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Clostridioides difficile toxins: mechanisms of action and antitoxin therapeutics

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Abstract

Clostridioides difficile is a Gram-positive anaerobe that can cause a spectrum of disorders that range in severity from mild diarrhoea to fulminant colitis and/or death. The bacterium produces up to three toxins, which are considered the major virulence factors in *C. difficile* infection. These toxins promote inflammation, tissue damage and diarrhoea. In this Review, we highlight recent biochemical and structural advances in our understanding of the mechanisms that govern host–toxin interactions. Understanding how *C. difficile* toxins affect the host forms a foundation for developing novel strategies for treatment and prevention of *C. difficile* infection.

Clostridioides difficile, the causative agent of *C. difficile* infection (CDI), causes mild to severe diarrhoea and can result in life-threatening conditions such as colonic perforation, pseudomembranous colitis and toxic megacolon[1]. It is one of the leading causes of health-care-associated infection and diarrhoea in many countries and is associated with more than 200,000 hospitalizations and $1 billion in health-care costs each year in the United States alone[2,3]. The US Centers for Disease Control and Prevention has listed *C. difficile* as an urgent threat among the antibiotic-resistant pathogens[3].

C. difficile is a Gram-positive anaerobic bacterium that is transmitted via the faecal–oral route in the form of spores. Upon entering the small intestine, spores germinate in the presence of primary bile salts (reviewed in detail by Shen[4]). Conditions in which the microbiota has been disrupted, typically by antibiotics, can promote colonization in the colon and growth of *C. difficile* vegetative cells[4]. Many *C. difficile* sequence types contain

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Author contributions

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genes that encode up to three different toxins (BOX 1), which have been linked to the onset of clinical symptoms. Toxin A (TcdA) and toxin B (TcdB) are glucosyltransferases, which belong to the large clostridial toxin (LCT) family. Other LCT members include *Paeniclostridium sordellii* toxins TcsH and TcsL, *Clostridium perfringens* toxin TpeL and *Clostridium novyi* toxin TcnA[5]. When expressed and secreted within the colon, TcdA and TcdB bind host cell receptors, are endocytosed by host cells and inactivate Rho-family GTPases via glucosylation. Inactivation of Rho GTPases disrupts the host cytoskeleton and accelerates the breakdown of epithelium barrier function. The third toxin, *C. difficile* transferase (CDT; or binary toxin), is an ADP-ribosyltransferase (ADPRT) that binds host cell receptors, is endocytosed by host cells and catalyses the depolymerization of actin[6]. Together, the toxins can disrupt the colonic epithelium to cause fluid secretion, inflammation and tissue damage, the hallmark characteristics of CDI. Concurrent with toxin production, many bacteria will initiate a genetic programme for sporulation, thus adopting a dormant and oxygen-tolerant form that can infect another host.

Although the enzymatic functions of the toxins are well established, the understanding of toxin–host cell receptor binding and toxin entry, as well as the individual contributions of the toxins to CDI, is less clear. In this Review, we describe recent structural and mechanistic advances in understanding the *C. difficile* toxins, and how this knowledge forms a foundation for considering novel strategies for CDI treatment and prevention.

TcdA and TcdB

The LCTs TcdA and TcdB are considered the major virulence factors in CDI pathogenesis. Recent developments in toxin receptor biology, combined with toxin structure–function studies, have strengthened our mechanistic understanding of *C. difficile* intoxication. In this section, we provide an overview of toxin mechanisms from production within the bacterium to effects on the mammalian host.

The pathogenicity locus.

The genes encoding TcdA and TcdB are found in a 19.6-kb DNA region termed the ‘pathogenicity locus’ (PaLoc) (FIG. 1a). The PaLoc contains genes for four additional proteins, TcdR, TcdE, TcdL and TcdC, which are thought to play a role in the regulation of toxin expression and secretion. The *tcdA* and *tcdB* genes are transcriptionally activated by TcdR, a member of the σ70 family of RNA polymerase σ-factors[7]. TcdR acts as a positive regulator of toxin production and stimulates its own transcription to further increase LCT expression[8–10]. The activity of TcdR is modulated by activators and repressors and is influenced by environmental changes such as changes in temperature and nutrient availability[11]. Conversely, TcdC is thought to function as a membrane-bound anti-σ-factor and negative regulator of toxin expression[12]. Following toxin expression, TcdA and TcdB are secreted through TcdE, a protein predicted to adopt a holin-like function[13–15]. Phage holins are typically expressed in tandem with an endolysin protein that breaks linkages in the peptidoglycan layer of the cell wall. Analysis of PaLoc sequences revealed a small fragment of an endolysin gene, *tcdL*, maintained directly downstream of *tcdE.* While the endolysin function is no longer present, one study proposed TcdL may bind TcdB to

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facilitate toxin secretion[16]. More experiments are needed to fully elucidate the mechanisms of toxin secretion.

Overview of TcdA and TcdB structures.

TcdA and TcdB are 308 and 270 kDa in size, respectively, and share 47% sequence identity. Both contain four functional domains: an amino-terminal (N-terminal) glucosyltransferase domain (GTD), an autoprotease domain (APD), a delivery domain with receptor binding, pore formation and cargo translocation functions, and a domain formed by combined repetitive oligopeptide sequences (CROPS). The TcdA and TcdB holotoxins are elongated molecules containing a 'multilobed' head and two tails, a short one and a long one[17]. The short tail corresponds to the delivery domain and the long tail corresponds to the carboxy-terminal (C-terminal) CROPS domain, which can adopt a variety of conformations relative to the rest of the toxin. The flexibility of the CROPS domain has rendered structural studies of the toxins difficult. In 2016, a 3.25-Å crystal structure of $TcdA_{1-1,802}$ was obtained by deletion of the CROPS. This structure was then docked into a 20-Å map of TcdA holotoxin obtained by negative stain electron microscopy[18] (FIG. 1b). In 2019, the Jin laboratory crystallized the TcdB holotoxin by adding nanobodies to shore up flexible elements[19]. In this structure, the CROPS domain is flipped up, demonstrating the breadth of conformations that TcdA and TcdB can assume at the delivery domain–CROPS domain junction (FIG. 1c).

TcdA and TcdB mechanism of action.

TcdA and TcdB intoxicate host cells through a multistep mechanism: (1) receptor binding and endocytosis, (2) pore formation and translocation of the GTD and APD across the endosomal membrane, (3) autoprocessing and release of GTD into the cytosol and (4) glucosylation of host GTPases (FIG. 2).

The TcdA and TcdB CROPS domains are thought to mediate toxin attachment to the cell surface via glycan binding interactions. Initial studies in animal models found that TcdA binds trisaccharides with the Galα1,3Galβ1,4GlcNAc moiety; however, αGal1,3Galβ linkages are not found in humans, and most people produce antibodies to these carbohydrates[20–22]. A later study discovered TcdA bound I, X and Y carbohydrate antigens and highlighted the conserved core containing branched Galβ1–4GlcNAc structures[23]. A recent glycan array study indicated that the TcdA and TcdB CROPS domains can bind diverse antigens and carbohydrate structures[24].

Two glycoproteins have been reported as TcdA receptors, sucrase-isomaltase (SI) and soluble glycoprotein 96 (gp96), although SI is not expressed within the colon and gp96 is predominantly found in the endoplasmic reticulum[25,26]. Recently, a CRISPR–Cas9 screen using TcdA lacking the CROPS domain revealed additional receptor targets, including sulfated glycosaminoglycans and members of the low-density lipoprotein receptor (LDLR) family[27]. The LDLR family includes LDLR, LDLR-like protein 1 (LRP1) and several proteins with shared domains that function in binding LDLR ligands. Knockdown of either LDLR or LRP1 attenuated TcdA-induced cytopathic effects, hinting at a role for these proteins in cellular endocytosis[27,28]. In addition, LRP1 was found to bind TcdA directly[28]. Current thinking suggests TcdA engages multiple cell surface receptors simultaneously to

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achieve high-affinity entry into cells, but more studies are needed to explore this possibility in a physiological context.

