Passive immunization against invasive salmonella enterica

Angelene Richards

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PASSIVE IMMUNIZATION AGAINST INVASIVE SALMONELLA ENTERICA

By

Angelene F. Richards

A Dissertation
Submitted to the University at Albany, State University of New York
In Partial Fulfillment of
The Requirements for the Degree of
Doctor of Philosophy

School of Public Health
Department of Biomedical Sciences
2021
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*Important note for the reader, Charlie is a golden retriever.*

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DEDICATION

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<tbody>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>CCR6</td>
<td>C-C chemokine receptor 6</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>c-di-GMP</td>
<td>cyclic dimeric guanosine monophosphate</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CHO cell</td>
<td>Chinese Hamster Ovary cell</td>
</tr>
<tr>
<td>CI</td>
<td>competitive index</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>cryogenic electron microscopy</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DGC</td>
<td>diguanylate cyclase</td>
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<tr>
<td>dlgA</td>
<td>dimeric IgA</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EPS</td>
<td>exopolysaccharide</td>
</tr>
<tr>
<td>ETEC</td>
<td>enterotoxigenic Escherichia coli</td>
</tr>
<tr>
<td>Fab</td>
<td>antigen-binding fragment</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment crystallizable region</td>
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<tr>
<td>FAE</td>
<td>follicle-associated epithelium</td>
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<td>gut-associated lymphoid tissue</td>
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<td>gastrointestinal</td>
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<td>H&amp;E</td>
<td>hematoxylin &amp; eosin</td>
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<td>HBC</td>
<td>hyperimmune bovine colostrum</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>immunoglobulin A</td>
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IgG: immunoglobulin G
IgM: immunoglobulin M
IHC: immunohistochemistry
IL: interleukin
iNTS: invasive nontyphoidal Salmonella
i.p.: intraperitoneal
J-chain: joining chain
LB: Luria Bertani
LPS: lipopolysaccharide
M cell: Microfold cell
mAb: monoclonal antibody
mCherry: derivative of monomeric red fluorescent protein
mg: milligram
mlgA: monomeric IgA
mL: milliliter
NTS: nontyphoidal Salmonella
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
plgA: polymeric IgA
plgR: polymeric immunoglobulin receptor
SC: secretory component
SED: subepithelial dome
SEM: scanning electron microscopy
SGF: Simulated Gastric Fluid
SPI: Salmonella pathogenicity island
SlgA: secretory IgA
S_IgM: secretory IgM

STEC: Shiga like toxin-producing *E. coli*

STm: *Salmonella enterica* serovar Typhimurium

STy: *Salmonella enterica* serovar Typhi

T3SS: type III secretion system

*T*_FH: T follicular helper cell

TLR: Toll-like receptor

WHO: World Health Organization

WT: wildtype

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ABSTRACT

The Gram-negative bacterium, *Salmonella enterica* is a prominent etiologic agent of both diarrheal disease and enteric fever that encompasses over 2500 serovars, including *S. Typhimurium* (STm) and *S. Typhi* (STy). *S. enterica* is transmitted through contaminated food and water and, following ingestion, invades the gastrointestinal (GI) mucosa. The bacterium uses flagellar-based motility to target microfold (M) cells overlying gut-associated lymphoid tissues known as Peyer’s patches in the small intestine. Entry into Peyer’s patch tissues is a pivotal step in the infection process, as the bacterium can then disseminate systemically in the host. Given the rise in antibiotic resistance amongst *S. enterica* serovars and a lag in vaccine development, there is a push to investigate alternative biologics for therapeutic use. As such, the goal of my dissertation was to investigate the potential of passively administered antibodies to limit *S. enterica* infection, examining STm and STy as pathogens of interest.

Secretory IgA (SIgA) is the primary antibody found in mucosal secretions that line the gastrointestinal tract. It is also the predominant antibody in breastmilk, where it affords maternal immunity to newborns. SIgA antibodies specific for *S. enterica* LPS are postulated to facilitate host protection by arresting bacterial motility, reducing type III secretion system (T3SS)-mediated entry into epithelial cells, and by preventing access to the epithelium through bacterial agglutination. One such IgA antibody is Sal4, a well-characterized monoclonal antibody (mAb) that binds the O-antigen region of STm lipopolysaccharide (LPS), specifically at the acetylated abequose moiety known as the O5 epitope. While a number of biological activities have been attributed to Sal4 *in vitro*, the possible mechanisms of protection by Sal4 *in vivo* have yet to be fully elucidated.
In Chapter 3, I developed a mouse model to examine the potential of Sal4 to passively immunize animals against oral STm infection via reduced bacterial invasion into Peyer’s patches, the primary site of invasion for STm in the mouse gut. In this model, oral delivery of Sal4 IgA significantly blocked STm invasion into Peyer’s patch tissues of infected animals, which led us to explore the capacity of other antibody isotypes to impede STm pathogenesis. I evaluated an IgG1 variant of Sal4 and found that Sal4 IgG treatment arrested flagellar-based motility and blocked STm invasion into epithelial monolayers to the same extent as Sal4 IgA in vitro at equivalent concentrations. However, unlike Sal4 IgA, Sal4 IgG failed to prevent wildtype invasion into Peyer’s patches when employed in vivo. I determined that the diminished efficacy was at least in part due to instability in the mouse GI tract, as Sal4 IgG was readily degraded in adult simulated gastric fluid (SGF) in vitro and its activity was partially restored in vivo after buffering.

As a follow up to our invasion studies, in Chapter 4 I sought to utilize our in vivo model to determine the window of protection of Sal4 by examining the kinetics of STm after administration, using human recombinant Sal4 SIgA. By isolating STm colony forming units (CFUs) from the fecal samples of infected animals over time, I demonstrated that Sal4 mAbs significantly reduce wildtype STm colonization 21 hours after infection. In our tracking studies, I determined that bacterial transit time in the mouse is rapid, with STm cells detected at the rectal end of the GI tract 60 minutes after gavage by immunohistochemistry (IHC). When provided at time of challenge, Sal4 mAbs localized STm cells to the mucus layer overlying the epithelium, compared to PBS control groups where STm cells were freely dispersed in the lumen. These data
highlight a likely mechanism of elimination by SIgA. Based on these results, I conclude that Sal4 mAbs can facilitate clearance of colonized STm via entrapment, but that timing for protection is limited due to dilution in the lumen from accelerated GI movement.

Predominantly, IgA found on mucosal surfaces is in the form of SIgA, a complex heavily glycosylated molecule made up of two monomers bound by joining (J) chain and associated with secretory component (SC). In Chapter 5, to further elucidate the importance of human SIgA in mucosal protection from STm, I tested humanized recombinant Sal4 SIgA, as well as dIgA and mIgA isoforms, in parallel in the *Salmonella* invasion model. I found that human Sal4 SIgA successfully prevented wildtype STm invasion into Peyer’s patches and promoted robust agglutination of STm in the small intestines of infected animals. Sal4 SIgA-induced aggregates were resistant to detection by IHC despite rigorous antigen retrieval methods. Based on these results, I hypothesized that Sal4 SIgA treatment induced the production of exopolysaccharide (EPS) *in vivo*, which was recapitulated *in vitro* as crystal violet (CV) staining of borosilicate glass tubes and determined to be cellulose-dependent. Taken together, these data validate the efficacy of oral Sal4 SIgA to passively immunize the mucosa from STm infection and postulate agglutination and extracellular matrix induction as protective mechanisms by secretory antibodies.

In addition to facilitating typhoid fever, STy produces an A$_2$B$_5$ toxin intracellularly following internalization into host cells that contributes to STy pathogenesis and disease progression. In Chapter 6, to examine the potential of antibodies to passively immunize against typhoid toxin, I developed a panel of 11 IgG mAbs by immunizing mice intraperitoneally with recombinant typhoid toxoid and generating hybridoma cell lines
from antigen-specific splenic B cells. We found that the majority (10) of the mAbs bound the binding subunit, PltB, and identified three distinct hybridoma clones; TyTx1, 3, and 4. Each TyTx mAb was then evaluated in vitro by our collaborators at Cornell University for the ability to block toxin binding to human epithelial cells. TyTx 1 and 4 bound separate epitopes of PltB, which accounted for differences in antibody avidity, cell surface binding inhibition, and efficacy in vivo. Overall, these data demonstrate that TyTx mAbs passively protect against intoxication by typhoid toxin through multiple mechanisms and provide important insight into the future design of antibody-based intervention strategies.

The results of this dissertation highlight the ability of mAbs to passively immunize against multiple serovars of S. enterica, by either arresting bacterial access in the lumen, or through direct toxin neutralization. Indeed, while oral delivery of SIgA protected the GALT from STm infection, the window of protection was determined to be limited. It is evident from these studies that robust agglutination by SIgA antibodies is required for protection from STm, which empathizes the need for adequate local concentrations of antibody at the time of infection. In the advent of transitioning these biologics for use in humans, sufficient dosing and stabilization of SIgA mAbs would be a prerequisite. Taken together, these studies emphasize the capacity of mAbs to defend against an arsenal of virulence factors that enteric bacterial pathogens may employ and define the challenges that must be overcome for future therapeutic use in humans.
CHAPTER 1 : INTRODUCTION
INTRODUCTION TO S. ENTERICA

Despite advances in science and medicine, diarrheal disease remains a prominent public health issue globally. At-risk populations, such as immunocompromised individuals and young infants and children are most at-risk for morbidity, mortality, and long-term health deficits. Acute diarrheal disease remains one of the leading causes of death in children under the age of five globally, predominantly in regions of limited sanitation and access to reliable medical resources (Kotloff et al., 2019). A number of enteric pathogens circulating in regions of Southeast Asia and sub-Saharan Africa can induce diarrheal disease, including enterotoxigenic Escherichia coli (ETEC), Shigella flexneri, Vibrio cholerae, and Salmonella enterica (Troeger et al., 2017).

Salmonella enterica is the etiologic agent of both nontyphoidal salmonella (NTS) and typhoid fever. Salmonella enterica subspecies enterica encompasses over 2500 serovars of Salmonella known to facilitate disease in humans, which are typically divided into typhoidal and nontyphoidal serovars (Grimont & Weil, 2007). The vast majority of serovars are nontyphoidal and cause disease that is characterized by severe vomiting, diarrhea, fever, and intestinal inflammation (Coburn et al., 2007). The CDC estimates that NTS cause approximately 1.35 million infections and over 26,000 hospitalizations in the United States annually (Centers for Disease Control and Prevention, 2021). Globally, occurrences of nontyphoidal gastroenteritis disease were estimated to be around 95.1 million, contributing to 3.1 million disability-adjusted life-years (DALYs) lost (Roth et al., 2018). Typical gastroenteritis induced by NTS is commonly self-limiting with the infection resolving after several days. However, within
NTS subtypes are circulating strains that exhibit a more invasive phenotype within the host, known as invasive NTS (iNTS) (Gilchrist & MacLennan, 2019). Unlike common NTS infections, iNTS bacterial strains progress to systemic infection after entering the human gut, resulting in typhoid-like symptoms and bacteremia (Feasey et al., 2012). iNTS infections are associated with higher morbidity and mortality rates, particularly within vulnerable populations such as young children and adults with HIV (Roth et al., 2018). In a global survey of invasive nontyphoidal disease, it was estimated that 535,000 cases occurred in 2017 alone, with the majority of cases localized to sub-Saharan Africa (Stanaway et al., 2019). The vast number of circulating endemic Salmonella serovars further complicates the development of a cross-protective vaccine against NTS infections.

*Salmonella enterica* serovar Typhi (STy) is a major causative agent of typhoid fever, which is restricted to the human host (Johnson et al., 2018). Typhoidal serovars include *S. Typhi* (STy) and *S. Paratyphi*, though disease is more severe in cases of STy. Like iNTS pathogenesis, STy infection leads to systemic dissemination and bacteremia after ingestion of contaminated food or water (Dougan & Baker, 2014). Unlike STm which circulates in both developed and developing regions, STy is primarily endemic in countries with compromised drinking water and sanitation (Khan et al., 2017). The World Health Organization estimates that anywhere between 11 and 20 million people contract typhoid fever annually with 128,000 to 161,000 global deaths, although those estimates are likely underrepresented due to current diagnostic methods (Parry et al., 2011; World Health Organization, 2018). Considering the continuing
infections of both STm and STy around the globe, *S. enterica* remains today a prominent public health threat.

**SALMONELLA ENTERICA SEROVARS TYPHIMURIUM AND TYPHI**

*Salmonella enterica* is a Gram-negative, rod-shaped, motile bacterium that is a member of the Enterobacteriaceae family that includes other prominent enteric pathogens *S. flexneri*, *E. coli*, and *V. cholerae* (Lanata et al., 2013). The vast number of *Salmonella enterica* serovars are characterized by distinct differences in surface flagellar and lipopolysaccharide (LPS) antigens (Grimont & Weil, 2007). The bacterial lipopolysaccharide component of the *S. enterica* outer membrane consists of a lipid A region, core oligosaccharide, and repeating O-polysaccharide unit that extends above the bacterial LPS (Raetz & Whitfield, 2002). Two of the most prominent *S. enterica* NTS serovars in the United States, *S. Typhimurium* (STm) and *S. Enteritidis*, are partially identified by the O-antigen that can consist of up to 40 repeating units (Lerouge & Vanderleyden, 2002). STm is defined as O (1, 4, [5], 12) while *S. Enteritidis* is characterized by the presence of O-antigens (O:1, 9, 12) (Grimont & Weil, 2007). The STm O-antigen is made up of tetrasaccharide repeats consisting of galactose, rhamnose, and mannose moieties within the side chain that are bound to abequose (O4) at the mannose sugar (**Figure 1.1**) (Lindberg & Le Minor, 1984; Slauch et al., 1995). The O5 epitope is defined by the acetylation of the O4 abequose residue at the 2-hydroxyl group, which is not present in all STm strains, but is an immunodominant epitope when available (Kim & Slauch, 1999; Slauch et al., 1995). The O-antigen of *S. enterica* has been identified as a prominent virulence factor, which can provide
STm LPS consists of a lipid A region, core oligosaccharide, and O-antigen repeating regions(s) (repeat indicated by “n”). As part of the O-antigen, STm specifically expresses the O1, O12, O4 and/or O5 epitopes. Monoclonal antibodies (mAbs) Sal4 and PeA3 used in this study both bind the O5 epitope of STm LPS. Abbreviations: KDO = keto-deoxyoctulosonate; Hep = heptose; Glu = glucose; Gal = galactose; GluNAc = N-acetylglucosamine; Rha = rhamnose; Man = mannose; Abe = abequose; OAc = O-acetylation. Based on schematics by (Gunn, 2008) and (Ernst et al., 1999). Diagram created using BioRender.
resilience to antimicrobial activity within the host (Ilg et al., 2009; Rondini et al., 2013).

As such, antibodies directed against the O-antigen region of STm have been shown to facilitate infection in both mice and humans (Baliban et al., 2017; Rondini et al., 2013).

Along with expressing O-epitopes (O:1, 9, 12), STy is characterized by the additional presence of the Vi capsule, which is largely restricted to STy and S. Paratyphi C (Dougan & Baker, 2014; Grimont & Weil, 2007). Like the O-antigen for NTS serovars, the Vi capsule affords an evolutionary advantage to STy by providing resistance to both complement-mediated and phagocyte-mediated killing in sera (Hart et al., 2016). Anti-Vi capsule antibodies are correlated with protection in humans and currently licensed vaccines target the Vi polysaccharide as a protective epitope (Jin et al., 2021). At present, there are two typhoid vaccines approved for use in humans that induce antibodies against the Vi capsule: an oral live-attenuated deliverable and an intramuscular Vi polysaccharide vaccine. While the vaccines demonstrate moderate efficacy, there are constraints for broad-scale use (Yang, Chong, et al., 2018). For one, there is little immunogenicity observed in young children and no vaccination strategy is currently available for children under the age of 2 (Khan et al., 2017). Additionally, the window of protection is limited following immunization by either the oral or injectable vaccine, with the intramuscular vaccine and the oral deliverable affording 3 years and 7 years of protection, respectfully (Khan et al., 2017).

STy is unique to typhoid serovars in that the bacteria also produces a toxin as a virulence factor within the host. Typhoid toxin is an A₂B₅ toxin that was first identified in vitro for its ability to induce G2 cell cycle arrest of human epithelial cells (Haghjoo & Galan, 2004). It was later shown to recapitulate the acute phase of typhoid symptoms in
mice following systemic administration (Song et al., 2013). The toxin consists of two covalently linked A subunits, CdtB and PltA, that are bound to a pentameric B subunit, PltB (Song et al., 2013). PltB facilitates toxin binding and internalization by recognizing specific glycan and sialic acid residues on the surface of host cells (Chong et al., 2017), while the CdtB subunit possesses nuclease activity and facilitates cell cycle arrest and cell death upon internalization (Spanò et al., 2008). Expression of the toxin by STy during infection has been associated with promoting persistence in a humanized mouse model of typhoid (Song et al., 2010). Additionally, it has been observed that sera from human patients who have recently recovered from STy infection contain typhoid toxin-specific antibodies, suggesting a potential role for anti-toxin immunoglobulins in immunity (Charles et al., 2010). Considering the need for alternative typhoid containment measures, there is a growing interest in exploring typhoid toxin as a possible protective target.

**S. ENTERICA PATHOGENESIS**

STm is primarily transmitted through the digestive tract, where upon entry into the small intestinal lumen, the bacteria utilize flagella-based motility to reach the epithelium. In invasive STm infection (Figure 1.2), such as in mice and in iNTS human infections, the bacteria preferentially invade Peyer’s patches, the gut-associated lymphoid tissues (GALT) of the intestine (Carter & Collins, 1975). STm cells traverse the intestinal mucus layer to identify microfold (M) cells overlying Peyer’s patch tissues,
Figure 1.2. Modes of pathogenesis by *S. Typhimurium* and *S. Typhi*.

(Left) Nontyphoidal *Salmonella* (NTS), like *S. Typhimurium*, predominantly induce gastroenteritis-type disease that is characterized by bacterial colonization of the lower intestine, resulting in inflammation. Bacteria replicate within the intestinal lumen and are shed from the host. In severe cases, or cases of invasive NTS (iNTS), the bacteria invade M cells of the epithelium to enter GALT. After invasion, STm cells are taken up by phagocytes, like macrophages, and replicate intracellularly within *Salmonella*-containing vacuoles (SCVs), resulting in systemic dissemination. (Right) Typhoidal *Salmonella* serovars, such as *S. Typhi*, invade the intestinal mucosa at M cells and do not typically induce intestinal inflammation or diarrheal symptoms. Following uptake by phagocytes and intracellular replication within host cells, STy bacteria disseminate into the systemic compartment, causing fever and bacteremia. STy cells also secrete...
typhoid toxin within SCVs at this stage of infection, which is transported extracellularly by host cell vesicle intermediates (Chang et al., 2016). In persistent cases of STy, the bacteria can colonize the gallbladder and facilitate long-term shedding in the gut. Diagram modeled after Dougan & Baker's work (Dougan & Baker, 2014). Image designed using BioRender.
which are identifiable by their reduced brush border and glycocalyx (Neutra et al., 1996). M cells are evolutionarily designed to select luminal antigen for presentation to the underlying immune system. Considering their unique structure, M cells act as efficient points of entry for invasive STm to reach the systemic compartment. To facilitate invasion, STm utilize a specific pathogenicity island-1 (SPI-1) encoded type III secretion system (T3SS-1) to inject effector proteins that induce profound cytoskeletal rearrangement of the epithelial cell (Diard & Hardt, 2017). Invasion of M cells by STm is considered the critical step in invasive pathogenesis (Jones et al., 1994). T3SS-2, which is encoded by SPI-2, has been further implicated as a necessary virulence factor in bacterial traversal within epithelial cells to the basolateral surface, allowing for entry into the Peyer’s patch (Muller et al., 2012). Once inside the lamina propria, STm cells are engulfed by phagocytic cells, specifically either neutrophils, dendritic cells (DCs), or macrophages. Engulfed STm cells can further reside and replicate intracellularly within Salmonella-containing vacuoles (SCVs) (Fabrega & Vila, 2013). Migration of STm-infected macrophages or DCs facilitates bacterial spread to the mesenteric lymph node (MLN) and spleen, contributing to further dissemination of infection (Rydström & Wick, 2007). Alternatively, STm cells can adhere to the epithelium and colonize the intestine, promoting inflammation. In an antibiotic-treatment mouse model of Salmonella colonization, STm cells make contact with the epithelium via adhesins, flagella, and/or type-I fimbriae (Bäumler et al., 1996; Hapfelmeier et al., 2004). Similar in vivo models of STm-induced colitis demonstrate that the bacteria reside and replicate in the lower intestine, facilitating shedding of the pathogen over time (Kaiser et al., 2012). STm
T3SS-1 activity via effector proteins SipA and SopE during colonization further drives this inflammatory response (Muller et al., 2009; Wall et al., 2007).

STy pathogenesis is similar to the preliminary stages of STm infection, in that the bacteria infect the distal ileum and seek out M cells overlying GALT for invasion (Dougan & Baker, 2014). After entry into the mucosa, the bacteria reside within Peyer’s patches and replicate until a critical threshold has been reached for dissemination into the bloodstream, approximately 7 to 14 days later (Parry et al., 2002; Smith et al., 2016). In persistent infections, STy cells that have spread systemically can colonize the gallbladder, leading to long-term shedding in what is called a “carrier state” (Dougan & Baker, 2014).

**SECRETORY IGA**

Secretory immunoglobulin A (SIgA) is the predominant antibody isotype in the gut as well as in other mucosal compartments, such as the respiratory tract. SIgA is a dimeric, heavily glycosylated antibody that acts as the “first line of defense” to pathogens attempting to access epithelial surfaces. In humans alone, the average adult is capable of shedding secretory IgA at a rate of 3 grams per day (Macpherson et al., 2012). SIgA is a dimeric molecule consisting of two monomeric IgA immunoglobulins bound together by joining (J) chain at the tailpiece (Bastian et al., 1992). The antibody is further defined by the presence of secretory component (SC), which is the cleaved portion of pIgR that is covalently associated with the molecule following transcytosis (Brandtzaeg, 1978). There are two subclasses of IgA, IgA1 and IgA2, that differ in the length of the hinge region as well as in their glycosylation profiles (Woof & Russell,
In the serum, the chief IgA subclass is IgA1, which is primarily monomeric and accounts for ~90% of the IgA pool (Woof & Russell, 2011). However, in the gut the subclasses are found in somewhat equal proportions, with IgA2 antibodies making up 60% of the total IgA. IgA1 has an elongated hinge between Fab regions compared to IgA2, and also possess several O-linked glycosylation sites along the hinge region. The longer hinge region of IgA1 is suggested to provide a greater advantage towards crosslinking pathogens (Woof & Kerr, 2006). However, this distinction also renders the IgA1 antibody more susceptible to bacterial proteases, such as those secreted by Streptococcus pneumoniae in the nasal cavity (Proctor & Manning, 1990). Structural differences between the two subclasses have been shown to subtly influence proinflammatory responses as well (Steffen et al., 2020).

The Peyer’s Patch

Intestinal SIgA antibodies are generated within gut-associated lymphoid tissues, known as Peyer’s patches, which act as sites of immune surveillance along the gastrointestinal tract. In mice, Peyer’s patches are relatively dispersed along the small intestine, while in humans, the structures are largely clustered at the terminal ileum (Cornes, 1965). Structurally, Peyer’s patches are organized into three major regions: the follicle-associated epithelium (FAE), the subepithelial dome (SED), and the B cell follicle (Reboldi & Cyster, 2016). The FAE consists of a single layer of columnar epithelial cells overlying the Peyer’s patch. Within the FAE are specialized antigen sampling M cells that are designed to efficiently deliver luminal antigen (e.g., food or microbial) to the underlying lamina propria. M cells are uniquely structured to perform
this task due to their reduced brush border and invaginated pocket that facilitates antigen delivery to underlying phagocytic cells (Mabbott et al., 2013). The so-called subepithelial dome region (SED) of the lamina propria beneath the FAE overlies the B cell follicle of the Peyer’s patch. Antigen-presenting cells (APCs), such as dendritic cells (DCs) and phagocytic cells, reside within the SED prepared to accept antigen from M cells for further delivery to adjacent follicles. Evidence has also suggested that DCs can directly select antigen from the intestinal lumen by dendrite extension through epithelial tight junctions (Rescigno et al., 2001). Presentation of antigen by lamina propria DCs, specifically CCR6+ DC subsets, facilitates follicular B and T cell priming within the Peyer’s patch (Lycke, 2012). Activated B cells within germinal centers of the follicle are promoted towards IgA class-switch recombination, as IgA is the predominant isotype in the gastrointestinal tract (Bemark et al., 2012). This IgA class-switching in the follicle is encouraged largely by the presence of transforming growth factor β (TGF-β) (Cazac & Roes, 2000).

Following production in the lamina propria by resident plasma cells, dimeric IgA (dlgA) is transported on to the surface of the intestinal lumen by way of plgR. dlgA antibodies bind to plgR expressed on the basolateral side of intestinal epithelial cells by way of joining (J) chain region, a 15 kDa protein that has a high affinity for the receptor (Johansson et al., 2000). Upon binding, dlgA-plgR complexes are actively transcytosed across the epithelial cell, resulting in the catalytic cleavage of the ectodomain of plgR (Kaetzel, 2005). The portion of the glycoprotein receptor that is cleaved remains bound to IgA, referred to as SC. The presence of SC is postulated to afford a number activities
to SIgA on the mucosal surface, such as enhanced pathogen binding and stability in the gastrointestinal environment (Phalipon et al., 2002).

**Passive Immunity by SIgA Antibodies**

SIgA is the predominant immunoglobulin on mucosal surfaces, but it is also the most prevalent antibody in human colostrum and breast milk (Brandtzaeg, 2003). Breastmilk transfers nutrients, antimicrobial peptides, and antibodies to a developing newborn, protecting the infant from environmental pathogens. A number of studies have identified breastfeeding as an efficient means of decreasing infant morbidity and mortality (Ip et al., 2007). Considering the advantages of breastmilk for infant health, the World Health Organization has recommended globally that mothers breastfeed children throughout the first six months of life (World Health Organization, 2021).

Colostrum is the milk first secreted within four days of delivery and it is highly concentrated in a number of bioactive proteins, including lactoferrin, lysozyme, and SIgA (Hennet & Borsig, 2016). Of the immunoglobulin pool, SIgA accounts for 90% of all colostrum-derived antibodies, suggesting a vital importance in passive immunity (Turin & Ochoa, 2014). Maternal IgA antibodies delivered via breastmilk contribute to the shaping of a healthy neonatal microbiota following delivery by binding to "pathogenic" commensal taxa and preventing colonization. Coating of inflammatory Enterobacteriaceae by maternal by SIgA in breastmilk has been shown to hinder the ability of the bacteria to establish a niche within the infant gut, protecting against neonatal colitis (Palm et al., 2014). Recent work has reinforced this protective capacity in also preventing pre-term infants from necrotizing enterocolitis (NEC) (Gopalakrishna
et al., 2019). However, much of the maternal SIgA is specific for previously encountered pathogens that affords passive immunity to the newborn. In a prospective study surveying infant disease from *Vibrio cholerae* in Bangladesh, Glass and colleagues observed that children breastfed from mothers with high amounts of *V. cholerae*-specific SIgA were protected from diarrhea compared to those that nursed from mothers with low levels (Glass et al., 1983). Neonatal protection from enteric viruses through breastmilk antibodies has also been demonstrated. In a randomized control trial, infants that breastfed for greater than two weeks had significantly fewer nonpolio enterovirus infections in the first year of age than children who breastfed for less than two weeks (Sadeharju et al., 2007).

Oral IgA antibodies have also been shown to facilitate protection from enteric pathogens experimentally. The delivery of recombinant yeast-derived VHH-IgA administered in dried food in a piglet model of ETEC has been shown to afford sufficient protection from bacterial colonization and shedding in infected animals (Virdi et al., 2019). In a similar study examining immunity to *C. jejuni* infection, orally administered human recombinant SIgA specific for the bacterial flagella successfully reduced *C. jejuni* shedding and intestinal inflammation in an enteric disease mouse model (Perruzza et al., 2020). In our own lab, transgenic dams secreting mAbs specific for *V. cholerae* LPS in the mammary gland were able to passively protect suckling mice from a lethal *V. cholerae* challenge (D. E. Baranova et al., 2020). Given the feasibility of milk-derived antibodies to combat enteric pathogens, there is an interest in pursuing oral antibody delivery further therapeutically.
Protection by SlgA Antibodies

The current paradigm of protection by SlgA is by way of “immune exclusion”, where SlgA antibodies facilitate pathogen crosslinking and cause agglutination. The aggregated microbes are rendered avirulent through this process, resulting in clearance of the pathogen via peristalsis (Stokes et al., 1975). SlgA is uniquely suited to facilitate agglutination given the polymeric nature of the antibody. Protection by SlgA antibodies via crosslinking has been attributed to immunity towards a number of diarrheal pathogens, such as Shigella flexneri, Campylobacter jejuni, and ETEC (Giuntini et al., 2018; Mathias et al., 2013; Perruzza et al., 2020). Specifically, agglutination has been attributed as a major mechanism of immunity to STm infection by limiting access to the FAE of Peyer’s patches in an ileal loop model of infection (Bioley et al., 2017). Alternatively, under low-density infection conditions, high avidity SlgA antibodies generated by oral vaccination can induce “enchained growth” of pathogenic bacteria in the gut (Moor et al., 2017). This function by SlgA prevents the segregation of daughter cells as the bacteria, either STm or E. coli, replicate in the intestinal lumen, leading to clonal extinction of the pathogen via peristalsis. However, there are a number of unique functions of IgA that can either enhance mechanisms of agglutination or impose virulence deficits independently of IgA-mediated agglutination. The IgA mAb, IgAC5, specific for S. flexneri LPS, has been shown to induce transient suppression of the bacterial T3SS (Forbes et al., 2011). Inhibition of T3SS activity in S. flexneri by IgA treatment was also associated with damage to the bacterial outer membrane in these experiments. Similar reports, from this lab and others, have identified a number of direct insults imparted on the fitness of STm by the IgA mAb, Sal4.
SAL4 ACTIVITY

Sal4 IgA is a well-characterized protective mAb that has been studied since the early 1990s. Sal4 was originally generated from IgA+ B cells isolated from Peyer’s patches of mice orally immunized with STm (Michetti et al., 1992). Its activity was first identified alongside a panel of other STm-specific mAbs for its ability to agglutinate STm cells in liquid culture. Sal4 was not observed to be bactericidal, which is congruent with the current literature regarding the effects of SIgA binding on pathogenic bacteria (Moor et al., 2017). The early studies indicated that Sal4 bound plgR by Western Blot, indicating the production of polymeric IgA by Sal4 hybridoma B cells. As such, the authors explored the potential of Sal4 to be transported to the intestinal lumen by way of a “backpack tumor” (Figure 1.3). In these experiments, Sal4 hybridoma cells were injected subcutaneously in vivo to facilitate the development of a hybridoma tumor that systemically secreted Sal4. Once substantial levels of Sal4 IgA were in circulation, delivery of the mAb was naturally transported by plgR into the intestinal lumen. Sal4 IgA delivered on mucosal surfaces via this model was shown to reduce STm entry into Peyer’s patches after oral infection. Subsequent studies demonstrated that Sal4 IgA treatment prevented STm invasion into polarized epithelial cells in vitro, an activity that correlated with the ability of the antibody to agglutinate STm in liquid culture. These early experiments demonstrated the ability of Sal4 to function independently of the mucus environment (Michetti et al., 1994).
Figure 1.3. Backpack tumor model.

(1) Hybridoma cells secreting IgA mAbs are subcutaneously injected in the backs of mice and allowed to develop. (2) After 10-14 days, tumors secrete sufficient levels of mAbs systemically into circulation. (3) Once in circulation, IgA mAbs reach the lamina propria of the intestine. Lamina propria IgA mAbs are actively transported across the epithelium by pIgR, resulting in delivery of the mAb on to the epithelial surface.
It was later determined that Sal4 binding was specific for the O5 epitope of STm LPS (Figure 1.1) (Slauch et al., 1995).

Follow-up studies in the Mantis lab have since focused on examining the direct effects on STm cells following Sal4 treatment. The presence of Sal4 in soft agar and in liquid culture was shown to induce rapid flagellar-based motility arrest under non-agglutinating conditions (Forbes et al., 2008). Congruent with the original experiments, Sal4 was shown to reduce STm invasion into epithelial monolayers in vitro. This phenotype was later determined to be the result of SPI-1 T3SS abrogation, along with the reduction in secreted effector proteins SlrP and SopB (Forbes et al., 2008; Forbes et al., 2012). In addition to arresting bacterial motility and imparting stress on the STm outer membrane, Sal4 was shown to induce the production of a polysaccharide “coat” under non-agglutinating conditions (Forbes et al., 2012). Further investigation by Amarasinghe suggested that the exopolysaccharide (EPS) produced by STm cells in response to Sal4 IgA treatment was likely a phenotype reminiscent of the beginning stages of biofilm formation (Amarasinghe et al., 2013). Sal4-induced extracellular matrix was found to consist of O-antigen capsule, colonic acid, and cellulose in these studies. This phenomenon was also determined to be dependent on cyclic dimeric guanosine monophosphate (c-di-GMP) signaling by STm. Taken together, these studies highlight the multiple mechanisms by which Sal4 IgA can combat STm through both antibody-mediated agglutination and direct effects on the bacterial cell.

In this dissertation project, I sought to investigate Sal4 and its potential to afford passive immunity to mice from oral STm infection. This was in pursuit of elucidating mechanisms of immunity by IgA immunoglobulins, as well as evaluating the capacity of
oral mAbs to passively immunize against enteric disease. I set out to develop an in vivo model to study passive immunity by oral antibodies utilizing a panel of Sal4 isoforms to assess the significance of isotype in protection. Additionally, I conducted in vivo tracking studies to resolve the pharmacokinetics of antibody delivery and define the window of protection for oral prophylaxis to STm. Given the evolutionary advantage of SIgA and its many roles in the mucosal compartment, the central hypothesis of my thesis is that orally administered polymeric IgA facilitates immunity to STm through enhanced crosslinking, prevent bacterial access to the GALT via agglutination as a major mechanism of protection.
CHAPTER 2: MATERIALS & METHODS
BACTERIAL STRAINS AND GROWTH CONDITIONS

*Salmonella enterica* serovar Typhimurium (STm) strains used in this study are described in Table 2.1. STm ATCC14028 was purchased from the American Type Culture Collection (Manassas, VA) (Jarvik et al., 2010). S. Typhimurium strains AR04 (zjg8101::kan oafA126::Tn10d-Tc fkpAlacZ) and AR05 (zjg8101::kan) are derivatives of ATCC14028, as described (Forbes et al., 2008; Forbes et al., 2012). Unless otherwise stated, single colonies were used to inoculate sterile LB broth and were incubated overnight at 37°C with aeration, then subcultured in fresh LB to mid-log phase (OD$_{600}$ 0.40) before use. AR05-mCherry contains an arabinose-inducible plasmid under gentamicin antibiotic pressure (10 μg/mL) that allows for expression of mCherry fluorescent protein by AR05 cells in the presence of arabinose (0.2% final concentration).

GENERATION OF MCHERRY-EXPRESSING STM STRAINS

A high copy plasmid harboring an arabinose-inducible mCherry cassette (pMW232) was engineered for expression in *Salmonella* Typhimurium. pMW232 was created by replacing the arabinose-inducible GFPmut3 ORF in pMQ8060 with a codon optimized variant of mCherry designed for expression in *Pseudomonas aeruginosa*. To accomplish this, pMQ80 was first subjected to restriction digestion with KpnI & HindIII (NEB) to remove the GFPmut3 ORF and associated Shine-Dalgarno sequence. The mCherry ORF was then amplified from pIDTSmart-Kan-mCherryPa (Integrated DNA Technologies) using Q5 DNA polymerase (NEB) and tailed primers.
(mcherry_F_SD_KpnI, 5’-
TCGGTACCCGGAGAAGGAGATATACATATGGTGAGCAAGGGCGAGGAGGA-3’ &
mcherry_R_HindIII, 5’-CAGAAGCTTTCTACTTGTACAGCTCGTCCATGCCG-3’) that
were designed to incorporate a 5’ E. coli Shine-Dalgarno consensus sequence and
flanking KpnI & HindIII restriction sites. The amplified DNA fragment was then digested
with KpnI & HindIII, ligated into similarly cut pMQ80, transformed into chemically
competent NEB 5 alpha cells (NEB), plated on LB agar supplemented with 10 μg/ml of
gentamicin, and then incubated overnight at 37°C. Gentamicin-resistant colonies that
emerged were subsequently screened for incorporation of the mCherry ORF via
culturing in LB supplemented with 10 μg/ml of gentamicin and 0.2% L-arabinose.
pMW232 was then harvested from a culture that turned pink after several hours of
growth using Qiagen’s Miniprep kit and provided protocol. Electrocompetent Salmonella
Typhimurium (AR05) cells were then prepared and transformed with pMW232 using
previously described methodology to generate the AR05-mCherry strain (O’Callaghan &

MONOCLONAL ANTIBODIES AND HYBRIDOMAS

Antibodies used in this study are listed in Table 2.2. The B cell hybridoma cell
lines secreting Sal4 IgA, specific for the O5-antigen, and 2D6 IgA, specific for V. cholerae Ogawa LPS, were originally obtained from Dr. Marian Neutra (Children’s
Hospital Boston) (Michetti et al., 1992). Purified dimeric Sal4 IgA (dIgA) and
recombinant human secretory component (rSC) were associated for 1 h at room
temperature to generate murine Sal4 SIgA, as described (Phalipon et al., 2002). The
PeA3 murine B cell hybridoma secreting a monoclonal IgA against the STm O5-antigen was generated from the Peyer's patches of BALB/c mice repeatedly immunized orally with STm, essentially as described (Michetti et al., 1992). The resulting hybridomas were screened by ELISA for reactivity with STm whole cells and purified LPS (Sigma-Aldrich, St. Louis, MO). Chimeric Sal4 IgG, PB10 IgG, and HD9-N IgG were provided by Mapp Biopharmaceutical (San Diego, CA). 2D6 IgA, PB10 IgG, HD9-N IgG, and human IgA from colostrum (Sigma-Aldrich, St. Louis, MO) were used as IgA, IgG, and SlgA isotype controls throughout the study.

