Unveiling Cholera Toxin binding and intoxication using Enteroids and Site-Specific Mutants

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Cover illustration: Cholera toxin protein structure highlighting the H18 and W88 residues. Rendered using the web-based - https://3dproteinimaging.com.

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Science can never solve one problem without raising ten more problems – George Bernard Shaw

Dedicated to my grandma

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Abstract

The diarrheal disease of cholera affects millions each year, causing over 100 000 deaths. This is caused by the bacteria *Vibrio cholerae* which produces the highly efficient Cholera toxin (CT) that exerts its function in the small intestine of humans. CT has long been considered to intoxicate using the glycolipid GM1 due to its extreme binding strength, but in the past 10 years an alternative binding site has been visualized to bind glycoproteins. Through subsequent findings, it has been shown that blocking either binding site can ablate the effects of CT on human tissue. I sought to expand upon these findings for my thesis, using human enteroids as a primary model. We also aimed to investigate the CT binding ligands in humans that can be functional, in parallel to understanding the binding site these are facilitated through. Finally, we investigated the effects of the passive defense of mucins on how they might affect CT binding and internalization. Initial work in this thesis focused on blocking CT binding using inexpensive simple polymers, where we show that a combined Fucose/Galactose polymer could both block CT binding and partially prevent CT intoxication, while a GM1 oligosaccharide could completely prevent CT intoxication. Using inhibitors in combination with CT B-subunit (CTB) binding deficient mutants, we found that fucose plays a dual role as both a component of binding decoy structures for CT and on functional ligands. Further, we found that O-linked, but not N-linked, glycoproteins play a key role in binding decoy ligands. Using holotoxin binding deficient mutants, we confirmed the requirement of both binding sites for CT to exert the diarrheal response. Inducing MUC17 also provided protection against CT, preventing expressing cells from binding CT and reducing overall internalization. It is evident that the diverse glycosylation in the human small intestine can provide layers of protection from CT, but the specific ligands that can function to facilitate intoxication are yet to be determined.

Keywords: Cholera toxin, Enteroids, GM1, Fucose, MUC17

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Sammanfattning

Kolera, en diarrésjukdom som drabbar upp till 5 miljioner människor varje år och som kan ha hög dödlighet utan behandling. Oftast är det i områden som Västra Bengal samt Östra Afrika som drabbas av kolerasmitta, men även vid naturkatastrofer eller vid konflikter kan hög smitta av kolera förekomma. Nuvarande behandling består av oral vätskeersättning eller vid allvarligare fall ges intravenös vätsketerapi ihop med antibiotika. Det finns vacciner mot kolera med ganska bra effektivitet men det rekommenderas inte att vaccinera barn under 1 år, och vaccinet är dessvärre inte tillgängligt i alla områden som är drabbade.

Det vi kallar kolera är effekten som koleratoxin har på våra kroppar, ett toxin som tillverkas av Vibrio cholerae.Bakterien koloniserar tunntarmen hos människor där den producerar koleratoxin som binder till molekyler (ligander) på cellens yta vilket resulterar i att den internaliseras. Efter internaliseringen, utövar toxinet sin effekt för att orsaka överdriven vätske- och saltsekretion från cellerna. Slutligen, får man risvattendiarré som kan bestå av mer än 10 liter vätskeförlust per dag vilket är karakteristiskt för kolera.

På 70-talet upptäcktes det att koleratoxin binder starkt med GM1-glyckolipiden (sockermolekyler kopplat till en fettkedja), som kan möjligöra funktionell upptagning av toxinet. Forskning har mestadels bedrivits under antagandet att förgiftningen drivs av GM1-bindning inom djur och cellinjer. Sedan upptäckten har det antagits att GM1 och liknande glykolipider är liganderna för koleratoxin, men på senare tid har det avslöjats att glykoproteiner (sockermolekyler på proteiner) också kan binda toxinet. Dessa glykoproteiner är lämpliga för en alternativ bindningsplats, men orsaken och funktionen för denna alternativa plats är för närvarande okänd. Dessutom har människor mycket låga nivåer av GM1 i tunntarmen, vilket föreslår att olika bindningsmekanismer kan vara ansvariga för koleradriven förgiftning av människor.

I denna avhandling använde vi organoider (miniatyrorgan) från friska mänskliga donatorer, för att undersöka hur koleratoxin binder till våra celler för och förgiftar människor med den mest fysiologiskt representativa metoden som finns tillgänglig för närvarande. Vi visar att enkla långa sockerkedjor av galaktos och fukos kan lindra effekten av koleratoxin och förhindra bindningen helt i mänskliga tunntarmceller. Samt visar vi att mucinet MUC17, som uttrycks på cellytan på tarmceller, skyddar tarmen från koleratoxin bindning och upptag.

Genom skapande av bindande defekta mutanter, har vi visat att fukos i tunntarmen finns som båda lockbeten och funktionella ligander. Vi har även visat att koleratoxin-förgiftning av tunntarmsceller behöver ligander riktad mot båda bindingsplatserna för att inducera jonutsödnring, Som inte reflekterats i klassiska cellinjer som hittills använts. Taget illsammans har denna avhandling avslöjat några av kraven på koleratoxin för att förgiftas, vilket kan leda till förbättrad behandling och vaccinutveckling mot kolera i framtiden.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Jakob Cervin, Andrew Boucher, Gyusaang Youn, Per Björklund, Ville Wallenius, Lynda Mottram, Nicole S. Sampson, and Ulf Yrlid.
Fucose-Galactose Polymers Inhibit Cholera Toxin Binding to Fucosylated Structures and Galactose-Dependent Intoxication of Human Enteroids

ACS Infect. Dis. 2020, 6, 1192−1203,
https://dx.doi.org/10.1021/acsinfecdis.0c00009

II. Akshi Singla, Andrew Boucher, Kerri-Lee Wallom, Michael Lebens, Jennifer J. Kohler, Frances M. Platt, and Ulf Yrlid.
Cholera intoxication of human enteroids reveals interplay between decoy and functional glycoconjugate ligands

Glycobiology, 2023, 00, 1–16,
https://doi.org/10.1093/glycob/cwad069

III. Andrew Boucher, Akshi Singla, Michael Lebens, and Ulf Yrlid
Binding Deficient Cholera Toxin Mutants Reveal Dependence on Both Binding Sites for Human Intoxication

Manuscript

IV. Sofia Jäverfelt*, Andrew Boucher*, Ulf Yrlid, and Thaher Pelaseyed
The glycocalyx-forming membrane mucin MUC17 prevents entry of cholera toxin in human enterocytes

Manuscript, * denotes equal contribution

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Abbreviations

2F-Fuc	2-Fluoro-L-Fucose
AC	Adenylate Cyclase
APC	Antigen Presenting Cell
BCR	B cell Receptor
cAMP	Cyclic Adenosine Monophosphate
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CT	Cholera Toxin
CTA	Cholera Toxin A-Subunit
CTB	Cholera Toxin B-Subunit
CTL	Cytotoxic T-Lymphocyte
DAMPs	Damage-Associated Molecular Patterns
DC	Dendritic Cell
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated protein Degradation
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GalNAc	N-acetyl Galactosamine
GlcNAc	N-acetyl Glucosamine
GM1os	GM1 Oligosaccharide
HBGA	Histo-Blood Group Antigen
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IHC	Immunohistochemistry
Le$^{a/b/x/y}$	Lewis$^{a/b/x/y}$

LN	Lymph Node
LPS	Lipopolysaccharide
LT	Heat-Labile Enterotoxin
LTB	Heat-Labile Enterotoxin B-Subunit
MHC	Major Histocompatibility Complex
NB-DGJ	N-(n-Butyl)deoxygalactonojirimycin
PAMPs	Pathogen-associated molecular patterns
PKA	Protein Kinase A
PRR	Pattern Recognition Receptor
RIP	Ribosome Inactivating Protein
Isc	Short Circuit Current
SA	Streptavidin
SI-EC	Small Intestinal Epithelial Cell
TCP	Toxin-Coregulated Pilus
TCR	T-Cell Receptor
TEER	Trans-Epithelial Electrical Resistance

INTRODUCTION

Cholera

Cholera is the commonly known diarrheal disease that has afflicted humans across the planet. This disease is caused by the gram-negative bacteria *Vibrio Cholerae (V. cholerae)*, presenting with the main symptoms of acute "rice water diarrhea" and vomiting. The excessive loss of fluid and electrolytes can easily last up to six days, and can kill within hours if left untreated. It is estimated that 3 – 5 million are afflicted by cholera each year, of which 200 000 to 1.2 million are reported to the World Health Organization (WHO), where over 100 000 cases are fatal [1-5]. Despite having cases of cholera, the overall number of those who are infected with *V. cholerae* is substantially higher, as 40-80% of carriers are asymptomatic [6].

The culprit of cholera symptoms is the exotoxin cholera toxin (CT) that is secreted by the bacteria once the small intestine is colonized [7, 8]. CT binding has extremely high affinity for the glycosphingolipid GM1 and has been considered the canonical ligand ever since its discovery in 1970 [9]. Subsequently, glycan oligosaccharides were found to bind with much lower affinity, but were only recently found to bind to a separate binding site on the toxin [10, 11]. Furthermore, the overwhelming share of binding occurs to these alternative binding ligands in the human small intestine, and can even facilitate intoxication [12].

Within the scope of this thesis, we aimed to identify the binding ligands used by CT in humans to build the groundwork for developing cost-effective treatments. Historically, previous CT research has been performed in animals or using transformed cell lines, which we have translated using tools we created. Additionally, we have used the more physiologically relevant primary tissue derived organoid model, to extend our knowledge of how CT intoxicates humans.

Epidemiology

V. cholerae is primarily transmitted through contaminated food and water sources, overwhelmingly affecting developing countries, especially in poorer regions where access to clean water is lacking. The natural reservoir for *V. cholerae* is primarily humans and bodies of water, with the Ganges delta of West Bengal commonly believed to be the ancestral epicenter [13-15]. With the movement of populations and the advancements in travel, cholera has become a widespread disease that affects significant portions of the developing world. Large parts of Asia, Africa, Southern America, and Central America are some of the areas most affected [1, 4].

Cholera is often regarded as an opportunistic disease during disasters such as wars or natural calamities, due to its mode of transmission. In these events, volunteers may carry the bacteria asymptomatically and inadvertently contaminate food and water sources while providing assistance to affected individuals. An example of this occurred during the recent major outbreak in 2010 following the Haiti earthquake disaster, when a body of drinking water was

inadvertently contaminated with *V. cholerae* by Nepalese soldiers deployed to provide aid [16]. Following the Yemeni civil war, a cholera outbreak occurred due to a mix of factors that were severely exacerbated by the conflict [17]. However, even in nations that are not beset by disaster, cholera cases often rise during increased rainfall and monsoon seasons [18, 19]. More recent disasters such as the global Covid-19 pandemic have also led to substantial rises in cholera cases [20].

Cholera has often persisted throughout history as separate pandemics. The first pandemic occurred in the Western Bengal region of Calcutta in 1817 and lasted until 1824 [21]. This pandemic caused hundreds of thousands of deaths across Asia, but also managed to spread via traders to Eastern Africa, the Middle east, Europe and Southeast Asia. There have been seven pandemics recorded, with high mortality rates observed across the world during the 2nd to the 6th pandemics [22-24]. The pandemics until this point were caused by the so-called *V. cholerae* O1 classical biotype. Currently, we are in the seventh recorded cholera pandemic, which began in 1961 [6]. The current pandemic originated in Indonesia, with the premiere of the El Tor biotype, which subsequently displaced nearly all traces of the classical biotype world-wide, except in Bangladesh [22, 25, 26]. The biotype of El Tor differs from classical in the differential expression of ~13% of the genome, as well as the production of hemolysins [27, 28]. The inclusion of hemolysins have been postulated to have a similar effect to that of CT, and therefore may be a method of diversifying to provide more strategies for survival [29]. The El Tor genome expression change likely has a selective advantage for growth and survival [29, 30]. Despite this, the El Tor biotype has the potential to produce more CT than that of the classical biotype, although the induction of CT requires different conditions [31]. In the current cholera pandemic, there are hundreds of El Tor biotype strains, all of which are independently evolving to geographical and antimicrobial challenges [32, 33].

In general, bacterial strains are distinctly categorized by different factors to establish lineage and function. The three most common for *V. cholerae* are the classifications of serogroup, serotype and biotype. Serotypes seek to classify based on the antigens expressed on the cell surface. Serogroup classification is broader and aims to define based on more drastic differences. Finally, the biotype is a characterization based on biological activity.

Even though *V. cholerae* produces CT to enact the fluid secretion it is well known for, only two serogroups out of over 200 produce the toxin. These serogroups are known as O1 and O139. The O1 serogroup is the first detected pathogenic CT-producing strain, while O139 arose in Bangladesh and India in 1992, and characteristically expresses a polysaccharide capsule [34-36]. Additionally, the O1 serogroups can be split into the classical and El Tor biotypes. There is strong evidence that the O139 serogroup originates from the O1 El Tor biotype [37]. Despite this, the polysaccharide capsule in combination with an effective LPS polysaccharide core substitution results in distinctive altered virulence factors [36, 38]. The El Tor biotype originally had substitutions in the *ctxB* gene at positions 115 and 203 that have subsequently reverted back to the classical biotype sequence [39, 40].

The O1 serogroup also has 3 known serotypes named Inaba, Ogawa and Hikojima. These are distinguished based on the antigenic determinant expression of their O-side chain lipopolysaccharides (LPS). All three serotypes have the A antigen in common, while Ogawa also has the B antigen, in contrast to Inaba that has the C antigen and Hikojima has all three [41, 42]. These serotypes can be readily identified through an agglutination in response to antibodies that target these antigens [43]. The difference in the immunological determinant between Ogawa and Inaba is the methylation state on the LPS. This is dependent on a functional methyltransferase, which is absent in the Inaba serotype [44, 45]. Additionally, it has been shown that Ogawa and Inaba can switch between serotypes, for unlcear reasons, but is suspected to be in response to selective pressures [46-49]. The Hikojima serotype characteristically exists in both unmethylated and methylated LPS states, likely a result of being in a transitional state between serotypes that does not occur in nature [50, 51].

While only two serogroups have been found to produce CT, other strains of *V. cholerae* have also been noted as pathogenic [52, 53]. Interestingly, all *V. cholerae* strains produce a second toxin known by its gene name of MakA [54]. This cytotoxin induces pore formation in host cell endosomes via cholesterol, resulting in disruptions to standard autophagy [54-57]. Unlike CT, MakA functions in a wide range of different organisms, providing *V. cholerae* with survival and proliferative benefits [54]. It has also been shown that MakA has a functional preference for cancerous or transformed cells compared to healthy cells of the intestine, providing a host of options for the toxin to be utilized as a treatment for tumors [58].

