Dietary Bovine Lactoferrin Alters Mucosal and Systemic Immune Cell Responses in Neonatal Piglets

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Abstract

Lactoferrin (LF) is a multifunctional immune protein found at high concentrations in human milk. Herein, the effect of dietary bovine LF (bLF) on mucosal and systemic immune development was investigated. Colostrum-deprived piglets were fed formula containing 130 (control [Ctrl]), 367 (LF1), or 1300 (LF3) mg of bLF/kg body weight · d. To provide passive immunity, sow serum was provided orally during the first 36 h of life. Blood, spleen, mesenteric lymph node (MLN), and ascending colon (Asc) contents were collected on day 7 (n = 10–14/group) and day 14 (n = 10–12/group). Immune cell populations were quantified by flow cytometry and immunoglobulins (Igs) were measured by ELISA. Additionally, immune cells were isolated from spleen and MLNs (n = 7/group) on day 7 and stimulated ex vivo with phytohemagglutinin or lipopolysaccharide (LPS) ± LF for 72 h. Secreted cytokine concentrations were quantified by multiplex assay. Lymphocyte populations [cluster determinant (CD)4, CD8, and natural killer cells] developed normally and were unaffected by dietary bLF. LF3 piglets tended to have 1.4 to 2 times more serum IgG than Ctrl piglets (P = 0.07) or LF1 piglets (P = 0.03), but IgA in Asc contents was unaffected by bLF. Asc IgA was 4 times higher on day 14 than day 7. Spleen cells from LF3 piglets produced 2 times more interleukin (IL)-10 and tumor necrosis factor (TNF)-α ex vivo than those from Ctrl or LF1 piglets. MLN cells from LF1 and LF3 piglets produced 40% more IL-10 and tended to produce 40% more IL-6 (P = 0.05) than those from Ctrl piglets. However, ex vivo bLF did not affect the cytokine response of spleen or MLN cells to LPS. In summary, dietary bLF alters the capacity of MLN and spleen immune cells to respond to stimulation, supporting a role for LF in the initiation of protective immune responses in these immunologically challenged neonates. J. Nutr. 144: 525–532, 2014.

Introduction

Clinical and epidemiologic evidence suggest that human infants fed formula have altered immune responsiveness compared with those fed human milk (1,2). It is well known that human milk and formula differ in composition (3,4). One protein whose concentration markedly differs between human milk and infant formula is lactoferrin (LF)7. Humancolostrum and mature milk contain ~7000 and 2500 mg LF/L, respectively (5,6). In contrast, bovine milk contains between 50 and 300 mg LF/L (7). This low concentration in bovine milk results in cow milk–based infant formulas containing small quantities of LF (8).

LF is an iron-binding protein that has immune-modulating effects (9), antimicrobial functionality (10), and antioxidant activity (8). LF has direct bacteriocidal effects, but may also directly interact with the host immune system through interactions with pathogen-associated molecules, such as CpG-rich DNA or LPS (11–14). Additional immune-modulating effects of LF may be receptor-mediated as activated lymphocytes express LF receptors (15–17). As a product of neutrophil granule release, LF likely plays an important role in the local microenvironment of an immune response (18). Orally administered bovine lactoferrin (bLF) has been demonstrated to influence human immunity (19,20). Thus, milk-derived LF may be important in neonatal immune response and development.

The neonatal piglet is a well-accepted model for human infant gastrointestinal function, growth, and metabolism. Sow milk contains LF (21), the LF receptors are present in the porcine intestine (22), and orally administered LF is absorbed by the neonatal piglet (23). For these reasons, we used the neonatal piglet to study how dietary bLF influences immune cell populations and...

1 Supported by Wyeth Nutrition, a Nestlé Business, King of Prussia, PA.
2 Author disclosures: N. Contractor was an employee of Wyeth Nutrition at the time of this study. S. M. Donovan and S. S. Comstock have consulted for Wyeth Nutrition. E. A. Reznikov, no conflicts of interest.
3 Supplemental Methods, Supplemental Table 1, and Supplemental Figures 1–5 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
4Abbreviations used: Asc, ascending colon; bLF, bovine lactoferrin; BW, body weight; CD, cluster determinant; Ctrl, control, 130 mg bLF/kg BW · d; IPP, ileal Peyer’s patch; LF, lactoferrin; LF1, 367 mg bLF/kg BW · d; LF3, 1300 mg bLF/kg BW · d; LFR, lactoferrin receptor; MLN, mesenteric lymph node; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PreLPS, LF pretreated with LPS.
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responsiveness in early life. We hypothesized that dietary bLF would improve the immune development of formula-fed neonates. We anticipated that bLF supplementation would increase cytotoxic T-cell numbers, prime stimulated immune cells to produce more cytokines, and increase immunoglobulin production.