**Generation of recombinant human Sal4 mlgA, dlgA, and SlgA mAbs**

Sal4 mlgA, dlgA, and SlgA mAbs were generated as described previously (Perruzza et al., 2020). In brief, Expi293 cells were transiently transfected with a construct containing the sequence for the Sal4 heavy chain variable domain (V_h) in a human IgA2 allotype m(2) alongside the corresponding Sal4 light chain (V_l). Sal4 dlgA and SlgA were co-transfected with a plasmid containing joining chain (J), with Sal4 SlgA additionally expressed with secretory component (SC). Supernatants were collected and purified using CaptureSelectTM IgA Affinity Matrix (Thermo Fisher).

**Generation of TyTx hybridomas and mAbs**

Mouse experiments were conducted following the protocol approved by the Wadsworth Center's institutional animal care and use committee (IACUC). Protocol #17-428 was assigned by the IACUC/ethics committee that approved the animal experiments performed for this study. The experiments were followed by IACUC and
AAALAC guidelines. Female BALB/c mice approximately 5-7 weeks of age were purchased from Taconic Biosciences (Albany, NY) and housed under conventional, specific pathogen-free conditions. Mice were immunized via intraperitoneal injections with 2 μg of ultrapure endotoxin-free recombinant typhoid toxoid without adjuvant at two-week intervals. Genetically engineered inactive forms of typhoid toxin were used in the study. Specifically, the toxoid carries point mutations in PltA$^{E133A}$ and CdtB$^{H160Q, D195S}$. The toxoid has the same $A_2B_5$ stoichiometry as WT typhoid toxin, as determined via size exclusion chromatography. Serum reciprocal endpoint titers were >100,000 following three immunizations.

B cell hybridomas were then generated by performing Hybri-Max PEG fusion procedures using the Sp2/0-Ag14 (ATCC® CRL-1581) myeloma. Similar to the procedure established previously (Van Slyke et al., 2018), the resulting hybridomas were seeded onto wells of 96-well cell culture-treated microtiter plates, and hybridomas were selected in DMEM media supplemented with 10% UltraCruz® Hybridoma Cloning Supplement (HCS) (Santa Cruz Biotechnology, Dallas, TX), 10% fetal calf serum, oxaloacetate, pyruvate, and insulin (OPI), hypoxanthine/aminopterin/thymidine (HAT), and penicillin/streptomycin. HAT in the culture media was gradually replaced with hypoxanthine-thymidine (HT), and surviving hybridomas secreting antibodies of interest were cloned by limiting dilution and expanded in DMEM media without HT, before being transitioned to either CD Hybridoma AGT (Invitrogen) or DMEM supplemented with 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 10% HCS, 20% Ultra Low IgG Fetal Bovine Serum (Invitrogen), penicillin, and streptomycin. B cell hybridomas were cloned by limiting dilution three times to ensure
clonality. Hybridoma clones were selected based upon a positive reaction to typhoid toxoid by direct ELISAs. Murine mAbs were purified from hybridoma supernatants using protein G chromatography and subjected to epitope characterization using ELISAs and Western blots.

**TYTX MAB AFFINITY DETERMINATION: SURFACE PLASMON RESONANCE (SPR) ASSAY**

TyTx mAb binding kinetics and affinity were determined by performing SPR assays using the Biacore T200 (GE Healthcare), as previously described (Rudolph et al., 2020). Series S CM5 chips were immobilized with WT typhoid toxin to obtain a target response bound of ~100-120 RU. Pilot experiments to optimize experimental parameters were then performed using 10-fold serial dilutions of each Ab injected at a flow rate of 50 μl/min with a contact time of 120 sec and dissociation time of 600 sec, which were conditions deemed appropriate by the Biacore T200 Evaluation software (GE Healthcare) for the affinity determination of each mAb. Following optimization of the concentration range and conditions, 4-fold serial dilutions of each mAb were input for replicate experiments. Sensograms for each replicate were analyzed to fit a bivalent binding model using the Biacore T200 Evaluation software. Data obtained from the Biacore T200 was imported into GraphPad Prism 8.3 for the generation of final sensograms.
SIZE-EXCLUSION CHROMATOGRAPHY ULTRA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (SEC-UHPLC)

Size exclusion chromatography was performed with a series of columns consisting of Acquity UPLC Protein BEH SEC Guard Column (WAT186006850); Acquity UPLC Protein BEH SEC, 200 A (WAT176003904 - 1.7 μm, 4.6 x 150), and Acquity UPLC Protein BEH SEC, 450 A (WAT176002996 - 2.5 μm, 4.6 x 150 mm) using an Agilent 1260 Infinity Quaternary Bio-inert LC. Purified mAbs were filtered with 0.22 μm filter and centrifuged for 5 min at 10,000 x g before 5 μl of each sample were injected in the columns at 30°C with a flow rate of 0.35 mL/min in PBS + 350 mM NaCl, pH 6.9. Protein detection was performed at 280 nm. BEH200 SEC Protein Standard Mix, (Waters 186006518) was used to benchmark the samples’ molecular weights.

ELISAS

For either direct or whole-cell ELISA experiments, all plates were developed using the appropriate HRP-labeled secondary antibody (final concentration of 0.5 μg/ml; Table 2.2) and SureBlue TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD). Plates were read by spectrophotometry (A$_{450}$) within 30 minutes of developing using a VersaMax microplate reader and SoftMaxPro 5.2 software.

Direct-binding ELISAs

For direct ELISAs, 96-well NUNC MaxiSorp plates (ThermoScientific, Waltham, MA) were coated with 0.1 ml of STm LPS (1 μg/ml in sterile PBS) overnight at 4°C. Wells were blocked with PBS containing 0.1% Tween-20 (PBST) and 2% goat serum.
for 2 h at room temperature before washing with PBST. Plates were developed as described above.

**Whole-cell ELISAs**

96-well NUNC MaxiSorp plates were coated with poly-L-lysine (10 μg/ml) overnight at 4°C. Overnight cultures of STm were washed twice with PBS and then placed into each well of the microtiter plate. The plates were centrifuged two times at 500 x g for 3 min (rotating 180° for the second spin), and then fixed with 2% paraformaldehyde (PFA) in PBS. The bacteria-coated plate was then treated with sterile glycine (0.1 M) to quench residual PFA and ELISAs were performed as described above.

**TyTx ELISAs**

To select positive TyTx hybridomas clones, 96-well Immulon® Microtiter™ plates were coated with 50 ng of purified typhoid toxoid or tagless PltB pentamer in 100 μl plate-coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6), and incubated overnight at 4 °C. Wells were washed with PBST and blocked as described above. Binding by purified mAbs was assessed by diluting antibodies in PBS containing 0.05% Tween 20 and 0.5% BSA and incubating for 2 h at 37 °C. After washing, bound antibodies were detected with HRP-conjugated anti-mouse immunoglobulin IgG (Southern Biotech) at a 1:10,000 dilution. Plates were developed and the results were assessed as described.
BACTERIAL AGGLUTINATION ASSAY

An aliquot of overnight liquid cultures of STm (100 μl) were mixed in equal ratio (v/v) with PBS containing a final concentration of 15 μg/ml of each mAb of interest (n = 6 per treatment), and then placed into individual wells of a U-bottom 96 well plate. The plate was incubated at 37°C and visually monitored every 15 minutes for clumping of cells, as described (Forbes et al., 2008).

SEMI-SOLID AGAR MOTILITY ASSAY

For the soft agar motility assays, LB medium with 0.3% Bacto agar (Becton Dickinson) was prepared with 15 μg/ml of each mAb of interest poured into 60 mm Petri dishes (n = 3 per treatment group) and allowed to set at room temperature for 30 min. Individual colonies of ATCC14028 STm were then picked from a freshly-streaked LB agar plate and stabbed directly into the center of the plate (K. Levinson et al., 2015). The plates were placed in a 37°C incubator and the diameters of the concentrically growing bacterial cultures were measured at 60-minute intervals.

HELA CELL INVASION ASSAYS

HeLa cells were obtained from the ATCC and maintained in Dulbecco's Modified Eagle Media (DMEM) with 10% fetal bovine serum at 37°C and 5% CO₂. Cells were seeded at 5 x 10⁵ cells/mL in 96-well plates and grown for 24 to 36 h to establish 70–90% confluency. Prior to STm infection assays, HeLa cells were washed three times with serum-free DMEM. Overnight cultures of AR05 and AR04 were subcultured in LB
at 37˚C with aeration and adjusted to an OD$_{600}$ of ~0.7. Strains were mixed 1:1 and washed twice by centrifugation (6,000 x g for 2 minutes) and resuspended in PBS. Bacteria were then diluted 1:10 in Hank’s Balanced Salt Solution (HBSS, Wadsworth Center Media Core) and an aliquot was plated on LB agar supplemented with kanamycin (50 μg/mL) and X-gal (40 μg/mL) to compute bacterial input.

For the invasion assay, bacterial mixtures were incubated with 15 μg/mL of mAbs for 15 min at 37˚C to minimize agglutination. Treated bacteria were applied to HeLa cell monolayers and centrifuged at 1,000 x g for 10 min (rotating the plate 180˚ at 5 min) to promote STm adherence to HeLa cell surfaces. The microtiter plates were then incubated for 90 minutes at 37˚C. Cells were washed three times with HBSS and treated with gentamicin (40 μg/mL) to eliminate extracellular bacteria. Finally, cells were washed with HBSS lysed with 1% Triton X-100 (in Ca$^{2+}$ and Mg$^{2+}$-free PBS), serially diluted, plated on LB agar containing kanamycin (50 μg/mL) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/mL) and incubated overnight at 37˚C. The competitive index [(%strain A recovered/% strain B recovered)/(%strain A inoculated/%strain B inoculated)] was calculated for each treatment group.

**IN VITRO DIGESTION ASSAY**

Sal4 IgA or Sal4 IgG mAbs were diluted to a final concentration of 0.1 mg/mL in simplified adult simulated gastric fluid (94 mM NaCl, 13 mM KCl; pH adjusted to 3.0 with 1M HCl) with or without pepsin (2000U/mL) on ice similarly as described (Hu et al., 2019; Menard et al., 2017; Minekus et al., 2014). Samples were incubated statically at 37˚C and aliquots were taken after 10 minutes, 30 minutes, and 60 minutes of
incubation and neutralized on ice to a pH of 7.0 to 7.4 using 1M NaOH. Following neutralization, all samples were analyzed for binding of purified STm LPS by ELISA as described above. Concentrations of Sal4 IgA and Sal4 IgG were quantified by establishing a standard curve using SoftMax Pro 5.2.

**ANIMAL CARE AND ETHICS STATEMENT**

The mouse experiments performed in these studies were reviewed and approved by the Wadsworth Center’s Institutional Animal Care and Use Committee (IACUC) under protocol #17–428. The Wadsworth Center complies with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and was issued assurance number A3183-01. The Wadsworth Center is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Obtaining this voluntary accreditation status indicates that Wadsworth Center’s Animal Care and Use Program meets all standards required by law and goes beyond the standards as it strives to achieve excellence in animal care and use. Mice were euthanized by carbon dioxide (CO₂) asphyxiation followed by cervical dislocation, as recommended by the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health.

**MICE**

Female BALB/c mice age 8-12 weeks were purchased from Taconic Biosciences (Rensselaer, New York) and cared for by the Wadsworth Center Animal Core Facility. All experiments were performed in strict ordinance of the approved Wadsworth Center’s
IACUC protocols, as described above.

**S. TYPHIMURIUM INTRAGASTRIC CHALLENGE MODEL**

Overnight cultures of AR05 and AR04 were subcultured to an OD$_{600}$ of 0.7, combined 1:1 (v/v) and resuspended in PBS, unless stated otherwise. An aliquot was plated on LB agar containing kanamycin (50 μg/mL) and X-gal (40 μg/mL) at the start of the experiment to determine bacterial input (CFUs). Before gavage, bacteria (~4 x 10$^7$ CFUs) were either incubated for 10 minutes with mAbs (30 μg/mL, unless stated otherwise), or mAbs were provided as a “chase” immediately before STm gavage (50 μg/mouse, unless stated otherwise). Twenty-four hours later, the mice were euthanized by CO$_2$ asphyxiation followed by cervical dislocation. For each mouse, a laparotomy was performed, and the small intestine was removed above the cecum. Approximately 7-10 Peyer’s patches were collected from each mouse using curved scissors (#14061-09, Fine Science Tools, Foster City, CA) and pooled in 1 mL ice-cold sterile PBS. Samples were then homogenized with a Bead Mill 4 Homogenizer (Fisher Scientific) three times for 30 seconds each. Homogenates were serially diluted, plated on LB agar containing kanamycin and X-Gal and incubated overnight at 37°C. Blue and white colonies were enumerated and the competitive indices (CI) were calculated as $CI = [(\% \text{ strain A recovered}/\% \text{ strain B recovered})/(\% \text{ strain A inoculated}/\% \text{ strain B inoculated})]$. Whole-plate dilutions (100 μl per plate) were required to observe enough colonies to calculate competitive indices. All samples that contained less than 30 CFUs (per 100 μl) were eliminated from the data set and considered “too few to count” (TFC). This pool of cells is more likely a representation of only a few bacteria that have successfully
invaded and replicated within the lymphoid tissues, as Peyer’s patch entry by Salmonella has been shown to be a bottleneck for further dissemination during infection (Lim et al., 2014).

MOUSE MODEL OF SYSTEMIC S. TYPHIMURIUM INFECTION

BALB/c female mice were administered 40 μg or 10 μg (unless otherwise indicated) of Sal4 IgA, Sal4 IgG1, or isotype control antibody in sterile PBS by intraperitoneal (i.p.) injection. An overnight culture of wildtype STm (ATCC14028) was subcultured, washed in sterile PBS, and diluted to a final concentration of $5 \times 10^4$ CFUs/mL. Twenty-four hours after passive immunization, mice were challenged with STm inoculum (1 x $10^4$ CFUs) by i.p. injection. An additional 24 h later, mice were euthanized, and spleens and livers were collected, weighed, and homogenized in 1 mL sterile PBS, as described above. Homogenates were serially diluted, plated on LB agar and incubated overnight at 37˚C. Total CFUs were counted and computed for $\log_{10}$ CFUs/gram (tissue).

TRACKING S. TYPHIMURIUM IN THE GASTROINTESTINAL TRACT BY IMMUNOHISTOCHEMISTRY

Oral Challenge and Digestive Tract Isolation

For analysis by histology and immunohistochemistry, overnight cultures of ATCC14028 were prepared as described above. Cohorts of BALB/c female mice were orally administered 200 μl of PBS, Sal4 IgG, or Sal4 SIgA at indicated concentrations before an oral challenge of ATCC14028 (~$4 \times 10^7$ CFUs). Groups of animals in each
treatment group were then euthanized either 20 min or 40 min post-infection. A laparotomy was performed on each animal and the entire gastrointestinal tracts were quickly removed, placed in histology cassettes, and fixed in buffered formalin for 24 h. After 24 h cassettes were transferred to 70% ethanol solution until paraffin-embedding.

**Immunohistochemistry of STm-inoculated mouse tissues**

Paraffin-embedded tissue samples were sectioned at a thickness of 3-4 μm on charged microscope slides. Samples were deparaffinized using CitriSolv (Decon Labs, Inc., King of Prussia, PA) and rehydrated sequentially in graded alcohols. Antigen retrieval was performed by incubating slides in 10 μg/mL proteinase K (MilliporeSigma) in PK buffer (0.6M Tris (pH 7.5)/0.1% CaCl2) for 10 min at RT. Blocking of endogenous peroxidase and alkaline phosphatase was performed by incubating slides in Rodent Block M (BioCare Medical, Pacheco, CA) followed by incubation with BLOXALL® Endogenous Blocking Solution (Vector Laboratories, Burlingame, CA). Slides were washed in TBS buffer and incubated with primary rabbit Salmonella O Antiserum (Group B Factors 1, 4, 5, 12, #BD 229481, Becton, Dickinson and Company) for 1 h at 1:5,000 dilution. Slides were then incubated in AP-polymer (Rabbit on Rodent AP-Polymer, BioCare Medical) followed by Vina Green Chromogen™ (BioCare Medical, Pacheco, CA) treatment. Tissues were finally counterstained with hematoxylin (BioCare Medical) before mounting with EcoMount (BioCare Medical). Slides were cured at 60-70°C for 15 min.
STM COLONIZATION BY FECAL SHEDDING

Overnight cultures of AR05 and AR04 were subcultured to an OD$_{600}$ of 0.7, combined 1:1 (v/v) and resuspended in PBS. An aliquot was plated on LB agar containing kanamycin (50 μg/mL) and X-gal (40 μg/mL) at the start of the experiment to determine bacterial input (CFUs). 50 μg of indicated mAbs diluted in PBS were provided by oral gavage immediately prior to bacterial challenge (~4 x 10$^7$ CFUs). At the indicated time points, animals were placed in individual collection chambers until 2-4 fecal pellets were obtained. Pellets were placed in PBS and homogenized by vortexing. Samples were serially diluted, plated on LB/kan/X-gal plates, and enumerated for CFUs and competitive indices, as described above.

AGGLUTINATION OF STM BY FLOW CYTOMETRY

AR05 cultures were subcultured to mid-log phase and washed twice with sterile PBS by pelleting cells at 6,000 x g for 3 min and were resuspended in PBS. STm cells were incubated with the indicated antibody treatments for 1 h at 37°C before being transferred to round-bottom polystyrene tubes for flow cytometry. Live samples were analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA), similarly as described (Mitsi et al., 2017). STm groups were gated in forward scatter (FSC) and side scatter (SSC) to visualize cell aggregate size and granularity. Untreated AR05 was used for thresholding and gating. Agglutination was calculated by adding FSCpositive and-SSC positive quadrants (Q2 + Q4) essentially as described (Habets et al., 2017). 10,000 events were acquired per sample using CellQuest Pro (BD Biosciences) and samples were analyzed in three biological replicates using GraphPad Prism 8 (San Diego, CA).
AGGLUTINATION OF STM BY FLUORESCENCE MICROSCOPY

AR05-mCherry overnight cultures were inoculated in LB broth containing 10 μg/mL gentamicin and 0.2% sterile arabinose and were incubated with aeration at 37°C. Cells were subcultured 1:50 in the same media until an OD$_{600}$ of ~0.5 was reached. Fluorescent STm were examined visually to confirm proper fluorescence intensity and cell morphology at the beginning of each experiment. AR05-mCherry STm were then treated with the indicated antibodies at room temperature. 10 μl of cells were spotted on to uncharged microscope slides at 10 min intervals for 30 min. 4 to 7 images were taken for each condition at each time point at 20X magnification in both the DIC and TexasRed (600 ms of exposure/frame) channels on a Nikon TI inverted microscope equipped with a CoolSnap HQ2 camera (Photometrics, Tucson, AZ). Mean fluorescence intensity (MFI) per aggregate (MFI = Mean Gray Value – (Background Mean Gray Value * Area)) was quantified using Fiji (Image J 1.52p)63, 64. To outline aggregates, the TexasRed channel was thresholded (minimum value 157). Using this thresholded image, mean gray value and area of individual aggregates was measured on the original TexasRed channel. MFI for all aggregates were averaged for each biological replicate.

STAINING OF STM-MAB COMPLEXES IN BOVINE THROMBIN CLOTS

ATCC14028 overnight cultures were prepared as described above. Cells were treated with PBS, Sal4 IgG, or SIgA at the indicated concentrations and pelleted at 6,000 x g for 3 min. Antibody treated pellets were resuspended in bovine plasma (Sigma-Aldrich) and dispensed into cryomolds pre-coated with bovine thrombin (Sigma-
Aldrich) on ice to initiate clotting, similarly as described (Balassanian et al., 2016). Plasma clots were incubated at 4°C for 20 min, removed from molds, and wrapped in lens paper prior to placement in histology cassettes. Cassettes were fixed in buffered formalin and transferred to 70% ethanol prior to paraffin-embedding and sectioning, as described above. Slides were counterstained using hematoxylin and eosin (H&E).

CRYSTAL VIOLET ASSAY

Crystal violet (CV) assays were done essential as described previously (Baranova et al., 2018). Indicated strains of Salmonella Typhimurium cells were grown to mid-log phase (OD600 ~0.6) and diluted 1:2 into LB medium containing indicated antibodies at 50 μg/mL for 1 h at 37°C in shaking conditions (220 RPM) in borosilicate glass tubes with cork stoppers. Tubes were subsequently washed three times with PBS, and then fixed with methanol for 15 min. Once dried, tubes were stained with 0.1% crystal violet dye for 5 min, and then rinsed with water. CV was solubilized with 30% acetic acid for 30 min. To quantitate CV staining, 200 μl of solubilized CV was transferred to a microtiter dish and the $A_{550}$ was read using a Versamax Microplate Reader. Images were taken after incubation with antibody before the first wash step and post-CV staining.
<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>O-Ag</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>ATCC14028</td>
<td>O5</td>
<td>Wildtype</td>
<td>(Jarvik et al., 2010)</td>
</tr>
<tr>
<td>AR05</td>
<td>O5</td>
<td>zjg8101::kan</td>
<td>(Forbes et al., 2008)</td>
</tr>
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<td>AR04</td>
<td>O4</td>
<td>zjg8101::kan oafA126::Tn10d-Tc fkpA-lacZ</td>
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<td>CS022</td>
<td>O5</td>
<td>pho-24 (PhoP constitutive)</td>
<td>(Murata et al., 2007)</td>
</tr>
<tr>
<td>SJF59</td>
<td>O4</td>
<td>phoP-24 oafA126:: Tn10d-Tc</td>
<td>(Richards et al., 2020)</td>
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<td>O5</td>
<td>zjg8101::kan; pMW232</td>
<td>This study</td>
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<tr>
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<td>O5</td>
<td>ΔbcsA</td>
<td>This study</td>
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<tr>
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<td>O5</td>
<td>bscE::kan; kanamycin cassette insertion mutation in bscE gene</td>
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**Plasmids**

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<th>Description</th>
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<td>pMW232</td>
<td>$P_{BAD}$-mCherryPa in pMQ80</td>
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Table 2.2. Antibodies used in this study

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<th>Source</th>
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<td>Sal4 IgA</td>
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<td>Hybridoma</td>
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<td>(Michetti et al., 1992)</td>
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<td>(Richards et al., 2020)</td>
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<td>Expi293F cells</td>
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<td>This study</td>
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<tr>
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<td>This study</td>
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<td>Rabbit</td>
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<td>(Richards et al., 2020)</td>
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CHAPTER 3: INHIBITION OF INVASIVE SALMONELLA BY ORALLY ADMINISTERED IG A AND IGG MONOCLONAL ANTIBODIES

The work in this chapter has been adapted from an article published in PLOS Neglected Tropical Diseases and is being included because they were part of a programmatic line of research that comprised the dissertation and including them provides a coherent and appropriately sequenced investigation.


Author Contributions: AFR and NJM conceived and design the experiments. AFR, JED, SAL, and JJV performed the experiments. GGW, PMW, KW, and LZ contributed reagents and materials. AFR and NJM analyzed the data and wrote the manuscript. AFR and NJM reviewed, edited, and submitted the final manuscript. Administration, supervision and funding acquisition were performed by NJM.
ABSTRACT

Non-typhoidal *Salmonella enterica* strains, including serovar Typhimurium (STm), are an emerging cause of invasive disease among children and the immunocompromised, especially in regions of sub-Saharan Africa. STm invades the intestinal mucosa through Peyer’s patch tissues before disseminating systemically. While vaccine development efforts are ongoing, the emergence of multidrug resistant strains of STm affirms the need to seek alternative strategies to protect high-risk individuals from infection. In this report we investigated the potential of an orally administered O5 serotype-specific IgA monoclonal antibody (mAb), called Sal4, to prevent infection of invasive *Salmonella enterica* serovar Typhimurium (STm) in mice. Sal4 IgA was delivered to mice prior to or concurrently with STm challenge. Infectivity was measured as bacterial burden in Peyer’s patch tissues one day after challenge. Using this model, we defined the minimal amount of Sal4 IgA required to significantly reduce STm uptake into Peyer’s patches. The relative efficacy of Sal4 in dimeric and secretory IgA (SIgA) forms was compared, as was a second lower avidity O5-specific IgA mAb that we produced from STm immunized mice. To assess the role of isotype in oral passive immunization, we engineered a recombinant IgG1 mAb carrying the Sal4 variable regions and evaluated its ability to block invasion of STm into epithelial cells *in vitro* and Peyer’s patch tissues. Our results demonstrate the potential of orally administered monoclonal IgA and SlgA, but not IgG, to passively immunize against invasive Salmonella. Nonetheless, the prophylactic window of IgA/SlgA in the mouse was on the order of minutes, underscoring the need to develop formulations to protect mAbs in the gastric environment and to permit sustained release in the small intestine.
INTRODUCTION

Enteric bacterial pathogens constitute a major burden on global health, especially in children younger than five years of age (Kotloff et al., 2013; O'Ryan et al., 2015). The Global Enteric Multicenter Study (GEMS) surveyed children ages 0-59 months in seven countries in sub-Saharan Africa and south Asia and identified the leading etiological agents of moderate-to-severe (MSD) and less-severe diarrhea (LSD) in this age group (Kotloff et al., 2019; Kotloff et al., 2013). Included on the list were Shigella species, Campylobacter jejuni, Vibrio cholerae and enterotoxigenic E. coli (ETEC) among others. Episodes of MSD and LSD can each have long term impacts on child health, most notably linear growth faltering. Other pathogens like multidrug resistant typhoid and invasive non-typhoidal Salmonella (iNTS) caused by different serovars of Salmonella enterica are also a source of infections in sub-Saharan Africa that can have short- and long-term health consequences (Feasey et al., 2012; Kariuki et al., 2019; Simon et al., 2011). The dire need for vaccines against enteric bacterial pathogens like C. jejuni, Shigella, ETEC and Salmonella has been recognized for decades, especially within military settings (O'Ryan et al., 2015). While there are numerous candidate vaccines under evaluation, the path forward remains challenging and alternative approaches need to be considered to combat C. jejuni, Shigella, ETEC and Salmonella in the immediate future.

With the advent of affordable and scalable platforms for the production of pathogen-specific IgG and secretory IgA (SIgA), the notion of oral passive immunization with polyclonal or monoclonal antibodies (mAbs) as a strategy to blunt diarrheal diseases in high-risk populations is gaining attention. For example, it was reported that
ingestion of polyclonal hyperimmune bovine colostrum (HBC), marketed as Travelan®, reduces experimental traveler’s associated ETEC infection (Otto et al., 2011). Sears and colleagues recently presented evidence that IgG and possibly IgA antibodies in Travelan® and a related HBC product (IMM-24E) may exert their protective effects through arresting ETEC motility and complement-mediated killing (Sears et al., 2017b). More recently, Guintini and colleagues demonstrated that oral administration of IgG or IgA mAbs targeting a single adhesin (CfaE) were able to reduce ETEC colonization by several orders of magnitude in a mouse model (Giuntini et al., 2018). In the case of invasive Salmonella enterica serovar Typhimurium (STm), Corthésy and colleagues reported that polyreactive secretory-like IgA/IgM mixtures were capable of reducing bacterial entry into Peyer’s patch tissues (Bioley et al., 2017; Corthésy et al., 2018). While these studies represent a proof of concept that oral immunoglobulins can abrogate Salmonella infection, the amount of IgA/IgM required to achieve a reduction in bacterial burden was excessive (i.e., ~10 mg of SIgA/IgM; ~ 500 mg/kg) and likely impractical if translated to a human setting. For that reason, we sought to investigate the potential benefit of a mAb-based passive immunization approach in blocking invasive Salmonella.

Sal4 is a well-characterized, dimeric IgA mAb originally isolated from a panel of B cell hybridomas derived from Peyer’s patch tissues of mice that had been immunized with an attenuated strain of STm (Michetti et al., 1994). Sal4 recognizes the O5-antigen of STm lipopolysaccharide (LPS) (Michetti et al., 1992). The O-antigen of STm is a tetrasaccharide consisting of galactose, rhamnose, and mannose, with an abequose (3,6 dideoxy-galactose) moiety on the mannose side chain. The O5 antigen is conferred
when the abequose residue is acetylated, while the O4 antigen is defined by the absence of acetylation modification (Slauch et al., 1995). Both STm O4 and O5 serotypes are invasive in mouse models of intragastric and parenteral challenge, although the actual lethal dose values vary slightly (Michetti et al., 1992).

In the so-called backpack tumor model, it was shown that Sal4 IgA, when actively transported into the intestinal lumen of mice in form of secretory IgA (SIgA), was able to reduce STm uptake into Peyer’s patch tissues (Michetti et al., 1992). Peyer’s patches represent the point of entry for invasive strains of Salmonella enterica and the bottleneck for systemic dissemination (Lim et al., 2014). Sal4 IgA’s protective capacity was limited to the gut, as even high levels of Sal4 IgA in circulation were unable to curtail STm systemic infection in the face of a parenteral bacterial challenge (Michetti et al., 1992). Thus, Sal4 IgA limits STm infection exclusively in the context of the gastrointestinal lumen. Although the exact mechanisms by which Sal4 IgA prevents bacterial uptake into Peyer’s patch tissues have not been fully resolved, Sal4 IgA strongly promotes bacterial agglutination in vitro and is a potent inhibitor of STm flagella-based motility in liquid and viscous media (Forbes et al., 2008). In this report, Sal4 IgA was chosen as a prototype to investigate the potential of orally administered mAbs to passively immunize against invasive Salmonella. We first established a robust mouse model of bacterial entry into Peyer patch tissues and then used the model to compare the efficacy of Sal4 as dimeric IgA, secretory IgA and even IgG, in limiting bacterial access to the intestinal mucosa. We also generated and characterized a second O5-specific IgA mAb and compared it to Sal4 IgA in vitro and in vivo.
RESULTS

*Oral administration of Sal4 IgA prevents infection of STm in mice*

To explore the benefit of passively administered Sal4 IgA on reducing the invasion of Peyer’s patch tissues by STm, we developed a competitive infection assay using two STm strains, AR05 and AR04 (Forbes et al., 2012). AR05 is a kanamycin resistant derivative of the type strain ATCC14028 (Table 2.1) that expresses the O5 antigen (O5-Ag). AR04 is a derivative of AR05 with a Tn10 insertion in the acetyl transferase gene (oafA126::Tn10d-Tc) that abolishes the bacterium’s ability to express the O5 Ag. Therefore, Sal4 IgA reacts with AR05 but not AR04 (Figure A1.1) (Forbes et al., 2008; Slauch et al., 1995). In addition, AR04 constitutively expresses β-galactosidase. Thus, AR05 (“white”) is readily distinguished from AR04 (“blue”) on when colonies grown on LB/X-Gal agar. The competitive index (CI) is simply the ratio of AR05 to AR04 in the inoculum compared to the ratio of AR05 to AR04 recovered from Peyer’s patch tissues, that allows for normalization in variations of challenge inoculum and Peyer’s patch number between mice (see Materials and Methods).

Adult BALB/c mice were challenged by gavage with a 1-to-1 mixture of AR05 and AR04 and 24 h later Peyer’s patches were collected along the entire length of the small intestine (Figure A1.2). It should be noted that at this time point, there was no gross evidence of inflammation or secretory diarrhea. Peyer’s patch tissues were normal in size and number and there was no evidence of fluid accumulation in the intestinal lumen. These observations are consistent with what has been reported in the literature (Carter & Collins, 1974; Palmer & Slauch, 2017; Santos et al., 2001).
The Peyer’s patch tissues were pooled and homogenized and the resulting homogenates were serially diluted onto LB agar containing kanamycin and X-Gal. Preliminary studies determined that an inoculum of $4 \times 10^7$ total CFUs (1:1 AR05 and AR04), which is roughly equivalent to $20 \times LD_{50}$ (Michetti et al., 1992), resulted in the reproducible recovery of $10^2$-$10^4$ CFUs from each mouse. The experiments also revealed AR05 was slightly more invasive than AR04, as evidenced by a CI of $\sim 1.2$ to 1.5. This minor difference in invasiveness between the two strains was only observable after computing the CI, as the total number of AR04 and AR05 CFUs in the Peyer’s patches were not significantly differ from each other (Figure 3.1). Furthermore, STm invasion of both AR05 and AR04 strains was confined to Peyer’s patch tissues. In a representative study, we found that there were 208 CFUs/mm of Peyer’s patch tissue, as compared to 0.1 CFUs/mm of proximal small intestine and 0.17 CFUs/mm distal small intestine. This $\sim 2000$-fold enrichment of STm in Peyer’s patch tissues is consistent bacterial uptake occurring primarily via M cells and agrees with what has been reported in the literature dating back almost 40 years (Carter & Collins, 1974; Clark et al., 1994; Jones et al., 1994).

We next examined the impact of Sal4 IgA on the ability of STm to invade Peyer’s patch tissues. Pre-treatment of the STm inoculum with Sal4 IgA resulted in a dose-dependent reduction in the number of AR05 recovered in Peyer’s patch tissues (Figure 3.1). The highest dose of Sal4 IgA tested (12 $\mu$g) resulted in $> 4 \log_{10}$ reduction in AR05 invasion efficiency, as compared to controls. In contrast, the number of AR04 CFUs recovered from the same Peyer’s patch tissues was unaffected by Sal4 IgA treatment. The relative impact of Sal4 IgA on AR05 versus AR04 on Peyer’s patch invasion was
Figure 3.1. Orally administered Sal4 IgA blocks STm invasion into mouse Peyer's patches.

Adult BALB/c mice were challenged by gavage with a one-to-one mixture of STm O5 and O4 strains STm (4 x 10^7 CFU total) co-administered with Sal4 IgA or an isotype
control. Peyer’s patches were collected ~24 h later and assessed for bacterial loads. (A) Competitive indices and (B) total CFUs of AR05 and AR04 STm. Shown are the combined results of five independent experiments with at least 4 mice per group. Each symbol represents an individual mouse. Statistical significance evaluated for each concentration over the isotype control, as determined by Kruskal-Wallis test and Dunn’s post-hoc test.
most apparent when the recovery values were expressed as a CI (Figure 3.1). By this metric, the addition of as little as 0.4 µg of Sal4 IgA rendered AR05 at a competitive disadvantage. The addition of greater amounts of Sal4 further reduced the CI with a maximal reduction occurring at concentrations above 1.2 µg Sal4 IgA. Invasion of Peyer’s patch tissues by AR05 and AR04 was unaffected by 2D6, an anti-Vibrio cholerae IgA mAb that served as the isotype control for these studies (K. Levinson et al., 2015; Michetti et al., 1992).

Inhibition of STm invasion of epithelial cells in vitro and Peyer's patch tissues in vivo by a second O5-specific IgA

A number of important biological activities have been ascribed to Sal4 IgA that likely contribute to its ability to limit bacterial uptake into Peyer’s patch tissues (Forbes et al., 2008; Forbes et al., 2012; Michetti et al., 1992; Michetti et al., 1994; Slauch et al., 1996; Slauch et al., 1995). Most notably is Sal4 IgA’s ability to arrest bacterial motility in liquid and semi-solid agar (Forbes et al., 2008). In addition, Sal4 IgA blocks Salmonella pathogenicity island 1 (SPI-1) type III secretion system (T3SS)-mediated entry of STm into epithelial cells and limits the translocation of T3SS effector proteins S1rP and SopB (Forbes et al., 2012). However, because Sal4 IgA is the only O5-specific IgA mAb that has been characterized in detail, it is not known these activities are unique to Sal4 shared by other O5-specific IgA antibodies. For that reason, we sought to generate an additional O5-specific mouse IgA mAbs and evaluate them in vitro and in vivo. To this end, groups of BALB/c mice were immunized with an attenuated STm mutant (Michetti et al., 1992). B cell hybridomas were generated from Peyer’s patch
lymphocytes from immunized mice and screened by ELISA for IgA reactivity with STm strain 14028. Despite numerous attempts, only a single stable B cell hybridoma secreting an O5-specific IgA mAb was identified, which we designated PeA3. PeA3 IgA bound STm LPS by ELISA (Figure A1.3). In liquid culture, PeA3 promoted agglutination of AR05, but not AR04, thereby demonstrating its specificity for the O5 epitope (Figure A1.3). Finally, PeA3 IgA significantly impeded STm flagella-based motility through soft agar.

