# Evaluation of Fucosylated Receptors for Cholera Toxin in the Human Small Intestine

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Cover illustration: *An interpretation of the cholera toxin B-subunit pentamer by*

***Dag Almqvist***

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Man will occasionally stumble over the truth, but usually manages to pick himself up, walk over or around it, and carry on…

-Winston Churchill

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ABSTRACT

Cholera toxin (CT) produced by *Vibrio cholerae* is the causative agent for the diarrheal disease cholera. Cholera is yearly afflicting millions and is estimated to kill over 100 000 people every year. In this thesis I aimed to better understand the role of noncanonical CT receptors, e.g. receptors other than the glycolipid GM1. Epidemiological studies have found a link between cholera severity and blood group indicating that histo-blood group antigens (HBGAs) could play a role as receptors for CT. The work presented in this thesis shows that CT readily binds to the HBGA Lewis X on cells and on some cells CTB binding correlates with the level of Lewis X. Furthermore, we show that other fucosylated glycans such as Lewis Y, A/BLewis Y and 2´-fucosyllactose (found in human breast milk) readily inhibit CT binding to cell lines and primary cells from human small intestine. In contrast, sialylated or non-fucosylated glycans did not show any inhibitory effect on CT binding to human cell lines indicating a fucose-dependent binding. This was further confirmed in blocking studies using long synthetic polymers displaying glucose, fucose, galactose or a mix of the latter two. Functional evaluation identified that the fucose-binding lectin AAL completely blocked the effect of CT, but so could the galactose-binding lectin PNA. The galactose-fucose polymers yielded a partial inhibition of CT intoxication of human small intestinal enteroids whereas GM1 glycan completely blocked the effect of CT. Hence, fucosylated glycans are involved in attachment of CT to the intestinal wall. However, if this binding assists or counteracts subsequent internalization by other receptors carrying terminal galactoses remains to be determined. Importantly, these receptors can be other glycans than GM1 as this thesis show GM1-independent CT-mediated intoxication.

**Keywords**: Cholera toxin, Lewis antigen, HBGA, HMO, fucose, GM1

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# SAMMANFATTNING PÅ SVENSKA

Kolera är en allvarlig diarrésjukdom som kan ha en dödlighet på över 50 % om de drabbade inte får sjukhusvård. Standardbehandlingen för kolera är oral vätskeersättning och vid svårare fall även intravenös vätska och antibiotika. Numera finns även vaccin mot kolera som har god effektivitet, men långt ifrån alla som har risk att drabbas av kolera är vaccinerade. Kolera drabbar främst människor i Gangesdeltat och länder i Afrika söder om Sahara.

Kolera orsakas av koleratoxin som utsöndras av bakterien *Vibrio cholerae.* Koleratoxin produceras först när bakterien har nått tunntarmen och där binder det till det yttersta cellagret. Under 1970-talet upptäcktes att en glykolipid (socker kopplat till en fettkedja) på cellens yta kallad GM1 kunde binda koleratoxin. GM1 kunde på så vis möjliggöra upptag av toxinet in i cellen. Väl inne i cellen så tar koleratoxin över delar av cellens signalsystem. Detta leder i sin tur till att stora mängder salter och vatten utsöndras i tunntarmen. Effekten av detta blir en mycket kraftig diarré som kan ge vätskeförluster på så mycket som en liter i timmen.

Majoriteten av de studier som är gjorda beträffande hur koleratoxin förgiftar celler med hjälp av GM1 är gjorda med cellinjer och djurförsök. Tyvärr kan dessa metoder inte fullt ut representera hur koleratoxin påverkar den mänskliga tunntarmen. Tidigare har det också visats att koleratoxin förutom GM1 också binder andra sockermolekyler. Denna typ av receptorer har inte utforskats i lika hög grad som GM1. Vi ville därför undersöka vilken roll den nya typen av receptorer har för koleratoxin. Dessa experiment utfördes därför med vävnad från mänsklig tunntarm. Vi använde oss även av möss som saknar förmågan att framställa glykolipiden GM1 i våra studier. Dessa möss liknar människor då vi har mycket låga nivåer av GM1 i våra tarmar.

I denna avhandling kan vi visa att sockermolekyler som är eller liknar blodgruppsmolekylerna i ABO-systemet har stor inverkan på koleratoxin-bindning till celler från mänsklig tunntarm. Detta medför att den stora majoriteten koleratoxin inte binder till GM1 som man tidigare trott.

Vi visar också att andra receptorer än GM1 påverkar diarrén i både mus och människa. Bindningen av koleratoxin till dessa receptorer är till mestadels beroende på sockret fukos men även sockret galaktos är viktigt.

Slutligen kan vi påvisa att bindningen av koleratoxin till celler från mänsklig tunntarm, kan blockeras av långa syntetiska kedjor med fukos och galaktos. Vi ser även indikationer på att dessa kedjor kan blockera effekten av koleratoxin. Detta gör att de är potentiella kandidater för att förbättra behandlingen med vätskeersättning vid kolera.

# LIST OF PAPERS

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

I. **Cervin J**, Wands AM, Casselbrant A, Wu H, Krishnamurthy S, Cvjetkovic A, *et al.*; **GM1 ganglioside-independent intoxication by Cholera toxin**

*PLoS Pathog 2018 14(2): e1006862. https://doi.org/10.1371/journal.ppat.1006862*

II. Amberlyn M. Wands, **Jakob Cervin**, He Huang, Ye Zhang, Gyusaang Youn, Chad A. Brautigam, Maria Matson Dzebo, Per Björklund, Ville Wallenius, Danielle K. Bright, Clay S. Bennett, Pernilla Wittung-Stafshede, Nicole S. Sampson, Ulf Yrlid, and Jennifer J. Kohler; **Fucosylated Molecules Competitively Interfere with Cholera Toxin Binding to Host Cells**

*ACS Infectious Diseases 2018 4 (5), 758-770 DOI: 10.1021/acsinfecdis.7b00085*

III. **Jakob Cervin**, Andrew Boucher, Gyusaang Youn, Xiaoxi Yo, Surita R. Bhatia, Per Björklund3, Ville Wallenius, Michael Lebens, 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**

*Manuscript*

Relevant paper not included in this thesis:

Amberlyn M Wands, Akiko Fujita, Janet E McCombs, **Jakob Cervin** *et al.*; **Fucosylation and protein glycosylation create functional receptors for cholera toxin**

*eLife 2015;4:e09545 doi: 10.7554/eLife.09545*

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| CT | Cholera toxin |
| CTA | Cholera toxin subunit A |
| CTB | Cholera toxin Subunit B |
| cAMP | Cyclic adenosine monophosphate |
| ORT | Oral rehydration therapy |
| LT | Heat-labile toxin |
| LTB | Heat-labile toxin subunit B |
| ER | Endoplasmic reticulum |
| Fuc | Fucose |
| Gal | Galactose |
| Glc | Glucose |
| FUT | Fucosyltransferase |
| HBGA | Histo-blood group antigen |
| TCP | Toxin regulated pilus |
| LPS | Lipopolysaccharide |

# INTRODUCTION

## CHOLERA

The diarrheal disease cholera is a well-known old plague of the human population and is caused by the Gram-negative bacteria *Vibrio cholerae* (*V. cholerae*). The extreme diarrheal fluid loss associated with cholera disease can kill a healthy adult human quickly if not treated. Annually it is estimated that 3 million people are affected by cholera, with about 100 000 deaths worldwide as a result. It's hard to get exact data on number of cases due to poor surveillance and a lot of cases occurring in areas with limited infrastructure or in areas of armed conflict (1,2).

The work presented in this thesis aim at a better understanding of the main cause behind cholera; cholera toxin. More specifically I was interested in how noncanonical receptors interact with cholera toxin (CT). The canonical receptor for CT has been known for about 50 years and is a glycolipid called GM1 with very high affinity for CT. Despite being able to facilitate CT intoxication GM1 is not the sole binder of CT on the cellular surface. For this reason, I was interested in further investigating these novel types of receptors and aimed to evaluate their functional role. I also identified a lack of data regarding CT binding to primary infected tissue in the human small intestine. Using this new knowledge, I was interested in evaluating different blocking agents to prevent CT intoxication.

## CHOLERA EPIDEMIOLOGY

Cholera primarily affects poor regions where there is limited access to clean drinking water and good sanitation. Africa is the most affected continent with 60% of recorded cases and almost 70% of deaths, but south Asia (primarily Ganges delta) and the Middle East as well as Haiti have endemic cholera (2). Outbreaks often occur during flooding when the drinking water supplies are contaminated with fecal matter or bacterium from otherwise isolated water containing *V. cholerae*. In fact, *V. cholerae* is normally resides in fresh and saline water all year round, mainly causing disease during the rainy season. This means that even endemic areas have a large variance over the year in the number of cholera cases similar to non-endemic outbreaks (3,4).

It is believed that cholera was originally only endemic in the Indian subcontinent (primarily the Ganges delta), but with the development of fast and frequent travel cholera has been able to spread and cause epidemics and also pandemics elsewhere. In fact, since 1816 there have been seven documented cholera pandemics responsible for the deaths of tens of millions of people. The first recorded pandemic (1816–1826) began in the Ganges delta and spread across India, China, Indonesia and as far west as the Caspian Sea. The following five pandemics of cholera (between 1829-1923) also had recorded large outbreaks in Europe and in the Americas (5-7). These six pandemics were caused by *V. cholerae* O1 of the "classical" biotype, whereas the seventh pandemic was started in the 1960s and which is currently ongoing, is caused by the other biotype "El Tor". This seventh pandemic which started in Indonesia and not India as the previous ones had, is the most extensive in regards to its duration and its geographical spread (8,9). A more detailed overview of the different *V. cholerae* strains can be found below.

The cause of the longevity of the current seventh pandemic can be explained by the modern changes in the speed and frequency of travel. The El Tor biotype has taken over as the primary cholera-causing organism from the classical biotype (7,9). It has been suggested that the El Tor biotype is better suited for surviving in the environment and hence will be more likely to infect humans than the classical biotype (10). This appears a more plausible explanation than differences in pathogenic capacity since infection with either El Tor or classical biotype O1 *V. cholerae* can be equally severe and immunological protection against reinfection are comparable (7,9,11).

The most common infection route is via contaminated water or food since a relative high number of bacteria ($10^3$-$10^8$) need to enter the stomach to successfully colonize the small intestine. The bacteria are sensitive to the acidic environment of the stomach so individuals with high stomach pH are far more susceptible, to infection (12,13). The infection is often cleared within a week by the host's own immune system and antibiotics are seldom needed as a part of the hospital treatment (1).

Another important risk factor for cholera is being a child under 5 years old. Also, previously unexposed adults run a great risk of contracting cholera during an outbreak (14). This is most likely due to the lack of preexisting immunity (as in young children) that adults living in endemic areas often have due to repeated exposures to *V. cholerae* (1,4,14). There have been some reports on cholera being more prevalent in HIV-infected individuals during outbreaks, but bigger studies have to be conducted to verify this link (15). However, this would be in line with the hypothesis that preexisting immunity to *V. cholerae* is a large contributing factor to why cholera outbreaks is not affecting even more people in endemic areas. An elevated risk of sever cholera can be seen in blood group O-positive individuals. The risk for infection is however not altered indicating that the blood group antigen could be involved in the cellular uptake of CT (16,17).

## VIBRIO CHOLERAE

Like for all bacterial species *V. cholerae* is a name that groups several genetically similar, yet distinct sub strains together. In order to understand the world around us we humans have a strong need to classify things into categories. This need for classification is very useful in studying all forms of life but in the case of bacteria it also becomes a huge and difficult task. Since bacteria have such a high reproduction rate the phenotypic and genetic variation is often large. To handle such large diversity there are several classification systems that work together to generate a more comprehensive picture of reality. In the case of *V. cholerae* it can be divided into serotypes on the basis of how they are aggregated by serum antibodies. This classification only considers the differences that strains have on the surface but is a good indicator that there is a significant genetic variation between strains.

Serotypes can be grouped into serogroups where only smaller differences can be detected. One can also take a genetic approach and classify genetically identical/very similar strains into biotypes. Since the difference between biotypes can sometimes only be noticeable on the inside of the cell, the same serogroup or serotype can have different biotypes. Ergo, biotype is the level of classification that has the most resolution but is also requires the most work to determine.

*V. cholerae* is usually an environmental-residing Gram-negative bacteria, and although cholera is a human exclusive disease, humans are not the only natural host. Several aquatic animals and alga are carrying the bacteria as a symbiont or as part of the commensal flora (18). In fact, out of the 200 serogroups that exists, only the O1 and O139 cause major outbreaks of cholera disease. The current pandemic is predominantly caused by serogroup O1 of the El Tor biotype (19). The serogroups O1 and O139 carry the bacteriophage (CTXΦ) coding for CT as well as other virulence factors. The vast majority of the over 200 *V. cholerae* serogroups do not cause cholera but might cause other diseases in humans. In fact, the vast majority of *V. cholerae* strains isolated from the environment around human settlements are not carrying all the virulence factors necessary to cause cholera (2,20-22).

CTXΦ is a filamentous bacteriophage that contains genes coding for CT and two other toxins as well as structural virion proteins (23,24). The two other toxins are accessory cholera enterotoxin (Ace) and zonula occludens toxin (Zot). Ace and Zot seem to be able to contribute to the diarrhea in cholera as shown in animal models and human cell lines (25,26). However, the vast majority of the diarrhea in cholera is caused by CT (23). The phage originally differed between the two major biotypes of cholera-causing bacteria. In the beginning of the seventh pandemic the emerging El Tor biotype had its own variant of CT where the B-subunit differs at amino acids 18 and 47. However, over time the classical CT-genes took over and now the El Tor biotype bacteria express the classical CT (27,28). This fact can lead to some confusion in the field about what type of CT to use in studies. We consistently used the classical CT in this thesis as we consider this one to be the most clinically relevant.

The *Vibrio cholerae* pathogenicity island (VPI) element carries genes necessary for the formation of the toxin-coregulated pilus (TCP), a type IV pilus that is thought to mediate adherence and colony formation during intestinal colonization of neonate mice and humans (29,30). The VPI element also carries genes that encode two transcriptional activators, Tox T and TcpP, which regulate the production of both TCP and CT. In addition, *V. cholerae* O1 and O139 have several other genetically encoded virulence factors, which enable the bacteria to effectively colonize and cause cholera disease in the small intestine. This includes a flagellum enabling the bacteria to swim and thus withstand the constant outward motion of the mucus layer fluid. It is also important that *V. cholerae* can penetrate the small intestinal mucus layer (31). This is accomplished with the aid of a mucus degrading protease (mucinase) (31,32). The ability to traverse the intestinal mucus layer is of a high importance to the bacterium, since closer proximity to the epithelium likely ensures better intestinal colonization (31). By entering the mucus layer *V. cholerae* also escapes the high concentrations of bile in the upper small intestine. Bile is bactericidal to *V. cholerae* and can also be sensed as a chemorepellent by the bacteria (31).

CT induced fluid secretion also correlates with rapid depletion of mucus production. This indicates that the mucus producing goblet cells are sensing and responding to the toxin onslaught but fail to eliminate the bacterial infection (33-35). The mucus release form goblet cells is also regulated by the same signal molecule that CT induces an overproduction of; cAMP. It is therefore possible that CT intoxicated goblet cells quickly release all stored mucus, but this needs to be proven experimentally (34,36).

The rather big family of *V. cholerae* strains can be divided into several subgroups with regards to reactivity to serum antibodies. First the strains are divided into serogroups where bacterial strains with similar reactivity cluster together. There are over 200 serogroups that can be further subdivided into serotypes Inaba or Ogawa. The only difference between Ogawa and Inaba serotypes is that Ogawa has a terminal methylation on its lipopolysaccharide (LPS) that Inaba lacks due to an inactive methyltransferase. This small change in the LPS synthesis has a great impact of bacterial fitness and ability to cause cholera. As to why the Ogawa strains of serogroup O1 have outcompeted the Inaba strains is still not fully understood (37,38).

## CHOLERA TOXIN

CT is a holotoxin with a binding (CTB) and a catalytically active part (CTA). CT is made up of one CTA subunit and five identical B-subunits that are organized in a pentamer ring with a hollow middle as seen on the cover of this thesis. The pentamer structure is sensitive to heat and pH and must be intact to enable binding of the main ligand GM1 (39). GM1 is a glycolipid and will be discussed in more detail in a later section. CTA is positioned in the hollow center of the CTB pentamer and can be further subdivided into CTA1 and CTA2. CTA2 is responsible for attaching CTA to the CTB pentamer. Upon internalization CTA1 is cleaved from CTA2 and is free to migrate inside the cell. CT intoxication will be covered in more detail later in this text (40,41).

CT gene expression is under complex regulation under the ToxR regulon cascade, and is turned on by sensors recognizing intra intestinal components such as bile (42-44). CTA and CTB are coded and translated separately and then assembled in the periplasmic space of *V. cholerae*. The level of translation does not seem to differ dramatically between the two subunits and therefore a surplus of CTA will build up that has to be handled by the bacteria (45). The fully assembled toxin is then secreted from the bacteria via a type II secretion system into the environment or, during infection, into the intestinal lumen (46,47).

In the early seventies the glycolipid GM1 was discovered to bind strongly to CT and proven to act as a receptor for uptake (48-50). The affinity between CTB and GM1 is very high for a protein glycan interaction. This high affinity stems from the branched nature of GM1. The GM1 glycan consists of a stem glycan with glucose, galactose and GalNAc terminating in a galactose. Attached to the core galactose is a sialic acid which provides two distinct "handles" for a protein to grab on to (51). Each subunit of CTB can bind the terminal galactose of GM1 via a deep pocket located on the basal side (when viewing CTA as protruding from the top of CTB) in the middle of the subunit (Fig 1) (40). The sialic acid pocket is a bit shallower and located in the junction between two CTB subunits. This double pocket binding of GM1 is the reason for the high affinity and is highly dependent on the pentameric organization of the CTB subunits (Fig 1). The bond is sometimes referred to as a two-finger grip with galactose as the index finger and sialic acid as the thumb (49,52,53).

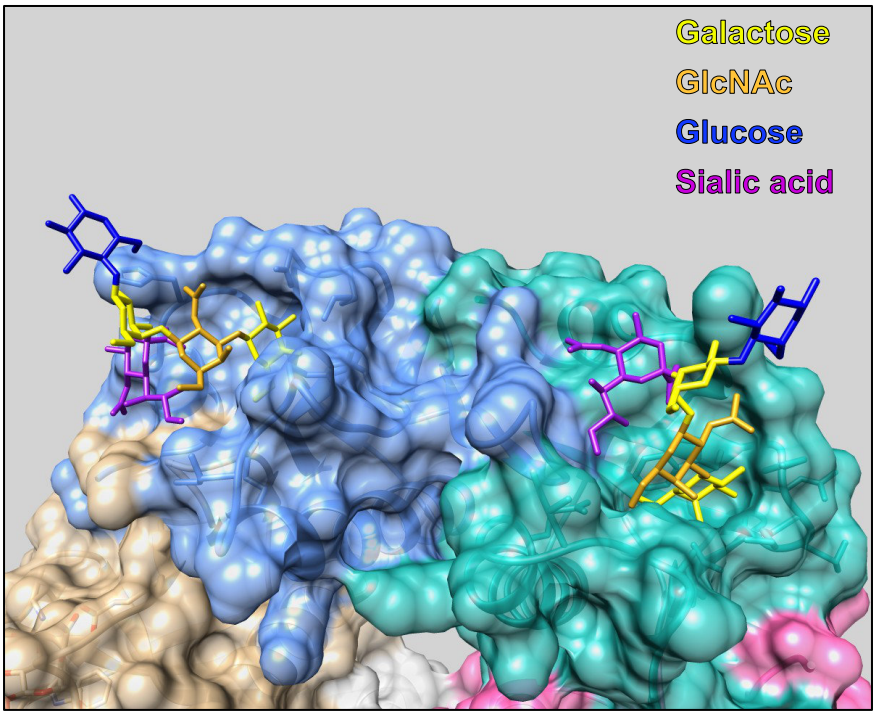


**Figure 1. CTB in complex with GM1.** A computer rendering of the surface of CTB obtained from X-ray crystallography with two GM1 glycans bound (PDB-ID: 2CHB). All the subunits for CTB are not fully shown but all have a distinct color to better visualize the transition between each. The individual sugars are color coded to visualize the galactose (yellow) and sialic acid (purple) binding pockets. The blue glucose residue is normally attached to ceramide in the cell membrane.

Most of the binding strength is dependent on galactose although sialic acid also plays an important role in stabilizing the binding. This site primarily binds to GM1 but also binds to other oligosaccharides with terminal galactoses such as asialo-GM1 with low affinity (54). GM1 has since long been confirmed as a functional receptor for CT intoxication in rabbit intestine and human cell lines (49,55-57). CT binding has been shown to correlate with the varying levels of GM1 in intestines of some species, but not for all cell types or tissues. It is therefore clear that other binders exists on cells of various tissues (49,58-60).

Interestingly, a noncanonical site has also been discovered but has been less well characterized for both receptor specificity and functional significance (61-63). It accepts several types of fucosylated oligosaccharides such as the histo-

blood group antigens (HBGAs) A and O as well as Lewis Y and X albeit at much lower affinities than for GM1 (61-64). Much of the work in this thesis has been focusing on understanding this new site and what role it plays in CT intoxication.

## EXTRACELLULAR BINDING

As mentioned above CT is produced by *V. cholerae*, assembled in the bacterial periplasm and then secreted into the surroundings via type II secretion system (65,66). The bacterial production of CT is initiated upon small intestinal infection. CT binding to epithelial cells is greatly increased by several virulence factors such as the flagella and a mucinase. These virulence factors together enable toxin secretion in closer proximity to the apical side of the epithelial cells.

For about 50 years the ganglioside GM1 has been considered the only receptor for CT uptake. The glycolipid GM1 consists of a constant glycan and a lipid tail that can vary in composition (67). The affinity of the CT-GM1 two-finger grip is storing and has a Kd in the mid pM to low nM range, varying somewhat depending on the assay used (54,68-71). A schematic drawing of how CT enters and intoxicates cells can be seen in figure 2. It has also been shown that the level of GM1 on a cell type correlates with CTB binding and that incorporating exogenous GM1 into cell membranes both increases CT binding and the intoxication level of the cells (49). Additionally, numerous groups have shown that GM1-os or GM1-like molecules readily block CT intoxication in both animals and human cell lines. Together this proves beyond a doubt that GM1 can act as a cellular receptor (72-74). However, not all cell types show a correlation between binding of CTB and the level of GM1. This indicates that there are one or several other receptors for CT and that the receptor repertoire may vary between different species and tissues (58-60).

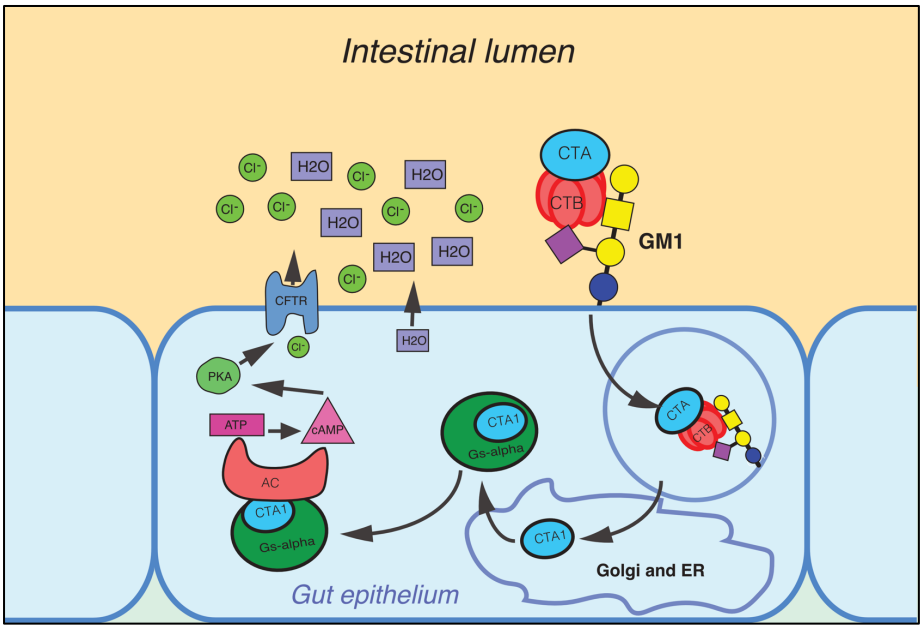


**Figure 2. Classical view on intoxication pathway for CT.** A schematic drawing of how todays consensus regarding CT intoxication of epithelial cells in the human small intestine.