Current treatments

The current gold standard treatment for cholera comes in the form of oral rehydration therapy (ORT). As the name implies, this therapy is typically administered orally and consists of glucose, sodium, potassium and chloride in clean water [3, 59, 60]. Furthermore, the inclusion of rice starches have been shown to improve therapy efficacy due to the slow release of glucose, leading to reduced stool output and overall shorter diarrheal duration [61]. In addition, rehydration therapies that are formulated with higher osmolarity have been shown to increase risk of hypernatremia and to be less effective in children, with current guidelines recommending a lower solute concentration especially for extremely malnourished children [62-64].

In more severe cases, where the patient may be vomiting from cholera or in response to ORT, or when they have lost consciousness, it is typically advised that the therapy is replaced preferentially with intravenous perfusion of Ringer's lactate IV - a solution containing sodium, chloride, potassium, calcium and lactate [3, 65]. In some extreme cases, antibiotics may also be administered to limit the continued bacterial proliferation, although this is strongly advised against due to the potential rise of antibiotic resistance [3, 66]. These therapies have gone a long way to improve overall survival rates to those afflicted, as they are low-cost, easy to transport and store, and require little training to administer effectively. Hence, the overall

mortality rate has reduced significantly from ~50% to lower than 1%, which is especially important in children who had fatalities exceeding 70% of all cases [3, 67].

Several alternative treatments have been explored to treat cholera with varying levels of success. Some of these treatments are currently under investigation and are being considered as potential primary therapies against cholera infection. A previous trial that had some promising results utilized the ganglioside GM1 adsorbed into charcoal, showing significant reductions in fluid loss [68]. However, the effectiveness was found to be time-sensitive relative to symptom onset [68]. Another avenue being explored is the use of bacteriophages. It has been shown that a phage targeting *V. cholerae* can effectively replicate and halt bacterial progress in mice. However, as phages are viruses, the host immune system may adapt to produce phage specific antibodies, while the bacteria may evolve to evade phage attacks [69, 70]. Probiotics are another approach being investigated. Multiple bacterial strains have shown to have positive effects on overall cholera infection outcomes, several of which are common in human or mice microbiota, from reducing CT production to disrupting biofilm construction [71-74]. Finally, another treatment avenue involves using a Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) inhibitor which has shown efficacy in blocking cholera disease symptoms in mice. Additionally, it has demonstrated to decrease bacterial survival and impede further growth [75]. Interestingly, those with cystic fibrosis are significantly protected from cholera symptoms, leading to speculation that cystic fibrosis may be a selective advantage in areas where cholera is common [76-78]. Consequently, CT has been hypothesized as a potential therapeutic agent for relieving symptoms in individuals afflicted by cystic fibrosis [76].

The current recommended treatment of ORT has provided a substantial increase in the overall survival rate, especially in children infected with cholera. However, they are primarily reactive, given once symptoms appear, and rely on the affected individual reaching a facility with aid readily available. Other treatments have explored the potential for a proactive approach to decrease overall morbidity and mortality, particularly for individuals who may not be able to access care promptly. While alternative treatments have been proposed, each with its own benefits, they have also been limited by various factors.

History of research

Cholera Toxin

The toxin produced by *V. cholerae* that induces fluid secretion in humans is CT. CT is composed of two main components: a single copy of the enzymatically active component of CTA, and 5 subunits arranged in a ring of the receptor binding portion known as CTB (Fig. 1A, B) [79]. The CTB pentameric ring is arranged in such a way that the CTA subunit fits inside comfortably. Together this is known as CT, weighing around 88 kDa. Interestingly, CT has around 80% homology to the heat-labile enterotoxin (LT) produced by some *E. coli* strains. The genes that encode for CTA and CTB are *ctxA and ctxB* respectively, and these genes are regulated by *toxT* within the ToxR regulon. The secondary main virulence factor is the toxin co-regulated pilus (TCP), which can be triggered by physiological sensing [80-82]. In addition to *toxT*, the expression of TCP has recently been revealed to be controlled by another, as-yet-unknown regulator [80]. Once expressed, CT is secreted by the bacteria through the Type-2 Secretory System (T2SS) pathway, and into the intestine where CT can then internalize and cause fluid efflux [83]. CT expression is believed to robustly clear out competing microbes in the gut. This creates opportunity for exploitation by *V. cholerae* to further proliferate, resulting in subsequent virulence factor repression and upregulation of detachment factors [84].

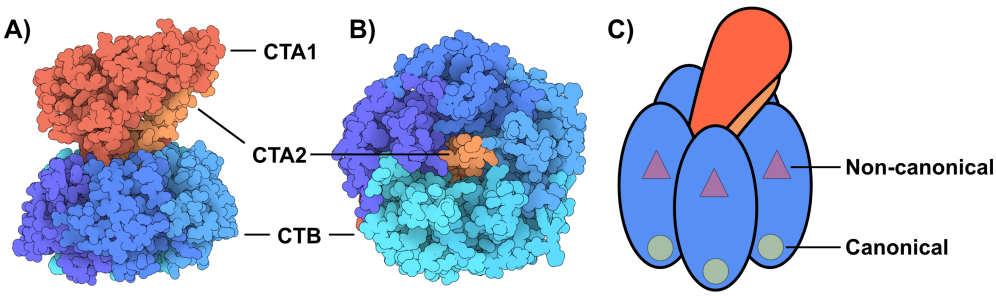

Fig. 1. CT structure and binding sites. Structural model A) side on and B) bottom view of whole CT, displaying the 5 CTB-subunits, CTA1 and CTA2 in complex. C) CT simplified diagram, displaying the Canonical (GM1) binding site and Non-canonical (Fucose dependent) binding site.

CTB is the portion of CT that binds to ligands on the cell surface to facilitate internalization and deliver the CTA payload. Pioneering research in the seventies demonstrated that CTB binds to the ganglioside GM1 [85-87]. This binding was found to occur on the basal side of each CTB subunit via a binding pocket that accepts a “two-finger” grip by the GM1 structure [88]. The binding strength of this grip is remarkably strong for a non-covalent bond and was therefore assumed to be the canonical binding ligand. Thus, this binding pocket is termed “the canonical binding site” (Fig. 1C). Despite this there is little detectable GM1 or other CTB binding gangliosides in the human small intestine [89]. In addition, glycoproteins and fucosylated structures were found to bind to CTB [10, 11, 90]. These ligands were found to bind to an alternative binding site known as the non-canonical binding site which cannot bind to GM1, and in contrast to the canonical binding site that binds GM1, this site is better suited for fucosylated structures and is situated on the lateral side of each CTB subunit (Fig. 1C) [10,

11, 91]. Most of the work in this thesis has focused on investigating these CTB binding sites and their role in attachment and intoxication of the human small intestine.

Regardless of the site used by CTB to bind for human intoxication, CT(B) binds to a ligand that can facilitate internalization. Once bound to the correct ligand(s), one of several potential pathways enable internalization and subsequently, intoxication. These range from clathrin-dependent mechanisms to clathrin-independent mechanisms such as caveolae and lipid rafts [92-96]. Studies have revealed that the uptake of CT does not always result in a functional toxin response, with the outcome likely dependent on the specific type of ligand to which CT binds (Fig. 2) [92]. Once bound to the correct ligand(s), CT is internalized by retrograde transport via endosomes to either the Golgi Apparatus or the Endoplasmic Reticulum (ER). At this point, CT can travel to the ER and the Golgi Apparatus to take advantage of different chaperones [97]. The Golgi was considered to be an integral part of the CT intoxication pathway, but has subsequently been shown to be optional [98, 99].

At this point, one of two proposed steps occur to activate intoxication. One theory postulates CTA dissociates from the pentameric CTB vehicle, allowing for the release of CTA1 from CTA2 through ER-degradation. Due to the low lysine content of CTA1, sufficient ubiquitination cannot occur, thereby preventing full Endoplasmic reticulum associated protein degradation (ERAD) [100]. During this process, protein disulphide isomerase (PDI) aids in reduction while Ero1 facilitates reoxidation to unfold and refold CTA1 respectively [101]. More recent studies suggest that PDI does not function to unfold CTA1 at all [102, 103]. In the ER, the CTA1 portion of the holotoxin is recognized by reduced PDI, which upon contact unfolds PDI [104]. Following a temperature dependent CTA1 separation from the holotoxin, CTA1 spontaneously unfolds releasing the unfolded PDI [103, 105, 106]. Following this, the misfolded CTA1 protein is recognized by a series of proteins such as ERdj3 and BiP, among others, for processing in the ERAD system to exit into the cytosol [107-109]. The initial toxin response is quite ineffective, but does stimulate an unfolded protein response, which causes a feedback loop increasing the efficacy of CT intoxication [110]. This now unfolded CTA1 is refolded by either Hsp70 or Hsp90 and seeks to ADP-ribosylate Adenylate Cyclase (AC) by binding to the heterotrimeric G protein subunit, $G_s\alpha$ [107, 111]. The activation of AC increases the turnover of adenosine triphosphate to cyclic Adenosine Monophosphate (cAMP) [112]. The cAMP released into the cytosol results in over-activation of Protein Kinase A (PKA), up to 100-fold greater than under homeostatic conditions, constitutively activating the cells CFTR [113, 114]. As the CFTR is an ion channel for chloride ion homeostasis in the cell, having CFTR channels constitutively activated causes a mass exodus of chloride ions and subsequently water from the cell will follow into the intestinal lumen (Fig. 2) [115].

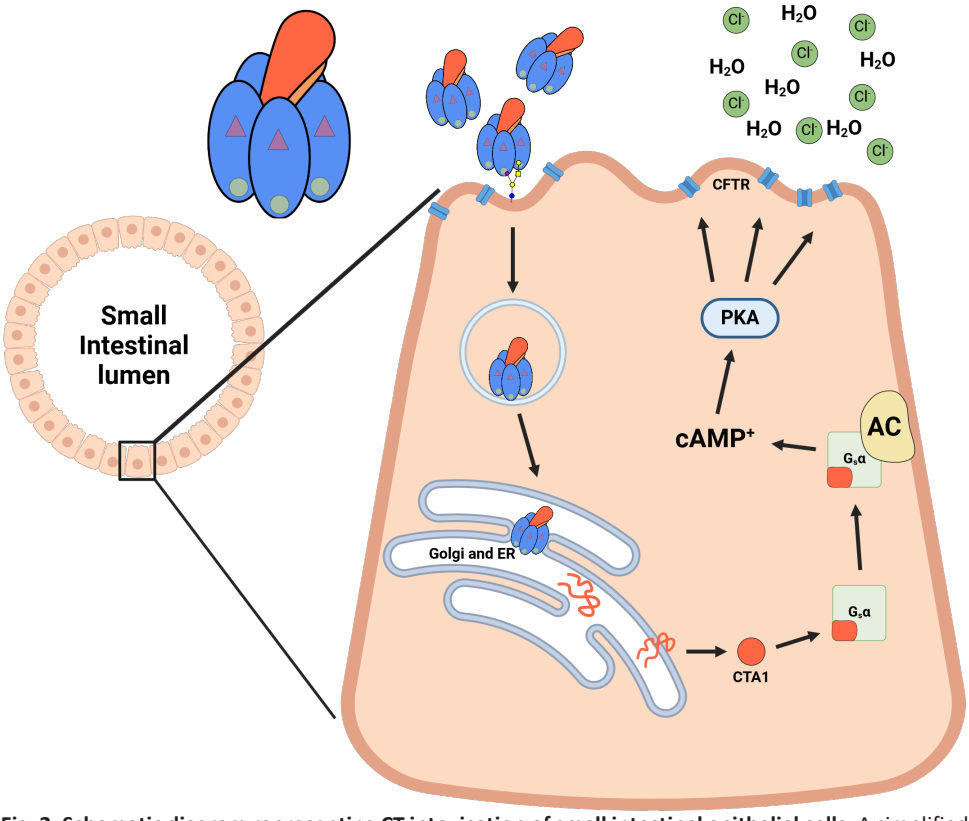

Fig. 2. Schematic diagram representing CT intoxication of small intestinal epithelial cells. A simplified schematic figure representing the current understanding of CT intoxication in the small intestine.

Cholera immune response

As the majority of this thesis is centered around the effector function of CT and how it initially binds, I have done minimal work with the toxin with respect to the human immune response. It is worth noting that there is a wealth of research in this field, and as our hope is that our findings may in contribute to the development of improved vaccines, I will provide a brief overview of the immune system and response to cholera and how vaccines can mitigate this.

The immune system is generally categorized into two separate arms, that often interact and coordinate to keep us safe. These are known as the innate and adaptive immune systems. The innate immune system is the first line of defense actively regimented by the body. This system is responsible for recognizing common structures that are parts of invading pathogens known as pathogen associated molecular patterns (PAMPs). Some of the most recurrent examples are dsDNA of viruses and bacterial LPS. Another role of the innate immune system is to remove damaged cells or debris through a similar detection method known as damage-associated molecular patterns (DAMPs). These PAMPs are recognized by a pattern recognition receptor (PRR) causing a cascade of signaling inside the cell, resulting in the promotion of

inflammation and recruitment of more innate immune cells. Innate immune cells specialized in taking up and digesting cells are called phagocytes that are then tasked with the uptake of the damaged cells or pathogenic material to help maintain a healthy cellular environment. This system is effective but has a limited repertoire of commonly expressed features that can be detected, and as the war between pathogens and hosts is a struggle for survival of both parties, the pathogens often mutate to develop new virulence mechanisms to avoid detection.

To combat this ever-evolving threat, the adaptive immune system has evolved as the name suggests. While this seems like it would be the only system needed, the adaptive immune system requires a lot of energy and generally requires a long time to mount a first response [116-118]. However, the adaptive immune system has immunological memory so that once a defense has been constructed, it can be rapidly deployed if the threat reappears. This immune memory is the principle that underlie vaccines, which will be discussed later. Even though we address these systems as two separate entities, there is significant crossover, with the adaptive immune system often working to aid the innate immune system to form a cohesive defense strategy. The adaptive immune system is typically initiated when the innate immunity proves insufficient. In this scenario specialized cells known as antigen presenting cells (APCs) are required to take up foreign material to present it to T lymphocytes. The adaptive immune response consists of T and also B lymphocytes, that are for simplicity, referred to as T and B cells. In addition, research has identified and characterized several subsets within these cell types.

Antigen presentation occurs through cell surface receptors known as major histocompatibility complex (MHC). Two major types of MHCs are present in humans where they present processed peptides to T-cell receptors (TCRs) of a cognate T cell. MHC-I is presented on all nucleated cells and functions as a check of cellular health. MHC-I displays peptides of proteins degraded within the cytosol of the cell, typically presenting the cell's own degraded proteins, which is then recognized by a $CD8^+$ cytotoxic T-lymphocyte (CTL) and judged to be healthy. When a pathogen has invaded an immune cell, some of its protein will be displayed and recognized as foreign material by the CTL. If expression of MHC-I is disabled by the pathogen, or for example in a tumor cell that is attempting to evade detection, natural killer (NK) cells will kill the transformed cell. In contrast, MHC-II is expressed by APCs that have phagocytosed a foreign agent, wherein protein material is processed for presentation upon MHC-II as a peptide for $CD4^+$ T-helper cell recognition.