To test whether PeA3 IgA impedes STm T3SS-mediated invasion into epithelial cells, a 1:1 mixture of AR05 and AR04 was treated with PeA3 IgA, Sal4 IgA, or an isotype control (2D6) before being applied to HeLa cells with gentle centrifugation to bypass the need for motility (Forbes et al., 2008). At the doses of mAb tested, PeA3 IgA treatment resulted in a significant reduction in AR05 uptake into HeLa cells (Figure 3.2).

To assess the ability of PeA3 IgA to block STm entry into mouse Peyer’s patch tissues, groups of BALB/c mice were challenged with a 1:1 mixture of AR05 and AR04 supplemented with a high (12 µg) or low (4 µg) dose of PeA3 IgA. As a control, Sal4 IgA was included in the experiments at the same doses as PeA3. At the high dose, PeA3 and Sal4 IgA were equally effective at blocking bacterial uptake into Peyer’s patch tissues, as evidenced by similar competitive indices (Figure 3.2). At the low dose, however, Sal4 IgA was ~five-fold more efficient than PeA3, as evidenced by mean CI values of 0.16 (± 0.30) versus 0.64 (± 0.20), respectively (Figure 3.2). The differences in mAb activities in vivo likely reflect different relative avidities of Sal4 and PeA3 for STm LPS. Nonetheless, these data demonstrate that another O5-specific IgA mAb,
Figure 3.2. PeA3 IgA blocks wildtype STm invasion in vitro and in vivo.

(A) A 1:1 mixture of AR04 and AR05 STm strains was treated with Sal4 IgA, PeA3 IgA, or isotype control antibody and applied to HeLa cells. Monolayers were then treated with gentamicin to eliminate extracellular bacteria and HeLa cells were lysed. The remaining cell lysate was enumerated for CFUs (n = 3 experiments, each done in triplicate). Panels B, C: Adult BALB/c mice were challenged by gavage with a 1:1 mixture of STm AR04 and AR05 (4 x 10^7 CFU total) in the present of 30 µg/mL (B) or 10 µg/mL (C) of indicated mAb. The mice were euthanized 24 h later and Peyer’s patches were assessed for bacterial loads. Shown are the combined results of two independent experiments with at least 4 mice per group. Statistical significance evaluated for each concentration over the isotype control, as determined by one-way ANOVA with either Dunnett’s (A, B) or Tukey’s (C) post-hoc tests.
besides Sal4, is able to inhibit STm invasion of epithelial cells \textit{in vitro} and Peyer’s patch tissues \textit{in vivo}.

\textit{Pre-exposure prophylactic activity of Sal4 IgA}

Given that human breast milk provides passive immunity to newborns primarily in the form of specific polyclonal SIgA (Brandtzaeg, 2003), there is interest in replicating this phenomenon utilizing mAbs for protection against enteric pathogens. \textit{In vivo}, antigen-specific oral IgA has already been shown to protect against rotavirus challenge in a suckling mouse model (Li et al., 2018) and oral feedings of recombinant VHH-IgAs, specific for the F4 fimbriae of ETEC, to piglets prior to bacterial challenge significantly reduced disease progression compared to control-fed groups (Virdi et al., 2013). After establishing Sal4 as a superior anti-O5 mAb, we sought to investigate its potential prophylactically in a model of STm infection.

We first addressed the degree to which Sal4 IgA administered to mice in advance of ST challenge retained its capacity to limit AR05 uptake into Peyer’s patch tissues. Sal4 IgA was delivered by gavage to mice 20 min prior to challenge with 1:1 mixture of AR05 and AR04. Under these conditions, Sal4 IgA’s activity was effectively lost. For example, Sal4 IgA (36 µg) given to mice by gavage 20 min before STm challenge afforded no protection against Peyer’s patch uptake (\textbf{Figure A1.4}). In an effort to overcome the possible deleterious effects of gastric pH and intestinal proteases like pepsin, the experiments were repeated with the addition of sodium bicarbonate (3% NaHCO$_3$) or protease inhibitors. The co-administration of Sal4 IgA with either sodium bicarbonate or protease inhibitors resulted in a 40-50% reduction in AR05 uptake into
the Peyer’s patches (Figure 3.3), consistent with pH and proteases as being factors influencing the half-life of Sal4 IgA in the gastric and intestinal lumen. While beyond the scope of the current study, these results underscore the necessity of identifying formulations capable of protecting orally administered antibodies like Sal4 IgA from the gastric environment.

**Benefit of SC on Sal4 IgA function in the mouse model**

SC imparts a number of biologically important activities upon SIgA in the context of the intestinal lumen, including protease resistance and mucus affinity (Corthésy et al., 2018; Gayet et al., 2017). We therefore expected that Sal4 SIgA would be significantly more effective *in vivo* than equivalent amounts of dimeric Sal4 IgA lacking SC. To test this hypothesis, purified, dimeric Sal4 (dIgA) and dIgA complexed with human recombinant SC (SIgA) were compared side-by-side in the mouse model of invasive STm. Analysis of bacterial burdens in intestinal tissues collected 24 h after challenge revealed that Sal4 dIgA and SIgA were equally effective at limiting uptake of AR05 into Peyer’s patches (Figure 3.4), indicating that the addition of SC did not enhance the function of Sal4 dIgA in this model.

We postulated that the advantage of SC may only be apparent when antibody interacts with the intestinal environment in advance of bacterial challenge. We therefore repeated the experiments in which Sal4 dIgA and SIgA were given to mice by gavage immediately before STm challenge. Once again, however, Sal4 SIgA was no more effective than Sal4 dIgA at reducing invasion of AR05 into Peyer’s patch tissues. We
Figure 3.3. Sodium bicarbonate and protease inhibitors improve Sal4 IgA prophylactic activity.

Adult BALB/c mice were gavaged with Sal4 IgA (50 µg) in (A) 3% NaHCO$_3$ or (B) a protease inhibitor cocktail 20 min or 1 min before STm challenge. The mice were euthanized 24 h later and Peyer’s patches were assessed for bacterial loads, as a readout of bacterial invasion. Shown are the results of three independent experiments with at least 5 mice per group. Each symbol represents an individual mouse. Statistical significance compared to the isotype control at each time point, as determined by unpaired Student’s $t$-test.
Figure 3.4. Comparison between Sal4 dimeric IgA (dIgA) and secretory IgA (SIgA) preparations in mouse model of STm infection.

Adult BALB/c mice were challenged by gavage with a one-to-one mixture of STm O5 and O4 strains (4 x 10⁷ CFU total). Dimeric IgA (dIgA) or secretory IgA (SIgA) forms of Sal4 were (A) premixed with the bacterial inoculum or (B) administered ~1 min prior to STm challenge. The mice were euthanized 24 h later and Peyer’s patches were assessed for bacterial loads. Shown are the results of two independent experiments with 4 mice per group. Statistical significance evaluated for each concentration over the isotype control, as determined by one-way ANOVA and Tukey’s post-hoc test.
conclude that, at least in this model of passive oral immunization, the potency of Sal4 IgA is not enhanced by the addition of SC (Figure 3.4).

Potential of orally administered Sal4 IgG to passively immunize mice

In clinical trials, ingestion of bovine milk- or colostrum-derived immunoglobulins consisting mainly of IgG from immunized dairy cows is sufficient to significantly reduce ETEC infection in adult volunteers (Otto et al., 2011; Tacket et al., 1988), indicating a role for IgG in passive oral immunizations. In fact, in a recent report, orally delivered anti-colonization factor antigen CFA/I IgG and SIgA human mAbs were equally effective at blocking ETEC infection in a mouse model (Giuntini et al., 2018).

To investigate the potential of orally administered IgG to prevent STm invasion of Peyer’s patch tissues, we engineered a Sal4 IgG chimeric antibody in which the V\text{H} and V\text{L} domains of the Sal4 IgA were grafted onto a human IgG1 framework (Figure A1.5). The resulting Sal4 IgG1 was expressed in *Nicotiana benthamiana* using so-called RAMP technology (K. Levinson et al., 2015). The chimeric IgG1 mAb reacted with STm LPS by ELISA (Figure A1.5) and promoted agglutination of STm in liquid culture. In a soft agar motility assay, Sal4 IgG limited bacterial spread over the course of the 6 h experiment, although slightly less effectively than Sal4 IgA. In the HeLa cell invasion assay, Sal4 IgG and Sal4 IgA were more or less equivalent in their abilities to block AR05 uptake (Figure 3.5). We therefore conclude that the Sal4 IgG1 molecule has expected the biological activities associated with Sal4 IgA, at least in vitro.
Figure 3.5. *In vitro* functionality of Sal4 IgG.

(A) Effect of Sal4 IgG on STm motility in soft agar. STm was stab-inoculated into 0.3% LB agar containing 15 µg/mL of IgA control, Sal4 IgA, IgG control, or Sal4 IgG antibody. Plates were incubated at 37°C and the diameter of bacterial swimming was measured every hour for 6 h (*n* = 3 experiments each done in triplicate). (B) A one-to-one mixture of AR05 and AR04 STm (10^7 CFUs total) were incubated for 10 min with 15 µg/mL of IgA control, Sal4 IgA, IgG control, or Sal4 IgG antibody before being applied to HeLa
cell monolayers (MOI ~10), as described in the Materials and Methods. After 1 h, the
HeLa cells were lysed and the CFUs were enumerated. Asterisks indicate significant
reduction in wildtype STm motility (A) or invasion (B) over the respective isotype control,
as determined by unpaired Student’s t-test (n = 2 experiments done in triplicate; P < 0.05).
We next investigated the potential of chimeric Sal4 IgG1 to passively immunize mice against STm infection in both systemic and oral challenge models. For the systemic challenge model, groups of BALB/c mice were administered Sal4 IgG1 or a chimeric IgG1 isotype control (PB10) by intraperitoneal injection and then challenged 24 h later with $10^4$ CFUs of wild type STm ATCC14028 by the same route. One day later, the mice were euthanized and CFUs in the spleens and livers were evaluated. As compared to the IgG1 control group, mice that received the high dose Sal4 IgG1 (40 µg) had 10 to 100-fold lower STm burden in the spleens and livers (Figure A1.6). Bacterial numbers were also reduced in mice that received a low dose (10 µg) of Sal4 IgG1, although to a lesser extent than the high dose group of animals. These results demonstrate that passively administered Sal4 IgG1 results in dose-dependent reduction in STm systemic infection.

To examine Sal4 IgG1’s activity in the context of intestinal immunity to STm, groups of BALB/c mice were gavaged with a 1:1 mixture of AR05 and AR04 supplemented with 30 µg/mL Sal4 IgA or IgG1 or the relevant isotype controls. Invasion of Peyer’s patch tissues was measured 24 h later. As observed previously, Sal4 IgA reduced AR05 invasion into Peyer’s patch tissues by several orders of magnitude (CI value of 0.04 ± 0.02) (Figure 3.6). In contrast, Sal4 IgG1 had no effect on STm invasion, as evidenced by a CI value of 0.95 ± 0.13 (Figure 3.6). The failure of Sal4 IgG1 to function in these studies could not be overcome by increasing antibody dose (e.g., >750 µg) or repeated administration over a 12 h period (Figure A1.7) or by high dose parenteral administration (Figure A1.8).
Figure 3.6. Sal4 IgG fails to inhibit STm invasion following oral infection *in vivo* but can be partially rescued upon stabilization.

BALB/c females were orally challenged with a competitive index of wildtype (AR05) and *oafA* mutant (AR05) STm (4 x 10⁷ CFUs) either (A) pre-incubated with 30 µg/mL of antibody or (B) administered antibody prior to STm challenge at the indicated time points. 24 hours (p.i.) mice were euthanized and Peyer’s patches were harvested and enumerated for CFUs and competitive indices. Statistical significance evaluated for each group over the isotype control, as determined by unpaired Student’s *t*-test (*n* = at least 5 mice per group).
We postulated that antibody stability in the gastric environment might account (at least in part) for the failure of Sal4 IgG1 to function in the oral passive immunization model (Corthésy et al., 2018; Hu et al., 2019). To address this experimentally, Sal4 IgA and IgG1 variants were incubated in an adult simplified Simulated Gastric Fluid (SGF), without and with pepsin, essentially as described (Menard et al., 2017; Minekus et al., 2014). After 10, 30, and 60 min at 37°C, aliquots were removed and tested reactivity with STm LPS by ELISA. In the presence of SGF, Sal4 IgG1 levels declined steadily over a 30 min time period, whereas the IgA1 variant was relatively stable (Table 3.1). Upon the addition of pepsin, however, IgG1 declined so precipitously that it was undetectable at 10 min. At the same time point (10 min), Sal4 IgA had declined to just ~10% of starting levels, but then remained detectable until 30 min. Collectively, these results confirmed the differential sensitivity of Sal4 IgG1 and IgA to the gastric environment.

The in vitro stability studies with Sal4 IgG prompted us to repeat the passive immunization studies with the addition of sodium bicarbonate plus protease inhibitors. Specifically, Sal4 IgG1 in sodium bicarbonate (3% NaHCO₃) plus protease inhibitors administered to mice by gavage 1 min or 20 min prior STm challenge. Under these conditions, Sal4 IgG1 did in fact block STm invasion into Peyer’s patch tissues, but only when given immediately before STm challenge (Figure 3.6). Collectively, these results suggest that ineffectiveness of Sal4 IgG1, as compared to Sal4 IgA, is due to its instability in the gut environment.
Table 3.1. Differences in antibody half-life during *in vitro* digestion between Sal4 IgA and IgG

<table>
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<td>60 min</td>
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</table>

<sup>a</sup>100 µg/mL Sal4 IgA and IgG were incubated at 37°C in adult simplified simulated gastric fluid (SGF) with or without pepsin (2000 U/mL) at pH 3.0

<sup>b</sup>Aliquots were taken after incubating for 10, 30, and 60 minutes and neutralized to pH 7.0 – 7.4 using 1M NaOH.

<sup>c</sup>Neutralized samples were assessed for ST-LPS binding ELISA and amount of Sal4 IgA and IgG binding remaining was calculated using a standard curve with SoftMax Pro 5.2 software.
DISCUSSION

In this study, we investigated the potential of orally administered mAbs to passively immunize mice against invasive Salmonella. The study was motivated by several factors. First is the rapid emergence of multi-drug resistance Salmonella infections, which constitute an increasing threat to public health in developing and developed countries (An et al., 2017; Arnott et al., 2018). Second, given the difficulty and extended timeline associated with vaccine development, there is pressure from federal and private foundations such as the Bill and Melinda Gates Foundation to explore alternative strategies as a means of protecting at risk individuals from debilitating enteric infections. With the remarkable advances in recombinant mAb engineering and scale-up using mammalian cells, transgenic animals, plants and even seed-based production platforms, the prospect of combatting diarrheal diseases through orally administered mAb cocktails is technically feasible and cost-effective (Tokuhara et al., 2013; Virdi et al., 2013; Virdi et al., 2019).

We found that direct administration of Sal4 IgA to adult mice by gavage overcomes many of the impediments associated with the so-called “backpack” tumor model that was used previously to study STm-IgA interactions (Jones et al., 1994). In the backpack model, antibody-secreting B cell hybridomas are implanted subcutaneously into mice, resulting in local tumor formation and the accumulation of antibodies at very high concentrations in serum and interstitial fluids, including the lamina propria (Michetti et al., 1992). Ultimately, hybridoma-derived, antigen-specific IgA is detected in intestinal secretions, presumably as a result of pIgR-mediated transcytosis (Haneberg et al., 1997; L. r. Winner et al., 1991). While this set-up resulted
in physiologic delivery of Sal4 IgA into the intestinal lumen, there are several drawbacks with the model. First, depending on how well the hybridoma “takes,” the amount of Sal4 IgA in serum and intestinal secretions varies widely from mouse to mouse, thereby confounding the ability to perform strict dose-response studies. Second, because hybridoma-derived antibodies accumulate at potentially very high levels in serum (1-10 mg/ml) and interstitial fluids, it is not always possible to delineate whether observed protection is due to intestinal (secretory) or interstitial antibodies (Haneberg et al., 1997). Finally, while it is assumed Sal4 IgA antibodies detected in the intestinal secretions in the backpack tumor mice are complexed with SC, the actual amount of Sal4 SIgA in the lumen has never been determined. Direct administration of Sal4 IgA of known molecular forms and at specific doses overcomes these concerns.

The other notable benefit of the challenge model employed here is that the primary readout is bacterial load (CFUs) in Peyer’s patch tissues, which are known to be the primary point of entry for invasive Salmonella (Carter et al., 1975; Hohmann et al., 1978). Uptake into Peyer’s patches occurs through M cells and is dependent on the SPI-1 T3SS. Moreover, Peyer’s patch invasion occurs in the presence of a normal gut microbiota. This is in contrast to models of Salmonella-induced inflammation where infection occurs primarily in the cecum and colon and involves pre-treatment of mice with antibiotics like streptomycin to deplete the gut microbiota (Barthel et al., 2003; Kaiser et al., 2012). For the purposes of this study, the challenge model granted us the ability to ask vital questions about IgA biology, such as the importance of SC in intestinal immunity.
Indeed, contrary to what we expected, the addition of SC did not augment Sal4 IgA activity in our mouse model of invasive STm. One possible explanation for this finding relates to the route of antibody delivery. Normally, dIgA is transported across the intestinal epithelium by the plgR, which is preferentially expressed by enterocytes in intestinal crypts. Following transport, SlgA localizes to the mucus layer overlying the epithelial barrier where SC plays a central role in anchoring IgA within this microenvironment and protecting the antibody from protease-mediated degradation (Duc et al., 2010; Johansson & Hansson, 2016; Phalipon et al., 2002). It is unclear if the physiologic distribution of SlgA is recapitulated when antibody is administered by gavage. Our attempts to track Sal4 SlgA, using immunohistochemistry, in the small intestine following oral delivery have not been successful to date. Another possible explanation for why SC did not impart a benefit to Sal4 IgA is that the rate-limiting determinant for antibody activity in this model is dilution effects upon gavage, not protease sensitivity or mucus anchoring, where SC would be expected to play an important role.

The comparison between PeA3 and Sal4 IgA mAbs in the mouse model of invasive Salmonella serves as an indirect demonstration of the importance of IgA avidity in protecting against invasive pathogens. By all accounts, Sal4 and PeA3 recognized the same or a very similar epitope but differed in their relative binding affinities for the O5 antigen by ~10 fold. The difference in binding activity correlated with differences in in vivo efficacy, at least when PeA3 and Sal4 IgA were given to mice at lower doses. At higher doses, Sal4 and PeA3 were equally effective at limiting STm uptake into Peyer’s patch tissues, underscoring that protection of the mucosal surface is due to the
interrelationship between IgA avidity and local antibody concentrations. As illustrated by Corthésy et al., even polyreactive SIgA is protective if present at sufficiently high concentrations (Corthésy et al., 2018). From the standpoint of passive immunization, however, higher affinity/avidity antibodies are clearly advantageous since much lower doses would be required to achieve protection. Indeed, in the case of respiratory infections, the selection for higher affinity mAbs resulted in correspondingly higher neutralizing activities and in vivo potency (Wu et al., 2007).

We found that a recombinant human IgG1 variant of Sal4 had only marginal capacity (when co-administered with protease inhibitors and sodium bicarbonate) to passively immunize mice against intragastric Salmonella infection. These findings are consistent with IgG1 instability in the gastric environment. It is likely that the heavily glycosylated nature of IgA provides an advantage upon direct delivery into the gut in terms of maintaining both direct antigen binding and crosslinking between multiple antigens (Duc et al., 2010), while the IgG mAb, with a lone pair of N-glycans on the Fc region (Kiyoshi et al., 2017), is outmatched. Other factors may also be at play. Sal4 IgG1, which is a monomer, likely differs from Sal4 IgA, which is a dimer, in its ability to promote bacterial agglutination. We cannot rule out the possibility that the nature of agglutination between IgG and IgA is quantitatively different considering that we did observe slight differences in the kinetics of microagglutination between to two antibody isotypes.

Ultimately, our study highlights some of the fundamental challenges associated with oral mAb delivery as a means to combat enteric diseases, especially in children. Foremost is the remarkably short apparent “half-life” of Sal4 IgA and Sal4 SIgA in the
gastric and intestinal environments. As noted above, our results suggest that the rapid 
decline of Sal4 IgA activity when given prophylactically to mice is likely due to a 
combination of physical degradation by local proteases and acid pH, coupled with rapid 
clearance/dilution in the intestinal lumen. Even modest declines in the local 
concentration of Sal4 IgA would be expected to impact antibody potency, considering 
that the ability of Sal4 IgA to block bacterial entry into Peyer’s patches is the result (at 
least in part) of bacterial agglutination in the intestinal lumen. For Sal4 or other IgA 
mAbs to be used clinically in the future will require a more thorough understanding of 
the pharmacokinetics of IgA and SIgA in mucosal tissues. Coupled with those efforts is 
a need to identify formulations and delivery strategies to ensure proper delivery and 
localization of IgA and SIgA in the regions of the gut where they are most needed. In the 
case of STm, that would be in the proximal small intestine, while for Shigella species the 
antibodies would need to reach the colon to exert their effects. Nonetheless, in spite of 
these barriers, specific oral IgG preparations from HBC have seen success 
therapeutically in clinical trials of children with rotavirus diarrhea (Sarker et al., 1998), 
prophylactically in rotavirus-infected mice (Pant et al., 2007) and ETEC challenged 
humans (Savarino et al., 2017; Savarino et al., 2019), suggesting the goal of using 
passive immunization to combat enteric disease is not far from reach.
CHAPTER 4 : SALMONELLA UPTAKE INTO GUT-ASSOCIATED LYMPHOID TISSUES: IMPLICATIONS FOR TARGETED MUCOSAL VACCINE DESIGN AND DELIVERY

The work in this chapter has been accepted for publication in the following form:


For the purpose of the dissertation thesis, an additional discussion section has been amended.

Author Contributions: AFR, FJTV, and NJM conceived and design the experiments. AFR and FJTV performed the experiments. AFR and NJM analyzed the data and wrote the manuscript. AFR and NJM reviewed, edited, and submitted the final manuscript. Administration, supervision and funding acquisition were performed by NJM.
ABSTRACT

Peyer’s patches are organized gut-associated lymphoid tissues (GALT) in the small intestine and the primary route by which particulate antigens, including viruses and bacteria, are sampled by the mucosal immune system. Antigen sampling occurs through M cells, a specialized epithelial cell located in the follicle-associated epithelium (FAE) that overlie Peyer’s patch lymphoid follicles. While Peyer’s patches play an integral role in intestinal homeostasis, they are also a gateway by which enteric pathogens, like *Salmonella enterica* serovar Typhimurium (STm), cross the intestinal barrier. Once pathogens like STm gain access to the underlying network of mucosal dendritic cells and macrophages they can spread systemically. Thus, Peyer’s patches are at the crossroads of mucosal immunity and intestinal pathogenesis. In this chapter, we provide detailed methods to assess STm entry into mouse Peyer’s patch tissues. We describe Peyer’s patch collection methods and provide strategies to enumerate bacterial uptake. We also detail a method for quantifying bacterial shedding from infected animals and provide an immunohistochemistry protocol for the localization of STm along the gastrointestinal tract and insight into pathogen transit in the presence of protective antibodies. While the protocols are written for STm, they are easily tailored to other enteric pathogens.
1. INTRODUCTION

Organized gut-associated lymphoid tissues (GALT) located along the length of the human gastrointestinal tract maintain a delicate balance between tolerance and immunity. On the one hand, GALT tolerate the billions of resident commensal microorganisms that inhabit the proximal small intestine, cecum and colon. Overreaction to the commensal flora results in chronic inflammation and intestinal dysbiosis. On the other hand, the GALT are poised to mount immune responses against pathogenic viruses, bacterial and fungi. An insufficient response to an enteric pathogen can result in debilitating disease and even death.

Nowhere is the balance between tolerance and immunity more precarious than within Peyer’s patches (Owen et al., 1991). Peyer’s patches are aggregates of organized lymphoid follicles concentrated in the small intestine. In mice Peyer’s patches are distributed throughout the proximal and distal small intestine, while in humans they are disproportionately clustered in the ileum (Cornes, 1965). The epithelium overlying Peyer’s patch follicles, the so-called follicle-associated epithelium (FAE), contains a unique cell type known as microfold (M) cells (Figure 4.1). M cells are the primary route by which particulate antigens in the intestinal lumen are sampled by Peyer’s patches. The apical aspects of M cells lack the brush border that defines neighboring enterocytes (Kim & Jang, 2014). M cells also have a reduced glycocalyx (Neutra et al., 1996) and express cell surface receptors for antigen uptake, such as glycoprotein 2 (GP2) (Nakamura et al., 2018). In essence, M cells are designed for optimal uptake and transcytosis of luminal antigens into the subepithelial dome (SED) region of Peyer’s patches. The SED consists of a network of macrophages and antigen-presenting cells.
Figure 4.1. Schematic of the Peyer’s patch.

Above the Peyer’s patch resides a specialized follicle-associated epithelium (FAE) containing antigen-surveilling microfold (M) cells that are identified by their shortened brush border and basolateral invagination. Follicular macrophages and dendritic cells occupy the region beneath the FAE, known as the subepithelial dome (SED) where they receive luminal antigen either via M cell delivery, or by reaching dendrites through epithelial tight junctions to directly sample the intestinal lumen. The Peyer’s patch follicle contains germinal centers where activated B cells primarily undergo IgA class-switching and expansion. IgA-specific plasma cells secrete polymeric IgA antibodies carrying J-chain into the lamina propria where they bind to the polymeric immunoglobulin receptor (pIgR) at the basolateral side of intestinal epithelial cells, resulting in transcytosis of secretory IgA (SIgA) into the lumen. Image generated with BioRender.
(APCs), such as dendritic cells (DCs), capable of capturing luminal antigens following M cell transport vesicles (Corthesy, 2007). SED DCs can also traffic antigens to germinal centers deeper within the Peyer’s patch (Brandtzaeg, 2009).

Peyer’s patch lymphoid follicles contain germinal centers with T follicular helper cells (T\textsubscript{FH}) and B cells undergoing somatic hypermutation and a bias towards class switch recombination to IgA, the predominant antibody isotype of the gut (Bemark et al., 2012). B cells emigrate from Peyer’s patch tissues and seed the surrounding lamina propria as IgA-secreting plasma cells. Gut-derived, IgA-class switched B cells can also home to the mammary gland (Lindner et al., 2015). IgA antibodies secreted from mucosal B cells into the lamina propria are predominantly dimers (dIgA) or higher molecular weight polymers (plgA) and are transported across columnar enterocytes (basolateral to apical) by the polymeric immunoglobulin receptor (pIgR). The ectodomain of pIgR, called secretory component (SC), remains covalently associated to dIgA after transport, resulting in the generation SIgA. SC, which is heavily glycosylated, enhances the stability of dIgA within the intestinal environment (Duc et al., 2010). SC is also proposed to anchor dIgA within the mucus layer architecture (Phalipon et al., 2002).

Given the importance of secretory antibodies in protecting the intestinal epithelium (Alvarez et al., 2013; Amarasinghe et al., 2013; Bioley et al., 2017; Blutt et al., 2012; Brandtzaeg, 2013; Gopalakrishna et al., 2019; Mantis & Forbes, 2010; Moor et al., 2017; Richards et al., 2020; Stoppato et al., 2020; Virdi et al., 2013; Virdi et al., 2019), targeting the Peyer’s patch is a vital strategy for mucosal vaccination. By taking advantage of M cells as an entry point, an oral vaccine can be delivered to preexisting
germinal centers without the need for transport to the lymphatic system (Lycke, 2012). Luminal antigens that make their way into the SED are captured by CCR6+ DCs that facilitate activation of antigen-specific CD4+ T cells, allowing for T cell-priming (Salazar-Gonzalez et al., 2006; Sheridan & Lefrancois, 2011). Mucosal vaccines targeted to Peyer’s patches would be expected to give rise to long-lived plasma cells and memory B cells (Lycke, 2012).

_Salmonella enterica_, a highly motile, facultative anerobic, Gram-negative bacterium, is a leading cause of disease in children and adults worldwide (Kotloff et al., 2019). _S. enterica_ encompasses over 2500 serovars, including two serovars of great importance, Typhi (STy) and Typhimurium (STm) (Fabrega & Vila, 2013). STy is the causative agent of typhoid fever, a disease acquired through contaminated food and water. The WHO estimates that there are between 10-20 million cases of typhoid fever annually (World Health Organization, 2018). STm infection is historically associated with acute gastroenteritis, especially in developed countries where the bacterium is frequently responsible for foodborne outbreaks. However, in certain parts of sub-Saharan Africa, there has been a rise over the past decade in the incidence of STm strains that cause invasive non-typhoidal salmonellosis (iNTS) (Feasey et al., 2012).

Much of our understanding of STy and STm pathogenesis has been derived from mouse models. In mice, STm utilizes flagella-based motility to approach the intestinal epithelium and specifically target M cells within the FAE (Clark et al., 1994). Invasion of M cells involves the SPI-1 type III secretion system (T3SS), resulting in transcytosis of the bacteria into the SED (Jones et al., 1994; Ohl & Miller, 2001). Once inside the SED, the bacteria replicate within host macrophages and DCs (Gorvel & Méresse, 2001)
before disseminating to regional lymph nodes and beyond (e.g., liver, spleen) (Vazquez-Torres et al., 1999). If mice are treated with antibiotics to clear resident microbiota, STm is able to colonize and trigger inflammation within the distal small intestine, cecum and colon (Barthel et al., 2003). Disease progression results in epithelial erosion, infiltration of leukocytes, and the generation of inflammatory mediators (Kaiser et al., 2012).

SIgA antibodies are of critical importance in protecting the gastrointestinal mucosa from enteric pathogens like STm and the prospect of oral passive immunization regimens to prevent disease in vulnerable populations is under investigation (D. E. Baranova et al., 2020; Corthésy et al., 2018; Giuntini et al., 2018; Richards et al., 2020; Stoppato et al., 2020). Indeed, it has been proposed that supplementing formula or even natural breast milk with a cocktail of antigen-specific monoclonal IgA antibodies might be a strategy to reduce disease incidence and the long-term consequences associated with even acute episodes of diarrheal diseases at a young age (Brandtzaeg, 2003; Turin & Ochoa, 2014).

In this chapter, we describe our effort to assess the potential of oral monoclonal antibodies (mAb) to passively immunize mice in a model of invasive Salmonella. We utilized the well-characterized mAb, Sal4 IgA, as our prototype. Sal4 IgA recognizes the O5-epitope of STm lipopolysaccharide (LPS) and has been shown, when provided systemically in high amounts via the “backpack tumor” model, to protect Peyer’s patch tissues from STm infection (Michetti et al., 1992; Michetti et al., 1994). We provide techniques to evaluate bacterial infiltration into Peyer’s patches, as well as shedding of colonized bacteria into fecal pellets. We describe a challenge model in which wild type STm strain (AR05), which expresses the O5-polysaccharide is mixed 1:1 with mutant
strain (AR04), which lacks the O5 epitope due to a mutation in the oafA gene. The relative ratio of AR05 and AR04 invasion of Peyer’s patches quantified by computing a competitive index reveals relative degrees of bacterial fitness (Michetti et al., 1992; Michetti et al., 1994; Richards et al., 2020; Slauch et al., 1996; Slauch et al., 1995). We include a protocol for staining orally administered STm by immunohistochemistry to visualize bacterial transit through the GI tract over a period of hours or even days.

The methods have been optimized to examine STm pathogenesis, but are applicable to invasive and non-invasive pathogens like Shigella flexneri (Sansonetti et al., 1996), Listeria monocytogenes (Disson et al., 2018), and Yersinia pseudotuberculosis (Clark et al., 1998). If the pathogen of interest predominantly colonizes rather than invades the mucosa, quantification of infectious microbes shed in the fecal pellet of infected animals can be used as a surrogate to measure colonized pathogens (Knipping et al., 2011; Perruzza et al., 2020; Stoppato et al., 2020).

Alongside this method, we provide an immunohistochemistry approach for detecting oral STm inoculum at different time points post-challenge, which can be implemented in alternative studies to examine pathogen localization after infection, or to gain further insight into the pharmacokinetics of experimental oral therapies.

2. MATERIALS

2.1 Quantifying Peyer’s Patch invasion

1. Laboratory mice: the protocol described here utilizes BALB/c female mice 8-12 weeks of age.
2. Bacterial strains of interest; here AR04 and AR05 are used (see Table 2.1 and Note 4.1).
3. Spectrophotometer (600 nm capability)
4. Centrifuge (capable of holding 50 mL conical tubes and reaching 6,000 x g)
5. UV-Cuvette Disposable Cuvettes (BrandTech Scientific, Inc., Essex, CT)
6. Headlamp/light source
7. Safety glasses
8. Fine, curved scissors (catalog #: 14061-09, Fine Science Tools, Foster City, CA)
9. Forceps
10. Surgical scissors
11. 70% ethanol
12. Luria-Bertani (LB) broth
13. Shaking 37°C incubator (capable of shaking at 200 RPM)
14. 15- and 50-mL conical tubes
15. Sterile serological pipets (5 mL, 10 mL, and 25 mL sizes)
16. 1 mL syringes
17. Feeding needles (catalog #: 01-208-87, Fisher Scientific)
18. Ice
19. Blue pads
20. Fisherbrand™ Bead Mill 4 homogenizer (Fisher Scientific)
21. 2 mL Bead Mill homogenizing tubes (Fisher Scientific)
22. Fisherbrand™ 2.8 mm homogenizing beads (Fisher Scientific)
23. LB agar plates containing kanamycin (50 μg/mL) and X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) (40 μg/mL)
24. 1X PBS (sterile)
25. Permeant lab markers (multiple colors)
26. 96-well dilution plates (catalog #: 83-2797, Corning)
27. 200 μl tips, sterile
28. Multichannel pipettor (10–100 μl range)
29. Glass plating beads (MP Biomedicals)
30. Bleach
2.2 Quantification of colonization by fecal shedding

1. Laboratory Mice (8-12 weeks)
2. Bacterial strains of interest; here AR04 and AR05 are used (see Table 2.1 and Note 4.1)
3. Centrifuge (capable of holding 50 mL conical tubes and reaching 6,000 x g)
4. Spectrophotometer (600nm capability)
5. UV-Cuvette Disposable Cuvettes (BrandTech Scientific, Inc., Essex, CT)
6. 70% ethanol
7. Luria-Bertani (LB) broth
8. Shaking 37°C incubator (capable of shaking at 200 RPM)
9. 1 mL syringes
10. Safety glasses
11. Feeding needles (catalog #: 01-208-87, Fisher Scientific)
12. Mouse containment chambers (empty 200 μl tip boxes used in this study)
13. Forceps
14. 1.5 mL microcentrifuge tubes
15. Benchtop vortexer
16. Ice
17. LB agar plates containing kanamycin (50 μg/mL) and X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) (80 μg/mL; see Note 4.3)
18. 1X PBS, sterile
19. Scissors
20. Glass plating beads
21. Bleach

2.3 Isolation and immunohistochemistry of intestinal samples containing S. Typhimurium

1. Laboratory mice (8-12 weeks)
2. Bacterial strain of interest; here ATCC14028 is used.
3. Spectrophotometer (600nm capability)
4. Centrifuge (capable of holding 50 mL conical tubes and reaching 6,000 x g)
5. UV-Cuvette Disposable Cuvettes (BrandTech Scientific, Inc., Essex, CT)
6. Luria-Bertani (LB) broth
7. Shaking 37°C incubator (capable of shaking at 200 RPM)
8. 15- and 50-mL conical tubes
9. 1 mL syringes
10. Feeding needles (catalog #: 01-208-87, Fisher Scientific)
11. Safety glasses
12. Headlamp/light source
13. Dissection tray and pins
14. Surgical scissors
15. Histopathology cassettes
16. Tissue bottle containing 10% buffered formalin
17. Fine Scissors (Fine Science Tools, Foster City, CA)
18. Anatomy probe (similar to catalog #: 10088-15, Fine Science Tools, Foster City, CA)
19. 60°C oven (used here, cat# 11-475-154, Fisher Scientific)
20. Positive charged microscope slides
21. Slide staining jars + slide rack (Fisher Scientific)
22. CitriSolv™ Hybrid Solvent and Clearing Agent
23. 70%, 80%, 95%, and 100% ethanol solutions
24. Deionized water, filtered
25. Proteinase K, PCR grade (MilliporeSigma)
26. Proteinase K (PK) buffer (0.6M Tris (pH 7.5)/0.1% CaCl₂)
27. TBS Wash Buffer, diluted to 1X (BioCare Medical, Pacheco, CA)
28. Rodent Block M (BioCare Medical, Pacheco, CA)
29. BLOXALL® Endogenous Blocking Solution, Peroxidase and Alkaline Phosphatase (Vector Laboratories, Burlingame, CA)
30. Difco Salmonella O Antiserum Group B Factors 1, 4, 5, 12 (catalog #: BD 229481, Becton, Dickinson and Company, Franklin Lakes, NJ)
31. Rabbit on Rodent AP-Polymer (BioCare Medical, Pacheco, CA)
3. METHODS

3.1 Quantifying Peyer’s Patch Invasion

Before starting, inoculate 5 mL of LB broth in borosilicate or polypropylene culture tubes with single colony of each bacterial challenge strain (e.g., AR04 and AR05). Incubate culture tubes at 37°C in rotary shaker (200 RPM) for ~16 h.

