Dietary Human Milk Oligosaccharides but Not Prebiotic Oligosaccharides Increase Circulating Natural Killer Cell and Mesenteric Lymph Node Memory T Cell Populations in Noninfected and Rotavirus-Infected Neonatal Piglets¹–³

Sarah S Comstock,¹ Min Li,¹ Mei Wang,¹ Marcia H Monaco,¹ Theresa B Kuhlenschmidt,⁵ Mark S Kuhlenschmidt,⁵ and Sharon M Donovan⁴*

¹Food Science and Human Nutrition and ⁵Pathobiology, University of Illinois, Urbana, IL

Abstract

Background: Human milk oligosaccharides (HMOs) have antimicrobial and immunomodulatory actions. It has previously been reported that these oligosaccharides contribute to the reduced duration of rotavirus-induced diarrhea in pigs. Objective: We measured the effects of HMOs and prebiotic oligosaccharides on immune cell populations from noninfected and rotavirus-infected pigs. We hypothesized that dietary HMOs would modulate systemic and gastrointestinal immunity. Methods: Colostrum-deprived newborn pigs were fed formula, formula with 4 g HMOs/L (2'-fucosyllactose, lacto-N-neotetraose, 6'-sialyllactose, 3'-sialyllactose, and free sialic acid), or formula with 3.6 g short-chain galactooligosaccharides/L and 0.4 g long-chain fructooligosaccharides/L. On day 10, half of the pigs were infected with the porcine rotavirus strain OSU. Peripheral blood mononuclear cell (PBMC), mesenteric lymph node (MLN), and ileal Peyer/C213s patch immune cell populations were assessed with the use of flow cytometry 5 d postinfection. Interferon-γ (IFN-γ)-producing cells were assessed with the use of Enzyme-Linked ImmunoSpot assay.

Results: Infection changed immune cell populations with more systemic natural killer (NK) cells, memory effector T cells, and major histocompatibility complex II cells in infected than noninfected pigs (P < 0.06). Regardless of infection status, HMO-fed pigs had nearly twice as many PBMC NK cells, 36% more MLN effector memory T cells, and 5 times more PBMC basophils than formula-fed pigs (P < 0.04). These populations were intermediate in pigs fed prebiotics. PBMCs from HMO-fed noninfected pigs had twice as many IFN-γ-producing cells as did those from formula-fed noninfected pigs (P = 0.017). The PBMCs and MLNs of formula-fed noninfected pigs had 3 times more plasmacytoid dendritic cells (pDCs) than those of HMO-fed noninfected and formula-fed infected pigs (P < 0.04). In the MLNs, the formula-fed noninfected pigs had more macrophages, pDCs, and mature DCs (P < 0.04) but fewer immature DCs than HMO-fed noninfected pigs (P = 0.022).

Conclusions: Dietary HMOs were more effective than prebiotics in altering systemic and gastrointestinal immune cells in pigs. These altered immune cell populations may mediate the effects of dietary HMOs on rotavirus infection susceptibility. J Nutr 2017;147:1041–7.

Keywords: human milk oligosaccharides, prebiotics, mucosal immunology, rotavirus, swine

Introduction

Although rotavirus vaccination programs are now widespread, rotavirus-associated diarrhea persists in many developing countries (1–5). In addition, in areas where vaccination is not common, rotavirus continues to be a major viral pathogen (6). The WHO rotavirus surveillance program reported that 46% of children aged <1 y who were hospitalized with diarrhea tested positive for rotavirus in countries where children are not vaccinated compared with 17% of those in countries where rotavirus vaccination is routine (7). Therefore, it is important to identify additional methods for preventing or reducing the impact of rotavirus infection.

Breastfed infants have a lower incidence of rotavirus infection than formula-fed infants (8), which has been attributed to...
the presence of Igs and other bioactive components in human milk (9). Until recently, little attention had been paid to the potential role of complex oligosaccharides in the immune response to rotavirus infection despite the fact that human milk oligosaccharides (HMOs)8 have been shown to have antimicrobial and immunomodulatory actions in vitro and are absent from most infant formulas (10–12).

In terms of immunomodulatory activity, several direct effects of HMOs on immune cells have been reported. When added to cultures of peripheral blood mononuclear cells (PBMCs), HMOs affected immune cell populations and functions, such as cellular proliferation and cytokine production (12). HMOs also stimulated ex vivo—cultured umbilical cord blood cells to produce cytokines (13) and modulated in vitro immune cell activation and extravasation (14, 15). These findings support the potential for HMOs to directly affect immune activity in rotavirus-infected neonates.