Three classes of protein receptors have been reported for TcdB: chondroitin sulfate proteoglycan 4 (CSPG4), Frizzled 1 (FZD1), FZD2, FZD7 and Nectin 3, also termed 'poliovirus receptor-like protein 3' (PVRL3)[29–31]. The use of the term 'receptor' is qualified in that none of the initial studies established a role for the host factor in receptor-mediated endocytosis, but these proteins undergo constitutive endocytosis and recycling through clathrin-dependent pathways and thus could promote toxin entry. The larger issue is that the study of the function of these receptors in TcdB intoxication was initially limited to experiments in tumour-derived cell lines, whose intoxication responses differ according to the receptor expression profile of the cell.

The role of these receptors in the physiological context of infection is not well understood. CSPG4 is not expressed on colonic epithelial cells, but it is expressed on colonic subepithelial myofibroblasts and therefore could be accessed once the epithelial barrier has been disrupted[30]. Recent infection experiments using CSPG4-knockout mice indicated a significant decrease in the severity of infection, although the mice still displayed symptoms of diarrhoea and weight loss[32]. Nectin 3 is highly expressed on the surface of the colonic epithelium but is typically described for its role in mediating the formation of adherens junctions during development[29]. Outside of development, its role in gut physiology has not been well characterized. By contrast, FZD proteins have been extensively studied, as they are key receptors of the Wnt-signalling pathway involved in stem cell differentiation in the gastrointestinal crypts[33]. Within the FZD1-FZD2-FZD7 subset, human FZD7 is expressed on the basolateral surfaces of healthy colonic crypt cells[33] (FIG. 3).

Members of the FZD protein family have seven transmembrane passes with a single extracellular domain called the cysteine-rich domain, which serves as the Wnt-binding site. A crystal structure of the FZD2 cysteine-rich domain bound to the TcdB delivery domain revealed the presence of a lipid in the interface[34] (FIG. 1d). The lipid resembles the palmitoleic modification of Wnt and suggests a mechanism for how TcdB inhibits Wnt signalling. The inhibition of Wnt signalling could represent a pathway for TcdB-induced crypt damage in the colon. Indeed, TcdB-dependent crypt damage has been observed in the mouse model of CDI using multiple *C. difficile* strains[35]. However, we now know that TcdB2 sequences from ST1 strains (see BOX 1 for an explanation of strain and toxin nomenclature) are not able to bind FZD1/2/7 (REFS[35–40]). The study by Mileto and colleagues suggests that TcdB2 is binding something other than FZD proteins to cause these changes, and there may be undiscovered TcdB receptors in this sequence type[35].

CSPG4 is a large (251 kDa) and highly glycosylated protein with roles in cell proliferation, adhesion and migration[41]. Recently, Jin and colleagues reported a 3.2-Å cryo-electron microscopy structure of the TcdB–CSPG4 binding interface[32]. The first of 15 consecutive CSPG4 repeats (CSPG4 residues 410–551) is bound in a groove between the APD and the delivery domain–CROPS hinge region (FIG. 1e). Mutagenesis and binding analyses confirmed that residues from both the APD and the hinge region contribute to binding, and the CSPG4-binding site is conserved in TcdB from ST1 strains.

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Upon binding its cognate receptor, each toxin is endocytosed. Despite their structural homology, TcdA and TcdB have distinct endocytic pathways, which ultimately use dynamin to facilitate scission of the formed endosome[42,43]. TcdB undergoes clathrin-mediated endocytosis[42], whereas TcdA is internalized by a novel clathrin-independent mechanism mediated by PACSIN2 (REF.[43]). Endosomal maturation results in a drop in pH, which induces a conformational change within the delivery domain of the toxins and the formation of a pore in the endosomal membrane. The pore permits translocation of the N-terminal enzymatic cargo into the cytosol[44] (FIG. 2).

The machinery for pore formation is located within the N-terminal half of the TcdA or TcdB delivery domain. Residues and sequence stretches involved in pore formation have been identified by a variety of biochemical and biophysical approaches[18,44–46]. In the crystal structures of soluble TcdA and TcdB, these elements are found in an extended series of α-helices that wrap around and span the length of the delivery domain[18,19]. Although the structure of the pore in the context of the membrane is not known, some of these residues were mobile in the TcdB structure determined at acidic pH[19], consistent with the hypothesis that the α-helices will become dislodged at a low pH to insert themselves into the endosomal membrane[47]. The mechanism is not entirely conserved, as TcdA requires cholesterol as a cofactor to form pores in the cell membrane and in artificial lipid bilayers, whereas TcdB does not[48]. On the other hand, a recent database analysis using the TcdB delivery domain as a search query revealed hundreds of $TcdB_{851-1,473}$ domain homologues[49]. This finding suggests the presence of an evolutionarily conserved translocase structure capable of delivering a diverse repertoire of cargo into target cells[49].

The toxins are proteolytically processed following pore formation and membrane translocation. The N-terminal GTD and APD are delivered through the endosomal membrane, and the chaperonin TRiC/CCT assists with toxin refolding in the cytosol[50,51]. Inositol hexakisphosphate then binds and induces a conformational change in the APD, which results in proteolytic cleavage and the release of the GTD[52–56]. TcdA and TcdB autoprocessing depends on the presence of a zinc ion bound to a conserved cysteine within the active site of the APD[18]. Currently, it is unclear whether the APD functions as a cysteine protease or a zinc protease, so the general term 'autoprotease' is used. Although both toxins share this mechanism, TcdB is more susceptible to autoproteolysis-induced cleavage by inositol hexakisphosphate than TcdA[57]. Autoprocessing of TcdA could be repressed owing to interdomain interactions between the CROPS and the N terminus[18,58,59].

Autoproteolysis provides the GTD with access to substrates located at the inner leaflet of the host cell membrane. In the case of TcdA and TcdB, these substrates include members of the Rho GTPase family such as CDC42, Rho and RAC1 (REFS[60,61]). The GTD catalyses the transfer of a glucose moiety from UDP-glucose onto the switch I region of the GTPase, resulting in its inactivation[62]. The N-terminal membrane localization domain of the GTD is thought to orient itself on the membrane for efficient catalysis[63]. Despite their similarities, several studies indicate that TcdA and TcdB may differ in terms of which GTPases are inactivated in the host[64–68]. This could result from differences in the kinetics of cellular entry, localization based on their distinct endocytic pathways and/or substrate recognition.

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Structures of the GTDs in complex with cognate GTPases would guide the discovery of how TcdA and TcdB discriminate among GTPases.

Effects of TcdA and TcdB on the host.

Inactivation of the Rho-family GTPases leads to changes in the actin cytoskeletal structure, the secretion of cytokines, cell cycle arrest and, eventually, cell death[69]. A detailed overview of the cellular effects induced by TcdA and TcdB and their underlying mechanisms can be found elsewhere[70]. The loss of cytoskeletal structure and the disruption of focal adhesions and tight junctions in epithelial cells are thought to contribute to a reduction of barrier function and the cause of diarrhoea in the colon[71–74] (FIG. 3). The secretion of pro-inflammatory chemokines and cytokines triggers an acute influx of myeloid-derived immune cells to the site of infection. These cells are also sensitive to the toxins, and their intoxication further amplifies the intestinal damage associated with CDI. GTPase inactivation is associated with cell cycle arrest, which prevents cellular proliferation. Eventually, intoxication leads to cell death, which may contribute to the formation of pseudomembranes, crypt damage and/or necrotic lesions within the colon[75–79].

There are multiple mechanisms of cell death depending on the cell type and toxin concentration. In myeloid-derived cells, toxin-induced Rho-GTPase inactivation leads to the activation of the pyrin inflammasome pathway, which results in pyroptosis[80]. In epithelial cells, pyrin is not expressed, and the toxins induce caspase 3/7-mediated apoptosis[81]. CDI studies using pyrin-knockout mice indicated that the pyrin pathway does not contribute to weight loss and diarrhoea, whereas the disruption of caspase 3/7 in intestinal epithelial cells of mice enhanced the severity of disease[81]. These data imply apoptosis in intestinal epithelial cells may have a protective role. Nevertheless, it is plausible to consider whether extensive cell death contributes to the formation of necrotic lesions and/or perforation of the bowel, sequelae observed in the severest forms of CDI. Extensive cell death could result from the inflammatory response or the accumulation of toxins in the colon. In vitro, TcdB induces a necrotic form of cell death (referred to by some as ‘pyknosis’) at concentrations greater than 0.1 nM (REFS[82–84]). The response is independent of glucosyltransferase and autoprocessing activities and still requires toxin pore formation[45,85], but the mechanistic basis of how this response is initiated remains unclear. While multiple reports have demonstrated a critical role for the glucosyltransferase activity (and autoprotease activity) in the establishment of disease[86–88], the necrotic response could contribute to the severe sequelae associated with TcdB-dependent modes and models of infection[89–91]. Further studies are required to fully understand cellular responses in the context of physiological tissue systems, as well as the consequences of targeting different cell types within the host.

