I, Sayali Karve, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Molecular Genetics, Biochemistry, & Microbiology.

It is entitled:
Structural and signaling aspects of Shiga toxin

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Abstract

Shiga toxin (Stx) producing *E. coli* (STEC) is a major cause of foodborne illnesses. Approximately 265,000 cases of STEC infections are reported annually in the United States, of which ~10% lead to the life-threatening complication, hemolytic uremic syndrome (HUS). The major virulence factor of STEC is Stx, an AB₅ toxin that consists of a single A-subunit with ribosomal RNA-cleaving activity, surrounded by a receptor-binding B-pentamer. Two major isoforms, Stx1 and Stx2, and Stx2 variants (Stx2a-h) significantly differ in toxicity. The reason for this toxicity difference is unknown, however different receptor binding preferences are speculated to be important. Previous studies reported binding of Stx1 and Stx2a toxoids to glycolipid receptors. We studied binding of holotoxin and B-subunits of Stx1, Stx2a, Stx2b, Stx2c and Stx2d to glycolipid receptors globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) in the presence of cell membrane components such as phosphatidylcholine (PC), cholesterol (Ch) and other neutral glycolipids. In the absence of PC and Ch, holotoxins of Stx2 variants bound to mixtures of Gb3 with other glycolipids but not to Gb3 or Gb4 alone. Binding of all holotoxins significantly increased in presence of PC and Ch. Stx2a has been previously shown to form a less stable B-pentamer compared to Stx1. However, its effect on receptor binding is unknown. We showed that the more stable B-pentamer of Stx1 bound better to glycolipids than the less stable B-pentamer of Stx2a. However, B-subunit mutant of Stx1 L41Q, which shows similar stability as Stx2a B-subunits, lacked glycolipid binding, suggesting that pentamerization is more critical for glycolipid binding of Stx1 than Stx2a.

STEC colonize the gastrointestinal tract and produce Stx. The mechanism by which Stx crosses the intestinal epithelial barrier to reach systemic circulation is unknown, since until
recently no animal or cellular models were available that closely resembled the human intestine. We investigated stem cell derived ‘induced human intestinal organoids’ (iHIO), which maintain the human intestinal architecture and function, to study the intestinal actions of Stx and STEC. Purified Stx2a and Stx2a producing *E. coli* were microinjected into iHIO lumens. As negative controls, phosphate buffered saline (PBS), inactivated Stx2a toxoid, and non-pathogenic ECOR13 strain of *E. coli* were microinjected. iHIO treated with PBS and Stx2a toxoid remained intact; however, Stx2a caused loss of iHIO epithelial barrier function. Cryosections of PBS treated iHIO displayed well-defined lumens and intercellular E-cadherin, whereas luminal extrusion of cells and altered E-cadherin localization was observed for Stx2a treated iHIO. iHIO supported rapid growth of *E. coli*. Intact iHIO were observed after infection with ECOR13, whereas STEC caused a very rapid and severe damage, resulting in loss of 3-dimensional arrangement of iHIO. In case of ECOR13, cryosections revealed short rods of *E. coli* in lumens, surrounded by a well-defined epithelial layer with intercellular E-cadherin and apical F-actin. Conversely, STEC treated iHIO displayed cryosections filled with filamentous *E. coli*, damaged epithelial cells displaying mislocalized E-cadherin and F-actin. Overall these results suggest that iHIO are sensitive to Stx and STEC, and thus represent the first humanized model to study intestinal interaction of STEC.
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<tr>
<td>STEC</td>
<td>Shiga toxin producing <em>E. coli</em></td>
</tr>
<tr>
<td>Stx</td>
<td>Shiga toxin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>HC</td>
<td>Hemorrhagic colitis</td>
</tr>
<tr>
<td>HUS</td>
<td>Hemolytic uremic syndrome</td>
</tr>
<tr>
<td>GSL</td>
<td>Glycosphingolipid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Dissociation constant</td>
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<tr>
<td>Gb3</td>
<td>Globotriaosylceramide</td>
</tr>
<tr>
<td>Gb4</td>
<td>Globotetraosylceramide</td>
</tr>
<tr>
<td>NMR</td>
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</tr>
<tr>
<td>TLR4</td>
<td>Toll like receptor 4</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human microvascular endothelial cells</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% lethal dose</td>
</tr>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% effective dose</td>
</tr>
<tr>
<td>ZO</td>
<td>Zona occludens</td>
</tr>
<tr>
<td>A/E</td>
<td>Attaching and effacing</td>
</tr>
<tr>
<td>LEE</td>
<td>Locus of enterocyte effacement</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type-3 section system</td>
</tr>
<tr>
<td>Tir</td>
<td>Translocated intimin receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated protein degradation pathway</td>
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<tr>
<td>JNK1</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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<tr>
<td>PLD1</td>
<td>Phospholipase D1</td>
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<tr>
<td>AUC</td>
<td>Analytical ultracentrifugation</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>BLI</td>
<td>Biolayer interferometry</td>
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<td>BEI</td>
<td>Biodefense and Emerging Infections Research Resources Repository</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>Ch</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
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<td>Bmax</td>
<td>Maximum binding</td>
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<td>h-value</td>
<td>Hill coefficient value</td>
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<td>Lyso-Gb3</td>
<td>Deacetylated Gb3</td>
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<tr>
<td>iHIO</td>
<td>Induced human intestinal organoids</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential media</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s modified eagle medium/ Ham’s F-12</td>
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<td>BCA</td>
<td>Bicinchoninic assay</td>
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Chapter 1.

Shiga toxin literature review
A. Background

Overview of Shiga toxin producing *E. coli*: Shiga toxin-producing *E. coli* (STEC) is a frequent cause of food and water borne gastrointestinal symptoms in humans with a substantial risk for more serious complications (2). STEC cause approximately 265,000 illnesses, 3,000 hospitalizations and 90 deaths annually in the United States (3). The virulence factor common to all STEC infections is a multi-subunit protein exotoxin called as Shiga toxin (Stx) (*Figure 1.1*), which is responsible for majority of the complications associated with STEC (4).

*E. coli* serotypes are classified based on the type of O and H antigens. The O antigen is the somatic antigen, determined by the repeating lipopolysaccharide (LPS) chains in the outer membrane. The H antigen is unique to the bacterial flagellum. Several in vitro and in vivo studies suggest that LPS contributes to STEC virulence. LPS has been shown to interact with host immune, and renal cells. This leads to proinflammatory cytokine and chemokine response, thereby exacerbating STEC pathogenesis (4, 5). The most prevalent STEC serotype is O157:H7. However, several studies suggest that up to 50% of STEC infections are caused by serotypes other than O157, of which there are over 100 (6-11). Recently, Food Safety and Inspection Service of the U. S. Department of Agriculture declared the non-O157 strains, O104, O145, O111, O45, O26 and O121 (also known as the “Big Six’) as adulterants (12). The diversity of different STEC serotypes in recent outbreaks is evident from *Table 1.1* (13-19).

Disease Progression and pathogenesis: STEC are zoonotic, for which ruminant animals such as cattle, sheep, deer and elk, are the natural reservoirs. These animals can harbor STEC in their guts asymptptomatically (20-22). The major source of human illness is cattle. Human infections can generally be traced to contamination of food or water with cattle manure.
Interestingly, cattle are not susceptible to STEC pathogenesis. O157:H7 have been primarily demonstrated to colonize at the recto-anal junction in the cattle, which is thought to promote asymptomatic long-term carriage of the bacteria (23, 24).

<table>
<thead>
<tr>
<th>Year of outbreak</th>
<th>Country</th>
<th>STEC serotype</th>
<th>Source</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>USA</td>
<td>O157:H7</td>
<td>Ground beef</td>
<td>19</td>
</tr>
<tr>
<td>2013</td>
<td>USA</td>
<td>O121</td>
<td>Frozen foods</td>
<td>35</td>
</tr>
<tr>
<td>2012</td>
<td>USA</td>
<td>O26</td>
<td>Clover sprouts</td>
<td>29</td>
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<tr>
<td>2011</td>
<td>Germany</td>
<td>O104:H4</td>
<td>Raw sprouts</td>
<td>852</td>
</tr>
<tr>
<td>2010</td>
<td>USA</td>
<td>O145</td>
<td>Lettuce</td>
<td>32</td>
</tr>
<tr>
<td>2009</td>
<td>Wales</td>
<td>O157:H7</td>
<td>Fast food outlet</td>
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<tr>
<td>2008</td>
<td>USA</td>
<td>O157:H7</td>
<td>Kroger Beef</td>
<td>49</td>
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</table>

Infections in humans begin with consumption of STEC contaminated food such as undercooked meat, unpasteurized juice, raw milk and raw vegetables. The clinical manifestations of STEC infections range from mild non-bloody diarrhea to more severe complications such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and death. The average interval between exposure and illness is about 5-7 days. Over 70% of patients report bloody diarrhea. Of these, about 3-7% develop the fatal disorder HUS. HUS is characterized by a triad of symptoms, which include acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia.
Reports have shown that the mortality rate of HUS is as high as 87% in children and elderly. 5-10% of the patients that resolve from HUS can experience permanent renal and neurologic sequelae (25-27).

**Shiga toxin genetics:** In *E. coli*, the genes encoding Stx are exclusively associated with lambdoid prophage (28-30). These stx genes are located in the late gene region of the phage chromosome and are under the control of the phage late gene promoter (31). As a result, the basal expression of Stx is very low and it is only expressed during the phage lytic cycle (32). When the lysogenized host bacterial cell undergoes stress such as DNA damage, the cellular stress response (also called as the ‘SOS’ response) activates the phage lytic cycle. This results in phage production coupled with Stx expression. Host intestinal antimicrobial response including production of reactive oxygen species, H$_2$O$_2$ (33) and antibiotics that target DNA synthesis, for example, ciprofloxacin and trimethoprim-sulfamethoxazole are capable of inducing the SOS response in *E. coli*. As a result, antibiotics complicate the treatment of STEC infections and have been reported to result in a more severe form of the disease (34). For STEC, on the other hand, the phage lysis is advantageous since it provides a metabolically and energetically inexpensive way of Stx secretion. The Stx-phages vary in size and gene composition. STEC are capable of carrying multiple Stx-phages, leading to production of several Stx variants. The presence of multiple Stx-encoding phages together in a single cell also increases the likelihood of genetic recombination between the phages, increasing the potential for creating new phages (35). The Stx-encoding phages are capable of lateral transfer of stx genes (36). During STEC infection, lysis of the *E. coli* cells and release of Stx, and Stx-encoding phage occur in the intestine. These
phages have the ability to infect the susceptible commensal *E. coli* in the intestine, resulting in amplified toxin production, further complicating the treatment of these infections (37, 38).

**Treatment of STEC Infections:** Currently there is no specific treatment of STEC infections. Centers for Disease Control and Prevention suggests supportive therapy, including hydration, hemodialysis or peritoneal dialysis for HUS. Antibiotics, especially DNA inhibitors such as ciprofloxacin, cannot be used to treat this infection, since it may increase the risk of HUS. Novel strategies are being designed for disease prevention or amelioration, as follows:

**a) Neutralization of Stx:** Since Stx is principally responsible for the clinical manifestations of STEC, in vivo neutralization of Stx is being investigated as a potential therapeutic strategy. Approaches exploiting the high specificity and strength of interaction between Stx and its glycosphingolipid (GSL) receptors are underway. An example of this type of agent is Synsorb-Pk, made up of the Stx receptor-mimicking trisaccharide galactose-galactose-glucose, covalently linked to silica via an 8-carbon spacer. The agent, developed by Armstrong et al, showed the ability to neutralize Stx from patient intestinal samples (39, 40). Unfortunately, oral administration of drug after the diagnosis of HUS could not ameliorate the disease course in the clinical trials (41). A limitation of orally administered Stx-neutralizing agents is that they might not be active against Stx that has already entered the systemic circulation. This gives these agents a very narrow therapeutic window of opportunity. More advanced, multi-branched Stx receptor analogs and polymers are being developed with the therapeutic potential for oral and intravenous application (42, 43). Another hurdle in the development of Stx-neutralizing agents is the production of agents with higher affinity for Stx than its receptor. This is particularly a problem for Stx2, which displays unique receptor recognition, as will be discussed later.
Moreover, the Stx neutralizing strategies have the potential to limit the severity or duration of the disease symptoms, but cannot be expected to reduce STEC transmission.

b) Vaccines: Considering the quick course of disease progression and unavailability of efficient diagnostic tests for STEC infections, developing vaccines against either STEC or Stx seems as an attractive approach. Since cattle are the reservoir of most STEC infections, vaccines that prevent colonization of STEC in the cattle are being developed. A currently available cattle vaccine consists of an O157:H7 bacterial extract. In a field trial, cattle immunized with this extract were shown to shed fewer O157:H7 than the controls (44). Although a vaccination strategy for humans has not been widely developed yet, efforts are being made to make safe and successful STEC/Stx vaccines for humans. Immunization with catalytically inactive Stx2e toxoid has been shown to protect piglets from the disease after an oral challenge with Stx2e-producing bacteria (45). Rabinovitz, et al. showed that systemic immunization of pregnant cows with catalytically inactivated Stx2 toxoid yielded bovine colostrum with increased levels of antibodies against O157:H7. These specific antibodies could be efficiently transferred to newborn calves by feeding them colostrum (46).

c) Stx antibodies: Humanized mouse monoclonal antibodies (mAbs) directed against the A- and B-subunits of Stx are currently being evaluated. Administration of Stx mAbs has been shown to rescue mice after an oral challenge of Stx2-producing *E. coli* (47). The antibodies are well tolerated in healthy adults and children with early STEC infections and a single injection at the beginning of symptoms is expected to last until the onset of HUS (48, 49).
**B. Shiga toxin- structural aspects**

**Structure and Function:** The Stx family of toxins is a group of structurally and functionally related protein toxins, which include Stx from *Shigella dysenteriae*-*I* and Stx produced from STEC strains (50). The toxins belong to the AB\(_5\) class of toxins, with an enzymatically active, monomeric A-subunit (MW: 32 kDa), which is non-covalently attached to the pentameric, binding B-subunit (Monomer MW: 7.8 kDa). The pentamer of identical B-subunits forms a doughnut shaped structure into which the C-terminus of Stx A-subunit inserts (Figure 1.1) (51).

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**Figure 1.1: Structure of Stx:** a. Pictorial representation of Stx holotoxin, showing Stx A-subunit with A\(_1\) (red) and A\(_2\) (green) fragments attached to pentamer of B subunits (blue). b. Cartoon representation of Stx1 crystal structure (PDB: 1DMO) c. Cartoon representation of Stx2 crystal structure (PDB: 1r4p).

The B subunit of Stx allows the toxin to bind to the host cell surface receptors, followed by its internalization. This enables the entry of the A-subunit across the cellular membranes. Once inside the cell, the A-subunit is cleaved by furin-like proteases into two fragments A\(_1\) and A\(_2\) (Figure 1.1) (52). The Stx A\(_1\)-fragment undergoes retrograde transport, eventually entering
the cytoplasm. The A₁ fragment possesses a specific N-glycosidase activity that acts to cleave an adenine residue at position 4324 on the sarcin/ricin loop of the 28S rRNA. The destruction of the 28S rRNA inhibits elongation factor-dependent amino acyl tRNA binding and subsequent amino acid chain elongation, rendering the ribosome incapable of further protein synthesis, resulting in cell death (53, 54).

**Shiga Toxin Variants:** Two immunologically distinct isoforms of Stx: Stx1 and Stx2 share about 57% amino acid sequence identity and an overall conserved structure (Figures 1.1b and 1.1c). Stx2 is further subtyped into 8 variants, Stx2a to Stx2h, based on the nucleotide and amino acid similarity (55, 56). In spite of the high structural similarity, these variants significantly differ in toxicity, with Stx2a being over 100-fold more toxic to mice than Stx1, and variant isoform Stx2b. STEC strains can express one or more Stx variants. However, strains producing Stx2a, Stx2c and Stx2d are more commonly associated with HUS in humans than those producing Stx1 or Stx2b (55). Stx2e, which is known to use different cellular receptors than the other the Stx variants, is not associated with human disease but causes fetal edema disease in neonatal piglets (57). The molecular basis of this difference in toxicities between the Stx variants is still not clear. Previous experiments with cell-free in vitro translation inhibition assays demonstrated that the A-subunits of Stx variants display similar activity, suggesting that the enzymatic activities of the A-subunits may not be responsible for the observed differences in cellular and animal toxicities (58). A study by Weinstein et al. suggested that the B-subunits determined cytotoxic specificities and localization of the toxin within the host cell, indicating that the toxicities could be related to B-subunits of the Stx variants (59). Analysis of the B-subunits of the Stx variants reveals differences in the regions involved in receptor binding,
suggesting that differential cell surface binding of Stx variants could be responsible for the
difference in cellular toxicity (Figure 1.2). In addition to the B-pentamer, the A2-subunit of Stx
is also thought to play a role in the cellular binding of Stx. This is especially true in the case of
Stx2. Unlike Stx1 the C-terminus of Stx2 A-subunit extends through the pore formed by the B-
pentamer (Figure 1.1c, shown in green. Note that Stx1 A-subunit does not extend through
the pore formed by the B-pentamer in Figure 1.1b). The extended portion of the A2-subunit
could affect the receptor interactions of the B-subunits. Cleavage of two terminal amino acids
from the C-terminus of the A2-subunit of Stx2d was reported to occur in the human intestine by
intestinal elastase, leading to activation of Stx2d. Infection studies in mice suggested that
removal of these C-terminal amino acids makes the B-pentamer more accessible to interact with
the cell surface receptors, resulting in increased potency (60-62). However, Fuller et al. reported
that the elastase treated form of Stx2d did not display a significant increase in potency when
purified toxin was administered by intraperitoneal injection to mice (55). Interestingly, in recent
binding studies Stx2d holotoxin preferred binding to the entire glycolipid receptor instead of just
the glycan portion, while the B-subunits of Stx2d showed similar binding to both glycolipids and
glycans, suggesting a role of A-subunit in mediating receptor interaction of Stx2d (63, 64).

