>
<td>2.64E-03</td>
<td>10.50nM</td>
<td>44.85</td>
<td>4.44</td>
<td>9.90</td>
</tr>
</tbody>
</table>

Figure 2.8 Surface plasmon resonance curves of the binding response for the toxin A and toxin B Fabs. Toxin A and B recombinant proteins were immobilized on the sensor chip surface and different concentration of Fabs was injected into the flow cell (analyte 1-5). The binding curves correspond to the SPR data in Table 2.3. A) Fab A1 against TcdA B) Fab A1 against TcdB C) Fab A3 against TcdA.
2.1.4 Epitope grouping of the Toxin A Fabs, Fab A1 and Fab A3.

To assess whether the Fabs bound to the same or different epitopes on the toxins, I carried out competitive ELISAs to determine if purified Fabs could compete with phage-Fab clones. Competing Fabs indicated that these Fabs bound to the same epitope and were grouped. When the phage-Fabs competed with their own corresponding purified Fabs, the epitope was occupied by the purified Fab and prevented the phage-Fab from binding with a consequent decrease in absorbance of bound phage-Fab. Maltose-binding protein (MBP) Fab was used as the non-competing Fab control and therefore, the phage-Fab should bind freely to the antigen.

From our analysis, the toxin A Fabs, A1 and A3, did not compete with each other and showed nearly equivalent absorbance as the non-competing phage-Fab control (Figure 2.9). As expected, they were able to carry out autoinhibition of binding with a > 2-fold decrease in absorbance compared to the non-competing phage-Fab control. The competitive ELISA suggests that Fab A1 and Fab A3 bind to different epitopes of RBD-toxin A.

Figure 2.9 Epitope grouping of Fab A1 and A3. Phage-Fab competed with purified Fabs for the epitope binding site of RBD fragment of toxin A. The Fabs indicated in the x-axis are purified Fabs that were competing with the phage-Fab. Phage-Fab binding was detected using anti-M13-HRP. The bar highlighted in white showed auto inhibition. A) Detection of phage-Fab A1 binding. Purified Fab A3 did not inhibit phage-Fab A1 binding (mean absorbance of phage-Fab binding ± SD with technical replicates of n = 3). B) Detection of phage-Fab A3 binding. Purified Fab A1 did not inhibit phage-Fab A3 (mean absorbance of phage-Fab binding with technical replicates of n = 3).
2.1.5  Affinity measurements of anti-toxin A Fab A3 on full-length toxin A by ELISA

It is functionally important to test binding of these Fabs on the full-length toxins and therefore, I performed an ELISA on the full-length TcdA and TcdB. From the ELISA, only Fab A3 was capable of binding to the full-length toxin A (Figure 2.10). Since Fab A3 was the only Fab that showed binding to the RBD of toxin A in SPR and binding to the full-length functional toxin A in ELISAs, I carried out a competitive ELISA only on Fab A3 against the full-length toxin to estimate the affinity of Fab A3. The EC\textsubscript{50} of the Fab against 2 ug/ml of full-length toxin A was first determined from the titration of the Fab against the protein by ELISAs. The EC\textsubscript{50} was estimated to be 90 nM but is most likely higher since the dose-curve did not reach saturation (Figure. 2.11A). The EC\textsubscript{50} of Fab A3 against the full-length toxin was also ~88 fold higher than the EC\textsubscript{50} against the truncated version of the toxin. Using the half-maximal concentration of Fab A3 for the competitive ELISA, the estimated affinity of the Fab was also much lower against the full-length toxin A compared to the truncated version of the toxin. With 100 nM of the toxin used in the competitive ELISA, the Fab was not significantly inhibited and was capable of binding to the immobilized toxin A (Figure 2.11B). The competitive ELISA suggests that the affinity of the Fab to the full-length toxin A is much lower than to the truncated version and that the K\textsubscript{D} could be > 100 nM.

![ELISA data for the binding specificity of Fab A1 and A3 against full-length toxin A and toxin B.](image)

In the ELISA, only Fab A3 bound to the full-length toxin. MBP Fab was used as a control to determine that only toxin specific Fabs bound to the toxins. A) ELISA against full-length Toxin A. B) ELISA against full-length Toxin B.
Figure 2.11 EC$_{50}$ and competitive ELISA binding curves of Fab A3 against full-length toxin
A) EC$_{50}$ binding curve. The EC$_{50}$ of Fab A3 was estimated to be 90 nM from the binding curve using Prism but it could be greater since a saturation plateau of Fab binding was not achieved at the highest concentration of ~1000 nM (mean absorbance of Fab binding at each Fab concentration ± SD with technical replicates of n = 3). B) Competitive ELISA binding curve. The half-maximal concentration of Fab A3 from the EC$_{50}$ binding curve was used in the competitive ELISA. Fab A3 binding to immobilized full-length toxin A was competed with 10-fold dilutions of toxin in solution. At a concentration of 100 nM of antigen, there was not a significant inhibition of Fab binding to immobilized antigen (mean absorbance of Fab binding when competed with antigen in solution ± SD with technical replicates of n = 3).

2.1.6 Cytotoxicity assay

In collaboration with Dr. Roman Melnyk, Fab A3 was tested for its protective properties against toxin A on cells. Vero cells, IMR90 cells, and Chinese hamster ovarian (CHO) cells were used in the assay as these cell-lines were shown previously to be affected by toxin A and toxin B intoxication and exhibited cell rounding upon the addition of the toxins. A dose-response curve was conducted to determine the minimum concentration of toxin A that could be used in the assay in order to allow the best possibility of seeing protective effects of the Fab. It was first determined that a concentration of 400 pM of toxin A was the minimum concentration of toxin to achieve cell rounding. However, when the functional assay was conducted using this concentration, the Fab did not protect the cells from toxin A and there was no difference in the cell viability between cells treated with the Fab and those treated with toxin alone. The assay was conducted using a high saturating concentration of the Fab (up to 40ug/ml).
2.2 Discussion

Pathogenic strains of *C. difficile* can produce two large clostridial toxins, toxin A and toxin B, which are the main contributors to the symptoms of CDI. In this chapter, I aimed to develop high-affinity synthetic antibodies against the receptor-binding domains of toxin A and toxin B using the phage display method instead of the traditional hybridoma method. However, it is important to note that my goal was not solely to develop neutralizing antibodies but also to develop antibodies that could be used as a potential diagnostic and research tools for the detection of these toxins, which current antibodies have failed in this respect due to the insensitivity of the antibodies.

From the phage display screen, I selected for Fabs that bound to the truncated recombinant toxins A and B receptor-binding domains. From two separate studies, it was known that those portions of the RBDs contain sites that are important for binding [121,122] and therefore using those specific portions for phage display should allow for selection of neutralizing antibodies. Fabs were isolated from selections on two differently tagged truncated RBD-toxin B proteins, His-tagged and GST-tagged RBD<sub>2286-2366</sub> Toxin B. Upon analysis of the binding specificity for these proteins using ELISAs, some Fabs bound to His<sub>6</sub>-RBD<sub>2286-2366</sub>-toxin B but not to GST- RBD<sub>2286-2366</sub>-toxin B. Since GST, with a molecular weight of ~26 kDa, is 2 times larger than RBD<sub>2286-2366</sub>-toxin B that has a molecular weight of ~13 kDa, it is possible that the GST tag masked some epitopes of RBD<sub>2286-2366</sub>-toxin B that were exposed on the His<sub>6</sub>-tagged RBD toxin B fragment. I am confident that at least five Fabs bind to the RBD of toxin B because these Fabs were able to bind to both His-tagged and GST-tagged recombinant proteins.

Unfortunately, I was unable to identify neutralizing antibodies against toxins A and B. There could be several factors that contributed to the difficulties encountered, including a factor that could arise when generating truncated proteins for phage display. One factor was that the affinity of the Fab was too low to inhibit toxin binding. The *K<sub>D</sub>* value for Fab A1 obtained from the SPR against RBD-toxin A and B fragments was in the nanomolar range; however, the binding response curves for this Fab contained a large amount of background noise and had a high % Chi<sup>2</sup> of R<sub>max</sub> value (Table 2.3) (Figure 2.8 A, B), suggesting that it may be unreliable. SPR is a good indication of the affinity of the Fabs but there are also pitfalls to consider when using SPR that may skew the true affinity of the Fabs[123]. One of the pitfalls is that the orientation of the
antigen of interest when immobilized on the sensor surface is unknown and the ligand-receptor interaction could be different in solution. Therefore, the epitopes for Fab binding can be masked in SPR. The stability and purity of the antigen should also be considered, as this may cause false positives and background noise, and therefore it may cause misinterpretation of the affinity of the protein. For these reasons, competitive ELISAs were also conducted, and from this assay it suggests that the affinities are higher than the affinities obtained in SPR.

Since the affinity of the Fabs was determined using the truncated toxins, I performed ELISAs on the binders Fab A1 and Fab A3 against the full-length toxins, and I expected that the Fabs would bind equally well. However, only Fab A3 bound to the full-length toxin A. The EC$_{50}$ and estimated affinity from the competitive ELISA of the Fab against the full-length toxin was much lower than that of the truncated protein, and at ~1000 nM Fab A3 binding did not reach saturation in the ELISA (Figure 2.11A). It is clear that Fab A3 has a lower affinity against the full-length toxin than expected. The full-length toxins A and B are much larger than the truncated toxins with a size of 300 kDa and 270 kDa, respectively. Based on the binding data from the ELISAs, I suspect that the remaining regions of the toxins were masking some of the epitopes found on the truncated toxins. Fab A3 could have been binding to an epitope that was partially masked by the remaining domains of the toxins and therefore weakening the interaction between the Fab and the toxin, and leading to a lower affinity measurement in the SPR.

Since the human receptor for toxins A and B is unknown, the affinity of the toxins for the receptor is not clearly defined. This uncertainty makes it difficult to predict an affinity of the antibody required to compete with the toxins for the receptor. However, from this study and other studies, it has been suggested that the affinity of bacterial toxins for their receptor is high and possibly within the nanomolar to picomolar range[106,124,125] and therefore, high affinity antibodies that are less than single-digit nanomolar affinities might be required to block toxin entry.

Lastly, in order to develop neutralizing antibodies, it is important that the Fabs bind to therapeutic epitopes; these are epitopes that when bound by an antibody will neutralize target antigen function (in the case of antagonistic antibodies)[126]. Although the human receptor for toxins A and B is unknown, it is suggested that the toxins bind to carbohydrate-containing receptors. In one study, the crystal structure of the C-terminal region containing the receptor-
The binding domain of toxin A was determined when bound to a synthetic derivative of a naturally occurring carbohydrate receptor, \( \alpha\text{-Gal}(1,3)-\beta\text{-Gal}(1,4)-\beta\text{-GlcNAcO(CH}_2\text{)}_8\text{CO}_2\text{CH}_3[122] \). From this crystal structure, multivalent binding of toxin A to the receptor was identified through seven putative binding sites. Current therapeutic monoclonal antibodies do not decrease the severity of CDI[106,119], which may be due to the multiple binding sites of toxin to the receptor and that the antibodies may have blocked only a few key binding sites, leaving the remaining binding sites available for receptor binding. This study verifies that generating antibodies against toxins A and B for complete neutralization could be difficult.

Overall, I have developed a Fab called Fab A3 that binds to the full-length toxin A, but I was unable to develop any anti-toxin B Fabs. Fab A3 binds to the full-length toxin at a low affinity of \( > 100 \text{ nM} \) based on the competitive ELISA data. The affinity of Fab A3 could be improved, however, via affinity maturation, and perhaps with a higher affinity, it might be possible for Fab A3 to neutralize the toxicity of the toxin on cells if it binds to a therapeutic epitope.

### 2.3 Materials and Methods

#### 2.3.1 Phagemid plasmid of Library F

The phagemid used to generate the library contains an origin of replication (dsDNA ori) for replication of double-stranded DNA and f1 site, which enables single-stranded DNA replication. Additionally, the phagemid contains a selectable marker (Amp\(^\text{r}\)) and a phoA promoter for protein purification (Appendix D. Figure 1). The phage library used in the selection was termed “Library F” and displayed Fabs of an antibody. The library was created by Dr. Helena Persson (a former postdoctoral fellow in Dr. Sidhu lab). In this library, CDRs VL3, VH1, VH2, and VH3 of the Fab were randomized via oligonucleotide-directed mutagenesis. A secretion signal is found at the N-terminus of both the light chain and heavy chain of the Fab, allowing the light chain and the heavy chain to be secreted separately and then associate in the periplasm of \( E.\text{coli} \) to form the functional Fab. The C-terminal of the heavy chain is fused to the pIII coat protein, which allows the expression of the Fab on the surface of the phage particles. The library has an estimated measured diversity of \( 10^{10} \) different antibodies. Detailed description of the library construction can be found in Fellouse & Sidhu (2007)[97].
2.3.2 Generation of expression plasmids for production of His$_6$-RBD$_{2304–2710}$-toxin A and His$_6$/GST-RBD$_{2286–2366}$-toxin B

Fragments of the receptor-binding domain of TcdA and TcdB (amino acid 2304-2710 of TcdA and amino acid 2286-2366 of TcdB, NCBI accession# NC_009089.1) using genomic DNA of C. difficile strain 90556-M6S as template for PCR were cloned into pGC297 vector and pHH0103 containing a isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter (Appendix D, Figure 2A, B). The RBD$_{2304–2710}$-toxin A and RBD$_{2286–2366}$-toxin B were cloned using a restriction-enzyme independent method developed by Walker et al. (2008). In this method, primers were designed to generate two sets of blunt-end PCR products containing the desired antibody sequence with different overhangs. DNA polymerases that have proofreading activity, such as Phusion® High-Fidelity DNA polymerase (NEB, cat# M0530), were used to generate these PCR products so that extra nucleotides were not added at the end of the products. The cycling protocol for generating the PCR products were as follows: 95 °C for 3 min; 95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min repeated 34 times; 72 °C for 5 min (primers listed in Appendix B, Table 1). The His$_6$-tagged vector, pGC297, was cut using KpnI (NEB, cat# R0142) and NcoI (NEB, cat# R0193). The GST-tagged vector, pHH0103, was cut using KpnI and NotI-HF (NEB, cat# R3189). The cut plasmids were dephosphorylated using Antarctic Phosphatase (NEB, cat# M0289) and inserts were phosphorylated using T4 Phosphonucleotide Kinase (NEB, cat# M0201) to improve the cloning efficiency. The ligation was performed using T4 ligase (NEB, cat# M0202). Successfully ligated vectors were confirmed using PCR method with the following cycling protocol: 95 °C for 3 min; 95 °C for 30 s, 55°C for 30 s, 72 °C for 1 min repeated 29 times; 72 °C for 5 min (primers listed in Appendix B, Table1).

