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Deciphering the domain specificity of *C. difficile* toxin neutralizing antibodies

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ABSTRACT

Clostridium difficile infection (CDI) is the principal cause of nosocomial diarrhea and pseudomembranous colitis associated with antibiotic therapy. The pathological effects of CDI are primarily attributed to toxins A (TcdA) and B (TcdB). Adequate toxin-specific antibody responses are associated with asymptomatic carriage, whereas insufficient humoral responses are associated with recurrent CDI. While the data supporting the importance of anti-toxin antibodies are substantial, clarity about the toxin domain specificity of these antibodies is more limited. To investigate this matter, combinations of human mAbs targeting multiple domains of TcdB were assessed using toxin neutralization assays. These data revealed that a combination of mAbs specific to all major toxin domains had improved neutralizing potency when compared to equivalent concentrations of a single mAb or a combination of mAbs against one or two domains. The function and toxin domain binding specificity of serum antibodies elicited by immunization of hamsters with a toxoid vaccine candidate was also assessed. Immunization with a toxoid vaccine candidate provoked toxin neutralizing antibodies specific to multiple domains of both TcdA and TcdB. When assessed in a toxin neutralization assay, polyclonal sera displayed greater activity against elevated concentrations of toxins than equivalent concentrations of individual mAbs. These data suggest a potential benefit of any antibody based therapeutic or prophylactic treatment that targets multiple toxin domains.

1. Introduction

Clostridium difficile is spore-forming toxin producing, Gram-positive anaerobic bacillus that when transmitted through the fecal–oral route can cause of pseudomembranous colitis and infectious diarrhea. *Clostridium difficile* infection (CDI) is a serious and increasingly common problem; in 2011 there were estimated to be half a million CDI and 29,000 deaths associated with CDI in the United States [1]. The primary virulence factors for *C. difficile* are the large clostridia toxins, TcdA and TcdB [1]. These two toxins are single chain proteins with 44% sequence identity and approximately 66% sequence similarity [2]. Both toxins are composed of four separate functional domains listed in order from N- to C-terminus of the protein: a glucosyltransferase domain (GTD), a cysteine protease domain (CPD), a translocation domain (TLD), which includes a pore forming region (PFR), and a C-terminal domain (CTD) composed of multiple smaller subdomains called combined repetitive oligopeptides (CROPs). The different toxin domains function collaboratively to inactivate small guanosine triphosphatases which leads to actin cytoskeleton disruption, destruction of tight junctions, induction of pro-inflammatory responses, and death of intestinal tissue [3–5]. While evidence for importance of antibody mediated toxin A and toxin B neutralization in protection against CDI associated symptomatic disease has been demonstrated in both preclinical [6–13] and clinical [14–19] studies, less is known about the specific epitopes associated with this antibody mediated protection. Recent studies in humans further emphasized the

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importance of toxin B specific antibody in protection [19]. To evaluate the contributions of different domain specific antibodies to the toxin neutralizing response, we used a panel of domain mapped human anti-TcdB mAbs to assess the neutralizing capacity of combinations of mAbs that were specific either to individual or to multiple toxin domains. While neutralization can be achieved by single mAb [20], combinations of mAbs that target multiple epitopes within a toxin domain or across multiple domains are able to neutralize far higher concentrations of toxin. Noting the potential importance of targeting multiple toxin domains, we assessed the domain specificities of antibodies induced by immunization with a *C. difficile* toxoid vaccine candidate. The majority of serum antibodies elicited by immunization of hamsters with this vaccine candidate bound to the CTD and GTD domains of TcdA and the GTD, CTD, and CPD + PFR domains of TcdB. Immunoabsorption experiments using recombinant fragments revealed that GTD and CTD fragments were required to impede the serum antibody neutralization of TcdA while a combination of GTD, CTD and CPD + PFR fragments was necessary for the inhibition of TcdB neutralization. This indicates that immunization with a toxoid vaccine candidate induces toxin neutralizing antibodies that bind epitopes located in multiple toxin domains.

In conclusion, while therapies that target individual toxin domains or epitopes are efficacious [21], these data suggest that targeting multiple epitopes or domains have the potential for even greater efficacy.

2. Methods

2.1. Recombinant C. difficile toxin fragment preparation

2.1.1. Generation of domain mapping reagents

Recombinant *C. difficile* toxin fragments used in the study are listed in Table 1. The CTDs of TcdA and TcdB as well as the GTD and TLD of TcdA and the GTD, TLD and CPD + PFR of TcdB were cloned, overexpressed, and purified as previously detailed [20]. Subfragments of the CTD; CROP B1, CROP B2, CROP B3, and CROP B4, were made as previously described [36]. Fragments designated B1, B2, B4 were defined by Kink and Williams [8]. Additional constructs used in the mapping studies were cloned and prepared in the procedures outlined below.

2.1.2. Cloning, expression, and purification of the MLD and ASE fragments of TcdB

The MLD fragment (amino acids 1–91) and ASE fragment (amino acids 95–586) were codon-optimized for expression in *E. coli* by Thermo Fisher Scientific (formerly GeneArt), cloned into pET28a + using the 5′ NcoI and 3′ XhoI restriction sites of the multiple cloning site, and expressed in BL21 Star (DE3). The resulting constructs had a His-tag with the sequence LPETGGHHHHHH following amino acid 91 and 586, respectively. Recombinant expression was induced in LB medium using 1 mM IPTG. Cultures were harvested after 4 h at 37 °C. Cells were pelleted by centrifugation, lysed by passage through a Y-110 Microfluidizer (Microfluidics, USA), and the soluble proteins were purified by nickel-affinity resin in batch mode followed by bind-and-elute chromatography on a HiTrap Q Sepharose HP column. The MLD and ASE fragments were concentrated on a Vivaspin 3 K spin column and exchanged into PBS using a Zeba column (Thermo). Purity was assessed at 95% by densitometry of the Coomassie-stained SDS-PAGE gels.

Table 1
C. difficile TcdA and TcdB recombinant fragments.

Toxin	Domain	Fragment name	Amino acids
B	GTD	MLD	1–91
	GTD	ASE	95–586
	GTD	B4	10–520
	CPD + PFR	B1	510–1110
	TLD	B2	1110–1530
	CTD	CROP B1	1834–2366
	CTD	CROP B2	1834–2101
	CTD	CROP B3	1949–2275
	CTD	CROP B4	2102–2366
A	GTD	GTD	300–660
	TLD	TLD	660–1100
	CTD	CTD	1811–2711

Toxin domain and recombinant fragment designations:
GTD - Glucosyltransferase Domain; MLD – Membrane Localization Domain; ASE - Enzymatic Subdomain; CPD - Cysteine Protease Domain; PFR – Pore Forming Region; TLD - Translocation Domain; CTD - C-Terminal Domain; CROP – Combined Repetitive Oligopeptide.

