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The Role of the Gut Microbiome in Shaping Immune Responses to *Vibrio cholerae*

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Dedication

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ABSTRACT OF THE DISSERTATION

The Role of the Gut Microbiome in Shaping Immune Responses to *Vibrio cholerae*

by

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Vibrio cholerae is the etiologic agent of cholera, a severe diarrheal disease that represents a significant burden on global health and productivity. The high morbidity of cholera demands effective prophylactic strategies, but oral cholera vaccines exhibit variable immunogenicity and efficacy in human populations. One variable factor in human populations is the gut microbiome, the resident community of microorganisms in the gut, which in cholera-endemic areas is strongly and repeatedly modulated by malnutrition, cholera, and non-cholera diarrhea. However, the role of microbiome variations in immune responses against *V. cholerae* and oral cholera vaccination is not well understood. We conducted fecal transplants from healthy human donors and model communities of either human gut microbes resembling those of healthy individuals or those of individuals recovering from diarrhea or malnutrition in various mouse models. We show microbiome-specific effects on host antibody responses against *V. cholerae*, and that dysbiotic human gut microbiomes,

commonly present in areas where malnutrition and diarrhea are common, suppresses the immune response against *V. cholerae* through the action of CD4+ lymphocytes. Our findings suggest that the composition of the gut microbiome at time of infection or vaccination may be pivotal for providing robust mucosal immunity, suggesting a target for the improvement of prophylactic and therapeutic strategies for cholera.

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CHAPTER 1: Introduction and Review of the Literature

Cholera pandemics

Over the last two centuries, *Vibrio cholerae* has caused seven major cholera pandemics, resulting in waves of terror and death across much of the world. The first major pandemic began in 1817 with subsequent pandemics in 1829, 1852, 1863, 1881, 1889, and 1961, with the pandemic starting in 1961 continuing until today (Harris et al., 2012).

During the third pandemic of 1852, cholera spread to England where the physician John Snow made his pivotal epidemiological discovery that transmission of the disease was waterborne via contaminated well water, as it was previously thought to be transmitted via bad air (Snow, 1856). The German microbiologist Robert Koch was initially credited with identifying the bacterium *V. cholerae* as the causative agent of the disease (Koch, 1884). However, his findings appear to be preceded by that of Italian microbiologist Filippo Pacini (Pacini, 1865).

While the first six pandemics had origins in the Ganges delta region of Bangladesh, the current 7th pandemic that began sixty years ago originated in Celebes, Indonesia. The *Vibrio cholerae* strain that initiated this pandemic arose from a different biotype - the El Tor biotype of *V. cholerae* O1, named after where it was isolated in El Tor, Egypt (Baine et al., 1974). It continued to spread around the world to India in 1964, Africa in 1970 (Cvjetanovic and Barua, 1972), Europe

in 1970 (Baine et al., 1974) and South America in 1991 (Swerdlow et al., 1992). It has since continued to establish itself endemically in regions of low sanitation, resulting in periodic epidemics, particularly in Africa and Asia.

In 1992, a new non-O1 serogroup *V. cholerae*, emerged in the southern port city of Madras, India, and was classified as O139 Bengal (Ramamurthy et al., 1993). It quickly spread to the northern city of Calcutta and to southern coastal areas such as Bangladesh. It was designated *V. cholerae* O139 because it was the 139th serotype to be classified and it became the only biotype other than O1 that was able to cause pathology. In 1993, it continued to spread in the India/Bangladesh region and displace *V. cholerae* El Tor, just as O1 El Tor had done to O1 Classical in the early 1960's. While O139 was potentially on the verge of becoming the new dominant pandemic strain, its presence has waned in recent years.

Both natural disasters and civil strife continue to be harbingers of cholera as they instigate poor sanitation and water quality if not properly managed, as evidenced by the cholera outbreak in Haiti in 2010 and the ongoing outbreak in Yemen. It was determined that the cholera outbreak in Haiti was caused by UN aid workers that had travelled to Haiti to offer assistance after the earthquake (Lantagne et al., 2014). Overall, the Haitian outbreak resulted in 697,256 cholera cases and 8,534 deaths (Population, 2014). The 2016 cholera outbreak in Yemen resulted from a civil war that further eroded an already decrepit sanitation system. It eventually resulted in 2.5 million cases to date and nearly 4,000

deaths, making it the worst outbreak in recorded history (World Health Organization (WHO), 2020). Cholera continues to be a major global public health burden as there are approximately 3,000,000 cases each year resulting in approximately 95,000 deaths (Ali et al., 2015).

V. cholerae life cycle and virulence mechanisms

Mainstream thought is that *V. cholerae* is only spread from infected people to others via fecal contents in contaminated water. However, *Vibrios* are capable of living in coastal brackish aquatic environments (Colwell et al., 1977). Indeed, they tend to multiply in association with chitinaceous zooplankton and shellfish (Colwell, 1996; Huq et al., 1983). The association with algal blooms is one reason for the seasonality of epidemics of *V. cholerae* in endemic areas, as coastal waters spread *V. cholerae* that preferentially attaches to chitinaceous plankton. As some plankton have the capacity to contain 10^4 *V. cholerae*, it is quite feasible for humans who are consuming undercooked shellfish or drinking small volumes of drinking water to obtain the infectious dose as low as 10^3 *V. cholerae*, as reported in some human volunteer studies (Colwell et al., 1996).

Once ingested, *V.* cholerae moves through the acidic environment of the stomach, where it then transits through the mucus layer of the small intestine and colonizes the surface of intestinal epithelial cells. *V. cholerae* causes pathogenesis through the induction of various virulence factors in a highly coordinated fashion. One genetic factor that distinguishes a pathogenic *V.*

cholerae from an innocuous strain is the presence of the genome of a lysogenic bacteriophage designated CTXΦ. CTXΦ encodes the genes for cholera toxin (CT) and the vibrio pathogenicity island (VPI), which carries genes for the toxin-coregulated pilus (TCP) (Waldor and Mekalanos, 1996).

The notion of a toxin being associated with the disease was initially postulated by Robert Koch in the 1800's (Robert Koch, 1987). However, it was not until 1959 that the presence of cholera toxin (CT) was demonstrated (De, 1959; Dutta et al., 1959) and in 1969 the toxin was isolated (Finkelstein and LoSpalluto, 1969). CT is an ADP-ribosylating toxin that is part of a larger family of A-B toxins (Finkelstein and LoSpalluto, 1969). CT consists of an inactive pentameric B subunit (CT-B) and the active monomeric subunit CT-A. CT-B binds to the GM_1 ganglioside on the cell surface where CT-A is then cleaved by host proteases into an A_2 subunit that enzymatically links the active A_1 subunit to the complex (Gill and King, 1975; Gill and Rappaport, 1979; King and Van Heyningen, 1973). The bound toxin is then internalized and proceeds by retrograde trafficking through the Golgi apparatus to the endoplasmic reticulum (Majoul et al., 1998). After processing, CT-A_1 is released into the cytosol where it catalyzes ADP-ribosylation of the Gsα subunit of adenylate cyclase. This reaction conforms adenylate cyclase into a GTP-bound activated state, resulting in increased cyclic AMP (cAMP) production (Mekalanos et al., 1979). Enhanced production of cAMP alters electrolyte transport in two ways. As a result of cAMP activation of protein kinase A (PKA), the intake of NaCl and water from the villous

cells is inhibited and in crypt cells phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) leads to the secretion of chloride ions and bicarbonate, leading to paracellular secretion of water (Field, 1979) (Field, 2003; Jan Holmgren, 1986). This secretion of water is the source of the severe watery diarrhea that is characteristic of cholera.

In addition to cholera toxin, toxin coregulated pilus (TCP) is an essential component for pathogenic *Vibrios*. TCP is a type IV bundle-forming pilus that is expressed during colonization of both the human and animal small intestine (Herrington et al., 1988; Taylor et al., 1987). The gene is part of the *tcp* operon, which is located within the vibrio pathogenicity island (VPI). TCP can cause cell-cell aggregation, although it has not been shown to have adhesive properties with epithelial cells (Kirn et al., 2000). Thus, it may mainly aid colonization by causing aggregation of *V. cholerae* cells rather than interaction with host epithelial cells.

Other features of *V. cholerae* that promote colonization are HapA, a soluble hemagglutinin/Zn-metalloprotease that facilitates bacterial penetration of the intestinal mucosal layer, as well as the flagellum that moves the bacteria through the mucus layer toward the epithelial surface. In addition to HapA, mucinase, and neuraminidase, downregulation of a type IV mannose-sensitive hemagglutinin (MSHA) pilus is required for successful colonization in infant mice. *V. cholerae* that were unable to do so exhibited increased binding and secretion by S-IgA (Hsiao et al., 2006). Transposon mutagenesis also identified another

set of accessory colonization factor (*acf*) genes, *acfA, acfB, acfC,* and *acfD.* The *acf* genes are necessary for colonization, however, the function of the proteins remains to be elucidated (Peterson and Mekalanos, 1988). Lastly, a toxin that is present in El Tor strains but not Classical is the repeats-in-toxin (RTX) toxin, which crosslinks with actin polymers, causing degradation of paracellular tight junctions. In a murine pulmonary model, *V. cholerae* with cholera toxin genes absent (*ctxAB*) still caused pathology, whereas the cholera toxin mutant *V. cholerae* in addition to deletions in the (RTX) toxin gene (*rtxA*) resulted in less severe pathology, suggesting that *rtxA* plays a role in mediating cholera pathogenesis.

The myriad of virulence factors are activated in a highly coordinated and regulated manner to ensure that transcription only occurs in the most optimal conditions. ToxT, a transcriptional activator and a member of the AraC family, directly activates transcription of the *ctx* and *tcp* genes as well as additional virulence genes within the VPI (DiRita et al., 1991; Higgins et al., 1992). To activate transcription, ToxT recognizes and binds to an area known as the *tox*box. Expression of ToxT is under tight regulation from the ToxR regulon. ToxR is a membrane-spanning protein consisting of a cytoplasmic localized DNA-binding domain, a transmembrane domain, and a periplasmic domain. ToxR requires the presence of another protein, ToxS, which is localized to the inner membrane (Miller and Mekalanos, 1984). Additionally, ToxR must act in conjunction with another protein, TcpP, which interacts with the membrane-

bound effector protein, TcpH (Hase and Mekalanos, 1998; Krukonis et al., 2000). In the absence of TcpH, TcpP is continually degraded, which occurs in conditions where virulence activation would be unfavorable (Murley et al., 1999). While ToxR expression alone cannot activate *toxT* expression, overexpression of TcpP can activate expression, suggesting in this scenario that ToxR acts as an enhancer for TcpP. Since *tcpPH* genes are encoded within the VPI, they are only found in pathogenic strains of *V. cholerae* whereas the genes for *ToxRS* are found in all *Vibrio* species (Childers and Klose, 2007).

Activation of the ToxR regulon is dependent on the induction of *tcpPH* and TcpPH is driven by the proteins AphA and AphB (Skorupski and Taylor, 1999). Various environmental signals influence AphA and AphB activity which can include quorum sensing and glucose availability. Quorum sensing allows bacterial members to monitor and regulate population densities through the secretion of small molecules called autoinducers. At low *V. cholerae* cell density, virulence genes are activated by the presence of AphA. However, at high cell density, heightened autoinducer levels initiate a signal cascade that results in increased expression of HapR, a regulatory protein that is normally degraded at low cell densities (Jobling and Holmes, 1997). Elevated levels of HapR then inhibit AphA and thus decrease TcpPH and ToxR stimulation of the virulence genes CT and TCP (Zhu et al., 2002). At the molecular level, virulence gene expression is a highly regulated process that provides perspective for the severe diarrhea that cholera causes at the clinical level.

Clinical manifestations of *V. cholerae* infection and management

If not treated properly, cholera in its most severe form can cause severe diarrhea resulting in dehydration and death. Before the advent of more modern rehydration approaches, case fatality rates were as high as 50% (Joo, 1974). However, better rehydration practices have reduced the mortality rate to 1%. Cholera symptoms occur within 18 hours - 5 days after infection, beginning with watery stools and vomiting. With continuous bouts of diarrhea, the stool can begin to have the appearance of rice water, with flaky mucus and a fishy odor. Typically, there are no symptoms of a fever, unless there is a co-infection. The most significant symptoms are those that are related to dehydration. In mild cases symptoms can include thirst and irritability but in more advanced stages symptoms include loss of skin turgor, diminished urine output, decreased blood pressure, and sunken eyes. If patients are severely dehydrated, it can lead to hypovolemic shock. Complications of cholera also include electrolyte imbalances such as: hypokalemia, hyponatremia, and metabolic acidosis, because of lost sodium, chloride, and bicarbonate.

Since the immediate concern for patients is dehydration, replacing lost water and electrolytes is essential. In most cases, oral rehydration solution, which contains water and appropriate concentrations of electrolytes is sufficient for the patient. In particular, the addition of glucose is essential to bypass the inhibited Na^+/H^+ exchanger and force sodium intake via the Na^+/glucose cotransporter SGLT1. This causes Cl^- anions and water to follow, reversing the

effects of cholera toxin. However, in severe cases, intravenous solutions are necessary to mitigate the symptoms. Administration of antibiotics reduces the total volume of stool passed as well as shorten the period of *V. cholerae* excretion. The type of antibiotic treatment varies geographically and by age. In regions with low levels of tetracycline resistance, doxycycline is used for adults. In other regions, azithromycin and ciprofloxacin are used as the secondary antibiotic of choice.

Animal models

Developing a tractable animal model for *V. cholerae* is essential to define the underlying molecular mechanisms that contribute to the severe diarrheal disease of cholera. Over the years, various animal models have been developed, each with their advantages and disadvantages. Achieving diarrhea and death, the most relevant clinical outcomes of cholera, has been challenging as demonstrated by Metchnikoff as he unsuccessfully induced infections in kittens, puppies, mice, or gerbils (Metchnikoff, 1894). It was not until 1954 that a successful model was developed in infant rabbits when under certain conditions, they were highly susceptible to *V. cholerae*, causing diarrhea and death (Dutta and Habbu, 1955). The newly developed infant rabbit model allowed for study of gross pathological characteristics of diarrhea and death, however, as the crypts and villi in infant rabbits are not fully developed, a modified approach was needed to properly examine *Vibrio* attachment and adherence in a more human

relevant system. The ligated ileal loop adult rabbit model filled this niche as it allowed for *Vibrios* to be examined in animals with more developed small intestines and reduced peristalsis. However, this method requires surgical expertise as segments of the small intestine are isolated with ligatures (De and Chatterje, 1953; Nelson et al., 1976). This technique was later modified so that the adult rabbit would also develop diarrhea and was known as the RITARD (for removable intestinal tie-adult rabbit diarrhea) model (Spira et al., 1981). For this procedure, the cecum was ligated close to the ileocecal junction and the small intestine was temporarily ligated, resulting in severe diarrhea for the challenged animals. While the ileal loop adult rabbit model enabled study of cholera in a physiologically and immunologically mature system, the technical surgical expertise required remains prohibitive for most labs. This led to the foundation of the infant mouse model as a main animal model to study *V. cholerae* virulence pathways.

A major benefit of the suckling mice model is that infant mice are susceptible to *V. cholerae* colonization and express various virulence mechanisms that are also present in *V. cholerae* colonization in humans (Klose, 2000). Notably, the infant mouse model was influential in elucidating the essential role of toxin-coregulated pili (TCP) during the pathogenesis of cholera as evidenced by several key studies (Attridge and Rowley, 1983; Herrington et al., 1988; Sharma et al., 1989; Taylor et al., 1987). Additionally, the suckling mouse model was used to effectively examine morphological changes of *V.*

cholerae in vivo (Krebs and Taylor, 2011) as well as additional colonization factors such as GbpA, which were found to modulate *V. cholerae* attachment to epithelial cells (Kirn et al., 2005). Although suckling mice do not display diarrhea, the model remains a very important *in vivo* model system for examining *V. cholerae* colonization factors (Klose, 2000) (Alavi and Hsiao, 2020).

There are several non-mammalian systems that have recently gained traction for being low cost, high-throughput, and genetically modifiable such as *Drosophila melanogaster*, *Caenorhabditis elegans,* and zebrafish, *Danio rerio*. The Drosophila model gained relevance when it was shown that flies are susceptible to oral *V. cholerae* infection and die within a day, exhibiting diarrheal symptoms similar to cholera (Blow et al., 2005). *Drosophila* also exhibit a simple microbiome, which enables researchers to examine *V. cholerae* infection in the context of host metabolism. Recently, a *Drosophila* study demonstrated that the type six secretion system (T6SS) reduces epithelial cell repair mechanisms in a microbiota-dependent manner (Fast et al., 2020). While this model is attractive for various reasons, it is important to note that flies have a very different microbiome from humans and lack an adaptive cellular immune system, and the cholera-like disease symptom is CT-independent, which demonstrates a different pathogenic mechanism of killing from that of humans (Davoodi and Foley, 2019).

As nematodes are natural predators of bacteria, *Caenorhabditis elegans* is a unique invertebrate model for *V. cholerae*. Indeed, several experiments have shown that two secreted factors of *V. cholerae*, the protease PrtV and the

hemolysin HlyA, have protective functions and cause lethality in *C. elegans* (Cinar et al., 2010; Vaitkevicius et al., 2006). Additionally, an elegant study utilizing recombination-based *in vivo* expression technology (RIVET), demonstrated that mannose-sensitive hemagglutinin (MSHA) is necessary to colonize the pharynx of *C. elegans* (List et al., 2018). Further, a novel cytotoxin named motility associated killing factor (MakA) was identified that is regulated by HapR and mediates *C. elegans* in a flagellin-dependent manner (Dongre et al., 2018). The summation of these studies demonstrates that *C. elegans* is a unique model that enables the study of *V. cholerae* accessory toxins in a very genetically tractable manner.

Lastly, *V. cholerae* naturally resides in an aquatic environment, which makes the zebrafish, *Danio rario*, an attractive model for studying cholera pathogenesis. Various strains of *V. cholerae* were shown to successfully colonize the zebrafish small intestine after a natural exposure route of infection and did so independently of TCP and CT (Runft et al., 2014). Moreover, zebrafish display diarrhea as measured by optical density, independent of several cholera accessory toxins including MARTX A, accessory cholera toxin, and zonula occludens toxin (Mitchell et al., 2017). While more work will need to be done to explore the underlying mechanisms of this model, the zebrafish provides an additional system to study both O1 and non-O1 strains of *V. cholerae*.

Over time, the suckling mouse model of cholera became an established model for studying *V. cholerae* pathogenesis. This model is advantageous

because *V. cholerae* virulence mechanisms that are activated in humans were also shown to be activated in the suckling mice (Klose, 2000). Indeed, the two main players for virulence, CT and TCP, were identified in the suckling mouse model (Klose, 2000).

While the infant mouse model is widely used, it is not a suitable model to study robust immune responses and vaccine candidates as only passive immunity can be examined. As mentioned previously, adult models of cholera such as the ligated loop adult rabbit model can be expensive and is less practical. Thus, an antibiotic treated adult mouse model would be ideal to study vaccine interactions. A study utilizing streptomycin treatment of mice showed that various immune correlates of protection were achieved using this system (Nygren et al., 2009). An additional alternative is using a germfree mouse model. In this scenario, germfree mice were orally inoculated with various strains of *V. cholerae* and developed robust antibody responses against cholera toxin (Butterton et al., 1996). However, a germfree setting is not totally ideal, as the immune system typically co-develops with the presence of microbiota. Thus, to examine immune interactions in a controlled environment that a germfree mouse model allows, animals were first colonized for two weeks prior to *V. cholerae* infection to allow for immune maturation (Hapfelmeier et al., 2010; Seedorf et al., 2014).

The diverse cholera animal models discussed have various benefits but also shortcomings; the ideal model would be in a physiologically and

immunologically mature animal with inoculation through the oral route, activation of virulence factors, and resulting in diarrhea. As such a model does not currently exist, it is necessary to choose the appropriate animal model for the hypotheses being tested.

Overview of immunity to *V. cholerae*

Unlike other enteric pathogens such as *Shigella* and *Salmonella*, which cause clinically apparent inflammation and disease after penetrating cells or the intestinal epithelium, *V. cholerae* is a non-inflammatory, noninvasive bacterium that causes pathology through the release of cholera toxin. However, cholera is associated with minute inflammatory changes such as widening of intracellular spaces, apical junction abnormalities, as well as infiltration of neutrophils, mast cells, and macrophages into the affected area (Mathan et al., 1995; Pulimood et al., 2008). Additionally, a recent study of cholera patients demonstrated a pronounced upregulation of genes encoding immune effectors and molecules. These include *DUOX2*, an NADPH oxidase involved in maintenance of intestinal homeostasis and *TLR8*, an endosomal Toll-like receptor expressed in lamina propria cells (Bourque et al., 2018). While oxidases are often employed as an immediate host defense response system, it may benefit *V. cholerae* by removing bacterial competition as *V. cholerae* engages inducible resistance to oxidative stress during colonization (Wang et al., 2017a). Signals from the innate immune response also coordinate and direct the appropriate adaptive immune

responses through the release of various cytokines. In cholera, the release of cytokines includes IL-1β, IL-6, and IL-17.

While there are minor innate immune responses, immunity to cholera is mainly due to humoral antibody responses with little implications of protector cellular immune responses. Protective antibody responses mainly fall under two categories: anti-toxoid against CT and anti-bacterial against LPS. The main immunomodulatory component of CT appears to predominantly be the CT-B subunit as opposed to the active CT-A subunit. While CT does initially stimulate a strong anti-CT antibody response, it is not long lasting and does not confer enduring immunity in humans (Levine et al., 1979; Lindholm et al., 1983; Peterson, 1979).

Additionally, while anti-toxin antibody responses are minimal and short-lived, functional anti-bacterial responses typically confer long-term protection for up to 3 years (Provenzano et al., 2006). Previous studies demonstrated that purified LPS of *V. cholerae* resulted in significant protection against cholera in both animals and humans (Provenzano et al., 2006). This protection was in part found to be correlated to antibodies against LPS, purified from hyperimmune sera to whole vibrios by affinity chromatography (Svennerholm, 1975). As *V. cholerae* does not penetrate intestinal mucosa, only antibodies present in the gastrointestinal lumen would serve to have a role in protecting against the vibrios. Indeed, Kaur et al. initially demonstrated that secretory IgA antibodies extracted from crypts of immunized rabbits had toxin-neutralizing capabilities

(Kaur et al., 1972). The mechanism of antibody protection was initially not well known, but it was found that the presence of IgA, IgG, and IgM antibodies are all capable of protection if they are present in the intestine (Steele et al., 1974). While mechanisms of antibody protection are still being elucidated, more recent developments have heightened understanding of antibody-mediated protection. The mechanism of antibody protection seems to be largely driven by inhibition of motility and entrapment of bacteria, preventing *V. cholerae* from accessing the intestinal epithelium. (Levinson et al., 2016; Levinson et al., 2015; Wang et al., 2017b). Long-term immunity appears to be driven by the presence of mucosal S-IgA memory B cells. Memory B cells do not produce any antibody but if exposed to antigen can rapidly expand and differentiate into plasmablasts. An experiment that provides evidence of this is that household contacts with circulating antigen-specific memory B cells were less likely to be infected with *V. cholerae*, even if there was no evidence of circulating antibody at time of exposure (Patel et al., 2012). As shown by numerous studies, protective luminal antibody is the main component of protection against *V. cholerae.* While there has been substantial work to elucidate the end-product of protection against *V. cholerae*, more experimentation is needed to better understand the signaling involved between bacterial metabolites and host-immune cell signaling that leads to differential immune protection.

The gut microbiome across the lifespan

A biological component that continues to be integral in moderating health and disease in the host is the gut microbiome. The human body exists in relative homeostasis with microbial cells that colonize both the skin and mucosal environments in numbers at least as great as somatic cells (Sender et al., 2016). Approximately 500-1,000 bacterial species inhabit the body (Turnbaugh et al., 2007), with each species and subspecies contributing a multitude of genetic potential, creating far more genetic diversity than the human body (Locey and Lennon, 2016). Various factors shape the gut microbiome which include diet, pharmaceuticals, geography, stage of life, birthing process, infant feeding method, and stress (Cresci et al., 2020).

The microbiota in early life is influenced by a variety of factors including method of delivery, antibiotic usage, and whether or not the infant was breastfed. The first significant exposure of microbes to a newborn occurs at delivery and varies whether the delivery was vaginal or by Caesarian section. Infants delivered vaginally are enriched in *Lactobacillus* spp. whereas infants delivered by C-section are instead colonized by common skin and environmental microbes such as *Staphylococcus, Streptococcus,* or *Propionibacterium* (Tamburini et al., 2016). The microbial consortium then shifts depending on food consumption, with *Bifidobacterium* and *Lactobacillus* being the primary constituents due to milk consumption before shifting to *Bacteroides* and *Clostridiales* with more solid food intake. Between 1-3 years of age, the gut microbiome then shifts to a more adult-

like state which is predominantly represented by the phyla Bacteroidetes or Firmicutes (Kundu et al., 2017).

Metabolic functions of the gut microbiota

The great diversity of the gut microbiome contains a distinct subset of genes and enzymes that are unique but complementary to mammalian hosts, allowing for digestion of nutrients that would not be possible otherwise. The contribution of additional enzymes from the microbiota allows for the breakdown of complex polysaccharides, polyphenols, and synthesis of vitamins. The intake of certain dietary components may shape the gut microbiota and resulting metabolites. These can either be indicators or induce the development of several gastrointestinal diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome, or immune responses (Rowland et al., 2018).

Bacteria in the colon utilize anaerobic fermentation of complex polysaccharides, resulting in the production of short chain fatty acids (SCFAs). The majority of SCFA's are acetate, propionate, and butyrate ranging in concentrations from 3:1:1 to 10:2:1 (Rowland et al., 2018). The metabolites serve as an energy source for epithelial cells but also persist in systemic circulation. These microbial metabolites are also shown to be important regulators for the gut-brain axis. For example, acetate was shown to reduce inflammatory signals in microglia primary cultures and butyrate has been shown to shift microglia to a more homeostatic profile and inhibit LPS-induced pro-inflammatory signals

(Soliman et al., 2012). Additionally, butyrate has been studied extensively as an immunomodulator for both innate and adaptive immunity processes. Addition of butyrate was shown to enhance antimicrobial activity of macrophages *in vivo* (Schulthess et al., 2019). Additionally, butyrate produced by *Clostridia* induced the differentiation of T_{reg} cells *in vitro* and *in vivo* and lessened the colitis in an adoptive transfer of T cells into *Rag1*$^{-/-}$ mice (Furusawa et al., 2013). Propionate on the other hand is mostly taken up by the liver and serves as an important energy source for hepatic cells and play a role in satiety signaling (De Vadder et al., 2014). While SCFAs are an integral component of bacterial metabolism and have systemic effects on the host.

Host-microbe interactions on the immune system

As mentioned previously, main contributions of the microbiota to the host include the digestion and fermentation of carbohydrates, the production of vitamins, and the development of gut-associated lymphoid tissues (GALT) and diversification of gut-specific lymphoid responses. A complex interplay exists between gut microbial populations and the immune system to maintain gut homeostasis.

The germ-free mouse model demonstrated the importance of the gut microbiota for normal gastrointestinal function and immune modulation. Primarily, the gut mucosa serves as the initial barrier protecting intestinal epithelium. In germ-free mice, the mucosal lining is severely diminished but can be restored with the administration of LPS (Petersson et al., 2011). The development and

maturation of GALTs which include Peyer's patches, crypt patches, and isolated lymphoid follicles (ILFs) is driven by intestinal bacteria. As shown in a germ-free rabbit model, the combination of *Bacteroides fragilis* and *Bacillus subtilis* was shown to be important to drive GALT development through stress responses (Rhee et al., 2004). Additionally, sensing of the microbiota with Toll-like Receptors (TLRs), a component of the innate immune system, has been shown to be influential in ILF maturation, as mice deficient in certain TLRs were shown to have immature mucosal immune structures (Lycke and Bemark, 2017). Intestinal epithelial cells (IECs) regulate innate immune processes as they can release various antimicrobial peptides. For example, Paneth cells release the antimicrobial compounds REGIIIγ and REGIIIβ, which are antimicrobial compounds that are reduced in mice that are deficient in the TLR signaling pathway, denoting the importance of microbial sensing.

The resident gut microbiome has also been implicated in serving as regulators in the development of various immune cell types. T helper 17 (T_H17) cells are a specific subset of CD4+ T_H cells that have been shown to be essential in combating bacterial, viral, and fungal infections mainly by the release of IL-17, IL-21, and IL-22 cytokines (Khader et al., 2009). T_H17 cells are shown to have increased frequencies in intestinal tissue, however, there are decreased populations in germ-free mice. Further, a seminal study by Ivanov and Littman demonstrated that certain microbial species can influence and induce immune cell populations as a particular species of *Clostridia*-related bacteria, called

segmented filamentous bacteria (SFB), induced the generation of T_H17 cell development (Ivanov et al., 2009). An additional subset of CD4+ T_H cells that accumulate in the intestine are regulatory T_{Reg} cells. Similar to T_H17, the gut microbiota is instrumental in the development of peripheral T_{Reg} cells as their populations are decreased in colonic tissue of germ-free mice. A consortium of bacteria including a mixture of *Clostridium* spp., altered Schaedler flora (ASF), which consists of 8 defined commensal bacteria, or *Bacteroides fragilis* was shown to induce development of IL-10 producing T_{Reg} cells, although the exact mechanism remains to be elucidated (Atarashi et al., 2011; Round and Mazmanian, 2009).

Lastly, IgA producing B cells and plasma cells constitute a large proportion of immune cells in mucosal tissues. Secretory IgA (S-IgA) released into the gastrointestinal lumen accounts for 2-3 grams of daily antibody production and high metabolic activity to maintain gut homeostasis. Nutrient uptake in the small intestines is essential, however, it is also an area that is easily accessible to potentially harmful bacterial metabolites and pathogens. A recent study highlights the importance of antibodies in setting boundaries to microbial metabolite penetration, demonstrating the important role of antibodies in maintaining gut homeostasis (Uchimura et al., 2018). Host-microbial mutualism is essential for functional IgA responses. Colonization with gut microbes ensures mature Peyer's patches, which are the main source of IgA-secreting plasma cells. Once IgA is secreted from the plasma cells, it is bound to the polymeric immunoglobulin

receptor (pIgR), which transports it within cell vesicles where it is released by proteolytic cleavage into the lumen leaving a small fragment attached, called the secretory component. S-IgA predominantly protects from toxins and pathogens via immune exclusion by binding to the pathogen and inhibiting attachment to the gastrointestinal epithelium (Figure 1.1) (Cho et al., 2021).

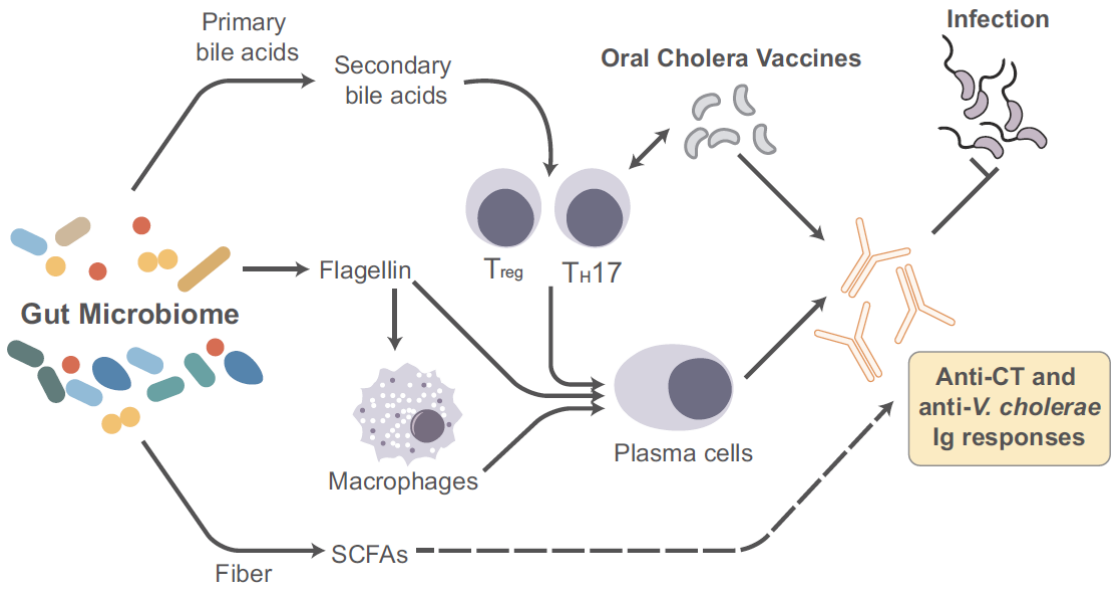

Figure 1.1. Interactions of the gut microbiome, host immunity, and *V. cholerae*. Production of specific metabolites by commensal microbes can influence the activity and abundance of specific cell subtypes in the host immune system, including effector and regulatory lymphocytes, macrophages, and plasma cells. These pathways include the production of secondary bile acids and short-chain fatty acids (SCFAs) from dietary fiber. Additionally, bacterial components such as flagellin directly stimulate both macrophages and plasma cells, allowing for a stronger antibody response. Together, these commensal metabolic activities lead to modulation of antibody responses against *V. cholerae* induced by infection and oral cholera vaccines.

Normal vs. dysbiotic microbiome

The resident human gut microbiome is an essential "organ" with the potential to influence host development, immune, and physiological functions. Characterization became the first step to understand function. Early studies observed that most organisms were members of the *Firmicutes* and *Bacteroidetes* phyla and a low abundance of *Proteobacteria* (Eckburg et al., 2005). With the advent of more improved and cost-effective sequencing technologies in the late 2000's, understanding microbiome populations on a global scale became much more feasible. Several landmark studies such as the Human Microbiome Project (HMP) (Human Microbiome Jumpstart Reference Strains et al., 2010) and MetaHIT (Li et al., 2014; Qin et al., 2010) established the baseline structure of the gut microbiome. A pivotal study by the Gordon group examined gut microbial populations of healthy children and adults from rural Malawi, US metropolitan areas and tribal populations of Venezuela and demonstrated that taxonomic changes occur as a function of age and population (Yatsunenko et al., 2012). The variation in gut microbial composition across the adult lifespan and geography underscores the influence of nutritional and environmental changes on human physiology and development.

While the adult microbiome is predominantly stable, extreme perturbations such as diarrhea can dramatically alter the composition of the gut microbiome. In cholera-endemic regions, diarrhea occurs from a multitude of factors including malnutrition, excessive antibiotic usage, and reduced host resistance to infection,

continuing the deleterious cycle. Indeed, multiple studies demonstrated that either *V. cholerae* or enterotoxigenic *Escherichia coli* (ETEC) infection can drive the microbiome to a dysbiotic state that is predominantly composed of *Enterococcus, Escherichia,* and *Streptococcus* (David et al., 2015; Hsiao et al., 2014). At 30 days post-infection, the gut microbiota returned to a state resembling healthy subjects, with *Prevotella* being dominant, which is indicative of a healthy microbiome in developing regions (David et al., 2015). Some of the processes that are involved in gut microbial succession include dispersal potential, resource availability, and changes in environmental stresses (Fierer et al., 2010). The prediction around dispersal potential is that microbes that are more motile can disperse more easily in the gut to fill vacant niches. A method for the dispersion is from microbes that are found on foods. In accordance with this idea, 24% of the Early-Stage taxa were found to be present on various food items (David et al., 2015). Environmental stresses such as oxygen are also a large factor in determining microbial succession. Oxygen levels are abnormally elevated after infection, allowing for facultative anaerobes such as *Escherichia, Enterococcus,* and *Streptococcus* to initially dominate. The last factor involved in microbial succession involves nutrient availability. At 7 days post-presentation in the mid-stage of succession, there was an enrichment of carbohydrate-associated genes particularly with *Bacteroides*, as 70% of these genes had at least 40% of their metagenomic reads mapping to the genus (David et al., 2015). Indeed, nutrient availability for enteric microbes is an important factor for gut

microbiome composition. A recent study examined the gut microbiomes of both healthy and malnourished children and found that severe acute malnutrition greatly contributes to microbiota immaturity (Subramanian et al., 2014). Gut microbial succession is also integral in host colonization resistance to pathogens such as *V. cholerae.* In particular, a study constructed artificial communities that are either representative of human gut bacterial species that are involved with the recovery of cholera in adults or healthy children in Bangladesh. The study noted that one of the species, *Blautia obeum,* was able to establish well in the gut with *V. cholerae* and reduce *V. cholerae* colonization levels by utilizing quorum-sensing repression of various *Vibrio* colonization factors (Hsiao et al., 2014). A recent follow up study observed that *B. obeum* is also able to reduce *V. cholerae* colonization by degrading the host produced virulence molecule taurocholate (Alavi et al., 2020). These various studies characterize the human gut microbiome's variability due to geography, age, and nutritional differences and the resulting implications of a normal versus dysbiotic microbiome.