Figure 1.2 shows the structural model of Stx2a B-subunit with each individual subunit
displaying polymorphisms observed in the Stx variants (Stx1 and Stx2b-2d), built using the
mutagenesis function of PYMOL on the Stx2a crystal structure (PDB: 1r4p). The structure is
oriented to display the receptor binding face. Although the Stx2 variants share extensive amino
acid similarity, it can be observed that several of the amino acid polymorphisms map to or near
the predicted receptor binding sites. This suggests that these polymorphisms could influence the
binding of the Stx variants to the cell surface receptors, eventually resulting in their different cellular toxicities.
Figure 1.2: Structural models and sequence alignments of B-subunits of Stx variants. The structures are oriented to show the receptor-binding side of Stx2a B-subunit with individual subunit displaying polymorphisms (shown in pink) found in the Stx variants with respect to Stx2a and are color coded as follows: Stx2a: green; Stx2b: yellow; Stx2c: blue; Stx2d: grey; Stx1: wheat; Stx2a A-tail: red. **a. Cartoon representation. b. Surface representation. c. Sequence alignment of Stx B-subunits.** Sequences were aligned using BLASTP (NCBI/BLAST), with periods indicating identity and dashes indicating absent amino acids. Numbering starts with the first amino acid of the mature peptide; numbering at the top corresponds to Stx2 variants; numbering at the bottom corresponds to Stx1 B-subunit. Amino acid polymorphisms are shown in red. Pk binding residues of Stx1 B-subunit have been highlighted and are color coded as follows: binding site 1, yellow; binding site 2, blue; binding site 3, green.
The Stx B-subunits are known to differ in their subunit stabilities. A study by Pina et al on the structural stability of Stx B-subunits as a function of temperature, protein concentration, pH and denaturant concentration demonstrates that the heat induced by unfolding of Stx B-subunits was a highly co-operative, two-state reversible process, populated predominantly by folded pentamers and unfolded monomers (65, 66). Kitova et al reported that the B-subunits of Stx1 were primarily thermostable pentamers at concentrations ranging from 5 – 85 µM. On the other hand, studies with B-subunits of Stx2a showed the presence of dimer, trimer, tetramer, and pentamer species at 65 µM, with predominantly lower-order species present at lower B-subunit concentrations (67, 68). Conrady et al confirmed these results using analytical ultracentrifugation (AUC). Whereas the molar concentration to achieve 50% pentamer assembly (EC$_{50}$) for Stx1 B-subunits was found to be 0.043 µM, Stx2a B-subunits showed the EC$_{50}$ value of 2.29 µM, indicating approximately 50-fold higher stability for the B-subunits of Stx1 compared to Stx2a in solution state (1). Additionally, using X-ray crystallography, Conrady et al. demonstrated that while Stx1 has mostly hydrophobic amino acids at the B-subunit interface, Stx2a has a polar destabilizing glutamine in an otherwise hydrophobic region at the B-subunit interface. Reverse mutations of these residues in Stx1 (L41Q) and Stx2a (Q40L) demonstrated a phenotypic reversal. Whereas Stx1 L41Q was now unstable (EC$_{50}$ of 0.69 µM compared to 0.0043 µM for WT Stx1), Stx2 Q40L produced a stable pentamer (EC$_{50}$ 0.11 µM, compared to 2.3 µM of WT Stx2) (1). Q40 has been found to be conserved among all the Stx2 variants, making it highly likely that the destabilization of the B-pentamer might impart a selective advantage to Stx2.

**Cellular receptors:** Shiga toxins are known to use the glycosphingolipids (GSL), present in the cell membrane, as their receptors. The best characterized receptor for Stx is
globotriaosylceramide (Gb3; also known as CD77) (69). Gb3 contains a tri-saccharide called as the Pk trisaccharide, made up of galactose $\alpha[1-4]$-galactose-$\beta[1-4]$-glucose. This is attached to the ceramide portion and a fatty acid chain (Figure 1.3). The ceramide portion of Gb3 is made up of a relatively invariant 18-carbon atom long mono-unsaturated sphingosine chain. The fatty acid chain varies in length, with the typical length in the range of 16-24 carbon atoms. Mice lacking the Gb3 synthase gene, which synthesizes Gb3 from lactosylceramide, have been shown to be completely resistant to Stx1 and Stx2a (70). Stx2e, on the contrary, is known to use globotetraosylceramide (Gb4) as the primary receptor, which is made up of N-Acetyl galactose $\beta[1-3]$-galactose $\alpha[1-4]$-galactose $\beta[1-4]$-glucose (71). These GSLs are primarily found in the microdomains of the cell membrane known as lipid rafts. Lipid rafts are evolutionarily conserved sphingolipid and cholesterol-rich structures that play roles in cell signaling. The spatial organization of GSLs, phosphatidylcholine and cholesterol in the lipid rafts plays an important role in the interaction of Stx with the cell membrane (72).

The expression of Gb3 and Gb4 in humans is restricted to certain cell types and is most commonly observed in the cells of kidney, intestine, central and peripheral nervous systems (73-
The binding of Stx to Gb3 is not only dependent on the number of Gb3 molecules present but also on the availability of other Gb3 isoforms and the plasma membrane components, as well as on the orientation of Gb3. A mixture of Gb3 with other membrane components such as phosphatidylcholine and cholesterol has been demonstrated to provide higher Stx affinity than Gb3 alone (76). The lipid moiety of Gb3 also affects toxin binding. Optimal binding to Gb3 has been shown to depend on the fatty acid chain length of Gb3 (77, 78). Binding of Stx2 has been demonstrated to be more dependent on the ceramide portion of Gb3 as compared to Stx1 (76).

Ling et al. determined the crystal structure of Stx1 B-subunit with the sugar portion (Pk) of Gb3 (79). The structure revealed that each B-monomer contains three Gb3 binding sites, suggesting that the holotoxin can crosslink up to 15 Gb3 molecules. This partly explains the high affinity of Stx for Gb3. All 15 Gb3-binding sites in the Stx1 B-subunit were present on the face of the B-pentamer distal to the A-subunit binding site, thus indicating the membrane interaction surface (Figure 1.4). Binding of Stx1 B-subunit to Pk is primarily mediated by weak hydrogen bonds between polar amino acids and hydroxyl residues on the sugar.

Binding site 1 in Stx1 is made up of residues from the adjacent B-monomers (Figures 1.2c and 1.4, shown in yellow), an interaction possible only in higher order B-subunit oligomers. The Pk trisaccharide is located perpendicular to the binding site 1. At this site, the amino acid residues Asp17, Thr21, Glu28 and Gly60 have been predicted to form hydrogen-binding interactions with Galα and Galβ of the Pk trisaccharide. In addition, a hydrophobic stacking interaction can be seen between the residue Phe30 and the B-face of Galβ. Binding site 2 (Figures 1.2c and 1.4, shown in blue) displays the highest occupancy, with electron density defining the position of the entire trisaccharide. This site is in an elongated shallow cleft and the Pk trisaccharide is oriented approximately parallel to the protein surface at this site, allowing a
direct interaction of all the three sugars of Pk with the protein. Binding site 2 involves residues Asp16, Asn32, Arg33, Asn55 and Phe63 forming hydrogen bonds with Gal1 and Gal2. Hydrophobic interactions can also be predicted from the crystal structure involving Thr1, Phe30, Thr31, Thr54, Asn55, Ala56, Gly62 and Ser64 residues and all the three sugars, Gal1, Gal2 and Glc of the Pk trisaccharide. Binding site 3 shows fewer contacts between Pk and the protein (Figures 1.2c and 1.4, shown in green). The hydrogen bonding interactions at site 3 involve residues Asp18, Trp34 and Asn35 bound to Gal1. Trp34 also forms hydrophobic interactions with Gal1. At this site, one face of indole ring of Trp34 interacts with Gal1 of the neighboring subunit and the other face stacks onto Gal2. Several site directed mutagenesis studies showed that Pk trisaccharide-binding sites 1 and 2 are more relevant for cytotoxicity and involve higher affinity binding than site 3, which mediates the low affinity Pk binding epitopes (80-82). Similarly NMR spectroscopy and fourier transform ion cyclotron resonance electrospray mass spectrometry studies showed that the affinity of Stx1 site 2 for the Pk trisaccharide was ~10 fold higher than for site 1, while site 3 was barely occupied in solution (67).

Several mutational analyses have been performed to determine the importance of the Gb3-binding sites for the interaction of Stx with Gb3 (83-85). However, interpretation of these mutational studies has been complicated. Many binding studies have been performed using only the sugar portion of Gb3, whereas the fatty acid chain is also known to be required for optimal binding. As a result, direct interpretation of these artificial binding conditions using soluble forms of Gb3 might result in misleading conclusions about the biological relevance of the binding sites.
Figure 1.4: Stx1 B-subunit (surface representation) bound to Pk trisaccharide (red sticks) (PDB: 1BOS). Gb3 binding sites are color coded as follows: site 1, yellow; site 2, blue; green, site 3, green.
The crystal structure of Stx2a holotoxin bound to a disaccharide analogue of Gb3, N-acetyl galactose α[1-4]-galactose, was recently published (86). In this disaccharide Gb3 analogue, α-GalNAc replaces the terminal α-Gal residue of Gb3. In spite of the high similarity between the B-subunits of Stx1 and Stx2a, the disaccharide Gb3 analogue occupied only 2 of the 15 Gb3 binding sites in Stx2a holotoxin predicted based on homology to Stx1 (Figure 1.5). In this crystal structure, the interactions between disaccharide and the Stx2a site 1 are similar to those observed between Pk trisaccharide and Stx1 B-pentamer binding site 1, where 7 of 8 residues are conserved. (Lys12, Asn14, Asp16, Thr18, Thr20, Glu27, Trp29, and Gly59) and Trp29 is substituted by Phe30. Stx2a binding site 2 is made up of 9 residues (Trp29, Ser31, Arg32, Ser53, Ser54, Thr55, Gly61, Phe62, Ala63) from one B-subunit, and Glu15 from the neighboring B-subunit. The orientation of disaccharide at site 2 of Stx2a is similar to that of Pk trisaccharide in the site 2 of Stx1. However, binding site 2 in Stx2a displays more polar and van der Waals interactions with the disaccharide than in site 2 of the Stx1-Pk trisaccharide complex.

Binding of carbohydrate in only two sites instead of predicted 15 sites was attributed to steric hindrance from C-terminus of A-subunit to the centrally located binding site 3 in Stx1 B-subunit-Pk trisaccharide crystal structure. This site is close to the B-pentamer pore and involves residues from adjacent B-subunits. As a result, the binding at this site could be easily occluded by the protruding A-tail of Stx2a, which is absent in Stx1.

Storck et al investigated the expression of Gb3 and Gb4 in human pancreatic ductal adenocarcinoma cell lines (87). They found no correlation between amount of Gb3 expression in these cells and sensitivity towards otherwise lethal concentrations of Stx2a, suggesting that the amount of Gb3 present on the cell surface does not solely mediate the cellular sensitivity of Stx. Existence of protein receptors for Stx, in addition to the GSLs, seems plausible. Both Stx1 and
Stx2 have been speculated to interact with proteins at the cell surface. Although the identity of these proteins is not known, surface binding of Stx has been shown to play a role in signal transduction and endocytosis of the toxin. In a study by Torgersen et al, Toll-like receptor 4 (TLR4) was identified as a co-receptor that facilitated the binding of Stx1 to Gb3. Depletion with siRNA of either TLR4 or Gb3 synthase markedly inhibited the cell surface binding and endocytosis of Stx1 holotoxin and B-subunit in SW480 colon carcinoma cells and primary HUVECs (88). This study has been reported using Stx1, and the relevance to Stx2 was not reported.

**Figure 1.5: Stx2a holotoxin bound to a diaccharide analogue of Gb3** (red sticks) (PDB: 4M1U). Stx2a B-pentamer is shown in wheat, while the protruding A-tail is shown in red. Binding sites are color-coded as follows: site 1, yellow; site 2, blue.
There is a huge disparity between the in vitro receptor binding data and in vivo toxicity of Stx. The reported $K_D$ (50% maximum binding) values of Stx1 and Stx2 to Gb3 (89, 90) generally range between $10^{-7}$ M and $10^{-9}$ M. The concentration of toxin in blood at 50% lethal dose ($LD_{50}$) in mice is approximately $10^{-9}$ M for Stx1 and $10^{-10}$ M for Stx2 (91). However, both Stx1 and Stx2 cause protein synthesis inhibition in primary human renal proximal tubular epithelial cells with a 50% effective dose ($ED_{50}$) of about $10^{-13}$ M and to the Vero monkey kidney cell line with an $ED_{50}$ of about $10^{-11}$ M, suggesting that the cellular binding of Stx is more efficient than binding solely to the GSLs. Gallegos et al. studied the ability of GSLs to neutralize the cellular toxicity of Stx to Vero cells. Although pre-incubation of Stx1 with Gb3 in the presence of phosphatidylcholine and cholesterol conferred protection to Vero cells, no significant loss of Stx2 toxicity was observed upon pre-incubation of the toxin with either Gb3 or Gb4, further indicating the possibility of a co-receptor, in addition to Gb3 (76). Such a dual receptor strategy has been previously observed with botulinum toxin, which uses both glycolipid and protein receptors to gain entry into the cell (92).
C. Shiga toxin and human intestine

Composition of the human intestine: The intestine interfaces with the outside world, serving to protect the body, and absorbing and digesting nutrients. The small and large intestines form a continuous lumen that is lined with a single layer of epithelial cells creating the largest of the body’s mucosal surfaces, covering approximately 300 m² area (93). The small intestine is divided into three parts, the duodenum being closest to the stomach, followed by jejunum, and then ileum. The large intestine begins at caecum, followed by ascending colon, transverse colon, descending colon, and then rectum. Different regions of the intestine have specific physiologic functions. Digestion and absorption of food mostly occurs in the small intestine. The presence of finger like projections known as villi, increase the surface area of the small intestine and aid the digestive process; the villi are absent from the caecum and the colon. The long villi of the small intestine are covered by a layer of microvilli, called microscopic brush border, which produce digestive enzymes and nutrient transporters. The brush border is largely absent in the large intestine, which plays little role in food digestion. The main function of the large intestine is to reabsorb water from feces, and to act as a barrier to the commensal microbiota. In all parts of the intestine, the epithelial layer is invaginated producing crypts of Lieberkühn. The crypts harbor intestinal stem cells and their progeny, transit amplifying cells, which upon multiple cell divisions give rise to different types of differentiated epithelial cells (94, 95).

Four types of specialized differentiated epithelial cells reside within the intestinal epithelium (94): enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. In addition M cells are present in the follicle-associated epithelia (96). Enterocytes are found throughout the length of the intestine. These are absorptive cells, and are mainly responsible for the transport of ions, water, peptides, sugars, vitamins, and lipids across the mucosa. In addition, these cells are
also responsible for secretion of immunoglobulins, especially IgA into the intestinal lumen (97). Goblet cells are glandular and their main function is to secrete gel-forming mucins, which are the central components of mucus (98). The mucus acts as a physical barrier to microbial and other toxic products. However, the polysaccharides and other mucus components can also serve as energy sources for the commensals. The frequency of goblet cells progressively increases moving down the gastrointestinal tract from the small to the large intestine. In parallel, the mucus layer is thickest in the colon. Enteroendocrine cells are specialized endocrine cells of the gut (99). They produce gastrointestinal hormones or peptides in response to various stimuli and release them into the bloodstream for systemic effect. Paneth cells secrete lysozyme and other antimicrobial peptides such as defensins and regenerating islet-derived protein IIIγ (100, 101). Paneth cells also help to maintain the intestinal homeostasis by producing pro-epidermal growth factor, WNT3 and Notch ligands, which are required for normal intestinal stem cell activity. In contrast to goblet cells, the Paneth cells are mainly found in the small intestine, where they are mostly located in the ileum.