2.2. Dot blotting

Dot blotting (DB) was performed to confirm binding of each mAb to full length TcdA and TcdB. It was also used to investigate binding of some mAbs to the CTD. Briefly, 0.2 μg of protein (TcdA, TcdB or their recombinant CTDs) in 5 μl of PBS was spotted onto a Nitrocellulose membrane with a 0.45 μm pore size. The membrane was blocked by incubation in 5% dry milk in phosphate buffered saline (PBS) with 0.1% Tween-20 (PBST), 1 h at room temperature (RT) with agitation or overnight (ON) at 4 °C without agitation. The membrane was probed with the indicated mAbs at a concentration of 0.0215 μg/mL in blocking solution for 1 h at RT with agitation, or overnight at 4 °C without agitation. The membrane was washed 3x for 5 min with PBST at RT with agitation prior to incubation with the appropriate secondary antibody (goat anti-human IgG, alkaline phosphatase-labeled) diluted 1:1000 in PBST. The membrane was once again washed 3× for 5 min with PBST at RT with agitation before development with the colorimetric substrate 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT).

2.3. SDS-PAGE and Western Blotting

SDS-PAGE and Western Blotting (WB) were performed to investigate mAb binding to purified, recombinant toxin fragments. All SDS-PAGE and WB reagents were from the Thermo Fisher Scientific NU-PAGE Bis-Tris system. *C. difficile* toxins and recombinant toxin fragments were prepared for analysis by diluting to a concentration of 1 μg/15 μl in 1× LDS loading buffer and 1× sample reducing agent and heating to 95 °C for 5 min. Samples were loaded and run under reducing conditions at 1 μg/lane on 4–12% Bis-Tris gradient gels in 1× MES running buffer with antioxidant, at 200 V for 40 min at RT. Blotting was performed in the iBlot dry transfer apparatus onto Nitrocellulose membrane using the iBlot transfer stack set for 6 min. Proteins were visualized after SDS-PAGE by staining with Coomassie Brilliant Blue SimplyBlue safe stain (Thermo) used per the manufacturer's instructions. The membrane was then blocked by incubation in 5% dry milk in PBST, 1 h at RT with agitation or ON at 4 °C without agitation. The membrane was probed with the indicated mAbs at a concentration of 0.0215 μg/mL in blocking solution for 1 h at RT with agitation or ON at 4 °C without agitation. The membrane was washed 3× for 5 min with PBST at RT with agitation prior to incubation with the appropriate secondary antibody (goat anti-human IgG, alkaline phosphatase-labeled) diluted 1:1000 in PBST. The membrane was washed 3× for 5 min with PBST at RT with agitation before development using the colorimetric substrate BCIP/NBT.

2.4. Fortébio Octet assessments

The binding of mAbs to recombinant toxin fragments was determined by Bio-Layer Interferometry (BLI) using a FortéBio

Octet® Red96 (Pall). Sensors coated with Protein A were first wet for 10 min in FortéBio kinetics buffer (KB), PBS pH 7.4 containing 0.002% Tween-20 and 0.1 mg/ml bovine serum albumin (BSA). The sensors were then transferred to wells containing mAb at a concentrations ranging from 5 to 10 μg/ml in KB and the accumulation of antibody on the sensors was measured for 300 s. Next, the sensors were transferred to wells containing only KB for 300 s to wash off nonspecifically bound proteins. Throughout the experiment, samples were assessed at 30 °C with 1000 rpm agitation. Changes in thickness of the protein layer were measured and binding was recorded if observed.

2.5. C. difficile toxoid vaccine candidate

This *C. difficile* toxoid vaccine formulation is a formalin-inactivated, highly purified preparation of toxoids A and B from *C. difficile* reference strain VPI 10,463 [37,38] presented as a freeze-dried formulation. For the immunization of hamsters, the vaccine candidate was reconstituted with diluent and mixed with aluminum hydroxide adjuvant using a previously described method [39].

2.6. Generation of C. difficile toxoid vaccine candidate-induced sera in hamsters

All animal experiments were performed in compliance with European Directive 2010/63 and national regulations. Studies were conducted in animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The protocols were approved by the Committee on the Ethics of Animal Experiments at Sanofi Pasteur, France and all efforts were made to reduce the use of animals and to minimize pain and distress. Female Golden Syrian hamsters (*Mesocricetus auratus*) from Charles River Laboratories (Germany), 70–90 g, were used for immunization and challenge studies. Hamsters were injected three times via the intramuscular route, two weeks apart with 5 μg of *C. difficile* toxoid vaccine candidate. Animals were bled and sera were collected on day 35. On day 41, 10 mg/kg of clindamycin-2-phosphate antibiotic solution was administered via the intraperitoneal route to disrupt the gut microbiota and render the animals susceptible to subsequent lethal challenge. Twenty four hours later, hamsters were challenged intragastrically, using a feeding needle, with a predetermined lethal dose of live *C. difficile* spore-enriched preparations of SP041 US strain, ribotype 027 or reference strain VPI 10,463 (ATCC 43255). *C. difficile* spore preparations were prepared as described previously [40]. After *C. difficile* challenge, animals were housed individually in cages with isocaps. Post challenge, animals were monitored at least twice a day for body weight, morbidity and mortality. All immunized animals survived challenge.

2.7. Indirect ELISA with recombinant toxin domain fragments

Mapping of the antibody-binding epitopes within candidate vaccine-induced sera was performed by indirect ELISA using the following recombinant *C. difficile* toxin fragments: GTD and CTD of TcdA and B4 (GTD), B1 (CPD + PFR), B2 (TLD), and CROP B1 (CTD) of TcdB (Table 1). Pre-challenge sera from the *C. difficile* vaccine candidate immunized animals were tested. The serum pool was created by combining equal volumes of serum samples from 11 animals that survived subsequent lethal *C. difficile* challenge. Levels of the IgG specific to any given recombinant toxin fragment were normalized to the total IgG titers bound to the respective full length toxin, which was considered 100%. Plates were coated with 30 nM of either native purified TcdA or TcdB of designated toxin fragments in carbonate–bicarbonate coating buffer, pH 9.6, overnight at 2°–8 °C. Plates were washed between each step with PBST–0.05%. After overnight incubation and washing blocking buffer containing 1% BSA was added to each well of the plate for 1 h at 37 °C. After washing, two-fold serial dilutions of serum samples were prepared in diluent 0.2% BSA in PBS–0.05% Tween 20 and added to the wells and incubated for 1 h. Bound antibodies were detected with (HRP)-conjugated secondary antibody goat anti-hamster IgG (H + L) (Southern Biotech) at 1:8000 dilution. Plates were developed with the addition of the TMB substrate (KPL) for 20 min at 37 °C and then read at 650 nm using a Molecular Devices plate reader and SoftMax Pro software. The endpoint titer was defined as the reciprocal of the highest serum sample dilution that generated OD reading above 0.1.