## INTERNALIZATION

Over the years there has been some controversies regarding CT uptake. One such argument is if CT uptake is clathrin-dependent or mediated by calveolar uptake in lipid rafts (75-78). However, the most reasonable standpoint when summarizing the findings is that CT can enter cells via various pathways and uptake systems. The uptake route clearly varies between different cell types but also within cells, again indicating that there are several types of receptors for CT (75,79). It is important to note, that only a minority of the CT that is taken up can effectively contribute to intoxication, which again is indicative of several uptake routes and/or several receptors also within the same cell (80). To my knowledge no one has thoroughly mapped CT uptake in primary cells from human small intestine. This would of course be of high interest since these are the cells that during natural infection are exposed to CT.

Upon internalization CT begins a complicated journey from the cell membrane via the Golgi and ER to eventually assert its effect in the cytosol

(Fig 2). First CT is retrogradely transported from the surface in endosomes to the trans-Golgi network. From there CT is further transported to the ER and from that point able to traffic back and forth between the ER and Golgi, awaiting recognition by chaperones. Although the CTA-chain is cleaved into CTA1 and CTA2 well before entering the ER by serine protease cleavage and disulfide-bond reduction they still stick firmly together (81). It is only with the help of protein disulfide isomerase that CTA1 can escape the rest of the toxin complex (82,83). In light of recent findings the exact role that the protein disulfide isomerase plays in the detachment of CTA1 is less clear and other proteins such as Hsp70, Hrd1 and BiP are also involved in the dissociation (84,85). Once free CTA1 is recognized as a miss-folded protein, it is transported into the cytosol for degradation. Upon entering the cytosol CTA1 escapes degradation and facilitates ADP-ribosylation of Gs-alpha with the help of ADP-ribosylation factor 6 (Fig 2) (80,85).

## TOXICITY

The toxic effect of CT begins with CTA1 facilitating activation of ADP-ribosylation of Gs-alpha (Fig 2). Activated Gs-alpha will in turn activate adenylate cyclase which converts adenosine triphosphate to cyclic adenosine monophosphate (cAMP) (86,87). Consequently, high levels of cAMP then activates protein kinase A, which phosphorylates and activates the protein cystic fibrosis transmembrane conductance regulator (CFTR) (Fig 2) (88). In turn, activated protein kinase A also triggers trafficking of CFTR from endosomal storage to the host cells surface, further increasing the ion secretion. CFTR specifically transports intracellular chloride ions into the intestinal lumen generating a huge osmotic imbalance (89). Trying to return to homeostasis the cells secrete other ions and water into the intestinal lumen. With this the full intoxication pathway of CT is completed (Fig 2).

In fact, CT is not necessarily toxic to the cells it enters and the intestinal barrier function is not greatly affected in mice when challenged with a moderate dose of CT (90). Some have reported aberrant translocation of molecules important for tight junctions in the small intestine with high doses of CT in mice and drosophila. Others have shown that barrier disruption can occur upon *V. cholerae* infection and high-dose CT challenge, but that this

phenomenon is strain specific (91,92). In human cell lines it has been shown that CT disrupts the formation of protein complexes important for the formation of tight junctions (93). The vast majority of the fluid comes from the intestinal blood vessels and can even be inhibited via interference with nerves responsible for vasodilatation and is actively secreted by enterocytes (94-96).

Given the general nature of intracellular cAMP-sensing and protein kinase A activity, several other cellular processes are also affected by CT intoxication (97-99). Those CT-induced effects include immunostimulatory effects as well as redistribution of intercellular adhesion molecules and metabolic changes. In fact, several attempts are currently ongoing to utilize mutated or modified versions of CT as adjuvants in oral vaccines. Oral vaccines are rare today but could prove beneficial since the immunity would be localized to the gastro-intestinal and airway tracts where most of the infections occur. In order to break tolerance and induce an immune response in the gut, a potent adjuvant is needed compared to intradermal injections that are common today (100-104).

# IMMUNITY AND CHOLERA

Cholera is fairly limited in the endemic areas compared to outbreaks in naïve populations. This can largely be attributed to development of immunity against *V. cholerae* and CT (1). This is also the reason that young children without a preexisting immune response are at much higher risk for developing cholera. To better understand the role of the immune system in regulating *V. cholerae* infection, we first need to go through the basics of immunology.

To deal with the constant onslaught from the microorganism, we need a complicated immune system devoting special cells for the task of defending the acquired recourses. Immunity can be divided into two parallel but connected subsystems called innate and adaptive immunity. The innate system has a set and limited number of receptors for recognition of microbes and stress signals from dying cells but does not have a long-term memory function. Innate immunity is built up of cells and secreted molecules. It has a fast response time and can eliminate microbes. This function can be enhanced by the help of cells of adaptive immune system (105-107). Innate immune cells also get rid of dying cells in the absence of a local inflammation that damages the surrounding tissue. Local inflammation is otherwise the cardinal sign of the immune system combating infectious organisms.

The cells of the adaptive system on the other hand only recognize one small part of a pathogen or foreign biomolecule called antigens. This means that the individual cells are highly specific and thereby effective at finding and eliminating the target. It also means that they are practically useless against all other pathogens. The large amount of energy spent on developing this adaptive cellular repose would be a waste if there was no memory function built into the system saving the effective cells for future reinfections (108). To generate an antigen specific cell takes a long time compared to cells of the innate immune system, but once generated they are long lived and can persist in the body for decades. This is the reason that vaccines can be effective for so long. Although the innate system is the older of the two, adaptive immunity can be found at least as far back on the evolutionary time scale as the emergence of vertebrates (109).

The two main types of cells in the adaptive immune system are B and T cells. The cell types directly translate to the concepts of humoral and cellular

immunity (110). B cells representing humoral immunity largely depend on the secretion of glycoproteins called antibodies as a defensive mechanism against invading pathogens and toxins (111). Upon activation, B cells produce and secrete large quantities of antibodies of various types, discussed further in a section below. Antibodies are designed for binding to specific targets and can thereby be used to mark pathogens for destruction or prevent pathogen or toxin binding to cells (111-113).

T cells on the other hand need to be in direct contact with other cells to elicit their effect. Instead of antibodies, T cells utilize the T cell receptor (TCR) to interact with an antigen presenting molecule present on all cells, the major histocompatibility complex (MHC) (114,115). TCR interaction with MHC enables specific presentation of intracellular antigens present inside the cells and act as a health check for cells. Infected or tumor cells will have an altered protein transcription profile and thereby present a different set of antigens on the MHC. Effector T cells then eliminate cells that are recognized as infected or tumor cells but lack the capacity to combat extracellular bacteria like *V. cholerae* (116).

Both B and T cells have the unique ability to recombine the DNA coding for antibodies and TCR respectively. This fact enables the creation of receptors that can recognize pathogens never before encountered (117). The recombination of these receptor gene segments (called V, D and J-segments) is a stochastic process in both B and T cells, resulting in an extreme diversity in receptors(118). Most of those clones are deemed unsuitable and deleted soon after the recombination event. However, a few are selected for after a rigorous process to avoid reactivity to own tissues (119). The now genetically unique B and T cells start to patrol the blood stream and various lymph nodes. In this state they are naïve an unable to respond effectively even if they encounter the antigens they are set to detect. To become activated and thereby fully functional they need professional antigen presentation in a lymph node (120-122).

Adaptive immunity depends on the innate system for selecting the right cells that recognize a certain pathogen. This crosstalk between the systems is mainly mediated via dendritic cells. Dendritic cells patrol tissues and upon encountering a suspicious material will transport this to the lymph nodes

(122). In the lymph nodes dendritic cells interact with a special type of T cells called T-helper cells and activates them. Activated T-helper cells can in turn interact with B cells presenting that antigen on MHC. The antigen has been internalized using its surface-bound antibody and is therefore a good representation of the antibody's ligand. T-helper cells can then selectively activate the right kind of B cells to combat the pathogen (119). Upon activation B-cells will undergo Ig class switch and start producing antibodies if IgG-, IgM-, IgA- or IgE-type (123).

For combating extracellular bacteria in the intestine like *V. cholerae* the most important type is IgA since it is actively secreted over mucosal surfaces (124). IgA therefore has the ability to neutralize pathogens and toxins before they reach the epithelial cells and thereby conferring complete immunity to the effects of a *V. cholerae* exposure (14). It has been shown that a significant portion of antibodies produced during a natural infection with *V. cholerae* are directed against CT (125,126). IgA antibodies against CT in breast milk also correlates with protection from cholera underlining the importance of antibodies, but possibly also other components in breast milk (127-129). Another common antigen for antibodies is the cell wall component LPS (125,126). LPS is sensed by the toll-like receptor 4 present on cells of the innate immune system resulting in direct activation and secretion of pro-inflammatory cytokines (130,131). LPS is hence highly immunogenic and if present inside the body, especially in humans. High levels of LPS in the blood can be lethal through the severity of the immune response. This phenomenon is called septic shock.

Since CT is so potent at eliciting an immune response at mucosal surfaces it has been subjected to extensive investigation as a vaccine adjuvant (132). An adjuvant is a necessary component in vaccines that are based around only a few components of a pathogen often called subunit vaccines. The components alone often lack a strong immune stimulatory property that the adjuvant instead can contribute with (133). To avoid adverse effects CT has been modified in several ways and sometimes the subunits of CT have even been used individually (132,134-136). It has been concluded in animal studies that the adjuvant effect of CT is dependent on expression of GM1 or GM1-related glycolipids in the dendritic cells (137). Why the adjuvant effect has this dependency is unclear and this expression dependence is exclusive to

dendritic cells. Possibly the specific uptake via GM1 could facilitate the necessary pathway for eliciting a response. Alternatively, GM1 is so abundantly expressed on GM1-competent dendritic cells and the lack therefore effectively renders GM1-incompetent cells insensitive to the dose used. So far the only licensed vaccine with CT components in it is Dukoral, used to vaccinate against cholera (138). In addition to inactivated bacteria this vaccine also contains the CTB subunit.

## VACCINES AND TREATMENTS

Currently there are two licensed vaccines against cholera; Dukoral and Shanchol. Both vaccines are oral solutions that give 50-75 % protection after immunization. However, booster doses of the vaccines or reoccurring *V. cholerae* exposure is required to maintain protective antibodies over long periods of time. Both Shanchol and Dukoral are vaccines based on bacterial components from inactivated *V. cholerae.* The main differences between the vaccines are that Dukoral has only bacteria from serogroup O1 and recombinant CTB, whereas Shanchol contains both serogroup O1 and O139 (138). The idea behind adding excess CTB to Dukoral is that this will ensure a strong antibody response to CTB and thus block CT's ability to bind to cells. This way, a strong response to CT can be raised without having the detrimental effects of CTA. Neutralizing antibodies against CT in the intestinal lumen are the main protective feature of the vaccines and natural immunity (138,139). Both vaccines are based on 3 different *V. cholerae* stains of classical and El Tor biotype and of both Inaba and Ogawa serotype, ensuring a response to the two types of LPS (138,140).

However, since a large part of the population in endemic areas lack vaccination the need for acute clinical treatment is urgent. Treating cholera can often be done using quite inexpensive oral rehydration therapy (ORT) consisting of clean water with salt and glucose (1,141). Addition of amylase-resistant rice starch can further increase the effectiveness of the ORT and shorten the period with watery diarrhea as well as reducing the loss of fluid. It is believed that this effect comes from increased colonic fluid uptake due to increased osmolality from the starch (142). Another possible theory is that rice starch is able to inhibit CT uptake, thus ameliorating the diarrheal

response upon infection. This theory could also explain why treatment with ORT and amylase-resistant rice starch shortens the duration of the diarrhea, as well as the reduction in fluid loss.

In hospitals rehydration can also administered intravenously using Ringer's lactate. Administrations of antibiotics can also be necessary to improve recovery in cases of severe diarrhea, although usage should be limited to avoid emergence of antibiotic resistance. Implementation of these relatively simple treatments has taken the case-fatality down to well below 1%, whereas untreated cholera can have a mortality rates of 50% or more (1,4).

As the case fatality rate is so low in hospitals it is challenging to further improve the survival rate in that setting. Treatment could however be improved from a morbidity-point, by trying to decrease the fluid loss or shortening the time with acute diarrhea. For such an endeavor a CT-binding agent might be employed to inhibit cellular uptake of CT. One aim of this thesis was focused on evaluating such inhibitors in various model systems of cholera. These studies were intended as a first step towards the possibility of an additive in ORT to reduce morbidity. Previous trials using immobilized GM1 on charcoal showed some promise in this regard but that particular method proved too inefficient (143).

# GLYCOSYLATION

A large number of pathogens and toxins are dependent on host glycans for binding, and CT is no exception. It is therefore key for understanding CT to know the basic mechanisms and patterns of expression with regards to cellular glycosylation. This section will aim at creating a basic understanding for glycosylation in mammals.

Glycosylation can be described as a one of several post-translational alterations to proteins, enabling a much greater diversity than the unmodified proteome would be able to achieve. Glycosylation is often more long-lived in nature compared to other modifications such as phosphorylation and often last for the majority of the protein lifetime. As the name implies glycosylation refers to the addition of sugars to a protein (or lipid discussed below). The main types of sugars used for glycosylation in mammals are mannose, glucose and galactose and the amino sugars N-acetyl-glucosamine (GlcNAc) or N-acetyl-galactosamine (GalNAc). These sugars are commonly used to build up chains of sugars called glycans (144-146). The Glucose-Galactose-based glycans are often further modified with additional sugars such as sialic acid or fucose. Sialic acid is clearly different from the other sugars by having 9 instead of 6 carbons and in non-human mammals comes in both Ac- and Gc-forms (147). Fucose is also somewhat special since it only occurs naturally in the steric L-form, as opposed to the other sugars that have the D-form. Other types of glycans also exists based around sugars like hyaluronic acid or those consisting of just one sugar like O-GlcNAc-modifications, but these will not be further discussed in this thesis (144,145).

Glycosylation is carried out by enzymes called glycosyltransferases, which link sugars via hydroxyl groups on sugar backbone carbons. Most glycosyltransferases specifically join exact carbons of two specific sugars adding an important layer of specificity in the glycan synthesis. Furthermore, glycosyltransferases also have specificity for the type of glycan chain it can act upon so that only the intended glycans are further modified. However, several glycosyltransferases might be able to modify the same substrate glycan chain. Therefore, there is a competition in the process of building complex glycans dependent on the respective affinities for the substrates and the efficiency of modification. This commonly leads to a mix of end products, resulting in varying degrees of completion for complex glycans. As there is no template

for glycosylation, as there is for protein synthesis, this is expected (145,146). In addition, external and internal physical, chemical and enzymatic onslaught can alter the finished glycans over the lifetime of the glycoprotein. Asides from being attached to proteins, glycans can also be secreted to act as food for commensal bacteria or decoy receptors and barriers against pathogens.

## *N*- AND *O*-LINKED GLYCOSYLATION

As mentioned above CT relies on binding to glycans for internalization and intoxication. Glycans are linked to proteins via the terminal nitrogen or oxygen on amino acid side chains. Nitrogen-linked glycans or oligosaccharides are only performed on asparagine and is called *N*-linked glycosylation. *O*-linked glycosylation as the name implies is performed on the terminal oxygen of serine or threonine (146,148). The enzymes required for *N*- and *O*-linked glycosylation are very different and will result in different types of glycans.

*N*-linked glycosylation always starts in the endoplasmic reticulum by attaching a prefabricated oligosaccharide with two core N-acetylglucosamines with nine mannoses and three glucoses (Fig 3A). Three glucoses and one mannose moieties are quickly cleaved from the oligosaccharide, while the remaining glycoprotein is transported to the Golgi for further trimming of the mannose moieties and extensions of other sugars. *N*-linked oligosaccharides are often divided into three main types: high mannose, complex and hybrid (Fig 3B). High mannose as the name implies mainly contain mannoses and lack further glycosylation. The complex variants have extensively reduced the mannose content and display a wide variety of different sugars usually in a branched fashion. *N*-linked glycosylation exclusively occurs on sites with a consensus sequence (Asn – XXX – Ser/Thr). In contrast, no such sequence has been identified for *O*-linked glycosylation (149). *O*-linked glycosylation is performed one sugar at the time gradually building branched or non-branched glycans.

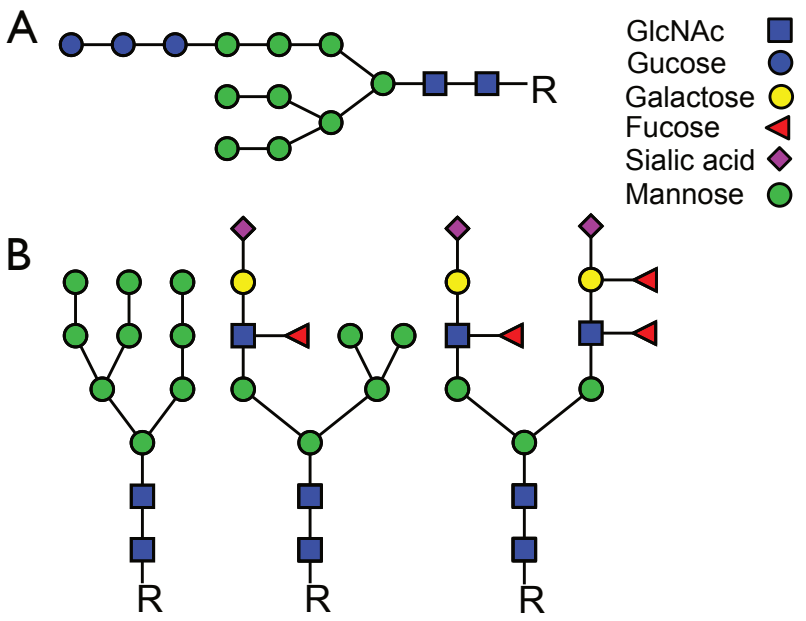


**Figure 3. Mammalian N-glycosylation.** A) Schematic drawing of the start glycan in *N*-glycosylation. B) Examples of the three types of N-glycans; high mannose, hybrid and complex, as seen from left to right. R represents attachment to asparagine on a protein chain.

## LIPID GLYCOSYLATION

The hydrophilic head-group of lipids can also be glycosylated similar to side chains on proteins. As for proteins the various roles that these glycan modifications play are too diverse to fully cover in this introduction. However, the glycolipid GM1 is commonly known to bind CT with a very high affinity and act as a functional receptor for CT. The natural and more constructive role of GM1 in the human body is not fully understood but it has been shown to facilitate neuronal survival, differentiation and proliferation (74). Other glycolipids have been shown to be involved in cellular adhesion and cell signaling. It is not uncommon that glycolipids are present in extracellular fluids and blood group antigen glycolipids are abundantly found in serum. Several glycolipids also acts as toxin receptors and anchors for bacterial adhesion (74,150-152). In this section I will mainly focus on describing the glycosphingolipids (GSLs) since these are of well-documented importance for CT binding and intoxication. All GSL glycosylation starts by

glucose being *O*-linked to a ceramide head group. The GSLs are then further glycosylated to form more complex glycans, either branched or non-branched. An important subclass of GSLs is gangliosides to which GM1 belongs. All gangliosides use the lactose-ceramide as a starting substrate and are made by adding galactose and glucose together with branching sialic acid/s (in most cases). Although CTB has the strongest affinity for GM1, other gangliosides also have a weaker affinity such as GM2 and GD1a to mention a few (54). In the absence of GM1 those other binders might act as receptors for CT.

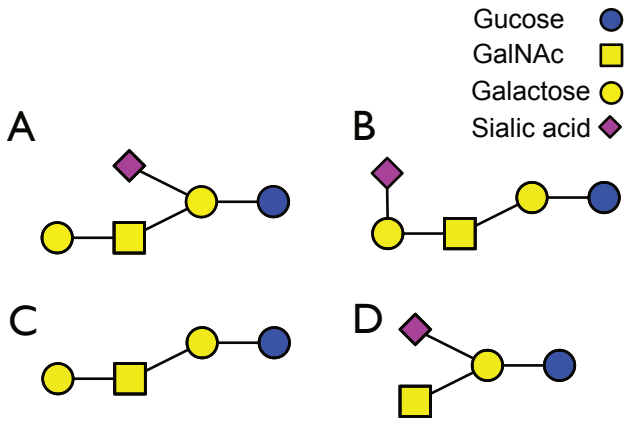


**Figure 4.GM1 and GM1-related gangliosides.** Schematic drawings of A) GM1a, B) GM1b, C) asialo-GM1 and D) GM2. The blue glucose is attached to the ceramide in the cell membrane.

Important to mention is that the "M" in GM1 refers to the fact that this ganglioside is mono-sialylated (as opposed to "D "and "T" referring to di- and tri-sialylation) of which there are two forms GM1a and GM1b (Fig 4A-B). GM1b has a sialic acid on the terminal galactose and does not bind well to CTB whereas GM1a has the sialic acid on the core galactose and binds strongly to CTB. When GM1 is mentioned in this text it infers to GM1a unless otherwise stated. As mentioned above the CTB-GM1 interaction is highly dependent on the two-finger grip provided by the terminal galactose (the index finger) and the core sialic acid (the thumb) shown by the fact that asialo-GM1 and GM2 (Fig 4C-D) have severely impaired ability to bind CTB. The nature of this binding was first elucidated in a crystal structure of CTB in complex with GM1. From the structure it is also clear why the sugar

orientation in GM1b is not able to mediate sufficient binding for CTB, since the terminal placement of sialic acid would disable the two finger grip (Fig1) (53,153).

## FUCOSYLATION AND HBGA SYNTHESIS

Fucosylated glycans are common in the human intestine, especially in the form of histo blood group antigens (HBGAs) (154). Interestingly, blood group O expression has been implied to increase sensitivity to cholera (17,155). Addition of fucose to glycans is performed by fucosyltransferases (FUTs) using GDP-L-fucose as donor. In humans thirteen FUT genes have so far been identified (156,157).

The known human FUTs catalyze $\alpha$(1,2)-, $\alpha$(1,3/4)- and $\alpha$(1,6)-fucosylation. The FUTs responsible for making $\alpha$(1,2)- and $\alpha$(1,3/4)-linked fucose do so terminally or sub-terminally on substrate glycans, whereas $\alpha$(1,6)-FUTs catalyzes core fucosylation at the innermost moiety of *N*-glycans. Core fucosylation is crucial for antibody function as it regulates antibody interaction with complement and Fc-receptors, but does not generate CT-binding glycans (156,158). The alpha-linkage is formed between two hydroxyl groups from carbons of the same spatial orientation resulting in a same-side, bond as opposed to the beta-linkage where the bond reaches from one side of sugar 1 to the other side of sugar 2. This enables beta-linkage to produce straight chains of sugars like those seen in cellulose, whereas alpha-linkage chains results in a spiral shape (159).

Terminal and sub-terminal FUTs are crucial for making HBGAs like the ABO- and Lewis-system glycans. Fucosyltransferase 2 (FUT2) is responsible for adding terminal 1,2 fucose on terminal LacNAc forming a blood group O glycan. Blood group O then acts as the precursor for blood group A and B glycans (Fig5) (160). FUT2 alleles have a high degree of diversity in humans and dysfunctional variants, and about 20% of the European population are so called non-secretors (161,162). FUT2, also call the secretor gene, is expressed in all mucosal tissues (160). Secretor negative individuals does not lack secreted glycans, as the name would suggest, but lack more complex LacNAc-based structures than Lewis A and Lewis X on core 1 and 2 chains

(160). Normally the FUT2 enzyme will be able to act upon virtually all LacNAc chains and thus inhibit the formation on Lewis X and A, and instead the most common types found in a blood group O positive individual are Lexis Y and B (Fig 5). The LacNAc chains may also only go down the path of making blood group A and B (160). This is done by adding a terminal galactose or GalNAc respectively, inhibiting further addition of sub-terminal fucose. However, Lewis Y and B may be acted upon by the blood group A and B enzymes resulting in ALewis Y/B or BLewis Y/B (Fig 5) (160).

The Lewis system glycans can be made from two forms of LacNAc called type 1 and 2 chains. Type 1 chains have a 1-3 linkage between the galactose and the GlcNAc whereas type 2 has a 1-4 linkage. This might seem like a small difference but has a dramatic impact on how the sugars are presented sterically. By default, a type 1 chain can only accept a subterminal (attached to the GlcNAc) fucose in a 1-4 linkage and this then becomes Lewis A, or Lewis B if a terminal fucose (attached to the galactose) was already attached. The same is true for fucosylation of type 2 chains but then the products are Lewis X and Y, which are the enantiomer counterpart to Lewis A and B respectively (163-165).

In the process of making HBGAs several FUTs are involved and they all have slightly different preferences for substrates. FUT7 and 9 can also fill the role of FUT3 in making Lewis Y/X, primarily acting on terminal LacNAc whereas FUT3 can act also on subterminal LacNAc. FUT7 only has the ability to fucosylate sialylated LacNAc, enabling further diversity but also regulation at the protein level of the expressed glycome (165,166). Therefore, the sialylated version of Lewis X is closely linked to FUT7 expression and detected in high amount on neutrophils. Sialylated Lewis X is also expressed at a low level in the human small intestine but other HBGA are more abundant compared to GM1 (Fig 5) (157,163,165,167). In fact, expression of GM1 in human small intestinal cells is very low (168). In addition, there is, as mentioned above, a strong correlation between blood group O and severity of cholera disease symptoms in humans. Hence, although GM1 is by far the most effective receptor for CT the HBGAs may be of importance due to the abundance in the small intestine. A schematic representation of HBGAs relevant to binding to CT can be seen in figure 5.