2.3.3 Expression and purification of His$_6$-tagged RBD$_{2304–2710}$-Toxin A and RBD$_{2286–2366}$-Toxin B

The plasmids were transformed into BL21 bacteria cells. The cells were induced with 1 mM of IPTG at OD$_{600}$ of 0.6 and the induction proceeded for 24 hours at 16 °C. The cells were then pelleted at 10,000 rpm for 20 minutes and resuspended in 40 ml of lysis buffer (50 mM Tris-pH 8.0, 500 mM NaCl, 10 mM imidazole-pH 8.0, 1 mM PMSF, and 1x protease inhibitor cocktail)
per 1 L of bacterial culture. The lysate was sonicated on ice with 5 s on / 5 s off pulses for 2 min at 40% amplification. The lysate was centrifuged twice for 10 min at 10,000 rpm at 4 °C. The supernatant was added to gravity flow columns that were packed with 1.5 ml of prewashed Ni beads slurry (Qiagen, cat# 30230) and allowed to flow through the columns. The columns were washed 3 times with 30 ml of wash buffer (50 mM Tris-pH 8.0, 500 mM NaCl, 20 mM imidazole). The protein was eluted with 400 ul of elution buffer (50 mM Tris-pH 8.0, 500 mM NaCl, 250 mM imidazole, 5% glycerol). The proteins were buffer exchanged with PBS using Thermo Scientific Slide-A-Lyzer Dialysis Cassette G2 20 kDa (cat# 87734) and 2 kDa (cat# 87717) for RBD_{2304–2710}-toxin A and RBD_{2286–2366}-toxin B, respectively.

2.3.4Expression and purification of GST-tagged RBD_{2286–2366}-Toxin B

The plasmids were transformed into BL21 bacteria cells. The cells were induced with 0.4 mM of IPTG at OD$_{600}$ of 0.6 and the induction proceeded for 24 hours at 16 °C. The cells were then pelleted at 10,000 rpm for 20 minutes and 50 ml lysis buffer (Phosphate Buffered Saline (PBS) buffer containing 1 mM EDTA, 1 mM DTT, 0.5% Triton, 1x protease inhibitor cocktail) was added per 250 ml of bacterial culture. The lysate was sonicated on ice with 5 s on / 5 s off pulses for 2 min at 40% amplification. The lysate was centrifuged twice for 10 min at 10,000 rpm at 4 °C. The supernatant was added to prewashed 300 ul of glutathione resin (GE Healthcare, cat# 17-0756-05) and incubated for 1 hour with gentle nutation. The beads were collected using a gravity flow columns. The columns were washed 3 times with the first wash using three column volumes of PBS, the second wash using PBS-150 mM NaCl solution, and the third wash using PBS. The protein was eluted with three column volumes of the elution buffer (10 mM glutathione, 50 mM Tris-pH 8.0). The protein was buffer exchanged with PBS using Thermo Scientific Slide-A-Lyzer Dialysis Cassette G2 20 kDa.

2.3.5Western blot for confirmation of RBD$_{2304–2710}$-toxin A and RBD$_{2286–2366}$-toxin B protein preps

Western blot was performed to confirm that the proteins were not degraded and were the correct estimated size. The recombinant proteins were separated by sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE) using 4–15% Mini-PROTEAN® TGX™ Precast Gel (Biorad, cat# 456-1086, 456-1083) and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Health, cat# RPN303F). The membrane was blocked with Tris-buffered saline-0.05% Tween 20, 5% skim milk for 1 hour. His6-RBD2304-2710-toxin A and His6-RBD2286-2366-toxin B were detected by 1:10000 anti-His6-horseradish peroxidase (HRP) (R&D, cat# MAB050H) and GST- RBD2286-2366-toxin B was detected using 1:10000 anti-GST-HRP (Sigma, cat# A7340) in PBS-5% skim milk. The blots were developed using Pierce ECL Western Blotting substrate (ThermoScientific, cat# 32106).

2.3.6 Phage display

A 96-well protein binding MaxiSorp® plate (Thermo Scientific, cat# 439454) was coated overnight at 4 °C with 100 ul of RBD2304-2710-toxin A and RBD2286-2366-toxin B (5 ug/ml in PBS). A second plate was coated with 100 ul of neutravidin, streptavidin, or GST (5 ug/ml) for the pre-absorption step. The next day the plates were blocked with PBS-0.5% BSA for at least 1 hour. A 0.5 ml glycerol stock of Library F containing 8x10^{12} phage/ml was precipitated with 5 ml 20% polyethylene glycol-8000 (w/v) / 2.5 M NaCl in 30 ml of PBS for 20 min on ice and centrifuged for 20 min at 12,000 rpm, 4 °C. The precipitated phage pellet was resuspended with PBS-0.5% BSA, 0.05% Tween 20 (PBT) buffer. The volume of PBT used to resuspend the phage pellet depended on the number of wells of the plate used. For example, for 8 coated wells, 1 ml of phage library was needed, 100 ul for each well and the remaining 200 ul to titre the input phage.

The phage library was first incubated on the pre-absorption plate for 1 hour at room temperature, and then transferred to the antigen plate and incubated for 1 hour at room temperature. For the selection on His-tagged proteins, the library was pre-absorbed on neutravidin and streptavidin, alternating with each round. For the selection on the GST-tagged protein, the library was pre-absorbed on GST for each round. The plates were washed 6 times with PBS-0.05% Tween 20 in the first round of selection with an increasing number of washes (8, 10, 12) in each round to increase the stringency of the selection. The phage was eluted using 0.1 N of HCL and neutralized with Tris-HCL pH 8.0. The eluted phage was then amplified in OmniMAX™ 2 T1 Phage-Resistant (T1\textsuperscript{R}) E. coli strain for the next round.
At the end of each round, the titre of the phage was examined for enrichment. A 10-fold serial dilution of the phage was carried out in PBS and 90 ul of mid-log OmniMAX E. coli strain was infected with the diluted phage in a 96-well plate (Corning, cat# 3596). The infection proceeded for 30 min at 37 °C. 5 ul of the infected E. coli was spotted on carbenicillin, kanamycin, and tetracyclin 2YT agar plates. The next day, the number of infected colonies on the carbenicillin plate was examined. The kanamycin was used to select for infection of the helper phage and the tetracycline was used to select for viability of the E. coli, which is tetracycline resistant.

2.3.7 Phage-Fab ELISA

Binding specificities of the phage-Fab clones were assessed by ELISA. 2 ug/ml of His6-RBD<sub>2304-2710</sub>-toxin A (57 nM) and His6/GST-RBD<sub>2286-2366</sub>-toxin B (57-100 nM) were immobilized on protein binding Nunc MaxiSorp® flat-bottom 384 well plates (Thermo Scientific, cat# 464718) overnight at 4 °C. Neutravidin and GST (2 ug/ml) were also immobilized on separate wells as protein controls. Single bacterial colonies were plated for round 3 and round 4 and each colony was inoculated in 600 ul 2YT/carbenicillin/M13K07 helper phage. The next day the ELISA plates were blocked with 60 ul of PBS-0.5% BSA solution for 1 hour. The overnight cultures were centrifuged at 8000 rpm for 10min, and 30 ul of each phage supernatant was aliquoted to each blocked well quadrant that was coated with the proteins. The phage supernatant was incubated with the coated wells for 1 hour at room temperature. Afterwards, the phage was removed and the wells were washed 6 times with PBS-0.05% Tween 20. The bound phage was detected with anti-M13-HRP (GE, cat#27-9421-01) and the signal was developed with 25 ul of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate after 6 times wash with PBS-0.05% Tween 20 buffer. The reaction was stopped with 25 ul 1 M H<sub>3</sub>PO<sub>4</sub> and the plate was read for absorbance at a wavelength of 450 nm.

2.3.8 Sanger sequencing for the CDRs of the Fabs

The VL and VH of the Fab were amplified using previously established primers (Appendix B. Table 2). The cycling protocol was 94 °C for 5 min; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min repeated 29 times; 72 °C for 5 min to amplify the variable regions of the light and heavy
chain of the Fabs. PCR cleanup was performed using an enzymatic approach. For every 4 ul of PCR product, 0.3 ul of ExoI (NEB, cat# M0293) and 0.3 ul of shrimp alkaline phosphatase (SAP) (NEB, cat# R0569) was added. Sequencing was performed by the Biobasic sequencing facility. CDR sequences of Fab A3 are shown in Appendix A.

2.3.9 Purified Fab generation

Protein expression of the antibody fragments from Library F is under the control of a phoA promoter and the expression of the proteins can be induced by phosphate depleted media. The Fabs can be purified as an entity without the phage particle by introducing a stop codon via oligonucleotide-directed mutagenesis at the junction between the gene encoding the pIII coat and the antibody fragment. However, the expression of the Fab under the phoA promoter is low. Alternatively, the Fab can be subcloned into a vector that can be expressed under an IPTG expression vector.

The Fabs were subcloned into P3-His-Stop-IPTG vector (given by Genetech) that was modified by Nicolas Economopoulos and Rachel Hanna (Appendix D. Figure 1B). The Fabs were subcloned using a restriction-enzyme independent method developed by Walker et al. (2008). The cycling protocol was: 98 °C for 30 s; 98 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min repeated 29 times; 72 °C for 10 min (refer to Appendix B. Table 3 for primer list). These products were purified using Qiagen PCR purification columns (cat# 28106). These products were then mixed at 1:1 ratio and reannealed using the cycling protocol: 94 °C 5 min, 50 °C 3 min, and 20 °C 3 min, to obtain sticky ends. The vector was cut using NcoI and XbaI (NEB, cat# R0145). The cut plasmid was dephosphorylated using Antarctic Phosphatase and inserts were phosphorylated using T4 Phosphonucleotide Kinase to improve the cloning efficiency. The ligation was performed using T4 ligase. The bacterial colonies were screened for successful ligation using PCR method with cycling protocol: 95 °C for 3 min; 95 °C for 30 s, 57 °C for 30 s, 72 °C for 2 min repeated 29 times; 72 °C for 10 min (primers listed in Appendix B. Table 3).

The expression was induced by 1 mM of IPTG; from there the protein could be collected either from the bacterial cells and/or the supernatant of the bacteria culture. If the Fabs were purified using the bacterial pellet, the bacteria was lysed (1% Triton X-100, 1:100,000 of 250U/ul
benzonase, 2 mM MgCl₂, 0.2 mM PMSF, and 100 mg/ml lysozyme) and the bacteria fragments were collected through centrifugation at 12000 rpm for 40 min. The Fabs present in the supernatant were captured at 4 °C for 2 hours with prewashed protein A-sepharose beads (GE Healthcare, cat# 17-1279-03), using 500 ul bead volume per 250 ml of supernatant. If the Fabs were purified using the supernatant from the bacteria culture, the supernatant was simply incubated with the protein A-sepharose beads (500 ul bead volume per 250 ml of supernatant). The protein A-sepharose bead column was washed 3 times with PBS and eluted with Fab elution buffer (50 mM NaH₂PO₄, 100 mM H₃PO₄, 140 mM NaCl, pH 2.8). The eluted Fabs were neutralized with 1 M Tris pH 10. The protein was then concentrated and buffer exchanged with PBS using Millipore Amicon Ultra-4 and -15 Centrifugal Filter Units – 10,000 NMWL as specified by the manufacturer.

2.3.10 Purified Fab ELISA for direct binding

The recombinant proteins, His₆-RBD₂₃₀₄₋₂₇₁₀-toxin A (57 nM) and His₆/GST-RBD₂₂₈₆₋₂₃₆₆-toxin B (57-100 nM), were coated overnight at 4 °C on protein binding Nunc MaxiSorp® flat-bottom 384-well plates and blocked the next day with 60 ul of PBS-0.5%BSA for 1 h at room temperature. For the full-length Toxin A protein, I coated 1 ug/ml (3 nM) of the protein. 30 ul of 1 ug/ml-5 ug/ml of Fab was added to the pre-blocked protein coated plates and incubated for 1 hour at room temperature. The plate was washed 6x with PBS-0.05% Tween 20, and 30ul of anti-flag-HRP (1:5000 in PBS-0.5% BSA-0.05% Tween 20) was added and incubated for 30 min at room temperature. The plate was then washed 6 times with PBS-0.05% Tween 20, and the plate was developed with 25 ul of 3,3’,5,5’-tetramethylbenzidine (TMB) substrate. The reaction was stopped with 25 ul 1 M H₃PO₄ and the plate was read for absorbance at a wavelength of 450 nm.

2.3.11 Determination of EC₅₀ for each Fab by generating a binding curve

A dose-response curve of each purified Fabs was generated to determine the sub-maximal concentration of Fab to use for the competitive ELISA and the EC₅₀ values. The recombinant proteins, His₆-RBD₂₃₀₄₋₂₇₁₀-toxin A (57 nM) and His₆/GST-RBD₂₂₈₆₋₂₃₆₆-toxin B (57-100 nM),
were coated overnight at 4°C on protein-binding Nunc MaxiSorp® flat-bottom 384-well plates and blocked the next day with 60 ul of PBS-0.5% BSA for 1 hour at room temperature. For the full-length toxin A protein, I coated 2 ug/ml of the protein. The Fabs were serial diluted in PBS-0.05% BSA and 30 ul of the diluted Fabs were added to the pre-blocked protein coated plates and incubated for 1 hour at room temperature. The plate was washed 6 times with PBS-0.05% Tween 20, and detected with anti-flag-HRP (1:5000 in PBS-0.5% BSA-0.05% Tween 20). The ELISA proceeded in the same manner as explained previously. The EC₅₀ values were determined using computer software called Prism.

2.3.12 Competitive ELISA for affinity measurements of Fabs

The recombinant proteins, His₆-RBD₂₃₀₄₋₂₇₁₀-toxin A (57 nM) and His₆/GST-RBD₂₂₈₆₋₂₃₆₆-toxin B (57-100 nM), were coated overnight at 4 °C on protein binding Nunc MaxiSorp® flat-bottom 384-well plates and blocked the next day with 60 ul of PBS-0.5% BSA for 1 hour at room temperature. During the 1 hour blocking step, submaximal concentration of Fabs were incubated with different concentrations of antigen in solution (0 nM to 100 nM) on protein non-binding plates (Corning, cat# 3641) for 1 hour at room temperature with gentle shaking. 30 ul of the mixture was then transferred to the blocked plates and incubated for 20-30 min at room temperature. The plates were washed 6 times with PBS-0.05% Tween 20 and detected with anti-flag-HRP (1:5000 in PBS-0.5% BSA-0.05% Tween 20). The assay proceeded as described previously.

2.3.13 Surface Plasmon Resonance

Binding kinetics of the purified Fab proteins were determined by surface plasmon resonance using PROTEON (Biorad) with antigen immobilized on GLC chips at a density sufficient to produce ~100 response units when saturated with Fab. The on-rate constant (kₐₙ) was measured by the change of refractive index when the analyte (antibodies) accumulated on the immobilized ligand (His₆-RBD₂₃₀₄₋₂₇₁₀-toxin A / His₆-RBD₂₂₈₆₋₂₃₆₆-toxin B) as it was injected in the flow cell. Serial dilutions of Fab proteins were injected at 100 ul/minute for 1 minute. The refractive index changed as PBS was injected in the flow cell and the antibody was dissociated from the ligand.
This change was the off-rate constant ($k_{\text{off}}$) of the antibody. PBS was injected at $100 \text{ ul/minute}$ for 10 minutes to observe dissociation. Binding responses were corrected by subtraction of responses on a blank flow cell. For kinetic analysis, a 1:1 Langmuir model of global fittings of $k_{\text{on}}$ and $k_{\text{off}}$ was used.