We tested the antirotavirus activity of HMOs in vitro, in situ, and in vivo. Sialic acid—containing HMOs (3’-sialyllactose and 6’-sialyllactose) inhibited the binding and infectivity of the sialic acid—dependent rotavirus to the host cells in vitro and both neutral and acidic HMO reduced rotavirus replication in an in situ acute rotavirus infection piglet model (11). In a piglet rotavirus infection model, we further demonstrated that dietary HMO reduced the duration of diarrhea (16).

We expand the research published by Li et al. (16) by presenting the effects of dietary HMOs on immune cell populations from the peripheral blood (PBMCs), mesenteric lymph nodes (MLNs), and ileal Peyer’s patches (IPPs) of healthy and rotavirus-infected pigs. Complex oligosaccharides, one member of a class of compounds called prebiotics, are currently being added to some infant formulas and are readily available for experimentation, but prebiotics have rarely been tested compared with HMOs in large animal models. Therefore, this research compared pigs fed a control formula, HMOs, or prebiotics. We hypothesized that feeding a mixture of HMOs would modulate systemic and gut mucosal cellular immunity. Prebiotic supplementation was expected to have an intermediary effect.

Methods
Animal care and dietary treatments. This study was approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. The study was conducted as described, and samples for this study were taken from the same pigs whose Ig response, intestinal cytokine production, and gut microbiota community composition have been described previously (16). Piglets (26 females and 24 males) were vaginally delivered but removed from the sow immediately after birth to avoid the ingestion of colostrum. Piglets were fed a commercial milk replacer formula [Advance Liquiwean; Milk Specialties (17); n = 16]; formula with 4 g HMOs/L that consisted of 40% 2’-fucosyllactose (Glycom), 35% lacto-N-neotetraose (Glycom), 10% 6’-sialyllactose (Carbosynth), 5% 3’-sialyllactose (Carbosynth), and 10% free sialic acid (Glycom) (n = 17); or formula with 4 g prebiotics/L consisting of 3.6 g short-chain galactooligosaccharides (Domo Vitalin GOS; FrieslandCampina) and 0.4 g long-chain fructooligosaccharides/L (Orafti HP; BENEO-Orafti) (n = 17). Lactose (4 g/L) (Cereal Byproducts) was added to the control formula diet (Supplemental Table 1). Piglets were weighed, and formula (18.3% solids) was provided as described previously (16). Pigs voluntarily consumed all provided milk. All pigs were passively immunized with sow serum (16). Serum was obtained from sows purchased from Midwest Research Swine’s High Health Status herd, which exceeds the standards set by the National SPF Swine Accrediting Agency. The sows were vaccinated against common porcine diseases except rotavirus. At postpartum day 10, half of the pigs in each dietary treatment group were orally gavaged with 5 × 10⁶ focus-forming units of the porcine rotavirus strain OSU/L prepared as described previously (11).

Sample collection. PBMCs, IPPs, and MLNs were collected 5 d postinfection (15 d postpartum). PBMCs were isolated with the use of density gradient centrifugation (400 × g; 30 min; at room temperature with the brake off) (12). IPPs and MLNs were dissociated as described previously (18). Resulting cell suspensions were treated as described previously (19).

Phenotypic identification of cells. PBMC, MLN, and IPP immune cell populations were assessed by flow cytometry with the use of fluorescently labeled antibodies as described previously (12, 18). Cell-staining antibody cocktails are presented in Supplemental Table 2. Some cell populations were not analyzed for some pigs because of a lack of sufficient isolated cells (i.e., basophils in infected pigs).

Enzyme-Linked ImmunoSpot assay. IFN-γ—producing cells were assessed with the use of the EL985 Enzyme-Linked ImmunoSpot assay (R&D Systems) per the manufacturer’s instructions. Cells (5 × 10⁶/well) were either unstimulated or stimulated with 10 µg purified rotavirus strain OSU/L for 24 h. Plates were analyzed by Cellular Technology Ltd. Data are expressed as fold change: the number of cells that secreted IFN-γ in response to rotavirus stimulation divided by the number of cells producing IFN-γ in unstimulated cultures.

