Studies of pathogenesis, innate immunity and therapeutics of human enteric viruses in gnotobiotic pigs

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ACADEMIC ABSTRACT

Norovirus and rotavirus are the most common viral causes of acute gastroenteritis among all age groups and in children under 5 years of age, respectively. Understanding the pathogenesis of the virus and correlates of protective immunity is fundamental to developing effective prevention and treatment strategies. Gnotobiotic (Gn) pigs are an attractive animal model for studying enteric viruses due to their similarities to humans, particularly in regards to the immune system and gastrointestinal anatomy and physiology. Here, to establish a reliable Gn pig model of human norovirus (HuNoV) infection and disease, we determined the median infectious dose (ID_{50}) of a GII.4 2006b variant in pigs. We also evaluated the effects of age and administration of the cholesterol-lowering drug simvastatin on susceptibility to NoV infection. In neonatal pigs (4-5 days of age, the ID_{50} was determined to be $\leq 2.74 \times 10^3$ viral RNA copies. The ID_{50} was increased in 33-34 day old pigs (6.43×10^4), but decreased to $<2.74 \times 10^3$ following simvastatin treatment in the same age group. Overall, the development of diarrhea, fecal virus shedding and small intestinal cytopathological changes confirmed the usefulness of the Gn pig as an appropriate animal model for studying HuNoVs. We also utilized the well-established Gn pig model of human rotavirus (HRV) infection and disease to evaluate adjunctive treatment options for HRV-induced diarrhea. We demonstrated that the anti-secretory drug racecadotril was capable of diminishing clinical signs of HRV infection and shortening duration of illness. Reduced dehydration in the racecadotril-treated pigs was evident by the significant gain in body

weight compared to controls during the course of the study. We also determined that a high dose of the probiotic *Lactobacillus acidophilus* NCFM (LA) was able to reduce RV diarrhea severity and duration compared to a low dose. The difference in therapeutic potential was attributed to divergent effects in innate immunity pre- and post-challenge. High dose of LA (HiLA) induced an anti-inflammatory dendritic cell (DC) profile, characterized primarily by upregulation of TLR2 expression and production of cytokine IL-10. Conversely, low dose of LA (LoLA) upregulated TLR3 and TLR9 and increased secretion of cytokine IL-6. Additionally, HiLA induced both IFN-α and TNF-α responses in DCs, but LoLA was only able to increase the frequency of TNF-α-producing DCs. These results provide further support of Gn pigs as a highly applicable animal model for studying pathogenesis, innate immunity and therapeutics of human enteric viruses.

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GENERAL AUDIENCE ABSTRACT

Norovirus and rotavirus are the most common viral causes of acute gastroenteritis among all age groups and in children under 5 years of age, respectively. Understanding the pathogenesis of the virus and correlates of protective immunity is fundamental to developing effective prevention and treatment strategies. Gnotobiotic (Gn) pigs are an attractive animal model for studying enteric viruses due to their similarities to humans, particularly in regards to the immune system and gastrointestinal anatomy and physiology. Here, we established a reliable Gn pig model of human norovirus (HuNoV) infection and disease. Overall, the development of diarrhea, fecal virus shedding and small intestinal cytopathological changes confirmed the usefulness of the Gn pig as an appropriate animal model for studying HuNoVs. We also utilized the well-established Gn pig model of human rotavirus (HRV) infection and disease to evaluate adjunctive treatment options for HRV-induced diarrhea. We demonstrated that the anti-secretory drug racecadotril was capable of diminishing clinical signs of HRV infection and shortening duration of illness. We also determined that a high dose of the probiotic *Lactobacillus acidophilus* NCFM (LA) was able to reduce RV diarrhea severity and duration compared to a low dose. These results provide further support of Gn pigs as a highly applicable animal model for studying pathogenesis, innate immunity and therapeutics of human enteric viruses.

Dedicated to Ernest and to all those who loved him, because that pretty much covers everyone I care about as well.

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ATTRIBUTIONS

Several colleagues contributed to the projects, research, writing, and editing of each chapter of this dissertation.

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Chapter 1

Acute viral gastroenteritis due to norovirus and rotavirus infections: Studies of pathogenesis, innate immunity, and therapeutics in a gnotobiotic pig model

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1.1 Introduction: Acute viral gastroenteritis

Acute gastroenteritis (AGE) presents as a sudden onset of diarrhea with or without vomiting, and can include symptoms of nausea and abdominal pain as well (1). While typically self-limiting in duration, cases of AGE still have a significant impact on health care and economic costs. Furthermore, diarrhea is currently the fourth leading cause of mortality among all ages globally, causing approximately 1.3 million deaths in 2015 (2, 3). Young children are especially affected by infectious causes of AGE; diarrhea is the second most common cause of death due to communicable disease worldwide in individuals under 5 years of age, with an estimated 578,000 deaths occurring in 2013 (4, 5). Among infectious causes of diarrhea, norovirus (NoV) and rotavirus (RV) are the predominant viral agents. NoVs are implicated in the majority of AGE cases in all age groups worldwide, while RVs are specifically the most common cause in children under 5 years of age (6, 7).

1.2 Norovirus (NoV)

NoVs belong to the *Caliciviridae* family and are small, non-enveloped, positive-stranded RNA viruses (8-12). The prototype of the virus, Norwalk virus (GI.1), was discovered following an outbreak of gastroenteritis in Norwalk, Ohio in 1968 (13, 14). Currently, six genogroups and >40 genotypes have been identified, with GII.4 strains being responsible for the majority of human NoV (HuNoV) infections (9). More recent outbreaks have also included GII.17 strains (15-18). Aside from GII, other genogroups that are known to infect humans include GI and GIV (12). Other animal NoVs include those that infect mice (GV), pigs (GII), cattle (GIII) and dogs (GIV, GVI) (19, 20).

1.2.1 Clinical signs of NoV infection

Transmission of the NoV primarily occurs through a fecal-oral route from contaminated food, water and persons (21, 22). However, NoV in vomitus may be a contributing factor in spreading of the virus as well, either by aerosolization or contamination of fomites (23, 24). Additionally, detection of NoV in nasopharyngeal swabs raises the question of whether respiratory transmission is possible (25). Overall, the infectious dose has been reported to be fairly low at 10-100 viral particles (26).

After a short incubation period of 12-48 hours (8, 9, 11, 21, 27), symptoms manifest as vomiting and non-bloody diarrhea with some individuals also experiencing, nausea, abdominal pain, mild fever and myalgia (8-10, 12, 21, 27, 28). Most cases of norovirus in healthy, immunocompetent individuals are self-limiting and symptoms cease after a typical period of 2-3 days, but can last for up to 6 days (8-11). Virus shedding in feces can start before clinical presentation (as early as 18 hours after infection) and tends to persist for much longer, though peak shedding has been reported at four days post-infection (28). In general, virus shedding can continue for three to eight weeks (8, 11, 27, 28). Asymptomatic shedding is also possible and has been reported in approximately 1/3 of cases (8, 29). In both symptomatic and asymptomatic infections, high shedding titers between 10^5-10^{11} viral copies per gram of feces can be expected (29). In a recent study, NoV titers in vomitus were reported as 8.0×10^5 and 3.9×10^4 viral copies per milliliter for GI and GII viruses, respectively (24). The combination of shedding in high titers, of long duration and in asymptomatic individuals, along with low infectious dose facilitates ease of NoV transmission.

While NoVs are capable of infecting all age groups, more severe presentations and even mortalities can occur in the young (<5 years of age), elderly (>65 years of age) or immunocompromised (9, 30, 31). As with most causes of diarrhea, severe dehydration is a common complicating factor. In the US, children under 5 years of age have higher rates of clinical visits pertaining to NoV-related illnesses (32). Longer duration of symptoms (7 versus 3.5 days) and greater severity of diarrhea has been reported in children <2 years compared to children between 2 and 5 years (33). Of significance, NoV infection has also been associated with necrotizing enterocolitis in neonates (34, 35), benign infantile seizures (36-38), and encephalopathy (39, 40). Increased duration of diarrhea is also noted in the elderly, and symptoms of anorexia, headache and abdominal pain may be more common (10, 30, 41). Furthermore, higher rates of mortality occur in the elderly population, especially in developed countries. In the period between 1999 to 2007 in the US, 90% of the approximately 800 deaths annually due to NoVs occurred in individuals 65 years or older (10, 42).

In several studies of immunocompromised patients, NoVs have been implicated as a common enteropathogen, resulting in chronic diarrhea and fecal virus shedding. NoV infections have been diagnosed in patients with inherited immune disorders (43), HIV (44), cancer (45, 46) and stem cell or organ transplants (47-54). Viral screening of stool from 62 children with combined or humoral immune deficiencies detected NoV in 11 out of 24 positive samples (43). Similarly, in solid organ transplant patients, 35% (67 out of 192) were positive for NoV in one study (50). Another study evaluating both solid organ and hematopoietic stem cell transplant recipients determined that 22% (25 out of 116) of patients were infected with NoV (54). These patients often experience clinical symptoms lasting weeks to months, with recurrence possible

(44, 54). In addition to severe dehydration, complications arising from chronic diarrhea can include disruption of the intestinal barrier, malnutrition, acute kidney injury and transplant/graft failure (48, 55). An increased occurrence of pneumatosis intestinalis, or gas within the intestinal wall, has been reported as well. Four out of five immunocompromised individuals with pneumatosis intestinalis were NoV-positive in one retrospective study (56). Virus shedding is prolonged compared to healthy, immunocompetent patients and has continued for over a year in certain cases (44, 57). Such long periods of shedding can possibly act as a reservoir for infection and allow for the emergence of new variants (55, 58).

NoV infections have also been rarely associated with various other unique presentations. Aside from necrotizing enterocolitis and neurological manifestations in young children, these include hemolytic-uremic syndrome (59), ischemic colitis (60), flare-ups of inflammatory bowel disease (61), post-infectious irritable bowel syndrome (62) and spontaneous bowel perforation (63). Viremia is not a common occurrence with NoV infections and may explain the predominance of intestinal disease. Viremia has not been reported in healthy adults; only in children (64), immunocompromised individuals (65) and gnotobiotic pigs (66, 67) .

1.2.2 Pathogenesis of NoV infection

Historically, the lack of an efficient cell culture system and small animal model for human NoVs (HuNoVs) has limited the understanding of pathogenesis. The most well-studied aspect is likely the binding of NoVs to histo-blood group antigens (HBGAs). HBGAs are complex carbohydrates that can be found on erythrocytes and various epithelial tissues, as well as

secreted freely in biological fluids of secretors (see below) (68). The specific phenotypic expression of HBGAs in an individual is dependent on different functioning glycosyltransferases. In humans, ABO, secretor and Lewis families are involved in the biosynthesis pathways that produce the major HBGAs (69). The *FUT1* gene encodes an α1,2fucosyltransferase that synthesizes H (O) antigen on erythrocytes, while the *FUT2* gene is responsible for the α1,2 fucosyltransferase that results in H antigen expression on mucosal epithelial surfaces and as free oligosaccharides in saliva, milk and intestinal contents (70, 71). Individuals with at least one functional *FUT2* allele are designated as 'secretors'. A and B antigens are produced through subsequent modification of the H antigen by A and B glycosyltransferases (ABO gene), respectively (71). Further addition of carbohydrate moieties by the *FUT3* gene-encoded α1,3/4 fucosyltransferase leads to expression of Lewis antigens in secretors (Le^b, Le^y) and non-secretors (Le^a, Le^x) (68, 69, 72). Altogether, individuals can be classified based on their ABO blood-type, as well as whether they are secretors or non-secretors and Lewis-positive or negative.

Based on the knowledge that rabbit hemorrhagic disease virus, which is a calicivirus, binds to a HGBA (73, 74), it was postulated that NoVs may use similar antigens as cellular receptors. Indeed, studies demonstrated HuNoV virus-like particles (VLPs) hemagglutinating and binding to HBGAS in saliva and on intestinal epithelial cells of secretors (75-78). Canine NoV VLPs have also been shown to bind HBGAs *in vitro* (20). The use of HBGAs as cellular ligands for NoV binding was first supported *in vivo* by a study reporting association of Norwalk virus (GI.1) infection in humans with ABO histo-blood type. The study revealed an increased risk of infection in those with O blood-type compared to B blood-type (79). Further characterization of

susceptibility confirmed that, in addition to blood-type, secretor status was an equally important determinant of symptomatic infections as non-secretors were resistant to infection with a GII NoV in one study (80). Many other investigations since then have also supported increased susceptibility to NoV infection in persons who are secretors (17, 72, 81-86). Resistance in some secretor individuals, as well as symptomatic infection in non-secretors (87-89), has been explained by recognition of several strain-specific binding profiles to ABO and Lewis antigens through human studies, *in vitro* assays and structural analysis of the NoV capsid protein (71, 90-94). In general, two major classifications of HBGA binding have been proposed and include those strains that bind A/B antigens and those that bind Lewis antigens (90).

Through X-ray crystallography, it has been determined that the P2 subdomain of the NoV capsid protein (VP1) is the primary site of binding interactions with HBGAs. The NoV capsid protein is comprised of shell (S) and protruding (P) domains, with the P domain being further subdivided into P1 and P2. The P2 subdomain is found on the outermost surface of VP1, making it an ideal target as a receptor interface. It has been suggested that the polymorphism of HBGA phenotypes in the human population and mutations of the P2 domain influence NoV evolution (95-97)

Other potential receptor candidates for HuNoV infection have included heparin sulfate (98), sialic acid (99) and β-galactosylceramide (100). For murine NoVs (MNV), in addition to sialic acid (101), glycolipid and glycoprotein receptors (102), recent evidence for proteinaceous cell receptors, CD300lf and CD300ld, has been demonstrated (103, 104).

The cellular tropism for NoVs remains incompletely understood, with both intestinal epithelial cells (IECs) or enterocytes and immune cells being viable candidates (105-109).

Initially, based on the multitude of evidence for HBGAs as cellular ligands and the nature of NoVs as enteric viruses, it was speculated that IECs expressing HBGAs were the primary targets of NoV infection. Detection of NoV antigen in IECs has been demonstrated in chronically infected human transplant patients (110), Gn pigs (66), Gn calves (111) and $STAT1^{-/-}$ mice (112), indicating that these cells can at least be infected in immunocompromised individuals. Yet, until recently, multiple attempts to culture HuNoV in epithelial cell lines have failed (113-116), even with the presence of HBGAs (117). Successful HuNoV replication has now been established utilizing stem cell-derived human intestinal enteroids with and without the inclusion of bile acid supplementation (118), though routine use of this model remains to be implemented. Still, the enteroid cell culture system provides strong evidence for the continued notion that, similar to other enteric viruses, NoVs can infect enterocytes.

Meanwhile, MNVs have been readily cultured in dendritic cells, macrophages and B cells (119, 120). *In vivo*, MNV antigen has also been detected in the aforementioned immune cells (112, 119-121) and intestinal viral titers were lower in B cell-deficient mice (122), further suggesting the importance of immune cells in the pathogenesis of MNV infection. It has been proposed that, in order to traverse the intestinal epithelial barrier and access immune cells, virus is trancytosed by microfold (M) cells (106, 107, 123). M cells are specialized intestinal cells that are heavily involved in immunosurveillance through the engulfment of microscopic material from the lumen, which also makes them a key entry portal for pathogens (124). In mice that were depleted of M cells, reduced viral titers were observed compared to controls (125).

Evidence of HuNoVs infecting immune cells also exists, though is more conflicting. In the

previously mentioned study demonstrating viral antigen detection in enterocytes of immunocompromised human transplant patients (110), antigen was also detected in macrophages, dendritic cells and T cells in intestinal biopsies. Yet, Norwalk virus (GI.1) was incapable of replicating in human monocyte-derived macrophages and dendritic cells (126). In terms of B cell infection, only the GII.4 Sydney strain among HuNoVs has been shown to replicate in B cells *in vitro* (120, 127). The situation *in vivo* is likely even more complicated, as similar incidence of NoV infection occurred in B cell competent (60%) and deficient (63%) children with severe combined immunodeficiency (SCID) (128). However, the viral titer in B cell deficient individuals, while still on the scale of millions of genomic copies per milliliter, was significantly reduced compared to those patients with B cells. In contrast, infection with a GII.4 HuNoV in RAG2/IL2RG deficient Gn pigs, which lack B cells, T cells and natural killer cells, proved to be of higher titer and longer duration versus wild type controls (67). As with previous studies in Gn pigs, detection of viral antigen occurred in enteroctyes of the small intestine (66, 129). Overall, it appears that regulation of NoV cell tropism is complex, possibly depending on virus strain, host species, immune status and other potentially undetermined innate or environmental factors.

Interestingly, the ability of NoV to replicate in the BJAB human B cell line was dependent on the presence of HBGA-expressing enteric bacteria as use of filtered stool samples for culture resulted in decreased titers (120). Addition of H type-positive *Enterobacter cloacae* to cultures subsequently reestablished NoV infectivity. In the same study, antibiotic treatment of mice prior to MNV infection significantly reduced viral titers. Another recent study also recognized GII.4 and GI.6 NoV binding to enteric bacteria, again possibly due to interactions with HBGA-like

moieties (130). Thus, HBGAs in the context of intestinal microbiota may be involved in regulating NoV pathogenesis as well. However, the exact interaction between virus and bacteria is still uncertain. A recent study in Gn pigs demonstrated reduced viral titers in pigs colonized with *E. cloacae* and subsequently infected with a GII.4 human norovirus (131). The authors suggested that HBGA-expressing bacteria may function to act as decoy receptors, thus blocking rather than enabling binding to viral receptors. Further studies will need to be conducted to clarify the role of HBGA-expressing bacteria in NoV infection.

Another unanswered question regarding NoV pathogenesis involves reconciling the relative lack of intestinal pathology and inflammation with the severity of diarrhea that can occur (108, 109). Most studies in both humans (132-134) and animal models (66, 111, 135) report fairly mild disruption of the intestinal epithelium, with blunting of villi being most common. Other changes observed include microvilli destruction, mitochondrial distension, cytoplasmic vacuolization, intercellular edema and cell apoptosis (129). Therefore, it is reasonable to assume that other mechanisms of diarrhea may be involved. One study of human duodenal biopsies reported a decrease in transepithelial resistance following NoV infection, likely secondary to a reduction in tight junction proteins occludin, claudin-4 and claudin-5 (133). Such functional impairments may contribute more to NoV diarrhea than structural damage of enterocytes. Further studies are needed to understand the pathophysiology of NoV diarrhea.

1.3 Rotavirus (RV)

RVs belong to the *Reoviridae* family and are non-enveloped, segmented, double-stranded RNA viruses (136, 137). Based on the structural protein VP6, RVs are classified into at least 8 different groups, denoted with letters A-H (136, 138). RVA, RVB and RVC cause infection in humans and certain animals, while RVD-RVH are exclusively associated with animal infections. Recently, new canine and bat species of RV have been suggested to comprise RVI and RVJ groups, respectively (139, 140). Within groups, RVs are further classified based on capsid proteins VP7 (glycoprotein - G type) and VP4 (protease sensitive protein - P type) (137). Among the RVA group, which is the most commonly involved in human infections, G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] are the most prevalent strains (141).

1.3.1 Clinical signs of RV infection

RV infection is most prevalent in infants and young children and is characterized by non-specific symptoms of acute gastroenteritis. Vomiting followed by watery, non-bloody diarrhea typically occurs within 48-72 hours of infection via the fecal-oral route and can be accompanied by fever, abdominal cramps, nausea and headaches (136, 142-146). Severity of symptoms can vary from mild to debilitating and diarrhea can persist for up to one week (146, 147).

Due to the onset of both vomiting and diarrhea, death can occur subsequent to extreme dehydration, electrolyte imbalances and cardiovascular failure if medical intervention is not available (136, 142). Dehydration is more common with RV infection compared to other bacterial or viral agents of gastroenteritis in children (143). Cases that require hospitalization and either oral or intravenous rehydration therapy are in the minority though, and RV infection

is more often self-limiting (147). Children between three months to three years of age are more likely to present with severe illness (145). Mortality rates are highest in underdeveloped regions, such as in Saharan Africa and southeast Asia, where access to appropriate medical care can be limited and malnutrition or coinfections with other enteropathogens exacerbate clinical symptoms (136, 147-149).

Virus shedding often precedes onset of clinical symptoms and can usually be expected to continue for up to 10 days, with an average of four days duration (145, 150). Peak levels of shedding in young children can produce as much as $10^{10\text{-}11}$ viral particles per gram of feces (142). Prolonged shedding has been reported with severe infections (150) and in immunocompromised individuals (145, 150, 151).

Asymptomatic RV infections are known to occur, both in young children and in adults. Though severe infections can occur in neonates, infections at this age are typically asymptomatic due to maternal antibody protection via placental transfer or breastfeeding (145, 152, 153). Furthermore, individuals encounter multiple RV infections with age and have less severe symptoms with successive infections due to partial immunity (145, 154-156). Fecal virus shedding continues to occur in these infections, but often at much lower titers than with symptomatic cases (142, 157) **.**

However, not all RV re-infections in the adult population are asymptomatic (158-160). In a recent study of adults requiring hospitalization for acute gastroenteritis in Denmark, RV was determined to be the second-most common (25/265 patients or 9.4%) enteropathogen implicated (161). Similarly, in the US, RV was detected as the cause of diarrhea in 18% of cases (19/106) from several emergency centers (162). Although elderly and immunocompromised

individuals are understandably more predisposed to infection (163, 164), outbreaks have been reported in young adults as well (159). These findings suggest that rotavirus should still be considered as a causative agent in presentations of acute gastroenteritis in adults. Symptoms in adults are similar to those observed in young children (142).

RV replicates in the small intestine and expectedly results in local disease, but there are several reported instances of extraintestinal manifestations. Seizures are the most commonly observed presentation of systemic infection (165-167), with both febrile and afebrile seizures being possible and generally occurring without lasting consequences. Meningitis and encephalitis are among the other prevalent neurological diseases occurring with RV infection (165, 168, 169). Though even rarer, tentative associations have also been proposed for cases of pneumonia (170), disseminated intravascular coagulation (171), exanthema (172), haemophagocytic lymphohistiocytosis (173) and autoimmune diseases such diabetes mellitus among others (174).

In general, it has become accepted that antigenemia and viremia frequently occur with acute RV infections, either with or without diarrhea (168, 175-178). Although usually clinically insignificant in the course of illness, this evidence may explain the ability of RV to cause disease outside of the gastrointestinal tract.

1.3.2 Pathogenesis of RV infection

Rotavirus is widely known to infect and replicate in mature enterocytes of small intestinal villi (136, 179, 180). How exactly RV binds to these cells and induces watery diarrhea

and vomiting has been the subject of multiple studies. From these observations, it is clear that the pathogenesis of RV infection is multifactorial and possibly incompletely understood.

The main capsid proteins of RV are VP4 and VP7, with VP7 comprising the outer shell of the capsid and VP4 forming the spike protein (137). As such, VP4, is primarily responsible for RV attachment to enterocytes. VP4 is enzymatically cleaved by trypsin into VP5* and VP8*, with VP5* forming the main stem and VP8* forming the head of the spike that directly encounters receptors on small intestinal cells (136, 181). Initially, sialic acid moieties were thought to be involved in RV binding, since interaction of some animal RV strains with cell surfaces is interrupted by sialidase treatment (182). However, it was eventually determined that human and most animal RV strains were sialidase-resistant and likely not dependent on sialic acid for cellular entry (181, 183). Based on multidisciplinary studies involving structural biology and glycobiolity, it became evident that RVs can use HBGAs as cellular receptors (181, 184-188).

As mentioned, these ligands have previously been determined to be responsible for the cellular binding of NoV (76, 92). Since NoV can infect enterocytes, it was speculated that RV may proceed similarly. Indeed, from *in vitro* assays using human saliva, milk synthetic oligosaccharides, rotavirus VP8* was demonstrated to bind to HBGAs (186). Current knowledge indicates RV strain-specific binding to A, H type 1 and/or Lewis^{b} antigens (185, 186). Most studies have also determined increased susceptibility to symptomatic infection in secretor-positive individuals (82, 189-191), though conflicting evidence of infection in non-secretors does exist (192). Yet, a recent study did demonstrate increased RV-specific salivary IgA titers in secretors (193).

HBGAs are not the only glycans that have been demonstrated to interact with RVs. VP8* also interacts with gangliosides in a strain-dependent manner (194). As stated previously, some animal strains do bind sialic acid as confirmed by their sensitivity to sialidases. These strains are able to bind the sialic acid found as a terminal moiety on the ganglioside GM3 (195, 196) and as terminal and internal moieties on ganglioside GD1a (197). In contrast, sialidase-insensitive RVs, which include most human strains, appear to interact with the ganglioside GM1a that lacks a terminal sialic acid (197). Of note, GM1a contains an internal sialic acid moiety that can interact with VP8* and it is not affected by sialidases.

In addition to VP8*, VP5* and VP7 have also been implicated for their role in cellular binding. VP5* has been demonstrated to bind integrin $\alpha 2\beta 1$ (198), while VP7 binds integrins $\alpha x\beta 2$ and $\alpha v\beta 3$ (198, 199). Heat shock cognate protein hsc70 (200) and tight junction protein JAM-A (201) are also involved in RV-host cell interaction. Once bound to enterocytes, RV has been shown to enter the cell via endocytosis, though the exact mechanism appears to be strain dependent (202-204).

Malabsorption is one of the fundamental mechanisms of RV-induced diarrhea. In the small intestine, mature villus enterocytes are non-replicating cells with numerous microvilli that are involved in both active and passive absorption of fluid and electrolytes, as well as enzymatic digestion of food. Conversely, intestinal crypt cells proliferate in self-renewal of intestinal epithelium and maintain a secretory capacity, releasing chloride ions into the intestinal lumen (179). With RV infection, structural damage to the absorptive mature enterocytes has long been accepted as a major contributor to the presence of watery diarrhea. Early histological study of duodenum samples form children diagnosed with RV infection identified blunting of villi,

vacuolization of epithelial cells, increase in cuboidal versus columnar epithelium, intestinal crypt hyperplasia and a mild influx of inflammatory cells (136, 179, 205-208). Intestinal villi damage has also been reported due to ischemia in mice, though the relevance to humans is unclear (209). Combined, the reduced absorptive capacity due to enterocyte loss results in increased intestinal fluid.