In a typical immune response, dendritic cells (DCs) are recruited as professional APCs to bridge the gap between the two systems and mediate crosstalk. These cells are typically few in number, residing in tissues amongst the front line, where they are constantly on the prowl for dubious matter. The DC will phagocytose anything of interest and migrate to a nearby lymph node (LN), where the material is processed into peptides. Various peptide sections are then displayed on the DC cell surface by MHC-II molecules and recognized by the TCR of cognate T-helper cells. The B cell receptor (BCR) is an immunoglobulin (Ig), expressed by mature B cells

in the form of either IgM or IgD. These BCRs (similar to TCRs) are constructed by an intricate recombination of genetic elements and thereby generate lymphocytes with different specificities. While the TCR only recognizes linear peptides displayed by MHC molecules on APCs, BCRs can bind not only proteins but also lipids and carbohydrates. Upon successful binding to the latter, some B cells will become activated and proliferate to become short-lived plasma cells that produce antibodies (secreted BCRs) of the isotype IgM, that has weak antigen specificity. In addition, protein antigens bound by B cells can also be captured by the BCR internalized and after degradation, presented on its own MHC-II to T cells [119-122]. If a T-cell recognizes the MHC-II + peptide, this initiates a cascade of events to prepare an effective antibody blueprint. During this cascade, B cells go through Ig class switch recombination to produce IgG, IgA or IgE which have higher affinity. Their Fc portions (which in turn defines the Ig sub class) direct antigens to different FcR expressing immune cells thereby tailoring the ensuing immune response. During class-switching, the B cells will form multicellular compartments in the LN known as germinal centres, where the B cells will undergo affinity maturation via a process known as somatic hypermutation, as well as a selection processes to result in non-autoreactive, specific antibodies. The B cells selected by the peptide-specific T cells present in the germinal center, will proliferate, and become memory B cells or long-lived plasma cells. Long lived plasma cells excrete the higher affinity monomeric IgA, IgE or IgG into the blood. Memory B cells go into a state of quiescence, surviving for decades, until the familiar antigen is recognized again, kickstarting the proliferation of the memory cell and rapid production of high affinity antibodies. The initial IgM response can often take up to a week to provide protection, and IgG can take over two weeks. It is for this reason that the two arms of the immune system work collaboratively to progressively mount a stronger defensive response to pathogens.

When *V. cholerae* colonizes and exerts its toxic effects in the small intestine, the secreted IgA antibody is suited for protection [123]. IgA is typically, secreted into the lumen as a dimer by plasma cells residing in the lamina propria, where the sIgA can neutralize extracellular pathogens and their secreted products [124]. IgA has been shown to provide substantial neutralizing effects against CT, and can even be transferred from mother to child through breast milk [125]. Cholera has such a devastating impact on humans, necessitating a strong immune response to prevent future infections. It has been shown that humans who have recovered from cholera have antibodies, against both CT and the O-specific polysaccharide of LPS of the bacteria [126-128]. This T cell-independent antibody mediated protection is limited to the Inaba and Ogawa strains, and confers no protection against the capsulated O139 serogroup. However, T cell-dependent CTB-specific antibodies neutralize CT [129].

The ability of the human immune system to mount a response to cholera has provided much incentive for the development of vaccines to protect against the disease. Additionally, due to the current absence of strong mucosal adjuvants and the robust mucosal immune response triggered by CT, there is ongoing exploration into utilizing the toxin as a potent adjuvant [130, 131]. However, as CT is extremely toxic to humans, alterations are required to make it safe

while ensuring that its adjuvanticity is retained. One example of this is mmCT, which incorporates multiple mutations to the CTA subunit, thereby detoxifying the enzymatic activity of the protein [132, 133]. CTB itself can sometimes function as an adjuvant and interestingly, if conjugated to antigens, may soften some autoimmune responses to the antigen [134].

Vaccines

There are currently three options for vaccination against cholera. These are the licensed oral vaccines; Dukoral and Shanchol/Euvichol-Plus, and the US-only Vaxchora. Each of these vaccines have their own advantages and disadvantages, but the length of vaccine efficacy (protection) is relatively short in all products, lasting up to 3 years without boosters. Dukoral is based on a killed whole-cell vaccine of O1 cholera mixed with recombinant CTB, while Shanchol combines killed whole-cell constituents from both O1 and O139 serogroups [135]. Finally, Vaxchora is comprised of a live-attenuated O1 Inaba serotype [136]. Dukoral was designed principally to form an immune response to both the bacteria and CTB to provide neutralizing antibodies against intoxication. Due to the similarity of CT and LT, Dukoral also provides some protection against ETEC infections [137]. The inclusion of recombinant CTB does incur an increase in vaccine cost [138]. With the addition of O139, Shanchol ensures that some protection against the capsulated *V. cholerae* serogroup is provided, but lacks the CTB component of Dukoral, resulting in no neutralizing CT/LT antibodies. Despite their differences, both vaccines contain both Classical Inaba and Ogawa serotypes, as well as one Inaba El Tor biotype, with an efficacy of 50-80% [139]. Vaxchora has the distinct advantage of only needing a single dose, providing protection within 10 days, but as of yet the extent of protection has only been measured up to 3 months-post vaccination [136, 139]. Additionally, the Vaxchora vaccine is very expensive, requires storage in cold conditions and has low shelf-life compared to the other two alternatives, making it unsuitable for widespread adoption in endemic areas [140].

Vaccines are vital in the battle against cholera, especially in the developing world where the disease is endemic. Despite this, there are large swathes of these populations that are unvaccinated. In part, this can be due to lack of accessibility to facilities that provide vaccination but can also be an issue for those who are immunocompromised or who fail to mount a vaccine response. Additionally, the currently available vaccines are advised to only dose children over 12 months old (24 months for Dukoral and Vaxchora) [135, 139]. Due to this there is still a substantial requirement for new safe vaccines to be developed, and cheap effective treatments that can help mitigate symptoms in humans.

Potential pro-active measures for those who cannot be vaccinated currently rest on the idea of CT-blocking therapies. Blockers have traditionally been designed to bind the CTB component to prevent entry into the cell. One such option is the GM1-oligosaccharide (GM1os), which binds with high affinity and can block intoxication, but is expensive and difficult to synthesize, making it unsuitable deployment as a therapy [12, 141]. There is also a

plethora of different carbohydrate-blocking molecules designed to mimic the CTB-GM1 interaction that have varying success in binding CTB, but as of yet, none have been trialed or are commercially available [142-147]. One common tactic is the use of multivalent blockers that target multiple GM1 binding sites, having hundreds of thousands fold higher affinity than monovalent GM1os [144-147]. Targeting the non-canonical site, fucosylated molecules such as lewis^x (Le^x) derivatives, free fucose and fucose containing polymers can block CT binding to cellular surfaces [12, 148, 149]. CT blockers need to be designed to be large enough that they are not internalized by the absorptive cells of the intestine, have proven efficacy in blocking CT intoxication with no or little side effects to the host, and must also be cost-effective. With a better understanding of the ligands that CT binds in order to initiate intoxication and via which binding site, better therapies might be developed that can provide protection to those who cannot be vaccinated in regions where cholera remains a persistent risk.

CTB binding partners

Glycosylation

Typically, in biology we often think of expressed proteins as the key determinants of cellular processes and interactions beyond the cell membrane. However, post-translational modifications can alter the behavior of a protein to a significant extent. One such type of biochemical reaction that frequently occurs on proteins or lipids is known as glycosylation, where a carbohydrate (glcycans) attaches to functional groups, resulting in the formation of a glycoconjugate. Once a protein has been transcribed, these further modifications are often made, allowing for a wide host of changes ranging from correct protein folding to altering function [150]. These post-translational sugar alterations present with considerable diversity, enabling thousands of potential glycoforms on proteins and lipids [151].

Glycosylation is typically built from a repertoire of commonly occurring sugars, which differ between mammals and other orders. In mammals, the most common sugars are glucose, mannose, galactose, fucose, as well as the amino sugar derived N-Acetylgalactosamine (GalNAc) and N-Acetylglucosamine (GlcNAc). It is by using various combinations, lengths, and branching constructions of these sugars that make glycan diversity so expansive.

The synthesis of protein glycans are covalently attached to terminal oxygen and nitrogen residues of amino acids in mammals and are thus termed as O-linked or N-linked glycans respectively. N-linked glycosylation occurs to asparagine residues, specifically in amino acid sequons of Asp-Xaa(not proline)-Ser/Thr [152, 153]. This process begins in the ER, where the mannose-rich dolichol-linked GlcNAc precusors are synthesized, trimmed, and attached to a protein. The glycoconjugate then moves to the golgi apparatus where, through a series of processing steps, the final composition of the glycoconjugate is synthesized [154]. This glycan processing results in the three major types of glycans: high-mannose, complex, and hybrid [154]. O-linked glycosylation also occurs in the ER, golgi apparatus and even cytosol, but in a much simpler process. O-linked synthesis starts with almost any sugar residue (except sialic acid) bound to Ser/Thr residues, followed by the construction of subsequent glycan residues through terminal sugar extension [155, 156]. This flexibility in initial sugar options provides the glycan a variety of properties such as protein activity regulation or stability to specific environmental conditions [156, 157].

Glycolipids

CTB was identified to bind to the ganglioside GM1 more than 50 years ago, and its strong binding led to its recognition as the primary surface ligand for CT intoxication [85, 87, 158, 159]. This also translates effectively to the capacity of GM1 to facilitate intoxication, where in rabbit ileal loops, GM1 was shown to be the ligand that binds and internalizes CT [86, 160]. Despite being a protein-carbohydrate interaction, the strength of the CT:GM1 bond is one of the strongest known in the natural world, measured at 4.6 x 10^{-12} M while biotin-streptavidin (SA) is measured close to 1 x 10^{-14} M [9, 141, 161]. This high binding affinity is typically attributed to the finger and thumb grip that GM1 specifically presents to bind to the canonical binding site of CTB, supplied by terminal residues of galactose on one finger and NeuAc (sialic acid) on the other [88]. Other gangliosides such as GM2, GD1A, GD1B, Fucosyl-GM1, GM3, asialo-GM1 and GT1B, are also able to bind CTB strongly, but at much lower affinity than that of GM1 (Fig. 3) [9, 162, 163].

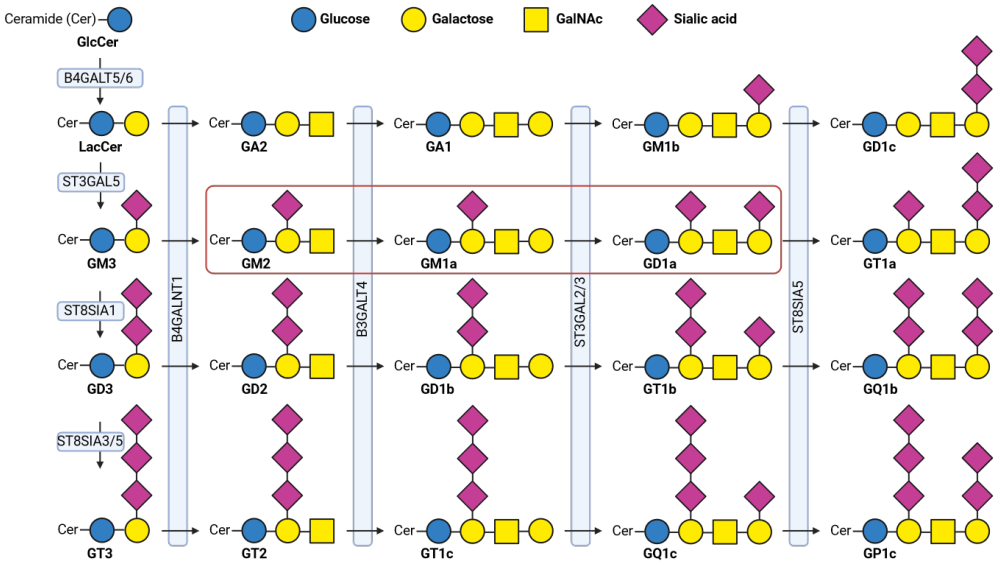

Fig. 3. Ganglioside synthesis and structure. Schematic diagram denoting ganglioside synthesis and structure. Circled in red, the highest affinity CTB binding gangliosides. Created with BioRender.com

The general structure of all gangliosides consists of a glycosphingolipid, comprising an oligosaccharide attached to a ceramide tail, with one or more sialic acids presented (Fig. 3). The ceramide tail is initially built upon with a glucose residue, followed by a galactose residue to create LacCer. This structure is further extended when sialyltransferase ST3GAL5 attaches a sialic acid, resulting in the formation of the mono-sialylated ganglioside GM3. The (M) letter nomenclature refers to the addition of a single sialic acid, and additional sialic acids would change to D(2), T(3), Q(4) or P(5). Upon addition of GalNAc to the terminal galactose by the glycosyltransferase B4GALNT1, GM2 is created, and further addition of a galactose residue by B3GALT4 results in GM1. This GM1, known as GM1a, has the sialic acid on the core galactose,

providing the finger and thumb grip. An alternative route can lead to GM1b synthesis, where the sialic acid residue is positioned on the terminal galactose, leading to deficient CTB binding.

Glycoproteins

Despite the evidence pointing towards the GM1 ganglioside being the de-facto ligand for CTB, just 5 years after discovering the GM1-CTB binding interaction, it was found that ganglioproteins or ganglioside-like glycoproteins could also bind CTB [164, 165]. It was later shown that glycoproteins of rat microvilli could also bind CTB [90]. Interestingly, it was discovered that individuals with blood group-O had significantly worse symptoms compared to others, and that those with blood group AB were significantly protected, becoming mostly asymptomatic [166-168]. Interestingly however, those with blood group O were found to have significantly less chance of being infected by *V. cholerae* O1 serotype [169]. Despite this, blood group-O individuals who are infected have more severe [169]. It was then demonstrated that type 2 core (β1-4 terminal galactose linkage) blood group determinants could function as ligands for a CTB/LTB hybrid. These ligands were reasoned to bind via an alternative laterally located binding site on CTB, distant from the canonical binding site [10]. This was also later confirmed by crystal structure analysis [10, 170]. It was shown that the histo-blood group antigen (HBGA) H determinant (Blood group O) binds with higher affinity than A determinants, and binds in both orientations while A determinants do not [11].

The lack of decoration on the H determinant was shown to also increase sensitivity to CT induced intoxication in CRISPR/Cas9 $A^{-/-}$ cells [171]. These complex HBGAs are only secreted as water soluble structures in secretor-positive individuals (those with functional a *FUT2* gene), leaving non-secretors even more susceptible to CT intoxication [172]. However, human small intestinal epithelial cells (SI-ECs) and mucosa express vast amounts of HBGAs, regardless of secretor status [173, 174]. Non-secretors have limited fucosylation, resulting in only being able to express Le^x and $Lewis^a$ (Le^a) on their cell surface, while secretors have additional modifications to the H-antigen allowing for the expression of $Lewis^y$ and $Lewis^b$ (Le^y/Le^b) and any respective blood group modification (Fig. 4) [173, 175, 176]. The terminal residue lacking Le^x as a protein was also shown to bind CTB, and that it was able to block CTB binding to cells [12], which was shown to bind to the non-canonical binding site with similar affinity as Le^y [91]. It was also shown that Le^y with or without blood group A or B modifications were able to block CTB binding to cells [149].