Statistical analyses. Data that were not normally distributed were transformed with the use of logarithmic or square root transformations. Data were analyzed with the use of SAS version 9.3 (SAS Institute). Proc GLM was used with diet, infection, and the interaction of diet and infection as terms in each model. Post hoc testing was conducted with the use of the pdiff option of the least-squares means statement in SAS. Multiple-comparison correction was based on Tukey’s method. When the model that included both diet and infection as main effects was not significant but a single effect (diet or infection) was significant, a parsimonious model that included only the significant main effect was used to analyze the data. Statistical significance was set at P ≤ 0.05, and comparisons with P values ≤ 0.1 are reported as trends. Data are expressed as means ± SEMs.

Results
Growth
Birth weights (P = 0.51), final weights (P = 0.33), and weight gains (P = 0.30) were similar for pigs in all treatment groups. Pigs weighed 1.45 ± 0.05 kg at birth. By the end of the study, pigs weighed 4.14 ± 0.12 kg. Pigs gained 2.69 ± 0.08 kg over the course of the study.

Immune cell populations
Basophils. Basophils play an important role in the early life immune system (20). Because of limited cell sample availability, basophil populations could only be determined for noninfected pigs. Among noninfected pigs, formula-fed pigs had fewer PBMC or MLN basophils than HMO-fed pigs (P < 0.02) (Table 1). Noninfected formula-fed pigs also tended (P < 0.10) to have fewer IPP basophils than HMO- or prebiotic-fed pigs (data not shown).

NK cells. Infection and diet had independent effects on NK cell populations. PBMC NK cells were increased (P = 0.05) by infection (Table 1). NK cell populations in the local intestinal environment, including the MLNs (P = 0.009) and IPPs (P = 0.011)}
TABLE 1  Immune cell populations 5 d postinfection in rotavirus- and noninfected piglets aged 15 d fed formula alone or formula supplemented with 4 g HMOs/L or 4 g prebiotics/L