However, these lesions are not always consistently observed and often do not necessarily correlate with clinical symptoms (146, 179). As such, a maldigestion component has also been implicated as a cause of RV diarrhea. A decrease in digestive function has been reported subsequent to a loss of disaccharidases that are located at villi tips (205, 208, 210, 211). This produces an abundance of carbohydrates, which act as osmotically active particles and further adds to the water being drawn into the intestinal lumen.

A unique aspect of RV-induced diarrhea is the role of nonstructural protein, NSP4, which acts as an enterotoxin. NSP4 is produced by infected enterocytes (146, 179, 208, 212, 213) and peptides are secreted into the intestinal lumen to further affect neighboring cells (214). NSP4 appears to have multiple effects on enterocytes that collectively contribute to intestinal fluid loss and secretion. It acts to release intracellular Ca^{2+} from cells, which can induce changes in cellular ion transport and permeability (208, 215, 216). It has been suggested that the Ca^{2+} signal transduction produces a mild, net chloride secretion through a cystic fibrosis transmembrane conductance regulator (CFTR)-independent process (208, 212, 217). NSP4 has also been demonstrated to inhibit the sodium-glucose transporter SGLT1, reducing water reabsorption (218). Additionally, there is some evidence that this signaling can modify actin

filaments of microvilli and disrupt intestinal tight junctions, possibly increasing intestinal permeability (216, 219, 220).

Furthermore, Ca^{2+} signaling can incite production of cytokines, prostaglandins and reactive oxygen species that have been postulated to stimulate the enteric nervous system (ENS) (208). Located in the intestinal wall, the ENS is conveniently able to sense changes in the intestinal environment and alter secretion of fluid and electrolytes in response (221). Serotonin (5-hydroxytryptamine or 5-HT) is an important neurotransmitter of the ENS and is secreted exclusively in the small intestine by enterochromaffin (EC) cells (207). In terms of gastrointestinal effects, it is responsible for gut motility, intestinal secretion, blood flow and vagally-mediated vomiting (207, 222). Recently, RV has demonstrated capability of infecting EC cells and subsequent NSP4 release activates serotonin synthesis to influence diarrhea, nausea and vomiting (207, 222). Additionally, serotonin antagonists have been shown to reduce RV diarrhea in mice, further confirming the role of ENS in RV pathogenesis (207, 223). In terms of ENS involvement in intestinal motility, the drugs loperamide and atropine, which function to increase intestinal transit time and allow for a greater period of fluid absorption, have been shown to attenuate diarrhea induced by RV infection (224).

1.4 Gnotobiotic (Gn) pig models for acute viral gastroenteritis

1.4.1 General overview

Domestic pigs (*Sus scrofa domesticus*) are being increasingly utilized as an animal model in biomedical research. While pigs, even minipig breeds, represent a greater husbandry

challenge due to their size, there are several factors that make them more ideal than rodent or other animal models for studying human diseases. In particular, pigs are anatomically, physiologically and immunologically fairly equivalent to humans (225-227). Many studies thus have exploited the similarities with the integumentary, cardiovascular, urinary and digestive systems (225). Additionally, the reproductive characteristics (short generation interval, large litter size), ease of adequate sample collection and availability of numerous outbred and inbred lines are also advantageous qualities (227).

In terms of utilizing pigs as an animal model for studying enteric viruses, the resemblance with the human gastrointestinal tract and immune system are especially appropriate. There are gross anatomical differences in the stomach, small and large intestine (225), but the digestive physiology, as well as susceptibility and response to disease are comparable (227-229). Furthermore, pigs have similar intestinal villi structure and cell classification (228) (230), enabling studies of disease pathophysiology. For immunological evaluation, it is especially beneficial that >80% of the immune parameters assessed are analogous to humans (231) and that all immune cell populations are similarly present in pigs (227).

Germ-free animals are those that are deemed microbiologically sterile by specific testing, while gnotobiotic (Gn) animals are those that are associated with known and identifiable organisms (232). Technically, germ-free animals can also be called Gn because the status of microorganisms is known. Gn mammals are derived by aseptic hysterectomy into sterile isolators, where they are fed a sterilized, commercial milk diet. Gn pigs have been available for biomedical research since the 1960's and present a unique opportunity to evaluate

host-pathogen interactions and related immunity. Unlike humans and mice, pigs have a diffuse epitheliochorial placentation, which prevents the transfer of maternal antibodies during intrauterine development (225, 233). Neonatal pigs acquire passive immunity via ingestion of colostrum within the first 36 hours after birth (234) to aid in protection against pathogens while their immune systems are still developing. The lack of maternal antibody interference, intestinal microbiota and unknown pathogens allows for specific evaluation of primary immune responses to chosen enteric microbes and vaccines.

Still, recent studies have highlighted the importance of the intestinal microbiota in influencing enteric immunity and response to infections (235, 236). To better elucidate these relationships, Gn pigs have been successfully transplanted with human gut microbiota (235-237). Additionally, with the development of the CRISPR/Cas9 system for gene editing, the manipulability of the Gn pig model has broadened even further (67).

Currently, in addition to several other Gn pig models of enteric pathogens (238-240), models for HuNoV and HRV have been well-established.

1.4.2 Gn pig model of HuNoV infection

As stated, efficient cell culture systems for HuNoV are lacking, making animal models even more vital for studying pathogenesis, immunity and response to vaccines and therapeutics. Aside from Gn pigs, other animal models include mice (241), Gn calves (111), and non-human primates (135, 242). However, Gn pigs have been suggested as a more suitable model due to presence of clinical signs, i.e. diarrhea, compared to mice and non-human

primates. Additionally, pigs have also been demonstrated to have similar intestinal HBGA expression and NoV binding as humans (243).

The Gn pig model of HuNoV infection and disease was first described in 2006 using a GII.4 strain (HS66) (66). The authors reported mild diarrhea in 74% of infected pigs, as well as fecal virus shedding as determined by RT-PCR and ELISA. NoV capsid antigen was detected in enterocytes of both the duodenum and jejunum, supporting occurrence of viral replication. Similar to findings in humans, only mild histopathological lesions of the intestines were observed, mainly consisting of duodenal villous atrophy and edema of the lamina propria. In a follow-up study with the same GII.4 strain, the group evaluated cytokine and antibody responses post-infection (244). It was noted that pigs with greater severity of diarrhea were more likely to seroconvert and had higher intestinal IgA and IgG titers. In terms of cytokine secretion, HuNoV infection induced a balanced Th1/Th2 (IFN-γ, IL-12, IL-4, IL-10) and delayed IFN-α response systemically, while only IFN-α and IL-12 were detected locally in intestinal contents.

Gn pigs continue to be used to evaluate NoV vaccine candidates, including both VLP and P particle-based vaccines (245, 246). Other studies have utilized the model to assess efficacy of immunomodulators and therapeutics, such as IFN-α, probiotics and rice bran (247, 248). As previously mentioned, recent pathogenesis studies also demonstrated that, in contrast to MNV, HuNoV does not have a tropism for B cells in Gn pigs and that *E. cloacae* inhibits infectivity instead (67, 131, 249).

1.4.3 Gn pig model of HRV infection

In contrast to HuNoVs, HRVs can be grown efficiently in cell culture and commercial vaccines are readily available. However, these vaccines have lowered efficacy in developing countries, thus the Gn pig model is still fairly relevant for studying RVs. Similar to HuNoVs, infection with HRV is capable of causing clinical disease in Gn pigs. Additionally, unlike mice and rabbit models, Gn pigs continue to be susceptible to RV-induced diarrhea until 6 weeks of age (250).

Gn pigs were first utilized to study HRVs starting in the 1970's (251, 252), demonstrating development of diarrhea and fecal virus shedding. Yet, it was not until 1996 when the Gn pig model of HRV infection and disease was fully established (253). This model was developed using a pig-adapted HRV Wa strain (G1P1A[P8]), which induced diarrhea in all pigs at a dose of ~10^5 focus-forming units (FFU) approximately 13 hours post-inoculation and lasting for an average of four days. The diarrhea was associated with histopathological lesions in the duodenum and jejunum (villous atrophy), seroconversion and fecal virus shedding up to 2 x 10^7 FFU/ml.

Since that time, the model has been utilized to determine immune responses to HRV infection and evaluation of vaccine and therapeutic candidates. A study conducted in Gn pigs was the first to demonstrate intestinal IgA antibody-secreting cells (ASCs) as a correlate of protective immunity in an HRV animal model (254). Two other Gn pig studies suggested that vaccine candidates for regions with infants having high maternal antibody levels (developing countries) may require specialized formulations and/or dosing regimens to circumvent maternal antibody interference on the development of protective ASC responses (255, 256). An additional study highlighted the importance of a balanced Th1/Th2 response induced by Wa

HRV in providing protection against subsequent re-challenge (257). Potential RV vaccines and therapeutics evaluated in Gn pigs include VLP-based vaccines (258, 259), chicken egg yolk IgY antibodies (260), llama-derived antibodies (261), probiotics (262-265) and rice bran (266, 267). Recently, a Gn pig model of enteric dysbiosis was established to elucidate the effects of human gut microbiota on immune responses induced by RV vaccination (235).

1.5 Role of dendritic cells in innate immunity against acute viral gastroenteritis

1.5.1 General overview

Dendritic cells (DCs) are specialized cells of the innate immune system that are particularly adapted for antigen capture in their immature state (268, 269). Recognition of antigenic stimuli by DCs are controlled by pattern recognition receptors (PRRs) that are associated with conserved pathogen associated molecular patterns (PAMPs) of microbes (268, 270). This contact prompts the secretion of cytokines and chemokines that can attract other innate immune cells (268). Upon maturation, the activated DCs migrate with the captured antigens to lymphoid tissues and interact with T, B and NK cells (271). Significantly, DC presentation of antigens to T cells results in guidance of the adaptive immune response to pathogens, inducing either an immunogenic or tolerogenic state (270). DCs are generally classified as either myeloid/conventional DCs (cDCs) or plasmacytoid DCs (pDCs) (272). cDCs are recognized primarily for antigen presentation, while pDCs are known as potent secretors of type I interferons (IFN-α and IFN-β) (273). In mice, cDCs are comprised of two major subsets,

$CD103^+CD11b^-$ and $CD11b^+$ cells (274, 275). Two cDC subsets are also present in humans and are mainly characterized as either $CD1c^+$ or $CD141^+$ (275, 276). As for pDCs, those in humans are known to express CD303 (CLEC4C), CD304 (neuropilin-1), and CD123 (IL3RA), while those in mice express B220, sialic acid-binding immunoglobulin-like lectin H (Siglec H), and intermediate levels of CD11c (275, 277). cDC and pDC populations in pigs have been defined as CD172a(SWC3a)$^+CD4^-CD11R1^+$ and CD172a(SWC3a)$^+CD4^+CD11R1^-$, respectively (278). It is important to note that CD11R1 in pigs, which is recognized by mouse anti-pig CD11R1 antibody clone MIL4, is analogous to CD11b in humans and thus also recognized by the cross-reactive anti-human CD11b clone TMG6-5.

DCs are found in several locations throughout the body where there is a higher incidence of encountering pathogens. Specifically, in the gastrointestinal tract, DCs are located in Peyer's patches of the small intestine and throughout the lamina propria (279, 280). In mice, the majority of the DCs in the lamina propria of the small intestine are characterized as $CD103^+CD11b^+$, while those in the colon are comprised of $CD103^+CD11b^-$ and $CD103^-CD11b^+$ populations (281). Studies of intestinal DC populations in humans have demonstrated that $CD103^+CD172a^+$ DCs are closely related to murine $CD103^+CD11b^+$ DCs. Similarly, $CD103^+CD172a^-$ DCs in humans are homologous to $CD103^+CD11b^-$ DCs in mice (282). In pigs, DCs in the lamina propria are defined as $CD11b^+CD172a^+$, while DCs in Peyer's patches are divided into $CD11b^-$ $CD172a^+$ in subepithelial domes and $CD11b^-CD172a^-$ in interfollicular regions. A fourth subset in pigs is represented by $CD11b^+CD172a^-$ DCs in mesenteric lymph nodes (283). As the gastrointestinal tract is a prime site for interactions with enteric pathogens, recent studies have been conducted to understand the role of DCs in NoV- and RV-induced immunity.

1.5.2 Dendritic cell responses to NoV infection

Studies of DC responses to NoV infection are limited and have primarily been conducted in mice thus far. In addition to their permissiveness to MNV infection, DCs have also been implicated in clearance of virus and influence of antibody responses. One study depleted cDCs from mice prior to MNV infection and reported increased virus shedding and decreased MNV-specific IgG titers (279). A more recent study associated the ability of DCs to control MNV replication with the secretion of Type I IFN (284). Mice with DCs that were unable to respond to Type I IFN due to a receptor deficiency demonstrated persistence of MNV infection. This persistence occurred despite compensatory increased humoral responses.

1.5.3 Dendritic cell responses to RV infection

While numerous studies have advanced understanding of adaptive immunity against RV infection, knowledge of innate immunity in terms of DC responses is still incomplete. Two features seem to be well-accepted from recent studies though. First, DCs are clearly capable of being activated by RV infection as demonstrated by up-regulation of CD40, CD86, MHCII, TLR3 and TLR4, cytokine secretion, and reduced binding to VLPs (285, 286). Second, Type I interferon secretion by pDCs (272) is important for immune defenses against RV infection. pDC secretion of IFN-α has been associated with stimulation of IFN-γ-producing T cell responses *in vitro* (287). Another *in vitro* study revealed the necessity of Type I IFN production by pDCs in regards to B cell activation (288). The same study also demonstrated that mice without functional pDCs

were unable to mount sufficient RV-specific antibody responses to control infection as these mice had increased virus shedding.

1.6 Prevention of acute viral gastroenteritis

1.6.1 Disinfection methods

Both NoV and RV are spread through the fecal-oral route and are fairly easily transmitted from person to person via contaminated food, water and fomites if proper hygiene and disinfection are not employed. Numerous studies have been conducted to determine the best sanitation practices to reduce spread of each virus.

Due to the lack of an efficient cell culture system for HuNoVs, most studies evaluating disinfection methods either utilize other calicviruses (MNV, FCV) as surrogates (289, 290) or measure reduction of HuNoVs by PCR methods (291, 292). NoV appears to be most successfully removed from hands via washing with regular soap and water (291-293). Different concentrations of alcohol-based hand sanitizers have proven either ineffective (291, 292) or possibly even detrimental. A survey of long-term care facilities found an association with the use of alcohol-based hand sanitizers and an increased risk of NoV outbreaks (294). Chlorhexidine is also considered ineffectual as a hand sanitizer against NoVs (295). In terms of surface disinfectants, it seems that NoVs are fairly resistant to quaternary ammonium compounds (293). Sodium hypochlorite or bleach at a minimum concentration of 500-1000 ppm has proven to be effective in NoV decontamination (296-298).

In contrast, simple handwashing with regular soap is not necessarily effective at removing RV. Studies have shown that alcohol-based hand sanitizers are more reliable at reducing RV loads from human hands (145, 299, 300) and should be utilized in food preparation and when contact with infected individuals or contaminated fomites is suspected. As far as surface disinfectants are concerned, these should also contain alcohol in high concentration as alcohol has been shown to damage the outer capsid of RV (291, 301). Chlorhexidine gluconate 0.5% in 70% ethanol, quaternary ammonium compounds containing >40% isopropyl alcohol, 0.1% o-phenylphenol with 79% ethanol or 95% ethanol alone are capable of inactivating RV (145, 302). Domestic bleach also has acceptable activity against RV (302).

1.6.2 NoV vaccine development

There is currently no licensed NoV vaccine available. The difficulty in developing a suitable NoV vaccine is multifactorial and includes the inability to efficiently culture HuNoVs efficiently and cost-effectively, the lack of appropriate animal models and the significant antigenic diversity of NoVs (2, 303, 304). Additionally, the diversity between genogroups and the continual evolution of the more prevalent GII.4 genotype has clouded understanding of how previous exposure to multiple NoV strains may influence the response to vaccines and whether long-lasting immunity exists (303, 305). Studies have suggested immune protection may lapse after 2 months to 2 years (306) or may persist for up to 8 years (307).

Progress has steadily been continuing to address these issues though and several candidates are being evaluated in pre-clinical and clinical trials (304, 305, 308, 309). Since HuNoV cell culture systems are not well-established, recombinant NoV vaccines are being

developed at this time. Particular interest has been afforded to VLP vaccines. VLPs are produced by expression of the major capsid protein VP1 in an appropriate system (304, 309), including insect cells (310), mammalian cell lines (311), *E. coli* (312), yeast (313), potatoes (314), tobacco (315) and tomatoes (316). All lead to formation of particles that are morphologically and antigenically comparable to the actual virus capsid itself (304, 317). When administered either orally (318, 319), intranasally (320) or intramuscularly (321), VLP vaccines have been capable of inducing homotypic and heterotypic humoral, mucosal and cellular immunities to a varying degree (309). Various adjuvants, including LT(R192G) toxin from *E. coli* (320), monophosphoryl lipid A (MPL) and chitosan (322), have thus been utilized to increase immune responses to VLP-based vaccines. Several preclinical studies have also considered the delivery of VLP vaccines via a viral vector to induce greater immunity (323-325), though concerns regarding biosafety and efficacy related to previous host exposure have reduced the relevance of such vaccines (309, 326).

Of the current NoV vaccine candidates, a VLP-based vaccine from Takeda Pharmaceuticals is the most advanced in clinical trial progress. To overcome the hurdle of antigenic diversity among NoVs, design of multivalent vaccines is considered imperative (327). The phase 2 clinical trial candidate is a bivalent GI.1/GII.4 vaccine that is adjuvanted with MPL and aluminum hydroxide; it is administered intramuscularly in two doses, 28 days apart (328-332). The vaccine is comprised of the Norwalk GI.1 strain VLP and a GII.4 VLP developed from a consensus sequence of three GII.4 variants. Various dosage formulations have been evaluated, with more recent results indicating a 15/50 (GI.1/GII.4) formulation producing robust serological responses following just one dose of vaccine, while maintaining tolerability (331).

After vaccination of human adults, IgA and IgG ASC responses peaked at day 7 post 1st dose of vaccine. All vaccine recipients showed >4-fold rises in ASCs from pre-vaccination levels at this time point and minimal increases in ASCs were observed following a second vaccine dose, indicating the immune responses are anamnestic in nature. GI.1- and GII.4-specific IgG memory B cells were detected 180 days post-vaccination. But it remains to be evaluated if these responses function as vaccine-induced correlates of protection (332). Overall, the highest GI.1 antibody levels (pan-Ig, IgA and HBGA-blocking titers) were seen with a 50/50 formulation, but the 15/50 formulation unexpectedly produced both good GI.1 responses and even better GII.4 responses. As GII.4 NoVs are the predominant circulating strains, this will most likely be the formulation moving forward with future studies. Current plans include determining whether the MPL adjuvant is absolutely essential.

The other recombinant protein utilized in NoV vaccine development is the P particle, which is a subviral particle formed from expression of the VP1 P domain in *E. coli* or yeast (333). As mentioned previously, the P domain, specifically the P2 subdomain, is responsible for host binding interactions (334). An advantage of the P particle is the ease of production, making them likely a more economical vaccine option than VLPs (334). Studies indicate P particles retain HBGA-binding properties (333) and are immunogenic in mice (335) and Gn pigs (246) following intranasal administration. The study conducted in pigs demonstrated 47% cross-variant protection against diarrhea compared to 60% with the same GII.4 strain-derived VLP vaccine. However, the authors noted that immunization with P particles induced greater T cell responses than with VLPs. Conversely, another study revealed that VLPs, but not P particles, were capable of priming T cells for IFN-γ production and inducing cross-reactive T and B cell responses in mice

(321). Questionable formation of the P particles used in the mouse study may have affected the immunological responses though (326, 336). Further studies are likely required to better characterize the immunity induced by P particle vaccination.

As multiple enteric pathogens can cause acute gastroenteritis, there has been considerable interest in developing combination vaccines that include NoV. In particular, inclusion of RV recombinant VP6 protein with NoV VLPs has been evaluated in several studies (337-339). Additionally, the P particle has proven to be an excellent platform for presentation of additional antigens (340); P particles have been developed with addition of RV (340), hepatitis E virus (341), influenza virus (342) and astrovirus (343) antigens.

It remains to be seen whether NoV vaccines will be capable of producing durable and adequate homotypic and heterotypic immune responses. It is possible that NoV vaccines will need to be reformulated as emergent strains appear.

1.6.3 RV vaccine development

RV vaccine development has been an important contributor to reducing disease burden. The first licensed oral RV vaccine was a tetravalent reassortant of a rhesus RV strain (G3) with VP7 from human G1, G2, and G4 (137, 344). RotaShield or RRV-TV was licensed in 1998 after proving to be safe and efficacious in studies conducted in the US, Finland and Venezuela (137, 344-347). Unfortunately, it was believed to be associated with an increased risk of intussusception, a condition when a part of the intestinal lumen folds into itself and may result in obstruction, ischemia and perforation. The incidence was greatest in the 3-7 days following the first dose and more often when the vaccine was administered to children between 3 and 9

months of age (344, 348, 349). Due to safety concerns, RotaShield was voluntarily withdrawn from the market in 1999, less than a year after licensure.

Since 2006, two new live, attenuated oral RV vaccines have been commercially available – RotaTeq (approved 2006) and Rotarix (approved 2008). Rotarix (GlaxoSmithKline Biologicals) is a monovalent human G1P1A[8] strain vaccine that was attenuated through serial cell culture passage (136, 137, 344). Upon development, the safety and efficacy was tested in 60,000 infants in Europe and Latin America (147, 350, 351). The vaccine is administered in 2 doses at 2 and 4 months of age. Due to the human strain origin of the vaccine, intestinal replication does occur and shedding following the first dose is well-observed. Immunity should be high enough by the second dose that shedding should be very minimal (147, 344).

RotaTeq (Merck) is a pentavalent, reassortant vaccine containing a bovine RV strain (WC-3, G6P[5]) backbone with VP7 of human G1, G2, G3 and G4 strains and VP4 of a P1A[8] strain (344). Similar to clinical trials of Rotarix, RotaTeq was evaluated in >60,000 infants in the US and Europe (147, 352). Most likely due to less intestinal replication than Rotarix, RotaTeq requires a 3-dose schedule of administration at 2, 4 and 6 months of age. In accordance with the limited replication, much lower incidences of shedding of the vaccine strain occur as well (137, 147).

Regional RV vaccines include the Lanzhou lamb RV vaccine (LLR) licensed in China, a monovalent human-bovine vaccine (Rotavac) in India and an attenuated G1P[8] strain vaccine (Rotavin) in Vietnam (353-356). More recently, an oral bovine-human reassortant pentavalent vaccine (Rotasiil) demonstrated a 66.7% efficacy against severe RV gastroenteritis with 3 doses in a randomized, placebo-controlled trial (357). The vaccine contains human RV serotypes G1,

G2, G3, G4, and G9 on a bovine RV G6P[7] backbone (BRV-PV). Of importance for use in resource-limited countries, the vaccine is heat-stable for 2 years at a temperature of 37°C and for 6 months at 40°C.

Both RotaTeq and Rotarix were demonstrated to induce good immunity in licensing trials, with efficacy against severe RV gastroenteritis ranging from 85-98% that seems to persist for a few years following immunization, at least in developed countries (350, 352, 358-362). As countries continue to include RV vaccination in standard immunization protocols as recommended by the World Health Organization (WHO), the true clinical impact is becoming better understood. As of May 2016, 81 countries have incorporated RV vaccination into their national immunization programs (363).

Decline in cases of RV diarrhea and related hospitalization has been observed in the majority of vaccine studies in both developed and developing countries. One study in the US reported a 74-90% decrease in the number of positive RV tests between 2010-2012 compared to prevaccine baselines (2000-2006) (364). Additionally, RV-associated hospitalizations in the US were decreased between 60-94% from 2007-2011 when compared with rates observed from 2001-2006 (365). Similarly, hospitalization rates in Europe were estimated to decline by 65-84% when reviewing data from 2006-2012 (366). Furthermore, evidence of herd immunity has emerged as decline in all-cause diarrhea hospitalizations have occurred in older children and adults since release of current RV vaccines (367, 368). Regarding reduction of RV burden in developing countries, a recent report from Rwanda indicated that introduction of RotaTeq vaccination reduced RV-related hospitalizations by 61-70% between 2011 to 2014 (369).

Likewise, a study from Ghana conducted during the first three years following Rotarix vaccine introduction demonstrated a 49% reduction in hospitalizations due to RV (370).

Despite the promising results from implementation of RV vaccine usage in developing countries, overall vaccine efficacy (50-64%) still remains lower than that of developed regions (354, 363). Reasons for this observation appear to be multifactorial and encompass interference by maternal antibodies, concurrent administration of oral polio vaccine, malnutrition, coinfections with other enteric pathogens and differences in gut microbiome (147, 353, 371). Addressing this issue may require alterations of vaccine administration schedules or development of parenteral vaccine candidates (147).

Since the introduction of RotaTeq and Rotarix, a couple of safety concerns have arisen, though neither have been serious enough to permanently remove either vaccine from the market. Despite careful safety evaluation in clinical trials, both vaccines have possibly been associated with a slight increase in intussusception risk in postlicensure studies in several countries, including the US, Australia, and Mexico (372-375). Similar to Rotashield, the incidence is most likely to occur in the first week following initiation of the vaccine series (376). However, the overall incidence is much lower than observed with Rotashield, equaling 1-5 excess cases for every 100,000 children vaccinated (147, 376).

Another, though temporary, concern was the discovery of porcine circovirus I (PCV-1) DNA in Rotarix and PCV-1 and PCV-2 DNA fragments in RotaTeq in 2010. PCV-1 DNA was found originating in the master seed virus of Rotarix, while contaminated trypsin used in the production of RotaTeq was implicated (344). Since porcine circovirus does not infect humans, use of both vaccines was allowed to continue while production of PCV-free vaccines was

initiated. Overall though, the benefits of RV vaccination in reduction of morbidity and mortality outweigh any perceived risks.