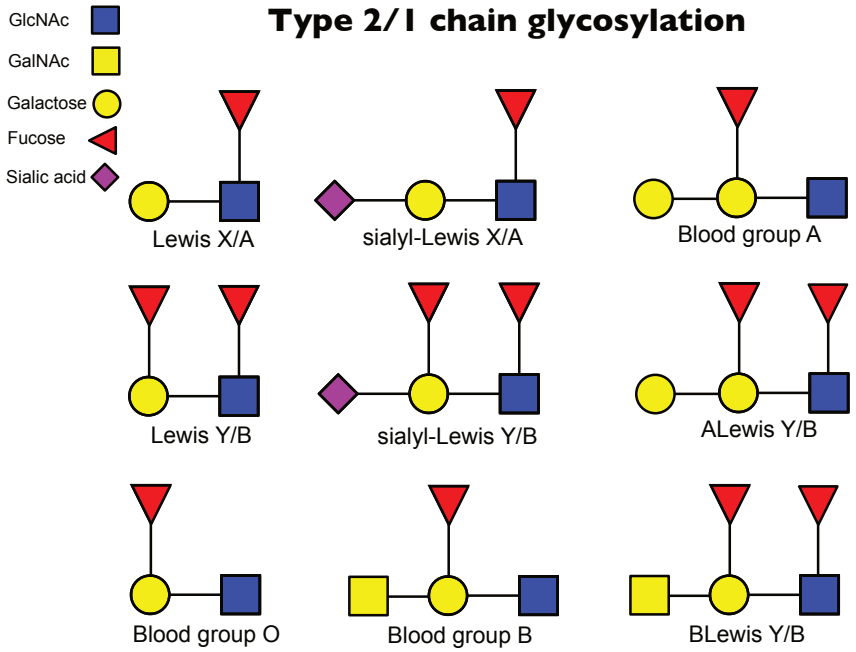


**Figure 5. Common intestinal HBGAs.** Schematic drawings of Lewis and ABO-antigens found in the human intestine. The difference between core 2 and 1 is the beta1-4 or beta1-3 linkage between the galactose and GlcNAc forcing the GlcNAc-liked fucose into the alpha1-3 or alpha1-4 linkage respectively.

## GLYCOSYLATION IN THE INTESTINE

As on all cells in the human body the intestinal barrier cells are heavily glycosylated. On the apical side several membrane-bound mucins are located effectively creating a physical barrier for the cells in addition to the secreted mucin layer discussed below (169,170). Both lipids and proteins are glycosylated with a broad spectrum of glycans serving multiple functions (171). Some glycans or glycoproteins, such as mucins, are also secreted into the intestinal lumen. All forms of glycans play different roles but in general they add an extra layer of complexity and diversity to the proteome and lipidome. This fact enables cells to deeply specialize and efficiently manage protein and lipid functions (171). More concrete examples of functions related to glycosylation are half-life extension, conferred by steric hindrance for

enzymes or glycans acting as the ligand in cell surface receptor interactions (146).

In the human intestine a significant proportion of the glycans contain fucose, and HBGAs are abundantly expressed (172-176). Several symbionts have adapted to this and carry fucosidases to efficiently extract nutrition from host-produced glycans, or fucose sensing proteins to adapt to the host milieu upon infection (177,178). This intricate and complicated interplay between microorganisms and the human host depend significantly on IL-22 production by immune cells sensing the bacterial presence and composition. IL-22 is then sensed by the epithelial cells and induces the production of more fucosylated structures (179). The commensal bacteria also seem to be able to influence the glycosylation via secreted metabolites sensed by the host (174).

This enables a crosstalk between organisms without the need for physical contact. This mode of communication is necessary since direct bacterial contact with the epithelium and the underlying immune cells would most likely trigger a severe immune response (35,180,181). To this end the mucus layer in the small intestine acts as an accumulation zone close to the crypts for antimicrobial peptides and IgA. This accumulation prevents bacteria reaching the epithelium but allows the mucus to be porous enough for efficient nutritional uptake (180). This highlights that the immune system, epithelium and microbiota all actively interact to maintain a delicate balance beneficial for all parties (174,181).

## MUCINS

The whole gastrointestinal tract is lined with a mucus layer protecting the underlying cells from dehydration and the environment. The mucus in the small intestine is mainly made up of Muc2 and is differentially organized in the different parts of the intestine (180). Colon has an inner and outer mucus layer where the inner layer is firmly attached to the cells, while the outer layer is more loosely packed and is more permeable to bacteria. This two-layer system ensures that the epithelial layer is protected from direct contact with the extensive colonic flora. The small intestine has a much lower bacterial burden and only produces a mucus layer corresponding to the

colonic outer layer (182). The mucus layer is constantly replenished via Muc2 release from goblet cells. Muc2 is heavily glycosylated with about 80% of the total weight from the added glycans (183,184). Muc2 is expressed in a gradient from duodenum, with lowest level, to colon with the highest (180,185). The high density of glycans on Muc2 enables the binding of a lot of water effectively forming a protective gel. Muc2 is so large that it is polymerized and folded inside the goblet cells until release. When secreted the polymer quickly expends forming a net-like sheet with a diameter several times bigger that the goblet cell (183,186). Although Muc2 has *N*-glycosylation the vast majority of the glycans are *O*-linked and several of the glycan chains include HBGA moieties (183,184,187). As mentioned above the *O*-glycans fill several functions like protecting the protein backbone and binding water to create the gel texture of mucus. Another function is that the glycans provide nutrition for commensal bacteria, with glycosylation being a result of crosstalk between the host and commensals (184,188). Muc2 also acts as a physical barrier and habitat for commensal bacteria having the potential, together with other secreted glycoproteins like IgA, to modulate colonization and to inhibit harmful bacterial and toxin binding to epithelial cells. As discussed below, glycans in human breast milk can also fill this function, likely acting in synergy with secreted maternal IgA antibodies to prevent infection and intoxication in infants.

## FUCOSYLATION IN THE IMMUNE SYSTEM

Many glycan structures important for cellular interactions contain fucose such as fucosylated glycans interacting with selectins. Selectins catch and enable rolling of leukocytes on endothelial surfaces in inflamed areas, leading to extravasation of immune cells from blood into the tissue (189,190). The selectins primarily interact with sialyl-Lewis X on glycoproteins such as CD44, where the relatively low affinity between glycan and protein allows for rolling to occur slowing down the leukocytes traveling in the blood stream without damage. Although cell adhesion to endothelium is the most studied function of fucosylation, it also plays a key role in other areas such as in the differentiation and linage plasticity of a several of immune cells. One example is core fucosylation in the Fc domain, which is important for the function of antibodies in antibody-dependent cell-mediated cytotoxicity (163).

CD44 expression is not limited to classical immune cells but is also expressed on stem cells in the intestine. In fact, the Kohler group recently published a paper showing an interaction between CTB and CD44(191). Mass spectrometry analysis of eluates from CTB pull-down experiments indicated that CD44 could act as a receptor in the colonic cell line T84. Further immunoprecipitation experiments confirmed an association between CD44 and CTB (191). Another promising CTB-binding candidate from that paper was CEACAM5 (also called CD66e), which has also been shown to have Lewis X glycans attached (192). As for CD44, CEACAM5 was proven to associate with CTB.

## GLYCANS IN HUMAN MILK AS TOXIN INHIBITORS

As discussed above, human breast milk contains large amount of IgA antibodies effectively protecting infants and breast feeding children from various pathogens such as *V. cholerae* (127-129). Brest milk is also protective for a different reason, namely that formula has to be mixed with water, and if not sterilized properly this water might harbor pathogens. Breast milk also contains several types of molecules other than antibodies that might affect pathogens, such as glycans. Therefore it is hard to assess the impact of maternal antibodies alone (193-196). However, association studies can be performed between antibody levels and risk of disease (128,129). Such studies cannot confirm any hypothesis but together with other functional studies IgA is proven to prevent infection and intoxication (197,198).

As stated above fucose plays a major role in bacterial colonization of the large and small intestine. Human milk contains a lot of different oligosaccharides (HMOs) that are fucosylated to a large degree (199). The individual HMO composition correlates with the intestinal microbiota in breastfeeding infants and clear differences in colonization can also be seen compared to formula-fed infants (194,200). The HMO composition also seems to affect the microbiota of the milk itself influencing the infant gut colonization (200,201). Although heterogeneously expressed HMOs are also able to bind to several virulence factors of pathogenic bacteria and viruses, potentially inhibiting those from infecting infants (190,195,196,199,202,203). Most formulas are based around cow milk, which contain few fucosylated oligosaccharides. The

addition of 1-2 linked fucosyllactose (2'FL), the major fucosylated HMO, to regular formula significantly normalized the levels of pro-inflammatory cytokines seen in formula feed infants compared to breastfed (190,203). Another example of the immune systems impact is the direct link seen between the dendritic cell marker DC-SIGN and HMOs, where fucosylated HMOs effectively protecting dendritic cells from infection by inhibiting pathogen adhesion to DC-SIGN (204). This suggests that the fucosylated HMOs not only modulate bacterial colonization and prevent pathogen establishment, but also regulate proinflammatory cytokine release. To complicate this even further the composition of HMOs varies between mothers and non-secretors lack the major HMO of secretors, 2'FL. Instead the major HMO in non-secretors is lacto-N-fucopentose II (LNFP II), where fucose is linked via 1-4 linkage and hence independent of FUT2 (196,199).

All the heterogeneity with regard to HMO composition highlights the impossibility of having an innate mechanism that can confer protection towards all pathogens. In fact, it is likely that some of the HMO glycans can facilitate pathogen infection when attached to lipids and proteins. Humans in different physical or temporal locations are subjected to different floras of pathogens. This would assert varying selection pressure for glycan expression. This theory would explain why humans have such a diverse glycome that also has a geographical/ethnic component (196,205).

Taken together human breast milk likely contains several molecules, like HMOs and IgA, that are effectively promoting normal gut flora as well as inhibiting pathogen colonization and aid in immune system development (194,200,201,204,206). HMOs are also able to bind and thereby reduce the effect of various bacterial toxins and viruses (207-210). This proposed protective mechanism would then fall under the innate immune system conferring some protection against yet not encountered pathogens. HMOs can be viewed as a complement to IgA, but IgA is likely to confer a more direct and effective neutralization of toxins and viruses.

# ALTERNATIVE RECEPTORS FOR CT

Although it is quite clear that GM1 can act as a receptor for CT and will do so efficiently, there are several findings indicating that other receptors might also be involved in CT intoxication. The main findings include a clear association between blood group O carriers and cholera severity in addition to the fact that GM1 is so lowly expressed on the epithelial surface in the human small intestine (16,168,211). I will here attempt to make an overview of previous work showing that CTB also binds other ligands than GM1 and create a context around the development of the "GM1-dogma".

It has long been known that the close relative to CTB, LTB has a more promiscuous binding pattern regarding glycolipids (212-214). LTB was also shown to bind to glycoproteins in the rabbit small intestine. In contrast to LTB, CTB was not shown to bind to proteins. However, this might be explained by the detection method used. Toxin binding to the protein fraction from the rabbit intestine was determined by its ability to block CTB/LTB binding to GM1-coated ELISA plates. This approach would effectively ignore all other possible biding sites on CTB/LTB (213). Later this study was repeated on human intestinal tissue with similar results and again with a strong GM1 bias for detection of protein-CTB/LTB interaction (214). In fact, since the identification of GM1 as a major ligand for CTB most detection of CTB binding has been assessed using GM1 in a unfortunately biased way (33,72,78,215-221). It is therefore clear, all be it in hindsight, that the conclusion that CTB exclusively bind to glycolipids was predicated on experiments that were unlikely to come to another conclusion given the extreme affinity in the CTB-GM1 complex.

Furthermore, recent (and not so recent) papers have shown a GM1-independent binding of CTB to both cells and in biochemical assays (62,63,222). As mentioned above, this binding has primarily been mediated via fucose on HBGAs that can occupy a distinct site from the GM1-site on CTB. This effectively means that there is potential for CTB being bound to both GM1 and HBGAs simultaneously (61-64,207). In fact, the CTB binding to cells has not always been found to correlate with or depend on GM1 or glycolipid expression (58-60). If this GM1-independent binding is always mediated via the noncanonical lateral HBGA-binding site, or if the canonical site is also involved was not elucidated in these publications.

There are several publications over the last few years both indicating and proving that HBGAs specifically bind to CTB (62,63,222). A relationship between CT binding and the A glycan of the ABO-system was identified in the late eighties (223). The same group later showed that the blood group A carrying mucus, but also A-negative could inhibit CT binding (224). Later it was also shown that both LTB and a CTB/LTB hybrid could interact with HBGAs (225-227). More evidence has been published using more defined glycans showing that both classical and El Tor CTB binds Lewis Y and all ABO-glycans (61-63). This HBGA-link also has functional evidence to support it, as human enteroids that are blood group A-positive are better protected against CT induced cAMP than A-negative cells (155). This, and other findings have been put forward as the most plausible explanation for the more severe cholera seen in blood group O individuals (17). A-glycans could be considered as decoy receptors for CT, preventing effective uptake via the canonical pathway for CTA. Due to similarity and experimental binding data, blood group A and B are considered to confer equally good protection, although this has not been experimentally tested in enteroid cultures (62). To date, there is no conclusive data regarding the effect that the Lewis glycans have on CT toxicity and more investigation is needed to fully understand the functional role of ABO-glycans in CT-mediated intoxication.

Given the substantial knowledge today regarding alternative ligands for CTB the scientific community should stop using CTB as a specific marker for GM1. An example of this misuse outside the primary field of cholera is evident in the field of neuroscience. CTB is commonly used as a marker for GM1 in many tissues like CNS. However, Lewis X is also highly expressed in the CNS complicating interpretations of CTB binding in the CNS. What can be said with confidence is that both Lewis X and GM1 readily binds CTB making CTB as a marker for GM1 highly unreliable (64,228-230).

In conclusion, non-GM1 binding of CT has been known for a long time, yet GM1 has not been properly questioned as the sole functional receptor. Therefore, functional experiments investigating the role of HBGAs (and/or other glycans) in CT toxicity would be valuable additions to the otherwise vast pool of knowledge about CT. My hope is that the work in this thesis will shed some more light onto the matter and also further spread the knowledge about alternative receptors for CT.

## ALTERNATIVE SELECTION FOR CTXΦ

A recent paper has described that CT can act as a bactericidal suggesting that CT production can generate a growth advantage for *V. cholerae* during intestinal infection. CT is then able to bind GM1-like glycans expressed by other bacteria such as *Campylobacter jejuni*. Interestingly, the same paper showed that the CTB-binding in the chicken small intestine is partially fucose-dependent and that fucosylated structures together with GM1 make up the CTB-binding potential (231). This important finding highlights the possibility that CT might have been selected for in inter-bacterial competition rather than, or in combination with, the effect that CT clearly has on the host. This new theory could explain why it is beneficial for *V. cholerae* to keep the CTXΦ phage also in the environment. Environmental *V. cholerae* is often found on water dwelling organisms such as shellfish and alga but also insect larva. A possible symbiotic relationship between *V. cholerae* and crustaceans has therefore been proposed (232). The theory was that CT produced in the gills would help the animal to adapt to the change in osmotic pressure when moving into more saline rich water. Meanwhile the bacteria would gain access to larger amounts of nutrition from the surrounding water. Alternatively, the biofilm of the bacteria could protect the shell from degradation in low pH. However, this area needs more investigation. A more likely reason for colonization and symbiosis could be that *V. cholerae* can use chitin as a carbon and nitrogen source, in return forming a pH resistant biofilm around the host shell (21,232,233).

Together these findings suggest a possibility that CT is not only a virulence factor in human infection but also plays a role in the environmental survival of *V. cholerae*. Further investigation into this topic could help us understand why the CTXΦ phage is selected for, not only in the infected human gut, but also in the environment.

# CLASSICAL MODEL SYSTEMS OF CHOLERA

## IN VIVO

One of the major challenges in studying cholera is the fact that *V. cholerae* is an obligatory human pathogen. The main "natural" infection model uses infant mice infected just after birth. The bacteria is then able to colonize the small intestine and induce watery diarrhea similar to that seen in human cholera cases (234). However, for a yet unknown reason, a few days after birth the mice become no longer susceptible to infection.

Other infection models exist for *V. cholerae*. One example is the ligated loop model, where loops of the intestine is surgically exposed and tied of with sutures. The loops are then injected with bacteria to mimic an infection. However, this method raises ethical concerns since animals are often woken from anesthesia after surgery to accommodate for the several hour long incubation before loop harvest. The advantage of this model is the ability to assess the exact amount of fluid secreted in response to CT (90). The loops can also be used to test the CT alone since it will readily elicit a forceful response in most mammals including mice, rabbits, pigs and dogs (88,235-238). Another *in vivo* method is also available where streptomycin resistant *V. cholerae* is used to infect mice treated with streptomycin. The infection is enabled by clearing a niche via the streptomycin challenge and unlike human infection this model is independent of TCP (239).

## CELL LINES

Human cell lines derived from various cancers (primarily colon cancers) have also been used extensively to study the effects of CT. This has the benefit of using tissue from the only species where the disease is observed, which is more practical and better from an ethical perspective. However, cell lines derived from cancers are not always recapitulating the original tissue correctly. In addition, *V. cholerae* primarily infects and acts upon the small intestine, not the large intestine (88,240,241). Patterns of protein expression and (importantly for CT) glycosylation have been known to be quite different in transformed cell lines compared to primary tissue (242-244). It has also been shown that growing cells with different concentrations of glucose greatly

affects glycosylation and protein expression. In fact most cell culture media contains glucose well above the normal blood sugar levels, while it is known that elevated blood sugar has a number of effects (242-244). There are culture mediums with glucose levels corresponding to normal blood sugar levels, but it requires more frequent media change due to nutritional depletion.

The cell lines also fail to recapitulate the diversity of cell types in the primary tissue. Some are able to differentiate into similar morphology and function as only one or a few cell types of the primary tissue. As an example, confluent Caco2 cells will over time form tight junctions and microvilli and thus closely recapitulate the intestinal enterocyte. However, other cell types such as goblet cells, Paneth cells and enteroendocrine cells are lacking. Using cells for experiments always runs the risk of missing crucial aspects of the primary tissue for all the reasons listed above.

## ENTEROIDS AS AN INTESTINAL MODEL

To combat these general problems, establishment of protocols to culture primary intestinal stem cells have been the priority for several groups. In the past 5-10 years several groups have been able to establish intestinal cell cultures from primary cells. Some have also used pluripotent stem cells and differentiated them into cells very much like intestinal stem cells - here called organoids. Others have isolated crypts from small intestinal biopsies and established stem cell cultures - here called enteroids (245). We have only used enteroids and all further discussion will be regarding such cultures. The idea with these cultures is to recapitulate most of the complexity of the mucosal barrier including several cell types and functions.

Indeed, the cultures show a much more diverse phenotype than cell lines with all the major cell types seen in the primary tissue and a mucus layer etc. (246,247). Using enteroid cultures to model *V. cholerae* infection is most likely as close as we can get to the natural human infection without actually infecting health volunteers. The system also offers the possibility of genome editing to knock out or knock in different glycosylation enzymes and thus study the effect of the glycome in cholera pathogenesis.

Enteroids are not transformed and are therefore very much like primary cells. For them to survive in culture several requirements needs to be met. Firstly a cocktail of growth factors such as epidermal growth factor and noggin needs to be added to the culture media to facilitate stem cell growth and renewal as well as maintenance of other cell types (245). To help the cells remain in a stem cell state the medium also contains the growth factor Wnt3A. By removing the Wnth3A from the medium the cells fully differentiate and stem cells are lost (245,246).

Another requirement is a 3D support in form of a gel for the cells so they can maintain their spatial relationship to each other. The gel might also contain hormones helping the cells to grow properly. The cells form small spheroids in the gel that grow more complex with time resembling miniature intestines with crypt-like spikes. In this culture state the apical side of the cells face the lumen so only the basolateral side is exposed to the outside. To study effects if microbiota and other intestinal exposures the spheroids need to be broken up and seeded in collagen-coated trans-well inserts. In the trans-wells the basolateral side will attach to the collagen and form a monolayer of cells with the apical side exposed for treatment. Several groups have recently used this culture system for both CT challenge and co-culture with macrophages seeded on the basolateral side. Using this system it can be observed that macrophages extend protrusions though the confluent enteroid monolayer in response to an apical bacterial infection and that enteroid cells readily respond to CT (155,246,248,249).

This is only the first step in fully recapitulating the diversity of cells present in the small intestine. To have a species specific model of the intestine would be of immense benefit for researchers both trying to find cures for intestinal diseases, but also for the general understanding of cellular functions such as mucus secretion and nutrient uptake. It would also be uniquely possible to study the full pathogenesis of *V. cholerae* in a co-culture model of enteroids and immune cells.

# AIMS

This thesis has two connected but distinct aims both addressing CT intoxication of the cells in the human intestine. They are based around exploration of noncanonical binding by CT to fucosylated glycans with the goal of understanding how and if these novel receptors act to facilitate CT intoxication or counteract it as decoys.

The specific aims of this thesis are:

- To characterize and evaluate the binding and the functional potential of fucosylated receptors for CT on epithelial cells in human and mouse small intestine. This also includes investigation of the role of the previously described GM1 or GM1-like receptors.

- To evaluate the effectiveness of polymer-linked fucose and/or galactose in inhibiting CT binding to intestinal cells and preventing CT-mediated intoxication.

# MAIN METHODS AND ETHICAL CONSIDERATIONS

Working in a lab focused on intestinal immunity and vaccines, it was natural to use methods like flow cytometry, enzyme-linked immunosorbent assay (ELISA) and animal challenge models. I have used these methods extensively throughout the thesis, but several other methods have also proved very useful. Here I will go through the main methods I have used while studying with CT. I will also give arguments for and against using the methods as well as why they were chosen. Some methods used in the papers will not be discussed here since I did not perform them. One clear example of this is the synthesis and biophysical characterization of the fucose polymers.

## FLOW CYTOMETRY

Without a flow cytometer this entire thesis would not have been possible. It is truly the backbone method of this work. Flow cytometry is in short a tool to investigate the presence and level of expression of any cellular component. Flow cytometry is used primarily for assessing proteins and glycans but also nucleic acids, ions and lipids. This is useful for characterizing cell types and how they are affected by various conditions and treatments. In this thesis I have primarily used flow cytometry to identify cell types and assess the relative binding of CTB to those cells.

A flow cytometry experiment starts by making a single cell suspension of the tissue or cultured cells of interest, to stain the cells with antibodies against various cell markers. The antibodies or other cell binding molecules have covalently liked fluorochromes used for detection in the flow cytometer. The stained cells are aspirated into the flow cytometer where they are made to pass one by one through a set of laser beams. The laser beams excite the fluorochromes and the emitted light is recorded. By using a set of different fluorochromes and light filters the expression of several markers can be assessed on each cell simultaneously. The ability to accurately assess multiple markers for both presence and level of expression is very useful and not possible with many other methods.

A method like fluorescence microscopy will give you similar capabilities but only with a few markers at the same time and has low resolution with regards to expression level. In flow cytometry cells are stained in a single cell suspension increasing the accuracy with regards to expression level compared to fluorescence microscopy. The reason for this is that the suspended cells are completely and homogenously surrounded by the staining liquid. This is not the case for tissue or fixed cells stained on a glass slide for microscope, since the cells are less likely to have equal access to the staining liquid. Due to this a substantial discrepancy in staining intensity is not uncommon.

One of the disadvantages of flow cytometry is that you often have problems with auto fluorescent cells and contamination of dead cells and small debris. This is especially the case for intestinal tissue whereas most peripheral blood mononuclear cells have low and well defined autofluorescence signals. Autofluorescence is not always a problem and can be avoided by not using the fluorochromes most affected for the autofluorescent cells. Using live/dead stains and excluding dead cells in the analysis helps as dying cells will not have an intact cell membrane and unspecific staining of intracellular proteins can occur. Relating to the live/dead-problem is the fact that the cytometer only records something called events or cell-like objects. Events are thought to be mostly cells but there is no clear way for the machine to distinguish cell-like objects from real cells. In this regard microscopy has a distinct advantage in enabling the operator to exclude suspected or confirmed non-cell objects from the subsequent analysis.

All considered the benefits of flow cytometry far outweigh the drawbacks making the choice easy to use it as the backbone method.

## ANIMAL STRAINS

The work in this thesis has only used mice of the commonly used inbred C57 black6/J main stain. These mice are also referred to as wild type (WT) in the following text the particular stain was chosen as it has been extensively used for cholera research in the past. This meant that there were several preexisting models for us to use in evaluating the role of CT and tolerated doses of the toxin were already established. We primarily used animals for *ex*

*vivo* work in determining the binding specificity of CTB under various conditions. We also used mice for *in vivo* toxin challenge experiments both by oral gavage and the ligated loop model. The *in vivo* work is discussed in more detail below.