2.3.14 Epitope grouping

Fresh phage-Fabs were prepared for competitive ELISAs to group Fabs that bind to different epitopes of the antigens. Each phage-Fab was re-infected in 5 ml of mid-log phase OmniMAX E. coli strain (OD = ~0.6) with helper phage, M13K07 (1:1000), and grown in carbenicillin/kanamycin 2YT media overnight at 37 °C with shaking at 200 rpm. The phage supernatant was collected by centrifugation at 8000 rpm for 10 min and precipitated with 5 ml of 20% polyethylene glycol-8000 (w/v) / 2.5 M NaCl on ice for 20 min. The phage precipitate was pelleted by centrifugation at 12,000 rpm for 20 min and resuspended in 1 ml PBS-0.5% BSA. The phage was first titrated in an ELISA to determine the concentration of the phage diluent at which the absorbance is at a sub-maximal level of ~0.8-1.2. 5 ul of the diluted phage-Fab was used in an ELISA and incubated with 2 ug/ml of immobilized recombinant protein on Nunc MaxiSorp® flat-bottom 384-well plates. The phage-Fab was detected by anti-M13-HRP and treated the same manner as previously stated.

Next, saturating concentration of purified Fab (10 ug/ml) was tested on a separate plate for binding using anti-flag-HRP (1:5000 in PBS-0.5% BSA-0.05% Tween 20) to detect the purified Fab. Once purified Fab binding was validated, 30 ul of 10 ug/ml of Fab was incubated with the immobilized recombinant protein on pre-blocked Nunc MaxiSorp® flat-bottom 384-well plates for at least 1 hour. 5 ul of phage-Fab was added to the 30 ul purified Fab solution on the plate and incubated 15 min. The plate was washed and treated the same manner as previously stated using anti-M13-HRP to detect phage-Fab binding.
2.3.15 Cytotoxicity assay

The cytotoxicity assay was conducted by John Tam from Dr. Roman Melnyk’s Lab at the Hospital for Sick Children in Toronto, ON. In the assay, 10000 cells of IMR90 cells (in EMEM media) was plated per well. The next day, the media was exchanged with serum-free EMEM with the addition of 1 uM cytotracker orange for 3 hours. On a separate plate, two-fold dilutions of Fab in serum-free media plus 400pM of native TcdA (List Biological Laboratories, Inc., cat# 152B) was pre-incubated for 1 hour and then the mixture was added to the cells by media exchange. After 24 hours (when cell rounding in controls became evident), the media was changed to complete media (EMEM with serum). Cell rounding was quantitated using the Cellomics array scan (Thermo Scientific).
Chapter 3
Developing high-affinity synthetic human antibodies for CDT
3 Developing high-affinity synthetic human antibodies for CDT

Considering that TcdA and TcdB are known to cause disease in animal models, much of the research on *C. difficile* has been mainly focused on toxins A and B, and developing therapeutics against these toxins. It is only recently that the binary toxin, CDT, has been taken notice in the research field due to increasing number of outbreaks involving CDT producing strains. The role of CDT is unknown in CDI, and it is mainly due to the lack of tools, such as inhibitors, required for assessing the importance of CDT and its role in virulence. Therefore, novel antibodies could be used as tools to better understand the pathogenicity of CDT and binding of the toxin to the newly discovered host receptor, lipolysis-stimulated lipoprotein receptor (LSR).

3.1 Results

3.1.1 Identification and binding of CDTb Fabs

After four rounds of phage display selection on GST-RBD-CDTb recombinant protein, the binding specificities of clones from rounds 3 and 4 were determined using ELISAs. There were 24 unique phage-Fab clones isolated from the selection that bound to GST-RBD-CDTb with an absorbance of > 1.0 compared to GST and BSA control wells, which had an absorbance of < 0.3 (Figure 3.1A). All clones showed > 5-fold difference in binding specificity compared to the controls. The heavy and light chains of these 23 phage-Fab clones were cloned into IPTG expression vectors and the Fab portions of the phage were purified as proteins. Phage-Fab A12 failed to clone into the expression vector after multiple attempts and thus was excluded. In order to verify that the purified Fabs retained their binding specificity, ELISAs were performed against the GST-RBD-CDTb as well as against the full-length CDT-His<sub>6</sub>. From the 23 purified Fabs, 8 Fabs did not bind to the full-length CDTb-His<sub>6</sub> while the remaining 15 CDTb Fabs bound to both the GST-RBD-CDTb and full-length CDTb-His<sub>6</sub> with > 14-fold difference in specificity compared to the controls (Figure 3.1B).
Figure 3.1 Binding specificities of phage-Fabs and purified Fabs against CDTb. A) Phage-Fab ELISA data against the receptor-binding domain of CDTb. All phage-Fabs bound specifically to the RBD of CDTb with > 5-fold difference between the GST and BSA controls. B) Purified Fab ELISA data against the RBD-CDTb and the full-length CDTb. All purified Fabs bound to the RBD of CDTb but only 15 purified Fabs bound to both RBD-CDTb and full-length CDTb. MBP was used as a control Fab.

3.1.2 CDTb-LSR inhibitory properties of CDTb Fabs

An ELISA-based in vitro binding assay was designed to determine and explore the inhibitory properties of the CDTb Fabs on recombinant LSR protein, the receptor required for CDT cellular entry. Firstly, I wanted to determine if the epitopes of the ECD-LSR-Fc recombinant protein for
CDTb binding were present and detectable by ELISA. The ECD-LSR-Fc was immobilized on the plate and incubated with CDTb-His$_6$, and the presence of CDTb-His$_6$ remaining bound on the plate after several washing steps was detected using anti-His$_6$-HRP (Figure 3.2A). From the assay, CDTb-His$_6$ was found at detectable levels on the ECD-LSR-Fc immobilized well (absorbance = ~2.5) compared to the well containing the background control, Fc protein, and a no CDTb-His$_6$ well (absorbance < 0.1) (Figure 3.2B). Additionally, a dose-curve was performed to determine the concentration of CDTb that gave an absorbance at sub-maximal level (EC$_{50}$) and was calculated to be 19 nM of CDTb (Figure 3.2C). The binding assay could then be manipulated to determine the inhibitory properties of the CDTb Fabs when these Fabs were pre-incubated with the submaximal concentration of CDTb before pre-incubation with immobilized ECD-LSR-Fc (Figure 3.2A).

![Diagram of the binding assay](image)

**Figure 3.2** Determination of CDTb concentration to use in the ELISA-based *in vitro* binding assay. A) Schematic of the binding assay. The binding assay was modified to determine if the Fabs can inhibit CDTb binding to LSR by pre-incubating the Fabs with CDTb for 1 hour and incubating the mixture on the immobilized LSR. B) Graph of the ELISA data on LSR-CDTb detection. CDTb was detected on the immobilized proteins with anti-His$_6$-HRP C) EC$_{50}$ binding curve for LSR-CDTb interaction.
From the binding assay, the CDTb Fabs were grouped into three categories. The first category contained CDTb Fabs that did not bind to the full-length CDTb and showed the same absorbance as MBP Fab and No Fab controls (absorbance = 0.8) (Figure 3.3A, B). The MBP Fab is a control Fab that should not bind to CDTb and thus served as a negative control in the assay. The second category contained CDTb Fabs that bound to CDTb but did not inhibit the CDTb-LSR interaction. Because the Fab was also His₆-tagged and could be detected by the anti-His₆-HRP along with CDTb-His₆, these wells showed an amplified absorbance (absorbance > 0.8) above MBP Fab control well and No Fab control well (Figure 3.3A, B)). The last category contained five CDTb Fabs that inhibited the CDTb-LSR interaction and showed an absorbance below MBP Fab control and No Fab control (absorbance < 0.8). The binding assay indicated that there were five CDTb Fabs that could inhibit the CDTb-LSR interaction in vitro.

![Figure 3.3](image)

**Figure 3.3 Data of the ELISA-based in vitro binding assay.** A) ELISA data on the binding assay. The red dotted line was the absorbance at which CDTb binding was detected in no Fab and MBP Fab controls. The absorbance above the line indicates the presence of both CDTb and Fab on the plate after the washing step. The absorbance below the line indicates that the Fabs were able to inhibit CDTb binding (mean absorbance of CDTb binding to LSR after Fab incubation ± SD with technical replicates of n = 3). B) The schematic displays the binding scenario of three groups of Fabs determined from the binding assay.
3.1.3 Affinity measurements of CDTb Fabs and IgGs using SPR

In order to measure the binding affinity of the antibodies to CDTb, surface plasmon resonance (SPR) was performed. The Fabs were also converted to the full-length IgG1 and the binding properties of the IgGs were also determined. The Fabs/IgGs were injected in the flow cell and the $k_{on}$ was measured as the change of the refractive index over time when the antibodies flowed over and accumulated on the immobilized CDTb. The $k_{off}$ was measured as PBS was injected into the flow cell and the antibodies dissociated from CDTb. These antibodies had a fast on-rate and a slow off-rate, indicating that the protein-protein interaction between the antibody and CDTb was very strong. This analysis showed that anti-CDTb Fabs (Table 3.1) and IgGs A3, B8, B9, B10 and B12 (Table 3.2) have an estimated affinity of a $K_D$ value of $<10 \text{nM}$, with some Fabs and IgGs having a higher estimated affinity with a $K_D$ value of $<1 \text{nM}$. The order of affinity from highest to lowest binding Fabs was found to be $B8 > A3 > B12 > B10 > B9$ while the order of affinity from highest to lowest as IgGs was found to be slightly different with $B12 > A3 > B8 > B10 > B9$. The binding curves of the SPR data (Figure 3.4 and Figure 3.5) showed a good and reliable fit to the curves.

<table>
<thead>
<tr>
<th>Fab</th>
<th>Antigen</th>
<th>$k_{on}$ (1/Ms)</th>
<th>$k_{off}$ (1/s)</th>
<th>$K_D$</th>
<th>$R_{max}$</th>
<th>Chi$^2$</th>
<th>% Chi$^2$ of $R_{max}$</th>
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<tr>
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<td>1.33E-04</td>
<td>636pM</td>
<td>56.91</td>
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<tr>
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<td>2.42E-04</td>
<td>571pM</td>
<td>44.88</td>
<td>8.02</td>
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<td>930pM</td>
<td>23.25</td>
<td>5.66</td>
<td>24.34</td>
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</table>

Table 3.1 Surface plasmon resonance data for CDTb Fabs. The association rate constant ($k_{on}$) was determined by the rate at which the Fab bound to CDTb when injected into the flow cell. The dissociation rate constant ($k_{off}$) was determined by the rate at which the Fab was released from the ligand surface when PBS was injected. The $K_D$ is the ratio of $k_{off}/k_{on}$. For kinetic analysis, a 1:1 Langmuir model of global fittings of $k_{on}$ and $k_{off}$ was used. $R_{max}$ and Chi$^2$ are displayed to show the confidence of the fit. For a good fit to the model, Chi$^2$ should be less than ~20% of $R_{max}$. 
Table 3.2 Surface plasmon resonance data for CDTb IgGs. The association rate constant \((k_{on})\) was determined by the rate at which the IgG bound to CDTb when injected into the flow cell. The dissociation rate constant \((k_{off})\) was determined by the rate at which the IgG was released from the ligand surface when PBS was injected. The \(K_D\) is the ratio of \(k_{off}/k_{on}\). For kinetic analysis, a 1:1 Langmuir model of global fittings of \(k_{on}\) and \(k_{off}\) was used. \(R_{max}\) and \(\text{Chi}^2\) are displayed to show the confidence of the fit. For a good fit to the model, \(\text{Chi}^2\) should be less than \(~20\%\) of \(R_{max}\).

<table>
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<tr>
<th>IgG</th>
<th>Antigen</th>
<th>(k_{on}) (1/Ms)</th>
<th>(k_{on}) (1/s)</th>
<th>(K_D)</th>
<th>(R_{max})</th>
<th>(\text{Chi}^2)</th>
<th>% (\text{Chi}^2) of (R_{max})</th>
</tr>
</thead>
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<tr>
<td>A3</td>
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<td>1.98E-04</td>
<td>306 pM</td>
<td>54.46</td>
<td>8.24</td>
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<td>25.14</td>
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3.1.4 Epitope grouping of CDTb affinity reagents

To assess whether the anti-CDTb Fabs bound to the same or different epitopes on CDTb, I carried out competitive ELISAs to determine if purified Fabs could compete with phage-Fab clones. Competing Fabs indicated that these Fabs bound to the same epitope and were grouped. The ELISAs showed that the purified Fabs competed effectively with their own corresponding phage-Fab clones and that Fabs A3, B9, B10, and B12 all competed with each other (Figure 3.6). Phage-Fab B8 binding, however, was not inhibited by purified Fabs A3, B9, B10, and B12, but the corresponding phage Fabs of A3, B9, B10, and B12 was inhibited by binding of purified Fab B8. The competitive ELISA data suggested that Fabs A3, B9, B10, and B12 share the same CDTb epitope and Fab B8 may not share the same binding site as Fabs A3, B9, B10, and B12.

Figure 3.5 Surface plasmon resonance curves of the binding response for the CDTb IgGs. CDTb recombinant protein was immobilized on the sensor chip surface and different concentration of IgGs was injected into the flow cell (analyte 1-5). The binding curves correspond to the SPR data in Table 3.2. A) IgG A3 B) IgG B8 C) IgG B9 D) IgG B10 E) IgG B12.
Figure 3.6 Epitope grouping of CDTb antibodies. Phage-Fab competed with purified Fabs for the epitope binding site of CDTb. The Fabs indicated in the x-axis are purified Fabs that competed with the phage-Fab. Phage-Fab binding was detected using anti-M13-HRP. The bar highlighted in white showed autoinhibition. All purified Fabs inhibited phage-Fab binding except for Fab B8 (mean absorbance of phage-Fab binding ± SD with technical replicates of n = 3). A) Phage-Fab A3 B) Phage-Fab B8 C) Phage-Fab B9 D) Phage-Fab B10 E) Phage-Fab B12 binding was detected.
3.1.5 Competitive cell binding experiments by flow cytometry

I performed flow cytometry to determine if the five CDTb Fabs and IgGs can inhibit CDTb binding on LSR expressing mammalian cells. In order to detect binding of CDTb on cells, our collaborators (Dr. Panagiotis Papatheodorou and Dr. Klaus Aktories), provided a fluorescent dye-conjugated CDTb, CDTb-Dylight 488, which is also fully functional for the intoxication of cells in the presence of CDTa. HeLa (+LSR) cells are LSR overexpressing cell line created by Dr. Panagiotis Papatheodorou, and was used in our flow cytometry assay along with an LSR endogenously expressing colon cell line, HCT116 (Figure 3.7). HeLa cells normally do not express LSR and this cell-line served as an additional negative control for the assay. The schematic in Figure 3.7 outlines the flow cytometry assay for the CDTb inhibiting Fabs.

Figure 3.7 Schematic of the flow cytometry assay for determining CDT inhibition on cells and western blot of LSR expression. A) Western blot of LSR expression on HeLa (-LSR), HeLa (+LSR), and HCT116 cell lines used in the flow cytometry assay. LSR was detected using rabbit anti-human LSR (1:1000). Mouse anti-actin was used to detect actin as the loading control. B) In the flow cytometry schematic, it depicts one tube of cells being treated with CDTb-Dylight 488 and CDT-inhibiting Fabs, and the second tube of cells being treated with CDTb-Dylight 488 and MBP Fab. There should be a peak shift for tube 2 but not in tube 1 because MBP Fab does not inhibit CDTb binding but the Fabs that inhibit CDTb binding to LSR should inhibit CDTb-Dylight 488 binding on cells. HCT116 cells express less LSR than Hela (+LSR) cells and therefore there should be less of a peak shift when treated with only CDTb-Dylight 488.
In the absence of CDTb Fab or IgG, a fluorescent peak shift for CDTb-Dylight 488-treated HeLa (+LSR) and HCT116 cells could be seen; with median fluorescence intensity values greater than CDTb antibody-treated cells. The anti-CDTb Fab/IgG-treated HeLa (+LSR) cells had the same fluorescence peak shift as the untreated cells and CDTb-Dylight 488-treated HeLa (-LSR) cells (Figure 3.8 and Figure 3.10). Notably, HCT116 cells express LSR at a lower level than the HeLa (+LSR) cells and therefore a smaller fluorescence shift compared to the LSR over-expressing cell line was expected and seen. Strikingly, the antibodies prevented the CDTb-Dylight 488 binding to HCT116 cells and thus showed a peak shift similar to the untreated cells (Figure 3.9 and Figure 3.11). The flow cytometry results were the same when I pre-incubated CDTb-Dylight with the antibody for 1 hour at 4 °C or when I added the CDTb-Dylight 488 and antibody simultaneously. From the flow cytometry analysis, I showed that all five CDTb Fabs and IgGs inhibited CDTb binding on HeLa (+LSR) and HCT116 cells compared to MBP Fab/IgG control.