1.7 Treatment of acute viral gastroenteritis

1.7.1 Oral rehydration solutions

Due to the lack of availability of NoV vaccines and the still under-utilized RV vaccines, adjunctive treatments remain significant. Most treatment modalities for NoV and RV are aimed at reducing clinical symptoms of disease and are thus similar between the two. The most important and beneficial aspect of treatment is appropriate rehydration therapy. Fatalities occur when intestinal water loss is severe enough to cause electrolyte imbalances and cardiovascular failure. WHO recommends the use of oral rehydration solution (ORS) in treatment of acute watery diarrhea of all causes. Use of ORS helps prevent life-threatening dehydration, though it does not alleviate symptoms of diarrhea or shorten duration. ORS contains glucose, electrolytes and citrate in an hypo-osmolar formulation to replenish losses and reduce acidosis associated with diarrhea and vomiting (377). The basis of ORS therapy is that glucose stimulates water and salt absorption in the small intestine via the Na^+/glucose cotransporter SGLT1 (378). In situations where oral rehydration is not adequate enough to counteract dehydration, then intravenous fluid therapy is necessary (379).

Since 2004, WHO has also recommended the use of zinc supplementation for two weeks in conjunction with ORS (380, 381). Zinc has been shown to be important for mucosal integrity of the intestine and inhibition of potassium channels involved in cyclic adenosine

monophosphate (cAMP) mediated chloride secretion (146, 382, 383). Consequently, low plasma zinc levels have reportedly been linked with increased risk of morbidity due to severe diarrhea (384) and supplementation with ORS can reduce diarrhea volume and duration (385).

1.7.2 General anti-diarrheal and anti-viral agents

Since ORS only serves to offset dehydration, non-specific adjunctive treatments continue to be proposed and studied with the aim of either reducing diarrhea or limiting viral replication. Probiotics are well-known for their gastrointestinal healthy benefit. Multiple probiotic strains have been evaluated for their ability to decrease duration of diarrhea with generally positive, though variable strain and dose-dependent responses (263, 386, 387). The most commonly administrated strains include *Lactobacillus* and *Bifidobacterium* species (388). The mechanism of action of probiotics is not fully defined, but may involve improving gut barrier function, regulating the intestinal environment and other enteric microbes and/or influencing the host immune system (389, 390).

In particular, *Lactobacillus rhamnosus* GG (LGG) has garnered the most supportive evidence for lessening clinical symptoms associated with acute gastroenteritis (391). In a clinical trial in Pakistan, LGG supplementation following rehydration elicited a response by the second day of treatment in children with acute, non-bloody gastroenteritis (392). By this time, 31% of children in the LGG group had continued diarrhea compared to the 75% in the placebo group. Similarly, in a clinical study conducted in Europe, LGG administration in conjunction with ORS reduced the duration of diarrhea in children with assumed viral gastroenteritis (no identifiable bacterial pathogens detected) (393). LGG has also demonstrated efficacy in preventing

nosocomial diarrhea in young children (394). Studies in Gn pigs have also confirmed specific efficacy of LGG against both HRV (265, 395) and NoV-induced diarrhea (248).

In relation to probiotic administration, the prebiotic rice bran has also been reported to ameliorate acute, watery diarrhea. Comprising the outer layer of rice, rice bran has been identified as a dietary source of bioactive compounds and has been suggested to be beneficial in intestinal health as well (396, 397). The mechanism of action includes promoting the growth of diarrhea-reducing probiotic species, maintaining gut barrier integrity and improving innate immunity (266). In Gn pigs infected with HuNoV and colonized with LGG and *E. coli* Nissle 1917, rice bran feeding reduced the incidence and duration of diarrhea, as well as virus shedding (248). Similarly in HRV-infected Gn pigs, rice bran feeding alone was able to decrease the incidence and severity of diarrhea, though did not affect virus shedding (266).

Commercial drugs are also available for the treatment of acute gastroenteritis. The anti-secretory drug racecadotril has proven to be effective in decreasing intestinal water loss and has been licensed for use in Europe (398, 399). Racecadotril acts to inhibit the enzyme neutral endopeptidase and, in doing so, increases the intestinal concentration of enkephalins, which have natural anti-secretory properties (400). Another drug that has been evaluated is the broad-spectrum anti-infective nitazoxanide (401, 402). Nitazoxanide was initially developed as an anti-parasitic agent, but has also shown activity against anaerobic bacteria (403) and viruses, including NoV (401, 404) and RV (401, 402, 405). Its use is particularly applicable in cases of NoV infection in immunocompromised individuals where reduction in immunosuppressive therapy or administration of intravenous immunoglobulin is not possible (404). In RV infection at least,

it appears that nitazoxanide inhibits VP7 maturation (406); the mode of action against NoV is unknown (407).

Recombinant llama-derived single chain antibody fragments (VHH) are an additional therapeutic agent that have been proposed for both NoV and RV-induced diarrhea. These so-called nanoantibodies are able to be produced against a specific antigen, including VP6 of RV (261) and VP1 of NoV (408, 409), and represent a potential immunotherapy.

1.7.3 RV-specific adjunctive treatments

For RV infection, other novel approaches to induce passive immunity include the use of hyperimmune bovine colostrum (410) and hyperimmune chicken egg yolk immunoglobulin (260). Additional potentially effective treatments include *N*-acetyl-cysteine and ergoferon. The antioxidant *N*-acetyl-cysteine has been demonstrated to reduce RV infectivity in cell culture (411) and resolve RV diarrhea in children after just two days of oral administration at 60 mg/kg/day (412). Ergoferon, which is composed of release-active polyclonal antibodies against IFN-γ, CD4 and histamine, was recently tested for antiviral activity against RV in cell culture with promising results (413). *In vivo* studies evaluating Ergoferon have yet to be conducted.

1.7.4 NoV-specific adjunctive treatments

Various compounds have been evaluated in attempt to disrupt the NoV replication cycle (407). In response to the well-established use of HBGAs as cellular receptors by NoVs, there has been considerable interest in blocking this binding interaction to limit infection (414-417).

Other anti-NoV drugs aim to reduce replication by targeting the viral protease (418-420) or RNA-dependent RNA polymerase (421-423).

1.8 Concluding remarks

My dissertation research involves investigations of pathogenesis, innate immunity and therapeutics in Gn pig models of NoV and RV infection and diarrhea. As discussed, pigs are a particularly suitable animal model for studying the pathogenesis of enteric viruses due to their similarities in anatomy, physiology and immune function with humans (225, 227-229). Utilization of Gn pigs enables evaluation of primary immune responses to virus, vaccination and/or therapeutics without confounding variables such as maternal antibody interference, prior antigenic exposure and possible co-infections with other enteric pathogens.

The first phase of my dissertation research was focused on the establishment of a reliable Gn pig model of NoV infection and disease. It has previously been demonstrated that Gn pigs are indeed susceptible to infection with HuNoVs using GII.4 and GII.12 strains (66, 424). However, these prior models failed to determine the median infectious dose (ID_{50}) of the strains used. This characterization enables more consistent incidence of infection for appropriately determining protective efficacy in vaccine and therapeutic trials. Based on the predominance of GII.4 strains in outbreaks of NoV gastroenteritis (8), a GII.4 2006b variant was selected as the challenge strain. To determine the effect of age on the ID_{50}, both neonatal (4-5 days of age) and older (33-34 days of age) were infected. We also confirmed whether

administration of a cholesterol-lowering drug, simvastatin, was able to increase susceptibility to NoV infection as reported previously (247, 425).

The second phase of my dissertation research involved utilizing the well-established Gn pig model of RV infection and disease to evaluate adjunctive treatments for RV-induced diarrhea. While RV vaccination has reduced morbidity and mortality, the efficacy of Rotarix and RotaTeq is still underwhelming in developing countries (354). An effective therapeutic to reduce diarrhea and shorten duration of illness would thus be especially beneficial.

Racecadotril is an attractive anti-diarrheal drug candidate due to its efficacy and tolerability (399, 400). As it decreases intestinal secretion, it addresses one of the possible mechanisms of diarrhea due to RV infection (208). Previous studies have reported generally favorable results in all-cause and RV-induced diarrhea, though conflicting evidence exists (426, 427), possibly due to confounding variables such as patient compliance and co-infections with other enteric pathogens. Subsequently, the Gn pig model presents an ideal opportunity to determine the specific efficacy of racecadotril against RV-induced diarrhea. Such evaluation is prudent before standardization of its use in treatment protocols.

Probiotics have also been highly studied for their anti-diarrheal effects. However, numerous strains are available and it is well-accepted that results may be both strain- and dose-dependent (263). Furthermore, the exact mechanism of action is ill-defined, though immunomodulation is likely involved (389). Again, the immunologically naïve background of Gn pigs facilitates elucidation of immune responses elicited by probiotic administration and the possible association with diarrhea reduction. Our previous studies evaluated the dose-dependent effects of *Lactobacillus acidophilus* NCFM (LA) on T cell responses in Gn pigs

receiving an oral HRV vaccine (428). Currently, limited studies have been conducted regarding the role of DCs in innate immunity to RV infection. As DCs in the gastrointestinal tract would expectedly encounter both probiotics and RVs, we elected to explore this interaction in Gn pigs administered either a low or high dose of LA, both before and after HRV infection.

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Chapter 2

Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age

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2.1 Abstract

Human noroviruses (NoVs), a major cause of viral gastroenteritis, are difficult to study due to the lack of a cell culture and a small animal model. Pigs share with humans the types A and H histo-blood group antigens on the intestinal epithelium and have been suggested as a potential model for studies of NoV pathogenesis, immunity and vaccines. In this study, the effects of age and a cholesterol-lowering drug, simvastatin, on the susceptibility of NoV infection and diarrhea were evaluated. The median infectious dose (ID_{50}) of a large inoculum pool of a GII.4 2006b variant was determined. The ID_{50} in neonatal (4-5 days of age) pigs is $\leq 2.74 \times 10^3$ viral RNA copies. In older pigs (33-34 days of age), the ID_{50} is 6.43×10^4 but decreased to $< 2.74 \times 10^3$ in simvastatin-fed older pigs. Evidence of NoV infection was obtained by increased virus load in the intestinal contents, cytopathological changes in the small intestine, including irregular microvilli, necrosis and apoptosis, and detection of viral antigen in the tip of villi in duodenum. This GII.4 variant was isolated in 2008 from a patient from whom a large volume of stool was collected. GII.4 NoVs are continuously undergoing selective pressure by human immunity, and antigenically different GII.4 NoV variants emerge every 1-2 years. The determination of the ID_{50} of this challenge virus is valuable for evaluation of protection against different GII.4 variants conferred by NoV vaccines in concurrence with other GII.4 variants in the gnotobiotic pig model.

2.2 Introduction

Members of the genus *Norovirus* in the family *Caliciviridae* are responsible for the majority of nonbacterial gastroenteritis outbreaks worldwide, with genogroup II, genotype 4 (GII.4) noroviruses (NoVs) being the predominant circulating strain (1, 2). Due to the highly infectious nature and environmental stability of NoVs, they are a serious concern in outbreaks under close quarter conditions, such as schools, hospitals, cruise ships, and retirement communities. Although a number of candidate vaccines have been proposed (3-9), no single NoV vaccine is currently commercially available. Vaccine development has been hampered by the absence of an efficient cell culture and small animal model.

In addition to human NoVs, which include GI, GII and GIV, animal NoVs also exist and include those known to infect pigs (GII) (10), mice (GV) (11) and cattle (GIII) (12, 13). Models utilizing these NoV-susceptible animals, along with a few species of non-human primates, have been described to study NoVs and vaccines (14-26). Ideally, an animal model for human NoVs would exhibit comparable clinical signs and immune responses following NoV infection. Of these animal models, the gnotobiotic (Gn) pig model shares significant similarities with humans in physiology, immunology, histo-blood group antigen (HBGA) phenotypes and virus binding patterns (19), providing an excellent animal model for studying human NoVs.

In this study, effects of age and a cholesterol-reducing drug, simvastatin on the susceptibility of NoV infection were evaluated. Our goal was to establish a reliable Gn pig model of human NoV infection and disease by determining the median infectious dose (ID_{50}) for a recently circulating GII.4 variant (27) in neonatal and older gnotobiotic pigs (4-5 and 33-34 days of age, respectively). The use of simvastatin and other cholesterol-lowering drugs have been

correlated with an increase in susceptibility to NoV infection (25, 28). We determined the ID_{50} of the GII.4 2006b variant (092895) in simvastatin-treated versus simvastatin-free pigs. One of the applications of the Gn pig model is to evaluate NoV vaccine efficacy; therefore ID_{50} at the time of challenge (33-34 days of age for Gn pigs) is an important parameter to determine. To our knowledge, this is the first study to determine the ID_{50} of a human NoV strain in a Gn pig model with or without simvastatin treatment.

2.3 Materials and Methods

2.3.1 Virus

A single pool of human stool containing the GII.4 2006b variant (092895) (GenBank accession number KC990829) was collected in Dr. Xi Jiang's laboratory in Cincinnati Children's Hospital Medical Center from a child with NoV gastroenteritis in 2008 and used to orally inoculate Gn pigs. The stool sample was diluted to 10% in diluent #5 (Minimal Essential Media with 1% penicillin-streptomycin and 1% HEPES) and treated by high-speed centrifugation to remove bacteria. Sterility was confirmed by culturing the treated inoculum on blood-agar plates and in thioglycollate medium. Absence of other contaminating viruses was confirmed by testing on a Virochip Microarray (University of California, San Francisco Viral Diagnostics and Discovery Center). Endotoxin levels were measured by a limulus amebocyte lysate test (ToxinSensor™ chromogenic LAL endotoxin assay kit, GenScript). The inoculum contains an endotoxin level of 1 EU/ml, which is below the recommended level for live attenuated vaccines (<200 EU/ml) (29).

2.3.2 Inoculation of Gn pigs

Near-term Large White cross pigs were derived by hysterectomy and maintained in germ-free isolator units as described (30). A+/H+ pigs were orally inoculated at either 4-5 or 33-34 days of age with 2.74 x 10^3 to 2.74 x 10^6 viral RNA copies of GII.4 variant 092895 suspended in 5 ml media (diluent #5). Control pigs received diluent #5 only. To neutralize stomach acids, 4 ml of 200 mM sodium bicarbonate were given 10 minutes prior to inoculation. Pigs were euthanized at post-inoculation day (PID) 3, 4, or 7 to collect intestinal contents and tissues. All animal experimental procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of Virginia Tech.

2.3.3 Simvastatin

To evaluate the effect of simvastatin on increasing virus infectivity (25, 28), a subset of pigs receiving 2.74 x10^3 to 2.74 x 10^6 viral RNA copies in the older pig groups were treated with simvastatin (Dr. Reddy's Laboratories, Ltd.), which was given orally at a dose of 8 mg/day for 11 days prior to virus inoculation with NoV. As the younger pigs were inoculated at 4-5 days of age, they did not receive any simvastatin treatment. Briefly, 80 mg tablets of simvastatin were dissolved in 100% ethanol to a concentration of 8 mg/ml, filtered for sterility purposes, and diluted in six ml of Diluent #5 prior to feeding. Serum cholesterol levels of subsets of pigs were measured by the VMRCVM Veterinary Hospital lab pre- and post-treatment with simvastatin to confirm reduction of cholesterol levels.

2.3.4 Blood typing of Gn pigs by PCR and immunofluorescence assay

Prior to inoculation, Gn pigs were confirmed to be A+ or H+ in blood type by PCR and immunofluorescence assay. It has been shown that histo-blood group antigen (HBGA) phenotype influences susceptibility to norovirus infection and that A+ or H+ pigs are more likely to be infected than A- or H- pigs (19, 31-37).

PCR blood typing was conducted with DNA isolated from whole blood using DNAzol Genomic DNA Isolation Reagent (Molecular Research Center, Inc., Cat. No. DN 127) and following the manufacturer's instructions for micro-isolation of DNA from whole blood. At least 50 ng of genomic DNA was required to perform the PCR reaction. Forward primer ABO4s (5'-AGCTGTTCCTGGAGACAGCGGAGA-3') and reverse primer ABO5a (5'-CAGGTGGCTCTCATCATGCCACAC-3'), designed by Revivicor and producing a 500 bp product, were used to determine whether the pigs were A+ or A-. Internal control primers Pig5' (5'-CCCTGGAACTCTGCCACTGTC-3') and Pig3' (5'CTGCACGTAGCACCAGGGTCT-3'), producing a 300 bp product, were also included. PCR conditions were as follows: initial denaturation at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and a final extension of 72°C for 10 min.

To determine the H phenotype of Gn pigs and confirm the PCR results, buccal cells were collected and immunofluorescence-stained as previously described (38). Briefly, the cheeks of Gn pigs were swabbed to collect buccal cells. Swabs were then swirled in 1 ml of PBS, centrifuged to pellet the cells, subsequently washed twice with PBS to remove any traces of milk, and resuspended in 20 μl of PBS. Slides were prepared using 2 μl drops of the buccal cell suspension. A, H1, and H2 histo-blood group antigens were detected with mouse anti-human A (Covance

Research Products, Inc., Cat. No. SIG-3311) (1:50 dilution), H1 (Covance Research Products, Inc., Cat. No. SIG-3313) (1:50 dilution) and H2 (Abcam, Inc., Cat. No. ab24224) (1:200 dilution). Slides were counterstained with propidium iodide (Invitrogen, Cat. No. P3566) and observed using a fluorescent microscope.

2.3.5 Assessment of NoV diarrhea

Gn pigs were rectally swabbed daily following inoculation with GII.4 human NoV variant 092895 to assess diarrhea and NoV shedding. The scoring system was adapted from Cheetham et al. (2006) and amended depending on the age of the pigs due to the change in consistency of the feces with maturity. Pigs inoculated at 4-5 days of age were scored based on the following system: 0) solid, 1) semi-solid, 2) pasty, 3) semi-liquid, and 4) liquid. Pigs inoculated at 33-34 days of age were scored based on a more stringent system: 0) solid, 1) pasty, 2) semi-liquid, and 3) liquid. For both scoring systems, a score of 2 or greater was considered diarrhea. Rectal swabs were also collected weekly for sterility test on blood-agar plates and in thioglycollate media.

2.3.6 Detection of NoV shedding by RT-PCR

Rectal swabs were swirled in 1 ml of PBS and large (LIC) and small intestinal contents (SIC) were diluted 1:10 in PBS before centrifugation at 10,000 x *g* for 5 min. The supernatant was collected and 200 μl used for viral RNA extraction using TRIzol (Invitrogen, Cat. No. 10296010) following manufacturer's instructions. The RNA was dissolved in 30 - 40 μl of RNAse-free molecular grade water for use in both conventional RT-PCR and TaqMan real-time RT-PCR.

For conventional RT-PCR, a two-step protocol was followed using the degenerate primer set p290HIJK/p289HI designed to detect NoVs and sapoviruses (39, 40). Superscript III Reverse

Transcriptase (Invitrogen, Cat. No. 18080-093) and primer p289HI were used to synthesize cDNA from 5 μl of RNA, according to manufacturer's instructions. PCR amplification was performed using 5 μl of cDNA and Platinum *Taq* DNA Polymerase (Invitrogen, Cat. No. 10966-018), following manufacturer's instructions. The PCR cycling conditions were: initial denaturation at 94°C for 3 min, followed by 39 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 2 min, and a final extension of 72°C for 15 min, producing PCR amplicons of 319 bp. Rectal swabs and LIC/SIC from mock-inoculated pigs were used as negative controls, while a known norovirus-positive human stool sample was used as a positive control.

2.3.7 Determination of NoV shedding titers by TaqMan real-time RT-PCR

To measure the NoV shedding titers, a one-step TaqMan real-time RT-PCR protocol adapted from the CDC using primer set COG2F/R and probe RING2 (41) was utilized. Briefly, 5 μl of RNA, extracted from either rectal swabs or LIC and SIC as described above, was used for the 25 μl reaction with the SensiMix Probe One-Step kit (Bioline, Cat. No. QT725-05). Cycling conditions were: reverse transcription at 42°C for 10 min, initial denaturation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 10s and annealing and extension at 58°C for 60s. Negative and positive controls were included as described above for conventional RT-PCR. A standard curve was included using plasmid DNA containing the COG2 amplicon serially diluted 10-fold from 1 x 10^7 to 1 genomic copy. Amplification data were collected and analyzed with Bio-Rad iQ5 optical system software, version 2.1. Virus shedding titers were reported as viral RNA copies/ml.

2.3.8 Determination of ID_{50} for NoV GII.4 variant 092895

The ID_{50} for the younger Gn pigs (inoculated at 4-5 days of age) and for the older Gn pigs (inoculated at 33-34 days of age) treated with versus without simvastatin were calculated using the Reed-Muench method (42). Pigs were considered infected if rectal swab or intestinal content samples tested positive by conventional RT-PCR or real-time RT-PCR. The following formula was used to determine the proportionate distance between dilutions above and below 50% endpoint:

$$\text{Proportionate distance (PD)} = \frac{(\text{Percentage infected at dilution next above } 50\% - 50\%)}{(\text{Percentage infected at dilution next above } 50\% - \text{Percentage infected at dilution next below } 50\%)}$$

The ID_{50} was then calculated based on the following formula:

log of the 50% endpoint = (log dilution above 50%) – (PD x log dilution factor)

Dilution factor is the fold difference between the titers for above 50% and below 50% infected.

2.3.9 Detection of NoV antigen in intestinal tissues by indirect immunofluorescence

Duodenum and jejunum samples were collected upon euthanasia and fixed in 4% paraformaldehyde-PBS, dehydrated in a graded ethanol series and embedded in paraffin blocks. Sections were cut and collected on positively charged slides. The staining procedure was adapted from previous studies (14, 25). NoV antigen was detected using guinea pig anti-NoV serum [against a mixture of recombinant virus-like particles from 9 GI and GII NoVs including VA-387 (GII.4), Grimsby (GII.4), MOH (GII.5), VA-207 (GII.9), (GII.1), Mexico (GII.3), Norwalk (GI.1), and Hawaii VA-115 (GI.3) produced in Dr. Xi Jiang's laboratory at the Cincinnati Children's Hospital Medical Center] diluted 1:3000.

2.3.10 Evaluation of cytopathological changes by transmission electron microscopy

Duodenum and jejunum samples collected upon euthanasia from both infected and mock-inoculated Gn pigs were fixed in 3% gluteraldehyde-PBS fixative solution for transmission electron microscopy (TEM) as previously described (14).

2.3.11 Statistical analysis

Proportions of virus shedding and diarrhea among treatment groups were compared using Fisher's exact test. Mean percent days of virus shedding and diarrhea along with peak titers shed among the treatment groups were compared using one-way ANOVA general linear model, followed by Duncan's multiple range test. A two-tailed paired Student's *t*-test was used to analyze changes in serum cholesterol levels following simvastatin feeding. Statistical significance was assessed at $P<0.05$ for all comparisons. All statistical analyses were performed using SAS program 9.3 (SAS Institute).

2.4 Results

2.4.1 Simvastatin feeding lowers the serum cholesterol levels

The cholesterol levels among a set of ten pigs tested in one trial were significantly lowered by simvastatin treatment at a dose of 8 mg/day for 11 days (Table 1). The mean serum cholesterol concentration was reduced from 98 to 79 mg/dL, a mean reduction of 17%. In another two trials with a set of six and seven pigs each, the same dosage of simvastatin treatment reduced the mean serum cholesterol from 142 to 80 mg/dL and 157 to 73 mg/dL, a mean reduction of 44%

Table 1. Cholesterol levels (mg/dL) after 11 days of simvastatin feeding (8mg/day)

Pig	Prior to simvastatin	Post simvastatin	% decrease	Infection status	Inoculum dose (viral RNA copies)	Peak virus titers shed (RNA copies/ml)
1	80	81	-1	no	2.74×10^3	0
2	88	67	24	yes	2.74×10^3	5.58×10^3
3	99	83	16	yes	2.74×10^3	5.61×10^5
4	79	74	6	yes	2.74×10^3	3.87×10^7
5	79	84	-6	yes	2.74×10^4	7.24×10^3
6	94	89	5	yes	2.74×10^4	1.72×10^5
7	112	92	18	yes	2.74×10^4	2.71×10^3
8	117	76	35	yes	2.74×10^5	1.37×10^4
9	101	78	23	yes	2.74×10^5	9.15×10^3
10	135	69	49	yes	2.74×10^5	6.57×10^3
Mean	**98**	**79**	**17**			

A paired Student's *t*-test was used to analyze changes in serum cholesterol levels following simvastatin feeding (P=0.008).

and 52%, respectively (data not shown).

2.4.2 Observation of diarrhea in both age groups of Gn pigs following inoculation with NoV

Neonatal pigs and both simvastatin and non-simvastatin fed older pigs exhibited mild diarrhea (mostly scores of 2) after NoV inoculation. In the neonatal pigs, 67% of those receiving 2.74 x 10^3 RNA copies of NoV showed signs of diarrhea (Table 2). The incidence of diarrhea increased to 100% in neonatal pigs receiving 2.74 x 10^5 RNA copies. Diarrhea was also present in older pigs, with or without simvastatin feeding (Table 3). We observed a tendency for the older pigs to have looser feces and consequently altered the diarrhea scoring system for these pigs to be more stringent than the scoring system utilized for the neonatal pigs. Among the infected older pigs in all dosage groups, there was a higher occurrence of diarrhea in the simvastatin-fed pigs (79%) compared to the non-simvastatin fed pigs (38%).

Not all of the pigs that shed virus developed clinical signs of diarrhea in this study. This probably reflects an asymptomatic infection that has been reported like-wise in humans (43, 44) and other animal models (15, 16, 21).

2.4.3 Gn pigs shed virus in feces as determined by RT-PCR and quantified by TaqMan real-time RT-PCR

Following inoculation, Gn pigs were confirmed to be infected with NoV by conventional RT-PCR and TaqMan real-time RT-PCR. Figure 1 depicts representative large intestinal content (LIC) samples that were positive for NoV shedding following inoculation with the 092895 variant.