<table>
<thead>
<tr>
<th></th>
<th>Noninfected Formula</th>
<th>Noninfected HMO</th>
<th>Noninfected Prebiotics</th>
<th>Infected Formula</th>
<th>Infected HMO</th>
<th>Infected Prebiotics</th>
<th>Model</th>
<th>Diet</th>
<th>Infection</th>
<th>Diet × infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basophils (CD16−“MHCIICD172a+”), % CD172a+ events</td>
<td>7.5 ± 1.1a,b</td>
<td>35 ± 7.1a</td>
<td>22 ± 7.8b</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>0.016</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>RBMC</td>
<td>14 ± 0.66b</td>
<td>46 ± 1.2a</td>
<td>32 ± 7.5b</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>0.003</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>MLN</td>
<td>4.6 ± 1.1b</td>
<td>9.1 ± 4.2a</td>
<td>7.7 ± 3.6b</td>
<td>7.0 ± 2.9b</td>
<td>13 ± 3.2a</td>
<td>9.7 ± 1.8b</td>
<td>0.04</td>
<td>0.02</td>
<td>0.05</td>
<td>0.83</td>
</tr>
<tr>
<td>NK cells (CD3−“CD4+ “CD8−”), % singletons</td>
<td>7.8 ± 1.3</td>
<td>6.1 ± 1.1</td>
<td>7.5 ± 1.1</td>
<td>4.4 ± 1.2</td>
<td>4.6 ± 1.1</td>
<td>4.5 ± 1.2</td>
<td>0.14</td>
<td>0.77</td>
<td>0.01</td>
<td>0.69</td>
</tr>
<tr>
<td>IPP</td>
<td>1.1 ± 0.10</td>
<td>1.6 ± 0.22</td>
<td>1.4 ± 0.23</td>
<td>0.73 ± 0.23</td>
<td>0.89 ± 0.19</td>
<td>0.11 ± 0.21</td>
<td>0.04</td>
<td>0.15</td>
<td>0.01</td>
<td>0.41</td>
</tr>
<tr>
<td>Memory effector T cells (CD4−“CD8+ “CD3+), % CD3+</td>
<td>7.8 ± 0.89</td>
<td>10 ± 0.76</td>
<td>8.3 ± 1.0</td>
<td>11 ± 1.4</td>
<td>11 ± 1.7</td>
<td>11 ± 1.8</td>
<td>0.29</td>
<td>0.68</td>
<td>0.04</td>
<td>0.56</td>
</tr>
<tr>
<td>RBMC</td>
<td>4.2 ± 0.58b</td>
<td>7.2 ± 0.64a</td>
<td>6.5 ± 1.1b</td>
<td>7.9 ± 0.98b</td>
<td>8.9 ± 1.3a</td>
<td>9.1 ± 1.2b</td>
<td>&lt;0.01</td>
<td>0.04</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>MLN</td>
<td>77 ± 2.2</td>
<td>83 ± 2.9</td>
<td>85 ± 1.4</td>
<td>88 ± 3.1</td>
<td>86 ± 2.2</td>
<td>85 ± 2.4</td>
<td>0.05</td>
<td>0.66</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>IPP</td>
<td>4.0 ± 0.82</td>
<td>2.7 ± 0.4</td>
<td>4.2 ± 1.4</td>
<td>1.2 ± 0.3</td>
<td>1.7 ± 0.4</td>
<td>1.9 ± 0.45</td>
<td>0.02</td>
<td>0.53</td>
<td>&lt;0.01</td>
<td>0.49</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>40 ± 17</td>
<td>46 ± 14</td>
<td>32 ± 8.4</td>
<td>110 ± 27</td>
<td>89 ± 25</td>
<td>78 ± 26</td>
<td>0.03</td>
<td>0.65</td>
<td>&lt;0.01</td>
<td>0.62</td>
</tr>
<tr>
<td>Monocytes (CD172a−“CD14+), % myeloid cells</td>
<td>29 ± 2.1</td>
<td>21 ± 1.2</td>
<td>29 ± 2.4</td>
<td>19 ± 1.3</td>
<td>20 ± 2.0</td>
<td>18 ± 1.0</td>
<td>&lt;0.01</td>
<td>0.34</td>
<td>0.01</td>
<td>0.07</td>
</tr>
<tr>
<td>MLN</td>
<td>33 ± 7.5</td>
<td>50 ± 6.3</td>
<td>33 ± 4.1</td>
<td>66 ± 8.0</td>
<td>60 ± 5.8</td>
<td>61 ± 6.7</td>
<td>&lt;0.01</td>
<td>0.38</td>
<td>&lt;0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>Macrophages (CD16+ “CD172a− “CD14+), % myeloid cells</td>
<td>10 ± 1.7</td>
<td>2.9 ± 1.3</td>
<td>5.8 ± 1.9</td>
<td>2.8 ± 1.5</td>
<td>2.4 ± 1.3</td>
<td>2.7 ± 1.0</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td>&lt;0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>CD14+, % myeloid cells</td>
<td>26 ± 2.0</td>
<td>26 ± 1.6</td>
<td>29 ± 2.5</td>
<td>22 ± 2.3</td>
<td>22 ± 1.4</td>
<td>20 ± 0.52</td>
<td>0.01</td>
<td>0.86</td>
<td>&lt;0.01</td>
<td>0.32</td>
</tr>
<tr>
<td>MLN</td>
<td>49 ± 5.3</td>
<td>54 ± 5.6</td>
<td>44 ± 3.8</td>
<td>80 ± 3.2</td>
<td>70 ± 5.3</td>
<td>71 ± 6.3</td>
<td>&lt;0.01</td>
<td>0.40</td>
<td>&lt;0.01</td>
<td>0.32</td>
</tr>
<tr>
<td>MHCIIC+, % singletons</td>
<td>36 ± 2.5</td>
<td>56 ± 5.3</td>
<td>45 ± 6.2</td>
<td>53 ± 6.5</td>
<td>53 ± 6.0</td>
<td>57 ± 3.4</td>
<td>0.04</td>
<td>0.18</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>RBMC</td>
<td>50 ± 3.8</td>
<td>55 ± 2.6</td>
<td>55 ± 2.6</td>
<td>59 ± 4.0</td>
<td>62 ± 3.6</td>
<td>60 ± 7.0</td>
<td>0.28</td>
<td>0.57</td>
<td>0.02</td>
<td>0.92</td>
</tr>
<tr>
<td>MLN</td>
<td>80 ± 2.3</td>
<td>85 ± 1.6</td>
<td>82 ± 3.0</td>
<td>86 ± 5.9</td>
<td>90 ± 2.5</td>
<td>89 ± 3.0</td>
<td>0.12</td>
<td>0.35</td>
<td>0.03</td>
<td>0.89</td>
</tr>
</tbody>
</table>

1 Values are means ± SEMs. The per diet/infection combination was as follows: PBMCs, n = 6–9; MLNs, n = 7–9, and IPPs, n = 5–9. The number of samples differed by tissue and treatment group because there was a limited number of total cells available from any one piglet from any one tissue, limiting the analyses that could have been done. All data collected were used in the analysis. Labeled means in a row without a common superscript letter differ, P ≤ 0.05. HMO, human milk oligosaccharide; IPP, ileal Peyer’s patch; MHC, major histocompatibility complex; MLN, mesenteric lymph node; NM, not measured; PBMC, peripheral blood mononuclear cell.

