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Select human milk oligosaccharides directly modulate peripheral blood mononuclear cells isolated from 10-d-old pigs

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Abstract

Infant formulas lack the complex mixture of oligosaccharides found in human milk. These human milk oligosaccharides (HMO) may be pivotal to the development of the neonatal immune system. Few comprehensive analyses of the effects of HMO on immune cells from neonates have been undertaken. Herein, the direct effects of HMO on immune cells were analysed *ex vivo*. Peripheral blood mononuclear cells (PBMC) isolated from 10-d-old sow-reared (SR) or colostrum-deprived formula-fed (FF) pigs were stimulated for 72 h with single HMO, mixtures of single HMO or a complex mixture of HMO isolated from human milk (iHMO). T-cell phenotype, cytokine production and proliferation were measured by flow cytometry, immunoassay and [^{3}H]thymidine incorporation, respectively. Stimulation with HMO had direct effects on PBMC. For instance, cells stimulated with iHMO produced more IL-10 than unstimulated cells, and cells stimulated with fucosylated HMO tended to proliferate less than unstimulated cells. Additionally, co-stimulation with HMO mixtures or single HMO altered PBMC responses to phytohaemagglutinin (PHA) or lipopolysaccharide (LPS) stimulation. Compared with PBMC stimulated with PHA alone, cells co-stimulated with iHMO and PHA proliferated more and had fewer detectable $CD4^+CD8^+$ T cells. Compared with PBMC stimulated by LPS alone, cells co-stimulated with a mixture of sialylated HMO and LPS proliferated more and tended to have fewer detectable $CD4^+$ T cells. Differences in the baseline responses of PBMC isolated from the SR or FF pigs were observed. In summary, HMO directly affected PBMC populations and functions. Additionally, *ex vivo* measurements of PBMC phenotype, cytokine production and proliferation were influenced by the neonate's diet.

Key words: Infants: Human milk: Oligosaccharides: Immunity: Pigs

Immune response, as evidenced by the differences in the rates of infection[1–6] and vaccination response[6,7], differs between breast-fed (BF) and formula-fed (FF) human infants. This difference may be due in part to dissimilarities in the composition of infant formulas and human milk. Cow milk-based formulas are devoid of many of the bioactive components that are present in human milk, including human milk oligosaccharides (HMO) (reviewed in Kunz *et al.*[8]). The complexity and quantity of oligosaccharides are unique to human milk. For example, after lactose and fat, HMO constitute the third most abundant component of human milk (10–15 g/l). Furthermore, up to 200 potential oligosaccharide structures have been identified in human milk, of which typically approximately 70 % are fucosylated[8,9]. In contrast, cows' milk contains less than 0·1 g oligosaccharides/l milk[8,9], and much less structural diversity (forty structures) and very few fucosylated oligosaccharides[10]. Accordingly, structurally complicated oligosaccharides are rare in cow milk-based infant formulas and, thus, constitute a major compositional difference in the diets of BF infants compared with FF infants.

Oligosaccharides are important with respect to the immune response (reviewed in Rabinovich *et al.*[11]). Based on their structural similarity to selectin ligands, it has been hypothesised that HMO could have immunomodulatory effects[9,12–14]. For instance, the P- and E-selectins recognise sialyl-Lewis X (sLeX)[15], a moiety also found on HMO[16]. Some immune protein–carbohydrate interactions, such as those mediated by selectins, have been shown to be modulated by HMO[17,18]. Oligosaccharides can also affect binding to, the quality of or the length of the association

Abbreviations: 2′-FL, 2′-fucosyllactose; 3′-FL, 3′-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; BF, breast-fed; FF, formula-fed; HMO, human milk oligosaccharides; IFN-γ, interferon-γ; IHMO, isolated human milk oligosaccharides; LNFPIII, lacto-*N*-fucopentaose III; LNnT, lacto-*N*-neotetraose; LPS, lipopolysaccharides; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; SA, sialic acid; SLeX, sialyl-Lewis X; SR, sow-reared; TriGal, galactotriose.

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between cell surface receptors and their ligands, thereby altering the signalling from the surface to nucleus of a cell[19]. Many immune receptors recognise the oligosaccharide structures of their glycoprotein ligands[11]. Thus, HMO could potentially alter immune responses in the neonate.

In order to directly affect systemic immune cells, HMO must be absorbed into the neonate's bloodstream. Because of their structural complexity and the high concentrations found in the faeces of BF infants[20], it has been suggested that these complex oligosaccharides may not be absorbed by infants. However, others have estimated that the blood concentration of HMO in human infants may be about 100–200 μg/ml[9,21]. Recently, between 1 and 3 mg of single HMO have been shown to be excreted in the urine of infants over the course of a day[22]. Urinary excretion of intact HMO indicates that these compounds are not only absorbed, but are also present in the blood in their typical conformation. This confirms that these complex oligosaccharides have the potential to exert systemic effects.

Although acidic HMO have been described to affect cytokine production by cord blood mononuclear cells[23], the direct effects of a large number of individual HMO on cells isolated from neonates have not been measured. In the present study, using the neonatal pig (*Sus scrofa*) model[24], the direct effects of HMO on the proliferation, phenotype and cytokine production of peripheral blood mononuclear cells (PBMC) isolated from 10-d-old pigs were analysed. Furthermore, the responses between PBMC isolated from piglets fed their own mother's milk *v.* those fed a cow milk-based sow milk replacer formula were compared.