Table 2. Summary of diarrhea and norovirus shedding in Gn pigs challenged with GII.4 2006b variant (inoculum 092895) at 4-5 days of age [*]

		Diarrhea [†]			Virus shedding			
Inoculum dose (RNA copies)	n	Percent diarrhea [‖]	Mean onset (days) [‡¶]	Mean percent of days with diarrhea [§‖]	Percent shedding [‖]	Mean onset (days) [‡¶]	Mean percent of days with shedding [§‖]	Geometric mean peak titer shed (RNA copies/ml) [¶]
2.74×10^3	6	67% (4/6) [AB]	4.5 (1.1) [B]	32% [A]	50% (3/6) [AB]	4.7 (1.5) [AB]	11% [B]	4.34×10^{1} [B]
2.74×10^5	3	100% (3/3) [A]	5.3 (0.3) [AB]	29% [A]	100% (3/3) [A]	3.3 (0.7) [B]	42% [A]	1.09×10^{4} [A]
Control	5	0% (0/5) [B]	8.0 (0) [A]	0% [B]	0% (0/5) [B]	8.0 (0) [A]	0% [B]	0 [B]

[*] Gn pigs were challenged with human norovirus 092895 at 4-5 days of age. Rectal swabs were collected daily after challenge to determine diarrhea and virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents.

[†] Diarrhea scoring system: 0) Solid, 1) Semi-solid, 2) Pasty, 3) Semi-liquid, 4) Liquid. Scores of 2 or greater were considered diarrhea.

[‡] In the groups where some but not all pigs had diarrhea or shedding, the onset of diarrhea or shedding for non-diarrheic/shed pigs were designated as 8 for calculating the mean days to onset [pigs were all euthanized before post-inoculation day (PID) 7]. Standard error of the mean is indicated in parenthesis.

[§] Calculated as percent of days with diarrhea or shedding from PID 0 until last sample was collected (PID 3, 4, or 7).

[‖] Proportions in the same column followed by different letters (A, B) differ significantly (Fisher's exact test, $p<0.05$); while shared letters indicate no significant difference.

[¶] Means in the same column followed by different letters (A, B) differ significantly (One way ANOVA, $p<0.05$); while shared letters indicate no significant difference.

Table 3. Summary of norovirus shedding in Gn pigs challenged with GII.4 2006b variant (inoculum 092895) at 33-34 days of age with and without simvastatin feeding [*]

(a) Non-simvastatin fed pigs

Inoculum dose (RNA copies)	n	Percent shedding [¶]	Mean onset (days) [‡#]	Mean percent of days with shedding [§¶]	Geometric mean peak titer shed (RNA copies/ml) [#]	# of pigs infected	Infected pigs with diarrhea [†]
2.74×10^3	4	25% (1/4) [AB]	6.8 (1.3) [A]	6.3% [B]	1.29×10^1 [BC]	1	1
2.74×10^4	5	40% (2/5) [AB]	5.8 (1.4) [A]	9.0% [B]	5.47×10^1 [BC]	2	0
2.74×10^5	3	67% (2/3) [A]	5.7 (1.2) [A]	11% [B]	3.31×10^2 [AB]	2	0
2.74×10^6	3	100% (3/3) [A]	1.0 (0) [B]	75% [A]	4.31×10^4 [A]	3	2
Control	11	0% (0/11) [B]	8.0 (0) [A]	0% [B]	0 [C]	N/A	N/A
Total # of virus inoculated pigs	15					8 (53%)	3 (38%)

(b) Simvastatin fed pigs [||]

Inoculum dose (RNA copies)	n	Percent shedding [¶]	Mean onset (days) [‡#]	Mean percent of days with shedding [§¶]	Geometric mean peak titer shed (RNA copies/ml) [#]	# of pigs infected	Infected pigs with diarrhea [†]
2.74×10^3	4	75% (3/4) [A]	3.8 (1.4) [B]	25% [B]	1.87×10^4 [A]	3	2
2.74×10^4	3	100% (3/3) [A]	3.3 (1.5) [B]	13% [BC]	1.50×10^4 [A]	3	3
2.74×10^5	3	100% (3/3) [A]	1.3 (0.3) [CΔ]	50% [A]	9.37×10^3 [A]	3	2
2.74×10^6	5	100% (5/5) [A]	1.0 (0) [C]	70% [A]	1.89×10^4 [A]	5	4
Control	11	0% (0/11) [B]	8.0 (0) [A]	0% [C]	0 [B]	N/A	N/A
Total # of virus inoculated pigs	15					14 (93%)	11 (79%)

[*] Gn pigs were challenged with 2.74×10^3 to 2.74×10^6 viral RNA copies of human norovirus 092895 at 33-34 days of age. Rectal swabs were collected daily after challenge to determine virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents.

[†] Diarrhea scoring system: 0) Solid, 1) Pasty 2) Semi-liquid, 3) Liquid. Scores of 2 or greater were considered diarrhea.

[‡] In the groups where some but not all pigs had shedding, the onset for non-shedding pigs were designated as 8 for calculating the mean days to onset [pigs were all euthanized before post-inoculation day (PID) 7]. Standard error of the mean is indicated in parenthesis.

[§] Calculated as percent of days with shedding from PID 0 until last sample was collected (PID 3, 4, or 7).

[||] Pigs were fed 8mg/day of simvastatin for 11 days prior to challenge.

[¶] Proportions in the same column followed by different letters (A, B, C) differ significantly (Fisher's exact test, $p<0.05$); while shared letters indicate no significant difference.

[#] Means in the same column followed by different letters (A, B, C) differ significantly (One way ANOVA, $p<0.05$); while shared letters indicate no significant difference.

[Δ] Indicates significant difference when compared between the simvastatin fed pigs and non-simvastatin fed pigs.

In both age groups without simvastatin treatment, pigs inoculated with a higher dose of virus inoculum displayed a higher, but not significant, rate of virus shedding (Tables 2 and 3a). This was often accompanied with longer duration of shedding, expressed as mean percentage of days with shedding from post-inoculation day (PID) 0 until the last rectal swab sample was collected (PID 3, 4, or 7). Mean percent of days of virus shedding and diarrhea are used in Tables 2 and 3 to depict the length of virus shedding or diarrhea because the mean number of days of virus shedding or diarrhea from PID 0 to 7 could not be calculated since a subset of pigs within the different dosage groups was euthanized on PID 3-4 for the pathogenesis study.

Pigs inoculated at 4-5 days of age with 2.74×10^5 RNA copies were all kept until PID 7. They all shed virus for 3-4 days (42% of the swab days) (Table 2). Only 3 of the 6 pigs inoculated with 2.74×10^3 RNA copies shed virus (all for only 1 day; 11% of the swab days). The 2.74×10^5 dosage group had significantly higher mean percentage of days with shedding and mean peak titers shed than the 2.74×10^3 group. Similarly, increased rates and duration of fecal virus shedding were observed in pigs inoculated at 33-34 days of age with 2.74×10^3 to 2.74×10^6 RNA copies without simvastatin feeding (Table 3a). The 2.74×10^6 group had a significantly earlier onset (PID 1) and longer duration of shedding (mean percentage of days with shedding from PID 0 – 75%) than the three lower dosage groups. The virus titers shed in the 2.74×10^6 group were also significantly higher compared to the two lowest dosage groups.

Among the simvastatin fed pigs, all dosage groups greater than 2.74×10^3 RNA copies exhibited a 100% shedding rate (Table 3b). The 2.74×10^6 and 2.74×10^5 groups had significantly

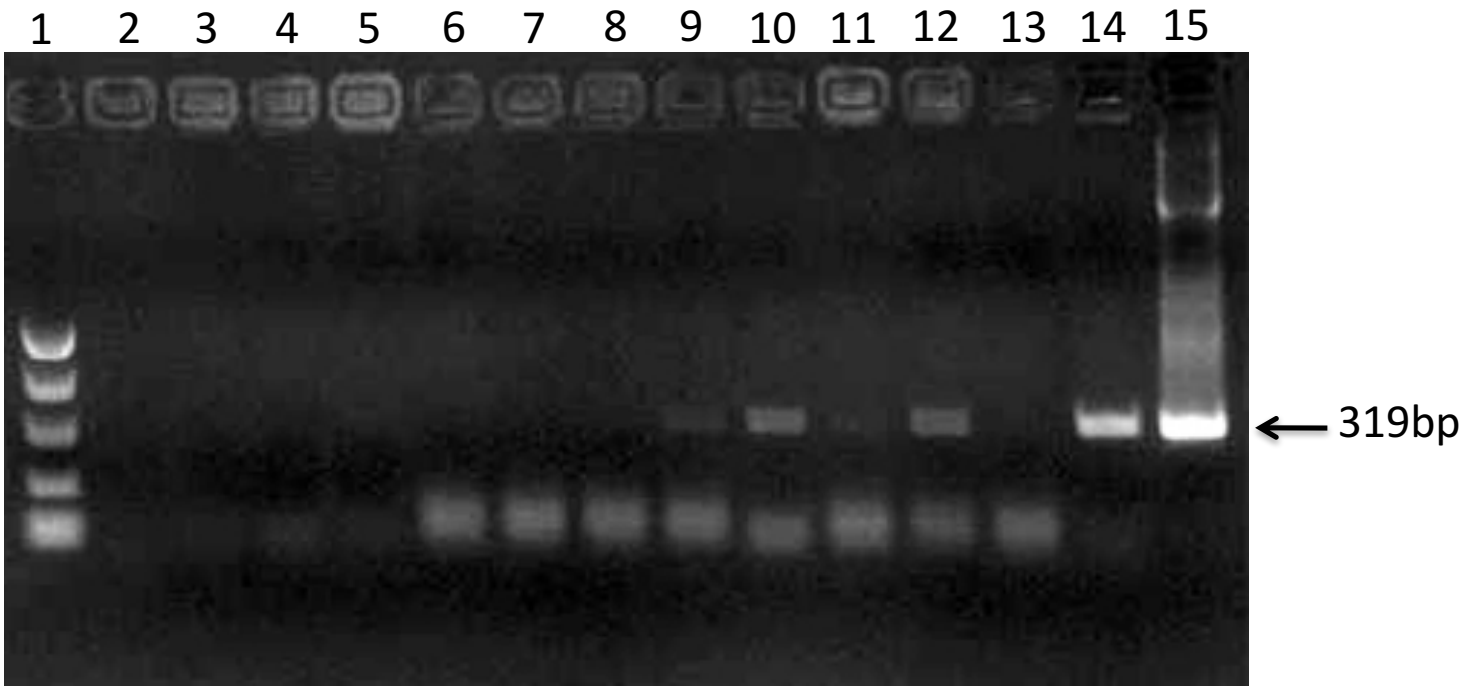


Figure 1. **RT-PCR to detect NoV shedding in large intestinal contents (LIC).** Primer pair p290HIJK/p289HI was used for two-step RT-PCR, producing a 319 bp product. LIC samples positive for virus shedding are present in lanes 9 – 12. LIC samples negative for virus shedding are in lanes 7, 8 and 13. Negative and positive controls are as follows: lane 1, DNA Ladder; lane 2, PCR negative control; lane 3, no reverse transcriptase control; lane 4, reverse transcription negative control; lane 5, RNA extraction negative control; lane 6, negative control LIC; lane 14, positive RNA control; lane 15, positive plasmid control.

longer duration of shedding (70% and 50% of swab days, respectively) than either of the two lowest dosage groups (13% and 25%, respectively). Significantly longer virus shedding was accompanied with significantly earlier mean onset of shedding (PID 1 and 1.3) in the two highest dosage groups compared with the two lowest dosage groups (PID 3.3 and 3.8).

2.4.4 Virus amplification in Gn pigs

It is important to note that the volume of intestinal contents needs to be considered when evaluating the virus load in the pigs. The average volume of intestinal contents in pigs at 33 to 34 days of age is approximately 100 ml (45). Based on this estimate, 18 out of 22 NoV-infected (eight non-simvastatin and 14 simvastatin-fed) pigs, shed a total amount of virus greater than the amount used for inoculation (Table 4). This confirms that virus replication indeed occurred.

2.4.5 Simvastatin increases susceptibility of NoV infection and the incidence of diarrhea in Gn pigs

Comparing virus shedding in simvastatin- versus non-simvastatin fed pigs at each dosage group, the mean percentages of shedding were higher, the mean onset was earlier, the mean percentage of days with shedding were higher, and the peak virus titers were higher in the simvastatin-fed pigs, except for the 2.74 x 10^6 dosage group (Table 3). These differences were not statistically significant except for the mean onset in the 2.74 x 10^5 dosage group, in which the simvastatin-fed pigs had significantly earlier onset of virus shedding than the simvastatin-free pigs (1.3 versus 5.7). The total incidence of infection among pigs in the simvastatin-fed group was 93% compared to 53% in the simvastatin-free group. The total incidence of diarrhea among the infected pigs in the simvastatin-fed group was 79% compared to 38% in the simvastatin-free

Table 4. Virus amplification in older Gn pigs with and without simvastatin feeding [*]

Non-simvastatin fed pigs					Simvastatin fed pigs [†]				
Pig	Inoculum dose (viral RNA copies)	Infection status	Peak virus titers shed (RNA copies/ml)	Total amount of virus shed at peak (RNA copies) [‡]	Pig	Inoculum dose (viral RNA copies)	Infection status	Peak virus titers shed (RNA copies/ml)	Total amount of virus shed at peak (RNA copies) [‡]
1	2.74×10^3	No	0	0	1	2.74×10^3	No	0	0
2	2.74×10^3	No	0	0	2	2.74×10^3	Yes	5.58×10^3	5.58×10^5
3	2.74×10^3	No	0	0	3	2.74×10^3	Yes	5.61×10^5	5.61×10^7
4	2.74×10^3	Yes	2.73×10^4	2.73×10^6	4	2.74×10^3	Yes	3.87×10^7	3.87×10^9
5	2.74×10^4	No	0	0	5	2.74×10^4	Yes	7.24×10^3	7.24×10^5
6	2.74×10^4	No	0	0	6	2.74×10^4	Yes	1.72×10^5	1.72×10^7
7	2.74×10^4	Yes	1.23×10^4	1.23×10^6	7	2.74×10^4	Yes	2.71×10^3	2.71×10^5
8	2.74×10^4	Yes	3.99×10^4	3.99×10^6	8	2.74×10^5	Yes	1.37×10^4	1.37×10^6
9	2.74×10^4	No	0	0	9	2.74×10^5	Yes	9.15×10^3	9.15×10^5
10	2.74×10^5	No	0	0	10	2.74×10^5	Yes	6.57×10^3	6.57×10^5
11	2.74×10^5	Yes	7.09×10^3	7.09×10^5	11	2.74×10^6	Yes	3.11×10^4	3.11×10^6
12	2.74×10^5	Yes	5.13×10^3	5.13×10^5	12	2.74×10^6	Yes	3.13×10^4	3.13×10^6
13	2.74×10^6	Yes	2.20×10^4	2.20×10^6	13	2.74×10^6	Yes	6.46×10^3	6.46×10^5
14	2.74×10^6	Yes	7.45×10^4	7.45×10^6	14	2.74×10^6	Yes	1.82×10^4	1.82×10^6
15	2.74×10^6	Yes	4.87×10^4	4.87×10^6	15	2.74×10^6	Yes	2.10×10^4	2.10×10^6

[*] Gn pigs were challenged with 2.74×10^3 to 2.74×10^6 viral RNA copies of human norovirus 092895 at 33-34 days of age. Rectal swabs were collected daily after challenge to determine virus shedding by conventional and real-time RT-PCR. Virus shedding was also detected in intestinal contents.

[†] Pigs were fed 8mg/day of simvastatin for 11 days prior to challenge.

[‡] Total amount of virus shed is based on an average intestinal volume of 100 ml.

group. All these data clearly indicate that simvastatin increased the susceptibility of Gn pigs to NoV infection and diarrhea. However, there were no significant reverse correlations between the peak virus titers shed and the cholesterol levels or the percentage reduction of cholesterol levels in the NoV-inoculated pigs at the time of virus inoculation (Table 1).

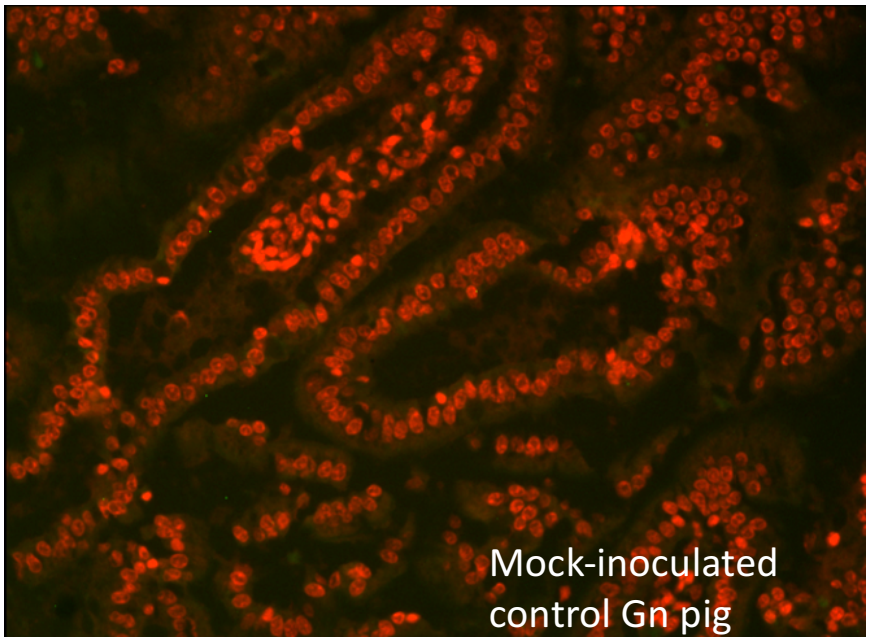
2.4.6 Determination of ID_{50} for the GII.4 092895 variant in neonatal and older pigs, and lowering of the ID_{50} in the older age group by simvastatin

From the titration of dosages administered to the Gn pigs, the ID_{50} was determined using the Reed-Muench method (42) (see calculation formula in Methods). Pigs were considered infected if rectal swab or intestinal content samples tested positive by conventional RT-PCR or real-time RT-PCR. The ID_{50} for neonatal pigs was equal to or less than 2.74×10^3 viral RNA copies as the lowest dose administered produced a 50% infection rate. Among the older pigs, simvastatin feeding lowered the ID_{50} compared to the simvastatin-free pigs. With simvastatin feeding, the ID_{50} for 33-34 day old pigs was below 2.74×10^3 RNA copies as 75% of the pigs were infected at this dose (Table 3). Simvastatin-free pigs at 33-34 days of age had a higher ID_{50} of 6.43×10^4 RNA copies.

2.4.7 Detection of NoV antigen in the duodenal villi of infected pigs

Presence of human NoV viral capsid protein in the duodenal villi of a pig receiving simvastatin and inoculated with 2.74×10^6 viral RNA copies and euthanized at PID 3 (37 days of age) was demonstrated in Figure 2. Prominent NoV antigen was detected at the damaged tips of

(a)



(b)

Figure 2. Detection of NoV antigen in intestinal tissue by immunohistochemistry. Paraffin-embedded sections of the duodenum of NoV 092895 infected or mock-infected Gn pigs were stained with guinea pig anti-NoV serum and FITC-conjugated rabbit anti-guinea pig IgG (green). Nuclei were stained with propidium iodide (red). (a) Duodenal tissue from a mock-inoculated Gn pig at 37 days of age. (b) Duodenal tissue from NoV infected Gn pig at PID3 (37 days of age), depicting NoV antigen in intestinal epithelial cells of the villi and in exfoliated enterocytes (arrow).

the duodenal villi. NoV antigen was not detected in the duodenum of the mock-inoculated Gn pigs (Figure 2a) or the jejunum of any pigs (data not shown). Other strains of human NoV have previously been indicated to preferentially infect the proximal small intestine in the Gn pig and calf models based on histopathological changes and occurrence of antigen in enterocytes (14, 15)

2.4.8 Cytopathological changes in the duodenum and jejunum in infected pigs

Histologically, no major changes were observed in hematoxylin and eosin stained intestinal sections of the infected pigs (data not shown). However, moderate to severe cytopathological changes were noted in both the duodenum and jejunum of infected neonatal and older pigs compared with mock-inoculated controls. Representative transmission electron microscope (TEM) images of these intestinal changes from pigs, along with mock-inoculated controls, are depicted in Figure 3. All of the images in Figure 3 are from simvastatin-fed pigs. Major changes that were most often observed and were indicative of enteric disease included irregularity, blunting and shortening of microvilli, and cytoplasmic vacuolization. These changes were sometimes associated with signs of necrosis and apoptosis of enterocytes. Mock-inoculated control pigs (simvastatin-fed) did not develop these cytopathological changes, suggesting that the cytopathological changes are not due to simvastatin.

2.5 Discussion

In this study, we confirmed that Gn pigs are able to be infected by human NoVs and that this susceptibility can be altered by simvastatin treatment (25). Furthermore, we have evaluated

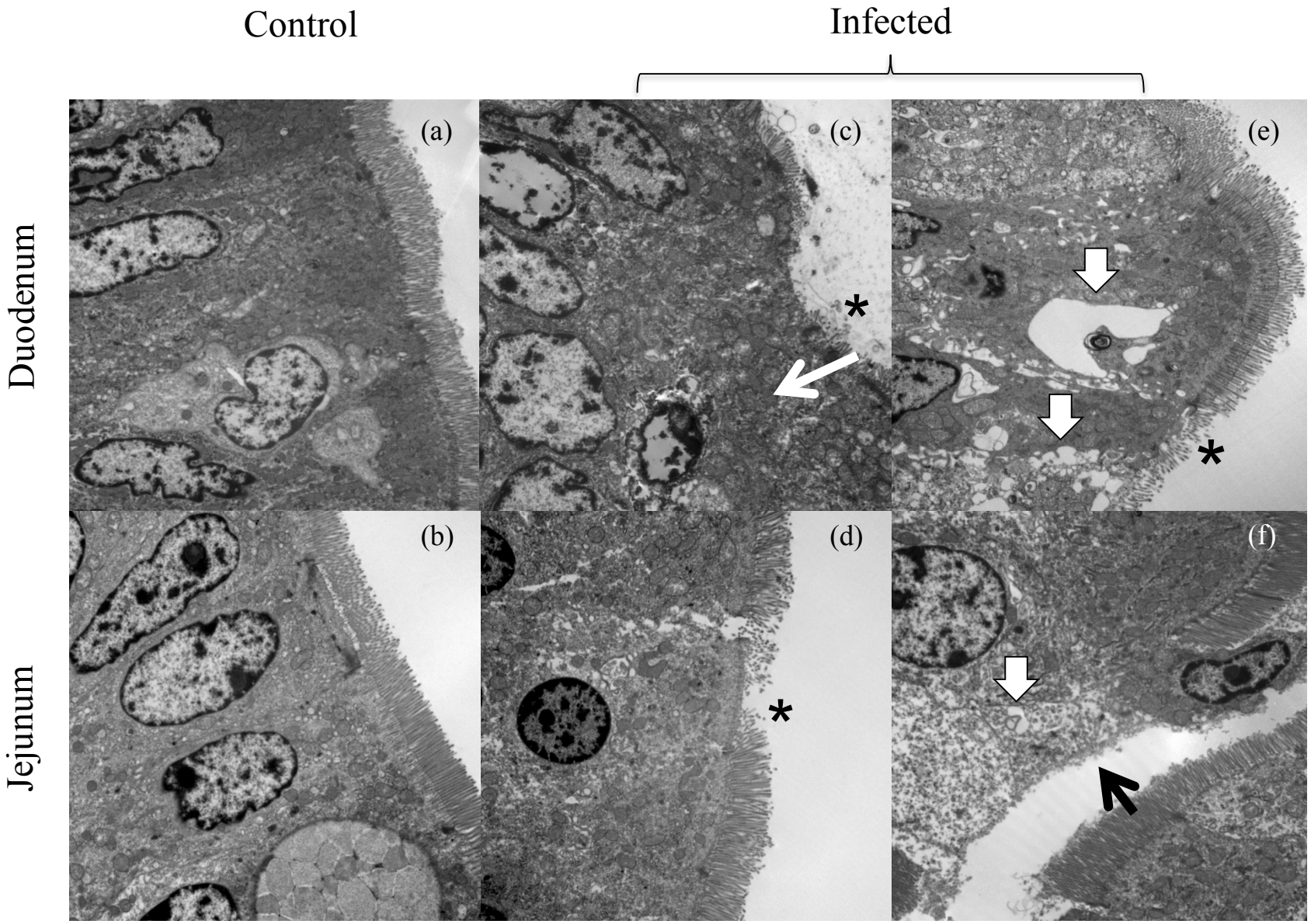

Control
Infected
Duodenum
Jejunum
(a)
(b)
(c)
(d)
(e)
(f)
*
*
*
5000x

Figure 3. Cytopathological changes in the duodenum and jejunum of Gn pigs infected with NoV 092895. Control pigs that received diluent #5 (a, b). Infected pigs (c-f) that received 2.74 x 10^6 RNA copies of NoV 092895. All the pigs were treated with simvastatin. Major changes observed in NoV-infected pigs included irregular microvilli (asterisk), cytoplasmic vacuolization (block arrows). Signs of apoptosis (white arrow) included shrunken and darker cells, condensed nuclei, chromatin margination. Signs of necrosis (black arrow) included swollen cells, disrupted cell membranes, loss of organelles, damaged mitochondria.

the ID_{50} of a particular strain of human GII.4 NoV both with and without simvastatin treatment. Determination of the ID_{50} is critical in using the animal model in vaccine studies to ensure confident measurements of protective efficacy. Gn pigs are one of the few candidates for an animal model of human NoV infection and disease. All pigs used in this study were A+, H1+ or H2+. The susceptibility of the pigs to the NoV 092895 variant infection did not differ among the A+, H1+ and H2+ types (data not shown). Differences in susceptibility of infection between the neonatal pigs and the older pigs (both free of simvastatin) was observed, likely reflecting the difference in the maturation status of the immune system of the two age groups. The neonatal pigs were more prone to infection, having a lower ID_{50} of equal to or less than 2.74×10^3 viral RNA copies. The ID_{50} was approximately 23-fold higher at 6.43×10^4 viral RNA copies in the older pigs. The higher dose required to infect the older pigs is probably due to the more developed innate immune system of these pigs. NoV has been reported to affect the young and the elderly to a greater degree, causing more severe symptoms and longer duration of illness (46-48).