2 Data on the percentage of basophils in blood from rotavirus-infected pigs were not available because of a lack of sufficient isolated cells.

(Table 1) of infected pigs, were smaller than those in noninfected pigs. Dietary HMOs increased PBMC NK cell populations independent of infection status. Formula-fed pigs had fewer PBMC NK cells than HMO-fed pigs (P = 0.023) (Table 1), with prebiotic-fed pigs being intermediate.

Memory effector T cells. Infection and diet had independent significant effects on memory effector T cell populations in the MLN (Table 1). MLN memory effector T cell populations were smaller in noninfected pigs than infected pigs (Table 1). Formula-fed pigs had fewer MLN effector memory T cells than HMO-fed pigs (P = 0.042) (Table 1), with prebiotic-fed pigs having an intermediate percentage of effector memory T cells. Infected pigs had larger PBMC memory effector T cell populations than noninfected pigs. IPP memory effector T cells were not affected by diet or infection (data not shown).

T helper cells. PBMC and MLN T helper cells were not affected by diet or infection (data not shown). Infected pigs had larger IPP T helper cell populations than noninfected pigs (P = 0.029) (Table 1).

Cytotoxic T cells. Diet did not affect cytotoxic T cell populations in PBMCs, MLNs, or IPPs (data not shown). Infected pigs had smaller populations of IPP cytotoxic T cells than noninfected pigs (P < 0.001) (Table 1).

T helper (CD4+ “cytotoxic T (CD8) cell ratio). Diet did not affect the T helper:cytotoxic T cell ratios in the PBMCs, MLNs, or IPPs (data not shown). Infection, however, increased the CD4:CD8 ratio in the IPPs (P = 0.001) (Table 1) but not in the PBMCs or MLNs (data not shown).

γδ T cells. Diet did not affect γδ T cell populations in the PBMCs, MLNs, or IPPs (data not shown).

Monocytes and macrophages. Diet did not affect total CD14+, monocyte, or macrophage cell populations in the PBMCs, MLNs, or IPPs (data not shown). Total MLN CD14+ cells and monocytes were decreased by infection, whereas total IPP CD14+ cells and monocytes were increased by infection (P < 0.01) (Table 1). Although the total CD14+ cell population and the monocyte population of MLN and IPP tissues responded...
differently to rotavirus infection, macrophage populations decreased in both the MLNs (P < 0.01) (Table 1) and IPPs (P = 0.024) (data not shown) in response to rotavirus infection.

B cells. PBMC, MLN, and IPP B cell populations were not affected by diet or infection (Table 1). When infection was considered as the only main effect, noninfected pigs had fewer major histocompatibility complex II* cells than infected pigs in PBMCs, MLNs, and IPPs (P < 0.043) (Table 1). Diet did not affect major histocompatibility complex II* cell populations (Table 1).

Dendritic cells. Dietary treatment affected systemic and mesenteric plasmacytoid dendritic cells (pDCs) in noninfected but not rotavirus-infected pigs (P < 0.029). Formula-fed noninfected pigs had more pDCs than did HMO-fed noninfected pigs, with prebiotic-fed noninfected pigs having intermediate-sized pDC populations in both the PBMCs (P = 0.012) (Figure 1A) and MLNs (P = 0.023) (Figure 1B). IPP pDC populations were not affected by diet or infection (Figure 1C). The immature DC populations in the MLNs was larger in formula-fed noninfected pigs than in all other groups (P < 0.022) (Figure 2A). Formula-fed noninfected pigs had smaller immature DC MLN populations than did HMO-fed noninfected and all infected pigs (P < 0.008) (Figure 2B). There were no effects of diet or infection on either mature or immature CD172a+ DCs in the PBMCs or IPPs (data not shown).

IFN-γ secretion
Dietary HMOs also affected the functional capability of PBMCs. In response to stimulation with rotavirus antigens, PBMCs from HMO-fed noninfected pigs experienced a 2-fold greater change in IFN-γ-producing cells than that experienced by PBMCs from formula-fed noninfected pigs (P = 0.017) (Figure 3). In addition, PBMCs from prebiotic-fed noninfected pigs tended to have a similar increase in fold change in IFN-γ-producing cells as that seen in PBMCs from HMO-fed noninfected pigs (P = 0.086). The fold change in IFN-γ-producing cells induced by rotavirus stimulation in PBMCs from infected pigs was similar to that induced in noninfected pigs. Neither diet (P = 0.60) nor infection (P = 0.45) affected the number of MLN cells that secreted IFN-γ in response to rotavirus stimulation (data not shown). No IFN-γ-producing cells could be detected in cultures of rotavirus-stimulated IPP cells (data not shown).