Experimental methods

Animals and housing

Vaginally delivered pigs were randomised into two groups at birth: sow-reared (SR, *n* 5) group or colostrum-deprived FF (*n* 5) group. Since the FF pigs received no maternally transferred antibodies, they were oro-gastrically administered with pregnant sow serum at birth (4 ml/kg body weight), 4 h (5 ml/kg body weight) and 22 h postpartum (10 ml/kg body weight) to provide passive immunity. The FF pigs were fed a sow milk replacer formula at a rate of 360 ml/kg body weight per d divided equally into twenty-two feedings (Milk Specialties Global Animal Nutrition), and were individually housed in environmentally controlled rooms (25°C) in cages capable of maintaining six piglets separated by Plexiglas partitions (Arkema). Radiant heaters were attached to the top of the cages to maintain an ambient temperature of 30°C. The SR piglets remained with their mothers in farrowing crates under environmentally controlled conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois. The institutional and national guidelines for the care and use of animals were followed.

Sample collection

On day 10 postpartum, the piglets were sedated with an intramuscular injection of Telazol® (Tiletamine HCl and Zolazepam HCl, 3·5 mg/kg body weight each; Pfizer Animal Health). After sedation, blood was collected by cardiac puncture into heparin-laced vials (BD Biosciences) for the isolation of mononuclear cells. The piglets were then euthanised by an intravenous injection of sodium pentobarbital (72 mg/kg body weight, Fatal Plus; Vortech Pharmaceuticals).

Isolation of mononuclear cells from peripheral blood and immune tissues

PBMC were isolated by density gradient centrifugation. Heparinised blood was diluted in Roswell Park Memorial Institute (RPMI)-1640 (Gibco; Life Technologies) and layered over Ficoll-Paque PLUS lymphocyte separation medium (GE Healthcare). PBMC were recovered after centrifugation (400 ***g***, 30 min) across the density gradient. Erythrocytes were lysed using ammonium chloride lysis buffer. The cells were washed (Hanks' balanced salt solution, 2% bovine serum albumin, 10 mM-HEPES, 50 μg gentamicin/ml, 1 IU (0·1 μg) penicillin/ml and 100 μg streptomycin/ml). Isolated PBMC were placed in complete medium (RPMI-1640, 10% fetal calf serum, 2 mM-L-glutamine, 1 IU (0·1 μg) penicillin/ml, 100 μg streptomycin/ml and 50 μg gentamicin/ml; Gibco). The cells were counted using a Countess automated cell counter (Life Technologies). The number of viable cells was assessed by trypan blue (Life Technologies) exclusion. The isolated cells were kept in complete medium at 4°C until use.

Human milk oligosaccharides

Lacto-*N*-fucopentaose III (LNFPIII) was purchased from Sigma. Lacto-*N*-neotetraose (LNnT) was provided by Abbott Nutrition. All other HMO, including sLeX, 3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), 2-fucosyllactose (2′-FL) and 3′-fucosyllactose (3′-FL), were purchased from V-Labs. Galactotriose (TriGal) and sialic acid (SA) were also obtained from V-Labs. TriGal was used as a negative control, and sLeX was used as a positive control for interactions with immune cells[18,25]. Isolated human milk oligosaccharides (iHMO) were purified, as described previously[26], from pooled preterm human milk (mean gestational age 24 (SD 2·3) weeks) provided by Dr Paula Meier (Rush University, Chicago, IL). The iHMO contained 30·9% fucosylated oligosaccharides, 31·6% sialylated oligosaccharides and 12·4% of both fucosylated and sialylated oligosaccharides, as determined by HPLC-chip time-of-flight MS[27].

Endotoxin reduction and measurement

Endotoxin (lipopolysaccharides; LPS) content in the oligosaccharide samples was measured by a quantitative limulus amebocyte lysate test (QCL-1000; BioWhittaker). A level of $<0{\cdot}5$ endotoxin units/ml of endotoxin was considered to be acceptable. If the level was higher, the endotoxin was removed using polymixin B columns (Pierce Thermo Scientific). The samples were retested to ensure a level of $<0{\cdot}5$ endotoxin units/ml.

Cell stimulation

PBMC were plated in ninety-six-well plates (2×10^5 cells/well) in a final volume of 200 μl complete medium at 37°C under 5% CO_2. Stimulants were added immediately (*n* 3 wells/sample per stimulant for proliferation or *n* 2 wells/sample per stimulant for cytokine and T-cell phenotyping). The cells were stimulated for 72 h with LPS (2 μg/ml), phytohaemagglutinin (PHA, 2·5 μg/ml) or single HMO at 125 μg/ml including sLeX, TriGal, LNFPIII, LNnT, 3′-SL, 6′-SL, 2′-FL and 3′-FL as well as SA. The iHMO (125 μg/ml) was also used alone and in combination with PHA and LPS. Additionally, the following mixtures were used to stimulate the cells: SL mix (10% 3′-SL, 40% 6′-SL and 50% SA) or FL mix (85% 2′-FL and 15% 3′-FL) with a total final concentration in culture of 125 μg/ml. The SL mix mimicked the total SA concentration of human milk[8,28–31]. 6′-SL (40%) and 3′-SL (10%) were added at the reported ratio to each other, and the remaining total SA was provided as free SA (50%), assuming that some lipid or protein-bound SA could be released by sialidases in the intestine[32] or by microbial fermentation[33].

Proliferation assay

[^{3}H]Thymidine (Perkin Elmer) was added 72 h after the initiation of mitogenic stimulation at a concentration of 1 μCi/well, and plates were incubated for an additional 18 h. The plates were stored at −80°C until analysis. Cells were harvested (Harvester 96 Mach III M; TomTech) onto a 1·5 μm glass fibre filter paper (Skatron Instruments) and placed in vials containing 7 ml Ultima Gold F scintillation fluid (Perkin Elmer). Samples were counted on a Beckman Coulter (LS 6500 Scintillation System). Data are expressed as counts per minute. The samples were analysed in triplicate. Data analysis was performed on log-transformed counts per minute.