A knockout (KO) strain of C57 black6/J mice, deficient in synthesis of GM1 and other more complex gangliosides, was also used. This particular strain has a partial deletion of the gene coding for β-1-4GalNAc-transferase. This deletion results in a limitation in ganglioside synthesis to only GM3, GD3 and GT3. Effectively this strain is a powerful control for GM1-independent CT intoxication and binding assessment. On a side note, the "GM1-KO" mice were originally acquired for a different research project but serendipitously lead to the discovery of GM1-independent intoxication. It should be noted that although WT and heterozygous (+/-) mice have been shown to make identical levels of GM1 in neuronal tissues and leukocytes, we could not detect any GM1 in the WT or +/- mouse small intestine by HPLC. This raises the interesting possibility that regarding the expression of GM1 in the intestinal mucosa, WT and KO mice could be very similar if not identical.

One can make a strong argument for using more strains of inbred and also outbred mice to validate the data collected from C57 black6/J mice. A more diverse set of inbred strains would likely eliminate any inherent bias in relation to CT response and receptor expression that the C57 black6/J stain might harbor. Such a bias would be impossible to foresee and could have a tremendous impact. Still there are good reasons for using only one strain as I have done. One is that the KO mice were generated in a C57 black6/J background. This means that if I were to include other strains, I would have to set up laborious and time consuming cross-breeding to transfer the KO gene to the other strains. This would in turn consume time and resources impacting other parts of the project such as collecting data from human primary tissue. From an ethical point this would also force me to use more animals and thereby increase the suffering generated for obtaining my data. The aim of the studies was not to intricately investigate the effect of CT in mice and therefore work related to human tissue got precedence.

## IN VIVO CT CHALLENGE

To efficiently test our hypotheses regarding GM1-independenct intoxication and the ability of our polymers to block the effect of CT we used two variants of *in vivo* CT challenge; ligated loops and oral gavage. Working with live animals is a valuable complement to human cells and primary tissue. The ability to accurately measure the ultimate response to CT, namely fluid secretion, could only be done using animals. For the ligated loop models a slight modification was done to the conventional method by using the proximal section of the small intestine as opposed to the commonly used distal section. This since we noticed a significantly higher response to CT in the proximal sections. The literature also supports this observation in several other animals and it is therefore unclear to me why the conventional way of performing CT challenge in ligated loops in the distal part was established (88,221,240,241,250).

Oral gavage is performed by feeding mice CT in a pH-buffered solution. After a few hours the mice are euthanized and the length and weight of the intestine is recorded to assess fluid accumulation. This is a very effective method, but it has some drawback as discussed below. The other method called ligated loops are in short created by surgically exposing the small intestine and carefully tying off sections that are injected with CT. After several hours under anesthesia the mice are euthanized and the length and weight of the loops are recorded. This is a difficult and time consuming operation but more exact than the oral gavage method. The exactness stems from the fact that all the net generated fluid will stay in the loop and can be accurately recorded. You also have the possibility to visually assess the fluid accumulation during the ongoing experiment to choose an appropriate time span to use. In the oral gavage method, the time becomes a greater issue since you have to terminate the experiment to weigh and measure the intestine. If you do this too early you miss all or part of the response and thereby assay resolution. Similarly, if the response has been ongoing for too long some fluid has already been discharged. Since the release of stool is sporadic and the volume released unclear, the certainty of the fluid measurement in the intestine becomes hard to determine. Both methods are viable options but must be used with the respective drawbacks in mind.

## ELISA

The relatively inexpensive and time efficient ELISA-method has been an invaluable tool for me in mapping the CTB binding to various types of glycans. Working in a group of immunologists it was impossible to overlook the ELISA-method as a tool for assessing CTB binding to receptor candidates. Strictly speaking most assays were not ELISAs since no antibodies were used but the principle was the same.

The main principle in an ELISA is to display receptors as a solid phase so that a liquid phase of ligands can bind and in turn be detected using reporter-tagged antibodies. This is a good way of assessing an antibody response to a vaccine component or the level of a second messenger after treating cells with a novel compound. For my experiments I have primarily used glycoproteins as the solid phase and then horseradish peroxidase (HRP) conjugated to CTB to assess the binding of CTB to various glycans in different modes of display. This reductionistic approach let me in detail study how individual glycans could interact with CTB in a way that in not possible on a cellular level. However, this also meant that I had to use a synthetic and high-density display of the glycans. This is obviously not how the glycans are displayed on the cell surface and it cannot be excluded that CTB binding specificity depend part of the protein or lipid as well as the glycan. Therefore, how the glycan is attached to the carrier protein or lipid can play a major role for the binding. Given those circumstances the resulting data cannot be used to determine the relative affinity for the glycans in relation to each other. The only thing that can be assessed is if the glycan in question binds CTB. A negative results does not mean that CTB does not bind to the glycan when naturally displayed on a cell. The data obtained from ELISA is still invaluable as a clean enough system to prove binding to a specific glycan in a cost- and time-efficient way. Together with other methods this helps create a better picture of binding specificity for CTB.

## HUMAN TISSUE

The main goal for the thesis was to further the knowledge about CT intoxication of humans. To this end it was paramount to use primary tissue from the human small intestine. With regards to assessing CT binding and

intoxication primary tissue must be considered the best available representation of reality, second only to CT-challenge of healthy volunteers. Experiments on humans come with considerable ethical and practical concerns and cannot be justified in this case. To justify such a trial a CT-blocking candidate of substantial potential (as well as subjection to extensive safety testing) would be necessary. Another possibility would be if a huge benefit for cholera patients was likely to be gained by performing the challenge. At no point during my studies did we fulfill any of these criteria. To make the most out of the donated tissue we employed several different methods aimed at understanding CTB binding and the receptor dependence for CT intoxication. In most cases cells were isolated by enzymatically degrading the tissue to analyze CTB biding using flow cytometry. Other more complex methods are described and discussed below.

## USSING CHAMBER

The idea behind an Ussing chamber is that ion transport over a mucosal surface will result in an electrical current. This also makes the method very well suited for assessing the effect of CT and to test the efficacy of CT inhibitors. The mucosa is mounted as a barrier between two chambers containing isotonic solution. By exposing the apical side to CT, the natural *V. cholerae* infection is mimicked and the resulting ion flux can be measured as a current over the mucosal membrane. The current was calculated from the cross-membrane voltage and epithelial resistance. The epithelial resistance, as opposed to the whole mucosal resistance was obtained by using the Ussing pulse method (251). In short, a set of electrical pulses charges up the epithelial cells effectively making them into capacitors. As the applied current is turned off the charge is gradually released. The voltage is measured to enable calculation of the resistance. Normally the Ussing chamber method measures resistance directly but this will include the non-specific resistance of the subepithelial tissue making the measurement less exact. The voltage together with the resistance was then used to calculate the short circuit current. The short circuit current is an indirect measurement of the ongoing ion flux over the tissue membrane. The main drawback with this method is that the cells starts to die within hours, so long term experiments are not possible. In fact, the response time of CT was usually 3-4 hours and the tissue often started

to deteriorate after 5-6 hours. The Ussing chamber was used in paper I to evaluate the ability of different lectins to block CT-induced ion secretion.

The method is very dependent on tissue accessibility and viability. Therefore, data acquisition took a long time and offered little room for titrating CT or the lectin to optimal concentrations. After completing paper I we therefore started to investigate alternative methods.

## CT CHALLENGE OF ENTEROID CULTURES

In paper III we used a variant of the Ussing chamber to overcome the viability restraints. To do this we established cultures of stem cells from human small intestine called enteroids. The enteroids were grown and expanded in a medium with Wnt3A inhibiting stem cell differentiation according to an established protocol. When enough cells had been generated Wnt3A was removed to enable the cells to differentiate into several types of mature types found in the intestine (246). This was confirmed by flow cytometry and fluorescence microscopy showing that several mature cell types were present after differentiation. The cells also showed the same CTB-binding pattern as the primary cells isolated from the corresponding fresh tissue.

Instead of using primary mucosal tissue to separate two chambers, enteroids were grown on membrane-mesh well inserts to complete confluence. The cells were then differentiated and treated apically with CT while the trans-membrane voltage and resistance were continuously recorded. This enabled us to calculate the short circuit current similar to a normal Ussing chamber experiment. However, a normal Ussing chamber apparatus would directly measure the short circuit current. Therefore, our method is less exact and requires taking the cells in and out of the incubator several times during the experiment. The only benefit compared to the Ussing chamber is throughput. With this adapted method there is no limitation in number of samples as opposed to regular Ussing chamber setups that usually only accommodate 4-8 samples at the same time.

# RESULTS AND DISCUSSION

This thesis is based on the work from 2 published papers and a manuscript in preparation. The papers are continuations of the work presented in another paper titled "*Fucosylation and protein glycosylation create functional receptors for cholera toxin*" (191). In the published paper I contributed with a minor part regarding data on CTB binding to primary cells from human colon. Although my contribution to the published paper appear minor, a lot of our own initial method development for the subsequent papers was done when acquiring the data. The paper did have a significant impact in the beginning of my studies but also contains a lot of data from experiments that I had no part in. I also did not contribute substantially to the intellectual process in writing the paper. For these reasons the paper was omitted from my thesis but is still significant enough to be acknowledged.

The main findings from the study mentioned above was that fucosylated protein receptors seemed to play a significant role in CTB binding to various cells (191). Moreover, some of the toxic effect of CT could be related to fucosylated glycans. This gave us a good starting point for further investigation into the role of fucosylated glycans on CT intoxication in the human small intestine. Wands *et al* also identified the glycoproteins CD44 and CD66e as potential receptors for CT giving us a set of markers to investigate for correlation with CTB binding.

# PAPER I - GM1 GANGLIOSIDE-INDEPENDENT INTOXICATION BY CHOLERA TOXIN

This paper is the main paper of the thesis where several important findings were made. It has as strong link to the Wands *et al* 2015 paper and is in large a direct continuation of that work. Paper I mainly focused on characterizing the main CTB binding requirements of various cell types and also to shed some light on how CT binding and intoxication occurs in the human small intestine. We also wanted to evaluate CTB binding to cells with different ABO-blood groups to investigate if the above mentioned link between cholera severity and blood group O could be directly attributed to CTB binding. Finally, we also wanted to evaluate CT intoxication in a GM1-free cell line and a GM1-deficient mouse model as more human-like models of CT intoxication.

## CTB BINDING CORRELATES WITH LEWIS X EXPRESSION

Whole human blood was stained with CTB to investigate binding to cells with different blood group antigens. The rationale for the experiment was to determine if the ABO-blood group of the donor had any effect on CTB binding to red blood cells. Surprisingly CTB binding was hardly detectable on red blood cells regardless of blood type. Instead granulocytes were shown to bind the most CTB by far of all blood cells (Fig 6A-B). This binding correlated with the Lewis X expression and was independent of the ABO-blood group of the donor (Fig 6A). Furthermore, the CTB binding could be blocked with L-fucose, the fucose-binding lectin AAL and the Lewis X glycan in both free form and attached to a protein (Fig 6C). This shows that CTB is able to interact with Lewis X or similar fucosylated structures on primary human cells. We could also show that CTB immunoprecipitated Lewis X-carrying proteins in a granulocyte cell line.

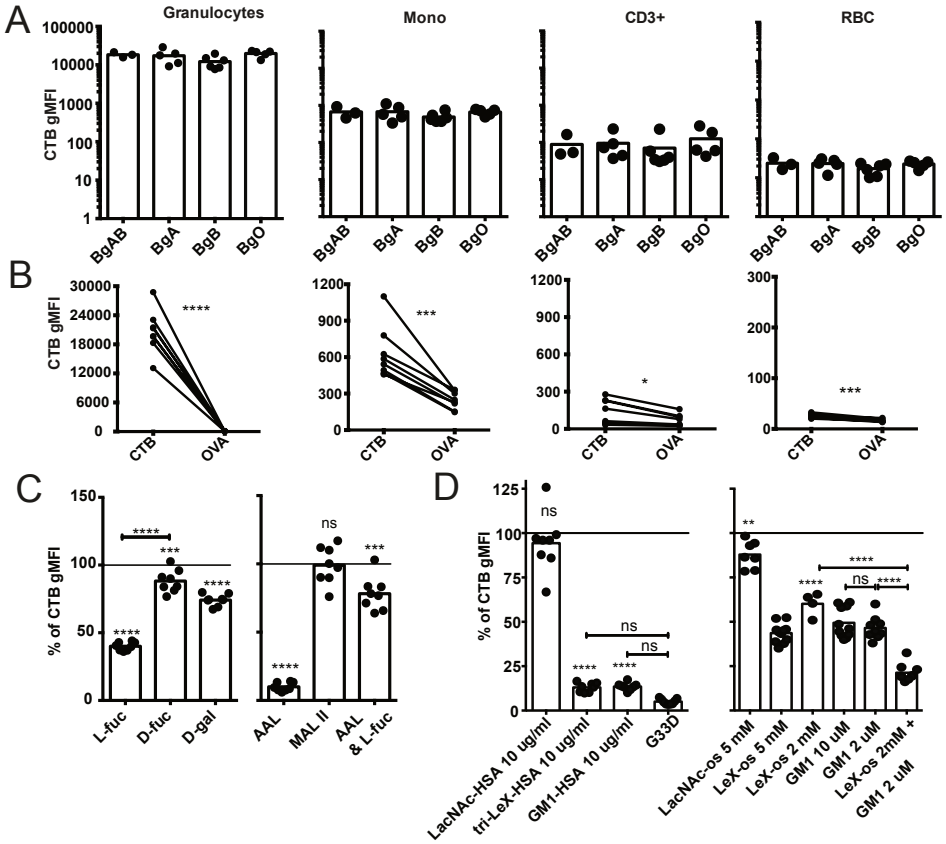


**Figure 6.** ***CTB binds to granulocytes independent of ABO-blood group.*** Whole human blood was stained with CTB and analyzed using flow cytometry. A) CTB binding to blood cell types with donors divided on ABO-blood group. B) CTB binding to blood cells compared to unspecific background, assessed using ovalbumin (OVA). C) CTB was preincubated with sugars or cells were preincubated with lectins before addition of CTB to granulocytes. Bars show % of unblocked CTB gMFI for the different treatments. D) CTB was preincubated with glycans or synthetic glycoproteins before addition of CTB to granulocytes.

The GM1 glycan could also partially block CTB binding to granulocytes but the effect was saturated at about 50 % of CTB binding. By adding Lewis X at a non-saturated concentration together with a saturated concentration of GM1 we observed an increase in CTB block compared to GM1 alone (Fig 6D). This strongly indicates the presence of two different binding sites since GM1 has a much higher affinity for CTB than Lewis X. GM1 could therefore not be outcompeted in the canonical GM1-site. This hypothesis was further

strengthened by data from ELISA experiments showing that Lewis X has no to little effect on CTB binding to GM1 and vice versa (Fig 7A).

A more sensitive variant of ELISA using radiolabeled CTB showed that the CTB-Lewis X interaction was not present when Lewis X was attached to a lipid in the solid phase (Fig 7B). This suggests that CTB-Lewis X interactions are dependent on multimeric display of Lewis X and/or a protein back bone acting as a spacer for the attached glycan. It could also be that the protein backbone actively interacts with CTB together with the glycan. The latter is unlikely for tri-Lewis X HSA since there is 18 mole of tri-Lewis X for each mole of HSA, effectively occluding most of the HSA surface.

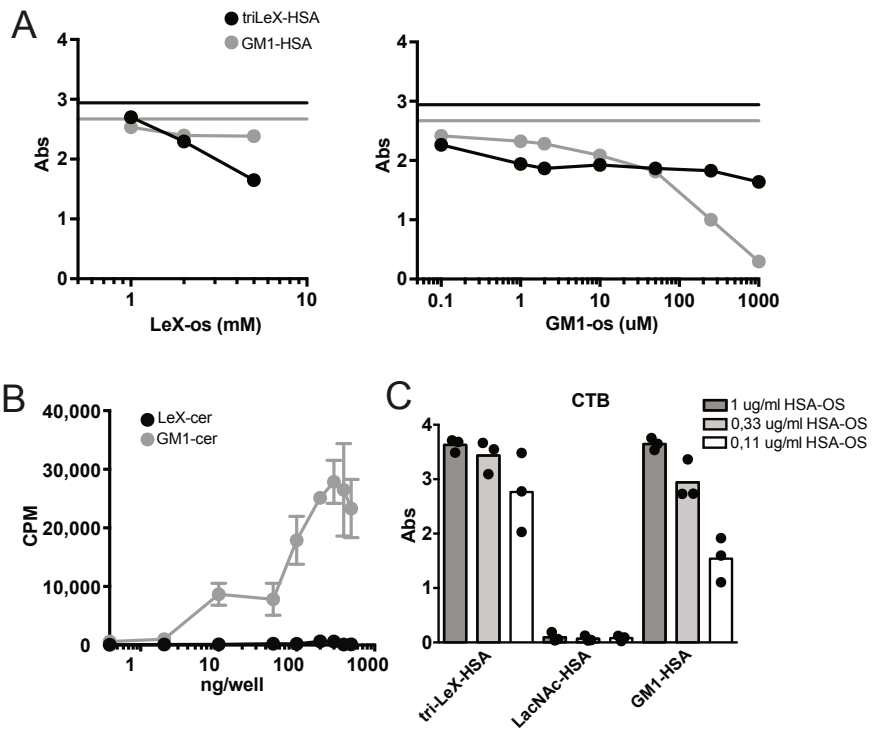


**Figure 7. CTB readily bind to protein- but not ceramide-linked Lewis X.** A) Results from ELISA with HSA-linked Lewis X (left) and GM1(right) as solid phase, detected with CTB-HRP with increasing concentrations of free Lewis X- or GM1-glycans. B) CTB binding to a solid phase of GM1 or Lewis X linked to ceramide. Detection was done by using inodine-125 labeled CTB. Relative binding was assessed from counts per minute (CPM). (C) Results from ELISA with varying concentrations of HSA-linked glycans as solid phase detected with CTB-HRP. Graph shows pooled data from three independent experiments.

## CTB BINDS TO FUCOSE IN THE HUMAN SMALL INTESTINE

The same pattern observed in blood was seen in cells freshly isolated from human small intestine. The epithelial cells bind CTB in a gradient from lowest on the villi to highest in the crypt. The CTB-binding also correlates with Lewis X and is completely fucose dependent (Fig 8A-B). We found that the GM1 glycan could block binding to epithelial cells from some donors but had the opposite effect on others. This phenomenon was not seen for any of the other blockers such L-fucose or Lewis X (Fig 8B). We speculate that this could be due to patient variation of surface glycosylation or glycosylation of mucus. As for the granulocytes the binding could be blocked by the fucose-binding lectin AAL and HSA-linked Lewis X and GM1, but not by the sialic acid-binding lectin MAL II or the galactose binding lectin PNA (Fig 8C-D). LacNAc, that is Lewis X without fucose (pre-Lewis X), did not show any interaction or blocking-capacity of CTB binding (Fig 7C and 8B).

Unexpectedly we could not observe any correlation between CTB binding and the levels of CD44 or CD66e, although both were expressed on the isolated cells and indicated as potential CTB binders in Wands *et al* (191). This indicates that protein glycosylation might differ between cell lines and fresh tissue on the same proteins. Another possibility is that so many other types of receptors were present on the cells that no single glycoprotein could be singled out as correlative.

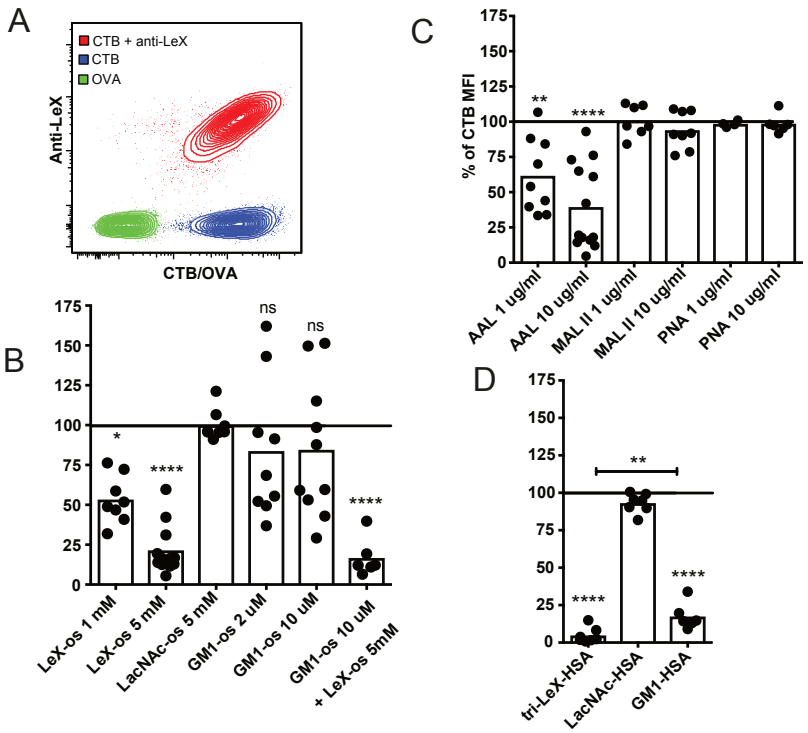


**Figure 8. CTB binding in human small intestine is fucose dependent.** A) Flow cytometry data of CTB and anti-Lewis X binding to EpCAM+ cells from a suspected secretor negative donor. B-D) Bar graphs of gMFI as % of CTB binding to small intestine epithelial cells after treatment with B) glycans, C) lectins and D) HSA-linked glycans. 100% represents CTB staining without any blocking and each dot represents a donor. Significance was calculated using a one-way-ANOVA with Tukey correction compared to CTB without block when not indicated otherwise (**** = p<0,0001, *** = p<0,005, ** = p<0,01 and * = p<0,05).

## GM1-INDEPENDENT INTOXICATION IN MICE AND CELL LINES

To confirm the findings of CTB binding functionally, we first used a KO mouse strain lacking all complex gangliosides including GM1 and challenged them orally with CT. The KO mice had a stronger response to CT than WT littermates (Fig 9A). This shows that CT *in vivo* can intoxicate cells devoid of GM1. We could also show that the WT mice lacked detectable levels of GM1 specifically in the small intestine, but that asialo-GM1 was present at a relatively high level (Fig 9B). Potentially asialo-GM1 acts as a decoy receptor for CT in wild type mice effectively protecting them from the more severe response seen in KO mice. Unexpectedly primary mouse epithelial cells almost exclusively depend on galactose for CTB-binding and AAL or Lewis X

has no to little blocking effect (Fig 9C). Furthermore, we could show that the GM1-negative rat cell line C6 was rendered more sensitive to CT when treated with the glycolipid synthesis inhibitor NB-DGJ. This was also true for the sialylation inhibitor 3Fax-Neu5Ac (Fig 9D). The increased sensitivity to CT after 3Fax-Neu5Ac is likely due to elevated levels of terminal galactose. Elevated levels of terminal galactose was confirmed by PNA stain (Fig 9E). This again shows that CT intoxication can occur in a GM1-independent way.

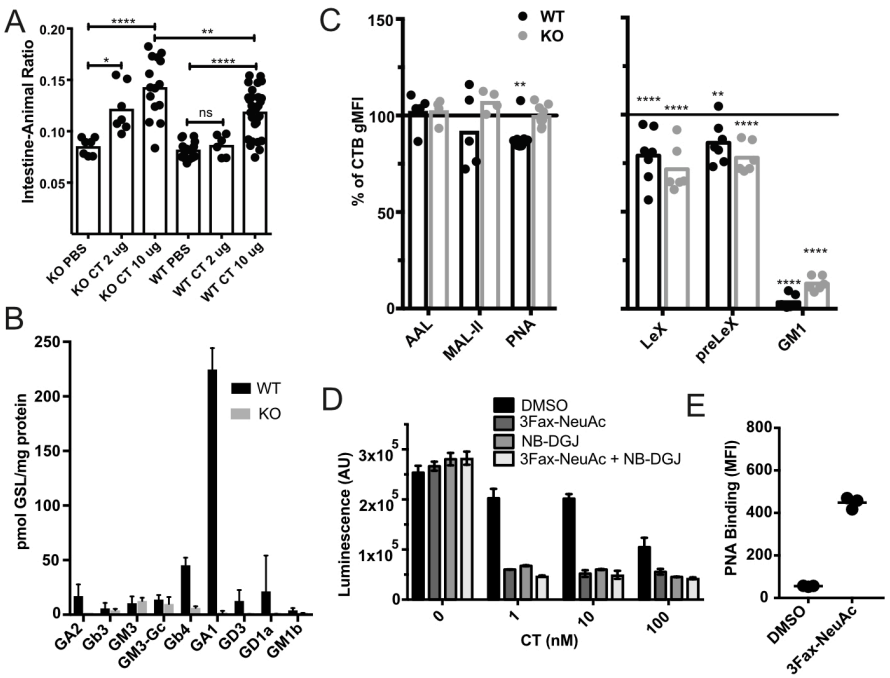


**Figure 9. GM1-independent intoxication both in vivo and in vitro.** A) Graph on intestine-animal weight ratio for mice gavaged with PBS solution with or without CT. The data is pooled from several experiments and each dot represents one animal. One-way-ANOVA with Tukey correction was used to calculate significance (**** = p<0,0001, *** = p<0,005, ** = p<0,01 and * = p<0,05). B) Graph on the concentration of all GSLs in middle section in murine small intestine. C) Bar graphs on % of CTB binding to small intestine epithelial cells (WT black and KO gray) following pretreatment of the cells with lectins or CTB with glycans. 100% represents CTB staining without blocking. D) C6 cells were exposed to CT for 1 h after which accumulated cAMP was measured. The signal is inversely correlated to cellular cAMP levels. E) C6 cells were stained with PNA and binding was assessed by flow cytometry.