Cholera vaccines

While improved infrastructural sanitary improvements would help abate the spread of cholera, vaccination is still an excellent form of prevention. Over the last century, there have been various efforts to develop cholera vaccines with the hope of offering robust universal protection.

Observations by Koch and colleagues in the 1880's noted that people who had cholera were protected from the disease during the same pandemic, indicative of naturally occurring immunity (Pollitzer and Burrows, 1955). The first cholera vaccine was developed by Ferran in 1885 and used in a vaccination campaign in Spain. The first oral cholera vaccine (OCV) was developed in 1893 when two researchers cultured agar-grown *V. cholerae* in broth and ingested the heat-killed culture (Pollitzer and Burrows, 1955).

In the early 1960's, parenteral whole cell killed vaccines continued to be developed and were administered in various trials. A whole cell killed (WCK) *V. cholerae* O1 (Ogawa+Inaba mixture) was tested in a double-blind controlled trial in Bangladesh in 1963-1964 and induced high levels of serum vibriocidal antibody (Benenson et al., 1968b). It also achieved 76% protective efficacy after 6 months in adults, but not for children and resulted in many reactogenic events such as fever and pain and swelling at the injection site (Benenson et al., 1968a). Additionally, a parenteral vaccine that was licensed in the US was administered in two doses. However, it provided only 50% efficacy for 6 months, deeming it not sufficient by the WHO ((WHO), 2001). Due to the limited duration of efficacy in subjects and the increased numbers of reactogenic events, cholera vaccine design then shifted to oral administration.

Moving towards oral, mucosal administration was beneficial for several reasons as it would mimic natural infection, is capable of inducing IgA immune responses, and is a needle-free vaccination, reducing the training required to

administer the vaccine and making it more suitable for deployment in developing regions. However, the oral route also has its downsides such as needing to potentially overcome mucosal tolerance, surviving oral delivery through stomach acid and digestive enzymes as well as retaining immunogenicity without reactogenicity.

There are primarily two killed OCVs. The first licensed killed WC oral vaccine was Dukoral in 1991. It consists of recombinant cholera toxin subunit B (CTB) and three strains of inactivated O1 Classical and one strain of O1 El Tor. The vaccine requires administration with a sodium bicarbonate buffer to protect the acid labile CTB component. A main component of oral tolerance is that the body's immune system would not respond to dead, foreign material, thus, a killed vaccine might not be optimum, however, at the time of production for this vaccine, it was discovered that CT itself was an adjuvant. Thus, there were great hopes that inclusion of CTB would act as a successful adjuvant with the vaccine.

Initially, the vaccine was made with purified B subunit of CT (Bs-WC) and was shown to be safe and immunogenic in endemic areas (Clemens et al., 1991). As the recombinant CTB (rCTB) was easier and less expensive to manufacture, it replaced the purified CTB and was shown to have similar safety and efficacy profiles (Sanchez and Holmgren, 1989).

The second widely approved killed WCK vaccine is Shanchol. Given the relative success of the Dukoral vaccine, Vietnamese scientists developed a killed OCV containing various *V. cholerae* strains including one O139 strain, two O1

Classical, and one O1 El Tor strain, however, it was produced without the addition of rCTB. The vaccine is given in a two-dose regimen over two weeks without buffer and was shown to be safe and immunogenic in children and adults (Trach et al., 2002) and demonstrated 50% protection 3-5 years after vaccination (Thiem et al., 2006).

There are also live attenuated cholera vaccines, which are considered to be more immunogenic and generate stronger mucosal vaccine responses as compared to whole cell killed (WCK) vaccines. Lending support to this idea, a study noted that live *V. cholerae* were taken up by M cells in Peyer's patches, whereas killed bacteria were not (Owen et al., 1986), suggesting that live bacteria are more readily sensed and acted upon by the immune system. The main concern for developing a live attenuated cholera vaccine and live attenuated vaccines in general is to have a balance between strong immunogenicity to develop a robust immune response but low reactogenicity to avoid detrimental adverse outcomes. As CT was identified as the dominant factor in mediating cholera pathogenesis, it was paramount to attenuate its effects. One of the first live-attenuated vaccines was Texas Star-SR. It consisted of an El Tor strain and produced no catalytic CT-A, only CT-B achieved by chemical mutagenesis. The vaccine resulted in seroconversion of 96% of the subjects, however, it resulted in high levels of diarrhea and the method of chemical mutagenesis was deemed unsuitable as the point-mutation could easily revert back to wild-type (Levine et al., 1984). A successful live attenuated vaccine was

developed at the University of Maryland's Center for Vaccine Development and was branded CVD 103-HgR. It consists of the *V. cholerae* O1 classical Inaba strain, with 94% of the *ctxA* gene deleted, leaving the *ctxB* gene intact, with a mercury resistance cassette inserted into the gene for hemolysin, to aid in identification purposes (Levine et al., 1988). The dose was given as a single dose and was found to be safe and immunogenic in Swiss volunteers (Cryz et al., 1990) (Table 1.1).

Table 1.1 Summary of significant findings of the gut microbiome's effect on immune response

Pathogen or Vaccine	Host	Significant Findings	Reference
V. cholerae El Tor N16961	Healthy adult volunteers	Variable *Vibrio* serum antibody responses in North American adults compared to Bangladeshi adults; at most time points, serum anti-CtxB IgA and IgM responses were greater in naïve North American population than Bangladeshi participants	Hossain et al, 2019
HaitiV (live attenuated Oral Cholera Vaccine)	Infant rabbits	HaitiV was constructed from a *V. cholerae* strain from Haiti outbreak and consists of 9 modifications, rendering less virulent but still capable of imparting protection in a probiotic-like fashion	Hubbard et al, 2018
Dukoral (killed Oral Cholera Vaccine)	Healthy adult volunteers	Seven different probiotic strains were given to healthy adults prior to and after vaccination with Dukoral. Serum specific IgG was increased in groups given *Bifidobacterium lactis* and *Lactobacillus acidophilus* compared to control	Paineau et al, 2008
killed Oral Cholera Vaccines	Swedish and Nicaraguan children	Serum anti-CTB IgA and serum vibriocidal titer was greatly increased in Swedish children as compared to the Nicaraguan children	Hallander et al, 2002
Trivalent Influenza Vaccine (TIV)	Antibiotic-treated mice	Flagellin-sensing by TLR5 is necessary to stimulate immune responses to TIV. Vaccine specific responses were reduced in antibiotic-treated and germ-free mice but restored upon bacterial colonization	Oh et al, 2014
Seasonal Influenza Vaccine	Healthy adult volunteers	Antibiotics given to subjects with low baseline titers prior to vaccination exhibited a reduced H1N1-specific IgG1 response. However, there was not difference for subjects that had high baseline titers	Hagan et al, 2019
Influenza A	Antibiotic-treated mice	Antibiotic treatment of adult mice prior to infection with influenza A virus resulted in a reduction of CD4 T-, CD8 T-, and B cell specific immune responses	Ichinohe et al, 2011
Inactivated PR8 influenza virus (IPR8)	Infant nonhuman primates	Infants vaccinated with flagellin-adjuvanted influenza virus yielded an increase in virus-specific T cells compared to infants given a mutant flagellin in conjuction with the inactive virus	Kim et al, 2015
D x RRV (oral rotavirus vaccine)	Infants	*Lactobacillus casei* strain GG (LGG) administered to 2-5 month old infants along with a live oral rotavirus vaccine increase in rotavirus-specific IgM secreting cells as well as greater IgA seroconversion compared to control	Isolauri et al, 1995
Rotarix (oral rotavirus vaccine)	Adult volunteers from Amsterdam	Targeted and broad antibiotic administration yielded no change in absolute anti-RV IgA over time compared to control. However, there was a boosting effect at d7 in the broad spectrum antibiotic-treated group	Harris et al, 2018
Rotarix (oral rotavirus vaccine)	South India infants	There were no consistent associations between seroconversion and microbial composition. As seroconversion was only 30%, it's possible that the microbiota structures of the overall study were inhibitory to seroconversion as compared to infants in developed regions	Parker et al, 2018

Most recently, the Waldor research group developed a live attenuated cholera vaccine based on the wild type HaitiV strain (Hubbard et al., 2018). The live-attenuated strain has nine modifications that render it less virulent, but it still imparts long term immunity. Some of the modifications included removal of the bacteriophage (CTXϕ) encoding CT, deletion of flagellin genes, as well as removing genes within the SXT integrative conjugative unit that would allow antibiotic resistance. Their results indicate that HaitiV mediates colonization

resistance to wild type *V. cholerae* when given 24 hours prior to wild type infection. While long-term protection was not measured, the vaccine exhibits probiotic-like protection. Vaccination remains an important measure to prevent and stem cholera outbreaks and must be optimized to provide maximum benefit.

The gut microbiome and oral cholera vaccine responses

Both live-attenuated vaccines and whole-cell killed vaccines are used for cholera prevention and control of outbreaks, however, there is notable variation of protective efficacy when the vaccines are given in developing vs. developed regions.

CVD 103-HgR, currently branded as Vaxchora, consists of the *V. cholerae* O1 classical Inaba strain, with 94% of the *ctxA* gene deleted, leaving the *ctxB* gene intact. While the vaccine was safe and immunogenic in US trials (Levine et al., 1988), it also had a less desirable outcome in a cholera endemic area. When tested in a large-scale field trial in Bangladesh, the vaccine exhibited an overall efficacy of 14% (Richie et al., 2000). While this result was certainly less than ideal, CVD 103-HgR was also tested later in an outbreak scenario in Micronesia and had an 80% efficacy after 10 days (Calain et al., 2004). Another promising live-attenuated vaccine is CholeraGarde, consisting of O1 El Tor Inaba with CT deleted; it was safe and immunogenic in US and Bangladeshi adults as well as Bangladeshi toddlers and infants, albeit with lower cholera toxin responses as seen in Bangladesh adults (Cohen et al., 2002; Qadri et al., 2007; Qadri et al.,

2005). While cholera vaccine efficacy has been variable in the past, there are promising vaccines which may offer more robust immune responses.

Both killed and live oral cholera vaccines have performed less well in populations of developing countries as compared to developed countries, particularly in younger children. The killed oral cholera vaccine Dukoral, consisting of recombinant cholera toxin subunit B (CTB) and three strains of inactivated O1 classical and one strain of O1 El Tor, showed a 50% efficacy against all age groups in a large field trial in Bangladesh (Clemens et al., 1990). while it resulted in significant vibriocidal titers in 89% of volunteers in the US (Black et al., 1987). Another study compared the vibriocidal responses in age matched Swedish children and although they had a lower baseline titer, there was a greater vibriocidal response (Hallander et al., 2002).

Shanchol, a similar inactivated vaccine that is composed of one O139 strain, two O1 classical, and one O1 El Tor strain demonstrated a response efficacy of 5 years, which is longer than the 3-year efficacy that is more commonly observed. In children aged 1-5 years, the vaccine had an efficacy of 43% after 5 years as compared to 65% in the 5-15 years of age group (Bhattacharya et al., 2013; Sur et al., 2011).

Gut microbial composition and metabolism plays an integral role in maintaining host physiological systems and is integral in mediating host immune responses. Data presented in the following chapters supplements the understanding of the gut microbiome-pathogen-host axis.

CHAPTER 2: A Dysbiotic Microbiome Suppresses Immune Responses Against *V. cholerae*

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SUMMARY

Cholera is a severe diarrheal disease that places a significant burden on global health. Cholera's high morbidity demands effective prophylactic strategies, but oral cholera vaccines exhibit variable efficacy in human populations. One contributor of variance in human populations is the gut microbiome, which in cholera-endemic areas is modulated by malnutrition, cholera, and non-cholera diarrhea. We conducted fecal transplants from healthy human donors and model

communities of either human gut microbes that resemble healthy individuals or those of individuals recovering from diarrhea in various mouse models. We show microbiome-specific effects on host antibody responses against *Vibrio cholerae*, and that dysbiotic human gut microbiomes representative of cholera-endemic areas suppresses the immune response against *V. cholerae* via CD4+ lymphocytes. Our findings suggest that gut microbiome composition at time of infection or vaccination may be pivotal for providing robust mucosal immunity, and suggest a target for improved prophylactic and therapeutic strategies for cholera.

Keywords: gut microbiome, cholera, immune responses, oral cholera vaccines, interpersonal variation

Introduction

Vibrio cholerae is the etiologic agent of cholera, a severe diarrheal disease affecting approximately 3 million people annually, resulting in approximately 100,000 deaths (Ali et al., 2015). The bacterial mechanisms through which *V. cholerae* causes infection *in vivo* have been extensively studied. *V. cholerae* preferentially colonizes the small intestine, where it releases cholera toxin (CT), which causes profuse watery diarrhea and loss of electrolytes. While the advent of oral rehydration therapy has dramatically reduced mortality from cholera, recent major outbreaks are reminders of the pressing global public health need to improve cholera prevention strategies. Although cholera is thought of as a non-inflammatory disease, potentially due to the action of the MARTX toxin in suppressing host inflammatory responses during infection (Woida and Satchell, 2020), the relationship of host immunity to cholera is of critical importance to the control of the disease, both for the outcome of infection but also the outcome of prophylactic strategies such as vaccination. Though several oral cholera vaccines (OCVs) have been developed, they have demonstrated high variance in protective efficacy in field trials (Levine, 2010); while OCVs have been shown to have protective efficacy of generally 80-90% in areas of good sanitation such as the United States and Europe, large field studies in cholera-endemic regions with less developed infrastructure such as Bangladesh and India have exhibited less overall efficacy of as little as 55% (Bishop and Camilli, 2011; Clemens et al., 1990; Clemens et al., 1991; Harris et al., 2017; Levine et al., 1988; Richie et al.,

2000). Cholera vaccine studies to optimize vaccine responses in endemic areas are ongoing, whether it be higher dosing of a live attenuated vaccine (Sow et al., 2017) or to understand the effects of single doses of killed oral cholera vaccines (Ali et al., 2021; Qadri et al., 2018; Qadri et al., 2016). We hypothesized that one contributor to this high level of geographical variation in OCV efficacy, and potentially antibody responses to *V. cholerae* infection, may be variation in the microbial populations of the gut, the gut microbiome.

Several studies have shown that gut bacterial populations can change due to diet and geography, (Subramanian et al., 2014; Yatsunenko et al., 2012) especially when comparing populations in the United States and Europe versus those in resource limited regions that have higher rates of enteric disease (Arumugam et al., 2011; Costea et al., 2018; Qin et al., 2010). After cholera diarrhea, the gut microbiome shifts to a taxonomically less diverse and dysbiotic state, largely composed of Streptococci, before transitioning to a conformation comparable to non-diarrheal individuals over the course of several weeks once the acute phase of the disease is over (Hsiao et al., 2014). This dysbiotic configuration has been observed in studies examining other enteric pathogens such as enterotoxigenic *E. coli* and rotavirus (David et al., 2015; Kieser et al., 2018) as well as other environmental insults such as malnutrition also commonly found in cholera-endemic areas (Subramanian et al., 2014). Recent work highlights the role of the gut microbiome in either conferring resistance or susceptibility to *V. cholerae* infection; key commensal microbes have been

shown to modulate resistance to *V. cholerae* infection via degradation of bile salts, which are critical in the virulence activation pathway (Alavi et al., 2020). While the presence of a normal murine microbiome has been implicated in antibody responses against viral vaccines (Oh et al., 2014), the effects of a human gut microbiome on host responses to *V. cholerae* or other enteropathogenic bacteria have not been well determined.

In this study, we sought to understand how variation in microbial communities affect immune responses upon infection with *V. cholerae.* We hypothesized that microbial dysbiosis from recurring environmental insult in cholera-endemic areas represents a recurring window of vulnerability to insufficient commensal-modulated immune responses to *V. cholerae*, and more broadly that interpersonal variation in microbiome structure can lead to individual-specific responses. We initially conducted fecal transplants from a small subset of human donors into germ-free mice in order to characterize immune correlates of protection when colonized with complete human fecal microbiomes. Based on these results, we moved beyond our fecal transplant observations to defined human model microbial communities in antibiotic-cleared mice. This allowed us to better understand the role of how interpersonal human microbiome variation at time of infection affects antibody responses. We show here that individual human gut microbiomes drive differential antibody responses to both wild-type and vaccine strains of *V. cholerae*, and that these immune responses are dampened by the presence of dysbiotic gut bacterial populations in a CD4+-cell-dependent

manner. These findings suggest that gut bacterial composition at time of infection may impact adaptive immune responses to *V. cholerae*, and suggests that oral cholera vaccine design and distribution may need to take into account gut microbiome structure for optimal efficacy.

Results

Human microbiomes drive variable immune responses to *V. cholerae* in an adult germ-free mouse model of infection

Preclinical studies in animal models are essential to elucidate the mechanisms underlying interactions of host immunity, pathogens, and commensal microbes during infection. Several animal models have been developed for studying the behavior of *V. cholerae in vivo,* the most widely used being the infant mouse cholera model (Klose, 2000). However, while the suckling animals can be used to study *Vibrio* colonization and virulence, they are poorly suited for immunological studies, as the infant mouse does not have a fully developed adaptive immune system, a limitation shared by the recently developed infant rabbit model of cholera (Hubbard et al., 2018; Ritchie et al., 2010). While adult conventionally-reared mice have been used to explore immune responses to infection (Nygren et al., 2008; Nygren et al., 2009), the murine gut microbiome differs dramatically from human gut commensal communities, and is highly refractory to the addition of human-associated bacterial species (Seedorf et al., 2014). Adult germfree

(GF) mice can be used to finely control microbial content, but exhibit reduced adaptive immunity in their axenic state. However, transient colonization with even a single bacterial species has been shown to restore immunoglobulin production to conventional levels (Hapfelmeier et al., 2010).

In order to evaluate the role of variation in human gut microbiomes in immune responses to *V. cholerae* infection, we used several experimental paradigms involving complete and defined model human gut microbiomes in adult mice. First, we transplanted several complete human fecal gut microbiomes into C57/BL6-Tac germfree (GF) mice via intra-gastric gavage. These fecal samples were part of a previously-established biospecimen repository consisting of fecal samples taken from a healthy adult cohort in the United States (Alavi et al., 2020). Individuals were 18-45 years of age, and at time of collection had not suffered recent diarrhea or undergone antibiotic usage, and did not report any ongoing inflammatory conditions of the gastrointestinal tract.

After two weeks to allow for the establishment of human microbial colonization and restoration of adaptive immune activity, animals receiving these complete human fecal microbial communities were equivalently colonized (Figure 2.1) prior to inoculation with ~5 x 10^9 CFU of *V. cholerae* C6706 El Tor. *Vibrio* shedding following infection was low and consistent across human microbiome contexts (Figure 2.2), perhaps due to the high density of pre-established commensals relative to *V. cholerae* inoculum and the lack of virulence-

associated clearance mechanisms such as diarrhea that is a characteristic of *V. cholerae* colonization in adult animals (Freter, 1956; Olivier et al., 2007).

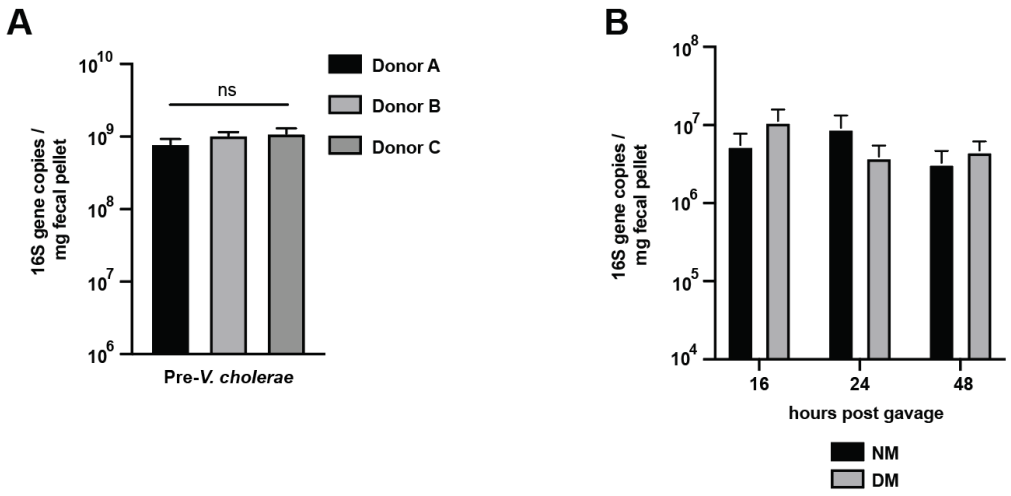

Figure 2.1. 16S gene copy number per mg fecal pellet for designated samples.(A) After 2 weeks of colonization in germ-free mice, little variation in bacterial abundance was observed by 16S qPCR prior to infection with *V. cholerae*. (B) Bacterial abundance in the antibiotic treated adult mouse model post gavage with indicated communities. ns, $p>0.05$, one-way ANOVA.

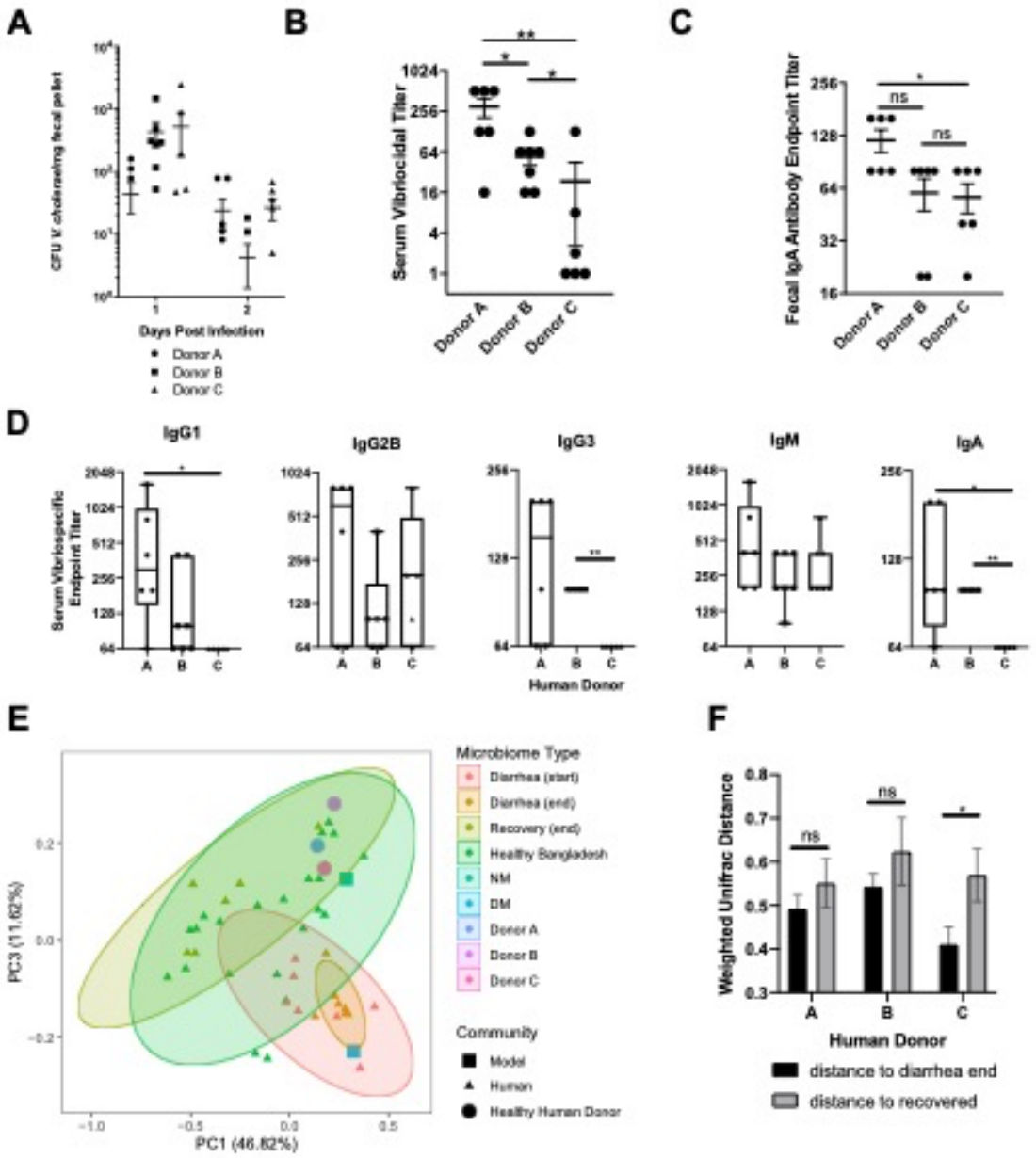
A
CFU V. cholerae/mg fecal pellet
Days Post Infection
Donor A
Donor B
Donor C
B
Serum Vibriocidal Titer
Donor A
Donor B
Donor C
C
Fecal IgA Antibody Endpoint Titer
ns
ns
D
IgG1
IgG2B
IgG3
IgM
IgA
Serum Vibriospecific Endpoint Titer
Human Donor
E
Microbiome Type
Diarrhea (start)
Diarrhea (end)
Recovery (end)
Healthy Bangladesh
NM
DM
Donor A
Donor B
Donor C
Community
Model
Human
Healthy Human Donor
PC3 (11.62%)
PC1 (46.62%)
F
Weighted Unifrac Distance
ns
ns
Human Donor
distance to diarrhea end
distance to recovered

Figure 2.2. *V. cholerae*-specific antibody levels in fecal and serum samples. (A) *V. cholerae* colonization in mice containing fecal microbiota of indicated human donors. (B) Serum vibriocidal titer in mice by donor community colonization after *V. cholerae* infection at 4 weeks post infection. (C) Fecal vibriospecific IgA endpoint titer in germ-free mice for human donors at 4 weeks post infection. (D) Serum vibriospecific endpoint titers for various types of antibody across the donors 4 weeks post infection. (E) Metanalysis of existing 16S ribosomal gene sequencing datasets to compare Donors A, B and C as well as defined communities (NM & DM) to an adult cholera cohort from Bangladesh and a cohort of healthy adults based on weighted UniFrac distance, % variance explained by axis shown in parentheses. Ellipses represent 95% confidence intervals. (F) Weighted Unifrac distances of US human donor communities versus Bangladeshi communities at diarrhea end or state of recovery *, $P<0.05$, **, $P<0.01$, Mann-Whitney *U* test. Error bars represent mean ± SEM. (1F) *, $P<0.05$, Student's t-test.

To evaluate the efficacy of anti-*V. cholerae* antibody responses, we examined levels of *Vibrio*-specific immunoglobulin responses in both serum and fecal samples from these animals. First, we used a serum vibriocidal assay, which is considered to be the best clinical correlate of protection for cholera (Haney et al., 2017; Harris, 2018; Qadri et al., 2005; Sow et al., 2017). The serum vibriocidal titer (SVT) from this assay is the reciprocal of the highest dilution of serum at which killing of *V. cholerae* is observed with the addition of exogenous complement. In humans, a clinically successful seroconversion as a result of vaccination is defined as a more than four-fold rise in serum vibriocidal titers compared to baseline pre-immune titer over two weeks, although there is no defined titer at which protection can be considered to be definitively achieved (Kanungo et al., 2015). Interestingly, after 4 weeks post infection, the serum vibriocidal titer data varied up to 12-fold in animals hosting gut microbiomes from different human donors, ranging from a mean titer of 300 in Donor A to 23.5 in Donor C. This suggested that gut microbial composition was a strong and personalized driver of immune responses to the introduction of *V. cholerae* (Figure 2.2B). Measurement of vibriospecific serum antibodies using a whole-cell ELISA assay yielded statistically significant differences by donor both in IgA, IgG1, and IgG3 isotypes (Figure 2.2D).

Although the serum vibriocidal titer is an important correlate of immunity after infection or vaccination, actual protection to subsequent challenge is mediated by secreted immunoglobulin at the gut mucosa (Strugnell and Wijburg,

2010). During the course of infection, class-switching to IgA and the secretion of antigen-specific secretory IgA (S-IgA) serves as the main means of protection by binding to *V. cholerae* and preventing pathogen access to epithelium, and neutralizing cholera toxin (Apter et al., 1993). Recent studies have also indicated that anti-O-specific polysaccharide antibodies in sera from humans surviving cholera can agglutinate *Vibrio* and prevent motility (Charles et al., 2020), and that expression of a monoclonal human anti-LPS IgA1 in mice can provide passive protection to infants from milk (Baranova et al., 2020). For an up-to-date article of cholera immunity, please read Holmgren J, Trop. Med. Infect. Dis., 2021 (Holmgren, 2021). A recent study highlights the capacity of a monoclonal IgA antibody to inhibit *V. cholerae* motility, preventing access to the intestinal epithelium (Levinson et al., 2015). The bulk of IgA in the body is secretory IgA (S-IgA) secreted in gram quantities per day onto the mucosa (Macpherson et al., 2012). We observed differences in antibody titers of *Vibrio*-specific IgA across weight-normalized fecal suspensions from mice colonized with different human donors, matching the pattern seen in serum vibriocidal responses; mice colonized with Donor A microbes showed the highest fecal IgA responses to *V. cholerae*, and Donor C the lowest (Figure 2.2C).

A comparative analysis of US donors with Bangladeshi cholera cohorts yields insights into overall gut microbiome structures

We performed a metanalysis using Principal Coordinates Analysis (PCoA) of existing 16S ribosomal RNA gene sequencing datasets to compare the microbial community structure of these complex human fecal microbiomes to an adult cholera cohort from Bangladesh (Hsiao et al., 2014) and a cohort of healthy adults from Bangladesh (Subramanian et al., 2014) (Figure 2.2E). In accordance with previous studies (Hsiao et al., 2014), the early- ("diarrhea start") and immediately post-diarrhea microbiome ("diarrhea end") was distinct from the state in the same individuals after 3 months of convalescence from diarrhea ("recovery"). Recovery samples in turn resembled a broader cohort of individuals that were healthy at time of sampling ("Healthy Bangladesh"). While the US Donors (A, B, C) overlapped in microbiome structure with healthy Bangladesh adults and with recovered diarrhea patients, the diversity among the ostensibly continuously healthy Bangladesh cohort was extremely high, and in some cases overlapped with dysbiotic post-diarrhea microbiomes (Figure 2.2E). This suggests that if gut microbiome dysbiosis as a function of diarrhea or malnutrition affects host immune responses to subsequent *V. cholerae* infection or vaccination, that the population impact in cholera endemic areas may be even more significant than the variance observed in US microbiomes. Interestingly, the microbiome of Donor C, which yielded the weakest SVT when transplanted into GF mice, was the only complex US donor community to be more similar to the

dysbiotic post-diarrhea state in Bangladesh (“diarrhea end”) than the same patients 3 months after recovery from acute diarrhea (“recovery”) (Figure 2.2F) using an abundance-weighted metric, weighted UniFrac distance.

Colonization of model communities of normal or dysbiotic gut microbiota results in differential immune response outcomes in mice

To expand upon our gnotobiotic studies we constructed several defined, simplified, model human gut microbiomes (Figure 2.3A) as shown in our previous studies (Alavi et al., 2020). One model community, “NM”, was characteristic of healthy human microbiomes found in the United States and Bangladesh, and contained *B. obeum* and a commonly found *Bacteroides, Bacteroides vulgatus*, and *Clostridium scindens*. As a comparison beyond “healthy” individuals, we constructed a second defined community (“DM”) that was representative of microbiomes suffering from dysbiosis found in cholera endemic areas (Alavi et al., 2020). A comparison against complex human microbiomes confirmed that the NM community was representative of healthy Bangladesh gut microbiomes, and the DM community was similar to the dysbiotic state found at the end of watery diarrhea (Figure 2.2E). Diarrhea of multiple etiologies, along with severe malnutrition, a common comorbidity of tropical diarrheas, drive the human gut microbiome to a characteristic low-diversity state dominated by bacteria such as Streptococci, Enterococci, and Proteobacteria (Alavi et al., 2020; David et al., 2015; Hsiao et al., 2014; Kieser et al., 2018; Subramanian et al., 2014). While

this state is able to recover over the course of weeks following the end of diarrhea or the application of therapeutic dietary interventions (David et al., 2015; Di Luccia et al., 2020; Hsiao et al., 2014), we hypothesized that this dysbiotic state represents a window where microbial community structure may lead to poor responses to *V. cholerae* antigen.

The logistical limitations of germfree mice limit the number and type of experimental human microbiomes and conditions to be tested. To address this in an immune-competent experimental system, we used adult conventionally reared CD-1 animals that had their native microflora cleared using treatment with antibiotics, as mouse-adapted microbes rapidly out-compete non-murine communities (Seedorf et al., 2014). Animals were given an antibiotic cocktail in drinking water for 1 week (See Materials and Methods) and then switched to streptomycin treatment alone 3 days before the gavage. The mice were then gavaged with *V. cholerae* C6706 O1 El Tor, which is resistant to streptomycin as well as the respective NM or DM communities. As measured by 16S qPCR, total bacterial load was consistent between NM and DM and persisted at least until 48 hours post gavage (Figure 2.1B). We observed that antibiotic treatment was in fact critical to observe robust antibody responses against infection by *V. cholerae* (Figure 2.3B).

A

Bacterium	Community	Strain Name	NCBI Taxonomy ID
Bacteroides vulgatus	NM	ATCC 8482	435590
Blautia obeum	NM	ATCC 29174	411459
Clostridium scindens	NM	ATCC 35704	411468
Enterococcus faecalis	DM	OG1RF	474186
Escherichia coli	DM	DH5αλpir	668369
Streptococcus infantarius subsp. *infantarius*	DM	ATCC BAA-102	471872
Streptococcus salivarius subsp. *salivarius*	DM	ATCC 13419	1304
Streptococcus salivarius subsp. *thermophilus*	DM	DSM 20617	1091038

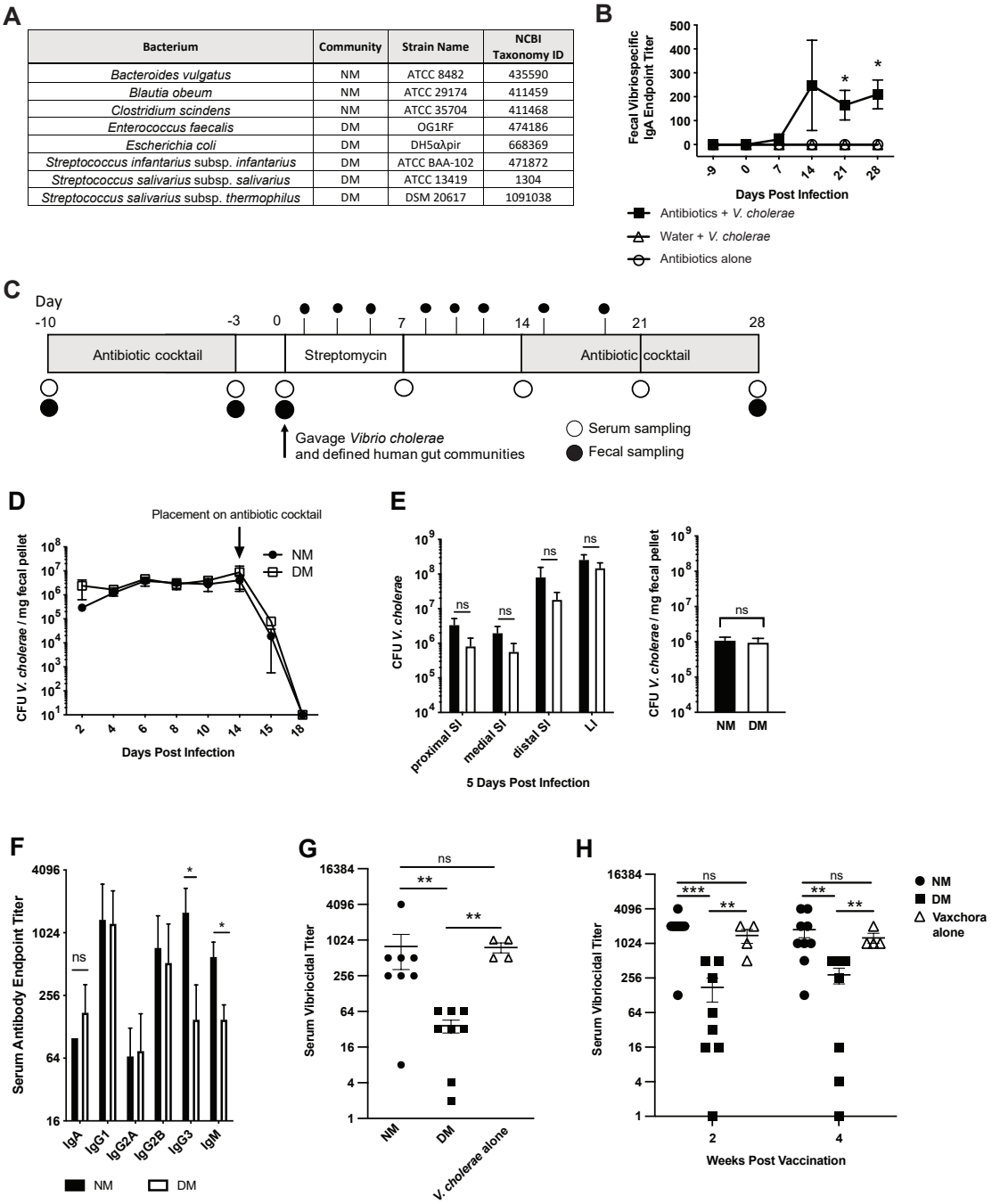

Figure 2.3. **Model community colonization and antibody responses in antibiotic treated adult mouse model.** (A) Composition of defined human communities. (B) Antibiotic treatment is required to elicit vibriospecific antibody responses. (C) Schematic of antibiotic treatment and bacterial introduction in SPF CD-1 mice. (D) *V. cholerae* colonization levels after co-gavage of NM and DM communities; antibiotic cocktail added at 14 days post infection. (E) *V. cholerae* colonization loads in proximal, medial, distal small intestine, large intestine and fecal pellet at 5 days post infection. (F) Serum antibody profiles against whole cell *V. cholerae* 4-weeks post-infection. (G) Serum vibriocidal titer 4-weeks post-introduction of *V. cholerae* and indicated defined communities and *V. cholerae* alone in antibiotic-treated mice. (H) Serum vibriocidal titer 2 and-4-weeks post-vaccination with CVD 103-HgR-SmR in the presence of indicated defined communities and the vaccine strain alone in antibiotic-treated mice. NM: normal model microbiome, DM: dysbiotic model microbiome. SI: small intestine, LI: large intestine. ns, $P>0.05$, *, $P<0.05$, **, $P<0.01$, ***$P<0.001$, Mann-Whitney U test. Error bars represent mean ± SEM. n=4-8 mice per group for all experiments.