2.8. Vero cell-based C. difficile toxin neutralization assay

The assay procedure was described in detail previously [20]. In brief, Vero cells were seeded in a 96 well plate at 2.5×10^4 cells/well and incubated overnight. Purified *C. difficile* toxinotype 0 TcdA and TcdB used in the assay were produced in-house from the reference strain VPI 10463 (ATCC 43255) as per internal procedures. One MC_{50} dose was 1.95 pM for TcdA and 0.016 pM for TcdB. Purified *C. difficile* toxinotype III TcdA and TcdB were purchased from tgcBIOMICS (Tcd-027, Bingen, Germany). One MC_{50} dose for TcdA and TcdB of toxinotype III was 1.43 pM and 0.1137 pM, respectively. For the antibody-dependent toxin neutralization assay, hamster immune sera or mAbs were mixed with the toxins at the concentrations noted in the figures. Even at the lowest concentrations of antibodies tested, there was at least 10-fold molar excess of antibodies over toxin. For the immunoabsorption assays, recombinant fragments of TcdA and TcdB were mixed at the indicated concentrations with hamster immune sera at final dilutions of 1:12000 or 1:14000 for TcdA assays or 1:8000 for TcdB assays and either TcdA or TcdB at final concentration of $4 \times MC_{50}$ which is 7.8 pM and 53.63fM, respectively. The immune hamster sera dilutions were near top of the linear range but not within the upper asymptote as was determined in prior toxin neutralization assays performed with a $4 \times MC_{50}$ concentrations of either toxin. An excess of recombinant toxin domain fragments were mixed with the immune sera to bind or absorb all of the antibodies specific to that domain thereby preventing those particular domain-specific antibodies from interacting with the toxin. The recombinant toxin fragments of the GTD, CTD of TcdA and B4 (GTD), B1 (CPD + FPR), and CROP B1 (CTD) of TcdB (Table 1) were used for inhibition studies. After 1 h of incubation, medium was removed from the Vero cell monolayer and 100 μl of antibody-toxin or antibody-toxin-toxin fragment mixture was added. After 72 h of incubation at 37 °C with 5% CO_2, the antibody-toxin or antibody-toxin-toxin fragment mixture was removed and the cells were washed twice with 120 μl of L-glutamine, FBS and phenol red-free MEM medium and 100 μl of the medium and 10 μl of AlamarBlue (Invitrogen) were added to each well. The plates were mixed and incubated at 37 °C for 4 h before reading fluorescence at 560 to 590 nm with a cutoff at 590 nm. The fluorescence results were plotted over antibody concentration or over concentration of the respective recombinant fragments for the fragment inhibition studies. Results in the Table 3 are shown as NT50 and maximum % completeness defined as antibody concentrations providing 50% neutralization and maximum neutralization at the highest antibody concentration tested. Testing was performed in at least three separate experiments. Intra-assay precision was 20%.

2.9. Transepithelial electrical resistance C. difficile TcdB neutralization assay

The assay procedure was described in detail previously [20]. In brief, T84 human colonic carcinoma cells (ATCC CCL-248) were

seeded into 0.4 μm polyester transwell plates (Costar) at a seeding density of 3.6×10^5 cells/cm^2. T84 cells are derived from a human colonic adenocarcinoma. The cells were maintained at 37 °C with 5% CO_2 in complete culture medium for 10–12 days until stable transepithelial resistance was achieved. Medium was replaced in both the upper and lower compartments daily from day 6 and on the day of assay. Purified *C. difficile* toxinotype 0 TcdB used in the assay was produced in-house from the reference strain VPI 10463 (ATCC 43255). For testing of neutralizing activity of mAbs, TcdB was combined with antibody at a 1:1 ratio by volume and incubated at 37 °C for 30 min before 100 μl of mixture being added to polarized T84 cells. TcdB TEER assays were performed by adding TcdB -antibody combinations to the lower compartment of the transwell. A final concentration of 0.83 nM TcdB of toxinotype 0 was used for the challenge dose and was equivalent to 15 $xTEER_{50}$. The controls consisted of at least one well per plate of toxin challenged without antibody and one well containing medium only. Transepithelial resistance was measured immediately (T_0) before toxin-antibody sample addition and then after 2.5 h to 5 h ($T_{2.5} - T_5$) of incubation at 37 °C and 5% CO_2. Percent TEER loss was determined using the equation $[(T_0 - T_{2.5})/T_0] \times 100\%$ - %TEER loss in negative well. The percent protection for antibody was calculated using the equation: (% TEER loss in toxin-only challenge) - (% TEER loss in antibody neutralized toxin challenge). The percent protection results were plotted versus mAb concentration. Results in the Table 3 are shown as NT50 and maximum % completeness defined as antibody concentrations providing 50% neutralization and maximum neutralization at the highest antibody concentration tested. Testing was performed in at least three separate experiments. Intra-assay precision was 20%.

3. Results

3.1. Mapping of human C. difficile toxin neutralizing mAbs at toxin domain level

In a prior publication the identification, selection, and generation of recombinant toxin-neutralizing human mAbs was described [20]. Using recombinant toxin domain fragments (Table 1) and a combination of techniques including Western immunoblotting, dot blotting and Fortébio Octet binding assessments, the domain specificity of 33 of these 39 mAbs was determined (Table 2). The 6 mAbs which bound to holotoxin but not to any of the recombinant domains were designated as unmapped. The majority of anti-TcdA mAbs (71%) bound to the CTD; 14% of the anti-TcdA mAbs bound to the TLD, and a smaller proportion (7%) recognized GTD. The remaining 7% of anti-TcdA mAbs could not be mapped to the available fragments. In contrast, the anti-TcdB mAbs bound more equivalently across the TcdB domains: 23% to GTD (B4 fragment), 19% to CPD + PFR (B1 fragment), 19% to TLD (B2 fragment) and 19% to CTD (CROP B1-B4 fragments). Nineteen percent of the anti-TcdB mAbs could not be mapped to a specific recombinant fragment.

The toxin neutralizing activities of individual mAbs were assessed in the Vero cell cytotoxicity and the T84 cell transepithelial electrical resistance (TEER) assays using toxinotype 0 toxins. The activities for the anti-TcdB mAbs used in the combination experiments (Figs. 1–4) are listed in Table 3. The activities for these mAbs ranged from 0 (no activity) to 100% (complete toxin neutralization). While both assays measured the ability of antibody to neutralize the impact of toxin, the activities of different mAbs were not always aligned between the two assays suggesting that

Table 2
Mapping of human *C. difficile* toxin neutralizing mAbs at toxin domain level.

Anti-TcdB mAbs				Anti-TcdA mAbs				Anti-TcdA/B mAb			
Anti-TcdB mAb	TcdB domains	TcdB fragment	Mapping method	Anti-TcdA mAb	TcdA domains	TcdA fragment	Mapping method	Anti-TcdA TcdB mAb	TcdA and TcdB domains	Tcd fragment	Mapping method
B1	GTD	ASE	WB	A1	CTD	CTD	DB/WB	A14/B26	CTD (A, B)	CTD (A), CROP B2 (B)	DB/WB (A), WB/Octet (B)
B2	GTD	MLD	WB	A2	CTD	CTD	DB/WB				
B3	GTD	ASE	WB	A3	CTD	CTD	DB/WB				
B4	TLD	B2	WB	A4	UM	N/A	N/A				
B5	TLD	B2	Octet	A5	CTD	CTD	WB				
B6	CTD	CROP B2/B3	WB/Octet	A6	CTD	CTD	DB				
B7	TLD	B2	Octet	A7	CTD	CTD	DB/WB				
B8	CTD	CROP B2	WB	A8	GTD	GTD	WB/Octet				
B9	GTD	ASE	WB	A9	CTD	CTD	DB/WB				
B10	UM	N/A	N/A	A10	CTD	CTD	DB/WB				
B11	UM	N/A	N/A	A11	CTD	CTD	DB/WB				
B12	CTD	CROP B3	DB/WB	A12	TLD	TLD	WB				
B13	CPD + PFR	B1	WB	A13	TLD	TLD	WB				
B14	UM	N/A	N/A								
B15	TLD	B2	Octet								
B16	UM	N/A	N/A								
B17	TLD	B2	Octet								
B18	CPD + PFR	B1	WB								
B19	GTD	MLD	WB								
B20	GTD	B4	Octet								
B21	CTD	CROP B3/B4	WB/ Octet								
B22	CPD + PFR	B1	WB								
B23	CPD + PFR	B1	WB								
B24	CPD + PFR	B1	WB								
B25	UM	N/A	N/A								