## CT INTOXICATION IS LIKELY DEPENDENT ON FUCOSE AND GALACTOSE IN THE HUMAN SMALL INTESTINE

To test if the fucosylated CTB-binders could act as functional receptors in the human intestine, we utilized the fucose binding lectin AAL to block fucosylated glycans on fresh jejunal tissue. The response to CT was measured in an Ussing chamber showing that AAL could completely inhibit the ion secretion induced by CT. This was also true for the galactose-binding lectin PNA (Fig 10A). For this reason, it was hard for us to conclude if galactose and/or fucose were acting as functional receptors. It is possible that PNA and AAL can bind to the same glycan when both galactose and fucose are presented terminally on different branches.

The argument can be made that fucosyl-GM1 could play an active role in CT intoxication, potentially acting as a ligand for both the canonical and the noncanonical, fucose-dependent, sites. However, to my knowledge the presence of fucosly-GM1 has not been shown in the small intestine of humans. In fact, only very low levels of regular GM1 can be detected (168). In a recent publication it was also shown by X-ray crystallography that fucosly-GM1 interact primarily with the canonical site on CTB and not the fucose-binding one (252).

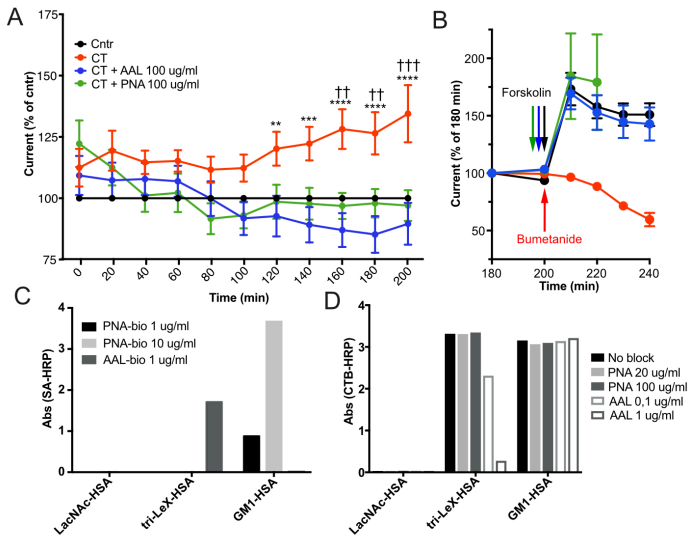


**Figure 10. Inhibition of CT intoxication by pretreating jejunal tissue with AAL and PNA.** Human jejunal mucosae was pre-incubated with or without AAL or PNA before

mounting in Ussing chamber and exposure to CT. A) Graph on percent difference of current in CT treated tissue compared to current of untreated tissue over time. Each dot represents a mean (4–7 donors tested in duplicates). Two-way-ANOVA with Tukey correction was used to calculated significance (compared to the CT only). * represent CT to CT + AAL comparison and † represent CT to CT + PNA comparison (**** = $p<0.0001$ and ** = $p<0.01$). (B) Graph on percent of start current for jejunal mucosae at 180 min. The tissue were exposed to forskolin (or forskolin analog NKH477) or bumetanide. Each dot represents a mean of 2–3 donors tested in duplicates. C-D) ELISA with as solid phase of HSA-linked glycans, detected with biotinylated lectins and streptavidin-HRP C) or blocked with lectins and probed with CTB-HRP D).

By using a lectin that binds directly to cells we might also introduce a lectin-dependent effect on the cells. It is possible that AAL and/or PNA have an effect opposite to that of CT and therefore falsely indicating an inhibition of CT intoxication. Controls using stimulatory chemicals were preformed and verified that the tissues were still viable and able to respond with the signal pathway induced by CT (Fig 10B). However, the controls cannot fully exclude the possibility of the abovementioned lectin effects. It can also be argued that PNA binds to GM1 and thereby inhibits CTB binding (Fig 10C). We therefore performed an ELISA with GM1-HSA as the solid phase. PNA as well as AAL was not able to inhibit CTB binding to GM1 (Fig 10D). We therefore conclude that the lectins are not affecting the canonical CT-GM1 binding and intoxication pathway.

## CONCLUSION

Paper I identifies that the majority of the CTB binding to primary intestinal epithelia cells is fucose-dependent and that fucose seems to be involved in the intoxication by CT. It also shows that GM1 and Lewis X binding occurred on at least two distinct sites on CTB. Moreover, we could show a GM1-independent intoxication by CT both *in vivo* in mice and in a rat cell line. Taken together this proves an alternative intoxication route complementary to GM1 in non-human mammals and the data suggests that this could also occur in humans. Finally, we present data indicating that fucose and non-GM1 galactose play a significant role in intoxication of human small intestinal tissue.

# PAPER II - FUCOSYLATED MOLECULES COMPETITIVELY INTERFERE WITH CHOLERA TOXIN BINDING TO HOST CELLS

Paper II focused on understanding the exact molecular properties and 3D-display necessary for biding to the noncanonical site on CTB and was done in tight collaboration with Jennifer Kohler's lab at University of Texas Southwestern. In addition, we were also interested in the effects that fucosylated and sialylated HBGA- and HMOs have on CTB-binding to various cell types. Therefore, a collaboration with Nicole Sampson's lab at Stony Brook University, New York was also initiated. The Sampson synthesized polymer chains with fucose and glucose attached to them. This enabled us to evaluate if synthetic blockers could inhibit CTB-binding and thereby the possibility to use them for intoxication inhibition. This ties in to the second aim of this thesis evaluating the possibility of developing a novel type of CT-blocking agent not based around the GM1 binding site.

## STERICAL DEPENDENCE OF HYDROXYL GROUPS FOR FUCOSE BINDING TO CTB

L-fucose readily blocks CTB binding to both Colo205 and T84 cells and is the superior blocker compared to both the enantiomer D-fucose and a diastereomer 6-deoxy-D-glucose. This is in line with the findings in paper I where D-fucose was found to be a far inferior blocker of CTB-binding (253). This underlines the specificity of the noncanonical site on CTB even though it has far lower affinity than the canonical site.

Using a structure-activity relationship approach for modifying the hydroxyl groups on L-fucose we could show that carbons 1, 4 and 6 are of great importance for CTB-binding. For the hydroxyl group on carbon 1 it is important to have it in an alpha-orientation for CTB binding. The hydroxyl group oxygen on carbon 4 seems to interact with CTB according to our data. Finally, we conclude that carbon 6 along with the hydroxyl group is also of importance for L-fucose-mediated blocking of CTB binding to cells (Fig 11A-D). The other 3 carbons were less sensitive to modification and we could not detect any shift in blocking capacity for these other "fucose variants".

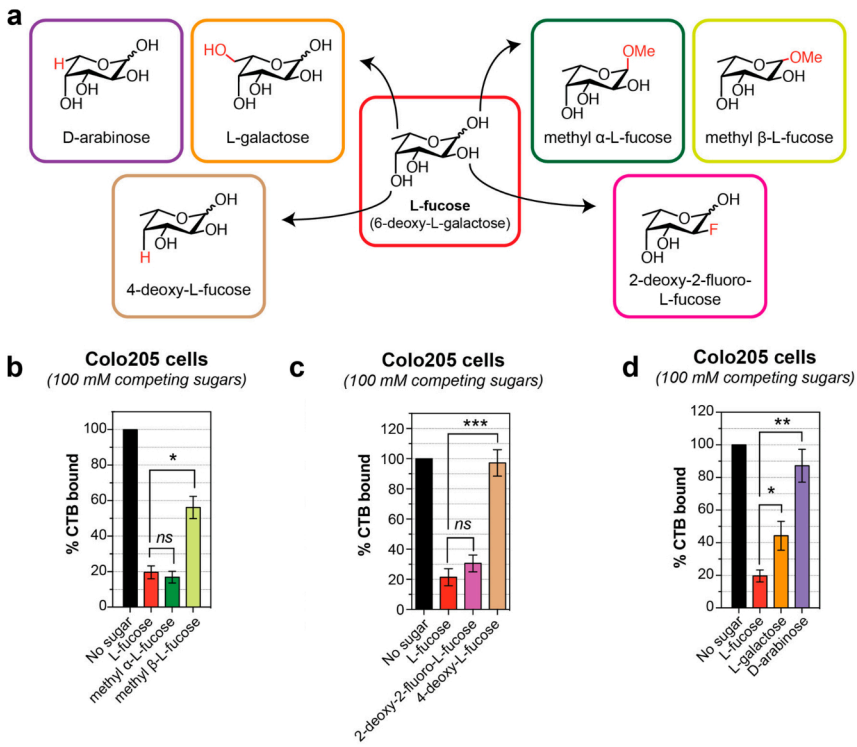


**Figure 11. Analysis of the importance of fucose hydroxyl groups for CTB binding.** A) Schematic drawing of the L-fucose analogues used in this study. B-D) Inhibition of CTB binding to Colo205 cells assessed by flow cytometry. Analogues of L-fucose varying at carbon 1 B), 2 and 4 C), and 5 and 6 D) (n = 3). Statistical significance was calculated using unpaired Welch's test: *** p value < 0.001, ** p value < 0.01, and * p value < 0.05.

## HMOS BLOCK BINDING OF CTB TO COLONIC CELL LINES

All above-mentioned experiments were made using mono-sugars but in the intestine CT would be interacting with complex glycans. As the next step we decided to use HMO-derived lactose with L-fucose or sialic acid additions. We choose to work with HMOs since they had been implicated in protection against diarrhea in breastfed infants (254,255). Lactose alone had a significant ability, albeit lower than L-fucose, to block CTB binding to Colo205, but less so to T84 cells (Fig 12A-C). The addition of L-fucose via an alpha1-2 linkage to the galactose in lactose (2´-FL) significantly increased the block of CTB binding compared to L-fucose alone. 2´-FL is present in high concentration in breast milk from secretor-mothers (2´-FL synthesis requires functional a

FUT2 gene) and is therefore not unlikely to affect CT intoxication in breast fed infants (196). However, this remains to be proven in functional studies.

Surprisingly, the Lewis X-like 3´-FL (lactose with L-fucose in 1-3 linkage to glucose) did not show better blocking efficacy than L-fucose alone. When fucose was exchanged for sialic acid in alpha2-3 and 2-6 linkage on the lactose backbone all CTB blocking effect was mitigated (Fig 12A-C). This suggests that the sialic acid pocket in the canonical GM1-site was not important for blocking CTB binding to Colo205 or T84 cells. GM1 on the other hand could effectively block CTB binding to Colo205 cells but only partially to T84 cells indicating a major galactose-dependence for CTB-binding to Colo205 cells.

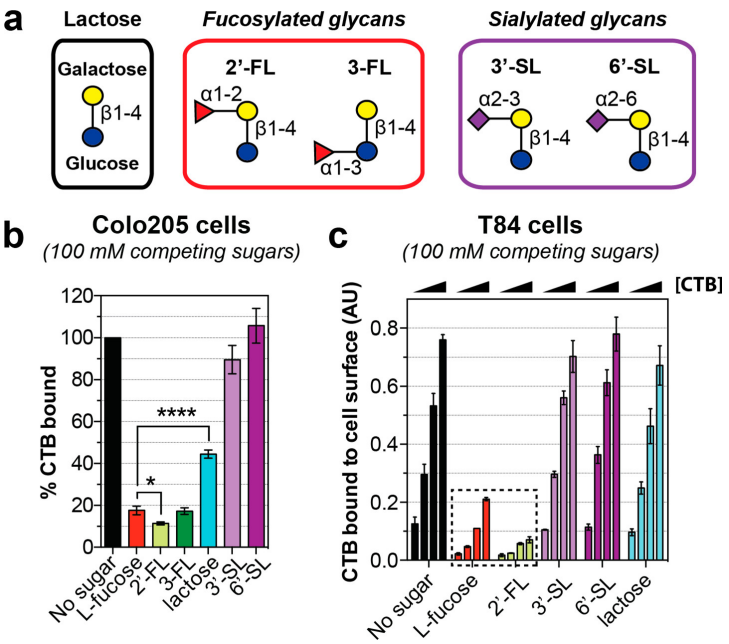


**Figure 12. HMOs can inhibit CTB binding to cell lines.** A) Schematic drawing of the HMOs used. B) Inhibition of CTB binding to Colo205 cells assessed using flow cytometry and related to unblocked CTB binding. Statistical significance calculated using unpaired Welch's test: **** p value < 0.0001 and * p value < 0.05. C) Inhibition of CTB binding to T84 cells was assessed using in-cell ELISA.

## LEWIS Y INHIBITS CTB-BINDING IN HUMAN SMALL INTESTINE

To further investigate if more complex glycans would better inhibit CTB binding, we took a stepwise approach going from 2´-FL to the more complex A/BLewis-Y glycans. All intermediate glycans were tested for inhibitory

properties against CTB and a schematic drawing can be seen in figure 13A. We observed a dramatic improvement in inhibition efficacy for Lewis Y and this effect was not further improved but maintained in A- and BLewis Y (Fig 13B). Further titration of Lewis Y and sialyl-Lewis X showed that both bifurcated glycans had a far better ability to block CTB binding than L-fucose and 2´FL (Fig 13C-D). This suggests that sialic acid is not always detrimental and might aid in glycan binding to CTB when oriented correctly.

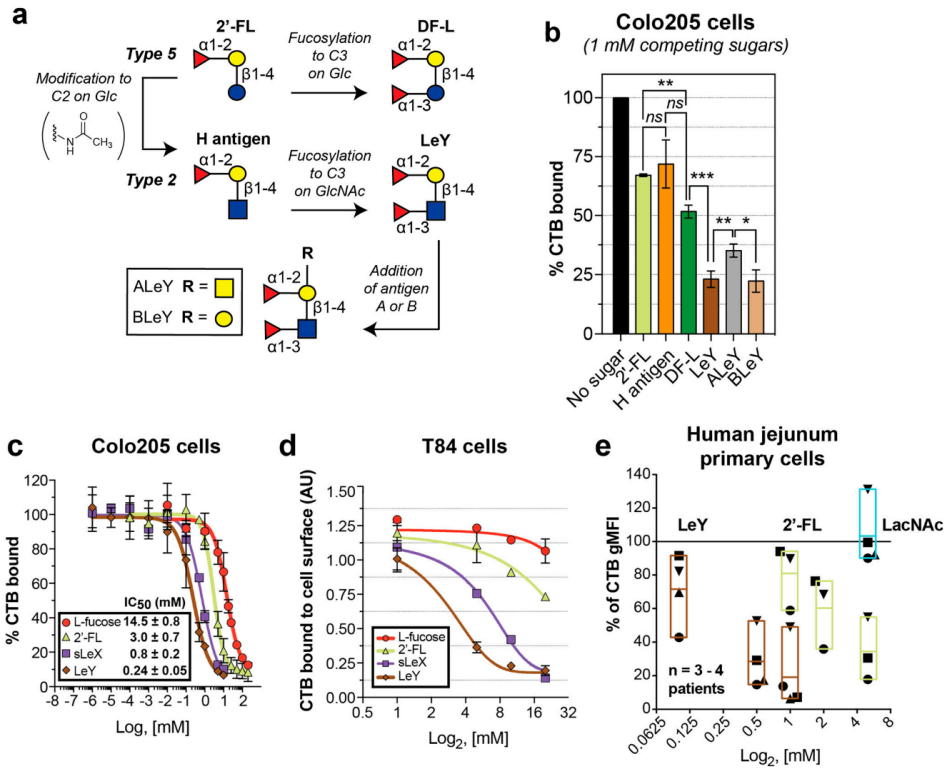


**Figure 13. Lewis Y act as a strong inhibitor of CTB binding.** A) Schematic drawing of the stepwise conversion of 2'-FL to A/BLeY. B) Inhibition of CTB binding to Colo205 cells assessed using flow cytometry. 100% is set to the fluorescence from CTB without blocking C) Graph on titration of several glycans that inhibit CTB binding to Colo205 cells assessed by flow cytometry. D) Graph on titration of several glycans that inhibit CTB binding to Colo205 cells assessed by in-cell ELISA. E) Inhibition of CTB binding to primary small intestine epithelial cells from humans assessed using flow cytometry. Different donors are represented by different symbols. Box plots encompassing low-high data points with the mean as an internal line.

The same pattern was observed in primary epithelial cells from human small intestine. Lewis Y was superior to 2´-FL in blocking CTB-binding. Surprisingly

the LacNAc backbone of Lewis Y had no effect on CTB binding to primary cells whereas lactose had a significant effect on CTB binding to Colo205 (Fig 13E). This indicates that the cell lines used were relatively similar to the primary tissue in human small intestine, but that galactose played a more dominant role in CTB binding to Colo205. Due to limited access to primary tissue we were not able to confirm all cell line findings on primary epithelial cells.

## FUCOSE POLYMERS EFFICIENTLY INHIBIT CTB-BINDING

Finally, we tested if synthetic polymers with glucose and fucose could block CTB binding to both cell lines and primary small intestinal epithelial cells. The fucose-glucose polymer was found to be 150-200 times more effective at inhibiting CTB-binding than Lewis Y, while the glucose-only polymer had no significant effect (Fig 14A-C). However, a fairer comparison of CTB-blocking would be to normalize the fucose content of the polymer to that of Lewis Y. After normalization the polymer still had a 3-4 times better efficacy than Lewis Y showing that the polymeric display of fucose has a synergetic effect. This is most likely due to the fact that a long polymer can aggregate several CTB pentamers. Aggregation would be made possible both by the length of the polymer as well as by the 5 binding sites for fucose on each CTB pentamer. Several CTB pentamers can bind to the same polymer chain and also individually pentamers are able to bind more than one polymer effectively creating a lattice.

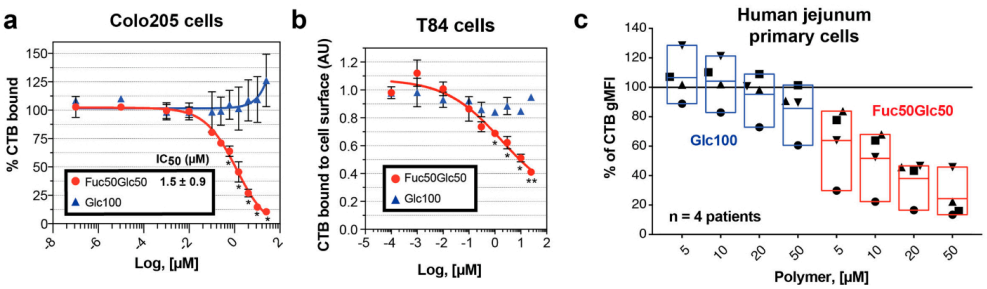


**Figure 14. Polymers with fucose block CTB binding to intestinal epithelial cells.** A) Titration of polymers to assess blocking of CTB binding to Colo205 cells by flow cytometry. B) Titration of polymers to assess blocking of CTB binding to Colo205 cells by in-cell ELISA. Statistical significance was calculated using unpaired Welch's test: *** p value < 0.001, ** p value

< 0.01 and * p value < 0.05. C) Assessment of CTB binding to primary small intestine epithelial cells from humans by flow cytometry. 100 % is set to gMFI of unblocked CTB binding with unique symbols for each donor. Box plots encompassing low-high data points with the mean as an internal line.

## CONCLUSION

To summarize we show in paper II that the CTB-fucose interaction is dependent the hydroxyl groups at carbons 1, 4 and 6. This effectively means that L-fucose must be linked via an alpha-linkage to the rest of the glycan chain in a cellular setting to bind CTB. It also means that the hydroxyl groups of carbon 4 and 6 must be oriented in such a way that they can interact with CTB. Furthermore, Lewis Y is a very potent natural blocker of CTB binding, but the HMO 2´-FL can also inhibit CTB binding at physiologically relevant concentrations (low mM) (196). This means that free glycans in both breast milk and the intestine could act as potent blockers of CT binding and thereby intoxication. Finally, we show that long synthetic polymer chains with fucose attached are very effective at inhibiting CTB binding to both cell lines and primary cells. The polymers therefore show promise as potential therapeutics for alleviating CT induced diarrhea.

# PAPER III - FUCOSE-GALACTOSE POLYMERS INHIBIT CHOLERA TOXIN BINDING TO FUCOSYLATED STRUCTURES AND GALACTOSE-DEPENDENT INTOXICATION OF HUMAN ENTEROIDS

This paper has a strong focus on evaluating the blocking capacity of glycosylated polymers, relating primarily to the second aim of the thesis. The polymers in paper III were a further development of the polymers used in paper II. These polymers has covalently attached fucose, galactose or a mix thereof to fully encapsulate binding to the canonical and noncanonical sites on CTB. Polymer blocking of CTB binding was evaluated using the same methods as for paper I and II, further building on the CTB binding requirements of cells from various tissues. The evaluation of functionally inhibiting CT by use of the polymers was done using human intestinal organoids.

## FUCOSYLATED POLYMERS EFFECTIVELY AGGREGATES CTB AND BLOCK LEWIS X BINDING

After synthesis the polymers were tested for binding to CTB by dynamic light scattering and small angle X-ray scattering. The Gal50Fuc50 mixed sugar polymer clearly interacts with CTB having superior affinity compared to the fucose (Fuc100) or galactose (Gal100) polymers. The effect of Gal50Fuc50 can be mimicked by mixing Fuc100 and Gal100 polymers (Fig 1, paper III). This suggests that by interacting with both the canonical GM1-site and the noncanonical HGBA-site the CTB interaction can be significantly stabilized. We provide evidence that the Gal50Fuc50 polymer forms aggregates with CTB, effectively crosslinking several CTB and polymer molecules. A schematic representation of this can be seen in figure 1C in paper III.

Furthermore, evaluation of the polymer efficacy for blocking CTB binding was done using ELISA. HSA-linked tri-Lewis X or GM1 was used as the solid phase to act as ligands for CTB. The fucose-containing polymers could readily block CTB binding to tri-Lewis X in a dose-dependent manner. The Gal50Fuc50 was by far the most potent blocker of CTB binding to tri-Lewis X but was

unable to block binding to GM1. The only polymer that could block CTB-binding to GM1 was Gal100, although only partially. In fact, the other polymers increased the CTB binding to GM1 indicating formation of polymer-CTB aggregates. We also investigated the effect of polymer length and concluded that the 100 sugar long polymers were far superior to the shorter ones in blocking of CTB-binding. This again supports the hypothesis of polymer aggregation of CTB and that this depends in part on the length of the polymer.

Next, we made a CTB mutant (W88K) that lacks affinity for galactose and therefore also GM1 resulting in a valuable tool for studying GM1-idependent binding. Using ELISA, we could show that W88K has a maintained affinity for tri-Lewis X indicating that the mutation has no effect on the noncanonical site (Fig 2D-E, paper III). Some residual binding to of W88K to GM1 was detected, indicating that the sialic acid pocket could still accept its ligand. Therefore, when interpreting W88K binding data we cannot exclude the possibility that sialic acid accounts for some of the observed binding.

To summarize, we show that the mixed sugar polymer Gal50Fuc50 is an effective inhibitor of CTB binding via the noncanonical site but is unable to block binding via the canonical GM1-site. We also provide further evidence that Gal50Fuc50 aggregates CTB. Finally, we show using W88K that CTB binding to galactose-pocket is independent of binding to the noncanonical HBGA-site.