In general, O-linked glycosylation and fucosylation have been shown to have a role in some ligands suitable for CTB binding and internalization [148]. Free L-fucose has also been shown to block CTB binding to both cell lines and human primary small intestinal tissue, but not to murine small intestinal tissue [12, 149]. Furthermore, using the α-1,3, α-1,6 targeting lectin AAL, human small intestinal tissue was protected from CT, denoting the importance of fucosylated ligands for intoxication [12]. Interestingly, it has been shown that expressed GSLs can function as decoys for CT, while expressed glycoproteins can functionally mediate CT intoxication [177].

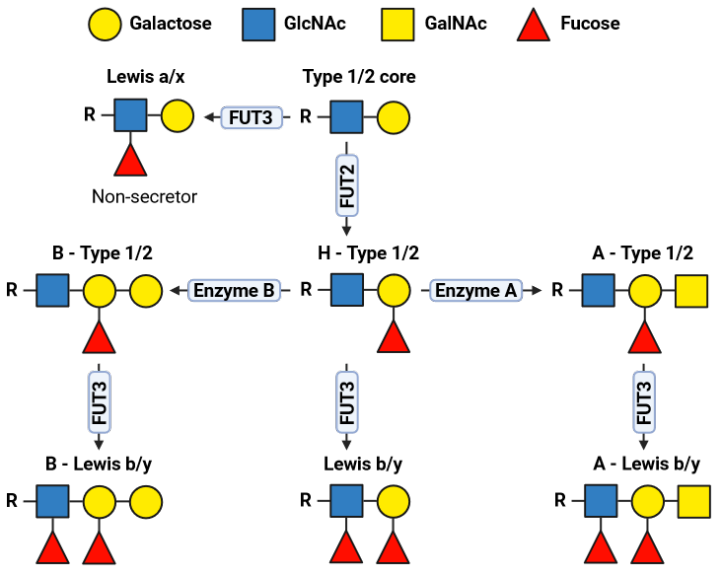

Fig. 4. HBGA synthesis and structures. Schematic diagram displaying the common HBGA's and their relation to blood group determinants. Created with BioRender.com

Alternative ligands

Despite GM1 and similar gangliosides being ideal ligands for CTB, there is increasing evidence that other galactosylated ligands are suitable for CT binding. This was demonstrated using resected human intestinal tissue, where the PNA lectin that specifically binds terminal galactose of Gal-β(1-3)-GalNAc, was able to prevent CT intoxication although it did not affect overall binding, similar to the previously mentioned AAL lectin [12]. Additionally, GM1os exhibits considerable variability in its capacity to block CTB binding [12]. β4GalNAc-transferase knockout mice which cannot add a GalNAc to the terminal galactose, resulting in complex ganglioside truncation, have similar or higher sensitivity to challenge of CT, while GM1os still abates CTB binding [12]. Additionally, the two colonic cell lines T84 and Colo205, lack measurable ganglioside-CTB binding, but have a detectable presence of glycoprotein-CT binding [148]. This glycoprotein binding can be partially blocked by the addition of L-galactose [149]. Hence, these studies indicate that alternative lower affinity galactosylated ligands on SI-ECs can act as functional receptors for CT. Additionally, it has been shown that if CTB binds to a higher affinity ligand, weaker ligands will then have an increased capacity to bind, in an effect called hetero-multivalency [163]. This efficiently enhances CTB binding to the cell membrane via other unoccupied binding sites [163].

Mucins

Some of the heaviest glycosylation occurs on mucins, which gives them an appearance similar to a bottle brush. Mucins constitute the major portion of our extracellular defense system. Mucins cover organs that are exposed to the external environment, such as the intestinal tract, the airways, and the female reproductive system. As the intestine has such a vast surface area that is constantly exposed to non-native microbes, the mucosa provides the first robust layer of defense [178-180]. The mucosa of the small intestine is primarily composed of the goblet cell-secreted MUC2 [178, 181]. This helps to form the main constituent of what is known as the gel-forming layer, which provides a motile barrier among the villi that can shift and be excreted upon peristalsis [178]. This layer also provides protection from the low pH of the digestive contents from the stomach [182]. MUC2 is secreted in a descending gradient along the entire length of the intestine, with the sections of the intestine that have greater villous density exhibiting greater secretion [183]. In comparison, the colon has two discrete gel-forming layers: one similar to the small intestine, and a denser layer close to the cell surface [184]. This dense layer provides protection from the increased presence of bacteria in the colon [184].

On the cell surface, anchored transmembrane mucins form the second layer of protection. In the SI, these are primarily made up of MUC13 and MUC17 which are extensively glycosylated, forming the aforementioned brush-like structures [181]. Together, the transmembrane-bound mucins extend from the tips of villi and form what is called the glycocalyx. MUC13 has been shown to have some protective effect towards ETEC infection, which itself down-regulates MUC13 expression [185]. In addition, MUC13 has been detected to bind CTB in T84 cells [148]. Interestingly, the O-glycans found on mucins also have structures that resemble the Lewis antigens [186]. MUC17 is 8 times longer than MUC13, and has also been shown to substantially decrease enteropathogenic *E. coli* binding when expressed in Caco2 cells [187]. Hence, it is evident that mucins contribute significant innate protection against pathogenic bacteria, and likely also contribute to protection against virulence factors produced by these microorganisms.

CT research

CTB mutations

Due to the potential applications of CT as both an adjuvant and a selective drug vehicle for targeted medications, it is of particular interest to investigate CTB binding. As CTB has two independent binding sites that target different ligands, mutational analyses and trials have been conducted multiple times over the years to determine the binding interactions of binding site-deficient CTB complexes [188-192]. CTB is also a protein that has a propensity for stability after numerous targeted mutations (Fig. 5A+B) [193]. Mutating the glycine (G) at amino acid position 33 in the GM1-binding pocket to a negatively charged residue such as aspartic acid (D) results in CTB unable to bind GM1 or intoxicate, while a positively charged residue results in retained binding with the inability to intoxicate (Fig. 5) [194]. Additionally, this residue was found to also affect the CTB-HBGA binding interaction due to its close proximity to the noncanonical binding site [12, 91]. A mutation at position 88 that converts the naturally occurring tryptophan (W) to a lysine (K) residue, resulted in a CTB mutant with a deficient capacity to bind GM1 [91, 188]. In addition, a holotoxin with mixed CTB subunits of mutated W88K and G33D, failed to induce an intoxicative response in T84 monolayers [191]. Substitution of histidine (H) residue 57 to an alanine (A) retains binding to GM1 but renders the CT unable to intoxicate [189, 190]. Amino acid substitutions targeting the non-canonical dependent binding pocket are less well researched. Only one previously successful mutation of H18 to an alanine has been identified, resulting in a defect in binding Le^x oligosaccharides. With a second mutation at H94, the affinity for Le^x was further diminished [91]. Since binding-deficient CTB mutants are key to studies investigating toxin-binding in human cells, throughout this thesis we focused on the development and use of mutated CTB mutants with impaired binding capacity. Specifically, mutants with W88K and H18L substitutions were created to disrupt canonical and non-canonical binding sites (Fig. 5C).

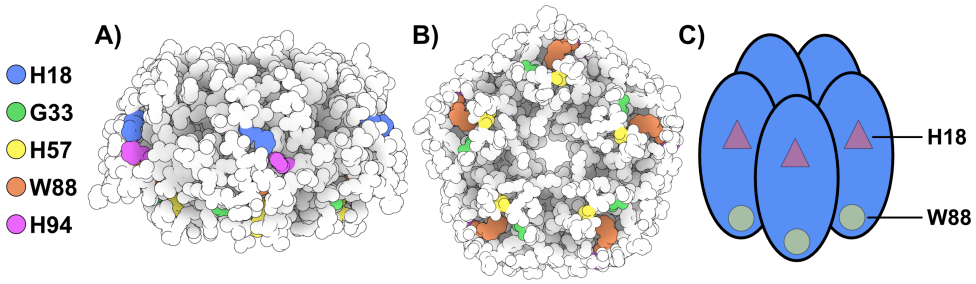

Fig. 5. CTB with common residues targeted for mutation. A) Side-on and B) bottom structural view of CTB, highlighting the most common residues targeted for mutation to incapacitate binding. C) Simplified CTB schematic displaying the binding sites affected by mutations to W88 and H18 (mutations used in this thesis).

Animals

Because cholera is a disease that primarily afflicts humans, conducting cholera research using a relevant in vivo model has historically been challenging. Animal models have previously included: canines, rabbits, chickens, rats, and mice, among others [113, 161, 195-199]. Such research has typically been done by providing animals with crude batches of *V. cholerae* broth, followed by a volume measurement of the resulting excretions. The colonization model has been proven to be an effective model for some animal models, yet in murine models this can only be achieved in neonatal mice without the use of specific strains or antibiotic ablation of the gut flora prior to infection [161, 195, 200].

Another commonly used methodology in animal research is the use of a ligated loop, where a defined section of the intestine of the animal is tied off, thereby creating an exclusive section of the lumen that is sealed off. This loop can then be perfused with bacteria or CT and weighed after several hours of incubation and then compared to the initial loop weight. Compared to the previous method, ligated loops offer a valuable approach to investigate specific sections of the intestine for the response to CT challenge, in a quantifiable manner. Moreover, it is applicable to many different animal models [161, 197, 199].

In contrast to *in vivo* methods, an alternative methodology to researching CT challenge comes in the form of the *ex vivo* Ussing chamber experiment. This method entails excising living tissue from the site of interest and mounting it in a specially designed chamber that creates a separation between two compartments containing isotonic fluid. The apical side of the tissue is then challenged with CT, while the transepithelial electrical resistance (TEER) and voltage over the membrane are measured. This technique is a valuable proxy for the diarrheal response recorded *in vivo,* as the values can then be calculated and give a value of the short circuit current (Isc), giving a measurable level of ion efflux across the membrane which can be tracked over time [201, 202].

Cell lines

Immortalized human cell lines have also been extensively used to study CT intoxication, binding mechanisms and the various ligands involved in cell-toxin interaction. Cell lines can overcome a few of the major hurdles that limit the potential of animal models, in that they are derived from human donors from specific tissues that can preserve their isolated phenotype. These cell line models are also able to provide a platform to cost-effectively test multiple conditions, while reducing the overall burden on animal usage. The drawback in this methodology is that cell lines originate from cancerous cells of a specific phenotype, which may result in cells that have undergone numerous mutations. This could potentially deviate from the phenotypic expression of the original cell, altering the response to treatment. Cancerous cells also have extensively altered glycosylation compared to healthy cells. O-glycosylation is typically affected in cancer cells, with increases in T and Tn antigens, which are common prognostic markers for cancers due to their relative absence in healthy tissue

[203, 204]. Additionally, sialic expression is typically much higher in cancerous cells, often as a prognostic indicator of low survival rates. These sialic acid moieties are present as a terminal addition on glycans, which can modulate the binding affinity to CTB, either positively or negatively [205-207]. As cell lines are derived from a single donor, they also lack the diversity present across the species, such as the glycoprotein profile associated with blood type and secretor status, which can significantly alter the severity to toxin challenge. Cell lines are also limited to a single cell type, often epithelial cells of colonic origin. Caco2 cells after several days of confluency will differentiate towards a small intestinal epithelial cell phenotype with microvilli, but still lack other key cell types such as goblet, tuft, or enteroendocrine cells, impacting the overall scope of the other cell types upon CT challenge.

The commonly used cell lines in CT research are the colonic epithelial cell lines Colo205, T84 and Caco2 [149, 208-210]. However these colonic cell lines often have differing ligand glycosylation expressed on their surface that do not fully encapsulate the ligands detected in human intestinal tissue [148], resulting in CT being endocytosed via different routes and further altering how the cells are able to process it [92]. In addition, *V. cholerae* primarily infects the small intestine of humans which primarily functions to aid in the final steps of digestion and nutrient uptake, whereas the colon is more specialized for water absorption and electrolyte uptake.

Organoids

One of the more exciting developments in models for human biological and medical research is the development of the organoid model. Organoids have a history extending back to the early 20th century [211], with the boom in current techniques originating in 2013 [212, 213]. Organoids are miniaturized versions of organs derived from stem cells, kept in a stem-like state for growth and can be differentiated to the cells of the organ of interest. These organs-in-a-dish have the capacity to maintain their heterogenous phenotypic cellular expression, differentiating to express epithelial cells, mixed with goblet, Paneth, and enteroendocrine cells furnished with a mucosa [214-216]. The organoids used throughout this thesis are derived from small intestinal tissue and are referred to as “enteroids” [217].

The establishment of organoids can be achieved with embryonic and induced pluripotent stem cells, or from donated primary tissue providing a diverse library of characteristics. Due to the lack of native muscle, connective tissue, and fat, organoids need some structural factors found in extracellular matrices (ECM) containing multiple structural factors such as Collagen and Laminin, as well as various growth factors [218, 219]. Stem cells or isolated crypt cells are seeded into ECM domes and allowed to harden, and subsequently grown in a specific media which contains the growth factors needed to sustain the cultures. To preserve the stemness of the organoids, Wnt3A protein is required to continually stimulate the LGR5^{+} expression of the adult stem cells. Upon removal of Wnt3A, the stem-positive cells will progressively differentiate giving rise to the heterogenous populations of the small intestine. Additionally,

R-spondin, Noggin, and Epidermal Growth Factor are all key to enteroid maintenance [215, 220-222].

While enteroids have the capacity to recapitulate the small intestine that they are derived from, the media used to grow organoids lacks some of the factors stimulated by the rest of the body and surrounding immune system interaction. This can be a limiting factor when assessing how the complete small intestinal niche might behave upon stimulation. Conversely, enteroids are advantageous in contexts where the goal is to determine the mechanisms or effects induced by certain ligands or cytokines, such as IL22 which stimulates MUC17 expression [223, 224].

Enteroids can be adapted to multiple different forms, depending on the research question. To create a polarized 2D platform, enteroids can be seeded onto a collagen-coated surface and grown to create confluent monolayers that can then be differentiated. Once ready, the monolayers can be stimulated either basally or apically in an easy and controllable manner [171, 215, 225, 226]. Additionally, organoids can have their ECM withdrawn, leading to eversion and the apical out model. It has been shown that this can be utilized to effectively study bacterial-host interactions while overcoming the need for micro-injection into the spheroid as is typically required for a 3D model [227-229].

While still not a perfect system, the organoid model does allow for a closer representation of the human small intestine, addressing some of the previously mentioned concerns. The inclusion of certain cytokines can help to fill out the diversity of expressed ligands and their glycome. In addition, to further increase the resemblance of the *in vivo* context, co-culture with immune cells and microbiota found in the small intestine could be performed [215, 223, 230].