In previous reports, Gn pigs infected by a GII.4 or GII.12 NoV strain developed mild diarrhea (14, 18, 19, 26). We demonstrated clearly that simvastatin increased susceptibility of infection and incidence of diarrhea induced by the GII.4 2006b variant in pigs. The ID_{50} of the simvastatin fed pigs inoculated at 33-34 days of age was lower than the simvastatin-free older pigs and possibly equal to or lower than that of the neonatal pigs. However, we did not reach the ID_{50} dose as the lowest inoculum of 2.74×10^3 RNA copies induced a 75% shedding rate. Simvastatin has been reported to be an immunosuppressive agent through down-regulation of innate cytokine IFN-α response and MHC class II dependent T cell activation, which may explain

its ability to increase susceptibility to human NoV infection and the incidence of diarrhea in Gn pigs (25, 49). Overall though, the ID_{50} of the human GII.4 2006b variant in Gn pigs is higher than what has been reported in humans since the human NoV has to overcome species barriers in order to infect pigs. For adult humans, Norwalk virus (GI.1) has a low ID_{50} of only 4.8 RT-PCR units (50, 51).

It is important to note that higher incidences of diarrhea were observed among the infected pigs treated with simvastatin compared to those not receiving simvastatin. In the study using GII.4 strain HS194 (99.6% capsid protein VP1 sequence identity with 092895) (25), it was observed that simvastatin alone caused mild diarrhea up to 5 days post treatment. It is possible that simvastatin alone might have contributed in part to the increased incidence of diarrhea in the simvastatin-fed pigs seen in this study. However, clinical signs of diarrhea were observed in the 092895-variant-infected simvastatin-free pigs, but not in the HS194-infected simvastatin-free pigs. A different GII.4 strain, HS66 (93.9% capsid protein VP1 sequence identity with 092895), used to infect Gn pigs in previous studies was able to cause mild signs of diarrhea (14, 18-20). A GII.12 strain, HS206, has also been reported to cause mild diarrhea (26). Therefore, it seems that NoV strains may vary in their ability to cause diarrhea in Gn pigs.

Strain variability may also affect the levels of virus shedding observed in feces of NoV infected Gn pigs. Previous NoV studies using the Gn pig model also reported low genomic copy numbers of virus shedding in feces (14, 18, 19). Inoculation of Gn pigs with approximately 2.7 x 10^6 genomic equivalents of NoV GII.4 variant HS66 led to 73% of pigs shedding virus in feces as detected by RT-PCR (14). Comparatively, in the older age group in the present study, 100% of pigs receiving 2.74 x 10^6 RNA copies of 092895 with or without simvastatin exhibited fecal virus

shedding. With simvastatin feeding prior to virus inoculation, a 100% shedding rate was seen in pigs receiving as few as 2.74 x 10^4 RNA copies. Even with the simvastatin feeding, the possibility does exist that the strain used in this study was better adapted to a porcine host than GII.4 variants used in previous studies and is more infectious in pigs. Yet, the virus shedding titers of 092895 in Gn pigs were much lower compared to that of humans, even after higher doses up to 10^6 viral copies were administered. In human volunteers inoculated with Norwalk virus, the median peak of virus shedding was 9.5 x 10^{10} genomic copies/g feces (51), whereas the peak titers of virus shed in Gn pigs ranged from 10^3 to 10^7 genomic copies/ml feces in the present study. An emerging GII.12 NoV strain induced shedding titers of 1 x 10^5 to 2 x 10^7 genomic equivalents/ml following challenge with 6 x 10^{10} genomic equivalents (26). Further adaptation may occur with serial passage of the intestinal contents and a subsequent increase in virus replication and shedding occurring in Gn pigs, although it may take many passages for this to progress. Increased susceptibility to human NoV infection was accomplished with administration of simvastatin prior to virus inoculation of the Gn pigs. The geometric mean peak titer shed in the simvastatin-treated animals did not vary greatly among the various infectious doses administered, which may reflect species barrier in infectivity of NoV. Continued use of simvastatin in conjunction with serial passaging of the intestinal contents in Gn pigs may improve the chance of selecting for a better porcine-adapted strain of 092895.

Presence of abundant NoV antigen after inoculation of Gn pigs with NoV strain 092895 was demonstrated by immunofluorescence staining in the atrophied villi of the duodenum at PID 3. Previous studies in Gn pigs also reported detection of NoV antigen in enterocytes of duodenum and jejunum following infection with either GII.4 strains HS66 or HS194 (14, 25), whereas studies

of biopsies from NoV-inoculated chimpanzees reported that NoV antigens were detected in scattered dendritic cells and B cells of lamina propria (21). The reason for the discrepancy is unknown. It has been shown that Norwalk virus does not replicate in human macrophages or dendritic cells derived from the peripheral blood of susceptible humans (52). One of the possibilities is that NoV replication was not occurring in dendritic cells and B cells but, instead, these cells took up viral capsid antigen by phagocytosis. In addition, there were differences in NoV genogroup and species in the pig model versus non-human primate model studies. Chimpanzee studies used Norwalk virus (GI.1) and Gn pig studies used GII.4 variants.

Supporting the occurrence of infection, the immunofluorescence and TEM images of the small intestinal tissues from 092895-infected pigs displayed severe cytopathological changes that are characteristic of gastroenteritis. In contrast, HS66-infected pigs showed only very subtle changes in intracellular morphology observable only by TEM (14). HS194-infected pigs also did not demonstrate histological changes in either the small or large intestine (25). These differences in cellular damage may be due to the better adaptability of strain 092895 in porcine hosts compared to HS66 and HS194 or possibly due to the use of simvastatin to increase susceptibility to NoV infection. Most probably, a combination of the two factors contributed to the severity of the cytopathological changes that were observed. In chimpanzees, although virus antigen was detected in the lamina propria, no histopathological changes were observed (21). In human volunteers inoculated with Norwalk (GI.1) or Hawaii virus (GII.1), histological changes were evident in proximal intestinal biopsy samples (53). The researchers described blunted villi with decreased length of microvilli, mitochondrial enlargement and paleness, cytoplasmic vacuolization, and intracellular edema. These changes are very similar to what we observed in

the duodenum and jejunum of 092895-infected pigs. Thus, the Gn pig 092895 variant challenge model more closely mimics the pathological changes in humans than the HS66- or HS194-Gn pig model and the Norwalk-virus chimpanzee model.

In conclusion, Gn pigs are a better animal model of human NoV infection and disease than other currently available candidates. Using the GII.4 variant 092895, we report similar findings to previous studies of human NoV pathogenesis in Gn pigs, including fecal virus shedding and mild diarrhea (14). We demonstrated that simvastatin increased susceptibility of infection and more importantly the incidence of diarrhea in Gn pigs inoculated with a GII.4 NoV variant. The presence of clinical signs and cytopathological changes following NoV infection is an advantage of this model compared to other animal models. Infection of mice with murine NoVs or non-human primates with human NoVs does not induce gastrointestinal disease (16, 21, 23). In addition, Gn pigs were inoculated orally whereas chimpanzees were inoculated intravenously, which does not mimic the natural route of NoV infection. Using this Gn pig challenge model, NoV vaccine-induced protection against infection and diarrhea can be evaluated, as well as the immune correlates of protective immunity.

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Chapter 3

Effects of racecadotril on weight loss and diarrhea due to human rotavirus in neonatal gnotobiotic pigs (*Sus scrofa domesticus*)

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3.1 Abstract

Diarrheal disease is the second leading cause of death in children younger than 5 y, and the most common cause of acute watery diarrhea in young children worldwide is rotaviral infection. Medicines to specifically reduce diarrhea would be a desirable adjunctive treatment to supportive fluid therapy to decrease the mortality rate of diarrheal diseases. In this study, we evaluated the efficacy of an antisecretory drug, racecadotril, in treating human rotavirus (HRV)-induced diarrhea in a neonatal gnotobiotic pig model. In total, 27 gnotobiotic pigs were randomly assigned (*n* = 9 per group) to receive either racecadotril, chlorpromazine (positive-control drug), or PBS (mock treatment) after inoculation with HRV. Pigs were weighed daily and rectal swabs were collected to determine fecal consistency scores and virus shedding. Rotaviral infection was confirmed by ELISA and cell culture immunofluorescence. Overall, the racecadotril-treated pigs had less severe illness than either the chlorpromazine- or mock-treated groups; this conclusion was supported by the lower fecal-consistency scores, shorter duration of diarrhea, and significant gain in body weight during the course of the study of the racecadotril-treated pigs. Through its influence on decreasing intestinal hypersecretion, racecadotril was better able to control the clinical signs of rotaviral infection in the gnotobiotic pigs. These results lend support for using racecadotril as a treatment for rotaviral diarrhea.

3.2 Introduction

Acute gastroenteritis is a common ailment, with approximately 179 million cases occurring in the United States annually (1). These infections are often self-limiting, but severe dehydration is a possible consequence and a potentially life-threatening concern, especially in

young children and the elderly. In young children, rotavirus is the main cause of acute viral diarrhea worldwide. Although effective vaccines (Rotarix and RotaTeq) exist, rotavirus accounts for 40% of hospitalizations for acute gastroenteritis in regions with limited implementation of vaccination (2). In general, the basis of treatment involves supportive care with oral rehydration solution to counteract intestinal losses of water and electrolytes. Intravenous fluid therapy and other medical interventions may be necessary in critical cases (3, 4).

Additional therapeutic drugs have been recommended in the treatment of acute gastroenteritis, specifically those with the ability to shorten the duration of diarrhea. Many of these medications (loperamide, codeine, morphine) are μ-opioid receptor agonists and reduce diarrhea by prolonging intestinal transit time. Unfortunately, potential side effects of decreased gut motility include constipation, abdominal discomfort, and bacterial overgrowth (5-8). These concerns have thus limited the use of loperamide to children older than 2 y(7). The neutral endopeptidase inhibitor racecadotril has been evaluated as a suitable alternative, because of its high safety profile and tolerability. Racecadotril reduces hypersecretion in the intestine through the inhibition of enkephalinases and subsequent increase in endogenous enkephalins, which have antisecretory effects. As such, it specifically addresses a common cause of intestinal water loss in acute infectious diarrhea without affecting gut motility (6, 9). Studies have reported similar efficacy of racecadotril compared with loperamide (7, 8, 10, 11).

In the current study, we assessed the efficacy of racecadotril in the treatment of human rotavirus (HRV)-induced diarrhea in a well-established gnotobiotic pig model of HRV diarrhea (12). Pigs represent an ideal species for evaluating gastrointestinal physiology, disease, and treatments. Although the gross anatomic configuration of the pig gastrointestinal tract may

differ compared with that of humans, numerous reports have highlighted the similarities in regard to metabolism, function, and susceptibility and response to disease (13-18).

Racecadotril was first approved for pediatric use in acute diarrheal cases in France in 1999 and has since been available in several other European countries but not in the United States (19, 20). However, because of the many causative agents of diarrhea, additional evaluation of the effectiveness of racecadotril in specifically reducing rotaviral diarrhea is warranted if the drug is to be used routinely in treatment protocols. Moreover, the gnotobiotic pig model allows for the direct assessment of racecadotril in treating HRV diarrhea with fewer confounding variables, including compliance, vaccine status, and possible coinfections, than in human clinical studies.

We compared the efficacy of racecadotril with that of chlorpromazine, which has been demonstrated to reduce diarrhea due to enterotoxigenic *E. coli* and transmissible gastroenteritis virus in piglets (21, 22). To our knowledge, this study is the first to specifically evaluate the effectiveness of racecadotril in a gnotobiotic pig model of rotaviral diarrhea.

3.3 Materials and Methods

3.3.1 Animals, diets, housing conditions, and health monitoring

All animal experimental procedures were conducted in accordance with protocols approved by the IACUC of Virginia Tech (protocol no. 13-187-CVM). Euthanasia was performed in accordance with recommendations from the *AVMA Guidelines for the Euthanasia of Animals*.

Near-term Yorkshire cross pigs (*Sus scrofa domesticus)* were derived by hysterectomy and maintained in custom HEPA-filtered germ-free isolator units as previously described (23). Immediately after derivation, all pigs received an intramuscular injection of iron dextran (150 mg) to prevent iron deficiency and subsequent anemia.

Pigs were housed in groups of 2 to 4 per isolator, with each pig in an individual section separated by stainless steel dividers. Stainless steel floors slotted with small holes (approximately 1 cm in diameter and spaced 2 cm apart) facilitated passage of waste material into a pan below the false bottom. Each pig was provided a sterile surgical towel for bedding. A rubber barbell toy was provided for stimulation and enrichment purposes. Environmental conditions were maintained as a 12:12-h light:dark cycle and room temperature of 93 to 95 °F (33.9 to 35.0 °C).

Throughout the study, pigs were fed commercial, sterile (ultra-high temperature-treated) cow's milk 3 times daily. The amount of milk fed increased in conjunction with age. One-week old pigs received a total of 295 mL of milk per day, while 2-wk old pigs received 354 mL. Pigs were kept for a maximum of 9 d of age in this study.

During the daily feedings, pigs were monitored for clinical signs of lethargy, inappetence, and dehydration. Because pigs were fed a set amount at each feeding, failure to finish the previous meal warranted increased concern. When a pig showed any of these signs, its health status was reevaluated every 2 to 4 h throughout the day. Continued lack of improvement necessitated euthanasia as a humane endpoint.

3.3.2 Sterility

Isolator units and all supplies requiring passage into the isolators were sterilized by using a solution of 0.06% peroxyacetic acid–0.08% H_2O_2 (catalog no. 6526Q8, Spor-Klenz Ready-To-Use Solution, STERIS Life Sciences, Mentor, OH)]. Sterility of equipment, reagents, and pigs was confirmed throughout the study through culturing on blood-agar plates and in thioglycollate medium. All gnotobiotic isolators were deemed sterile by swabbing both the bottom grates and top canopy. All inocula and medications were tested for sterility prior to passage into the isolators. Rectal swabs were collected from all pigs prior to inoculation and at euthanasia to confirm continued sterility.

3.3.3 Virus

The virulent HRV Wa strain (G1P1[8]) was passaged through gnotobiotic pigs; the intestinal contents from the 27th passage were collected and used for viral challenge at a dose of approximately 10^5 focus-forming units. The virus titer was determined with cell culture immunofluorescent assay and was reported as number of focus-forming units per milliliter, as described previously (24).

3.3.4 Drug treatment groups and inoculation of gnotobiotic pigs

In total, 27 pigs from 4 litters were randomly assigned to 3 treatment groups (*n* = 9 per group) to receive either (catalog no. 020441, United States Biological, Salem, MA) chlorpromazine (catalog no. 029700, MWI Veterinary Supply, Boise, ID) or PBS. Three pigs from each group were euthanized on postinoculation days (PID) 2, 3, and 4. Pigs were orally

inoculated with virulent Wa HRV at 5 d of age (PID0); 20 min prior to viral challenge, the gnotobiotic pigs received 4 mL 200 mM sodium bicarbonate to neutralize stomach acids. Treatments were initiated on PID1 and continued until the day of euthanasia. Gnotobiotic pigs in the racecadotril group orally received 80 mg/kg racecadotril diluted in 5 mL PBS every 8 h. The chlorpromazine-treated group received 2 mg/kg chlorpromazine (25 mg/mL) intramuscularly every 24 h; this dose of chlorpromazine was selected in light of previous studies (21, 22) that demonstrated efficacy in treating diarrhea caused by enterotoxigenic *E. coli* and transmissible gastroenteritis virus. Mock-treated pigs received 5 mL PBS orally every 8 h.

All oral inoculations were slowly administered in 1-mL boluses by using needleless syringe feeding, with pigs held in an upright position, and ensuring that swallowing had occurred prior to delivering the next bolus. The maximal volume of chlorpromazine for intramuscular injection did not exceed 0.18 mL.

3.3.5 Sample collection

Gnotobiotic pigs were rectally swabbed daily to assess clinical signs of infection (that is, diarrhea) and virus shedding. Rectal swabs were processed by swirling 2 swabs in 8 mL of MEM containing 1% penicillin and streptomycin and 1% HEPES (diluent no. 5), followed by centrifugation at 2100 rpm for 15 min at 4 °C. After euthanasia, the entire intestinal tract with contents was weighed, and the volumes of the small intestinal contents and large intestinal contents were measured. Intestinal contents were diluted 1:2 (small intestine) and 1:3 (large intestine) in diluent no. 5 and underwent the same centrifugation protocol as for the rectal swabs. The resulting supernatant was saved for evaluation of virus shedding. Blood samples at

euthanasia were collected in acid–citrate–dextrose to measure plasma levels of thiorphan (25) which is the active metabolite of racecadotril.

3.3.6 Assessment of rotavirus diarrhea and virus shedding

Rectal swabs were scored for fecal consistency (26) as follows: 0, normal consistency; 1, pasty; 2, semiliquid; and 3 liquid. Pigs with scores of 2 or greater were considered to have diarrhea. Prior to inoculation, all pigs were considered to have either normal or pasty stool (that is, no evidence of diarrhea). Swabs were collected each morning, at 2 h after the pigs' first meal. Dehydration in gnotobiotic pigs was monitored by following changes in daily weight. Pigs were weighed each morning, after rectal swab collection, by using a spring scale and rope fastened around the hock.

Viral antigen in daily rectal swabs, small intestinal contents, and large intestinal contents was detected by ELISA and the infectious virus titer determined by cell culture immunofluorescence, as previously described (24). Briefly, for the ELISA, 96-well microtiter plates were coated with goat antibovine rotavirus antibody (100 µL/well; catalog no. PA1-7241, ThermoFisher Scientific, Waltham, MA) diluted 1:250 in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The following day, plates were washed twice with PBS–0.05% Tween 20 (PBST), blocked with PBS (pH 7.4) containing 5% nonfat dry milk (300 µL/well), and incubated for 1 h at 37 °C. Samples diluted in PBS containing 0.1% BSA were then added (100 µL/well) in duplicate and incubated for 1 h at 37 °C, followed by the addition of goat antibovine rotavirus antibody conjugated with horseradish peroxidase (catalog no. PA1-73015, ThermoFisher Scientific) diluted 1:200 in PBS–1% BSA and incubation for 1 h at 37 °C. Plates

were developed by adding ABTS peroxidase substrate solution (100 µL/well; catalog no. 50-62-00, KPL, Gaithersburg, MD) and incubating for 15 to 30 min at room temperature and then adding ABTS stop solution (100 µL/well; catalog no. 50-85-01, KPL). Unless otherwise specified, plates were washed 3 times with PBST after each incubation period. Semipurified attenuated HRV antigen was used as a positive control; supernatant of mock-infected MA104 cells was used as a negative control. Optical density values were measured at 405 nm.

For cell-culture immunofluorescence, 96-well plates containing confluent African green monkey kidney cells (MA104 cells; catalog no. CRL-2378.1, ATCC, Vienna, VA) were washed with PBS and incubated with 100 µL Eagle minimal essential medium for 2 h at 37 °C with 5% CO_2. The media were then discarded, and 10-fold serial dilutions of samples were added (50 µL/well) in duplicate. The plate was centrifuged at 2000 rpm for 1 h at 21 °C with slow deceleration. Trypsin diluted in Eagle minimal essential medium was then added to a final concentration of 0.5 µg/mL and the plates incubated at 37 °C with 5% CO_2 for 18 to 24 h. After the samples were discarded, the plates were fixed with 80% acetone for 10 min at room temperature. The acetone was removed and the plates allowed to air-dry for 1 to 2 h. Fixed plates were stored at –20 °C for a maximum of 1 wk or were stained immediately.

Prior to immunofluorescence staining, the plates were rehydrated with PBST for 2 min at room temperature. Goat antibovine rotavirus antibody (50 µL/well; catalog no. PA1-7241, ThermoFisher Scientific) diluted 1:250 in PBST containing 2% nonfat dry milk was added and incubated for 1 h at 37 °C. Plates were washed 3 times with PBST, followed by the addition of FITC-conjugated rabbit antigoat IgG antibody (50 µL/well; catalog no. F7367, Sigma–Aldrich, St Louis, MO) diluted 1:400 in PBST containing 2% nonfat dry milk. Plates were incubated for 1 h

at 37 °C and then washed 3 times with PBS (pH 7.4) and once with PBS (pH 8.0). Mounting media (60% glycerol, 40% PBS [pH 8.0]) was added prior to evaluation by fluorescent microscopy.

Infection of all gnotobiotic pigs in the study was confirmed with no significant differences observed among groups in the onset or duration of shedding or in viral titer (data not shown).

3.3.7 Statistical analysis

Mean percentage of days with diarrhea was compared between treatment groups by using a linear mixed-effects model. The statistical analyses based on a linear mixed-effects model were performed by using R (R Development Core Team, 2015; www.r-project.org). Mean diarrhea scores and mean percentage of body weight gained were compared between treatment groups by using a one-way ANOVA general linear model, followed by the Duncan multiple-range test. Correlation between percentage of days with diarrhea and percentage of body weight gained was evaluated by using the Spearman rank correlation coefficient. The Kruskal–Wallis rank sum test was used to compare the AUC for diarrhea scores between treatment groups. Statistical significance for these tests was assessed at a *P* value of less than 0.05. Unless otherwise specified, statistical analyses were performed by using SAS version 9.4 (SAS Institute, Cary, NC) or Prism 6.0 (GraphPad Software, San Diego, CA).

3.4 Results

3.4.1 Detection of thiorphan in plasma after racecadotril administration

Because racecadotril is rapidly metabolized (3-h half-life in humans), we measured the plasma levels of its active metabolite, thiorphan (5). Pigs were euthanized approximately 9 h after receiving their last dose of racecadotril. Most pigs (7 of 9) had measurable levels of thiorphan, which ranged from 73 to 526 ng/mL (Table 1). The average plasma thiorphan level of these 7 pigs was 206.7 ng/mL.

3.4.2 Effects of racecadotril on the duration and severity of diarrhea in gnotobiotic pigs infected with HRV

HRV-induced diarrhea in gnotobiotic pigs occurs as early as 13 h after inoculation, and the severity peaks at PID2 to PID4 (27). Pigs were euthanized at different time points (PID2 to PID4) to ensure that the peak of clinical signs was not missed. The incidence of diarrhea was expressed as a percentage of days from PID1 until euthanasia for each subset of pigs (Table 2). In general, in each of these subsets of pigs, the mock group tended to have more days with diarrhea compared with either the chlorpromazine or racecadotril groups. There were no significant differences between groups for pigs euthanized on PID2, but the chlorpromazine-treated pigs had a significantly ($P < 0.05$) shorter mean duration of diarrhea (33.3% of days) compared with both the racecadotril and mock-infected groups (55.6% for both) on PID3. When pigs were retained for a longer duration (PID4), the racecadotril group had significantly ($P < 0.05$) fewer days with diarrhea (33.3%) than either of the other 2 groups. Interestingly, the pigs given chlorpromazine had diarrhea for the majority of the 4 d (91.7%), whereas the mock group experienced diarrhea for an average of 3 d (75%). However, when all gnotobiotic pigs within a

Table 1. Plasma thiorphan concentrations in Gn pigs treated with racecadotril (dose, 80 mg/kg)

Pig ID	Euthanasia day	Thiorphan concentration (ng/ml)
1	PID2	78.6
2	PID2	73
3	PID2	141
4	PID3	121
5	PID3	526
6	PID3	165
7	PID4	BLQ[b]
8	PID4	BLQ
9	PID4	342

BLQ = Below the lower limit of quantitation (4.62 ng/ml).
Blood samples were obtained at euthanasia approximately nine hours after racecadotril administration.

Table 2. Percentage of days with diarrhea (relative to PID1; mean ± SEM) in gnotobiotic pigs infected with human rotavirus and treated with PBS (mock), racecadotril, or chlorpromazine

Euthanasia day	Mean percentage of days with diarrhea		
	Mock	Racecadotril	Chlorpromazine
PID2	33.3 (16.7)[A]	16.7 (16.7)[A]	16.7 (16.7)[A]
PID3	55.6 (22.2)[A]	55.6 (11.1)[A]	33.3 (0.0)[B]
PID4	75.0 (14.4)[B]	33.3 (16.7)[C]	91.7 (8.3)[A]
Group mean	54.6 (10.9)	35.2 (9.4)	47.2 (12.6)

Each treatment group comprised 9 gnotobiotic pigs, 3 of which were euthanized on either PID2, 3, or 4.

For a given day, values with different letters differ significantly (linear mixed-effects model, $P < 0.05$).

Table 3. Summary of diarrhea data analysis using linear mixed-effects model

Comparison of group	Mean difference	P[a]
Mock vs. racecadotril	-28.255	0.00766
Mock vs. chlorpromazine	-8.310	0.60805
Racecadotril vs. chlorpromazine	-36.565	<0.001

[a]The number of mean percentage of days with diarrhea was compared between groups; $P < 0.05$ indicates a significant difference between groups.

specific treatment group were pooled and evaluated by using a linear-mixed effects model (Table 3) the racecadotril group had significantly ($P < 0.05$) shorter duration (35.2% of days with diarrhea) than pigs given either PBS (54.6%) or chlorpromazine (47.2%). There was no difference in duration of diarrhea between the PBS- and chlorpromazine-treated pigs.

In terms of severity of diarrhea, the racecadotril group was observed to have less watery stools. The daily mean diarrhea scores for each treatment group (Figure 1) indicate that the scores for the racecadotril group were nonsignificantly lower than those for mock-treated pigs and significantly ($P < 0.05$) lower than those for chlorpromazine-treated pigs on PID2 and PID4. No other significant differences were observed between any other groups on any other day. However, it is important to note that the mean daily diarrhea score of the racecadotril group never reached a '2,' which was the minimal score clinically associated with diarrhea in this study. Furthermore, although it did not achieve significant difference (Kruskal–Wallis rank sum test, $P < 0.05$), the AUC for the diarrhea scores was the lowest in the racecadotril-treated pigs (4.750), compared with 5.139 and 6.472 for the mock and chlorpromazine groups, respectively.