## GAL50FUC50 BLOCKS CTB BINDING AND CT-INDUCED FLUID ACCUMULATION IN MOUSE SMALL INTESTINE

To test the blocking efficacy of the polymers on live cells, we isolated various cell types from mouse tissues. CTB binding to B and T cells could be blocked by Gal100 and Gal50Fuc50 but not Fuc100 (Fig 3, paper III). When blocked with Fuc100 CTB binding to T cells increased, indicating binding to a strong CTB-ligand like GM1 that can maintain binding to heavy CTB-polymer aggregates. The lymphocyte blocking-pattern was largely repeated in epithelial cells from mouse small intestine, with the exception that Fuc100 had a weak blocking effect at low micromolar concentrations, compared to low

nanomolar concentrations for the other polymers (Fig 4, paper III). These results together indicate that the majority of CTB binding is not mediated via GM1, since Gal50Fuc50 could readily block binding. This conclusion is based on the fact that Gal50Fuc50 was unable to block CTB binding to GM1 in ELISA. The solid phase of GM1-coating in ELISA is of course not the same as the GM1-distrubution on the membrane of a living cell. However, I would still argue that the ELISA-data is representative of the cellular context since the affinity of CTB for GM1 is higher than that of the individual galactose-CTB interaction achieved on the polymer. Therefore, GM1 can easily, given time, outcompete the Gal50Fuc50 polymer one binding site at the time and thereby loosening up the aggregating effect.

The promising results from staining cells with polymer-blocked CTB prompted us to perform CT inhibition experiments *in vivo*. Therefore, we used mice and injected ligated small intestinal ligated loops with CT pre-incubated with Gal50Fuc50 and measured the fluid accumulation (Fig 4D-E, paper III). We observed a clear reduction in fluid accumulation with CT + Gal50Fuc50 compared to CT alone. However, CT + Gal50Fuc50 did not result in complete inhibition of fluid accumulation. This could indicate the presence of sialylated receptors, since the sialic acid pocket is left open by Gal50Fuc50. It could also be that other receptors are present in the small intestine that can compete with the polymer for CT binding. If so, it is highly unlikely that it is GM1 since we in paper I were unable to detect GM1 in the mouse small intestine by HPLC.

To summarize, we confirm results from paper I that CTB binding to mouse cells is not dependent on fucose but rather on galactose. Furthermore, the data suggests that CTB binding is mainly mediated by non-GM1 glycans, although GM1 likely is contributing to CTB binding to lymphocytes. We also show that Gal50Fuc50 can partially inhibit CT induced fluid accumulation in mice. This indicates that Gal50Fu50 has a therapeutic potential in inhibiting CT intoxication.

## CT INTOXICATION OF ENTEROID CULTURES IS ONLY PARTIALLY INHIBITED BY GAL50FUC50

Taking into account that CT is an obligatory human disease and that CTB-binding to mouse cells is fucose-independent, the findings in our mouse model required validation in a human model system. We first investigated how the polymers affected CTB binding to epithelial cells isolated from human small intestine. All polymers had a significant effect on CTB biding, although Gal100 was inefficient and never completely blocked CTB-binding at the concentrations tested. Gal50Fuc50 was also far superior to Fuc100 indicating that the aggregation effect and/or the blocking of both sites have a significant effect on CTB binding. Considering the GM1-block results presented in paper I, that showed a large spread, it is likely that the ability of Gal50Fuc50 to block both sites has different impact on cells from different donors. We also observed that Gal50Fuc50 has a similar blocking effect on LTB binding suggesting that LT intoxication could potentially be ameliorated by this polymer.

To test the polymer blocking of CT functionally we established long-term stabile enteroid cultures from some of the human donors. These cultures displayed various cell types present in the human small intestine (Fig 6A-D, paper III). They were therefore deemed a close representation of the freshly isolated in intestinal tissue. There are of course several issues with this assumption. This first and most obvious is that these cultures lack the connective tissue and undelaying immune cells as well as a luminal microbiome. It is known that the proteome and glycome of epithelial cells are affected by both immune cells and the intestinal flora (174,179). The enteroid cultures are therefore likely to suffer from several minor differences compared to the live tissue, which could impact CTB binding. Another possible source of discrepancy is the culturing itself. What cells are selected for/against, and if as a consequence subtypes of cell are lost, is impossible to control for at this point in time. The culturing conditions with high glucose levels are also likely to alter the proteome and glycome (242-244).

Given such concerns we stained the cultured cell in the same way as the fresh tissue. However, the results confirmed that the cultured enteroid cells behaved similarly to the freshly isolated epithelial cells with regards to CTB binding and polymer block thereof. This indicated that no major discrepancy

was introduced during culturing that affected CTB-binding (fig 6E-F, paper III). Aside from polymer data, we showed that the CTB binding was primarily fucose-dependent since the mutant W88K bound equally well as CTB (Fig 6 G-H, paper III). All this indicated that the enteroids could be readily used as a good representation of the epithelial tissue from the human small intestine.

We therefore continued with functional testing of polymer blocking of CT induced intoxication. Enteroid cells were grown on permeable membranes that, when confluent, effectively separated the apical and basal fluids. This enabled measurement of trans-epithelial resistance and voltage (Fig 7A, paper III). From that we calculated the current representing the CT-induced ion flow. This was a quite complicated procedure and we have so far only been able to reproduce reliable CT-intoxication on cells from one donor. The preliminary results from this donor show that Gal50Fuc50 can partially inhibit CT intoxication (Fig 7B, paper III). In contrast, GM1 glycan completely blocked all CT intoxication showing that binding does not correlate with intoxication in humans. This is in line with the data obtained from ligated loops in mice, indicating that although the overall CTB binding patterns are different the functional receptor/s in both models depend on galactose.

To summarize we have functional data from one donor strongly indicating that only a minority of the CTB binding to cells is actively taken up in a way that enables intoxication. This intoxication appears solely galactose-dependent, as opposed to what the CTB-binding data suggested with fucosylated glycans as the major binders. This hypothesis is further strengthened by the fact that CTB binding to enteroids is only minimally inhibited by GM1 glycan and the galactose-binding deficient mutant W88K binds as well as wildtype CTB (Fig 6E and G, paper III).

## CONCLUSION

In paper III we investigated and evaluated polymers based around the two main sugars described for CTB binding; galactose and fucose. We show that the mixed polymer Gal50Fuc50 is able to aggregate CTB pentamers and thereby block binding and partially block intoxication in mice and human cells. The results also confirm previous findings that CTB binding to murine cells is exclusively galactose dependent and that fucose is the main binder in the

human small intestine. Counterintuitively, we show that the fucose-dependence for CTB binding observed in human tissues is not reflected in CT intoxication. In cells from one donor CT intoxication is, as in mice, occurs exclusively in a galactose-dependent way, indicating the fucosylated receptors acts as decoys rather than functional receptors. To obtain a better and clearer picture of human intoxication enteroids from more donors should be tested. As seen in paper I the blocking with GM1 glycan had a variable impact on CTB binding possibly representing a high variability in CT-receptors among humans.

# THESIS CONCLUSION

Summarizing the work of this thesis brings back memories of my first fumbling steps of realizing that glycans actually matter in biological systems, apart from being carriers of energy. Five years later I must constantly fight the urge to ask any college if he or she has considered that glycosylation could play a crucial role in their work. Although glycosylation is often ignored in most fields of life science, it is no excuse for me to narrow my perspective this way. My exaggerated glyco-focus is one of the biggest biases I have noticed in myself. Another related bias of mine is the importance of fucose for CT intoxication (with some galactose contribution), with complete disregard for sialic acid. The third bias consists of an obsession with the idea that CTB binds to HBGAs and fucosylated HMOs, leading me to ignore other investigation into other glycans that might bind CTB.

For the reasons stated above the data in papers I-III lack thorough investigation of the role of sialic acid for CT intoxication. The few experiments done indicate that it has only a small, if any, role in CT intoxication. However, as shown in paper III binding of CTB does not always correlate with CT intoxication, e.g. I had no rational reason for excluding investigation of known CTB binders. Only in hindsight have I realized this error.

I also have to admit that the aims were only partially answered during my studies. For the first aim regarding the role of fucosylated glycans in CT intoxication the results from paper I and III were somewhat contradictive and no conclusion regarding whether the fucosylated receptors indeed facilitates or act as decoys could be drawn. For the same reason the second aim, investigating if synthetic polymers can block CT intoxication, was also left partially unanswered. The functional data was somewhat ambiguous showing that the GM1 glycan could completely block intoxication whereas the Gal50Fuc50 had a partial effect.

However, I could firmly conclude that CTB binding to human small intestinal epithelium is mainly mediated via the noncanonical site dependent on fucose for binding. I therefore speculate that CTB binding to these cells is mainly mediated via HBGAs such as Lewis X, Y, A/B-Lewis Y and blood group ABO-

antigens. Given that these antigens readily bind CTB and are known to be expressed by epithelial cells this is not an unfair assumption. In a subset of human donors (suspected to be secretor negative) we could also show that an antibody against Lewis X directly inhibited CTB binding (Fig S5E, paper 1).

Given our somewhat inconclusive data there are several possible roles for fucosylated receptors in CT intoxication. It can be argued that fucosylated receptors act to facilitate binding to GM1 by helping to "fish out" CT from 3D-solution in the intestinal lumen to the near-2D surface of the epithelial cells. This could potentially help facilitate CT intoxication by increasing the chance of CT-GM1 encounters. This hypothesis cannot be excluded given the available data, but neither can a decoy-hypothesis be excluded where fucosylated receptors actively counteract CT intoxication by preventing functional uptake.

I argue that the two alternatives above are less likely for CT mediated intoxication in the human small intestine. If fucosylated receptors facilitated intoxication in a significant way, one should expect the Fuc100 polymer to have an impact on intoxication by effectively eliminating the fucosylated helper-receptors. The opposite would be true if fucosylated receptors were decoys, as Fuc100 would eliminate this protective mechanism and thereby further sensitizing the cells to CT.

As we were unable to observe any of these proposed outcomes, I propose a third alternative. I believe that fucosylated receptors are largely inactive bystanders with limited impact on CT toxicity. It has also been shown, albeit in murine thymocytes, that as few as 10 CT molecules can elicit a significant cellular response (256). This indicates that only a small amount of CT has to enter a cell to elicit a response, most likely rendering weak affinity decoys ineffective against CT. With all this said I must again stress that I cannot prove my conviction using the experimental data at hand. Instead this subject needs further investigation to fully elucidate the role of fucosylated receptors in the human small intestine.

Furthermore, we show that galactosylated non-GM1-repetors can act as functional receptors for CT. This was proven in KO mice lacking the ability to synthesize GM1 since they were even more sensitive that WT mice. To add the WT mice did not have detectable levels of GM1 indicating that also

WT intoxication was due to non-GM1, yet galactosylated, receptors. A strong galactose-dependent intoxication was also observed in the GM1-free rat cell line C6. In C6 cells sialic acid had a detrimental effect on intoxication, whereas fucosylated receptors seemed to contribute to intoxication, albeit to a minor degree. Finally, a non-GM1 intoxication was indicated by the effectiveness of the two lectins AAL and PNA in inhibiting CT induced ion secretion in primary human tissue. AAL, binding fucose, and PNA, binding galactose were unable to inhibit CTB binding to GM1 in ELISA, leading me to conclude that CT-intoxication in human small intestine is likely not dependent on GM1. However, it can be argued that the lectins themselves might skew the data by eliciting their own effects. Therefore, functional data from more human donors is called for using various blocking methods.

To my knowledge, the level of GM1 on human small intestinal epithelial cells has only been measured on tissue from one donor. I therefore call for a more comprehensive study involving more donors to be able to conclude the level of GM1 and thereby the likelihood of it acting as a receptor of CT. To me it seems paramount that showing physiologically relevant levels of a candidate receptor in the target tissue must precede the development of a receptor dogma. For CT this procedure has clearly not been observed and results from animal studies have been extrapolated onto humans without sufficient evidence.

# FUTURE PERSPECTIVES

As scientists we are supposed to be seekers of truth and knowledge. It is at the very core of our profession. Often, I find that my thoughts still linger at the challenges at work long after I come home. This obviously leaves me less attentive, and therefore also a lesser husband and father. A significant cost to pay not just for me, but possibly therefore also a cost that is worthy of the task. Revealing truths that in turn enable subsequent gathering of more knowledge and truths in a never ending loop. To enable this loop is paramount since no scientist alone can solve the complex biological problems we are facing. We need to build on each other's work and step by step try to paint the full picture.

Therefore, all data published must hold a high quality as well as truthfully representing the phenomenon studied. In life science today I see a need for better access to good and truthful models. A solid and reproducible model is not by default going to produce data truthful to the aspect it is set out to mimic. In each case a decision has to be made if the model is good enough and not simply "the best we have right now". After over 8 years in life science I suspect that far too often the truthful answer to that question is "no". No, the model is not good enough, but it is the best we have right now. So, we use it regardless out of laziness and convenience.

We are supposed to be seekers of truth. It cannot be enough to use only one or two model systems with a " it's the best we have right now". A scientist should know that the devil is in the details. So why accept findings in a so-and-so model as truth? False data, although unintentional, has unlimited capacity to do damage in leading others endeavors astray. Old habits die hard, and so does published false data. Our standard must be held higher than that! How can we otherwise motivate spending huge amounts of tax money and private funds, if nothing real comes out of our work?

A good step in the direction of creating "good enough"-models I propose that a large biobank is set up with access to enteroids from a diverse set of donors. This would be very expensive but at the same time also a valuable resource in enabling truthful research. The biobank would represent at least part of the genetic diversity seen in humans and thereby be better suited than the inbred

mouse models or cell lines often used. By enabling a broad access to this biobank and training the scientists on how to handle the delicate enteroids, more groups could expand their model repertoire and validate findings form other cheaper and easier models. The enteroid cultures can also be expanded to encompass more cell types effectively including other fields such as immunology and neurobiology.

More verification of findings done in animal models and cell lines by usage of human-like models is in my mind a good and necessary way forward. This would be a small step, but in general I believe that this more systematic and large-scale approach is called for. By having access to large and diverse sets of human-like models scientist can systematically truly verify initial findings in simpler and cheaper systems without exposing humans to unnecessary risk. This verification would obviously be expensive and time consuming, giving that only larger research groups with sufficient manpower and founding could undertake this. Although unfair to smaller groups and possibly suffocating some of the current creativity, a consolidation would give the huge benefit of quickly reaching a well-founded conclusion for a given question. In my limited experience I find that the amount of creativity is not an issue for us scientists. Instead I have often found that the limiting factor is the physical means to truthfully verify or disprove a hypothesis.

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If you read this section **first,** I think I know **some** things about you.

You can read (and hopefully write) English

You "love" science

You love reading about yourself even more?

You also love **spikafika**

You love spikafika specifically because of **glucose**

Despite loving sugar, you see **glycobiology** as a boring and of little use

You are **right** about the first statement above, but not the second

You are therefore **wrong** as often as you are right

You need to **remember** that

You likely work at University of Gothenburg...

... or you are just a **family member** that did not bother to read Sara's thesis either. I mean blood, ribosomes and such, how fun can that be right ;-) Mine is at least about **exciting** stuff... maybe not, but still.

It is obvious that the work presented in a PhD thesis is far from a One-Man-Job. Ergo, I owe a huge amount of "thank you".

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# REFERENCES


1. Clemens JD, Nair GB, Ahmed T, Qadri F, Holmgren J. Cholera. Lancet. 2017 Mar 10;390(10101):1539–49.

2. Ali M, Nelson AR, Lopez AL, Sack DA. Updated global burden of cholera in endemic countries. Remais JV, editor. PLoS Negl Trop Dis. Public Library of Science; 2015;9(6):e0003832.

3. Shin S, Desai SN, Sah BK, Clemens JD. Oral vaccines against cholera. Clin Infect Dis. 2011 Jun;52(11):1343–9.

4. Nelson EJ, Harris JB, Morris JG, Calderwood SB, Camilli A. Cholera transmission: the host, pathogen and bacteriophage dynamic. Nat Rev Microbiol. Nature Publishing Group; 2009 Oct;7(10):693–702.

5. COCKBURN TA, CASSANOS JG. Epidemiology of endemic cholera. Public Health Rep. Association of Schools of Public Health; 1960 Sep;75(9):791–803.

6. Karaolis DK, Lan R, Reeves PR. Molecular evolution of the seventh-pandemic clone of Vibrio cholerae and its relationship to other pandemic and epidemic V. cholerae isolates. J Bacteriol. 1994 Oct;176(20):6199–206.

7. Siddique AK, Cash R. Cholera outbreaks in the classical biotype era. Curr Top Microbiol Immunol. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014;379(19):1–16.

8. MUKERJEE S. PRELIMINARY STUDIES ON THE DEVELOPMENT OF A LIVE ORAL VACCINE FOR ANTI-CHOLERA IMMUNIZATION. Bull World Health Organ. World Health Organization; 1963;29(6):753–66.

9. Cvjetanovic B, Barua D. The seventh pandemic of cholera. Nature. Nature Publishing Group; 1972 Sep 15;239(5368):137–8.

10. Pradhan S, Baidya AK, Ghosh A, Paul K, Chowdhury R. The El Tor biotype of Vibrio cholerae exhibits a growth advantage in the stationary phase in mixed cultures with the classical biotype. J Bacteriol. American Society for Microbiology Journals; 2010 Feb;192(4):955–63.

11. Clemens JD, van Loon F, Sack DA, Rao MR, Ahmed F, ChakrabortY J, et al. Biotype as determinant of natural immunising effect of cholera. Lancet. 1991 Apr 13;337(8746):883–4.

12. Hornick RB, Music SI, Wenzel R, Cash R, Libonati JP, Snyder MJ, et al. The Broad Street pump revisited: response of volunteers to ingested cholera vibrios. Bull N Y Acad Med. New York Academy of Medicine; 1971 Oct;47(10):1181–91.

13. Schmid-Hempel P, Frank SA. Pathogenesis, virulence, and infective dose. PLoS Pathog. Public Library of Science; 2007 Oct 26;3(10):1372–3.

14. Leung DT, Chowdhury F, Calderwood SB, Qadri F, Ryan ET. Immune responses to cholera in children. Expert Rev Anti Infect Ther. 2012 Apr;10(4):435–44.

15. Seidlein von L, Wang X-Y, Macuamule A, Mondlane C, Puri M, Hendriksen I, et al. Is HIV infection associated with an increased risk for cholera? Findings from a case-control study in Mozambique. Trop Med Int Health. John Wiley & Sons, Ltd (10.1111); 2008 May;13(5):683–8.

16. Swerdlow DL, Mintz ED, Rodriguez M, Tejada E, Ocampo C, Espejo L, et al. Severe life-threatening cholera associated with blood group O in Peru: implications for the Latin American epidemic. J Infect Dis. 1994 Aug;170(2):468–72.

17. Harris JB, LaRocque RC. Cholera and ABO Blood Group: Understanding an Ancient Association. Am J Trop Med Hyg. The American Society of Tropical Medicine and Hygiene; 2016 Aug 3;95(2):263–4.

18. Reidl J, Klose KE. Vibrio cholerae and cholera: out of the water and into the host. FEMS Microbiol Rev. 2002 Jun;26(2):125–39.

19. Hu D, Liu B, Feng L, Ding P, Guo X, Wang M, et al. Origins of the current seventh cholera pandemic. Proc Natl Acad Sci USA. 2016 Nov 29;113(48):E7730–9.

20. Morris JG. Cholera and other types of vibriosis: a story of human pandemics and oysters on the half shell. Clin Infect Dis. 2003 Jul 15;37(2):272–80.

21. Sakib SN, Reddi G, Almagro-Moreno S. Environmental role of pathogenic traits in Vibrio cholerae. DiRita VJ, editor. J Bacteriol. American Society for Microbiology Journals; 2018 Mar 26;200(15):e00795–17.

22. Kimani RW, Muigai AWT, Sang W, Kiiru JN, Kariuki S. Virulence factors in environmental and clinical Vibrio cholerae from endemic areas in Kenya. Afr J Lab Med. 2014;3(1):41.

23. Sears CL, Kaper JB. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiol Rev. American Society for Microbiology (ASM); 1996 Mar;60(1):167–215.

24. Faruque SM, Mekalanos JJ. Phage-bacterial interactions in the evolution of toxigenic Vibrio cholerae. Virulence. Taylor & Francis; 2012 Nov 15;3(7):556–65.

25. Trucksis M, Galen JE, Michalski J, Fasano A, Kaper JB. Accessory cholera enterotoxin (Ace), the third toxin of a Vibrio cholerae virulence cassette. Proc Natl Acad Sci USA. National Academy of Sciences; 1993 Jun 1;90(11):5267–71.

26. Trucksis M, Conn TL, Wasserman SS, Sears CL. Vibrio cholerae ACE stimulates Ca(2+)-dependent Cl(-)/HCO(3)(-) secretion in T84 cells in vitro. Am J Physiol, Cell Physiol. American Physiological SocietyBethesda, MD; 2000 Sep;279(3):C567–77.

27. Morita M, Ohnishi M, Arakawa E, Yamamoto S, Nair GB, Matsushita S, et al. Emergence and genetic diversity of El Tor Vibrio cholerae O1 that possess classical biotype ctxB among travel-associated cases of cholera in Japan. J Med Microbiol. Microbiology Society; 2010 Jun;59(Pt 6):708–12.

28. Faruque SM, Nair GB. Vibrio Cholerae: Genomics and Molecular Biology. Caister Academic Press; 2008. 1 p.

29. Davis BM, Waldor MK. Filamentous phages linked to virulence of Vibrio cholerae. Curr Opin Microbiol. 2003 Feb;6(1):35–42.

30. Mukhopadhyay AK, Chakraborty S, Takeda Y, Nair GB, Berg DE. Characterization of VPI pathogenicity island and CTXphi prophage in environmental strains of Vibrio cholerae. J Bacteriol. American Society for Microbiology Journals; 2001 Aug;183(16):4737–46.

31. Almagro-Moreno S, Pruss K, Taylor RK. Intestinal Colonization Dynamics of Vibrio cholerae. Bliska JB, editor. PLoS Pathog. 2015 May;11(5):e1004787.

32. Crowther RS, Roomi NW, Fahim RE, Forstner JF. Vibrio cholerae metalloproteinase degrades intestinal mucin and facilitates enterotoxin-induced secretion from rat intestine. Biochim Biophys Acta. 1987 Jun 22;924(3):393–402.

33. Leitch GJ. Cholera enterotoxin-induced mucus secretion and increase in the mucus blanket of the rabbit ileum in vivo. Infection and Immunity. American Society for Microbiology (ASM); 1988 Nov;56(11):2871–5.

34. Birchenough GMH, Johansson MEV, Gustafsson JK, Bergström JH, Hansson GC. New developments in goblet cell mucus secretion and function. Mucosal Immunol. Nature Publishing Group; 2015 Jul;8(4):712–9.

35. Pelaseyed T, Bergström JH, Gustafsson JK, Ermund A, Birchenough GMH, Schütte A, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immunol Rev. John Wiley &

Sons, Ltd (10.1111); 2014 Jul;260(1):8–20.

36. Kim JJ, Khan WI. Goblet cells and mucins: role in innate defense in enteric infections. Pathogens. Multidisciplinary Digital Publishing Institute; 2013 Feb 4;2(1):55–70.

37. Villeneuve S, Boutonnier A, Mulard LA, Fournier JM. Immunochemical characterization of an Ogawa-Inaba common antigenic determinant of Vibrio cholerae O1. Microbiology (Reading, Engl). Microbiology Society; 1999 Sep;145 ( Pt 9)(9):2477–84.

38. Karlsson SL, Thomson N, Mutreja A, Connor T, Sur D, Ali M, et al. Retrospective Analysis of Serotype Switching of Vibrio cholerae O1 in a Cholera Endemic Region Shows It Is a Non-random Process. Ryan ET, editor. PLoS Negl Trop Dis. 2016 Oct;10(10):e0005044.

39. Zrimi J, Ng Ling A, Giri-Rachman Arifin E, Feverati G, Lesieur C. Cholera toxin B subunits assemble into pentamers--proposition of a fly-casting mechanism. Hofmann A, editor. PLoS ONE. 2010 Dec 21;5(12):e15347.

40. Zhang RG, Scott DL, Westbrook ML, Nance S, Spangler BD, Shipley GG, et al. The three-dimensional crystal structure of cholera toxin. J Mol Biol. 1995 Aug 25;251(4):563–73.

41. Lönnroth I, Holmgren J. Subunit structure of cholera toxin. J Gen Microbiol. Microbiology Society; 1973 Jun;76(2):417–27.

42. Peterson KM, Gellings PS. Multiple intraintestinal signals coordinate the regulation of Vibrio cholerae virulence determinants. Pathog Dis. 2018 Feb 1;76(1):4111.

43. Yang M, Liu Z, Hughes C, Stern AM, Wang H, Zhong Z, et al. Bile salt-induced intermolecular disulfide bond formation activates Vibrio cholerae virulence. Proc Natl Acad Sci USA. 2013 Feb 5;110(6):2348–53.

44. Howard MF, Bina XR, Bina JE. Indole Inhibits ToxR Regulon Expression in Vibrio cholerae. Payne SM, editor. Infection and Immunity. American Society for Microbiology Journals; 2019 Mar;87(3):3028.

45. Rasti ES, Brown AC. Cholera Toxin Encapsulated within Several Vibrio cholerae O1 Serotype Inaba Outer Membrane Vesicles Lacks a Functional B-Subunit. Toxins. Multidisciplinary Digital Publishing Institute; 2019 Apr 6;11(4):207.