Aims

Aim I – To evaluate the capacity of fucose/galactose polymers to block CT binding and intoxication

Aim II – To delineate the binding site requirements for CT binding, internalization and intoxication of human small intestinal enteroids

Aim III – To determine the role of MUC17, a surface-bound mucin, in protection of the small intestine against CT

Methods and ethical considerations

The work throughout this thesis was performed mostly at the Department of Microbiology and Immunology, giving us unique access to tools often overlooked for CT research. Despite this, we decided to limit the use of animals as much as possible for the studies in this thesis, choosing to only use mice as an experimental model when alternative options would not suffice. This decision was taken in part due to the ethical considerations to abide by the 3 Rs (Replacement, Reduction and Refinement). Additionally, by using cell lines and enteroids that could be assayed using the same techniques, our results have greater relevance for human contexts and therefore offers a more direct comparison. In this section I will briefly go through the key methods I used, the benefit of using these techniques, as well as some comments on ethical considerations surrounding them if applicable.

Cell culture

We chose to use a variety of different cell lines throughout this thesis from human origin, due to various different properties that could help explain CT binding and intoxication. The main focus of this thesis is on the interaction between CT and the human small intestine. The primary model we used throughout this thesis was the primary tissue derived organoid cultures. These were established from donated tissue following gastric bypass surgery of otherwise healthy individuals, for which we acquired ethical permission. Aside from the knowledge of the surgery, these donors are completely anonymous, and no identifying information or other samples were given to us. In addition, we used several cell lines that have been extensively used in CT research to investigate the binding and intoxication profile of CT. For this, we decided to use the monolayer forming T84 and Caco2 cells, as well as Colo205, Jurkat, HL-60 and THP-1 cells. Wherever possible, we also used recipes for growth media containing less animal serum, or as is the case with organoids, we switched to a completely serum-free option once it was commercially available.

CT/B mutants

The CTB mutants and subsequent holotoxin mutants that we generated were essential tools for a large amount of our findings in this thesis. Briefly, we designed the CTB mutants W88K, H18L, and a double mutant H18L/ W88K which prevented binding at either binding site (Fig. 5C). The CTB mutants were generated by inducing changes to a high expression *ctxB* plasmid that was created in-house. Using basic primer-based PCR techniques to induce random single point mutations, classic enzyme restriction, and transformation, we were able to find a mutation that incapacitated the non-canonical binding site without affecting the canonical binding site. The various CTB mutants were first validated using ELISA capture techniques to ensure specific binding sites were knocked out. These were then further validated using murine splenocytes and human granulocytes to test canonical and non-canonical site dependent binding respectively. The holotoxin variants were expressed on a plasmid alongside *ctxA* and transformed into an *E. coli* strain. These were again validated for deficient binding, as well as successful CTA integration by sandwich ELISA.

Flow cytometry

One of the most frequently used methods in this thesis is flow cytometry. Flow cytometry is typically used to identify different proteins expressed on a cells surface by use of fluorescent antibodies, and in turn by using specific combinations of antibodies targeting these proteins, the types of cells can be determined in a mixture of cells. Throughout my thesis work, we have used flow cytometry as a measurement of how much CTB binding has occurred to the cell type of interest, or alternatively, the expression level of certain carbohydrates targeted by specific lectins.

In simplified terms, a flow cytometer works by shining a series of lasers on a stream of single cells. These cells have been incubated with antibodies targeting specific proteins, or in our case CTB, that are themselves or by way of another antibody, covalently attached to a fluorescent chemical compound known as a fluorophore. If the wavelength of a given laser meets the excitation spectrum of any fluorophore, then that fluorophore will emit light in typically another wavelength, which is then captured by a set of light filters so that it can be recorded. By using multiple different fluorophores, lasers and light filters, multiple different markers can be discerned on each cell, allowing for identification of the cell type. As most cells express proteins that are also expressed on other cell types, this multi stain approach is often needed, especially in immunology. The relative amount fluorescence detected by each tagged marker is recorded as a value for each cell, which we have used as the metric to compare the amount of each CTB mutant that binds to each cell. When analyzing this data we sort cells by "gating", where we look at the general intensities of our cells and define cut-offs for each parameter.

Several cell lines were used in combination with CTB to study the binding ligands expressed on the cells, and further to study the binding site(s) of CTB used to bind them. To explore this, cells were spun down into serum-free PBS, while adherent cells were detached from their growth cultureware prior to centrifugation. Cells were then ready to be stained for flow cytometry. Enteroids were grown as everted enteroids that were subsequently made into single cell suspensions and stained like other cell lines.

Flow cytometry gave us many advantages over other experimental methods that would be impractical or impossible at the scale of different variables that we tested. Firstly, the process of staining cells for flow cytometry is relatively quick. For our work, it took less than 2 hours to complete a full stain; with acquisition also being typically quick and efficient. This has advantages over techniques such as Immunohistochemistry (IHC), as this method gives us an easily quantifiable assessment of CTB binding. Additionally, the CTB and mutants used for staining in this thesis were biotinylated, allowing for the use of SA conjugated to a fluorophore in a two-step stain. Biotinylated CTB with detecting SA was used primarily as CTB binding via the non-canonical site rendered the epitope of primary antibodies inaccessible, resulting in limited CTB detection. However, the biotin-SA detection is not affected by this issue.

Despite this, flow cytometry is not a perfect technique, but for most of the concerns, there are different management techniques to compensate. One of the major issues with flow cytometry is the occurrence of overlapping emission spectra or bleed-through, where one fluorophore's emitted light can be detected in multiple channels, which can limit the viable antibodies you can use. This can mostly be overcome by either using fewer fluorophores and ensuring they do not overlap, or by limiting the recorded spectra to a specific band for each fluorophore. Autofluorescence can also be of concern, where a cell fluoresces when a laser is applied even without a fluorophore. This can generally be solved by a little trial and error, and avoiding using a fluorophore in that spectrum. Cell aggregates or cells that have not dissociated into single cells, can give incorrect data as multiple cells will be recorded as singular events. This misleading data can be avoided by gating cells based on their size in two different dimensions, leaving only single cell sized events. The last major concern is that of dead cells and debris. When we record our data, we want to just look at binding to fully intact cells, which we can generally ensure by using a marker that stains DNA and by some careful gating, as whole cells won't allow this staining chemical inside the cell.

ELISA

ELISA is another method that we have used within this thesis that has been key to our study of CTB binding. This method is robust as well as inexpensive and can be scaled up to test multiple different conditions at the same time. Principally, ELISA is used to study the binding efficacy of molecules in a liquid phase to immobilized ligands. We know from previous literature that CTB binds to specific ligands at each binding site. Hence, we have used ELISA to investigate the capacity of blocking molecules to prevent CTB binding their target. In addition, we have used this method to confirm the site-specific ablation of each of the binding sites in the created CTB mutants.

For our ELISA experiments we used a variation on the capture ELISA method. We immobilized glycoproteins and glycolipids into 96-well plates which were then treated with either CTB or binding-deficient CTB, and titrated across more wells of the same ligand. These different conditions were then detected by binding antibodies (specific for CTB or CTA) that were finally detected by secondary antibodies conjugated to horseradish peroxidase (HRP). A substrate is then added, which rapidly changes color upon contact with HRP allowing for a quantification of bound CTB. This approach was used as a pilot binding experiment to investigate canonical and non-canonical site-dependent binding. ELISA has the advantage over using a cell line in that we can specify the ligand assayed in the experiment, whereas when we investigate using cell lines through flow cytometry, despite knowing that certain cell lines mostly have ligands that bind to a specific binding site, we cannot control for diverse ligand expression. This comes with the inherent limitation that a ligand attached to a plate is in a solid state and the dynamics of the membrane and how that impacts binding to the ligand is not recapitulated [231]. This also limits binding to only half of the available binding sites, so any potential cooperative hetero-multivalent binding that may be a property of CTB binding to a cell is lost. Nevertheless,

this approach to assessing CTB binding still offers value, especially when confirming blocked binding of specific binding sites.

Saporin Assay

In paper III we started using a new assay in an attempt to identify site-specific internalization of CTB. Saporin itself is a ribosome inactivating protein (RIP), and as such its primary mode of action is to cause cell death by preventing protein synthesis. Some RIPs such as abrin or ricin have secondary subunits with specific carbohydrate ligand binding properties, making them lectins, and thus have a delivery system into the cell. However, saporin lacks this and is thus considered unable to enter the cell on its own. Using a SA tagged saporin, we were able to couple this with our CTB binding deficient mutants to study internalization by assessing the level of cell death. This assay has the inherent advantage that this can be performed on any cell line and does not require cells that are adherent to make a monolayer. Additionally, using this assay provides the ability to investigate CTB binding ligands that could facilitate internalization. This however is a double-edged sword, as it has been suggested that CT may be taken up by cells through different pathways, and in some of those pathways CT may be inactive [95]. In addition, unlike other RIP, saporin does not have a lectin based binding subunit limiting internalization to most cells. However, intestinal cells, especially small intestinal cells are absorbative cells designed to take up nutrients including proteins, allowing for direct cell mediated uptake and thus initiating ribosome inactivation leading to cell death without initial binding to the cell surface. We were able to limit this by coupling saporin with our double binding site deficient CTB mutant, but we were not able to eradicate this entirely. Despite this, one must be aware that absorptive, pinocytic and phagocytic cells may have considerable background "death" using this assay, while cells with no inherent uptake such as the T-cell derived Jurkat cells are limited to toxin bound cell death.

Apical CT challenge assay

Another method we have used throughout this thesis is the apical CT challenge assay. This method uses a similar principle to the previously discussed Ussing chamber method. To achieve this, we grew monolayer-forming cells of human enteroids, T84 or Caco2 cells on suspended polyester membranes known as transwells, that has growth media on both sides. This growth method allows for the cells to attach, form a monolayer and polarize, giving definitive access to the basal and apical side of the monolayer. Using a probe that is inserted into the apical and basal media simultaneously, the resistance and voltage of the monolayer was measured over time to calculate current as a measure of ion efflux across the cellular membrane. Cells were first visually inspected every two days for monolayer formation, followed by TEER measurements of the monolayer every 2 days to confirm confluency, up to a value of 1000 ohms/cm^2, Caco2 cells would then be further differentiated for two-three weeks. Human enteroids were grown to a confluency of 600 ohms/cm^2, and then cultured with differentiation media. Once confluent, CT was then applied to the apical side of the monolayer and the cells were allowed to incubate at 37°C.

One of the primary advantages of using this method is that we are able to tangibly measure the level of intoxication. Additionally, this assay can be repeated using the same cell type and scaled up to have several conditions in the same experiment, while an Ussing chamber is limited to fresh tissue that can only be used once and only for a single condition per experiment. This experimental technique has been integral to the first three papers of this thesis, proving to be a repeatable method that is adaptable to numerous toxin conditions.

Immunohistochemistry

The last major technique used in this thesis was immunohistochemistry (IHC). For this technique, enteroids were grown in transwells to confluency before being stained with antibodies specific for differential cellular expression labeled with fluorescent markers. Additionally, enteroids or Caco2 cells were treated with CTB-biotin (detected by SA conjugated fluorophore) and stained with an antibody targeting MUC17 to investigate cellular protection from CTB binding. This method uses lasers and filters similar to flow cytometry, but this is done on the whole unified sample and the resulting emission is displayed as an image. Using IHC, images of whole membranes can be established showing the expression of specific cells and binding of CTB or lectins in a spatial plane. This is especially useful for cells like enteroids that have heterogenous cell expression, including rare cell types. Additionally, as cells are often specialized towards specific tasks, the protective effect of cells expressing structures such as mucins can be visualized, which might be lost with other techniques such as flow cytometry. Furthermore, by looking at cells stained using confocal microscopy, a third dimension can be added to the visualization, giving deeper insights into the positional binding of CTB or expression of certain markers like MUC17. This is however a very time-consuming method and is not as scalable as flow cytometry. This method is also not particularly easy to quantify without the aid of subjective threshold-set algorithms. Thus, this method is more suited to visualizing expression and spatial arrangement in comparison to flow cytometry.

Key findings

This thesis is composed of four main papers, that in part are based on the findings in several recent papers [11, 12, 91, 148, 149]. One of these papers was published from the same laboratory that this work was performed in, and is the foundational study that we aimed to expand upon our investigation [12]. The findings of these papers are key to our first steps to understand the non-canonical binding site and the impact it might have on CT in the human small intestine. I have also contributed to an additional paper that is currently under work for resubmission [177]. Although this is of great relevance to this thesis, it was not included as a paper in the thesis due to my more limited lab work contributions towards the final manuscript.

Paper I – Fucose-Galactose Polymers Inhibit Cholera Toxin Binding to Fucosylated Structures and Galactose-Dependent Intoxication of Human Enteroids

The first paper expands on the previously reported CT-blocking molecules, by designing simple and cost-effective sugar polymers to prevent CT intoxication. This primarily focused on exploring the first aim of this thesis, but also the second aim to a lesser extent. This was achieved by synthesizing simple sugar polymers displaying a total of 100 residues of either galactose or fucose, or a racemic of both, targeting the canonical site, non-canonical site or both sites of CTB. The blocking efficacy of the polymers was then tested against CTB on primary tissue from both murine and human SI, and further assessed for their capacity to abate CT intoxication of human enteroids and *in vivo* in mice.

Fucose containing polymers block CTB binding to Le^x by forming aggregates

The blocking efficacy of these polymers in inhibiting CTB binding to immobilized triLe^x or GM1 ligands was first evaluated by ELISA. As expected, the Fuc100 (fucose) polymer blocked binding of CTB to the immobilized non-canonical site Le^x ligand, while the Gal100 (galactose) polymer efficiently blocked CTB binding to the canonical site GM1 ligand (Paper I Fig. 1). Mixing both the Fuc100 + Gal100 polymers or using the Gal50Fuc50 (fucose + galactose) polymer was sufficient to prevent CTB binding to the low affinity Le^x, but had an arguably positive effect on GM1 binding. The overall enhancement in CTB-GM1 binding detected upon addition of all polymers (except Gal100) is likely an effect of CTB aggregation. This is supported by the observation that the shortened Gal5Fuc5 (10 sugar residues) and Gal15Fuc15 (30 sugar residues) polymers, were unable to show similar increases in CTB binding to GM1.

Galactose containing polymers block CTB binding and Gal50Fuc50 diminishes CT-induced fluid secretion in murine small intestine

The previous experiment showed that binding to ligands suitable for either site could be prevented, however ligands on cells can have different properties due to the natural membrane fluidity, which allows multivalent binding [231]. We first tested our polymers for their capacity to block CTB binding to murine WT SI-ECs (Paper I Fig. 2). Co-incubating the Fuc100 polymer with CTB only resulted in a detectable inhibition of CTB binding when the polymer was used at high concentrations. In contrast, both the Gal100 and the Gal50Fuc50 polymer resulted in a dose-dependent reduction in CTB binding. This indicated that almost all CTB binding to the murine small intestine is canonical site driven, but likely not by GM1 or related higher affinity ligands. The existence of alternative canonical site ligands that are not complex gangliosides was confirmed by using β4GalNAcT KO mice. The blocking profile of our sugar polymers on the ganglioside KO murine SI-ECs showed a similar trend to that observed

of WT SI-ECs. As the Gal50Fuc50 polymer was not just unable to prevent, but also enhanced CTB binding to immobilized GM1, it is likely that the ligand(s) targeted by CTB in the murine small intestine are lower affinity than the galactose displayed on our polymer. The Gal50Fuc50 polymer was then premixed with CT and trialed *in vivo*, using ligated loops of mouse small intestine. The racemic polymer was able to partially prevent CT-induced fluid secretion. The incomplete CT inhibition with the Gal50Fuc50 polymer may be due to the relatively low threshold required for CT intoxication, where some CT molecules may not be mopped up and aggregated by the sugar polymer.