CDT toxin

Between 17% and 23% of *C. difficile* strains produce an additional toxin, CDT, which belongs to a family of bipartite ADP-ribosylating clostridial toxins[6,92]. The role of CDT in infection is unclear, although its prevalence in epidemic sequence types, such as ST1 and ST11, suggests it may promote severe CDI[93–95] (BOX 1). In this section, we highlight

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recent discoveries in CDT production and host intoxication with an emphasis on structure and function.

The CDT locus.

The genes encoding CDT are located within a 6.2-kb region termed the 'CDT locus' (CDTloc)[96]. The CDTloc harbours the genes encoding three proteins: CDTa and CDTb, which constitute CDT, and CdtR, a regulatory protein (FIG. 4a). *cdtR* encodes a LytTR-family response regulator that is part of a two-component signal transduction system[97]. An unidentified histidine kinase sensor phosphorylates CdtR and activates transcription of *cdtA* and *cdtB*[98,99]. Alternative regulators of the CDTloc likely exist, as CDT is still produced in ST11 strains, which contain nine non-synonymous polymorphisms within *cdtR*, including a premature stop codon[99]. The mechanism of CDT secretion is currently unknown. CDT lacks canonical secretory signals, and, unlike the PaLoc, no genes are associated with a pore-forming mechanism of transport within the CDTloc[96].

Overview of CDT structure.

CDT is composed of CDTa and CDTb, which are 43 and 99 kDa in size, respectively. CDTa consists of ADPRT and pseudo-ADPRT (pADPRT) domains[100,101] (FIG. 4b). The non-enzymatic pADPRT domain mediates binding to CDTb, whereas the enzymatic ADPRT domain catalyses the ADP-ribosylation of globular actin[92,100,102]. CDTb shares structural and functional homology with the anthrax toxin protective antigen (PA) and promotes host cell binding, pore formation and CDTa translocation. CDTb and PA adopt similar structures in which four domains are conserved (D1, D2, D3 and D4). A fifth domain, located between D3 and D4, is unique to CDTb and is therefore referred to as 'D3′'[103,104] (FIG. 4c). Host cell intoxication requires (1) receptor binding by CDTb and oligomerization, (2) association of CDTa with the CDTb oligomer, (3) pore formation and endocytosis, (4) translocation of CDTa into the host cytosol and (5) ADP-ribosylation of host actin (FIG. 5).

CDT mechanism of action.

After secretion, the CDTb D4 domain engages the extracellular domain of lipolysis-stimulated lipoprotein receptor (LSR) on the host cell surface[103,105]. Proteolytic activation by host serine proteases releases a 20-kDa peptide from the N terminus of CDTb, which enables the remaining protein to oligomerize into a heptamer[106]. The resulting CDTb heptamer then engages the pADPRT of one CDTa molecule[102] (FIG. 4d).

The CDT complex (seven CDTb protomers with one protomer of CDTa) enters the cell by endocytosis. Like PA, CDTb undergoes a series of conformational changes to create a β-barrel pore in the host cellular membrane that enables cargo translocation into the host cytosol. A pore-forming loop (residues 312–383 of D2) from each of the seven CDTb protomers dislodges from a pocket next to D3′ and undergoes a conformational change to insert itself into the membrane, with each pore-forming loop contributing two strands to a 14-stranded β-barrel[103,104] (FIG. 4c,d). Following CDTb insertion and exposure to the low-pH environment of the endosome, CDTa translocates through the lumen of the pore[106] (FIG. 5). HSP90 and cyclophilin A assist with CDTa refolding in the cytosol[107].

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Effects of CDT on the host.

The ADPRT ADP-ribosylates G-actin at Arg177, thereby preventing G-actin polymerization[108]. ADP-ribosylated actin also acts as a capping protein that binds to the fast-growing barbed end of F-actin to prevent further polymerization[109] (FIG. 5). ADP-ribosylation alters host cellular morphology, including the detachment of tight junctions connecting epithelial cells, and culminates in cellular rounding and epithelial tissue shedding.

ADP-ribosylation of host actin also drives the formation of microtubule protrusions on the apical host cell surface[110] (FIG. 5). Actin beneath the cell membrane prevents cytoskeletal expansion by microtubules. Actin depolymerization caused by CDTa impedes this crucial regulation and allows the growth of long microtubules (greater than 150 μm)[110]. Septin proteins are translocated to the apical host membrane to guide and support the aberrant outgrowths of microtubules[111]. The microtubule protrusions significantly augmented the adherence of *C. difficile* to colonic epithelial cells and increased adherence fourfold in control-treated mice compared with mice treated with a CDT-neutralizing antiserum[110,112] (FIG. 3).

CDT can stimulate the nuclear factor-κB (NF-κB) pathway and pro-inflammatory responses in murine bone marrow-derived dendritic cells in a TLR2/6-dependent manner[113,114]. This activity could represent a priming step for TcdA and TcdB-induced activation of the inflammasome and may contribute to cytokine production in the host. CDT activity has also been associated with suppression of the otherwise protective colonic eosinophilic responses in vivo[113]. The mechanism of how synergistic toxin activities suppress the eosinophil response remains unclear.

Therapeutic strategies against *C. difficile* toxins

Antibiotics (vancomycin, metronidazole and fidaxomicin) are standard treatment options for patients with confirmed CDI[115]. Although often effective, antibiotic treatment prolongs the state of dysbiosis of the intestinal microbiota, and there is a high rate of recurrent disease[116]. Faecal microbiota transplantation is effective against refractory and recurrent CDI, but has inherent risks associated with the lack of standardization[117]. Immune-based therapies have proven effective in clinical trials, and bezlotoxumab, a human monoclonal antibody (mAb) to TcdB, is in clinical use for the prevention of recurrent CDI[118]. Vaccine trials using inactivated TcdA and TcdB as antigens are also ongoing. Here we provide some recent examples of how understanding toxin mechanism informs treatment strategies and, conversely, how promising therapeutic leads inform our knowledge of toxin mechanism.

Protein-based inhibitors.

Several studies have reported antibodies capable of neutralizing toxin activity in cell culture and animal models of infection[118–128]. Antibodies have been cloned from patients who have recovered from CDI, vaccinated animals and in vitro screens, and include mAbs, single-chain variable fragments and single variable domain (VHH) ‘nanobodies’. In some cases, the divalent binding of mAbs may induce aggregation of the toxin. In other cases,

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mAbs can be proteolytically digested into antigen-binding fragments (Fabs) still capable of potent neutralization. In the absence of induced multimerization or Fc-mediated antibody effector functions, the Fabs, single-chain variable fragments and nanobodies neutralize the toxin either by blocking a key mechanistic step in intoxication or by inducing a non-productive conformational change in its structure. Structural analyses of these antibodies in complex with either TcdA or TcdB have provided the opportunity to define key epitopes and mechanisms of neutralization.

The anti-TcdA mAb actoxumab binds multiple repeats within the TcdA CROPS, and both the mAb and the Fab of actoxumab block binding of TcdA to Vero cells[119] (FIG. 6a). This antibody was tested extensively in combination with bezlotoxumab and showed promise in animals and early-stage clinical testing[121,122], but ultimately did not have efficacy regarding recurrent CDI in phase III clinical trials[118]. The lack of efficacy has left many open questions for the research community. Is TcdA not a relevant target for the prevention of CDI recurrence? Would actoxumab have helped in another clinical context, for example as a therapeutic or in the prevention of initial infection? Could another mAb, one recognizing a different TcdA epitope, offer protection against recurrence even though actoxumab did not? Although the financial barriers to clinical testing are substantial, a growing number of neutralizing antibodies are being identified that recognize distinct epitopes of TcdA. For example, Murase and colleagues determined a structure of a TcdA CROPS domain fragment in complex with A26.8, a camelid VHH nanobody shown in prior work to have potent neutralization activity against TcdA[123,124]. Notably, the nanobody bound an epitope unique to the C terminus of TcdA, distinct from previously observed carbohydrate-binding sites (FIG. 6a). Another study demonstrated the Fab of PA50, another potent neutralizer of TcdA activity, also had binding sites at the extreme C terminus[125] (FIG. 6a). These studies have implied a specific role for the C terminus of the TcdA CROPS domain in intoxication.