Since even very transient presence of *V. cholerae* was able to induce strong antibody responses in a microbiome-dependent manner in GF mice, and to prevent any sustained differences in *V. cholerae* colonization, as well as the resurgence of murine commensals, we placed these animals back on an antibiotic cocktail after 2 weeks post introduction of *V. cholerae* (see Figure 2.3C for experimental layout). The extended presence of streptomycin and restoration of antibiotic cocktail in this experimental system prevented major effects of the host microbiomes on *V. cholerae* that might be expected from previous studies of the effect of human commensals on colonization resistance (Alavi et al., 2020), thus standardizing the amount of *V. cholerae* able to interact with host immunity across groups. In order to confirm that the load of *V. cholerae* does not contribute to subsequent immune outcomes, we measured CFU *V. cholerae* per mg fecal pellet including after placement on an antibiotic cocktail at 14 days post infection (Figure 2.3D). We observed no variation in *V. cholerae* levels in the mice that were given the NM or DM communities at time of infection. Additionally, in order to evaluate whether *V. cholerae* colonization load may affect gut mucosal antibody responses, we examined proximal, medial, and distal small intestine as well as large intestine and fecal pellet *V. cholerae* colonization levels 5 days post infection while the mice were maintained on streptomycin and before replacement on the antibiotic cocktail and found no statistically significant differences in pathogen load in this system (Figures 2.3D-E). Taken together, these data suggest that any differences in host immune responses by the

presence of model human microbes during infection will not be due to accessibility of antigen in a colonization-dependent manner.

At 4 weeks post introduction of *V. cholerae*, serum and fecal samples were collected from antibiotic-cleared mice containing NM and DM human microbiomes. Serum vibriospecific ELISA showed that levels of IgG3 and IgM, strong complement fixing antibodies, were decreased in the DM group as compared to the NM group (Figure 2.3F). Additionally, vibriospecific antibody levels were examined at 0 days post infection and 2 weeks post infection but showed no significant differences as a function of microbiome at time of introduction of *V. cholerae* (Figure 2.4). We observed that serum from animals bearing the (DM) microbiome at time of infection exhibited a statistically significant reduction in serum vibriocidal activity compared to that from animals infected in the presence of the (NM) microbiome as well as *V. cholerae* alone (Figure 2.3G), suggesting that the presence of members of the dysbiotic community at time of infection may hinder the development of a robust serum antibody response.

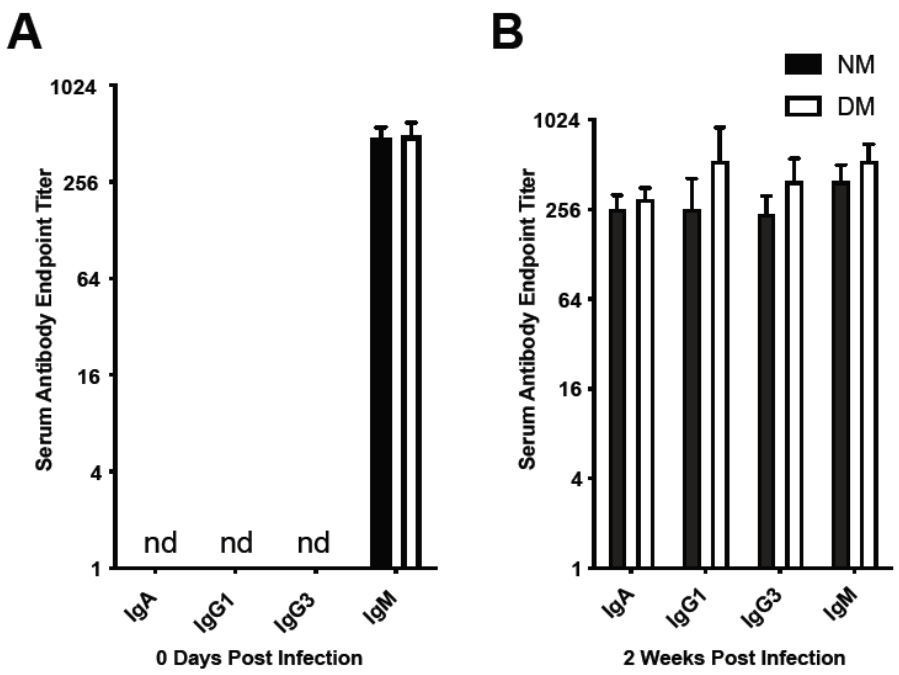

Figure 2.4. Serum antibody profiles at designated timepoints. (A) Serum antibody profiles against whole cell *V. cholerae* 0 days post infection and (B) 2-weeks post-infection. nd, not detected.

Fecal Ig from *V. cholerae* infected mice bearing the DM community are less protective in an infant passive protection mouse model

Although the vibriocidal assay represents an established correlate of protection in humans, we examined the ability of mucosal antibodies to affect *V. cholerae* infection, as secreted immunoglobulins are likely the direct mediators of protection following immunity due to natural infection or immunization. We therefore used a passive protection assay to determine the efficacy of fecal antibodies in protection against *V. cholerae*. Fecal antibody generated by animals containing different model microbiomes were enriched from other fecal

constituents using protein L purification (See Methods). These antibody pools were predominantly IgA with low levels of IgM (Figure 2.5A). In order to examine whether enriched antibody preparations or fecal water had any intrinsic effects on *V. cholerae* growth, we conducted an *in vitro* growth inhibition assay. We observed no alteration in growth between enriched and non-enriched fecal water for our respective communities, confirming the absence of inhibitory components in enriched antibody preparations (Figure 2.5B). Enriched Ig from both groups was combined with *V. cholerae* grown overnight and incubated for 1 hour before being gavaged into 4-day old infant CD-1 mice. Suckling animals were used as conventionally-reared adult animals without antibiotics are highly resistant to *V. cholerae* colonization (Olivier et al., 2009; Olivier et al., 2007). After 18 hours of infection, the small intestines were homogenized and plated on selective medium. We observed that pre-treatment with antibody from animals bearing the dysbiotic microbiota led to colonization nearly 2-log greater than pre-infection treatment with antibody from animals with the NM microbiome (Figure 2.5C). Taken together, these findings suggested that oral infection in a DM microbiome context led to a significantly less effective anti-*Vibrio* antibody response.

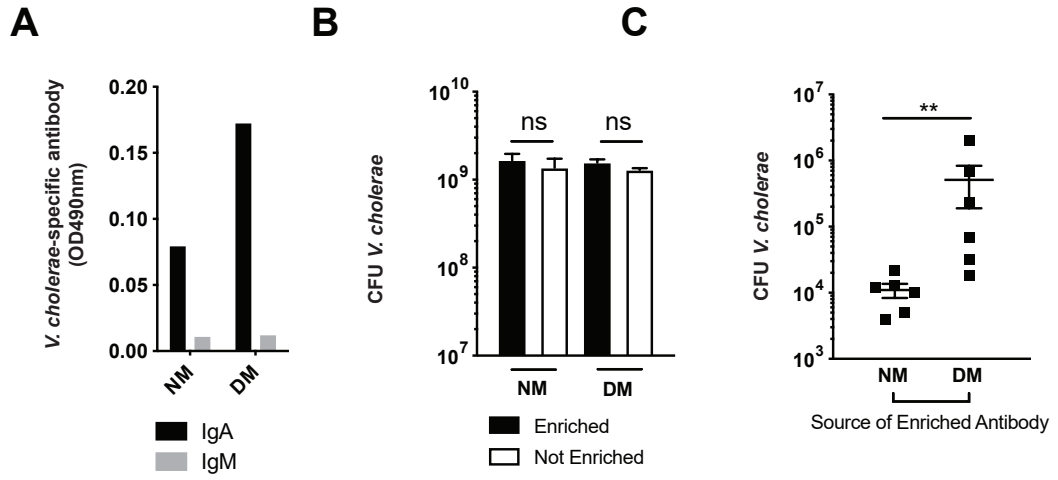

Figure 2.5. Enriched fecal antibody of infected NM, but not DM animals, can passively protect suckling animals from *V. cholerae* infection. (A) Isotype distribution of pooled, enriched fecal antibodies from NM and DM mice. (B) Survival of *V. cholerae* incubated with either enriched or not enriched fecal antibody preparations for 6 hours. (C) Colonization of suckling CD-1 mice by *V. cholerae* pre-incubated with enriched fecal antibody from infected mice bearing NM and DM microbiomes. Input was normalized so that *V. cholerae* used to colonize either NM and DM groups were incubated with equivalent amounts of IgA. **, $P<0.01$, Mann-Whitney *U* test. Error bars represent mean ± SEM.

Live members of DM community exhibit dominant suppressive effects on antibody responses

To determine whether the NM or DM anti-*Vibrio* antibody phenotype would be dominant when the bacterial communities are combined, we infected mice with *V. cholerae* in the presence of either NM, DM, or NM+DM microbiomes. At 4 weeks post infection, the NM+DM group showed a low serum vibriocidal titer comparable with the DM group, while the NM group had significantly higher titer levels than NM+DM (Figure 2.6A). These data suggested that the dysbiotic

microbiome may have a role in suppressing the host antibody response against *V. cholerae.* Due to the reduced vibriocidal titer levels observed in the NM+DM group, we wanted to determine whether or not live members of the susceptible community were required to mediate this effect. Accordingly, we heat inactivated all the members of the respective communities and again infected mice with live *V. cholerae.* We observed that the serum vibriocidal titer increased in the heat-killed DM group were to similar levels with the NM group (Figure 2.6B), suggesting that live members of the dysbiotic community are necessary at time of infection in order to mediate suppression of anti-*Vibrio* antibody protection.

While the DM microbiome exhibited a dominant reduced anti-*Vibrio* antibody phenotype in the NM+DM group, we wanted to determine whether the NM group could potentially rescue the DM phenotype under different circumstances. To study this, we initially introduced the DM community into antibiotic-treated animals 4 days prior to infection with *V. cholerae*. To model a targeted modification of the gut microbiome shortly after infection or immunization with OCVs, we either co-gavaged the NM with *V. cholerae* + NM or *V. cholerae* + DM. At 2- and 4-weeks post infection, we observed that serum vibriospecific Ig was significantly increased in the DM->Vc+NM group as compared to the DM->Vc+DM group, suggesting that the presence of NM microbes was able to partially rescue the DM phenotype using specific treatment conditions (Figure 2.6C).

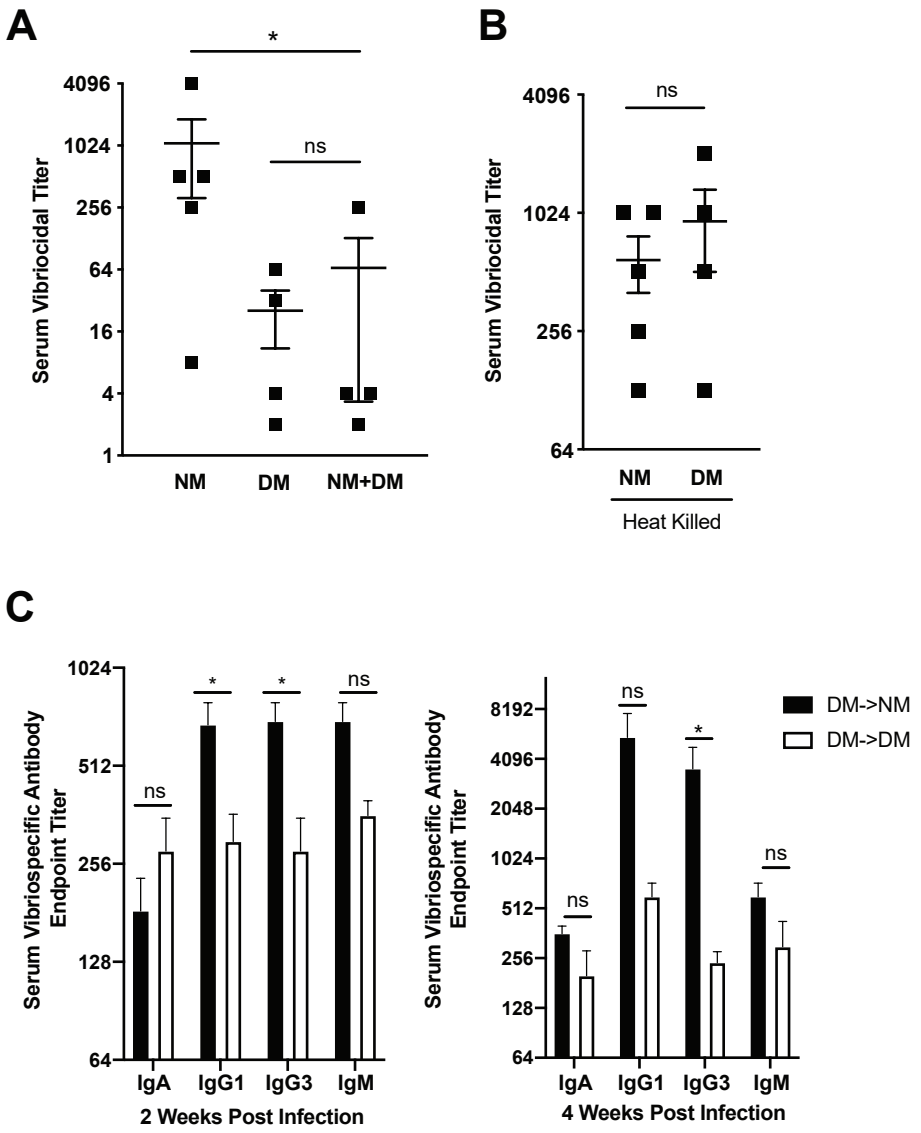

Figure 2.6. The effect of DM microbes is dominant on infection outcomes, and requires the presence of live bacteria during infection. (A) Serum vibriocidal titers 4-weeks post-infection in CD-1 mice infected with *V. cholerae* and bearing indicated human model microbiomes. (B) Vibriocidal titers of mice gavaged with indicated heat-killed communities at time of infection with live *V. cholerae*. (C) Profiles of anti-whole cell *V. cholerae* serum antibody 2 weeks (left) and 4 weeks (right) post-infection in mice that were pre-colonized for 4 days with DM communities and subsequently given either NM or DM at time of infection. *, $P<0.05$, Mann-Whitney *U* test. ns, $P>0.05$, Mann-Whitney *U* test. Error bars represent mean ± SEM.

Depletion of CD4+ cells restores *Vibrio*-specific immune response in mice colonized with DM defined community

In general, immune responses to *V. cholerae*, whether in the context of infection or immunization, have yet to be fully elucidated. Initial OCV responses appear to be driven by TLR-2-dependent interactions that can cause $CD4^+$ proliferation, and it has been shown in natural *V. cholerae* infection that $CD4^+$ T cells are also instrumental in stimulating long-term memory B cell responses (Bhuiyan et al., 2009; Kuchta et al., 2011; Sirskyj et al., 2016; Weil et al., 2009). Even though the overall levels of T lymphocytes remained constant during colonization with different microbiomes, B-cell expansion depends on the action of numerous types of cells such as antigen-presenting dendritic cells, M Cells as well as CD4+ cells, including T_{FH} cells and T_{Reg} cells (Cerutti and Rescigno, 2008; Perez-Lopez et al., 2016). To determine if CD4+ cells were responsible for mediating immune system effects of different microbiomes, we used antibiotic-depleted mice bearing NM and DM model microbiomes under CD4+ cell depletion. We were able to ablate CD4+ cell populations through intraperitoneal injection with anti-CD4 monoclonal antibodies every 4 days during antibiotic treatment. After verifying depletion of $CD4^+$ cells by flow cytometry analysis of whole blood (Figures 2.7A-B), animals were gavaged with live defined microbial communities and *V. cholerae* as previously described. Levels of serum anti-*V. cholerae* IgA were severely reduced in both groups compared to non-depleted animals. Similarly, serum anti-*V. cholerae* IgG3 and IgM were decreased in the NM group

compared to non-depleted animals (Figures 2.3A, 2.7C). Depletion of CD4+ cells yielded no statistically significant differences in levels of serum IgG and IgM, but strikingly, the vibriocidal titer of the DM group increased to levels comparable to the NM group after $CD4^+$ cell depletion (Figure 2.7D). This level was also comparable to that observed in NM group without depletion, suggesting that $CD4^+$ cells are not required for the development of serum vibriocidal responses, and interactions between these host cell populations and members of the dysbiotic gut microbiome leads to suppression of subsequent development of specific antibody responses.

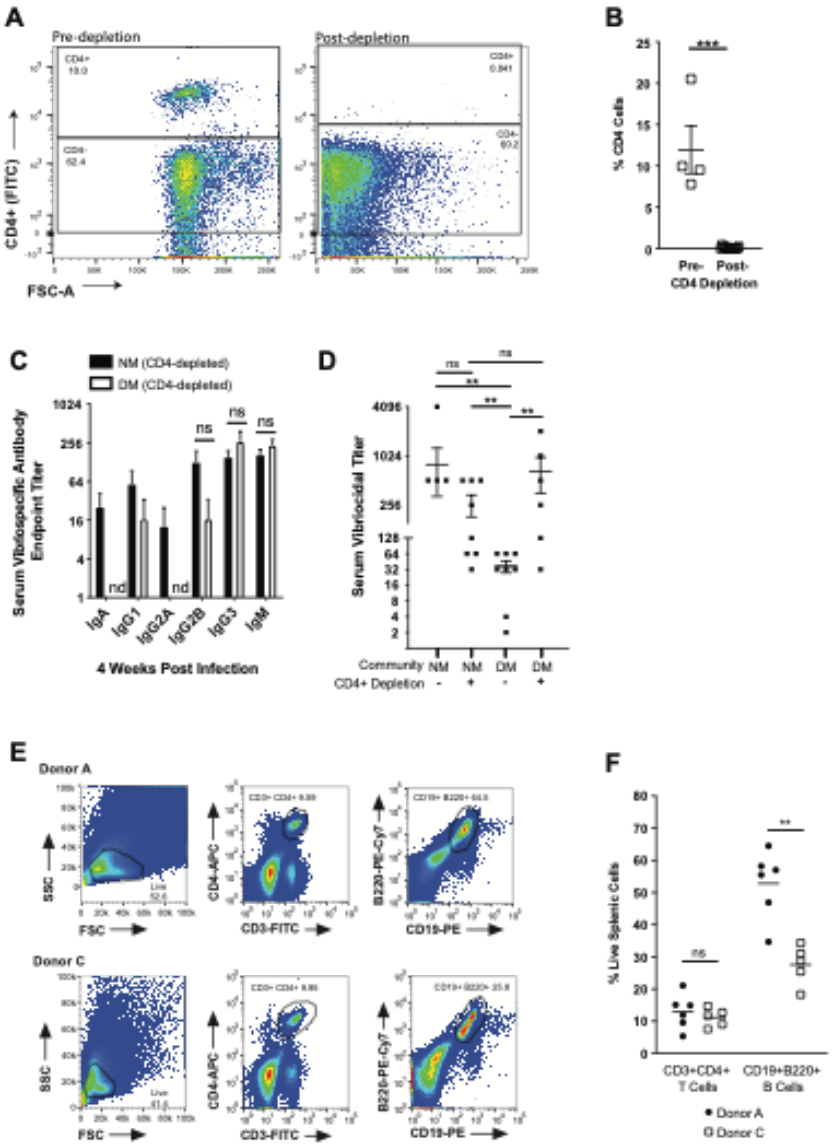

Figure 2.7. DM community effects are mediated *in vivo* by CD4⁺ cell populations. (A and B) % of CD4 cells pre and 7-days post depletion in blood. (C) Serum antibody levels against *V. cholerae* 4-weeks post-infection in the presence of indicated human model microbiomes in CD4+ depleted mice. (D) Comparison of vibriocidal titer levels in NM and DM groups 4-weeks after infection with or without CD4⁺ cell depletion. (E and F) Analysis of splenic T cells and B cells in germ-free mice bearing human donor microbiomes. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$, ns, $P>0.05$, Mann-Whitney U test, nd, not detected. Error bars represent mean ± SEM.

Interpersonal microbiome variation results in variable splenic B cell populations

We next extended these studies to GF mice colonized with complex human donor microbiomes and challenged with *V. cholerae.* We focused our studies on mice with donor A and C microbiomes, as these communities were associated with the highest and lowest vibriocidal antibody titers upon *V. cholerae* introduction. In animals with A and C donor microbes, levels of splenic T-lymphocytes (CD3+ CD4+) were not statistically significant (Figures 2.7E-F). However, we observed higher levels of CD19+B220+ B-cells in spleens of donor A mice compared to Donor C animals (Figures 2.7E-F). This is consistent with the higher levels of fecal and serum antibodies generated by donor A mice in contrast to donor C mice. These data are strong indicators of the influence of microbial communities on impacting immune responses to *V. cholerae*.

Suppressed immune response in DM mice given modified CVD 103-HgR

To broaden the applicability of our previous observations detailing microbiome compositional changes affecting immune outcomes to *V. cholerae* infection in mice, we utilized the strain used in the FDA-approved live-attenuated vaccine Vaxchora. CVD103-HgR is an O1 Inaba strain containing a 94% deletion of the cholera toxin enzymatic subunit gene *ctxA* and shows high immunogenicity in US populations (Chen et al., 2016). We isolated an isolate of CVD103-HgR

demonstrating spontaneous resistance to streptomycin (CVD 103-HgR-SmR) for inoculation into our antibiotic treated adult mouse model. Using CVD 103-HgR-SmR, we observed similar immune responses in the NM and DM groups as compared to infection with wild-type C6706, albeit at 2 weeks post vaccination (Figure 2.3H). The DM group given the vaccine strain showed a 7-11-fold decrease in SVT as compared to mice given the NM communities and a 4-8-fold decrease as compared to mice only given CVD 103-HgR-SmR. More studies will need to be carried out to further the observations in these results, however, these data describe the impact transient colonization of the gut microbiome impacts downstream vaccine-specific antibody responses.

Discussion

In humans, the gut microbiome enters a DM-like state transiently after infectious diarrhea or severe malnutrition, due to repeated infection by multiple pathogens, ranging from cholera to pathogenic *Escherichia coli* and rotavirus, a state that is likely to be much more frequently attained in cholera-endemic areas (David et al., 2015; Hsiao et al., 2014; Subramanian et al., 2014). Malnutrition, another common public health concern often co-occurring with recurrent infectious diarrhea, induces a DM-like state for much longer periods, and is refractory to therapeutic nutritional intervention (Smith et al., 2013). Previous work has demonstrated that this transient DM-like state represents a risk factor for *V. cholerae* colonization (Alavi et al., 2020). Our work suggests that this dysbiosis

may also represent a risk factor for poor immune responses to *V. cholerae* beyond infection; the composition of the human gut microbiome at time of exposure to *V. cholerae* in antibiotic-cleared and GF animals can suppress resultant antibody-mediated immune responses.

These findings also have significant implications for the use of OCVs. Efficacy of vaccination against enteric pathogens has been shown to be highly variable on a geographical and per-study basis, including for rotavirus (Harris et al., 2017), *Salmonella* (Eloe-Fadrosh et al., 2013), polio (Huda et al., 2014)and cholera (Levine, 2010). One of the potential reasons for the variability may be due to interpersonal variations in gut microbiomes (Sack, 2008). Previous studies sought to identify the relative abundance of certain species that were either positively or negatively correlated with protection from infection (Midani et al., 2018), but few studies have examined how microbiome composition affects host immune responses to infection in experimental models. We observed in our studies that individuals who were healthy overall and whose microbiomes aligned well with the healthy Bangladeshi group, exhibited variable immune response outcomes in our germ-free mouse model. In order to move to a more experimentally tractable, reductionist animal model, we designed simple model communities that are representative of gut microbiomes present in healthy versus diarrhea endemic populations.

Our DM model community is similar to these dysbiotic microbiomes in humans both by overall community diversity and types of characteristic

organisms; human microbiomes during fulminant diarrhea and early recovery from diarrhea can be dominated by 99% *Streptococcus* species by relative abundance (Hsiao et al., 2014). Live, as opposed to heat-killed, DM community organisms, were able to suppress serum and fecal antibody responses to introduction of *V. cholerae*. One prior study shows that *Sutterella* species are capable of degrading the stabilizing peptide of s-IgA, leading to decreased levels of IgA (Moon et al., 2015). The mechanism in our studies is likely different, as our microbial populations are only transiently present, and overall antibody levels are comparable across different model microbiomes. Our results suggest that even brief differences in microbiome structure may have important consequences, for example in OCV effectiveness, where microbiome dysbiosis at time of immunization can jeopardize outcomes (Levine, 2010).

This suppressed host antibody response can be reversed in DM-colonized animals through subsequent microbiome modification by microbes that are more characteristic of the healthy human gut microbiota. While the definition for what specific taxa constitutes a truly “healthy” microbiome is not settled, our NM model community is very broadly reflective of healthy human communities at higher taxonomy levels and by PCoA analysis. The introduction of the NM community at time of *Vibrio* infection of mice colonized by DM microbes was able to partially rescue the generation of robust anti-*Vibrio* serum Ig. This has significant translational implications as it suggests that a normal microbiota consortium may be used improve OCV outcomes.

Our antibiotic treated adult mouse experimental system is a robust model to study gut microbiota interactions in the host. In contrast to previous studies (Butterton et al., 1996), we are able to transplant actual human microbes into an immune-competent animal system, shortening the loop from initial observations to potentially clinically-relevant conclusions. Results with simplified defined microbiomes, similarly to complex human fecal microbiomes in germfree mice, exhibited interpersonal/inter-community differences in driving anti-*Vibrio* immune responses. However, additional human fecal communities, including those from cholera endemic areas, will be necessary to more robustly probe temporal variations in interactions between the host and the broad range of microbiome structures seen in healthy humans induced by temporal and intrapersonal variation in individuals with complex microbiomes.

Unlike other enteric pathogens such as *Shigella* and *Salmonella*, which cause clinically apparent inflammation and disease after penetrating cells or the intestinal epithelium, *V. cholerae* is thought to cause a non-inflammatory, noninvasive infection. However, cholera is associated with inflammatory changes such as widening of intracellular spaces, apical junction abnormalities as well as an infiltration of neutrophils, mast cells, and macrophages into the affected area (Mathan et al., 1995; Pulimood et al., 2008). While innate immune cells such as neutrophils were shown to be essential for containment of *V. cholerae,* protection is mainly derived from adaptive immunity (Queen and Satchell, 2012). To begin teasing apart the host mechanism behind our observed microbiome-dependent

antibody response phenotypes, we examined the role of $CD4^+$ T cells, which are important cellular regulators of B cell maturation into antigen specific IgA secreting plasma cells (Cerutti and Rescigno, 2008). Upon depletion of $CD4^+$ cells, we observed decreased levels of serum IgA after infection in both NM and DM mice, potentially indicating decreased seroconversion (Figure 2.7C). However, serum vibriocidal titer in DM, $CD4^+$-depleted animals increased to levels comparable to the NM mice (Figure 2.7D). These data show that $CD4^+$ cells are integral in mediating microbiome-dependent changes in an infection-induced antibody response. These results are surprising as one would expect $CD4^+$ T cell depletion to substantially reduce the vibriocidal titer but our data suggests that there are compensatory, non-$CD4^+$ mediated mechanisms to aid in seroconversion. A recent clinical study evaluating the efficacy of the oral cholera vaccine Shanchol in human immunodeficiency virus (HIV)-infected individuals demonstrated that while vibriocidal titer was lower in HIV-infected individuals with depleted $CD4^+$ T-cell populations there was still seroconversion in 65-74% of the subjects (Ivers et al., 2015). While the study population was not completely depleted of $CD4^+$ T cells, it demonstrates vibriocidal titers can be elicited even in a highly-$CD4^+$ cell-depleted state, albeit to a lesser degree. Furthermore, while our analysis of splenic T cell populations yielded no differences as a function of complex donor microbiome colonization, our phenotype may depend on change in certain specific T cell types such as Regulatory T cells ($T_{REG)}$ or Follicular Helper T cells (T_{FH}); further experimentation will be required to define these

specific T cell subtypes. Interestingly, there was an increase in splenic B cells in the mice given fecal transplants from Donor A as compared to Donor C, indicating that the gut microbial community in Donor A was associated with more robust immune responses including highly proliferative B cell populations.

We extended our observations from wild-type C6706 *V. cholerae* to a live-attenuated vaccine strain, Vaxchora. Because the native murine microbiome is refractory to *V. cholerae* colonization (Freter, 1956), we utilized an isolate of CVD 103-HgR that was spontaneously resistant to streptomycin. The DM and NM communities are associated with similar host SVT responses to the vaccine strain as with wild-type *V. cholerae* (Figure 2H). Additionally, the NM community shares a similar SVT profile to the vaccine strain alone, suggesting that NM does not significantly boost response above that of the vaccine strain. Our studies in mice reflect a recent human clinical study that compared SVT data in age matched North America and Bangladesh adults that were voluntarily infected with *V. cholerae* O1 Inaba. Notably, anti-CtxB IgA and IgM responses were greater in the North American group compared to the Bangladeshi participants (Hossain et al., 2019). These findings support the notion that specific human gut microbial populations can result in varied humoral immune responses to *V. cholerae.* Antibody-mediated protection to natural infection is both anti-toxin and anti-bacterial cell (Weil et al., 2019), whereas vaccine-mediated immunity is predominantly against LPS (Svennerholm, 1975). While much remains to be elucidated in relation to the effects of the gut microbiome on cholera vaccine

responses, our data adds to the ever-increasing literature of the role of gut bacteria modulating mucosal vaccine immune responses.

In order to more fully understand the correlations between bacterial communities, *V. cholerae*, and host interactions, more work will need to be done to study the biochemical underpinnings of microbiome-host interaction as it impacts host immunity. The precise molecular interface between DM microbes and the immune system is yet to be defined; the inability of heat-killed DM communities to influence infection outcomes suggest that an active interaction with host tissue, or the production of active compounds *in vivo* are required for this. At the host level, while we investigated the role of $CD4^+$ T cells in this phenotype, other immune cell types such as antigen presenting cells may act as more direct intermediaries between host immunity and microbial composition. As mentioned previously, Helper T cells are integral in stimulating and guiding B cell responses, so it would be beneficial to further define $CD4^+$ subsets involved such as follicular helper T cells or regulatory T cells as well as B-cell subtypes.

Taken together, our data advances how gut microbiome structure may alter the immune pathways resulting in a weakened humoral response. Ultimately, our studies on the influence of bacterial composition at time of introduction of *V. cholerae* to the gastrointestinal tract will help delineate the host contributors to infection response, as well as the immune response to introduced antigen such as with live attenuated OCVs. Variability in the gut microbiome may

thus contribute to both individualized disease outcomes, and the high observed variability in oral cholera and other mucosal vaccines.

Limitations of the study

While our data further advances the understanding and impact of gut bacterial composition on immune outcomes to natural infection with *V. cholerae* or vaccination, it is important to acknowledge several limitations to our study's approach and animal modeling. Although we examined immune correlates of protection of cholera from US stool donors in germ-free mice, it would be an informative comparison to do a similar analysis with stool samples from populations where enteric disease is common. Additionally, mouse models are an imperfect lens through which human disease and immune biology can be viewed. Some human-associated microbes do not successfully engraft into the mouse gut, and the distribution of these microbes vary from rodent to human, especially in complex fecal microbiomes. To gain more translational insights, further analyses and modulation of complex human fecal microbiomes in the context of OCV administration would ultimately be required.

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Author contributions: All authors helped design and analyze experiments; J.C.M., R.L., and S.A. performed experiments; J.C.M and A.H. wrote the paper.

Declaration of Interests: The authors declare no competing interests.

STAR Methods

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Ansel Hsiao (ansel.hsiao@ucr.edu).

Materials Availability

Unique strains and reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

- This paper analyses existing, publicly available data. These accession numbers for the datasets are listed in Table 2.1-2.2.

- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Table 2.1. Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
InVivoMab anti-mouse CD4 antibody Clone GK1.5	BxCell	Cat#BE0003-1
Rat anti-mouse CD16/32 Clone 2.4G2	BD Pharmingen	Cat#553142
APC anti-mouse CD4 Clone RM4-5	Invitrogen	Cat#17-0042-82
FITC anti-mouse CD4 Clone RM4-5	Invitrogen	Cat#11-0042-82
FITC rat anti-mouse CD3 Clone 17A2	BD Pharmingen	Cat#561798
PE rat anti-mouse CD19 Clone 1D3	BD Pharmingen	Cat#557399
PE-Cy7 Anti-mouse B220 Clone RA3-6B2	Invitrogen	Cat#25-0452-82
Goat anti-mouse IgA-HRP	Southern Biotech	Cat#1040-05
Goat anti-mouse IgG1-HRP	Southern Biotech	Cat#1071-05
Goat anti-mouse IgG2A-HRP	Southern Biotech	Cat#1081-05
Goat anti-mouse IgG2B-HRP	Southern Biotech	Cat#1091-05
Goat anti-mouse IgG3-HRP	Southern Biotech	Cat#1101-05
Goat anti-mouse IgM-HRP	Southern Biotech	Cat#1021-05
Bacterial and virus strains		
Vibrio cholerae C6706 El Tor	Hsiao Lab stock	C6706
CVD 103-HgR-SmR	This paper	Vaxchora/CVD 103-HgR-SmR
Escherichia coli	Hsiao Lab stock	DH5α- λpir
Streptococcus salivarius subsp. *salivarius*	ATCC	ATCC 13419
Blautia obeum	ATCC	ATCC 29174
Clostridium scindens	ATCC	ATCC 35704
Bacteroides vulgatus	ATCC	ATCC 8482
Streptococcus infantarius subsp. *infantarius*	ATCC	ATCC BAA-102
Streptococcus salivarius subsp. *thermophilus*	DSMZ	DSM 20617
Enterococcus faecalis	Hsiao Lab Stock	OG1RF
Escherichia coli	Hsiao Lab Stock	BW30045
Biological samples		
Human volunteer donor fecal sample	Alavi, et al, 2020	Donor A
Human volunteer donor fecal sample	Alavi, et al, 2020	Donor B
Human volunteer donor fecal sample	Alavi, et al, 2020	Donor C
Chemicals, peptides, and recombinant proteins		
Ampicillin sodium salt	Fisher Bioreagents	Cat#BP1760

Table 2.2. Key Resources Table Continued

Chemicals, peptides, and recombinant proteins		
Ampicillin sodium salt	Fisher Bioreagents	Cat#BP1760
Neomycin trisulfate salt hydrate	Sigma-Aldrich	Cat#N1876
Vancomycin hydrochloride	Alfa Aesar	Cat#J62790.06
Streptomycin sulfate	VWR Life Sciences	Cat#0382
Guinea pig complement serum	Sigma-Aldrich	Cat#234395
Critical commercial assays		
iQ SYBR Green Supermix	Biorad	Cat#170882
SuperScript IV First-Strand Synthesis System	Invitrogen	Cat#18091200
Protein L Purification Kit	ThermoScientific	Cat#88849
Deposited data		
Short-read sequencing data for meta-analysis	European Nucleotide Archive	See Table 2.3 for accession numbers
Experimental models: Organisms/strains		
Mouse: C57/BL6-Tac Inbred	UCR gnotobiotic facility	N/A
Mouse: CD1 IGS	Charles River Laboratories	N/A
Oligonucleotides		
16S F PCR Primer forward: 5'-CTCCTACGGGAGGCAGCAG-3'	IDT	N/A
16S R PCR Primer reverse: 5'-TTACCGCGGCTGCTGGCAC-3'	IDT	N/A
Recombinant DNA		
Software and algorithms		
QIIME	Caporaso et al., 2010	http://qiime.org/
Graphpad Prism	Graphpad software (CA, USA)	N/A
FlowJo	BD Biosciences	N/A
Other		
Lab diet	Newco Distributors	Cat# 5K52

Experimental model and subject details

Animal and Human Studies

Female CD-1 mice were purchased from Charles River Laboratories, and generally used at 5-9 weeks of age. 4-day old suckling CD-1 mice were purchased from Charles River Laboratories. Germfree C57/BL6Tac animals were bred and reared in the gnotobiotic facility at the University of California, Riverside. Male and female C57/Bl6Tac mice were used generally at 5-9 weeks of age. No differentiation was observed between sexes and animal data were pooled by sex where applicable. Animals in the study were treated and housed under specific-pathogen-free or germfree conditions. All animal protocols were approved by University of California, Riverside's Institutional Animal Care and Use Committee. All human samples were part of a study approved by the University of California, Riverside's Institutional Review Board.

Human study design and sample collection

Human stool samples from a cohort of healthy adult individuals were collected at the University of California, Riverside using an IRB-approved protocol. Inclusion criteria were: between 18-40 years old, ability to provide informed consent, and willing and able to provide a stool specimen. Exclusion criteria were: systemic antibiotic usage (oral, intramuscular, or intravenous) 2 weeks prior to stool collection, acute illness at time of enrollment, diarrhea or very loose stools within

2 weeks prior to collection, active uncontrolled GI disease such as Crohn's disease, ulcerative colitis, gastritis, constipation, major surgery of the GI tract (excluding cholecystectomy and appendectomy). Fecal samples were stored at −80°C until further processing. Stocks of fecal slurries for subsequent experimentation were prepared by re-suspending samples at 1:3 weight/volume in sterile reduced PBS and adding sterile glycerol to a final concentration of 25% volume/volume.

Method details

Germ-free and gnotobiotic mouse experiments

Germ-free C57/BL6Tac mice were bred and maintained in plastic gnotobiotic isolators at University of California, Riverside. Mice were fed an autoclaved, low-fat plant polysaccharide-rich mouse chow (Lab Diet 5K52) and were 6-13 weeks old at time of gavage. We used real-time PCR and universal 16S primers to normalize human fecal slurries so that each adult mouse received approximately 20 µg of microbial genomic DNA. Reactions comprised 2 µL of extracted DNA (200ng/reaction) as template, 12.5 µL SYBRGreen Master Mix (BioRad), 10 µL PCR-grade water, and 0.25 µL of forward and reverse primers at 10µM (forward: 5'-CTCCTACGGGAGGCAGCAG-3', reverse: 5'-TTACCGCGG CTGCTGGCAC-3'). Cycle conditions were 95°C for 3 min, followed by 39 cycles (95°C for 10 sec, 55°C for 30 sec, 95°C for 10 sec, 65°C for 5 sec, 95°C for 5 sec). Mice were

fasted for two hours prior to introduction of bacteria, and stomach pH was buffered by intra-gastric gavage of 100 µL 1M $NaHCO_3$, followed by gavage with 150 µL of fecal slurries. 2 weeks after human commensal colonization, each group was infected with ~5 x 10^9 CFU *V. cholerae* O1 El Tor C6706. Fecal samples were suspended in 500 µL of PBS and homogenized using a bead beater (BioSpec) at 1,400 RPM for 30 seconds. CFU enumeration of *V. cholerae* was done on LB agar containing 200 µg/mL streptomycin.

Bacterial strains and growth conditions

All human gut commensal strains used are listed in Figure 2A. Unless otherwise noted, human gut strains were propagated in LYHBHI liquid medium (BHI supplemented to 5g/L yeast extract, 5mg/L hemin, 1mg/mL cellobiose, 1mg/mL maltose and 0.5mg/mL cysteine-HCl). Cultures were then grown in a Coy anaerobic chamber (atmosphere 5% H_2, 20% CO_2, balance N_2) or aerobically at 37ºC. All *V. cholerae* strains were derived from the C6706 El Tor pandemic isolate and propagated in LB media with appropriate antibiotics at 37°C. Vaxchora (CVD 103-HgR) was grown in LB and CVD 103-HgR-SmR was derived from an isolate that exhibited resistance to streptomycin. It was propagated in LB media with streptomycin at 37°C.

Preparation of bacteria for inoculation into antibiotic treated mice

Female adult CD-1 mice were given an antibiotic cocktail *ad libidum* (1 g/L ampicillin, 1 g/L neomycin, and 125 mg/L vancomycin)(Ichinohe et al., 2011; Rakoff-Nahoum et al., 2004) for 1 week as described previously with modifications as mice refrained from drinking water with metronidazole (Reikvam et al., 2011). 2.5 g/L of Splenda was added as well to make the cocktail more palatable. 3 days prior to gavage with *V. cholerae*, the cocktail was replaced with 2.5 g/L streptomycin and 2.5 g/L Splenda. Each anaerobic human gut bacterium was cultured from glycerol stocks in LYHBHI media for 24 hours at 37°C, and then diluted (1:50) in fresh LYHBHI media. *Enterococcus faecalis* and *Escherichia coli* were grown aerobically in LYHBHI and LB, respectively, for 24 hours at 37°C, and then diluted (1:50) in respective media. After growth for an additional 48 hours, cultures were normalized for density by OD_{600}. For inoculation into adult mice, normalized mixtures were prepared so the equivalent total of 300 μL of OD_{600}=0.4 culture divided evenly across the respective strains for each community was pooled, centrifuged, and resuspended in LYHBHI. The suspension was prepared so that each mouse received 50 μL of the bacterial community mixture, as well as 50 μL containing ~5 x 10^9 *V. cholerae* O1 El Tor C6706. Prior to bacterial introduction, the mice were fasted for 3 hours and then gavaged with 100 μL of 1 M $NaHCO_3$, to buffer stomach acid, after which the bacterial communities and *V. cholerae* were inoculated via oral gavage.

DNA extraction

DNA extraction from fecal pellets was done using a combination of mechanical disruption and phenol/chloroform isolation followed by isopropanol precipitation. In brief, fecal pellets were added to sterile 1.8 mL o-ring tubes with 0.1 mm zirconia/silica beads (BioSpec). Then, 500 μL of 200 mM NaCl, 200 mM Tris, and 20 mM EDTA was added along with 210 μL of 20% SDS and 500 μL phenol:chloroform:isoamyl alcohol (25:24:1) (Fisher Biosciences). The microbial cells were lysed via mechanical disruption with a bead beater (BioSpec) for 4 minutes at 2500 RPM. After density separation by centrifugation, the supernatant was again extracted with phenol:chloroform:isoamyl alcohol. The DNA was precipitated with isopropanol with the addition of 3M sodium acetate at -80C for 1 hour followed by a wash of 100% ethanol and resuspension in nuclease-free water.

Quantification of 16S copy number density by qPCR

DNA was extracted from fecal pellets as previously described. The reaction consisted of 2 μL of genomic DNA (20 ng per reaction), 10 μL of SYBR Green Master mix (Biorad), 6 μL of nuclease free water, and 1 μL of 10 uM (forward: 5'-CTCCTACGGGAGGCAGCAG-3'), and 1 μL of 10 uM R primer (reverse: 5'-TTACCGCGG CTGCTGGCAC-3'). Cycle conditions were 95°C for 3 min, followed by 39 cycles (95°C for 10 sec, 55°C for 30 sec, 95°C for 10 sec, 65°C

for 5 sec, 95°C for 5 sec). A standard curve was generated as described in Ritalahti and Loffler, et al., 2006 (Ritalahti et al., 2006) using *E. coli* BW30045 as the construct.

Human gut microbiome 16S meta-analysis

In order to compare the human gut microbiome in Bangladesh under the dysbiotic pressure of diarrhea, and to compare defined model communities with complete human gut microbiomes, we performed a meta-analysis of existing 16S ribosomal RNA gene sequencing studies. Raw sequencing data of the V4 region of the 16S rRNA gene from published studies were used (for accession numbers, see Table 2.3). We compared samples taken from different phases of cholera in an adult cohort, examining the earliest sample taken during diarrhea after clinical presentation, the last time points of diarrhea, and a sample taken 3 months into recovery from diarrhea. Fecal samples collected from healthy parents of malnourished Bangladesh children were selected as a healthy adult Bangladesh control (Subramanian et al., 2014). Defined community inputs were calculated on the basis of even distribution of all strains in the specific community (NM: 3000 reads/species; DM: 2000 reads/species). All of the sequencing data were collected together and analyzed using the QIIME 1.9.1 software package (Caporaso et al., 2010).

Table 2.3. Public datasets used for 16S rRNA meta-analysis, Related to STAR Methods

Run Accession	Fecal Sample ID	ENA Accession Number	Group
ERR520665	A.diarrhea.016	PRJEB6358	Diarrhea (start)
ERR520694	A.diarrhea.end	PRJEB6358	Diarrhea (end)
ERR520710	A.recovery.d088	PRJEB6358	Recovery (end)
ERR520711	B.diarrhea.018	PRJEB6358	Diarrhea (start)
ERR520728	B.diarrhea.end	PRJEB6358	Diarrhea (end)
ERR520744	B.recovery.d088	PRJEB6358	Recovery (end)
ERR520745	C.diarrhea.011	PRJEB6358	Diarrhea (start)
ERR520825	F.diarrhea.005	PRJEB6358	Diarrhea (start)
ERR520851	F.diarrhea.end	PRJEB6358	Diarrhea (end)
ERR520867	F.recovery.d087	PRJEB6358	Recovery (end)
ERR520868	G.diarrhea.005	PRJEB6358	Diarrhea (start)
ERR520884	G.diarrhea.028	PRJEB6358	Diarrhea (end)
ERR498974	Bgtw10.F.m13	PRJEB5482	Healthy Bangladesh
ERR499132	Bgtw10.M.m13	PRJEB5482	Healthy Bangladesh
ERR499151	Bgtw11.M.m12	PRJEB5482	Healthy Bangladesh
ERR498802	Bgtw12.F.m4	PRJEB5482	Healthy Bangladesh
ERR499027	Bgtw2.M.m24	PRJEB5482	Healthy Bangladesh
ERR499445	Bgtw3.F.m16	PRJEB5482	Healthy Bangladesh
ERR499505	Bgtw3.M.m22	PRJEB5482	Healthy Bangladesh
ERR498846	Bgtw4.F.m13	PRJEB5482	Healthy Bangladesh
ERR499050	Bgtw4.F.m13	PRJEB5482	Healthy Bangladesh
ERR498850	Bgtw4.M.m20	PRJEB5482	Healthy Bangladesh
ERR499054	Bgtw4.M.m20	PRJEB5482	Healthy Bangladesh
ERR498702	Bgtw5.F.m13	PRJEB5482	Healthy Bangladesh
ERR499227	Bgtw5.M.m22	PRJEB5482	Healthy Bangladesh
ERR498551	Bgtw6.F.m10	PRJEB5482	Healthy Bangladesh
ERR499464	Bgtw6.M.m10	PRJEB5482	Healthy Bangladesh
ERR499518	Bgtw6.M.m10	PRJEB5482	Healthy Bangladesh
ERR498561	Bgtw7.F.m7	PRJEB5482	Healthy Bangladesh
ERR498884	Bgtw7.M.m16	PRJEB5482	Healthy Bangladesh
ERR499088	Bgtw7.M.m16	PRJEB5482	Healthy Bangladesh
ERR499476	Bgtw8.F.m13	PRJEB5482	Healthy Bangladesh
ERR499530	Bgtw8.F.m13	PRJEB5482	Healthy Bangladesh
ERR499249	Bgtw8.M.m13	PRJEB5482	Healthy Bangladesh
ERR498905	Bgtw9.F.m13	PRJEB5482	Healthy Bangladesh
ERR499109	Bgtw9.F.m13	PRJEB5482	Healthy Bangladesh
ERR498908	Bgtw9.M.m13	PRJEB5482	Healthy Bangladesh
ERR499112	Bgtw9.M.m13	PRJEB5482	Healthy Bangladesh
ERR3190459	SA.Human1.Sample	PRJEB31497	Donor A
ERR3190460	SA.Human2.Sample	PRJEB31497	Donor B
ERR3190463	SA.Human6.Sample	PRJEB31497	Donor C

Serum vibriocidal assay

Mouse whole blood was collected via tail vein bleeds using heparinized Caraway collection tubes (Fisher Scientific) or cardiac puncture. Blood was centrifuged at 9,000 x g for 10 minutes, and the serum fraction was isolated and stored at -

20°C. The vibriocidal titer measurement was done as previously described with minor modifications (Son and Taylor, 2011). In brief, mouse serum was heat inactivated for 30 minutes at 56°C. The heat-inactivated serum was then serially diluted two-fold with phosphate-buffered saline (PBS). Separately, PBS, guinea pig complement serum (Sigma-Aldrich), and ~ 5 x 10^8 CFU *V. cholerae* were combined at a ratio of 7:2:1, respectively. The above mixture was then added to the wells containing serially diluted serum and incubated at 37°C for two hours. The resulting dilutions were then plated onto streptomycin (200 μg/mL) LB plates. The vibriocidal titer is the reciprocal of the highest serum dilution which displayed no *V. cholerae* growth.

Fecal pellet collection

Fresh fecal pellets were collected from mice, weighed, and placed in 600 μL of PBS in a 2.0 mL screw cap tube. The pellets were disrupted by agitation without beads in a bead beater (BioSpec) for 30 seconds at 1400 RPM. 10-fold serial dilutions of the resulting fecal slurry were then plated onto LB agar with streptomycin to enumerate *V. cholerae* colonization.

Analysis of antibody responses by ELISA

100 μL dense overnight culture of *V. cholerae* grown in LB was plated onto high-binding, clear, flat bottom Costar 96 well plates (Corning, Inc) ELISA plates and allowed to bind overnight. 3% bovine serum albumin (BSA) in PBS was used as

a blocking solution. Serum was added at a 1:100 dilution and then diluted two-fold. Alternatively, to measure total antibody levels, serum was added at a 1:100 dilution to plates previously coated with unlabeled goat anti-mouse IgA, IgG, IgM (Southern Biotech) and allowed to bind at 37°C for 3 hours. Next, the plates were washed with PBS with 0.001% Tween-20 and PBS. 100 μL of goat anti-mouse HRP conjugated antibodies of either IgA, $IgG_{1,2A,2B,3}$ or IgM (Southern Biotech) were added to 96 well plates at a dilution of 1:4,000 in 3% BSA and incubated overnight at 4°C. After several washes, the plates were developed with the addition of 5 mg o-phenylenediamine dihydrochloride (Thermo Scientific) and stable peroxide substrate buffer (Thermo Scientific); 1 N HCl was used as a stop solution. The plates were read at 490 nm on a Synergy HTX multi-mode reader (BioTek). Endpoint titer was calculated as the observed signal two standard deviations above background signal.

Growth inhibition and passive immune protection assay

From a fresh overnight culture of *V. cholerae,* 1 μL of culture was added to LB with enriched or not enriched antibody and incubated for 6 hours at 37°C for 6 hours. After incubation, samples were plated on streptomycin-LB plates in order to enumerate *V. cholerae* growth. Fecal samples from infected animals bearing the NM and DM communities was collected and processed as described previously. Total IgA/IgM fecal antibody was enriched using Protein L magnetic

beads according to the manufacturer's protocol (Pierce Biotech). 50 ng of enriched antibody was bound to ~1.25 x 10^6 CFU *V. cholerae* and allowed to bind at 37°C for 1 hour. 4-day old suckling CD-1 mice were gavaged with 30-gauge plastic tubing with 50 µL of antibody/*V. cholerae* mixture. After 18 hours of infection, the animals were sacrificed, and intestines homogenized for *V. cholerae* CFU enumeration on selective medium.

Preparation of heat-killed commensal bacteria

Strains from the NM and DM communities were grown in pure cultures and the bacterial suspension was prepared as previously mentioned. The respective bacterial communities were killed by heating in a heat block for 1 hour at 100°C. Bacterial death was confirmed by plating onto solid media and observing lack of growth.

Rescue experiment

Adult CD-1 mice were placed on an antibiotic cocktail of ampicillin (1 g/L), neomycin (1 g/L), and vancomycin (125 mg/L) for 1 week to deplete the native murine microflora as previously described. Prior to introduction of model communities, the mice were switched to streptomycin (2.5 g/L). The mice were pre-colonized with the DM model community 4 days before infection with *V. cholerae*. At time of infection, one group was gavaged with the NM group while

the other was gavaged with the DM group. At 2 weeks post infection, the mice were placed back on the ampicillin, neomycin, and vancomycin antibiotic cocktail.

in vivo depletion of $CD4^+$ cells

In order to deplete $CD4^+$ cells *in vivo*, 100 μg of GK1.5 antibody (Bio X Cell) was administered intraperitoneally every four days. Depletion of $CD4^+$ cells in blood was confirmed using a FACS Canto flow cytometer (BD Biosciences) and FITC rat-anti-mouse CD4 (BD Biosciences). Red blood cell lysis was done using ACK lysis buffer and anti-CD16/32 was used as an Fc block. Analysis was done using Flow Jo (BD Biosciences) and Prism (GraphPad). Mice were treated with ampicillin, neomycin, and vancomycin as previously mentioned. 3 days prior to infection, the mice were placed on streptomycin water alone. The mice were infected with ~5 x 10^9 CFU *V. cholerae* and serum vibriospecific ELISAs and vibriocidal assays were performed as previously described.

Flow cytometry analysis

Upon animal sacrifice, spleens were mechanically broken down with surgical scissors and ground through a 40 μm strainer with a plastic plunger of a 1 mL syringe into a 50 mL conical tube. The strainer was washed with 5 mLs of FACS Buffer (PBS with 3% w/v bovine serum albumin (BSA)). After centrifugation at 176 x g, cells were resuspended in 2 mLs Pharmlyse Buffer for 2 minutes in a 37

C water bath. After incubation, 40 mLs of FACS buffer were added to the samples. Cell viability was assessed using Trypan Blue. To minimize non-specific Fc receptor binding, rat anti-mouse CD16/32 (BD Pharmingen) was used as an Fc block. Splenic cells were stained with PE rat anti-mouse CD19 (BD Pharmingen), PE-Cy7 anti-mouse B220 (Invitrogen), FITC rat anti-mouse CD3, and APC anti-mouse CD4 (Invitrogen).

Quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism Software (v9). If data were deemed normally distributed, Student's *t*-test were performed. If data were deemed not normally distributed, Mann Whitney *U* tests were performed. Statistical details of the experiment can be found in the figures and figure legends.

CHAPTER 3: Interpersonal microbiome variation drives divergent immune responses in human cholera vaccine studies

Introduction

As mentioned in previous chapters, the diarrheal disease cholera caused by *V. cholerae* results in approximately 100,000 deaths per year (Ali et al., 2015). It remains endemic in southeast Asia and areas of Africa and can resurface due to outbreaks as a result of civil war or natural disasters causing inadequate sanitation and water infrastructure.

A primary tool used to prevent infection and controlling cholera in outbreak settings are the use of oral cholera vaccines (OCVs). There are predominantly two types of OCVs: Vaxchora, formerly known as CVD 103-HgR (Emergent Biosolutions) and Shanchol (Shantha Biotechnics, India or Euvichol, Eubiologics, South Korea). Vaxchora is a live attenuated vaccine with 94% of *ctxA* gene removed and is the only approved vaccine for use in the United States. Shanchol is a killed whole-cell *V. cholerae* vaccine that consists of 5 different formulations of *V. cholerae* strains in addition to recombinant CTB. Unfortunately, protection has been shown to be variable with protective efficacy ranging from 50-90% (Bishop and Camilli, 2011; Kabir, 2014).

Several hypotheses exist for this high level of variation such as pre-existing immunity, diet, or alterations in the gut microbiome (Levine, 2010). Other mucosal vaccines have this phenomenon as well including rotavirus (Madhi et al., 2010) and Shigella (Katz et al., 2004; Rahman et al., 2011). While several

studies have implicated the role of the gut microbiome on an influenza vaccine model in mice (Hagan et al., 2019; Oh et al., 2014), the role of the gut microbiome in oral cholera vaccines and other bacterial vaccines remains poorly understood. These findings demonstrate an avenue of research to determine the microbiota and microbial products that influence immune responses to infection or vaccination.

Our initial approach to study the role of human gut microbiome variation was initially two-pronged in nature. The first study would take place utilizing generally healthy US adult cohorts. Our hypothesis is that the structure of individual gut commensal communities at time of vaccination confer individualized levels of vaccine protective efficacy when these individuals are given OCVs. We aim to determine whether differences in the gut microbiome between individuals can mediate this change by correlating gut microbiota composition with serum *Vibrio*-specific responses and cellular transcription levels. Additionally, we are aiming to transplant the individual microbiome communities into germ-free and antibiotic treated mice, followed by infection or vaccination with the vaccine strain in order to confirm the phenotype in an experimental animal model. While this study had the potential to help elucidate mechanisms by which human gut microbiota influence vaccine responses, it was cancelled mainly due to 3 reasons related to the COVID-19 pandemic. First, Emergent Biosolutions halted manufacture of Vaxchora, our doses purchased expired June 30th 2021. Second, we did not receive final approval from UCR's

IRB at the time because our proposal was deemed non-essential according to the current school restrictions. Lastly, the study was to be funded from non-restricted funds that were set to expire Fall, 2021. Please see the methods section for overall planned methods for the US study.

Bangladesh cholera vaccine study

The second prong of our approach was to conduct a cholera vaccine study in an area where cholera is endemic. Fortunately, Dr. Rashidul Haque and his project scientist, Dr. Jasmin Akter, collaborators of Dr. Ansel Hsiao at icddr,b were eager at the prospect of conducting a cholera vaccine study with the intent of also examining the microbiome. An analysis of different vaccines would be insightful, we settled on using the killed whole cell vaccine Shanchol (Shantha) as it is the vaccine that has regulatory approval and is most often used in controlling cholera in Bangladesh and other resource limited areas (Bhattacharya et al., 2013; Saha et al., 2011; Sur et al., 2011).

Multiple studies examined the immunogenicity of Shanchol in adults and children from cholera endemic and non-endemic areas (Bhattacharya et al., 2013; Lopez et al., 2014; Sur et al., 2011) and reported that the cumulative efficacy of the vaccine at five years was 65%, however, 42% in children 5 years and under, the age group most susceptible to cholera. Additionally, high variability in immunological responses were observed in children aged 5-9 years from areas of endemic cholera and in volunteers from low socioeconomic

backgrounds with poor hygiene status (Gotuzzo et al., 1993; Simanjuntak et al., 1993; Suharyono et al., 1992).

The microbial community of the gastrointestinal tract, or the gut microbiome, is highly diverse and impacts vaccine immunogenicity and efficacy. Recent findings demonstrate the influence of nutrition and the gut microbiome on oral vaccination measures. In one study, germ-free mice were given fecal microbial transplants from malnourished Bangladeshi children along with a nutritional supplement and immunized orally with cholera toxin. The gut microbiome that was responsive to the nutritional supplement exhibited increased CT-specific fecal IgA antibody. Also, gut microbiota from the responsive group were capable of invading the hyporesponsive microbiome and augment CT-specific IgA (Di Luccia et al., 2020). However, it remains poorly understood how the microbiota influences immune responses to vaccination. A recent study on Rotavirus vaccine (RVV) in Ghanaian infants showed that the vaccine efficacy significantly was associated with overall microbiome composition in RVV responders versus non-responders (Harris et al., 2016). It has also been observed that immunogenicity is lower in populations from countries with poor sanitation against cholera (Hallander et al., 2002; Levine, 2010) rotavirus (Lopman et al., 2012) and polio (Grassly et al., 2009), where a higher incidence of faecal-oral bacterial exposure exist, and where the structure of the microbiota differs from that observed in the United States or Europe.

Several studies have attempted to understand the impact of the gut microbiome on responses to vaccines. Studies of influenza vaccine responsiveness, either through comparisons of germfree and conventionally raised mice, or humans treated with antibiotics have revealed a role for the microbiome in influencing vaccine responses (Hagan et al., 2019; Oh et al., 2014). However, these studies examined the effects of large-scale depletion of microbiome which is not representative of most human contexts, and significant variance in vaccine responses were observed in individuals harboring intact gut microbiomes. Other works have correlated microbiome features and rotavirus vaccine efficacy (Harris et al., 2017; Parker et al., 2018), but these studies do not establish causality via experimental microbiome manipulation. Finally, while microbiota-induced immunosuppressive regulatory T-cells (Tregs) specific to gut bacteria have been shown to limit responses to orally administered *E. coli* heat labile toxin (Bhattacharjee et al., 2020), these studies did not identify the specific microbiome members responsible for the generation of these immune cell populations. Thus, there remains a dearth of information regarding how specific microbial taxa modulate oral vaccine responsiveness through interaction with specific immune cell subtypes and subpopulations.

Therefore, it is important to understand the role of the gut microbiome in the vaccine efficacy. Here, we aim to study the role of human gut microbiome variation at time of vaccination with the oral cholera vaccine Shanchol in healthy Bangladeshi individuals. Our hypothesis is that the structure of individual human

gut commensal communities confers individualized levels of vaccine protective efficacy. We aim to determine whether differences in the gut microbiome between individuals are capable of mediating this change by correlating gut microbiota composition with serum and faecal vibriocidal and vibriospecific titer responses and cellular transcription levels. Additionally, we aim to transplant the individual microbiome communities into germ-free and antibiotic treated mice, followed by infection or vaccination with a *V. cholerae* vaccine strain to confirm the immunomodulatory phenotype in an experimental animal model. Final approval has been received by both iccdr,b and Bangladesh's regulatory board and enrollment is set to begin in January 2022.

Transplantation of Bangladesh fecal samples 605 and 613 into C57/Bl6-Tac and Swiss-Webster germ-free mice and vaccinated with CVD 103-HgR-SmR (Vaxchora)

At the conclusion of the Bangladesh OCV study, it is our goal to transplant pre-vaccinated human stool microbiomes into germ-free mice and determine whether observed human correlates of protection yield similar patterns in the setting of a tractable animal model. Hence, further microbial manipulation and analyses can be carried out in an animal model isolated from other variables to further determine the attributes of certain microbiota and microbial metabolites.

Our collaborator Dr. Ana Weil at the University of Washington previously conducted a Shanchol vaccine study during which stool samples were collected

prior to one or two dose Shanchol administration. Fortunately, we received aliquots of stool samples from several of the participants that had lower serum vibriocidal titers. Overall, transplanting human microbiomes into germ-free mouse models assists in establishing causality between microbial populations and host phenotypical variations.

Results

Findings for the human cholera studies are currently minimal as the US study was halted and the Bangladesh study is set to begin in January 2022. However, there are some results available from the preliminary pilot experiment.

The approach of our preliminary studies is two-fold. First, since we were given very minimal amounts of human stool samples, we desired to expand the microbial cultures utilizing both *in vitro* and *in vivo* expansion. For *in vitro* expansion, the fecal expansion was plated onto a variety of agar plates including: LYHBHI, BHI + inulin + vitamin K, beef, and blood agar. For *in vivo* expansion, fecal suspensions were gavaged into one donor germ-free mouse. As mice are coprophagic, the donor microbiota was allowed to colonize in the donor mouse and colonize recipient mice over a period of 4 days after which the donor mouse was switched to subsequent cages. Percent bacterial genera recovered from cecal contents and fecal pellets was then calculated to determine similarity to the donor mouse and to the input stool sample. Second, we sought to determine whether trends of immune correlates of protection that were conducted for the

human subjects held true for our germ-free mouse model of vaccination. Following this methodology and utilizing the Prospector system, it would be possible to isolate single colonies of interest and create a repository of pure cultures isolated from Bangladesh patients that are either positively or negatively involved with immune outcomes. Hence, confirmed primary isolates with immunomodulatory properties would serve as an important foundation for developing probiotics to aid OCV outcomes.

As shown in Figure 3.1, there was considerable variation in serum vibriocidal titer across the individuals. Vibriocidal titer was measured against Inaba and Ogawa *V. cholerae* strains as both serotypes are seasonal and protection against each is not very well determined. Interestingly, subjects 612 and 613, have a higher relative ratio of Streptococcus, which is similar to our dysbiotic model community. In contrast, subject 605 had a low proportion of Streptococcus and higher serum vibriocidal titer. Thus, we selected these donors 605 and 613 for our pilot transplantation experiment (See Figure 3.1C for experimental layout). While we have been using germ-free C57/Bl6-Tac mice as the main germ-free animal model, there are several limitations as they typically have smaller litters and the dams are prone to cannibalize their offspring, making it difficult to carry out frequent experiments. To improve this experimental limitation, we started a colony of Swiss-Webster germ-free mice which often have larger litter sizes, are more docile, and do not cannibalize offspring as frequently.

Therefore, a third aim for this experiment was to compare mouse strain to strain variation of bacterial and immune phenotypes.

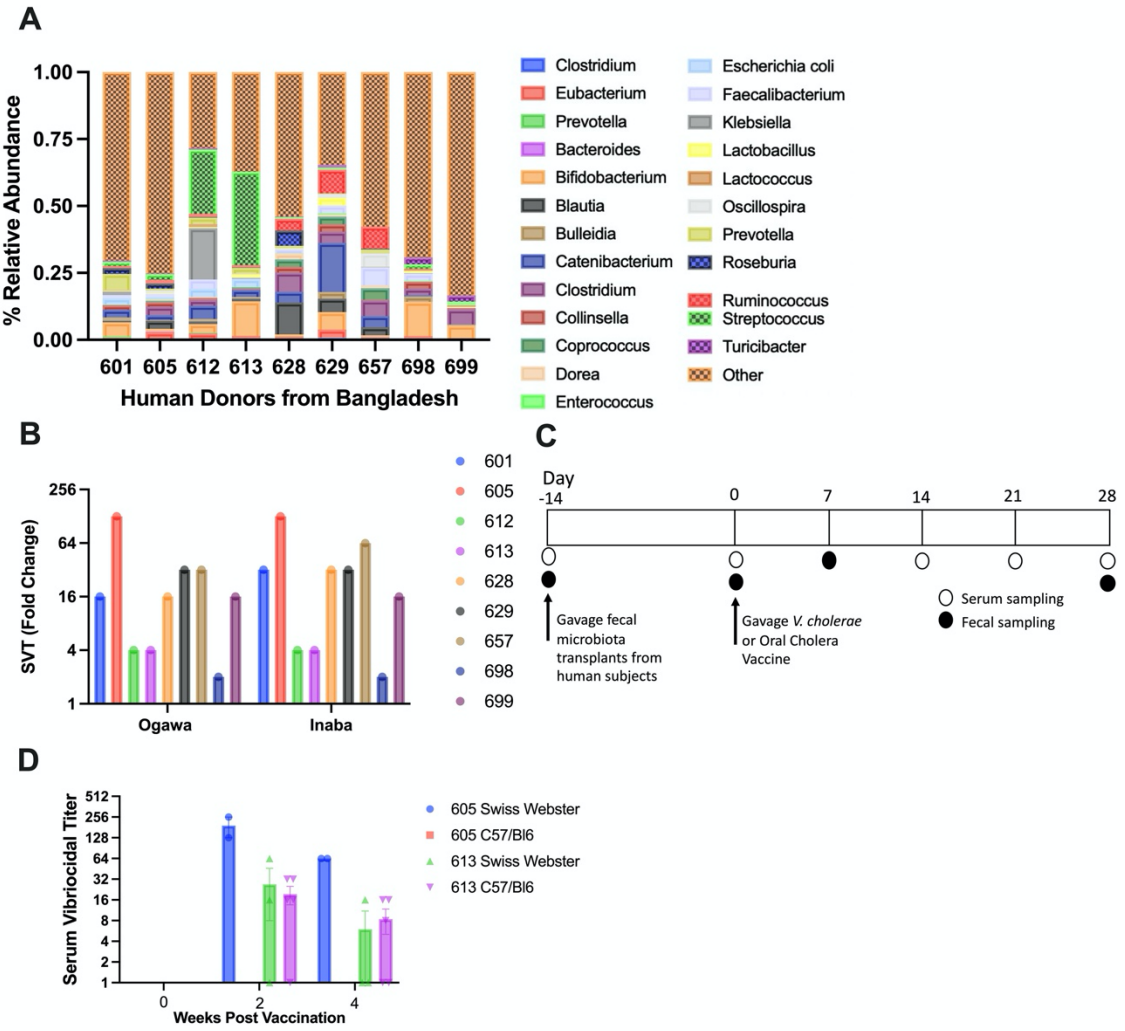

Figure 3.1. Transplantation of adult Bangladeshi stool samples into germ-free mice. (A) 16S relative abundance of adult Bangladeshi donors pre-OCV administration. (B) Serum vibriocidal fold change of adult Bangladeshi donors post OCV administration. (C) Experimental layout for transplantation of Bangladeshi stool transplants into germ-free mice. (D) Serum vibriocidal titer data for transplantation of donor 605 and 613 into either germ-free Swiss Webster or C57/Bl6-Tac mice. Error bars represent mean ± SEM.

Discussion

Oral cholera vaccines remain the primary preventative measure for preventing cholera, a very severe diarrheal disease resulting in 90,000 deaths each year (Ali et al., 2015). Indeed, the International Vaccine Institute in coordination with the World Health Organization is devoted to the creation of a OCV stockpile and has administered 25 million doses to more than 25 countries worldwide. Although OCVs are crucial in preventing morbidity and mortality, they have been shown to have variable efficacy depending on sanitation infrastructures. One of the reasons for this may be due to variability in gut microbiome composition (Levine, 2010). A growing number of studies have examined the interplay between the microbiota and vaccine specific responses. Several studies have implicated/explored the role of the gut microbiome to predominantly rotavirus vaccinations, however, there have been few studies examining the role of the gut microbiome for bacterial vaccines, in particular for cholera. A recent study by Hossain et al. examined populations of North American volunteers and a Bangladeshi cohort (Hossain et al., 2019). Interestingly, there were decreased serum correlates of protection at most time points in the Bangladeshi group as compared to the US group. Moreover, recent studies by Midani discuss the role of various classes of microbiota in predicting outcomes of cholera infection (Midani et al., 2018). However, a recent cholera vaccine study in Bangladesh examined gut microbiome composition at time of vaccination with Shanchol between responders and non-responders, according to their ability to activate

IgA-secreting memory B cells. While overall gut microbial diversity measures were not associated with particular memory B cell responses, individuals with a higher abundance of *Clostridiales* and lower abundance of *Enterobacterales* maintained a greater probability to develop memory B cell responses (Chac et al., 2021).

