Protein Malnutrition Alters Tryptophan and Angiotensin-Converting Enzyme 2 Homeostasis and Adaptive Immune Responses in Human Rotavirus-Infected Gnotobiotic Pigs with Human Infant Fecal Microbiota Transplant

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ABSTRACT  Malnutrition leads to increased morbidity and is evident in almost half of all deaths in children under the age of 5 years. Mortality due to rotavirus diarrhea is common in developing countries where malnutrition is prevalent; however, the relationship between malnutrition and rotavirus infection remains unclear. In this study, gnotobiotic pigs transplanted with the fecal microbiota of a healthy 2-month-old infant were fed protein-sufficient or -deficient diets and infected with virulent human rotavirus (HRV). After human rotavirus infection, protein-deficient pigs had decreased human rotavirus antibody titers and total IgA concentrations, systemic T helper (CD3^+ CD4^+) and cytotoxic T (CD3^+ CD8^+) lymphocyte frequencies, and serum tryptophan and angiotensin I-converting enzyme 2. Additionally, deficient-diet pigs had impaired tryptophan catabolism postinfection compared with sufficient-diet pigs. Tryptophan supplementation was tested as an intervention in additional groups of fecal microbiota-transplanted, rotavirus-infected, sufficient- and deficient-diet pigs. Tryptophan supplementation increased the frequencies of regulatory (CD4^+ or CD8^+ CD25^+ FoxP3^+) T cells in pigs on both the sufficient and the deficient diets. These results suggest that a protein-deficient diet impairs activation of the adaptive immune response following HRV infection and alters tryptophan homeostasis.

KEYWORDS  ACE2, gnotobiotic, malnutrition, rotavirus, tryptophan, fecal microbiota

Malnutrition leads to wasting and stunted growth and is associated with almost half of all under-5 deaths worldwide (1, 2). The causative association between malnutrition and infectious diseases has long been known (3, 4), and malnourished children frequently have increased diarrheal episodes resulting in exacerbated energy loss and death (1, 4, 5). It is estimated that enteric infections with overt diarrhea account for 25% of stunting cases in children (6), and exposure to multiple enteric pathogens over an extended period of time, with or without diarrhea, may account for up to 43% of stunting cases (7). The common enteric pathogen rotavirus (RV) is a major cause of gastroenteritis in children worldwide, leading to more than 215,000 deaths per year (8, 9). RV infection results in diarrhea due to intestinal net fluid secretion via mechanisms including malabsorption due to villous atrophy (10).
Previously, murine models have been used to show that the enteric pathogens Cryptosporidium (11) and enteroaggregative Escherichia coli (12) exacerbate growth impairment and intestinal damage associated with malnutrition. The neonatal piglet is similar to human infants in gastrointestinal physiology, micro- and macronutrient metabolism, and mucosal immunity. Further, like children, germfree (GF) pigs are susceptible to repeated infections by multiple RV serotypes with ensuing disease and intestinal lesions (13–15). Finally, pigs are outbred, eliminating the strain bias common in mouse model research. We have used the GF pig to study controlled exposure to microorganisms and physiologically relevant and economically feasible intervention strategies (15–22). Recently, investigators have successfully transplanted GF pigs with human gut microbiota (23) and used this model to examine the effects of probiotics on human rotavirus (HRV) pathogenesis and immunity (24–26). The pig has also been used as a translational model for malnutrition, which disrupted intestinal barrier function and exacerbated RV-induced disease in colostrum-deprived neonatal piglets (27–31).