**Intestinal microbiota and epithelial barrier function:** The intestine is continuously exposed to approximately $1 \times 10^{14}$ commensals of more than 500 different species, as well as potential pathogens (102). The number of commensals generally increases going down the length of the gastrointestinal tract. About 100-1,000 bacteria per ml are found in the acidic stomach environment, the upper part of the small intestine contains about $10^5$ bacteria per ml, and the maximum bacterial population of up to $10^{12}$ per ml is found in the terminal ileum and colon (103, 104). There is a distinct distribution of aerobic and anaerobic bacteria along the length of the gastrointestinal tract. Aerobes and facultative anaerobes are predominant in the upper parts of the
intestine, while the low oxygen tension in the colon favors anaerobic bacteria (105). Interestingly, it has been shown that aerobes and/or facultative anaerobes first colonize the newborn gut, and consume oxygen in the gastrointestinal tract creating a suitable environment for the anaerobes (106). The commensal microbiota exists in a mutually beneficial relationship with the host. The commensals thrive in the relatively stable environment conditions of the intestine, and use undigested fibers, polysaccharides and other mucus components as energy sources, producing enzymes and essential metabolites such as biotin, and vitamin K. The host exploits these enzymes and nutrients to break down complex carbohydrates into small absorbable units, thereby increasing energy uptake. Moreover, the commensals act to prevent colonization of invasive pathogens, and stimulate the host immune defenses, thus preserving the niche for commensal bacteria and assisting the host in preventing infections.

A very important function of the intestinal epithelium is to prevent the commensal microorganisms, pathogens and their products from entering the systemic circulation, while allowing the entry of essential nutrients (107). The epithelium maintains its selective barrier function through the formation of protein-protein junctions that mechanically link adjacent cells and seal the intercellular spaces (108, 109). Two main types of junctions have been identified in the intestinal epithelium: tight junctions and adherens junctions (110). These junctions consist of

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**Figure 1.6:** Schematic representation of intestinal epithelium showing location of tight and adherens junction proteins.
transmembrane proteins that interact extracellularly with proteins from adjacent cells and intracellularly with adaptor proteins that link to the cytoskeleton (111-113). Most apical of these junctions are tight junctions, which seal adjacent epithelial cells in a narrow band just beneath the apical surface and create a semi-permeable diffusion barrier (114, 115). Forty different proteins are known to be located in the tight junctions, including members of claudin, occludin, and zona occludens (ZO) families, and several unidentified phosphoproteins (116). Below the tight junctions, on the lateral side of the epithelial cells are the adherens junctions, which constitute the strongest physical link between adjacent epithelial cells (117, 118). The most important and common adherens junctions are formed by cadherin-catenin complexes, established through calcium-dependent homophilic binding of E-cadherin molecules, which are in turn connected to the actin cytoskeleton through α- and β-catenin (119). Both tight and adherens junctions play an active role in maintaining polarity of the epithelial cells and regulating epithelial cell migration and proliferation. In addition to these junction complexes, desmosomes provide additional mechanical attachments between adjacent epithelial cells, while gap junctions allow intercellular communications to occur (108).

Various physiological and pathological stimuli affect the synthesis, assembly and disassembly of the junction complex proteins, resulting in enhancement of or damage to the intestinal barrier function (120). Several pathogens and their toxins have been shown to downregulate the function of the junction proteins. For example, Bacteroides fragilis disrupts tight junctions of intestinal epithelium by proteolytic degradation of the junction proteins (121) and Clostridium difficile toxins A and B cause endocytosis of tight junction proteins resulting in decreased epithelial cell adhesions (122). Candida albicans and Porphyromonas gingivalis secrete proteases that cleave E-cadherin, resulting in invasion of the epithelial barrier by
affecting the adherens junctions (123, 124). Bacterial toxins such as botulinum toxin and cholera toxin also target E-cadherin to disrupt the intestinal epithelium and enter the systemic circulation (125, 126). On the other hand many microorganisms are known to boost the intestinal barrier function. Probiotics such as *Lactobacillus acidophilus*, *L. permentum*, and *L. gasseri* improve the function of adherens junction protein E-cadherin by activating protein kinases, thereby stabilizing the cadherin-catenin complex (127). Treatment of epithelial cells with *E. coli* Nissle 1917, a human fecal isolate and widely used probiotic, has been shown to reduce barrier disruption caused by enteropathogenic *E. coli* by increasing the expression of tight junction protein ZO-2 and redistributing ZO-2 from the cytosol to cell boundaries in vitro (128, 129).

**STEC colonization in intestinal mucosa:** Intestinal mucosa is the main site of colonization of STEC following ingestion of contaminated food or water. The ability to adhere to the intestinal mucosa is a key determinant to STEC toxicity (130). The number of STEC required to establish an infection can be as low as 100 (131). STEC are assumed to colonize the colon and the distal parts of the small intestine, however the exact colonization site has not been demonstrated directly (132). A common trait of STEC, particularly the enteropathogenic O157:H7, is the formation of attaching and effacing (A/E) lesions on the intestinal epithelial cells (133). During A/E lesion formation, the bacteria attach tightly to the host cell membrane causing disruption of the cell surface and effacement of microvilli. Beneath the attached bacterium there is cytoskeletal rearrangement mediated by F-actin and other cytoskeletal proteins, which results in outgrowth of cuplike pedestals at the adherence site (134). For O157:H7 and other enteropathogenic *E. coli*, the genes necessary for A/E lesion formation are located on a pathogenicity island termed the locus of enterocyte effacement (LEE) (135). A
cluster of genes in the LEE encode for the type III secretion system (T3SS). The T3SS mediates the secretion of other LEE encoded effector proteins, including intimin and translocated intimin receptor (Tir/EspE) (136). Interaction of Tir, translocated by the T3SS into the host cell membrane with the bacterial outer membrane protein intimin is critical for pedestal formation (137). Other T3SS effector proteins including EspA, EspB and EspD trigger intracellular signaling cascade in the target cells, causing the release of inositol triphosphate, phosphorylation of myosin light chains, and tyrosine phosphorylation of certain epithelial cell membrane proteins (138).

Although most of the STEC are characterized by formation of A/E lesions, a direct correlation between A/E lesions and development of HC and HUS has not been demonstrated, and lately a significant minority of STEC have been identified that do not produce A/E lesions (132, 139, 140). For example the STEC serotype O104:H4 responsible for the outbreak in Germany in 2011, maintained the adherence properties of enteroaggregative E. coli, and produced Stx, but was negative for A/E lesion formation (141). It is possible that these strains produce additional, as of yet uncharacterized virulence factors to compensate for the absence of A/E lesions. In addition, whether Stx, the common factor produced by all STEC, is directly responsible for the intestinal actions of STEC is not clearly known.

**Stx interaction with intestinal epithelium:** Low oxygen levels, the intestinal epithelial cells, and commensal microflora of the intestine increase STEC virulence by directly or indirectly affecting Stx production. Low oxygen levels are not directly related to Stx production, but lead to increased STEC adherence to host cells (142). Some probiotic bacteria such as *Bacteroides thetaiotaomicron*, inhibit Stx2 production by repressing the phage lytic cycle (143).
On the other hand, Stx2 phage infection of susceptible commensal *E. coli* causes increased Stx2 production (37, 38). Production of hydrogen peroxide by human neutrophils and intestinal epithelial cells has been shown to activate the Stx phage elaborating Stx production (33). On the other hand, nitric oxide produced by activated intestinal epithelial cells has been suggested to inhibit bacterial SOS response and Stx production (144).

Intestinal epithelium forms the first point of contact of Stx released from STEC, and furthermore forms a barrier by preventing the access of Stx to the systemic circulation. How Stx overcomes this intestinal epithelial barrier to reach the distant targets such as kidneys and neurons is unknown. It is important to note that while mice are very susceptible to Stx when injected IP or IV, they are completely resistant to purified Stx introduced via the intestinal tract. In addition, while highly virulent *E. coli* O157:H7 can colonize the mouse intestinal tract to very high levels, no disease is observed (37). To date, no small animal model is available that can replicate the human intestinal architecture. A model of human intestine is critical to understand the pathways used by Stx to enter the human blood circulation. While immortalized intestinal epithelial cells have been used to study the action of Stx, these cells differ from healthy intestinal epithelium in many ways. In addition, the human intestinal epithelium contains a wide variety of cells with different functions, such as goblet cells, Paneth cells, enterocytes and enteroendocrine cells, whereas immortalized cells are clonal. For example, Caco-2 cells resemble enterocytes (145), and T84 cells maintain both enterocyte and goblet cell phenotypes (146). Different cell types show different responses to Stx. While Paneth cells have been shown to express Gb3 and bind Stx, T84 cells do not express Gb3 and are insensitive to Stx. Another major drawback of immortalized cancer cell lines is that they vary in Gb3 expression (87, 147). This can cause serious artifacts while studying the action of Stx. Appropriate experimental model systems need
to be developed which are based on native human intestinal mucosal conditions rather than cancer-derived cell lines or small animal models. A better understanding of Stx-related events in the human gut is necessary and important, as it will lead to the development of early intervention strategies for the treatment of HUS.
D. Intracellular Signaling of Shiga toxin

Retrograde Transport of Shiga toxin: The predominant action of Stx is to inhibit protein synthesis by acting on the ribosomal RNA of the target cells. To reach this cytoplasmic target, Stx undergoes retrograde transportation. Following steps are involved in the retrograde transport of Stx (Figure 1.7):

**Figure 1.7: Intracellular Signaling of Stx.** 1. Stx can interact with the cell surface either as a holotoxin or it can oligomerize at the cell surface. 2. Stx binding to plasma membrane is followed by toxin endocytosis. 3. Toxin binding to plasma membrane can potentially activate different signaling pathways. 4. The toxin then undergoes retrograde sorting into the endosomes, which is followed by transfer to the trans-Golgi network. 5. Stx is finally transferred from Golgi into the endoplasmic reticulum and then Stx A-subunit is retro-translocated into the cytosol.
i. **Endocytosis:** Through multivalent binding to the GSL receptors present in the lipid rafts of the cell membrane, Stx1 has been shown to induce lipid clustering and changes in the membrane properties. This induces tubular membrane invaginations (148). Some reports suggest that Stx1-induced invaginations are then processed by different cellular mechanisms, resulting in toxin uptake and activation of several signaling pathways. These include activation of tyrosine kinases such as SYK in HeLa cells (149), YES in ACHN renal cells (150) and LYN in Burkitt’s lymphoma Ramos cells (151). Activation of these kinases leads to rapid tyrosine phosphorylation and activation of several proteins, which eventually results in cellular uptake of Stx in a clathrin-mediated endocytic pathway. It is believed that the toxin-receptor complex might interact with other plasma membrane proteins recruited to the clathrin-coated pits. Identity of these proteins remains unknown.

ii. **Retrograde transport:** An important aspect for Stx cell toxicity is transport of the toxins to the Golgi apparatus after internalization. Cells such as macrophages and dendritic cells that bind to but do not allow sorting of Stx from endosomes to Golgi have been shown to be resistant to Stx intoxication (152). Following its entry into the cell, Stx localizes into the early and recycling endosomes, where retrograde tubules are formed and Stx preferentially localizes to this tubular environment. Stx bypasses the scission of the retrograde tubules in the late endocytic pathway and is transferred directly from the early and recycling endosomes to the *trans*-Golgi network and from here to the endoplasmic reticulum (ER) (52). The mechanisms by which Stx escapes the late endocytic pathway and lysosomal degradation are not completely understood. At the stage of early endosomes an endopeptidase called as furin recognizes the consensus sequence R-X-X-R between residues 242-261 of Stx A-subunit and cleaves the A moiety into A1 and A2 fragments (153). The amino acids surrounding the R-X-X-R recognition sequence are also
known to be important for processing and activation of Stx by furin (154). Furin is primarily localized in the trans-Golgi network in most cells, but small amounts can also be found in endosomes and at the cell surface. Furin cleavage of the A-subunit is important for activity of Stx and elimination of the furin-sensitive site has been shown to cause significant reduction in protein synthesis inhibitory activity of Stx. In addition to furin, other proteases such as trypsin are also known to cleave Stx, although to a much lesser extent. After cleavage of the A-subunit the A₁ and A₂ fragments still remain attached by the virtue of a disulfide linkage between Cys242 and Cys261 of the A₁ and A₂ fragments, respectively, until the toxin reaches the ER.

iii. Retro-translocation: Characterization of the processes by which Stx translocates from ER into the cytosol is an area of active study. Once inside the ER, the disulfide linkage between the A₁ and A₂ fragments of Stx is reduced, possibly by ER chaperones and enzymes such as protein disulfide isomerase, releasing the enzymatically active A₁ fragment (155). Reduction of the disulfide bond of Stx A-subunit has been thought to be the rate-limiting step for the retro-translocation of the toxin into the cytosol. Tam et al (156), have shown that most of the cell-associated Stx A-subunit is cleaved into A₁ and A₂ fragments by proteases, mostly furin, but they remain attached, as the disulfide bond between the two fragments fails to be reduced. Consequently, only a small fraction of the endocytosed toxin is thought to reach the cytosol. In furin containing Vero cells, only 4% of the A₁ fragments of the internalized Stx1 were translocated within 4 hours of toxin exposure. Studies suggest that the free A₁ fragment uses the cellular ER-associated protein degradation (ERAD) pathway to gain entry into the cytosol. In a series of deletion mutation experiments on the A₁ fragment of Stx1, LaPointe et al (157) demonstrated that the residues 240-251 in A₁-subunit are essential for translocation. Since this region is mostly made up of hydrophobic amino acids, it was proposed that owing to this
hydrophobic portion, the cleaved A₁ fragment is recognized by ERAD pathway as a misfolded protein and is thus translocated to the cytoplasm. The A₁ fragment has been shown to interact with ER lumen resident chaperones such as HEDJ and BiP. It is believed that the chaperone-A₁-subunit complex recruits Sec61 translocon for its transport across the ER membrane. Once in the cytosol, the A₁ fragment is thought to escape the proteasomal degradation due to the lack of lysine residues (155).

**Other cellular actions of Shiga toxin:** In addition to inhibiting protein synthesis, Stx is also capable of triggering other signaling cascades such as cytokine secretion, which have been suggested to contribute to the cytotoxic effects of Stx (158). Katagiri et al showed that treatment of human renal tubular cells with Stx holotoxin resulted in activation of tyrosine kinases within several minutes after toxin treatment, and protein synthesis inhibition ensued after a few hours (150). Upon entry into the cytosol, Stx triggers several cytosolic kinases such as c-Jun NH2-terminal kinase (JNK1) and p38 (159-161).

B subunits of Stx, by themselves, are also capable of inducing a variety of lipid raft-dependent signaling mechanisms, such as cytoskeletal remodeling, secretion of blood clotting factors and retrograde trafficking of Stx. Nolasco et al. showed that Stx1 and Stx2 could promote human endothelial-cell secretion and impair ADAMTS13-induced cleavage of von Willebrand (vWF) factor multimers (162). Increased secretion of vWF or deficiency in ADAMTS13 activity is related to the formation of ultra-large multimers of vWF, which is associated with blood clotting disorders such as thrombocytopenic purpura and HUS. Huang et al showed that the B-subunits of both Stx1 and Stx2, in the absence of the A-subunit were capable of causing an acute secretion of vWF in human endothelial cells and thrombotic microangiopathy in mice models,
occasionally leading to death (163). More studies suggested that the B-subunits of Stx1 and Stx2 induce different signaling cascades. Whereas Stx1 B-subunits increase intracellular Ca\(^{2+}\) and activate protein kinase C\(\alpha\), Stx2 B-subunits activate protein kinase A (164). Later, phospholipase D1 (PLD1) was shown to be important for the induction of vWF secretion by the b-subunits of both Stx1 and Stx2. In agreement with the previous report, this study also indicated that Stx1 B-subunits and Stx2 B-subunits activated different signaling pathways in human endothelial cells, which converge on PLD1 to induce vWF secretion (165). These studies were performed using human umbilical vascular endothelial cells, which are not directly implicated in human pathogenesis. Whether the B-subunits display similar effects in more relevant cell lines and disease models remains to be seen.
E. Conclusion

Our knowledge about Stx and their role in human infections has greatly expanded in the past few years. However, many questions regarding Stx signaling and Stx variants still remain unanswered. Majority of the studies on Stx are performed on the less toxic Stx1, and not much is known about the more toxic isoform Stx2. Although the Stx variants share about 60% amino acid sequence identity and an overall conserved structure, they significantly differ in cellular toxicities. The molecular basis of this difference in toxicities is still unclear. We need a more detailed understanding of binding, cellular entry and intracellular trafficking of Stx in order to fully understand how the toxins function. Many of the studies on Stx are performed using cells that are not the primary targets of Stx, such as HeLa and THP-1 cells. Consequently, the reported results may not be applicable to the toxin sensitive cells lines such as kidney, intestinal and endothelial cells.

How Stx, produced in the intestine, crosses the intestinal barrier to reach the distant targets such as kidneys and neurons is still not clearly known, since until recently, a model that resembles the cellular architecture and function of a human intestine was not available. It is critical to fill these gaps in our understanding of Stx pathogenesis to develop therapeutic, preventive and diagnostic strategies to combat the life threatening complications associated with Stx.
Chapter 2

Glycolipid Binding Preferences of Shiga Toxin Variants

1 Parts of the following work have been previously published in:


A. Introduction

Shiga toxin producing *E. coli* (STEC) (66), including serogroups O157:H7 and non-O157, are one of the leading causes of food poisoning worldwide (166). Ingestion of as few as 30 bacteria is enough to produce disease symptoms (131). STEC infections result in a range of symptoms from mild diarrhea to hemorrhagic colitis (26, 27). About 10% of the infected progress to the life-threatening kidney disorder called as hemolytic uremic syndrome (HUS) (88, 167-172). Currently there is no specific treatment for HUS and conventional antibiotic treatment is known to worsen HUS symptoms (173).