Cytokine production

Cell culture supernatants were collected 72 h after the initiation of culture and frozen at −80°C until analysed. Supernatants were analysed for interferon-γ (IFN-γ), IL-12p70, IL-4, IL-10 and TNF-α by Aushon BioSystems using SearchLight, a chemiluminescent technology based on a multiplexing sandwich-ELISA system. Assays were specific for porcine cytokines. When the value for a sample fell below the limit of detection of the assay, the values were set to the limit of detection in pg/ml (IFN-γ, 3·1; IL-12p70, 1·1; IL-10, 1·6; TNF-α, 0·9; IL-4, 1·1).

Phenotypic identification of mononuclear cells

Cells were collected 72 h after the initiation of culture and resuspended in flow staining buffer (PBS, 1% bovine serum albumin and 0·1% sodium azide). The phenotypes of T-lymphocyte subpopulations from PBMC were determined by flow cytometry using fluorescently labelled monoclonal antibodies. T lymphocytes were identified by mouse anti-pig CD3:PE-Cy5 (Clone PPT3; Southern Biotech). To further differentiate T-cell populations, cells were stained with mouse anti-pig CD4–fluorescein isothiocyanate (Clone 74-12-4; Southern Biotech) and mouse anti-pig CD8:PE (Clone 76-2-11; Southern Biotech) antibodies. All staining procedures took place on ice and care was taken to prevent unnecessary exposure to light. Briefly, 1 million cells/well were blocked with anti-pig CD16 (0·2 μg, G-7; AbD Serotec) for 5 min, followed by incubation with 5% mouse serum (Southern Biotech) for 5 min. Next, the cells were incubated for 15 min in a total of 10 μl anti-CD3 (0·2 μg). The cells were then centrifuged at 2000 rpm for 5 min at 4°C and supernatants were removed. The cells were incubated for 15 min in a total volume of 20 μl anti-CD4 (0·16 μg) and anti-CD8 (0·05 μg). The cells were washed twice with PBS/1% bovine serum albumin/0·1% sodium azide, and then fixed with 2% paraformaldehyde. Staining was assessed using an LSRII flow cytometer (BD Biosciences). The relative size of T-cell subpopulations was determined using FlowJo 7.0 software (FlowJo). $CD3^+$ events were considered to be T cells. $CD3^+CD4^+CD8^-$ events were considered to be T-helper cells. $CD3^+CD8^+CD4^-$ events were considered as cytotoxic T cells. $CD3^+CD4^+CD8^+$ events were considered as double-positive T cells.

Statistical analyses

Statistical analyses were performed using SAS 9.2 (Cary, NC). Data were analysed by two-way ANOVA using the generalised linear model procedure to determine the effects of diet, stimulant and the interaction between diet and stimulant. When a main effect was significant, a *post hoc* Tukey test was used. If the model containing both diet and stimulant was not significant, but a single factor was significant, a one-way ANOVA was performed on the single factor. Statistical significance was defined as $P < 0{\cdot}05$, and $P \leq 0{\cdot}10$ accepted as a trend. Data are presented as means and standard deviations.

Results

T-cell proliferation

Both piglet diet and *ex vivo* stimulation with HMO affected proliferation. PBMC isolated from the FF piglets proliferated more than those isolated from the SR piglets (Fig. 1; Table S1, available online). Fucosylated compounds tended ($P \leq 0{\cdot}10$) to inhibit proliferation. PBMC stimulated with 2′-FL (Fig. 1(A)) or FL mix (Fig. 1(B)) tended to proliferate less than the unstimulated cells. Compared with PBMC stimulated with PHA alone, cells co-stimulated with PHA and iHMO proliferated more (Fig. 2(A)). Co-stimulation with SL mix increased PBMC proliferation in response to LPS stimulation (Fig. 2(B)). The diet had no effect on proliferation in response to PHA stimulation (Fig. 2(A)). However, when stimulated with LPS, PBMC isolated from the FF piglets proliferated more than those from the SR piglets (Fig. 2(B)). Neither TriGal nor sLeX stimulation affected cellular proliferation.

T-cell phenotype

T-helper cell ($CD3^+CD4^+CD8^-$) populations were affected by both piglet diet and *ex vivo* stimulation with HMO. Compared

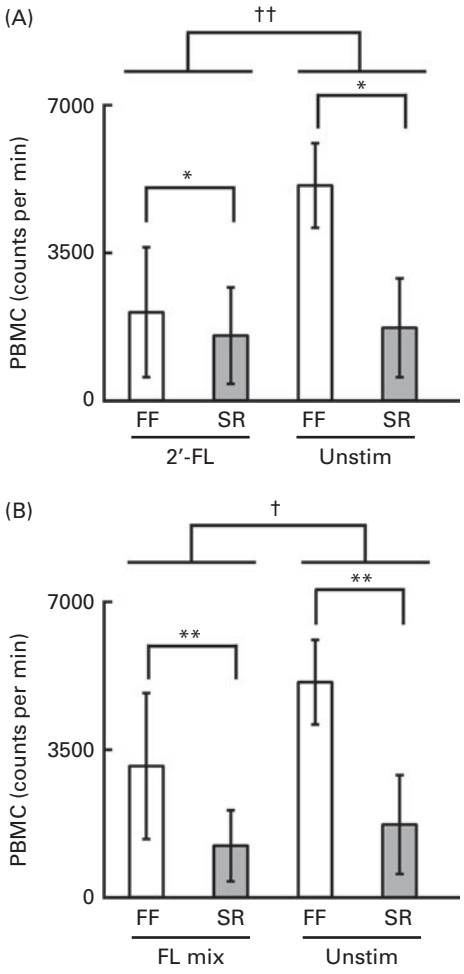

Fig. 1. Peripheral blood mononuclear cells (PBMC) from sow-reared (SR) piglets proliferate less than those from formula-fed (FF) piglets, and fucosylated human milk oligosaccharides tend to inhibit PBMC proliferation. (A) PBMC stimulated with 2′-fucosyllactose (2′-FL) tended to proliferate less than the unstimulated (Unstim) PBMC. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model (P=0·05); stimulant (†† P=0·07); diet (* P=0·07); stimulation × diet interaction (P=0·19). (B) PBMC stimulated with the FL mix tended to proliferate less than the unstimulated PBMC. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model (P=0·01); stimulant († P=0·10); diet (** P=0·001); stimulant × diet interaction (P=0·54).