While our overall study design is similar to the aforementioned study, there are several notable variations that we will utilize that may improve the overall clarity of the gut microbiota in driving divergent immune responses to vaccination. Primarily, we will be enrolling a cohort of children aged 3-5 years old as well as an adult cohort. As children are the most at risk for adverse outcomes to cholera, they are an essential age group to study microbiota-vaccine responses. Additionally, the compositional diversity of this age group is likely different from the adult cohort, and thus would be pivotal to study for future prophylactic or preventative strategies. Moreover, we aim to enroll and prioritize mother-child subjects for administration of either cholera vaccine or saline placebo. Several studies have shown that maternal gut microbiota is often transferred at time of the infant's birth. The effects of intergenerational transfer of microbiota from mother to child are inadequately studied yet this is potentially an important avenue for studying the implications that the gut microbiome may have in early life and affecting immune responses to vaccination. Lastly, we aim to transplant stool from subjects that exhibit either high or low serum vibriocidal titers into germ-free mice. Transplanting pre-vaccination stool samples into germ-

free mice, vaccinating after a period colonization, and carrying out analyses of humoral responses allows for a repeatable and tractable animal model of disease. Further, these animal experiments coupled with the technology of GALT, which can conduct high throughput plating of single colonies, allows us to obtain pure cultures of strains that either have beneficial or detrimental effects for cholera or more broadly for other gastrointestinal diseases. Once the physiological importance of particular strains is known, further analyses can be conducted to determine the role of microbial metabolites on immune responses. Indeed, several recent studies have shown the impact of short chain fatty acids on modulating immune outcomes including in macrophages, cytokine production, and antibody responses (Chang et al., 2014; Chemudupati et al., 2020; Kim et al., 2016; Parada Venegas et al., 2019; Yang et al., 2020).

The end-goal of these proposed experiments may be long-term in nature, however, the data obtained may have significant implications for understanding the breadth of the gut microbiome for physiological processes.

Materials and Methods for US Study

Recruitment

Recruitment will occur through a variety of platforms and will potentially involve recruitment of UC Riverside students as well as the greater public in Southern California. We will post recruitment advertisements using our own personal social media platforms, most likely Facebook Groups including: What's happening in

Riverside, Jobs in Riverside, Moreno Valley, Corona. What's Really Going on in Riverside County.

Finally, we are working in concert with non-UCR clinical collaborators at Riverside Medical Clinic, so the type of recruitment may vary depending on their procedures.

Compensation

Total potential compensation offered will be $60. This will be divided into increments of $20 in cash at the successful completion of each visit. For a successful 1st visit, participant must provide a stool sample, have 5 mLs of blood drawn, and take the vaccine. If the subject is unable to provide a stool sample at the first visit, it will be rescheduled. If the participant is unable to provide a stool sample at the 2nd and 3rd visits, they will still be given payment of $20 per visit.

Study procedures

In order to reduce physical interactions, informed consent will initially take place virtually over Zoom or a different video platform between the participant and a trained member of the research team. We will adhere to any campus, city, county, and state guidelines and adapt our protocols accordingly. The timeline of the research visits including estimated duration is listed in the table below:

Table 3.1. Description of procedures and timing for subjects' visits

Visit #	**Procedures**
Recruitment Visit-30 min	Consent, demographics
1st Visit (baseline)-30 min	Signing of consent, stool sample and blood sample, vaccine administration
2nd Visit / 1 week after baseline-30 min	Stool and blood sample collections
3rd Visit/ 4 weeks after baseline-30 min	Stool and blood sample collections

Recruitment visit and demographic information collection

At the recruitment visit, we will go over demographic information we will ask of them and describe the informed consent process. After the recruitment visit, we will send them a Google Form to complete several demographic questions including: Full Name, Age, Gender, Blood Type (if known), ethnicity, and level of English fluency, and preferred method of contact. Depending on their preference for method of contact, we will notify them two days before the appointment as well as the morning of the appointment.

Please see Appendix C Recruiting script for a list of the questions.

After the virtual recruitment visit, the participant will be able to ask any questions of the research staff prior to signing the physical informed consent form at first visit. At first visit, it is expected that the participant will be able to give consent to be enrolled in the study.

Vaccine administration

Clinical personnel at Riverside Medical Clinic will administer the vaccine. Dr. Andrew Hwang and Eric Choi will be overall clinical supervisors at RMC for this study. The vaccine consists of the lyophilized vaccine in one packet and the buffer in a different packet. The two packets will then be mixed together in a cup with bottled water and then given to the participant.

Staff will observe the participant for any adverse reactions. Since the study will be done in a clinical setting, healthcare staff will be able to take appropriate measures. Severe Adverse Reactions rarely occur for this vaccine. Symptoms mostly include: tiredness, headache, abdominal pain, nausea/vomiting, lack of appetite, diarrhea, and fever. However, if after vaccination, the participant is experiencing unexpected symptoms, they will be directed to go to their closest urgent care facility or emergency department if necessary. Medical records are not being accessed; vaccine information will not be included in medical records.

Blood draws

5 mLs of blood will be drawn via venipuncture per study visit. This will be done by a trained phlebotomist at Riverside Medical Clinic.

Stool samples

The stool collection is a self-administered process that consists of a tub-like device that fits onto the toilet seat. It has instructions clearly stated on the lid that state the following:

A. Place collection unit under toilet in center of rear bowl
B. Close toilet seat to hold system. Attention: Stool collection only-no urine with stool sample.
C. Snap lid on the container tightly after collection

The collection will take place in a restroom at Riverside Medical Clinic.

Sequencing

Stool samples: We will conduct 16S ribosomal RNA sequencing or shotgun sequencing in the study. Anything mapping to human DNA from fecal samples will be deleted.

Peripheral Blood Mononuclear Cells (PBMCs): We will be conducting transcriptional profiles of cell types to help determine cellular functions. We will not be conducting any genetic analysis on DNA of the samples or genome sequencing.

Withdrawal

Participants will be advised of their ability to withdraw from the study as part of the informed consent form. Participants will be able to contact the investigator

directly to request withdraw from the study (contact provided on consent form). Participants will be able to withdraw up until 3 months after their last sample. Up until this point, samples (stool, extracted nucleic acids or other molecules, cells) that are associated with the individual will then be destroyed. 3 months after last sample collection, all samples and corresponding participant data will be de-identified, and thus we will be unable to withdraw the subject's samples/data.

Privacy, confidentiality, and data

Participants will provide information to a member of the Hsiao research team. Identifying information to be collected will be limited to: 1) age, b) sex, c) blood type, and d) ethnicity. We will also be collecting email addresses and phone numbers for scheduling purposes for the study. Hardcopy consent forms will be kept in a locked filing cabinet that is accessible only to members of the study. Research data will be stored on a password-protected computer in the Hsiao lab within the protected building of MRB1. Samples will be stored in a locked -80C freezer either at Riverside Medical Clinic or at MRB1 and accessible only to members on the study team.

Consent forms will be copied for the participants, and originals stored in a locked filing cabinet at the Hsiao laboratory at UCR, access to which is restricted to the general public. Names and other identifying information will be coded as sample identifiers in electronic files to be encrypted and stored on password-protected computers at the Hsiao laboratory. The document that lists the code #

and corresponding identity will be stored separately from research data, on a different password-protected computer and/or locked filing cabinet. Any samples (fecal, blood, nucleic acid or molecular extracts of fecal samples) will be appropriately coded. Information will be coded up until 3 months after collection of the subject's last sample. After this period, all coded information will be de-identified so that there are no longer any links between the subject and samples.

Coded data would only be accessed by the principal investigator in the event that a participant would want to withdraw their samples from the study so that the associated samples could be destroyed.

For individuals who take part in the screening visit, but who are not eligible for the study, their information will be stored for 1 month after their screening visit and then shredded and/or deleted.

Possible risks

The study utilizes an FDA-approved live cholera vaccine, Vaxchora. The potential risks are those associated with oral administration of the live cholera vaccine, possible reactions to the vaccine and having blood drawn.

The documented potential risks include but are not limited to: tiredness (30%), headache (28%), abdominal pain (18%), nausea/vomiting (17%), lack of appetite (16%), diarrhea (4%), and fever (0.6%). (Information from Vaxchora package insert).

Clinical trials found no related serious adverse reactions to the Vaxchora vaccine. Rarely, a subject may experience an allergic reaction, which produces rash, hives, or difficulty breathing. Vaccine products have been shown to be shed in stool for at least one week following administration. Prior to vaccination, we will inform them that if at any time they experience extreme reactions to go to their local urgent care or emergency facility, depending on the extent of their symptoms.

Blood sample collection involves transient discomfort and may cause fainting, which is managed by having the subject lie down. The blood draw sight may bruise, and this can be ameliorated by holding pressure to this site following the blood draw. The sites of blood are potential sites of infection, but this risk is made very unlikely by the use of proper sterile technique.

For stool collection, potential risks include temporary contamination of the skin with some of the stool from the collection container.

There is a potential risk administration of the vaccine would increase symptoms present due to COVID-19 such as shortness of breath or difficulty breathing, fever or chills, cough, fatigue, muscle or body aches, headache, new loss of taste or smell, sore throat, congestion or runny nose, nausea or vomiting, diarrhea.

There is also a risk that someone outside the project could learn information about participants, such as a computer system breach. The study team may also be required by law to disclose any information.

If the participant is injured as a result of being in this study, the University of California will provide the necessary medical treatment. The costs of the treatment may be billed to the participant, or their insurer, just like any other medical costs, or covered by the University of California or the study sponsor, depending on a number of factors. The University and the study sponsor do not normally provide any other form of compensation for injury.

Incidental findings risk

This stool collection and blood draw are done for research purposes only. The stool collection and blood draw being done are designed to answer research questions, not to medically examine you or provide a clinical diagnosis. The stool collection and blood draw are not a substitute for ones a physician would order. It may not show problems that would be picked up by a medical stool collection or blood draw test. The researchers are not professionally qualified to act as the medical provider. However, if we see something unusual in a subject's stool, we will inform them so that they can obtain appropriate follow-up evaluation by their physician. We will also provide their physician with a copy of the stool results upon request. Any follow-up evaluation or treatment that they seek will be at the subject's own expense. Even if their physician rules out any problems, the participant may be unnecessarily worried if a problem is suspected.

Materials and Methods for Bangladesh Study

Study design and settings

This is a randomized clinical trial of a killed oral cholera vaccine response study which will be conducted in healthy Bangladeshi individuals living in Mirpur slum, Dhaka. The total participants population of 80 will be categorised into four arms based on age and treatment: a) 20 children aged 3-5 years will receive oral cholera vaccine Shanchol; b) 20 control children aged 3-5 years will receive saline, and c) 20 adult aged 18-40 years will receive the oral cholera vaccine Shanchol and 20 adult aged 18-40 years will receive saline (Figure 3.2).

First, consent or assent will be taken from participants who are willing to participate in the study according to their age. Screening will be done based on PCR test for cholera. Enrolment of participants will be confirmed if they are negative for cholera PCR test. Once enrolled on the first visit the participant's metadata (age, sex, ethnicity, previous exposure to cholera, blood type; any child/mother or child/parent pairings for collected samples will be noted); and their stool and 5.0ml blood samples will be collected. The participants will then receive oral cholera vaccine or saline. Three follow-up visits will be conducted approximately 1, 2 (just before 2nd dose of vaccination) and 6 weeks after the first visit. A home visit will be made within 2 days of receiving each study vaccine mainly to ensure safety i.e. to note and record any adverse events.

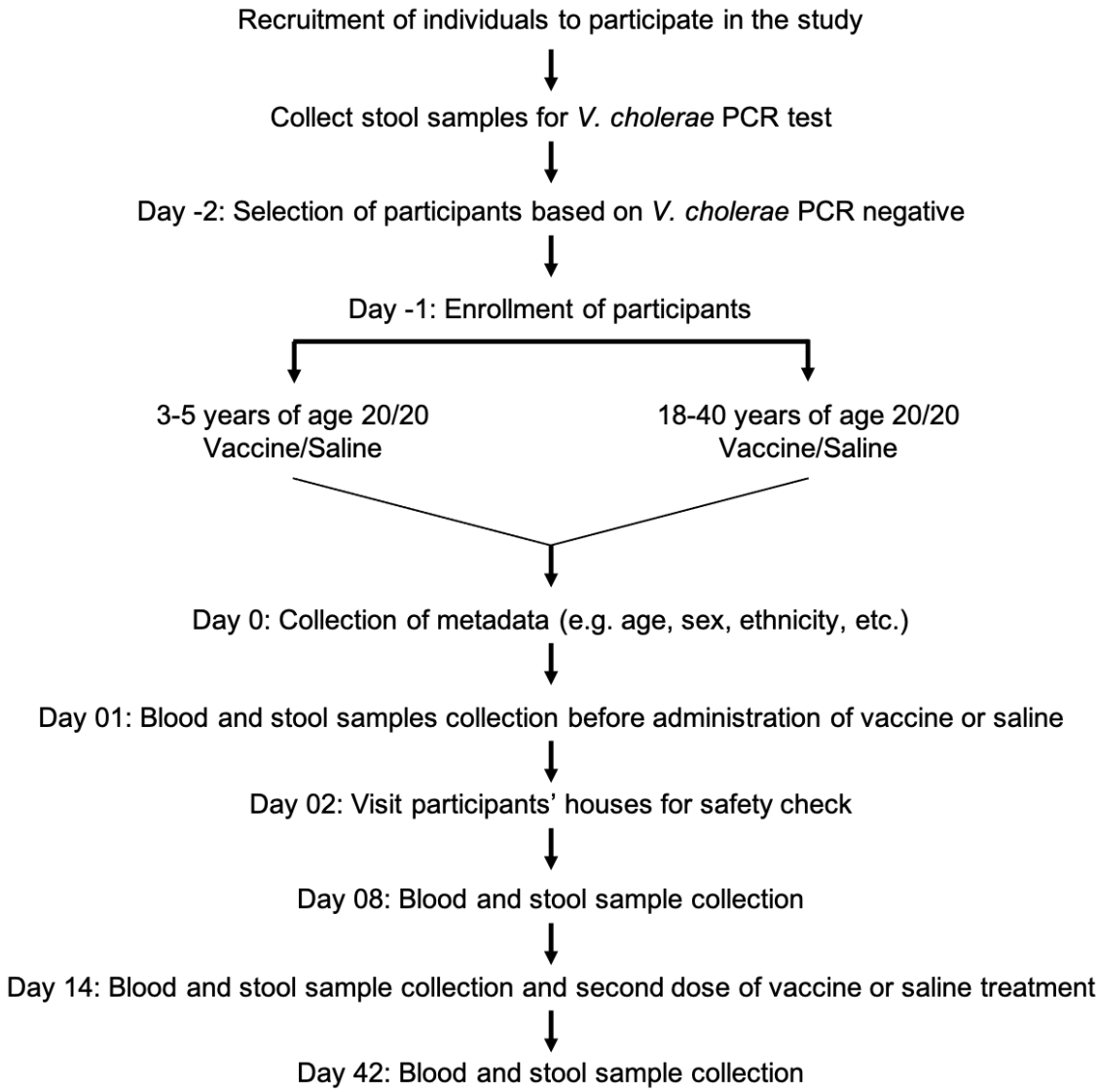

Figure 3.2 Schematic for Euvichol gut microbiome study in Bangladesh

During follow-up visits, data on any side effects, exposure to cholera, faecal and 5.0 ml blood samples will be collected from study participants as well. Two weeks after first dose of vaccine/saline treatment participants will get the second dose of vaccine/saline according to their age group. Faecal and blood

samples will be processed immediately after collection and stored at -80C and liquid nitrogen as per requirement of future experiments.

Inclusion criteria

The following set of criteria will be utilized for selecting the participants in the study:

For Adults:

1) Male or female 18-40 years of age
2) Must be able to provide signed and dated informed consent
3) Healthy subjects willing and able to provide stool specimen

For Children:

1) Male or Female 3-5 years of age
2) Must have a parent or guardian to provide dated informed consent
3) Healthy subjects willing and able to provide stool specimen

Exclusion Criteria:

1) Systemic antibiotic usage (oral, intramuscular, or intravenous) in the week prior to sampling
2) COVID-19 related symptoms two weeks prior to sampling and vaccination including: shortness of breath or difficulty breathing, fever or chills, cough, fatigue, muscle or body aches, headache, new loss of taste or smell, sore throat, congestion or runny nose, nausea or vomiting, diarrhoea
3) Have an acute illness within 72 hours before vaccination

4) Diarrhoea (liquid or very loose stools not associated with a change in diet) in the weeks prior to sampling
5) Active or uncontrolled GI disorders including:

   Inflammatory bowel disease (IBD) including ulcerative colitis, Crohn's disease, or indeterminate colitis

   Persistent, infectious gastroenteritis, colitis, gastritis

   Chronic constipation
6) Major surgery of the GI tract, excluding appendectomy but including major bowel resection at any time
7) Have a suppressed immune system as a result of illness, immunosuppressive medication, chemotherapy, or radiation therapy within 3 years prior to study vaccination.
8) Reside with individuals under the age of 2 or with immunocompromised individuals.
9) Have a known history of autoimmune disease
10) Have a history of Guillain-Barre Syndrome
11) Has previously received a cholera vaccine or have a known history of cholera infection.
12) Have donated blood or blood products within 56 days before study vaccination, plan to donate blood at any time during the 56-day duration of subject study participation, or plan to donate blood within 56 days after the last blood draw.

13) Have known hypersensitivity or allergy to any component of the vaccine or history of anaphylaxis with a vaccine or vaccine component.
14) Are pregnant or breastfeeding or plan to within one month of vaccination
15) Have received any vaccine within the previous 21 days.
16) Positive PCR test for cholera at enrollment

Laboratory procedure

Each collected specimen will be investigated according to the following research framework.

Detection of *Vibrio cholerae* by polymerase chain reaction (PCR)

A single-plex PCR will be performed to detect *Vibrio cholerae* using the species-specific gene *ompW*, (encoding outer membrane protein OmpW) and extracted bacterial DNA from fecal sample. The PCR primer and methods were described in (Nandi et al., 2000), briefly, bacterial cells will be grown overnight at 37°C on Luria agar (LA) plates. Next, isolated colonies will be picked up and mix with 100 ml of normal saline, and bacterial cells will be pelleted by centrifugation. The cell pellet will be resuspended in 100 ml of double-distilled water and boil for 10 min. Cell debris will be removed by centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was used as a template for PCR. The PCR primer sequences for

gene *ompW* are Forward 5'-CACCAAGAAGGTGACTTTATTGTG-3' and *ompW* Reverse 5'-GGTTTGTCGAATTAGCTTCACC-3'.

PCR amplification of the target DNA will be carried out in a thermal cycler using 200-µl PCR tubes with a reaction mixture volume of 25 µl. Samples (3 µl) will be added to the PCR mixture containing 2.5 µl of each primer (10 pmol/µl), 2.5 µl of 2.5 mM deoxynucleoside triphosphates, 0.3 µl (5 U/µl) of Taq DNA polymerase (Takara Shuzo Co., Ltd.), 2.5 µl of 10X reaction buffer containing 20 mM MgCl2 (Extaq; Takara), and 11.8 ml of distilled water to obtain the final reaction volume 25µl. The reaction mixture will be subjected to an amplification of 30 cycles, each of which consist of three steps in the following order: denaturation of template DNA at 94°C for 30 s, annealing of the template DNA at 64°C for 30 s, and extension of the primers at 72°C for 30 s. Before initiation of the first cycle, the reaction mixture was heated at 94°C for 5 min to allow complete denaturation of the template. PCR products, thus obtained, will be electrophoresed through 1.5% (wt/vol) agarose gel to resolve the amplified products which will be visualized under UV light after ethidium bromide staining.

Separation of plasma and PBMC from blood samples

Plasma and PBMCs (peripheral blood mononuclear cells) will be obtained from the same blood sample for all participants using Ficoll-HyPaque (Pharmacia Biotech, Quebec, Canada) gradient centrifugation, as previously reported (Corkum et al., 2015). Briefly, anticoagulated blood will be centrifuged at

800 × *g* for 30 min and the top layer containing plasma will be removed. The remaining blood will be diluted with an equal volume of phosphate-buffered saline, pH 7.4 (PBS), containing 0.05 M ethylenediaminetetraacetic acid (EDTA; Invitrogen). 12.5 ml of diluted blood will be layered over 25 ml of the Ficoll-Paque PLUS (GE Healthcare). Gradients will be centrifuged at 400 × *g* for 30 min at room temperature in a swinging-bucket rotor without the brake. The PBMC interface will carefully be removed by pipetting and washing with PBS-EDTA by centrifugation at 250 × *g* for 10 min. PBMC pellets will be suspended in ammonium-chloride-potassium (ACK) lysing buffer (Invitrogen) and incubated for 10 min at room temperature with gentle mixing to lyse contaminating red blood cells (RBC). This will be followed by a wash with PBS-EDTA. PBMC pellets will then be counted and the percentage viability estimated using Trypan blue staining. Cells can be used immediately or cryopreserved in liquid nitrogen in fetal calf bovine (FBS; Invitrogen) containing 10 % dimethyl sulfoxide (DMSO; Thermo Fisher Scientific) and stored until required for downstream analyses. We will define CD4+ T cell phenotypes by measuring via flow cytometry T-bet (Th1), GATA3 (Th2), Th17 (RORγt) and Treg (FoxP3), as well as PD-1, CXCR5 and BCL-6 to identify Tfh cells.

Vibriocidal assay and vibriospecific antibodies by ELISA

Vibriocidal antibody assay for V. cholera serogroups O1 and O139, will be performed as described in (Benenson et al., 1968c; Qadri et al., 1995) and levels

of antitoxin to *V. cholerae* will be measured by IgG enzyme-linked immunosorbent (ELISA) as previously described (Levine et al., 1985). Briefly, for vibriocidal assays with *V. cholerae* serogroups O1 and O139, bacterial suspensions will be adjusted to an OD at 600 nm of 0.3 (approximately 2 X 10^9 to 3 X 10^9 CFU/ml), and 150 µl of the suspension (final dilution of 1:20 containing 1 X 10^8 CFU/ml) will be used in a final volume of 3 ml of the assay mixture (containing guinea pig complement at 1:10 dilution and physiological saline). The mixture will be applied to microtiter plates (25 µl per well) (Nunc, Roskilde, Denmark) containing serially twofold diluted (25 µl per well plasma samples (starting dilution, 1:10), and this mixture will incubate in a shaker incubator (40 rpm) at 37°C for 1 h. After adding 150 µl of brain heart infusion broth, the plates will again incubate at 37°C for about 3 to 4 h. The ODs of the plates will be measured at 595 nm. The vibriocidal titer will be defined as the reciprocal of the highest plasma dilutions causing a greater than 50% reduction of the OD at 595 nm when compared with the OD of the control wells without plasma.

DNA sequencing for microbial diversity

For DNA from human fecal samples, ~200 mg (average wet weight) fecal sample will be suspended in 600µl PBS. ~500 µl 0.1mm glass beads (BioSpec), 210µl SDS %20, and 500µl neutral phenol:chloroform:isoamyl-alcohol (24:24:1, Fisher Scientific) are then added to each sample, and bacterial cells are then lysed by bead-beading and DNA purified by partitioning to the aqueous phase by

centrifugation followed by ethanol precipitation. The V4 variable region of bacterial 16S ribosomal RNA genes will be amplified in 25µl total volume reactions comprising 1µl of extracted DNA as template, 10µl Platinum Hot Start PCR Master Mix (ThermoFisher), 13µl PCR-grade water and 0.5µl of forward and reverse primers (10 µM). Cycling conditions were 94°C for 3 min, followed by 33 cycles (94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec), and 72°C for 10 min. An equal amount of each amplicon (~240 ng) was pooled into libraries, which were then purified using QIAquick PCR purification columns (Qiagen) and subjected to sequencing using the Illumina MiSeq platform. Paired-end 150nt reads will then be assembled, de-multiplexed, rarefied to >900 reads per sample, and analyzed using the QIIME software package.

We have used Illumina V4 with great success in identifying up to species level taxa (Alavi et al., 2020; Hsiao et al., 2014). More recent analytical pipelines using exact sequence variants have been used with Illumina V4 sequencing to identify taxa down to strain level. While long-read sequencing platforms such as PacBio for 16S sequencing is a promising approach, the Illumina pipelines are robust and already established in our collaborators' group at UCR. DNA extracted from samples collected in the study can also be used for PacBio sequencing in follow-up analyses in the event that V4 sequencing is unable to discriminate microbial taxa at the resolution necessary, or DNA prepared for shotgun metagenomic sequencing followed by metagenome assembly and identification of taxa at strain level.

Primary and secondary variables

The proposed study will have four arms and the total sample size is 80 participants:

Children aged 3-5 years / 20 subjects will receive vaccine

Children aged 3-5 years / 20 subjects will receive saline

Adult aged 18-40 years / 20 subjects will receive vaccine; and

Adult aged 18-40 years / 20 subjects will receive saline.

The main purpose of this work is to examine the relationship between the configuration and magnitude of gut microbiota on the variability in the host's immune response to OCV. To our knowledge, such OCV studies have never before been done in human populations, and hence it is not possible to estimate the required sample size let along power calculations for this 'proof-of-concept 'study, which will have an arbitrary sample size. Preliminary studies have demonstrated 16-fold serum vibriocidal titer (SVT) response variations in the US human faecal microbiomes when transplanted into germfree mice followed by introduction of *V. cholerae*, and the ability to discriminate species-level taxa of transplanted human faecal microbiomes in an animal allows for a more mechanistic understanding of the microbiota. We are hopeful that the data generated from this study will enable enumeration of microbiome variations and compare them with OCV efficacy, which could be used in the estimation of sample size and power calculations in future studies.

Data analysis

Microbial content of fecal samples will be analysed by collaborators at UCR, with input from the study team at icddr,b. We will identify both the distribution and abundance of individual bacterial taxa in study participant fecal microbiomes at the time of OCV administration using Illumina sequencing of 16S rRNA gene amplicon libraries (V4 region), and qPCR for 16S gene abundance, allowing us to estimate the actual abundance of individual microbial taxa at exact sequence variant (ESV) level in human fecal samples. Ecological measures of species abundance will be analysed by QIIME2 (Bolyen et al., 2019). We will separate pre-vaccination fecal samples into high (>4-fold increase in SVT) and low (<4-fold increase in SVT) groups. We will then perform Indicator Species analysis to identify the microbial taxa most characteristic of the microbiome of each group, which has the advantage of considering both the relative abundance and presence/absence of individual taxa across all samples of a given cohort (Hsiao et al., 2014). We will also employ several regression analyses approaches to identify individual taxa that correlate to SVT outcomes across the entire dataset to identify microbial correlates of strong and weak SVT responses in humans. PBMC populations will be analyzed by antibody staining and flow cytometry for the presence of lymphocyte populations. All analyses across the dataset will take place taking account sex, age, and vaccine/placebo treatment.

Chapter 4: Preliminary analyses of interactions between commensal bacteria, *V. cholerae*, and host immune cells

Introduction

Shaped by thousands of years of co-evolution, bacteria have adapted to inhabit the guts of various host species, in a generally mutualistic relationship, in which the microbiota contributes to a range of physiological processes and in turn the host provides nutrients and a niche to survive. The main contributions of the microbiota are to metabolize indigestible nutrients but also to provide colonization resistance against pathogens and to help train the immune system (Round and Mazmanian, 2009). The major contributions to immune system development has been extensively studied, primarily in germ-free mice. In particular, germ-free mice have been shown to have markedly reduced production of secretory IgA (S-IgA) as well as underdevelopment of gut associated lymphatic tissues (GALTs) such as Peyer's patches, crypt patches, and isolated lymphoid follicles (Kamada et al., 2013; Round and Mazmanian, 2009). In the last several decades, extensive work has been done to study both the composition of the human microbiota (Qin et al., 2010) and the impact of microbial metabolites on the host (Uchimura et al., 2018). These studies helped pave the way for more direct functional effects that certain bacterial species may induce in the host. Indeed, a pivotal study by Ivanov et al. describes that segmented filamentous bacterium (SFB) promote Th17 development in the intestine of a mouse model (Ivanov et

al., 2009). While these studies implicate end-stage functional consequences of specific bacterial species, the underlying mechanisms causing differentiated outcomes remains to be determined. In a similar fashion, my studies indicate that the presence of a dysbiotic microbiota may dampen antigen-specific antibody responses to *V. cholerae* infection or vaccination strategies. In this chapter, the preliminary experiments and data to understand underlying mechanistic pathways between the gut microbiota, *V. cholerae*, and host cells are discussed. However, interactions of the gut microbiota can be quite complex with many variables, making it difficult to ascertain definitive mechanisms. In order to further examine the effects of the microbiota and in particular, the normal microbiota (NM) or dysbiotic microbiota (DM) simple model communities, our approach spanned several different functional aspects to grasp bacterial community interactions. Our results discussed in chapter 2, figure 2.7E-F, suggest that *in vivo* depletion of CD4+ cells restores immune correlates of protection for cholera. Therefore, we sought to determine the role of various CD4+ T cell subsets including T_{Reg}, T_{FH}, and Th17 cells as well as Th1 vs Th2 responses. Moreover, as interactions of S-IgA with *V. cholerae* in conjunction with mucin within the gastrointestinal tract are critical for protection, we modeled this physiological process in a simple *in vitro* model. Lastly, *V. cholerae* exerts its pathogenicity through the production of various virulence factors, which may be differentially regulated depending on the milieu of surrounding bacteria and bacterial metabolites. Teasing apart underlying mechanisms of pathogenicity and

immunogenicity may be challenging, the data in this chapter introduces several potential avenues for future research.

Results

Depletion of Treg cells fails to fully restore serum vibriocidal responses

A subset of Helper T cells that we wanted to explore the functional role of in our experimental system are Treg cells. Previous studies examined the beneficial role of Tregs in autoimmune settings such as Inflammatory Bowel Disease. Accordingly, the researchers observed that Treg cells releasing IL-10 could lessen the pro-inflammatory milieu present in this debilitating autoimmune disease. Moreover, they observed that PSA released from *B. fragilis* induced the Treg response (Round and Mazmanian, 2010). In contrast to the beneficial effects due to the presence of Tregs in autoimmune individuals, the presence of Tregs in a pathogen-mediated immune response has been shown to hamper antibody mediated protection in multiple infectious disease models such as Friend retrovirus infection and *H. pylori* (Lundgren et al., 2005; Moore et al., 2018). Therefore, we hypothesized that the DM community may induce IL-10 secreting Treg cells, leading to an unfavorable environment for the production of a robust antigen-specific B cell response.

To determine the effect of an anti-*Vibrio* antibody-mediated protection in the absence of Treg cells, we conducted a targeted depletion using a monoclonal antibody against CD25, one of the defining extracellular factors of differentiated

Treg cells. Figure 4.1 shows the gating strategy used to define the splenic Treg cells prior to and post-depletion. While other measures of Tregs were also examined such as FoxP3, the overall measurement of depletion was done from examining the CD4+CD25+ populations of splenic cells. While there was a slight increase in mean SVT for the CD25 depleted group, it was not significantly different than the non-CD25 depleted SVT titer. Similarly, there were slight increases in serum vibriospecific IgA, IgG1, IgG3, and IgM, but they were not significantly different than the non-CD25 depleted group treated with DM. Using a monoclonal antibody against CD25 did result in a reduction of CD4+CD25+ T cells, however, it may not have been enough to result in a full restoration of anti-*Vibrio* responses. An alternative approach would be to utilize mice deficient in IL-10 (*Il10*$^{-/-}$), a key functional cytokine for Treg cells.

An even better scenario would be to derive *Il10*$^{-/-}$ mice in a germ-free mouse system. Indeed, our collaborator Dr. Philip Ahern at the Cleveland Clinic maintains *Il10*$^{-/-}$ germ-free mice and is in the process of gathering preliminary data with our model microbiome communities.

Approaches to understand IgA-*V. cholerae* binding characteristics

As mentioned previously, functional protection from *V. cholerae* is due to antigen specific S-IgA antibodies that are released from B cells in the lamina propria that then bind to epitopes on the V. cholerae cell. Binding thus inhibits *V. cholerae*

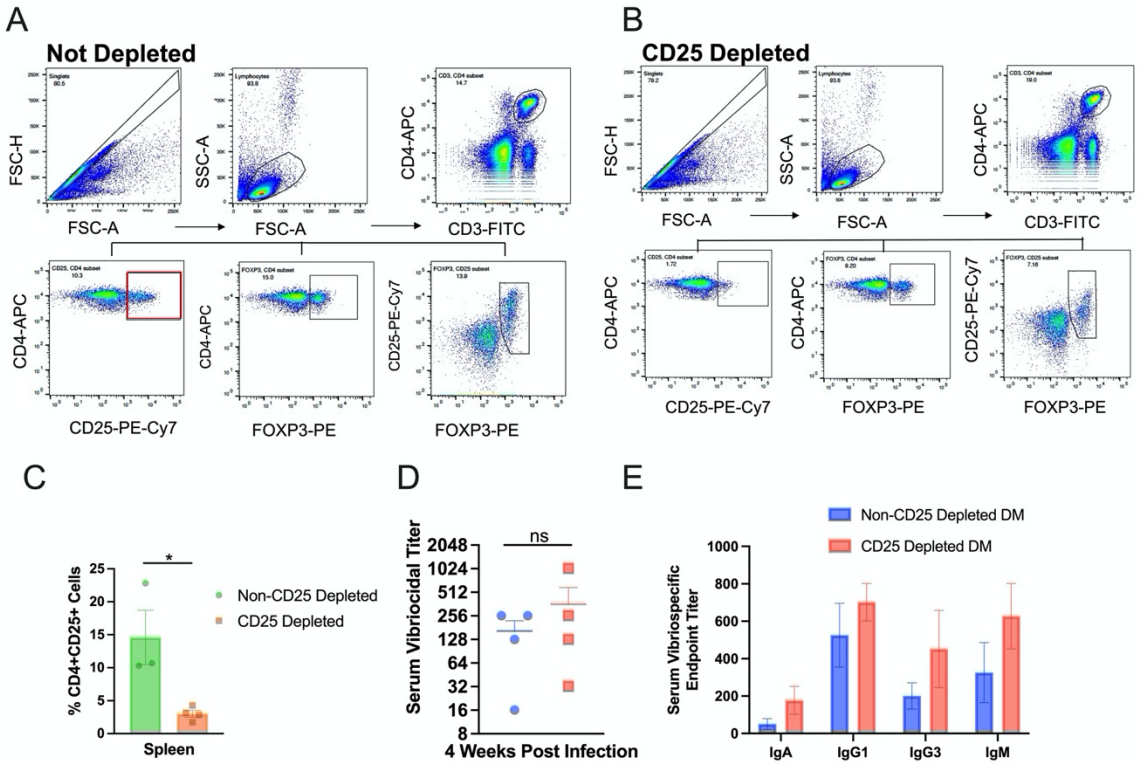

Figure 4.1. Depletion Strategy for CD25+ T_{Reg} cells and corresponding correlates of protection. (A-B) Flow Cytometry gating strategy for not depleted and CD25 depleted mice. (C) Percentage of CD4+CD25+ splenic cells that were CD25 depleted or non-depleted. (D) Serum vibriocidal titer of animals bearing the DM community according to respective depletion strategies at 4 weeks post *V. cholerae* infection. (E) Serum vibriospecific endpoint titer against whole cell *V. cholerae* for indicated antibody subtypes. *, $P<0.05$. Mann Whitney *U* test. Error bars represent mean ± SEM.

from gaining access to the epithelium and bacterial cell-SIgA entrenchment also enables further “elution” from the gastrointestinal tract.

An *in vitro* experiment we conducted to further define the functional protection of fecal antibody was a mucin penetration assay. As *V. cholerae* transits the gastrointestinal tract, it also moves through the mucin layer as part of its final stages of pathogenesis to release cholera toxin. In addition to various

antimicrobial peptides and other innate immune factors, S-IgA is also present in the mucin and binds to *V. cholerae*, preventing further access to the epithelium. To mimic this scenario in an *in vitro* setting, briefly, 1% submaxillary porcine mucin was added to a sterile syringe. Fresh *V. cholerae* was then pre-treated with fecal water from the respective communities and then layered on top of the mucin layer. After an incubation period, the mixture was separated into fractions to delineate the percentage of *V. cholerae* that moved through the column. Our preliminary results as shown in Figure 4.2 suggest that the antibody derived from animals bearing the DM microbiome are not as capable of preventing migration through the column as fecal antibody derived from the NM animals. While these results are promising, more studies will need to be done to fully appreciate the protective or detrimental effects of the antibodies involved. While the assays previously mentioned may be indicative of function, they do not fully resemble biological mechanisms that would take place in an animal or human model of infection. As described in Chapter 2 Figure 2.5, we utilized a passive protection model using infant mice to better understand the functional protection of the antibodies in a model more closely representative of human infection.