Bezlotoxumab targets the N-terminal domain of the TcdB CROPS[126] (FIG. 6b). X-ray crystallography has shown the Fabs are capable of binding two adjacent sites on the CROPS domain, yet it remains unclear whether both sites can be bound simultaneously by the mAb. The mAb prevents TcdB binding to CSPG4 on cells[129]. The recent CSPG4–TcdB structure suggests bezlotoxumab binding induces an allosteric change in TcdB, which disrupts the CSPG4-binding site[32]. Bezlotoxumab is not thought to affect interactions with FZD1/2/7 or Nectin 3, as these receptors do not require the presence of the CROPS for binding[29,30,130]. Bezlotoxumab was tested in adults receiving antibiotic treatment for primary CDI or recurrent CDI in two global phase III trials (MODIFY I and MODIFY II)[118]. The addition of bezlotoxumab compared with placebo led to significant reductions in the rate of recurrent CDI (17% vs 28% in MODIFY I and 16% vs 26% in MODIFY II; $P < 0.001$)[118]. This efficacy can be viewed from two perspectives. On the one hand, the potency of bezlotoxumab may reflect an important role for the TcdB–CSPG4 interaction in the context of human CDI, at least in terms of recurrence. On the other hand, would a different mAb or a mAb combination offer even greater efficacy? A continued consideration of antibodies that block various sites on TcdB could advance the understanding of physiological mechanisms and expand therapeutic choices for clinicians managing complex CDI cases.

In the past few years, several examples of biologics that effect neutralization by binding regions outside the CROPS have been identified and characterized by X-ray crystallography. PA41 neutralizes TcdB as either a mAb or a Fab by binding the GTD and blocking its translocation across the endosomal membrane[127] (FIG. 6b). Three additional epitopes were identified by crystallization of the TcdB holotoxin with three neutralizing nanobodies, 5D, E3 and 7F[19,131] (FIG. 6b). 5D binds the delivery domain and likely prevents pH-driven conformational changes necessary for pore formation[19]. E3 binds the membrane localization domain of the GTD and impedes Rho glucosylation, likely due to impaired GTD localization to the inner leaflet of the cell membrane[19,131]. 7F binds the C terminus of the GTD and is predicted to obstruct the APD movement required for GTD cleavage[131]. Recently, the Feng group engineered the strain *Saccharomyces boulardii* to secrete a genetic fusion of four neutralizing nanobodies, two against TcdB (5D and E3) and two against TcdA. The strain was orally administered as a probiotic and effectively prevented primary and recurrent CDI in both prophylactic and therapeutic mouse models of disease[128]. Nanobodies against CDT have also been identified, including five that inhibit CDTa ADP-ribosylation, three of which are specific for the active site[120]. Biological alternatives to antibodies, such as designed ankyrin repeat proteins (DARPins), inhibited TcdB interactions with CSPG4 and FZD2 and offered protection in vivo during a systemic toxin challenge as well[40,132].

Other studies have reported a role for antimicrobial peptides in neutralizing toxin function. An early study showed human α-defensins neutralized the effects of TcdB[133]. These results were later expanded upon to show that α-defensin 1 and α-defensin 5 can inactivate TcdA, TcdB and CDT[134,135]. α-Defensins are cysteine-rich cationic peptides that are expressed in human neutrophils and intestinal Paneth cells. While considered key elements of the innate immune response for their role in inactivating pathogenic bacteria, the potential that these peptides could also reduce the burden of active toxin in the gut is intriguing. In addition to the *C. difficile* toxins, α-defensins are capable of inactivating a large repertoire of bacterial toxins (for example, anthrax toxin and diphtheria toxin). Despite functional diversity, the toxins share a conformational plasticity that enables them to insert themselves into the membrane to form a pore. It is thought that the inherent instability of the toxins contributes to α-defensin-induced unfolding and subsequent aggregation[136]. Curiously, the Ballard laboratory identified a $TcdB_{1,769–1,779}$ peptide, part of the delivery domain, which interacts with the TcdB CROPS to induce destabilization and aggregation, similarly to α-defensins[137]. Other efforts at developing peptide-based inhibitors include efforts to break up toxin–receptor interactions, such as between the FZD7 cysteine-rich domain and TcdB[138] or by using soluble CSPG4 ectodomain as a decoy[32].

Small-molecule inhibitors.

Concurrent with discovery of biologics, considerable effort has been invested in identifying small-molecule inhibitors of toxin function. Some of these efforts have been directed towards the glucosyltransferase[139–142] or autoprocessing[143,144] activities, whereas others have been directed against cellular targets in the host[85,145,146]. One of the promising leads from these studies is ebselen, an organoselenium molecule identified independently by two groups, one using cell-based screening for inhibition of glycosyltransferase function[141] and

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the other with a targeted screen for activity-based inhibition of the APD[144]. Ebselen reduced recurrence rates, inflammation and colitis in the context of a relapsing CDI hamster model and enhanced microbiota recovery in mice with CDI[147].

Other intriguing small-molecule therapeutics against the toxins have emerged from high-throughput screens. In a cell-rounding phenotypic screen, the bile acid derivative methyl cholate and the naturally occurring flavonoid phloretin were found to indirectly bind and inhibit TcdB[141]. Although structural characterization is required to evaluate the site selectivity of these molecules, a follow-up study showed that multiple secondary bile salts induce structural changes in TcdB that limit its function[148]. Another small-molecule screen found that calcium channel blockers with a dihydropyridine core interfered with TcdB-induced calcium signalling and diminished reactive oxygen species production and subsequent necrosis[85]. Other screens have identified molecules that inhibit acidification of the endosome[145,146]. For example, the anthelmintic drug niclosamide ablated the cytotoxicity of TcdA, TcdB and CDT by targeting host endosomal pH through a proton-shuttle mechanism and, thus, prevented translocation of toxin cargo. Notably, niclosamide was preferentially distributed in the colon and prevented primary disease and recurrent CDI in murine models while leaving the microbiota unaltered, which highlights its promise as an effective oral therapeutic against CDI[146].

Conclusion and future perspectives

In this Review, we have summarized our current structural and mechanistic understanding of the three *C. difficile* toxins. Advances in research from the past 5 years include the determination of the holotoxin structures of TcdA and TcdB, the structures of TcdB–receptor complexes and the multiple conformational states associated with CDT function. Similarly, large-scale panning and screening efforts have identified host receptors for the toxins, as well as biological and chemical molecules capable of inhibiting toxin function. There are still many questions that remain, however.

The subject of how the toxins bind host cells is likely to remain an active area of investigation. In addition to the multiple cell surface proteins, both TcdA and TcdB can bind a wide spectrum of glycans, suggesting that the toxins may be able to engage cells through multiple mechanisms[20–24,27]. In the case of CDTb, only LSR has been identified as a receptor[105]. Although CDTb was also recently revealed to engage glycans, there is no evidence that these interactions contribute to cellular binding[103].

Once the toxins are bound, it is currently unclear whether the toxins enter the host cell through an active endocytic mechanism or constitutive protein recycling. It is possible that TcdA and TcdB bind and enter cells variably depending on the cell type. A thorough understanding of this complexity will require transitioning from cell culture models to more complex environments. The entry mechanisms for TcdB may be further complicated by sequence variation among clades[35,37].

Although the mechanism of TcdA and TcdB pore formation remains unclear, it is certain to be dissimilar to that of CDTb. Structural analysis of CDTb points to an orderly progression

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of structural conformations that culminate in the formation of a 14-stranded β-barrel[103,104]. In the case of TcdA and TcdB, there has been no evidence to indicate the formation of a stable transmembrane pore. The current model suggests that an extended stretch of hydrophobic α-helices is displaced from the delivery domain structure in the presence of endosomal membranes and a low-pH environment[18,19,44–46]. The absence of any direct evidence of a pore structure may reflect a dynamic process for translocating the APD and GTD into the cytosol.