Materials and Methods

Piglets, housing, and care. Pregnant sows (n = 10) were supplied by the Swine Research Center at the University of Illinois, Urbana-Champaign. Vaginally delivered piglets (n = 92; n = 2–17 per sow) were removed from the sow immediately, prior to consumption of colostrum, and were washed with a povidone-iodine microbicidal solution (Henry Schein Animal Health). Because piglets receive no maternally transferred antibodies prenatally, piglets were administered pregnant sow serum at birth [4 mL/kg body weight (BW)] and 5 mL/kg BW at 8 h, 22 h, and 36 h postnatal age by oro-gastric gavage to provide passive immunity. Pigs were fed a sow milk replacer formula (Animix; Supplemental Table 1) at a rate of 360 mL/kg BW.d divided equally into 22 feedings. Piglets were assigned to 1 of 3 dietary treatment groups about 1 L of human milk/d with an LF concentration of 1850 mg/L (24). The bLF preparation was 97% pure LF (DMV). Piglets were individually housed as described (25). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois. Institutional and national guidelines for the care and use of animals were followed.

Sample collection. On day 7 (n = 36) or 14 (n = 32) postpartum, piglets were sedated with an intramuscular injection of Telazol (Tiletamine HCl and Zolazepam HCl, 3.5 mg/kg BW each; Pfizer Animal Health, Fort Dodge, IA). After sedation, blood was collected by cardiac puncture into EDTA-laced vials (BD Biosciences, Franklin Lakes, NJ) for isolation of mononuclear cells. Piglets were then killed by an intravenous injection of ketamine/xylazine (Asc) contents were collected.

Isolation of mononuclear cells from peripheral blood and cells from immune tissues. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (26). Supplemental Methods provides detailed isolation procedures. Briefly, spleens, MLNs, and IPPs were washed. PBMCs were incubated in DTT/HBSS and then with EDTA/HBSS. Spleens, MLNs, and IPPs were rinsed and single cell suspensions were made through a combination of enzymatic and mechanical digestion. Resulting cell suspensions were treated as described (25).

Cell stimulation. Cells from 7-d-old piglets were plated (96-well plate, 2 × 10^5 cells/well) in a final volume of 200μL complete Roswell Park Memorial Institute at 37°C under 5% CO2. Stimulants were added immediately (n = 2 wells per sample per stimulant). Cells were stimulated with LPS, a toll-like receptor 4 agonist, (2 μg/mL); phytohemagglutinin (PHA), a lectin that binds and crosslinks the T-cell receptor, (2.5 μg/ml); or bLF (50 μg/mL). In some cases bLF was added with polymixin B (10 μg/mL) to control for potential endotoxin in the bLF solution. Additionally, the following mixtures were used to stimulate cells: LPS + LF, LF + polymixin B, and LF pretreated with LPS (PreLPS) for 1 h prior to addition to cells (PreLPS + LF). Cell culture supernatants were collected 72 h after initiation of culture and frozen at −80°C until analyzed.

Cytokine production. Supernatants were analyzed for IL-6, IL-10, IFN-γ, IL-12p40, IL-4, and TNF-α by Panomics using a Luminex-based assay specific for porcine cytokines. When the value for a sample fell below the limit of detection of the assay, values were set to 0.

Phenotypic identification of cells. Cells were resuspended in flow staining buffer (PBS, 1% BSA, 0.1% sodium azide). Cell phenotypes were determined by flow cytometry as previously described (26). To differentiate T and NK cells, cells were stained with anti-pig cluster determinant (CD) 3, anti-pig CD4, and anti-pig CD8 (27). B lymphocytes were identified by anti-pig CD21 and anti-pig swine leukocyte antigen class II DR. Polymorphonuclear cells were identified by anti-pig CD163, anti-pig CD172a, and anti-pig swine workshop cluster 8 (28). Detailed information is included in Supplemental Methods. Staining was assessed by a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo 7.0 (FlowJo).

Immunoglobulin and bLF concentrations. Serum IgG, serum bLF, and Asc content IgA were determined using ELISA antibody sets (Bethyl Labs). The manufacturer’s instructions were followed with the exception that PBS was used as the basis for assay buffers. Prior to analysis, Asc contents were extracted (1:3) using a PBS/protease inhibitor solution (Roche). IgA concentrations are reported as μg IgA per mg total protein in the extract.