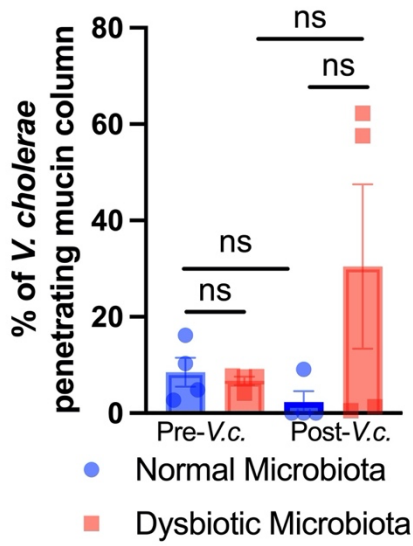

Figure 4.2. Simple *in vitro* mucin penetration assay to study *V. cholerae*-IgA binding characteristics. (A) Comparison of *V. cholerae* movement through a 1% porcine mucin column that were exposed to fecal antibody from mice bearing either NM or DM microbiomes pre- and post-*V. cholerae* infection. ns>0.5, Mann Whitney *U* test. Error bars represent mean ± SEM.

Role of complement in the gastrointestinal tract

While the complement pathway is not traditionally involved in the gastrointestinal lumen, several recent studies have highlighted its potential importance. In a model of ischemia and reperfusion, the authors observed that depletion of gut commensal bacteria led to a decrease in both IgM and C3 deposition within the gut lumen (Yoshiya et al., 2011). This led us to explore whether gut bacterial communities can differentially induce expression of various components of the complement pathway. We observed that in a germ-free state, very little complement is secreted into the gastrointestinal lumen (Figure 4.3). As bacterial presence and innate immune pathways are often intertwined, we examined whether and how the addition of a complex microbiota from human stool

transplants might alter the presence of C3 in the lumen. After 2 weeks of colonization, fecal pellets were collected and levels of C3 were examined via ELISA across the 3 donors. Interestingly, the levels of C3 were significantly increased after addition of the complex human microbiota (Figure 4.3). Moreover, the levels of C3 were inversely proportional to the level of protection as seen by serum vibriospecific titer and vibriospecific fecal IgA. Thus, levels of C3 could potentially be indicative of a more inflammatory response, leading to subsequently poor antibody protection.

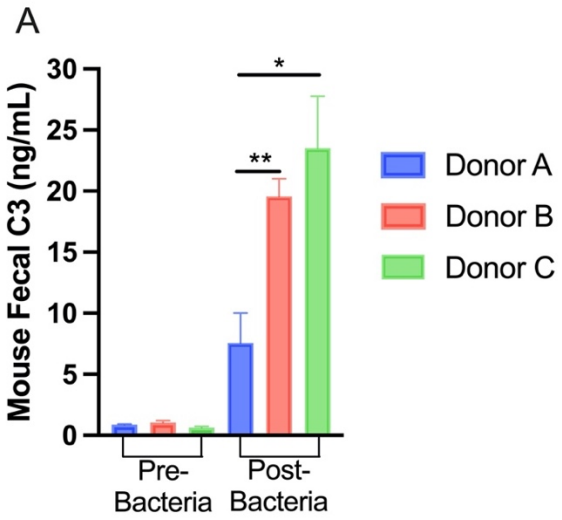

Figure 4.3. Concentration of fecal C3 in germ-free mice given fecal transplants from indicated donors. Levels of C3 in germ-free mice pre- and post-fecal transplants from indicated human donors. *, $P<0.05$; **, $P<0.01$, Mann-Whitney *U* test. Error bars represent mean ± SEM.

Heat inactivated killing factor

In early experimentation studies, we examined whether fecal water containing antibodies could mediate killing with the addition of exogenous complement. For

this experimental set up, fecal water was combined with *in vitro* grown *V. cholerae* before the addition of exogenous complement serum. Interestingly, we observed killing of *V. cholerae* in the presence of fecal water alone prior to the addition of complement. While killing was evident before the addition of complement, after the addition of complement there was a dramatic reduction of *V. cholerae*, particularly in the NM group as compared to mice bearing the DM group at time of infection. Moreover, when the fecal water was heat-inactivated, the killing of *V. cholerae* ceased. This is indicative of a heat-labile killing factor present in the fecal water (Figure 4.3A). These results may be indicative of complement playing a role in killing. While these results are indicative of a component killing *V. cholerae* independent of a protective antibody-mediated response, these results were not replicated. Next, as shown in Figure 4.4B, we enriched fecal water for antibody and conducted a similar *Vibrio* killing assay. We observed that enriched antibody from the DM group were less capable of mediating vibriocidal activity with or without the presence of complement. Additionally, we thereafter examined the ability of antibody to mediate killing via complement by a titer of the serum rather than amount of *V. cholerae* remaining after a given amount of serum, which is often considered the gold standard for clinical correlate of protection.

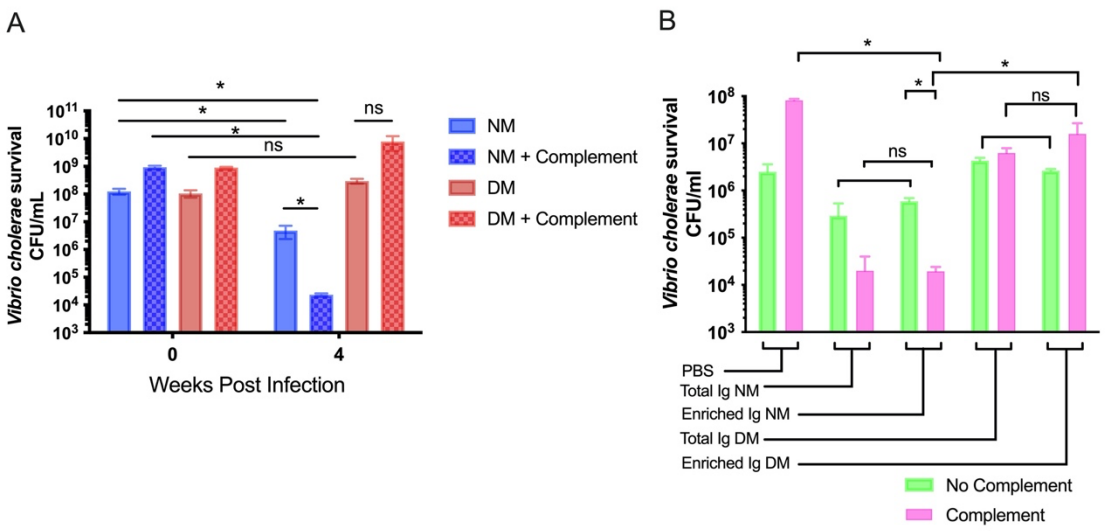

Figure 4.4. Measurement of *in vitro* grown *V. cholerae* survival utilizing fecal water from antibiotic treated adult mice. (A) Levels of survival for *in vitro* grown *V. cholerae* incubated with fecal water from NM or DM communities with or without the addition of guinea pig complement. (B) Levels of survival for *in vitro* grown *V. cholerae* incubated with **enriched antibody** from NM or DM communities with or without the addition of guinea pig complement. *, $P<0.05$, Mann-Whitney *U* test. Error bars represent mean ± SEM.

Transcription factor Peyer's patches analysis

Peyer's patches serve as important antigen sampling sites for luminal contents transiting the gastrointestinal tract. Sampling is mainly achieved by M cells, which then transfer absorbed antigens to surrounding antigen-presenting cells and lymphocytes. Activated cells then migrate to mesenteric lymph nodes to become mature, after which they migrate to other mucosa-associated lymphatic tissues. Peripheral sites of immunity are becoming an ever more important area of study, especially in relation to bacterial metabolites (Uchimura et al., 2018). Therefore, we sought to determine whether the introduction of different bacterial communities could alter the overall expression of cytokines within Peyer's

patches or the activation of various effector T cells. We examined the following cytokines as shown in Table 4.1 that are associated with the following T cell subsets: Th1, Th2, Th17, and Treg cells.

Table 4.1 T cell subsets with associated transcription factors and effector cytokines.

T cell subsets	Transcription factors	Effector Cytokines
Th1	T-bet	IFN-γ, TNF-α
Th2	GATA3	IL-4, IL-5, IL-13
Tfh	Bcl-6	IL-21, IFN-γ, IL-4
Th17	RORγt	IL-17, IL-22
Treg	Foxp3	TGF-β, IL-10

In animals that were given either NM, DM, or vaccine alone, Th2 and Th17 cytokines were more highly expressed in the NM mice than the DM mice, relative to mice given only Vaxchora. These data may suggest that the NM group is more capable of inducing a Th2 stimulated humoral immune response than the DM or Vaxchora alone groups. However, at present, these studies are only an *n* of 2 for the NM group due to experimental and technical reasons. More experimentation will need to be done to further analyze cytokine effects as shown by previous studies (Lo et al., 2004). While examining overall cytokine expression in Peyer's patches is beneficial, a more targeted analysis examining which cell types are actively producing indicated cytokines would be beneficial. One method to achieve this would be to examine cytokine expression of different cell types via flow cytometry. I attempted to examine different cell populations using these

experimental conditions, however, further optimization is required to better examine the cytokine expression.

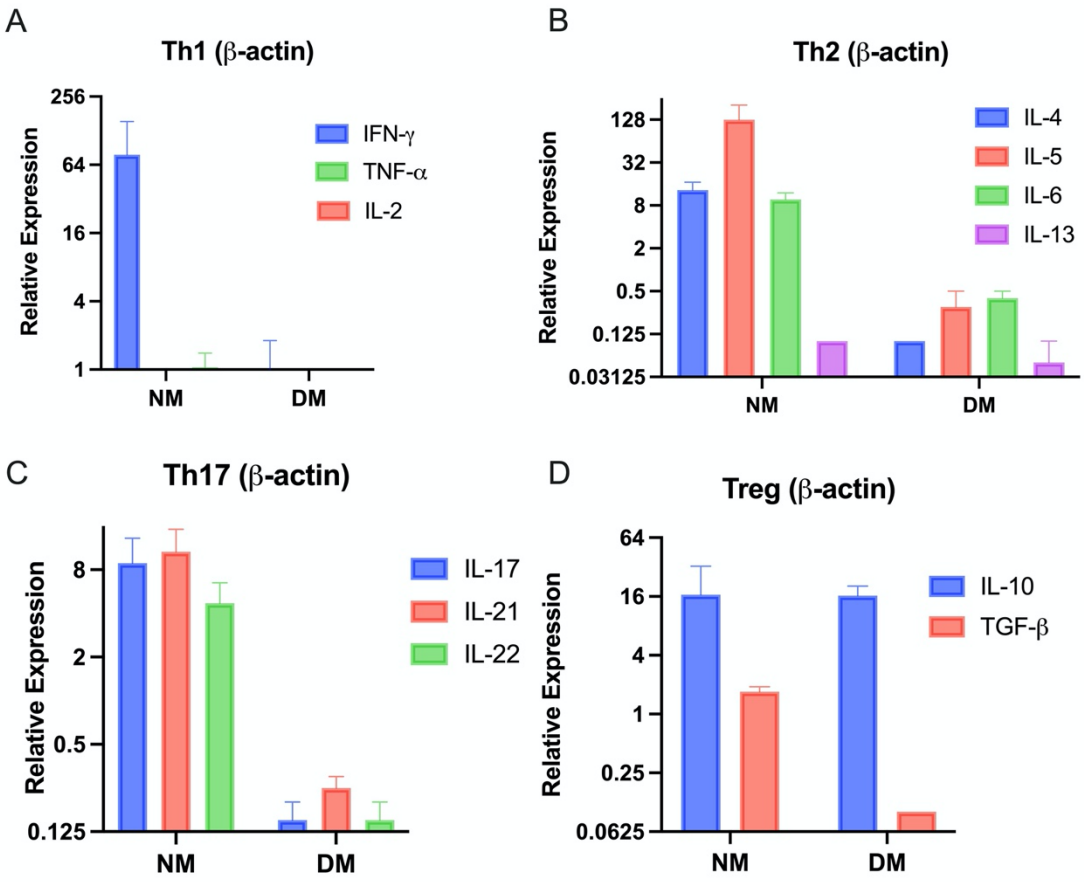

Figure 4.5. A comparison of cytokine expression observed in mice given model communities (A-D) Relative transcriptional expression of indicated cytokines in Peyer's patches from antibiotic-treated adult mice bearing either NM or DM communities in comparison to animals only administered Vaxchora at 4 weeks post vaccination. Error bars represent mean ± SEM. *n*=2-5 mice per group.

Besides cytokine expression, another defining factor of Helper T cells is the expression of certain nuclear transcription factors (See Table 4.1). In our analysis, we examined the expression of various helper T cell subsets in both

Peyer's patches and splenic tissue. First, we observed negligible differences between the percentage of helper T cells in either spleen or Peyer's patches across the different treatment groups of NM, Vaxchora alone, and DM (Figure 4.6-7). Further, while there was no significant difference in T-bet expression across the different treatment groups, T-bet in general was more highly expressed than GATA3 or RORγT in both the positive and negative CD3+CD4+ populations. This trend was similarly observed in Peyer's patches also, with greater T-bet expression in comparison to the other transcription factors as shown in Figure 4.6.

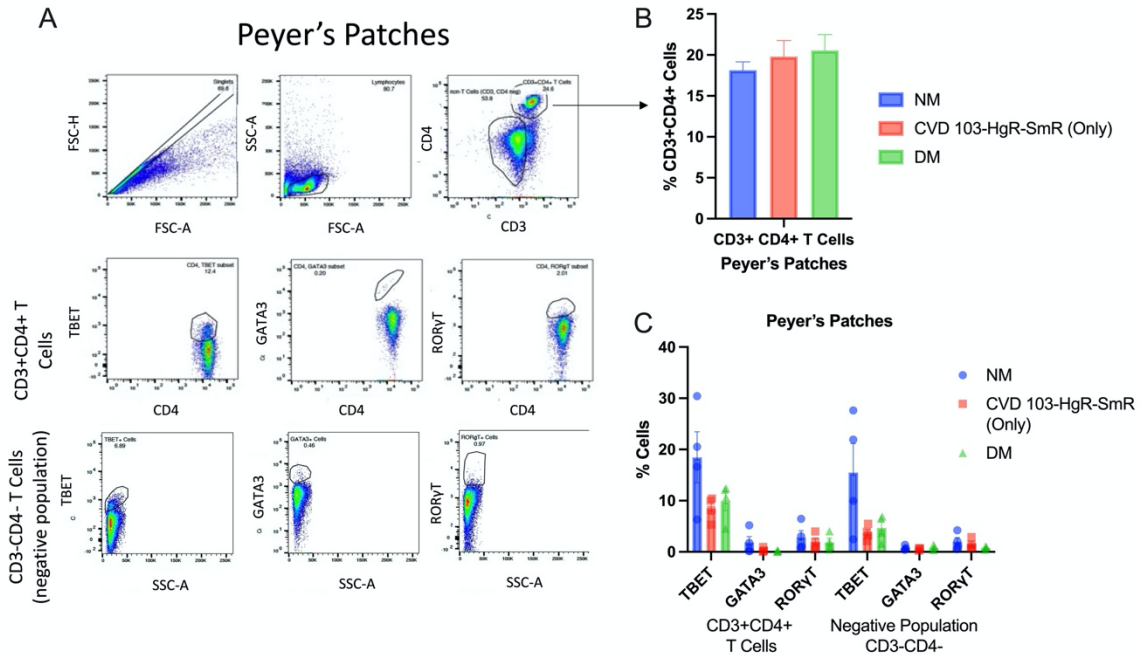

Figure 4.6. Intranuclear transcription factor staining of immune cell subsets within Peyer's patches. Intracellular staining of nuclear transcription factors in Peyer's patches of antibiotic-treated adult mice given indicated bacterial communities and vaccinated with Vaxchora or given Vaxchora alone. Error bars represent mean ± SEM. *n*=3-4 mice per group.

These data are different than what one might expect as T-bet expression is typically associated with a Th1 response with prototypical IFNγ secretion. A more nuanced approach will be required to further understand the underpinnings of the potential role of the microbiota in differentially stimulating different Helper T cell populations.

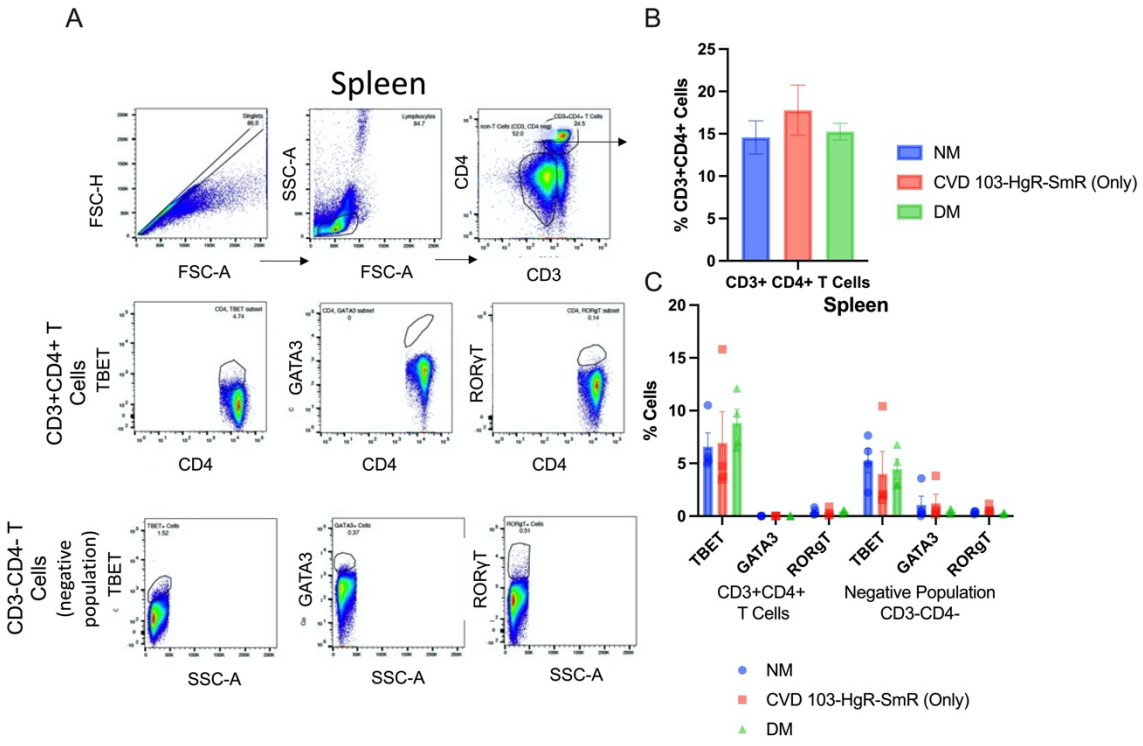

Figure 4.7. Intranuclear transcription factor staining of splenic cells. Intracellular staining of nuclear transcription factors in Peyer's patches of antibiotic-treated adult mice given indicated bacterial communities and vaccinated with Vaxchora or given Vaxchora alone. Error bars represent mean ± SEM. *n*=3-4 mice per group.

However, there are several other accessory toxins present as well, which may be differentially regulated in the presence of different microbial communities that ultimately results in variable antibody-mediated protection. Additional

accessory toxins include Repeat Toxin (RTX), hemolysin (HlyA), and Hemagglutinin (HA)/protease, see table 4.2 for a description of their functions.

Table 4.2 Associated virulence factors with *V. cholerae*.

Toxin	Main host cellular effects
Cholera Toxin (CT)	Activation of adenylate cyclase, increase in cAMP, active secretion of electrolytes and water
Repeat Toxin (RTX)	Pore formation, depolymerization of the actin cytoskeleton
Hemolysin (HlyA)	Extracellular pore forming toxin
Hemagglutinin (HA)/protease	Mucinase, disturbs the paracellular barrier

Genetic expression of these accessory toxins in addition to cholera toxin were examined in the context of NM, DM, and NM+DM. Because RTX is still present in most oral cholera vaccines, we hypothesized that the presence of DM microbes could lead to increased expression of RTX in comparison to the NM microbes or mixed setting. As seen in Figure 4.8, expression of *hlyA* and *rtxA* was highly variable across the different treatment groups. However, expression of *ctxA* and *hapA* was significantly increased in NM in comparison to both DM and NM+DM. These findings are contrary to results that one might expect as one would predict virulence factors to be decreased in mice bearing the NM group instead of DM. However, it is possible that heightened expression of the virulence genes may ultimately lead to stronger humoral immune responses.

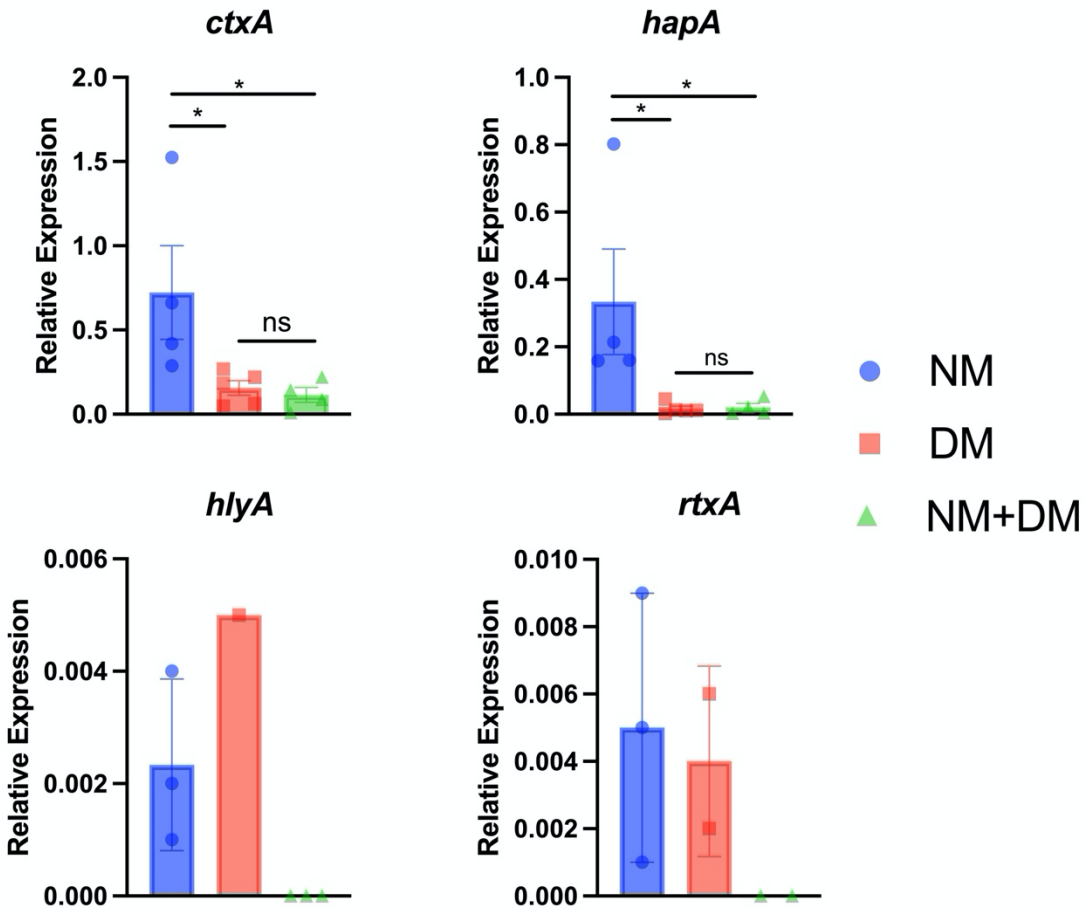

Figure 4.8. Relative expression of *V. cholerae* virulence factors. A comparison of virulence expression in *V. cholerae* in comparison to *recA* in mice either given NM, DM, or NM+DM communities at time of infection with *V. cholerae.* *, $P<0.05$, Mann-Whitney *U* test. Error bars represent mean ± SEM. n=3-4 mice per group.

Isolators for germ-free mice are most ideal for controlling experimental settings for germ-free experiments. However, experimentation is time and resource limiting. Therefore, we explored whether we could replicate our findings using germ-free mice taken from our breeding isolators and conduct the experiment in a specific pathogen free environment with limited user interaction, in line with

previous studies (Butterton et al., 1996). Both stool from human donors and model microbial communities were gavaged into the germ-free mice at time of removal from germ-free environment. After 4 days of colonization, all animals were given Vaxchora with a control group receiving only Vaxchora. As shown in Figure 4.9, while mice from Donor I exhibited a slight increase in SVT response, there was little difference observed across the groups.

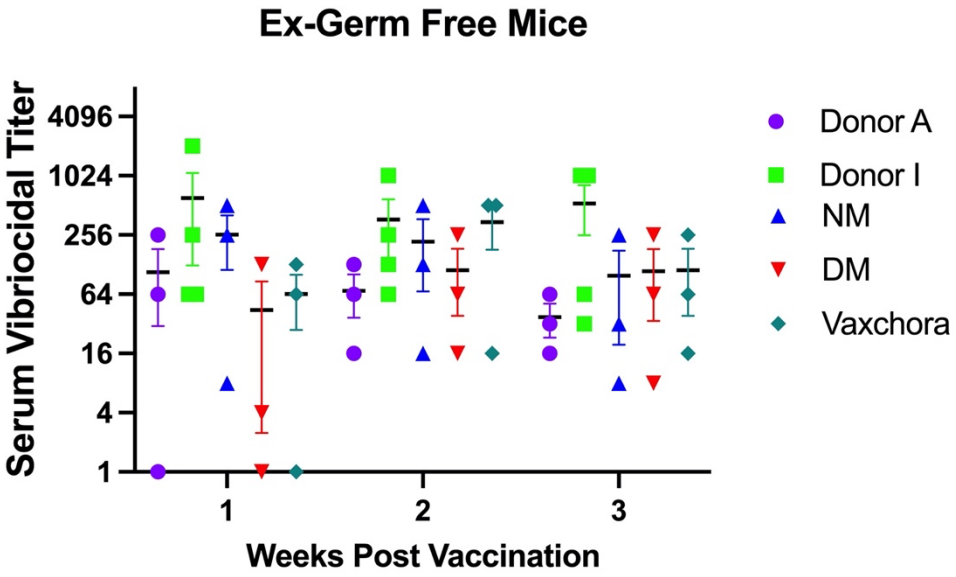

Figure 4.9. Serum vibriocidal titer in ex-germ free mice at 1-3 weeks post vaccination. Serum vibriocidal titer data of C57/Bl6-Tac germ-free mice that upon immediate removal from isolators were given Vaxchora, fecal transplants of human donors, model communities, or vaccine alone. Error bars represent mean ± SEM. *n*=3-4 mice per group.

Discussion

The gut microbiota has a profound impact on the human body, including aiding in digestion of compounds, training and altering the immune system as well as

potentially altering behavioral patterns with the further development of the gut-brain axis. In chapter, 2 we established how the overall presence of microbial populations at time of infection or vaccination can affect overall effector functions of a primarily humoral antibody mediated response. The experiments conducted in this chapter aim to tease apart the underlying interactions between *V. cholerae*, microbial populations, and host cellular responses.

As mentioned in chapter 2, upon *in vivo* depletion of CD4+ T cells, the serum vibriocidal titer of animals bearing the DM microbiota at time of infection increased at 4 weeks post infection to comparable levels with animals given the NM microbiota. Surprisingly, this suggests that CD4+ T cells may have a suppressive effect on the development of robust antibody response in infectious pathogen scenarios. Regulatory T cells (T_{REGS}) are important in reducing inflammation that may occur during autoimmune diseases such as inflammatory bowel disease or rheumatoid arthritis. However, their presence may also increase pathogen persistence and reduce vaccine-induced immunity in infectious disease scenarios. Indeed, regulatory T cells were found to suppress virus-specific responses in a retrovirus infection (Moore et al., 2018) as well as suppress memory T cell responses to *H. pylori* in humans (Lundgren et al., 2005). These studies suggest that either excess number or function of T_{reg} cells may prevent effector immune responses and be disadvantageous to the host. Therefore, we hypothesized that the absence of regulatory T cells would restore antigen-specific antibody mediated protection in mice bearing the DM

community. While there was a significant decrease in CD4+CD25+ splenic cells after depletion, the incomplete depletion may not have been enough to fully restore antibody responses. Alternatively, there may also be other suppressive factors independent of Treg cells. In determining the potential role of Tregs, transcriptional profile analyses were conducted of prototypical Treg cytokines IL-10 and TGF-β on Peyer's patches in animals given NM, DM, or Vaxchora alone (Figure 4.5). While there was no difference between the groups, it is possible that an analysis of total transcription levels within Peyer's patches is too broad of an approach and a more targeted cell-specific analysis is required. Indeed, I attempted to examine cytokine expression via flow cytometry, however, minimal cytokine expression was observed (data not shown) and more protocol optimization is required. It is likely that the cells were not stimulated or incubated for the proper duration of time.

Beyond examining T cell subsets, it would also be beneficial to examine changes in other cell populations including dendritic cells, macrophages, and potentially neutrophils. Additionally, it would be crucial to examine functional changes in B cells as well between the communities and *V. cholerae* alone. Additionally, all observations were done in a post-*V. cholerae* infection or vaccination scheme, it would be intriguing to examine the effects of the model microbial communities themselves on host responses. However, it may be that an immune stimulus in the form of *V. cholerae* is required.

The complement activation pathway is an important intermediary between innate and adaptive immune responses. The activation of the complement pathway is a highly regulated pathway with several components such as C3a and C5a serving as potent mediators of pro-inflammatory pathways, including further activation of macrophages, and other cells. It is known that IgG and IgM antibody subtypes are potent activators of the complement activation pathway via C1q, resulting in formation of a membrane attack complex within the bacterial cell membrane and subsequent bacterial lysis. However, little is known regarding the presence of complement and antibodies in the gastrointestinal tract and the overall role of the microbiota. Dr. Wen Zhen, a member of Dr. Dennis Kasper's lab, explored the potential role of the gut microbiota to stimulate the presence of complement proteins in the gastrointestinal tract. In her doctoral work, she demonstrated that germ-free mice exhibit minimal concentrations of C3 in the gut, however, upon colonization with gut microbiota, levels of C3 increased (Zheng, 2017). Our results confirm this observation as there was little complement present in germ-free mice pre-colonization. Interestingly, after colonization of human donors A, B, and C, C3 concentrations increased and was inversely proportional to levels of vibriospecific fecal IgA. Thus, increased C3 may be indicative of a heightened inflammatory state and a decreased antigen-specific IgA response. This scenario would be contradictory to what one might expect as several studies suggest that animals deficient in C3 have impaired immune responses (Fischer et al., 1998; O'Neil et al., 1988). To more definitively

study the effects of the gut microbiota regulating complement and resultant antibody mediated protection, it would be ideal to utilize mice deficient in C3, however, it would be difficult to derive that mouse strain germ-free. The potential role of complement in the gastrointestinal tract remains an area of potential for future research.

Defining the interactions between S-IgA, *V. cholerae,* and the gut mucosa is integral in understanding both overall protection and clearance mechanisms of bacterial pathogens from the gut. In addition to mucin preventing access to the gut epithelium, S-IgA antibodies become enmeshed within the mucin layer thereby serving as an additional level of protection by binding and preventing bacterial movement and access. We sought to replicate this in an *in vitro* setting, similar to (Liu et al., 2008), utilizing porcine mucin and measure the percentage of *V. cholerae* that penetrate through the mucin column in the presence of fecal antibody from either NM or DM bearing animals. As shown in Figure 4.2, the pooled antibody from DM exhibited a greater % of bacteria transiting through the column than the NM group. Immune exclusion via agglutination, entrapment, or clearance of microbial pathogens or toxins is one of the primary functions of S-IgA (Mantis et al., 2011). Additionally, a study by Macpherson et al. details how S-IgA limits uptake of microbial metabolites to the rest of the body (Uchimura et al., 2018). While our simple *in vitro* mucin penetration approach may give some insight into antibody, mucin, bacterial pathogen interactions, an *in vivo* approach in which co-localization of mucin, *V. cholerae*, and S-IgA is examined would be

more informative. As S-IgA binding to *V. cholerae* in the lumen is considered to be the primary protective mechanism, it is essential to further define the role of gut commensal bacteria and metabolites in driving differential humoral antibody responses.

Materials and Methods

Treg Depletion Experiment

In line with previous CD25 depletion experiments (Christensen et al., 2015; Clemente-Casares et al., 2016), animals were administered 300 μg of rat anti-mouse CD25 monoclonal antibody (PC61.5.3, BioXcell) via intraperitoneal injections at -14 and -7 days prior to infection. Treg cell populations were examined via splenic cells in non-depleted animals and after 2 i.p. injections in CD25 depleted animals. The following antibodies were used for analysis via flow cytometry: rat anti-mouse CD3-FITC (17A2, BD Pharmingen), anti-mouse CD4-APC (RM4-5, Invitrogen), anti-mouse CD25-PE-Cy7 (3C7, Biolegend), anti-mouse Foxp3-PE (FJK-16s, Invitrogen) (Table 4.3).

RNA extraction and cDNA synthesis

At animal sacrifice, tissue samples such as Peyer's patches were immediately snap-frozen on dry ice to preserve RNA integrity and subsequently stored at -80C until sample processing. At time of total RNA extraction, Trizol was added to the tissue and immediately homogenized with a homogenizer. Total RNA

extraction was done according to the manufacturer's guidelines. To verify RNA integrity, RNA was run using 1% bleach gel electrophoresis at 100 mV for 35 minutes and imaged using Gel red on a Biorad image analyzer (Aranda et al., 2012). DNAase treatment of the total RNA was done using Baseline-ZERO (Lucigen). 500 ng of total RNA was used for cDNA synthesis using SuperScript IV (Invitrogen) with oligo$(dT)_{12\text{-}18}$ primers.

Table 4.3 Fluorescence-Labeled Antibodies Used for Flow Cytometry

Host	Target	Clone	Fluorophore	Manufafcturer	Cat. No.
Rat anti-mouse	CD3	17A2	FITC	BD Pharmingen	561798
Anti-Mo	CD4	RM4-5	APC	Invitrogen	17-0042-B2
Anti-Mouse	CD8a	53-6.7	PE	eBioscience	12-0081-82
Anti-Mouse	CD25	3C7	PE-Cy7	Biolegend	101916
Anti-Human	CD3	SK7	PE-Cy7	Biolegend	344815
Rat anti-mouse	CD16/32	2.4G2		BD Pharmingen	553142
Rat anti-mouse	CD3	17A2	APC-Cy7	BD Pharmingen	560590
Anti-Mouse	T-bet	4B10	FITC	Biolegend	644811
Anti-mouse	GATA3	16E10A23	PerCP/Cy5.5	Biolegend	653811
Anti-mouse	Foxp3	FJK-16s	PE	Invitrogen	12-5773-80
Anti-Mouse	ROR gamma (t)	B2D	PE-Cy7	Invitrogen	25-6981-80
Anti-Mouse	PD-1	J43	PE-Cy7	Invitrogen	25-9985-80
Anti-Mouse	CXCR5	2G8	FITC	BD Biosciences	BDB56057
Rat anti-mouse	IgA	11-44-2	APC	Southern Biotech	1165-11
Anti-Mouse	IL-4	11B11	PerCP/Cy5.5	Biolegend	504123
Anti-Mouse	IL-17A	TC11-18H10.1	PE-Cy7	Biolegend	506921
Anti-Mouse	IL-10	JES5-16E3	PE	Biolegend	505007
Anti-Mouse	IFN-g	XMG1.2	FITC	Biolegend	505805
Anti-Mouse	Bcl-6	K112-91	PE	BD Biosciences	561522
Anti-Mouse	B220	RA3-6B2	PE-Cy7	Invitrogen	25-0452-82
Anti-Mouse	CD3	17A2	PerCP/Cy5.5	Biolegend	100217
Anti-Mouse	CD45.1	A20	APC-e700	Invitrogen	47-0453-82
Anti-Mouse	CD19	1D3	PE	BD Pharmingen	557399
Anti-Mouse	CD45R/B220	RA3-6B2	FITC	Biolegend	103205

Real-time quantitative PCR

The real-time quantitative PCR (qPCR) assays were performed using the Biorad CFX96 instrument. Amplifications were carried out in 20 μl reactions containing 10 μl iQ SYBR Green Supermix (BioRad), 2 μl first-stranded cDNA (diluted 1:10), 1 μl of each specific primer (10 μM), and 6 ul of nuclease-free water. Cycle conditions were 95°C for 3 min, followed by 39 cycles (95°C for 10 sec, 55°C for 30 sec, 95°C for 10 sec, 65°C for 5 sec, 95°C for 5 sec). Primer sequences for cytokine expression are available in table 4.4. The relative transcript levels of the target genes were compared to Vaxchora alone controls with either GAPDH or β-actin used as the reference genes according to the Livak method (2^{-DDCT}) method (Livak and Schmittgen, 2001). Student's *t*-test was used to compare the treatment groups with significance reported for $P < 0.05$.