3.4.3 Efficacy of racecadotril in reducing dehydration in gnotobiotic pigs infected with HRV

The body weight of the gnotobiotic pigs was monitored daily, and a decrease in weight was considered to be indicative of diarrhea and subsequent water loss. Conversely, weight gains demonstrated a reduction in sensible water loss through diarrhea and possibly allocation of energy toward growth as well. The daily mean body weight of individual pigs for each treatment group (Figure 2) and shows an upward trend in body weight for the pigs receiving

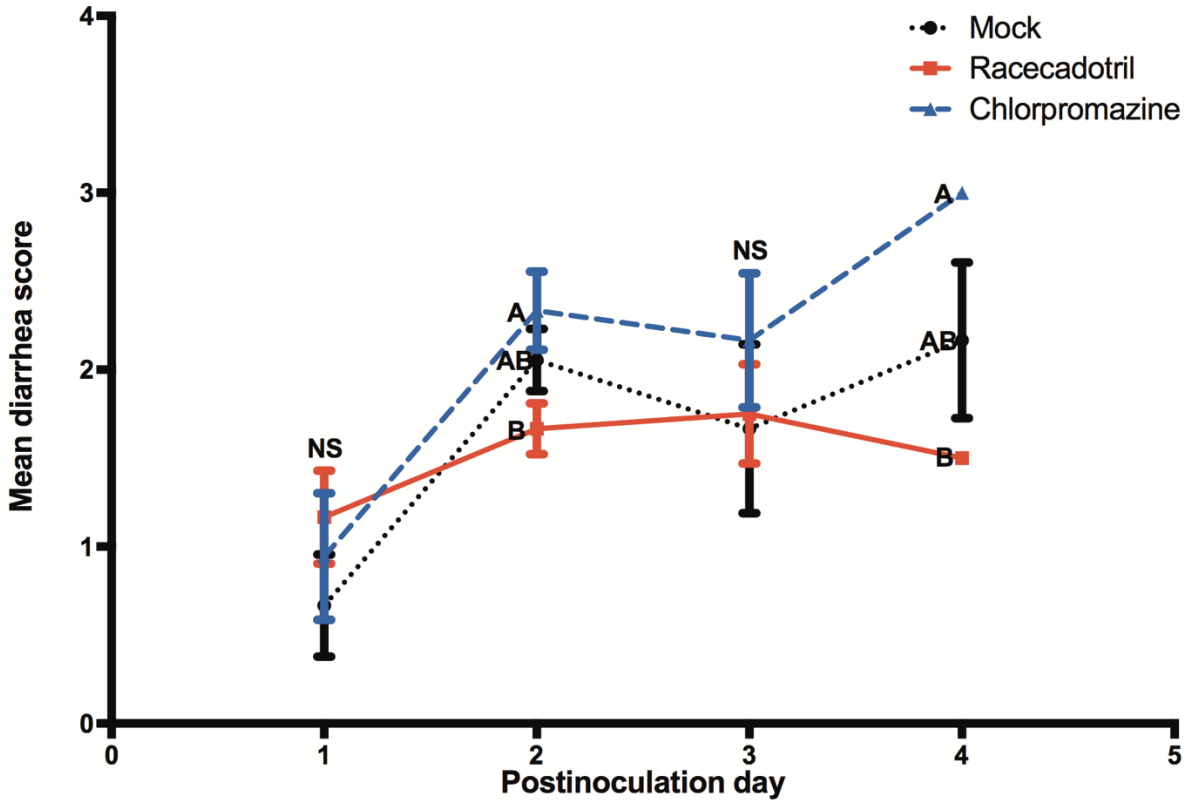

Figure 1. Mean diarrhea score after infection with human rotavirus and treatment with PBS (mock), racecadotril, or chlorpromazine. After inoculation, fecal consistency scores (0, solid; 1, pasty; 2, semiliquid; 3, liquid) were determined daily until euthanasia at PID2, 3, or 4. Pigs with scores of 2 or greater were considered to have diarrhea. The mean diarrhea score for each treatment group was then calculated for a given PID (PID1, *n* = 9; PID2, *n* = 9; PID3, *n* = 6; PID4, *n* = 3). Statistical significance was determined by one-way ANOVA, followed by the Duncan multiple-range test. Different letters (A, B) on the same PID indicate scores that differ significantly ($P < 0.05$). Error bars indicate SEM.

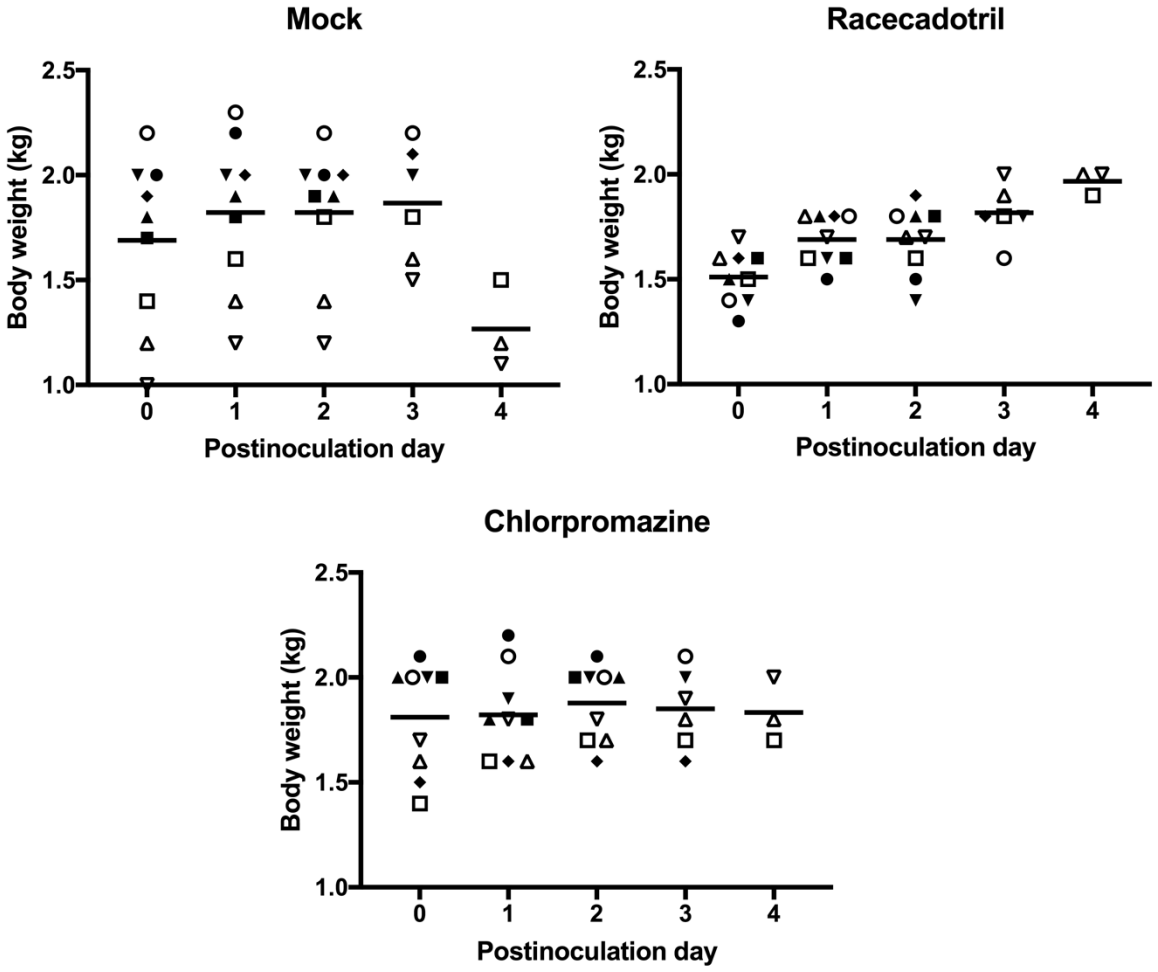

Figure 2. Changes in body weight (kg) after infection with human rotavirus and treatment with PBS (mock), racecadotril, or chlorpromazine. Gnotobiotic pigs were weighed daily from PID0 until euthanasia at PID2, 3, or 4. The daily body weight (kg) of individual pigs from each treatment group (PID1, *n* = 9; PID2, *n* = 9; PID3, *n* = 6; PID4, *n* = 3) is shown. The black bars indicate the mean body weight of each group on each day.

racecadotril. Chlorpromazine-treated pigs appeared to maintain their body weight throughout the study, whereas the mock-treated pigs experienced a noticeable decrease in body weight after PID3, following an increase in diarrhea severity (Figure 1).

Table 4 depicts the changes in body weight during the course of the study as a percentage of initial body weight at PID0. For each subset of pigs euthanized on PID2 to PID4, the racecadotril group gained a greater percentage of their initial body weight. This increase was significant ($P < 0.05$) at all time points when compared with the mock-treated pigs and on PID2 and PID3 compared with the chlorpromazine-treated pigs. Thus, the racecadotril-treated pigs were overall able to gain significantly more weight (19.2%; $P < 0.05$) relative to their weight on PID0, compared with the mock (5%) and chlorpromazine (7%) groups.

As anticipated, these changes in body weight were associated with the duration of diarrhea, because the groups with a higher percentage of days with diarrhea (Table 2) had a subsequently decreased gain in body weight (Table 4). These measures were significantly correlated (Spearman rank correlation coefficient, $r = 0.7501$, $P < 0.05$) in the chlorpromazine group, although not in the mock or racecadotril groups. However, with the significant increase in weight observed in the racecadotril-treated pigs, it is reasonable to conclude that the drug was effective in reducing dehydration due to its antisecretory effects.

Table 4. Body weights (% relative to PID0, mean ± SEM) in gnotobiotic pigs infected with human rotavirus and treated with PBS (mock), racecadotril, or chlorpromazine

Euthanasia day	Mean percentage of body weight gained		
	Mock	Racecadotril	Chlorpromazine
PID2	5.8 (3.4)^{B}	16.0 (2.2)^{A}	0.0 (0.0)^{B}
PID3	3.5 (3.5)^{B}	18.5 (5.1)^{A}	3.9 (2.0)^{B}
PID4	5.7 (3.0)^{B}	23.1 (2.8)^{A}	17.2 (2.6)^{A}
Group mean	5.0 (1.7)^{B}	19.2 (2.1)^{A}	7.0 (2.8)^{B}

Each treatment group comprised 9 gnotobiotic pigs, 3 of which were euthanized on PID2, 3 or 4.

For a given day, values with different letters differ significantly (one way ANOVA, $P<0.05$)

3.5 Discussion

Overall, the results of this study highlight the benefit of racecadotril in the treatment of acute diarrhea caused by rotavirus infection. In accordance with its antisecretory effects, racecadotril ameliorated HRV-induced diarrhea and prevented weight loss due to dehydration.

The diarrhea caused by rotavirus infection has been characterized as both osmotic and secretory in nature (28, 29). Malabsorption occurs due to the disruption of intestinal villi and decrease in absorptive surface area, leading to osmotic diarrhea. In addition, NSP4 enterotoxin has been suggested to contribute to a secretory component of rotavirus-induced diarrhea by producing a net chloride secretion. Therefore, the HRV-induced diarrhea in the gnotobiotic pigs was expected to be responsive to racecadotril treatment.

Racecadotril has been recognized as a potential adjunctive treatment for acute diarrhea due to its antisecretory properties. Several studies have reported improved resolution of diarrhea compared with placebo control groups in both adults (30) and children (19, 31, 32). In addition, racecadotril has demonstrated comparable efficacy to loperamide treatment (7, 8, 10, 11). However, a couple of studies determined that racecadotril did not significantly improve diarrhea in children with acute gastroenteritis (9) or diarrhea in adults with severe cholera (33). These studies may represent both extremes of diarrhea severity, making the inclusion of racecadotril treatment inconsequential to the resolution of symptoms. The authors of a pediatric study (9) attributed the lack of response to the addition of racecadotril to oral rehydration solution as a result of the mild to moderate cases of diarrhea treated in their study. In those cases, the diarrhea may have been sufficiently self-limiting to not require additional treatment. In contrast to studies that enrolled children requiring hospitalization (31, 32), the

previous authors (9) treated and observed outpatient children; compliance was thus another confounding variable in that study. The authors of the adult study (33) suggested that the lack of benefit due to the addition of racecadotril to treatment protocols might reflect the substantial secretory diarrhea induced by cholera in these cases, which prevented racecadotril from reaching adequate therapeutic levels.

In the current study and others of rotoviral infection in gnotobiotic pigs, the diarrhea most likely would be characterized as mild to moderate in presentation. Thus, we were not necessarily concerned with inadequate drug absorption of the orally administered racecadotril. Indeed, the drug was detectable as its active metabolite thiorphan in 7 of the 9 pigs approximately 9 h after administration. Unfortunately, racecadotril pharmacokinetic studies in animal species other than humans are few (5) and are unavailable in pigs. Presuming that the metabolism in pigs is similar to humans might explain the lack of measurable levels in the 2 pigs euthanized on PID4. Because the drug does not accumulate in the body (34), these results do not imply lack of absorption. Rather, due to the short half-life and length of time that had passed prior to obtaining blood samples, the drug might simply have been eliminated in these particular pigs. The possibility of enterocyte destruction and malabsorption is not completely excluded, however. In a previous pathogenesis study of virulent Wa HRV in gnotobiotic pigs (26), the observed mild to moderate villous atrophy occurred 24 to 48 h after inoculation. Because the 2 pigs that had undetectable levels of thiorphan were in the PID4 group, villous atrophy is an additional potential explanation.

It is somewhat difficult to directly compare the current study with other studies of racecadotril treatment in humans because none exclusively evaluated rotavirus-induced

diarrhea. However, the authors of 2 previously mentioned pediatric studies (31, 32) observed that rotaviral status had no effect on differences between treatment groups. That is, similar efficacy was demonstrated in both rotavirus-infected and noninfected children. In contrast, a recent study conducted in both a hospital and community-setting in India found that racecadotril did not alter treatment outcomes compared with oral rehydration solution alone in either setting, regardless of rotavirus infection status (35). Treatment delay or coinfections with enteric pathogens causing nonsecretory diarrhea may have influenced the efficacy of racecadotril in that study (35). Therefore, the effect of racecadotril on rotavirus-induced diarrhea warrants further clarification in animal models.

As stated, the gnotobiotic pig model of HRV infection and disease presents a prime opportunity to evaluate the specific effect of racecadotril on HRV-induced diarrhea. Pigs in particular are well suited for comparative studies of human gastrointestinal illness owing to their analogous digestive physiology and susceptibility to disease. In addition to HRV, these similarities have been exploited to develop several disease models, including short bowel syndrome (36), intestinal ischemia–reperfusion injury (37), stress-induced intestinal dysfunction (38), and neonatal necrotizing enterocolitis (39) as well as *Helicobacter pylori* (40), *Cryptosporidium parvum* (41) and noroviral (42) infections. In terms of diarrheal diseases, the similarities between humans and pigs in intestinal villi structure, cell classification, and transit times (13, 14, 17) aid in elucidating pathophysiology. Furthermore, the mucosal permeability of gnotobiotic pigs has been reported to be similar to that in humans as well (43).

The results of the current study support the findings of clinical human trials (7, 8, 10, 11, 19, 30-32), which observed positive outcomes with racecadotril treatment of acute diarrhea.

Advantages of our study include the exclusive evaluation of rotavirus diarrhea without concerns regarding confounding coinfections and patient compliance. Following HRV infection, racecadotril reduced the severity of diarrhea (lower mean diarrhea scores; decreased AUC) and decreased the duration of symptoms (lower percentage of days with diarrhea) compared with the control pigs. More importantly, the racecadotril-treated gnotobiotic pigs consistently gained more weight, indicating reduced effects of dehydration due to diarrhea.

Like racecadotril, the antisecretory drug chlorpromazine led to fewer days with diarrhea and a subsequently greater increase in body weight compared with those of mock-treated pigs. However, the chlorpromazine-associated improvements in clinical presentation were not as pronounced as those observed in the racecadotril group. Given that both of these drugs ultimately reduce intestinal secretions by decreasing intracellular cAMP (9, 44), the side effects of chlorpromazine may have diminished its beneficial, antisecretory effects. Being a phenothiazine derivative, sedation, hypothermia, and hypotension are among the possible outcomes that can occur with chlorpromazine use. These effects can be dose-dependent: in a previous study (22) pigs were heavily sedated at a 5 mg/kg dose but less so at 2 mg/kg. However, this lower dose still exhibited beneficial antisecretory action, which thus influenced our dose selection. Because we did not expect any serious side effects at this dose, we did not specifically measure these parameters in our study. However, such undesirable effects have previously been reported in piglets given chlorpromazine to reduce diarrhea caused by coinoculation with enterotoxigenic *E. coli* and transmissible gastroenteritis virus(21, 44) Coinfection with these pathogens in pigs tends to result in much more severe diarrhea than HRV, which may have exacerbated the effects of chlorpromazine in these previous studies. Still,

it is reasonable to speculate that, due to biologic variation, the PID4 group of pigs may merely have been more susceptible to these negative effects, thus eliminating any favorable effects of chlorpromazine treatment.

As mentioned, one of the major advantages of racecadotril as an antidiarrheal drug is the minimal side -effects encountered. Indeed, in studies involving both children and adults, no more adverse effects than placebo have been reported (31, 33). For this reason, racecadotril has been licensed for use in children older than 3 mo. The combination of efficacy and safety makes racecadotril an ideal therapeutic drug, especially for use in young children, which is the most common age group affected by rotavirus. In addition, a 2012 study in the United Kingdom evaluated the cost effectiveness of racecadotril for treatment of acute watery diarrhea in children younger than 5 y (20). Using computer modeling analysis, the authors concluded that the use of racecadotril in conjunction with oral rehydration solution reduced costs associated with prolonged treatment and referrals. Therefore, the use of racecadotril in a similar adjunctive capacity may be advantageous in developing countries where HRV vaccine efficacy is problematic.

Limitations of this study include the inability to record the total number of bowel movements or total stool output (weight) per day due to the set-up of the germ-free isolators, where waste passes through the grated bottom. In addition, because only 3 pigs were included at each time point in each treatment group, significant differences might have been overlooked. Future studies would require additional pigs in each treatment group to confirm the effect of racecadotril in alleviating symptoms of diarrhea. The secretory component of rotaviral diarrhea and its response to racecadotril might be further elucidated by comparing the

moisture content of fecal samples (45) and monitoring changes in intestinal cAMP levels or ion channels. In addition, the model might be used to specifically evaluate the effect of racecadotril on other relevant human enteric pathogens, such as norovirus, as well.

In summary, racecadotril lessened the severity and duration of rotaviral diarrhea in gnotobiotic pigs. Clinical improvement or, alternatively, reduced dehydration was interpreted as a greater proportional increase in body weight gained. These findings support the use of racecadotril as an effective ancillary treatment for rotaviral diarrhea.

3.6 References

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Chapter 4

Divergent impacts of *Lactobacillus acidophilus* dosage on intestinal and systemic dendritic cells in gnotobiotic pigs infected with human rotavirus

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4.1 Abstract

Although commercial oral rotavirus vaccines are currently available, there are still many countries that have yet to incorporate these vaccines into their standard immunization protocols. Additionally, these vaccines have demonstrated reduced efficacy in developing countries. Therefore, development of successful treatments for rotavirus-induced diarrhea have the potential to make a valuable health impact. Particular interest has been afforded to the use of probiotics to reduce diarrhea in cases of acute gastroenteritis. The mode of action of probiotics is not completely understood and different strains and doses have been demonstrated to have varied effects. In this study, we investigated the influence of two different doses of *Lactobacillus acidophilus* NCFM (LA) strain on the modulation of dendritic cell (DC) responses in gnotobiotic (Gn) pigs before and after infection with virulent human rotavirus (VirHRV). Starting at three days of age, Gn pigs were administered either a low dose of LA (LoLA, accumulative dose of 2.11×10^6 CFU) or a high dose of LA (HiLA, accumulative dose of 2.22×10^9 CFU). Non-fed LA (ZeroLA) pigs served as a control. A subset of pigs from each group was challenged with VirHRV at 33 days of age [post-challenge day (PCD) 0]. Rectal swabs were collected daily to determine fecal consistency scores and virus shedding. Ileum, spleen and peripheral blood were collected at euthanasia on PCD 0 or 7 to isolate MNCs and evaluate plasmacytoid DC (pDC) and conventional DC (cDC) responses pre- and post-challenge by flow cytometry. HiLA, but not LoLA, significantly reduced clinical signs of diarrhea and this different effect was associated with the divergent innate immunomodulatory effects of the two doses. HiLA pigs induced overall higher TLR2 DC responses in most tissues pre- and post-challenge, while LoLA induced higher TLR3 and TLR9 responses in ileum and spleen. In terms of cytokine

production, IL-6 tended to be upregulated by LoLA pre-challenge, while HiLA more consistently upregulated IL-10 both pre- and post-challenge. HiLA was also able to induce both IFN-α (post-challenge) and TNF-α (pre- and post-challenge) responses in DCs, while LoLA only increased TNF-α-producing DCs pre- and post-challenge. These results indicated that LA at different dosages can induce different innate immune responses characterized by an anti-inflammatory DC profile induced by HiLA and a pro-inflammatory profile by LoLA with or without VirHRV infection. The findings provide new knowledge on dose-dependent influence of probiotics on host innate immune responses.

4.2 Introduction

Among acute gastroenteritis cases in young children worldwide, rotavirus infection is still the predominant cause and contributed to 215,000 deaths in 2013 (1). Since their commercial licensing in 2006 and 2008, the use of oral rotavirus vaccines (Rotarix and RotaTeq) has reduced morbidity and mortality, though the efficacy has been lower than desired in developing countries of Africa and Asia (2). Thus, interest has continued in identifying applicable adjunctive treatments for severe diarrhea and prevention of life-threatening dehydration associated with rotavirus infection.

Probiotics have long been considered beneficial in gastrointestinal health, among other functions (3, 4). The exact mechanism by which probiotics can alter the course of gastrointestinal infections and reduce diarrhea is incompletely understood, though may be related to their interactions with other microorganisms and the intestinal environment, impact on intestinal barrier integrity and influence on the host immune system (3, 5). Lactobacilli and

bifidobacteria are the most well-known probiotics in use (6) and their effects have been the focus of various studies, including those evaluating their immunomodulatory potential. The general consensus has been that these effects are both strain and dose-dependent (7-12).

Indeed, our previous study demonstrated that administration of a low dose of *Lactobacillus acidophilus* NCFM (LA) significantly increased IFN-γ producing T cell responses and downregulated regulatory T (Treg) cell responses in both intestinal and systemic lymphoid tissues of gnotobiotic (Gn) pigs immunized with an oral human rotavirus (HRV) vaccine. Conversely, high dose LA increased the frequencies of Treg cells in the same model (13). Another subsequent study demonstrated that only an intermediate dose of LA significantly enhanced rotavirus-specific antibody, antibody secreting cell and memory B-cell responses, as well as reduced rotavirus diarrhea (14).

In this present study, we hypothesize that different doses of probiotics LA differentially modulate innate immune responses, specifically dendritic cells (DCs), during rotavirus infection, leading to different effects on rotavirus diarrhea severity and development of adaptive immune responses. DCs are specialized innate immune cells that are capable of capturing antigens at peripheral sites in their immature state, migrating to lymphoid tissues upon maturation to present such components to T cells and subsequently manipulating adaptive immune responses (15, 16). In the gastrointestinal tract, especially, DCs are exposed to a multitude of different antigenic stimuli and utilize pattern recognition receptors (PRRs) [i.e. Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-1) like receptors, lectins, etc.] to recognize conserved pathogen associated molecular patterns (PAMPs) of microbes (8, 16-19). Thus, it has been proposed that probiotics exert their immunomodulatory effects via DC activity (7, 20-24). Here,

we evaluated the profiles of intestinal and systemic plasmacytoid DC (pDC) and conventional DC (cDC) responses induced by LA feeding at two different doses (low and high) before and after rotavirus infection in Gn pigs, as well as the ability of different dosage regimens to ameliorate rotavirus diarrhea.

4.3 Materials and Methods

4.3.1 Virus and probiotics

The virulent human rotavirus (VirHRV) Wa strain was propagated through neonatal Gn pigs and the intestinal contents from the 23rd passage were pooled and used as inoculum. The virus titer in FFU/milliliter was determined by cell culture immunofluorescence assay (CCIF) (25). The *Lactobacillus acidophilus* NCFM (NCK56) strain (LA) was propagated in lactobacilli MRS broth (Weber). LA inoculums were titrated (CFU/mL) via serial dilutions in 0.1% peptone water (BD Biosciences) and plating on MRS agar (Dot Scientific, #7543A) as described (26). Prior to dilution to the specified CFU/mL for feeding, frozen bacterial stocks were thawed and washed twice with 0.1% peptone water by centrifuging at 2000 rpm for 10 min at 4°C (26).

4.3.2 Treatment groups and inoculation of Gn pigs

Neonatal Gn pigs (Yorkshire crossbred) were derived by hysterectomy from near-term sows and maintained in sterile isolator units (27). Pigs were fed commercial sterile milk and confirmed germ-free prior to LA feeding. Pigs (both males and females) were randomly assigned to different treatment groups as follows: low dose LA (LoLA; n=6), high dose LA (HiLA; n=9) and non-LA-fed control (ZeroLA; n= 7). The detailed LA dosing regimen and VirHRV challenge are shown in Table 1 as described previously (14). Starting at postpartum day (PPD) 3, pigs were orally administered LA suspended in 3 mL of 0.1% peptone water by using a needle-

Table 1. Probiotic dose and feeding regimens and VirHRV challenge

Age (PPD)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	33	34	40	Total no. of feedings	Accumulative dosage
PCD																		0	1	7		
High dose (CFU)				10^3	10^3	10^4	10^4	10^5	10^5	10^6	10^6	10^7	10^7	10^8	10^8	10^9	10^9				14	2.22×10^9
Low dose (CFU)				10^3		10^4		10^5		10^6		10^6									5	2.11×10^6
VirHRV challenge (FFU)																		1×10^5				

PPD, post-partum day; PCD, post-challenge day; CFU, colony-forming units; FFU, fluorescent focus-forming units; VirHRV, virulent human rotavirus.

less syringe as previously described (26). Non-LA-fed pigs received an equal volume of 0.1% peptone water alone. At PPD 33 or postchallenge day (PCD) 0, a subset of pigs from each group were orally challenged with VirHRV at 10^5 FFU. Twenty minutes prior to virus challenge, 4 mL of 200 mmol/L of sodium bicarbonate was administered to reduce gastric acidity. Subsets of pigs from ZeroLA, LoLA and HiLA groups were euthanized at PCD 0 and PCD 7. Ileum, spleen and peripheral blood (PBL) were collected for isolation of MNCs as previously described (28). All animal experimental procedures were conducted in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee of Virginia Tech.

4.3.3 Assessment of sterility

Prior to initial LA feeding, rectal swabs from all pigs were collected to confirm germ-free status. Swabs were plated on blood agar plates and in thioglycollate broth at 37°C for 72 h. Thereafter, all ZeroLA pigs were checked weekly to ensure sterility.