3.1.1 Preparation of Bacteria

1. Day 1. Dilute overnight cultures of each bacterial strain (AR04 and AR05) 1:50 into fresh LB broth and incubate at 37°C.
2. Incubate with aeration until cultures each reach OD_{600} of ~0.7. Adjust the OD_{600} of both strains to 0.7.
3. Mix equal volumes of each strain via pipetting followed by light vortexing. Subject bacterial mixtures to centrifugation for 2 min at 6,000 x g.
4. Decant supernatant and resuspend remaining pellet in sterile PBS. Repeat centrifugation, decant, and resuspension.
5. Centrifuge, decant, and resuspend in half of the original volume of PBS to concentrate the STm inoculum (see Note 4.2).

3.1.2 Gavage

1. Starting with one mouse at a time, gavage 200 μl of antibody mixture.
2. Next, gavage 200 μl of STm inoculum to the same mouse (see Note 4.2). This will correspond to ~4 x 10^7 CFUs/mouse.
3. Following gavage, dilute and plate input (100 μl/plate) on LB/Kan/X-gal plates and incubate O/N at 37°C. This will serve as the “input” or challenge inoculum.

3.1.3 Collection and Processing of Peyer’s Patches

1. *Day 2.* Remove input plates from 37°C incubator and store at 4°C until end of the experiment.
2. Euthanize animals by group via CO₂ asphyxiation followed by cervical dislocation. Perform a laparotomy and remove the small intestine by an incision above the cecum and a second incision below the pyloric sphincter.
3. Collect Peyer’s patches from each mouse using curved scissors. Pool tissues in 1 mL cold PBS (in homogenizing tubes) on ice (Figure 4.2).
4. Following collection of Peyer’s patches from all animals, homogenize samples with a BeadMill Homogenizer three times, for 30 seconds at 3 m/s. Incubate tubes on ice between each homogenization.
5. Serially dilute homogenates (to $10^{-2}$) and plate each dilution on LB/Kan/X-gal plates (100 μl/plate). Incubate plates overnight at 37°C.

3.1.4 Count Colonies and Compute Competitive Indices (CIs)

1. *Day 3.* Count blue (O5-mutant) and white (wildtype) colonies the following morning for each mouse, as well as the starting input plate.
2. Compute competitive indices for each mouse using the following formula:

\[
CI = \frac{(%\text{strain } A \text{ (white) recovered} / %\text{strain } B \text{ (blue) recovered})}{(%\text{strain } A \text{ (white) inoculated} / %\text{strain } B \text{ (blue) inoculated})}
\]

3.2 Quantification of Colonization by Fecal Shedding

Before starting, inoculate overnight cultures of each bacterial strain (AR04 and AR05) by mixing one colony into 5 mL of LB broth. Incubate at 37°C at 200 RPM with aeration for ~16 h.
Figure 4.2. Peyer’s patch isolation.

(A) The small intestine is separated from the cecum and the mesentery is removed delicately. A final cut is made at the duodenum just below the stomach. (B) Peyer’s patches are identified along the intestine and are located opposite to the mesenteric vasculature. (C) Peyer’s patches are removed and pooled per mouse, taking precaution to avoid the surrounding tissue during isolation.
3.2.1 Preparation of Bacteria

1. *Day 1.* Subculture overnight cultures of each bacterial strain (AR04 and AR05) 1:50 and incubate at 37°C.
2. Incubate with aeration until OD$_{600}$ of ~0.7 is reached. Adjust the OD$_{600}$ of both strains to 0.7.
3. Mix equal volumes of each strain via pipetting and light vortexing. Centrifuge subculture for 2 min at 6,000 x g.
4. Decant supernatant and wash with sterile PBS by resuspending. Repeat centrifugation, decant, and resuspension for an additional wash (total of 2 washes).
5. Centrifuge, decant, and resuspend in half of the original volume of PBS to concentrate the STm inoculum.

3.2.2 Collection of Fecal Pellets

1. *Day 2.* Place one mouse in a box or chamber (with air holes) and monitor mice. Keep mice in chambers until there are ~3 usable pellets. Return mice to cage after pellets are collected.

3.2.3 Processing of Fecal Pellets

1. Add 2 ceramic homogenizing beads to each 1.5 mL tube containing fecal pellets.
2. Homogenize samples by vortexing briefly. Shake each tube by hand to further homogenize samples.
3. Serially dilute homogenates by cutting the tip off of the pipet tip to obtain the neat dilution. Cut tip as needed for subsequent dilutions. Plate each dilution on LB/Kan/X-gal plates (100 μl/plate). Incubate plates overnight at 37°C (see Note 4.3).

3.2.4 Count Colonies and Compute Competitive Indices (CIs)

1. *Day 3.* Count blue (O5-mutant) and white (wildtype) colonies the following morning for each mouse, as well as the starting input plate.
2. Compute competitive indices for each mouse using the following formula:

\[
CI = \left[ \frac{\% \text{strain A (white) recovered}}{\% \text{strain B (blue) recovered}} \right] \div \left[ \frac{\% \text{strain A (white) inoculated}}{\% \text{strain B (blue) inoculated}} \right]
\]

3. Using this protocol, antibody-mediated effects can be observed on STm shed by infected animals (Figure 4.3).

3.3 Isolation and immunohistochemistry of intestinal samples containing S. Typhimurium

Before starting, inoculate an overnight culture (ATCC14028) by mixing one colony into 5 mL of LB broth. Incubate at 37°C at 200 RPM with aeration for ~16 h.

3.3.1 Preparation of Bacteria and Gavage

1. Day 1. Divide mice into control and antibody treatment groups for the desired timepoints. Here, we used (1) PBS 20 min & (2) 40 min; (3) Sal4 SIgA 20 min & (4) 40 min; (5) Sal4 IgG 20 min & (6) 40 min.

2. Gavage 200 μl of antibody treatment (PBS, Sal4 SIgA, or Sal4 IgG) and then immediately follow with 200 μl of STm inoculum. Set a timer and euthanize mice at 20 min and 40 min post-gavage. This will correspond to ~4 x 10^7 CFUs/mouse.

3.3.2 Collection of Digestive Tract (stomach, small intestine, cecum, large intestine)

1. Euthanize each group after the indicated amount of time via CO₂ inhalation and cervical dislocation.

2. Before dissection, set aside three histological cassettes for each mouse.

3. Mount the mouse on a dissection board and perform a laparotomy. Begin by carefully removing the stomach from the esophagus and continue to remove the small intestine using your hands. Cut when you reach the cecum (see Note 4.4).

4. Cut the stomach away from the small intestine (now isolated from the animal) above the pyloric sphincter. Before placing the lid on the cassette, make 2 cuts with fine scissors in the excised stomach and cecum. This will prevent the organs
Figure 4.3. Anti-LPS monoclonal antibodies (mAbs) significantly reduce wildtype STm in fecal pellets of infected mice 21 h post-infection.

(A) STm cells are shed in the fecal pellets of animals orally infected with $4 \times 10^7$ CFUs of AR05 over time. (B) Oral administration of the anti-LPS mAbs Sal4 IgG and Sal4 SIgA significantly reduced wildtype STm shedding at 21 h post-infection, as demonstrated by a reduction in the competitive index. 50 μg of either control mAb, Sal4 IgG, or Sal4 SIgA was administered in PBS to BALB/c female mice just before oral inoculation of a 1:1 mixture of STm strains AR04 and AR05. Fecal pellets collected at each time point were homogenized and plated for CFUs on LB agar containing kanamycin (50 μg/mL) and X-gal (80 μg/mL) to distinguish between AR04 and AR05 strains. n = 3 separate experiments of at least 3 mice per group.
from rupturing during processing. Place the lid on the cassette and put in formalin.

5. Take the small intestine and arrange it in the cassette in a zig-zag orientation using a metal surgical probe (Figure 4.4). Place lid on cassette and place in buffered formalin for 24 h (see Note 4.4).

6. Excise the cecum from the mouse and place in a fresh cassette. Remove the remaining colon and place in the same cassette. Do not to overload the cassette with tissues, as more than one cassette may be needed.

7. Repeat for each of the remaining mince.

8. Back in the lab, dilute out the bacterial inoculum and plate on LB plates for CFU enumeration (4 quadrants, three 20 μl droplets per dilution). Incubate O/N at 37°C.

9. **Day 2.** Remove input plates from 37°C incubator and count to confirm CFUs.

10. 24 h after samples have fixed in formalin, transfer cassettes to 70% ethanol so that all of the samples are completely submerged for at least an additional 24 h.

11. Send samples to a core facility for paraffin-embedding and cutting. Cut samples at a thickness of 3-4 μm on charged slides.

### 3.3.3 IHC: Deparaffinization and Tissue Rehydration

1. Heat slides in slide holder at 60°C for 20 min (see Note 4.5).

2. Immerse slides in 3 changes of CitriSolv™ for 10 min each time (total time 30 min).

3. Remove from the CitriSolv™ and let the tissue sections air-dry in a chemical hood until the tissue becomes pearly white.

4. Immerse the tissues in 2 changes of 100% ethanol for 3 min each time (total time 6 min).

5. Immerse tissues in 95% ethanol for 3 min.

6. Immerse tissues in 80% ethanol for 3 min

7. Immerse tissues in 70% ethanol for 3 min

8. Rinse tissues in DI water. Leave in DI water until the antigen retrieval process.
Figure 4.4. Preparation of the small intestine for histology.

The small intestine is isolated from the stomach using scissors and is separated from the mesentery by hand to avoid tissue damage, before being cut above the cecum. The tissue is rehydrated with sterile PBS and then oriented in a zig-zag direction across the width of the cassette using a metal surgical probe. Once properly positioned, the lid is placed on the cassette and the sample in transferred to buffered formalin for 24 h.
3.3.4 IHC: Antigen Retrieval (Proteinase K Method)

1. Apply 10 μg/mL proteinase K in PK buffer to tissue sections and incubate for 10 min at room temperature.
2. Rinse in 3 changes of DI water. Leave the slides in the container during the final wash for 5 min.
3. Wash in TBS buffer 3 times.

3.3.5 IHC: Blocking (Rodent Block M and BLOXALL®)

1. Apply Rodent Block M for 30 min. If doing by hand, apply enough solution sufficient to cover all tissues (3 drops; left, right, and middle to ensure solution is spread evenly).
2. Rinse in 2 changes of TBS wash buffer
3. Apply BLOXALL® in a similar manner as Block M and incubate for 10 min.
4. Rinse in 2 changes of TBS wash buffer.

3.3.6 IHC: Primary Antibody Application

1. Apply primary antibody for 60 min at room temperature. Use a large enough volume to fully cover all of the tissues (see Note 4.5). For Rabbit anti-Salmonella, apply at 1:5,000 dilution.
2. Rinse in 2 changes of TBS wash buffer.

3.3.7 IHC: AP-Polymer and Chromogen Application

1. Apply AP-polymer for 30 min (see Note 4.5).
2. Rinse in 2 changes of TBS wash buffer.
3. Rinse slides in Warp Red Buffer for 1 minute. If doing by hand, apply appropriate volume of buffer to each slide and set a timer. When 1 minute has passed, dab slides on a blue pad to remove excess buffer, and then apply Warp Red.
4. Incubate with Warp Red Chromogen for 5 min.
   a. Add 2 drops of Warp Red Chromogen to 5 mL Warp Red Buffer
5. Rinse in DI water.
Figure 4.5. Immunohistochemistry of orally administered STm approaching the Peyer’s patch.

BALB/c female mice were orally challenged with $4 \times 10^7$ CFUs (ATCC14028) in PBS. 40 minutes post-infection, animals were sacrificed, and the small intestines isolated, fixed, paraffin-embedded, and sectioned for immunohistochemistry. STm cells are shown in red. Original magnification: 20X. Abbreviations: (L) lumen, (FAE) follicle-associated epithelium, (SED) subepithelial dome.
3.3.8 IHC: Counterstain
   1. Immerse tissues in Tacha’s Hematoxylin for 3 min.
   2. Wash tissues in running DI water for 1 min.
   3. Immerse in bluing solution for 1 min.
   4. Wash slides in DI water.

3.3.9 IHC: Dehydrate Tissue and Apply Coverslips
   1. Dry slides (in slide rack) at 60°C for 30 min or at room temperature overnight.
   2. Apply 1-2 drops of EcoMount to the tissues and apply coverslips.
   3. Bake slides at 60-70°C for 15 min.
4. DISCUSSION NOTES

4.1. Selection of Bacterial Strains

As mentioned previously, this protocol is designed to examine STm entry into the Peyer’s patch, which is the predominant site of invasion in mice that possess an intact intestinal microbiota (Fabrega & Vila, 2013). Here, we have optimized the model to examine the efficacy of the anti-LPS mAb, Sal4, at preventing STm entry into Peyer’s patch tissues when provided at time of bacterial challenge. Sal4 IgA has been shown to have specific affinity for the O5-polysacharide epitope of STm LPS, as a mutant strain containing a disruption in the acetyl transferase gene, oafA, that generates this epitope does not bind Sal4 (Michetti et al., 1992; Michetti et al., 1994; Slauch et al., 1995). To examine Sal4-dependent activity in this model, we employ a 1:1 mixture of bacterial strains AR05, which expresses the O5-antigen, and AR04, which is unaffected by Sal4, and calculate a competitive index to quantify differences in the Peyer’s patches versus the original input. AR04 also encodes lacZ for constitutive expression of β-galactosidase that allows for discrimination between AR04 and AR05 from tissue homogenates by blue-white screening on LB agar containing X-gal. Each STm strain also contains a kanamycin resistance cassette, aiding in isolation of the challenge inoculum from the mouse microbiota. This optimized model has demonstrated that Sal4 IgA treatment significantly impedes wildtype STm invasion into Peyer’s patches in a dose-dependent fashion when administered at time of initial challenge (Richards et al., 2020).

It is important to note that the competitive index employed in these experiments is specifically suited to examine antibody-mediated protection to Salmonella bacteria expressing the O5 antigen. However, the model can feasibly be adjusted to investigate
immunity to other NTS serovars, as long as the corresponding mutant strain is not
defective in infectivity. Other potential S. enterica serovars of public health interest
include S. Enteritidis (O:1, 9, 12) or S. Montevideo (O:6, 7, 14, [54]), which continue to
be a source of acute diarrheal disease in the United States (Grimont & Weil, 2007).

4.2. Oral Gavage and Peyer’s Patch Model

In the original experiments characterizing in vivo activity by Sal4 IgA, intestinal
Sal4 was shown to protect mouse Peyer’s patches from oral STm infection in a
“backpack tumor model” (Michetti et al., 1992). This was demonstrated experimentally
by injecting Sal4-secreting hybridoma cells subcutaneously into animals and facilitating
their growth into tumors, which resulted in systemic secretion and plgR-mediated
transport (presumably) of Sal4 IgA into the gut. STm challenge was then performed
once detectable levels of Sal4 in the fecal samples of treated animals were reached.
While these experiments demonstrated a proof-of-concept for Sal4 IgA, the cohorts
used in the study were limited and Sal4 secretion from tumors varied between animals.
As such, these early experiments supported the further exploration of Sal4 in an oral
capacity.

In the experimental procedures described in this section, the bacterial input is
resuspended in half the original volume following centrifugation to reduce the volume
required for oral gavage. While 400 μl is often used in oral administration protocols, in
our hands, the animals respond better to smaller dose administrations. To quickly
administer mAbs at time of STm challenge, we perform the gavage using two people.
One animal user administers antibody treatment, while the other administers the bacterial inoculum.

In these studies, we utilize a model of invasive STm to resolve requirements for protection by Sal4 mAbs. This is advantageous in terms of investigating antibody efficacy because there is a defined readout for protection, as determined by CFUs in Peyer’s patches. To obtain an accurate readout of bacterial invasion in these experiments, we determined it was required to use a whole-plate dilution (i.e., 100 μl) as opposed to a smaller volume, as smaller volume dilutions resulted in low CFU counts that skewed competitive indices, adding variability to the assay. This was not surprising, since it is known that bacterial load in the GALT after STm infection is more representative of a bottleneck for further dissemination within the host, which typically progresses after several days (Lim et al., 2014). Therefore, this method of STm quantitation in Peyer’s patches is more of an early indicator of mucosal defense. Additionally, this mode of pathogenesis is likely representative of currently circulating iNTS strains that are of public health concern (Ao et al., 2015). However, Salmonellosis in developed regions primarily results in severe inflammation, diarrhea, and vomiting and does not present as systemic disease (Coburn et al., 2007). Considering this difference in disease pathology, it would be of interest to investigate Sal4 in a model of STm-induced colitis to evaluate if correlates of protection are comparable (Barthel et al., 2003).
4.3. Isolating STm from Fecal Samples

We sought to investigate the effect of oral Sal4 mAbs on STm colonization to determine if additional protective effects could be observed after oral passive immunization in vivo. To quantify colonization of animals over time, STm strains AR04 and AR05 were isolated from fecal samples of inoculated animals and competitive indices were calculated to assess Sal4-dependent effects. Similar methods have been employed to characterize colonization of the gut by *C. jejuni* and ETEC, though without the employment of a competitive index (Perruzza et al., 2020; Virdi et al., 2019). In the protocol described in this report, blue-white screening was performed on LB agar plates containing X-gal to discern changes in STm colonization following antibody treatment. Plating media also contained kanamycin, which was vital for eliminating other bacterial species from fecal samples. Indeed, we observed significant reductions in wildtype AR05 shedding in Sal4 SIgA and IgG treated animals at 21 hours post-infection compared to vehicle control treated animals. However, the total bacterial burden in terms of CFUs remained unchanged (data not shown). These data would suggest that as opposed to substantially reducing STm colonization in the gut, Sal4 mAbs are imparting a fitness deficit on the wildtype population which can be detectable as a skew towards AR04 shedding via the competitive index. This observation was in contrast to previous work demonstrating little activity by Sal4 IgG in the gastrointestinal compartment (Richards et al., 2020). It is possible in these experiments that Sal4 IgG attenuates STm virulence through motility arrest early on in infection, which is enough to reduce STm niche establishment in the lumen, but not enough to prevent entry into Peyer’s patches. Previous work indicates that STm motility accounts for about 0.5 Log_{10}
of CFUs in the total bacterial burden following infection in mice, but that motility arrest is not sufficient enough to fully protect (Moor et al., 2017). Considering these data, it is possible that Sal4 IgG-mediated motility arrest is at play in these experiments.

However, colonization by fecal shedding in this model may not accurately represent the replicating bacterial population within the lumen. With the murine microbiota still intact, STm cells predominantly invade the intestinal mucosa as opposed to residing in the gut (Hapfelmeier & Hardt, 2005). Typical models investigating colonization as a measure of protection require the ablation of commensal bacteria to facilitate an available niche for administered STm (Barthel et al., 2003). Therefore, there are limitations with our conclusions regarding the influence of Sal4 on fecal shedding in this model compared to other models used in the literature. Despite these limitations, we did observe significant changes in wildtype shedding due to Sal4 treatment, suggesting that any residual STm cells in the intestinal lumen that have not invaded can still be influenced by antibody administration.

There are some limitations to employing blue-white screening in a fecal shedding assay. For example, when thoroughly homogenized fecal specimens containing AR04 are plated on 40 μg/mL X-gal, residual host lactase from the gastrointestinal tract in the sample utilizes the X-gal media so that blue-positive colonies are difficult to identify from white AR05 CFUs. To avoid this, fecal samples are vortexed as opposed to homogenized, which reduces the amount of host lactase released in the sample in our hands. Additionally, we employed a higher concentration of X-gal to account for residual enzyme reacting with the substrate. These aspects could be improved upon by generating an alternative reporter system that is not reliant on β-galactosidase activity.
4.4. Isolation of Gastrointestinal Tract for Immunohistochemistry

Isolation of all tissues of the gastrointestinal tract for immunohistochemistry should be performed gently, using gloved hands, with the exception of the necessary cuts used to isolate the organs. This is to avoid tissue damage that can occur from using forceps or other instruments that would be observed during histopathological evaluation. Isolation of the tissue must also be performed quickly to avoid significant autolysis of epithelium and drying out of the specimens; therefore, the tissue must be placed in formalin as soon as possible following euthanasia. Tissue hydration can be maintained by applying sterile PBS throughout the procedure, which will also make it easier to position in the correct orientation within the histology cassette. If timing becomes a problem, then cut each curve of the intestine with scissors once the tissue is placed in a cassette. Doing this will allow for formalin perfusion through each end of the now individual tissue rods.

4.5. Optimization of Staining for STm Tracking Studies

We performed tracking studies of orally administered STm to elucidate gastrointestinal transit time and bacterial localization within the gut after infection. This was to answer two main questions: (1) how are Sal4 mAbs influencing STm in the intestinal lumen during infection and (2) what is the window of protection to STm infection in mice? Original experiments examining the prophylactic potential of Sal4 IgA demonstrated that efficacy was dramatically reduced after the time between challenge and antibody dosing was extended to 20 minutes (Richards et al., 2020). As such, we were interested in establishing a timeline of STm passage through the intestine. Our
data indicated that STm transit through the gut was fast, with STm cells detected at the terminal ileum of infected animals after 20 minutes (Figure 4.5). Cells were detected along the FAE overlying mouse Peyer’s patches in these sections, a step preceding invasion via T3SS-mediated entry into M cells (Jones et al., 1994). These data were congruent with pilot experiments in our lab, as well as published reports, highlighting that invasion into GALT at the distal intestine occurs quickly and is the first step in disease progression of murine typhoid (Carter & Collins, 1974).

We successfully developed methods to detect STm cells by immunohistochemistry 20, 40, and 60 minutes after initial infection. An important note to make during the procedure is to use an oven that produces uniform heat, as opposed to a hot plate, during the deparaffinization and tissue rehydration process, as the latter will create significant background. After rehydration of tissue samples for immunohistochemistry, make sure to always keep the slides wet to prevent the tissue from drying out. Liquid volumes required for proteinase K treatment, primary antibody incubation, AP-polymer, and chromogen application are not provided in this protocol. This is because the amount of tissue per slide can vary and the use of an autostainer can add further variability to the liquid volume required per slide. Therefore, we recommend that a liquid volume be used in these steps that is sufficient to fully cover all tissues during the allotted incubation time to prevent the samples from drying out.

Indeed, we observed that oral STm reached the end of the gastrointestinal tract by 60 minutes using these assays. Considering that our previous data suggested that a local concentration of Sal4 is required for protection of Peyer’s patches in vivo, it is likely that dilution via gastrointestinal transit accounts for a lack of efficacy prophylactically in
this model. Transit time and dilution effects may be issues for protection to STm specifically, as other reports indicate that a single bolus of oral monoclonal SIgA is sufficient to reduce *C. jejuni*-induced diarrhea when administered 3 hours in advance (Perruzza et al., 2020). However, other groups have demonstrated feasible protection to oral STm using plasma-derived antibodies in milligram quantities over multiple doses (Corthésy et al., 2018). These studies suggest that oversaturation of the gut with therapeutic mAbs may be able to overcome issues with gastrointestinal pharmacokinetics. Taken together, the methods and procedures described in this report demonstrate an array of techniques that can be employed to further define correlates of protection by oral antibodies to invasive STm. While these assays have been optimized to investigate *S. enterica* pathogenesis specifically, they can easily be employed to examine immunity to other enteric pathogens.
CHAPTER 5: RECOMBINANT HUMAN SECRETORY IGA INDUCES SALMONELLA TYPHIMURIUM AGGLUTINATION AND LIMITS BACTERIAL INVASION INTO GUT-ASSOCIATED LYMPHOID TISSUES

The work in this chapter has been accepted for publication in the following form:


Author Contributions: AFR and NJM conceived and design the experiments. AFR, DEB, JED, and FJTV performed the experiments. MSP, JS, GGW, FB, and DC contributed reagents and materials. AFR, DEB, FJTV, and NJM analyzed the data. AR and NJM wrote and submitted the manuscript. Administration, supervision and funding acquisition were performed by NJM.
ABSTRACT

As the predominant antibody type in mucosal secretions, human colostrum, and breast milk, secretory IgA (SIgA) plays a central role in safeguarding the intestinal epithelium of newborns from invasive enteric pathogens like the Gram-negative bacterium, Salmonella enterica serovar Typhimurium (STm). SIgA is a complex molecule, consisting of an assemblage of two or more IgA monomers, J-chain, and secretory component (SC), whose exact functions in neutralizing pathogens are only beginning to be elucidated. In this study, we produced and characterized a recombinant human SIgA variant of Sal4, a well-characterized monoclonal antibody (mAb) specific for the O5-antigen of STm lipopolysaccharide (LPS). We demonstrate by flow cytometry, light microscopy, and fluorescence microscopy that Sal4 SIgA promotes the formation of large, densely packed bacterial aggregates in vitro. In a mouse model, passive oral administration of Sal4 SIgA was sufficient to entrap STm within the intestinal lumen and reduce bacterial invasion into gut-associated lymphoid tissues by several orders of magnitude. Bacterial aggregates induced by Sal4 SIgA treatment in the intestinal lumen were recalcitrant to immunohistochemical staining, suggesting the bacteria were encased in a protective capsule. Indeed, a crystal violet staining assay demonstrated that STm secretes an extracellular matrix enriched in cellulose following even short exposures to Sal4 SIgA. Collectively, these results demonstrate that recombinant human SIgA recapitulates key biological activities associated with mucosal immunity and raises the prospect of oral passive immunization to combat enteric diseases.
INTRODUCTION

Globally, diarrheal diseases remain a leading cause of morbidity and mortality among children (Troeger et al., 2017). The Global Burden of Diseases (GBD) investigative group determined that in 2019, for example, gastrointestinal infections resulting in prolonged intestinal inflammation and malnutrition were the third leading cause of disability-adjusted-life years (DALY) lost by children under the age of ten (Vos et al., 2020). The majority of these cases are concentrated within regions of high socioeconomic disparity, such as Sub-Saharan Africa and Southeast Asia (Liu et al., 2012). A number of enteric pathogens have been attributed to high diarrheal incidence in these areas, including enterotoxigenic Escherichia coli (ETEC), Shigella sp., Campylobacter jejuni, and Vibrio cholerae (Lanata et al., 2013). Salmonella enterica is also on the list of pathogen-specific sources of severe diarrhea, accounting for 95.1 million cases worldwide in 2017 (Stanaway et al., 2019). While nontyphoidal Salmonella serovars typically cause self-limiting gastroenteritis, there is an emergence of invasive nontyphoidal strains (iNTS) that cause severe systemic infection (Gilchrist & MacLennan, 2019). iNTS are associated with antibiotic resistance and increased mortality rates, often disproportionately impacting vulnerable populations of HIV-infected adults and young children (Gilchrist & MacLennan, 2019; Oneko et al., 2015; Rondini et al., 2013).

Secretory IgA (SIgA) is the predominant immunoglobulin on mucosal surfaces and serves as a formidable barrier against bacterial and viral pathogens. SIgA is also the primary antibody found in human colostrum and breast milk (Brandtzaeg, 2010; Trégoat et al., 2001). At its core, SIgA consists of two IgA monomers (mIgA) covalently
attached at their C-termini by joining (J) chain (15 kDa) (Brandtzaeg, 1974; Kumar Bharathkar et al., 2020; Woof & Russell, 2011). Humans have two IgA isotypes, IgA1 and IgA2, that differ structurally in their hinge regions and degrees of O-glycosylation (de Sousa-Pereira & Woof, 2019). Dimeric (dIgA) and some higher molecular weight polymers (pIgA) are produced by plasma cells in the intestinal lamina propria. Dimeric IgA is selectively transported across the intestinal epithelium in a basolateral-to-apical direction by the polymeric immunoglobulin (plgR) receptor (Brandtzaeg, 2013). Following transcytosis, the ectodomain of plgR is proteolytically cleaved and remains associated with the Fc regions of dIgA, generating a complex known as SlgA (Kumar et al., 2020; Woof & Russell, 2011). The cleaved ectodomain of plgR is referred to as secretory component (SC) (Brandtzaeg, 1978). The plgR is also expressed in mammary epithelial tissues and is responsible for delivery of IgA into colostrum and breast milk.

Once in mucosal secretions and breast milk, SlgA is proposed to protect the intestinal epithelium through a process known as “immune exclusion” in which SlgA promotes antigen and pathogen crosslinking, entrapment in the intestinal lumen, and eventual clearance from the gastrointestinal tract through peristalsis (Brandtzaeg, 2013; Corthésy, 2013; Stokes et al., 1975). By restricting access to the intestinal epithelium, SlgA effectively prevents pathogens like *E. coli*, *C. jejuni*, and iNTS from colonizing and invading the gut mucosa. SlgA is uniquely suited to perform this function, as the molecule is surrounded by a “glycan shield” (Royle et al., 2003). SC alone has seven N-linked glycosylation sites (Norderhaug et al., 1999; Stadtmueller et al., 2016). The substantial glycosylation renders SlgA relatively stable in the acidic and proteolytic conditions of the gut, compared to other antibody isotypes like IgG (Crottet & Corthésy,
The extensive carbohydrate side chains also anchor SIgA in intestinal mucus on epithelial surfaces (Gibbins et al., 2015; Phalipon et al., 2002).

These unique attributes have garnered interest in SIgA as an alternative or supplement to antibiotic-based therapeutics for enteric diseases. Recent advancements in the generation of recombinant antibodies via multi-vector mammalian expression systems has enabled the expression of SIgA monoclonal antibodies (mAbs) (Perruzza et al., 2020; Stoppato et al., 2020; Virdi et al., 2019). While there are examples in which passively administered mouse IgA or SIgA have been shown to afford immunity against experimental shigellosis and cholera, the use of human SIgA is only beginning to be explored (D. E. Baranova et al., 2020; Corthésy et al., 2018; Mathias et al., 2013; Michetti et al., 1992). For example, we recently showed in a mouse model of invasive Salmonella infection that Sal4 IgA, an anti-lipopolysaccharide (LPS) mouse mAb, was sufficient at reducing invasion of Salmonella enterica serovar Typhimurium (STm) into Peyer’s patches tissues (Richards et al., 2020). Sal4 SIgA was superior to Sal4 IgG (Richards et al., 2020). Our results demonstrated that while both Sal4 isotypes were functional in vitro, only Sal4 mouse IgA was able to prevent STm from entry and dissemination in the mouse gut.

In this report we sought to examine whether human SIgA version of Sal4 is also effective at limiting STm infection in the mouse model. We were prompted by recent studies from other groups showing that human SIgA mAbs specific for a bacterial adhesin and flagellar protein subunits prevent intestinal colonization from ETEC and C. jejuni, respectively (Giuntini et al., 2018; Stoppato et al., 2020). We report that human
recombinant Sal4 SIgA is a potent inducer of STm agglutination in vitro and in vivo and that this activity likely contributes to bacterial entrapment in intestinal lumen and limits invasion into gut-associated lymphoid tissues.

RESULTS

Recombinant human Sal4 SIgA induces STm agglutination

As a first step in investigating the potential of recombinant human SIgA to protect against STm infection, we generated a chimeric form of Sal4 IgA in which the mouse VH region was grafted onto a human IgA2 allotype m(2) backbone, with the VL element onto a human kappa light chain. Transient co-transfection of Expi293 cells with heavy and light chain constructs gave rise to monomeric IgA, as measured by size-exclusion chromatography (SEC) (Figure A2.1). Triple co-transfection with a plasmid encoding human J-chain resulted in the formation of IgA products that by SEC were consistent with dimer formation, while quadruple transfection with the addition of a vector encoding human SC resulted in the appearance of a product with a molecular weight of >280 kDa, consistent with the formation of SIgA. All three Sal4 IgA variants (mIgA, dIgA, SIgA) were affinity-purified, as described (Perruzza et al., 2020). All forms of IgA bound to STm LPS by ELISA, as detected with goat anti-human IgA secondary antibodies (Figure 5.1). Sal4 SIgA was specific for the O5-antigen, as demonstrated by ELISA (Figure A2.2).
Figure 5.1. Sal4 mAbs bind STm lipopolysaccharide (LPS).
Sal4 (A) IgG, (B) mlgA, (C) dIgA, and (D) SlgA binding to purified STm LPS as measured by ELISA. Filled circles represent Sal4 mAb reactivity, while empty circles represent isotype control antibody. ELISA graphs depict two technical replicates and are representative of two biological replicates.
To further assess antibody functionality, Sal4 SIgA was examined by flow cytometry for the ability to induce bacterial agglutination (Mitsi et al., 2017; Moor et al., 2017; Moor et al., 2016). Mid-log phase cultures of STm were treated with increasing amounts of Sal4 SIgA for 1 h at 37°C, and then analyzed by forward scatter (FSC) and side scatter (SSC) to quantify the size and frequency of STm-antibody complexes. We defined agglutination as the percentage of total events located in quadrants 2 (Q2) and 4 (Q4). AR05 cells treated with isotype control antibodies (or saline) had a maximal agglutination index of <1% (Table 5.1). Treatment of cells with Sal4 SIgA resulted in a dose-dependent increase in bacterial FSC and SSC that achieved an agglutination index of >65% in the presence of 200 µg/mL of antibody (Table 5.1; Figure 5.2). The maximal agglutination index achieved with Sal4 IgG (200 µg/mL) was just ~33%, or roughly half of that observed for SIgA. It is notable that both Sal4 mIgA and dIgA variants were as effective as SIgA in promoting bacterial agglutination, as measured by flow cytometry (Table 5.1; Figure A2.3).

We next used fluorescence microscopy to capture STm agglutination in real time. We used a strain of STm AR05 expressing mCherry under the control of an arabinose-inducible promoter. STm cells were grown to mid-log phase in the presence of 0.2% arabinose, treated with Sal4 IgA and then spotted on microscope slides. Four to seven images were taken at 20X magnification and mean fluorescence intensity (MFI) of STm-antibody complexes was measured over time. Bacteria treated with an isotype control antibody remained motile and uniformly dispersed within the liquid medium. There was no evidence of aggregation at any time point among control groups (Figure 5.3). When
Table 5.1. Agglutination of STm cells by Sal4 mAbs by flow cytometry

<table>
<thead>
<tr>
<th>Sal4 (μg/mL)(^a)</th>
<th>2</th>
<th>20</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>5.82% ± 4.50</td>
<td>19.19% ± 11.18</td>
<td>33.51% ± 14.81</td>
</tr>
<tr>
<td>mlgA</td>
<td>11.05% ± 3.39</td>
<td>31.46% ± 4.99</td>
<td>79.04% ± 2.46</td>
</tr>
<tr>
<td>dlgA</td>
<td>12.41% ± 5.20</td>
<td>38.85% ± 10.01</td>
<td>91.12% ± 3.07</td>
</tr>
<tr>
<td>SIgA</td>
<td>10.30% ± 2.78</td>
<td>23.39% ± 7.85</td>
<td>66.38% ± 4.98</td>
</tr>
</tbody>
</table>

\(^a\)Mid-log phase AR05 cultures were washed in PBS and incubated with indicated amounts of Sal4 mAbs for 1 h at 37°C. 10,000 events per sample were analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA) by forward scatter (FSC) and side scatter (SSC) to visualize aggregate size and granularity.  
\(^b\)Gating was set on untreated AR05 cells and agglutination was defined by SSC-positive FSC-positive cells (Q2 + Q4). STm cells treated with isotype control antibodies resulted in Q2 + Q4 values > 1%.  
\(^c\)Results represent data from three separate biological experiments.
Figure 5.2. Sal4 mAbs agglutinate live STm cells by flow cytometry.

Mid-log phase cultures of AR05 were washed in PBS and incubated with 2, 20, or 200 μg/mL Sal4 SIgA or IgG or isotype control antibodies for 1 h at 37°C. 10,000 events per sample were analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA) by forward scatter (FSC) and side scatter (SSC) to visualize aggregate size and granularity. Gating was set on untreated AR05 cells and agglutination was defined by SSC-positive FSC-positive cells (Q2 + Q4), similarly as described (Mitsi et al., 2017). (A) Diagram demonstrating antibody-to-bacterium ratio for each of the concentrations.
examined. (B & C) Representative flow cytometry plots showing FSC and SSC for (B) Sal4 SIgA, (C) Sal4 IgG and isotype control groups. STm cells treated with isotype control antibodies resulted in Q2 + Q4 values > 1%. Results represent data from three separate biological experiments.
Figure 5.3. Sal4 SIgA significantly agglutinates mCherry-expressing STm cells.