Recombinant fragments designations:
GTD - Glucosyltransferase Domain; CPD - Cysteine Protease Domain; PFR – Pore Forming Region; TLD - Translocation Domain; CTD - C-Terminal Domain; MLD – Membrane Localization Domain; ASE - Enzymatic Subdomain; CROP – Combined Repetitive Oligopeptide; UM – Unmapped.
Mapping methods designations:
WB –Western Blot; DB-Dot Blot; N/A – not applicable.
For all indicated fragments, as the sequences of some fragments are embedded within other fragments, only the smallest fragment bound is listed.

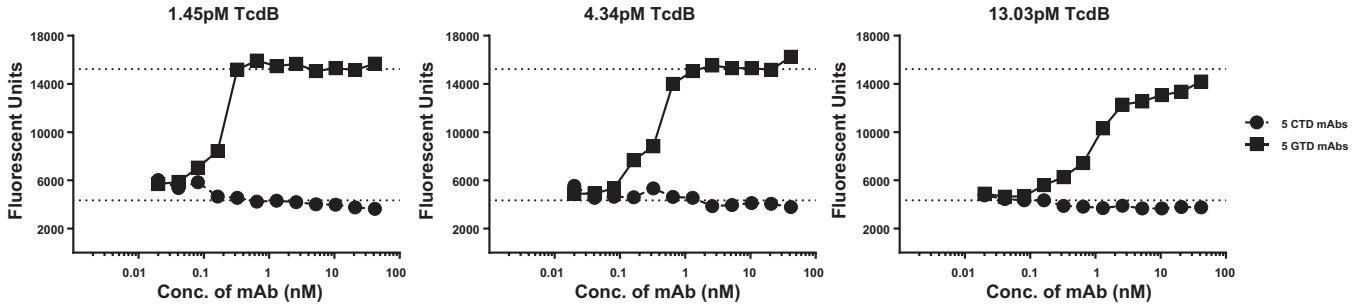

Fig. 1. The 5 GTD-specific mAb combination showed superior neutralizing potency in comparison to the 5 CTD-specific mAbs combination in the Vero cell neutralization assay. The concentrations of *C. difficile* TcdB toxinotype 0 used in the Vero cell neutralization assays are indicated in the graphs. Total concentrations of mAb combinations are shown in X axes. Upper and lower dotted lines indicate limits of the assay, fluorescent signals for positive and negative controls. The upper line marks the fluorescent signal for Vero cells in the absence of toxin which defines 100% neutralization of the toxin and the lower line marks the signal for Vero cells mixed with TcdB in the absence of neutralizing antibodies which defines 0% neutralization of TcdB. Monoclonal Ab designations: 5 GTD mAbs: B1, B2, B9, B19, B20; 5 CTD mAbs: B6, B8, A14/B26; B12, B21.

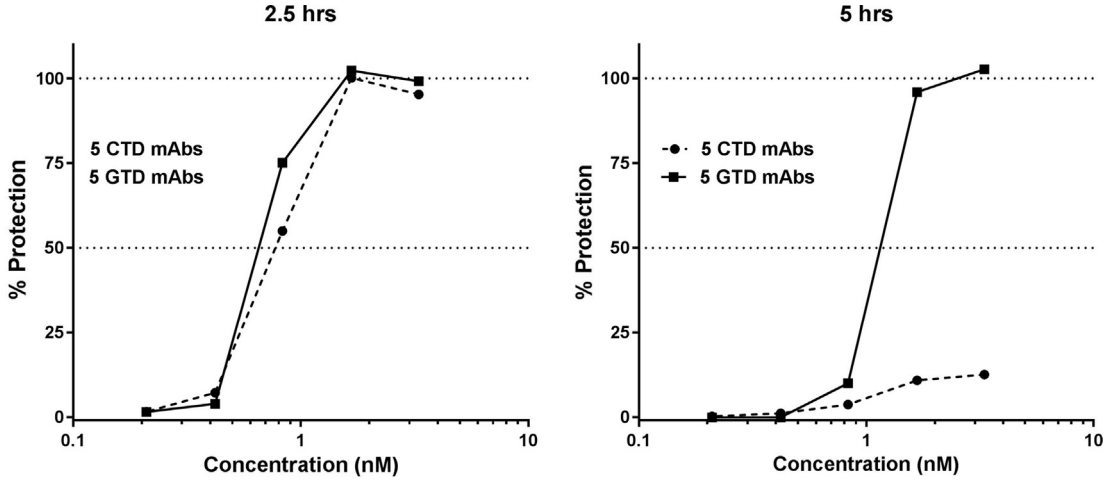

Fig. 2. While not distinguishable after 2.5 h of exposure to the TcdB, after 5 h of exposure, the 5 GTD-specific mAb combination showed superior neutralizing potency in comparison to the 5 CTD-specific mAbs combination in the TEER assay. Exposure time (2.5 h or 5 h) to *C. difficile* TcdB, toxinotype 0 is indicated in the graphs. Total concentrations of the mAb combinations are shown on the X axes. Upper and lower dotted lines indicate 100% and 50% protection marks respectively. Monoclonal Ab designations: 5 GTD mAbs: B1, B2, B9, B19, B20; 5 CTD mAbs: B6, B8, A14/B26; B12, B21.

different mechanisms of toxin action are being assessed. These differences could stem from differences in the assays themselves including the different cells used, toxin concentrations, or functional readouts for each assay.