with the SR piglets, the T-cell populations in PBMC isolated from the FF piglets consisted of a larger percentage of T-helper cells (expressed as a percentage of total $CD3^+$ events; Table S2, available online). The co-stimulation of PBMC with iHMO (Fig. 3(A)) or FL mix (Fig. 3(B)) significantly decreased T-helper cell populations in response to LPS stimulation. The treatment with SL mix (Fig. 3(C)) tended to decrease T-helper cell populations in response to LPS stimulation.

Piglet diet, but not *ex vivo* stimulation with HMO, affected cytotoxic T-cell ($CD3^+CD4^-CD8^+$) populations. Compared with the FF piglets, the SR piglets had a larger percentage of cytotoxic T cells within their $CD3^+$ PBMC (Table S2, available online). The only stimulant with independent effects on cytotoxic T-cell populations within PBMC was PHA. Stimulation with PHA decreased the percentage of cytotoxic T cells for both the FF and SR piglets.

Consistent with their larger percentage of T-helper cells and smaller percentage of cytotoxic T cells, the ratio of T-helper:cytotoxic T cells was greater within the $CD3^+$ PBMC of the FF piglets than those of the SR piglets (Table S2, available online). Again, the only stimulant that consistently affected the T-helper:cytotoxic T-cell ratio was PHA. PHA stimulation increased the ratio of T-helper:cytotoxic T cells.

Changes in the double-positive T-cell population ($CD3^+CD4^+CD8^+$) depended on specific stimulation conditions. In the unstimulated cultures of PBMC, the double-positive T-cell population was small (1–5 %) and did not differ upon stimulation with HMO compounds or by diet (Table S2, available online). Co-stimulation with either SL mix (Fig. 4(A)) or iHMO (Fig. 4(B)) decreased the percentage of double-positive T cells compared with that produced upon stimulation with PHA alone. Upon stimulation with either PHA or LPS, $CD4^+CD8^+$ T-cell populations differed by diet (Fig. 4). Stimulation with PHA resulted in double-positive T-cell populations that were larger in the SR piglets than in the FF piglets (Fig. 4(A) and (B)). Stimulation with LPS resulted in double-positive T-cell populations that were larger in the FF piglets than in the SR piglets (Fig. 4(C)). Only co-stimulation with iHMO (Fig. 4(C)) reduced the size of the double-positive T-cell population produced in response to LPS stimulation. Neither TriGal nor sLeX stimulation affected any T-cell populations.

Cytokine production

IL-10 production was affected by piglet diet as well as by *ex vivo* stimulation with HMO. The PBMC isolated from the FF pigs produced more IL-10 than those isolated from the SR pigs (Table S3, available online). *Ex vivo* stimulation with iHMO increased IL-10 production by PBMC independent of the diet (Fig. 5).

Both piglet diet and *ex vivo* stimulation with HMO affected TNF-α production. When all the treatments were analysed simultaneously, TNF-α production was a function of both diet and *ex vivo* stimulation (Table S3, available online). TNF-α production did not differ by diet or stimulant when the cells were unstimulated or stimulated with HMO alone (data not shown). However, when stimulated with LPS or PHA, the PBMC isolated from the SR pigs produced more TNF-α than those isolated from the FF pigs (Table S3, available online). Co-stimulation with LNnT increased TNF-α production in response to PHA stimulation by about 1·5-fold (Fig. 6).

In general, IL-4 production by *ex vivo* cultured PBMC was low; however, it was affected by piglet diet. IL-4 production under all the conditions, except the stimulation with PHA, ranged between the limit of detection and 17·3 pg/ml. Under PHA stimulation, IL-4 production ranged from 57·4 to 1052 pg/ml and averaged 370·5 pg/ml. The PBMC isolated from the SR piglets produced more IL-4 than those isolated from the FF piglets (Table S3, available online).

Neither neonatal diet nor stimulation with HMO affected IFN-γ (Table S3, available online) or IL-12p70 production by

PBMC (data not shown). IFN-γ production ranged from the limit of detection to 1992 pg/ml. For the cells unstimulated or stimulated only by HMO, the range was from the limit of detection to 155·5 pg/ml with an average production of 22·0 pg/ml. Stimulation with either LPS ($P<0·0001$) or PHA ($P<0·0001$) increased IFN-γ production compared with the production by unstimulated cells (Table S3, available online). For the cells stimulated with LPS with or without HMO co-stimulation, the range was from 4·1 to 272·3 pg/ml with an average IFN-γ production of 73·1 pg/ml. For the cells stimulated with PHA with or without HMO co-stimulation, the range was from 60·5 to 1992 pg/ml with an average IFN-γ production of 431·8 pg/ml. IL-12p70 production by PBMC isolated from 10-d-old piglets incubated under various co-stimulation conditions for 72 h was low. It ranged from the limit of detection to 20·4 pg/ml. PHA stimulation did significantly increase IL-12p70 production ($P=0·001$). However, even under PHA stimulation, the amounts of IL-12p70 produced reached a maximum of 20·4 pg/ml with an average production of 3·9 pg/ml. Neither TriGal nor sLeX stimulation affected cytokine production by PBMC.