4.3.4 Assessment of LA colonization

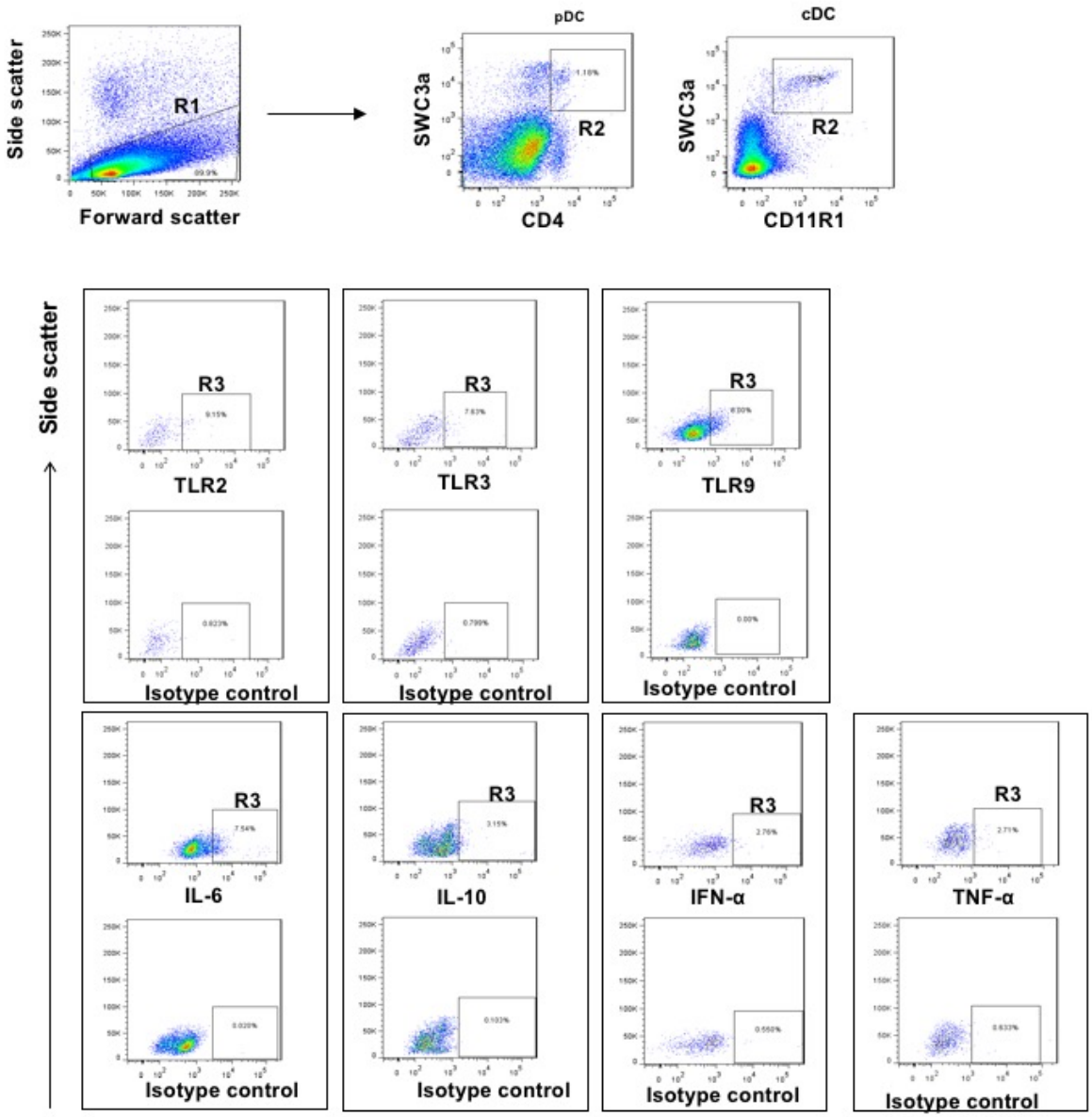
Fecal swabs from Gn pigs were collected weekly throughout the experiment for enumeration of LA as previously described (26). Briefly, rectal swabs were swirled in 4 ml of 0.1% peptone water to produce a fecal suspension. Samples were then further serially diluted in 0.1% peptone water and plated onto MRS agar (Dot Scientific, #7543A). Plates were incubated in BBL GasPak jars (Fisher, Hanover Park, IL, USA) containing GasPak EZ anaerobe sachets (BD Diagnostic Systems, #260678) at 37°C for 24 h. Fecal LA shedding was expressed as CFU/mL.

4.3.5 Assessment of rotavirus diarrhea and virus shedding

Following VirHRV challenge, Gn pigs were rectally swabbed daily (PCD 0-6) to evaluate clinical signs of diarrhea and virus shedding. Rectal swabs were scored for fecal consistency as follows: (0) normal, (1) pasty, (2) semi-liquid and (3) liquid. Diarrhea was considered present with scores of 2 or greater. Swabs were consistently collected each morning, two hours after the pigs' first meal. Fecal virus shedding was assessed by ELISA and CCIF as previously described (25).

4.3.6 Assessment of frequencies of cytokine and TLR expression in pDCs and cDCs by flow cytometry

Flow cytometry was used to determine frequencies of cytokines IL-6, IL-10, IFN-α or TNF-α producing and TLR2, 3 or 9 expressing pDCs (SWC3+CD4+) or cDCs (SWC3+CD11R1+) from ileum, spleen and blood of Gn pigs. Analysis of the stained cells was performed using a FACSAria flow cytometer (BD Biosciences), with at least 100,000 cells acquired. Data were analyzed using FlowJo 7.2.2 software (Tree Star, Ashland, Oregon). Representative dot plots are depicted in Figure 1. The frequencies of cytokine producing or TLR expressing DCs were described as a percentage among total pDCs or cDCs. All mean frequencies were calibrated with subtraction of the background frequencies.


Side scatter
R1
Forward scatter
pDC
SWC3a
R2
CD4
cDC
SWC3a
R2
CD11R1
Side scatter
R3
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R3
TLR3
Isotype control
R3
TLR9
Isotype control
R3
IL-6
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R3
IL-10
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R3
IFN-α
Isotype control
R3
TNF-α
Isotype control

Figure 1. Representative dot plots of frequencies of TLRs and cytokines among SWC3a+CD4+(pDC) and SWC3a+CD11R1+(cDC) cells

To investigate the frequencies of CD80/86, MHCII, cytokine and TLR expression on pDCs and cDCs in tissues of pigs from different LA dose groups, flow cytometry analysis was performed on freshly isolated MNC of ileum, spleen and blood. MNCs were stained freshly with antibodies against SWC3 (also known as CD172a) and CD4 or CD11R1 for the discrimination of pDC and cDC and among them the expression of TLR2, TLR3, TLR9, IL-6, IL-10, IFN-α and TNF-α, were stained respectively. MNC R1 area was gated from forward and side scatter. From R1 area, pDCs (SWC3+CD4+) or cDCs (SWC3+CD11R1+) were defined as R2 area. Side scatter and TLRs (TLR2, TLR3 or TLR9) or cytokines (IL-6, IL-10, IFN-α, and TNF-α) dot plots (R3) were performed within the R2 area to define frequencies of TLR or cytokine expression among total pDCs or cDCs. Cells stained with isotype controls were presented as control dot plots. The panmyeloid marker SWC3a is not only expressed on DCs, but also on granulocytes. However, gating on R1 eliminated the most SWC3+ granulocytes. Therefore, the MNC stained as SWC3+CD4+ and SWC3+CD11R1+ are mainly pDCs and cDCs, respectively.

The MNCs ($2x10^6$ cells/tube) were stained freshly on the same day of MNC isolation. For cytokine staining, the cells were first permeabilized with cytofix/cytoperm buffer (BD Biosciences, #554714) for 15 min at 4°C. This was then followed by staining with mouse anti-pig IL-6 (IgG2b, R&D Systems, #MAB6861) and FITC conjugated rat anti-mouse IgG2b (BD Pharmingen, #553395) for IL-6; mouse anti-pig IFN-α (IgG1, R&D Systems, #27105-1) and APC conjugated rat anti-mouse IgG1 (BD Biosciences, #550874) for IFN-α; and mouse anti-porcine IL-10 (IgG2b, R&D Systems, #MAB693) and FITC conjugated rat anti-mouse IgG2b (BD pharmingen, #553395) for IL-10 in separate tubes. All cells were then incubated with a mixture of phycoerythrin (PE) conjugated mouse anti-pig SWC3a (IgG1, Southern Biotech, #4525-09), Spectral Red (SPRD) conjugated mouse anti-pig CD4 (IgG2b, Southern Biotech,#4515-13) and pacific blue conjugated mouse anti-human CD11b (IgG1, BD Biosciences, #558123), which cross-reacts with porcine CD11R1 (29). All incubation steps after permeabilization were performed for 30 min at 4°C. The isotype-matched irrelevant antibody control tubes used mouse IgG1 (eBioscience, #8012-4714-025) as primary antibody and APC conjugated rat anti-mouse IgG1 (BD pharmingen, #550874) as secondary antibody.

For extracellular TLR2 detection, cells were first stained with a mixture of PE conjugated mouse anti-pig SWC3a (IgG1, Southern Biotech, #4525-09), SPRD conjugated mouse anti-pig CD4 (IgG2b, Southern Biotech, #4515-13), pacific blue conjugated mouse anti-human CD11b (IgG1, BD Biosciences, #558123) and PE conjugated mouse anti-human TLR2 (IgG1, eBioscience, #12-9024-82). This was incubated for 30 min at 4°C, followed by fixation with BD cytofix/cytoperm buffer for 15 min at 4°C. For staining of intracellular TLR3 and TLR9, permeabilization was first performed with BD cytofix/cytoperm buffer for 15 min at 4°C. Then

PE conjugated mouse anti-human TLR3 (IgG1, eBioscience, #12-9039-80) or TLR9 (IgG1, eBioscience, #12-9099-80) in perm/wash buffer was added and incubated for 30 min at 4°C. Finally, staining for CD4, SWC3a and CD11b was performed as described above for cytokine staining. Appropriate isotype controls were included.

All stained cells were resuspended in staining buffer and kept in the dark at 4°C before flow cytometry analysis. All antibodies were titrated and used at optimal concentrations.

4.3.7 Statistical analysis

Mean duration of diarrhea, as well as cumulative diarrhea scores and area under the curves (AUCs) for scores were compared among treatment groups using ANOVA-GLM, followed by Duncan's multiple range test. Non-parametric Kruskal-Wallis rank sum test was used to compare LA counts and frequencies of cytokine and TLR expressing pDCs or cDCs among treatment groups. When differences among the groups were detected, the same test was used in a pairwise fashion to identify the nature of the differences. All statistical significance was assessed at $p<0.05$. All statistical analysis was performed using SAS program 9.4 (SAS Institute, Cary, NC).

4.4 Results

4.4.1 LA colonization

Bacterial enumeration of fecal swabs for LA shedding was performed at 7, 12, 23 and 29 days after the initial LA feeding (corresponding to 10, 15, 26 and 32 days of age). The mean

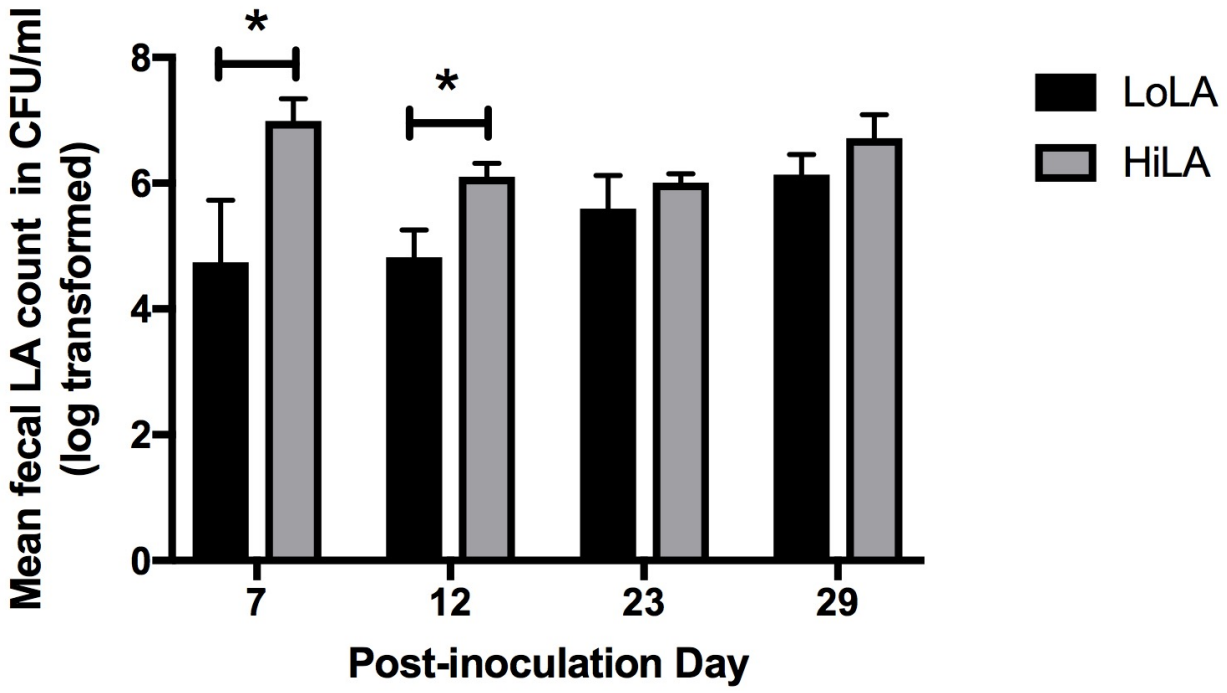

Figure 2. LA colonization in LoLA and HiLA dosage groups

Pigs were fed either low dose (LoLA, 5 feedings with an accumulative dose of 2.11 x 10^6 CFU) or high dose (HiLA, 14 feedings with an accumulative dose of 2.22 x 10^9 CFU). LA enumeration from fecal swab samples was performed on days 7, 12, 23 and 29 after initiation of feeding. Fecal LA shedding was expressed in CFU/mL. Analysis was performed on log transformed data and asterisk indicates significant difference between dosage groups (Kruskal-Wallis rank sum test; $p < 0.05$).

fecal LA counts in CFU/ml for LoLA and HiLA groups are depicted in Figure 2 as log transformed data. HiLA counts were significantly higher than LoLA on post-inoculation days 7 and 12 (PPD 1 and 15). No significant differences were noted on the remaining days (PID 23 and onward) as the LA colonization appeared to reach an equilibrium. ZeroLA pigs remained sterile for the duration of the study.

4.4.2 Rotavirus shedding and diarrhea

All of the pigs with or without LA feeding were infected after challenge with VirHRV as confirmed via virus shedding in daily fecal swab samples. There were no significant differences in mean duration or mean peak titers of virus shedding (data not shown). Clinically, all of the pigs developed diarrhea following VirHRV challenge as well. The diarrhea summary for all treatment groups is shown in Table 2. Overall, the HiLA dose appeared to attenuate clinical signs of VirHRV infection, demonstrating significantly shorter duration of diarrhea compared to ZeroLA (3.3 vs. 5.5 days). Additionally, the HiLA group also had both significantly lower mean cumulative diarrhea score (9.9) and calculated AUC (8.5) compared to both ZeroLA (14.4; 12.3) and LoLA (13.3; 11.6).

4.4.3 Higher overall TLR2 responses were observed in HiLA pigs, while LoLA induced higher TLR3 and TLR9 responses in ileum and spleen

TLR2, 3 and 9 responses in pDC and cDC in ileum, spleen and PBL pre- and post-challenge are depicted in Figure 3. In terms of TLR2 responses, HiLA pigs had significantly higher expression compared to ZeroLA in PBL cDCs pre-challenge and in both pDCs and cDCs of ileum post-challenge. Greater frequencies of TLR2-expressing pDCs and cDCs were also observed

Table 2. Protection against rotavirus diarrhea after VirHRV challenge.

Treatment groups	*n*	Mean duration of diarrhea (days) [#]	Mean cumulative score [*]	Area under the curve [*]
ZeroLA	4	5.5 (0.3[$])[AΨ]	14.4 (1.0)[A]	12.3 (1.0)[A]
LoLA	3	4.7 (0.3)[AB]	13.3 (0.4)[A]	11.6 (0.5)[A]
HiLA	4	3.3 (0.9)[B]	9.9 (1.0)[B]	8.5 (0.9)[B]

[#]Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semi-liquid; 3, liquid. Scores of 2 or greater were considered diarrheic.

[*]Mean cumulative score and area under the curve calculation included all pigs for 6 days postchallenge in each group.

[$]Standard error of the mean in parentheses.

[Ψ]Means in the same column with different superscript letters differ significantly (ANOVA-GLM and Duncan's multiple range test, $p<0.05$); shared letters indicate no significant difference.

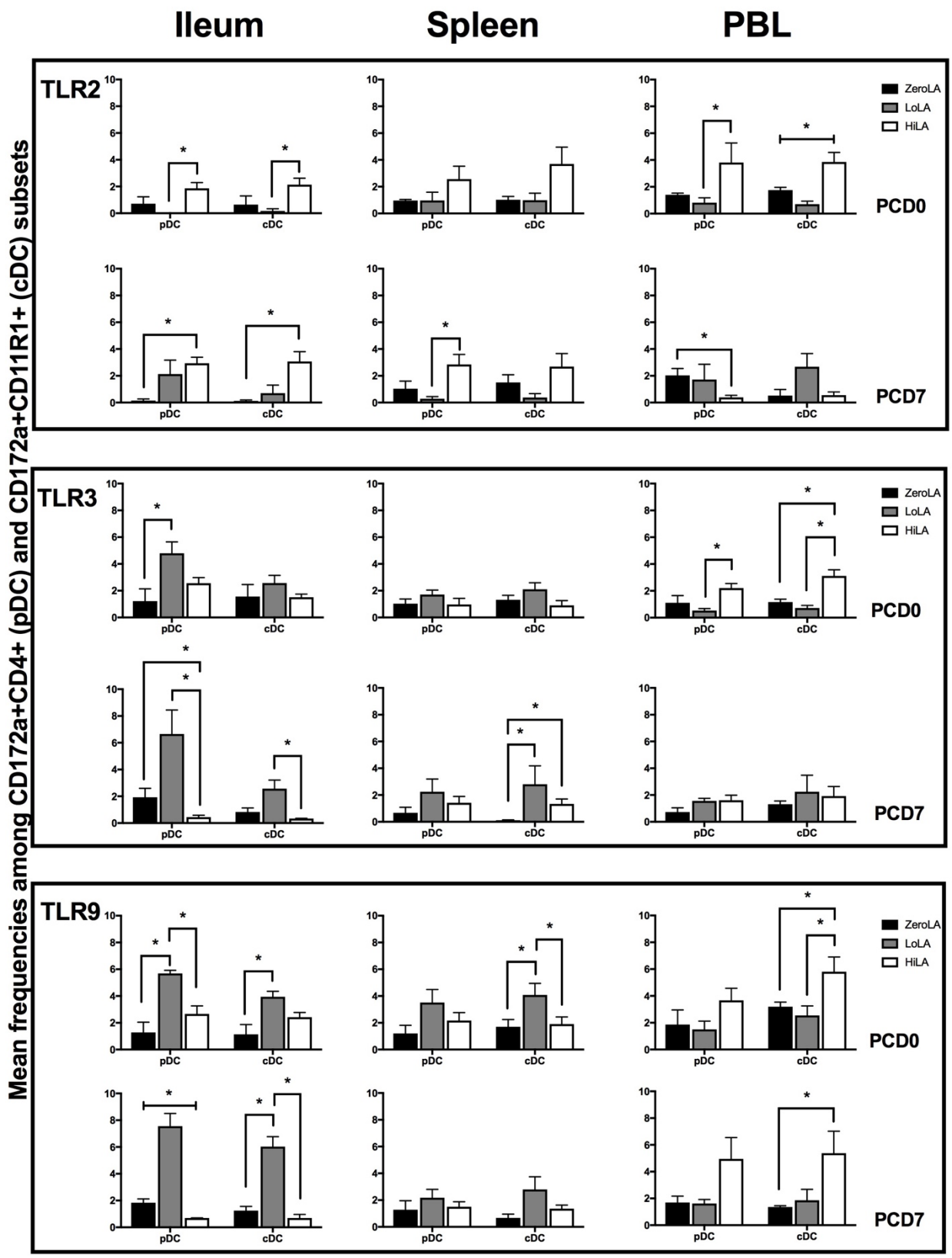
Ileum
Spleen
PBL
TLR2
TLR3
TLR9
PCD0
PCD7
ZeroLA
LoLA
HiLA
pDC
cDC
Mean frequencies among CD172a+CD4+ (pDC) and CD172a+CD11R1+ (cDC) subsets

Figure 3. TLR expression profile of DCs from ileum, spleen and PBL of Gn pigs pre- (PCD 0) and post-challenge (PCD 7) with VirHRV.

Pigs were fed with either high or low dose of LA. Data are presented as mean frequency ± standard error of the mean (n = 3-4). Frequencies of cells expressing TLR2, TLR3 and TLR9 among SWC3+CD4+ (pDC) and SWC3+CD11R1+ (cDC) subsets from intestinal (ileum) and systemic (spleen, PBL) lymphoid tissues were compared among control and different dose LA fed pig groups at PCD 0 and PCD 7. Bars with asterisk indicate significant difference among the pig groups (Kruskal-Wallis rank sum test; $p < 0.05$).

compared with LoLA in ileum and PBL pre-challenge. Post-challenge, there were significantly higher TLR2-expressing pDCs in spleen of HiLA pigs. No differences were noted in spleen pre-challenge. The only occurrence of significantly lower TLR2 expression in HiLA pigs was in PBL pDCs post-challenge.

TLR3-expressing DCs were significantly higher in the LoLA group compared to ZeroLA in ileal pDCs pre-challenge, as well as splenic cDCs post-challenge. TLR3-expressing pDCs and cDCs in ileum of HiLA pigs were significantly lower compared to LoLA pigs and pDCs compared to ZeroLA pigs post-challenge. HiLA pigs had significantly greater TLR3-expressing DCs in systemic tissues. This was noted in pDCs (vs. LoLA only) and cDCs (vs. ZeroLA and LoLA) in PBL pre-challenge and cDCs (vs. ZeroLA only) in spleen post-challenge. No differences were observed in either spleen pre-challenge or PBL post-challenge.

LoLA pigs were also observed to induce higher frequencies of TLR9-expressing DCs in ileum and spleen. Significance was observed compared to ZeroLA in pDCs and cDCs of ileum both pre- and post-challenge and in cDCs of spleen pre-challenge. The LoLA group also had greater TLR9 responses compared to HiLA in all DC populations of the ileum except for cDCs pre-challenge. Higher frequencies of TLR9-expressing splenic cDCs compared to HiLA were noted pre-challenge as well. HiLA only had significantly increased TLR9-expresing DCs in PBL, with the highest number of cDCs pre-challenge and higher cDCs compared to ZeroLA post-challenge. No differences were observed in spleen after challenge.

4.4.4 Higher IL-6 responses were observed in LoLA pigs pre-challenge and in HiLA pigs post-challenge, while IL-10 was more consistently upregulated in DCs of the HiLA group

IL-6 and IL-10 responses in pDC and cDC in ileum, spleen and PBL pre- and post-challenge are depicted in Figure 4. IL-6 expression in both DC populations in all tissues were significantly increased in LoLA pigs compared to ZeroLA pre-challenge. This upregulation was also significantly greater than HiLA pigs pre-challenge excluding ileal cDCs. Ileal DCs, both pDCs and cDCs, were the only populations where HiLA had significantly higher IL-6 production than ZeroLA prior to virus challenge. Conversely, post-challenge, HiLA pigs had significantly increased cDCs compared to ZeroLA in ileum, as well as the highest frequencies of both pDCs and cDCs in spleen that were significantly higher than both LoLA and ZeroLA pigs. No differences were observed in PBL post-challenge.

IL-10 responses were most abundant in the HiLA group. Compared to ZeroLA pigs, significantly higher IL-10 expression was observed in both DC populations in ileum and spleen of HiLA pigs prior to VirHRV challenge. Post-challenge, HiLA significantly enhanced frequencies of IL-10-producing pDCs and cDCs in ileum and cDCs in PBL compared to ZeroLA. Additionally, HiLA induced higher frequencies of IL-10-producing splenic and PBL cDCs pre -challenge, as well as pDCs in ileum and cDCs in PBL post-challenge compared to LoLA. LoLA only significantly increased IL-10 responses compared to ZeroLA, with higher pre-challenge responses in ileal cDCs and splenic pDCs and post-challenge responses in ileal pDCs. No differences were noted in spleen post-challenge.