Mid-log phase AR05-mCherry cells were induced in 0.2% arabinose and treated with 15 μg/mL (A) control IgA, (B) Sal4 SIgA, (C) control IgG, or (D) Sal4 IgG at room temperature. Cells were spotted on to uncharged microscope slides at 10 min intervals
for 30 min. 4 to 7 images were taken at 20X for each condition and time point in the TexasRed channel. Images were analyzed for Mean fluorescence intensity (MFI) per aggregate using Fiji, as described in the Materials & Methods. Arrows indicate primarily single cells present in control groups compared to bacterial aggregates in Sal4 treatment groups. (E) Quantification of mean MFI for Sal4 SIgA (red) and Sal4 IgG (blue) treatment groups. Isotype control values for each antibody are shown as shaded bars. Data represents three biological replicate experiments. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc multiple comparisons test. Asterisk (*) on bar indicates p < 0.05 compared to isotype control. *P < 0.05 between treatment groups.
treated with Sal4 IgG, STm formed granular aggregates within 10 min (Figure 5.3). The MFI of STm-Sal4 IgG aggregates at 10 min was ~3,400 and by 30 min reached ~5,000 (Figure 5.3). Sal4 SIgA was even more efficient at inducing bacterial aggregation, as evidenced by an MFI of ~7,000 at 10 min and ~11,800 by 30 min (Figure 5.3). The higher MFI values associated with Sal4 SIgA treatment were the result of the formation of both larger and more dense aggregates than observed following Sal4 IgG.

Sal4 SIgA inhibits invasion of Peyer’s patch tissues by STm in a mouse model

We next evaluated Sal4 SIgA for the ability to block STm invasion into Peyer’s patch tissues, which represent the primary portal of entry for STm the intestinal mucosa (Carter & Collins, 1974; Santos et al., 2001). As shown in Figure 5.4, BALB/c mice were gavaged with a 1-to-1 mixture (4 x 10⁷ CFUs per mouse) of kanamycin-resistant STm strains AR05 and AR04. AR04 is a derivative of AR05 that constitutively expresses β-galactosidase and lacks the O5 epitope due to a transposon insertion within oafA (Slauch et al., 1995). As such, AR04 does not react with Sal4 and serves as an internal control (Richards et al., 2020). Mice were euthanized ~24 h later and Peyer’s patch tissues were removed and homogenized. The homogenates were plated onto LB agar containing kanamycin and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to differentiate AR04 (LacZ⁺) from AR05 (LacZ⁻) by blue-white screening. The ratio of AR05 to AR04 in the challenge dose (input) was compared to the ratio of AR05 to AR04 in Peyer’s patch lysates (output) to yield a competitive index (CI).
Figure 5.4. Sal4 SIgA blocks STm entry into Peyer’s patches.

(A) Schematic of STm infection model. BALB/c female mice are orally administered Sal4 or isotype control antibody in PBS immediately prior to a 1:1 mixture of AR04 and AR05 STm strains (~4 x 10^7 CFUs). 24 h later mice are sacrificed, and a laparotomy is performed to isolate Peyer’s patches from the small intestine. Peyer’s patches from each mouse are pooled in 1 mL ice-cold sterile PBS and homogenized and plated for CFUs on LB agar containing kanamycin and X-gal. (B-D) STm invasion into Peyer’s patches of mice treated with (B) 50 μg of Sal4 mIgA, dIgA, or SIgA, (C) 10 μg of Sal4 mIgA or dIgA, or (D) 50 μg of IgG at time of STm challenge. Shown are the combined results of two separate experiments with at least 4 mice per group. Statistical
significance was assessed by one-way ANOVA followed by Tukey’s post hoc multiple comparisons test.
In the mouse model, Sal4 SIgA reduced AR05 invasion of Peyer’s patch tissues by several orders of magnitude, as demonstrated by a CI value of 0.09 +/- 0.07. The same concentration of Sal4 mIgA and dIgA variants also significantly inhibited AR05 infection, as evidenced by CI values of 0.34 and 0.04 respectively (Figure 5.4). At lower antibody doses, dIgA was slightly more effective than the mIgA, revealing the contribution of antibody valency in limiting bacterial entry into Peyer’s patch tissues (Figure 5.4). Sal4 IgG, by contrast, only marginally influenced STm AR05 infection, as evidenced by a CI of 0.7 +/- 0.164 (Figure 5.4). This result agrees with a previous study in which even >250 ug of Sal4 IgG has no significant impact on STm entry into Peyer’s patches (Richards et al., 2020).

To examine whether Sal4 SlgA can function prophylactically, Sal4 SlgA (50 μg per animal) was administered to mice by gavage at 40, 20 or 1 min prior to STm challenge. When administered immediately prior to bacterial challenge, Sal4 SlgA significantly reduced AR05 entry into Peyer’s patch tissues (Figure A2.4). However, administration of Sal4 SlgA at 40 min or 20 min prior to bacterial challenge did not have any appreciable effect on AR05 infection. Co-administration of SlgA with sodium bicarbonate (to buffer gastric pH) and protease inhibitors (to neutralize gastric and intestinal proteases) did not significantly improve Sal4 SlgA prophylactic activity (Figure A2.4), suggesting that antibody degradation was not a limiting factor. Collectively, we conclude from these studies that recombinant human Sal4 SlgA, Sal4 dIgA, and to some degree mIgA inhibit the earliest steps in STm infection of gut-associated lymphoid tissues, possibly due to bacterial agglutination and entrapment in the intestinal lumen.
Recombinant human Sal4 SlgA promotes STm agglutination in the intestinal lumen

Several lines of evidence suggest that Sal4 SlgA-mediated agglutination of STm is qualitatively and quantitatively different than agglutination induced by Sal4 IgG. For example, by flow cytometry, Sal4 SlgA induced an agglutination index that was approximately twice that of Sal4 IgG (Table 5.1). In macro-agglutination assays performed in microtiter wells, Sal4 SlgA (but not Sal4 IgG) promoted the formation of bacterial “mats” that were impervious to vigorous pipetting (Richards et al., 2020). Finally, scanning electron microscopy (SEM) analysis of bacterial aggregates induced by mouse Sal4 IgA hybridoma supernatants revealed gross alterations in cell morphology, especially at points of cell-cell contact (Mantis & Forbes, 2010).

To visualize the nature of the bacterial aggregates induced by recombinant human Sal4 SlgA, mid-log phase STm cultures were treated with 50 µg of Sal4 IgG or SlgA, collected by centrifugation, and then entrapped in bovine thrombin-plasma clots, essentially as described (Balassanian et al., 2016). The clots were embedded in paraffin, sectioned, and counterstained with H&E to further distinguish STm-antibody complexes. Saline-treated STm cells were uniformly dispersed within the fields of view across multiple sections of the clots (Figure 5.5). Sal4 IgG-treated STm cells were clustered in small, loosely packed aggregates that had a lacy appearance (Figure 5.5). In contrast, Sal4 SlgA-treated cells formed large masses that encompassed entire fields of view under low magnification (Figure 5.5). Closer inspection indicated that the cells were densely packed to the point where individual bacteria were not readily discernable.

To determine whether similar aggregates occur in vivo, we gavaged mice with Sal4 SlgA, Sal4 IgG, or vehicle alone (PBS) followed immediately by a bolus of STm
Figure 5.5. H&E staining of STm agglutination by Sal4 SlgA and IgG.

Mid-log phase ATCC14028 STm cells were treated with (A & B) PBS or 50 μg (C & D) Sal4 IgG or (E & F) Sal4 SlgA, pelleted, and immobilized in bovine-thrombin plasma clots, as described in the Materials & Methods. Clots were fixed and prepared for paraffin-embedding and sectioning. Samples were counterstained with H&E to visualize STm-antibody aggregates. (A & B) STm cells in PBS were uniformly dispersed as single cells. (C & D) Sal4 IgG generated numerous clusters of STm bacteria primarily arranged
in lacy and irregular patterns that appeared loosely packed. (E & F) Sal4 SIgA treatment induced large sheets of tightly packed bacteria that spanned the field of view on the microscope. Single-cells were rarely observed in SIgA-treated cells compared to PBS and IgG groups. Scale bars depict 10 μm (low magnification) and 5 μm (high magnification), as indicated.
(~4 x 10^7 CFUs). The mice were euthanized 20 or 40 min later and the lengths of the GI tract from the pyloric sphincter to rectum were excised, fixed in paraformaldehyde, embedded in paraffin blocks, and sectioned for immunohistochemistry (IHC), as described in the Materials and Methods. Tissue sections were deparaffinized, rehydrated and subjected to antigen retrieval by Proteinase K incubation. To visualize STm \textit{in situ}, paraffin sections were stained with rabbit \textit{Salmonella} Group B-specific antisera followed by AP-polymer and chromogen application.

STm was localized to the jejunum and upper portion of the ileum in tissues collected at 20 min (data not shown). After 40 min, STm was detected in the distal ileum and cecum (Figure 5.6). Bacteria were interspersed in the intestinal lumen, with occasional cells sequestered in the mucus layer. Most of the inoculum, however, was present as single cells or in small clusters (Figure 5.6). The pattern was similar in mice treated Sal4 IgG. STm cells were uniformly distributed in the lumen and occasionally found in small, loosely packed clusters in the terminal ileum and cecum (Figure 5.6). In contrast, in Sal4 SlgA-treated mice, STm appeared in large, dense clusters in the ileum and cecum (Figure 5.6). The STm aggregates were refractory to IHC with anti-LPS polyclonal antibody, as evidenced by Vina Green Chromogen™ staining around periphery but not in the center of the cell clusters (Figure 5.6; Figure A2.5). Even aggressive antigen retrieval methods such as heat-induced epitope retrieval with citrate buffer followed by proteinase K treatment did not render these clusters accessible to antibody staining (data not shown). These results are consistent with Sal4 SlgA promoting cell-cell crosslinking \textit{in vitro} and \textit{in vivo} that likely renders STm incapable of access Peyer's patch tissues.
Figure 5.6. Sal4 SlgA agglutinates STm cells in the intestines of infected mice at 40 min. BALB/c female mice were orally administered (A & B) PBS or 50 μg (C & D) Sal4 IgG or (E & F) Sal4 SlgA prior to ~4 x 10^7 CFUs of STm wildtype strain ATCC14028. Animals
were euthanized 40 min post-infection and the mouse gastrointestinal tracts were isolated, fixed, and prepared for immunohistochemistry. Tissue samples were subjected to antigen retrieval via proteinase K incubation and STm cells were stained using rabbit *Salmonella* Group B-specific antiserum (BD Difco) and AP-polymer and chromogen application. Positively stained STm cells are depicted in green and were predominately stained in tissue samples from PBS and Sal4 IgG-treated animals (A-D). STm cells in Sal4 SlgA-treated animals were found in large aggregates in the intestinal lumen, with only a portion of cells positively staining by IHC (E & F). Positive cells in Sal4 SlgA-treated animals were primarily at the periphery of STm aggregates. Scale bars depict 50 μm (low magnification) and 10 μm (high magnification), as indicated.
**STm extracellular matrix (ECM) production following Sal4 SlgA treatment**

The recalcitrant nature of the STm aggregates induced by Sal4 SlgA are reminiscent of extracellular matrix (ECM)-encased bacterial mats induced during in the early stages of bacterial biofilm formation (MacKenzie et al., 2017; Mantis et al., 2011; Zogaj et al., 2001). Indeed, we have reported that mouse Sal4 IgA stimulates STm to secrete an ECM containing (but not limited to) cellulose, colonic acid and O-antigen (Amarasinghe et al., 2013). To examine whether human Sal4 SlgA triggers STm to secrete ECM, mid-log phase cultures of AR05 grown in borosilicate glass tubes with aeration were treated with Sal4 SlgA (50 μg/mL) or an isotype control for 1 h at 37°C, and then subjected to crystal violet (CV) staining. As compared to controls, Sal4 SlgA-treated cells secreted copious amounts of ECM, particularly at the air-liquid interface (Figure 5.7). We employed available STm strains with mutations in cellulose biosynthesis (ΔbcsA, ΔbcsE) (Ahmad et al., 2011; Fang et al., 2014), colonic acid production (ΔwcaA) and O-antigen capsule formation (ΔyihO) (Gibson et al., 2006) to dissect which (if any) of these components contributed to CV staining observed following Sal4 SlgA. We also examined the role of CsgD, a known transcriptional regulator of Salmonella biofilm formation, in ECM expression in response to Sal4 SlgA (Liu et al., 2014). While the STm ΔwcaA, ΔyihO, and ΔcsgD mutants produced CV levels similar to the wild type strain (Figure 5.7), the STm ΔbcsA and ΔbcsE mutants each secreted less ECM than the control (Figure 5.7). The phenotype was more pronounced with ΔbcsE mutant than the ΔbcsA mutant, which is interesting considering that BcsE binds c-di-GMP and is required for optimal cellulose production (Fang et al., 2014). In preliminary studies, we found that treatment of STm by Sal4 IgG also
Figure 5.7. CV staining of biofilm mutants in response to Sal4 SIgA.

STm wildtype strain AR05 and biofilm mutant strains ΔbcsA and ΔbcsE (cellulose), ΔwcaA (colanic acid), ΔyihO (O-Antigen capsule), and ΔcsgD (biofilm regulator) were grown to mid-log phase and incubated in borosilicate glass tubes with 50 μg/mL of Sal4 SIgA or control SIgA for 1 h at 37°C under shaking conditions. Tubes were washed in PBS, fixed with methanol, and stained with 0.1% crystal violet (CV). (A) Representative images
depict CV staining following antibody treatment. (B) Solubilized CV was quantified at $A_{550}$. Data represents three biological replicates each performed with three technical replicates. Statistical significance was determined by two-way ANOVA followed by Tukey’s post hoc multiple comparison test. *$P < 0.05$. 


stimulated CV activity. However, the ECM profiles between Sal4 SIgA and Sal4 IgG were different in that SIgA triggered cellulose-dependent ECM, while IgG induced cellulose-independent ECM (data not shown).

Our results suggest that Sal4 SIgA promotes the formation of STm aggregates encased in cellulose and possibly other ECM components, thereby rendering the bacteria entrapped in the intestinal lumen and unable to penetrate Peyer’s patch tissues. If cellulose was limiting bacterial entry into Peyer’s patch tissues, then we predicted that the \textit{bscA} or \textit{bcsE} mutant would be more invasive than wild type strain in Peyer’s patch tissues in the presence of Sal4 SIgA. To test this hypothesis, the \textit{bscA} and \textit{bcsE} mutants were tested for Peyer’s patch invasion in the mouse model in the absence and presence of Sal4 SIgA. We found that Sal4 inhibited the invasion of the STm \textit{bscA} or \textit{bcsE} mutants to a similar degree as the wild type strain, indicating that the cellulose mutants did not “escape” the effects of Sal4 SIgA (data not shown). We speculate that cellulose secretion may play a role in other aspects of STm pathogenesis aside from invasion, such as survivability within the gut or host transmission.

**DISCUSSION**

In this study, we produced and characterized a recombinant human SIgA form of the monoclonal antibody, Sal4. Sal4 was originally isolated from a B cell hybridoma generated from mice that had been orally immunized with attenuated strains of STm. Sal4 IgA is specific for the immunodominant O5-antigen of STm LPS and is capable of preventing invasion of polarized epithelial monolayers by virulent STm. In mice, delivery of Sal4 IgA via the “backpack tumor” model or by gavage are each sufficient to limit
bacterial uptake into Peyer’s patch tissues (Michetti et al., 1992; Richards et al., 2020). It is also reported that Sal4 IgA treatment inhibits STm motility and abrogates the STm SPI-1 type III secretion system (T3SS), each of which contribute to bacterial entry into epithelial cells (Forbes et al., 2008). The motivation behind our current endeavor was to investigate whether a recombinant human SlgA form of Sal4 retains biological activity and is able to block bacterial entry into gut associated-lymphoid tissues when administered passively.

Historically, recombinant SlgA has been difficult to produce because of the complex nature of the molecule (Virdi et al., 2016). Corthésy and colleagues were one of the few teams who successfully employed SEC to purify mlgA, dlgA and plgA from B cell hybridoma supernatants (Favre et al., 2003). They were able to reconstitute SlgA in vitro by complexing dlgA and plgA with recombinant SC (Phalipon et al., 2002). Other groups have expressed recombinant SlgA in transgenic plants (Ma et al., 1998), but only recently have mammalian cell-based strategies proven fruitful (Giuntini et al., 2018; Perruzza et al., 2020; Stoppato et al., 2020). Moreover, the structure of SlgA has been revealed through X-ray and cryo-EM techniques (Kumar Bharathkar et al., 2020; Kumar et al., 2020; Stadtluex et al., 2016). By all accounts, the recombinant human Sal4 SlgA produced in our study has potent biological activity in vitro and in vivo.

Our results are consistent with immune exclusion as being a primary mechanism by which recombinant human SlgA Sal4 limits STm uptake into mouse Peyer’s patch tissues. In our studies, large and densely packed aggregates of STm were evident in the intestinal lumen of mice that had been pre-treated with Sal4 SlgA. By contrast, there was no such evidence for STm aggregation in mice treated with Sal4 IgG. Rather, in
those mice, STm was generally observed as individual cells throughout the lumen and dispersed within the mucosa. These observations are essentially in accordance with SIgA (but not IgG) being the primary mediator of immune exclusion (Stokes et al., 1975). However, we acknowledge that there is certainly more to the story. As noted above, we have reported that Sal4 IgA inhibits STm’s flagella-based motility by a mechanism that may involve membrane depolarization and/or dinucleotide signaling cascades (Amarasinghe et al., 2013; Forbes et al., 2008; Forbes et al., 2012; Richards et al., 2020). Moor and colleagues reported in a mouse model that high avidity polyclonal IgA elicited by mucosal vaccination of STm promotes enchained bacterial growth within the gut (Moor et al., 2017). Enchained growth is proposed to primarily act in low-density bacterial environments where agglutination with neighboring cells is infrequent.

Nonetheless, one caveat worth noting is that SIgA given via gavage is unlikely to assume the normal distribution pattern of SIgA that is transported into the gut via pIgR. In effect, pIgR-mediated delivery of SIgA into the gut results in an “inside-to-outside” diffusion pattern, whereas oral delivery would be subjected to the opposite (“outside-to-inside”). Moreover, orally delivered SIgA is subject to different transit times through the gastrointestinal tract and is likely diluted significantly within minutes after being deposited into the stomach (Woting & Blaut, 2018). Collectively these factors may account for why Sal4 SIgA pretreatment at -20 and -40 min was largely ineffective at blocking STm infectivity in our model.

Based on the results of our current study, we postulate that STm, upon exposure to Sal4 SIgA becomes encased in a bacterium-derived ECM that is reminiscent of early
biofilm formation. As described by Gunn and colleagues, bacteria like STm produce complex extracellular matrices consisting of a myriad of exopolysaccharides, extracellular DNA, and proteins (e.g., amyloids, flagella) (Gunn et al., 2016). In the case of STm, the major ECM components include cellulose, colanic acid, and O-antigen capsule. Using STm mutants deficient in the synthesis of these substances, we identified cellulose as the likely candidate contributing to ECM production in response to Sal4 SIgA. This is consistent with previous findings from our laboratory (Amarasinghe et al., 2013). We can only speculate that the secretion of ECM by STm in response to Sal4 SIgA is a defensive mechanism by which the bacteria render themselves recalcitrant to further attack by other components of the mucosal immune system (Gunn et al., 2016). We are currently investigating whether STm “senses” an antibody attack through a signaling pathway involving a cyclic di-guanulate monophosphate (c-di-GMP), known to regulate motility and cellulose production (Ahmad et al., 2011; Amarasinghe et al., 2013; Forbes et al., 2012; Le Guyon et al., 2015; Mantis et al., 2011).

Ultimately, our study highlights the opportunities and formidable challenges associated with passive oral immunization with recombinant human SIgA. In the literature, orally delivered antibodies have been demonstrated to protect against diarrheal pathogens, such as ETEC (Freedman et al., 1998). Similar prophylactic potential has been reported by pooled plasma-derived antibodies in alleviating intestinal inflammation following mucosal STm infection in mice (Bioley et al., 2017). However, these studies, as well ours, underscore that adequate dosing is a major barrier to the use of SIgA prophylactically. As noted to earlier, we speculate that protection against STm invasion of Peyer’s patch tissues is only achieved when Sal4 SIgA levels exceed a
critical threshold concentration required to promote bacterial agglutination. Indeed, in a controlled human infection model, multiple doses of hyperimmune bovine colostrum (HBC) before and after oral ETEC challenge were necessary to prevent diarrhea (Savarino et al., 2019). In mice, preincubation of the ETEC with SIgA mAbs was required to reduce bacterial colonization (Giuntini et al., 2018). Similar issues with achieving sufficient local antibody concentrations in the gut apply in the pursuit of using oral mAbs for therapeutic use for STm. While in a mouse model, human plasma-derived secretory antibodies have been reported to promote survival after STm challenge, the window of protection was limited to 8 hours after initial inoculation, and a number of the animals still succumbed to infection at the end of the study (Corthésy et al., 2018). Despite limitations, these results emphasize the developing potential of recombinant SIgA as an alternative approach for combatting enteric pathogens.
CHAPTER 6: THE STRUCTURAL BASIS OF SALMONELLA A2B5 TOXIN 
NEUTRALIZATION BY GLYCAN-RECEPTOR BINDING SITES-TARGETING ANTIBODIES

The work in this chapter has been submitted for publication in the following form:


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Author Contributions: T.N. conducted experiments shown in Figs. 2, 3, 4, 7b-e, and mAb sequencing experiments. A.F.R. generated and screened hybridoma clones and conducted experiments shown in Fig. 5 and N.J.M. supervised this work. D.P.N. conducted experiments shown in Fig. 1e-f and 6. J.R.F. analyzed cryo-EM data shown in Fig. 2 and J.C.F. supervised this work. Y.-A.Y. conducted experiments shown in Fig. 1d and contributed to experiments shown in Fig. 1a-c and g. J.H.S. conducted
experiments shown in Fig. 1a-c and g. H.B. conducted experiments shown in Fig. 1c. S.L. contributed to mAb sequencing and conducted experiments shown in Fig. 7a. C.A. conducted ELISAs shown in Fig. 7f. G.V.S. was involved in hybridoma cell maintenance. J.S. supervised this study and prepared the manuscript with input from all authors. This manuscript was left intact to fully convey my contributions to the study. Additional methods not performed by me, but by the Song Lab at Cornell University, are included as supplemental material in (APPENDIX 3: SUPPLEMENTAL METHODS CHAPTER 6).
ABSTRACT

Many bacterial pathogens secrete $\text{A}_2\text{B}_5$ toxins comprising two functionally distinct yet complementary ‘A’ and ‘B’ subunits to benefit the pathogens during infection. The lectin-like ‘B’ subunit pentamer recognizes specific sets of host glycans and sialic acids to deliver the toxin into target host cells. Here, we provide the structural basis explaining the mechanism by which neutralizing antibodies with the potential to block toxin glycan-binding sites were unable to do so. Cryo-EM studies explain their corresponding differences in toxin binding and neutralization. Through toxin-antibody complex particle analysis, we found that the skewed positioning of the ‘A’ subunit(s) toward one side of the ‘B’ pentamer inhibited antibody-binding to the corresponding glycan-binding sites and therefore made these binding sites available for the toxin binding and endocytosis. These results highlight new features in the interactions between toxins and glycan-binding site-targeting antibodies.
INTRODUCTION

The ongoing global spread of antibiotic-resistant bacterial pathogens poses a great public health challenge, and if effective intervention strategies are not implemented on time, this spread will significantly increase the associated morbidity and mortality rates (Feasey et al., 2015; Hernando-Amado et al., 2019; Klemm et al., 2018; Parkhill et al., 2001; U.S. Department of Health and Human Services, 2019; Yang, Chong, et al., 2018). Bacterial A$_2$B$_5$ toxins are asymmetrical multiprotein complex virulence factors secreted by many pathogens causing respiratory, gastrointestinal, and systemic diseases (Beddoe et al., 2010). Notable examples include cholera toxin from *Vibrio cholerae* for gastrointestinal disease, pertussis toxin from *Bordetella pertussis* for whooping cough, Shiga toxin from *Shigella dysenteriae* and Shiga like toxin-producing *E. coli* (STEC) for severe diarrhea and hemolytic uremic syndrome, labile enterotoxin (LT) from *E. coli* for diarrhea, and subtilase cytotoxin from a subset of STEC (ST) for diarrhea (Beddoe et al., 2010; Fan et al., 2000; Kitov et al., 2000; Zuverink & Barbieri, 2018). These bacteria secrete their AB$_5$ toxins during infection to benefit the pathogens (Beddoe et al., 2010). The secreted toxins are stable in the local and systemic circulations and recognize specific target host cell types that are either in the infection site or in some cases distant from infected host cells (Lee et al., 2020).

In A$_2$B$_5$ toxins, the enzymatic A subunit(s) intoxicates host cells by directly altering the function of their target host proteins. The lectin-like pentameric B subunits recognize specific sets of host glycans and sialic acids displayed on the surface of target host cells, which therefore determines host cell specificity. This B-subunit recognition of the specific glycan receptors on host cells is also responsible for the
delivery of the A subunits to cellular organelles where host cellular target proteins are located (Lee et al., 2020). Consequently, the specific glycan recognition by the B subunits is associated with toxin tropism, since different cells, tissues, and hosts express structurally different sets of glycans and sialic acids. Furthermore, toxin tropism is often niche-specific, corresponding to the primary infection sites of the bacterium producing the toxin, although in some cases particularly with toxins produced by bacterial pathogens causing systemic infection, toxins can target a broad range of host cells (Lee et al., 2020; Yang, Lee, et al., 2018). In the interaction between toxin and host glycan-receptor, the homopentameric configuration of the toxin B subunits enables for accommodating high-avidity multivalent interactions between the toxin and host glycan-receptors, contributing significantly to the toxin tropism to specific sets of host cells at the whole-body level (Yang, Lee, et al., 2018).

The recent technological advances in the field of toxin/protein biochemistry and glycobiology enabled us to begin to determine the interactions between bacterial A2B5 toxins and host glycan receptors in great detail. Notable examples include Salmonella A2B5 toxins, typhoid toxin and its epithelial cell niche-specific homolog Javiana toxin (Lee et al., 2020; Nguyen et al., 2020; Yang, Lee, et al., 2018). Typhoid toxin is the first discovered Salmonella A2B5 toxin that is secreted by Salmonella enterica serovar Typhi (S. Typhi), the causative agent of typhoid fever (Deng et al., 2014; Galán, 2016; Song et al., 2013). We recently demonstrated that S. Javiana, a nontyphoidal Salmonella serovar (NTS), secretes an intestinal epithelial cell niche-specific typhoid toxin homolog called Javiana toxin where the amino acid sequence variations found in the glycan-
receptor binding JαPltB pentamer play a primary role for the niche-specificity of this toxin (Lee et al., 2020).

Typhoid toxin is a pyramid-shaped heptamer consisting of two enzymatic ‘A’ subunits, CdtB and PltA arranged in tandem, and a homopentamer of receptor-binding ‘B’ subunits, PltB on the base of the assembled toxin structure (Song et al., 2013). Pentameric PltB subunits serve as a base of the combined AB toxin complex, where the hydrophobic channel located at the center of the donut-shaped PltB pentamer connects to PltA ( mono-ADP ribosyltransferase, mART) through the C-terminal α-helix structure of PltA (Song et al., 2013). Besides the two Cys residues conserved across the mART family members, PltA contains additional Cys residue that links the PltA-PltB₅ complex to CdtB (DNase-like (Spanò et al., 2008)), resulting in its A₂B₅ stoichiometry (Song et al., 2013). Therefore, there is no direct interaction between PltB pentamer and CdtB located at the base and the vertex of the assembled toxin complex, respectively.

PltB (receptor-binding) and CdtB (nuclease) subunits of the secreted assembled toxin available in the extracellular milieu are responsible for typhoid toxin-mediated cellular and in vivo toxicities (Song et al., 2013). In particular, the cellular toxicity of typhoid toxin can be objectively quantified through cell cycle profiling analysis of intoxicated host cells, which was used in this study when evaluating the antibody-mediated neutralizing efficacies (Spanò et al., 2008). On the other hand, the secreted assembled typhoid toxin mutant containing catalytically-inactive PltA available in the extracellular milieu still endocytosed and induced host cell cycle arrests in G2/M and typhoid toxin-mediated in vivo toxicities, indicating that the enzymatic activity of PltA does not directly affect typhoid toxin-mediated endocytosis trafficking and intoxication of
host cells, although PltA does play an important role at different stages of pathogenesis (unpublished data) (Song et al., 2013; Yang, Lee, et al., 2018).

There is a total of 15 spatially separated glycan-binding sites on the PltB pentamer of typhoid toxin and, in each PltB monomer, the glycan-receptor binding site BS1 is located on the lateral side of PltB whereas the other two glycan-binding sites BS2 and BS3 are located on the bottom side of PltB (Lee et al., 2020). The BS1 can accommodate both α2-3 sialosides and α2-6 sialosides with higher affinities, whereas the BS2 and BS3 are located on the bottom side of the donut-shaped PltB pentamer which can accommodate only α2-3 sialosides (Lee et al., 2020; Nguyen et al., 2020; Song et al., 2013; Yang, Lee, et al., 2018) (Supplementary Table 2). In this study, we took advantage of the well-characterized glycan-binding sites at the atomic level on the B subunits of typhoid toxin in dissecting the neutralizing mechanisms by antibodies targeting glycan-binding sites on bacterial A2B5 toxins. We then applied the structural basis explaining the different toxin neutralization outcomes by a set of PltB’s glycan-binding sites-targeting antibodies to other bacterial A2B5 toxins by conducting secondary structure matching (or SSM) analyses to obtain insights into whether this newly discovered mechanism is also applicable to other toxin-antibody interactions.

RESULTS

Monoclonal antibodies (mAbs) recognizing glycan-receptor binding PltB subunits of Salmonella A2B5 typhoid toxin display different toxin neutralization efficacies

To recover a set of neutralizing antibodies targeting glycan-receptor binding PltB subunits of typhoid toxin, we immunized a group of mice with recombinant typhoid
toxoid and, using splenic B cells, generated a collection of B cell hybridomas producing mAbs against typhoid toxin PltB. In brief, after we obtained recombinant typhoid toxoid preparations, we validated the same A$_2$B$_5$ configuration of the toxoid as wild-type (WT) typhoid toxin via size-exclusion chromatography (SEC). After immunizations when mouse serum reciprocal endpoint titers were >100,000, we generated B cell hybridomas using Hybri-Max polyethylene glycol (PEG)-mediated fusion to Sp2/0-Ag14 myeloma cells, as previously described (Van Slyke et al., 2018). B cell hybridomas were cloned by limiting dilution three times to ensure clonality, resulting in a total of ten PltB-positive hybridomas, named TyTx1-10. However, the characterization of hybridoma culture supernatants indicated that TyTx2 was a weak binder to the PltB homopentamer and excluded from further studies. All of the remaining nine antibodies, TyTx1, 3-10, recognized effectively both typhoid toxin and PltB homopentamer evaluated by conducting enzyme-linked immunosorbent assays (ELISAs) (Figure A4.1). To select hybridoma clones producing different antibodies targeting glycan-receptor binding PltB subunits, we sequenced the antigen-recognizing variable regions of 9 mAbs by conducting a modified reverse transcription-polymerase chain reaction (RT-PCR) followed by Sanger sequencing, as previously described (Meyer et al., 2019) (Table A5.1). We found from sequencing analysis that seven hybridoma clones producing TyTx4-10 belong to the same family, and therefore TyTx1, 3, and 4 (5-10) are three distinct hybridoma clones producing antibodies targeting PltB (Figure A4.2, Figure A4.3). To perform detailed characterizations of antibodies in the context of interactions between toxins and host glycan-receptors, hybridomas producing TyTx1, 3, and 4 were cultured on a milligram scale, and corresponding mAbs were purified using Protein G
agarose columns (Figure 6.1, Figure A4.4), after which we validated that purified TyTx1, 3, and 4 recognize PltB subunits, indicated via Western blots using purified typhoid toxin separated by SDS-PAGE (Figure 6.1).

PltB subunits recognize specific sets of host glycans expressed on the surface membranes of target cells and subsequently initiate the retrograde toxin trafficking to deliver the toxins into target host cells (Song et al., 2013). We, therefore, evaluated whether TyTx1, 3, and 4 could inhibit typhoid toxin binding and toxin trafficking. Quantitative fluorescent microscopy was carried out to measure mAb-mediated inhibition of Alexa Fluor 555-conjugated typhoid toxin binding to host cells. TyTx1 and 3 inhibited toxin binding (200 ng toxin used) to Henle-407 cells modestly, whereas TyTx4 resulted in a drastic inhibition of the toxin binding to host cells (Figure 6.1). Comparable results of mAb-mediated inhibition of the typhoid toxin binding to host cells were also observed with a lower dose (100 ng) of typhoid toxin (Figure A4.5). Consistently, the toxin amounts delivered inside host cells were slightly decreased in the presence of TyTx1 or TyTx3, whereas TyTx4 markedly decreased toxin delivery into host cells, as compared to typhoid toxin only, which was assessed 4 hrs after the treatment (Figure 6.1). We then investigated whether the different outcomes in toxin binding and trafficking inhibition among anti-PltB antibodies correlate to the neutralization outcomes against typhoid toxin-mediated cellular toxicity, by analyzing cell cycle profiles of Jurkat cells via flow cytometry. Typhoid toxin induces cell cycle arrests in G2 or M stages in intoxicated host cells due to CdtB-mediated host cell DNA damage (Spanò et al., 2008). Consistent with the binding and trafficking inhibition results (Figure 6.1), the effects of TyTx1 and 3 were modest, while the strong neutralizing effect was induced by TyTx4.
Figure 6.1. mAbs recognizing PltB subunits display different toxin neutralization efficacies.

(A) Purified mAbs were separated on SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). HC, heavy chain. LC, light chain. Superdex 200 chromatograms of these purified mAbs are shown in along with standards used. (B) Typhoid toxin (200 ng) was separated on SDS-PAGE and stained with CBB (left panel), when indicated, followed by Western blot analysis for each mAb to determine their binding subunits among the three subunits of typhoid toxin (right). At least three independent experiments were performed. Representative blot results are shown. (C) Quantification of mAb-mediated inhibition of typhoid toxin binding to Henle-407 cells. Henle-407 cells grown on coverslips were precooled to 4 °C, incubated with 200 ng of typhoid toxin-AF555 in the presence or absence of mAb for 30 min, counterstained with DAPI, and analyzed by fluorescent microscopy. Bars represent the mean ± SEM obtained from three
independent experiments. Each dot reflects the typhoid toxin signal intensity per image. n=65-89. ****, p<0.0001, compared to the TyT only group. Unpaired two-tailed t-tests. Representative fluorescent microscope images of Henle-407 cells incubated with 200 ng of typhoid toxin-AF555 ± mAbs for 4 hrs. Henle-407 cells were pre-treated with media containing 10 mM NH₄Cl for 20 min at 37 °C, before chilling and binding of anti-typhoid toxin MAbs and fluorescent labeled-typhoid toxin. TyT, typhoid toxin (red); DAPI (blue), DNA. (E-F) Jurkat cells were treated with purified typhoid toxin ± mAbs for 18 hrs. Cell cycle profiles were analyzed via flow cytometry to assess the typhoid toxin-induced host cell cycle arrest in G2/M. (E) Representative cell cycle profiles of Jurkat cells treated with typhoid toxin ± mAbs. (F) Percent of cells in the G2/M cell cycle from three independent experiments. Bars represent average ± SEM. **** p<0.0001. n=9 per group. Unpaired two-tailed t-tests. (G) Competition ELISA results between TyTx1 and TyTx4. TyTx4 (100 ng) immobilized on Protein G ELISA plates was incubated with a mixture of 10 ng biotinylated PltB pentamer and indicated doses of competitor mAbs. The graph shows the values for the mean ± SEM. Three independent experiments were performed.
(Figure 6.1), indicating that TyTx1, TyTx3, and TyTx4 are indeed three different antibodies targeting PltB with different neutralizing efficacies. Since neutralizing outcomes by TyTx1 and TyTx3 were similar, which is significantly different from TyTx4, we selected TyTx1 and TyTx4 for further detailed atomic level structure and function analysis. Competition ELISA results further supported the selection of TyTx1 and 4, since the binding of biotinylated PltB homopentamer to TyTx4 immobilized on ELISA plates was competed in the presence of TyTx4 but not TyTx1 (Figure 6.1), indicating that TyTx1 and TyTx4 recognize spatially separated epitopes on PltB.