Discussion
We have previously shown that the consumption of formula containing either 4 g HMOs/L or 4 g prebiotics/L by the neonatal piglets used in this study reduced the duration of diarrhea after rotavirus infection compared with the consumption of the control formula (16). Although both HMOs and prebiotics reduced the duration of diarrhea, some immune mechanisms differed between the groups (16). Specifically, HMOs enhanced ileal mucosal cytokine (IFN-γ, IL-10) mRNA expression 5 d postinfection, whereas prebiotics promoted rotavirus-specific IgM response to the infection (16). To expand upon this previous work, we investigated the impact of dietary HMOs and prebiotics on the cellular immune responses of these pigs. The many independent effects of infection on immune cell populations were expected because these analyses were conducted 5 d postinfection with rotavirus (16, 21). Some dietary effects, including increased PBMC NK cells and MLN memory effector T cells in HMO-fed pigs, were observed in both noninfected and infected pigs. Changes induced by dietary prebiotics were typically intermediate to those induced by dietary HMOs.

Some HMO-induced immune changes reported herein potentially contributed to the shortened duration of diarrhea in rotavirus-infected pigs reported previously (16). For instance, PBMCs isolated from HMO-fed noninfected pigs were more likely to produce IFN-γ than were those isolated from formula-fed noninfected pigs. In a gnotobiotic pig model of rotavirus infection, the induction of IFN-γ-producing cells before infection resulted in greater protection from rotavirus diarrhea upon rotavirus challenge (22). Others have shown that rice bran, which can function as a prebiotic because it is a source of complex oligosaccharides (23), effectively decreased rotavirus infection in gnotobiotic pigs by inducing more IFN-γ-producing PBMCs as well as more IgM-producing B cells (24). In this study,
Specifically recognize foreign viral material, such as toll-like receptors such as galectins (25), and the cellular receptors that nonspecifically recognize HMO and prebiotic glycan structures, responsive to rotavirus stimulus than those from formula-fed PBMCs from HMO-fed noninfected pigs may have been more responsive to the presence of viral material. The effects of HMO and prebiotic feeding would result in cells that are more responsive in the MLNs of HMO-fed noninfected pigs were larger than those of formula-fed noninfected pigs. Thus, the HMO-induced increases in monocytes, macrophages, and DCs in the MLN could improve the pig’s ability to limit the clinical signs of the rotavirus infection through the induction of a robust local helper 1 response.

A small body of work has examined the role of DCs in rotavirus infection. Human DCs internalize rotavirus-like particles (30). In addition, rotavirus particles have been found in DCs isolated from infected animals, with rotavirus replication occurring in at least some rotavirus-associated DCs (29, 31, 32). Once internalized, double-stranded rotavirus RNA binds intracellular toll-like receptor 3 in DCs, leading to the activation of immature DCs (28, 32). Rotavirus infection increases DC IL-6 production and enables DCs to convert naïve CD4+ T cells into activated IFN-γ-secreting T helper 1 cells. In this model, monocytes and macrophages and DC populations in the MLNs of HMO-fed noninfected pigs were larger than those of formula-fed noninfected pigs. Thus, the HMO-induced increases in monocytes, macrophages, and DCs in the MLN could improve the pig’s ability to limit the clinical signs of the rotavirus infection through the induction of a robust local helper 1 response.

HMO-induced changes in monocytes, macrophages, and DC populations in the MLNs of HMO-fed noninfected pigs were larger than those of formula-fed noninfected pigs. Thus, the HMO-induced increases in monocytes, macrophages, and DCs in the MLN could improve the pig’s ability to limit the clinical signs of the rotavirus infection through the induction of a robust local helper 1 response.