Taken together, these data show that murine cell surface ligands for CT are primarily targeted by the canonical site and are unlikely to be GM1 or related glycolipid-based. This suggests that galactosylated glycoproteins are the target ligands in mice. This also shows that while incapable of completely inhibiting fluid secretion following CT intoxication, the racemic polymer was more efficient than either simple sugar polymer.

The Gal50Fuc50 partially prevents CT intoxication of human enteroids, while GM1os ablates efflux

Finally, we moved to human primary small intestinal cells, as *V. cholerae* is primarily an obligate human pathogen [232]. First, we explored the inhibition of CTB binding to isolated human SI-ECs by use of the previously mentioned polymers (Paper I Fig. 3). CTB binding was blocked dose-dependently by the Fuc100 polymer, but only a partial block was detected at high concentrations using the Gal100 polymer. Furthermore, CTB binding was most efficiently inhibited from binding to human SI-ECs when incubated with the Gal50Fuc50 polymer in a dose-dependent manner. The overall ineffectiveness of the Gal100 polymer in blocking CTB binding, contrasted with the effective inhibition by Fuc100, suggests that CTB primarily binds to the human small intestine via the non-canonical site. This is consistent with the lack of detectable GM1-like gangliosides, and the previously identified free $Le^{x/y}$ and L-fucose block of CTB binding to human SI-ECs [12, 89, 149].

To investigate if our polymers could prevent functional CT internalization, we wanted to employ a model that would allow us to reliably repeat results from the same donor, while also capable of capturing the heterogeneity of cell types found in the SI. We decided to establish a library of enteroid lines from donated primary tissue. To validate the presence of the major cell types of the small intestine, we stained for epithelial, Paneth, and goblet cells by IHC, and confirmed that each cell type was indeed present (Paper I Fig. 4). Enteroid cells were then incubated with our sugar polymer-CTB mixtures and assessed for CTB binding. The Fuc100 polymer was able to block binding at higher concentrations, while the Gal100 polymer partially blocked binding at high concentrations. Conversely the Gal50Fuc50 polymer blocked CTB binding even at low concentrations, similar to the blocking detected on SI-ECs isolated from fresh tissue. Due to the high binding affinity, GM1os was also tested, but premixing the carbohydrate with CTB had no detectable effect on CTB binding.

We finally wanted to test the polymers functionally using human small intestine enteroids. To test this, enteroids were grown on permeable transwell membranes and differentiated once confluent, allowing for TEER and voltage measurements between basal and apical fluids to be made. Our CT challenge conditions were added to the apical side of the membrane and measurements taken every hour to calculate a current value (Paper I Fig. 5). We were successfully able to intoxicate our enteroid monolayers with CT, an effect that was unaffected by pre-mixing with the Fuc100 polymer. The Gal100 polymer had a minimal, but significant impact on measured CT ion efflux, while the Gal50Fuc50 partially blocked intoxication. However, pre-incubation with the GM1os completely ablated CT intoxication.

Overall, the blocking of CT(B) to human small intestine cells by our polymers suggests that CTB binding is vastly dominated by fucosylated (and non-canonical site) binding. However, CT intoxication does have some requirement for galactosylated (canonical site-binding) ligands. The GM1os did not prevent CTB binding to any detectable extent but was able to abolish intoxication. Finally, Gal50Fuc50 polymer blocked almost all CTB binding, but only partially blocked intoxication. Together with the ELISA data this would suggest that although the Gal50Fuc50 polymer is effective, the blocking detected is likely due to aggregation of CT(B), as the racemic polymer effectively produces large aggregates of CTB [233].

Conclusion

We aimed to generate simple cost-effective sugar polymers that could be of potential use as a therapy in a non-vaccinated population afflicted with cholera. These polymers were evaluated for their capacity to block CTB binding and prevent CT intoxication in both mice and humans. We show that in mice, CTB binding is dominated by canonical site ligands that our racemic polymer effectively blocked. This blocking partially translated to be a partial blocker of CT intoxication in mice. Conversely, we show that CTB binding to human SI-ECs and the enteroids from primary human tissue, is largely dependent on fucosylated ligands to the non-canonical site. Despite this, CT intoxication was not inhibited by a fucose only containing polymer but by a Gal50Fuc50 polymer (Fig. 6). However, pre-incubation with GM1os was able to fully prevent intoxication, while having no effect on overall binding. The blocked CTB binding seen by the Fuc100 inhibitor, but not the Gal100 inhibitor, and subsequent role reversal upon CT challenge suggests that fucosylated binding in the human small intestinal may serve an alternative function to that of intoxication. We reason that this data on human SI-ECs suggests that the fucosyl-dependent CTB binding may be expressed on ligands that function as decoy receptors for CT.

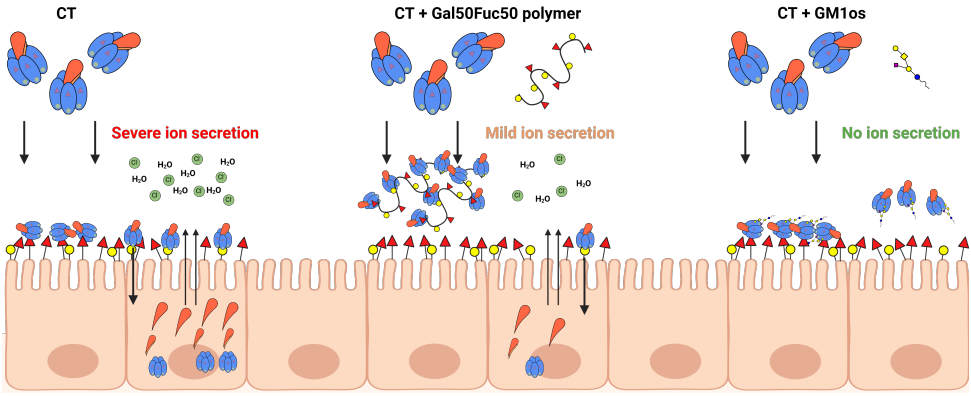

Fig. 6. Schematic summary of findings from paper I. CT binding and intoxication alone binds via the non-canonical binding site and has severe ion secretion, pre-incubation with the Gal50Fuc50 polymer ablates CT binding and reduces ion secretion, incubation with GM1os has no detectable effect on CT binding but ablates all ion secretion.

Paper II – Cholera intoxication of human enteroids reveals interplay between decoy and functional glycoconjugate ligands

Following the results from paper I, which suggest that fucosylated ligands functioned to bind CTB as decoy ligands, and considering that canonical site CT binding to cell surface ligands is required for intoxication, we decided to investigate the glycoprofile of the human small intestine from a different angle. This paper primarily explores aim II of the thesis, namely to determine the role of different groups of glycans expressed in the human small intestine as targets for CTB binding. To investigate this, we modulated enteroid cellular expression of glycosphingolipids and glycoproteins using inhibitors targeting specific glycan groups. In addition, we created CTB mutants defective in the canonical, non-canonical, or both binding sites, to identify which binding site each of these ligands targeted. Further, enteroid monolayers that had expression of their specific glycan groups modulated, were treated apically with CT to clarify which of these ligands were functional or served as potential decoys.

Human CT intoxication can occur via glycoprotein binding and fucose is present on decoy-like ligands for CT

As our previous results indicated that ligands for the canonical site appear to give CT functionality, we chose to first try enteroids treated with the glucosylceramide synthase inhibitor, N-(n-Butyl)deoxygalactonojirimycin (NB-DGJ), for 3 days leading up to experiments. In 4 different enteroid donors, CT challenge of NB-DGJ treated monolayers showed no significant difference compared to no inhibitor (Paper II Fig. 1). However, HPLC measurements of GSL expression showed no detectable GM1a and only trace levels of GM2, which upon NB-DGJ treatment showed a limited additional decrease of GSLs on enteroids (Paper II SI-Fig. 3).

Following this, we chose to investigate the fucosylation that was implicit in non-canonical site decoy-like ligands, by treating enteroid cells with the Fucosyltransferase inhibitor, 2F-Peracetyl-Fucose (2F-Fuc) (Paper II Fig. 2). Treatment of 2F-Fuc efficacy was confirmed with the lectins UEA-1 (α-1,2 linked fucose specific) and AAL (α-1,3 and α-1,6 linked fucose targeting) both of which showed reduced binding to 2F-Fuc-treated enteroids compared to untreated. When enteroid monolayers treated with 2F-Fuc were challenged with CT, significant increases in sensitivity to CT in both enteroid donors tested were observed. Additionally, when probed with the PNA lectin (targets terminal galactose and Galβ1-3GalNAc), 2F-Fuc-treated enteroids displayed substantially higher levels of PNA binding.

Together, this indicates that the fucosylated CTB binding ligands could function as decoys. Alternatively, or in addition, the inhibition of fucosylation causes an increase in detectable Gal/GalNAc ligands that are functional ligands for intoxication. As experiments performed in

mice genetically deficient in GM1 indicated, complex gangliosides appear to have a limited presence and impact on CT-mediated intoxication of human enteroids.

CTB binds primarily to SI-ECs via the non-canonical binding site

To further understand the CTB-binding ligand groups and their behaviors, we created CTB mutants that had either their canonical site or non-canonical site incapacitated by a single point mutation. These CTB mutants were then confirmed for their binding deficiencies on the immobilized ligands GM1 or triLe^x (Paper II Fig. 3). The CTB mutant with a defective canonical site (W88K) had severely diminished binding to the high affinity ligand GM1, but demonstrated non-canonical binding similar to the WT-CTB. Conversely the non-canonical site defective mutant was unable to bind triLe^x but maintained binding to the canonical binding-site ligand GM1. As a negative control we also designed and confirmed the double deletion mutant that contains both single defect mutations in the same strain. These mutant CTBs were further assayed against cells with known binding properties such as: murine leukocytes (predominantly canonical site binding) or human granulocytes (primarily non-canonical site binding) and the specificity of the mutations also concerning ligand expression on cell surfaces were confirmed.

With a new tool to assess the binding ligands of CTB, we set out to explore the binding site and the type of ligand that binds each site using the established enteroids. Consistent with the findings in paper I and the previously reported CTB blocking data, most CTB binding to human SI-ECs occurs via the non-canonical site, as the H18L containing mutant displayed background levels of binding to the enteroids (Paper II Fig. 4) [12]. Despite this, the W88K mutation also conferred significant impact on CTB binding, denoting that the canonical site binding is also of importance. Paper I showed that high concentrations of the Gal100 polymer could induce a slight blocking effect on CTB, while it has also been shown that while GM1os does not block CTB binding, GM1-HSA can block CTB binding to human jejunal cells.[12] Enteroid cells inhibited with 2F-Fuc demonstrated an overall decrease in CTB binding, and the complete loss of binding detected using the W88K mutation indicated that they were non-canonical ligands. Interestingly, 2F-Fuc inhibition also resulted in substantial increases in canonical site binding, detected by the H18L mutation. The increased canonical site binding reflects the increased PNA binding detected (Paper II Fig. 2) and are thus likely glycans that are usually decorated with fucose, that upon 2F-Fuc inhibition resemble a ligand similar to the Core 1/T antigen. The increased sensitivity to CT following fucosyl inhibition is therefore likely canonical site driven, with fucosylated structures functioning as decoy-like structures.

CTB non-canonical site binding decoys are O-linked glycans, that confer a reduction to CT sensitivity

Following our characterization of fucosylation in response to CT, we sought to explore glycoproteins, which are also potential functional ligands expressed by enteroids [90, 148]. We treated enteroids with inhibitors targeting N-linked glycans (Kifunensine) or O-linked glycans (Benzyl-α-GalNAc) (Paper II Fig. 5). Inhibition of N-linked glycoproteins showed no significant effect on overall CT intoxication but had some effect on non-canonical site binding of CTB, in one donor more than the other. In contrast, the Benzyl-α-GalNAc-treated enteroids displayed a significant increase to CT sensitivity, which similar to fucosyl inhibition, resulted in substantial increases in canonical site ligands for CTB. In addition, as observed with 2F-Fuc treated enteroids, the Benzyl-α-GalNAc also resulted in significant increases in terminal Gal/GalNAc structures detected by PNA. In contrast, Kifunensine treatment caused significant decreases in terminal Gal/GalNAc residues. Interestingly, enteroids derived from one of the donors that was treated with Benzyl-α-GalNAc had a reduction, while the other had increased detectable α-1,2 fucosylated structures. The Benzyl-α-GalNAc-driven increase in PNA binding is likely resulting in the increased expression of T- or core-1 antigens, which may be suitable for CTB binding [234].

O-linked fucosylated glycans are also functional receptors for CT

The promising indication of fucosylated and O-linked decoy-like ligands we identified, lead us to explore if combining inhibition of both could further increase sensitivity to CT. We found that Benzyl-α-GalNAc and 2F-Fuc-inhibited enteroid monolayers had an unexpected significant decrease in susceptibility to CT challenges compared to Benzyl-α-GalNAc inhibition alone (Paper II Fig. 6). Despite this, the overall level of binding and non-canonical site binding was not significantly different upon double inhibition, yet canonical site-dependent binding was significantly higher than after Benzyl-α-GalNAc inhibition alone. Consistent with single inhibition, there was also a significant increase in Gal/GalNAc ligands detected by PNA. There was also a substantial decrease in α-1,2 fucosylated structures compared to O-linked inhibition alone. Benzyl-α-GalNAc inhibition paired with NB-DGJ also reduced sensitivity to CT. This addition of NB-DGJ (causing limited reduction in GSL expression) had no effect on overall CTB binding or expression of Gal/GalNAc ligands and only minimally increased the availability of α-1,2 fucose.

The decreased sensitivity to CT upon simultaneous inhibition, of O-linked glycosylation and fucosylated structures, compared to single inhibition would lend evidence to ligands that are fucosylated being implicit both as functional CT ligands, and in the creation of decoy-like ligands suitable for preventing CT intoxication.

Conclusion

We created CTB site-specific mutants that effectively omit binding to ligands relevant to their designed defective binding sites. Complementing these with inhibitors, showed that the majority of CTB binding to human enteroids occurs through the non-canonical site to fucosylated O-linked glycans (Fig. 7). Using a combination of our tools, we postulate that fucosylated structures play a double role as functional ligands facilitating CT intoxication, and as part of decoy ligands that bind CTB but do not aid in CT uptake. As it appears that considerable GSL turn-over in enteroids is longer than the 3-day time-period used in this study, it is hard to directly determine what role GSLs have on CT intoxication. However, if GSLs are indeed involved, then the contribution of the canonical-site ligand GM1 is very limited as its expression in enteroids was below the level of detection, an observation which has also been previously reported in a study primary human tissue [89].