Discussion

Human milk contains a complement of HMO that are unique in their quantity and composition relative to other species[9,10]. Although others have studied the effects of some HMO on immune cells[17,18,23], this is the first investigation of the effects of a large variety of single HMO and combinations of HMO on cells isolated from neonates. Rather than using cells isolated from adult human subjects[17,18], cell lines[34,35] or human cord blood mononuclear cells[23], the present study examined the effects on primary PBMC isolated from neonatal pigs fed either their own mother's milk or formula. Although not identical to HMO, porcine milk oligosaccharides include predominantly sialylated HMO with some fucosylated compounds, sLeX, SL and LNnT[36,37]. We observed that HMO have the potential to directly affect lymphocytes isolated

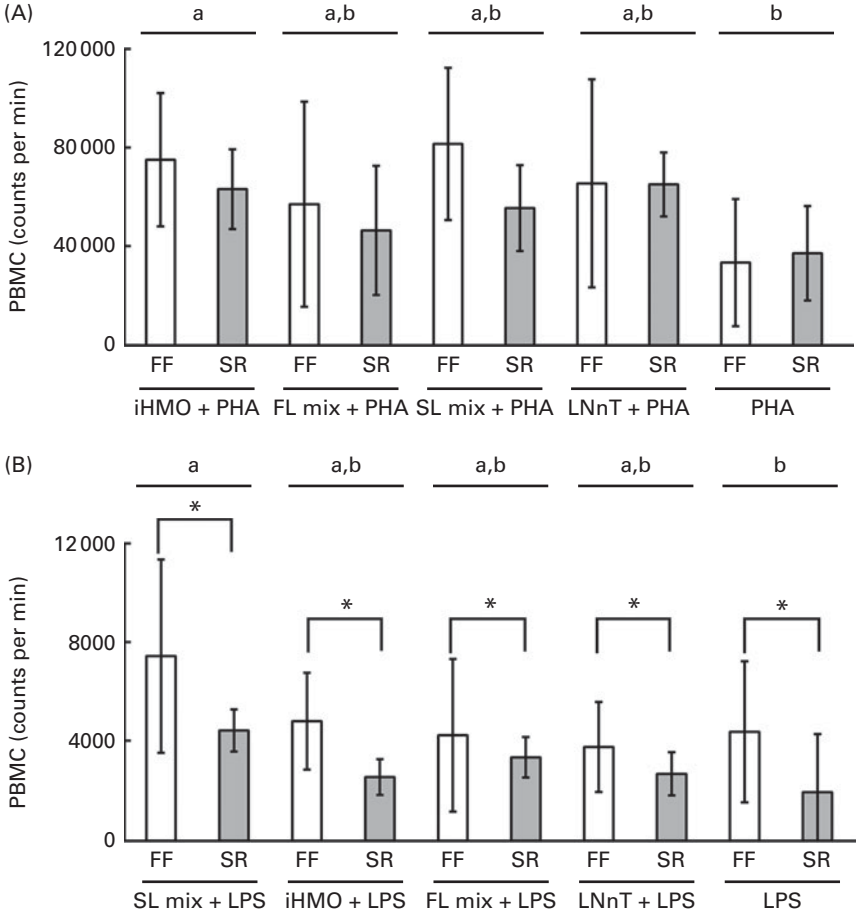

Fig. 2. Co-stimulation with human milk oligosaccharides increased peripheral blood mononuclear cell (PBMC) proliferation in response to both phytohaemagglutinin (PHA) stimulation and lipopolysaccharide (LPS) stimulation. (A) Co-stimulation with isolated human milk oligosaccharides (iHMO) increased PBMC proliferation in response to PHA stimulation. Values are means, with standard deviations represented by vertical bars. The full model was not significant ($P=0·18$), but the stimulant had a significant effect in the full model ($P=0·03$). Therefore, a one-way ANOVA was performed with the stimulant being the factor ($P=0·02$). [a,b] Stimulants with unlike letters were significantly different ($P=0·03$). (B) Co-stimulation of PBMC with sialyllactose (SL) mix and LPS resulted in greater proliferation than stimulation with LPS alone. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model ($P=0·02$); stimulant ([a,b] $P=0·02$); diet (* $P=0·01$); stimulant × diet interaction ($P=0·39$). [a,b] Stimulants with unlike letters were significantly different ($P=0·02$). FF, formula-fed; SR, sow-reared; FL, fucosyllactose; LNnT, lacto-*N*-neotetraose.