3.2. Enhancement of neutralizing activity of human anti-TcdB mAbs to different toxin domains when used in combination

We had previously shown that the combination of two mAbs to the GTD domain of TcdB (B1 and B2) provided improved *in vitro* toxin neutralizing potency and *in vivo* efficacy over individual mAbs [20]. In order to define the most potent combination of mAbs, we examined the impact of combining more than two mAbs to a single domain as well as combining mAbs specific to multiple domains. All the mAbs used in these combinations neutralized toxins in at least one of the two *in vitro* assays (Table 3), therefore in order to differentiate the neutralizing potencies of combinations of anti-TcdB mAbs, a more stringent "stress test" version of *in vitro* toxin neutralization assays was needed. For the Vero cell cytotoxicity assay, the combination of mAbs were evaluated at TcdB concentrations up to 117. 29 pM. In the "stress" TEER assay both the toxin concentration (0.83 nM) and time of exposure (5 h) were increased over what was used for the assessment of individual mAbs. When assessed using the "stress" Vero cell test, none of the individual mAbs exhibited neutralizing activity against TcdB concentrations of >1.45 pM (data not shown). We first examined the impact of increasing number of mAbs targeting a single domain by creating cocktails of 5 GTD only mAbs (B1, B2, B9, B19, B20) or 5 CTD only mAbs (B6, B8, A14/B26, B12, B21). The combination of mAbs specific to the GTD domain showed the best neutralizing potency (Fig. 1) while the combination of only CTD-specific mAbs showed no activity against the elevated TcdB concentrations. This overall ranking for the mAb combinations was confirmed in a stress version of the TEER assay. The potencies of these mAb combinations were not distinguishable after 2.5 hrs of exposure to the TcdB, however, by 5 hrs of exposure, the GTD only combination continued to exhibit a potent neutralizing response, whereas the cocktail of the CTD-specific mAbs showed considerable impairment of its ability to protect the cell monolayer against intoxication (Fig. 2). To examine the limits of increasing the domain coverage we assessed the impact of adding a single anti-CTD mAbs (B6) to the already potent cocktail of the five GTD-specific mAbs (B1, B2, B9, B19, and B20) (Fig. 3) in the Vero cell cytotoxicity assay. The GTD-only mAbs cocktail fully protected against toxin concentrations of up to 4.3 pM, however, the addition of a single anti-CTD mAb increased the protective capacity up to 39.1 pM. This result was particularly striking as the B6 mAb had no activity in the Vero cell assay when tested independently at 0.064 pM TcdB (Table 3). We next examined the impact of targeting domains beyond the highly immunogenic CTD and GTD domains as well as the impact

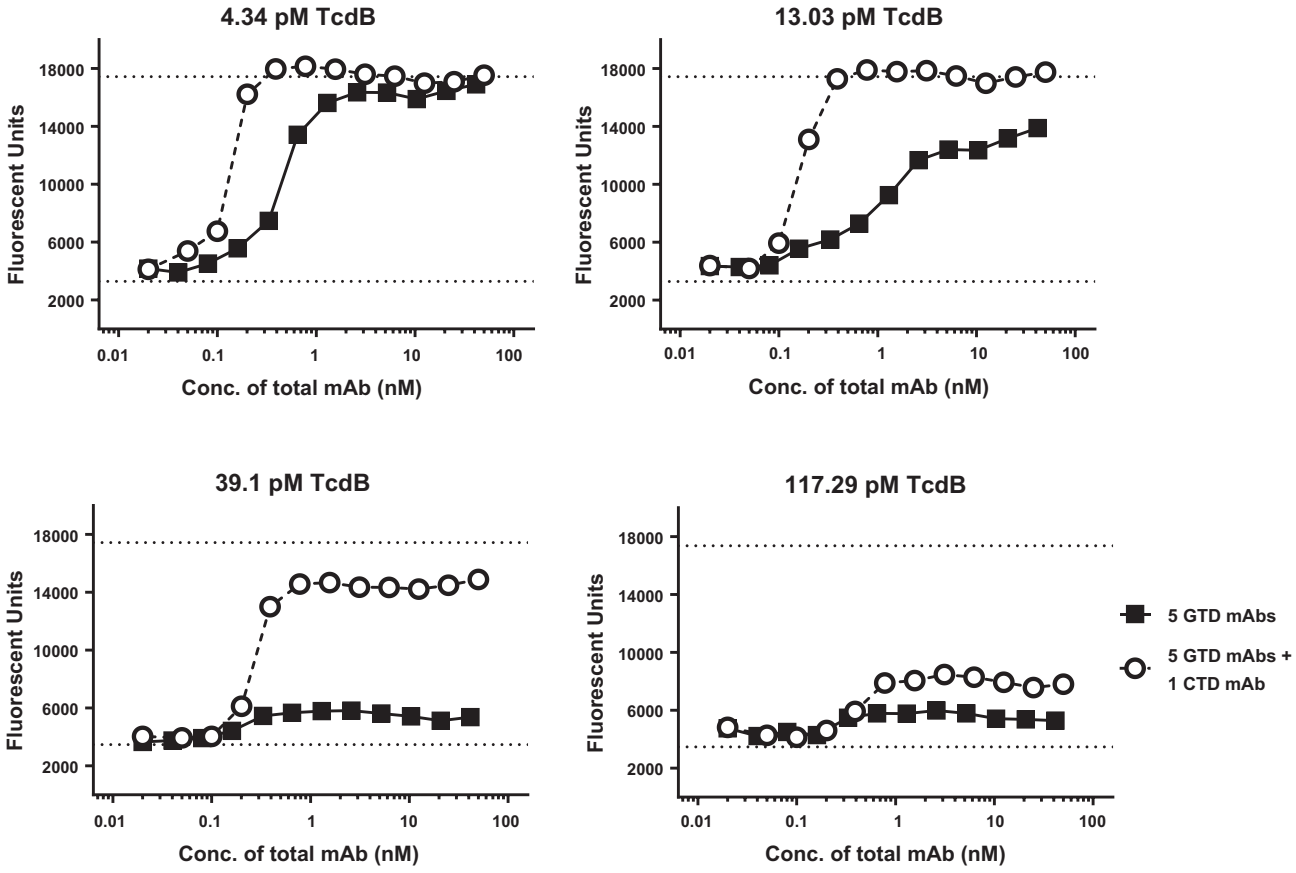

Fig. 3. The addition of a single CTD mAb greatly increases the neutralizing activity of an already potent combination of 5 GTD-specific mAbs. The concentrations of *C. difficile* TcdB, toxinotype 0 (4.34 pM, 13.03 pM, 39.1 pM, and 117.29 pM) used in the Vero cell neutralization assays are indicated in the graphs. Total concentrations of mAb combinations are shown in X axes. Upper and lower dotted lines indicate limits of the assay, fluorescent signals for positive and negative controls. The upper line marks the fluorescent signal for Vero cells in the absence of toxin which defines 100% neutralization of the toxin and the lower line marks the signal for Vero cells mixed with TcdB in the absence of neutralizing antibodies which defines 0% neutralization of TcdB. Monoclonal Ab designations: 5 GTD mAbs: B1, B2, B9, B19, B20; 5 GTD mAbs + 1 CTD mAb: B1, B2, B9, B19, B20 + B6.

of targeting different epitopes within a domain. When assessed at 4.34 pM of TcdB in the Vero cell assay, the previously identified GTD-specific B1 + B2 mAb combination [20] showed no neutralizing activity. However the addition of either the TLD-specific mAbs B5 or B7 or the CTD-specific mAb B6 greatly increased the neutralizing activity and the CPD + PFR mAb B24 as well as the TLD mAb B4 minimally increased the neutralizing activity of the new mAb combination (Fig. 4). The differential impact of the TLD mAbs B4, B5 and B7 suggests that the impact on neutralizing activity is epitope as well as domain specific. Finally, as there was a clear impact of increasing coverage to two domains, we wanted to assess the impact of combining mAbs to multiple domains. Therefore we tested a combination of mAbs specific to the GTD (B1 and B2), TLD (B7 and B4), CPD + PFR (B24) and CTD (B6). This increased domain coverage increased the neutralizing potency compared to what was observed after targeting only two domains (Fig. 4).