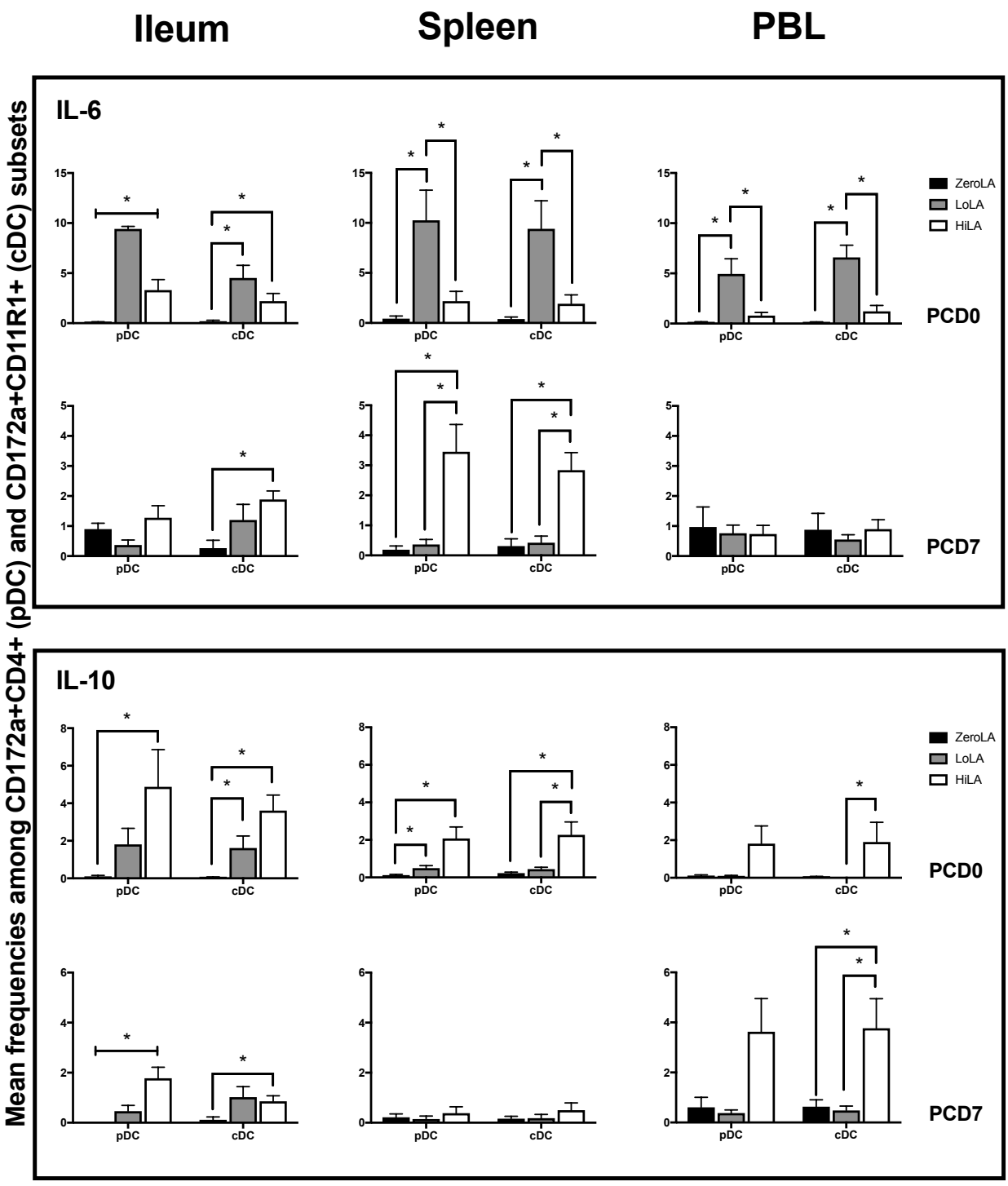
Ileum
Spleen
PBL
IL-6
IL-10
Mean frequencies among CD172a+CD4+ (pDC) and CD172a+CD11R1+ (cDC) subsets
ZeroLA
LoLA
HiLA
PCD0
PCD7
pDC
cDC

Figure 4. IL-6 and IL-10 expression profile of DCs from ileum, spleen and PBL of Gn pigs pre-(PCD 0) and post-challenge (PCD 7) with VirHRV.

Pigs were fed with either high or low dose of LA. Data are presented as mean frequency ± standard error of the mean (n = 3-4). Frequencies of the cells expressing IL-6 (a) or IL-10 (b) among CD172a+CD4+ (pDC) and D172a+CD11R1+ (cDC) subsets from intestinal (ileum) and systemic (spleen, PBL) lymphoid tissues were compared among mock control and different dose LA fed pig groups at PCD 0 and PCD 7. Bars with asterisk indicate significant difference among the pig groups (Kruskal-Wallis rank sum test; $p < 0.05$).

4.4.5 Increased production of IFN-α by DC populations occurred more frequently in HiLA pigs post-challenge, while TNF-α production was observed in both LoLA and HiLA pigs pre- and post-challenge

IFN-α and TNF-α responses in pDC and cDC in ileum, spleen and PBL pre- and post-challenge are depicted in Figure 5. Pre-challenge, the only significant difference was between LoLA and ZeroLA in spleen, where LoLA induced greater frequencies of IFN-α-producing pDCs. The majority of significant increases in IFN-α production by DCs were observed post-challenge in systemic tissues of HiLA pigs. This group had the highest frequencies of both pDCs and cDCs in PBL, as well as higher frequencies of pDCs compared in spleen to LoLA. No differences were observed in ileum or PBL prior to virus challenge, nor in ileum after challenge.

For TNF-α production by DCs, LoLA induced significant upregulation in systemic tissues as frequencies of pDCs and cDCs in spleen were increased compared to those of ZeroLA pre-challenge, while increased pDCs in PBL were observed post-challenge. HiLA also increased TNF-α-producing DC populations in cDCs of ileum and spleen pre-challenge and pDCs of PBL post-challenge compared to ZeroLA. No differences were seen in PBL pre-challenge or in ileum and spleen post-challenge.

4.5 Discussion

In this study, by comparing the profiles of intestinal and systemic pDC and cDC responses induced by HiLA, LoLA and ZeroLA feeding before and after rotavirus infection in Gn pigs, we demonstrated that dosage of probiotics has significant modulatory effects on innate immune responses. HiLA pigs induced overall higher TLR2 DC responses in most tissues

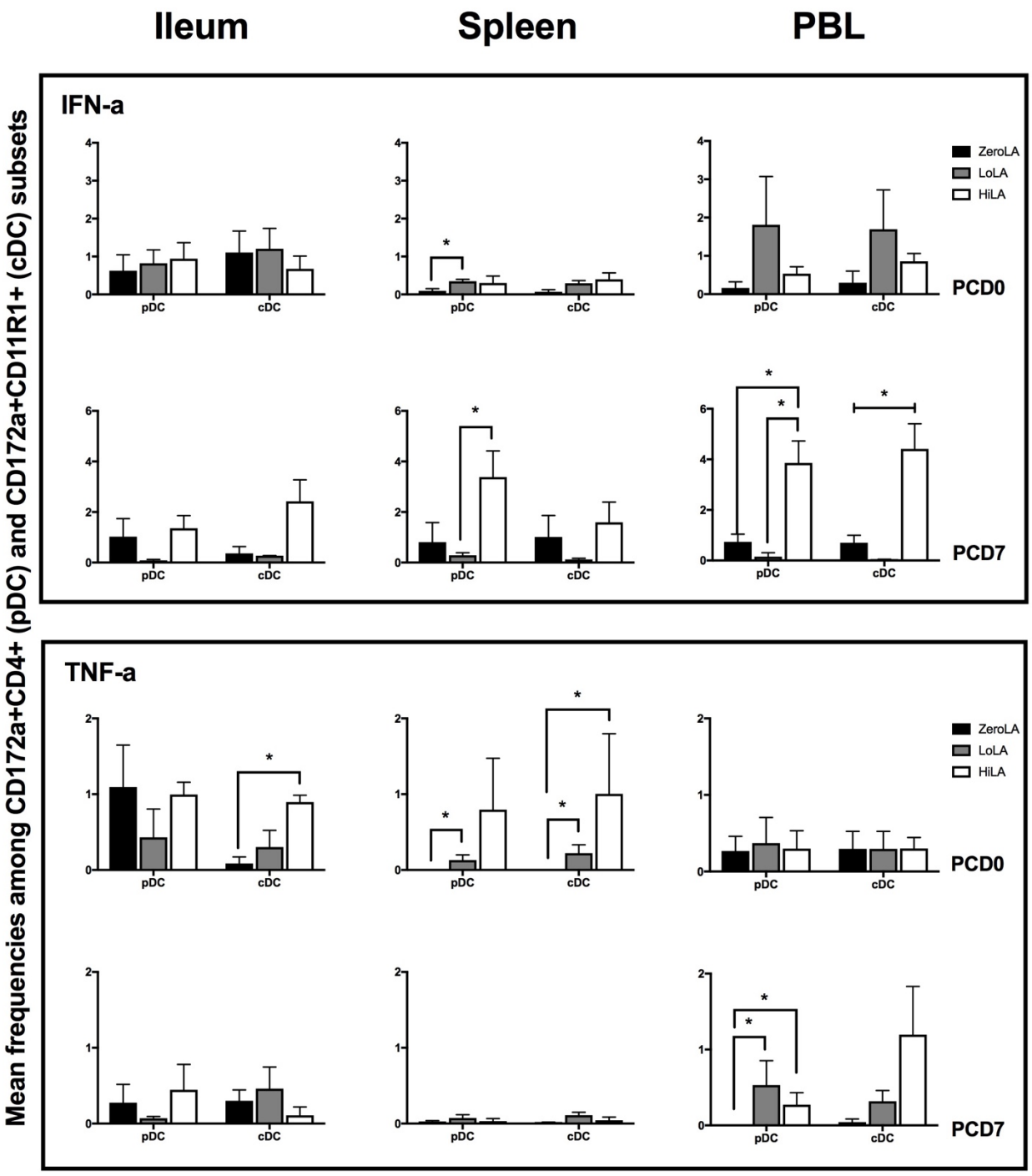
Ileum
Spleen
PBL
IFN-a
TNF-a
ZeroLA
LoLA
HiLA
PCD0
PCD7
pDC
cDC
Mean frequencies among CD172a+CD4+ (pDC) and CD172a+CD11R1+ (cDC) subsets

Figure 5. IFN-α and TNF-α expression profile of DCs from ileum, spleen and PBL of Gn pigs pre-(PCD 0) and post-challenge (PCD 7) with VirHRV.

Pigs were fed with either high or low dose of LA. Data are presented as mean frequency ± standard error of the mean (n = 3-4). Frequencies of the cells expressing IFN-α (a) or TNF-α (b) among CD172a+CD4+ (pDC) and D172a+CD11R1+ (cDC) subsets from intestinal (ileum) and systemic (spleen, PBL) lymphoid tissues were compared among mock control and different dose LA fed pig groups at PCD 0 and PCD 7. Different letters on top of bars indicate significant difference among the pig groups (Kruskal-Wallis rank sum test; $p < 0.05$); while shared letters indicate no significant difference.

pre- and post-challenge, while LoLA induced higher TLR3 and TLR9 responses in ileum and spleen. In terms of cytokine production, IL-6 tended to be upregulated by LoLA pre-challenge, while HiLA more consistently upregulated IL-10 both pre- and post-challenge. HiLA was also able to induce both IFN-α (post-challenge) and TNF-α (pre- and post-challenge) responses in DCs, while LoLA only increased TNF-α-producing DCs pre- and post-challenge. Overall, the results from this study support previous evidence of dose-dependent immunomodulation by various probiotic strains (7, 30).

TLRs are critical in the innate immune system for the detection of microbes via numerous conserved molecular components. Different TLR ligands contained in microbes can stimulate DC activation and specific cytokine profiles, subsequently augmenting adaptive T cell responses (31). In this study, we focused on TLR2, TLR3 and TLR9, which recognize peptidoglycans and lipoproteins of Gram-positive bacteria, double-stranded RNA and unmethylated CpG motifs in DNA, respectively (16). Of interest, HiLA and LoLA induced different TLR expression in both systemic and lymphoid DCs. Overall, HiLA pigs had increased TLR2 responses, while greater TLR3 and TLR9 responses occurred in the LoLA pigs. This divergent upregulation was understandably most consistently observed in the ileum both pre- and post-challenge as the gastrointestinal tract is the initial site of direct encounter of probiotics and rotavirus by DCs. TLR2 and TLR9 are well-known to be associated with recognition of lactobacilli (8), while TLR3 is activated during rotavirus infection (32, 33). There is evidence that certain TLRs can either act in a synergistic or suppressive manner to influence expression of other TLRs, which may explain the TLR profiles detected here. Indeed, TLR2 suppression of TLR9 responses has been described in DCs of Peyer's patches in mice (34). This

could possibly rationalize the decreased TLR9 expression in HiLA-stimulated DCs and, conversely, the increased TLR9 expression in DCs of LoLA pigs. The mechanism of TLR2 suppression of TLR9 in the previous mice study was not reliant on secretory factors, though cytokine production has been shown to contribute to TLR crosstalk. Of particular relevance, it has previously been reported that the same lactobacillus strain used in this current study was able to increase TLR3 expression on DCs via TLR2-dependent IFN-β production (35). Since bacteria do not contain double-stranded RNA, cytokine secretion by probiotic-stimulated immune cells may have mediated the increased TLR3 expression of DCs prior to VirHRV challenge of Gn pigs in this study and previously in monocytes/macrophages (33). Further studies would be necessary to elucidate the exact pathway of TLR crosstalk observed here.

As stated, different DC cytokine profiles can be stimulated by various TLR ligands and the results of this study reflect the potential for either pro- or anti-inflammatory responses, depending on the dose of LA. In general, *L. acidophilus* has been demonstrated to influence secretion of pro-inflammatory Th1 cytokines (IL-12, IFN-γ) *in vitro* (36) and consequently has shown adjuvant properties *in vivo* (26). However, the dosage can alter immunomodulatory effects as determined by our recent Gn pig study where HiLA induced Treg cells, while LoLA promoted IFN-γ T cell responses (13). Similarly, in the current study, HiLA induced higher frequencies of IL-10-producing DCs compared to ZeroLA in most tissues, except for spleen post-challenge. IL-10 is considered an anti-inflammatory cytokine that can be induced through the TLR2-signalling pathway and subsequently is capable of stimulating Treg cells (15, 34). As such, it is reasonable to postulate that high doses of LA increased regulatory immune responses via increased TLR2 expression of DCs and secretion of IL-10.

Despite the overall immunoregulatory profile induced by HiLA feeding, higher TNF-α responses were observed pre-challenge, and higher IL-6 and IFN-α responses post-challenge. These responses may have been involved in the antagonism of the IL-10-induced suppressive immune response. Probiotic immunomodulation is not necessarily skewed exclusively to either pro- or anti-inflammatory responses. Balanced responses are possible and again may be both strain and dose-dependent. A recent study of *Escherichia coli* Nissle 1917 colonization in Gn pigs demonstrated upregulation of IFN-α, IL-12 and IL-10 production by DCs (37). Similarly, two strains of *Lactobacillus gasseri* increased IL-12, TNF-α and IL-10 secretion by DCs (11).

In contrast, LoLA induced higher pro-inflammatory IL-6 responses in all tissues pre-challenge, as well as IFN-α and TNF-α responses systemically (spleen). Post-challenge, higher TNF-α production was observed in pDCs of PBL. It has been reported that the stimulation of TLR3 and TLR9 can induce pro-inflammatory immune responses (15, 38, 39) and IL-6, IFN-α and TNF-α are cytokines involved in the pro-inflammatory or anti-viral function (3, 26). Therefore, it is possible that LoLA promoted increased frequencies of IFN-γ T cells observed in our previous study (13) through the combined signal pathways of TLR3 and TLR9 and pro-inflammatory cytokine production from DCs.

While HiLA induced higher IFN-α responses systemically (spleen and PBL) post-challenge, robust IFN-α responses were not observed in ileum or in any tissues of ZeroLA and LoLA pigs. Lactobacilli are known to stimulate type I interferon production by pDCs (40) and interferons are important for anti-viral activity of the innate immune system, including limiting rotavirus infection (41). However, rotaviruses have methods of suppressing interferon

expression, including antagonism by NSP1 (32). This may have contributed to neither HiLA nor LoLA significantly reducing virus shedding.

Still, HiLA was able to significantly reduce VirHRV diarrhea in Gn pigs in terms of duration and severity scores. The slight increase in IFN-α production post-challenge could have played a small role in this reduction. However, the exact mechanism of rotavirus-induced diarrhea is incompletely understood, and may include villus blunting, decrease in digestive enzymes, enterotoxin NSP4, increase in intestinal permeability and secretion, as well as involvement of the enteric nervous system (ENS) (42). Probiotics have been heavily preferred as possible adjunctive treatments for reducing diarrhea in acute gastroenteritis, including rotavirus (43). In this study, TLR and cytokine responses from DCs in the HiLA group may have favored anti-inflammatory conditions that reduced intestinal epithelial injury and beneficially influenced the ENS to decrease diarrhea. In particular, the increased TLR2 signaling induced by HiLA may have preferentially maintained epithelial barrier function. A previous study demonstrated that TLR2 stimulation regulated tight junction barrier integrity of intestinal epithelial cells *in vitro,* as well as *in vivo* in a dextran sodium sulphate (DSS)-induced model of colitis in mice (44). Additionally, TLR2 signaling is involved in regulation of the ENS, which controls intestinal motility and fluid secretion that may contribute to rotavirus diarrhea (45, 46). In particular, it has been demonstrated that TLR2 deficient mice had altered intestinal motility and mucosal secretion. Subsequent restoration of these ENS dysfunctions with glial cell line-derived neurotrophic factor alleviated DSS colitis (47). Therefore, the predominant anti-inflammatory responses by HiLA dosing may have diminished VirHRV pathogenicity and ameliorated diarrhea in Gn pigs.

Intriguingly, the intestinal colonization by either low or high dose feeding regimens did not differ by the end of the study as determined by fecal LA shedding. Similar results were obtained in our previous study (13), where initial shedding was significantly higher in the HiLA group before equilibrating and ultimately decreasing compared to the LoLA pigs. Again, in a separate study, using an intermediate dose of LA did not significantly alter LA shedding compared to LoLA or HiLA (14). Despite the lack of variation in overall LA colonization in the pigs, we observed obvious modification of the innate immune response based on dosage administered. Perhaps the initial differences in bacterial load and secreted mediators was sufficient to produce differential effects in terms of TLR and cytokine profiles of DCs. In recent years, it has been suggested that the innate immune system is capable of being primed based on interactions with pathogens, which can lead to a non-specific, enhanced immune state (48, 49). This phenomenon has been termed “trained immunity” and is thought to occur due to epigenetic reprogramming of cells, with the change in response lasting weeks to months. It is still unclear whether commensal microorganisms or probiotic species participate in such reprogramming, though evidence supports that this is a distinct possibility as PRRs are involved. An *in vitro* study of human monocytes demonstrated that dose-dependent stimulation of PRRs, including TLRs, resulted in either enhanced or tolerant functional programming in terms of cytokine production (50). In general, they observed that high doses of microbial ligands of various TLRs influenced a tolerant state, while low doses prompted a pro-inflammatory response, which was similar to the effect of LoLA vs. HiLA on DCs in Gn pigs. The TLR ligands tested included lipoteichoic acid (Pam3CSK4), poly(I-C) and CpG, which stimulate TLR2, TLR3 and TLR9, respectively. Ligands of TLR2 and TLR3 both modulated cytokine production by

monocytes in the dose-dependent manner described, while CpG induced tolerance at any dose. A separate study observed tolerance induced by CpG upon subsequent challenge with the same or different TLR ligand [lipoteichoic acid or lipopolysaccharide (TLR4)] *in vitro* (51). However, CpG did not produce cross-tolerance *in vivo*, but instead increased TNF-α production and liver damage in mice, demonstrating that TLR9 stimulation can induce a pro-inflammatory state as observed in this current study. Overall, these findings suggest that innate immune cells can undergo differential functional modification depending on the concentration of a specific microbial ligand. Such training may explain the differences we observed between LoLA and HiLA groups despite similar endpoint colonization or fecal shedding, as the primary stimulus seems capable of inducing these functional changes for at least a few weeks. Further studies would need to be conducted to elucidate the training patterns of DCs as they express different profiles of PRRs compared to monocytes.

In summary, this study demonstrated that LA modulated the innate immune responses in a dose-dependent manner in Gn pigs by influencing how DCs express TLRs and cytokines in response to probiotic treatment before and after rotavirus infection. The findings provided insights into the mechanism of probiotic dose-dependent influence on the adaptive immune responses we reported previously (13, 14) and guidance for manipulating the host innate and adaptive immune responses using different dosages of probiotics. Based on our serial studies, we can conclude that dosages of probiotics have significant effects on their immunomodulatory functions. Optimized dosing regimen of probiotics is beneficial to the host by inducing a more balanced innate immune response for effectively removing invading pathogens with limited damage to the host and a protective adaptive immune response after infection or vaccination.

The dosage that produces a preferable effect may be strain-dependent and needs to be determined separately for different probiotic strains.

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Chapter 5

General conclusions

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5.1 General conclusions

In the first phase of this study, we successfully established a Gn pig model of HuNoV infection and diarrhea using a G11.4 2006b variant (092895) (1). Advantages of this model compared to other NoV animal models includes similarity to humans in HBGA expression, route of transmission, clinical signs and intestinal pathological changes. Both A^+ and H^+ Gn pigs were equally susceptible to infection, as previously demonstrated (2). Neonatal (4-5 days of age) and older (33-34 days of age) pigs were successfully infected via oral inoculation and developed mild diarrhea, unlike infection observed in mice (3) and non-human primates (4, 5). Virus antigen was detected in enterocytes of the duodenum, with cytopathologic changes consisting of microvilli damage, cytoplasmic vacuolization and enterocyte necrosis and apoptosis occurring in both the duodenum and jejunum. An advantage of this model compared to other Gn pig models includes the determination of ID_{50} of the challenge strain. The ID_{50} of neonatal pigs was $\leq 2.74 \times 10^3$ viral RNA copies, while the ID_{50} of older pigs was higher at 6.43×10^4 viral RNA copies. The lowered infectivity in older pigs is likely representative of a more developed innate immune system. Similarly, NoV can cause more severe illness in humans with weaker immune responses, such as the young, elderly and immunocompromised (6, 7). Interestingly, simvastatin administration in older pigs reduced the ID_{50} to a comparable level ($<2.74 \times 10^3$) as neonatal pigs. Simvastatin is a cholesterol-reducing drug that that is widely used in populations at risk for cardiovascular events and has been associated with increased susceptibility to NoV infection (8, 9). As this population may include individuals already more susceptible to NoV infections due to age, the implications of simvastatin use may be significant and merit further

investigation. Overall, this first phase highly reinforces the applicability of the Gn pig model for studying enteric viruses.

In the second phase of this study, we evaluated proposed therapeutics for the treatment of RV-induced diarrhea in a Gn pig model. Again, the insight gained from these latter studies relied heavily on the advantages of the Gn pig model. The resemblance to humans in anatomy, physiology, immunology and response to gastrointestinal disease were vital in assessing efficacy and mechanism of action of the selected treatments. In the first of these two studies, we initiated treatment with the anti-secretory drug racecadotril in neonatal pigs infected with a virulent HRV strain (Wa HRV) (10). Racecadotril is capable of reducing intestinal water loss by inhibiting enkephilnase enzymes, thereby increasing the availability of endogenous enkephalins that inherently reduce secretions in the gastrointestinal tract (11). A particular benefit of racecadotril compared to other anti-diarrheal agents, such as loperamide, is its high safety profile (12). Treatment with racecadotril significantly shortened the duration of diarrhea compared to control groups receiving PBS or chlorpromazine, which is a drug that has been shown to be effective against diarrhea due to enterotoxigenic *E. coli* and transmissible gastroenteritis virus in pigs (13, 14). In association with amelioration of diarrhea, racecadotril-treated pigs gained significantly more weight than both PBS- and chlorpromazine-treated pigs. These results supported the use of racecadotril as a treatment for RV-induced diarrhea.

In the second of the therapeutic studies, we examined the ability of *Lactobacillus acidophilus* NCFM (LA) to reduce diarrhea in the Gn pig model of RV infection and disease. Probiotics have been highly studied for their beneficial impacts on gastrointestinal illnesses, though complete understanding of the mechanisms involved is still absent (15). Conflicting

results from different studies may be explained by the various strains and dosages administered (16). We sought to investigate the immunomodulatory potential of different doses of LA on conventional and plasmacytoid DC responses pre- and post-infection with Wa HRV. Our prior study indicated that a low dose of LA potentiated IFN-γ producing T cell responses and downregulated Treg responses locally and systemically following HRV vaccination, while a high dose increased Treg cell frequencies (17). Similar divergent effects were observed in the current study, with low dose LA inducing primarily pro-inflammatory DC responses and high dose LA eliciting an anti-inflammatory profile. Of note, only the pigs receiving a high dose of LA demonstrated a reduction in severity and duration of diarrhea. Anti-inflammatory conditions may have decreased intestinal epithelial injury or altered intestinal motility and fluid secretion involved in the pathogenesis of RV-induced diarrhea (18, 19). These results indicate that probiotic dosage selection is an important consideration prior to implementation of use in treating acute gastroenteritis.

In summary, my studies examined the various approaches that can be used to modulate viral diarrhea. Simvastatin increases NoV diarrhea through downregulating innate immunity; high dose probiotic LA reduces RV diarrhea through enhancing innate immune defenses and downregulating inflammatory responses; and racecadotril non-specifically reduces diarrhea by controlling hypersecretion via increased availability of anti-secretory enkephalins in the intestine. Our findings further validated the Gn pig model for the studies of viral diarrhea. Importantly, it will facilitate mechanistic studies on viral diarrheal pathophysiology and the development of additional interventional approaches to reduce the burden of viral diarrhea, which is one of the leading causes of infant mortality.

5.2 Future directions

There remains a considerable wealth of knowledge to gain in understanding both NoV and RV pathogenesis and innate immunity. Such information will undoubtedly influence the development of efficacious vaccines and therapeutics to reduce disease burden. In regards to NoV, recent advances have occurred in understanding immune cell tropism and influence of intestinal microbiota (20, 21). However, these discoveries were initially observed with MNV and it is unknown if all HuNoVs will behave similarly. In this capacity, the Gn pig model for HuNoV infection is an invaluable tool for further investigations. Similar studies with a GII.4 HuNoV in a Gn pig model have so far yielded conflicting results compared to MNV studies (22, 23). Continued studies of NoV pathogenesis in animal models will hopefully direct the path towards development of an efficient and robust cell culture system for HuNoVs.

While RV vaccines are available, there exists a need to determine the cause of reduced efficacy in developing countries. Current speculation is that maternal antibody interference, malnutrition, co-infections with other enteric pathogens and/or the influence of commensal microorganisms may affect vaccine efficacy (24, 25). Again, the Gn pig model is ideal for evaluation of these multifactorial mechanisms. In particular, the Gn pigs can be transplanted with human gut microbiota (25-27). A recent study colonizing Gn pigs with human stool samples from children designated as having healthy versus unhealthy gut microbiomes (dysbiosis) demonstrated that diversity and composition are crucial characteristics of microbiome influence on oral RV vaccine immunogenicity (25). Additional advances in the RV field include the development of a reverse genetics system (28). This system will be advantageous in further understanding RV pathogenesis, as well as developing new vaccines

and therapeutics. The Gn pig model will expectedly continue to be useful in assessing novel approaches to RV prevention and treatment.

5.3 References

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