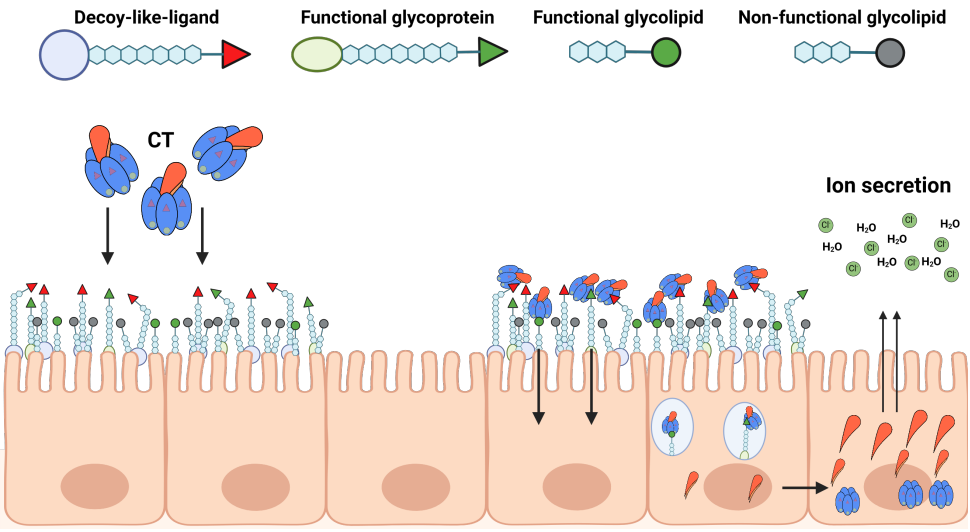

Fig. 7. Schematic summary of findings from paper II. CT binding mostly occurs to fucosylated ligands via the non-canoncial site, some of which are decoy ligands and some functional ligands. The presence of functional glycolipid ligands may contribute to intoxication, while most glycolipid ligands do not bind CT and are not functional.

Paper III – Binding Deficient Cholera Toxin Mutants Reveal Dependence on Both Binding Sites for Human Intoxication

The primary focus of paper III was to expand upon our findings in paper II using the CTB mutant tools we had created, to investigate human intoxication using a more direct approach. As with the previous two papers, our goal was to use our biologically relevant human enteroids, alongside the CTB mutants to investigate the specific site required for uptake using a tagged toxin (saporin). In addition, we decided to design in-house holotoxin mutants to explore the binding site used for intoxication. Furthermore, we wanted to examine the historically used cell lines in CT research and how they compare to human enteroids. The latter was done to determine the most appropriate cell lines to use as alternative to enteroids for large-scale experiments that require vast cellular material and to help decipher results by other research groups obtained using cell lines.

CTB uses both binding sites for internalization into most intestinal cells

Using the CTB mutants from the previous study, we first set out to establish the binding site utilized across several different human cell lines. First, we wanted to assess the use of the SA-tagged toxin, saporin (ZAP) for its potential as a marker for cellular intoxication in combination with our CTB mutants. Saporin is a RIP that has no natural receptor for internalization alone. However, when paired with the biotinylated vehicle CTB, will result in cell death upon internalization following continued incubation at 37°C. We confirmed this method on three leukocyte cell lines that have varying expression levels of canonical site or non-canonical site ligands that bind CTB. The Jurkat cell line is known to express GM1 and has been shown to bind CTB using this ligand [148, 235]. The granulocytic cell line HL-60 has a dependence on both fucosylated and O-linked glycosylation for CTB binding [12]. Finally, the THP-1 monocytic cell line was chosen for its dependence on both binding sites. By first examining CTB mutant binding by flow cytometry, and subsequently assessing the overall level of cell death when coupled with SA-tagged saporin, we were able to show that our site-specific deficient mutants were feasible tools to investigate CTB internalization via either binding site (Paper III Fig. 1).

With this, we endeavored to investigate internalization using human intestinal cells. For this we wanted to compare the classically used CT cell lines of T84, Colo205 and differentiated Caco2 alongside our human tissue-derived enteroids (Paper III Fig. 2). CTB binding to all three intestinal cell lines was severely impacted by the H18L mutation of the non-canonical site. In contrast, the W88K mutation of the canonical site had little impact on CTB binding to the T84 cell line. This designated a complete reliance on the non-canonical site of CTB that also directly facilitated internalization of CTB by the T84 cell line. This finding is also consistent with the previous report of the absence of detectable GM1 on the surface of these cells [148]. Conversely, the W88K mutation of the canonical site caused significant reductions in CTB

binding to both Colo205 and Caco2 cell lines. Internalization of CTB by Colo205 cells was mirrored by the CTB binding site requirements. Caco2 cells displayed an overall decrease in uptake by both defects, but the W88K mutation of the canonical site had more impact on the amount of CTB internalized than that of the non-canonical site (H18L). Analyses of binding of CTB to the enteroids established from two donors revealed similar requirement for both binding sites as detected with the Colo205 and Caco2 cells, with more emphasis on non-canonical site ligands. The effects of the site-specific mutations on CTB internalization by the two enteroids were similar to that observed with Caco2 cells where mutation of the canonical site appeared to have a larger impact.

In summary, we show that we have a method of determining CTB internalization using our site-specific defective mutants. Using this technique, we observed that almost all CTB binding and internalization of the extensively used T84 cell line is non-canonical site driven. We determine that CTB has a reliance on both sites for binding to and internalization by the commonly used Colo205 cell line. Finally, we show that of the selected cell lines, differentiated Caco2 cells appear to best reflect the donor derived enteroids in terms of CTB site binding and internalization.

Treatment with inhibitors imply that Caco2 cells are a closer model to human enteroids than T84 cells

To extend our exploration of how similar the previously used cell lines are to human enteroids, we decided to investigate the ligand expression in response to incubation with inhibitors. As we wanted to understand this behavior in the context of CT, the monolayer forming cell lines T84 and Caco2 were chosen so that the type of ligand utilized for intoxication could also be determined. The T84 cells had vast amounts of fucosylated and O-linked glycans as evidenced by 2F-Fuc and Benzyl-α-GalNAc inhibition, and were suitable for binding to either site of CTB (Paper III Fig. 3). This inhibition also revealed an increase in galactosylated ligands that were detected with PNA that did not translate to increased CTB binding. Inhibition of N-linked glycans had little impact on overall CTB binding but did result in a substantial increase in canonical site binding, despite the decrease in detectable galactosylated ligands i.e. PNA binding. In contrast, while fucosyl inhibition had an overall decrease in CTB binding to Caco2 cells, this was isolated to non-canonical site ligands. In addition, the O-linked glycan inhibition also led to an overall decrease in non-canonical site ligands but resulted in a steep mean increase in canonical site ligands. N-linked inhibition had little to no effect on overall binding of CTB to Caco2 cells. In paper II, we concluded that enteroids displayed increased binding of CTB by the canonical site upon fucosyl and O-linked inhibition, while a limited donor dependent effect following inhibition of N-linked glycans was observed (Paper II Fig. 4-5). Hence, comparing the T84 cells to the Caco2 where only the latter showed increased presence of canonical site ligands upon O-linked glycan inhibition, again suggests that the surface ligand expression of Caco2 more closely resembles enteroids than the commonly used T84 cells.

We next explored monolayers of these cells upon inhibition and apical CT challenge to compare back to the intoxication results obtained in paper II. Following inhibition of fucosyl, O-linked or N-linked glycans, the sensitivity of T84 cells drastically decreased (Paper III Fig. 4). In contrast, Caco2 cells consistently displayed a substantial increase in CT response following fucosyl inhibition, while either glycan inhibition had a limited effect on sensitivity. Taken together, we determined that the substantial decrease of potential toxicity upon inhibition of any non-canonical site ligand of the T84 cells did not reflect what was found using enteroids in paper II. In contrast, the increase in canonical site ligands upon O-linked inhibition, and the increased sensitivity upon fucosyl inhibition to CT challenge of the Caco2 cells more adequately reflect what we discovered in human enteroids.

CT requires both binding sites to intoxicate human enteroids, while only the non-canonical site of CTB is utilized for T84 cells

Finally, we tested our binding deficient mutants with the enzymatically active CTA inserted on monolayers of T84 and our human enteroids to determine the binding site required for intoxication (Paper III Fig. 5). In T84 cells, the W88K mutation had a comparable amount of measured ion efflux compared to the WT holotoxin; a property that was recapitulated when incubating WT with the GM1os. The H18L and double defective H18LW88K holotoxins however were significantly reduced in the amount of overall detectable intoxication. Conversely, mutations to either binding site significantly reduced the overall capacity of CT to intoxicate human enteroids to levels close to that seen with the PBS control. As observed before (Paper I Fig. 5), pre-incubation with GM1os also completely blocked intoxication. It is likely that the hetero-multivalent requirement of CT binding suggested in paper II, is the major contributing factor when either binding site of CT is defective, resulting in a toxin that cannot internalize through a pathway that will result in efficient intoxication.

Conclusion

A large amount of CTB binding to human enteroids occurs via non-canonical site ligands. This may have little impact on overall CT internalization, but both sites are required for CT to be enter the cell via the correct pathway to result in intoxication (Fig. 8). These requirements do not appear to be the same in the T84 cells, which bind CTB almost exclusively through the non-canonical site, but also facilitates internalization and intoxication of CT using the same binding site. Hence the overall binding, types of ligands present and pattern of internalization of differentiated Caco2 cells more accurately, represent the epithelial cells of the human small intestine in response to CT challenge compared to the colonic T84 cells.

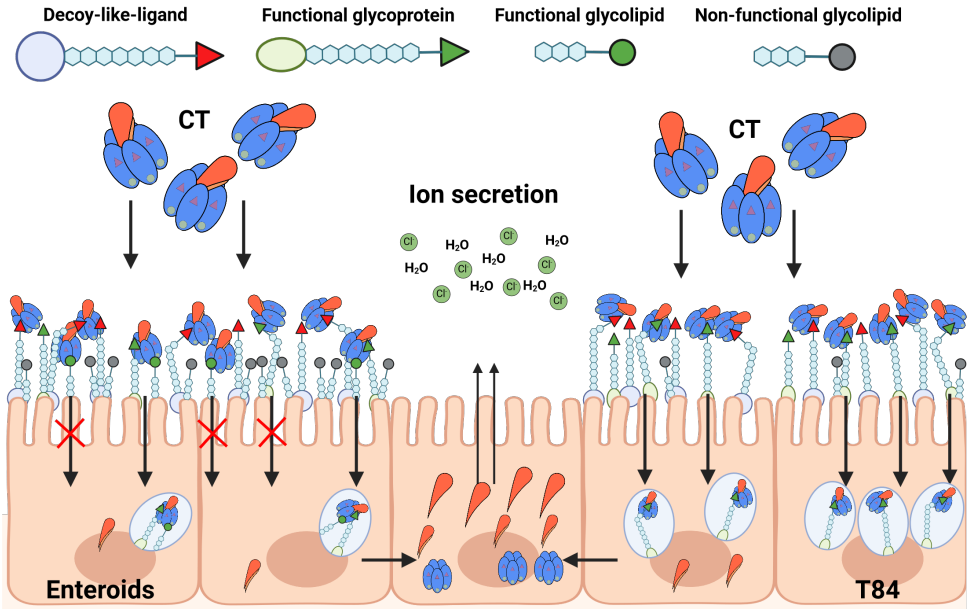

Fig. 8. Schematic summary of findings from paper III. CT binding to human enteroids requires binding to both canonical and non-canonical binding sites to induce intoxication, while the T84 cell line intoxicates solely through the non-canonical binding site.

Paper IV - The glycocalyx-forming membrane mucin MUC17 prevents entry of cholera toxin in human enterocytes

Investigating CTB binding is of vital importance when trying to understand the initial mechanisms that aid *V. cholerae's* success, as this could give us insight into potential treatments that could be developed to protect those who are already infected. However, equally important is the human body's own defenses against *V. cholerae* and other pathogens. Paper IV aimed to investigate how the glycocalyx expression of MUC17 may provide protection from CT intoxication. To investigate this, Caco2 cells expressing a recombinant form of MUC17 were investigated to determine their potential protective phenotype against CT. We further investigated mucin dependent CT protection in our enteroids upon stimulation of MUC17 expression. This study was performed in collaboration with Thaher Pelaseyed's lab at the University of Gothenburg.

MUC17 prevents cell surface CTB binding in recombinant MUC17 expressing Caco2 cells and enteroid cells

We have previously shown that CTB readily binds to differentiated Caco2 and human enteroid cells, both models necessitating engagement of both binding sites to achieve full CTB binding (Paper III Fig. 2). This offered a compelling incentive to study the mucin MUC17 by utilizing the previously designed recombinant Caco2 strain expressing 3F-MUC17(7TR) [236]. Upon incubation with CTB, a significant amount of binding to both WT Caco2 and a MUC17Δ strain was detected by confocal IHC staining (Paper IV Fig. 4). Conversely the 3F-MUC17(7TR) recombinant strain showed no detectable CTB binding to the brush border. When WT and 3F-MUC17(7TR) Caco2 cells were cultured together, CTB was only able to bind to the WT cells, displaying the cell specific protective effect of MUC17 expression.

Using flow cytometry to track the site-specific CTB mutants developed in Paper II, we were able to show that the majority of CTB binding occurs via the non-canonical binding site in this WT Caco2 cell strain, because despite the large reduction in binding with the W88K mutant compared to WT CTB, the H18L mutation resulted in further reduction in (Paper IV Fig. 5). However, the H18L mutation demonstrates that some level of canonical site-driven binding can still occur, where mutations to both sites result in background levels of binding. Consistent with the confocal staining, the MUC17 expressing Caco2 cells display a drastic decrease in WT CTB staining, which is completely abrogated when probed with either of the CTB mutants. Additionally, when stained using a polyclonal antibody targeting the cytosolic domain of MUC17, the increased expression over the relatively low levels expressed in WT Caco2 cells was detectable.

To validate this in a more heterogenous model that more closely represents what is established in humans, we treated 2 representative donor-derived human enteroids with IL22

to induce MUC17 expression. When assessed by flow cytometry, IL22 treatment resulted in a notable reduction in overall CTB binding in enteroids from one of the two donors, while no difference was detected cells from the other donor. Despite the decrease in CTB binding, there was no significant increase in stained MUC17 after IL22 treatment in either donor. The comparatively modest reduction in CTB binding detected in the IL22-treated enteroids is likely due to heterogenous cellular expression of MUC17, resulting in some cells still having considerable detectable CTB-binding, a property that can be hard to detect in a diverse cellular population.