3.3. Mapping toxoid vaccine candidate-induced sera antibodies at toxin domain level

Having shown the benefit of combining toxin neutralizing mAbs to multiple domains, we wanted to assess the domain specificity of the antibodies elicited by immunization with a toxoid *C. difficile* vaccine candidate. Immune pre-challenge sera from hamsters that survived subsequent challenge with lethal dose of *C. difficile* were used. Mapping of the serum antibody-binding epitopes was performed by indirect ELISA using recombinant *C. difficile* toxin fragments (Table 1). Out of all antibodies binding a full length TcdA, 54% and 9% were attributed as being specific to CTD and GTD regions of TcdA, respectively. Response to TcdB was attributed to the three domains: CTD (34%), GTD (16%) and CPD + PFR (9%) (Fig. 5). We did not detect binding of immune sera to the TcdB TLD fragment.

3.4. Inhibition of neutralizing activity of immune sera with C. difficile toxin recombinant fragments

As toxoid vaccine candidate immunization elicits antibodies that bind to multiple toxin domains, we wanted to assess the contribution of these domain-specific antibodies to toxin neutralization. Immunoabsorption with recombinant fragments was performed to determine the contribution of antibodies specific to different toxin domains to the neutralizing activity of polyclonal sera. An excess of recombinant toxin fragments was mixed with the immune sera to absorb all of the antibodies that bound to that domain thereby preventing those particular domain-specific antibodies from interacting with the toxin. If those antibodies were necessary for toxin neutralization, the addition of the recombinant fragment(s) should inhibit the antibody mediated toxin neutralization. A combination of the GTD and CTD TcdA fragments, but neither alone, completely inhibited neutralizing activity of vaccine candidate-induced sera against TcdA. In contrast, only the combination of the GTD, CTD, and CPD + PFR, but not individual (GTD and CTD are shown) or combination of any two (GTD + CTD is

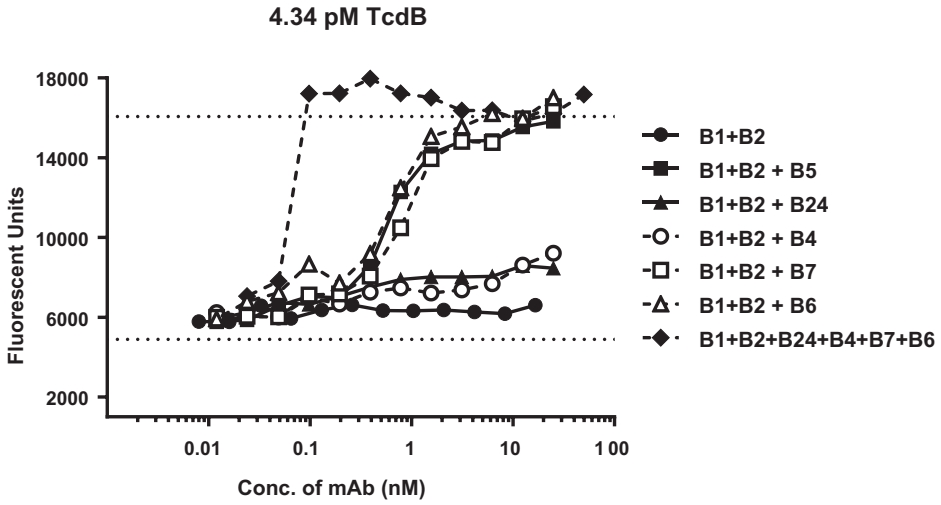

Fig. 4. A combination of mAbs with specificity to the GTD (B1 and B2), TLD (B4 and B7), CPD + PFR (B24) and CTD (B6), showed the best neutralizing potency in the Vero cell neutralization assay. *C. difficile* TcdB, toxinotype 0 was used at a concentration of 4.34 pM in the Vero cell neutralization assay. Total concentrations of mAb combinations are shown on the X axis. Upper and lower dotted lines indicate limits of the assay, fluorescent signals for positive and negative controls. The upper line marks the fluorescent signal for Vero cells in the absence of toxin which defines 100% neutralization of the toxin and the lower line marks the signal for Vero cells mixed with TcdB in the absence of neutralizing antibodies which defines 0% neutralization of TcdB. Individual mAbs showed no detectable neutralizing activity. mAbs used in each combination are indicated in the figures.

Table 3
Neutralization activity of anti-TcdB mAbs against *C. difficile* TcdB in Vero cell and TEER neutralization assays.

	Vero Cell Neutralization Assay		TEER Assay	
ID	NT50 (pM)	Maximum % completeness	NT50 (pM)	Maximum % completeness
B1	33	90	270	100
B2	33	70	70	100
B4	ND	0	130	100
B5	270	95	190	65
B6	ND	0	100	95
B7	330	100	130	90
B8	470	100	1300	70
B9	300	80	200	95
B12	ND	0	200	95
B19	ND	0	690	67
B20	ND	0	2666	30
B21	ND	0	6300	78
B24	370	80	130	80

ND- tested, but not detected.
The individual mAbs were assessed at 0.064 pM for TcdB toxinotype 0 in the Vero cell assay and at 0.3 nM for TcdB toxinotype 0 in the TEER assay.

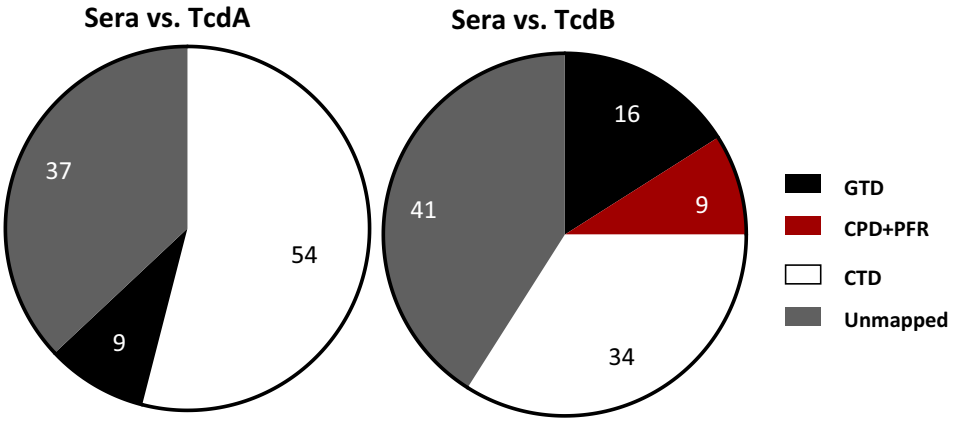

Fig. 5. Specificity of hamster polyclonal antibodies elicited after immunization with a *C. difficile* toxoid vaccine candidate was not distributed evenly across the full toxin molecules and the domain specificity differed between the two toxins. Hamster immune sera were mapped by indirect ELISA using the indicated *C. difficile* toxin fragments. Relative proportions (%) of serum IgG binding to a designated recombinant toxin fragment relative to binding of the respective holotoxin are shown. GTD - Glucosyltransferase Domain; CPD + PFR - Cysteine Protease Domain with Pore Forming Region; CTD – C Terminal Domain.

shown) fragments of TcdB, were able to abrogate the neutralizing activity of vaccine candidate-induced sera against TcdB (Fig. 6). In the absence of immune serum, fragments of either toxins alone or in combination did not have any neutralizing effect on the respective toxin (data not shown). These results suggest that immunization with *C. difficile* toxoids induces neutralizing antibodies that are specific to the GTD and CTD domains of TcdA and the GTD, CTD, and CPD + PFR domains of TcdB.