46. Sandkvist M. Type II secretion and pathogenesis. Infection and Immunity. American Society for Microbiology Journals; 2001 Jun;69(6):3523–35.

47. Sandkvist M, Michel LO, Hough LP, Morales VM, Bagdasarian M, Koomey M, et al. General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in Vibrio cholerae. J Bacteriol. 1997 Nov;179(22):6994–7003.

48. Holmgren J, Lönnroth I, Svennerholm L. Fixation and inactivation of cholera toxin by GM1 ganglioside. Scand J Infect Dis. Taylor & Francis; 1973;5(1):77–8.

49. Holmgren J, Lönnroth I, Månsson J, Svennerholm L. Interaction of cholera toxin and membrane GM1 ganglioside of small intestine. Proc Natl Acad Sci USA. National Academy of Sciences; 1975 Jul;72(7):2520–4.

50. Holmgren J. Comparison of the tissue receptors for Vibrio cholerae and Escherichia coli enterotoxins by means of gangliosides and natural cholera toxoid. Infection and Immunity. American Society for Microbiology (ASM); 1973 Dec;8(6):851–9.

51. Antoine T, Priem B, Heyraud A, Greffe L, Gilbert M, Wakarchuk WW, et al. Large-scale in vivo synthesis of the carbohydrate moieties of gangliosides GM1 and GM2 by metabolically engineered Escherichia coli. Chembiochem. John Wiley & Sons, Ltd; 2003 May 9;4(5):406–12.

52. Merritt EA, Kuhn P, Sarfaty S, Erbe JL, Holmes RK, Hol WG. The 1.25 A resolution refinement of the cholera toxin B-pentamer: evidence of peptide backbone strain at the receptor-binding site. J Mol Biol. 1998 Oct 9;282(5):1043–59.

53. Merritt EA, Sarfaty S, van den Akker F, L'Hoir C, Martial JA, Hol WG. Crystal structure of

cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. Protein Sci. John Wiley & Sons, Ltd; 1994 Feb;3(2):166–75.

54. Kuziemko GM, Stroh M, Stevens RC. Cholera toxin binding affinity and specificity for gangliosides determined by surface plasmon resonance. Biochemistry. American Chemical Society; 1996 May 21;35(20):6375–84.

55. Holmgren J, Lönnroth I, Svennerholm L. Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids. Infection and Immunity. American Society for Microbiology (ASM); 1973 Aug;8(2):208–14.

56. Holmgren J, Lönnroth I. Cholera toxin and the adenylate cyclase-activating signal. J Infect Dis. 1976 Mar;133 Suppl(Supplement 1):64–74.

57. Spiegel S. Insertion of ganglioside GM1 into rat glioma C6 cells renders them susceptible to growth inhibition by the B subunit of cholera toxin. Biochim Biophys Acta. 1988 May 13;969(3):249–56.

58. Yanagisawa M. Letter to the Glyco-Forum: Cholera toxin B subunit binding does not correlate with GM1 expression: a study using mouse embryonic neural precursor cells. Glycobiology. 2006 May 24;16(9):19G–22G.

59. Blank N, Schiller M, Krienke S, Wabnitz G, Ho AD, Lorenz H-M. Cholera toxin binds to lipid rafts but has a limited specificity for ganglioside GM1. Immunol Cell Biol. 2007 Feb 27;85(5):378–82.

60. Kirkeby S. Cholera toxin B subunit-binding and ganglioside GM1 immuno-expression are not necessarily correlated in human salivary glands. Acta Odontol Scand. Taylor & Francis; 2014 Nov;72(8):694–700.

61. Heggelund JE, Haugen E, Lygren B, Mackenzie A, Holmner Å, Vasile F, et al. Both El Tor and classical cholera toxin bind blood group determinants. Biochemical and Biophysical Research Communications. Elsevier Inc; 2012 Feb 24;418(4):731–5.

62. Vasile F, Reina JJ, Potenza D, Heggelund JE, Mackenzie A, Krengel U, et al. Comprehensive analysis of blood group antigen binding to classical and El Tor cholera toxin B-pentamers by NMR. Glycobiology. 2014 Jun 25;24(8):766–78.

63. Mandal PK, Branson TR, Hayes ED, Ross JF, Gavín JA, Daranas AH, et al. Towards a structural basis for the relationship between blood group and the severity of El Tor cholera. Angew Chem Int Ed Engl. WILEY-VCH Verlag; 2012 May 21;51(21):5143–6.

64. Heggelund JE, Burschowsky D, Bjørnestad VA, Hodnik V, Anderluh G, Krengel U. High-Resolution Crystal Structures Elucidate the Molecular Basis of Cholera Blood Group Dependence. Satchell KJF, editor. PLoS Pathog. 2016 Apr 15;12(4):e1005567–19.

65. Korotkov KV, Sandkvist M, Hol WGJ. The type II secretion system: biogenesis, molecular architecture and mechanism. Nat Rev Microbiol. Nature Publishing Group; 2012 Apr 2;10(5):336–51.

66. Reichow SL, Korotkov KV, Hol WGJ, Gonen T. Structure of the cholera toxin secretion channel in its closed state. Nat Struct Mol Biol. Nature Publishing Group; 2010 Oct;17(10):1226–32.

67. Yu RK, Tsai Y-T, Ariga T, Yanagisawa M. Structures, biosynthesis, and functions of gangliosides--an overview. J Oleo Sci. NIH Public Access; 2011;60(10):537–44.

68. Aman AT, Fraser S, Merritt EA, Rodigherio C, Kenny M, Ahn M, et al. A mutant cholera toxin B subunit that binds GM1- ganglioside but lacks immunomodulatory or toxic activity. Proc Natl Acad Sci USA. 2001 Jul 17;98(15):8536–41.

69. Shi J, Yang T, Kataoka S, Zhang Y, Diaz AJ, Cremer PS. GM1 clustering inhibits cholera toxin binding in supported phospholipid membranes. J Am Chem Soc. 2007 May 9;129(18):5954–

61.

70. Margheri G, D'Agostino R, Trigari S, Sottini S, Del Rosso M. The β-subunit of cholera toxin has a high affinity for ganglioside GM1 embedded into solid supported lipid membranes with a lipid raft-like composition. Lipids. John Wiley & Sons, Ltd; 2014 Feb;49(2):203–6.

71. Seo JH, Kim CS, Lee HY, Kawai T, Cha HJ. Interactive configuration through force analysis of GM1 pentasaccharide-Vibrio cholera toxin interaction. Anal Chem. American Chemical Society; 2011 Aug 1;83(15):6011–7.

72. Kumar V, Turnbull WB. Carbohydrate inhibitors of cholera toxin. Beilstein J Org Chem. Beilstein-Institut; 2018;14(1):484–98.

73. Das S, Angsantikul P, Le C, Bao D, Miyamoto Y, Gao W, et al. Neutralization of cholera toxin with nanoparticle decoys for treatment of cholera. Habib AG, editor. PLoS Negl Trop Dis. 2018 Feb;12(2):e0006266.

74. Aureli M, Mauri L, Ciampa MG, Prinetti A, Toffano G, Secchieri C, et al. GM1 Ganglioside: Past Studies and Future Potential. Mol Neurobiol. 2016 Apr;53(3):1824–42.

75. Fishman PH, Orlandi PA. Cholera toxin internalization and intoxication. J Cell Sci. The Company of Biologists Ltd; 2003 Feb 1;116(Pt 3):431–2–authorreply432–3.

76. Torgersen ML, Skretting G, van Deurs B, Sandvig K. Internalization of cholera toxin by different endocytic mechanisms. J Cell Sci. 2001 Oct;114(Pt 20):3737–47.

77. Orlandi PA, Fishman PH. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. J Cell Biol. 1998 May 18;141(4):905–15.

78. Lencer WI, Hirst TR, Holmes RK. Membrane traffic and the cellular uptake of cholera toxin. Biochim Biophys Acta. 1999 Jul 8;1450(3):177–90.

79. Broeck DV, Lagrou AR, De Wolf MJ. Distinct role of clathrin-mediated endocytosis in the functional uptake of cholera toxin. Acta Biochim Pol. 2007;54(4):757–67.

80. Wernick NLB, Chinnapen DJF, Cho JA, Lencer WI. Cholera Toxin: An Intracellular Journey into the Cytosol by Way of the Endoplasmic Reticulum. Toxins. 2010 Mar;2(3):310–25.

81. Mekalanos JJ, Collier RJ, Romig WR. Enzymic activity of cholera toxin. II. Relationships to proteolytic processing, disulfide bond reduction, and subunit composition. Journal of Biological Chemistry. 1979 Jul 10;254(13):5855–61.

82. Cherubin P, Garcia MC, Curtis D, Britt CBT, Craft JW, Burress H, et al. Inhibition of Cholera Toxin and Other AB Toxins by Polyphenolic Compounds. Mantis NJ, editor. PLoS ONE. 2016 Nov 9;11(11):e0166477–18.

83. Taylor M, Banerjee T, Ray S, Tatulian SA, Teter K. Protein-disulfide isomerase displaces the cholera toxin A1 subunit from the holotoxin without unfolding the A1 subunit. J Biol Chem. 2011 Jun 24;286(25):22090–100.

84. Nowakowska-Gołacka J, Sominka H, Sowa-Rogozińska N, Słomińska-Wojewódzka M. Toxins Utilize the Endoplasmic Reticulum-Associated Protein Degradation Pathway in Their Intoxication Process. Int J Mol Sci. Multidisciplinary Digital Publishing Institute; 2019 Mar 15;20(6):1307.

85. Cherubin P, Guyette J, Taylor M, O'Donnell M, Herndon L, Burress H, et al. Protein disulfide isomerase does not act as an unfoldase in the disassembly of cholera toxin. Biosci Rep. 2018 Oct 31;38(5):BSR20181320.

86. Sanderson IR, Xu Z, Chu SW, Xie QY, Levine LJ, Walker WA. Developmental differences in the expression of the cholera toxin sensitive subunit (Gs alpha) of adenylate cyclase in the rat small intestine. Gut. 2nd ed. 1996 Jun;38(6):853–8.

87. Simonds WF. G protein regulation of adenylate cyclase. Trends Pharmacol Sci. 1999

Feb;20(2):66–73.

88. Carpenter CC, Sack RB, Feeley JC, Steenberg RW. Site and characteristics of electrolyte loss and effect of intraluminal glucose in experimental canine cholera. Journal of Clinical Investigation. American Society for Clinical Investigation; 1968 May;47(5):1210–20.

89. Ameen N, Silvis M, Bradbury NA. Endocytic trafficking of CFTR in health and disease. J Cyst Fibros. 2007 Jan;6(1):1–14.

90. Sawasvirojwong S, Srimanote P, Chatsudthipong V, Muanprasat C. An Adult Mouse Model of Vibrio cholerae-induced Diarrhea for Studying Pathogenesis and Potential Therapy of Cholera. Picardeau M, editor. PLoS Negl Trop Dis. Public Library of Science; 2013 Jun;7(6):e2293.

91. Alisson-Silva F, Liu JZ, Diaz SL, Deng L, Gareau MG, Marchelletta R, et al. Human evolutionary loss of epithelial Neu5Gc expression and species-specific susceptibility to cholera. Satchell KJF, editor. PLoS Pathog. Public Library of Science; 2018 Jun;14(6):e1007133.

92. Satitsri S, Pongkorpsakol P, Srimanote P, Chatsudthipong V, Muanprasat C. Pathophysiological mechanisms of diarrhea caused by the Vibrio cholerae O1 El Tor variant: an in vivo study in mice. Virulence. 2016 Oct 2;7(7):789–805.

93. Guichard A, Cruz-Moreno B, Cruz-Moreno BC, Aguilar B, van Sorge NM, Kuang J, et al. Cholera toxin disrupts barrier function by inhibiting exocyst-mediated trafficking of host proteins to intestinal cell junctions. Cell Host Microbe. 2013 Sep 11;14(3):294–305.

94. Cassuto J, Siewert A, Jodal M, Lundgren O. The involvement of intramural nerves in cholera toxin induced intestinal secretion. Acta Physiol Scand. John Wiley & Sons, Ltd (10.1111); 1983 Feb;117(2):195–202.

95. Eklund S, Sjöqvist A, Fahrenkrug J, Jodal M, Lundgren O. Somatostatin and methionine-enkephalin inhibit cholera toxin-induced jejunal net fluid secretion and release of vasoactive intestinal polypeptide in the cat in vivo. Acta Physiol Scand. John Wiley & Sons, Ltd (10.1111); 1988 Aug;133(4):551–7.

96. Sjöqvist A, Fahrenkrug J, Jodal M, Lundgren O. The effect of splanchnic nerve stimulation and neuropeptide Y on cholera secretion and release of vasoactive intestinal polypeptide in the feline small intestine. Acta Physiol Scand. John Wiley & Sons, Ltd (10.1111); 1988 Jul;133(3):289–95.

97. Ravnskjaer K, Madiraju A, Montminy M. Role of the cAMP Pathway in Glucose and Lipid Metabolism. Handb Exp Pharmacol. Cham: Springer International Publishing; 2016;233(3):29–49.

98. Zhang L, Seitz LC, Abramczyk AM, Chan C. Synergistic effect of cAMP and palmitate in promoting altered mitochondrial function and cell death in HepG2 cells. Exp Cell Res. 2010 Mar 10;316(5):716–27.

99. Seyedi SY, Salehi F, Payandemehr B, Hossein S, Hosseini-Zare MS, Nassireslami E, et al. Dual effect of cAMP agonist on ameliorative function of PKA inhibitor in morphine-dependent mice. Fundam Clin Pharmacol. John Wiley & Sons, Ltd (10.1111); 2014 Aug;28(4):445–54.

100. Terrinoni M, Holmgren J, Lebens M, Larena M. Requirement for Cyclic AMP/Protein Kinase A-Dependent Canonical NFκB Signaling in the Adjuvant Action of Cholera Toxin and Its Non-toxic Derivative mmCT. Front Immunol. 2019;10:269.

101. Terrinoni M, Holmgren J, Lebens M, Larena M. Proteomic analysis of cholera toxin adjuvant-stimulated human monocytes identifies Thrombospondin-1 and Integrin-β1 as strongly upregulated molecules involved in adjuvant activity. Scientific Reports 2017 7. Nature Publishing Group; 2019 Feb 26;9(1):2812–3.

102. Larena M, Holmgren J, Lebens M, Terrinoni M, Lundgren A. Cholera toxin, and the related nontoxic adjuvants mmCT and dmLT, promote human Th17 responses via cyclic AMP-

protein kinase A and inflammasome-dependent IL-1 signaling. J Immunol. American Association of Immunologists; 2015 Apr 15;194(8):3829–39.

103. Mattsson J, Schön K, Ekman L, Fahlén-Yrlid L, Yrlid U, Lycke NY. Cholera toxin adjuvant promotes a balanced Th1/Th2/Th17 response independently of IL-12 and IL-17 by acting on Gsα in $CD11b^{+}$ DCs. Mucosal Immunol. Nature Publishing Group; 2015 Jul;8(4):815–27.

104. Mattsson J, Yrlid U, Stensson A, Schön K, Karlsson MCI, Ravetch JV, et al. Complement activation and complement receptors on follicular dendritic cells are critical for the function of a targeted adjuvant. J Immunol. 2011 Oct 1;187(7):3641–52.

105. Yoon KW. Dead cell phagocytosis and innate immune checkpoint. BMB Rep. Korean Society for Biochemistry and Molecular Biology; 2017 Oct;50(10):496–503.

106. Perez-Lopez A, Behnsen J, Nuccio S-P, Raffatellu M. Mucosal immunity to pathogenic intestinal bacteria. Nat Rev Immunol. Nature Publishing Group; 2016 Mar;16(3):135–48.

107. Bain CC, Mowat AM. Macrophages in intestinal homeostasis and inflammation. Immunol Rev. John Wiley & Sons, Ltd (10.1111); 2014 Jul;260(1):102–17.

108. Segerstrom SC. Stress, Energy, and Immunity: An Ecological View. Curr Dir Psychol Sci. NIH Public Access; 2007;16(6):326–30.

109. Hirano M, Das S, Guo P, Cooper MD. The evolution of adaptive immunity in vertebrates. Adv Immunol. Elsevier; 2011;109:125–57.

110. McCoy KD, Ronchi F, Geuking MB. Host-microbiota interactions and adaptive immunity. Immunol Rev. 2017 Sep;279(1):63–9.

111. Jennewein MF, Alter G. The Immunoregulatory Roles of Antibody Glycosylation. Trends Immunol. 2017 May;38(5):358–72.

112. Saphire EO, Schendel SL, Gunn BM, Milligan JC, Alter G. Antibody-mediated protection against Ebola virus. Nat Immunol. Nature Publishing Group; 2018 Nov;19(11):1169–78.

113. Lu LL, Suscovich TJ, Fortune SM, Alter G. Beyond binding: antibody effector functions in infectious diseases. Nat Rev Immunol. Nature Publishing Group; 2018 Jan;18(1):46–61.

114. Weidle UH, Georges G, Tiefenthaler G. TCR-MHC/peptide interaction: prospects for new anti-tumoral agents. Cancer Genomics Proteomics. 2014 Nov;11(6):267–77.

115. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T cell antigen receptor recognition of antigen-presenting molecules. Annu Rev Immunol. Annual Reviews; 2015;33(1):169–200.

116. Hall BM. T Cells: Soldiers and Spies--The Surveillance and Control of Effector T Cells by Regulatory T Cells. Clin J Am Soc Nephrol. 2015 Nov 6;10(11):2050–64.

117. Roth DB. V(D)J Recombination: Mechanism, Errors, and Fidelity. Craig, Chandler, Gellert, Lambowitz, Rice, Sandmeyer, editors. Microbiol Spectr. American Society of Microbiology; 2014 Dec;2(6):313–24.

118. Janeway C. Immunobiology Five. Garland Publishing; 2001. 1 p.

119. Nemazee D. Receptor selection in B and T lymphocytes. Annu Rev Immunol. 2000;18(1):19–51.

120. Cantrell D. Signaling in lymphocyte activation. Cold Spring Harb Perspect Biol. Cold Spring Harbor Lab; 2015 Jun 1;7(6):a018788.

121. Gasteiger G, Ataide M, Kastenmüller W. Lymph node - an organ for T-cell activation and pathogen defense. Immunol Rev. 2016 May;271(1):200–20.

122. Bekiaris V, Persson EK, Agace WW. Intestinal dendritic cells in the regulation of mucosal immunity. Immunol Rev. John Wiley & Sons, Ltd (10.1111); 2014 Jul;260(1):86–101.

123. Stavnezer J, Guikema JEJ, Schrader CE. Mechanism and regulation of class switch recombination. Annu Rev Immunol. Annual Reviews; 2008;26(1):261–92.

124. Svennerholm A, Lange S, Holmgren J. Correlation between intestinal synthesis of specific immunoglobulin A and protection against experimental cholera in mice. Infection and Immunity. American Society for Microbiology (ASM); 1978 Jul;21(1):1–6.

125. Rahman A, Rashu R, Bhuiyan TR, Chowdhury F, Khan AI, Islam K, et al. Antibody-secreting cell responses after Vibrio cholerae O1 infection and oral cholera vaccination in adults in Bangladesh. Clin Vaccine Immunol. 2013 Oct;20(10):1592–8.

126. Kauffman RC, Bhuiyan TR, Nakajima R, Mayo-Smith LM, Rashu R, Hoq MR, et al. Single-Cell Analysis of the Plasmablast Response to Vibrio cholerae Demonstrates Expansion of Cross-Reactive Memory B Cells. MBio. American Society for Microbiology; 2016 Dec 20;7(6):e02021–16.

127. Glass RI, Stoll BJ. The protective effect of human milk against diarrhea. A review of studies from Bangladesh. Acta Paediatr Scand Suppl. 1989;351:131–6.

128. Qureshi K, M Ibak KR, Sandstr m A, Kofoed P-E, Rodrigues A, Dias F, et al. Breast Milk Reduces the Risk of Illness in Children of Mothers With Cholera. The Pediatric Infectious Disease Journal. 2006 Dec;25(12):1163–6.

129. Jertborn M, Svennerholm AM, Holmgren J. Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. J Clin Microbiol. American Society for Microbiology (ASM); 1986 Aug;24(2):203–9.

130. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol. Nature Publishing Group; 2005 Jan;3(1):36–46.

131. Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN, Peri F. TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. Vaccines (Basel). Multidisciplinary Digital Publishing Institute; 2017 Oct 4;5(4):34.

132. Lebens M, Terrinoni M, Karlsson SL, Larena M, Gustafsson-Hedberg T, Källgård S, et al. Construction and preclinical evaluation of mmCT, a novel mutant cholera toxin adjuvant that can be efficiently produced in genetically manipulated Vibrio cholerae. Vaccine. 2016 Apr 19;34(18):2121–8.

133. Shah RR, Hassett KJ, Brito LA. Overview of Vaccine Adjuvants: Introduction, History, and Current Status. Methods Mol Biol. New York, NY: Springer New York; 2017;1494(1):1–13.

134. Lycke N, Lebrero-Fernández C. ADP-ribosylating enterotoxins as vaccine adjuvants. Curr Opin Pharmacol. 2018 Aug;41:42–51.

135. Agren L, Löwenadler B, Lycke N. A novel concept in mucosal adjuvanticity: the CTA1-DD adjuvant is a B cell-targeted fusion protein that incorporates the enzymatically active cholera toxin A1 subunit. Immunol Cell Biol. 1998 Jun;76(3):280–7.

136. Lycke N. From toxin to adjuvant: basic mechanisms for the control of mucosal IgA immunity and tolerance. Immunol Lett. 2005 Mar 15;97(2):193–8.

137. Gustafsson T, Hua Y-J, Dahlgren MW, Livingston M, Johansson-Lindbom B, Yrlid U. Direct interaction between cholera toxin and dendritic cells is required for oral adjuvant activity. Eur J Immunol. 2013 May 27;43(7):1779–88.

138. Sinclair D, Abba K, Zaman K, Qadri F, Graves PM. Oral vaccines for preventing cholera. Cochrane Infectious Diseases Group, editor. Cochrane Database Syst Rev. John Wiley & Sons, Ltd; 2011 Mar 16;13(3):CD008603.

139. Glass RI, Svennerholm AM, Stoll BJ, Khan MR, Hossain KM, Huq MI, et al. Protection against cholera in breast-fed children by antibodies in breast milk. N Engl J Med. Massachusetts Medical Society; 1983 Jun 9;308(23):1389–92.

140. Holmgren J, Czerkinsky C, Lycke N, Svennerholm AM. Strategies for the induction of immune responses at mucosal surfaces making use of cholera toxin B subunit as immunogen, carrier, and adjuvant. Am J Trop Med Hyg. 1994;50(5 Suppl):42–54.

141. Gregorio GV, Gonzales MLM, Dans LF, Martinez EG. Polymer-based oral rehydration solution for treating acute watery diarrhoea. Cochrane Infectious Diseases Group, editor. Cochrane Database Syst Rev. John Wiley & Sons, Ltd; 2016 Dec 13;12(1):CD006519.

142. Ramakrishna BS, Venkataraman S, Srinivasan P, Dash P, Young GP, Binder HJ. Amylase-resistant starch plus oral rehydration solution for cholera. N Engl J Med. 2000 Feb 3;342(5):308–13.

143. Stoll BJ, Holmgren J, Bardhan PK, Huq I, Greenough WB, Fredman P, et al. Binding of intraluminal toxin in cholera: trial of GM1 ganglioside charcoal. Lancet. 1980 Oct 25;2(8200):888–91.

144. Fukuda M. Glycobiology. Elsevier; 2006. 1 p.

145. Varki A, Chrispeels MJ. Essentials of Glycobiology. CSHL Press; 1999. 1 p.

146. Reily C, Stewart TJ, Renfrow MB, Novak J. Glycosylation in health and disease. Nat Rev Nephrol. 2019 Jun;15(6):346–66.

147. Varki A. Sialic acids in human health and disease. Trends Mol Med. 2008 Aug;14(8):351–60.

148. Dell A, Galadari A, Sastre F, Hitchen P. Similarities and differences in the glycosylation mechanisms in prokaryotes and eukaryotes. Int J Microbiol. Hindawi; 2010;2010(7):148178–14.

149. Mellquist JL, Kasturi L, Spitalnik SL, Shakin-Eshleman SH. The amino acid following an asn-X-Ser/Thr sequon is an important determinant of N-linked core glycosylation efficiency. Biochemistry. American Chemical Society; 1998 May 12;37(19):6833–7.

150. Boyd B, Magnusson G, Zhiuyan Z, Lingwood CA. Lipid modulation of glycolipid receptor function. Availability of Gal(alpha 1-4)Gal disaccharide for verotoxin binding in natural and synthetic glycolipids. Eur J Biochem. John Wiley & Sons, Ltd (10.1111); 1994 Aug 1;223(3):873–8.