The primary virulence factor of STEC is Shiga toxin (Stx), which belongs to the AB₅ group of toxins (174, 175). The A-subunit is responsible for inhibiting protein synthesis of the target cells by cleaving the N-glycosidic bond of adenine 4324 in 28S rRNA and preventing tRNA binding (176). The A-subunit is non-covalently attached to a pentamer of identical B-subunits, which bind to host cell surface receptors mediating cytoplasmic delivery of the A-subunit (4, 53, 177, 178). Stx includes two immunologically distinct isoforms, Stx1 and Stx2, which share about 60% amino acid identity and a highly conserved general structure. Stx2 is further subtyped into 8 variants (Stx2a-Stx2h), which display approximately 90% amino acid identity (Figure 2.1). In spite of the high structural similarity, these variants significantly differ in toxicity, with Stx2a being over 100-fold more toxic to mice than Stx1, and variant isoform Stx2b (179-184). STEC strains can express one or more Stx variants. However, strains producing Stx2a, Stx2c and Stx2d are more commonly associated with HUS in humans than those producing Stx1 or Stx2b (55). Previously, in cell free in-vitro translation inhibition assays A-subunits of Stx variants displayed similar activities (58). This suggested that the enzymatic activities of A-subunits are not likely responsible for the toxicity differences between Stx
variants. On the contrary, Stx B-subunits have been shown to display differences in receptor recognition, and influence cellular toxicity (55, 58, 59, 185-187).

The B-subunits of Stx recognize cell surface glycolipid globotriaosylceramide (Gb3) (188) and to a lesser extent globotetraosylceramide (Gb4) as receptors (55, 189) (Table 2.1). Gb3 is composed of a tri-saccharide (Galα1-4Galβ1-4Glc), called Pk trisaccharide, which is attached to the lipid, ceramide. Gb4 is derived from Gb3, and is composed of a tetra-saccharide (GalNAcβ1-3Galα1-4Galβ1-4Glc), called P trisaccharide, which is also attached to ceramide. These glycolipids are generally located in phosphatidyl choline (PC)- and cholesterol (Ch)-rich cell membrane microdomains called lipid rafts (72, 190-193).

Previous studies examined binding of purified Stx1 and Stx2a to the neutral glycolipids, alone or in mixtures and each variant displayed a unique binding profile (76). Similarly, differences in receptor recognition of Stx2 variants are known to mediate host specificity. Stx2a, associated with human disease, prefers binding to Gb3, while Stx2e, associated with swine disease, prefers Gb4 (194). Glycolipid-binding sites and preferences of highly toxic Stx2 variants including Stx2c and Stx2d, or weakly toxic variants Stx2b have not yet been reported.

In a report, Gb3 was found to be present in low quantities in colonic epithelial cells in vivo; whereas Gb4 was found abundantly (195). Low affinity binding of Stx1 to Gb4 has been reported (195), while Stx1 and Stx2a have been shown to bind to both Gb3 and Gb4 with equal affinities in the presence of other cell membrane components (76). However, in vitro binding affinities do not correlate with cellular or in vivo toxicity. While Stx affinity for Gb3 is in the nanomolar range, cellular and in vivo toxicity are in the picomolar range, suggesting that other factors might also mediate Stx toxicity in vivo and at cellular level (89-91).
Figure 2.1: Comparison of B-subunits of Stx variants: (A) Amino acid sequence comparison. Amino acid sequences of Stx B-subunits were aligned using BLASTP (NCBI/BLAST). Periods indicate identity and dashes indicate absent amino acids. Amino acid differences with respect to Stx2a are denoted in bold. Numbering starts with the first amino acid of the mature peptide. (B) Structural comparison. The mutagenesis function of PYMOL was used to substitute amino acids of the Stx variants into the crystal structure of disaccharide bound Stx2a (PDB: 4M1U). The structures are oriented to display the receptor binding face of the B-subunits, with an individual subunit representing a different Stx variant. Color-coding is as follows: wheat, Stx1; green, Stx2a; yellow, Stx2b, blue, Stx2c, blue, bound disaccharide; red, A-tail of Stx2a; pink, amino acid polymorphisms with respect to Stx2a.

Note that the Stx B-pentamer is made up of identical B-subunits.
Crystal structure of Stx1 B-subunit with the Pk trisaccharide has been determined. It indicates the presence of three Pk binding sites per B-monomer, for a total of approximately 15 Pk-binding sites per B-pentamer (79). The affinity of an individual binding site for its glycan receptor is very weak (81, 187), and tight binding is achieved by avidity, or the ability to simultaneously engage multiple receptor binding sites. Recently, Jacobson et al published the crystal structure of Stx2a holotoxin bound to a Pk derivative, NHAc-Pk. Only two sites on the B-pentamer displayed density for NHAc-Pk, (86), suggesting that Stx1 and Stx2a significantly differ in their receptor recognition as well as the number of potential binding sites.

While avidity is necessary for high affinity receptor binding, paradoxically studies using analytical centrifugation (AUC), mass spectrometry and circular dichroism indicate that B-subunits of Stx exist as lower order oligomers and not as pentamers at physiologically relevant concentrations. Moreover, Stx1 and Stx2a were shown to differ in their abilities to form a stable pentamer (1, 67). Conrady et al identified a glutamine (Q40) in Stx2a within an otherwise hydrophobic B-subunit interface. The corresponding amino acid in Stx1 was a hydrophobic leucine (L41). Interchanging these residues (Stx1-L41Q and Stx2a-Q40L) reversed the stability phenotypes of Stx1 and Stx2a. Interestingly, the destabilizing amino acid, Q40 is conserved among all Stx2 variants (Figure 2.1), suggesting that destabilization of the B-pentamer might impart a selective advantage to Stx2. Holotoxins of other AB₅ toxins are highly stable, and display different interacting residues that provide strong interactions between A- and B-subunits. For example, cholera toxin and subtilase cytotoxin contain a hydrophobic patch on top of the B-pentamer that plays a dominant role in binding to the A-subunit (196). In case of Stx, especially Stx2, this hydrophobic patch is much smaller, and displays hydrophilic residues near
the central pore of the B-subunits, further suggesting a decreased tendency of Stx to stay in a holotoxin conformation in solution state.

In this study, using enzyme linked immunosorbent assay (ELISA) and biolayer interferometry we characterized the glycolipid binding profiles of holotoxins and B-subunits of Stx variants, and discuss the implications of this binding on Stx activity. In addition, we determined that stabilities of the B-subunits are important determinants of glycolipid binding affinities. Taken together, this report gives information about receptor preferences of Stx variants and the role of B-subunits in these receptor interactions.
**B. Materials and methods**

**Glycolipids and other lipids:** The glycolipids used in this study were purchased from Matreya Inc. (Pleasant Gap, PA) and have been listed in **Table 2.1**.

**Antibodies:** Mouse monoclonal antibody against Stx1 A-subunit and rabbit polyclonal antibody against Stx2 A-subunit were obtained from Biodefense and Emerging Infections (BEI) Research Resources Repository and Meridian Bioscience respectively. Mouse monoclonal antibody against Stx1 B-subunit was obtained from BEI resources. Chicken polyclonal antibody against Stx2 B-subunit was obtained from Lampart Biologicals. Peroxidase-conjugated goat anti-mouse, anti-rabbit and anti-chicken IgG’s were purchased from MP Biomedicals.

<table>
<thead>
<tr>
<th>Name (Abbreviation)</th>
<th>Structure</th>
<th>Formula</th>
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<tbody>
<tr>
<td>Galactosyleceramide (Gal-cer)</td>
<td>Gal-ceramide</td>
<td>C₄₈H₉₃NO₉</td>
</tr>
<tr>
<td>Lactosyleceramide (Lac-cer)</td>
<td>Galβ[1-4]Glc-Ceramide</td>
<td>C₅₅H₁₀₁NO₁₃</td>
</tr>
<tr>
<td>Glucosyleceramide (Glc-cer)</td>
<td>Glc-ceramide</td>
<td>C₄₆H₈₉NO₈</td>
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**Production of Stx Holotoxin Supernatants:** The Stx strains used in this study are summarized in **Table 2.2**. Starter cultures of Stx holotoxins were grown in Mueller-Hinton (MH) broth. Overnight starter cultures were diluted 1/100 in fresh MH broth and grown with shaking at 37 °C until the optical density at 600 nm reached approximately 1. Stx expression was induced
by treating the cultures with ciprofloxacin (10 ng/ml) to induce the phage lytic cycle and the cultures were shaken overnight at 37 °C. The cells were subsequently removed by centrifugation and supernatants containing Stx holotoxins were filter-sterilized. Presence of both A- and B-subunits in the supernatants was confirmed by Western blots using antibodies against Stx A- and B-subunits. Vero monkey kidney cell line (197) (a gift from Alison O’Brien), transfected to express luc2p, a gene for destabilized luciferase (34), was used to confirm the protein synthesis inhibitory activity of the Stx supernatants.

<table>
<thead>
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<th>Table 2.2. Sources of Stx-producing strains used in this study</th>
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<tr>
<td>Protein Accession no. (NCBI)</td>
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<tr>
<td>Toxin</td>
</tr>
<tr>
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</tr>
<tr>
<td>Stx1</td>
</tr>
<tr>
<td>Stx2a</td>
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<tr>
<td>Stx2b</td>
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<td>Stx2c</td>
</tr>
<tr>
<td>Stx2d</td>
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<tr>
<td>Δ Toxin</td>
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**Toxin Quantification:** Western blots were performed using crude supernatants of unknown concentration, along with purified Stx1 and Stx2a holotoxins of known concentrations. Monoclonal antibodies against Stx1 and Stx2 A-subunits were used for the Western blots. The band densities corresponding to the A-subunit of the toxins with known concentrations were recorded using ImageJ software and were considered as standards. Concentrations of the toxin supernatants were then determined by comparing their band densities with the standards using
Analysis program of the ImageJ software. Supernatant from *E. coli* cells with Stx gene deleted from the λ phage (Δ toxin) was also prepared as the negative control.

<table>
<thead>
<tr>
<th>Table 2.3. Sources of B-subunit plasmids used in this study</th>
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<tbody>
<tr>
<td><strong>Plasmids</strong></td>
</tr>
<tr>
<td>pMFUC-20</td>
</tr>
<tr>
<td>pSHUC-5</td>
</tr>
<tr>
<td>pMFUC-21</td>
</tr>
<tr>
<td>pSHUC-6</td>
</tr>
<tr>
<td>pCF-6</td>
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<tr>
<td>pCF-7</td>
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**Expression and Purification of Stx B-subunits:** Expression and purification of B-subunits of Stx variants was performed as previously described. Briefly, pET21b(+) expression plasmids encoding the B-subunits of Stx variants (**Table 2.3**) were transformed into *E. coli* BL21(DE3)pLysS (Novagen). Transformants were cultured in Luria-Bertani broth containing ampicillin (250 µg.ml-1) and chloramphenicol (34 µg.ml-1). This was followed by cold-shock induction of the Stx B-subunits with 0.1 mM IPTG and 20% ethanol at 20 °C. Proteins were extracted by freeze-thaw, sonication and purified by ammonium sulfate precipitation (40-70%), Q-sepharose™ Fast Flow ion exchange chromatography (GE Healthcare, Uppsala, Sweden), Superdex™ 75 HiLoad 26/60 size exclusion chromatography (GE Healthcare) and UnoQ™ Q6R ion exchange chromatography (Bio-Rad, Hercules, CA). Presence of B-subunits in the preparations was confirmed by Western blot. Protein purity was verified by the presence of a
single band at 8 kDa on Coomassie stained SDS-PAGE gels, corresponding to the molecular weight of a single B-subunit. Bicinchoninic Acid Protein Assay (Pierce, Rockford, IL) was used to calculate the protein concentrations.

**Glycolipid ELISA:** We used ELISA to study glycolipid binding of Stx holotoxin supernatants and Stx B-subunits at equilibrium. Stock suspensions of glycolipids, PC and Ch were made in a 1:1 mixture of chloroform and methanol. Working mixtures of glycolipids, PC and Ch were made from the stock suspensions in the molar ratio of 1:3:3 respectively in methanol, as previously described [32]. 50 µl per well of single or mixed glycolipids, with or without PC and Ch were added to hydrophobic Mictotiter™ plates (Microfluor™ 1, Thermo Scientific) and allowed to dry in the fume hood overnight in order to facilitate immobilization. Wells coated with PC, Ch, PC+Ch, and methanol alone were used as the negative controls. Before starting the experiment, the plates were cooled down at 4 °C for at least 1 hour. The cooled plates were blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS; 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 128 mM NaCl, 2.7 mM KCl), pH 7.4. Half log dilutions of Stx holotoxin supernatants or purified B-subunits were prepared in PBS and subsequently added to the wells. The plates allowed to incubate for 1 hour at 37 °C, followed by extensive washing. The bound proteins were then incubated with respective primary and secondary antibodies. Finally, the plates were developed with QuantaBlue™ fluorogenic peroxidase substrate (Pierce, Rockford, IL) and read using FL600 microplate fluorescence reader (Biotek™). The plates were washed between each step with ice cold PBS containing 1% BSA and all the steps were performed at 4°C, unless otherwise specified. The signal was recorded as Relative Fluorescence Units (RFU’s). During analysis, the RFU’s corresponding with the negative controls were subtracted from the RFU’s corresponding with the proteins. Binding
curves were plotted using Prism 5.0™ (GraphPad software, La Jolla, CA). Statistical analyses were performed on three individual repeats.

**Vero Protection Assay.** Gb3 and Gb4 mixtures with Ch and PC were made in methanol and coated onto hydrophobic microtiter plates as described above. Unbound surfaces on the wells were blocked with Minimal Essential Medium 1X (Invitrogen) supplemented with 10% fetal bovine serum, vitamins (Sigma-Aldrich) and glutamine (Sigma). Stx1 and Stx2a (Biodefense and Emerging Infectious Diseases Research Resources Repository, Manassas, VA) were serially diluted in phosphate buffered saline (PBS) and added to the wells, starting with $10^{-8}$ M of toxin. The plates were incubated at 37°C for 1 hour. As negative controls, toxin was incubated in wells coated with methanol alone, coated with mixtures of PC + Ch alone, or not pre-treated. After incubation the amount of unbound toxin was determined using the Luc2P Vero assay, as previously described. Toxin samples were added to tissue culture treated 96 well plates (Corning Inc.), and Luc2P Vero cells were added to the wells ($10^4$ cells/well). After 4 hours of incubation at 37°C and 5% CO₂, the cells were washed with PBS and 25 µl/well of SuperLight luciferase substrate was added. Luminescence was measured using a luminometer (Thermo Labsystems Lumisoskan Ascent; Helsinki, Finland). The results were reported as percentage of maximum signal from cells incubated with PBS, without any toxin. Effective dose to inhibit 50% of protein synthesis (ED₅₀) was calculated using the two points above and below the midpoint.

**A₂ subunit peptides.** Peptides corresponding to A₂-subunits of Stx2a wild type (A₂-WT), Stop mutant (A₂-Stop) and W277R mutant (A₂-W277R) were purchased from Peptide 2.0 (Chantily, VA). Stock solutions were prepared by re-suspending the peptides in distilled water. Kaplan Meier survival curves were plotted, using GraphPad Prism 5.0.
**Biolayer interferometry.** To determine binding of B-subunits to A₂ peptides, the peptides were biotinylated on the cysteine residue (C261) using maleimide-PEG2-Biotin (Pierce), and immobilized on streptavidin biosensors (ForteBio, Cat. # 18-5019). Tips were incubated with unconjugated maleimide-PEG2-Biotin (0.6 µg.ml⁻¹) to block unbound sites and prepare negative control tips. Binding to Stx2a B-subunit (purified as previously described) was assessed at 37° C in PBS. In association studies, biosensors were immersed in 10 µM Stx2a B-subunit and shaken at 100 rpm. In dissociation studies, biosensors were transferred to PBS and shaken at 500 rpm. Final binding curves were calculated using ForteBio Data Analysis 7.0 software after subtracting the response units of the negative control.

To study glycolipid binding of A₂ peptide and B-subunit mixtures, Gb₃ (4 µg.ml⁻¹), PC and Ch were mixed in the molar ratio of 1:3:3 in methanol and immobilized on hydrophobic amino propylsilane biosensors (ForteBio, Cat. # 18-5045) using the Sidekick (ForteBio) at room temperature. Unbound glycolipids were removed by washing in PBS. Association and dissociation studies monitored binding of unbiotinylated A₂-peptides, B-subunit, and 1:1 mixtures of A₂-peptides and B-subunits. Binding was assessed at 37° C in PBS containing 100 µM dithiothreitol to avoid non-specific inter A₂-peptides interactions. Binding of proteins to uncoated tips was used as negative control. Final binding curves were obtained by using ForteBio Data Analysis 7.0 software after subtracting the response units of the negative controls.
C. Results

**Glycolipid binding of Stx holotoxins:** Stx1 and Stx2a display significant differences in glycolipid binding (76); we wanted to determine if the Stx2 variants also display differences in glycolipid recognition. We used ELISA to examine binding of Stx1, Stx2a, Stx2b, Stx2c and Stx2d using combinations of Gb3, Gb4, and other neutral glycolipids, Gal-Cer, Glc-Cer, and Lac-Cer. Slight binding to Gb3 and Gb4 alone was observed (Figures 2.2A and E). Among the Stx variants tested, Stx1 showed the highest ‘maximum RFU’s upon Stx binding’ (Bmax) for binding to Gb3 alone (Figure 2.2A); however the dissociation constant (K_D) values for Stx1-Gb3 and Stx2a-Gb3 were similar (Table 2.4). None of the Stx variants bound to Glc-Cer, Lac-Cer, and Gal-Cer alone (data not shown).