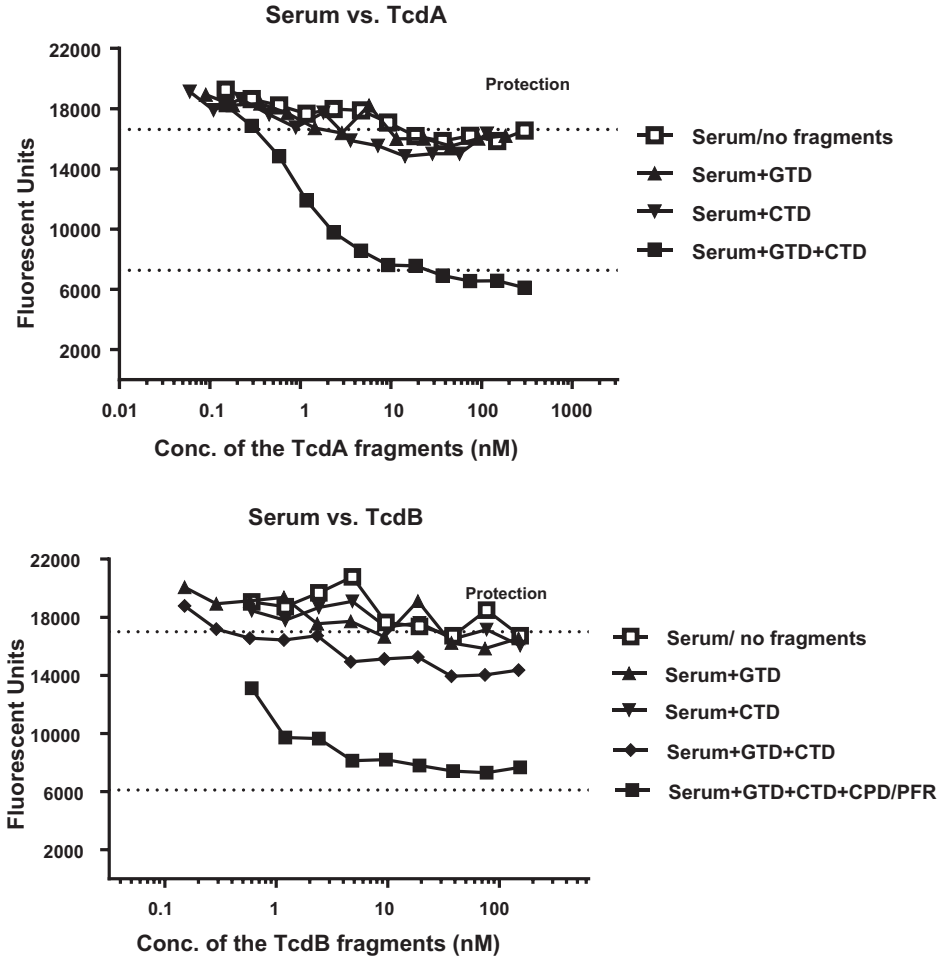

Fig. 6. Immunoabsorption experiments demonstrate that immunization with a C. *difficile* toxoid vaccine candidate elicits neutralizing Abs against the GTD and CTD domains of TcdA and GTD, CTD, and CPD + PFR domains of TcdB. Neutralizing activity of the toxoid vaccine candidate-induced hamster sera against *C. difficile* TcdA and TcdB of toxinotype 0 was assessed in the presence of the indicated recombinant toxin fragments. Upper and lower dotted lines indicate limits of the assay, fluorescent signals for positive and negative controls. The upper line marks the fluorescent signal for Vero cells in the absence of toxin which defines 100% neutralization of the toxin and the lower line marks the signal for Vero cells mixed with either TcdA or TcdB in the absence of neutralizing antibodies which defines 0% neutralization of the toxin. Recombinant fragment designations: GTD - Glucosyltransferase Domain; CPD + PFR - Cysteine Protease Domain with Pore Forming Region; CTD – C Terminal Domain.

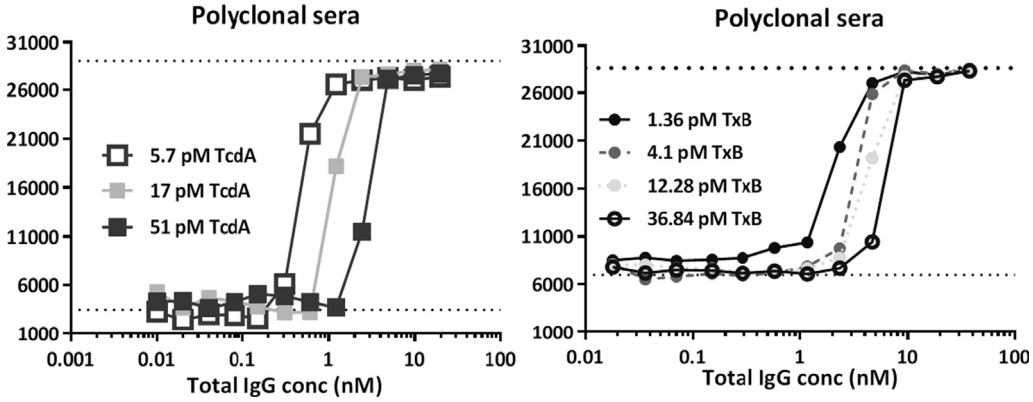

Fig. 7. Hamster immune sera maintain neutralizing activity at increasing concentrations of TcdA and TcdB. Concentrations of *C. difficile* TcdA and TcdB, ribotype 027, toxinotype III are indicated in the graphs. Upper and lower dotted lines indicate limits of the assay, fluorescent signals for positive and negative controls. The upper line marks the fluorescent signal for Vero cells in the absence of toxin which defines 100% neutralization of the toxin and the lower line marks the signal for Vero cells mixed with either TcdA or TcdB in the absence of neutralizing antibodies which defines 0% neutralization of the toxin.

3.5. High neutralizing potency of vaccine candidate-induced polyclonal sera against elevated concentrations of both C. difficile toxins

We next tested these polyclonal sera in the toxin neutralization "stress" test. We hypothesized that polyclonal serum containing a mixture of different toxin domain specific neutralizing antibodies would be very effective against increasing concentrations of purified native TcdA and TcdB of toxinotype III. Polyclonal vaccine candidate-induced sera retained protective activity against toxin concentrations of 51 pM for TcdA and 36.84 pM for TcdB (Fig. 7). These data suggest that a mixture of polyclonal antibodies against multiple toxin domains possess higher gram-to-gram potency than a mAb against a single domain.