Previous work with samples from these pigs demonstrated a significant increase in IL-8, IFN-γ, and IL-10 mRNA expression in the ileum of rotavirus-infected piglets (16). In this immune cell analysis, we detected larger populations of IPP monocytes in rotavirus-infected pigs than in noninfected pigs. This observation is consistent with the greater IL-8 expression in the ileum of these pigs because IL-8 is a chemoattractant for monocytes (33). Although changes in IPP pDC populations did not reach the level of statistical significance in response to diet or infection in this analysis, the pattern of IPP pDC population sizes was similar to that seen in the pattern of ileal IL-8, which is consistent with the

PBMCs from HMO-fed noninfected pigs may have been more responsive to rotavirus stimulus than those from formula-fed noninfected pigs because of an interaction between the cellular receptors that recognize HMO and prebiotic glycan structures, such as galectins (25), and the cellular receptors that nonspecifically recognize foreign viral material, such as toll-like receptors (26–28). Cell signaling through galectin ligation could result in the upregulation of toll-like receptor expression. In this way, HMO and prebiotic feeding would result in cells that are more responsive to the presence of viral material. The effects of HMO on the cellular response to viral material could be direct effects on IFN-γ-producing cell types or indirect effects whereby these mechanisms cause some cell types to produce mediators that trigger IFN-γ production by other cells. For instance, HMO and prebiotic glycan and rotavirus signaling could cause NK cells or pDCs to produce IFN-α, which then triggers IFN-γ production by cells of the adaptive immune system. Because of the IFN-γ production and stimulation capacity of NK cells, HMO-induced increases in PBMC NK cells may be pivotal in shortening the duration of rotavirus-induced diarrhea seen in infected pigs. If similar increases in circulating NK cell populations and IFN-γ-producing cells are observed in HMO-fed human infants, including HMOs in infant formulas could potentially protect infants from viral infections.

HMO-induced changes in MLN cell populations are also likely to be important in the resolution of clinical signs of rotavirus infection. The MLN is the most important site of the immune response to rotavirus (29). If an increase in memory effector T cells occurs in the MLN before infection, pigs fed HMOs could respond to rotavirus infection and other immunologic challenges faster than formula-fed pigs. Memory effector T cells also produce IFN-γ. However, the HMO-induced increase in MLN memory effector T cells did not result in a corresponding increase in MLN IFN-γ-producing cells. Similar to all rotavirus-infected pigs, HMO-fed noninfected pigs had fewer pDCs, fewer mature DCs, and more immature DCs in their MLNs at postpartum day 15 than did formula-fed noninfected pigs. Combined, these changes in MLN cell populations could indicate a general increased surveillance capacity of the intestinal immune system in HMO-fed noninfected pigs. Improved surveillance capacity would contribute to a more rapid response to intestinal challenges such as viral infections.

FIGURE 2  Mature (A) and immature (B) DC populations in the MLNs from rotavirus-infected and noninfected piglets aged 15 d fed FF alone or FF supplemented with 4 g HMOs/L or 4 g PREs/L. Samples were collected 5 da after rotavirus infection. Data are means ± SEMs (n = 2–5). Within a panel, labeled columns without a common letter differ, P < 0.05. D, diet; DC, dendritic cell; FF, formula; HMO, human milk oligosaccharide; I, infection; MHC, major histocompatibility complex; MLN, mesenteric lymph node; PRE, prebiotic.

FIGURE 3  IFN-γ–producing PBMCs from rotavirus-infected and noninfected piglets aged 15 d fed FF alone or FF supplemented with 4 g HMOs/L or 4 g PREs/L. PBMCs were collected 5 d after rotavirus infection and remained unstimulated or were stimulated with 10 μg rotavirus/mL for 24 h. Data are means ± SEMs (n = 6–9). Labeled columns without a common letter differ, P < 0.05. D, diet; FF, formula; HMO, human milk oligosaccharide; I, infection; PBMC, peripheral blood mononuclear cell; PRE, prebiotic.
fact that pDCs secrete IL-8 (34). The increased CD4+CD8+ T cell ratio observed in the IPPs of infected pigs is consistent with enhanced ileal IL-10 expression in the infected pigs but not with greater ileal IFN-γ expression in infected pigs (35). The higher IFN-γ mRNA expression in the ileum of infected pigs (16) was also inconsistent with the presence of fewer NK cells in the IPPs of infected pigs. These inconsistencies potentially resulted from the time point at which these analyses were conducted (5 d postinfection) and could also indicate that an alternative cellular source of IFN-γ is present in the ileum of these pigs.