Next we examined Stx binding to mixtures of Gb3 and Gb4 with Gal-Cer, Lac-Cer and Glc-Cer. Stx1 displayed dose dependent binding to 1:1 mixtures of Gb3 with Gal-Cer (Figure 2.2B), Glc-Cer (Figure 2.2C) and Lac-Cer (Figure 2.2D). Among the Gb4 combinations, Stx1 showed slight binding to Gb4 mixed with Glc-Cer (Figure 2.2G). No significant Stx1 binding was observed for Gb4 mixed with Gal-Cer (Figure 2.2F) or Lac-Cer (Figure 2.2H).

Compared to Gb3 alone (Figure 2.2A), binding of Stx2 variants considerably increased when Gb3 was presented in a 1:1 mixture with other glycolipids. Binding profiles of Stx2a, Stx2c and Stx2d were similar for Gb3+Glc-Cer (Figure 2.2C). On the other hand, Stx2a and Stx2d bound better than Stx2c to Gb3+Gal-Cer (Figure 2.2B) and Gb3+Lac-Cer (Figure 2.2D). Stx2b marginally bound to Gb3 alone and did not bind to any of the Gb3 mixtures. None of the Stx2 variants bound to Gb4 mixtures at the concentrations tested (Figures 2.2F-H).
Figure 2.2: Binding of Stx holotoxins to glycolipid mixtures in absence of PC and Ch.

Binding was assessed by ELISA at 37 °C using serial dilutions of Stx variants. A. Gb3; B. Gb3+Gal-Cer; C. Gb3+Glc-Cer; D. Gb3+Lac-Cer; E. Gb4; F. Gb4+Gal-Cer; Gb4+Glc-Cer; Gb4+Lac-Cer. Mixtures of glycolipids were prepared in methanol in the ratio of 1:1 of the two glycolipids. Total concentration of 200 ng glycolipid was added per well. As negative control toxins were incubated with plate sham-coated with methanol. In all experiments, background RFU values obtained in methanol were subtracted from each value. The RFU signal is the mean of three independent experiments and error bars indicate standard deviation (SD).
Since glycolipids are generally located in the PC- and Ch-rich lipid rafts of the cell membrane, glycolipid binding of Stx was assessed in the presence of PC and Ch. Stx variants did not bind to the monosaccharide or disaccharide glycolipids, Gal-Cer, Glc-Cer or Lac-Cer even in the presence of PC and Ch (data not shown). However, presence of PC and Ch increased binding of all Stx variants to both Gb3 (Figures 2.2A-D and 2.3A-D) and Gb4 (Figures 2.2E-H and 2.3E-H) mixtures. Stx1 bound to almost all glycolipid combinations tested; however, 1:1 mixture of Gb4+Lac-Cer was not able to capture Stx1 even in the presence of PC and Ch (Figure 2.3H). Among the Stx2 variants, Stx2a and Stx2d showed comparable glycolipid binding profiles, followed by Stx2c. The least toxic variant Stx2b bound only to Gb3+PC+Ch and to Gb3+Glc-Cer+PC+Ch (Figures 2.3A and C). In general, at high toxin concentrations (1 µM), glycolipid binding of Stx1 was equivalent to Stx2a. However, at lower concentrations Stx2a bound better than Stx1 to most of the glycolipid combinations tested.

**Biological effect of glycolipid binding of purified Stx.** Stx causes toxicity by cleaving 28S rRNA of target cells, thereby inhibiting protein synthesis (198, 199). While binding of Stx to glycolipids has been reported to occur at as low as 10^-9 M, cellular toxicity occurs at much lower concentrations (91). We assessed the ability of glycolipids to neutralize cellular toxicity of Stx in a protein synthesis inhibition assay. The residual toxicity of Stx1 and Stx2 to Luc2P-Vero monkey kidney cells was assessed after incubating the toxin with Gb3 and Gb4 mixed with PC+Ch (Figure 2.4A) and the effective doses to achieve 50% protein synthesis inhibition, ED_{50} of Stx were compared for different pre-incubations (Figure 2.4A, inset).
Figure 2.3: Binding of Stx holotoxins to glycolipid mixtures in presence of PC and Ch.

Binding was assessed by ELISA at 37 °C using serial dilutions of Stx variants. A. Gb3; B. Gb3+Gal-Cer; C. Gb3+Glc-Cer; D. Gb3+Lac-Cer; E. Gb4; F. Gb4+Gal-Cer; Gb4+Glc-Cer; Gb4+Lac-Cer. Mixtures of glycolipid 1, glycolipid 2, PC and Ch were prepared in ratio of 1:1:3:3, respectively, to make 200 ng of total glycolipid concentration per well. Binding was assessed as described in Figure 2. The RFU signal is the mean of three independent experiments and error bars indicate SD.
Results for Stx1 are shown in Figure 2.4A. Stx toxicity to Vero cells without pre-incubation with glycolipids is represented by the untreated control (open diamonds). Pre-incubation of Stx with controls: PC+Ch (open triangles) or methanol (open inverted triangles) did not decrease toxicity, as seen by no change in ED$_{50}$ concentrations compared to the untreated control. Pre-incubation of Stx1 with Gb3+PC+Ch (open circles), showed significant neutralization of its toxicity, and resulted in about 10 fold increase in the ED$_{50}$ of Stx1 compared to untreated toxin. On the other hand, pre-incubation of Stx1 with Gb4+PC+Ch (open squares) was not able to protect Vero cells, as seen by no significant change in ED$_{50}$ relative to the untreated control.

Pre-incubation of Stx2a (Figure 2.4B) with either Gb3 (open circles) or Gb4 (open squares) immobilized mixtures did not decrease protein toxicity compared to the untreated control. Comparison of ED$_{50}$ concentrations of treated and untreated toxin showed no significant difference (Figure 2.4B, inset). These results suggest that other factors might be playing an important role in Stx cellular binding.

Figure 2.4. Vero protection studies. Stx cellular toxicity was assessed by using luciferase activity of Luc2p Vero cells after a pre-incubation with glycolipid mixtures. The results are the average of three independent experiments. Statistical difference was calculated by the two-tailed Student's t-test using GraphPad Prism™ 5.
Glycolipid binding of Stx B-subunits. Stx binds to the target cell surface mainly via its B-subunits and this binding is suggested to be an important step in Stx mediated toxicity (59). As a result it is important to understand the details of B-subunit interaction with the cell surface receptors. In this study using different combinations of neutral glycolipids, we examined the glycolipid receptor interactions of Stx B-subunits.

Figure 2.5 shows binding of purified Stx B-subunits to Gb3 (Figures 2.5A and C) and Gb4 (Figures 2.5B and D) in presence or absence of PC and Ch. B-subunits of Stx1 displayed stronger glycolipid binding compared to Stx2 variants, as seen by a lower K_D for Stx1 (Table 2.4). Among the Stx2 variants, the B-subunits of Stx2a, Stx2c and Stx2d displayed similar glycolipid binding affinity. The presence of PC and Ch did not significantly change binding of the Stx B-subunits to Gb3 and Gb4. This was in contrast to the holotoxins, which preferred binding to Gb3 and Gb4 in the presence of PC and Ch.

Previous studies reported the molar concentration of B-monomer required to achieve 50% assembly (EC_{50}) indicating the B-pentamer stabilities (Table 2.4) (1). In our ELISA experiments the glycolipid binding of the B-subunits correlated with their pentamer stabilities. Binding reached saturation at the concentrations of the B-subunits above the reported EC_{50} for pentamerization (Figure 2.5). In order to further investigate the role of pentamerization in B-subunit receptor recognition, we tested the glycolipid binding of Stx1 mutant with decreased B-pentamer stability, L41Q and Stx2a mutant with increased B-pentamer stability, Q40L. The destabilized Stx1 mutant L41Q displayed significantly reduced glycolipid affinity than wild type B-subunits of both Stx1 and Stx2a (Figure 2.5 and Table 2.4). On the other hand, the stabilized Stx2a mutant Q40L displayed increased glycolipid affinity compared to the wild type of Stx2a (Figure 2.5 and Table 2.4). Glycolipid binding profile of Q40L resembled Stx1 B-subunits.
Next we determined the Hill coefficients (h) for glycolipid binding of the B-subunits. Hill coefficients are a measure of cooperativity in binding. A Hill coefficient value of 1 indicates no cooperativity; a value of greater than 1 indicates positive cooperativity, where binding of one ligand facilitates binding of subsequent ligands; a value of less than 1 suggests negative cooperativity, where binding of one ligand suppresses the binding of subsequent ligands. The h-values for glycolipid binding of the B-subunits were significantly different. The h-value for binding of Stx1 B-subunits to the Gb3 mixture was close to 1, whereas B-subunits of Stx2a, and

Figure 2.5. Glycolipid binding of Stx B-subunits. Serial dilutions of Stx B-subunits were titrated against immobilized glycolipids to obtain the dose response curves. A. Gb3, B. Gb3+PC+Ch, C. Gb4, D. Gb4+PC+Ch. The RFU signal is the mean of at least three independent experiments and error bars indicate SD. Data was analyzed with Prism5 (GraphPad software, La Jolla, CA).
Stx2c bound to glycolipids with h-values much greater than 1 (Table 2.4). Interestingly, the stability mutant of Stx2a, Q40L bound with a h-value more similar to Stx1, or around 1. On the other hand, the h-value of the destabilized mutant of Stx1, L41Q was 2.0, more similar to Stx2a.

| Table 2.4 Glycolipid binding dissociation constants for Stx holotoxin and B-subunits |
|---------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Glycolipid Binding, $K_D$ in $\mu$M (Hill coefficient) | AUC(1) | Holotoxin |
|                                | Gb3+PC+Ch | Gb4+PC+Ch | Gb3   | Gb4   | EC$_{50}$ (\mu M) |
| Stx1                           | 0.046 (1) | 0.105 (1) | 0.139 (1.2) | 0.308 (1.2) | - |
| Stx2a                          | 0.025 (0.7) | 0.035 (0.8) | 0.074 (0.8) | N.D. | - |
| Stx2b                          | 0.094 (0.8) | N.D. | 0.308 (0.7) | N.D. | - |
| Stx2c                          | 0.210 (0.7) | 0.915 (0.8) | 0.192 (0.8) | N.D. | - |
| Stx2d                          | 0.032 (0.9) | 0.653 (0.9) | N.D. | N.D. | - |
| B-subunits                     | | | | | |
| Stx1                           | 0.018 (1) | 0.011 (1.1) | 0.027 (1) | 0.026 (1.1) | 0.043 |
| Stx1-L41Q                      | 3.372 (1.6) | 2.329 (2.1) | 2.234 (2) | 2.584 (2.1) | 1.060 |
| Stx2a                          | 0.141 (1.6) | 0.235 (1.4) | 0.418 (2.7) | 0.559 (2.2) | 2.290 |
| Stx2a-Q40L                     | 0.003 (0.8) | 0.005 (1.0) | 0.005 (0.7) | 0.128 (0.8) | 0.693 |
| Stx2c                          | 0.583 (1.6) | 0.453 (1.6) | 0.117 (1.2) | 0.778 (1.9) | - |

(N.D.: Not determined due to insignificant binding)
Role of ceramide in Stx glycolipid interaction. Previous studies using purified toxoids showed that the ceramide portion of Gb3 is critical for binding of Stx2a; but is dispensable for binding of Stx1 (76). We investigated the requirement of ceramide for Gb3 binding of Stx variants in the holotoxin form. Deacetylated Gb3 (Lyso-Gb3), which lacks a carbonyl and a fatty acid chain in the sphingosine of Gb3, was used. Figure 2.6A shows binding of Stx holotoxins to Lyso-Gb3 in the presence of PC and Ch by ELISA. Crude supernatant of Stx1 holotoxin displayed binding to Lyso-Gb3+PC+Ch (Figure 2.6A). On the other hand, similar to the Stx2a toxoid, none of the Stx2 holotoxins bound to Lyso-Gb3+PC+Ch (Figure 2.6A). Next we determined whether B-subunits show similar ceramide requirement for binding to Gb3. Binding of Stx B-subunits to Lyso-Gb3+PC+Ch was studied using ELISA. Stx1-B bound equally to both Gb3 and Lyso-
Gb3. Unlike holotoxins, the B-subunits of both Stx1 and Stx2a bound very well to lyso-Gb3+PC+Ch. (Figure 2.6B). Binding of the B-subunits to Lyso-Gb3 mixtures was very similar to their binding to Gb3 (Figure 2.3A). Similar results were obtained for the stabilized Stx2a B-subunit mutant, Q40L, which showed similar binding to Lyso-Gb3 and Gb3 mixed with PC and Ch.

**Effect of A2 peptides on B-subunit glycolipid interaction.** We examined the structural basis for A- and B-subunit association. The Stx A-subunit is proteolytically processed, forming two domains joined by a disulfide bond (153). The N-terminal A1 domain possesses the catalytic activity, while the C-terminal A2 domain mediates association with the B-pentamer. The crystal structure of Stx2a (Figure 2.7A) reveals that A2 associates with the B-pentamer via the 19-amino acid tail that extends through a pore in B-pentamer and via tryptophan 277, which binds a hydrophobic pocket on a single B-subunit. We obtained peptides (Figure 2.7B) corresponding to the wild type A2 subunit (A2-WT), a truncated version lacking the C-terminal tail (A2-Stop), and one with the hydrophobic tryptophan replaced by bulky, hydrophilic arginine (A2-W277R).

Binding of purified B-subunit to immobilized A2 peptide was assessed using bio-layer interferometry; during association, binding was assessed in the presence of the B-subunit; during dissociation, residual binding was assessed after transfer to buffer without B-subunit (Figure 2.7C). Strongest binding was observed with immobilized A2-WT peptide, as seen by rapid association, slow dissociation, and highest binding levels. Less binding to immobilized truncated A2-Stop peptide and the A2-W277R peptide was seen, suggesting that both regions are important for optimal B-subunit association. Binding to the Stx glycolipid receptor Gb3 was also assessed. Mixture of Gb3+PC+Ch was immobilized on the biosensor tip (Figure 2.7B). A2 peptides
increased Gb3 binding of B-subunits. The trend for binding to Gb3 was similar to A- and B-subunit binding to each other. B-subunit plus A2-WT peptide displayed the strongest binding. Less binding was seen for the truncated A2-Stop peptide and A2-W277R peptide. These studies suggest that A- and B- subunit assembly plays a role in glycolipid binding of Stx, and both the C-terminal tail and W277 are needed for optimal association.
Figure 2.7. Effect of A\textsubscript{2}-peptides on B-subunit-Gb3 interactions. (a) Crystal structure of Stx2a, showing A\textsubscript{1} enzymatic subunit (gray), B-pentamer (red) and A\textsubscript{2}-subunit (blue and green). Trp-277 (green) sits in a hydrophobic pocket at the B-subunit interface. Image generated from PDB 1R4P using PyMOL. (b) Amino acid sequences of A\textsubscript{2}-peptides (purchased from Peptide 2.0, Chantily, VA). (c) Binding of 10 μM Stx2a B-subunits to A\textsubscript{2} peptides was assessed by BLI (ForteBio Octet, San Francisco, CA). Binding curves were calculated using ForteBio Data Analysis 7.0 software after subtracting the response units of the negative control. Means of three independent repeats are plotted. (d) Glycolipid binding of A\textsubscript{2}-peptide and B-subunit mixtures.
D. Discussion

Previous reports suggested that B-subunit activities such as receptor binding and toxin internalization play an important role in determining Stx toxicities (58, 200). Receptor interaction differences of purified variants of Stx1 and Stx2a have been previously reported (76). However, not much information is available about the receptor interactions of Stx2 variants, which significantly differ in toxicity. Here we report, for the first time, the glycolipid receptor binding preferences of holotoxins and B-subunits of Stx2 variants.

The Stx variants displayed distinct glycolipid binding profiles. In most cases the forms most toxic to humans, Stx1, Stx2a, Stx2c and Stx2d showed strong glycolipid binding, whereas the weakly toxic form, Stx2b, showed very weak glycolipid binding. This property has diagnostic implications. Capturing Stx by host cell receptors provides a new diagnostic approach to identify and differentiate strains producing Stx variants, which are highly toxic to humans from variants, which are not toxic to humans.

Binding of B-subunits prepared from Stx2a clone was identical to B-subunits prepared from Stx2d clone, consistent with the fact that the amino acid sequences of B-subunits of these variants were identical. In contrast, the holotoxin of Stx2a bound slightly better than holotoxin of Stx2d. The C-termini of the A-subunits differ for Stx2a and Stx2d. Whereas A-subunit of Stx2a possesses a basic lysine at the C-terminus, Stx2d contains an acidic glutamate. Crystal structures of Stx2a and glycan-bound Stx2a suggest that the C-terminus of the A-subunit might take part in receptor recognition. As a result, the slight differences observed between Stx2a and Stx2d holotoxin binding could be due to the A-subunit.