4. Discussion

The impact of antibody domain specificity on toxin neutralization was investigated using domain mapped human *C. difficile* toxin-neutralizing mAbs and serum from toxoid vaccine candidate immunized hamsters. As prior *forté*BIO Octet analyses showed that these 39 human mAbs had similar binding affinity to their respective toxins [20]; this suggested that the differences observed in neutralizing activity resulted from differences in the epitopes bound and not mAb binding affinity. In order to differentiate combinations of mAbs, "stress" versions of the Vero cell and TEER assays were used. Assessment of increasing toxin concentrations provided the "stress" that enabled the differentiation of combinations of mAbs in the Vero cell neutralization assay while increasing the time of incubation at a single toxin concentration enabled differentiation of mAb combinations in the TEER assay. A prior publication showed that the TcdB concentration in stool samples collected from CDI patients prior to treatment ranged from 4.9 to 413.1 pM [22]. The highest TcdB concentrations assessed in the stress versions of these toxin neutralization assays were 117.29 pM and 0.83 nM for the Vero cell and TEER assays respectively. While the toxin concentration in stool may not be equivalent to the concentration in the intestinal lumen, using concentrations of TcdB that fall within the range of or even exceed the values from stool samples from untreated CDI patients suggests that our *in vitro* assays involved clinically relevant concentrations.

We created cocktails of non-overlapping mAbs that bound epitopes located in either one, two, or all the major domains of the TcdB. The activity of a multi-domain combination was much greater than an equivalent concentration of individual mAbs or a combination of mAbs to two toxin domains. The toxin neutralization that results from the combination of mAbs indicates a true synergistic effect and the increase was not simply additive, but multiplicative or better. Interestingly the activity of the individual mAbs in the Vero cell neutralization assay (Table 3) was not fully predictive of the impact mAbs in combination of mAbs (Fig. 4) as neither mAb B4 nor B6 demonstrated activity in the Vero cell neutralization assay, yet the addition of the B6 to the two GTD specific mAbs B1 + B2 (Fig. 4) was far more impactful in terms of increasing the neutralizing activity than the addition of B4. In contrast, B5, B7, and B24 all showed very potent neutralizing activity when tested individually in the Vero cell neutralization assay, but the mAbs B5 and B7 were far more impactful when tested in combination with B1 + B2 than B24. These data suggest that full understanding of the neutralizing activity of anti-toxin mAbs should include assessment both alone and in combination as it appears possible that the binding of one mAb could impact the confirmation of the toxin thereby impacting and perhaps even improving the binding of additional mAbs.

These mAb combining experiments used only TcdB as the majority of the identified TcdA mAbs were specific for a single domain, CTD (Table 2) and the two TLD mAbs and one GTD mAb showed no activity in the Vero cell cytotoxicity assay and weak activity in the TEER assay (data not shown). However published data suggest that this synergistic impact observed with the TcdB mAbs could also apply to TcdA mAbs. The combination of two murine mAbs to the CTD region of TcdA demonstrated significantly more toxin neutralization than the same concentration of the two mAbs individually [23]. A2, the most potent anti-TcdA mAb we had previously identified, binds multiple A/VTGWQTI sites within the CTD [20] and the toxin neutralizing mAb actoxumab binds to two distinct sites in the in the CROPs of the CTD of TcdA [24]. Taken together these data create a picture that suggests that multiple antibody binding events, whether spread across a single domain or across the entire toxin molecule, can produce synergistic protection against intoxication and that the toxin neutralizing benefit of targeting multiple epitopes is a concept that applies to both TcdB and TcdA.

Having demonstrated a synergistic impact of combining mAbs across domains, we next examined sera from *C. difficile* toxoid vaccine candidate immunized hamsters to assess the role that antibody domain specificity plays in sera-mediated toxin neutralization. Mapping and immunoabsorption studies demonstrated that immunization with a toxoid vaccine candidate elicited neutralizing antibodies that were specific to multiple toxin domains. Immunoabsorption experiments that mapped the domain specificities of these neutralizing antibodies demonstrated that neutralizing polyclonal antibodies were not distributed evenly across the full toxin molecules and that the domain specificity differed for the two toxins. For TcdA, the toxin neutralizing epitopes were located in the CTD and GTD domains. For TcdB, the toxin neutralizing epitopes were spread across 3 domains, CTD, GTD, and CPD + PFR. While we identified toxin neutralizing human mAbs specific to the TLD of TcdB, the results of the immunoabsorption experiment indicated that anti-TLD neutralizing antibodies were not detected in the serum of hamsters immunized with a toxoid vaccine candidate. Therefore while our mAb results clearly indicate that anti-TLD mAbs can neutralize TcdB, these data suggest that this toxoid vaccine candidate may not be inducing TLD-specific neutralizing antibodies in hamsters. With the impact of targeting multiple toxin domains demonstrated, the next question is how are these antibodies functioning in concert to mediate this synergistic effect. Three distinct cell surface receptors have been identified for TcdB and each toxin binding site could contribute to the overall avidity of the toxin for the gut cell surface [25–31]. TcdB binds to the cell surface receptor chondroitin sulfate proteoglycan 4 (CSPG4) at the junction between the translocation and CROP domains and binding involves the first three oligopeptide repeats of this CROP domain. The clinically efficacious mAb bezlotoxumab [21] mediates its impact by blocking those sites and preventing TcdB binding to CSPG4 [32]. In contrast, TcdB binding to PVRL3 is independent of the CROP domain [31]. The existence of a CROP independent receptor had previously been suspected as prior studies had demonstrated that TcdA and TcdB which lack the CROP domains are still able to bind to and intoxicate sensitive cells [25,26]. Secondary cellular membrane binding motifs have also been identified in the GTD [33,34] and in amino acid residues 1371–1848 of the TLD [35], and data indicate that these regions can mediate endocytic uptake of the toxin. Therefore one way antibodies to multiple domains could be impacting toxin activity is by blocking these multiple paths of toxin association to the cell surface. Another potential mechanism is that attached antibodies could be internalized into cells with the toxin and then subsequently block toxin functions downstream of cell adhesion.

In conclusion, this work has extended knowledge about the domain specificity of toxin neutralizing antibodies. We demonstrated that while individual mAbs specific to multiple toxin domains can neutralize toxin there is a synergistic impact of

targeting multiple domains. We showed that vaccination with a toxoid vaccine candidate induced neutralizing antibodies to multiple toxin domains specially the CTD and GTD domains of TcdA and the GTD, CTD, and CPD + PFR of TcdB. We also demonstrated that increasing the domain coverage enables neutralization of higher concentrations of toxin. Taken together these data support a rationale for preventative or therapeutic *C. difficile* treatments that involve a polyclonal response against multiple toxin domains and that this multi domain targeting approach could be particularly impactful in ameliorating CDI cases involving high levels of toxin production.

Declaration of Competing Interest

LEC, LL, JZ, KP, FM, JZ, SM, YY, LQ, HK, SFA, and NGA are current or former employees of Sanofi Pasteur involved in pre-clinical development of *C. difficile* vaccine candidate. The study was funded by Sanofi Pasteur. UJ, LB, CR, and NB have no conflicts of interest.

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Author Contributions

LEC, JZ, SM, HK, SFA, and NGA developed the study concept design, performed data analysis and interpretation and prepared the manuscript. LL, UJ, JZ, KP, FM, YY, LB, CR, LQ, and NB were involved in key reagents and test articles preparation, data acquisition and critical revision of manuscript.

Role of the funding source

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