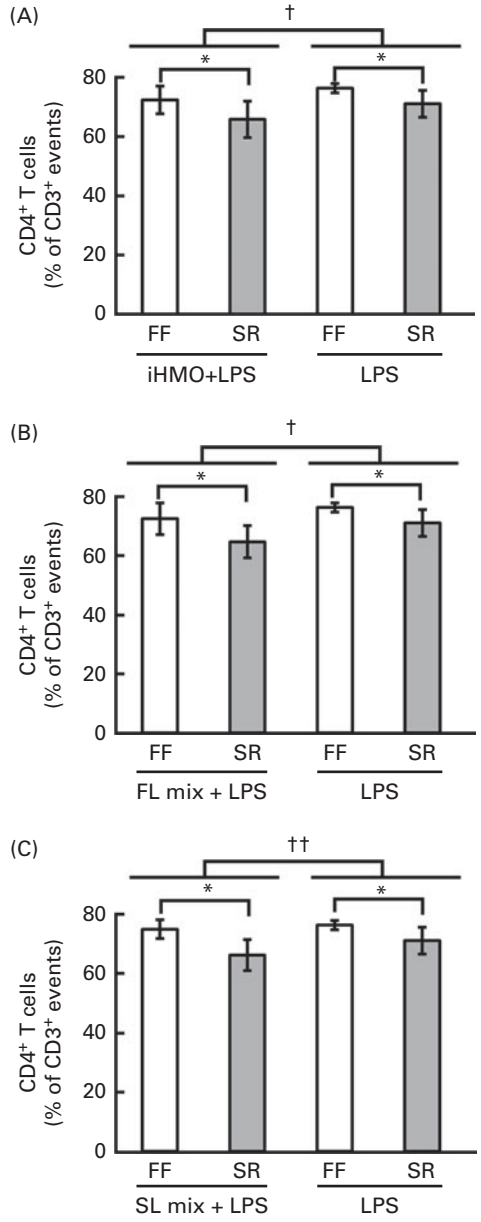

Fig. 3. Neonatal diet and *ex vivo* human milk oligosaccharide co-stimulation affected T-helper cell populations in peripheral blood mononuclear cells (PBMC). Sow-reared (SR) piglets had smaller T-helper cell populations. *Ex vivo* co-stimulation with human milk oligosaccharides decreased T-helper cell populations in response to lipopolysaccharide (LPS) stimulation. (A) Co-stimulation with isolated human milk oligosaccharides (iHMO) decreased T-helper cell populations in PBMC stimulated with LPS. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model ($P=0{\cdot}02$); stimulant († $P=0{\cdot}04$); diet (* $P=0{\cdot}01$); stimulant × diet interaction ($P=0{\cdot}77$). (B) Co-stimulation with fucosyllactose (FL) mix decreased T-helper cell populations in PBMC stimulated with LPS. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model ($P=0{\cdot}02$); stimulant († $P=0{\cdot}04$); diet (* $P=0{\cdot}01$); stimulant × diet interaction ($P=0{\cdot}57$). (C) Co-stimulation with sialyllactose (SL) mix tended to decrease T-helper cell populations in PBMC stimulated with LPS. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model ($P=0{\cdot}004$); stimulant (†† $P=0{\cdot}097$); diet (* $P=0{\cdot}001$); stimulant × diet interaction ($P=0{\cdot}35$). T-helper cells are $CD3^+CD4^+CD8^-$. These cells are expressed as a percentage of $CD3^+$ events.

from neonatal pigs. Additionally, neonatal diet altered the proliferative capacity, cell populations and cytokine production of PBMC. However, when an effect of *ex vivo* stimulation with HMO was observed, the effect was independent of the neonatal diet and typically required a mixture of HMO to be applied.

Both neonatal diet and *ex vivo* stimulation with HMO altered lymphocyte responses to LPS and PHA. Since LPS and PHA bind to PBMC cell surface glycoprotein receptors, HMO may affect ligand–receptor interactions and alter the proliferation, phenotype and cytokine production of PBMC in response to stimulation with these mitogens. Indeed, co-stimulation with HMO changed PBMC responses to LPS and PHA stimulation. In the case of co-stimulation, HMO increased immune system activation as demonstrated by increased proliferation (iHMO and SL mix), decreased T-helper cell population size (iHMO, FL mix and SL mix; $P=0{\cdot}097$) and increased TNF-α production (LNnT). Increased proliferation is typically an indication of enhanced immunocompetence. Decreases in T-helper cell populations indicate a shift in the balance of T-cell immune responses towards effector functions. TNF-α production is important in the innate immune response, and increased cytokine production also indicates enhanced immunocompetence. Together, these responses to co-stimulation with HMO suggest that in conditions of activation, HMO can potentiate an immune response.

HMO may affect PBMC phenotype and function in the absence of PHA or LPS co-stimulation due to their similarity to sequences that bind L-selectins[14,38–41], a subclass of carbohydrate-binding proteins involved in immune function. Because some HMO have been shown to inhibit epithelial cell proliferation[34,35,42], we predicted that they would also affect PBMC proliferation. Indeed, both 2′-FL ($P=0{\cdot}07$) and the combination of fucosylated HMO (FL mix; $P=0{\cdot}10$) tended to inhibit proliferation. Whether HMO affect immune cell proliferation through the regulation of cyclin expression, as has been demonstrated in epithelial cells[34,42], or through a more novel mechanism, such as fucose modification of Notch receptors or ligands[43], is unknown and should be tested. In terms of cytokine production, stimulation of PBMC with iHMO doubled IL-10 production by PBMC. Thus, under non-stimulatory conditions, HMO compounds generated a regulatory or neutral immune response through decreased proliferation (2′-FL and FL mix) and increased IL-10 production (iHMO). In the case of either co-stimulation or independent stimulation, HMO were more effective when present in combination. Notably, only 2′-FL had effects when present alone, and LNnT was the only single HMO to change proliferation or TNF-α production in response to PHA stimulation.