Individual glycan binding sites on Stx display low affinity binding, and host cell recognition is thought to be due to avidity, or the ability to engage multiple glycan receptors.
Consistent with this notion, the B-pentamer of Stx1 has been shown to be more stable than the B-pentamer of Stx2a (1, 67, 68), and the B-subunits of Stx1 displayed stronger glycolipid binding than the B-subunits of Stx2a variants, suggesting that pentamer stability affects receptor binding (58, 85, 201). This was further confirmed by increased glycolipid affinity of the Stx2a mutant, Q40L, which forms a more stable pentamer compared to wild type Stx2a B-subunits (Figure 2.5 and Table 2.4). Previously using AUC, Conrady et al showed that the destabilized Stx1 B-subunit mutant, L41Q, was less stable than Stx1, however more stable than Stx2a. Based on this, we had expected the L41Q mutant to show decreased glycolipid affinity than B-subunits of Stx1, but still higher than Stx2a B-subunits. To our surprise, the Stx1 L41Q mutant showed the weakest glycolipid binding of all B-subunits tested (Figure 2.5 and Table 2.4). It is possible that Stx1 B-subunits are capable of binding to glycolipids only as a stable pentamer; Stx2 B-subunits on the other hand can bind to glycolipids even in lower order oligomeric states.

The Hill coefficients for Gb3 binding of the B-subunits were significantly different. Whereas Stx1 B-subunits bound to Gb3+PC+Ch with a h value of 1 suggesting no cooperativity, Stx2a B-subunits bound with a h value of 2.4 suggesting strong positive cooperativity. Previous studies by AUC showed that at high concentrations (8 µM) Stx2a B-subunits predominantly exist as pentamers, while a small proportion exists in the form of lower order oligomers. On the other hand, predominantly lower order oligomers exist at concentrations lower than 2 µM. Positive binding cooperativity observed with Stx2 B-subunits suggests that binding of these lower order oligomers may occur in two steps, initially B-subunits bind as monomers, and binding of one B-subunit promotes binding of additional B-subunits to form higher order oligomers, ultimately forming pentamers. Since the pentamer formed by Stx1 B-subunits is more stable, this effect is
not seen as prominently as with Stx2 B-subunits. Overall, this suggests that the B-subunits of Stx are capable of associating at the glycolipid interface.

Holotoxins of Stx2 variants bound only to the intact glycolipid, and no binding was observed to Lyso-Gb3, which lacked carbonyl and a fatty acid chain of Gb3. On the other hand, Stx1 holotoxin and Stx2a B-subunits, irrespective of the pentamer stabilities, did not differentiate between Gb3 and Lyso-Gb3, suggesting that the B-subunits are flexible about fatty acid requirement. Crystal structures of Stx holotoxins show that the C-terminus of A-subunit of Stx2 extends through the pore formed by the B-pentamer and could occlude receptor binding to a region defined as site 3 in Stx1 (174). Consistently, in the recently reported co-crystal structure only two NAcPk disaccharide densities were reported on the B-subunit of Stx2a holotoxin (86). It was speculated that the A-subunit interfered with binding to the glycan, which lacked the ceramide. It is therefore possible that the ceramide portion of Gb3 is important for engaging the A-tail of Stx2a, thereby opening glycan-binding sites on the B-subunits.

An enormous disparity exists between the binding observed using biochemical assays compared to cellular susceptibility. The $K_D$ values of Stx1 and Stx2 to Gb3 from this and previously published studies (89, 90) generally range between $10^{-7}$ M and $10^{-9}$ M. The concentration of toxin in blood at 50% lethal dose in mice is approximately $10^{-9}$ M for Stx1 and $10^{-10}$ M for Stx2 (91). However, both Stx1 and Stx2 are toxic to primary human renal proximal tubular epithelial cells of the kidney with an ED$_{50}$ of about $10^{-13}$ M and to the Vero monkey kidney cell line with an ED$_{50}$ of about $10^{-11}$ M. Since we are unable to observe any binding in vitro at these low doses, we examined the ability of toxin preincubated with glycolipid to protect Vero cells from Stx-mediated inhibition of protein synthesis. Even though nearly identical $K_D$ values were observed for Stx1 and Stx2 binding to Gb3 and Gb4 in the presence of PC and Ch,
Stx1 but not Stx2 was neutralized by preincubation with Gb3 mixed with PC+Ch (Figure 2.4), suggesting living cells display additional components missing in vitro biochemical systems. It is possible that cells express other receptors with a higher affinity than Gb3 or Gb4, for example in previous studies (202, 203), Stx1 bound better to glycans containing GlcNAc at the third position instead of Glc (Galα1-4Galβ1-4GlcNAc), while Stx2 preferred a Pk mimic (NAcGalα1-4Galβ1-4Glc). While neither glycan is found on glycolipids, both glycans are found on glycoproteins. It is possible that Stx recognizes protein receptors. Other bacterial toxins use both a glycan receptor and a protein co-receptor; botulinum toxin is a classic example of a toxin that uses both (204-209).

Taken together, this report gives the first account of glycolipid binding of Stx2 variants. The knowledge of receptor binding preferences of Stx variants will not only provide understanding of the different toxicities of these highly related variants but it will also provide a means to detect and differentiate these variants during a STEC outbreak.
Chapter 3

Human Intestinal Organoids to Study the Intestinal Action of Shiga Toxin

Producing *Escherichia coli*
Shiga toxin (Stx) producing *E. coli* (STEC), including *E. coli* O157:H7, is a leading cause of food-borne illnesses. Each year STEC causes about 265,000 cases of infectious watery and bloody diarrhea, which progress to the life-threatening kidney disorder, hemolytic uremic syndrome (HUS) in about 10% of the infected individuals (2, 166). The primary virulence factor of STEC is Stx, which belongs to the AB₅ group of toxins (67, 174, 175). The A-monomer inhibits protein synthesis of the mammalian target cells. The B-pentamer binds to the target cell surface receptors, mostly to the glycolipid globotriaosylceramide (Gb3), and mediates internalization of the A-subunit (178). The genes for the Stx A- and B-subunits are encoded in the late-gene region of lysogenic bacteriophages. These genes are silent until viral lytic replication is triggered by the bacterial SOS stress response. Stx and new viral particles are released by bacterial lysis. The primary target cells of Stx include kidney epithelial and endothelial cells, neurons, and intestinal epithelial and micro-vascular endothelial cells (73, 130, 210-213). The effects of Stx on kidney epithelial and micro-vascular endothelial cells are well studied (75, 162, 214). However, much is unknown about the action of Stx and STEC on intestinal epithelial cells.

Intestinal mucosa is the main site of colonization of STEC following ingestion of contaminated food or water (130, 215). The ability to adhere to the intestinal mucosa is a key determinant to STEC toxicity (130). STEC are assumed to colonize the colon and the distal parts of the small intestine, however the exact colonization site has not been demonstrated directly (132). Upon colonization, STEC, especially the enteropathogenic O157:H7, are suggested to form F-actin mediated intimate attachment with the intestinal epithelium, resulting in activation of host antibacterial response (4, 134, 137, 216). This intimate contact with the intestinal
epithelium, along with the host antibacterial reaction is thought to induce expression of Stx through activation of SOS response in STEC (217, 218). Intestinal epithelium, therefore, forms the first point of contact and a barrier to the entry of Stx to the systemic circulation. How Stx overcomes this intestinal epithelial barrier to reach the distant targets such as kidneys and neurons is unknown. Improved human intestinal models are critical if we are to understand the pathways used by Stx to enter the human blood circulation. There is no small animal model for examining human intestinal STEC infection. It is important to note that although mice are very susceptible to Stx when injected by intraperitoneal or intravenous routes, they are completely resistant to purified Stx introduced via the intestinal tract. In addition, highly virulent E. coli O157:H7 can colonize the mouse intestinal tract to very high levels without causing disease (37). Immortalized human intestinal epithelial cells have been used to study the

Figure 3.1: Components of iHIO. A. Schematic diagram of iHIO showing different cell types typically found in an organoid, which is embedded in extracellular matrix to maintain the 3-dimensional arrangement. B. Picture of an iHIO in the process of microinjection. C. Histology staining for different intestinal cell markers. Images were captured using Zeiss LSM710 Live Duo confocal microscope and merged using ImageJ software.
intestinal action of Stx and STEC (215, 219). However, these cells differ from the healthy intestinal epithelium in many ways, including the amount of receptor Gb3 expression (147, 220). In addition, the human intestinal epithelium contains a wide variety of cells with different functions, such as goblet cells, Paneth cells, enterocytes and enteroendocrine cells, while the immortalized cells are clonal (145, 146). Different immortalized cell types show different responses to Stx (221). Human tissue explants are differentiated tissues, but they display limited viability after removal from the host, and are therefore unsuitable for long-term studies. As a result, there is a huge gap in our knowledge of STEC pathogenesis, and it is important to study STEC in a model that closely resembles the human intestine.

We used stem cell derived ‘induced human intestinal organoids’ (iHIO) to study the action of Stx2a, a highly toxic variant of Stx, and Stx2a producing E. coli O157:H7. iHIO are developed by directed differentiation of human embryonic stem cells, in a process very similar to the development of human intestine during embryogenesis (222-224). The organoids closely resemble the distal parts of the small intestine, as seen by higher GATA6 nuclear expression than GATA4 (222, 225). The iHIO contain epithelial cells organized around the luminal cavities and mesenchymal cells surrounding the epithelial cells (Figure 3.1A). The polarized, columnar epithelium contains functional absorptive enterocytes as well as the major secretory lineages including goblet cells, Paneth cells and enteroendocrine cells, in a crypt-villous arrangement (Figure 3.1C). The stratified mesenchyme contains smooth muscle and intestinal sub-epithelial fibroblast cells.

Our results indicate that Stx2a damages the epithelial barrier function of the iHIO. Moreover, iHIO display a very severe and rapid loss of structural and barrier integrity in response to STEC infection. iHIO therefore represent the first tractable humanized model to
study the intestinal interaction of STEC.
B. Materials and Methods

Reagents and equipment used in iHIO culture: Matrigel basement membrane matrix (BD Biosciences, cat. 356234), and extracellular matrix gel (Sigma, cat. E1270) were used to embed the iHIO in order to support development of 3-dimensional architecture. Gut media for iHIO culture was prepared using advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) media (Gibco, Invitrogen, cat. 12634-028) supplemented with B27 insulin (Invitrogen, cat. 17504044), N2 supplement (Invitrogen, cat. 17502048), L-glutamine (Fisher, cat. SH3003401), HEPES buffer (Invitrogen, cat. 15630080), epidermal growth factor (R&D Systems, cat. 236-EG-200), human noggin (R&D Systems, cat. 6057-NG-100) and either penicillin/streptomycin (Invitrogen, cat. 15140-122) or penicillin alone (Amresco, cat. E480-20ML). iHIO were maintained in tissue culture treated Nucleon delta 4-well (Nunc, cat. 176740) and 2-well (Lab-Tek, cat. 155380) dishes.

Antibodies: Antibodies used in this study and their sources are enlisted in Table 3.1.

Purified Stx and E. coli strains: Purified Stx2a was obtained from BEI resources. Inactivated Stx2a toxoid was prepared as described in a previously study (76). Pathogenic E. coli O157:H7, PT29, capable of producing Stx2a and other type 3 secretion system effectors was previously isolated from a patient (38). ECOR13 was a non-pathogenic human E. coli isolated from a healthy person in Sweden. This strain was obtained from the Michigan State University STEC Center ECOR collection (37).

iHIO culture: iHIO prepared by directed differentiation of H1 human embryonic stem cell line (NIH registry number 0043) (222) were obtained from Pluripotent Stem Cell Facility and Organoid Core at Cincinnati Children’s Hospital and Medical Center. iHIO were maintained in reconstituted gut media, which was renewed every four days. The iHIO were passaged as
previously described (223). Briefly, the organoids were removed from the Matrigel, washed in ice-cold phosphate buffered saline (PBS), and cut using a sterile scalpel. Cut organoids were washed in gut media to remove loose cells, and suspended in fresh Matrigel. The Matrigel was allowed to solidify at 37 °C for 15 minutes, followed by addition of reconstituted gut media.
### Table 3.1. List of antibodies and stains used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cellular Localization/ Cell type</th>
<th>Clone/Dye</th>
<th>Source (Catalogue number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>Cell membrane/ epithelial</td>
<td>HEC-1</td>
<td>abcam (ab1416)</td>
</tr>
<tr>
<td>F-actin</td>
<td>Cytoskeleton</td>
<td>Texas-red phalloidin</td>
<td>Invitrogen (T7471)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Cytoplasm/ mesenchymal</td>
<td>EPR3776</td>
<td>abcam (ab92547)</td>
</tr>
<tr>
<td>Villin</td>
<td>Cytoskeleton/ epithelial</td>
<td>SP145</td>
<td>abcam (ab130751)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Cell membrane/ epithelial- enterocyte</td>
<td>EPR4477</td>
<td>abcam (ab108337)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Secreted/ epithelial- Paneth</td>
<td>EPR2994(2)</td>
<td>abcam (ab108508)</td>
</tr>
<tr>
<td>Mucin 2</td>
<td>Cytoplasmic and cell surface/ epithelial- goblet</td>
<td>Ccp58</td>
<td>abcam (ab118964)</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>Secretory granules/ epithelial- enteroendocrine</td>
<td>LK2H10+PHE5</td>
<td>abcam (ab715)</td>
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<tr>
<td>Cleaved caspase 3</td>
<td>Cytoplasmic/ apoptotic cells</td>
<td>5A1E</td>
<td>Cell Signaling Technology (9664S)</td>
</tr>
<tr>
<td>DNA</td>
<td>Nucleus/cytoplasm</td>
<td>Hoechst 33342</td>
<td>Invitrogen (H-3570)</td>
</tr>
<tr>
<td>Gb3</td>
<td>Cell membrane/ Stx target cells</td>
<td>38-13</td>
<td>Accurate Chemical and Scientific Corporation (YSRTMCA579)</td>
</tr>
<tr>
<td>Stx2a</td>
<td>-</td>
<td>11F11</td>
<td>BEI resources repository</td>
</tr>
</tbody>
</table>
**Microinjections:** Drummond glass capillaries (Fisher, cat. 21-171-4) were pulled with micropipette puller (Sutter Instrument Company). The tips of the capillaries were cut using Cuterz glass scissors, and the capillaries were loaded onto Nanoject II auto-nanoliter injector (Fisher, cat. 13-681-455). Microinjections were performed, and before and after injection images of iHIO were obtained using stereomicroscope (Leica). iHIO were injected with PBS, 300 ng of Stx2a, 300 ng of inactivated Stx2a toxoid and the proteins mixed with 2.5 mg/ml of fluorescent dye fluorescein isothiocyanate (FITC). The iHIO were incubated in reconstituted gut media containing penicillin/streptomycin at 37 °C in a humidified chamber containing 5% CO₂ for 5 days. For bacterial infections, approximately $10^3$ *E. coli* cells were microinjected into the iHIO lumen. The iHIO infected with *E. coli* were incubated in reconstituted gut media containing penicillin at 37 °C in a humidified chamber with 5% CO₂ for 1 day. Images of the injected iHIO were collected using Zeiss LSM710 Live Duo Confocal Microscope.

**Growth of *E. coli* in organoids:** Approximately $10^3$ *E. coli* cells were microinjected into the iHIO lumen. After indicated incubation times at 37 °C with 5% CO₂ in a humidified chamber, the organoids were removed from the 3-dimensional culture matrix, transferred to an eppendorf tube, and washed with ice cold PBS. The organoids were then transferred to a sterile 2 ml tissue homogenizer, disrupted, and suspended in 100 µl PBS. Subsequent dilutions of this suspension were plated on L-agar plates, and incubated at 37 °C overnight. The total number of bacteria per organoids was calculated based on the colony forming units (CFU) observed on the agar plates on the next day.

**Cryosections and staining:** At the end of the indicated time points, the iHIOs were fixed in 4% formalin for 2-4 hours, and immersed in 30% sucrose solution, followed by freezing in Tissue Freezing Medium (Fisher, cat. 15-183-13). 10-20 µm thick cryosections of the frozen
organoids were obtained using BD Cryotome FSE Cryostat. Prior to staining, the cryosections were fixed using ice-cold acetone for 10 minutes, and rinsed with distilled water. A boundary surrounding the sections was drawn using a hydrophobic barrier pen, the slides were placed in a humidified chamber, and the sections were blocked with PBS containing 10% goat serum, 1% bovine serum albumin (BSA) and 0.01% Triton X-100 for 2 hours at room temperature. The slides were allowed to drain for a few seconds, and primary antibody diluted in wash buffer (PBS with 0.1% BSA and 0.025% Triton X-100) was applied. The sections were allowed to incubate with the primary antibody in the humidity chamber at 4 °C overnight. Next day, the sections were rinsed twice with wash buffer. Secondary antibody was diluted in PBS and was applied to the sections. The sections were allowed to incubate with the secondary antibody for 2 hours in dark at room temperature. The sections were washed with PBS and DNA was counterstained with Hoechst dye for 2 minutes in dark. Finally the sections were dehydrated and mounted using VectaMount permanent mounting medium. Analysis was performed using Zeiss LSM710 Live Duo Confocal Microscope. Merged images were generated using ImageJ software.