One overarching question is why *C. difficile* would invest resources in expressing both TcdA and TcdB, two large exotoxins with very similar functions. TcdA and TcdB are thought to have synergistic effects, and perhaps their ability to engage unique receptors provides an expanded set of cell types for intoxication or even host range. It is also possible that their GTDs can modify distinct GTPase subsets. A structure of either GTD bound to a GTPase target would accelerate the understanding of how these toxins discriminate among cellular substrates. Moreover, why would the bacterium benefit from CDT, as the LCT glucosyltransferase activity already has an impact on actin cytoskeletal structure? The proposed role for CDT in promoting *C. difficile* adherence through the formation of microtubule protrusions is intriguing and merits further investigation in vivo[110–112]. Other studies suggest CDT primes the host immune system for inflammasome activation by TcdA and TcdB and prevents a protective eosinophil response[113,114]. Together, these studies suggest a complex relationship in how the host immune system deals with CDI.

The work described in this Review is occurring within a larger environment of scientists investigating mucosal immunity, the role of the microbiota in restricting and permitting *C. difficile* growth, and the environmental cues for germination, sporulation and toxin production in the host[4,149,150]. While this Review discussed antitoxin therapeutics, many efforts are ongoing to develop new prevention and therapeutic strategies against various aspects of CDI. We view the breakthroughs in toxin biology as a central part of this effort and, when integrated, should lead to discoveries that benefit human health.

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Glossary

Pseudomembranous colitis

Inflammation of the colon characterized by raised yellow-white plaques of discarded epithelial and immune cells

Toxic megacolon

A life-threatening condition characterized by non-obstructive, inflammatory dilation, expansion and distension of the colon

Sequence types

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Unique combinations of alleles grouped together on the basis of multilocus sequence typing

Pyroptosis

A lytic and pro-inflammatory form of caspase 1-dependent programmed cell death

Pyknosis

Irreversible chromatin condensation and nuclear dissolution during necrosis or apoptosis

Two-component signal transduction system

A signal transduction pathway comprising a sensor that phosphorylates a response regulator in response to an environmental stimulus to elicit an effector function

Designed ankyrin repeat proteins

(DARPins). Small protein scaffolds engineered to bind an antigen with high specificity and affinity, similar to monoclonal antibodies

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Box 1 |

Genotypic and toxigenic heterogeneity in *Clostridioides difficile*

Accurate depiction of genome and toxin heterogeneity is crucial for understanding *Clostridioides difficile* epidemiology and for developing new diagnostic tests and therapeutics. *C. difficile* contains a highly mosaic pangenome of 17,000 genes[151–154]. Historically, *C. difficile* was classified through toxinotyping, based on variations in *tcdA* and *tcdB*, and genotypic PCR ribotyping[155]. However, these methods do not sufficiently detect diversity within the *C. difficile* pangenome and lack the resolution to delineate toxin structure–function relationships and evolution[154,156].

Other genetic analysis tools, such as multilocus sequence typing (MLST) and average nucleotide identity (ANI) analysis, have enhanced *C. difficile* categorization. MLST discriminates *C. difficile* strains on the basis of nucleotide sequences of housekeeping gene fragments. Bacteria with unique combinations of alleles are assigned sequence type (ST) numbers[157,158]. More accurate analysis of these STs can be accomplished with ANI analysis, which calculates whole-genome sequence similarity, with more than 96% ANI between samples representing a species[154]. MLST analysis of *C. difficile* identified eight monophyletic clades: five of which (C1–C5) are commonly studied, whereas three are considered ‘cryptic’ due to the atypical architecture of their toxin loci (CI–CIII) (see the table)[152,157,159]. Recent ANI analysis found CI–CIII fell below the threshold (ANI 89–94%) and represent ancestral clades of *C. difficile*[154]. Much remains unknown about CI–CIII, including whether STs are culturable[151,154,160,161].

Whole-genome sequencing has also enabled classification of *C. difficile* toxins. Alignments and hierarchal clustering of ~8,800 toxin sequences revealed seven distinct subtypes for toxin A (TcdA; TcdA1–TcdA7) and 12 for toxin B (TcdB; TcdB1–TcdB12) (data available in the online database DiffBase)[156]. Whereas TcdA receptors remain conserved, TcdB subtypes (TcdB1–TcdB4) have differences in receptor specificity[39], translocation ability[162], inflammatory responses[36] and pathological outcomes[39,163] (see the table). Universally conserved neutralization epitopes on TcdB variants have also been suggested and discovered[127,156,164]. Movement towards research inclusive of *C. difficile* genome and toxin heterogeneity will potentiate epidemiological and therapeutic advancements. For the sake of continuity, we address experimental strains on the basis of their ST in this Review.

Clade	Representative STs (RT, TcdB variant, receptor and disease severity)	Refs
C1: $TcdA^{+}TcdB^{+}CDT^{+}$ $TcdA^{+}TcdB^{+}CDT^{-}$ $TcdA^{-}TcdB^{+}CDT^{-}$ $TcdA^{-}TcdB^{-}CDT^{-}$	ST54 (RT630): TcdB1 variant; binds CSPG4 and FZD; induces severe disease ST2, ST7, ST46, ST48, ST145 (RT087): TcdB1 variant; binds CSPG4 and FZD; induces severe disease	32,34,153,165–167
C2: $TcdA^{+}TcdB^{+}CDT^{+}$	ST1 (RT027): TcdB2 variant; binds CSPG4; induces strong oedema ST1 (RT036): TcdB4 variant; binds FZD or CSPG4 weakly; induces mild disease	32,34,168
C3: $TcdA^{+}TcdB^{+}CDT^{+}$	ST5 (RT023)	169

Clade	Representative STs (RT, TcdB variant, receptor and disease severity)	Refs
C4: $TcdA^{-}TcdB^{+}CDT^{-}$	ST37 (RT017): TcdB3 variant; binds FZD; induces severe inflammation	170
C5: $TcdA^{+}TcdB^{+}CDT^{+}$ $TcdA^{-}TcdB^{+}CDT^{+}$	ST11 (RT078)	152,171
CI: $TcdA^{-}TcdB^{+}CDT^{-}$	ST200, ST311, ST620	155
CII: $TcdA^{-}TcdB^{+}CDT^{-}$	ST181, ST314, ST360	155
CIII: Variable	ST342, ST343, ST369	155

This is not an exhaustive list, and some STs may differ in their characteristics. CDT, *C. difficile* transferase; CSPG4, chondroitin sulfate proteoglycan 4; FZD, Frizzled; RT, ribotype.