Self-control asymmetry of $A_2B_5$ typhoid toxin interferes with antibodies recognizing epitopes located on the lateral side of pentameric B subunits

To understand the molecular mechanism by which TyTx1-mediated inhibition of toxin binding and neutralization is different from those by TyTx4, we used cryo-electron microscopy (cryo-EM) to analyze multiple different complex particles consisting of typhoid toxin and TyTx1, and to determine the amino acid sequence of epitopes on PltB subunits recognized by TyTx1. To eliminate the potential ambiguity in the stoichiometry analysis of complex particles, we prepared TyTx1 Fab fragments for monovalent binding to each PltB subunit, as TyTx mAb would likely result in bivalent interactions with pentameric PltB subunits. TyTx1 Fab fragments were prepared by carrying out papain digestions of TyTx1 IgG, completing Protein G-mediated removal of undigested mAb and Fc, and performing SEC of the samples containing Fab using Superdex 75. The prepared TyTx1 Fab fragments were then incubated together with purified typhoid toxin overnight, resulting in many toxin-TyTx1 Fab complex particles. A neutral isotonic
buffer (15 mM Tris-HCl, pH 7.5, and 150 mM NaCl) was used to mimic physiologically relevant conditions.

We analyzed 741,088 cryo-EM complex particles consisting of typhoid toxin and TyTx1 Fab (Figure 6.2, Figure A4.6, and Table A5.1). Note that 13% of the 741,088 particles were grouped into a “junk class” since we had insufficient cryo-EM density required for determining the precise location on PltB homopentamer that TyTx1 Fab recognizes (Figure 6.2, Figure A4.6). In the holotoxin structure, the position of PltA leans toward one side of the donut-shaped pentameric PltB subunits (PDB 4K6L)(Song et al., 2013), resulting in self-control asymmetry of pentameric PltB subunits when PltB subunits are part of the combined holotoxin. Due to this A-subunit-induced asymmetry, five subunits of the donut-shaped PltB pentamer are called in this manuscript (as well as in PDB 4K6L crystal structure) in a clockwise manner, chain B (the closest to PltA), chain D, chain E (the second-most distant from PltA), chain C (the most distant from PltA), and chain A. We found from analyzing 644,747 complex particles (741,088 minus 13% undefinable junk class) that approximately 72.4% of the complexes with sufficient density contained one typhoid toxin molecule and one TyTx1 Fab molecule where a single Fab was found to bind to either the C chain (the most distant from PltA) in ~48.3% of the complexes or the E chain (the second most distant from PltA) in ~24.1% of the complexes (Figure 6.2). The rest (27.6%) of the complexes with sufficient density consisted of typhoid toxin bound to two TyTx1 Fabs via binding of both C and E chains, the most distant and the second-most distant PltB subunits from PltA, respectively (Figure 6.2). These results are in support of the hypothesis that the toxin complexed
Figure 6.2. Self-control asymmetry of A$_2$B$_5$ typhoid toxin interferes with antibodies recognizing epitopes located on the lateral side of pentameric B subunits.

(A) Quantifications of the complexes consisting of typhoid toxin and TyTx1 Fab. See Figure A4.6 for details. (B) Sharpened cryo-EM density map (grey) of typhoid with
TyTx1 Fab (grey) with ribbon diagram of the refined structure of PltB pentamer (green) bound to variable regions of the light chain (VL, blue) and the heavy chain (VH, purple). (C) Ribbon diagram of the interface between PltB subunits (C chain, green and E chain, grey) and TyTx1 VL and VH. (D-E) Close-up views of the interactions between PltB subunits and TyTx1 VL (D) and PltB subunits and TyTx1 VH (E). Ala60\* (water-mediated), Gly62\* (water-mediated), Tyr93, and Gly96\* (water-mediated) in the PltB E chain form hydrogen bonds with the TyTx1 VL chain Arg35, Lys36, Arg33, and Arg35, respectively. Asterisk indicates H-bond via their main chains. Tyr33\*, Tyr33, Ser35, Asp36, Lys59, Asn61\*, Gly62\*, Ser63\*, and Thr131\* of the PltB C chain interact with Asp55 (VH chain), Tyr54 (VH, π stacks), Arg100 (VL), Tyr50 (VH), Asp57 (VH), Ser59 (VH), Gln62 (VH), Ser59 (VH), and Arg100 (VL) via H-bonds or π stacks, respectively. Key residues in the glycan-receptor binding site BS1 of PltB subunits are highlighted in bold.
skewed position of the A subunit(s) in the three-dimensional A$_2$B$_5$ toxin structure results in self-control asymmetry of pentameric B subunits and prevents antibodies binding to all five epitopes available on pentameric B subunits.

To better explain the principle governing this toxin’s A subunit-mediated interference of antibody-binding, we refined the Class I complex map (TyTx1 Fab bound to the C chain) to 3.0 Å to determine the amino acid sequence of the epitope that TyTx1 recognizes (Figure 6.2 and Figure A4.7). Typhoid toxin and PltB subunits in the refined cryo-EM structure align well with typhoid toxin and PltB pentamer crystal structures available, PDB 4K6L and 4RHR, respectively (Figure 6.2, Figure A4.6, and Figure A4.7). The overall and enlarged interfaces between PltB and the variable regions in the heavy and light chains of TyTx1 (VH and VL) are shown in Figure 6.2, along with their cryo-EM density maps (Figure A4.7). There are more interactions between PltB and the heavy chain of TyTx1 than the light chain (Figure 6.2). Intriguingly, many PltB amino acid residues recognized by TyTx1 overlap with PltB amino acid residues used for interacting with its glycan receptors α2-3 and α2-6 sialosides that are expressed on host cell surfaces.

We found that TyTx1 Fab directly interacts with many key residues in glycan-binding site BS1 on PltB, including Ser35 and Lys59 that are the most critical amino acid residues on BS1 (Figure 6.2, Figure A4.7, and Table A5.2). Specifically, among the PltB E chain residues, Ala60* (water-mediated), Gly62* (water-mediated), Tyr93, and Gly96* (water-mediated) form hydrogen bonds with the TyTx1 VL chain Arg35, Lys36, Arg33, and Arg35, respectively. Asterisk indicates H-bond via their main chains. Similarly, Tyr33*, Tyr33, Ser35, Asp36, Lys59, Asn61*, Gly62*, Ser63*, and Thr131* of
the PltB C chain interact with Asp55 (VH chain), Tyr54 (VH, π stacks), Arg100 (VL), Tyr50 (VH), Asp57 (VH), Ser59 (VH), Gln62 (VH), Ser59 (VH), and Arg100 (VL) via H-bonds or π stacks, respectively (Figure 6.2). Note that some E chain residues also contribute to TyTx1 binding to the C chain, although these are mostly water-mediated interactions that are in line with the distance to TyTx1 bound to the C chain.

*Interference is specific for antibodies recognizing epitopes located on the lateral side of glycan-receptor binding PltB subunits*

If the A subunit-mediated asymmetry of pentameric PltB subunits is indeed the primary reason for the interference with TyTx1 recognition of its epitopes laterally-located on PltB, the counterpart antibodies recognizing epitopes located on the far side of PltB subunits should not be interfered by the A subunit-mediated asymmetry. Competition ELISA results indicated that TyTx1 and TyTx4 recognize different epitopes on PltB, and TyTx1 and TyTx4 resulted in different levels of inhibition in toxin binding and neutralization (Figure 6.1). These results support the hypothesis that, unlike TyTx1 recognizing amino acid residues located on the lateral side of PltB subunits, the epitope recognized by TyTx4 may be located on the far side of PltB pentamer. We tested this hypothesis by solving the structure of typhoid toxin complexed with TyTx4 Fab via cryo-EM (Fig. 3 and Table 1). We found that TyTx4 Fab indeed recognizes amino acid residues located on the far side of the PltB pentamer (Fig. 3A-D and Supplementary Fig. 8). Specifically, 83% of the 309,475 complex particles composed of one typhoid toxin molecule and five TyTx4 Fab molecules, and the remaining 17% contained one
Figure 6.3. Interference is specific for antibodies recognizing epitopes located on the lateral side of glycan-receptor binding PltB subunits.

(A) Quantifications of the complexes consisting of typhoid toxin and TyTx4 Fab. (B) Sharpened Cryo-EM C1-symmetry density map (grey) of typhoid toxin complexed with TyTx4 Fab with ribbon diagram of the refined structure of PltB pentamer (green) bound to variable regions of the light chain (VL, light blue) and the heavy chain (VH, pink). (C) Top view of ribbon diagram of the overall structure of the complex between PltB pentamer and 5 TyTx4 VLs and VHs. (D) Close-up views of the interactions between
PltB subunit and TyTx4 VL and VH. Glu24, Asp48, Asp48, Gln75, Gln75, Gln104, Asn106, Thr109, Thr109*, 493 and Tyr110 of PltB subunits forming either H- bond or π stacks by interacting with Tyr100 (VH), Ser51 (VH), Gly53* (VH), Asp32 (VH), Gly52* (VH), Tyr105 (VH), Tyr50 (VL), Asn104 (VH), Tyr58 (VH), and Tyr56 (VH, π stacks), respectively. Key residues in the glycan-receptor binding sites BS2 and BS3 of PltB subunits are highlighted in bold.
typhoid toxin molecule and four TyTx4 Fab molecules (Figure 6.3, Figure A4.8). These results are in sharp contrast to the stoichiometry of the TyTx1 Fab-typhoid toxin complexes and, therefore, are in support of the specificity of self-control asymmetry-mediated interference towards the laterally-located epitopes (Figure 6.2, Figure 6.3, Figure A4.6, and Figure A4.8).

To determine the amino acid sequence that TyTx4 recognizes, we refined the Class I complex structures containing one typhoid toxin molecule and five TyTx4 Fab molecules to 3.1 Å (Figure 6.3, Figure A4.8). The epitope sequences for TyTx4 are Glu24, Asp48, Gln75, Gln104, Asn106, Thr109, and Tyr110 on PltB subunits (Figure 6.3, Figure A4.9). Significant inter-molecular interactions between PltB and TyTx4 Fab are Glu24, Asp48, Asp48, Gln75, Gln75, Gln104, Asn106, Thr109, Thr109*, and Tyr110 of PltB subunits forming either H-bond or π stacks by interacting with Tyr100 (VH), Ser51 (VH), Gly53* (VH), Asp32 (VH), Gly52* (VH), Tyr105 (VH), Tyr50 (VL), Asn104 (VH), Tyr58 (VH), and Tyr56 (VH, π stacks), respectively (Figure 6.3). Similar to TyTx1 mAb, most interactions were through the heavy chain of TyTx4 (Figure 6.3). Intriguingly, TyTx4 recognition residues also include many amino acid residues critical for glycan-receptor bindings situated 235 on the glycan binding sites BS2 and BS3 that can accommodate α2-3 sialosides (Lee et al., 2020). In particular, Q75, T109, and Y110 in the BS2 and E24 and D48 in the BS3 participate in the direct interaction with α2-3 sialosides, which are located on the far side of pentameric PltB subunits (Lee et al., 2020). Therefore, cryo-EM results are in support of the specificity of self-control asymmetry-mediated interference towards the laterally-located epitopes.
To further validate the findings, we prepared TyTx1, TyTx3 (one exhibited the similar phenotypes in toxin binding inhibition and toxin neutralization to TyTx1), and TyTx4 Fabs, incubated them with either typhoid toxin or PltB pentamer, conducted SECs to estimate the stoichiometry of the complexes formed (Figure 6.4, Figure A4.10). In line with cryo-EM and other characterization results, SEC analyses indicate that TyTx1 Fab and TyTx3 Fab bound to typhoid toxin in a ratio of 1~2 Fab molecule(s) to 1 typhoid toxin molecule, while TyTx4 Fab bound to the toxin in a ratio of 4~5 TyTx4 Fab molecules to 1 typhoid toxin molecule (Figure 6.4). In contrast, when pentameric PltB subunits without the A subunits were used, we found complexes containing Fabs and PltB pentamer in a ratio of 3~5 TyTx1 Fab and TyTx3 Fab molecules to 1 PltB pentamer and 4~5 TyTx4 Fab molecules to 1 PltB pentamer (Figure 6.4, Figure A4.10). Therefore, the SEC results are also in support of the specificity of ‘A’ subunit-induced, self-control asymmetry-mediated interference toward epitopes laterally-located on pentameric B subunits. We further validated the binding of TyTx3 to the lateral side of the PltB subunit by conducting the ELISA-based binding assays using two types of recombinant PltB pentamer preparations, tagless PltB5 and PltB-His6 at the C-terminal end that is located on the lateral side of PltB pentamer in 3-dimensional structure (PDB 4K6L). TyTx3 bound to tagless PltB but not to PltB-His6 tagged at the C-terminal end of PltB.
Figure 6.4. A-subunit(s)-mediated asymmetry is required for toxin interference with antibody binding to the laterally-located epitopes on PltB.

(A-D) Size-exclusion chromatography (SEC) of typhoid toxin complexed with TyTx1 Fab, TyTx3 Fab, or TyTx4 Fab. (A) SD200 chromatograms of typhoid toxin complexed with TyTx1 Fab (blue line), typhoid toxin complexed with TyTx3 Fab (green line), and typhoid toxin complexed with TyTx4 Fab (orange line). (B-D) SDS-PAGE analyses of peak fractions of TyTx1 Fab bound to typhoid toxin (B), TyTx3 Fab bound to typhoid toxin (C), and TyTx4 bound to typhoid toxin (D). Fraction names match with the start elution volume of 0.5 mL collections (e.g., F12.0 means the 0.5 ml collection corresponding to elution volume 12.0-12.5 ml. Mw, molecular weights of standards. Fab (LC), the light (kappa) chain of Fab. Fab (HC), the heavy chain of Fab. Protein bands with asterisks in c and d, Fc contamination. (E-H) SEC of PltB pentamer complexed with TyTx1 Fab, TyTx3 Fab, or TyTx4 Fab. E, SD200 chromatograms of tagless PltB pentamer complexed with TyTx1 Fab, TyTx3 Fab, or TyTx4 Fab. E, SD200 chromatograms of tagless PltB pentamer complexed with TyTx1 Fab (blue line), tagless PltB pentamer complexed with
TyTx3 Fab (green line), and tagless PltB pentamer complexed with TyTx4 Fab (orange line). F-H, SDS-PAGE analysis of peak fractions of TyTx1 bound to PltB pentamer (F), TyTx3 bound to PltB pentamer (G), and TyTx4 bound to PltB pentamer (H).
mAbs recognizing laterally-located epitopes on typhoid toxin PltB exhibit markedly reduced toxin binding avidities

We hypothesized that self-control asymmetry-induced interference towards laterally-located epitopes correlates to lower binding avidities for relevant antibodies, compared to antibodies that recognize epitopes on the far side from the A-subunit-mediated interference. TyTx1 and TyTx3 recognize epitopes on the lateral side of PltB, while TyTx4 binds to the far side of PltB subunits. To determine the apparent overall affinities or avidities of mAbs to typhoid toxin, we carried out surface plasmon resonance (SPR) analyses, as previously described (Rudolph et al., 2020). Typhoid toxin was immobilized on sensor chips followed by flowing 4-fold serial dilutions of each mAb over the toxin for the apparent affinity/avidity determination of TyTx1, TyTx3, and TyTx4. Consistently with cryo-EM and SEC data indicating ratios of 4~5 TyTx4 Fabs to 1 typhoid toxin (equivalent to 2~3 TyTx IgGs per toxin) and 1~2 TyTx1 Fabs to 1 typhoid toxin (equivalent to 1 TyTx1 IgG per toxin), SPR results indicate that the apparent affinity of TyTx1 to typhoid toxin was ~71 nM and the apparent avidity of TyTx4 to typhoid toxin was ~ 7 pM, indicating ~10,000-fold binding affinity/avidity difference between these two antibodies (Figure 6.5, Table A5.3). Like TyTx1, TyTx3 recognizes the laterally-located epitope. The binding affinity of TyTx3 to typhoid toxin was ~145 nM, which is ~20,000-fold lower than the apparent avidity of TyTx4 (Figure 6.5, Table A5.3). To better understand the precise contributions of the estimated monovalent vs. pentavalent bindings observed between mAbs recognizing the laterally-located epitopes and mAbs recognizing the far-side located epitopes to the overall binding affinity/avidity of these mAbs, we aimed to set up additional SPR assays using PltB monomer.
Figure 6.5. mAbs recognizing laterally-located epitopes on typhoid toxin PltB exhibit markedly reduced toxin binding avidities.

(A-C) Kinetics of TyTx1 (A), TyTx3 (B), and TyTx4 (C) binding to typhoid toxin by surface plasmon resonance (SPR). Mean sensorgrams from TyTx mAbs binding with typhoid toxin-coated Series S CM5 chips (5-10 μg/ml) over a range of high (silver) and low (green) concentrations (nM). Sensograms were normalized to fit a bivalent binding model using the Biacore T200 Evaluation software (GE Healthcare). Calculated KD values are indicated in the graph.
Unfortunately, however, we were unable to obtain the full-length PltB monomer as a soluble protein that can allow for obtaining the undisturbed affinity between mAbs and PltB monomer. Therefore, although we were unable to determine the precise contributions of mono- and pentavalent bindings of TyTx1 and TyTx4 towards their observed binding affinities to typhoid toxin, we demonstrated that the binding affinities of TyTx1 and TyTx3, recognizing epitopes laterally-located on the PltB pentamer and therefore interference, are markedly lower than the overall binding avidity of TyTx4, recognizing epitopes located on the far side of the PltB pentamer and therefore no interference.

*mAbs recognizing laterally-located epitopes on typhoid toxin PltB exhibit markedly reduced toxin neutralization efficacies*

We next investigated whether self-control asymmetry of typhoid toxin resulted in a decrease in relevant mAb-mediated toxin neutralization efficacies in the context of infection. We established *in vitro* infection assays using *S. Typhi* and, therefore, in this system typhoid toxin was produced continuously by *S. Typhi* during infection (Chang et al., 2016; Lee et al., 2020; Yang, Lee, et al., 2018). Indeed, we found that TyTx4 that is undisturbed by self-control asymmetry neutralized Salmonella A₂B₅ toxins much more effectively than TyTx1 and TyTx3, ones that are disturbed by self-control asymmetry (*Figure 6.6*). The difference became more apparent when a higher dose infection was used (*Figure 6.6*). Specifically, when a multiplicity of infection (m.o.i.) of 30 was used for *S. Typhi* infection, TyTx4 induced a marked toxin neutralization but TyTx1 and
Figure 6.6. mAbs recognizing laterally-located epitopes on typhoid toxin PltB exhibit markedly reduced toxin neutralization efficacies.

(A-C) Measurements of MAb-mediated toxin neutralization against typhoid toxin continuously produced by S. Typhi during infection. Representative cell cycle histograms (A) and percent cells in the G2/M cell cycle that indicates the typhoid toxin-mediated toxicity (B-C). Henle-407 cells were left uninfected or infected with S. Typhi (STy) at a multiplicity of infection of 30 (A-B) or 50 (C) for 3 days in the absence or presence of indicated mAbs. A mutant S. Typhi containing CdtBH160Q (STy CdtB mt) was used as an isogenic control for typhoid toxin-mediated G2/M cell cycle arrest. Cell cycle profiles were analyzed via flow cytometry. Three independent experiments were performed. Bars represent average ± SEM. ****, p<0.0001. n=9 per group. Unpaired two-tailed t-tests.
TyTx3 showed a mild effect in toxin neutralization (Figure 6.6). In comparison to ~50% host cells in G2/M in the 30 m.o.i. infection, 50 m.o.i. infection of S. Typhi made ~70% host cells arrested in G2/M (Figure 6.6). In this condition, the neutralization effects elicited by TyTx1 and TyTx3 were modest, which was not significantly different from the toxin-only samples without mAbs (Figure 6.6). An isogenic S. Typhi strain harboring the CdtB$^{H160Q}$ catalytic mutant subunit indicated that cell cycle arrests in G2/M were specific to the toxicity induced by typhoid toxin (Figure 6.6).

Toxin interference with antibody-binding to the laterally-located epitopes on pentameric ‘B’ subunits may be universal among AB$_5$ and A$_2$B$_5$ toxins.

Javiana toxin is an epithelial cell niche-specific typhoid toxin homolog exhibiting A$_2$B$_5$ stoichiometry and contains the pentameric JaPltB subunits that are highly homologous to typhoid toxin PltB since there are only 3 amino acid variations per PltB monomer between these two, although these small sequence variations resulted in their host cell tropism differences (Lee et al., 2020; Yang, Lee, et al., 2018). The epitope sequences recognized by TyTx1 and TyTx4 are present in JaPltB. Purified recombinant Javiana toxin was used to evaluate the efficacy of mAbs for the Javiana toxin neutralization. Like their neutralization efficacies to typhoid toxin, TyTx4 showed the greatest neutralization effect against Javiana toxin, whereas TyTx1 and TyTx3 showed little protection against Javiana toxin (Figure 6.7). These results indicate that the A subunit-mediated interference with antibody-binding to the laterally-located epitopes on the B pentamer is also applicable to another A$_2$B$_5$ Javiana toxin.
Figure 6.7. Toxin interference with antibody-binding to the laterally-located epitopes on pentameric ‘B’ subunits may be universal among $A_2B_5$ toxins.

(A) mAb-mediated neutralization of Javiana toxin, an epithelial cell niche-specific typhoid toxin homolog from $S$. Javiana. Percent of cells in the G2/M cell cycle indicate the Javiana toxin-mediated toxicity. Henle-407 cells were treated for 48 hrs with PBS (Untreated) or Javiana toxin (0.6 pM) in the absence (JaT only) and the presence of indicated antibodies (300 pM). Cell cycle profiles were analyzed via flow cytometry. Three independent experiments were performed. Bars represent average ± SEM. **,
**p**<0.01, ****, **p**<0.0001. n=9 per group. Unpaired two-tailed t-tests. (B) SSM analyses of typhoid toxin match with the data obtained from the cryo-EM studies, indicating antibodies recognizing the laterally-located epitopes can bind to up to 2 PltB subunits (indicated as 2 x Fab1, pink volume), as opposed to antibodies recognizing the bottom-located epitopes can bind to 5 PltB subunits (5 x Fab4, grey volume). The left panel, top view of typhoid toxin (4K6L), Fab1, and Fab4; the middle panel, side view of typhoid toxin complexed with Fab1; the right panel, side view of Fab1 clashed with the toxin A subunit (brown). (C-E) E. coli PltAB toxin (C), cholera toxin (D), and pertussis toxin (E) with TyTx1 Fabs (pink volume; representing antibodies recognizing the lateral side of the B pentamer) and TyTx4 Fabs (grey volume; representing antibodies recognizing the far side of the B pentamer). Total numbers of Fabs that can bind to the indicated toxins without clash are indicated in the top view images that overlay toxin, Fabs of TyTx1, and Fabs of TyTx4. (F) ELISAs determining the relative antibody abundance of antibodies recognizing the laterally-located epitopes and antibodies recognizing the far side-located epitopes on PltB. Serum samples from 10 individual mice received 2 injections with a 2-week interval of typhoid toxoid via the subcutaneous (SC) route were analyzed. Bars represent the average of the three independent measurements.
Next, to provide insight into whether this toxin interference mechanism with antibody-binding to laterally-located epitopes is universal among A(2)B₅ toxins, we carried out SSM analyses of four additional AB₅ toxins, *E. coli* PltAB toxin, cholera toxin, Shiga toxin, and pertussis toxin (Figure 6.7, Figure A4.11, and Table A5.4). In 3D structures, like typhoid toxin, the A-subunits of these toxins are asymmetrically positioned toward one side of their donut-shaped pentameric B subunits. Consistently, SSM analyses indicate that antibodies recognizing laterally-located epitopes on the B pentamer (like TyTx1) could not be placed for all five B subunits due to the clashes with the asymmetrically positioned A-subunits (Figure 6.7, Figure A4.11, and Table A5.4). In contrast, such clashes did not occur with antibodies recognizing the far side of the B pentamer (like TyTx4), indicating that the asymmetrically located A subunit-mediated interference with specific groups of antibody-mediated toxin neutralization may also occur in the case of other AB₅ toxins. Lastly, modified ELISAs were carried out to obtain insight into the relative abundance of two types of glycan-receptor subunit-targeting antibodies in immunized mice. Plasma samples from 10 immunized mice were probed for purified PltB, PltB pre-complexed with TyTx1 Fab, and PltB pre-complexed with TyTx4 Fab to capture and quantify total anti-PltB antibodies, antibodies recognizing the far side-located epitopes on PltB, and antibodies recognizing the lateral side-located epitopes on PltB, respectively, indicating all ten immunized mice had both types of anti-PltB antibodies (Figure 6.7).
DISCUSSION

Little is known about the A2B5 toxin neutralizing mechanism by glycan-receptor binding sites targeting antibodies. Using both wet- and dry-approaches integrating structural, biochemical, and biological, and computational characterizations, we unraveled for the first time the structural basis explaining the different toxin neutralizing efficacies among glycan-receptor binding sites-targeting antibodies at the atomic and molecular level. Due to the asymmetrical positioning of the A-subunits toward one side of the pentameric B subunits, A2B5 toxins were able to interfere with specific groups of antibody-binding – antibodies recognizing laterally-located epitopes on the B pentamer. As a result, this interference made the corresponding glycan-binding sites remained available for toxin binding and endocytosis processes into target host cells, indicating that A2B5 toxins can tolerate up to a certain threshold of laterally-located epitope-binding antibodies. This is supported by the observed differences in toxin binding and trafficking inhibition and neutralization outcomes, as well as apparent binding affinity differences to the toxins among PltB-targeting antibodies.

This toxin intrinsic interference mechanism was specific to epitopes laterally-located on the B pentamer of typhoid toxin, consistent with the skewed positioning of the A-subunit toward one side of the pentameric PltB subunits. In support, antibodies recognizing epitopes located on the far side of the B pentamer indeed bound to all five epitopes available on the B pentamer. Moreover, the observed interference with antibodies recognizing epitopes laterally located on PltB was no longer observed when the A-subunits were removed in the assembled toxin complex, indicating the
asymmetrical positioning of the A-subunit is essential in this toxin intrinsic interference against specific groups of neutralizing antibody-binding.

Other \( A_2B_5 \) toxins likely use this toxin interference mechanism against specific groups of antibodies. Another Salmonella \( A_2B_5 \) Javiana toxin contains the identical epitopes on JaPltB, due to the sequence similarity between the two. Anti-PltB mAbs also resulted in different toxin neutralizing efficacies against Javiana toxin, since we observed the modest toxin neutralization by mAbs recognizing laterally-located epitopes on JaPltB and the profound neutralizing outcomes by mAbs recognizing far-side-located epitopes on JaPltB. Intriguingly, we also demonstrated that cholera toxin, pertussis toxin, \( E. \ coli \) toxin, and Shiga toxin have their A-subunits asymmetrically positioned toward one side of the pentameric B subunits. Consistently, SSM analysis was conducted using the crystal structures of these toxins and TyTx1 Fab and TyTx4 Fab structures as model antibodies representing mAbs recognizing laterally-located and far-side-located epitopes on the B pentamer, respectively. The SSM analysis results indicate that this newly revealed molecular feature may be broadly applicable to the interactions between asymmetrical \( A_2B_5 \) toxins and B-subunit-targeting antibodies.

Both types of glycan-receptor binding sites-targeting antibodies against typhoid toxin were available at the similar level in all typhoid toxoid-immunized mice, as evaluated by conducting modified ELISAs designed for learning about the relative antibody abundance of two types of anti-PltB antibodies. In this experiment, inactive typhoid toxoid was used for immunizing mice that received 2 injections of the toxoid via a subcutaneous route. So, this study did provide insight into the relative antibody titers and repertoire about anti-PltB antibodies induced after toxoid immunization but didn’t
provide information about whether the ratio of these two types of anti-PltB antibodies in other conditions such as natural infection and different vaccination regimens (e.g., different routes and numbers of boosters). However, it is intriguing to speculate that active typhoid toxin secreted by infected S. Typhi during natural infection may result in significantly different antibody titers and repertoire since active typhoid toxin can modulate host immune cell functions (Lee et al., 2020; Nguyen et al., 2020; Yang, Lee, et al., 2018). It is also intriguing to learn more in the future about whether the adjustment of toxoid vaccination regimes changes the antibody titers and repertoire toward the prolonged, more efficacious humoral immune responses. The toxin interference phenomenon unraveled in this study would be valuable in interpreting data obtained from those future research endeavors with typhoid toxin and other A2B5 toxins.

It is also worthwhile to note that the neutralizing epitopes that we discovered in this study are well conserved among S. Typhi clinical isolates, as well as non-typhoidal Salmonella serovars that harbor an epithelial cell niche-specific typhoid toxin homolog. For instance, all S. Typhi clinical isolates identified thus far, including multidrug-resistant (MDR) and extensively drug-resistant (XDR) S. Typhi strains, encode identical typhoid toxin PltB subunits (Supplementary Table 5). No sequence variation in the typhoid toxin subunits has been observed among several thousand of S. Typhi clinical isolates since all S. Typhi clinical isolates encode the identical typhoid toxin PltB subunits at the amino acid level (Supplementary Table 5), suggesting that the neutralizing epitopes characterized in this study would be able to neutralize typhoid toxin produced by drug-sensitive and resistant S. Typhi during human infection.
In summary, we conducted a series of structural and functional studies designed for learning about, for the first time, fundamentals of the molecular interactions between bacterial A₂B₅ toxins, glycan receptors, and glycan-receptor binding sites-targeting antibodies. The principle governing the toxin intrinsic interference phenomenon to A₂B₅ toxin neutralization offers insights into the advanced understanding of bacterial AB toxin biology in the context of host-pathogen interactions and the design of the more effective and prolonged anti-toxin strategies which relate to today’s significant public health concerns driven by antibiotic-resistant bacterial pathogens.
CHAPTER 7 : DISCUSSION
I. SUMMARY

Invasion of the intestinal mucosa by *Salmonella* Typhimurium (STm) is a complex process that involves bacterial transit through the small intestine, penetration of the mucus layer, adherence to the follicle-associated epithelium (FAE) of Peyer’s patch tissues, and entry into M cells to gain access to underlying phagocytes for further bacterial replication (Ohl & Miller, 2001). STm plays an active role in each of these steps through the expression of an array of virulence factors such as the O-antigen, which renders the bacterium resistant to antimicrobial peptides, flagella-based motility, which enables the bacterium to penetrate viscous environments, and the SPI-1 T3SS that facilitates uptake into epithelial cells. Other serovars of *S. enterica*, namely, STy, produce a toxin that is proposed to induce neurological complications. It is therefore remarkable that a single monoclonal IgA, namely Sal4, directed against O-antigen, is able to effectively prevent STm invasion of Peyer’s patch tissues. Michetti and others previously demonstrated *in vivo* that Sal4 IgA was able to reduce bacterial uptake into Peyer’s patches when transported passively through a so-called “backpack tumor” (Michetti et al., 1992). Sal4 IgA also blocked STm invasion of polarized epithelial monolayers that correlated with bacterial agglutination (Michetti et al., 1994). It has subsequently been demonstrated that Sal4 has a number of other effects on virulence *in vitro*, including inhibition of bacterial motility, suppression of SPI-1-mediated T3SS activity, alteration of outer membrane (OM) permeability and promotion of a biofilm-like phenotype that correlates with c-di-GMP production. Thus, Sal4 has been assigned a number of protective effects that could contribute to mucosal immunity to STm *in vivo*. 
The goal of my dissertation was to investigate the mechanisms by which Sal4 IgA limits STm entry into Peyer's patch tissues in a mouse model. This was to expand the knowledge on both the mechanisms of Sal4, as well as the potential of monoclonal antibodies to protect against a diarrheal pathogen as a deliverable biologic, since vaccination strategies against nontyphoidal Salmonella (NTS) are lacking. As described in Chapter 3, I developed a robust in vivo model of Peyer's patch invasion that relied on the administration of O4- and O5-polysaccharide STm strains in parallel to normalize the bacterial challenge inocula across animals. Furthermore, I found that a single dose of Sal4 IgA reduced uptake into Peyer's patches in a dose-dependent manner. I defined the minimal dose required for protection, which correlated with ability agglutinate in liquid culture. Using this model, I tested another O5-specific mAb, PeA3 IgA, which demonstrated Sal4-like activities, including motility arrest in soft agar and prevention of epithelial cell entry in vitro. Indeed, PeA3 IgA was of lower avidity than Sal4, as demonstrated by dot blot and LPS-ELISA. Upon titration in dose-response studies, I found that PeA3 was significantly less effective than Sal4 at protecting mouse Peyer's patches from STm entry. These results suggest that IgA avidity is critical in protection, possibly due to agglutination potential.

I used the STm infection model to examine the contribution of isotype in protection by testing a chimeric IgG1 variant of Sal4 alongside Sal4 IgA. Sal4 IgG was observed to bind purified STm LPS and whole bacterial cells by ELISA and attenuated STm virulence in in vitro assays equally as well as Sal4 IgA. However, Sal4 IgG was not able to prevent STm invasion into gut-associated lymphoid tissues (GALT) during
infection *in vivo*. This was due in part to half-life and degradation in the intestinal environment, though it is likely that valency was also a major contributor.

In Chapter 4, I performed tracking studies and demonstrated that STm cells primarily occupy the terminal ileum in the 20 minutes following infection and are predominantly detectable in distal Peyer’s patches at 3 hours, which is consistent with previously published reports (Carter & Collins, 1974). Tracking studies indicated that transit time through the intestine is rapid, with orally administered STm cells detected at the end of the gastrointestinal tract after 60 minutes. The kinetics of gavaged STm gave insight into experiments examining the prophylactic potential of Sal4, which was significantly reduced after 20 minutes. These data suggest that in a prophylactic approach, the window of protection is limited, likely due to an inability to sufficiently crosslink infectious STm after gastrointestinal dilution.

Considering the importance of STm crosslinking by Sal4 in these studies, I also examined recombinant variable region matched SIgA, dIgA, mIgA, and IgG forms of Sal4 in parallel (Chapter 5). I demonstrated by flow cytometry and fluorescence microscopy that IgA mAbs induced significantly larger STm aggregates than Sal4 IgG at equal concentrations. I confirmed *in vivo* that Sal4 SIgA reduced STm entry into mouse Peyer’s patches by several orders of magnitude, which was associated with dense agglutination of administered STm in the intestinal lumen as detected by immunohistochemistry. Collectively, these results emphasize the importance of dimeric IgA in protection against invasive STm over other antibody forms and reinforce agglutination by IgA as a major mechanism of protection to STm infection in the gut.
II. BIOLOGICAL ACTIVITIES OF SAL4

*Agglutination is a Major Driver of Protection by Polymeric Sal4 mAbs*

The results from my thesis work emphasize that agglutination by orally administered Sal4 is a primary mechanism of protection from invasive STm *in vivo*. In an examination of Sal4 mAbs of varying isotype and isoform (**Figure 7.1**), we consistently observed that the polymeric mAbs Sal4 dIgA and SIgA had the greatest efficacy in shielding the intestinal mucosa from STm infection. Reduced STm entry into mouse Peyer’s patches by IgA mAbs was further associated with greater agglutination of STm *in vitro* by flow cytometry and fluorescence microscopy-based assays. These data reinforce the paradigm of “immune exclusion” by IgA antibodies as a predominant mechanism of protection on mucosal surfaces in the gastrointestinal tract.

By way of natural immunity, antigen-specific SIgA antibodies positioned within the outer mucus layer are postulated to act by binding and sequestering pathogens and/or toxins through recognition of target epitopes (Corthésy, 2013; Stokes et al., 1975). Aggregation by SIgA can render infectious microbes avirulent through multiple mechanisms and further prevent access to epithelial surfaces. Once agglutinated, immune complexes are thought to be cleared from the host through mechanical forces such as peristalsis and mucociliary action (Brandtzaeg, 2013). This method of clearance of IgA appears to be specific for pathogenic microbes though, since IgA has been shown to coordinate with the intestinal microbiota to facilitate beneficial commensal colonization in the gut (Donaldson et al., 2018; Macpherson et al., 2018; Schofield & Palm, 2018).
Table: Sal4 mAbs used throughout this study.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Source</th>
<th>Species</th>
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<tr>
<td>Sal4 IgA</td>
<td>Hybridoma</td>
<td>Mouse</td>
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<tr>
<td>Sal4 IgG1</td>
<td><em>Nicotiana benthamiana</em></td>
<td>Human-Murine Chimera</td>
</tr>
<tr>
<td>Sal4 IgG</td>
<td>Hybridoma + Recombinant SC</td>
<td>Murine IgA Human SC</td>
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<td>Sal4 mlgA</td>
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<td>Human</td>
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<tr>
<td>Sal4 dlgA</td>
<td>Exp293 Cells</td>
<td>Human</td>
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<tr>
<td>Sal4 SlgA</td>
<td>Exp293 Cells</td>
<td>Human</td>
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**Figure 7.1. Sal4 mAbs used throughout this study.**

Yellow bars indicate identical heavy and light chain variable regions ($V_H$ and $V_L$ respectfully) across all Sal4 mAbs. Legend indicates presence of joining (J) chain in polymeric mAbs and secretory component (SC) on SlgA.
Indeed, multiple mechanisms are at play in antibody-mediated agglutination of STm in the gut. Moor et al. have demonstrated that aggregation by high-avidity IgA results in the enchained growth of segregating STm daughter cells, leading to clonal loss of STm inocula and a pathogenic disadvantage to the bacterial population (Moor et al., 2017). Enchained growth is proposed to occur in low density infections though, compared to high density conditions that are typically observed in mouse models.