**Flow cytometry analysis:** iHIO were removed from the Matrigel, and washed with ice-cold PBS. Pre-warmed 0.25% trypsin/EDTA was added to the iHIOs, followed by pipetting the iHIOs to loosen the cellular attachments. iHIOs were allowed to incubate with trypsin for ~10 minutes at 37 °C, followed by more pipetting of the iHIOs. The loose cells were then washed in ice-cold PBS, spun down at 300 x g for 5 minutes, and suspended in PBS. The cells were allowed to pass through a 40 µm cell strainer to remove any cell aggregates. The filtrate containing single cells was washed as described above, suspended in 300 µl PBS, and chilled on ice for 5 minutes. To determine Gb3 distribution, the cells were incubated on ice with primary
anti-Gb3 antibody (Table 1) for 1 hour, followed by 1-hour incubation with the secondary antibody. To determine Stx2a binding, the cells were incubated with 300 ng Stx2a on ice for 1 hour, followed by incubation with primary and secondary antibody (Table 1) on ice. Between each incubation, the cells were washed with ice-cold PBS, spun at 300 x g, and resuspended in 300 µl PBS. Finally the cells were suspended in 1 ml ice-cold PBS and analyzed using FACSCalibur flow cytometer (BD Biosciences). Data was analyzed and figures were generated using FCS Express software (DeNovo Software).
C. Results

**iHIO express Gb3 and bind to Stx2a:** Stx uses glycolipid receptor Gb3 to enter the target cells. We investigated the distribution of Gb3 in iHIO using flow cytometry (Figure 3.2A). Two different sized cell populations, likely corresponding to the epithelial and mesenchymal cell types, were observed in controls, where the cells were incubated only with the secondary antibodies. Approximately 13% of total iHIO cells displayed positive staining for Gb3, mostly observed in the smaller sized cells, suggesting the presence of Gb3 on some of the iHIO cells.

**Figure 3.2. Flow cytometry analysis of iHIO.** iHIO were treated with trypsin/EDTA, followed by pipetting, and passing the cells through a 40 µm cell strainer to obtain single cell suspension for flow cytometry analysis. A. **Histogram** (X-axis: staining on FL-2 channel; Y-axis: cell count) and **dot plots** (X-axis: staining on FL2 channel; Y-axis: forward scatter, indicating cell size) for Gb3 distribution in iHIO. B. **Binding of Stx2a to iHIO.**

Data was collected using FACSCalibur flow cytometer, and analyzed using FCS Express software.
We also investigated the binding of Stx2a to iHIO (Figure 3.2 B). Approximately 9% of total iHIO cells displayed increased antibody staining when incubated with Stx2a, compared to iHIO incubated with primary and secondary antibody controls. Two patterns of Stx2a staining were observed. About 8% of the cells displayed low intensity staining, while about 1% of the total iHIO cells displayed very high intensity staining. High intensity staining was mostly observed in smaller sized cells, suggesting that this binding could be to epithelial cells.

**Stx2a damages the epithelial barrier function of iHIO:** To investigate the effect of Stx2a on the luminal epithelial barrier function, we microinjected (Figure 3.1 B) FITC mixed with Stx2a, PBS or catalytically inactivated Stx2a toxoid into the iHIO lumen, and followed the intensity of FITC fluorescence with time. Intensity of FITC within the lumen did not change significantly in iHIO injected with either PBS, or the inactivated Stx2a toxoid, for up to 5 days post-injection (Figure 3.3 A). On the other hand, iHIO injected with Stx2a + FITC mixture displayed a time-dependent loss in FITC intensity starting at about 48 hours post-injection. These results suggest that Stx2a affects the epithelial barrier function of iHIO, allowing the FITC to diffuse out of the lumen. Moreover, it appears that the catalytic action of Stx2a A-subunit is important for this activity, since injection of Stx2a toxoid did not affect the FITC intensity in iHIO. To further understand the luminal conditions, we stained the cryosections of the iHIO for DNA using Hoechst stain (Figure 3.3 B). Cellular regeneration occurs in the healthy intestine and old epithelial cells are shed off into the lumen, maintaining a tightly packed luminal epithelial layer at all times (226). In the PBS controls, the epithelial cells were tightly packed with very few cells in the lumen, consistent with the old cells shed off during the normal process of regeneration. On the other hand, the luminal epithelial cell packing in iHIO treated with Stx2a appeared to be disturbed, and a large number of cells were extruded in the lumens of these iHIO,
suggesting extensive injury to the luminal epithelium. These results suggest that Stx2a damages the epithelial cell arrangement in the iHIO lumens, and can impair the luminal barrier.
Figure 3.3: Stx2a affects the epithelial barrier function of iHIO. A. Stx2a affects the morphology of the intact organoid. PBS, 300 ng Stx2a toxoid or 300 ng Stx2a mixed with FITC were microinjected in iHIO and FITC intensity (green) was followed for 5 days. iHIO injected with PBS or Stx2a toxoid displayed no significant change in FITC intensity, while Stx2a injected iHIOS displayed a time dependent loss of FITC intensity. Quantification of FITC intensity is plotted as a mean of three independent experiments for PBS and Stx2a, two experiments for Stx2a toxoid. Statistical significance was calculated for PBS and Stx2a using two-tailed Student’s t-test using GraphPad Prism 5.0. B. Stx2a damages organoid epithelial layer. Cryosections of iHIO treated with PBS or Stx2a for 5 days were stained with DNA stain Hoechst (blue). Lumens were mostly clear and epithelial cells were tightly packed for PBS treated iHIOS. In contrast large numbers of cells were extruded in the lumens of iHIOS treated with Stx2a. Representative images from at least 3 independent experiments are shown.
**Stx2a causes altered distribution of epithelial junction proteins.** In healthy human intestine, the mucosal epithelium acts as a selective barrier (107). This epithelial barrier controls the exchange between lumen and systemic circulation, and protects the host from pathogens and their virulence factors (109). Proper functioning of the epithelial barrier depends on protein complexes that form different intercellular junctions, including tight junctions and adherens junctions (110, 117, 121). Tight junctions are the most apical, which seal adjacent epithelial cells in a narrow band just beneath the apical surface. Below the tight junctions are adherens junctions, which form strong mechanical attachments between adjacent cells and are responsible for holding epithelial cells together (110, 115, 117, 118).

Since Stx2a appeared to affect the iHIO barrier function, we examined the effect of Stx2a on iHIO junction proteins. Figure 3.4: Stx2a causes mislocalization of E-cadherin. iHIO injected with A. PBS and B. Stx2a, showing DNA (blue), E-cadherin (green), vimentin (red), and merged images of all three stains. Representative images from at least 3 replicates are shown.
**Adherens junction protein E-cadherin:** Cryosections of PBS-treated iHIO showed strong expression and normal intercellular distribution of E-cadherin at all time points tested. Moreover, a distinct boundary between epithelial and mesenchymal layers was observed, as seen by a clear distinction between the staining for the epithelial E-cadherin and the mesenchymal vimentin (Figure 3.4 A). Similar to PBS treated controls, intercellular E-cadherin staining was also observed for cryosections of iHIO at 4 hours post-Stx2a injection. However, at 48 hours E-cadherin appeared to be mislocalized, and the single cell epithelial layer appeared to be replaced by several layers of loosely associated cells, some of which were surrounded by E-cadherin. The E-cadherin mislocalization appeared to occur concurrently with the extrusion of cells in the lumens. At 96 hours, E-cadherin expression was greatly reduced, and not continuous. The distinct boundary between epithelial and mesenchymal staining was lost, and interestingly, some of the cells in the lumen stained positive for vimentin, suggesting that those could be of the mesenchymal phenotype (Figure 3.4 B).

**Tight junction proteins:** We also stained the cryosections for the tight junction proteins including occludin, claudin-2, claudin-3, zona-occludens (ZO)-1 and ZO-2. All tight junction proteins displayed mostly

![Figure 3.5: 300 ng Stx2a affects tight junction proteins. iHIO at 5 days post-injection with A. PBS and B. Stx2a, showing merged images of DNA in blue, occludin, claudin-2, ZO-1 in green, and claudin-3, ZO-2 in red. Images represent one repeat of experiment.](image)
intercellular epithelial staining in PBS treated controls, as would be expected in a healthy intestine (Figure 3.5 A). Similar to E-cadherin, the epithelial layer of the Stx2a-treated iHIO displayed improper or decreased staining for claudin-3, ZO-1 and ZO-3 (Figure 3.5 B). Interestingly, cells extruded into the lumen of Stx2a-treated iHIOs displayed strong staining with ZO-3, while ZO-3 staining was mostly absent in cells from the epithelial layer. In contrast, occludin and claudin-2 staining was not significantly different between the two treatment groups. Overall, these studies demonstrate that Stx2a alone is capable of breaking the epithelial barrier.

**iHIO support the growth of *E. coli***: To examine the ability of iHIO to serve as an appropriate model of human intestinal STEC infections, we determined whether *E. coli* can persist in iHIO lumen. We used a non-pathogenic ECOR13 strain of *E. coli* and a pathogenic O157:H7 serotype of Stx2a producing *E. coli* (PT29). Approximately $10^3$ bacteria were microinjected into iHIO lumen under conditions that restricted bacterial growth to the lumen, without allowing the bacteria to grow in the tissue culture media. iHIO were able to support the growth of both *E. coli* strains, as seen by increased number of bacterial CFU recovered 1-day post infection (Figure 3.6), while no visible bacterial growth was observed in the tissue culture media. Higher CFU were observed for ECOR13 at 24 hours than PT29 at 18 hours post injection.
Pathogenic PT29, but not non-pathogenic ECOR13, alters iHIO morphology: We determined the effect of bacterial infection on iHIO morphology and luminal epithelial layer. The morphology of iHIO infected with the non-pathogenic ECOR13 was very different than iHIO infected with pathogenic PT29. iHIO injected with $10^3$ non-pathogenic ECOR13 E. coli resembled in morphology to PBS injected organoids, and remained intact during the 1 day course of infection. On the other hand, injection of $10^3$ PT29 E. coli displayed a rapid damage to iHIO morphology (Figure 3.7 A). The iHIO morphology was altered and appeared to loose the 3-dimensional architecture.

To investigate the effects of both strains of E. coli on the iHIO lumen, cryosections of iHIO were stained for DNA dye Hoechst. While the lumen of iHIO injected with the non-pathogenic ECOR13 was clearly defined and appeared intact, no clearly defined lumens were observed in the cryosections of PT29 treated iHIO (Figure 3.7 B).

PT29, but not ECOR13, severely damages iHIO lumen: O157:H7 and other enteropathogenic E. coli are known to form intimate attachment with intestinal epithelial cells mediated by the cytoskeletal protein F-actin. This intimate attachment is thought to cause
damage to the mucosal epithelium and induce SOS stress response in STEC. We determined if PT29 display this phenomenon in iHIO, by examining the staining of E-cadherin and F-actin in the cryosections of iHIO treated with both ECOR13 and PT29. Luminal epithelial layer of iHIO infected with ECOR13 was well defined at 24 hours post-infection; intercellular E-cadherin, and apical F-actin staining was observed (Figure 3.8 A). Normal short rods of *E. coli* were observed inside the lumen, some of which were closer to the epithelial layer, while others were deeper inside the luminal cavity. Intimate attachment was not observed with these *E. coli*.

The luminal epithelial layer of iHIO treated for 1 hour with $10^3$ PT29 *E. coli* was also well defined (Figure 3.8 B). Few bacteria were observed in the lumen, consistent with the CFU count of $\approx 10^3$ observed at the end of 1 hour post infection with PT29 (Figure 3.6). Bacteria were found closely associated with the epithelium, and some *E. coli* cells appeared to be embedded into F-actin, possibly representing intimate bacterial attachment to the iHIO epithelial cells (Figure 3.8 B, white arrowheads). At 4 hours post infection with PT29, more bacteria were found in the iHIO lumen (Figure 3.8 C), consistent with the increased bacterial CFU at 4 hours (Figure 3.6). The luminal epithelial layer appeared less defined at 4 hours, with incorrectly localized E-cadherin in some areas. The lumens showed increased number of extruded cells and cellular debris. Similar to the 1-hour time-point, *E. coli* intimately embedded within the F-actin layer were observed in the cryosections of the iHIO at 4 hours post infection (Figure 3.8 C, white arrowheads). Moreover at this time point, while most of the bacteria appeared as short rods, some were displayed a filamentous phenotype (Figure 3.8 C, yellow arrows), seen by elongated and end-to-end attached bacteria. Much worse condition of the iHIO was seen in the cryosections at 18 hours post PT29 infection; dispersed mammalian cells, and cell debris were found throughout the iHIO (Figure 3.8 D). No defined luminal epithelial layer was seen and
patches of mislocalized E-cadherin and F-actin staining were observed in some areas. Intimately attached bacteria were not observed at this time-point, possibly due to lack of a proper epithelial layer. Moreover, most of the *E. coli* cells were found in long filaments throughout the iHIO (Figure 3.8 D, yellow arrows), suggesting activation of the bacterial SOS response.

**Figure 3.8:** PT29, but not ECOR13, severely damages iHIO lumen. Cryosections of iHIO treated with A. ECOR13 for 24 hours, B. PT29 for 1 hour, C. PT29 for 4 hours, D. PT29 for 18 hours, were stained for DNA (blue), E-cadherin (green), F-actin (red). Merged images are shown in the fourth column. Zoomed images are shown in the orange box in the right-most column. Representative images from duplicate experiments are shown. White arrowheads indicate bacteria intimately attached to F-actin, while yellow arrows point to filamentous *E. coli*. 
**D. Discussion**

We show that iHIO express Gb3, can bind to Stx2a, and are sensitive to the protein synthesis inhibitory action of purified Stx2a. Schuller et al have reported that Paneth cells express Gb3 and can bind to Stx2a (221). It is possible that Stx2a staining in iHIO also occurs on Paneth cells. However, more detailed experiments using Paneth cell specific markers, and Stx2a and Paneth cell co-staining would be required to confirm this. Different binding patterns of Stx2a observed on iHIO cells seem consistent with different Gb3 microenvironments on different cells. Previous studies have shown that the lipid raft microenvironment surrounding Gb3 strongly influences the affinity of Stx2a for Gb3 (64, 76, 77, 227, 228). It is therefore likely that the different binding patterns of Stx2a are a result of different affinities of Stx2a to Gb3, depending on the surrounding lipid raft composition of the cell.

The viability of intestinal epithelial cells is dependent on correct localization and interaction with neighboring cells. Failure to receive survival signals from neighboring cells can result in excessive shedding of epithelial cells into the lumen, and cause damage to the epithelium in a process called anoikis (229). The action of purified Stx2a on iHIO seems consistent with this phenomenon. Based on the few Stx2a-binding cells observed in iHIO, it is possible that damage induced by protein synthesis inhibitory action of Stx2a to these target cells results in failure of the neighboring cells to receive survival signals, resulting in slow damage to the epithelial barrier over a few days. The phenomenon of epithelial cell extrusion in response to Stx2a was previously reported human intestinal explants treated with Stx2a (219). However, due to the limited viability of tissue explants, their studies could not be extended past 24 hours, and the effect of Stx on epithelial barrier function was not reported. We were able to overcome this limitation using iHIO, and could determine the effect of Stx on iHIO for 5 days. To our
knowledge, this is the only intestinal model currently available to study the long-term intestinal effects of the toxin in tissue culture.

Several intestinal pathogens and their toxins have been shown to damage the intestinal mucosa by targeting epithelial cell-junction proteins. *Bacteroides fragilis* disrupts tight junctions of intestinal epithelium by proteolytic degradation of the junction proteins (121) and *Clostridium difficile* toxins A and B cause endocytosis of the tight junction proteins (122) resulting in decreased epithelial cell adhesions. *Candida albicans* and *Porphyromonas gingivalis* secrete proteases that cleave the adherens junction protein E-cadherin, resulting in invasion of the epithelial barrier by affecting the adherens junctions (123, 124). Bacterial toxins such as botulinum toxin and cholera toxin also target E-cadherin to disrupt the intestinal epithelium and enter the systemic circulation (125, 126). Similarly, our studies show that both Stx and STEC cause mislocalization of adherens and many tight junction proteins.

In addition to studying purified Stx, we developed techniques to contain the growth of viable *E. coli* inside the iHIO lumens, without contaminating the tissue culture media. Non-pathogenic ECOR13 were able to grow inside the iHIO lumens to very high numbers within 24 hours, suggesting that the iHIO lumen must be a nutrient rich environment. The lower CFU observed after overnight infection with PT29, as compared to ECOR13, could be the result of filamentous *E. coli* growth, indicating anomalous cell division, which typically results when the bacterial cells continue to elongate without actually dividing. The filamentous PT29 *E. coli* could have resulted in lower CFU, as a single chain can only form one CFU, in spite of possessing multiple nucleoids. This is consistent with the bacterial morphology observed in the cryosections at different time points post PT29 infection. The PT29 *E. coli* appear mostly as short rods at 1 and 4 hours post infection, at which points a rapid increase in CFU is seen.
Bacteria are known to assume filamentous morphology in response to environmental stress signals, including activated SOS stress response (230). The SOS signal is necessary for induction of the phage lytic cycle and Stx expression, and is suggested to be activated in response to free radicals, such as reactive oxygen species and reactive nitrogen species (31). In the human intestine, reactive oxygen species serve as an antimicrobial defense as well as signaling molecules to alert other intestinal cells to the presence of pathogens (231). Intimate attachment of bacteria to the intestinal epithelium is also known to activate bacterial SOS response. The presence of filamentous forms of PT29 strain suggests that it activated the host defenses, while the absence of filamentous forms of the non-pathogenic ECOR13 suggests that it failed to activate host defenses. Overall, these results suggest that iHIO are not only sensitive to the action of STEC, but they can also activate defense mechanisms specifically in response to bacteria that are pathogenic to the human intestine.