Recently, we established a neonatal gnotobiotic (Gn) pig model of childhood protein malnutrition in conjunction with human infant fecal microbiota (HIFM) colonization that reproduces the effects of kwashiorkor protein malnutrition (26). In this model, stunting was evident at 1 week of age and clinical features mimicking kwashiorkor protein malnutrition (edema of the face, neck, feet, and abdomen) appeared, in addition to changes in blood parameters, including hypoglycemia, hypoproteinemia, and hypoalbuminemia, commonly seen in malnourished children (26). Analysis of fecal samples and the microbiome in multiple regions of the intestine 1 week following the HIFM transplant found that over 99% of the operational taxonomic units (OTUs) from the original HIFM sample were present in the pigs and that the microbial community structure of the transplanted HIFM was qualitatively representative of the original sample (A. Kumar, A. N. Vlasova, L. Deblais, H.-C. Huang, A. Wijeratne, S. Kandasamy, D. D. Fischer, S. N. Langel, F. C. Paim, M. A. Alhamo, L. Shao, L. J. Saif, and G. Rajashekara, submitted for publication). Additionally, HRV-infected, protein-deficient, HIFM-transplanted pigs (deficient HIFM/HRV) had increased diarrhea and shed virus at higher titers after virulent human rotavirus (vIRV) inoculation in comparison to their sufficient-diet, HIFM-transplanted, vIRV-infected (sufficient HIFM/HRV) counterparts (26). Furthermore, deficient HIFM/HRV pigs had increased microbiota diversity in systemic tissues in comparison to sufficient HIFM/HRV pigs, indicating increased translocation from the intestine (Kumar et al., submitted).

To better understand the relationship between malnutrition, immune activation and responses to RV, and homeostatic control of selected amino acids, we compared adaptive immune responses (antibody production and T cell frequencies), concentrations of angiotensin I-converting enzyme 2 (ACE2; a regulator of dietary amino acid homeostasis), and levels of the essential amino acid tryptophan. ACE2 is associated with the neutral amino acid transporter B⁰AT1 and is required for expression of B⁰AT1 on the luminal surface of intestinal epithelial cells (IECs) (32–34). Knockout of ACE2 in mice resulted in increased susceptibility to intestinal inflammation and colitis, decreased serum tryptophan, and alteration of the gut microbiome (35). Restoring tryptophan levels reduced inflammation and increased the production of antimicrobial peptides by the intestinal epithelium, showing that ACE2 regulates dietary amino acid homeostasis, innate immunity, inflammation, and gut microbial ecology in a tryptophan-dependent manner (35). Furthermore, children at high risk for stunting often have limited tryptophan in their diets (36), and it has been shown previously that stunted children have lower serum concentrations of all nine essential amino acids, including tryptophan (37). Low plasma tryptophan is a predictor of poorer subsequent growth in boys and is associated with biomarkers of barrier disruption and intestinal and systemic inflammation in children of both genders (38). Tryptophan catabolism via the indoleamine 2,3-dioxygenase (IDO) pathway has immunoregulatory effects, and its depletion has been well studied in the context of chronic infections (39); however, the interrelationship between tryptophan and the adaptive immune response to RV and other acute infections is less clear, particularly in the context of host protein malnutrition.
In this study, we report that protein deficiency affects the adaptive immune response to RV and disrupts tryptophan homeostasis and catabolism in the neonatal gnotobiotic (Gn) pig model of childhood malnutrition, involving transplantation of pigs with human infant fecal microbiota (HIFM).

RESULTS

Protein deficiency reduced HRV IgA and IgM antibody isotype titers and total IgA concentrations following HRV inoculation, but increased total IgG and IgM concentrations. To evaluate the effect of protein deficiency on systemic and local antibody production, HRV-specific antibody titers and total IgA concentrations were measured. IgA HRV antibody titers were reduced in serum (Fig. 1A, \( P = 0.06 \)) and significantly reduced in small intestinal contents (Fig. 1B, \( P < 0.05 \)) of deficient HIFM/HRV pigs compared to sufficient HIFM/HRV pigs, suggesting that protein deficiency compromised mucosal and systemic viral antibody responses. Additionally, in deficient HIFM/HRV pigs, IgM HRV antibody titers (Fig. 1D, \( P < 0.05 \)) were significantly decreased compared with those in sufficient HIFM/HRV pigs. IgG HRV antibody titers were not significantly decreased in the deficient HIFM/HRV pigs. Reductions in HRV antibodies were seen despite higher rotavirus shedding in the deficient HIFM/HRV pigs (26).