Table 4.4. Primer sequences for qPCR.

Primer	Sequence	F or R	Organism
IL-1β (Mouse)	GAA GGG AGG TTT GCT GAC TAT C	F	Mouse
IL-1β (Mouse)	CAG GTC CAC ACC ATC ATC TAA A	R	Mouse
IL-2 (Mouse)	GTG CTG CCT TCT CTT GAT TAG A	F	Mouse
IL-2 (Mouse)	TTC TGT GGC CTA GAG GAG TAA TA	R	Mouse
IL-4 (Mouse)	CTC TCC AGA TAC CCA GGT GAT A	F	Mouse
IL-4 (Mouse)	CTC TTG AGC TTT GTC CCT AGT C	R	Mouse
IL-5 (Mouse)	GCT TCC TGT CCC TAC TCA TAA A	R	Mouse
IL-5 (Mouse)	GAC ACA CAC AAC ACC AGA AAG	R	Mouse
IL-6 (Mouse)	CCT AGG TCT GGG TGT GTA GAA	F	Mouse
IL-6 (Mouse)	GGA AGG GTC TGT GCC ATT ATC	R	Mouse
IL-10 (Mouse	GGA GCA GGT GAA GAG TGA TTT	F	Mouse
IL-10 (Mouse)	CAC ACT CCA GGT GCA GAA TAG	R	Mouse
IL-13 (Mouse)	GCA CTG GAT GCT GTC TTA TCT	F	Mouse
IL-13 (Mouse)	GGC CTC AGG TGA GAT TCT TAA A	R	Mouse
IL-17A (Mouse)	GTC CCT TTG TTG TCT CCT GT	F	Mouse
IL-17A (Mouse)	GAC CAT AGT GAG GTG AGG ATT G	R	Mouse
IL-21 (Mouse)	CTA AGG AGA GGA GTG GCT TCT A	F	Mouse
IL-21 (Mouse)	GTC TGT GTT TCC TGG CTA CTT	R	Mouse
IL-22 (Mouse)	GAA CAG AGC GAG GGA AGA TAA G	F	Mouse
IL-22 (Mouse)	CCT TCC CAG ACT TCC TTT GTT A	R	Mouse
IFN-γ (Mouse)	AAG GGT AGA AGA CGA GGA TGA	F	Mouse
IFN-γ (Mouse)	CTG CAT AGC ACT AGA GGC TAA C	R	Mouse
TGF-β1(Mouse)	CTA CCT TGT TGC CTC CTC TTT	F	Mouse
TGF-β1(Mouse)	GAG CAG AGG TTC AGT GAT GTA G	R	Mouse
TNF-α (Mouse)	CTA CCT TGT TGC CTC CTC TTT	F	Mouse
TNF-α (Mouse)	GAG CAG AGG TTC AGT GAT GTA G	R	Mouse
β-actin (Mouse)	GAG GTA TCC TGA CCC TGA AGT A	F	Mouse
β-actin (Mouse)	CAC ACG CAG CTC ATT GTA GA	R	Mouse
GAPDH(Mouse)	GAA GCA GCA TTC AGG TCT CT	F	Mouse
GAPDH(Mouse)	CAA GGA TAG GAC TCA GGG AAT A	R	Mouse
rtxA	CGG AAT GAG TGA GAA AGA CC	F	*V. cholerae*
rtxA	TTT ATC GAC AGC CTA GAG AGT A	R	*V. cholerae*
hlyA	GTC AAT ATC CGC CCA ATC TAT AA	F	*V. cholerae*
hlyA	CT TAC GGT CGA TTG GGA TC	R	*V. cholerae*
HapA	TAA ACC GTT ACT GCC GTA TTC	F	*V. cholerae*
HapA	GAT AAA GTA GAA CGG ACG CG	R	*V. cholerae*
tcpA	GAA GAA GTT TGT AAA AGA AGA ACA CG	F	*V. cholerae*
tcpA	CGC TGA GAC CAC ACC CAT A	R	*V. cholerae*
ctxA	CAC TAA GTG GGC ACT TCT CA	F	*V. cholerae*
ctxA	TGA TCA TGC AAG AAG AAC TCA	R	*V. cholerae*
RecA	ATT GAA GGC GAA ATG GGC GAT AG	F	*V. cholerae*
RecA	TAC ACA TAC AGT TGG ATT GCT TGA GG	R	*V. cholerae*

Mucin-Penetration Assay

Mucin columns were prepared by adding varying concentrations of bovine submaxillary mucin (Sigma) at the top of 1 mL syringes. 50 μl of fecal water containing antibodies from mice that were bearing either the NM or DM communities at time of infection were combined with 50 μl of *in vitro* grown *V. cholerae* C6706 and placed on top of the mucin columns. The columns were incubated for 30 minutes at 37°C. 100 μl fractions were then collected from the bottom of the mucin columns, serially diluted, and plated onto LB-Streptomycin (200 μg/ml) agar to count *V. cholerae* CFU.

T_{FH} Cell Staining

Whole blood and spleen were collected at sacrifice 4 weeks post infection with *V. cholerae* and the bacterial communities. Tissues were processed into single-cell suspensions by mashing the tissues through a 0.44 micron strainer into 50 mL Falcon Tubes. The strainers were washed with 5 mLs PBS to collect extra cells. Red blood cell lysis was conducted on the spleen by adding 3-5 mLs of ACK lysis buffer per sample and incubated on ice for 5 minutes. To stop the reaction, 40 mLs of PBS was added to the mixture and then centrifuged at 400 x g for 5 minutes at 4°C. Splenic cells were then resuspended in 5 mLs of RPMI media. Cell counts were conducted on a Biorad TC20 automated cell counter and approximately 2.5 x 10^6 cells were used for staining the samples and ~1.0 x 10^6 cells were used per unstained and single-stained control. The following

antibodies were used to stain for T_{FH} cells: anti-mouse CD4-APC (Invitrogen/RM4-5), anti-mouse CXCR5-FITC, (BD Biosciences/Clone: 2G8), anti-mouse Bcl-6-PE (Biolegend/Clone: K112-91).

Transcription factor staining via flow cytometry

Single cells suspensions were made of immune tissues as previously described on page: CD16/32 was added as an Fc block to all the samples and incubated for 15 minutes on ice. Afterward, the extracellular surface markers were stained as follows: Rat anti-mouse CD3-FITC and anti-mouse CD4-APC. Intracellular staining was performed using the eBioscience Foxp3/Transcription Factor Staining Buffer set as recommended by the protocol. The following intracellular cytokine antibodies were used: anti-mouse T-bet-FITC (Biolegend/Clone 4B10), anti-mouse GATA3-PerCP/Cy5.5 (Biolegend/Clone 16E10A23), anti-mouse Foxp3-PE (Invitrogen/FJK-16s), anti-mouse RORγ (t)-PE-Cy7 (Invitrogen/B2D).

Chapter 5: Conclusion

Summary

Regardless of etiology, whether from severe malnutrition or infectious diarrhea, the gut microbiome enters a state of dysbiosis, whereby overall community structure is disrupted. This state is more frequently achieved in cholera endemic areas. In contrast, in the absence of diarrhea, the gut microbiome provides a more robust barrier to pathogens either via increased nutrient acquisition or in regulating immune responses; therefore, studying gut microbial composition is essential to understand resultant host immune responses.

Results in chapter 2 demonstrates the ability of a simple dysbiotic model microbiome in suppressing *V. cholerae* specific antibody mediated protection in an antibiotic treated mouse model. First, we demonstrate that fecal transplants of relatively healthy US donors drive divergent immune responses to *V. cholerae* in a germ-free mouse model. From these observations and previously published data in our lab (Alavi et al., 2020), we established model microbial communities representing either a normal microbiota (NM) or a dysbiotic model microbiota (DM) as well as utilized a more immunologically mature, antibiotic treated adult mouse model. Our findings suggest that live members of the DM community suppress serum vibriocidal responses to infection or vaccination in comparison to the NM community. Additionally, enriched fecal Ig from the DM community is less protective in limiting *V. cholerae* colonization in a passive immune protection model. Moreover, our current data suggests that interestingly CD4+ T cells are

involved with the suppressive phenotype as *in vivo* depletion restores correlates of protection. Here, we provide evidence for the suppressive effects the gut microbiome has on the development of a robust humoral immune response to bacterial infection or vaccination.

In chapter 3, we discuss plans and preliminary results for conducting cholera vaccine studies in the United States and in Bangladesh. Unfortunately, our plans to conduct a cholera vaccine study utilizing a live-attenuated vaccine were halted due to several factors. However, the gut microbiome-cholera vaccine study has received final regulatory approval and is set to commence in January 2022. This study will consist of a children's cohort (aged 3-5 years of age) receiving either Euvichol, a killed oral cholera vaccine equivalent to Shanchol, or saline control. Additionally, there will be an adult cohort (aged 18-40 years of age who will either receive Euvichol or saline control. Additionally, we are prioritizing the recruitment of mother-child pairs to examine the gut microbiome from a cross-generational and household approach. From a different collaborator, we received limited quantities of pre-vaccine stool samples from subjects who later exhibited poor immunogenicity responses to a killed oral cholera vaccine. In a pilot experiment, we conducted stool transplants of 2 Bangladeshi donors into germ-free mice. The results for this experiment are still being analyzed, however, it serves as an important proof-of-concept experiment that will lead to the creation of pure bacterial isolates that have physiological potential and are

derived from endemic cholera regions. Therefore, this approach may be pivotal for the development of next generation probiotic therapies.

Finally, data discussed in chapter 4 details preliminary data to tease apart the causal immunomodulatory pathways involved between the gut microbiota, *V. cholerae*, and host cells. As mentioned previously in chapter 2, reduction of CD4+ cells *in vivo* surprisingly restored serum vibriocidal responses in the DM group as compared to NM. Since increased frequency of T_{Reg} cells have been implicated in reduced antibody-specific responses (Lundgren et al., 2005; Moore et al., 2018), we aimed to reduce T_{Reg} cells *in vivo* via targeted depletion of CD25+ cells. However, depletion was potentially not complete and correlates of protection were not fully restored. Additionally, we examined splenic and Peyer's patches and observed an increased expression of T-bet transcription factor, which was unexpected as that is mostly associated with a Th1 response. We also examined transcriptional profiles of various cytokines that are often associated with driving the effector functions of different T cell subsets. We observed no difference in expression of *IL-10* and *TGF-β* between animals bearing NM and DM communities after vaccination with a live-attenuated cholera vaccine. Functional protection of luminal antibody was demonstrated via a passive protection assay as described in chapter 2. We expanded upon these observations by carrying out an *in vitro* mucin penetration assay, however, further modifications and replication is needed. Lastly, we examined the regulation of several *V. cholerae* virulence associated genes. Our results show

that virulence factors *ctxA* and *HapA* are decreased in DM and NM + DM in comparison to the NM group, suggesting that presence of cholera toxin may stimulate innate immune responses leading to more effective adaptive immune responses as observed in NM treated group. Overall, these experiments lay the foundation for further studies for the intersection of the microbiota, *V. cholerae*, and host responses.

Future Studies

The *in vivo* and *in vitro* studies discussed provide promising insights into the role of the gut microbiome in altering antibody-mediated protection against both *V. cholerae* infection and vaccination, yet more studies are needed to strengthen the translational applications of this research.

The vibriospecific endpoint titers carried out in these experiments are beneficial because they are against whole-cell *V. cholerae* and therefore inclusive of all epitopes that antibodies may be bound to. However, it would be discerning to understand which epitope targets of *V. cholerae* are most impactful for protection. While it would be insightful to measure antibodies against cholera toxin, previous studies highlight the importance of S-IgA antibodies binding to O-specific polysaccharide (OSP) segment of LPS to aid in the clearance of *V. cholerae* (Charles et al., 2020). Additionally, as antibodies not only bind to pathogenic bacteria but all bacteria, measuring binding characteristics of antibodies against the bacterial members of the NM and DM communities would

potentially offer insight into the level of impact specific individual bacterial species may have. This could be done experimentally by conducting whole-cell ELISA's against individual NM and DM species. Once particular species of interest are identified from either the NM or DM community, mono-colonization in a germ-free mouse model would provide further evidence of either immunostimulatory or immunosuppressive potential of individual bacterial strains. However, it may be that the phenotype may only occur in the presence of other bacterial interactions as well. Moving beyond bacterial species of interest, identifying the microbial metabolites involved that mediates innate and adaptive immune responses would increase overall understanding. To achieve this, cell-free supernatant from a bacterial species of interest can be applied to a monoculture of dendritic cells and incubated for 2 hours or RPMI alone as a control. Following incubation, supernatant derived from the cell free supernatant of primed or unprimed dendritic cells can be applied to Th cell cultures with the addition of T-cell activator CD3/CD28 Dynabeads (Gibco) to activate the T cells and incubated for 72 hours at 37 C with 5% CO_2. The supernatant from the activated T cells can then be collected for downstream applications.

Similar to these studies, examining co-localization of IgA, mucin, and bacterial species of interest in a mouse model would be an additional measure to visualize host-pathogen responses. Indeed, in a study by Rogier et al., colons were preserved with Carnoy's fixative to preserve the mucus layer and sections were stained by immunofluorescence to visualize Muc2 and IgA (Rogier et al.,

2014). Further, a study by Mantis et al. utilized scanning confocal microscopy to examine *Lactobacillus*-IgA interactions within a Peyer's patch in a mouse-ligated ileal loop model (Mantis et al., 2011).

Examining overall antibody changes as described thus far allows for analyses of broad changes, but examining antigen-specific primary cells lends greater resolution for functional consequences. Several techniques exist for studying antigen-specific B cell responses that have various advantages and disadvantages. For example, in the B cell enzyme linked immunospot (ELISPOT), antibody secreting cells (ASCs) are added to plates coated with an antigen of interest. Antigen specific antibodies will then bind within close proximity to the B cells secreting the antibodies. Enzyme labeled secondary antibodies can then be added to visualize spots of antigen-specific secretion. The limitation for this technique is that the antigen of interest must be known ahead of time and the B cells identified are not available for downstream analyses. An extension of the ELISPOT is to utilize flow cytometry. If the antigen can be conjugated to a fluorochrome, the primary B cells can be further characterized by cell surface markers and intracellular staining. These selected antigen bound cells can then be cell sorted and used for downstream analyses such as BCR sequencing and cloning, *in vitro* proliferation, and transcriptional profiling (Boonyaratanakornkit and Taylor, 2019). Ultimately, an adoptive transfer of B cells of interest into mice with allelic variation in CD45 or mice devoid of B cells would be a fairly definitive mechanism to observe the established phenotypes.

Similar to examining antigen specific B cells, the ELISPOT assay can be used to detect antigen specific T cells as well as intracellular cytokine staining to examine cytokines trapped within Golgi/ER. However, both of these methods have limitations as one must decide cytokines of potential interest beforehand. For ELISPOT, only a few parameters can be measured at a time and the source of the secreted cytokines is not definitive; for intracellular cytokine staining, the user is limited by the number of fluorochromes and assay optimization.

Conclusions and Implications

The data presented in this dissertation expands upon the ever-growing body of work implicating the gut microbiome in human health and disease. These studies show that a gut microbiome in dysbiosis suppresses antibody-mediated protection against cholera, whether it be via wild-type infection or via vaccination with a live-attenuated oral cholera vaccine. Experiments conducted in both animal models and human vaccine studies implicate disparate gut microbiomes with diverging outcomes to vaccination (Levine, 2010). Our strategy to introduce human associated-microbiota into either germ-free or antibiotic treated mouse models allows for a fairly tractable and efficient model for human disease. It is important to note that while murine models are mostly beneficial, there are various limitations.

Data discussed here suggest that in our model the DM community has a suppressive effect on vaccine responses as compared to vaccine alone or

vaccine in the presence of NM, as opposed to the NM group having a “boosting” effect. However, this does not preclude the possibility that the addition of a normal microbiota may invade a dysbiotic microbiota and rescue or boost vaccine responses. Indeed, in a study by Di Luccia, et al, mice that were initially colonized by bacteria that had a decreased response to CT showed improved CT-IgA responses after colonization with a 5-member responder community (Di Luccia et al., 2020). Identifying the compounds of microbial metabolism that are responsible for the immunomodulatory effects would be pivotal for establishing a therapeutic for children who are most at-risk for cholera related severities.

Gut microbes serve an integral role in regulating pathogenesis of infectious diseases. Currently, the most promising gut microbial therapeutic is for treatment of recurrent *Clostridioides difficile* infection. The therapeutic RBX2660 by Ferring Pharmaceuticals offers great promise. Results from a multi-center study suggest that after administration of the therapeutic, RBX2660 was capable of shifting the taxonomic structures of recipients’ microbiomes to a healthier state, resembling that of the therapeutic (Kwak et al., 2020). The success of this story however is tempered by results of a different microbiome-based therapeutic SER-287 by Seres Therapeutics. In a phase II trial of patients with mild to moderate ulcerative colitis, remission rates were around 10-11% for patients who received two doses of SER-287 vs 11% for those who received placebo (Mullard, 2021). This was an unfortunate setback for the microbiome therapeutics

community and underscores that microbiome therapeutics are still at preliminary stages for improvement of human health.

Overall, the gut microbiome serves as an additional organ of the body and has crucial functions of mediating colonization resistance of pathogens, aiding in metabolism, modulating nervous and immune system function, as well as synthesizing vitamins and amino acids. This work discusses a subset of those functions and provides additional understanding for how gut bacteria modulate immune responses to pathogens. Utilizing the gut microbiome for treatment of various ailments of the body has great promise and will likely help resolve or treat numerous conditions in the coming decades.

References

(WHO), W.H.O. (2001). Cholera Vaccines, Weekly. Epidemiol Rec.

Alavi, S., and Hsiao, A. (2020). Protocol for Microbiome Transplantation in Suckling Mice during Vibrio cholerae Infection to Study Commensal-Pathogen Interactions. STAR Protoc *1*, 100200.

Alavi, S., Mitchell, J.D., Cho, J.Y., Liu, R., Macbeth, J.C., and Hsiao, A. (2020). Interpersonal Gut Microbiome Variation Drives Susceptibility and Resistance to Cholera Infection. Cell *181*, 1533-1546 e1513.

Ali, M., Nelson, A.R., Lopez, A.L., and Sack, D.A. (2015). Updated global burden of cholera in endemic countries. PLoS Negl Trop Dis *9*, e0003832.

Ali, M., Qadri, F., Kim, D.R., Islam, M.T., Im, J., Ahmmed, F., Khan, A.I., Zaman, K., Marks, F., Kim, J.H.*, et al.* (2021). Effectiveness of a killed whole-cell oral cholera vaccine in Bangladesh: further follow-up of a cluster-randomised trial. Lancet Infect Dis.

Apter, F.M., Michetti, P., Winner, L.S., 3rd, Mack, J.A., Mekalanos, J.J., and Neutra, M.R. (1993). Analysis of the roles of antilipopolysaccharide and anti-cholera toxin immunoglobulin A (IgA) antibodies in protection against Vibrio cholerae and cholera toxin by use of monoclonal IgA antibodies in vivo. Infect Immun *61*, 5279-5285.

Aranda, P.S., LaJoie, D.M., and Jorcyk, C.L. (2012). Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis *33*, 366-369.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.M.*, et al.* (2011). Enterotypes of the human gut microbiome. Nature *473*, 174-180.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y.*, et al.* (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. Science *331*, 337-341.

Attridge, S.R., and Rowley, D. (1983). The specificity of Vibrio cholerae adherence and the significance of the slime agglutinin as a second mediator of in vitro attachment. J Infect Dis *147*, 873-881.

Baine, W.B., Mazzotti, M., Greco, D., Izzo, E., Zampieri, A., Angioni, G., Di Gioia, M., Gangarosa, E.J., and Pocchiari, F. (1974). Epidemiology of cholera in Italy in 1973. Lancet *2*, 1370-1374.

Baranova, D.E., Chen, L., Destrempes, M., Meade, H., and Mantis, N.J. (2020). Passive Immunity to Vibrio cholerae O1 Afforded by a Human Monoclonal IgA1 Antibody Expressed in Milk. Pathog Immun *5*, 89-116.

Benenson, A.S., Joseph, P.R., and Oseasohn, R.O. (1968a). Cholera vaccine field trials in east Pakistan. 1. Reaction and antigenicity studies. Bull World Health Organ *38*, 347-357.

Benenson, A.S., Mosley, W.H., Fahimuddin, M., and Oseasohn, R.O. (1968b). Cholera vaccine field trials in east Pakistan. 2. Effectiveness in the field. Bull World Health Organ *38*, 359-372.

Benenson, A.S., Saad, A., and Mosley, W.H. (1968c). Serological studies in cholera. 2. The vibriocidal antibody response of cholera patients determined by a microtechnique. Bull World Health Organ *38*, 277-285.

Bhattacharjee, A., Burr, A.H.P., Overacre-Delgoffe, A.E., Tometich, J.T., Yang, D., Huckestein, B.R., Linehan, J.L., Spencer, S.P., Hall, J.A., Harrison, O.J.*, et al.* (2020). Enteropathy-induced regulatory T cells inhibit intestinal CD4+ T cell responses against oral vaccines. bioRxiv, 2020.2006.2003.130831.

Bhattacharya, S.K., Sur, D., Ali, M., Kanungo, S., You, Y.A., Manna, B., Sah, B., Niyogi, S.K., Park, J.K., Sarkar, B.*, et al.* (2013). 5 year efficacy of a bivalent killed whole-cell oral cholera vaccine in Kolkata, India: a cluster-randomised, double-blind, placebo-controlled trial. Lancet Infect Dis *13*, 1050-1056.

Bhuiyan, T.R., Lundin, S.B., Khan, A.I., Lundgren, A., Harris, J.B., Calderwood, S.B., and Qadri, F. (2009). Cholera caused by Vibrio cholerae O1 induces T-cell responses in the circulation. Infect Immun *77*, 1888-1893.

Bishop, A.L., and Camilli, A. (2011). Vibrio cholerae: lessons for mucosal vaccine design. Expert review of vaccines *10*, 79-94.

Black, R.E., Levine, M.M., Clements, M.L., Young, C.R., Svennerholm, A.M., and Holmgren, J. (1987). Protective efficacy in humans of killed whole-vibrio oral cholera vaccine with and without the B subunit of cholera toxin. Infect Immun *55*, 1116-1120.

Blow, N.S., Salomon, R.N., Garrity, K., Reveillaud, I., Kopin, A., Jackson, F.R., and Watnick, P.I. (2005). Vibrio cholerae infection of Drosophila melanogaster mimics the human disease cholera. PLoS pathogens *1*, e8.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F.*, et al.* (2019). Author Correction: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol *37*, 1091.

Boonyaratanakornkit, J., and Taylor, J.J. (2019). Techniques to Study Antigen-Specific B Cell Responses. Front Immunol *10*, 1694.

Bourque, D.L., Bhuiyan, T.R., Genereux, D.P., Rashu, R., Ellis, C.N., Chowdhury, F., Khan, A.I., Alam, N.H., Paul, A., Hossain, L.*, et al.* (2018). Analysis of the Human Mucosal Response to Cholera Reveals Sustained Activation of Innate Immune Signaling Pathways. Infect Immun *86*.

Butterton, J.R., Ryan, E.T., Shahin, R.A., and Calderwood, S.B. (1996). Development of a germfree mouse model of Vibrio cholerae infection. Infection and immunity *64*, 4373-4377.

Calain, P., Chaine, J.-P.P., Johnson, E., Hawley, M.-L.L., O'Leary, M.J., Oshitani, H., and Chaignat, C.-L.L. (2004). Can oral cholera vaccination play a role in controlling a cholera outbreak? Vaccine *22*, 2444-2451.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I.*, et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. Nature methods *7*, 335-336.

Cerutti, A., and Rescigno, M. (2008). The biology of intestinal immunoglobulin A responses. Immunity *28*, 740-750.

Chac, D., Bhuiyan, T.R., Saha, A., Alam, M.M., Salma, U., Jahan, N., Chowdhury, F., Khan, A.I., Ryan, E.T., LaRocque, R.*, et al.* (2021). Gut Microbiota and Development of Vibrio cholerae-Specific Long-Term Memory B Cells in Adults after Whole-Cell Killed Oral Cholera Vaccine. Infect Immun *89*, e0021721.

Chang, P.V., Hao, L., Offermanns, S., and Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A *111*, 2247-2252.

Charles, R.C., Kelly, M., Tam, J.M., Akter, A., Hossain, M., Islam, K., Biswas, R., Kamruzzaman, M., Chowdhury, F., Khan, A.I.*, et al.* (2020). Humans Surviving Cholera Develop Antibodies against Vibrio cholerae O-Specific Polysaccharide That Inhibit Pathogen Motility. mBio *11*.

Chemudupati, M., Kenney, A.D., Smith, A.C., Fillinger, R.J., Zhang, L., Zani, A., Liu, S.L., Anderson, M.Z., Sharma, A., and Yount, J.S. (2020). Butyrate Reprograms Expression of Specific Interferon-Stimulated Genes. Journal of virology *94*.

Chen, W.H., Cohen, M.B., Kirkpatrick, B.D., Brady, R.C., Galloway, D., Gurwith, M., Hall, R.H., Kessler, R.A., Lock, M., Haney, D.*, et al.* (2016). Single-dose Live Oral Cholera Vaccine CVD 103-HgR Protects Against Human Experimental Infection With Vibrio cholerae O1 El Tor. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *62*, 1329-1335.

Childers, B.M., and Klose, K.E. (2007). Regulation of virulence in Vibrio cholerae: the ToxR regulon. Future Microbiol *2*, 335-344.

Cho, J.Y., Liu, R., Macbeth, J.C., and Hsiao, A. (2021). The Interface of Vibrio cholerae and the Gut Microbiome. Gut Microbes *13*, 1937015.

Christensen, A.D., Skov, S., Kvist, P.H., and Haase, C. (2015). Depletion of regulatory T cells in a hapten-induced inflammation model results in prolonged and increased inflammation driven by T cells. Clin Exp Immunol *179*, 485-499.

Cinar, H.N., Kothary, M., Datta, A.R., Tall, B.D., Sprando, R., Bilecen, K., Yildiz, F., and McCardell, B. (2010). Vibrio cholerae hemolysin is required for lethality, developmental delay, and intestinal vacuolation in Caenorhabditis elegans. PLoS One *5*, e11558.

Clemens, J.D., Sack, D.A., Harris, J.R., Van Loon, F., Chakraborty, J., Ahmed, F., Rao, M.R., Khan, M.R., Yunus, M., Huda, N.*, et al.* (1990). Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet *335*, 270-273.

Clemens, J.D., van Loon, F., Sack, D.A., Chakraborty, J., Rao, M.R., Ahmed, F., Harris, J.R., Khan, M.R., Yunus, M., Huda, S.*, et al.* (1991). Field trial of oral cholera vaccines in Bangladesh: serum vibriocidal and antitoxic antibodies as markers of the risk of cholera. J Infect Dis *163*, 1235-1242.

Clemente-Casares, X., Blanco, J., Ambalavanan, P., Yamanouchi, J., Singha, S., Fandos, C., Tsai, S., Wang, J., Garabatos, N., Izquierdo, C.*, et al.* (2016). Expanding antigen-specific regulatory networks to treat autoimmunity. Nature *530*, 434-440.

Cohen, M.B., Giannella, R.A., Bean, J., Taylor, D.N., Parker, S., Hoeper, A., Wowk, S., Hawkins, J., Kochi, S.K., Schiff, G.*, et al.* (2002). Randomized, controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. Infect Immun *70*, 1965-1970.

Colwell, R.R. (1996). Global climate and infectious disease: the cholera paradigm. Science *274*, 2025-2031.

Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A., and Levine, M.M. (1996). Viable but non-culturable Vibrio cholerae O1 revert to a cultivable state in the human intestine. World J Microbiol Biotechnol *12*, 28-31.

Colwell, R.R., Kaper, J., and Joseph, S.W. (1977). Vibrio cholerae, Vibrio parahaemolyticus, and other vibrios: occurrence and distribution in Chesapeake Bay. Science *198*, 394-396.

Corkum, C.P., Ings, D.P., Burgess, C., Karwowska, S., Kroll, W., and Michalak, T.I. (2015). Immune cell subsets and their gene expression profiles from human PBMC isolated by Vacutainer Cell Preparation Tube (CPT™) and standard density gradient. BMC Immunology *16*, 48.

Costea, P.I., Hildebrand, F., Arumugam, M., Backhed, F., Blaser, M.J., Bushman, F.D., de Vos, W.M., Ehrlich, S.D., Fraser, C.M., Hattori, M.*, et al.* (2018). Publisher Correction: Enterotypes in the landscape of gut microbial community composition. Nat Microbiol *3*, 388.

Cresci, G.A.M., Lampe, J.W., and Gibson, G. (2020). Targeted Approaches for In Situ Gut Microbiome Manipulation. JPEN J Parenter Enteral Nutr *44*, 581-588.

Cryz, S.J., Jr., Levine, M.M., Kaper, J.B., Furer, E., and Althaus, B. (1990). Randomized double-blind placebo controlled trial to evaluate the safety and immunogenicity of the live oral cholera vaccine strain CVD 103-HgR in Swiss adults. Vaccine *8*, 577-580.

Cvjetanovic, B., and Barua, D. (1972). The seventh pandemic of cholera. Nature *239*, 137-138.

David, L.A., Weil, A., Ryan, E.T., Calderwood, S.B., Harris, J.B., Chowdhury, F., Begum, Y., Qadri, F., LaRocque, R.C., and Turnbaugh, P.J. (2015). Gut microbial succession follows acute secretory diarrhea in humans. mBio *6*, 15.

Davoodi, S., and Foley, E. (2019). Host-Microbe-Pathogen Interactions: A Review of Vibrio cholerae Pathogenesis in Drosophila. Front Immunol *10*, 3128.

De, S.N. (1959). Enterotoxicity of bacteria-free culture-filtrate of Vibrio cholerae. Nature *183*, 1533-1534.

De, S.N., and Chatterje, D.N. (1953). An experimental study of the mechanism of action of Vibriod cholerae on the intestinal mucous membrane. J Pathol Bacteriol *66*, 559-562.

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A., Backhed, F., and Mithieux, G. (2014). Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell *156*, 84-96.

Di Luccia, B., Ahern, P.P., Griffin, N.W., Cheng, J., Guruge, J.L., Byrne, A.E., Rodionov, D.A., Leyn, S.A., Osterman, A.L., Ahmed, T.*, et al.* (2020). Combined Prebiotic and Microbial Intervention Improves Oral Cholera Vaccination Responses in a Mouse Model of Childhood Undernutrition. Cell Host Microbe *27*, 899-908 e895.

DiRita, V.J., Parsot, C., Jander, G., and Mekalanos, J.J. (1991). Regulatory cascade controls virulence in Vibrio cholerae. Proc Natl Acad Sci U S A *88*, 5403-5407.

Dongre, M., Singh, B., Aung, K.M., Larsson, P., Miftakhova, R., Persson, K., Askarian, F., Johannessen, M., von Hofsten, J., Persson, J.L.*, et al.* (2018). Flagella-mediated secretion of a novel Vibrio cholerae cytotoxin affecting both vertebrate and invertebrate hosts. Commun Biol *1*, 59.

Dutta, N.K., and Habbu, M.K. (1955). Experimental cholera in infant rabbits: a method for chemotherapeutic investigation. Br J Pharmacol Chemother *10*, 153-159.

Dutta, N.K., Panse, M.V., and Kulkarni, D.R. (1959). Role of cholera a toxin in experimental cholera. J Bacteriol *78*, 594-595.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A. (2005). Diversity of the human intestinal microbial flora. Science *308*, 1635-1638.

Eloe-Fadrosh, E.A., McArthur, M.A., Seekatz, A.M., Drabek, E.F., Rasko, D.A., Sztein, M.B., and Fraser, C.M. (2013). Impact of oral typhoid vaccination on the human gut microbiota and correlations with s. Typhi-specific immunological responses. PLoS One *8*, e62026.

Fast, D., Petkau, K., Ferguson, M., Shin, M., Galenza, A., Kostiuk, B., Pukatzki, S., and Foley, E. (2020). Vibrio cholerae-Symbiont Interactions Inhibit Intestinal Repair in Drosophila. Cell Rep *30*, 1088-1100 e1085.

Field, M. (1979). Mechanisms of action of cholera and Escherichia coli enterotoxins. Am J Clin Nutr *32*, 189-196.

Field, M. (2003). Intestinal ion transport and the pathophysiology of diarrhea. J Clin Invest *111*, 931-943.

Fierer, N., Nemergut, D., Knight, R., and Craine, J.M. (2010). Changes through time: integrating microorganisms into the study of succession. Res Microbiol *161*, 635-642.

Finkelstein, R.A., and LoSpalluto, J.J. (1969). Pathogenesis of experimental cholera. Preparation and isolation of choleragen and choleragenoid. J Exp Med *130*, 185-202.

Fischer, M.B., Ma, M., Hsu, N.C., and Carroll, M.C. (1998). Local synthesis of C3 within the splenic lymphoid compartment can reconstitute the impaired immune response in C3-deficient mice. J Immunol *160*, 2619-2625.

Freter, R. (1956). Experimental enteric Shigella and Vibrio infections in mice and guinea pigs. J Exp Med *104*, 411-418.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T.*, et al.* (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature *504*, 446-450.

Gill, D.M., and King, C.A. (1975). The mechanism of action of cholera toxin in pigeon erythrocyte lysates. J Biol Chem *250*, 6424-6432.

Gill, D.M., and Rappaport, R.S. (1979). Origin of the enzymatically active A1 fragment of cholera toxin. J Infect Dis *139*, 674-680.

Gotuzzo, E., Butron, B., Seas, C., Penny, M., Ruiz, R., Losonsky, G., Lanata, C.F., Wasserman, S.S., Salazar, E., Kaper, J.B.*, et al.* (1993). Safety, immunogenicity, and excretion pattern of single-dose live oral cholera vaccine CVD 103-HgR in Peruvian adults of high and low socioeconomic levels. Infect Immun *61*, 3994-3997.

Grassly, N.C., Jafari, H., Bahl, S., Durrani, S., Wenger, J., Sutter, R.W., and Aylward, R.B. (2009). Mucosal Immunity after Vaccination with Monovalent and Trivalent Oral Poliovirus Vaccine in India. The Journal of Infectious Diseases *200*, 794-801.

Hagan, T., Cortese, M., Rouphael, N., Boudreau, C., Linde, C., Maddur, M.S., Das, J., Wang, H., Guthmiller, J., Zheng, N.Y.*, et al.* (2019). Antibiotics-Driven Gut Microbiome Perturbation Alters Immunity to Vaccines in Humans. Cell *178*, 1313-1328 e1313.

Hallander, H.O., Paniagua, M., Espinoza, F., Askelof, P., Corrales, E., Ringman, M., and Storsaeter, J. (2002). Calibrated serological techniques demonstrate significant different serum response rates to an oral killed cholera vaccine between Swedish and Nicaraguan children. Vaccine *21*, 138-145.

Haney, D.J., Lock, M.D., Simon, J.K., Harris, J., and Gurwith, M. (2017). Antibody-Based Correlates of Protection Against Cholera Analysis of a Challenge Study in a Cholera-Naive Population. Clin Vaccine Immunol.

Hapfelmeier, S., Lawson, M.A., Slack, E., Kirundi, J.K., Stoel, M., Heikenwalder, M., Cahenzli, J., Velykoredko, Y., Balmer, M.L., Endt, K.*, et al.* (2010). Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science *328*, 1705-1709.

Harris, J.B. (2018). Cholera: Immunity and Prospects in Vaccine Development. J Infect Dis *218*, S141-S146.

Harris, J.B., LaRocque, R.C., Qadri, F., Ryan, E.T., and Calderwood, S.B. (2012). Cholera. Lancet *379*, 2466-2476.

Harris, V.C., Armah, G., Fuentes, S., Korpela, K.E., Parashar, U., Victor, J.C., Tate, J., de Weerth, C., Giaquinto, C., Wiersinga, W.J.*, et al.* (2017). Significant Correlation Between the Infant Gut Microbiome and Rotavirus Vaccine Response in Rural Ghana. J Infect Dis *215*, 34-41.