151. Chiricozzi E, Pomè DY, Maggioni M, Di Biase E, Parravicini C, Palazzolo L, et al. Role of the GM1 ganglioside oligosaccharide portion in the TrkA-dependent neurite sprouting in neuroblastoma cells. J Neurochem. 2017 Dec;143(6):645–59.

152. Kopitz J. Lipid glycosylation: a primer for histochemists and cell biologists. Histochem Cell Biol. Springer Berlin Heidelberg; 2017 Feb;147(2):175–98.

153. Turnbull WB, Precious BL, Homans SW. Dissecting the cholera toxin-ganglioside GM1 interaction by isothermal titration calorimetry. J Am Chem Soc. American Chemical Society; 2004 Feb 4;126(4):1047–54.

154. Monedero V, Buesa J, Rodríguez-Díaz J. The Interactions between Host Glycobiology, Bacterial Microbiota, and Viruses in the Gut. Viruses. Multidisciplinary Digital Publishing Institute; 2018 Feb 24;10(2):96.

155. Kuhlmann FM, Santhanam S, Kumar P, Luo Q, Ciorba MA, Fleckenstein JM. Blood Group O-Dependent Cellular Responses to Cholera Toxin: Parallel Clinical and Epidemiological Links to Severe Cholera. Am J Trop Med Hyg. The American Society of Tropical Medicine and Hygiene; 2016 Aug 3;95(2):440–3.

156. Becker DJ, Lowe JB. Fucose: biosynthesis and biological function in mammals. Glycobiology. 2003 Jul;13(7):41R–53R.

157. de Vries T, Knegtel RM, Holmes EH, Macher BA. Fucosyltransferases: structure/function studies. Glycobiology. 2001 Oct;11(10):119R–128R.

158. Ma B, Simala-Grant JL, Taylor DE. Fucosylation in prokaryotes and eukaryotes. Glycobiology.

2006 Dec;16(12):158R–184R.

159. Berg JM, Tymoczko JL, Stryer L. Biochemistry, Fifth Edition. W. H. Freeman; 2002. 1 p.

160. de Mattos LC. Structural diversity and biological importance of ABO, H, Lewis and secretor histo-blood group carbohydrates. Rev Bras Hematol Hemoter. 2016 Dec;38(4):331–40.

161. Jaff MS. Higher frequency of secretor phenotype in O blood group - its benefits in prevention and/or treatment of some diseases. Int J Nanomedicine. Dove Press; 2010 Nov 2;5:901–5.

162. Mottram L, Wiklund G, Larson G, Qadri F, Svennerholm A-M. FUT2 non-secretor status is associated with altered susceptibility to symptomatic enterotoxigenic Escherichia coli infection in Bangladeshis. Scientific Reports 2017 7. Nature Publishing Group; 2017 Sep 6;7(1):10649–7.

163. Li J, Hsu H-C, Mountz JD, Allen JG. Unmasking Fucosylation: from Cell Adhesion to Immune System Regulation and Diseases. Cell Chem Biol. 2018 May 17;25(5):499–512.

164. Ewald DR, Sumner SCJ. Blood type biochemistry and human disease. Wiley Interdiscip Rev Syst Biol Med. 3rd ed. 2016 Nov;8(6):517–35.

165. Mondal N, Dykstra B, Lee J, Ashline D, Reinhold VN, Rossi DJ, et al. Distinct human α(1,3)-fucosyltransferases drive Lewis-X/sialyl Lewis-X assembly in human cells. J Biol Chem. 2018 Mar 28;:jbc.RA117.000775.

166. Nishihara S, Iwasaki H, Kaneko M, Tawada A, Ito M, Narimatsu H. Alpha1,3-fucosyltransferase 9 (FUT9; Fuc-TIX) preferentially fucosylates the distal GlcNAc residue of polylactosamine chain while the other four alpha1,3FUT members preferentially fucosylate the inner GlcNAc residue. FEBS Lett. 1999 Dec 3;462(3):289–94.

167. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science. American Association for the Advancement of Science; 2015 Jan 23;347(6220):1260419–9.

168. Breimer ME, Hansson GC, Karlsson KA, Larson G, Leffler H. Glycosphingolipid composition of epithelial cells isolated along the villus axis of small intestine of a single human individual. Glycobiology. 2012 Nov 1;22(12):1721–30.

169. Williams SJ, Wreschner DH, Tran M, Eyre HJ, Sutherland GR, McGuckin MA. Muc13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. Journal of Biological Chemistry. American Society for Biochemistry and Molecular Biology; 2001 May 25;276(21):18327–36.

170. Gum JR, Crawley SC, Hicks JW, Szymkowski DE, Kim YS. MUC17, a novel membrane-tethered mucin. Biochemical and Biophysical Research Communications. 2002 Mar 1;291(3):466–75.

171. Varki A. Biological roles of glycans. Glycobiology. 2017 Jan;27(1):3–49.

172. Ravn V, Dabelsteen E. Tissue distribution of histo-blood group antigens. APMIS. John Wiley & Sons, Ltd (10.1111); 2000 Jan;108(1):1–28.

173. Björk S, Breimer ME, Hansson GC, Karlsson KA, Leffler H. Structures of blood group glycosphingolipids of human small intestine. A relation between the expression of fucolipids of epithelial cells and the ABO, Le and Se phenotype of the donor. Journal of Biological Chemistry. 1987 May 15;262(14):6758–65.

174. Goto Y, Uematsu S, Kiyono H. Epithelial glycosylation in gut homeostasis and inflammation. Nat Immunol. Nature Publishing Group; 2016 Oct 19;17(11):1244–51.

175. Croce MV, Isla-Larrain M, Rabassa ME, Demichelis S, Colussi AG, Crespo M, et al. Lewis x is highly expressed in normal tissues: a comparative immunohistochemical study and literature revision. Pathol Oncol Res. 2007;13(2):130–8.

176. Gampa A, Engen PA, Shobar R, Mutlu EA. Relationships between gastrointestinal microbiota

and blood group antigens. Physiol Genomics. American Physiological Society Bethesda, MD; 2017 Sep 1;49(9):473–83.

177. Yu Z-T, Chen C, Newburg DS. Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes. Glycobiology. 2013 Nov;23(11):1281–92.

178. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, Moreira CG, et al. Fucose sensing regulates bacterial intestinal colonization. Nature. Nature Publishing Group; 2012 Dec 6;492(7427):113–7.

179. Pham TAN, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, et al. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. Cell Host Microbe. 2014 Oct 8;16(4):504–16.

180. Johansson MEV, Hansson GC. Immunological aspects of intestinal mucus and mucins. Nat Rev Immunol. Nature Publishing Group; 2016 Oct;16(10):639–49.

181. Cornick S, Tawiah A, Chadee K. Roles and regulation of the mucus barrier in the gut. Tissue Barriers. Taylor & Francis; 2015;3(1-2):e982426.

182. Johansson MEV, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol. Nature Publishing Group; 2013 Jun;10(6):352–61.

183. Arike L, Hansson GC. The Densely O-Glycosylated MUC2 Mucin Protects the Intestine and Provides Food for the Commensal Bacteria. J Mol Biol. 2016 Aug 14;428(16):3221–9.

184. Arike L, Holmén-Larsson J, Hansson GC. Intestinal Muc2 mucin O-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases. Glycobiology. 2017 Apr 1;27(4):318–28.

185. Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics. American Society for Biochemistry and Molecular Biology; 2014 Feb;13(2):397–406.

186. Asker N, Axelsson MA, Olofsson SO, Hansson GC. Dimerization of the human MUC2 mucin in the endoplasmic reticulum is followed by a N-glycosylation-dependent transfer of the mono- and dimers to the Golgi apparatus. Journal of Biological Chemistry. 1998 Jul 24;273(30):18857–63.

187. Larsson JMH, Karlsson H, Sjövall H, Hansson GC. A complex, but uniform O-glycosylation of the human MUC2 mucin from colonic biopsies analyzed by nanoLC/MSn. Glycobiology. 2009 Jul;19(7):756–66.

188. Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol. 2012 Apr 11;10(5):323–35.

189. Muller WA. Getting leukocytes to the site of inflammation. Vet Pathol. 2013 Jan;50(1):7–22.

190. Bode L, Jantscher-Krenn E. Structure-function relationships of human milk oligosaccharides. Adv Nutr. 2012 May 1;3(3):383S–91S.

191. Wands AM, Fujita A, McCombs JE, Cervin J, Dedic B, Rodriguez AC, et al. Fucosylation and protein glycosylation create functional receptors for cholera toxin. Elife. eLife Sciences Publications Limited; 2015 Oct 29;4:e09545.

192. Samsen A, Bogoevska V, Klampe B, Bamberger A-M, Lucka L, Horst AK, et al. DC-SIGN and SRCL bind glycans of carcinoembryonic antigen (CEA) and CEA-related cell adhesion molecule 1 (CEACAM1): recombinant human glycan-binding receptors as analytical tools. Eur J Cell Biol. 2010 Jan;89(1):87–94.

193. Liu B, Newburg DS. Human milk glycoproteins protect infants against human pathogens. Breastfeed Med. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA; 2013 Aug;8(4):354–62.

194. Plaza-Díaz J, Fontana L, Gil A. Human Milk Oligosaccharides and Immune System Development. Nutrients. Multidisciplinary Digital Publishing Institute; 2018 Aug 8;10(8):1038.

195. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology. 2012 Sep;22(9):1147–62.

196. McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, et al. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. The American Journal of Clinical Nutrition. 2017 May 1;105(5):1086–100.

197. Murthy AK, Chaganty BKR, Troutman T, Guentzel MN, Yu J-J, Ali SK, et al. Mannose-containing oligosaccharides of non-specific human secretory immunoglobulin A mediate inhibition of Vibrio cholerae biofilm formation. Sturtevant J, editor. PLoS ONE. 2011 Feb 9;6(2):e16847.

198. Majumdar AS, Dutta P, Dutta D, Ghose AC. Antibacterial and antitoxin responses in the serum and milk of cholera patients. Infection and Immunity. American Society for Microbiology (ASM); 1981 Apr;32(1):1–8.

199. Azad MB, Robertson B, Atakora F, Becker AB, Subbarao P, Moraes TJ, et al. Human Milk Oligosaccharide Concentrations Are Associated with Multiple Fixed and Modifiable Maternal Characteristics, Environmental Factors, and Feeding Practices. J Nutr. 2018 Nov 1;148(11):1733–42.

200. Moossavi S, Atakora F, Miliku K, Sepehri S, Robertson B, Duan QL, et al. Integrated Analysis of Human Milk Microbiota With Oligosaccharides and Fatty Acids in the CHILD Cohort. Front Nutr. Frontiers; 2019;6:58.

201. Korpela K, Salonen A, Hickman B, Kunz C, Sprenger N, Kukkonen K, et al. Fucosylated oligosaccharides in mother's milk alleviate the effects of caesarean birth on infant gut microbiota. Scientific Reports 2017 7. Nature Publishing Group; 2018 Sep 13;8(1):13757–7.

202. Newburg DS. Human milk glycoconjugates that inhibit pathogens. Curr Med Chem. 1999 Feb;6(2):117–27.

203. Goehring KC, Marriage BJ, Oliver JS, Wilder JA, Barrett EG, Buck RH. Similar to Those Who Are Breastfed, Infants Fed a Formula Containing 2'-Fucosyllactose Have Lower Inflammatory Cytokines in a Randomized Controlled Trial. J Nutr. American Society for Nutrition; 2016 Dec;146(12):2559–66.

204. Triantis V, Bode L, van Neerven RJJ. Immunological Effects of Human Milk Oligosaccharides. Front Pediatr. Frontiers; 2018;6:190.

205. Garratty G, Glynn SA, McEntire R, Retrovirus Epidemiology Donor Study. ABO and Rh(D) phenotype frequencies of different racial/ethnic groups in the United States. Transfusion. John Wiley & Sons, Ltd (10.1111); 2004 May;44(5):703–6.

206. Lin AE, Autran CA, Szyszka A, Escajadillo T, Huang M, Godula K, et al. Human milk oligosaccharides inhibit growth of group B Streptococcus. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2017 Jul 7;292(27):11243–9.

207. Prudden AR, Liu L, Capicciotti CJ, Wolfert MA, Wang S, Gao Z, et al. Synthesis of asymmetrical multiantennary human milk oligosaccharides. Proc Natl Acad Sci USA. National Acad Sciences; 2017 Jul 3;114(27):6954–9.

208. El-Hawiet A, Kitova EN, Klassen JS. Recognition of human milk oligosaccharides by bacterial exotoxins. Glycobiology. 2015 Aug;25(8):845–54.

209. Morrow AL, Ruiz-Palacios GM, Jiang X, Newburg DS. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. J Nutr. 2005 May;135(5):1304–7.

210. Morozov V, Hansman G, Hanisch F-G, Schroten H, Kunz C. Human Milk Oligosaccharides as

Promising Antivirals. Mol Nutr Food Res. 2018 Mar;62(6):e1700679.

211. Glass RI, Holmgren J, Haley CE, Khan MR, Svennerholm AM, Stoll BJ, et al. Predisposition for cholera of individuals with O blood group. Possible evolutionary significance. Am J Epidemiol. 1985 Jun;121(6):791–6.

212. Holmner Å, Mackenzie A, Okvist M, Jansson L, Lebens M, Teneberg S, et al. Crystal structures exploring the origins of the broader specificity of escherichia coli heat-labile enterotoxin compared to cholera toxin. J Mol Biol. 2011 Feb 25;406(3):387–402.

213. Holmgren J, Fredman P, Lindblad M, Svennerholm AM, Svennerholm L. Rabbit intestinal glycoprotein receptor for Escherichia coli heat-labile enterotoxin lacking affinity for cholera toxin. Infection and Immunity. American Society for Microbiology (ASM); 1982 Nov;38(2):424–33.

214. Holmgren J, Lindblad M, Fredman P, Svennerholm L, Myrvold H. Comparison of receptors for cholera and Escherichia coli enterotoxins in human intestine. Gastroenterology. 1985 Jul;89(1):27–35.

215. Chiricozzi E, Mauri L, Ciampa MG, Prinetti A, Sonnino S. On the use of cholera toxin. Glycoconjugate J. Springer US; 2018 Apr;35(2):161–3.

216. Yu RK, Usuki S, Itokazu Y, Wu H-C. Novel GM1 ganglioside-like peptide mimics prevent the association of cholera toxin to human intestinal epithelial cells in vitro. Glycobiology. 2015 Sep 24;:cwv080–11.

217. Becker PM, Widjaja-Greefkes HCA, van Wikselaar PG. Inhibition of binding of the AB5-type enterotoxins LT-I and cholera toxin to ganglioside GM1 by galactose-rich dietary components. Foodborne Pathog Dis. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA; 2010 Mar;7(3):225–33.

218. Terada Y, Hoshino Y, Miura Y. Glycopolymers mimicking GM1 gangliosides: Cooperativity of galactose and neuraminic acid for cholera toxin recognition. Chem Asian J. John Wiley & Sons, Ltd; 2019 Feb 27.

219. Tuosto L, Parolini I, Schröder S, Sargiacomo M, Lanzavecchia A, Viola A. Organization of plasma membrane functional rafts upon T cell activation. Eur J Immunol. 2001 Feb;31(2):345–9.

220. Wolf AA, Jobling MG, Saslowsky DE, Kern E, Drake KR, Kenworthy AK, et al. Attenuated Endocytosis and Toxicity of a Mutant Cholera Toxin with Decreased Ability To Cluster Ganglioside GM1 Molecules. Infection and Immunity. 2008 Mar 18;76(4):1476–84.

221. Rivera FP, Medina AM, Bezada S, Valencia R, Bernal M, Meza R, et al. Bovine lactoferrin decreases cholera-toxin-induced intestinal fluid accumulation in mice by ganglioside interaction. Wu M, editor. PLoS ONE. Public Library of Science; 2013;8(4):e59253.

222. Holmner Å, Mackenzie A, Krengel U. Molecular basis of cholera blood-group dependence and implications for a world characterized by climate change. FEBS Lett. 2010 Jun 18;584(12):2548–55.

223. Bennun FR, Roth GA, Monferran CG, Cumar FA. Binding of cholera toxin to pig intestinal mucosa glycosphingolipids: relationship with the ABO blood group system. Infection and Immunity. American Society for Microbiology (ASM); 1989 Mar;57(3):969–74.

224. Monferran CG, Roth GA, Cumar FA. Inhibition of cholera toxin binding to membrane receptors by pig gastric mucin-derived glycopeptides: differential effect depending on the ABO blood group antigenic determinants. Infection and Immunity. American Society for Microbiology (ASM); 1990 Dec;58(12):3966–72.

225. Barra JL, Monferran CG, Balanzino LE, Cumar FA. Escherichia coli heat-labile enterotoxin preferentially interacts with blood group A-active glycolipids from pig intestinal mucosa and A- and B-active glycolipids from human red cells compared to H-active glycolipids. Mol Cell

Biochem. Kluwer Academic Publishers; 1992 Sep 22;115(1):63–70.

226. Balanzino LE, Barra JL, Galván EM, Roth GA, Monferran CG. Interaction of cholera toxin and Escherichia coli heat-labile enterotoxin with glycoconjugates from rabbit intestinal brush border membranes: relationship with ABH blood group determinants. Mol Cell Biochem. 1999 Apr;194(1-2):53–62.

227. Angström J, Bäckström M, Berntsson A, Karlsson N, Holmgren J, Karlsson KA, et al. Novel carbohydrate binding site recognizing blood group A and B determinants in a hybrid of cholera toxin and Escherichia coli heat-labile enterotoxin B-subunits. J Biol Chem. 2000 Feb 4;275(5):3231–8.

228. Satoh J, Kim SU. Differential expression of Lewis(x) and sialyl-Lewis(x) antigens in fetal human neural cells in culture. J Neurosci Res. 1994 Mar 1;37(4):466–74.

229. García-Vallejo JJ, Ilarregui JM, Kalay H, Chamorro S, Koning N, Unger WW, et al. CNS myelin induces regulatory functions of DC-SIGN-expressing, antigen-presenting cells via cognate interaction with MOG. J Exp Med. 2014 Jun 30;211(7):1465–83.

230. Lai B-Q, Qiu X-C, Zhang K, Zhang R-Y, Jin H, Li G, et al. Cholera Toxin B Subunit Shows Transneuronal Tracing after Injection in an Injured Sciatic Nerve. Di Giovanni S, editor. PLoS ONE. Public Library of Science; 2015;10(12):e0144030.

231. Patry RT, Stahl M, Perez-Munoz ME, Nothaft H, Wenzel CQ, Sacher JC, et al. Bacterial AB5 toxins inhibit the growth of gut bacteria by targeting ganglioside-like glycoconjugates. Nat Commun. 3rd ed. Nature Publishing Group; 2019 Mar 27;10(1):1390–13.

232. Almagro-Moreno S, Taylor RK. Cholera: Environmental Reservoirs and Impact on Disease Transmission. Atlas RM, Maloy S, editors. Microbiol Spectr. American Society of Microbiology; 2013 Dec;1(2):149–65.

233. Conner JG, Teschler JK, Jones CJ, Yildiz FH. Staying Alive: Vibrio cholerae's Cycle of Environmental Survival, Transmission, and Dissemination. Microbiol Spectr. American Society of Microbiology; 2016 Apr;4(2):593–633.

234. Matson JS. Infant Mouse Model of Vibrio cholerae Infection and Colonization. Methods Mol Biol. New York, NY: Springer New York; 2018;1839(Chapter 13):147–52.

235. Forsyth GW, Hamilton DL, Goertz KE, Johnson MR. Cholera toxin effects on fluid secretion, adenylate cyclase, and cyclic AMP in porcine small intestine. Infection and Immunity. American Society for Microbiology (ASM); 1978 Aug;21(2):373–80.

236. Triadafilopoulos G, Pothoulakis C, Weiss R, Giampaolo C, Lamont JT. Comparative study of Clostridium difficile toxin A and cholera toxin in rabbit ileum. Gastroenterology. 1989 Nov;97(5):1186–92.

237. Reddy S, Taylor M, Zhao M, Cherubin P, Geden S, Ray S, et al. Grape extracts inhibit multiple events in the cell biology of cholera intoxication. Ratner AJ, editor. PLoS ONE. 2013;8(9):e73390.

238. Watanabe K, Kato J, Zhu J, Oda H, Ishiwata-Endo H, Moss J. Enhanced sensitivity to cholera toxin in female ADP-ribosylarginine hydrolase (ARH1)-deficient mice. Roop RM, editor. PLoS ONE. 2018;13(11):e0207693.

239. Nygren E, Li B-L, Holmgren J, Attridge SR. Establishment of an adult mouse model for direct evaluation of the efficacy of vaccines against Vibrio cholerae. Infection and Immunity. 2009 Aug;77(8):3475–84.

240. Carpenter CC, Greenough WB. Response of the canine duodenum to intraluminal challenge with cholera exotoxin. Journal of Clinical Investigation. American Society for Clinical Investigation; 1968 Dec;47(12):2600–7.

241. Banwell JG, Pierce NF, Mitra RC, Brigham KL, Caranasos GJ, Keimowitz RI, et al. Intestinal fluid and electrolyte transport in human cholera. Journal of Clinical Investigation. American

Society for Clinical Investigation; 1970 Jan;49(1):183–95.

242. Liu K, Liu H, Zhang Z, Ye W, Xu X. The role of N-glycosylation in high glucose-induced upregulation of intercellular adhesion molecule-1 on bovine retinal endothelial cells. Acta Ophthalmol. John Wiley & Sons, Ltd (10.1111); 2016 Jun;94(4):353–7.

243. Hernández-Sánchez F, Guzmán-Beltrán S, Herrera MT, Gonzalez Y, Salgado M, Fabian G, et al. High glucose induces O-GlcNAc glycosylation of the vitamin D receptor (VDR) in THP1 cells and in human macrophages derived from monocytes. Cell Biol Int. 2017 Sep;41(9):1065–74.

244. Fukami K, Asano E, Ueda M, Sekiguchi F, Yoshida S, Kawabata A. High glucose induces N-linked glycosylation-mediated functional upregulation and overexpression of Cav3.2 T-type calcium channels in neuroendocrine-like differentiated human prostate cancer cells. J Pharmacol Sci. 2017 Jan;133(1):57–60.

245. Sato T, Clevers H. SnapShot: Growing Organoids from Stem Cells. Cell. 2015 Jun 18;161(7):1700–1.

246. Noel G, Baetz NW, Staab JF, Donowitz M, Kovbasnjuk O, Pasetti MF, et al. A primary human macrophage-enteroid co-culture model to investigate mucosal gut physiology and host-pathogen interactions. Scientific Reports 2017 7. Nature Publishing Group; 2017 Mar 27;7:45270.

247. Sato T, Stange DE, Ferrante M, Vries RGJ, Van Es JH, Van den Brink S, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology. 2011 Nov;141(5):1762–72.

248. Haksar D, de Poel E, van Ufford LQ, Bhatia S, Haag R, Beekman J, et al. Strong Inhibition of Cholera Toxin B Subunit by Affordable, Polymer-Based Multivalent Inhibitors. Bioconjugate Chemistry. American Chemical Society; 2019 Jan 24;:acs.bioconjchem.8b00902.

249. Zomer-van Ommen DD, Pukin AV, Fu O, Quarles van Ufford LHC, Janssens HM, Beekman JM, et al. Functional Characterization of Cholera Toxin Inhibitors Using Human Intestinal Organoids. J Med Chem. American Chemical Society; 2016 Jul 28;59(14):6968–72.

250. Tabrizi NM, Amani J, Ebrahimzadeh M, Nazarian S, Kazemi R, Almasian P. Preparation and evaluation of chitosan nanoparticles containing CtxB antigen against Vibrio cholera. Microb Pathog. 2018 Nov;124:170–7.

251. Björkman E, Casselbrant A, Lundberg S, Fändriks L. In vitroassessment of epithelial electrical resistance in human esophageal and jejunal mucosae and in Caco-2 cell layers. Scandinavian Journal of Gastroenterology. 2012 Aug 31;47(11):1321–33.

252. Heim JB, Hodnik V, Heggelund JE, Anderluh G, Krengel U. Crystal structures of cholera toxin in complex with fucosylated receptors point to importance of secondary binding site. Scientific Reports 2017 7. Nature Publishing Group; 2019 Aug 22;9(1):12243–14.

253. Cervin J, Wands AM, Casselbrant A, Wu H, Krishnamurthy S, Cvjetkovic A, et al. GM1 ganglioside-independent intoxication by Cholera toxin. Coombes BK, editor. PLoS Pathog. 2018 Feb;14(2):e1006862.

254. Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, et al. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. The Journal of Pediatrics. 2004 Sep;145(3):297–303.

255. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr. Annual Reviews; 2005;25(1):37–58.

256. Holmgren J, Lindholm L, Lönnroth I. Interaction of cholera toxin and toxin derivatives with lymphocytes. I. Binding properties and interference with lectin-induced cellular stimulation. J Exp Med. 1974 Apr 1;139(4):801–19.