Figure 3.8 Flow cytometry of CDTb-Dylight 488 binding on HeLa (+LSR) cells when treated with Fabs. Fabs with CDTb-Dylight 488 were incubated on cells for 30 minutes and unbound CDTb was washed away. The fluorescence of the cells was then read. CDTb-Dylight 488 binding displayed a peak shift when cells were treated with only the CDTb. The Fab treated cells inhibited CDTb binding, as there was either a slight or no peak shift and it overlapped the peak representing the untreated cells. With the MBP Fab control treatment, there was a peak shift that overlapped the CDTb-Dylight 488 alone. HeLa cells do not express the receptor and therefore there was no peak shift. G = gated cell population. The table on the left shows the frequency of the gated cell population and the median fluorescent intensity (MFI) in area G of each treated samples. (MBP Fab-CDTb-Dylight 488 treated HeLa (+LSR) cells = 25.03 MFI, CDTb Fabs-CDTb-Dylight 488 treated HeLa (+LSR) cells < 12 MFI).
Figure 3.9 Flow cytometry of CDTb-Dylight 488 binding on HCT116 cells when treated with Fabs. Similar flow cytometric evaluation was performed as HeLa (+LSR) cells in Figure 3.8, and the effect of CDTb antibody inhibition on toxin binding was similar. G = gated cell population. The table on the left shows the frequency of the gated cell population and the median fluorescent intensity (MFI) in area G of each treated samples. (MBP Fab-CDTb-Dylight 488 treated HCT116 cells = 12.86 MFI, CDTb Fabs-CDTb-Dylight 488 treated HCT116 cells ≤ 11.44 MFI).

Figure 3.10 Flow cytometry of CDTb-Dylight 488 binding on HeLa (+LSR) cells when treated with IgGs. IgGs with CDTb-Dylight 488 were incubated on cells for 30 minutes and unbound CDTb was washed away. The fluorescence of the cells was then read. CDTb-Dylight 488 binding displayed a peak shift when cells were treated with only the CDTb. The IgG treated cells inhibited CDTb binding, as there was either a slight or no peak shift and it overlapped the peak representing the untreated cells. With the MBP IgG control treatment, there was a peak shift that overlapped the CDTb-Dylight 488 alone. HeLa cells do not express the receptor, and therefore there was no peak shift. G = gated cell population. The table on the left shows the frequency of the gated cell population and the median fluorescent intensity (MFI) in area G of each treated samples. (MBP IgG-CDTb-Dylight 488 treated HeLa (+LSR) cells = 24 MFI, CDTb IgGs-CDTb-Dylight 488 treated HeLa (+LSR) cells < 12 MFI).
Figure 3.11 Flow cytometry of CDTb-Dylight 488 binding on HCT116 cells when treated with IgGs. Similar flow cytometric evaluation was performed as HeLa (+LSR) cells in Figure 3.10, and the effect of CDTb antibody inhibition on toxin binding was similar. G = gated cell population. The table on the left shows the frequency of the gated cell population and the median fluorescent intensity (MFI) in area G of each treated samples. (MBP IgG-CDTb-Dylight 488 treated HCT116 cells = 14.3 MFI, CDTb IgGs-CDTb-Dylight 488 treated HCT116 cells < 10.5 MFI).

### 3.1.6 Cytotoxicity assay

To determine if my antibodies could functionally prevent the toxic effects of CDT in vivo, I carried out an cytotoxicity assay by treating HeLa (+LSR) cells with CDT (CDTa and CDTb) in the presence of the antibodies. The treatment was performed in serum-free media so that no other factors would affect the survival or death of the cells. The concentration of antibodies used was estimated by the in vitro binding assay and percent cell viability was measured using the cell viability reagent, PrestoBlue®. Prior to testing the antibodies for their protective properties, a dose-curve was conducted to determine the concentration of CDT that resulted in ~10-30% cell viability relative to untreated cells. A concentration of 1 nM CDT was found to be sufficient to kill ~70-90% of the cells and provided a window at which I could observe a change of cell survival when I treated the cells with the antibodies (Figure 3.12).
Figure 3.12 Cell viability dose-response curve of HeLa (+LSR) and HCT116 cells when treated with decreasing amount of CDT. HeLa (+LSR) and HCT116 cells were treated with decreasing concentration of CDT for 1.5 hours and cell viability was measured using PrestoBlue® after 24 hours (mean % cell viability ± SD with technical replicates of n = 2). A concentration of 1 nM of CDT (1 nM CDTa and 1 nM CDTb) as indicated by the arrows was the minimum amount of toxin that showed visible cell rounding and provided ~10-40% cell viability. 1 nM of CDT was chosen to be used in the functional assays.

Fabs/IgGs A3, B8, B9, B10, and B12-treated cells showed significant difference in cell survival against the toxin compared to the cells treated with MBP Fab/IgG control (p-value < 0.004, mean % cell viability ± SD with technical replicates of n = 3, student’s t-test). When CDT was pre-incubated with the Fab, the cell viability was 80-90% relative to the untreated cells. The cells that were treated with the control antibody, MBP Fab/IgG, did not show any protection against CDT and had a cell viability of 20%, which was equivalent to the cells treated with CDT alone (Figure 3.15). Fab A2 was previously shown in the in vitro binding assay to bind to CDTb but did not inhibit toxin-receptor interaction and it was used as an additional control in the cytotoxicity assay. Cell rounding was evident only with cells that were treated with MBP Fab/IgG, Fab A2, and CDTb alone (Figure 3.13 and Figure 3.14). Interestingly, when the CDT and IgG mixture was added directly to the cells without the pre-incubation step, the results were similar to the ones when I pre-incubated the CDT with the Fab (Figure 3.15).
Figure 3.13 Images of cells from the cytotoxicity assay that were treated with Fabs. Images of HeLa (+LSR) cells were taken using a bright field microscope after 1.5-hour treatment of pre-incubated CDT and Fab mixture. Cell rounding was evident in the panels with MBP Fab + CDT treated cells and CDT alone treated cells. Fab A2 was previously shown to bind to CDTb but did not inhibit toxin binding. Cells that were treated with Fab A2 + CDT served as an additional control along with the MBP Fab and showed cell rounding. Anti-CDTb Fab treated cells in the lower panels prevented the toxic effects of CDT and the cells exhibited the same morphological shape as the untreated cells. Each corner of the panels has an enlarged image of the cells.
Figure 3.14 Images of cells from the cytotoxicity assay that were treated with IgGs. Images of HeLa (+LSR) cells were taken using a bright field microscope after 1.5-hour treatment of CDT and IgG without the pre-incubation step. Cell rounding was evident in the panels with MBP IgG + CDT treated cells and CDT alone treated cells. Cells that were treated with MBP IgG alone served as a control and did not show cell rounding and therefore, it did not attribute to the cell rounding that was exhibited in the MBP IgG + CDT treated cells. Anti-CDTb IgG treated cells in the lower panels prevented the toxic effects of CDT and the cells exhibited the same morphological shape as the untreated cells. Each corner of the panels have enlarged image of cells.
Figure 3.15 Cell viability measurements from the cytotoxicity assays. The percent cell viability was calculated relative to the cell viability of untreated cells and student’s t-test was used to measure the significance of the difference in cell viability between anti-CDTb antibody treatments and MBP antibody control. A) 1 nM CDT was pre-incubated with 91 nM Fabs for 1 hour prior to treating the cells, and PrestoBlue® was used to measure the cell viability after treatment. Fab A2 was previously shown to bind to CDTb but did not inhibit toxin binding and was used as an additional control. All cells treated with the CDTb inhibiting Fabs showed cell viability similar to untreated and had greater cell viability than MBP Fab (*p-value < 0.001, mean % cell viability ± SD with technical replicates of n = 3) and Fab A2 treated cells.

B) 1 nM CDT and 100 nM IgG was used to treat HeLa (+LSR) cells without a pre-incubation step, and PrestoBlue® was used to measure the cell viability after treatment. All cells treated with the CDTb inhibiting IgGs showed cell viability similar to untreated and had greater cell viability than MBP IgG (***p-value < 0.004, mean % cell viability ± SD with technical replicates of n = 3).

From the cytotoxicity assay, I determined that ~100 nM of anti-CDTb Fab/IgG prevented intoxication of 1 nM CDT and increased cell survival but the minimum concentration of antibody that could provide the same level of protection against 1 nM of CDT was unknown.
Therefore, I carried out a cytotoxicity assay and decreased the concentration of antibodies by serially diluting the antibodies by two-fold. Strikingly, there was a correlation between the amount of added antibody and level of cell viability, and that there was a gradual decrease in cell viability, as the concentration of antibodies decreased. As a Fab, the ranking of the antibodies for their protective properties against CDT on HeLa (+LSR) cells was B12 > B8 > A3 > B9 > B10 (Figure 3.16A). Although Fab A3 initially had the highest cell viability from the dose-curve, the cell viability dropped more rapidly as the concentration of A3 decreased after 91 nM of Fab. Unlike Fab A3, I observed a steady drop of viable cells for Fabs B8 and B12 treatments with a decrease in effectiveness at 11 nM and 6 nM of treatment, respectively. The dose-curve was also performed on the HCT116 cell line and although the protective effects from Fab treatments were not as dramatic as on HeLa (+LSR) cells, I also observed decreased cell survival corresponding to Fab concentration (Figure 3.16B). As an IgG, the antibodies showed a similar trend in their effectiveness against CDT. The ranking of the IgGs for their protective properties on HeLa (+LSR) cells was A3 > B8 > B12 > B9 > B10 and generally showed increased cell survival compared to the Fab treatments as expected upon reformatting from monovalent Fabs to bivalent IgGs (Figure 3.17).

Figure 3.16 Cell viability dose-response curve of HeLa (+LSR) and HCT116 cells when treated with 1 nM of CDT and decreasing concentration of Fabs. A) HeLa (+LSR) cells that were treated with MBP Fab maintained a cell viability of 30-35% as the concentration of the Fab decreased. The CDTb Fabs displayed decreased cell viability from 60-98% to ~35% of viable cells as the Fab concentration decreased. CDT alone treated cells had a cell viability of ~12%. Fabs B9 and B10 provided the lowest protection, Fab A3 provided an intermediate protection, and Fabs B8 and B12 provided the highest protection against CDT intoxication. B) HCT116 cells that were treated with MBP Fab maintained a cell viability of 25% as the concentration of the Fab decreased. The CDTb Fabs displayed decreased cell viability from 90% to 25% of viable cells as the Fab concentration decreased. Fabs B9 and B10 provided the lowest protection, Fabs A3 and B12 provided an intermediate protection, and Fab B8 provided the highest protection against CDT intoxication.
Figure 3.17 Cell viability dose-response curve of HeLa (+LSR) cells when treated with 1 nM of CDT and decreasing concentration of IgGs. Cells that were treated with MBP IgG maintained a cell viability of 30-35% as the concentration of the IgG decreased. The CDTb IgGs displayed decreased cell viability from 70-98% to 35% of viable cells as the IgG concentration decreased. IgG B9 and B10 provided the lowest protection, IgG B12 provided an intermediate protection, and IgG B8 and A3 provided the highest protection against CDT intoxication (mean % cell viability for each concentration of IgG ± SD with technical replicates of n = 3).

Lastly, since I removed the toxins and antibodies after 1.5 hours of intoxication after cell rounding could be observed in the CDT only treated cells, I wanted to determine whether the antibodies could provide the same level of protection against CDT during longer periods of time. The cytotoxicity assay was carried out using 100 nM of the antibodies and 1 nM of CDT, and the CDT/antibody mixture remained on the cells for time points of 3 hours, 6 hours, and 12 hours. Interestingly, the cell viability of the treated cells at these time points remained the same at ~95% cell viability or more, suggesting that the antibodies could protect cells from 1 nM CDT longer term (Figure 3.18). Cytotoxicity assay that was conducted for 12 hours showed a significant difference in cell viability when treated with IgG A3, B8, B9, B10, and B12 from the MBP IgG control (p-value < 0.05, mean % cell viability ± SD with technical replicates of n = 2, student’s t-test).
Figure 3.18 Time course for CDT intoxication on HeLa (+LSR) cells with anti-CDTb IgG treatments. CDT (1nM) intoxication was conducted on HeLa (+LSR) cells with the addition of IgGs (100nM) for four time points: 1.5 hours, 3 hours, 6 hours, and 12 hours. After each time point, the CDT/IgG mixture was removed, and cell viability was measured using PrestoBlue® viability reagent after 24 hours. The percent cell viability was calculated relative to the cell viability of untreated cells and student’s t-test was used to measure the significance of the difference in cell viability between anti-CDTb IgG and MBP IgG control. As displayed, all the five anti-CDTb IgGs provided protection against CDT for all time points with a statistically significant increase in cell viability compared to the control (*p-value < 0.05, mean % cell viability ± SD with technical replicates of n = 2), except for IgG A3 at time point 1.5 hours and IgG B8 at time point 6 hours with p-values of 0.051 and 0.062, respectively (mean % cell viability ± SD with technical replicates of n = 2).

3.1.7 Immunofluorescence on F-actin of CDT and Fab-treated cells

Although I did not observe cell rounding in the Fab-treated cells, I could not conclude that the Fabs prevented all CDT molecules from entering the cell and prevented intoxication at the physiological level. Therefore, I assessed whether the Fabs could prevent actin depolarization in mammalian cells when treated with unconjugated CDTb or CDTb-conjugated Dylight 488 with CDTa by fluorescence staining of F-actin. In order to observe actin depolarization without causing complete cell death in the CDT only-treated cells, the incubation time for the treatments was decreased by half and the cells were fixed and stained with phalloidin-Alexa Fluor® 555. Phalloidin binds specifically to F-actin and thus the fluorescent label can detect the amount of F-actin present. There was less phalloidin-Alexa Fluor® 555 in MBP Fab + CDT treated-cells and CDT only-treated cells compared to the Fab + CDT-treated cells or untreated cells.
Additionally, the F-actin appeared fragmented in the cells that were not treated with anti-CDTb Fabs (Figure 3.19). The immunohistochemistry on both HeLa and HCT116 cells demonstrated that the cells retained the ability to polymerize G-actin to F-actin when treated with the Fabs as compared to untreated cells. Notably, the CDTb-DyLight 488 was seen present as clustered puncta in the cytoplasm of only MBP Fab + CDT-treated HCT116 cells and the CDT only-treated cells (Figure 3.20). By visualization of F-actin, I demonstrated that CDTb Fabs could prevent CDT entry into cells at a level that could also prevent the actin depolymerization caused by the toxin.