Harris, V.C., Armah, G., Fuentes, S., Korpela, K.E., Parashar, U., Victor, J.C., Tate, J., de Weerth, C., Giaquinto, C., Wiersinga, W.J.*, et al.* (2016). Significant Correlation Between the Infant Gut Microbiome and Rotavirus Vaccine Response in Rural Ghana. The Journal of Infectious Diseases *215*, 34-41.

Hase, C.C., and Mekalanos, J.J. (1998). TcpP protein is a positive regulator of virulence gene expression in Vibrio cholerae. Proc Natl Acad Sci U S A *95*, 730-734.

Herrington, D.A., Hall, R.H., Losonsky, G., Mekalanos, J.J., Taylor, R.K., and Levine, M.M. (1988). Toxin, toxin-coregulated pili, and the toxR regulon are essential for Vibrio cholerae pathogenesis in humans. J Exp Med *168*, 1487-1492.

Higgins, D.E., Nazareno, E., and DiRita, V.J. (1992). The virulence gene activator ToxT from Vibrio cholerae is a member of the AraC family of transcriptional activators. J Bacteriol *174*, 6974-6980.

Holmgren, J. (2021). An Update on Cholera Immunity and Current and Future Cholera Vaccines. Trop Med Infect Dis *6*.

Hossain, M., Islam, K., Kelly, M., Mayo Smith, L.M., Charles, R.C., Weil, A.A., Bhuiyan, T.R., Kovac, P., Xu, P., Calderwood, S.B.*, et al.* (2019). Immune responses to O-specific polysaccharide (OSP) in North American adults infected with Vibrio cholerae O1 Inaba. PLoS Negl Trop Dis *13*, e0007874.

Hsiao, A., Ahmed, A.M., Subramanian, S., Griffin, N.W., Drewry, L.L., Petri, W.A., Haque, R., Ahmed, T., and Gordon, J.I. (2014). Members of the human gut microbiota involved in recovery from Vibrio cholerae infection. Nature *515*, 423-426.

Hsiao, A., Liu, Z., Joelsson, A., and Zhu, J. (2006). Vibrio cholerae virulence regulator-coordinated evasion of host immunity. Proc Natl Acad Sci U S A *103*, 14542-14547.

Hubbard, T.P., Billings, G., Dorr, T., Sit, B., Warr, A.R., Kuehl, C.J., Kim, M., Delgado, F., Mekalanos, J.J., Lewnard, J.A.*, et al.* (2018). A live vaccine rapidly protects against cholera in an infant rabbit model. Sci Transl Med *10*.

Huda, M.N., Lewis, Z., Kalanetra, K.M., Rashid, M., Ahmad, S.M., Raqib, R., Qadri, F., Underwood, M.A., Mills, D.A., and Stephensen, C.B. (2014). Stool microbiota and vaccine responses of infants. Pediatrics *134*, e362-372.

Human Microbiome Jumpstart Reference Strains, C., Nelson, K.E., Weinstock, G.M., Highlander, S.K., Worley, K.C., Creasy, H.H., Wortman, J.R., Rusch, D.B., Mitreva, M., Sodergren, E.*, et al.* (2010). A catalog of reference genomes from the human microbiome. Science *328*, 994-999.

Huq, A., Small, E.B., West, P.A., Huq, M.I., Rahman, R., and Colwell, R.R. (1983). Ecological relationships between Vibrio cholerae and planktonic crustacean copepods. Appl Environ Microbiol *45*, 275-283.

Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S., and Iwasaki, A. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A *108*, 5354-5359.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V.*, et al.* (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell *139*, 485-498.

Ivers, L.C., Charles, R.C., Hilaire, I.J., Mayo-Smith, L.M., Teng, J.E., Jerome, J.G., Rychert, J., LaRocque, R.C., Xu, P., Kovac, P.*, et al.* (2015). Immunogenicity of the Bivalent Oral Cholera Vaccine Shanchol in Haitian Adults With HIV Infection. J Infect Dis *212*, 779-783.

Jan Holmgren, A.L., Roland Mollby (1986). Development of Vaccines and Drugs against Diarrhea. Chartwell-Bratt.

Jobling, M.G., and Holmes, R.K. (1997). Characterization of hapR, a positive regulator of the Vibrio cholerae HA/protease gene hap, and its identification as a functional homologue of the Vibrio harveyi luxR gene. Mol Microbiol *26*, 1023-1034.
Joo, I. (1974). Cholera vaccines. In Burua D, Burrows W, eds Cholera, 333-335.

Kabir, S. (2014). Critical analysis of compositions and protective efficacies of oral killed cholera vaccines. Clinical and vaccine immunology : CVI *21*, 1195-1205.

Kamada, N., Seo, S.-U.U., Chen, G.Y., and Núñez, G. (2013). Role of the gut microbiota in immunity and inflammatory disease. Nature reviews Immunology *13*, 321-335.

Kanungo, S., Desai, S.N., Saha, J., Nandy, R.K., Sinha, A., Kim, D.R., Bannerjee, B., Manna, B., Yang, J.S., Ali, M.*, et al.* (2015). An Open Label Non-inferiority Trial Assessing Vibriocidal Response of a Killed Bivalent Oral Cholera Vaccine Regimen following a Five Year Interval in Kolkata, India. PLoS Negl Trop Dis *9*, e0003809.

Katz, D.E., Coster, T.S., Wolf, M.K., Trespalacios, F.C., Cohen, D., Robins, G., Hartman, A.B., Venkatesan, M.M., Taylor, D.N., and Hale, T.L. (2004). Two studies evaluating the safety and immunogenicity of a live, attenuated Shigella flexneri 2a vaccine (SC602) and excretion of vaccine organisms in North American volunteers. Infect Immun *72*, 923-930.

Kaur, J., McGhee, and Burrows, W. (1972). Immunity to cholera: the occurrence and nature of antibody-active immunoglobulins in the lower ileum of the rabbit. J Immunol *108*, 387-395.

Khader, S.A., Gaffen, S.L., and Kolls, J.K. (2009). Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. Mucosal Immunol *2*, 403-411.

Kieser, S., Sarker, S.A., Sakwinska, O., Foata, F., Sultana, S., Khan, Z., Islam, S., Porta, N., Combremont, S., Betrisey, B.*, et al.* (2018). Bangladeshi children with acute diarrhoea show faecal microbiomes with increased Streptococcus abundance, irrespective of diarrhoea aetiology. Environ Microbiol *20*, 2256-2269.

Kim, M., Qie, Y., Park, J., and Kim, C.H. (2016). Gut Microbial Metabolites Fuel Host Antibody Responses. Cell Host Microbe *20*, 202-214.

King, C.A., and Van Heyningen, W.E. (1973). Deactivation of cholera toxin by a sialidase-resistant monosialosylganglioside. J Infect Dis *127*, 639-647.

Kirn, T.J., Jude, B.A., and Taylor, R.K. (2005). A colonization factor links Vibrio cholerae environmental survival and human infection. Nature *438*, 863-866.

Kirn, T.J., Lafferty, M.J., Sandoe, C.M., and Taylor, R.K. (2000). Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by Vibrio cholerae. Mol Microbiol *35*, 896-910.

Klose, K.E. (2000). The suckling mouse model of cholera. Trends Microbiol *8*, 189-191.
Koch, R. (1884). An Address on Cholera and its Bacillus. Br Med J *2*, 453-459.

Krebs, S.J., and Taylor, R.K. (2011). Protection and attachment of Vibrio cholerae mediated by the toxin-coregulated pilus in the infant mouse model. J Bacteriol *193*, 5260-5270.

Krukonis, E.S., Yu, R.R., and Dirita, V.J. (2000). The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Mol Microbiol *38*, 67-84.

Kuchta, A., Rahman, T., Sennott, E.L., Bhuyian, T.R., Uddin, T., Rashu, R., Chowdhury, F., Kahn, A.I., Arifuzzaman, M., Weil, A.A.*, et al.* (2011). Vibrio cholerae O1 infection induces proinflammatory CD4+ T-cell responses in blood and intestinal mucosa of infected humans. Clin Vaccine Immunol *18*, 1371-1377.

Kundu, P., Blacher, E., Elinav, E., and Pettersson, S. (2017). Our Gut Microbiome: The Evolving Inner Self. Cell *171*, 1481-1493.

Kwak, S., Choi, J., Hink, T., Reske, K.A., Blount, K., Jones, C., Bost, M.H., Sun, X., Burnham, C.D., Dubberke, E.R.*, et al.* (2020). Impact of investigational microbiota therapeutic RBX2660 on the gut microbiome and resistome revealed by a placebo-controlled clinical trial. Microbiome *8*, 125.

Lantagne, D., Balakrish Nair, G., Lanata, C.F., and Cravioto, A. (2014). The cholera outbreak in Haiti: where and how did it begin? Curr Top Microbiol Immunol *379*, 145-164.

Levine, M.M. (2010). Immunogenicity and efficacy of oral vaccines in developing countries: lessons from a live cholera vaccine. BMC biology *8*, 129.

Levine, M.M., Black, R.E., Clements, M.L., Lanata, C., Sears, S., Honda, T., Young, C.R., and Finkelstein, R.A. (1984). Evaluation in humans of attenuated Vibrio cholerae El Tor Ogawa strain Texas Star-SR as a live oral vaccine. Infect Immun *43*, 515-522.

Levine, M.M., Kaper, J.B., Herrington, D., Ketley, J., Losonsky, G., Tacket, C.O., Tall, B., and Cryz, S. (1988). Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet *2*, 467-470.

Levine, M.M., Nalin, D.R., Craig, J.P., Hoover, D., Bergquist, E.J., Waterman, D., Holley, H.P., Hornick, R.B., Pierce, N.P., and Libonati, J.P. (1979). Immunity of cholera in man: relative role of antibacterial versus antitoxic immunity. Transactions of the Royal Society of Tropical Medicine and Hygiene *73*, 3-9.

Levine, M.M., Young, C.R., Black, R.E., Takeda, Y., and Finkelstein, R.A. (1985). Enzyme-linked immunosorbent assay to measure antibodies to purified heat-labile enterotoxins from human and porcine strains of Escherichia coli and to cholera toxin: application in serodiagnosis and seroepidemiology. J Clin Microbiol *21*, 174-179.

Levinson, K.J., Baranova, D.E., and Mantis, N.J. (2016). A monoclonal antibody that targets the conserved core/lipid A region of lipopolysaccharide affects motility and reduces intestinal colonization of both classical and El Tor Vibrio cholerae biotypes. Vaccine *34*, 5833-5836.

Levinson, K.J., De Jesus, M., and Mantis, N.J. (2015). Rapid effects of a protective O-polysaccharide-specific monoclonal IgA on Vibrio cholerae agglutination, motility, and surface morphology. Infect Immun *83*, 1674-1683.

Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S., Arumugam, M., Kultima, J.R., Prifti, E., Nielsen, T.*, et al.* (2014). An integrated catalog of reference genes in the human gut microbiome. Nat Biotechnol *32*, 834-841.

Lindholm, L., Holmgren, J., Wikstrom, M., Karlsson, U., Andersson, K., and Lycke, N. (1983). Monoclonal antibodies to cholera toxin with special reference to cross-reactions with Escherichia coli heat-labile enterotoxin. Infect Immun *40*, 570-576.

List, C., Grutsch, A., Radler, C., Cakar, F., Zingl, F.G., Schild-Prufert, K., and Schild, S. (2018). Genes Activated by Vibrio cholerae upon Exposure to Caenorhabditis elegans Reveal the Mannose-Sensitive Hemagglutinin To Be Essential for Colonization. mSphere *3*.

Liu, Z., Miyashiro, T., Tsou, A., Hsiao, A., Goulian, M., and Zhu, J. (2008). Mucosal penetration primes Vibrio cholerae for host colonization by repressing quorum sensing. Proceedings of the National Academy of Sciences of the United States of America *105*, 9769-9774.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Lo, D., Tynan, W., Dickerson, J., Scharf, M., Cooper, J., Byrne, D., Brayden, D., Higgins, L., Evans, C., and O'Mahony, D.J. (2004). Cell culture modeling of specialized tissue: identification of genes expressed specifically by follicle-associated epithelium of Peyer's patch by expression profiling of Caco-2/Raji co-cultures. Int Immunol *16*, 91-99.

Locey, K.J., and Lennon, J.T. (2016). Scaling laws predict global microbial diversity. Proc Natl Acad Sci U S A *113*, 5970-5975.

Lopez, A.L., Gonzales, M.L., Aldaba, J.G., and Nair, G.B. (2014). Killed oral cholera vaccines: history, development and implementation challenges. Ther Adv Vaccines *2*, 123-136.

Lopman, B.A., Pitzer, V.E., Sarkar, R., Gladstone, B., Patel, M., Glasser, J., Gambhir, M., Atchison, C., Grenfell, B.T., Edmunds, W.J.*, et al.* (2012). Understanding Reduced Rotavirus Vaccine Efficacy in Low Socio-Economic Settings. PLOS ONE *7*, e41720.

Lundgren, A., Trollmo, C., Edebo, A., Svennerholm, A.M., and Lundin, B.S. (2005).
Helicobacter pylori-specific CD4+ T cells home to and accumulate in the human Helicobacter pylori-infected gastric mucosa. Infect Immun *73*, 5612-5619.
Lycke, N.Y., and Bemark, M. (2017). The regulation of gut mucosal IgA B-cell responses: recent developments. Mucosal Immunol *10*, 1361-1374.

Macpherson, A.J., Geuking, M.B., and McCoy, K.D. (2012). Homeland security: IgA immunity at the frontiers of the body. Trends in immunology *33*, 160-167.

Madhi, S.A., Cunliffe, N.A., Steele, D., Witte, D., Kirsten, M., Louw, C., Ngwira, B., Victor, J.C., Gillard, P.H., Cheuvart, B.B.*, et al.* (2010). Effect of human rotavirus vaccine on severe diarrhea in African infants. N Engl J Med *362*, 289-298.

Majoul, I., Sohn, K., Wieland, F.T., Pepperkok, R., Pizza, M., Hillemann, J., and Soling, H.D. (1998). KDEL receptor (Erd2p)-mediated retrograde transport of the cholera toxin A subunit from the Golgi involves COPI, p23, and the COOH terminus of Erd2p. J Cell Biol *143*, 601-612.

Mantis, N.J., Rol, N., and Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal immunology *4*, 603-611.

Mathan, M.M., Chandy, G., and Mathan, V.I. (1995). Ultrastructural changes in the upper small intestinal mucosa in patients with cholera. Gastroenterology *109*, 422-430.

Mekalanos, J.J., Collier, R.J., and Romig, W.R. (1979). Enzymic activity of cholera toxin. I. New method of assay and the mechanism of ADP-ribosyl transfer. J Biol Chem *254*, 5849-5854.

Metchnikoff, E. (1894). The Comparative Pathology of Inflammation. Nature, 194-195.

Midani, F.S., Weil, A.A., Chowdhury, F., Begum, Y.A., Khan, A.I., Debela, M.D., Durand, H.K., Reese, A.T., Nimmagadda, S.N., Silverman, J.D.*, et al.* (2018). Human Gut Microbiota Predicts Susceptibility to Vibrio cholerae Infection. J Infect Dis *218*, 645-653.

Miller, V.L., and Mekalanos, J.J. (1984). Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. Proc Natl Acad Sci U S A *81*, 3471-3475.

Mitchell, K.C., Breen, P., Britton, S., Neely, M.N., and Withey, J.H. (2017). Quantifying Vibrio cholerae Enterotoxicity in a Zebrafish Infection Model. Appl Environ Microbiol *83*.

Moon, C., Baldridge, M.T., Wallace, M.A., D, C.A., Burnham, Virgin, H.W., and Stappenbeck, T.S. (2015). Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. Nature *521*, 90-93.

Moore, T.C., Messer, R.J., and Hasenkrug, K.J. (2018). Regulatory T cells suppress virus-specific antibody responses to Friend retrovirus infection. PLoS One *13*, e0195402.

Mullard, A. (2021). Failure of Seres's phase II ulcerative colitis programme renews microbiome concerns. Nat Rev Drug Discov *20*, 658.

Murley, Y.M., Carroll, P.A., Skorupski, K., Taylor, R.K., and Calderwood, S.B. (1999). Differential transcription of the tcpPH operon confers biotype-specific control of the Vibrio cholerae ToxR virulence regulon. Infect Immun *67*, 5117-5123.

Nandi, B., Nandy, R.K., Mukhopadhyay, S., Nair, G.B., Shimada, T., and Ghose, A.C. (2000). Rapid Method for Species-Specific Identification ofVibrio cholerae Using Primers Targeted to the Gene of Outer Membrane Protein OmpW. J Clin Microbiol *38*, 4145.

Nelson, E.T., Clements, J.D., and Finkelstein, R.A. (1976). Vibrio cholerae adherence and colonization in experimental cholera: electron microscopic studies. Infect Immun *14*, 527-547.

Nygren, E., Holmgren, J., and Attridge, S.R. (2008). Murine antibody responses following systemic or mucosal immunization with viable or inactivated Vibrio cholerae. Vaccine *26*, 6784-6790.

Nygren, E., Li, B.L.L., Holmgren, J., and Attridge, S.R. (2009). Establishment of an adult mouse model for direct evaluation of the efficacy of vaccines against Vibrio cholerae. Infection and immunity *77*, 3475-3484.

O'Neil, K.M., Ochs, H.D., Heller, S.R., Cork, L.C., Morris, J.M., and Winkelstein, J.A. (1988). Role of C3 in humoral immunity. Defective antibody production in C3-deficient dogs. J Immunol *140*, 1939-1945.

Oh, J.Z., Ravindran, R., Chassaing, B., Carvalho, F.A., Maddur, M.S., Bower, M., Hakimpour, P., Gill, K.P., Nakaya, H.I., Yarovinsky, F.*, et al.* (2014). TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. Immunity *41*, 478-492.

Olivier, V., Queen, J., and Satchell, K.J. (2009). Successful small intestine colonization of adult mice by Vibrio cholerae requires ketamine anesthesia and accessory toxins. PLoS One *4*, e7352.

Olivier, V., Salzman, N.H., and Satchell, K.J. (2007). Prolonged colonization of mice by Vibrio cholerae El Tor O1 depends on accessory toxins. Infect Immun *75*, 5043-5051.

Owen, R.L., Pierce, N.F., Apple, R.T., and Cray, W.C., Jr. (1986). M cell transport of Vibrio cholerae from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. J Infect Dis *153*, 1108-1118.

Pacini, F. (1865). Du Choléra Asiatique. Tr Janssens E Brussels: Librarie Médicale de H Manceaux.

Parada Venegas, D., De la Fuente, M.K., Landskron, G., Gonzalez, M.J., Quera, R., Dijkstra, G., Harmsen, H.J.M., Faber, K.N., and Hermoso, M.A. (2019). Corrigendum: Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. Front Immunol *10*, 1486.

Parker, E.P.K., Praharaj, I., Zekavati, A., Lazarus, R.P., Giri, S., Operario, D.J., Liu, J., Houpt, E., Iturriza-Gomara, M., Kampmann, B.*, et al.* (2018). Influence of the intestinal microbiota on the immunogenicity of oral rotavirus vaccine given to infants in south India. Vaccine *36*, 264-272.

Patel, S.M., Rahman, M.A., Mohasin, M., Riyadh, M.A., Leung, D.T., Alam, M.M., Chowdhury, F., Khan, A.I., Weil, A.A., Aktar, A.*, et al.* (2012). Memory B cell responses to Vibrio cholerae O1 lipopolysaccharide are associated with protection against infection from household contacts of patients with cholera in Bangladesh. Clin Vaccine Immunol *19*, 842-848.

Perez-Lopez, A., Behnsen, J., Nuccio, S.P., and Raffatellu, M. (2016). Mucosal immunity to pathogenic intestinal bacteria. Nat Rev Immunol *16*, 135-148.

Peterson, J.W. (1979). Protection against experimental cholera by oral or parenteral immunization. Infect Immun *26*, 594-598.

Peterson, K.M., and Mekalanos, J.J. (1988). Characterization of the Vibrio cholerae ToxR regulon: identification of novel genes involved in intestinal colonization. Infect Immun *56*, 2822-2829.

Petersson, J., Schreiber, O., Hansson, G.C., Gendler, S.J., Velcich, A., Lundberg, J.O., Roos, S., Holm, L., and Phillipson, M. (2011). Importance and regulation of the colonic mucus barrier in a mouse model of colitis. Am J Physiol Gastrointest Liver Physiol *300*, G327-333.

Pollitzer, R., and Burrows, W. (1955). Cholera studies. IV. Problems in immunology. Bull World Health Organ *12*, 945-1107.

Population, M.r.d.l.S.P.e.d.l. (2014). Rapport journalierMSPP du 07 Janvier 2014. Port-au-Prince: Ministe`re de la Sante´ Publique et de laPopulation.

Provenzano, D., Kovac, P., and Wade, W.F. (2006). The ABCs (Antibody, B cells, and Carbohydrate epitopes) of cholera immunity: considerations for an improved vaccine. Microbiol Immunol *50*, 899-927.

Pulimood, A.B., Ramakrishna, B.S., Rita, A.B., Srinivasan, P., Mohan, V., Gupta, S., Perakath, B., Kang, G., Chandy, G., and Balasubramanian, K.A. (2008). Early activation of mucosal dendritic cells and macrophages in acute Campylobacter colitis and cholera: An in vivo study. J Gastroenterol Hepatol *23*, 752-758.

Qadri, F., Ali, M., Lynch, J., Chowdhury, F., Khan, A.I., Wierzba, T.F., Excler, J.L., Saha, A., Islam, M.T., Begum, Y.A.*, et al.* (2018). Efficacy of a single-dose regimen of inactivated whole-cell oral cholera vaccine: results from 2 years of follow-up of a randomised trial. Lancet Infect Dis *18*, 666-674.

Qadri, F., Chowdhury, M.I., Faruque, S.M., Salam, M.A., Ahmed, T., Begum, Y.A., Saha, A., Al Tarique, A., Seidlein, L.V., Park, E.*, et al.* (2007). Peru-15, a live attenuated oral cholera vaccine, is safe and immunogenic in Bangladeshi toddlers and infants. Vaccine *25*, 231-238.

Qadri, F., Chowdhury, M.I., Faruque, S.M., Salam, M.A., Ahmed, T., Begum, Y.A., Saha, A., Alam, M.S., Zaman, K., Seidlein, L.V.*, et al.* (2005). Randomized, controlled study of the safety and immunogenicity of Peru-15, a live attenuated oral vaccine candidate for cholera, in adult volunteers in Bangladesh. The Journal of infectious diseases *192*, 573-579.

Qadri, F., Mohi, G., Hossain, J., Azim, T., Khan, A.M., Salam, M.A., Sack, R.B., Albert, M.J., and Svennerholm, A.M. (1995). Comparison of the vibriocidal antibody response in cholera due to Vibrio cholerae O139 Bengal with the response in cholera due to Vibrio cholerae O1. Clin Diagn Lab Immunol *2*, 685-688.

Qadri, F., Wierzba, T.F., Ali, M., Chowdhury, F., Khan, A.I., Saha, A., Khan, I.A., Asaduzzaman, M., Akter, A., Khan, A.*, et al.* (2016). Efficacy of a Single-Dose, Inactivated Oral Cholera Vaccine in Bangladesh. The New England journal of medicine *374*, 1723-1732.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T.*, et al.* (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature *464*, 59-65.

Queen, J., and Satchell, K.J. (2012). Neutrophils are essential for containment of Vibrio cholerae to the intestine during the proinflammatory phase of infection. Infect Immun *80*, 2905-2913.

Rahman, K.M., Arifeen, S.E., Zaman, K., Rahman, M., Raqib, R., Yunus, M., Begum, N., Islam, M.S., Sohel, B.M., Rahman, M.*, et al.* (2011). Safety, dose, immunogenicity, and transmissibility of an oral live attenuated Shigella flexneri 2a vaccine candidate (SC602) among healthy adults and school children in Matlab, Bangladesh. Vaccine *29*, 1347-1354.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell *118*, 229-241.

Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S.K., Nair, G.B., Shimada, T., Takeda, T., Karasawa, T., Kurazano, H., Pal, A.*, et al.* (1993). Emergence of novel strain of Vibrio cholerae with epidemic potential in southern and eastern India. Lancet *341*, 703-704.

Reikvam, D.H., Erofeev, A., Sandvik, A., Grcic, V., Jahnsen, F.L., Gaustad, P., McCoy, K.D., Macpherson, A.J., Meza-Zepeda, L.A., and Johansen, F.E. (2011). Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PLoS One *6*, e17996.

Rhee, K.J., Sethupathi, P., Driks, A., Lanning, D.K., and Knight, K.L. (2004). Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J Immunol *172*, 1118-1124.

Richie, E.E., Punjabi, N.H., Sidharta, Y.Y., Peetosutan, K.K., Sukandar, M.M., Wasserman, S.S., Lesmana, M.M., Wangsasaputra, F.F., Pandam, S.S., Levine, M.M.*, et al.* (2000). Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. Vaccine *18*, 2399-2410.

Ritalahti, K.M., Amos, B.K., Sung, Y., Wu, Q., Koenigsberg, S.S., and Loffler, F.E. (2006). Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple Dehalococcoides strains. Appl Environ Microbiol *72*, 2765-2774.

Ritchie, J.M., Rui, H., Bronson, R.T., and Waldor, M.K. (2010). Back to the future: studying cholera pathogenesis using infant rabbits. MBio *1*.

Robert Koch, K.C.C. (1987). Essays of Robert Koch. Greenwood Press.
Rogier, E.W., Frantz, A.L., Bruno, M.E., and Kaetzel, C.S. (2014). Secretory IgA is Concentrated in the Outer Layer of Colonic Mucus along with Gut Bacteria. Pathogens *3*, 390-403.

Round, J.L., and Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol *9*, 313-323.

Round, J.L., and Mazmanian, S.K. (2010). Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A *107*, 12204-12209.

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., and Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr *57*, 1-24.

Runft, D.L., Mitchell, K.C., Abuaita, B.H., Allen, J.P., Bajer, S., Ginsburg, K., Neely, M.N., and Withey, J.H. (2014). Zebrafish as a natural host model for Vibrio cholerae colonization and transmission. Appl Environ Microbiol *80*, 1710-1717.

Sack, D.A. (2008). Achieving the millennium development goals for health and nutrition in Bangladesh: key issues and interventions--an introduction. J Health Popul Nutr *26*, 253-260.

Saha, A., Chowdhury, M.I., Khanam, F., Bhuiyan, M.S., Chowdhury, F., Khan, A.I., Khan, I.A., Clemens, J., Ali, M., Cravioto, A.*, et al.* (2011). Safety and immunogenicity study of a killed bivalent (O1 and O139) whole-cell oral cholera vaccine Shanchol, in Bangladeshi adults and children as young as 1 year of age. Vaccine *29*, 8285-8292.

Sanchez, J., and Holmgren, J. (1989). Recombinant system for overexpression of cholera toxin B subunit in Vibrio cholerae as a basis for vaccine development. Proc Natl Acad Sci U S A *86*, 481-485.

Schulthess, J., Pandey, S., Capitani, M., Rue-Albrecht, K.C., Arnold, I., Franchini, F., Chomka, A., Ilott, N.E., Johnston, D.G.W., Pires, E.*, et al.* (2019). The Short Chain Fatty Acid Butyrate Imprints an Antimicrobial Program in Macrophages. Immunity *50*, 432-445 e437.
Seedorf, H., Griffin, N.W., Ridaura, V.K., Reyes, A., Cheng, J., Rey, F.E., Smith, M.I., Simon, G.M., Scheffrahn, R.H., Woebken, D.*, et al.* (2014). Bacteria from diverse habitats colonize and compete in the mouse gut. Cell *159*, 253-266.

Sender, R., Fuchs, S., and Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol *14*, e1002533.

Sharma, D.P., Thomas, C., Hall, R.H., Levine, M.M., and Attridge, S.R. (1989). Significance of toxin-coregulated pili as protective antigens of Vibrio cholerae in the infant mouse model. Vaccine *7*, 451-456.

Simanjuntak, C.H., O'Hanley, P., Punjabi, N.H., Noriega, F., Pazzaglia, G., Dykstra, P., Kay, B., Suharyono, Budiarso, A., Rifai, A.R.*, et al.* (1993). Safety, immunogenicity, and transmissibility of single-dose live oral cholera vaccine strain CVD 103-HgR in 24- to 59-month-old Indonesian children. J Infect Dis *168*, 1169-1176.

Sirskyj, D., Kumar, A., and Azizi, A. (2016). Mechanisms Underlying the Immune Response Generated by an Oral Vibrio cholerae Vaccine. Int J Mol Sci *17*.

Skorupski, K., and Taylor, R.K. (1999). A new level in the Vibrio cholerae ToxR virulence cascade: AphA is required for transcriptional activation of the tcpPH operon. Mol Microbiol *31*, 763-771.

Smith, M.I., Yatsunenko, T., Manary, M.J., Trehan, I., Mkakosya, R., Cheng, J., Kau, A.L., Rich, S.S., Concannon, P., Mychaleckyj, J.C.*, et al.* (2013). Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. Science *339*, 548-554.

Snow, J. (1856). On the Mode of Communication of Cholera. Edinb Med J *1*, 668-670.

Soliman, M.L., Puig, K.L., Combs, C.K., and Rosenberger, T.A. (2012). Acetate reduces microglia inflammatory signaling in vitro. J Neurochem *123*, 555-567.

Son, M.S., and Taylor, R.K. (2011). Vibriocidal assays to determine the antibody titer of patient sera samples. Curr Protoc Microbiol *Chapter 6*, Unit6A 3.

Sow, S.O., Tapia, M.D., Chen, W.H., Haidara, F.C., Kotloff, K.L., Pasetti, M.F., Blackwelder, W.C., Traore, A., Tamboura, B., Doumbia, M.*, et al.* (2017). Randomized, Placebo-Controlled, Double-Blind Phase 2 Trial Comparing the Reactogenicity and Immunogenicity of a Single Standard Dose to Those of a High Dose of CVD 103-HgR Live Attenuated Oral Cholera Vaccine, with Shanchol Inactivated Oral Vaccine as an Open-Label Immunologic Comparator. Clin Vaccine Immunol *24*.

Spira, W.M., Sack, R.B., and Froehlich, J.L. (1981). Simple adult rabbit model for Vibrio cholerae and enterotoxigenic Escherichia coli diarrhea. Infect Immun *32*, 739-747.

Steele, E.J., Chaicumpa, W., and Rowley, D. (1974). Isolation and biological properties of three classes of rabbit antibody to Vibrio cholerae. J Infect Dis *130*, 93-103.

Strugnell, R.A., and Wijburg, O.L. (2010). The role of secretory antibodies in infection immunity. Nat Rev Microbiol *8*, 656-667.

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M.A., Benezra, A., DeStefano, J., Meier, M.F., Muegge, B.D.*, et al.* (2014). Persistent gut microbiota immaturity in malnourished Bangladeshi children. Nature *510*, 417-421.

Suharyono, Simanjuntak, C., Witham, N., Punjabi, N., Heppner, D.G., Losonsky, G., Totosudirjo, H., Rifai, A.R., Clemens, J., Lim, Y.L.*, et al.* (1992). Safety and immunogenicity of single-dose live oral cholera vaccine CVD 103-HgR in 5-9-year-old Indonesian children. Lancet *340*, 689-694.

Sur, D., Kanungo, S., Sah, B., Manna, B., Ali, M., Paisley, A.M., Niyogi, S.K., Park, J.K., Sarkar, B., Puri, M.K.*, et al.* (2011). Efficacy of a low-cost, inactivated whole-cell oral cholera vaccine: results from 3 years of follow-up of a randomized, controlled trial. PLoS Negl Trop Dis *5*, e1289.

Svennerholm, A.M. (1975). Experimental studies on cholera immunization. 4. The antibody response to formalinized Vibrio cholerae and purified endotoxin with special reference to protective capacity. Int Arch Allergy Appl Immunol *49*, 434-452.

Swerdlow, D.L., Mintz, E.D., Rodriguez, M., Tejada, E., Ocampo, C., Espejo, L., Greene, K.D., Saldana, W., Seminario, L., Tauxe, R.V.*, et al.* (1992). Waterborne transmission of epidemic cholera in Trujillo, Peru: lessons for a continent at risk. Lancet *340*, 28-33.

Tamburini, S., Shen, N., Wu, H.C., and Clemente, J.C. (2016). The microbiome in early life: implications for health outcomes. Nature medicine *22*, 713-722.

Taylor, R.K., Miller, V.L., Furlong, D.B., and Mekalanos, J.J. (1987). Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci U S A *84*, 2833-2837.

Thiem, V.D., Deen, J.L., von Seidlein, L., Canh, D.G., Anh, D.D., Park, J.K., Ali, M., Danovaro-Holliday, M.C., Son, N.D., Hoa, N.T.*, et al.* (2006). Long-term effectiveness against cholera of oral killed whole-cell vaccine produced in Vietnam. Vaccine *24*, 4297-4303.

Trach, D.D., Cam, P.D., Ke, N.T., Rao, M.R., Dinh, D., Hang, P.V., Hung, N.V., Canh, D.G., Thiem, V.D., Naficy, A.*, et al.* (2002). Investigations into the safety and immunogenicity of a killed oral cholera vaccine developed in Viet Nam. Bull World Health Organ *80*, 2-8.

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., and Gordon, J.I. (2007). The human microbiome project. Nature *449*, 804-810.

Uchimura, Y., Fuhrer, T., Li, H., Lawson, M.A., Zimmermann, M., Yilmaz, B., Zindel, J., Ronchi, F., Sorribas, M., Hapfelmeier, S.*, et al.* (2018). Antibodies Set Boundaries Limiting Microbial Metabolite Penetration and the Resultant Mammalian Host Response. Immunity *49*, 545-559 e545.

Vaitkevicius, K., Lindmark, B., Ou, G., Song, T., Toma, C., Iwanaga, M., Zhu, J., Andersson, A., Hammarstrom, M.L., Tuck, S.*, et al.* (2006). A Vibrio cholerae protease needed for killing of Caenorhabditis elegans has a role in protection from natural predator grazing. Proc Natl Acad Sci U S A *103*, 9280-9285.

Waldor, M.K., and Mekalanos, J.J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. Science *272*, 1910-1914.

Wang, H., Naseer, N., Chen, Y., Zhu, A.Y., Kuai, X., Galagedera, N., Liu, Z., and Zhu, J. (2017a). OxyR2 Modulates OxyR1 Activity and Vibrio cholerae Oxidative Stress Response. Infect Immun *85*.

Wang, Z., Lazinski, D.W., and Camilli, A. (2017b). Immunity Provided by an Outer Membrane Vesicle Cholera Vaccine Is Due to O-Antigen-Specific Antibodies Inhibiting Bacterial Motility. Infect Immun *85*.

Weil, A.A., Arifuzzaman, M., Bhuiyan, T.R., LaRocque, R.C., Harris, A.M., Kendall, E.A., Hossain, A., Tarique, A.A., Sheikh, A., Chowdhury, F*., et al.* (2009). Memory T-cell responses to Vibrio cholerae O1 infection. Infect Immun *77*, 5090-5096.

Weil, A.A., Becker, R.L., and Harris, J.B. (2019). Vibrio cholerae at the Intersection of Immunity and the Microbiome. mSphere *4*.

Woida, P.J., and Satchell, K.J.F. (2020). The Vibrio cholerae MARTX toxin silences the inflammatory response to cytoskeletal damage before inducing actin cytoskeleton collapse. Sci Signal *13*.
World Health Organization (WHO), E.M.R.O. (2020). Cholera Situation in Yemen.

Yang, W., Yu, T., Huang, X., Bilotta, A.J., Xu, L., Lu, Y., Sun, J., Pan, F., Zhou, J., Zhang, W*., et al.* (2020). Intestinal microbiota-derived short-chain fatty acids regulation of immune cell IL-22 production and gut immunity. Nat Commun *11*, 4457.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R.N., Anokhin, A.P*., et al.* (2012). Human gut microbiome viewed across age and geography. Nature *486*, 222-227.

Yoshiya, K., Lapchak, P.H., Thai, T.H., Kannan, L., Rani, P., Dalle Lucca, J.J., and Tsokos, G.C. (2011). Depletion of gut commensal bacteria attenuates intestinal ischemia/reperfusion injury. Am J Physiol Gastrointest Liver Physiol *301*, G1020-1030.

Zheng, W. (2017). Intestinal Complement Modulates Colitis by Targeting the Gut Microbiota. Harvard University.

Zhu, J., Miller, M.B., Vance, R.E., Dziejman, M., Bassler, B.L., and Mekalanos, J.J. (2002). Quorum-sensing regulators control virulence gene expression in Vibrio cholerae. Proceedings of the National Academy of Sciences *99*, 3129-3134.