The general protective effect detected in enteroids from one donor, combined with the understanding of the heterogenous nature of the small intestine/enteroids lead us to investigate CTB binding by an alternative method. Enteroid monolayers were grown and differentiated normally with IL22 and incubated with CTB and stained for MUC17 (Paper IV Fig. 6). We observed that the enteroids derived for the donor that exhibited a protective effect upon IL22 treatment had a considerable non-uniform increase in cells stained for MUC17. Strikingly, we detected that cells expressing high levels of MUC17 were protected from CTB binding and that the overall amount and intensity of CTB binding decreased in this donor. The enteroids from the other donor showed more modest increase in MUC17 expression and overall CTB protection. To further examine if this protective effect of IL22 was a result of increased mucin expression, IL22^{+} monolayers were treated with the O-glycosylated mucin glycoprotease, StcE, in order to remove Muc17 (and other mucins) prior to CTB treatment. Upon visual inspection, the intensity and frequency of cells stained with CTB increased upon StcE treatment, suggesting that MUC17 provides protection from CTB binding. These experiments need to be repeated and the level of binding appropriately quantified.

MUC17 expression also protects against CTB internalization

Blocking CTB binding is a key first step in preventing intoxication, but relatively few CT molecules are required for a full-scale intoxicative response [237]. This was first explored by immunoblot, where WT and 3F-MUC17(7TR) Caco2 cells were incubated with CTB and the lectin WGA (cell membrane detection), where they were then stained for surface-bound and internalized proteins (Paper IV Fig. 3). WT cells had detectable levels of both surface-bound and internalized CTB, while the 3F-MUC17(7TR) Caco2 showed only WGA binding, and no detectable CTB binding. To investigate this further, cAMP assays of CT challenged Caco2 cells of WT, 3F-MUC17(7TR) and the MUC17Δ strains were tested. This showed that the MUC17^{+} Caco2 cells were able to completely negate the increased cAMP levels detected in the WT Caco2 cells. In the MUC17Δ cells, the cAMP levels were similar to the WT Caco2 cells.

To adapt the cAMP to a method that we have confirmed to work with enteroids, we decided to employ the conjugated saporin assay method (Paper IV Fig. 7). We first sought to replicate the results of the cAMP assay using the three cell lines tested. In the two experiments performed so far, we observed that the 3F-MUC17(7TR) Caco2 cells lines appeared to be protected while the MUC17Δ Caco2 cells displayed almost WT levels of CTB-internalization.

We next assessed if IL22 treatment of enteroids which led to increased but patchy MUC17 expression, could affect internalization of CTB. Consistent with the IL22-induced expression of MUC17 (Paper IV Fig. 6) and the protective capacity of MUC17 overexpression in Caco2 cells (Paper IV Fig. 3), IL-22 treated enteroids derived from both donors displayed reductions in internalized CTB, albeit by varying amounts, as detected by saporin-mediated cell death in the limited number of experiments conducted thus far.

Conclusion

In summary we have shown that MUC17 expressed as a recombinant construct in Caco2 cells (only seven tandem repeats compared to the naturally expressed sixty) effectively negates productive binding of CTB. We have also shown that enteroids express MUC17 across a heterogenous distribution of cells, and cells that express this in high levels prevent CTB binding (Fig. 9). We observed that MUC17 expression not only prevented CTB binding, but also internalization and subsequent intoxication by CT. This would suggest that the externally expressed surface-bound MUC17, doesn't necessarily bind CTB robustly enough to be detected by our methods, but does manage to keep CTB from binding to functional ligands on the cell surface.

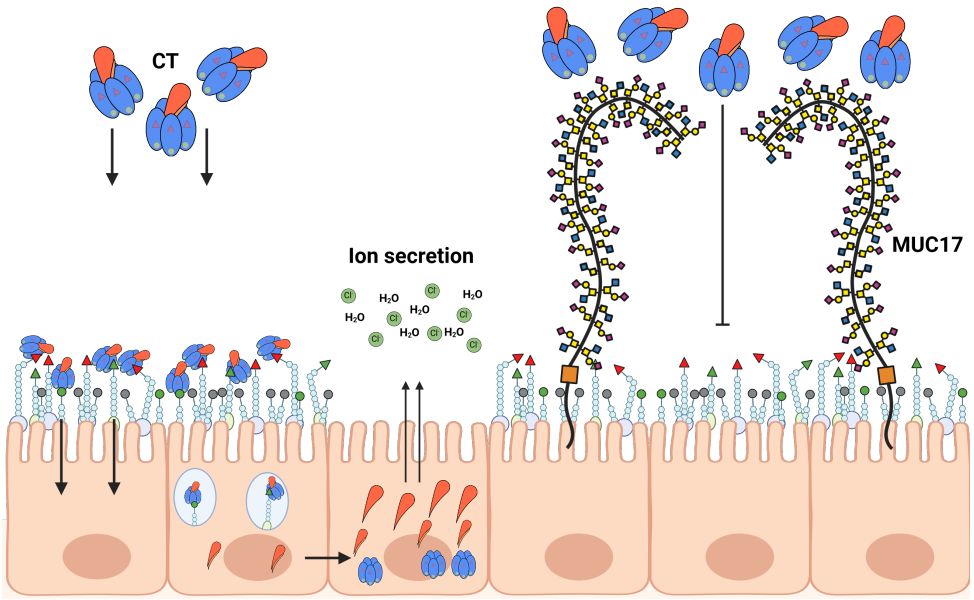

Fig. 9. Schematic summary of findings from paper IV. CT binding and intoxication occurs as normal in the absence of mucins such as MUC17, while induced expression of MUC17 provides protection from CTB binding and further CT intoxication in small intestinal cells.

Thesis conclusions

Starting the work presented in this thesis, I had little to no prior knowledge of cholera (beside the symptoms). As I started work with CT, I came to learn that there are two binding sites and that one bound GM1 with high affinity while the other targeted fucosylated structures. Initially the scope of what is presented in this paper was thought to have been covered by paper I, where we would then move onto developing more efficient blocking therapies and then look into CT as a vaccine adjuvant. In fact, this continued after we published our second paper, where we realized that inhibitors do not give us a clearcut answer either. At this point we decided to go for the more direct approach, where we would use binding deficient mutants that have CTA in what we think will be a more definitive answer, but I have no doubt that this will instead lead to more questions. Throughout the work on this thesis, I have thought of CT and my work as a puzzle where we already understand the end product, but the number of pieces, what they all look like, and how they fit together are unknown.

One area that my eyes were opened to upon investigation of paper II is how much glycosylation appears to be involved in CT research, a point that is heavily overlooked throughout literature, especially in other fields of research such as immunology. The CTB binding affinity for the GM1 ligand is so high that it has long been considered the de-facto binding ligand, and it is easy to dismiss non-canonical ligands as playing an important role in CTB binding. However, recent data has questioned this dogma, and convincingly shown that this alternative binding site is indeed of importance to cholera's story in humans [11, 12, 91, 148, 149].

Within the first paper of this thesis, we found that the synthesized Gal50Fuc50 polymer that targeted both CTB binding sites could efficiently block CTB. We then moved to functional experiments, where we found that the polymer could partially block intoxication. However, the GM1os could completely ablate intoxication despite having no detectable effect on CTB binding. Hence, this led us to postulate that CT intoxication in humans required some kind of hand-off from longer fucosylated structures that bind CT to allow to ligands close to the cell surface, where scarcely expressed ligands for the canonical site could bind to enable intoxication.

With this in consideration, in paper II we decided to use inhibitors for ligands that we believed CTB targeted. We determined that there are fucosylated structures in the human small intestine that contribute to the majority of CTB binding. This fucosylation can function to protect us in the form of decoy ligands that only bind CTB, but do not internalize it through an intoxicating pathway. We also discovered that O-linked glycosylated structures provide a level of protection via inhibition. However, to our surprise, when we thought to inhibit both O-linked glycosylation and fucosylation, we found that fucosylation can also be implicit in facilitating functional uptake of CT.

In paper III we decided to take the more direct approach, while also investigating some of the historically used cell lines for CT research. We discovered that CT requires engagement of both binding sites to intoxicate the human small intestine, a finding that our lab had observed several years earlier, but was questioned due to the potential toxicity of the lectin blockers that were employed. This combined with our findings of paper II, suggest that the functional fucosylated structures and canonical site ligands that are likely galactosylated, work in tandem as CT requires hetero-multivalent binding for successful intoxication.

Our interest in the apparent protection provided by O-glycosylation lead us to collaborate with a group working with mucins. From previous studies we already knew that CT could bind MUC13 [148], but the knowledge of the apparent epitopes present on MUC17 that are similar to that of the Lewis antigens was unknown to us, and of great interest as a potential answer to our CTB decoy finding. Together we have shown that CTB can be efficiently blocked from binding the cell surface where MUC17 is expressed, also providing protection from internalization. However, it is difficult to conclude how MUC17 expression is providing protection, but I speculate that it may be a result of canopy like protection from the mucin, changes to ligand distribution on the cell surface, or functioning as decoys that detach when CTB has bound.

As far as my literature search can find, there is only one account of the measured levels of GM1 and associated glycolipids, and that was performed on tissue from a single donor.[89] The glycolipid profile found from 2 enteroid donors appears to be consistent with this finding, with only trace levels of detected GM3 and GM2-expression. While more research is certainly warranted, it appears that despite the high binding to GM1, the canonical site binding in the human small intestine is likely to an alternative lower affinity ligand.

If all the findings of this thesis are taken together, the story of CT (and likely more) intoxication in the human small intestine occurs through an intricate system. This combines an initial defense of secreted mucous that provides an ever-replenished layer of protection. Further down, transmembrane mucins such as MUC17 provide the next layer of protection against CT that penetrates the secreted mucous layer. Closer to the cell surface, fucosylated O-linked ligands bind large quantities of CT, leaving little CT that can bind functional ligands. This CT binding requires ligands for both binding sites to initiate internalization and subsequently intoxication (Fig. 10).

From the start of my work on this thesis we have built up the use of enteroids as the current gold standard for CT research. Enteroids have proven to be a good heterogenous functional model, that we have been able to adapt to a multitude of experiments. One limitation that became apparent however is that of scale. We found that assays that require vast amounts of cellular material such as glycolipid extractions are difficult to accomplish with enteroids. This is in part due to cost, but also in the time and the amount of work required to reach such a scale. For this reason, we also sought to investigate some of the previously used cell lines in

CT research to find a scalable analogue that might be suitable for CT research. While we have yet to clarify this, we found that the extensively used T84 cells do not bind CT by similar ligands, and do not have the same ligand requirements to become intoxicated. Intoxication being driven in T84s almost entirely by non-canonical site binding, regardless of whether the canonical site is genetically destroyed or if the canonical GM1 ligand is blocking the site. A significant body of research using the T84 cell lines has been operating under the assumption that CTB binding is occurring to GM1. However, both previous studies and our own work suggest that this assumption is likely incorrect[148].

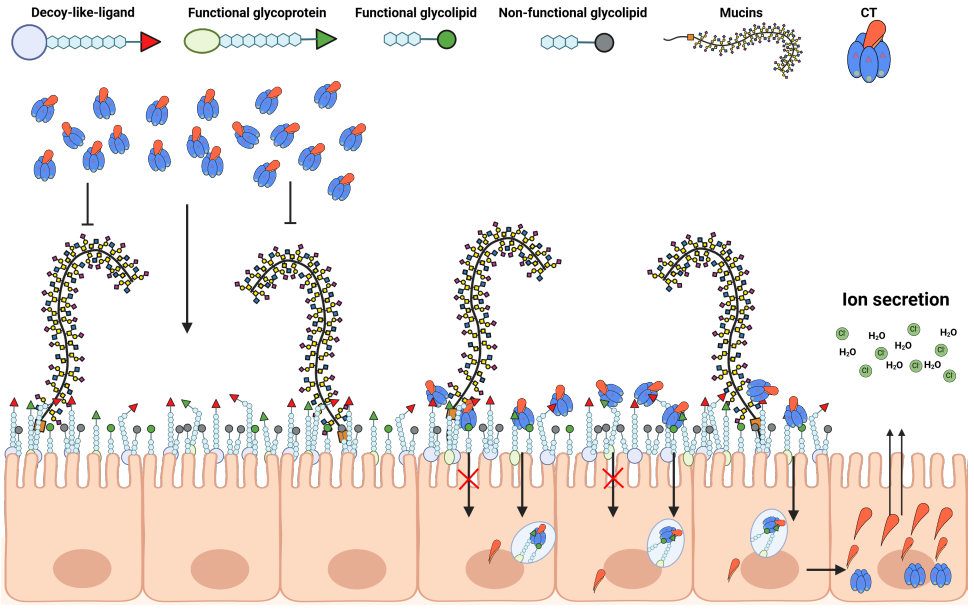

Fig. 10. Schematic summary of CT binding in the human small intestine. Large membrane bound mucins and likely secreted mucins prevent the vast amount of CT reaching the cellular surface, the CT molecules that do reach the surface often bind via the non-canonical site to fucosylated ligands that do not result in intoxication. Full intoxication requires binding ligands to both the canonical and non-canonical site of CT.

Future perspectives

Even with all these interesting findings condensed into this thesis, there still remains a wealth of relevant information to uncover regarding cholera. It has become clear that over time human evolution has attempted to protect itself from pathogenic bacteria, producing elaborate structures that bind and prevent intoxication, or serve as barriers against deflect incoming toxin-subunits. Thus, it is apparent that looking at CTB binding alone is not enough to determine a ligand that CT may bind to intoxicate. This is likely also true of other toxins, bacteria and other pathogenic microorganisms. Our research has suggested that CTB internalization does not equate to intoxication. It is evident that some CT binding results in uptake, but through the "incorrect" pathway for the toxin to enact its effect. Therefore, we advise fellow researchers in the field to exercise caution in their interpretation, depending on the specific aspects investigated. For example, if investigating CT functionality, it would be pertinent to look at binding, internalization and/or intoxication.

It has also become apparent that the choice of model needs to be carefully considered. Colonic cell lines have been extensively used despite the primary site of cholera infection being in the small intestine. Despite this, Caco2 cells have the capacity to become more small intestine-like after differentiation, but still lack the heterogeneity found in the human gut. A vast amount of research has also been explored using animals. While it is likely that some animals exhibit glycan expression similar to humans, careful consideration is warranted when choosing a model organism. For example, mice for example bind CT almost exclusively via the canonical site, yet SI-EC a complex ganglioside knock-out mouse binds very little CT, while CT induced intoxication of the knock-out mouse is similar to its WT counterpart [12].

Currently, our best model is that of primary tissue-derived enteroids, yet these are limited as they are isolated from the rest of the systems present in a human that might impact CT intoxication. It would be of interest to further our understanding by co-culturing human enteroids with microbiota and immune cells of the small intestine. Indeed, this endeavor may appear ambitious with our current techniques this seems like a tall order, but it holds the potential to significantly enhance our understanding of CT intoxication by elucidating the interplay between the toxin, the microbiota's defensive mechanisms, and the immune system's response to this threat.

As a final note, there is a substantial amount of research left to be done on CT to determine the scope of intoxication. The limited detection of glycolipids in the human small intestine, but requirement of a canonical (and non-canonical) site ligand for intoxication suggests that there are galactosylated structures that are yet unknown that facilitate this. My hope is that my findings may contribute to the development of accessible treatments or enhance the utilization of CT as a vaccine adjuvant, ultimately aiming to serve the well-being of humanity.

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