Figure 3.19 Immunofluorescence images of F-actin stained HeLa (+LSR) cells after anti-CDTb Fab and CDT (CDTb and CDTa) treatments. Treatments as indicated above the images were performed prior to staining of F-actin with phaloidin-Alexa Fluor® 555 (red). The nucleus was stained with Hoechst 33342 (blue). ~91 nM of Fab and ~1 nM of CDT (1 nM CDTb and 1nM CDTa) was used to treat the cells for 45 min. The panel with untreated cells was not treated with Fabs or CDT. Treatments with CDT in the presence of anti-CDTb Fabs, A3, B8, B9, B10, and B12 showed that the anti-CDTb Fabs prevented F-actin depolymerization as compared to the untreated cells. The panels with CDT only and MBP Fab-treated cells showed fragmented F-actin indicating F-actin depolymerization. MBP Fab is a Fab that binds to maltose binding protein and it was served as a control.
Figure 3.20 Immunofluorescence images of F-actin stained HCT116 cells after treatment with anti-CDTb Fab and CDT (CDTb-Dylight 488 and CDTa). Treatments as indicated above the images were performed prior to staining of F-actin with phalloidin-Alexa Fluor® 555 (red). The nucleus was stained with Hoechst 33342 (blue). ~727 nM of Fab and ~100 nM of CDT (100 nM CDTb-Dylight 488 and 100 nM CDTa) were used to treat the cells for 45 min. The panel with untreated cells was not treated with Fabs or CDT. Treatments with CDT in the presence of anti-CDTb Fabs, A3, B8, B9, B10, and B12 showed that the anti-CDTb Fabs prevented F-actin from depolymerization as compared to the untreated cells. The panel containing cells with CDT alone and MBP Fab treatments showed a depletion of F-actin indicating F-actin depolymerization. Additionally, CDTb-Dylight 488 (green) appeared as localized puncta in the cytoplasm of CDT only and MBP Fab-treated cells. MBP Fab is a Fab that binds to maltose binding protein and it served as a control.

3.2 Discussion

CDT is one of the known toxins produced by *C. difficile*, but its virulence role is not clear compared to the large clostridial toxins, toxin A and toxin B. Studies have shown that CDT is toxic to cultured cells that express the host receptor, LSR[73]; however A´B´CDT+ strains do not cause disease in an animal model[43]. Since CDT is not well understood, my objective was to generate inhibitors against CDT as research tools to understand its role in human disease. These inhibitors could be used to address the essentiality of CDT for *C. difficile* infection and it would
provide valuable insight into developing strategies to treat CDI. In this study, I used phage
display to develop synthetic human IgGs that bound to the receptor-binding domain of CDTb. By developing antibodies that target the receptor-binding domain of CDTb, I hypothesized that these antibodies could prevent CDT from entering the cells, and thus relieve the toxic effects of CDT on cultured cells.

From the antibody phage display selection on purified CDTb, I isolated five Fabs that bound and inhibited CDTb binding to the purified receptor, LSR, in an in vitro ELISA-based binding assay. Like toxins A and B, there were Fabs that bound to the receptor-binding domain of CDTb but not to the full-length CDTb. These Fabs may have bound to an epitope that was exposed in the truncated form of CDTb that may be masked by the folding of the missing portions in the full-length CDTb.

The CDTb-inhibiting Fabs have unique CDR sequences with very different amino acids in the VH3 and VL3 regions (Appendix A) but were shown to bind to the same epitope when phage-Fabs were competed with each purified Fabs for the binding site on CDTb in ELISAs. Binding of CDTb phage-Fabs, A3, B9, B10, and B12 to the immobilized CDTb were inhibited by binding of each corresponding purified Fabs which suggest that these Fabs bind to the same binding site or epitope. Fab B8 seemed to bind to the same epitope when purified form of B8 competed with phage-Fabs A3, B9, B10, and B12, however, this competition was unidirectional, as the phage-Fab of B8 could not be inhibited by purified Fabs A3, B9, B10, and B12. There could be two possible scenarios for this observation. It is possible that binding of purified Fab B8 occurred on a different epitope and this binding resulted in changes to the conformation of CDT in such a way as to alter phage-Fabs A3, B9, B10, and B12 epitope binding (Figure 3.21A). Thus, the phage-Fabs of A3, B9, B10, and B12 would not be able to bind. When purified Fabs A3, B9, B10, and B12 bound their epitopes, a similar alteration of the B8 epitope might not have occurred and allowed Fab B8 to bind to the epitope (Figure 3.21B). A second scenario could be that Fab B8 bound to the same epitope as Fabs A3, B9, B10, and B12 but bound to a slightly different position on the epitope from the other Fabs thereby allowing binding inhibition of phage-Fabs A3, B9, B10, and B12 due to steric hindrance by Fab B8 (Figure 3.21C). The binding of purified Fabs A3, B9, B10, and B12, however, did not cause steric hindrance for Fab B8 and allowed the phage-Fab to bind as suggested from the ELISA data (Figure 3.21D).
Figure 3.21 Hypothetical scenarios for binding in the epitope grouping ELISA data. A) Depicts the scenario where purified Fab B8 binds to its epitope (black circles) and changes the conformation of CDT and this conformational change masks some epitopes (green circles) that prevents the phage-Fabs A3, B9, B10, and B12 from binding to the antigen in the ELISA. B) Depicts the scenario where purified Fabs A3, B9, B10, and B12 bind to the same epitope that does not change the conformation and allowing phage-Fab B8 to bind. C) Depicts the scenario where purified Fab B8 binds to a different position of the same epitope than Fabs A3, B9, B10, and B12 and preventing the binding of these phage-Fab clones due to steric hindrance. D) Depicts the scenario where purified Fabs A3, B9, B10, and B12 bind to a different position of the epitope than Fab B8 but allows Fab B8 to bind.

Although there is a crystal structure of the enzymatic component of the toxin[127], x-ray crystallography of CDTb has not been reported to this date and much of the structural information is predicted from the iota toxin, Ib, and the protective antigen (PA) domains 1’ and 2’ of the anthrax toxin, both belonging to the binary toxin family[64,65]. Certainly, solving the structure of CDT in complex with the antibody would elucidate this issue and determine the exact epitope binding site of the Fabs. This structure would also provide a clue on how this toxin interacts with the receptor. Nevertheless, the competitive ELISAs suggest that there are possibly one or two epitopes on CDT that are readily accessible and essential for the toxin-receptor interaction. However, I cannot exclude the possibility of the presence of more than two epitopes required for the CDT-LSR interaction. A clear example is shown with neutralizing monoclonal
antibodies that have been developed to target the receptor-binding domain of toxins A and B produced by *C. difficile* [106,119,121]. These antibodies bind to different epitopes on the toxins and it has been shown that when the antibodies are combined as a cocktail it provides a synergistic effect [106].

The Fabs that bound to full-length CDTb but did not inhibit CDTb binding were not explored further, however, my data strongly suggests that these Fabs bound to different epitopes that were not involved in binding with the receptor, or inhibited CDTb binding at an insignificant level. Certainly, these Fabs can be used as potential tools for the detection of toxin in patient stools in combination with toxin A Fabs, potentially as bispecific IgGs. Additionally, non-inhibiting CDTb binders have a future prospect as tools to track CDTb inside cells. It has been shown that it is possible for IgGs to be internalized by the cell through receptor-mediated endocytosis, and has been explored as a tool to deliver drugs into cancer cells [128-130]. Therefore, my CDTb Fabs definitely hold promise in CDTb detection and other areas of *C. difficile* research.

All five Fabs that showed inhibition in the *in vitro* assay also showed inhibition in CDT cytotoxicity assays on mammalian cells expressing the receptor. As expected, Fab A2, which showed binding to the full-length CDT without inhibiting binding to LSR *in vitro* was not able to prevent cell rounding and cell death upon CDT intoxication on HeLa (+LSR) cells. The affinity of the Fabs correlated to the degree of protection against CDT in the does-curve. Fabs A3, B8, and B12 which have a pM-range affinity also provided the highest protection in the cytotoxicity assay compared to the nM-range affinity binders, Fabs B9 and B10, which showed a lesser degree of protection against CDT. These Fabs did not require a pre-incubation step with CDT in order to inhibit toxin entry in the assays but will likely provide a greater protective effect, if CDT was pre-incubated with the antibodies. Thus, the affinities obtained from the SPR can be trusted to be true affinities of the antibodies. HCT116 cells are less adherent than HeLa cells, and the cells were easily washed off and therefore the results were more variable compared to the HeLa cell lines. Nevertheless, the Fabs were able to prevent intoxication of 1nM of CDT at 91nM of Fab on the HCT116 cells. Additionally, the Fabs were able to prevent CDT intoxication at the physiological level for both the Hela and HCT116 cells, as there was no indication of aberrant actin polymerization in the CDT and Fab treated cells compared to untreated cells in the immunofluorescence stains. From immunofluorescence stains, it is apparent that the Fabs can completely block CDT entry by blocking CDTb binding. It also
validates that CDTa requires CDTb to bind to the receptor in order for CDTa to enter the cell and prevent polymerization of F-actin. Previously, my collaborators (Dr. Panagiotis Papatheodorou and colleagues) have shown that CDTa is the component that is essential for endocytosis of the CDTb/CDTa complex (unpublished work).

Immunoglobulin G is bivalent, leading to higher affinity compared to monovalent Fab due to avidity effects. As expected, the data from the SPR indicated that the IgG form had a higher affinity than the Fab. Additionally, the data from the dose-curve of the IgG + CDT treated HeLa (+LSR) cells showed that IgG can provide protection against CDT at a lower concentration than Fab. Not only could IgG block CDTb more effectively than Fab, it was also shown that they could provide protection for at least 12 hours against CDT on cells at a concentration of 100 nM. This result was not surprising since IgGs are quite stable and it has been shown that most IgGs are thermally stable as high as ~80 °C [106].

Taken together, it is apparent that my CDTb antibodies isolated from phage display selections can functionally block CDTb from binding to the receptor and prevent CDT entry. These are novel antibodies that can be useful as a tool to understand the role of CDT in virulence by determining if there is a synergistic protection against C. difficile infection when neutralizing anti-toxin A and anti-toxin B monoclonal antibodies in combination with my CDTb antibodies are used in an animal model. The CDTb antibodies may not only be used as research tools but perhaps also be used as a potential therapeutic upon further testing.

3.3 Materials and Methods

3.3.1 Recombinant CDTa and CDTb production and purification

The protein was kindly given to us by Dr. Carsten Schwan and Dr. Klaus Aktories from the Institute for Experimental and Clinical Pharmacology and Toxicology in Freiburg, Germany. The full-length CDTa and CDTb were cloned into pHIS1522 Bacillus megaterium expression vector where the C-terminal of the protein was tagged with 6x histidine tag. The toxin was expressed in Bacillus megaterium and the purification of the toxin was performed by Ni-affinity chromatography following an ion-exchange fractionation as described previously by my collaborators [73,131,132]. The full-length CDTb was treated with trypsin to obtain the
activated form of the toxin. The receptor-binding domain of CDTb (amino acid 677-876) was cloned into a pGEX-4T3 vector backbone containing GST tag located at the C-terminal end of the protein.

3.3.2 Recombinant LSR production and purification

LSR has several conserved cysteine residues that was predicted to form disulfide bonds that stabilize the protein. Previously, beta-mercaptoethanol was used in the lysis buffer for cell lysis in bacterial expression of LSR. This resulted in the destabilization of the protein due to the reduction of the disulfide bonds and subsequently the yield of the protein was poor, with the presence of nonspecific proteins. Therefore, expression of LSR was switched from a bacterial system to a mammalian system. The extracellular domain of LSR (amino acids 49-240, Accession # Q86X29) was cloned by Sheng Xue into a fusion Fc-vector, pFUSE-hIgG1-Fc2, (Invivogen, cat# pfuse-hg1fc2) and the protein was expressed in HEK293F cells. Protein A-sepharose beads were used to capture and purify ECD-LSR-Fc.

3.3.3 Phage display using Library F

The same protocol, as mentioned previously, was used except the library was pre-absorbed on GST (5 ug/ml) coated plates for 1 hour at room temperature with gentle shaking for each round and then transferred onto the antigen plate that was immobilized with 5 ug/ml of GST-RBD-CDTb.

3.3.4 ELISA for direct binding using Phage-Fab

The ELISA for determining the specificity of the phage-Fab clones was conducted the same manner as explained previously, except that the 384-well plates were coated with 2 ug/ml of GST-RBD-CDTb (41 nM), and GST control protein (77 nM) overnight at 4 °C.
3.3.5 Sanger sequencing for the CDRs of the Fabs

The PCR preparation of the phage samples were conducted the same manner as previously described for Sanger sequencing. CDR sequences of CDTb Fab A3, B8, B9, B10, and B12 are shown in Appendix A.

3.3.6 Purified Fab production

Purified Fabs were produced the same manner as explained previously.

3.3.7 Anti-CDTb IgG1 production

The heavy chains of the Fabs were subcloned into a mammalian IgG expression vector, pFUSEss-CHIg-hG1 (Invivogen, cat# pfuse-hchgl). The light chains of the Fab were subcloned into a separate mammalian IgG expression vector, pFUSE2ss-CLIg-hk (Invivogen, cat# pfuse2ss-hclk). Vector maps of these two plasmids are available on the Invivogen website. These Fabs were subcloned using a restriction enzyme-independent method developed by Walker et al. (2008). The cycling protocol for generation of the PCR products was: 94 °C for 3 min; 94 °C for 30 s, 60 °C for 10 s, 72 °C for 10 s repeated 35 times; 72 °C for 5 min. These products were purified using Qiagen PCR purification columns. These products were then mixed at 1:1 ratio and reannealed using the cycling protocol: 94 °C for 5 min, 50 °C for 3 min, and 20 °C for 3 min, to obtain sticky ended inserts that could be ligated into the vector of interest. The vector, pFUSEss-CHIg-hG1 for expressing the heavy chain was cut with EcoRI (NEB, cat# R0101) and NheI (NEB, cat# R0131), and the vector, pFUSE2ss-CLIg-hk, for expressing the light chain was cut with EcoRI and NcoI. The cut plasmid was dephosphorylated using Antarctic Phosphatase and inserts were phosphorylated using T4 Phosphonucleotidase Kinase to improve the cloning efficiency. The ligation was performed using T4 ligase. Successful ligated vectors were confirmed using PCR method with cycling protocol of: 94 °C for 3 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min repeated 24 times, and 72 °C for 7 min.

The plasmids containing the inserts of the desired heavy chain (500 ug) and light chain (500 ug) of the antibody were transfected into 293F cells using 293fectin transfection reagent (Invitrogen cat# 12347-019) and Opti-MEM (Invitrogen cat# 31985-062). The expression of the antibody
was controlled by the CMV promoter, which causes high and constitutive expression in mammalian cells. The protein was harvested from the media and purified 3-5 days after transfection. Culture supernatant was collected and incubated with protein A-sepharose beads for at least 1 hour at room temperature. The beads were collected using 20ml gravity flow columns and washed with 100x bead volume of 1x PBS. The antibody was eluted with 10 ml of 0.2 M Arginine, pH 3.0 and neutralized with 1 ml of 1 M Tris, pH 9.0. The antibodies were concentrated and buffer exchanged with PBS using the Amicon 30 kDa concentrators. For use on in vivo assays, the protein samples were sterile filtered using 0.22 uM Spin-X 700 ul spin filters (Corning, cat# 8160) and endotoxin testing was conducted on the samples.