In deficient HIFM/HRV pigs, mean concentrations of total IgA in the serum were lower at post-virHRV inoculation day 9 (PID9) and were reduced at PID14 (Fig. 2A, \( P = 0.06 \)) in comparison to sufficient HIFM/HRV pigs. Mean total IgA concentrations were also lower (Fig. 2B, \( P = 0.06 \)) in the small intestinal contents of deficient HIFM/HRV pigs at PID14 compared with the sufficient HIFM/HRV pigs. In deficient HIFM/HRV pigs, mean total IgG and IgM concentrations were higher in the serum pre- and postinoculation,
IgG at post-fecal microbiota transplant day 10 (PFMTD10)/PID0 (Fig. 2C, *P/H11005 0.05) and IgM at PID9 (Fig. 2D, *P/H11021 0.05).

Protein deficiency reduced systemic T helper and cytotoxic T cell frequencies. To evaluate the impact of protein deficiency on Th cells, the frequencies of CD3+/CD4+ Th cells in blood, spleen, duodenum, and ileum mononuclear cells (MNCs) were measured by flow cytometry at PID14 in deficient and sufficient pigs infected with HRV (Fig. 3A). Systemic CD3+/CD4+ Th cell frequencies were decreased systemically in the blood (*P/H11005 0.09, Fig. 3A) and significantly in the spleen (*, *P/H11005 0.02). No decreases or only slight decreases in Th frequencies were seen in the intestine (ileum or duodenum, respectively). Similarly, CD3+/CD8+ cytotoxic T lymphocyte (CTL) frequencies were decreased systemically in the spleen (P = 0.06) and significantly decreased in the blood (P = 0.02) but increased in the ileum (P = 0.08) in deficient HIRM/HRV pigs in comparison with the sufficient HIFM/HRV pigs (Fig. 3B).

Dietary tryptophan supplementation increased regulatory T cell frequencies in deficient- and/or sufficient-diet pigs infected with HRV. The frequencies of CD4+/CD25+Fox3+ (Fig. 4A) and CD8+/CD25+Fox3+ (Fig. 4B) regulatory T cells (Tregs) in blood, spleen, duodenum, and ileum MNCs were measured by flow cytometry at PID14 in tryptophan-supplemented and unsupplemented deficient and sufficient HIRM pigs infected with HRV. CD4+/CD25+Fox3+ cell frequencies were higher, but not significantly different, in the blood, spleen, and ileum of unsupplemented deficient HIRM/
HRV pigs compared with unsupplemented sufficient HIFM/HRV pigs. Deficient HIFM/HRV pigs supplemented with tryptophan had significantly increased frequencies of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs in the blood (P < 0.01), spleen (P < 0.001), and duodenum (P < 0.05) compared with unsupplemented deficient HIFM/HRV pigs. Sufficient HIFM/HRV pigs supplemented with tryptophan had significantly increased CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg frequencies only in the gut (ileum [P < 0.05] and duodenum [P < 0.05]) compared to unsupplemented sufficient HIFM/HRV pigs. The overall frequencies of CD8<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs were mostly lower in all tissues than those of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs. As for CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs, deficient HIFM/HRV pigs supplemented with tryptophan had highly significant increases (P < 0.001) in the frequencies of CD8<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs in the blood, spleen, and duodenum compared to unsupplemented deficient HIFM/HRV pigs. Also, sufficient HIFM/HRV pigs supplemented with tryptophan had highly significant increases (P < 0.001) in CD8<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cell numbers in the blood, spleen, ileum, and duodenum in comparison with their unsupplemented counterparts. Dietary tryptophan supplementation had no de-

FIG 3  CD3<sup>+</sup> CD4<sup>+</sup> T helper cell and CD3<sup>+</sup> CD8<sup>+</sup> cytotoxic T cell frequencies as a percentage of total MNCs in the blood, spleen, ileum, and duodenum in deficient and sufficient HIFM/HRV pigs at PID14, determined by flow cytometry. (A) CD3<sup>+</sup> CD4<sup>+</sup> T helper cells. (B) CD3<sup>+</sup> CD8<sup>+</sup> cytotoxic T cells. Results are ± standard errors of the means (SEM). *, significant (P < 0.05) change in deficient HIRM/HRV pigs compared to sufficient HIFM/HRV pigs (Mann-Whitney test).

FIG 4  T regulatory cell frequencies as a percentage of total MNCs in the blood, spleen, ileum, and duodenum of deficient and sufficient HIFM/HRV pigs with or without tryptophan supplementation at PID14, determined by flow cytometry. (A) CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells (scale, 0 to 50%). (B) CD8<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells (scale, 0 to 12%). Results are ± standard errors of the means (SEM). Asterisks show significant (*, P < 0.05; **, P ≤ 0.01; ***, P ≤ 0.005) increases in tryptophan-supplemented compared to unsupplemented pigs (two-way ANOVA and Bonferroni’s multiple-comparison test).
Table effects on HRV antibody isotype titers, total antibody concentrations, CD3⁺/CD4⁺ Th cell frequencies, or CD3⁺/CD8⁺ CTL frequencies.

Protein deficiency altered serum tryptophan concentrations. Serum tryptophan concentrations were measured (Fig. 5) preinoculation and during the acute and convalescent phases of virHRV infection. At 9 or 10 days PFMT, prior to virHRV inoculation (PFMTD9 or PFMTD10/PID0), deficient pigs had significantly lower (P < 0.005) levels of the essential amino acid tryptophan in serum than did sufficient HIFM/HRV pigs, indicating disruption in amino acid homeostasis consistent with hypoproteinemia (26). In the acute phase of virHRV infection (PID2) and convalescent phase (PID14), serum tryptophan levels rose steadily in deficient-diet pigs. In sufficient-diet pigs, serum tryptophan levels dropped significantly (**, P < 0.01) in the acute phase of virHRV infection and then rose significantly (**, P < 0.01) during the convalescent phase, consistent with immune activation and virus clearance.

Protein deficiency decreased serum ACE2 concentrations. Serum ACE2 concentrations were measured (Fig. 6) preinoculation and during the acute and convalescent phases of virHRV infection. Pre-virHRV inoculation (PFMTD9 or PFMTD10/PID0), serum ACE2 concentrations were significantly lower (P < 0.05) in deficient HIFM/HRV pigs than in sufficient HIFM/HRV pigs. Serum ACE2 levels were decreased in sufficient-diet pigs, but they remained higher than those in deficient-diet pigs during the acute phase (PID2) of virHRV infection (ACE2 levels also decreased in a manner coincident with that of tryptophan [Fig. 5]) and were restored to a significantly higher (P < 0.01) concentration during the convalescent (PID14) phase of infection. Deficient/HIFM pigs not challenged with virHRV had lower (but not statistically significantly lower) serum ACE2 concentrations than unchallenged sufficient/HIFM pigs at PID14 (data not shown).

DISCUSSION

Because malnutrition and malnutrition-associated infections are a leading cause of childhood deaths, it is critical to distinguish the interrelated factors that lead to increased infections or impaired immune responses and to identify targets for intervention. In this study, we report that in HIFM-transplanted pigs, protein deficiency impairs adaptive immune responses to HRV, lowers serum levels of tryptophan, impairs
its catabolism during the acute phase of HRV infection, and lowers serum ACE2 compared with sufficient-diet pigs. In addition, we found that whereas Th cells and CTLs were significantly decreased systemically only in the protein-deficient pigs, dietary tryptophan supplementation increased systemic and intestinal Treg frequencies in both deficient and sufficient animals.

The presence and titers of HRV-specific IgA antibodies in the feces/intestinal contents and serum of humans (40–43) and pigs (44–46) strongly correlate with protection from HRV infection in multiple studies (47). In this study, protein deficiency decreased HRV IgA antibodies in both the serum and small intestinal contents, despite HRV replicating to higher titers in deficient HIFM/HRV pigs, indicating impaired mucosal and systemic HRV antibody responses. Additionally, HRV IgM antibody titers and total IgA concentrations were reduced in serum at PID14 in the deficient HIFM/HRV pigs, further suggesting that protein deficiency compromises intestinal and systemic antibody responses to viral infection. Our findings showing that deficient HIFM/HRV pigs have decreased levels of total and HRV IgA antibody in the intestinal contents are in agreement with the previous findings that protein malnutrition impairs secretory IgA production in humans (48, 49) and mice (50, 51). It has been shown previously that total serum IgM, IgG, and IgA are elevated in humans with the protein malnutrition disorders kwashiorkor and marasmus (52–56). Our findings of increased total serum IgM and IgG concentrations in the deficient-diet pigs at PFMTD9 or PFMTD10/PID0 are consistent with these findings; however, it is unclear why we did not find elevated serum IgA at this time point, in contrast to the findings of others. The increased total concentrations of serum IgA postinoculation in the sufficient HIFM pigs post-HRV infection are likely due to the increased stimulation of mucosal IgA in response to HRV infection.

Protein deficiency also decreased the frequencies of systemic (blood and spleen) CD3⁺ CD4⁺ Th cells and CD3⁺ CD8⁺ CTLs among total MNCs in the HIFM/HRV pigs,
confirming alterations of systemic T cell immune responses. The significant decrease in splenic Th cell frequencies and the downward trend in blood Th cells in deficient HIFM/HRV pigs are consistent with the decreased production of HRV antibodies and total IgA concentrations, as Th cells are necessary for activation of B cells and subsequent antibody production. The decrease in systemic CTLs in deficient HIFM/HRV pigs suggests an impaired response to extraintestinal RV. Our results indicating that protein deficiency does not affect the levels of CD3^+ CD4^+ Th cells in the ileum and duodenum or CD3^+ CD8^+ CTLs in the duodenum are consistent with the findings of Zijlstra and colleagues, who found that malnutrition did not significantly alter the numbers of CD4^+ or CD8^+ T cells in the jejunum of conventional pigs infected with porcine rotavirus (27). The increase of CTLs in the ileum in the deficient HIFM/HRV pigs might be due to the high replication of the rotavirus in the ileum of pigs (14) or the altered and less diverse gut microbiota in these pigs (Kumar et al., submitted). An additional possibility is that *Turicibacter* species, which can modulate CD8^+ T cells (57), were found to be more abundant in the intestines of sufficient HIFM/HRV animals (Kumar et al., submitted). These decreases in T cells were also consistent with the decreased number and function of several innate immune cell types in deficient HIFM/HRV pigs (26), including CD103^+ dendritic cells (DCs), which are necessary to prime and confer intestinal homing of B and T lymphocytes (58).

Serum tryptophan concentrations were reduced in deficient HIFM/HRV pigs pre-HRV inoculation and at 2 weeks postinoculation compared with sufficient-diet pigs, indicating disruption of tryptophan amino acid homeostasis during protein deficiency. In sufficient HIFM/HRV pigs, during the acute phase of HRV infection, serum tryptophan concentrations dropped significantly from preinoculation levels before rising significantly again at PID14. In contrast, serum tryptophan concentrations in deficient HIFM/HRV pigs rose steadily throughout the experiment, without a decrease in concentrations at PID2, suggesting that tryptophan was not catabolized efficiently during the antiviral response. Decreased tryptophan concentrations have been found to inhibit the growth of certain bacteria (59) and parasites (60) and are associated with antiviral immune responses during measles (61), herpes simplex virus (HSV) 1 and 2 (62, 63), and vaccinia virus (64) infections (39). To our knowledge, this study is the first to show that HRV infection decreased serum tryptophan concentrations. Tryptophan also decreases during chronic viral infection with hepatitis B and C, HIV, herpesvirus, and cytomegalovirus (reviewed in reference 39). During these instances where virus is not cleared, IDO is persistently activated and may serve to balance tissue damage by inhibiting antiviral T cell responses and enhancing Tregs, leading to removal of the virus from immune surveillance, creating tolerance (39).