Timing is critical in an immune response, and thus the analysis of a single time point postinfection limited our ability to detect other dietary-driven changes in cell populations. Five days postinfection is just past the time of peak innate immune response (day 3) and just before the time of peak adaptive immune response (day 7). By day 5 postinfection in neonatal piglets, the T lymphocyte response begins to peak, rotavirus viral DNA production begins to decrease, and lactase activity begins to return to preinfection levels, although villus height is still blunted (36, 37). The main interactions between diet and infection were seen in myeloid cell populations but not in lymphocyte populations, potentially because of the timing of our analysis. A prebiotic supplementation study in mice demonstrated that NK cell numbers were higher in prebiotic-fed mice than mice fed control diets at 3 d postinfection, whereas no difference in NK cell populations existed at either 0 or 7 d postinfection (38). Rotavirus antigen has been detected in the IPPs, MLNs, and spleens of mice ≤20 d after rotavirus infection (29, 31). In these organs, rotavirus antigen was typically localized to the cytoplasm of DCs but could also be found in macrophages and B cells. A similar study found rotavirus antigen in macrophages and B cells but not in CD11c+ DC cells (39). All 3 cell types (DC, B cells, and macrophages) have the capacity to present viral antigen to rotavirus-specific T cells. However, it has also been shown that DCs and possibly macrophages and B cells support rotavirus replication (29). Thus, the day 5 postinfection effects on myeloid cells but not T lymphocytes may have been detected because myeloid cells are responsible for the innate response to rotavirus infection and because myeloid cells can support rotavirus replication.

HMO-induced effects on other immune cells could have been detected if the analyses had taken place at a different postinfection time point.

The mechanism by which dietary complex oligosaccharides, such as HMOs, change immune cell populations and the immune response to immunologic challenges remains to be determined. However, these effects are likely mediated by a combination of 4 interrelated consequences of feeding HMOs: 1) direct stimulation of immune cells (12, 13, 15, 40, 41), 2) alteration of intestinal bacterial populations (17, 42), 3) modulation of intestinal barrier function (43–47), and 4) alteration of viral pathogenicity (11, 48). Prebiotic oligosaccharides may be less effective than HMOs in eliciting these alteration of viral pathogenicity (11, 48, 49). Prebiotic oligosaccharides, such as HMOs, change immune cell populations and the immune response to immunologic challenges remains to be determined. However, these effects are likely mediated by a combination of 4 interrelated consequences of feeding HMOs: 1) direct stimulation of immune cells (12, 13, 15, 40, 41), 2) alteration of intestinal bacterial populations (17, 42), 3) modulation of intestinal barrier function (43–47), and 4) alteration of viral pathogenicity (11, 48). Prebiotic oligosaccharides may be less effective than HMOs in eliciting these changes because of differing structural moieties (50) or differential utilization and thus differential promotion of specific intestinal bacteria (17, 51). We have not addressed intestinal barrier function or viral pathogenicity but have previously reported (16) that the gut microbial communities of the pigs fed 4 g HMOs/L had an increased abundance of Lachnospiraceae and reduced abundance of Ruminococcaceae. Infection and HMO feeding explained 9.1% and 2.7%, respectively, of the total variability in the gut microbial communities of these pigs. Furthermore, intestinal IFN-γ mRNA and unclassified Lachnospiraceae were positively correlated in HMO-fed pigs. Unclassified Lachnospiraceae were only correlated with pDC in MLNs from HMO-fed pigs (r = 0.51; P = 0.04). Thus, future studies are needed to investigate how HMO-associated changes in the microbiome regulate neonatal cellular immune function.

In conclusion, complex oligosaccharides in human milk affect infant immune development and improve neonatal response to infection. There is a substantial clinical need for understanding the bioactivities of HMOs to inform the design of improved infant formulas and to identify potential clinical interventions for infants at risk for rotavirus infection. Although others have assessed the effect of bovine-sialylated milk oligosaccharides on growth in a gnotobiotic piglet model (52), this preclinical study is among the first, to our knowledge, to assess the effects of a combination of dietary HMOs on cellular immunity in the context of pathogenic challenge in a large animal model. As such, it is a first step toward understanding the in vivo bioactivities of HMOs.

Acknowledgments
We thank Kilia Liu for assistance with piglet care and sample processing. The authors’ responsibilities were as follows—SGC, ML, MW, and SMD: designed the in vivo study; SSC and SMD: designed the ex vivo studies, and analyzed and interpreted the data; ML and MHH: conducted the in vivo study; SSC, ML, and MW: conducted the ex vivo experiments; TBK and MSK: provided the rotavirus and expertise regarding swine rotavirus infections; SSC: drafted the manuscript; SMD: had responsibility for final content; and all authors: read and approved the final manuscript.

References

1046 Comstock et al.