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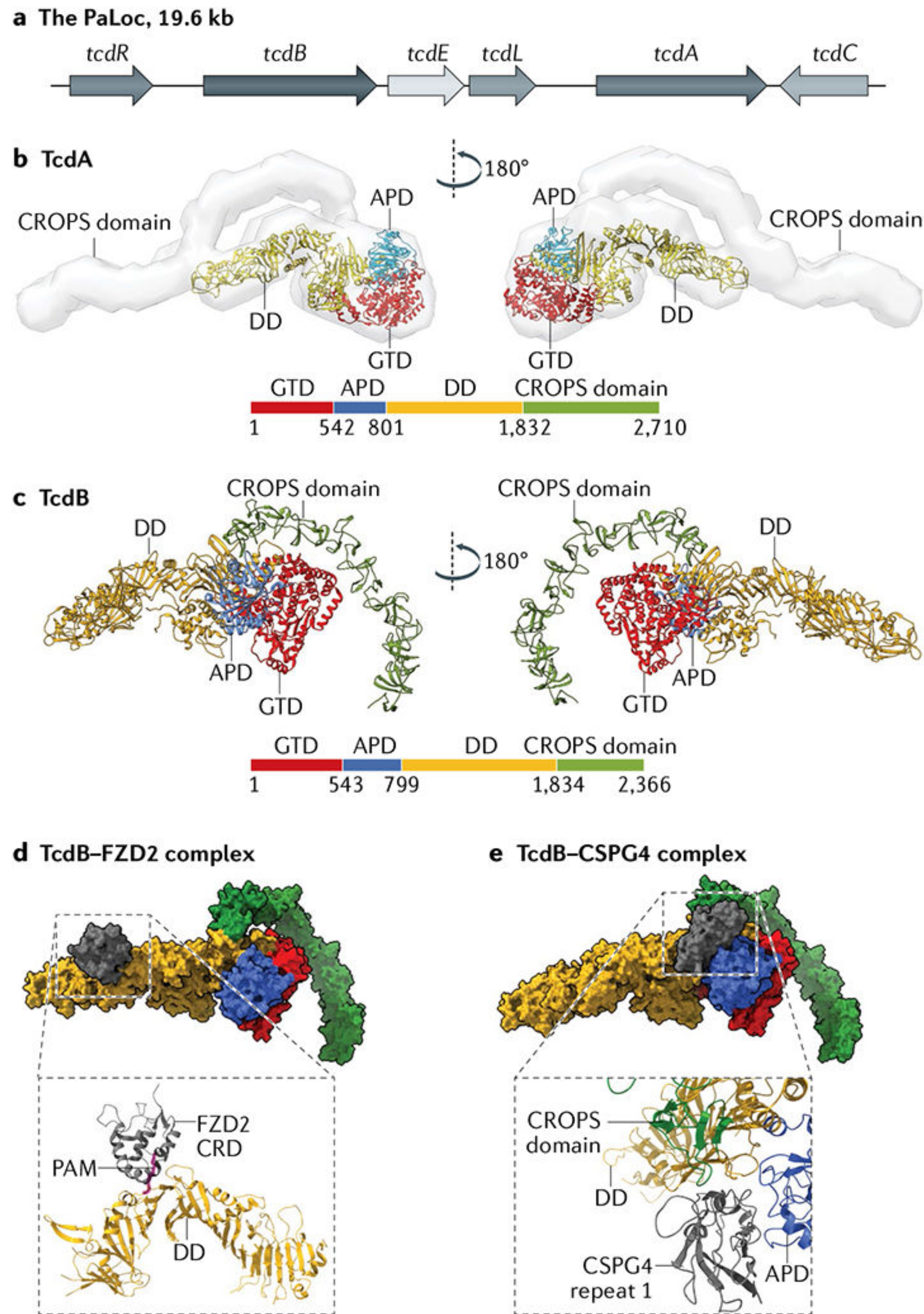

Fig. 1 |. Structures of TcdA and TcdB.

a | The pathogenicity locus (PaLoc). The PaLoc contains genes that encode the large clostridial toxins A (TcdA) and B (TcdB), the positive toxin regulator TcdR, the negative toxin regulator TcdC, the holin TcdE and the endolysin fragment TcdL. **b |** Crystal structure of $TcdA_{1-1832}$ (Protein Data Bank (PDB) identifier (ID) 4R04) docked into an electron density map of the holotoxin. **c |** Structure of TcdB (PDB ID 6OQ5). **d |** Structure of the Frizzled 2 (FZD2) cysteine-rich domain (CRD) bound to the TcdB delivery domain (DD) (PDB ID 6C0B) and docked onto the TcdB holotoxin structure (PDB ID 6OQ5).

The magnified view highlights the empirical structure containing palmitoleic acid (PAM) in magenta (PDB ID 6C0B). **e** | Structure of chondroitin sulfate proteoglycan 4 (CSPG4) repeat 1 bound to TcdB (PDB ID 7ML7) docked onto the TcdB holotoxin structure (PDB ID 6OQ5). The magnified view highlights the binding interface (PDB ID 7ML7). APD, autoprotease domain; CROP domain, combined repetitive oligopeptide domain; GTD, glucosyltransferase domain.

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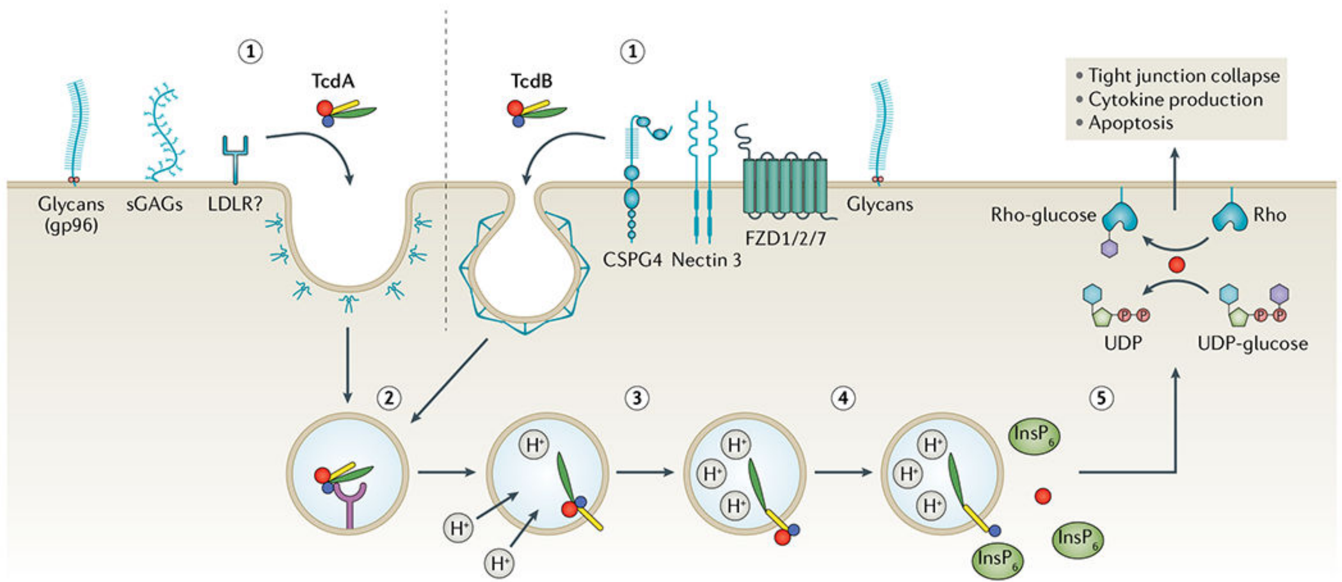

Fig. 2 |. Intoxication mechanism of TcdA and TcdB.

(1) Toxin A (TcdA) and toxin B (TcdB) bind distinct surface receptors. TcdA is thought to bind glycans (and proteoglycans such as glycoprotein 96 (gp96)), sulfated glycosaminoglycans (sGAGs) and/or members of the low-density lipoprotein receptor (LDLR) family. TcdB can bind chondroitin sulfate proteoglycan 4 (CSPG4), Nectin 3, Frizzled 1 (FZD1), FZD2, FZD7 and a variety of glycans. (2) Following receptor binding, TcdA and TcdB are internalized. TcdA uses a novel clathrin-independent entry mechanism through PACSIN2, whereas TcdB enters via clathrin-mediated endocytosis. (3) An influx of protons lowers the pH of the endosome, which induces transmembrane pore formation in the toxins. (4)The autoprotease domain (APD) and the glucosyltransferase domain (GTD) are translocated through the endosomal membrane into the cytosol. Inositol hexakisphosphate ($InsP_6$) activates the APD and initiates autoproteolysis and release of the GTD. (5) The free GTD monoglucosylates host GTPases, resulting in downstream cellular changes such as tight junction collapse, cytokine stimulation and apoptosis.