Deficient HIFM/HRV pigs may have been unable to catabolize tryptophan post-HRV inoculation for a number of reasons (Fig. 5). First, gamma interferon (IFN-γ) production may be impaired in deficient HIFM/HRV pigs, not reaching concentrations high enough to trigger IDO1 and the tryptophan pathway. We measured IFN-γ concentrations in the serum and tested for IFN-γ-secreting T cells; however, the low sample sizes showed inconsistent trends. A second mechanism may be impaired Toll-like receptor (TLR) signaling/reduced antigen-presenting cells. Stimulation of TLR2, -3, -4, -7, -8, and -9 can induce kynurenine production via tryptophan catabolism, while stimulation of TLR3 (activated by double-stranded RNA, the component of RV genomes) can also increase the levels of downstream metabolites (65). In mice, protein malnutrition decreases the number of macrophages (66), and we found reduced plasmacytoid dendritic cells and CD103^+ MNCs in deficient HIFM/HRV pigs (26). Finally, while during the initial stages of protein deficiency reserve proteins and amino acids are metabolized for energy, in extended periods of protein deficiency continued loss of cell proteins would be lethal. Therefore, protein turnover and amino acid degradation pathways are suppressed (reviewed in reference 67). In humans and rats who consume protein-deficient diets, there is a suppression of protein turnover and amino acid degradation in muscle (68–70). Furthermore, children with severe edematous malnutrition have been shown to have slowed breakdown of body protein, which decreases the availability of many
To confirm sterility from bacterial or fungal contamination prior to HIFM transplant, aerobic and fat, and carbohydrates, while protein levels were half the recommended levels (7.5% versus 15% of diet). were given a diet of 50% Parmalat and 50% sterile water, which fell within NRC standards for calories, bovine milk (Parmalat) that met or exceeded the National Research Council (NRC) Animal Care Commit-
only a subset of pigs. Protein-sufficient pigs received 100% ultrahigh-temperature-pasteurized (UHT) protein-deficient diet with tryptophan supplementation (\(g\)). pigs at a dose of 10^6 fluorescent focus units (FFU) in 3 ml of minimum essential medium (MEM; Thermo overcame the immune impairment. such as nutritional supplements and immunomodulatory probiotics can be used to
homeostasis. In future studies, this model will be used to test if intervention strategies
intestinal and systemic inflammation, intestinal permeability, and malabsorption and thereby alleviating the negative effects of malnutrition.
In conclusion, our results indicate that protein malnutrition in HIFM-transplanted pigs infected with HRV compromised adaptive immune responses and amino acid homeostasis. In future studies, this model will be used to test if intervention strategies such as nutritional supplements and immunomodulatory probiotics can be used to overcome the immune impairment.

MATERIALS AND METHODS

**Human infant fecal microbiota.** The collection and use of infant fecal samples were approved by the Ohio State University Institutional Review Board (IRB). With parental consent, human infant fecal samples were collected from the soiled diapers of a healthy, 2-month-old, exclusively breastfed, vaginally delivered infant. The fecal samples were pooled, weighed, diluted 1:20 (wt/vol) in phosphate buffer solution containing 0.05% (wt/vol) cysteine and 30% sterile glycerol, and stored at \(-80^\circ C\) as described previously (26).

**Virus.** The Gn pig-adapted (passage 23) HRV Wa G1P[8] strain (virHRV) was administered orally to Gn pigs at a dose of 10^9 fluorescent focus units (FFU) in 3 ml of minimum essential medium (MEM; Thermo Fisher Scientific, Waltham, MA, USA), immediately after oral administration of 3 ml of 100 mM sodium bicarbonate to reduce gastric acidity as described previously (14).