Intestinal infections with STEC result in serious complications involving multiple distant organs including kidneys and neurons. Until now, the lack of complete understanding of intestinal interactions of STEC, due to unavailability of an appropriate human intestinal model, has limited our ability to develop treatments for the life threatening HUS and neurological symptoms associated with STEC infections. This study demonstrates that iHIO can serve as the first tractable and physiologically relevant model of human intestinal complications associated with STEC infections.
Chapter 4

Intracellular Signaling of Shiga Toxin
A. Introduction

Shiga toxin-producing *E. coli* (STEC) is a major cause of diarrheal disease, and Shiga toxin (Stx) is primarily responsible for hemolytic uremic syndrome (HUS), the life-threatening complication following intestinal infection by STEC. Stx belongs to the AB\textsubscript{5} group of toxins. The A-monomer inhibits protein synthesis of mammalian target cells, while the B-pentamer binds to the host cell surface receptor, the glycolipid globo tria osylceramide (Gb3), and mediates internalization and cytoplasmic delivery of the A-subunit. The toxicity of Stx to intestinal epithelial, and endothelial cells, kidney cells, and neurons has been known to contribute to HUS pathogenesis. However, the exact mechanisms of pathogenesis are debated. While cellular death is primarily due to protein synthesis inhibition by the A-subunit, it is now clear that both A- and B-subunits can activate signaling cascades that can mediate toxicity without directly causing cellular death.

The Stx A-subunits are highly specific N-glycosidases, which cleave a single adenine residue from the 28S rRNA component of eukaryotic ribosomes. Signaling pathways activated by the catalytically active Stx have been previously reported. Treatment of human colonic epithelial cells with Stx\textsubscript{1} holotoxin, but not inactivated Stx\textsubscript{1}, resulted in stimulation of JNK and p38 MAPK signaling cascades initiating apoptotic pathways, suggesting an action mediated by the A-subunit catalytic activity of Stx\textsubscript{1} (232). Some reports suggest induction of signaling cascades leading to apoptosis in human monocytic THP1 and intestinal epithelial cell lines mediated by the ribocytotoxic stress induced by the A-subunit after entry into the cytosol. (159, 232). While for the A-subunit induced signaling, entry into the cytosol seems important, the B-subunits are suggested to induce rapid cell surface signaling cascades, occurring soon after Stx interacts with the cell surface receptors. A report by Katagiri et al showed that Stx induced
activation of tyrosine kinase YES in ACHN kidney cells within a few minutes of cell binding in a Gb3 dependent manner (150). Although this study used holotoxin, inhibition of protein synthesis was seen a few hours after Stx treatment. The immediate activation of kinases suggested that cell-surface signaling occurred independently of protein synthesis inhibition. In addition, Stx has been shown to activate several other tyrosine kinases such as SYK in HeLa cells (149), and LYN in Burkitt’s lymphoma Ramos cells (151). Activation of these kinases is thought to cause rapid tyrosine phosphorylation, and stimulation of several cytoskeletal proteins, cytokines and chemokines (233). For example, treatment of human intestinal epithelial cells with sublethal concentrations of Stx1 has been shown to stimulate IL-8 secretion (234). Moreover, Stx1 has been shown to activate tumor necrosis factor α (TNF-α) secretion in THP-1 monocytic cells in a tyrosine kinase dependent manner (159). B subunits of Stx, by themselves, are also capable of inducing a variety of lipid raft-dependent signaling mechanisms, such as cytoskeletal remodeling, secretion of blood clotting factors and retrograde trafficking of Stx (162-165).

Currently, most of the studies with Stx-related signaling mechanisms have been performed using the less toxic isoform Stx1, and not much information is available on the more toxic Stx2a mediated intracellular signaling events. Moreover, studies have been performed using cells such as HeLa, monocytes, and lymphoma cell lines, which are not the targets of Stx in disease. As a result, the physiological relevance of these reports to Stx pathogenesis is unknown. In this chapter we examined the ability of the more toxic Stx2a to induce activation of tyrosine kinases in Vero monkey kidney cells, which are extremely sensitive to the protein synthesis inhibitory action of Stx. We also report the effect of tyrosine kinase inhibition on the action of Stx on the Vero monkey kidney cells.
B. Materials and methods

Cell line and reagents: Vero monkey kidney cells (a gift from Alison O’Brien) previously transfected to express luc2p, a gene for destabilized luciferase, were used in this study (34). Purified Stx2a was obtained from Biodefense and Emerging Infections (BEI) Research Resources Repository. Biotin-conjugated mouse monoclonal antibody to phospho-tyrosine (clone 4G10) was obtained from Millipore. Peroxidase-conjugated streptavidin (Pierce) was used to detect the biotin-conjugated primary antibody. Genistein was obtained from Indofine Chemical Company, Hillsborough, New Jersey, and 100 mg/ml stock solutions were prepared in DMSO. Mouse monoclonal antibody to tubulin (clone DM1A) was obtained from Cell Signaling Technology, and peroxidase conjugated goat anti-mouse antibody was purchased from MP Biomedicals.

Determination of tyrosine phosphorylation: Vero monkey kidney cells were harvested, washed once with Hank's Balanced Salt Solution (HBSS) (Gibco) and suspended in serum-free Minimal Essential Media (MEM). The cells were warmed to 37 °C, and approximately 10^4 cells were incubated with serum-free MEM or 320 ng Stx2a at 37 °C with 5% CO2 for indicated times. Samples were subsequently washed with ice-cold PBS and lysed in lysis buffer containing 1% NP-40, 0.1% sodium dodecyl sulfate, 0.1% deoxycholate, 150 mM NaCl, 50 mM Tris, 20 mM Na3VO4, and Complete protease inhibitor tablet (Roche). The total protein content in the cell lysates was determined using bicinchoninic acid (BCA) assay. Approximately 75 μg of total protein was mixed with Laemmli loading buffer, and electrophoresed in 15-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Western blots were performed by transferring the gels to PVDF membranes, and probing with anti-phosphotyrosine primary and corresponding secondary antibody. Western blot for α-tubulin was performed as the loading control.
**Effect of tyrosine kinase inhibition on Stx activity:** Vero monkey kidney cells were washed once with HBSS and incubated with indicated concentrations of the tyrosine kinase inhibitor genistein in complete MEM at 37 °C with 5% CO₂ for 30 minutes. Serial dilutions of Stx1 and Stx2a were added to luminometer-compatible white tissue culture treated, Falcon 96-well microtiter plates (Becton Dickinson, Franklin Lakes, NJ). Vero cells (10⁴), pre-incubated with genistein or control cells were added to the wells and incubated with Stx2a for 4 hours at 37 °C with 5% CO₂. After 4 hours, the cells were washed three times with PBS, and 25 µl of Superlite luciferase substrate (Bioassay Systems, Hayward, CA) was added, and light measured using Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland).
C. Results

**Determination of tyrosine phosphorylation:** Previous studies have shown that Stx1 activates tyrosine kinases in various cell types (150). In this study, we examined the effect of Stx2a on tyrosine kinase levels in Vero monkey kidney cells by examining the accumulation of phosphorylated tyrosine residues upon Stx2a treatment. Stx2a treatment caused a time-dependent increase in tyrosine phosphorylation corresponding to a 32 kDa band about 20 minutes after incubation, and after 40 minutes about 20-fold increase in tyrosine phosphorylation compared to untreated cells was seen, based on the band intensity on the western blot (Figure 4.1). Levels of the loading control, α-tubulin were unaltered during the time course of the experiment, suggesting that the increase in intensity was specific for phosphorylated tyrosine residues.

Figure 4.1 Stx2a activates phospho-tyrosine residues. Vero monkey kidney cells were treated with Stx2a, and activation of tyrosine kinases was determined by observing phosphorylation of tyrosine residues by western blot. Levels of α-tubulin were determined as the loading control.
Effect of tyrosine kinase inhibition on Stx activity: Stx causes protein synthesis inhibition in Vero monkey kidney cells with an ED<sub>50</sub> value of 63 pg/ml for Stx1, and 461 pg/ml for Stx2a (55). Previous studies showed that inhibition of tyrosine kinase SYK in HeLa cells reduced the cellular uptake of Stx1 B-subunits (149). We determined the effect of tyrosine kinase inhibition on the catalytic activity of Stx1 and Stx2a holotoxins in Vero cells, using a known specific tyrosine kinase inhibitor genistein (235). Since tyrosine kinases serve many different

![Figure 4.2](image_url)

**Figure 4.2 Effect of genistein on Stx activity.** Vero cells were incubated with tyrosine kinase inhibitor genistein for 30 minutes, followed by incubation with Stx for 4 hours. Inhibition of protein synthesis was assessed as a measure of Stx activity. **A.** Protein synthesis inhibition at increasing concentrations of Stx after incubation with indicated genistein concentrations. **B.** Comparison of protein synthesis inhibition at highest Stx concentration. The results are an average of three independent experiments and error bars indicate standard deviation. Statistical differences in B was calculated by the two-tailed Student's t-test using GraphPad Prism 5.
functions in a cell, prolonged inhibition of tyrosine kinases could be deleterious to the cell. The luc2P transfected Vero cells allow measurement of protein synthesis in short time periods of 4 hours. As a result, it was possible to determine the effect of genistein on the protein synthesis inhibitory activity of Stx, while avoiding the unwanted side effects of prolonged tyrosine kinase inhibition in Vero monkey kidney cells. Pre-incubation of Vero cells with genistein protected the cells from the protein synthesis inhibitory effect of both Stx1 and Stx2a in a dose dependent manner. Maximum effect was at 100 µg/ml of genistein, at which no inhibition of protein synthesis was observed in Vero cells at the highest toxic concentrations of Stx tested (Figure 4.2).
D. Discussion

The studies showing activation of tyrosine kinases reported in this chapter were performed with Stx holotoxins, and not individual subunits. However, considering the short time required for tyrosine kinase activation, it is most likely that this is an effect of Stx-cell surface receptor interaction mediated by the B-subunits. A potential candidate for the 32 kDa protein could be DAPP1 (for dual adaptor for phosphotyrosine and 3-phosphoinositides), an adaptor protein that possesses a Src homology domain and a pleckstrin homology domain. DAPP1 displays broad cellular expression, including kidney cells, and interacts with different tyrosine kinases including YES, which has been previously shown to be activated by Stx (150). DAPP1 has been shown to co-localize with lipid rafts, clathrin, and actin filaments (236). Moreover studies suggest a link between DAPP1, cytoskeletal rearrangement, and receptor trafficking (237). Thus DAPP1 is in the right place to play a role in Stx internalization. Taken together, these results suggest that tyrosine kinases activated by Stx play an important role in the catalytic action of Stx on protein synthesis inhibition.
Chapter 5

Summary and Future Directions
Since their initial recognition in the 1980’s, the food-borne outbreaks of STEC appear to be increasing and Stx related disorders have emerged as a serious threat, especially when mass-produced and mass-distributed foods are concerned. Currently no treatment options exist for the life threatening complications such as HUS and neurological symptoms associated with STEC infections, mainly due to limited knowledge about Stx and incomplete understanding of interaction of Stx and STEC with different organs, especially the human intestine. In this thesis we worked to answer some important questions regarding Stx-receptor binding, and interaction of Stx and STEC with the human intestine. The results from this thesis can take the following directions in the future:

1. **Stx assembly and receptor interaction:**

   Results from this thesis suggest that A- and B-subunits of Stx are capable of assembling at the Gb3 receptor interface. Mixture of B-subunits with A$_2$-subunit peptides increased Gb3-interaction of the B-subunits. Moreover, we identified the residues on the A-subunit required for interaction with the B-subunit. In another study from our lab, the A$_2$-subunit peptides were shown to rescue mice from toxicity associated with Stx2a holotoxin (data not yet published). This suggests that the interaction of the A$_2$-subunit peptides with the B-subunits can be used to develop treatments for Stx-associated complications. However, this requires a more detailed understanding of inter-subunit interactions, and the effect of A$_2$-subunit peptides on B-subunit assembly and Gb3-interaction. Studies need to be performed using mutants altered for subunit association, for example comparing Stx2a B-subunit wild-type, stable Q40L, and an unstable B-pentamer, possibly Q40K, which fails to oligomerize and always stays in monomeric state. Effect of the A-subunit peptides on the assembly states of these B-subunit mutants can be determined using AUC. Moreover, due to the detection limit of BLI, the assembly studies in this
thesis were performed at concentrations of Stx much higher than the physiologically observed concentrations. As a result, use of more sensitive techniques to determine inter-subunit assembly would be useful. A possible approach could be to fluorescently label the A2-peptides at the Cys residue and study their effect on B-subunit assembly using fluorescence polarization, to study assembly at lower concentrations of the subunits.

2. Use of iHIO to understand intestinal interactions of Stx and STEC:

We have shown that iHIOs are sensitive to the catalytic action of Stx, and can replicate the suggested steps in the intestinal colonization and pathogenesis of STEC. iHIOs can therefore serve as an excellent model to answer the following questions about STEC pathogenesis:

i. **Stx target cells**: Our studies suggest that Stx targets a specific set of cells in the iHIO. Future experiments are necessary to determine the identity of these cells. Since Paneth cells have been previously implicated to be the intestinal targets of Stx, these seem to be the most likely candidate cell types. Co-staining experiment using flow cytometry can be performed to determine co-localization of Stx with Paneth cells, using cluster of differentiation, CD-24 marker for Paneth cells. Additionally, another co-staining experiment can be performed to determine whether Paneth cells express Gb3.

ii. **Variants of STEC**: iHIOs are sensitive to the action of Stx; however infection with Stx2a-producing O157:H7 (PT29) induces very rapid damage to the iHIOs. Since in addition to Stx2a, PT29 produces other toxic effectors mediated by the type-3 secretory system (T3SS), it is important to understand the individual actions of Stx versus the T3SS effectors on iHIOs. A Δ-toxin variant of PT29 can be used, in which Stx2a gene in the bacteriophage
will be replaced with GFP as the late phage gene reporter. Additionally, C600 *E. coli* lysogenized with Stx2a phage (C600::933W), which produce only Stx2a, without other toxic effectors produced by O157:H7, can be used. Determining the effect of these strains on SOS induction, bacterial filamentous growth and epithelial barrier breach will identify the contribution of Stx and other STEC effectors in inducing iHIO damage.

3. **Identification of tyrosine kinases activated by Stx:**

   We showed that Stx activates a tyrosine kinase of approximately 32 kDa in Vero monkey kidney cells, and inhibition of tyrosine kinase activity in Vero monkey kidney cells resulted in protection from Stx-mediated protein synthesis inhibition. Studies to identify this tyrosine kinase can be performed. A possible candidate for the 32 kDa protein is DAPP1, a cytoskeletal tyrosine kinase known to localize with the lipid rafts. The effects of pre-treatment of Vero cells with an siRNA directed against DAPP1 on Stx activity can be determined as an initial experiment. If knockdown of DAPP1 protects Vero cells from protein synthesis inhibitory action of Stx, this could lead to identification of a key player in cellular trafficking of Stx. Currently, much is unknown about the signaling pathways induced by Stx, and their role in overall Stx pathogenesis, mainly due to use of incorrect models to study Stx. As a result understanding the signaling events associated with Stx in correct disease cell targets would be extremely helpful.
Bibliography


13. CDC. Multistate outbreak of Shiga toxin-producing *Escherichia coli* O121 infections linked to raw clover sprouts: CDC; 2014 [updated 08/01/2014; cited 2014 1 August].

14. CDC. Multistate outbreak of Shiga toxin-producing *Escherichia coli* O121 infections linked to Farm Rich brand frozen food products CDC *E. coli* Web Page2013 [updated 05/30/2013].

15. CDC. Investigation update: outbreak of Shiga toxin-producing *E. coli* O104 (STEC O104:H4) infections associated with travel to Germany 2011 [updated 07/08/2011].

16. CDC. Multistate outbreak of Shiga toxin-producing *Escherichia coli* O26 infections linked to raw clover sprouts at Jimmy John's restaurant 2012 [updated 04/03/2012].
17. CDC. Investigation update: multistate outbreak of human *E. coli* O145 infections linked to shredded romaine lettuce from a single processing facility 2010 [updated 05/21/2010].


19. CDC. Investigation of multistate outbreak of *E. coli* O157:H7 infections 2008 [updated 07/18/2008].


164. Liu F, Huang J, Sadler JE. Shiga toxin (Stx)1B and Stx2B induce von Willebrand factor secretion from human umbilical vein endothelial cells through different signaling


198. Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K. Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic