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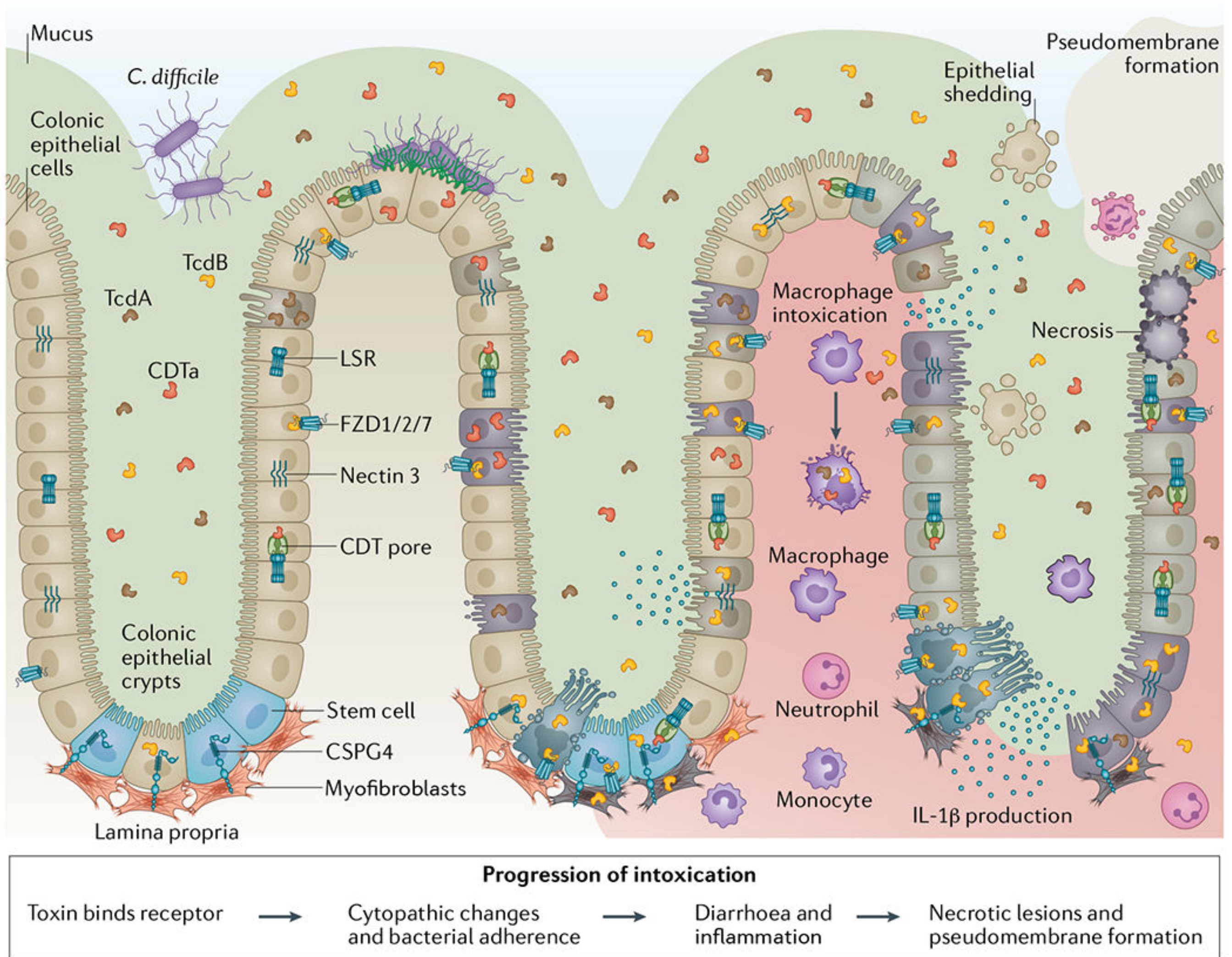

Fig. 3 |. Progression of colon intoxication by the *Clostridioides difficile* toxins.

Clostridioides difficile colonizes the colon and produces the large clostridial toxins A (TcdA; brown) and B (TcdB; yellow), as well as the *C. difficile* transferase (CDT; CDTa in red and CDTb in green). TcdA binds glycoproteins to enter host cells. TcdB binds Frizzled 1 (FZD1), FZD2 and FZD7 (blue) on the basolateralside of host epithelia, Nectin 3 (blue) at cellular junctions and chondroitin sulfate proteoglycan 4 (CSPG4; blue) on myofibroblasts within the lamina propria. CDTb binds lipolysis-stimulated lipoprotein receptor (LSR; blue) and oligomerizes to enable CDTa binding. Increasing concentrations of TcdA, TcdB and CDT within host cells cause the disruption of focal adhesions and tight junctions, loss of cellular polarity and cytoskeletal breakdown. These effects culminate in cellular rounding, stem cell apoptosis at the base of colonic crypts, epithelial cell shedding and oedema. Damaged epithelial cells release cytokines and chemokines, which recruit neutrophils and other immune cells to the tissue. The intoxication of monocytes and macrophages can promote IL-1β production and pyroptosis, which leads to further inflammation and damage within the tissue. Dead epithelial cells and immune cells contribute to the formation

of pseudomembranous plaques, a hallmark of *C. difficile*-associated pseudomembranous colitis.

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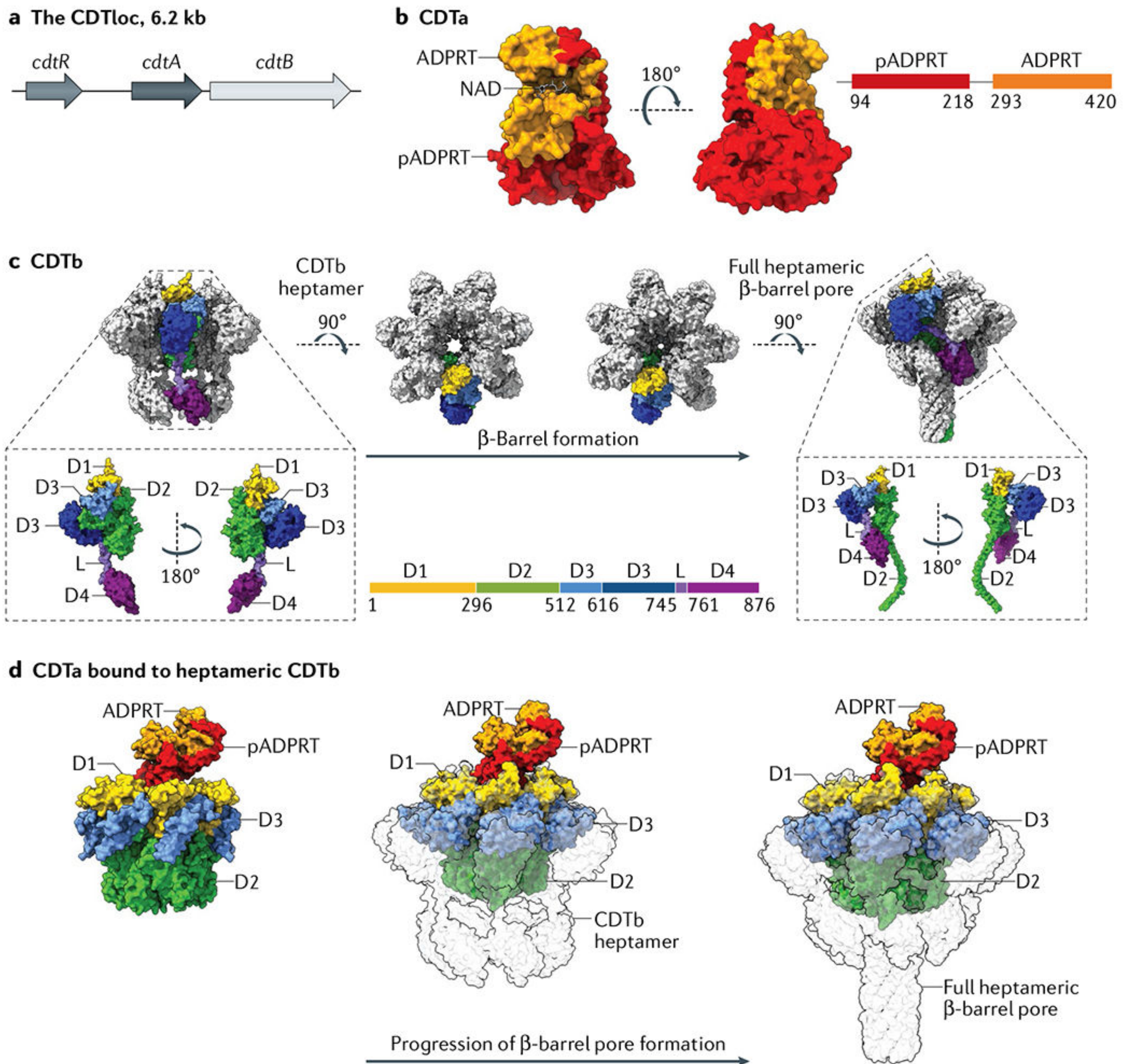

Fig. 4 |. Structure of the *Clostridioides difficile* transferase toxin.

a | The *Clostridioides difficile* transferase (CDT) locus (CDTloc). The CDTloc contains genes that encode CDT, *cdtA* and *cdtB*, as well as the positive toxin regulator CdtR, *cdtR*. **b |** Structure of CDTa bound to nicotinamide adenine dinucleotide (NAD) (Protein Data Bank (PDB) identifier (ID) 2WN6). CDTa comprises the ADP-ribosyltransferase (ADPRT) and pseudo-ADPRT (pADPRT) domains. **c |** Structures of heptameric CDTb with and without full β-barrel extension (PDB ID 6O2N). CDTb comprises domains D1–D3, D3’ and D4, with a linker (L) between D3’ and D4. Magnified views highlight one protomer of CDTb. **d |** Structure of CDTa bound to heptameric CDTb (PDB ID 6V1S) (left). Transparent surface representations of heptameric CDTb with and without full β-barrel extension (PDB

ID 6O2N) are shown in the same orientation after superimposition of CDTa bound to heptameric CDTb (PDB ID 6V1S). Colour schemes of domains are continued from FIG. 4b,c. D3’, L, and D4 are not visible.