**Animal experiments.** All animal experiments were approved by the Institutional Animal Care and Use Committee at The Ohio State University. Near-term pregnant sows (Landrace \times Yorkshire \times Duroc cross-bred) from The Ohio State University swine center facility were used to derive the Gn pigs by cesarean section, and the Gn pigs were maintained in sterile isolators as described previously (73). Pigs were randomly assigned to one of four groups: (i) protein-sufficient diet (\(n = 10\)), (ii) protein-deficient diet (\(n = 18\)), (iii) protein-sufficient diet with 0.4-g/day tryptophan supplementation (\(n = 3\)), or (iv) protein-deficient diet with tryptophan supplementation (\(n = 3\). T cell experiments were conducted in only a subset of pigs. Protein-sufficient pigs received 100% ultrahigh-temperature-pasteurized (UHT) bovine milk (Parmalat) that met or exceeded the National Research Council (NRC) Animal Care Committee’s guidelines for calories, fat, protein, and carbohydrates in suckling piglets. Protein-deficient pigs were given a diet of 50% Parmalat and 50% sterile water, which fell within NRC standards for calories, fat, and carbohydrates, while protein levels were half the recommended levels (7.5% versus 15% of diet). To confirm sterility from bacterial or fungal contamination prior to HIFM transplant, aerobic and
an aerobic cultures of rectal swabs were performed. Additionally, all piglets were confirmed negative for the enteric viruses—rotavirus, transmissible gastroenteritis, porcine epidemic diarrhoea, calicivirus/sapovirus, astrovirus, and kobavirus—prior to HIFM inoculation. Four days postderivation, Gn pigs were inoculated orally with HIFM. Nine to 10 days were allowed for the establishment and stabilization of the fecal microbiota transplant (26; also Kumar et al., submitted). At postfcal microbiota day 9 or 10 (PFMTD9 or PFMTD10, respectively), pigs were inoculated with virHRV (PID0). All pigs were humanely euthanized by electrocution following anesthesia at PID14.

**Antibody, tryptophan, and ACE2 ELISAs.** IgM, IgG, and IgA HRV antibody geometric mean titers (GMTs) and total IgA concentrations in serum were measured by enzyme-linked immunosorbent assay (ELISA) pre-virHRV inoculation (PFMTD9 or PFMTD10/PID0), post-virHRV inoculation (PID9), and at the termination of the experiment (PID14) as described previously (17). The same parameters were measured in the small intestinal contents at PID14. Serum tryptophan and ACE2 concentrations were quantitatively determined by competitive ELISA pre-virHRV inoculation (PFMTD9 or PFMTD10/PID0), 48 to 72 h post-virHRV inoculation (PID2), and at PID14, by comparison of the absorbance of the unknown samples with a reference curve generated from known standards according to the manufacturer’s instructions (tryptophan ELISA kit from Rocky Mountain Diagnostics, Colorado Springs, CO, and porcine ACE2 ELISA kit from NeoBioLab, Cambridge, MA).

**Isolation of MNCs and flow cytometry.** Blood, duodenum, ileum, and spleen were collected to isolate mononuclear cells (MNCs) as described previously (17). Result acquisition was done using a MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany), and analysis was performed using FlowJo software version 10 (FlowJo LLC, Ashland, OR). To determine Th cells (CD3+/CD4+), and cytotoxic T lymphocytes (CTLs [CD3+/CD8+]), we stained each sample with anti-porcine CD3+/fluorescein isothiocyanate (FITC; clone PPT3; Southern Biotech, Birmingham, AL), CD4+ phycoerythrin (PE; clone 74-12-4; BD Biosciences, San Jose, CA), and CD8+ spectral red (SPRD; clone 76-2-11; BD Biosciences) for 15 min at 4°C. The frequency of T cells was expressed as the percentage among total MNCs (determined based on forward and side scatter characteristics). The frequencies of natural regulatory T cells (Tregs; CD4+/CD25+Foxp3+ and CD8+/CD25+Foxp3+) were determined as described previously (17).

**Statistics.** Results were tested for normality using the D’Agostino-Pearson omnibus test. Differences in HRV antibody isotype titers, total IgA concentrations, CD3+/CD4+ Th cell and CD3+/CD8+ cytotoxic T cell frequencies, and ACE2 were analyzed by the Mann-Whitney test. Differences in tryptophan concentrations between and among groups were analyzed with unpaired t tests, and differences in CD4+/CD25+Foxp3+ and CD8+/CD25+Foxp3+ frequencies were calculated using 2-way analysis of variance (ANOVA) and Bonferroni’s multiple-comparison test. All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc., CA, USA). P values of <0.05 were considered statistically significant for all comparisons.

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