Evidence indicates that agglutination by specific antibodies is an important mechanism of immunity for other mucosal pathogens aside from STm. Antibody crosslinking by purified IgG and IgA has been shown to be required for protection from Streptococcus pneumoniae colonization in the nasal carriage of infected animals during passive immunization (Roche et al., 2015). Following enzymatic digestion of administered antibodies into monovalent Fabs, protective activity was lost, highlighting the need for pathogen crosslinking to confer immunity (Roche et al., 2015). In vivo activity was determined to be Fc-independent in these experiments, further supporting that antibody binding and pathogen sequestration is a main mechanism of protection. This has been reinforced by other groups linking agglutination by mucosal antibodies to protection from nasal colonization in human samples (Binsker et al., 2020; Mitsi et al., 2017). Similar reports have linked agglutination by IgA to epithelial protection from Shigella flexneri and Vibrio cholerae (K. J. Levinson et al., 2015; Mathias et al., 2013).

In my studies I hypothesized that the degree of STm aggregation by Sal4 IgA was a major factor in preventing STm cells from invading Peyer’s patches in vivo. This was partly because earlier experiments demonstrated significant differences in in vivo protection between Sal4 IgA and IgG mAbs. Indeed, I observed that reduced Sal4 IgG
activity in the gut was partially due to instability of the antibody in the intestinal environment. However, instability alone did not fully account for differences between Sal4 IgA and IgG treatment groups, since buffered Sal4 IgG never reached equivalent levels of activity observed by polymeric Sal4 IgA, even in excessive amounts. This led me to hypothesize that insufficient crosslinking of STm in the gut by Sal4 IgG was a contributing factor in reduced activity in vivo. This was supported by preliminary in vitro data demonstrating comparable effects by Sal4 IgA and Sal4 IgG in motility and invasion assays, but qualitative disparities in STm agglutination in liquid culture. Upon further investigation, I reported significant differences in the magnitude of agglutination by Sal4 IgA and IgG antibodies by fluorescence microscopy and flow cytometry. These results were in accordance with studies highlighting an insufficient capacity of nonspecific plasma-derived IgG antibodies to sufficiently aggregate STm cells in an ileal loop model of infection (Bioley et al., 2017). While descriptive, I did also observe distinct patterns of STm clustering between Sal4 IgG and Sal4 SIgA in intestinal sections of infected animals. Taken together, these data emphasize agglutination of STm as an important mechanism of protection by orally administered Sal4.

**Agglutination Independent Mechanisms of Sal4 Activity**

My work supports previous reports from our lab demonstrating that Sal4 IgA imparts a biological impairment on STm that is independent of bacterial crosslinking. In addition to prompting rapid motility arrest (< 1 min), Forbes’ original studies showed that Sal4 IgA binding forces the production of a “fuzzy coat” by STm that is observable by transmission electron microscopy (TEM) and cryo-EM (Forbes et al., 2012). In these
experiments, the extracellular matrix (ECM) was produced relatively quickly after Sal4 treatment, detectable after only 15 minutes. As a follow-up, Amerasinghe et al. demonstrated that further prolonged exposure (> 4 h) of STm to Sal4 IgA led to the release of ECM that consisted of O-Antigen, colonic acid, and cellulose, resulting in a biofilm-like phenotype (Amarasinghe et al., 2013). The production of ECM by Sal4 IgA was associated with increased intracellular levels of the intracellular signaling molecule c-di-GMP, suggesting the transition to a nonmotile biofilm-type state (Ahmad et al., 2011; MacKenzie et al., 2017). In my studies examining Sal4 SIgA treatment in vivo, I observed a similar phenomenon in small intestinal sections of animals challenged with oral STm. Sal4 SIgA-STm complexes observed 40 minutes post-challenge were densely agglutinated and recalcitrant to detection by immunohistochemistry (IHC) staining, even when rigorous antigen-retrieval methods were employed. While qualitative, we hypothesized that the inaccessibility of STm cells by IHC was due to the production of a “fuzzy coat” observed in previous Sal4 studies. However, the phenotype in vivo was specific to Sal4 SIgA, as STm in vehicle and Sal4 IgG treated animals exhibited no resistance to primary antibody detection.

In vitro, we confirmed that Sal4 SIgA treatment significantly induced EPS production by STm after incubation for 1 hour, as measured by crystal violet (CV) staining. By comparing a panel of in-house STm biofilm mutants previously employed in the Amarasinghe studies, we determined that ECM triggered by Sal4 SIgA was partially composed of cellulose, as the cellulose biosynthesis mutants ΔbcsA and ΔbcsE were reduced in their EPS production compared to wildtype Sal4 SIgA-treated cells. However, further attempts to detect individual EPS components in STm-SIgA immune
complexes by IHC and additional staining methods proved to be ineffective. Therefore, additional experiments are necessary to elucidate and confirm the role of cellulose production in response to Sal4 SIgA. Indeed, Sal4 IgG treatment under similar *in vitro* conditions produced ECM on borosilicate glass tubes that was qualitatively different from Sal4 SIgA in density and distribution. Mutant strains treated with Sal4 IgG that were employed in this assay, including ∆*bcsA* and ∆*bcsE* mutants, were not significantly affected in EPS production compared to wildtype-treated cells. However, this *in vitro* assay can generate variability, and therefore further follow-up is required to investigate EPS phenotypes in response to Sal4 binding.

Based on these data, it is likely that immediate actions imparted by Sal4 render the bacteria avirulent prior to antibody-mediated agglutination. In the context of the mouse gut, STm cells that are rendered immobile and avirulent by direct antibody binding could be more susceptible to aggregation by dimeric antibodies, supplemented by mechanical forces within the host. While direct antibody binding by Sal4 under non-agglutinating conditions disrupts virulence, flagellar-based motility, and STm membrane integrity, it is clear that antibody crosslinking is required for protection. Under similar conditions *in vitro*, Sal4 IgG is capable of rendering STm cells immobile in soft agar and liquid culture at equivalent concentrations to Sal4 IgA. Additionally, Sal4 IgG is capable of blocking motility-independent invasion of STm into epithelial monolayers *in vitro* to the same capacity as Sal4 IgA. These data would suggest that direct antibody binding effects between the two isotypes are similar. Granted, this interpretation is limited to the experiments performed in this study. For example, I cannot conclude that Sal4 IgG treatment has identical effects on the outer membrane or intracellular c-di-GMP
production as Sal4 IgA (Amarasinghe et al., 2013; Forbes et al., 2012). Given that, it is possible that refined differences in the physiologic response and downstream effects to antibody binding do exist between Sal4 isotypes.

There is still much to be explored in the investigation of Sal4-mediated immunity to STm. The final experiments studying Sal4 activity demonstrated the induction of cellulose-dependent EPS by Sal4 SlgA treatment, but not by Sal4 IgG. It is possible that the two isoforms are promoting differences in downstream signaling of c-di-GMP, since c-di-GMP regulated genetic mutants bcsA and bcsE exhibited variable phenotypes between the two treatment groups (Fang et al., 2014). It is possible that binding to specific SlgA in the gut, like Sal4, promotes the transition to a biofilm-like phenotype through c-di-GMP signaling as a strategy to exit an immune host (MacKenzie et al., 2017; Pontes et al., 2015). Considering the potential transition in response to SlgA but not IgG, the effect of Sal4 SlgA and IgG on transmission of STm to other hosts should be examined.

*Proposed Model of Polymeric Sal4 IgA Activity In vivo*

Based on the previous Sal4 studies and my findings in this dissertation, I propose the following model of Peyer’s patch protection by orally administered dimeric Sal4 IgA (*Figure 7.2*). (1) Following STm challenge, STm cells encounter Sal4 dIgA/SlgA antibodies in the upper digestive tract and induce rapid motility arrest (< 1 min) upon binding, imploring a virulence deficit on the bacteria. (2) Further exposure to Sal4 and movement through the gut facilitates dense agglutination of the STm challenge inoculum. Dense agglutination of STm cells prevents access to M cells overlying Peyer’s patches, blocking invasion. Sal4-mediated inhibition of SPI-1-mediated T3SS
Figure 7.2. Proposed model of activity by orally administered dimeric Sal4 IgA in vivo.

SI = small intestine; EPS = exopolysaccharide. Schematic generated using BioRender.
activity independent of antibody-crosslinking also supports host protection at this step
(Forbes et al., 2008; Forbes et al., 2012). (3) Prolonged binding (> 15 min) to Sal4 in
IgA-mediated aggregates forces the production of EPS by STm cells in the small
intestinal lumen. The consequence of Sal4-induced EPS may have further implications
in STm transmission and/or survival outside of the host (MacKenzie et al., 2017).

III. PASSIVE IMMUNITY BY ORALLY ADMINISTERED ANTIBODIES

Feasibility of Oral Antibodies to Protect Against Enteric Disease

The current paradigm of passive immunity by oral antibodies is that by delivery of
breastmilk to a developing newborn, a neonate will receive maternal antibodies specific
for a number of infectious agents that the infant will likely encounter following delivery
(Brandtzaeg, 2010). This is believed to be primarily facilitated through SlgA, which
accounts for roughly 90% of the total antibody pool in human colostrum (Turin & Ochoa,
2014). For example, protection of newborns from V. cholerae diarrhea by SlgA
antibodies in maternal milk has been demonstrated in breastfed infants previously in
population studies (Glass et al., 1983). Considering the potential of oral antibodies to
protect the developing gut, a major goal of my dissertation was to establish a model of
passive immunity by mAbs to evaluate mechanisms of protection to enteric disease,
with an emphasis on examining the potential of orally administered IgA.

Experimentally, orally delivered antibodies have also demonstrated the potential
to protect against diarrheal pathogens, such as enterotoxigenic E. coli (ETEC) and
Vibrio cholerae. Using milk-derived immunoglobulins from vaccinated dairy cows, oral
prophylaxis of IgG specific for the colonization antigen (CFA) CFA/I has been shown to
provide up to 90% protection against ETEC-induced diarrhea in human challenge studies (Freedman et al., 1998). In these human ETEC experiments though, the supplied antibody is largely polyclonal (Otto et al., 2011; Savarino et al., 2019; Sears et al., 2017a).

Oral passive immunization strategies in avian and mouse models have provided proof-of-concept for antibodies to protect against mucosal *Salmonella enterica* infection. In ducklings, oral delivery of *S. Enteritidis*-specific polyclonal antibodies derived from eggs was sufficient to prevent *Salmonella* colonization and dissemination when supplied in drinking water (Fulton et al., 2002). Chicken yolk antibodies have been confirmed to protect against gastrointestinal infection by both STm and *S. Enteritidis* in mice as well when given via oral administration (Yokoyama et al., 1998). However, it goes without saying that these examples are limited to the use of chicken-derived antibodies in the mucosal compartment and likely do not fully recapitulate any beneficial effects facilitated by secretory antibodies, such as mucus layer localization or potential interactions with the microbiota (Gibbins et al., 2015; Pabst et al., 2016).

At the beginning of this study, the most promising example of oral passive immunization to STm infection was reported by Bioley and others. In a series of experiments, the authors demonstrated the capacity of pooled polyreactive antibodies from human plasma to reduce intestinal inflammation and STm invasion of the mucosa by oral delivery of antibody-STm immune complexes (Bioley et al., 2017). Oral plasma-derived antibodies that were polymeric significantly blocked STm entry into Peyer’s patches and reduced bacterial dissemination into the spleens and mesenteric lymph nodes (MLNs) of infected animals in these experiments. A major caveat of these
studies, though, is that the antibody repertoire provided is not specific to protective epitopes of STm. Consequently, a larger dose of plasma-derived immunoglobulin was required to facilitate efficacy (≥ 1 mg), as well as in a pre-mixing administration strategy. This left an avenue of questions waiting to be explored regarding the protective capacity of STm-specific monoclonal antibodies by way of oral delivery.

Sal4 IgA as a Prototypic Protective mAb

In this thesis, I established a mouse model to examine passive immunity to mucosal STm infection conferred by monoclonal antibodies, using Sal4 as a model protective antibody. I demonstrated that a single dose of Sal4 IgA, either pre-complexed or pre-administered with STm was sufficient at reducing STm invasion into mouse Peyer’s patches. This is considerable in the field in an oral delivery strategy, as other groups require multiple doses of antibody to achieve protection from oral Salmonella (Corthésy et al., 2018; Fulton et al., 2002; Yokoyama et al., 1998). The robustness of the competitive index in normalizing the challenge inocula provided enough reproducibility between animals to reliably titrate Sal4 levels and establish a minimal protective dose. As little as 0.4 μg per animal by co-administration was sufficient to significantly reduce STm invasion (Chapter 3), compared to the ≥ 1 mg required observed in the studies by Bioley and others.

These data demonstrate two major points: (1) that mAbs specific for known protective STm epitopes are more efficient than polyreactive antibodies at protecting the gut during infection, likely due to enhanced avidity and subsequent agglutination, and (2) that employing a competitive index provides greater resolution experimentally into
the ability of antibodies to protect Peyer’s patches than total CFU burden alone. Presumably, enhanced protection by Sal4 is due to greater antibody avidity for its antigenic target, as high-avidity IgA generated from oral vaccination has been shown to facilitate clearance and protection from STm in the gut (Moor et al., 2017). Like Sal4, the protective IgA was also largely dependent on targeting the STm O-Antigen in these studies. However, it is clear that epitope specificity does not guarantee strong avidity, as PeA3 IgA was found to have a lower avidity than Sal4 by dot blot and ELISA despite also targeting the O5-polysaccharide. The weaker avidity demonstrated by PeA3 was later found to correlate with reduced activity in vivo at lower concentrations. As shown in Chapter 3, while the highest doses of Sal4 IgA in complex with STm were capable of reducing wildtype STm Peyer’s patch burden by multiple orders of magnitude over the O4-mutant strain, this effect was not observable following further titration below 4 μg. Therefore, it is reasonable to believe that minute amounts of high-avidity Sal4 IgA (< 4 μg) are imparting a deficit in STm fitness locally in the gut that are not discernable by CFUs alone at a log_{10} scale. The employment of a competitive index assay, like the one I used to study Sal4, is not novel for investigating enteric pathogen virulence in the gut (D. E. Baranova et al., 2020; Kohli et al., 2018). However, these data do make the case for using a competitive index strategy to investigate minute dynamics in bacterial fitness that influence protection, such as bacterial entry into the GALT or elsewhere. Taken together, these studies illuminate the requirements for oral mAbs to protect the gut from STm infection and emphasize that avidity of the antibody is critical for protection as an oral deliverable, which have implications for future therapeutic antibody design.
Implications for Future Use

My studies investigating Sal4 act as a proof-of-concept for the potential of oral antibodies to protect the gut from diarrheal pathogens but indicate that there are a number of hurdles that must be overcome biologically in order to achieve real efficacy. In this model, passive immunization is mediated by oral delivery of antibodies that must migrate through the digestive tract to the site of infection. The tracking studies described in Chapters 4 and 5 revealed that antibody transits through the digestive tract quickly, which likely restricts antibody retention in the gut and prevents long-term localization of administered mAbs. Attempts to track orally gavaged antibodies by immunohistochemistry were largely unsuccessful, indicating either dilution or transit of Sal4 IgA through the mouse gut. This lack of sufficient local Sal4 also reasonably accounts for the dearth of meaningful prophylactic activity by Sal4 in Chapter 3. Considering these issues, a greater amount of antibody and additional formulation techniques to maintain a local GI presence would be required for future use clinically. Another consideration is that the potential use of Sal4 IgA as an oral therapeutic would be highly restricted, since Sal4 is only functional for S. enterica serovars that express the O5-polysaccharide. S. enterica serovars are partially defined by their O-antigen modifications on LPS, and of the over 2500 serovars of S. enterica, only a subset present the O5 modification (Grimont & Weil, 2007). Even within the serovar Typhimurium, non-O5-bearing strains circulate and observe no significant deficit in infectivity in the absence of the O5-epitope (Slauch et al., 1995). As such, the actual therapeutic use of Sal4 to treat Salmonella infections is limited. However, these studies highlight the robustness of the oral challenge model to examine refined mechanisms of
antibody-mediated immunity, which could feasibly be adapted to study oral passive immunization to other *S. enterica* serovars.

*Limitations of the Model*

A major caveat to these studies is that only one STm epitope, the O5-Antigen of the bacterial LPS, was examined for its role in immunity. Anti-LPS antibodies are known to protect against STm, as the O-polysaccharide grants resistance to host antimicrobial molecules and assists in evasion of the immune system by impeding access to the bacterial outer membrane by way of its multiple repeating units (Dominguez-Medina et al., 2020; Peterson & McGroarty, 1985; Rondini et al., 2013; Slauch et al., 1995; Watson et al., 1992). While the LPS of NTS serovars is thought to be the predominantly protective epitope, antibodies directed against STm flagellin subunits, outer membrane proteins, and components of the T3SS have also demonstrated protective activity *in vitro* and protection *in vivo* (Baliban et al., 2018; Jneid et al., 2020; Kurtz et al., 2014; Reddy et al., 2020). Nevertheless, my studies, and others, indicate that agglutination by polymeric antibodies is a major mechanism of protection against STm in the gut and is demonstrated in the context of an anti-LPS humoral response (Forbes et al., 2008; Iankov et al., 2001; Michetti et al., 1992; Mitov et al., 2003). It is likely that the mechanisms of protection differ between antibodies directed against LPS versus other epitopes. One could speculate that anti-LPS antibodies primarily act through motility arrest and agglutination, while other antibodies, such as those directed towards T3SS-associated proteins, are likely functioning through direct neutralization of bacterial virulence factors.
As discussed throughout, the intragastric challenge model uses Peyer’s patch entry as a means of establishing a threshold of protection, which is representative of a bottleneck for further dissemination within the host (Lim et al., 2014). This limits the scope of conclusions on determinants of protection to that of invasion. However, in developed regions NTS infections predominantly present as self-limiting gastroenteritis that is characterized by severe inflammation, diarrhea, and vomiting (Coburn et al., 2007). Pathologically, in humans it has been observed that the bacteria primarily colonize and induce inflammation of the ileum and large intestine (Boyd, 1985). Similar observations of STm colonization in a mouse model of streptomycin-induced colitis have been reported (Barthel et al., 2003). Knowing this, it would be beneficial to further explore the capacity of oral Sal4 IgA to protect against STm colonization and intestinal inflammation in a similar model.

Antigen-specific IgA initiated through vaccination has been demonstrated to prevent inflammation induced by STm in mice (Moor et al., 2017; Proietti et al., 2019), and oral dosing of SIgA mAbs is sufficient to reduce Campylobacter jejuni tissue disruption and the intestinal inflammatory marker lipocalin-2 (LCN2) (Perruzza et al., 2020). Therefore, I would hypothesize that Sal4 could protect under similar conditions, albeit at experimentally comparable doses.

IV. ANTIBODY ISOTYPE IN IMMUNITY TO ORAL STM

Contribution of Sal4 IgG

One of the goals of these studies was to test whether IgG antibodies specific for STm could protect in the mucosal compartment as an oral deliverable. It is largely
recognized that SIgA antibodies are the predominant antibody isotype involved in protection to STm infection at the mucosal surface of the gut (Corthésy, 2010; Mantis & Forbes, 2010; Michetti et al., 1992). High-avidity IgA antibodies induced by oral vaccination of STm are sufficient to protect against intestinal inflammation and colitis in a streptomycin-induced model of STm cecum colonization (Moor et al., 2017). However, if STm infection progresses invasively, the bacteria will likely encounter humoral immunity of the systemic compartment in the form of IgG antibodies. Protection of invasive NTS (iNTS) by antigen-specific IgG in the serum has been well-documented in preventing extracellular growth through complement activation during bacteremia (MacLennan et al., 2008). STm-specific mAbs administered by intraperitoneal (i.p.) injection protect against systemic STm challenge in mouse studies (Colwell et al., 1984). These reports emphasize the ability of IgG antibodies to protect against STm in both mice and humans, suggesting a possible role for Sal4 IgG in my passive immunity model. By generating a Sal4 IgG mAb and testing it in parallel alongside Sal4 IgA, I could answer two questions independent of Fab-antigen binding: (1) what is the role of antibody isotype in protection, and (2) what is the importance of antibody crosslinking in Peyer’s patch protection by oral mAbs? Additionally, functional studies examining the activity of Sal4 IgG provided insight into the feasibility of using IgG antibodies in the gastrointestinal compartment. This is of particular importance considering that technological platforms to generate large amounts of IgG mAbs for therapeutic use are already established (Lu et al., 2020).

Considering the studies validating the utility of polyclonal and monoclonal IgG to prevent diarrheal disease in the gut, as well as the body of literature confirming the
protective efficacy of IgG mAbs to STm, I hypothesized that an IgG form of Sal4 would have some activity against STm in my in vivo model. While quantities of IgG on the mucosal surface are believed to be minimal, orally administered IgG antibodies have experimentally demonstrated efficacy to prevent diarrheal disease prophylactically. Oral delivery of the IgG mAb ZAC-3, specific for the LPS of V. cholerae, was shown to reduce bacterial colonization after challenge in neonatal mouse experiments (Levinson et al., 2016). The recent ETEC studies in humans demonstrating protection from diarrhea by hyperimmune bovine colostrum (HBC) products are largely facilitated by oral dosing of IgG (Savarino et al., 2019; Sears et al., 2017a). However, my results in Chapters 3 and 5 show that Sal4 IgG has little activity at preventing STm invasion in vivo compared to other Sal4 isotypes. This was not due to a lack of biological activity in vivo though, as Sal4 IgG dosing by (i.p.) injection significantly reduced the STm burden in the spleens and livers of systemically infected animals by multiple orders of magnitude. I partially attributed the reduced activity to instability in the gut, which is supported by previous literature suggesting reduced half-life within the gastrointestinal tract as a pharmacological detriment (Hu et al., 2019; Lee et al., 2012; Volpatti et al., 2019). Differences in the pH and enzymatic composition of the gut may account for why an IgG mAb, like ZAC-3, prevents V. cholerae colonization via the peroral route, since the neonatal gastrointestinal conditions are less developed than those of an adult in mice and humans (Bourlieu et al., 2014; Neal-Kluever et al., 2019).
Role of IgA Isoform in Sal4 Activity

I observed a hierarchy of Sal4 activity in these studies, with Sal4 dIgA and SIgA mAbs exhibiting the greatest activity in terms of preventing STm entry into Peyer’s patches. As discussed previously, enhanced efficacy by polymeric Sal4 is likely due to antibody crosslinking and agglutination. Contrary to my original hypothesis, I did not observe any additional benefit afforded by secretory component (SC) in the ability of Sal4 to block STm entry in vivo. SC is reported to afford a number of benefits to SIgA molecules within the intestinal environment, such as enhanced stability and pathogen binding via glycosylation (Crottet & Corthésy, 1998; Mathias & Corthésy, 2011). However, in the instance of either a vaccination approach or natural immunity, SIgA is transported across the epithelium and favorably positioned in the mucus layer alongside other innate factors like antimicrobial peptides (AMPs) and commensal bacteria (Chairatana & Nolan, 2017). Considering the oral delivery strategy, we cannot be certain that oral IgA would retain any coordinative benefits with other intestinal components.

I found that mucosal protection from STm by Sal4 was more complex than simple bacterial crosslinking by dimeric mAbs. To demonstrate the importance of antibody isotype, I utilized a panel of recombinant human Sal4 IgA mAbs of varying isoforms (Chapter 5) and characterized them in parallel (Figure 7.1). Despite having the same number of Fab bindings sites as Sal4 IgG, Sal4 mIgA was able to significantly block STm invasion into mouse Peyer’s patches in vivo to equivalent levels as Sal4 dIgA at a high dose (50 μg). Sal4 mIgA antibodies were also capable of significantly agglutinating STm inoculum to a greater magnitude than Sal4 IgG, as demonstrated two different
methodologies (e.g., flow cytometry and fluorescence microscopy). These findings contrasted with my original hypothesis, where I anticipated similar levels of protection and agglutination activity between Sal4 mIgA and IgG, since the valencies of the antibodies were the same. My results also deviate from what has been reported in S. pneumoniae experiments, where purified mIgA mAbs were determined to be insufficient at agglutinating bacterial cells compared to polymeric IgA forms (Fasching et al., 2007). However, differences in epitope availability, downstream antibody binding effects, or differing host niches between the two pathogens could account for these discrepancies. Additionally, glycosylation of Sal4 mIgA may be playing a role in agglutinating STm and preventing entry into Peyer’s patches in vivo. Sal4 mIgA is an IgA2 m(2) isoform and possesses four N-glycosylation sites, while IgG only contains one (Steffen et al., 2020; Woof & Burton, 2004). N-glycans on secretory IgA have been assigned a number of functions thought to improve efficacy of the antibody, which could be attributed to the activity observed by Sal4 mIgA in these experiments (Mathias & Corthésy, 2011; Raskova Kafkova et al., 2020; Royle et al., 2003). While further investigation into the exact mechanism is needed, these experiments demonstrate that there is an intrinsic benefit to IgA in the gut independent of valency.

V. PASSIVE IMMUNITY TO TYPHOID TOXIN BY IGG MABS

Another goal of this thesis project was to investigate the potential of IgG mAbs to passively immunize against typhoid toxin, and to determine protective epitopes on the toxin molecule. This project was in collaboration with Dr. Jeongmin Song’s lab at Cornell University, who had previously resolved the structure of typhoid toxin as well as its
mode-of-action in human epithelial cells in vitro and toxicity in mice (Chang et al., 2016; Deng et al., 2014; Song et al., 2013). Typhoid toxin-specific antibodies have been detected in human sera following resolution of STy infection, suggesting a potential role for antibodies in immunity to the toxin during STy infection (Charles et al., 2010; Liang et al., 2013). At the beginning of the study, little was known regarding the activity of anti-typhoid toxin antibodies or of any possible neutralizing epitopes on the toxin. However, other groups have recently reported about the potential of mAbs to neutralize typhoid toxin, further supporting the investigation of mAbs for therapeutic use of typhoid fever (Jiao et al., 2020).

In collaboration with the Song lab, we generated a panel of mAbs against the varying subunits of typhoid toxin using hybridoma technology and characterized the neutralizing and binding activity in vitro and in vivo. The Song lab independently sought to resolve the crystal structures of mAb-toxin complexes to elucidate identify neutralizing epitopes on the toxin. IgG mAbs generated from mice immunized with purified recombinant typhoid toxoid were predominantly directed against the pentameric binding subunit, PltB. This was not surprising, considering that the PltB subunit is responsible for facilitating entry into host cells after binding to specific glycans on the cell surface, promoting internalization (Song et al., 2013). After sequencing the original B cell hybridomas, we determined that the panel of 9 total mAbs originated from three separate B cell clones, despite originating from different animals. This highlighted the immunodominance for PltB following vaccination.

Given specificity for the binding subunit, it was determined that anti-PltB TyTx mAbs primarily neutralized typhoid toxin by blocking access to host cell surfaces and
preventing subsequent intracellular trafficking \textit{in vitro}. mAbs specific for the binding subunit of other toxins, such as ricin toxin’s RTB subunit, have demonstrated efficacy \textit{in vitro} and \textit{in vivo} by blocking toxin binding to cell surfaces in a similar manner (Lemley et al., 1994). Of the three TyTx mAbs, TyTx1 and TyTx3 were found to be modest neutralizers, while TyTx4 had strong neutralizing activity. These data were associated with different PltB binding epitopes between the mAbs, with TyTx1 and TyTx3 binding the laterally-located side of the pentamer and TyTx4 binding the far-side epitope, as resolved by the Song lab by cryo-EM. Based on these data, it is likely that the TyTx4 epitope facilitates more efficient binding and accessibility than the laterally-located epitopes occupied by TyTx1 and 3. This was supported by stronger binding avidity of TyTx4 for typhoid toxin, as measured by surface plasmon resonance (SPR), as well as a higher stoichiometric binding ratio (~4-5 TyTx4 Fabs to 1 toxin molecule) than TyTx1 and 3 mAbs. In a separate study examining the ability of TyTx mAbs to protect against intoxication \textit{in vivo}, all TyTx mAbs significantly protected mice from a lethal challenge of typhoid toxin, with TyTx4 requiring substantially less antibody than TyTx1 and 3 to achieve protection. Considering the possibility that typhoid toxin asymmetry may render neutralizing epitopes inaccessible by the humoral immune response, these data provide insight into protective targets required for either antibody-based therapeutic design or vaccination strategies.

While these results emphasize the capacity of mAbs to protect against typhoid toxin and illuminate mechanisms of immunity, there are limitations in the interpretations of the study. For one, STy is a human-restricted pathogen, and the \textit{in vivo} experiments are performed in mice. \textit{In vivo}, a lethal dose of toxin is administered to measure
protection by TyTx mAbs (Ahn et al., 2021), but it is not known if these concentrations recapitulate those observed in natural human infection. An additional consideration is that typhoid toxin is produced and secreted by STy intracellularly after bacterial invasion of host cells (Chang et al., 2016). Therefore, any therapeutic benefit of antibody treatment would likely be limited to neutralization of the toxin and not the bacterial pathogen. Indeed, studies examining the role typhoid toxin during STy infection in humanized mice indicated that the presence of the toxin helped facilitate persistent STy infection, so additional benefits of therapeutic antibody use may exist (Song et al., 2010). Despite the limitations, these data emphasize the capacity of mAbs to passively immunize against the effects of typhoid toxin intoxication and support the use of antibodies as potential therapeutics for typhoid fever.

VI. CONCLUSIONS

The findings from my dissertation research contribute three main points to the field of passive immunity to STm: (1) oral mAbs are capable of protecting the gut from STm infection, (2) immunity is dependent on antibody molecular form, i.e., IgA, and (3) agglutination is a major mechanism of protection for the intestinal mucosa by orally administered antibodies. My data provide additional insight into the mechanisms of Sal4-mediated immunity and how direct binding effects in vitro are recapitulated in vivo. I also showed for the first time the importance of mAb isoform in oral immunity to STm by characterizing multiple human recombinant variants in parallel. The studies presented in my thesis emphasize the potential of mAbs to protect against multiple serovars of Salmonella enterica, highlighting the ability of antibodies to attenuate
virulence by toxin neutralization and direct effects on the bacterial cell. Finally, these results demonstrate the feasibility of oral passive immunization by antibodies, while revealing barriers in terms of formulation and delivery that must be overcome for future therapeutic use.
APPENDIX 1: SUPPLEMENTAL FIGURES CHAPTER 3
Figure A1.1. Sal4 IgA binding to AR04 and AR05 by whole-bacteria ELISA.

Sal4 IgA reactivity to STm strains AR04 and AR05 by whole-cell ELISA, as described in the Materials and Methods.
Figure A1.2. Schematic of workflow for STm intragastric challenge model.

(1) A 1:1 mixture of wildtype (AR05) and mutant (AR04) STm is incubated with antibody for 10 minutes. (2) BALB/c mice are challenged with antibody treated STm inoculum (4 x 10^7 CFUs total per mouse). (3) 24 h post-infection mice are euthanized and Peyer’s patches from each mouse are collected and homogenized. (4) Tissue homogenates are plated on LB agar containing kanamycin (50 µg/mL) and X-Gal (40 µg/mL) to evaluate antibody-dependent changes on STm infection by blue-white screening. Images generated using Microsoft Office suite.
Figure A1.3. PeA3 IgA in vitro characterization.

A) Sal4 IgA and PeA3 IgA reactivity to STm purified LPS (Sigma), STm strains CS022 and SJF59, and S. Enteritidis by ELISA. (+) indicates positive binding, while (-) indicates no binding detected above background levels. (B) Agglutination of STm liquid culture by 15 μg/mL of Sal4 IgA and PeA3 IgA after incubation at 37°C for 60 minutes. (C) Effect of Sal4 IgA and PeA3 IgA (15 μg/mL) on STm motility in 0.3% soft agar. Plates were incubated at 37°C and the diameter of bacterial swimming was measured every hour for 6 hours. Asterisks indicate significant reduction in wildtype STm motility over the isotype control, as determined by Kruskal-Wallis and Dunn’s multiple comparisons tests at each
time point (P < 0.05). (D) Binding of Sal4 IgA and PeA3 IgA to purified STm LPS by ELISA. For additional experimental details see Materials and Methods.
Figure A1.4. Sal4 IgA prophylactic activity in PBS.

BALB/c mice were passively immunized orally with either control (2D6 IgA) or Sal4 IgA antibody treatment in PBS at the indicated doses. 20 minutes later mice were challenged with a 1:1 mixture of STm strains AR04 and AR05 (4 x 10⁷ CFUs/mouse). 24 h post-infection Peyer’s patches were harvested and enumerated for CIs, as described in the Materials and Methods.
Figure A1.5. Sal4 IgG binds to STm O5-Antigen and competes with Sal4 IgA.

(A) Sal4 IgG reacts to STm strain AR05, but not AR04, as determined by whole-cell ELISA (described in the Materials and Methods). (B) Sal4 IgG binding to purified STm LPS by ELISA and Sal4 IgG competition ELISA with Sal4 IgA. Sal4 IgA at the indicated concentrations was applied to purified STm LPS-coated plates and incubated for 1 h at...
room temperature. Plates were washed three times with PBST, and 10 µg/mL of Sal4 IgG was applied and incubated for an additional hour. Plates were then developed using goat anti-human HRP-conjugated secondary IgG antibody and SureBlue TMB Microwell Peroxidase Substrate to evaluate Sal4 IgG inhibition by Sal4 IgA.
Figure A1.6. Sal4 IgG and IgA significantly reduce bacterial burden in systemic organs following systemic STm challenge.

BALB/c mice were passively immunized with (A and B) Sal4 IgG or (C and D) Sal4 IgA at the indicated doses by intraperitoneal injection 24 h prior to a systemic lethal challenge of STm (1 x 10^4 CFUs). Control mice received isotype control-matched mAbs
(PB10 IgG, 2D6 IgA) as described in the Materials and Methods. For technical reasons, the 2D6 IgA treatment group received only 21 μg as opposed to 40 μg. 24 h post-infection, mice were euthanized, and the spleens and livers were harvested, homogenized, and plated for CFUs on LB agar. Asterisks indicate significant reduction bacterial burden compared to isotype control treatment as determined by one-way ANOVA and Tukey’s post-hoc test; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. 
Figure A1.7. Sal4 IgG does not significantly block invasion *in vivo* after multiple administrations.

BALB/c mice were orally administered 190 μg of isotype control (PB10 IgG) or Sal4 IgG antibody treatment in PBS in multiple doses at 2.5 h and 20 min before STm challenge (4 x 10⁷ CFUs of AR04 and AR05) and 15 min and 4.5 h following challenge for a total dose of 760 μg per mouse. 24 h post-infection Peyer’s patches were harvested and enumerated for CFUs and CIs as described in the Materials and Methods. No statistical significance between the control and Sal4 IgG treatment groups was observed, as determined by unpaired Student’s *t*-test (*P* = 0.35).
Figure A1.8. Intraperitoneal administration of Sal4 IgG does not significantly block invasion \textit{in vivo} after oral STm challenge.

200 μg of either Sal4 IgG or isotype control antibody (PB10 IgG) was administered via intraperitoneal injection. This corresponded to an average of 39.9 μg/mL (± SD of 5.8) of Sal4 IgG present in the serum of mice at the time of challenge as determined by ELISA (samples from \( n = 3 \) mice). 24 h after antibody administration, mice were challenged orally with STm inoculum containing a 1:1 mixture of strains AR04 (mutant) and AR05 (wildtype). 24 h post-infection, mice were euthanized, and Peyer’s patches harvested and enumerated for CIs, as described in the Materials \& Methods section. No statistical significance between the control and Sal4 IgG treatment groups was observed, as determined by unpaired Student’s \( t \)-test (\( P = 0.74 \)).
APPENDIX 2: SUPPLEMENTAL FIGURES CHAPTER 5
Figure A2.1. Characterization of human recombinant Sal4 mlgA and dIgA mAbs.

(A) SEC-UHPLC of purified (A) Sal4 mlgA, (B) dIgA, and (C) SlgA confirming correct isoform size.
Figure A2.2. Sal4 mAbs are specific for the O5-polysaccharide.

Sal4 mAbs (A) Sal4 mlgA, (B) dIgA, and (C) SIgA, react with STm wildtype strain AR05 (filled circles) but not mutant strain AR04 (open circles), which lacks the O5 epitope, by whole-cell ELISA. Graph depicts two technical replicates and are representative of two biological replicates.
Figure A2.3. Sal4 mlgA and dIgA agglutinate live STm cells by flow cytometry.