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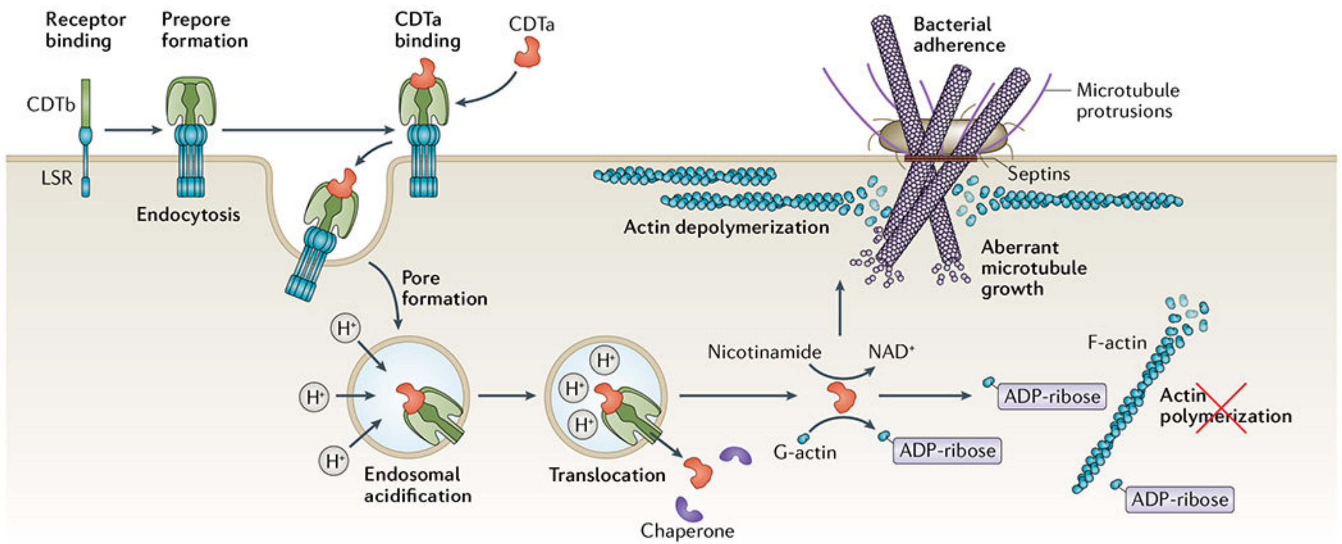

Fig. 5 |. Intoxication mechanism of *Clostridioides difficile* transferase toxin.

Monomeric *Clostridioides difficile* transferase b (CDTb; green) binds to lipolysis-stimulated lipoprotein receptor (LSR; blue) on the gut epithelium. Oligomerization results in a heptameric prepore state, upon which CDTa (red) can bind. The CDTb prepore and CDTa are then endocytosed. The CDTb prepore transitions into a pore-forming state, with a β-barrel pore that spans the endosomal membrane. Endosome acidification triggers the translocation of CDTa into the cytosol. CDTa ADP-ribosylates G-actin (blue), which acts as a cap and inhibits its polymerization. The depolymerization of F-actin (blue) at the apical host cell surface promotes aberrant microtubule protrusion (purple), supported by septin proteins (red). The protrusions can envelope *C. difficile* cells to augment adherence to the host. NAD^+, oxidized nicotinamide adenine dinucleotide.

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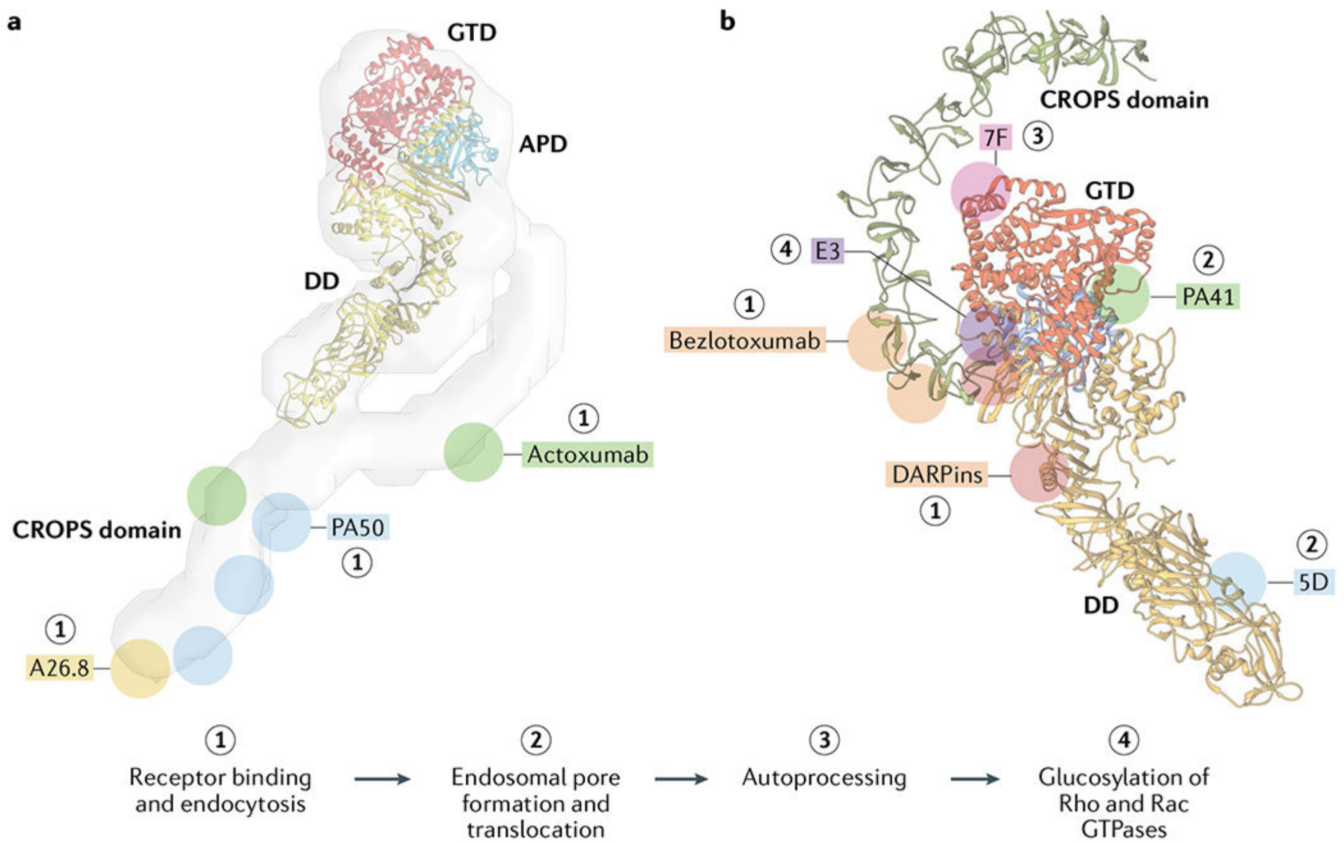

Fig. 6 |. Biological therapeutic binding locations on TcdA and TcdB.

The positions of biological molecules that bind toxin A (TcdA; Protein Data Bank identifier 4R04; panel **a**) or toxin B (TcdB; Protein Data Bank identifier 6OQ5; panel **b**) are shown in coloured circles with numbers that correspond to their proposed mechanism of inhibition. The autoprotease domain (APD) on the TcdB structure is hidden and therefore unlabelled. CROPS, combined repetitive oligopeptide sequences; DARPins, designed ankyrin repeat proteins DD, delivery domain; GTD, glucosyltransferase domain.