3.3.8 ELISA for direct binding using purified Fabs

Binding specificities of the purified Fabs were assessed by ELISAs. 2 ug/ml of full-length CDTb (27 nM), GST-RBD-CDTb (41 nM), and GST control protein (77 nM) were coated overnight at 4 °C on protein-binding Nunc MaxiSorp® flat-bottom 384-well plates and incubated with 2 ug/ml of purified Fabs. The remaining steps were conducted in the same manner as explained previously.

3.3.9 ELISA-based binding assay of LSR-CDTb interaction

In order to determine whether the Fabs could inhibit LSR-CDTb interaction in vitro, 2 ug/ml (53nM) of human ECD-LSR-Fc was immobilized overnight at 4 °C on protein binding Nunc MaxiSorp® flat-bottom 384-well plates. The next day the wells were blocked with 60 ul of PBS-0.5% BSA solution for 1 hour. At the same time, 1 ug/ml (14 nM) of CDTb-His6 was pre-incubated with 10 ug/ml (182 nM) of Fabs in PBS-0.5% BSA on non-binding protein plate for 1 hour at room temperature. After the pre-incubation step, the CDTb/Fab mixture was transferred onto the blocked ECD-LSR-Fc immobilized plate and incubated for 30 min at room temperature. It was then washed 8 times with PBS-0.05% Tween 20 buffer and detected with anti-His6-HRP (1:5000 in PBS-0.5% BSA-0.05% Tween 20). The signal was developed with 25 ul of TMB substrate after 6 times wash with PBS-0.05% Tween 20 buffer. The reaction was stopped with 25 ul 1 M H3PO4 and the plate was read for absorbance at wavelength of 450 nm.
3.3.10 Surface Plasmon Resonance

Binding kinetics of the purified Fab proteins and IgG were determined by surface plasmon resonance using PROTEON (Biorad) with antigen immobilized on GLC chips at a density sufficient to produce ~100 response units when saturated with Fab/IgG. The SPR was conducted the same manner as previously described.

3.3.11 Epitope grouping

Epitope grouping was conducted the same manner as previously stated except that the immobilized protein was full-length CDTb.

3.3.12 Cell cultivation

HeLa (+/-LSR) cells were grown in DMEM (Invitrogen Gibco, cat# 11995) supplemented with 10% Fetal bovine serum (FBS), penicillin (4 mM), and streptomycin (4 mM). HCT116 cells were grown in McCoy’s 5A modified medium (Multicell, cat# 317-010-CL) supplemented with 10% FBS, penicillin (4 mM), and streptomycin (4 mM). All cells were incubated at 37 °C with 5% CO₂ under humidified conditions. These conditions were used for further cell-based assays.

3.3.13 Whole cell lysate preparation and Western blot

Adherent cells were washed 2 times with PBS. 1 ml of 1x lysis buffer (20 mM Tris-HCL pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 ug/ml leupeptin) with addition of PMSF (final concentration of 1 mM) was added per 10-cm dish and incubated on ice for 5 minutes. The cells were scraped and transferred to ice-cold 1.5 ml microfuge tubes. The cell suspension was passed through a 25-gauge needle 5-7 times. The extract was centrifuged for 10 min at 14,000 x g at 4 °C and the supernatant was transferred to new 1.5 ml tubes. Bradford protein assay (Thermo Scientific) was performed to determine the protein concentration. Protein sample buffer (2x
Laemmili, 5% beta-mercaptoethanol was added to the lysate in 1:1 ratio) and boiled for 10 min. The lysate protein was separated by 6-10% SDS-PAGE (Biorad) and transferred onto a polyvinylidene difluoride (PVDF) membrane. LSR was detected by using a polyclonal rabbit anti-LSR antibody (Santa Cruz Biotechnology, cat# sc-133765) and actin with monoclonal mouse anti-β actin antibody (Abcam, cat# ab13772).

3.3.14 CDTb-Dylight 488 inhibition on cells by flow cytometry

A concentration of 5 ug/ml (68 nM) of CDTb-Dylight 488 was pre-incubated with 40 ug/ml (727 nM) of Fabs for 1.5 hour at 4 °C in the dark. Cells were lifted by EDTA solution (1 mM disodium EDTA, 137 mM NaCl, 9 mM NaHCO₃, 5.6 mM dextrose, and 5.4 mM KCl, and filtered sterilized) and collected with PBS-0.1% BSA. 0.2-0.5 x 10⁶ cells were aliquoted per sample and washed with PBS-0.1% BSA once. The cells were incubated with the CDTb-Dylight/Fab mixture for 30 min at room temperature in the dark. The cells were then washed twice with 1 ml of PBS-0.1% BSA before re-suspended in PBS-0.1% BSA. The cell samples were subjected to flow cytometry analysis immediately using BD FACS Calibur and statistical analysis was conducted using Cell Quest.

Using the same concentration as stated previously, I combined CDTb-Dylight 488 and Fabs on cells at time 0 and incubated for 30 min at room temperature in the dark. The cells were then washed twice with 1 ml of PBS-0.1% BSA before re-suspended in PBS-0.1% BSA. The cell samples were subjected to flow cytometry analysis immediately using BD FACS Calibur and statistical analysis was conducted using Flow Jo and Cell Quest.

Flow cytometry of inhibition of CDTb-Dylight 488 using IgGs was conducted in the same manner as the Fabs. The concentration of the IgGs and CDTb-Dylight 488 used in the assay was 40 ug/ml and 5 ug/ml, respectively. The cell samples were subjected to flow cytometry analysis immediately using BD FACS Calibur and statistical analysis was conducted using Flow Jo.
3.3.15 Cytotoxicity assay

A dose-curve of CDT was conducted to determine the concentration of CDT that resulted in ~20-30% cell viability. The cells were serum-starved for 3 hours. CDT was serial diluted from 10 nM to 0.00001 nM in serum-free media and used to treat the cells for 1.5 hours. The toxin was removed and the media was exchanged with 10% serum-containing media. Cell viability was measured the next day by adding 1x PrestoBlue® reagent (Invitrogen, cat# A13261) and the fluorescent was read at 560-620 nm using a plate reader (Biotek). Percent cell viability was calculated by (treated cells / untreated cells) * 100%. Statistical analysis was conducted using the t-test.

5 ug/ml (91 nM) of Fab and 1nM of CDT (1nM CDTa and 1nM CDTb) were pre-incubated in serum-free media for 1 hour at room temperature. The mixture was added to cells that were starved in serum-free media for 3 hours. After 1.5 hours of intoxication, when cell rounding was evident in CDT alone well, the toxins and Fabs were removed and exchanged with 10% serum containing media. Cell viability was measured using PrestoBlue® after 24 hours and images were taken.

Based on the results in the cytotoxicity assay using the CDTb Fabs, I used 16 ug/ml of the IgGs which was equivalent to 100nM of IgGs for the assay. The assay was conducted the same manner as the CDTb Fabs.

3.3.16 Dose-response curve of antibodies in cytotoxicity assay

The Fabs were diluted by two-fold from 5 ug/ml (91 nM) to 0.3125 ug/ml (5.7 nM) in serum-free media and used to treat the cells with CDT (1nM CDTa and 1nM CDTb) after the cells were starved in serum-free media for 3 hours. After 1.5 hours of intoxication, when cell rounding was evident in the CDT only well, the toxins and Fabs were removed and exchanged with 10% serum containing media. Cell viability was measured using PrestoBlue® after 24 hours.

The dose-curve for IgGs were conducted using the HeLa cells and were diluted by two-fold from 16 ug/ml (100 nM) to 0.5 ug/ml (3.125 nM) in serum-free DMEM media and treated cells
with CDT (1 nM CDTa and 1 nM CDTb) after the cells were starved in serum-free media for 3 hours. The intoxication proceeded as stated previously.

3.3.17 Time-course for the cytotoxicity assay using IgGs

The intoxication of HeLa-LSR cells was conducted as stated previously. The toxins and IgGs were removed at time points 1.5 hours, 3 hours, 6 hours, and 14 hours, and replaced with serum-containing DMEM media. Cell viability was measured using PrestoBlue® after 24 hours.

3.3.18 Immunofluorescence

7000 HeLa (+LSR) cells and 15000 HCT116 cells were seeded on 96-well glass plates or coverslips in DMEM and McCoy’s 5A media, respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (4 mM), and streptomycin (4 mM). All cells were incubated at 37 °C with 5% CO₂ under humidified conditions. Prior to treatment, the cells were starved in respective serum-free DMEM and McCoy’s 5A media for 3 hours. Fabs (90 nM) and CDT (1 nM CDTa and 1 nM CDTb) in serum-free media were used to treat the cells for 45 min, followed by fixing the cells in 3.7% paraformaldehyde for 15 min and permeabilizing the cells with 0.1% Triton X-100 for 3 min. For the treatment of the HCT116 cells with CDTb-Dylight 488, I used 40 ug/ml (727 nM) of Fabs and 100nM of CDT (100 nM CDTa and 100 nM CDTb-Dyalight 488). Post-fixation and permeabilization, the cells were blocked in PBS-1% BSA for 30 min at room temperature and stained with phalloidin-Alexa Fluor® 555 (Invitrogen, cat# A34055) in PBS-1% BSA (1:1000) for 30 min in the dark at room temperature. Following this, the cells were washed with PBS twice and then stained with 2 ug/ml of Hoechst 33342 (Molecular Probes, cat# H3570). The cells were then washed and imaged using the Quorum confocal microscope (Quorum Technologies Inc.) at 63x magnification.
Chapter 4
Conclusions
4 Conclusions

4.1 Summary

Current neutralizing anti-toxin A and anti-toxin B monoclonal antibodies prevent recurrence of CDI but they do not decrease the severity of CDI or provide complete protection against *C. difficile* toxins in animal models[119] and clinical trials[105]. Additionally, there are no known inhibitors or antibodies that can block the third known but not well understood toxin, CDT. CDA1 and MDX1388 were developed using hybridoma technology, which is a traditional method for generating monoclonal antibodies[119]. Here, my aim was to use a newer technique called phage display to generate higher affinity and more specific antibodies against toxins A and B and novel antibodies against CDT.

In chapter 2, I covered my work on the recombinant protein production of truncated fragments of the RBD of toxins A and B, and using these fragments to select for anti-toxin A and anti-toxin B antibodies from the existing phage-Fab library called Library F, which was developed by Dr. Helena Persson. I described the selection process of phage display that I undertook to generate these antibodies and the *in vitro* binding assays used to determine the binding properties of the Fabs. In particular, I focused on Fab A3, which was the only Fab that bound to the full-length Toxin A but did not neutralize the toxicity of the toxin on polarized cells, Vero and CHO cells. I concluded that due to its low affinity Fab A3 was not able to compete with the receptor for the binding site of toxin A. However, Fab A3 could potentially be used as a tool for toxin A detection. I also outlined two other reasons that I was not able to develop neutralizing Fabs: 1) Fabs did not bind to a therapeutic epitope and 2) some epitopes present on the truncated fragments of the RBD of the toxins may have been masked by the remaining regions of the full-length toxin. Hence, these factors must be considered when attempting to develop antibodies that cause a functional change on a biological system.

In chapter 3, I outlined the steps of developing antibodies against CDT by using phage display. I presented two main groups of Fabs: 1) Fabs that bound to CDT but did not inhibit CDT-LSR interaction 2) Fabs that bound to CDT and inhibited the interaction. This data further emphasized the importance of binding to a “therapeutic epitope” in order to see a biological effect. I further described the binding properties and functional properties of the five Fabs that inhibited CDT-LSR interaction in the *in vitro* assay. These binders had subnanomolar and
picomolar affinities in SPR. With these high affinity measurements, it is not surprising that they completely blocked CDTb when 1 nM of toxin was used and prevented CDT intoxication on cells both as a Fab and as an IgG. I also illustrated that the anti-CDTb IgG can provide a long lasting protection for at least 12 hours from CDT intoxication.

Although I was not successful in generating antibodies that inhibit Toxin A and Toxin B entry, I developed a Fab that can bind to toxin A and perhaps by affinity maturing Fab A3 and converting to an IgG it could potentially be used as a high affinity binder for toxin A detection. By establishing antibodies that can block cellular entry of CDT, the antibodies could be used as a tool in *C. difficile* research and provide a stepping stone in understanding this toxin and its role in virulence.

### 4.2 Future experiments for CDTb antibodies

Although I have successfully generated CDTb antibodies that can protect cultured cells from intoxication from CDT, further validation experiments must be performed to determine the neutralization activity or any protective activity that these antibodies may have in an animal model and to further investigate the specificity of the antibodies towards CDT in the presence of other related toxins.

One of the key experiments that can be carried out with the CDTb antibodies is to test for cross reactivity against binary toxins from related species in the Clostridial family. As discussed, binary toxins from *C. perfringens* and *C. sporiforme* share a high sequence identity of 84% with CDT and from recent studies by Papatheodorou et al, these toxins also share the same host receptor[73,81]. Therefore, it would not be surprising if the CDTb antibodies can also inhibit entry of these toxins. Interestingly, preliminary studies from my collaborators have shown that my CDTb IgGs did not inhibit binding of the toxin from *C. sporiforme* in a flow cytometry experiment but perfectly inhibited binding of CDT. Further exploration of the cross-reactivity of these antibodies can prove valuable for inhibiting entry of multiple toxins produced by disease causing pathogens such as *C. perfringens* using a single antibody. Additionally, antibodies that are specific for only CDT can have other potential uses for diagnostic purposes.
From a recent publication, my collaborator (Dr. Papatheodorou) have shown using time-lapse and immunofluorescence microscopy that CDT causes changes in the morphology of the host cell[44]. In the study, upon CDT intoxication on human colon carcinoma cell line, Caco-2, CDT induced the formation of microtubule-based protrusions. It was proposed that these protrusions wrap the bacteria onto the surface of the intestinal cell and increases the adherence of the bacteria[44]. As one of the future experiments, I would like to use these antibodies to determine if they can decrease the formation of these microtubules and therefore, decrease *C. difficile* colonization in an *in vitro* and *in vivo* model that was established by Dr. Papatheodorou. The experiment would further validate the virulence role of CDT and prove advantageous as a therapeutic target.

Lastly, in order to further determine if CDT has a role in disease I can investigate if there is synergistic protection when current neutralizing anti-toxin A and anti-toxin B antibodies[119] in combination with my CDTb IgG antibodies are administered in BI/NAP1/027 infected mice. Increased protection when there is an addition of CDTb antibodies will indicate that CDT may have an important role in CDI.

### 4.3 Factors to consider for therapeutic use

The CDTb antibodies can potentially be used as a therapeutic to inhibit the entry of CDT toxins produced by *C. difficile*. Since the notorious BI/NAP1/027 strain produces all three toxins, it suggests that CDT may contribute to the severity or recurrence of the infection, which is a trait of the BI/NAP1/027 strain[22]. If CDT has a role in CDI, there are several main factors to consider in using the antibodies as a therapeutic.

When developing therapeutics, the route of administration of the antibodies is a factor to consider when treating the site of the infection. *C. difficile* infects the epithelial cells of the lower intestine and treatments directed to the intestine via oral delivery prove to be difficult due to the gastric acid and digestive enzymes. Like most proteins, antibodies will unfold in a highly basic or acidic environment and thus far, oral delivery of antibodies has not been successfully developed but studies in this field suggest that a fraction of the immunoglobulin may remain active and neutralize the target antigen[133,134]. Currently, antibodies are commonly delivered
intravenously. Although the site of CDI is in the lower intestine, CDA1 and MDX1388 antibodies can neutralize toxins A and B when administered using this route[119].

Once administered, the half-life and immunogenicity of the antibodies should be considered when engineering antibody therapeutics. The length of time the antibodies remain stable or in circulation before it is removed can affect the effectiveness of the treatment and the number of repeated doses that a patient must receive. However, stability of the immunoglobulin can be modified through mutations of key residues that provide a more stable framework without affecting the binding of the antibody[108,109,135,136].

The administration of therapeutic antibodies into patients without causing a serious immune response against the drug is a challenge that the field is gradually overcoming. Although the antibodies generated in the lab are fully human, which significantly reduces the risk of immunogenicity, there are still some immunological risks such as aggregation of the antibodies[137,138], cytokine release syndrome, and presence of CD4+ T cell epitopes[139] that should not be ignored and should be taken into consideration.

It is also important to note that the antibodies are primarily used to relieve the symptoms of CDI caused by the toxins until the bacterium is removed. To increase the efficacy, these antibodies are most likely would be used in combination with other treatments such as fecal transplantation to allow the microflora in the intestine to return to adequate levels in order to outcompete C. difficile while the symptoms dissipate.

4.4 Final remarks

Exploration of developing antibodies that target CDT receptor, LSR, and other domains of the toxins can be considered as a future direction for the lab that can help C. difficile research. Phage display is a powerful tool for developing specific antibodies but there are also improvements that can be made to increase the chances of developing functional antibodies. Improving antibody phage display selection by initially screening antibodies for functionality instead of just binding has been implicated in obtaining agonistic antibodies in a recent study in 2012[140]. If we can employ this technique to cell surface proteins such as LSR and obtain
antagonistic antibodies, then it will provide a rapid screening system for obtaining functional antibodies.
References


Appendices

**Appendix A: CDR sequences of the antibodies**

**CDRs of the VH and VL regions of Toxin A Fab A3**

Nucleotide VL (ToxA\_A3) CDRL3 variable region

GATATCCAGATGCCACCGTGCTGCTCCCTCTGTTGCCGCTCTGTTGGGCGATAGG
CACCATCACCTGGCGGTGGCCAGTCAGTCCTCAGCAGGAGCTGAGCTGGATCACA
GAAACAGGAAAAAGCTCCGAAGCTTCTGATGATTACTCGGCATCCAGCTCTACTC
GAGTCCCTTCTCGCTTCTCTGTTAGCCGTTYCGCCTCAGAGGATTTACATCTGACC
ACATCA GCAGTCTGACAGCAAGAACCCACCTGCAACCTTATTACTGTCAGCAAGCT
TCTCAGCAGCTGGGACAGGATACCAAG

Amino acid VL (ToxA\_A3) CDRL3 variable region

DIQMTQSPSSLSASVGRVTITCRASQSVSSAVAWYQKPGKAPKLIYSAASSLYSGVP
RSFGSRSGTDFTLTISSLQPFEDFYTYCQQAFYGSPFQGQK

Nucleotide VH (ToxA\_A3) CDRH1-3 variable region

GAGGTTCCAGCTGGGTGGGCTGGGCTGGTGGGCTGGCAGCAGGGGACTCAGACTCA
TTTGTCCTGGGCAGCTGTCGCTGCTTCAAACATCTCTTTATTATTATGACGACTGG
GTGGGCTAGGGGCAAGGGCTGAGGAGCTGAGCATTATTACCTATGTCTTATTATCT
GATATTATGCGATGCAGCTGACGGCAGGCTTTACTGAAAGGCTCTACTGACTACG
AACACAGCCCTACCTAAATGAACACGCTAAAGAGCTGAGGACACTGGGTCAAGGAA
CCCTGGTCACGGGACCGCCTCGG

Amino acid VH (ToxA\_A3) CDRH1-3 variable region

EVQLVESGGGLVQPGSSRLSCAASGFSNISYYMHWVRQAPKGLEWVASIPYSSYT
YYADSVKGRFTISADSKNTAYLQMNSLRAEDTAAYYCARSYWASWHAMDYWGQG
TLTVSS


Appendix A. Figure 1 Complementarity determining regions of Toxin A Fab A3 and wild-type VL and VH region.

CDRs of the VH and VL regions of CDTb inhibiting antibodies

Nucleotide VL (CDTb_A03) CDRL3 variable region

GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCTCTGTGGGCGATAGGGT
CACCCATCACCTGCGTGCGCTAGTCAGTCCCTGGTGCCACGGCTGTAGCTGTGATCACAA
GAAACCGAGAAAGCTCGGAAGCTTCTGATTATTAATCGGCATCCAGCCCTCTACTCCTG
GAGTCCCTTCTCCGTCTTTCTGTTAGCGCCTCGCGAGCTCCGATTCTCAGACCATCA
GCAGTCTGACGCCGGAAGACTTCGCAACTTATTACTGTACGAATTTACACGCTGGTG
ACCTGATCAGTTCGGGACAGGGTACCAAG

Amino acid VL (CDTb_A03) CDRL3 variable region

DIQMHTQSPSSLASAVGRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPS
RFSGSRSGTDFTLTISSLQPEDFAYYYCQQSYAGYLiTFGQGTK

Nucleotide VH (CDTb_A03) CDRH1-3 variable region

GAGGTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCG
TTGTCTCTGTGCACTCTCTGTGCCCTCAACCTCTTATATTTCTTCTTATATGGTGGCT
CAGGGCCCCGGTAAGGGCCTGGAATGGGTTGACATATATTCCCTTATATTGCTAT
ACTTTCTATGCGATAGCCGTCAAGGGGCTTCGATTTTACTATAAGCGCAGACACATCCAA
AAACACAGCCTACCTACAAATGGAACAGTAAAGCCTAGCAGGACACTGCGGTCTATT
ATTGTGCTCGCCCCTCTCTCCTCCGGCTTGCTGATGCTTGCTTCTACATCCGCTGGTG
GGCGTGGGACTACTGGGGTCAGGAAACCTGGCTACCGCCTCTCCCGC

Amino acid VH (CDTb_A03) CDRH1-3 variable region

EVQLVESGGGLVQPGGSLRLSCAASGFNSLYYSSIHWVRQAPGKGLEWVAYISSSYGYT
SYADSVKGRFTISADTKNTAYLQMNSLRAEDTAAYVYCARPSPPFPGHGSHYPVVAL
DYWGQGTLVTVSS
Nucleotide VL (**CDTb_B08**) CDRL3 variable region

GATATCCAGATGACCAGTCCCGAGTCCCCCTGTCCGCTCTGTGGCGATAGGT
CACCACACCTGCGGCTGAGTCCAGTCCGCTCCGCTCTGTAGCTTCATCAACA
GAAAACCAGGAAAAAGCTCCGAGGCTTTCTGTATTTACTCGGACATCAGGCTCTCT
GAGTCCCTTCGCTTTCTCTGTAGCCGCTTTCCGGGACGCCGATTCACTCTGACCACTCA
GCAGTCTCGACCCCGAAAGACCTCGCAACTACTACTGTCAAGCAATACACTACACTTCT
GGTCTTCTCCGACTACCTCGGACACGGGTACCAAG

Amino acid VL (**CDTb_B08**) CDRL3 variable region

DIQMTQSPSSLSASVGRVITCRASQSVSSAVAWYQQKPGKAPKLIYSASSLYSGVPS
RFSGSRSTDTFTLTISSLQPEDFYCYCQYYYFWSSPITFGQGTK

Nucleotide VH (**CDTb_B08**) CDRH1-3 variable region

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CAGGCCCCCGGTAAGGGCTGGAATGTTGTCTACTATTATTATTATTCTGCTATAT
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ATTTGCTCCTGCTACTACATTGGGTTTCGTGTATTTGACTACTGGGTCAAGGACACC
TTGCACCGTCTCTCTG

Amino acid VH (**CDTb_B08**) CDRH1-3 variable region

EVQLVESGGGLVQPGGSLRLSCASASGNFISISYYMYWHRQAPGKGLEWVSAIYSGVY
TYRYADVKGRFTISADTSKNTAYLMNLSRAEDTAVYCYCARYYHWGSIGDYWYGQLTV
VTSS

Nucleotide VL (**CDTb_B09**) CDRL3 variable region

GATATCCAGATGACCAGTCCCGAGTCCCCCTGTCCGCTCTGTGGCGATAGGT
CACCACACCTGCGGCTGAGTCCAGTCCGCTCCGCTCTGTAGCTTCATCAACA
GAAAACCAGGAAAAAGCTCCGAGGCTTTCTGTATTTACTCGGACATCAGGCTCTCT
GAGTCCCTTCGCTTTCTCTGTAGCCGCTTTCCGGGACGCCGATTCACTCTGACCACTCA
GCAGTCTCGACCCCGAAAGACCTCGCAACTACTACTGTCAAGCAATACACTACACTTCT
GGTCTTCTCCGACTACCTCGGACACGGGTACCAAG

Amino acid VL (**CDTb_B09**) CDRL3 variable region

DIQMTQSPSSLSASVGRVITCRASQSVSSAVAWYQQKPGKAPKLIYSASSLYSGVPS
RFSGSRSTDTFTLTISSLQPEDFYCYCQYYYFWSSPITFGQGTK

Nucleotide VH (**CDTb_B09**) CDRH1-3 variable region

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ATTTGTGCTCGGCAGCGCTTACTACTACGCTGTTGGACTA TGACTGGGTTCAAGGAAACCC
TGGTCACC CTCCCTCG

Amino acid VH (CDTb_B09) CDRH1-3 variable region

EVQLVESGGGLVQPGSSRLSCAASGFNLNYYSYMHWVRQAPGKGLEWVASISPYSGYT
YYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARAAAYYAGLDYWGQGTL
VTSS

Nucleotide VL (CDTb_B10) CDRL3 variable region

GATATCCAGATGACCAGCAGCCCGAGCCTCCTGTCCGCCCTGTGTTGGGCGATAGGGT
CACCATCACCTGCGTGCGAGTCAGTCCTGCTCTCCAGGCTCTGAGCTGGTATCAAAC
GAAACACAGGAAAAAGCTCCGAAAGCTTCTGATTTATCTCAGCATCAGCTCCTACCTCG
GAGTCCCTTCTCTGCCGTTATCCCGGACTTCCAGCAGATTTCACTCTGCCATCACA
GCAGTCTGAGCCCGAAGACTTCGCAACTTATTACTGTCAGCAATCTACTACCCGT
CTCTGTCCAGGTCCAGCAGGTGTCAAGG

Amino acid VL (CDTb_B10) CDRL3 variable region

DIQMTQSPSSLSASVGRDRVITCRASQSVSSAVAWYQQKPGKAPKLIYSASSLYSGVPS
RFSGRSGTDFTLTISSLQPEDFYYCYQQSYYPSLETFFGQGTK

Nucleotide VH (CDTb_B10) CDRH1-3 variable region

GAGGTTCACTGTGGTGAGTCTGCGCCGGTGCTCTGGCTGCAGCCAGGGGCTCAGTCCCG
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CAGGCCCCGGGTAAGGGGCGATGTTGCATATATATTCTCTCTACTCTATGCTATAT
ACTTCTATTGCAGGATAGCGGGCCACCTTTGAATAAGCCACACATCACA
AAACACAGGCTATCCACAAATGAAACAGCAGCTAAGAGCTGGTAGGACACTTGCCGGTCTATT
ATTGTGCTCGGCAGCTTCTACCCAGCGCTGACTGACTGGGTCAAGGAAACCCCTGG
TCACCCTCTCTCG

Amino acid VH (CDTb_B10) CDRH1-3 variable region

EVQLVESGGGLVQPGSSRLSCAASGFNLNYYSYMHWVRQAPGKGLEWVASISPYSGYT
YYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARAFYPYAMYDLYWGQGTLVT
VTSS

Nucleotide VL (CDTb_B12) CDRL3 variable region

GATATCCAGATGACCAGCAGCCCGAGCCTCCTGTCCGCCCTGTGTTGGGCGATAGGGT
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GAAACACAGGAAAAAGCTCCGAAAGCTTCTGATTTATCTCAGCATCAGCTCCTACCTCG
GAGTCCCTTCTCTGCCGTTATCCCGGACTTCCAGCAGATTTCACTCTGCCATCACA
GCAGTCTGAGCCCGAAGACTTCGCAACTTATTACTGTCAGCAATCTACTACCCGT
CTCTGTCCAGGTCCAGCAGGTGTCAAGG
Amino acid VL (CDTb_B12) CDRL3 variable region

DIQMTQSPSSLSASVGSQVSRVFTCATCRASQSIVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPS
RFSGSRSGTDFTLTISSLQPEDFATYYCQQAYPPLSLTFQGQGTK

Nucleotide VH (CDTb_B12) CDRH1-3 variable region

GAGGTTTCAGCTGGTGAGTCTGCGGCTGGCCTGGTGAAGCCAGGGGCTCACTCCG
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ACTTCTATTGCGGATAGGCTACAGGGCGGATTCACTATAAGGCGGAAACACTCCG
TTTCTTCTTCTTCTTCTTCTTTATTATGCTAT

Amino acid VH (CDTb_B12) CDRH1-3 variable region

EVQLVESGGGLVQPSMGVRSLSGAGFGFHYYYSMHWVRQAPGKGLEWVASISSYGGY
TSYADSVKGRFTISADTKNTAYLQMNLSRAEDTAVYYCARYYGLDYWGQTLVTVSS

Appendix A. Figure 2 Complementarity determining regions of CDTb Fabs/IgGs and wild-type of VL and VH region.
## Appendix B: Primer list

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<th>Description</th>
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<td>Forward primer for bacterial colony screen</td>
<td>GGGCTGGCAAGCCACGTTTGGTG</td>
</tr>
</tbody>
</table>

**Appendix B. Table 1** Primers used for cloning RBD fragments of toxin A and toxin B into IPTG expression vector.

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Description</th>
<th>Oligonucleotide DNA Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP19</td>
<td>Forward primer for sequencing VL</td>
<td>TGTAAAACGACGGCCAGTCTGTCATAA AGTTGTACCGG</td>
</tr>
<tr>
<td>HP20</td>
<td>Reverse primer for sequencing VL</td>
<td>CAGGAAACAGCTATGCCCTTTGGTAC CCTGTCCG</td>
</tr>
<tr>
<td>HP21</td>
<td>Forward primer for sequencing VH</td>
<td>TGTAAAACGACGGCCAGTGGACGCATC GTGCCCTTA</td>
</tr>
<tr>
<td>HP22</td>
<td>Reverse primer for sequencing VH</td>
<td>CAGGAAACAGCTATGCCCTTTGGTG AGGCCGAG</td>
</tr>
</tbody>
</table>

**Appendix B. Table 2** Primers used for sequencing VL and VH from Library F vector.

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Description</th>
<th>Oligonucleotide DNA Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH98</td>
<td>Reverse primer for PCR product A for subcloning into IPTG vector</td>
<td>ATGTGTGAGTTTTTGTCACAAGAT</td>
</tr>
<tr>
<td>RH99</td>
<td>Reverse primer for PCR product B for subcloning into IPTG vector</td>
<td>CTAGATGTGAGTTTTTGTCACAAGAT</td>
</tr>
<tr>
<td>RH100</td>
<td>Forward primer for PCR product A for subcloning into IPTG vector</td>
<td>CATGGCATTCCGATATCCAGATGCCCA GTC</td>
</tr>
<tr>
<td>RH101</td>
<td>Forward primer for PCR product B for subcloning into IPTG vector</td>
<td>GCATCCGATATCCAGATGCCCAAGTC</td>
</tr>
<tr>
<td>NE059</td>
<td>Forward primer for bacterial colony screen</td>
<td>GTAAACGACGGCCAGTTCATCGGCT TATAATGTG</td>
</tr>
<tr>
<td>NE069</td>
<td>Reverse primer for bacterial colony screen</td>
<td>CAGGAAACAGCTATGACCGACAGAAT CAAGTTTGCC</td>
</tr>
</tbody>
</table>