Mid-log phase cultures of AR05 were washed in PBS and incubated with 2, 20, or 200 μg/mL Sal4 mlgA, dIgA, or control SIgA for 1 h at 37°C. 10,000 events per sample were analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA) by forward scatter (FSC) and side scatter (SSC) to visualize aggregate size and granularity, as described previously. Representative flow cytometry plots showing (A) Sal4 mlgA and (B) Sal4 dIgA. Results represent data from three separate biological experiments.
Figure A2.4. Sal4 SIgA blocks STm entry into Peyer’s patches at time of challenge.

(A & B). BALB/c female mice were orally administered 50 μg Sal4 SIgA or isotype control antibody in (A) PBS or (B) sodium bicarbonate and protease inhibitors either immediately before, 20 or 40 min before a 1:1 challenge with AR04 and AR05 STm strains (~4 × 10⁷ CFUs). 24 h post-infection Peyer’s patches were isolated, homogenized, and plated, as described. Shown are the results of two separate experiments with at least 4 mice per group. Statistical significance was assessed by one-way ANOVA followed by Tukey’s post hoc multiple comparisons test.
Figure A2.5. Sal4 SlgA-treated STm cells are refractory to detection by immunohistochemistry \textit{in vivo}.

(A-D) Sal4 SlgA treatment induced robust agglutination in the intestines of mice challenged with STm strain ATCC14028 40 min post-infection and was associated with reduced staining of STm cells by rabbit Salmonella Group B-specific antiserum (BD Difco). Positively stained cells (green) were primarily found at the periphery of STm aggregates within the lumen. Cells at the center of aggregates were resistant to staining and chromogen detection. Scale bars indicate 5 μm.
APPENDIX 3: SUPPLEMENTAL METHODS CHAPTER 6
S. Typhi strains and growth conditions

For S. Typhi experiments, wildtype and CdtB catalytic mutant S. Typhi strains ISP2825 have been described previously (Song et al., 2013). Prior to infection experiments, S. Typhi strains were grown at 37 °C in 2 mL LB broth containing 0.3M NaCl to an OD$_{600}$ of ~0.9 after inoculation from an overnight culture in 2 ml LB broth at a dilution of 1:50.

TyTx competition ELISA

Protein G ELISA plates were incubated with 1 μg/ml of capture mAb in 100 μl of 50 mM carbonate-bicarbonate buffer, pH 9.6 for overnight at 4 °C. After washing with PBST and blocking for 1 hr at 37 °C, the plates were incubated with 100 ng/ml of biotinylated PltB homopentamer and 0.1-10 μg/ml of competitor mAb diluted in block for 2 h at 37°C. Bound biotinylated PltB was detected with streptavidin-HRP-conjugated anti-mouse IgG (Bio-Rad Laboratories) at a 1:10,000 dilution, as detailed in the Materials and Methods section (Chapter 2).

Mammalian cell culture conditions for STy experiments

Human intestinal epithelial Henle-407 cells and human peripheral blood T lymphocyte Jurkat cells were cultured in DMEM high glucose (Invitrogen) and RPMI-1640 (Invitrogen) supplemented with 10% FBS (Hyclone cat# SH30396.03, Lot# AD14962284), respectively. Sialic acid contents of the FBS used were validated, which was ~99% Neu5Ac and less than 1% Neu5Gc. Cells were kept at 37°C in a cell culture
incubator with 5% CO2. Mycoplasma testing was conducted regularly as part of the cell maintenance practice.

**Recombinant typhoid toxin, typhoid toxoid, and PltB pentamer**

Overexpression and purification of typhoid toxin (containing the CdtB-His6), typhoid toxoid (containing CdtB-His6), and PltB-His6 homopentamer were carried out as previously described (Abreu, 2010; Deng et al., 2014; Song et al., 2013; Yang, Lee, et al., 2018). When indicated, purified toxins were fluorescently labeled with either Alexa Fluor-555 dye (Molecular Probes, Thermo Fisher Scientific) according to the vendor’s recommendation.

**Cloning, expression, and purification of tagless PltB pentamer**

The tagless PltB expression construct, named pJS0051, was generated by removal of the A subunit genes from the pSB5022 plasmid for expressing wildtype typhoid toxin complex with 6xHis tagged CdtB (Song et al., 2013). The following primer pairs were used for the process: F: 5’-GCTTTGGACCCAAGTAATGAGATCCGGCTGCTAACAAAGC-3’ and R: 5’-CTCATTACTTTGGGTCCAAGAGC-3’. Gibson assembly was used to generate the construct and the sequence was confirmed via Sanger sequencing by the Cornell Biotechnology Resource Center Genomic Core. The following procedures were conducted to purify the tagless PltB pentamer.
Preparation of TyTx1 antibody-conjugated agarose resin

TyTx1 antibody was conjugated onto resin using NHS activated agarose slurry (ThermoFisher Scientific, Pierce #26200) using the provided protocol with minor modification for gravity column chromatography. In brief, 1 ml of settle resin was transferred, washed with PBS, and incubated with PBS containing 14 mg of TyTx1 for 1 hr at room temperature with light rocking for the conjugation to occur. After washing to remove unconjugated proteins, the resin was then quenched by repeated washing with 10 ml of 0.5 M Tris-HCl, pH 8.0, and stored at 4°C until use.

Purification of tagless PltB

E. coli BL21(DE3) ΔendA ΔrecA (E. coli Acella strain; Edgebio) carrying pJS0051 was grown in LB broth to approximately OD600 = 0.7 at 37°C, transferred to 28°C, added with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubated for 16 hr. The harvested bacteria pellet was resuspended in a buffer containing 15 mM Tris-HCl, pH 8.0 and 150 mM NaCl, 1x EDTA-free protease inhibitor cocktail, 0.2 mg/ml lysozyme, and 80 μg/ml DNAse I, subjected to 3 times of the freeze-thaw step in liquid nitrogen and lysed by sonication. The clarified lysate was mixed with the prepared TyTx1 conjugated agarose and incubated at 4°C for 30 minutes with gentle rocking. The resin was recaptured by passing the lysate through a gravity column and washed with 15 mM Tris-HCl, pH 8.0, 150 mM NaCl. The protein was eluted with 5 ml of 100 mM Glycine, pH 3.0, and the eluted protein was quickly neutralized with 1 ml of 1 M Tris-HCl, pH 8.8. The purified protein was examined via 15% SDS-PAGE, SEC, and ELISA.
TyTx mAb variable region sequencing

Antibody sequencing was conducted based on a recently published method (Meyer et al., 2019) except for TyTx1 HC where a different reverse transcriptase and PCR reactions were used. In brief, total RNA was extracted from the hybridoma cells using the RNeasy mini kit (Qiagen). The cDNA 674 synthesis and PCR amplification of antibody variable regions were performed. The iScript Select cDNA Synthesis Kit (Bio-Rad) and mouse IgG reverse transcription primers (Table A5.2) were used for reverse transcription. A touch-down/step-down PCR was occurred with universal forward primer and reverse PCR primer based on the antibody chain (Table A5.2) to amplify the antibody variable regions. The amplicon appeared between 550-600 base pairs on 1 % agarose gel. DNA was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). The extracted DNA was Sanger-sequenced using the IgG PCR reverse primers (Table A5.2). For the TyTx1 heavy chain variable region, total RNA isolated from hybridoma cells was subjected to RT-PCR with AffinityScript (Agilent) reverse transcriptase together with Herculase II polymerase (Agilent) as described in the one-step RT-PCR protocol provided by the vendor. DNA band corresponded to the expected antibody variable region was excised, extracted, further amplified using Herculase II polymerase using the nested primers pair, and Sanger-sequenced.

Typhoid toxin intoxication assays

Jurkat cell intoxication assays: Jurkat cell intoxication assays were performed to evaluate mAb-mediated neutralization in the context of purified typhoid toxin treatment. In brief, Jurkat cells were cultured in RPMI1640 + 10% FBS (HyClone) + 1 mM sodium
pyruvate (Invitrogen) + 10 mM HEPES (Invitrogen), and kept at 37°C in a cell culture incubator with 5% CO₂. Cells were seeded at 1x10⁵/well into 12-well culture plates and treated with either 0.3 pM 6xHis-tagged typhoid toxin alone or a mixture of typhoid toxin and 30 pM mAbs. After incubation for 18 hrs, the cells were collected, washed, and fixed for 2 hrs in ~70% ethanol/PBS at -20°C. Fixed cells were washed with PBS and resuspended in 500 μl of PBS containing 50 μg/ml propidium iodide, 0.1 mg/mL DNase-free RNase A, and 0.05% Triton X-100. After incubation for 40 min at 37°C, stained cells were washed with PBS, resuspended in 150 μl PBS, filtered, and read using BD Accuri C6 Plus (BD Biosciences), followed by cell-cycle arrest profile analysis using FlowJo V10 software (Treestar Inc).

Henle-407 cell intoxication assays

Henle-407 cell intoxication assays were performed to evaluate mAb-mediated neutralization in the context of S. Typhi infection. In brief, Henle-407 human intestinal epithelial cells were cultured in DMEM high glucose + 10% FBS (HyClone) and kept at 37°C in a cell culture incubator with 5% CO₂. Cells were seeded at 3x10⁴/well into 12-well culture plates and incubated overnight. The next day, cells were infected with either WT or CdtB catalytic mutant S. Typhi harboring the cdtB<sup>H160Q</sup> gene in place of the WT cdtB gene at a multiplicity of infection (moi) of 30 or 50 for 1 hr in HBSS (Invitrogen), treated with 100 μg/mL gentamicin to kill all extracellular bacteria for 45 min, and washed with PBS. Infected cells were then incubated in the complete cell culture medium containing 10 μg/ml gentamicin for 72 hrs in the presence or absence of 10 nM mAbs. Fresh media containing 10 μg/ml gentamicin ± mAb were provided to the cells.
every 24 hrs during incubation. After incubation for 72 hrs, cells were collected, washed, and fixed for 2 hrs in 70% ethanol/PBS at -20°C. Other downstream procedures were the same as Jurkat cell assays.

Typhoid toxin binding assay

For typhoid toxin binding assay, Henle-407 cells were seeded onto sterile glass coverslips a day before the experiment. On the following day, cells were cooled to 4°C for 30 min before the addition of anti-typhoid toxin mAbs (2 μg/coverslip) and incubated for an additional 30 min. The coverslips were then stained with Alexa Fluor-555 conjugated typhoid toxin (200 ng/coverslip) for another 30 min. The coverslips were rinsed with cold PBS three times, fixed with 1% paraformaldehyde (PFA), and the nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI). For fluorescent images, Henle-407 cells were pre-treated with media containing 10 mM NH₄Cl for 20 min at 37 °C, before chilling and binding of anti-typhoid toxin mAbs and fluorescent labeled-typhoid toxin. After rinsing the coverslips with PBS, cells were then treated with warm media containing 10 mM NH₄Cl and transferred to a 37 °C incubator for 4 hrs. After the 4 hr-incubation, the cells were fixed with 1% PFA and stained with DAPI for nuclei counterstaining. Fluorescent images were acquired with a Leica DMI6000B/DFC340 FX fluorescence microscope system. 1,600 × 1,200-pixel full-frame pictures of various channels were recorded as 16-bit TIFF files with ×20 (numerical aperture (NA) 0.5) or ×40 (NA 0.75) objectives. The filter wavelengths were as follows: Alexa Fluor-555 – excitation filter 545/30 nm, emission filter 610/75 nm; DAPI – excitation filter 340~380 nm, emission filter 425 nm. The fluorescent signal intensity of images was quantified
using the measure function of ImageJ (National Institutes of Health, USA) after subtracting the background. Recorded images were merged using the ImageJ merge channels function and processed further with Adobe Photoshop to adjust the brightness and contrast equally for all recordings.

**Cryo-EM, data collection and structure determination**

*Preparation of Fab and typhoid toxin complex*

Purified mAbs were concentrated to 20 mg/ml in 0.1 ml of a buffer containing 20 mM sodium phosphate, pH 7.0, and 10 mM EDTA, followed by enzyme digestion using immobilized Papain (Thermo Fisher Scientific) overnight at 37°C. Digested mAbs samples were eluted with 10 mM Tris-HCl, pH 7.5, and the Fabs were differentially purified with Protein G resins away from Fc and undigested mAb. Further purification of Fab was carried out using Superdex 75 10/300 Increase column (GE Healthcare) using a constant flow of 0.5 ml/min of a buffer containing 10mM Tris-HCl, pH 7.5. Purified Fab was incubated together with purified typhoid toxin (PltB, PltA^{E133A}, CdtB^{H160Q}) overnight at 4 °C and submitted to Superdex 200 10/300 increase column (GE Healthcare) using a constant flow of 0.5 mL/min of a buffer containing 15 mM Tris-HCl, pH 7.5 and 150 mM NaCl. Fractions corresponding to the Fab-toxin complex were collected and used immediately for Cryo-EM grid/sample preparations for TyTx1 and TyTx4.

*Cryo-EM grid/sample preparation and data acquisition*

For TyTx1 and TyTx4, freshly prepared Fab toxin complex samples were diluted with a buffer containing 15 mM Tris-HCl, pH 7.5 and 150 mM NaCl to a final concentration of
Samples (3.5 μl) were applied to glow-discharged copper Quantifoil R1.2/1.3 300 Mesh cryo-EM grid (EMS) mounted, blotted, and plunge frozen in liquid ethane using a Vitrobot (ThermoFisher) at 100% humidity at 4 °C. Data were acquired at a nominal magnification of 49,000 x for TyTx1 and TyTx4 using a Talos Arctica (Thermofisher) operating at 200 kV equipped with a Gatan energy filter set to a slit width of 20 eV and K3 detector operating in super-resolution counting mode using a defocus range of -0.8 to -1.8 μm. Fifty frame movies with a super-resolution pixel size of 0.83 were collected over a 4-second exposure for both datasets. Both datasets were collected using a similar exposure and dose rates resulting in a total dose of 53 e- Å-2 (1.06 e- Å-2/frame) for the TyTx1/Toxin complex and 51.5 e- Å-2 (1.03 e- Å-2/frame) for the TyTx4/Toxin complex.

Image processing
Motion correction of each image stacks was performed using MotionCor2 function in RELION (Nakane et al., 2018; Zheng et al., 2017).

TyTx1/Toxin complex (Supplementary Fig.6)
Following CTF estimation, 676 images with GCTF (Zhang, 2016) estimated resolution better than 4 Å were selected from a total of 747 movies and subjected to automated Laplacian-of-Gaussian particle picking in RELION 3. A total of 3,159,007 particles were extracted with a binned pixel size of 2.53Å and randomly divided into 3 subsets for faster processing. To generate a reference map, particles from the first 76 micrographs were extracted with a pixel size of 1.66Å and subjected to iterative rounds of heterogeneous and homogenous refinement in CryoSparc 2 (Punjani et al., 2017);
Zheng et al., 2017) using the toxin crystal structure (PDB: 4K6L) lowpass-filtered to 60 Å as an initial reference model. The particle subsets were processed and sorted using several rounds of 3D classification, refinement, per-particle CTF, and beam tilt refinement. Particles from each subset extracted with a pixel size of 0.83Å were subjected to Bayesian polishing and combined to produce a reconstruction from 504,702 particles with a 0.143 FSC cutoff resolution of 3.1 Å. Additional 3D classifications seeking to remove particles containing complexes with two bound antibody molecules or a single antibody bound to an alternative PltB monomer resulted in a lower resolution anisotropic reconstruction, suggesting that these alternate states provide additional particle orientations. 2D classification at this stage revealed 2 classes containing ~81,000 (out of ~500,000) particles that appear to contain complexes with two bound antibody molecules. To improve the reconstruction in the region of interest (PltB subunits and the bound antibody), a mask was created around the density attributed to the PltA subunits and particle subtraction was performed. 3D classification without image alignment of the subtracted particles, using a mask around the PltB subunits and antibody molecule, produced a class of 326,766 particles. Subsequent 3D refinement and CTF refinement of the unsubtracted particle images resulted in a final reconstruction with a 0.143 FSC cutoff resolution of 3.0 Å. We note this reconstruction surpasses the physical Nyquist limit of 3.32 Å.

To assess the distribution of particles containing one versus two bound antibody fragments without introducing bias from masking, particles from the full dataset extracted with a binned pixel size of 2.53Å were subjected to 2D classification discarding only the classes containing obvious junk particles. A subset of 911,028
particles was selected by excluding particles that originated from micrographs with a GCTF estimated resolution worse than 3.0 Å. This subset was subjected to multiple rounds of 3D classification with a 210 Å circular mask using the toxin crystal structure as a reference model. Only particles in low resolution and low distribution junk classes were discarded after each round of classification. The final round of 3D classification contained 741,088 particles separated into two classes each with a single antibody molecule bound to the PltB(C) subunit or PltB(E) subunit (42% and 21%, respectively), one class containing two antibody molecules bound to the PltB(C) and PltB(E) subunits (24%), and two low-resolution junk classes (7% and 6%). Each of the three main classes failed to further refine to high resolution and produced maps characteristic of orientation bias, again confirming that including particles from alternate states were required to produce isotropic reconstructions.

TyTx4/Toxin complex

Following CTF estimation, a total of 572 movies collected during data acquisition were subjected to Laplacian-of-Gaussian particle picking in RELION 3. Approximately 1000 particles were manually picked, extracted, and 2D classified to serve as initial templates for automated particle picking in RELION 3. 2D classification of the resulting 903,271 particles was used to remove incorrectly picked particles. CryoSparc 2 was used to generate an ab-initio model from the cleaned particle set. This ab-initio model was used as a reference map for subsequent 3D classifications and refinement in RELION 3. Initial 3D classification without symmetry imposed reveals two of five classes contain only 4 Fabs bound constituted for approximately 36% of the initial particles. To exploit the C5 symmetry present when there are 5 Fabs bound, the class with the highest
resolution that contains 5 Fabs was used for further refinements. After per-particle CTF refinements and 3D classifications, a Bayesian polishing step followed a final 3D refinement step yield a final reconstruction using 97,594 particles were generated after the masked refinement imposing C5 symmetry. The final 3D reconstruction has a 0.143 FSC cutoff resolution of 3.13 Å according to RELION. We note this reconstruction also surpasses the physical Nyquist limit of 3.32 Å.

Model building and refinement
Chimera (www.cgl.ucsf.edu) was used to place the crystal structures of typhoid toxin (PDB 4K6L) and PltB pentamer (PDB 4RHR) into the sharpened reconstructions as preliminary atomic models. Based on their sequence similarity to the actual sequences of the TyTx1 and TyTx4 antibodies, crystal structures of Fab light and heavy chains were fit into the sharpened reconstructions to use as preliminary atomic models (TyTx1 light chain: PDB 1MHH; TyTx1 heavy chain: PDB 4H20; TyTx4 heavy chain: PDB 4M48, TyTx4 heavy chain: 2OZ4. Coot (www.biop.ox.ac.uk/coot) was then used to manually rebuild these preliminary models. The flexibility of the antibody constant regions, CdtB subunit, and the majority of the PltA subunit prevented us from confidently building these portions of the maps, so they were left unbuilt. Models were refined in Phenix using the real-space refinement module (phenix-online.org). The quality of the final models was validated using MolProbity (molprobity.biochem.duke.edu). Figures were generated using Chimera and Pymol Electronic copy available at: https://ssrn.com/abstract=3745287
Cryo-EM density maps and refined complex structures were deposited under EMD-22699 and PDB 7K7H for the TyTx1 Fab-toxin complex and EMD-22700 and PDB 7K7I for the TyTx4 Fab-toxin complex (Table A5.1).

**SEC analysis of TyTx1, TyTx3, TyTx4 Fabs binding to tagless PltB pentamer and typhoid toxin**

Before analysis, purified tagless PltB pentamer or typhoid toxin was incubated with a 5-fold molar excess of Fabs and incubated for 16 hours at 4°C. The time was staggered so that the size exclusion chromatography was executed at 16 hours mark. After 16 hours of incubation, the mixture was loaded onto a Superdex 200 Increase 10/300 GL column (Cytiva scientific, 28990944) equilibrated with PBS. The size exclusion chromatography was carried out at a flow rate of 250 μl/min. Fractions containing peak of interest were analyzed with 15% SDS-PAGE. The elution volume matching the center of the peaks was used to calculate the corresponding molecular weight of the native complex eluted using calibration curves analysis in comparison to known protein standards. Note that standard calibrated with globular protein, non-globular or poorly-folded protein migration might be slower.

**SSM analyses of bacterial AB₅ toxins**

The iterative Cα alignment SSM Algorithm of Eugene Krissinel (www.ebi.ac.uk/msd-srv/ssm) which is part of Coot was used to superpose the known toxin structures: S. Typhi typhoid toxin (4K6L), E. coli PltAB toxin (4Z9C), V. cholerae cholera toxin (1XTC), E. coli Shiga toxin type 2 (1R4P) and B. pertussis pertussis toxin (1PRT) together with
the two Fab-toxin complexes of TyTx1 and TyTx4 (Table A5.6). The SSM algorithm was also used to superpose glycan-bound toxin structures onto the respective toxins: 6P4M onto the typhoid toxin structure, 5ELB and 2CHB onto the cholera toxin structure, 1BOS onto the Shiga toxin structure, and 1PTO onto the pertussis toxin structure (Table A5.6). The SSM algorithm was used to generate 4 additional Fabs of the TyTx1-toxin complex for C5 symmetrized overlay purposes. The coordinate of the superposed structures was exported from Coot and figures were then generated via Pymol. The region of toxin encroaching the TyTx1 Fab volume was manually identified and highlighted.

Quantification and statistical analysis

Data were tested for statistical significance with GraphPad Prism 6 or 8.3 software. The number of replicates for each experiment and the statistical test performed are indicated in the figure legends. Image analysis and quantification and cell cycle profile analysis and quantification were performed using ImageJ and FlowJo V10 software, respectively.
APPENDIX 4: SUPPLEMENTAL FIGURES CHAPTER 6
mAb ELISAs were performed to determine their specificities towards typhoid toxin (A) and tagless PltB pentamer (B). Bars represent the mean values of three replicates, which were obtained by measuring the absorbance at 450 nm.
Figure A4.2. Amino acid sequences containing the light (kappa) chain variable region of TyTx1-10.

Highlighted in bold are the MAbs whose epitopes have been defined via cryo-EM in this study. The first amino acid residues indicated in the cryo-EM structures deposited are highlighted in red.
Figure A4.3. Amino acid sequences containing the light (kappa) chain variable region of TyTx1-10.

Highlighted in bold are the MAbs whose epitopes have been defined via cryo-EM in this study. The first amino acid residues indicated in the cryo-EM structures deposited are highlighted in red.
Figure A4.4. Size-exclusion chromatograms of mAbs and standards.

A, SD200 chromatograms of TyTx1 (left panel), TyTx3 (middle), and TyTx4 (right). B, SD200 chromatogram of a mixture of seven different recombinant proteins indicated in the table. Superdex 200 Increase prepacked columns (GE Healthcare) was used. The same column and running buffer were used for (A) and (B).
Figure A4.5. Quantification of MAb-mediated inhibition of typhoid toxin binding to Henle-407 cells.

Henle-407 cells grown on coverslips were precooled to 4 °C, incubated with 100 ng of typhoid toxin-AF555 in the presence or absence of 2 μg mAb for 30 min, counterstained with DAPI, and analyzed by fluorescent microscopy. Bars represent the mean ± SEM obtained from at least three independent experiments. Each dot reflects the typhoid toxin signal intensity per image. n=60-88. ****, p<0.0001, compared to the TyT only group. Two-tailed unpaired t-tests were performed.
Figure A4.6. Flow chart depicting the cryo-EM workflow of the TyTx1 Fab-toxin complexes.

Details are described in the experimental procedure section under cryo-EM, data collection, and structure determination.
Figure A4.7. Cryo-EM density maps of typhoid toxin PltB pentamer bound to TyTx1 Fab variable regions.

A, Overall cryo-EM density maps of PltB pentamer bound to TyTx1 variable regions of the light chain (VL, blue) and the heavy chain (VH, purple). B-C, Density maps of the
interface between PltB subunits (C chain, green and E chain, grey) and TyTx1 VL (blue) (B) and between PltB subunits and TyTx1 VH (purple) (C).
Figure A4.8. Flow chart depicting the cryo-EM workflow of the TyTx4 Fab-toxin complexes.
Details are described in the experimental procedure section under cryo EM, data collection, and structure determination.
Figure A4.9. Cryo-EM density maps of typhoid toxin PltB pentamer bound to TyTx4 Fab variable regions.

A-B, Side (A) and top views (B) of overall cryo-EM density maps of PltB pentamer bound to TyTx4 variable regions of the light chain (VL, light blue) and the heavy chain (VH, pink). C, Density maps of the interface between PltB pentamer (green) and TyTx4 VL (light blue) and VH (pink).
Figure A4.10. Purified recombinant tagless PltB

SDS-PAGE (a) and size-exclusion chromatograms (b) of purified recombinant PltB (tagless). Superdex 200 Increase prepacked columns (GE Healthcare) were used. See also the experimental procedure section for details.
Figure A4.11. Secondary structure matching (SSM) analyses of bacterial AB5 toxins.
A-E, Typhoid toxin (A), E. coli PltAB toxin (B), cholera toxin (C), Shiga toxin type 2 (D), and pertussis toxin (E) are complexed with TyTx1 Fabs (pink volume) and TyTx4 Fabs (grey volume). The region of toxin encroaching the TyTx1 Fab volume was manually identified and highlighted in brown (clashes with the A subunit). Total numbers of Fabs that can bind to the indicated toxins without clashes are indicated in the top view images that overlay toxin, Fabs of TyTx1, and Fabs of TyTx4 (the far-right panel). Glycan receptor moieties are also indicated if known.
Table A5.1. Cryo-EM data collection, refinement, and validation statistics.

<table>
<thead>
<tr>
<th></th>
<th>TyTx1-Toxin Complex (EMDB-22699) (PDB 7K7H)</th>
<th>TyTx4-Toxin Complex (EMDB-22700) (PDB 7K7I)</th>
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</thead>
<tbody>
<tr>
<td><strong>Data collection and processing</strong></td>
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<td></td>
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<tr>
<td>Magnification</td>
<td>49,000</td>
<td>49,000</td>
</tr>
<tr>
<td>Voltage (kV)</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Electron exposure (e−/Å²)</td>
<td>53.0</td>
<td>51.5</td>
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<tr>
<td>Defocus range (μm)</td>
<td>-0.8 to -1.5</td>
<td>-0.8 to -1.5</td>
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<td>Pixel size (Å)</td>
<td>1.66</td>
<td>1.66</td>
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<tr>
<td>Symmetry imposed</td>
<td>C1</td>
<td>C5</td>
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<td>Initial particle images (no.)</td>
<td>3,159,007</td>
<td>903,271</td>
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<tr>
<td>Final particle images (no.)</td>
<td>326,766</td>
<td>97,594</td>
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<tr>
<td>Map resolution (Å)</td>
<td>3.00</td>
<td>3.13</td>
</tr>
<tr>
<td>FSC threshold</td>
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<td>0.143</td>
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<tr>
<td>Map resolution range (Å)</td>
<td>2.6 to 3.4</td>
<td>3.0 to 3.7</td>
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<td><strong>Refinement</strong></td>
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<td></td>
</tr>
<tr>
<td>Initial models used (PDB code)</td>
<td>4RHR, 1MHH, 4H20</td>
<td>4RHR, 2OZ4, 4M48,</td>
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<tr>
<td>Model resolution (Å)</td>
<td>3.00</td>
<td>3.13</td>
</tr>
<tr>
<td>FSC threshold</td>
<td>0.143</td>
<td>0.143</td>
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<tr>
<td>Model resolution range (Å)</td>
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<td>2.97 to 3.69</td>
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<tr>
<td>Map sharpening B factor (Å²)</td>
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<td>101.11</td>
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<td>Model composition</td>
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<td>Non-hydrogen atoms</td>
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<td>13195</td>
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<tr>
<td>Protein residues</td>
<td>816</td>
<td>1705</td>
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<tr>
<td>Ligands</td>
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<td>0</td>
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<td>B factors (Å²)</td>
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<td></td>
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<td>Protein</td>
<td>16.79</td>
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<td>Ligand</td>
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<td>R.m.s. deviations</td>
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<td>Bond lengths (Å)</td>
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<td>Bond angles (°)</td>
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<td>Validation</td>
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<td>MolProbity score</td>
<td>1.93</td>
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<td>Clashscore</td>
<td>7.97</td>
<td>11.29</td>
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<td>Poor rotamers (%)</td>
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<td>0</td>
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<tr>
<td>Ramachandran plot</td>
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<tr>
<td>Favored (%)</td>
<td>91.88</td>
<td>87.82</td>
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<td>Allowed (%)</td>
<td>8.12</td>
<td>12.18</td>
</tr>
<tr>
<td>Disallowed (%)</td>
<td>0</td>
<td>0</td>
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</table>
Table A5.2. PltB amino acid residues known to interact with glycan-150 receptors.

Note that binding pocket BS1 is located on the lateral side of PltB pentamer, whereas binding pockets BS2 and BS3 are located on the bottom side of PltB pentamer.

<table>
<thead>
<tr>
<th>Glycan receptors*</th>
<th>Methods used</th>
<th>Residues in BS1**</th>
<th>Residues in BS2**</th>
<th>Residues in BS3**</th>
<th>References</th>
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<tbody>
<tr>
<td>Neu5Ac</td>
<td>Molecular docking</td>
<td>Y33, S35, K59</td>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td>Neu5Acα2-6Galβ1-4GlcNAc</td>
<td>Co-crystal structure</td>
<td>Y33, Y34, S35, K59, R100</td>
<td></td>
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<tr>
<td>Neu5,9Acα2-6Galβ1-4GlcNAc</td>
<td>Co-crystal structure</td>
<td>Y34, S35, K59, T65, R100</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Neu5,9Acα2-3Galβ1-4GlcNAc</td>
<td>Co-crystal structure</td>
<td>Y33, Y34, S35, K59, N61, T65, Q75, I107, W108, T109, Y110, F113</td>
<td>Q75, I107, W108, T109, Y110</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Neu4,5Acα2-3Galβ1-4GlcNAc</td>
<td>Co-crystal structure</td>
<td>Y33, Y34, S35, D36, K59, T65, Q75, W108, T109, Y110</td>
<td>Q75, W108, T109, Y110</td>
<td></td>
<td>3</td>
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<tr>
<td>GD2 glycan; GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc</td>
<td>Co-crystal structure</td>
<td>Y33, Y34, S35, K59, T65, R100</td>
<td></td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

* Neu5Ac, N-acetylmuramamic acid; Gal, galactose; GlcNAc, N-acetylglucosamine; Neu5,9Acα2, 9-O-acetyl Neu5Ac; Neu4,5Acα2, 4-O-acetyl Neu5Ac; GalNAc, N-acetylgalactosamine; Glc, glucose.

** Residues in bold, their importance was experimentally confirmed through mutagenesis studies in references indicated.
Table A5.3. Primers used in Chapter 6

<table>
<thead>
<tr>
<th>Application</th>
<th>Primer name</th>
<th>Forward or reverse</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>Template-switch olgo</td>
<td>Universal forward primer</td>
<td>AGGCAGTGTTATCAACGCAGAGTACATGrGrGr</td>
</tr>
<tr>
<td>mIGK RT</td>
<td>Reverse primer for kappa chain</td>
<td>TTGTTCCTCACTGCATCAATC</td>
<td></td>
</tr>
<tr>
<td>mIGL RT</td>
<td>Reverse primer for lambda chain</td>
<td>GGGGTACCACCTACCTACCTCCAG</td>
<td></td>
</tr>
<tr>
<td>mIGHG RT</td>
<td>Reverse primer for heavy chain</td>
<td>AGCTGGGAAGGTGTGCACAC</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>ISPCR</td>
<td>Universal forward primer</td>
<td>AAGCAGTGTTATCAACGCAGAG</td>
</tr>
<tr>
<td>mIGK PCR</td>
<td>Reverse primer for kappa chain</td>
<td>ACATTGATGTTTTGAGGTAGAAG</td>
<td></td>
</tr>
<tr>
<td>mIGL PCR</td>
<td>Reverse primer for lambda chain</td>
<td>ATCGTACACACCAGTGCTGCG</td>
<td></td>
</tr>
<tr>
<td>mIGHG PCR</td>
<td>Reverse primer for heavy chain</td>
<td>GGGATCCAGAGTTCAGGTCC</td>
<td></td>
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</table>

GrGrGr: 3 riboguanines. See Meyer et al, 2019 for details.
Table A5.4. Kinetics of TyTx mAbs binding to typhoid toxin by surface plasmon resonance (SPR) assays.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>Target</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)*</th>
<th>$k_d$ (s$^{-1}$)*</th>
<th>$K_D$ (M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TyTx1</td>
<td>IgG1</td>
<td>PltB</td>
<td>9.39 x 10$^4$</td>
<td>6.64 x 10$^{-3}$</td>
<td>7.11 x 10$^{-8}$</td>
</tr>
<tr>
<td>TyTx3</td>
<td>IgG1</td>
<td>PltH</td>
<td>1.73 x 10$^3$</td>
<td>1.68 x 10$^{-2}$</td>
<td>1.45 x 10$^{-7}$</td>
</tr>
<tr>
<td>TyTx4</td>
<td>IgG2a</td>
<td>PltH</td>
<td>3.83 x 10$^3$</td>
<td>8.53 x 10$^{-3}$</td>
<td>2.28 x 10$^{-10}$</td>
</tr>
</tbody>
</table>

*Mean values of three technical replicates.
Table A5.5. Secondary structure matching analyses of bacterial AB5 toxins.

<table>
<thead>
<tr>
<th>Toxin name</th>
<th>Holotoxin PDB</th>
<th>Glycan complex PDB (glycan name)(^1)</th>
<th>TyTx1-like IgGs per toxin (shoulder-located epitopes)</th>
<th>TyTx4-like IgGs per toxin (bottom-located epitopes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhoid toxin (A-B(_5))</td>
<td>4K6L</td>
<td>6P4M (Neu5Aca2-3Galβ1-4GlcNAc)</td>
<td>1 MAb (2 Fabs)</td>
<td>3 MAb (5 Fabs)</td>
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<tr>
<td>E. coli PltAB toxin (AB(_5))</td>
<td>4Z9C</td>
<td>Unknown(^2)</td>
<td>1 MAb (2 Fabs)</td>
<td>3 MAb (5 Fabs)</td>
</tr>
<tr>
<td>Cholera toxin (AB(_5))(^3)</td>
<td>1XTC</td>
<td>5ELB (Lewis Y glycans); 2CHB (GM1 glycans)</td>
<td>1 MAb (2 Fabs)</td>
<td>3 MAb (5 Fabs)</td>
</tr>
<tr>
<td>Shiga toxin type 2 (AB(_5))(^4)</td>
<td>1R4P</td>
<td>1BOS (α-D-galactopyranose-((1→4))-β-D-galactopyranose-((1→4))-β-D-glucopyranose)</td>
<td>2 MAbs (4 Fabs)</td>
<td>3 MAbs (5 Fabs)</td>
</tr>
<tr>
<td>Pertussis toxin (AB(_5))(^5)</td>
<td>1PRT</td>
<td>1PTO (Neu5Aca2-6-β-D-galactopyranose)</td>
<td>1 MAb (2 Fabs)</td>
<td>3 MAb (5 Fabs)</td>
</tr>
</tbody>
</table>

\(^1\)Additional glycan binding pockets may exist.

\(^2\)No glycan bound structure is unknown.

\(^3\)Similar to typhoid toxin, glycan-binding pockets are located in both the shoulder and the bottom.

\(^4\)Glycan binding pockets are located on the bottom side of the B pentamer.

\(^5\)Glycan binding pockets on the shoulder regions in S2 and S3. Heteropentameric B subunits consisting of S2, S3, 2S4, and S5 can also be considered as an intrinsic immune subversion mechanism because their epitopes in the B heteropentamer are different.
Table A5.6. Comparison of *S. Typhi* pltB sequences deposited in NCBI.

Shown are the first 100 hits from the BLAST search using *S. Typhi* CT18 *pltB*. Note that all *S. Typhi* strains encode *pltB* on their chromosomes and their DNA sequences are identical.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Sequence ID</th>
<th>PltB gene location</th>
<th>Sequence Homology (%)</th>
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</thead>
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<tr>
<td>CT18</td>
<td>Sty1891</td>
<td>1788687-1788274</td>
<td>100</td>
</tr>
<tr>
<td>R19_2839</td>
<td>CP046249.1</td>
<td>2684230-2683817</td>
<td>100</td>
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<td>2018K_0756</td>
<td>CP044007.1</td>
<td>2313144-2312731</td>
<td>100</td>
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<td>WGS1146</td>
<td>CP040575.1</td>
<td>2684661-2684248</td>
<td>100</td>
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<td>Ty2 4316STDY6559669</td>
<td>LR590081.1</td>
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</tr>
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<td>311189_217156</td>
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<td>1787549-1787136</td>
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