Due to the nature of the analysis, changes in the cell surface expression of CD4 or CD8 in response to stimulation may be indicative of cellular activation rather than a shift in the T-cell population. Future experiments detailing the activation status of stimulated cells are needed to determine the mechanism by which HMO affect cell populations in culture. HMO may activate cells, causing them to down-regulate cell surface markers and stimulate specific cell populations to proliferate,

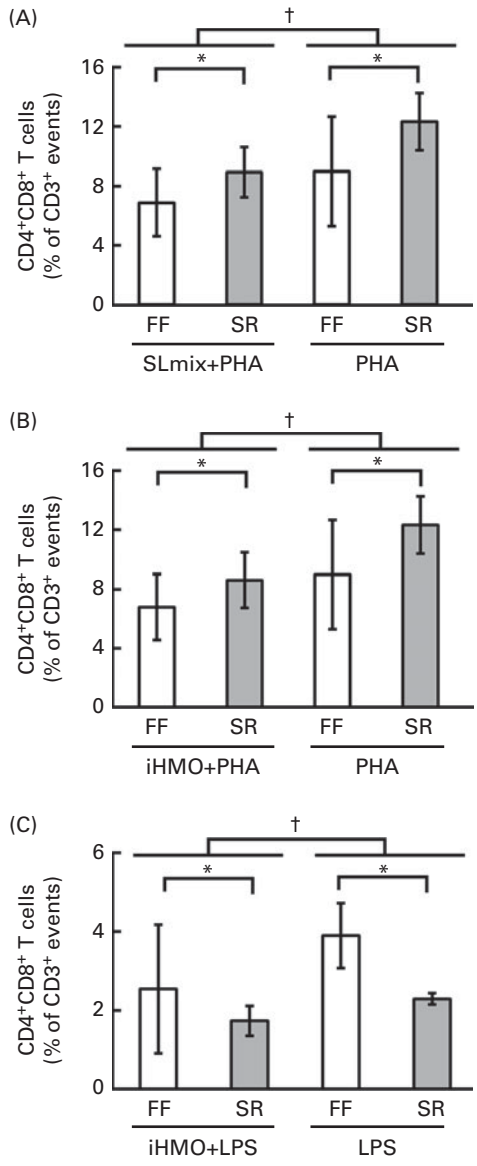

Fig. 4. Neonatal diet and *ex vivo* human milk oligosaccharide co-stimulation affected double-positive T-cell populations under the phytohaemagglutinin (PHA) or lipopolysaccharide (LPS) stimulation conditions. (A) Upon PHA stimulation, double-positive T-cell populations were larger in peripheral blood mononuclear cells (PBMC) from sow-reared (SR) piglets than those from formula-fed (FF) piglets. Co-stimulation with sialyllactose (SL) mix decreased double-positive T-cell populations. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model ($P=0{\cdot}03$); stimulant († $P=0{\cdot}03$); diet (* $P=0{\cdot}03$); stimulant × diet interaction ($P=0{\cdot}57$). (B) Co-stimulation with isolated human milk oligosaccharides (iHMO) decreased double-positive T-cell populations in response to PHA stimulation. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model ($P=0{\cdot}03$); stimulant († $P=0{\cdot}03$); diet (* $P=0{\cdot}03$); stimulant × diet interaction ($P=0{\cdot}59$). (C) Upon LPS stimulation, double-positive T-cell populations were larger in PBMC isolated from the FF piglets than those from the SR piglets. Co-stimulation with iHMO decreased double-positive T-cell populations in PBMC. Values are means, with standard deviations represented by vertical bars. Analysis of effects by PROC GLM: model ($P=0{\cdot}02$); stimulant († $P=0{\cdot}04$); diet (* $P=0{\cdot}01$); stimulant × diet interaction ($P=0{\cdot}57$). Double-positive T cells are $CD3^+CD4^+CD8^+$. These cells are expressed as a percentage of $CD3^+$ events.

thus driving down the size of other populations as a percentage of total or target-specific cells for apoptosis and driving up the size of other populations as a percentage of total cells. Thus, although it is clear that HMO affect the relative cell populations, the mechanism(s) by which they cause these changes is unknown.

Some of the observed T-cell changes could affect infant health. It has recently been reported that high levels of non-3′-SL HMO in human milk could protect infants from mother-to-child HIV transmission[44]. $CD4^+CD8^+$ T cells have been demonstrated to protect individuals from HIV infection[45]. We observed that co-stimulation with SL mix or iHMO decreased $CD4^+CD8^+$ T-cell populations under conditions that would normally stimulate T cells. This is a small, but important, piece of evidence to suggest that the effects of HMO on the immune system play a role in mother-to-child HIV transmission.

PBMC proliferation, phenotype and cytokine production were influenced by the neonatal diet. A large body of evidence has demonstrated differences in immune parameters between BF and FF human infants[46,47]. Maternal colostrum and milk protect against gastrointestinal and respiratory diseases[3,48] and have been shown to promote the maturation of the developing intestinal epithelium[49]. There is evidence from both developed and developing countries that breast-feeding provides a protective effect in the first 4–6 months of life[50,51]. Human milk may provide this protection as dietary composition has been shown to shape the development and competence of the immune system[52,53], and components of human milk signal the immune system and initiate immune development[48,54]. In addition, human milk is a source of cytokines and immune cells[55,56] and can stimulate the release of cytokines from PBMC[57]. Furthermore, BF infants have increased natural killer cell counts[58], higher antibody titres[58], increased vaccination response[54] and lower morbidity and mortality rates than their FF peers[5,51]. Lymphocytes from FF infants proliferate more in response

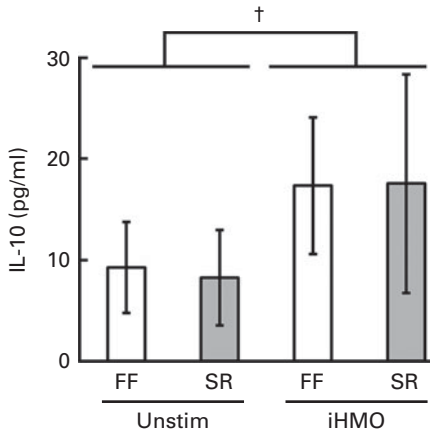

Fig. 5. Stimulation with isolated human milk oligosaccharides (iHMO) increased IL-10 production in peripheral blood mononuclear cells. Values are means, with standard deviations represented by vertical bars. The full model was not significant ($P=0{\cdot}12$), but the stimulant had a significant effect in the full model ($P=0{\cdot}02$). A one-way ANOVA was performed with the stimulant being the factor. Stimulants were significantly different († $P=0{\cdot}02$). FF, formula-fed; SR, sow-reared; Unstim, unstimulated.