**Appendix B. Table 3** Primers used for amplification of PCR products for subcloning Fab into IPTG expression vector.

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Description</th>
<th>Oligonucleotide DNA Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sb116_Ig-G-HF1</td>
<td>Forward primer for heavy chain PCR product A for subcloning into IgG vector</td>
<td>AATTCAGAGGTTCAGCTGGTGAGTCC</td>
</tr>
<tr>
<td>Reference</td>
<td>Description</td>
<td>Sequence</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>sb117_Ig G-HF2</td>
<td>Forward primer for heavy chain PCR product B for subcloning into IgG vector</td>
<td>CAGAGGTTTCAGCTGGTGGAGGT</td>
</tr>
<tr>
<td>sb118_Ig G-HR1</td>
<td>Reverse primer for heavy chain PCR product A for subcloning into IgG vector</td>
<td>CCGAGGAGACGGTGACCAGG</td>
</tr>
<tr>
<td>sb119_Ig G-HR2</td>
<td>Reverse primer for heavy chain PCR product B for subcloning into IgG vector</td>
<td>CTAGCCGAGGAGACGGTGACCAGG</td>
</tr>
<tr>
<td>sb120_k-LF1</td>
<td>Forward primer for light chain PCR product A for subcloning into IgG vector</td>
<td>AATTCAGATATCCAGATGACCAGTC</td>
</tr>
<tr>
<td>sb121_k-LF2</td>
<td>Forward primer for light chain PCR product B for subcloning into IgG vector</td>
<td>CAGATATCCAGATGACCAGTC</td>
</tr>
<tr>
<td>sb122_hk-LR1</td>
<td>Reverse primer for light chain PCR product A for subcloning into IgG vector</td>
<td>GCTTTGATCTCCACCTTGGTACC</td>
</tr>
<tr>
<td>sb123_hk-LR2</td>
<td>Reverse primer for light chain PCR product B for subcloning into IgG vector</td>
<td>CATGGCTTTGATCTCCACCTTGGTACC</td>
</tr>
<tr>
<td>sb141_pF USE M13F</td>
<td>Forward primer for colony screen for heavy and light chains of the antibodies</td>
<td>TGTAACGACGCCATGTAAAGCTCA GGTGAGACCC</td>
</tr>
<tr>
<td>sb142_hG 4 M13R</td>
<td>Reverse primer for bacterial colony screen for the heavy chain of the antibodies</td>
<td>CAGGAAACAGCTATGACCAAGGTGTC ACAGCGCTTG</td>
</tr>
<tr>
<td>sb144_hK M13R</td>
<td>Reverse primer for bacterial colony screen for the light chain of the antibodies</td>
<td>CAGGAAACAGCTATGACCTAGGTGCTG TC CTTGCTGTCC</td>
</tr>
</tbody>
</table>

**Appendix B. Table 4** Primers used for subcloning Fab into IgG expression vector.
Appendix C: Absorbance fold change between target protein and controls in ELISAs

<table>
<thead>
<tr>
<th>Name of Fab</th>
<th>$A_{450\text{nm}}$ His-RBD toxin A</th>
<th>$A_{450\text{nm}}$ Neutravidin</th>
<th>$A_{450\text{nm}}$ BSA</th>
<th>$A_{450\text{nm}}$ His-RBD toxin A / $A_{450\text{nm}}$ Neutravidin</th>
<th>$A_{450\text{nm}}$ His-RBD toxin A / $A_{450\text{nm}}$ BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxA_A1</td>
<td>2.184</td>
<td>0.053</td>
<td>0.067</td>
<td>41.2</td>
<td>32.6</td>
</tr>
<tr>
<td>ToxA_A2</td>
<td>0.478</td>
<td>0.055</td>
<td>0.064</td>
<td>8.7</td>
<td>7.5</td>
</tr>
<tr>
<td>ToxA_A3</td>
<td>3.229</td>
<td>0.062</td>
<td>0.078</td>
<td>52.1</td>
<td>41.4</td>
</tr>
<tr>
<td>ToxA_A4</td>
<td>3.077</td>
<td>0.108</td>
<td>0.073</td>
<td>28.5</td>
<td>42.2</td>
</tr>
</tbody>
</table>

Appendix C. Table 1 Fold changes of toxin A Fabs binding to His$_6$-RBD$_{2304-2710}$-toxin A in ELISA compared to the background controls. 1 ug/ml of Fab was used.

<table>
<thead>
<tr>
<th>Name of Fab</th>
<th>$A_{450\text{nm}}$ His-RBD toxin B</th>
<th>$A_{450\text{nm}}$ Neutravidin</th>
<th>$A_{450\text{nm}}$ BSA</th>
<th>$A_{450\text{nm}}$ His-RBD toxin B / $A_{450\text{nm}}$ Neutravidin</th>
<th>$A_{450\text{nm}}$ His-RBD toxin B / $A_{450\text{nm}}$ BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxB_A5</td>
<td>0.25</td>
<td>0.061</td>
<td>0.055</td>
<td>4.1</td>
<td>4.6</td>
</tr>
<tr>
<td>ToxB_A6</td>
<td>1.363</td>
<td>0.073</td>
<td>0.07</td>
<td>18.7</td>
<td>19.5</td>
</tr>
<tr>
<td>ToxB_A7</td>
<td>0.545</td>
<td>0.067</td>
<td>0.063</td>
<td>8.1</td>
<td>8.7</td>
</tr>
<tr>
<td>ToxB_A8</td>
<td>1.283</td>
<td>0.081</td>
<td>0.063</td>
<td>15.8</td>
<td>20.4</td>
</tr>
<tr>
<td>ToxB_A9</td>
<td>2.027</td>
<td>0.166</td>
<td>0.11</td>
<td>12.2</td>
<td>18.4</td>
</tr>
<tr>
<td>ToxB_A10</td>
<td>2.428</td>
<td>0.056</td>
<td>0.058</td>
<td>43.4</td>
<td>41.9</td>
</tr>
<tr>
<td>ToxB_A11</td>
<td>0.832</td>
<td>0.061</td>
<td>0.065</td>
<td>13.6</td>
<td>12.8</td>
</tr>
<tr>
<td>ToxB_A12</td>
<td>0.232</td>
<td>0.15</td>
<td>0.099</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>ToxB_B1</td>
<td>0.215</td>
<td>0.055</td>
<td>0.063</td>
<td>3.9</td>
<td>3.4</td>
</tr>
<tr>
<td>ToxB_B2</td>
<td>0.064</td>
<td>0.055</td>
<td>0.056</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>ToxB_B3</td>
<td>2.125</td>
<td>0.057</td>
<td>0.056</td>
<td>37.3</td>
<td>37.9</td>
</tr>
</tbody>
</table>

Appendix C. Table 2 Fold changes of Toxin B purified Fabs binding to His$_6$-RBD$_{2286-2366}$-Toxin B in ELISA compared to the background controls. 1 ug/ml of Fab was used.

<table>
<thead>
<tr>
<th>Name of Fab</th>
<th>$A_{450\text{nm}}$ GST-RBD toxin B</th>
<th>$A_{450\text{nm}}$ GST</th>
<th>$A_{450\text{nm}}$ BSA</th>
<th>$A_{450\text{nm}}$ GST-RBD toxin B / $A_{450\text{nm}}$ GST</th>
<th>$A_{450\text{nm}}$ GST-RBD toxin B / $A_{450\text{nm}}$ BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxB_A5</td>
<td>0.266</td>
<td>0.061</td>
<td>0.059</td>
<td>4.4</td>
<td>4.5</td>
</tr>
<tr>
<td>ToxB_A6</td>
<td>1.367</td>
<td>0.249</td>
<td>0.1</td>
<td>5.5</td>
<td>13.7</td>
</tr>
<tr>
<td>ToxB_A7</td>
<td>0.716</td>
<td>0.169</td>
<td>0.071</td>
<td>4.2</td>
<td>10.1</td>
</tr>
<tr>
<td>ToxB_A8</td>
<td>1.919</td>
<td>0.243</td>
<td>0.078</td>
<td>7.9</td>
<td>24.6</td>
</tr>
<tr>
<td>ToxB_A9</td>
<td>1.823</td>
<td>0.974</td>
<td>0.384</td>
<td>1.9</td>
<td>4.7</td>
</tr>
<tr>
<td>ToxB_A10</td>
<td>1.699</td>
<td>0.078</td>
<td>0.056</td>
<td>21.8</td>
<td>30.3</td>
</tr>
<tr>
<td>ToxB_A11</td>
<td>0.103</td>
<td>0.163</td>
<td>0.069</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>ToxB_A12</td>
<td>0.32</td>
<td>0.437</td>
<td>0.139</td>
<td>0.7</td>
<td>2.3</td>
</tr>
<tr>
<td>ToxB_B1</td>
<td>0.124</td>
<td>0.073</td>
<td>0.056</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Name of Fab</td>
<td>$A_{450\text{nm}}$ His-RBD toxin B</td>
<td>$A_{450\text{nm}}$ Neutravidin</td>
<td>$A_{450\text{nm}}$ BSA</td>
<td>$A_{450\text{nm}}$ His-RBD Toxin B / $A_{450\text{nm}}$ Neutravidin</td>
<td>$A_{450\text{nm}}$ His-RBD toxin B / $A_{450\text{nm}}$ BSA</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
<td>-----------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>ToxB_B2</td>
<td>0.058</td>
<td>0.059</td>
<td>0.06</td>
<td>1.0</td>
<td>0.97</td>
</tr>
<tr>
<td>ToxB_B3</td>
<td>0.088</td>
<td>0.087</td>
<td>0.06</td>
<td>1.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Appendix C. Table 3** Fold changes of Toxin B purified Fabs binding to GST-RBD$_{2286-2366}$-Toxin B in ELISA compared to the background controls. 1 ug/ml of Fab was used.

<table>
<thead>
<tr>
<th>Name of Fab</th>
<th>$A_{450\text{nm}}$ His-RBD Toxin A</th>
<th>$A_{450\text{nm}}$ GST</th>
<th>$A_{450\text{nm}}$ BSA</th>
<th>$A_{450\text{nm}}$ His-RBD toxin A / $A_{450\text{nm}}$ GST</th>
<th>$A_{450\text{nm}}$ His-RBD toxin A / $A_{450\text{nm}}$ BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxA_A1</td>
<td>1.476</td>
<td>0.056</td>
<td>0.06</td>
<td>26.4</td>
<td>24.6</td>
</tr>
<tr>
<td>ToxA_A2</td>
<td>0.34</td>
<td>0.054</td>
<td>0.066</td>
<td>6.3</td>
<td>5.2</td>
</tr>
<tr>
<td>ToxB_B3</td>
<td>2.125</td>
<td>0.057</td>
<td>0.056</td>
<td>37.3</td>
<td>37.9</td>
</tr>
</tbody>
</table>

**Appendix C. Table 4** Fold changes of cross-reactive Fabs binding to His$_6$-RBD$_{2304-2710}$-Toxin A in ELISA compared to the background controls. 1 ug/ml of Fab was used.

<table>
<thead>
<tr>
<th>Name of Fab</th>
<th>$A_{450\text{nm}}$ His-RBD Toxin A</th>
<th>$A_{450\text{nm}}$ GST</th>
<th>$A_{450\text{nm}}$ BSA</th>
<th>$A_{450\text{nm}}$ His-RBD toxin A / $A_{450\text{nm}}$ GST</th>
<th>$A_{450\text{nm}}$ His-RBD toxin A / $A_{450\text{nm}}$ BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxA_A1</td>
<td>2.184</td>
<td>0.075</td>
<td>0.067</td>
<td>29.1</td>
<td>32.6</td>
</tr>
<tr>
<td>ToxA_A2</td>
<td>0.478</td>
<td>0.061</td>
<td>0.064</td>
<td>7.8</td>
<td>7.5</td>
</tr>
<tr>
<td>ToxB_B3</td>
<td>2.111</td>
<td>0.098</td>
<td>0.065</td>
<td>21.5</td>
<td>32.5</td>
</tr>
</tbody>
</table>

**Appendix C. Table 5** Fold changes of cross-reactive Fabs binding to His$_6$-RBD$_{2286-2366}$-Toxin B in ELISA compared to the background controls. 1 ug/ml of Fab was used.
Appendix D: Vector maps

A) Phagemid contains the genes for the light chain (VL-CL) and heavy chain (VH-CH1) of an antibody that can be expressed under the Pho A promoter. Due to the complexity of the Fab, the light chain is expressed separately from the heavy chain which is fused to a pIII coat protein. The Fab also has a hinge region and a flag tag. The phagemid has a selectable marker (Amp^r), dsDNA origin of replication for maintaining the plasmid in bacteria, and a f1 ori site for ssDNA replication and phage packaging.

B) The antibody fragment was subcloned into P3-His-Stop-IPTG vector for purified Fab expression in bacteria. The fragment was cloned into the site before the His_6 tag and the stop codon so that the fragment is expressed without the coat protein. The expression is under the Tac promoter and the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) induces the expression of the protein. LacIq has a mutation in the promoter that causes increased expression of the Lac repressor so that the fragment is expressed only in the presence of IPTG.

Appendix D. Figure 1 Phagemid of Library F and IPTG vector used for purified Fab expression. A) Phagemid contains the genes for the light chain (VL-CL) and heavy chain (VH-CH1) of an antibody that can be expressed under the Pho A promoter. Due to the complexity of the Fab, the light chain is expressed separately from the heavy chain which is fused to a pIII coat protein. The Fab also has a hinge region and a flag tag. The phagemid has a selectable marker (Amp^r), dsDNA origin of replication for maintaining the plasmid in bacteria, and a f1 ori site for ssDNA replication and phage packaging. B) The antibody fragment was subcloned into P3-His-Stop-IPTG vector for purified Fab expression in bacteria. The fragment was cloned into the site before the His_6 tag and the stop codon so that the fragment is expressed without the coat protein. The expression is under the Tac promoter and the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) induces the expression of the protein. LacIq has a mutation in the promoter that causes increased expression of the Lac repressor so that the fragment is expressed only in the presence of IPTG.
Appendix D. Figure 2 Expression vectors used for protein expression of the RBD fragments of Toxin A and B. A) The fragments were subcloned into the site before the Fc tag. The His<sub>6</sub> tag and stop codon was incorporated in the insert via oligonucleotides so that the fragment is expressed without the Fc tag and coat protein. The expression is under the Tac promoter and the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) induces the expression of the protein. LacIq has a mutation in the promoter that causes increased expression of the Lac repressor so that the fragment is expressed only in the presence of IPTG. The plasmid has a selectable marker (Amp<sup>r</sup>), dsDNA origin of replication for maintaining the plasmid in bacteria, and an f1 ori site. B) The RBD fragment of Toxin B was cloned into pHH0103. The vector is similar to pGC297 except it does not have an Fc tag. A His<sub>6</sub> tag and a GST tag are present at the 5’ end of the insert.