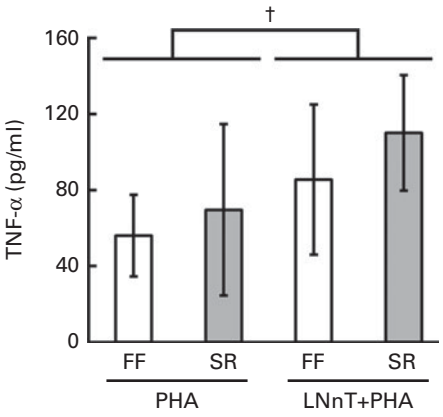

Fig. 6. *Ex vivo* stimulation with lacto-*N*-neotetraose (LNnT) increased TNF-α production in response to phytohaemagglutinin (PHA) stimulation. Values are means, with standard deviations represented by vertical bars. The full model was not significant ($P=0{\cdot}09$), but the stimulant had a significant effect in the full model ($P=0{\cdot}02$). A one-way ANOVA was performed with the stimulant being the factor. Stimulants were significantly different († $P=0{\cdot}02$). FF, formula-fed; SR, sow-reared.

to mitogens[59,60]. Additionally, FF infants have more T-helper cells than BF infants[61], and the CD4:CD8 ratio in PBMC isolated from BF infants is lower than that in FF infants[62,63]. These differences are similar to the results presented herein where PBMC isolated from the FF piglets proliferated more, consisted of more T-helper cells, and had greater T-helper: cytotoxic T-cell ratios than those isolated from the SR piglets. Thus, the differential responses of PBMC isolated from the SR and FF piglets were expected and mimic those of human neonatal PBMC.

Unexpectedly, sLeX stimulation did not affect proliferation, T-cell populations or cytokine production in PBMC isolated from 10-d-old pigs. The cells were similarly unresponsive to TriGal stimulation, which was the expected outcome. Much of the work with sLeX and selectin binding has been conducted using cells isolated from adults or in cell culture systems. Cells from young piglets may not yet express the cell surface molecules necessary to mediate interactions with sLeX or similar oligosaccharide moieties. Additionally, it is most well known that sLeX mediates the adhesion, rolling and extravasation of lymphocytes[13,17]. The role of sLeX in proliferation, cell populations and cytokine production is less well characterised. Although sLeX was expected to affect these functions, few reports have been published on these outcomes using PBMC from such young mammals.

Some limitations of the results exist. Because PBMC isolated from neonatal pigs were used in these experiments, it is possible that different results would be obtained when cells from human neonates are used. Porcine and human lymphocytes demonstrate similar proliferative responses to some stimulants[64]; however, this has neither been tested with neonatal cells nor have cytokine production or T-cell phenotype changes been directly compared between the species. The present findings show that in many respects, piglet immune cells respond to HMO similarly to human cells. Further advantages of neonatal piglets include the ease with which they can be reared independently of their mothers, the capacity to test defined dietary regimens and the ability to collect sufficient sample for multiple assays. It would be unethical and impractical to collect sufficient volumes of blood from human infants to conduct these experiments in a short time frame, under highly uniform conditions. In addition, pigs closely resemble humans for >80% of the immune parameters analysed (*v.* <10% when humans and mice are compared)[24]. The lymphoid cell populations in the pig are consistent with those of other vertebrates, especially humans[65], but pigs express greater proportions of $CD4^+CD8^+$ T lymphocytes[66–69]. Dietary administration of HMO was not evaluated. Rather, these compounds were screened for potential effects using *ex vivo* techniques and at a single dose. This dose probably reflects the local and, potentially, the systemic concentrations *in vivo* that PBMC may encounter. It is probable that similar effects will be seen with dietary administration, since similar quantities of non-metabolised HMO have been detected in the circulation and urine of human infants[22]. Because total PBMC were stimulated, the specific cells mediating the response have not been identified. The results from the stimulation of PBMC differ from those resulting from the stimulation of lymphocytes isolated from other tissues. Finally, since the present study included multiple comparisons, the differences that were detected will need to be validated in future studies. In light of these limitations, the present study represents a demonstration of potential outcomes that may result from dietary administration of HMO or *ex vivo* HMO stimulation of human cells. Future investigations should determine the effects of dietary administration of HMO, identify the specific cell types affected, define the mechanisms through which these compounds act and measure the effects of these compounds under immune stimulatory conditions such as vaccination or infection.

Approximately 75% of infants in the USA[70,71] and between 60 and 90% worldwide[72] consume infant formula at some time in their first year of life. Optimally, cow milk-based formulas should be formulated to mimic, as closely as possible, the biological functions of breast milk on immune system development. However, cow milk-based formulas are currently devoid of complex oligosaccharide structures similar to those found in human milk. Herein, HMO were shown to have direct effects on neonatal PBMC phenotype and function. Therefore, inclusion of HMO in bovine milk-based formulas should be considered.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513003267

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S. S. C., S. M. D. and M. W. designed the study. S. S. C., M. W. and S. N. H. performed the experiments. M. L. purified the complex mixture of HMO (iHMO) used in the experiments and with M. W. removed endotoxins. S. S. C. and M. W. analysed the data and completed the statistics. S. S. C. interpreted the data and wrote the manuscript. All authors commented on and contributed to the final manuscript.

S. N. H. worked as an intern for Abbott Nutrition. S. M. D. consulted for Abbott Nutrition. The rest of the authors have no conflicts of interest.

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