Amounts of bLF in diets were analyzed by semiquantitative Western blot at Wyeth Nutrition. Supplemental Methods provides details.

Statistical analyses. Data were analyzed using SAS 9.2. Detailed information about statistical analyses is in Supplemental Methods. Proc general linear model was used in most cases with day, diet, and day × diet as factors, or diet, stimulant, and diet × stimulant as factors. Post hoc testing was least-squares means. For IgG, sow had an effect in the Proc general linear model, so Proc MIXED was used with fixed effects of diet and day and random effects including sow and replicate. If data were not normally distributed, they were log transformed to be normally distributed or, when log transformation failed, analyzed by nonparametric tests. LF intake was analyzed with Wilcoxon’s rank-sum test to compare days and by Kruskal-Wallis 1-factor ANOVA to compare diets. Serum bLF concentration was analyzed using a nonparametric Friedman 2-factor ANOVA. For survival analysis, the LIFETEST procedure was used to generate Kaplan-Meier survival curves and to compare survival curves among the dietary treatment groups. Differences were considered significant at P < 0.05 and trends at P < 0.10. Data are expressed as means ± SEMs.

Results

Bovine lactoferrin intake

During the first 7 d, piglets consumed 103 ± 13 (Ctrl), 321 ± 26 (LF1), and 1170 ± 39 (LF3) mg bLF/kg BW.d, respectively. Throughout the 14-d study, piglets consumed 114 ± 14 (Ctrl), 413 ± 27 (LF1), and 1270 ± 37 (LF3) mg bLF/kg BW.d, respectively. Thus, 7-d-old and 14-d-old piglets were consuming similar amounts of bLF on a per-kg BW basis, but bLF intake differed across diets (P < 0.0001). However, on an absolute basis, piglets analyzed at day 7 consumed 256 ± 25 (Ctrl), 836 ± 111 (LF1), and 3130 ± 201 mg bLF/d (LF3) during the 3 days prior to sampling. Piglets analyzed at day 14 consumed 496 ± 82 (Ctrl), 1790 ± 159 (LF1), and 5130 ± 335 mg bLF/d (LF3) during the 3 d prior to sampling. Thus, the absolute dose of bLF was much higher just prior to sampling on day 14 than on day 7 within the Ctrl (P = 0.002), LF1 (P = 0.0003), and LF3 (P = 0.0002) groups. The absolute dose of LF also differed across diets (P < 0.0001).

Piglet growth and health status

Birth weights were similar (1.6 ± 0.04 kg). Neither absolute body weight nor weight gain of piglets was affected by dietary bLF. On day 7, piglets weighed 2.5 ± 0.07 kg. On day 14, piglets weighed 4.2 ± 0.11 kg. Spleen weight was unaffected by dietary
bLF but was affected by age, with the spleens of 14-d-old piglets (2.6 ± 0.2 g/kg BW) being larger than those of 7-d-old piglets (2.0 ± 0.1 g/kg BW) (P = 0.003). Liver weight (41.5 ± 0.6 g/kg BW) was unaffected by dietary bLF and age.

Because piglets were colostrum-deprived, there was some mortality during the study (29). Pairwise (LF3 vs. Ctrl, LF1 vs. Ctrl) comparisons were conducted. Dietary bLF at 367 mg/(kg BW · d) (LF1) was not sufficient to alter survival (Supplemental Fig. 1A). However, piglets fed the highest bLF dose [1300 mg/(kg BW · d), LF3] were more likely to survive than those consuming the control diet (P = 0.03) (Supplemental Fig. 1B). When survival of piglets in all 3 treatment groups was compared simultaneously, diet tended (P = 0.08) to affect survival.

**Bovine lactoferrin concentration in serum**

To determine whether dietary LF was being absorbed, bLF concentration in piglet serum was measured. Most samples were below the linear range (500–7.8 μg/L) of the assay. For piglets with quantifiable serum bLF (1 day 7 LF3, 3 day 14 LF3, and 1 day 14 LF1), the concentration was 16.1 ± 3.6 μg/L serum. Because few samples contained concentrations of bLF within the linear range of the assay, absorbance values above assay background were used for statistical analysis by ranks. LF3 piglets had higher serum bLF concentrations than Ctrl or LF1 piglets (P < 0.0001, Supplemental Fig. 2). LF1 piglets had higher serum bLF concentrations than Ctrl piglets (P = 0.0001).

**Immunoglobulin concentrations**

Total serum IgG concentrations were similar on day 7 and day 14. However, LF3 piglets had higher total serum IgG than LF1 piglets (P = 0.03) and tended to have higher total serum IgG than Ctrl piglets (P = 0.07, Fig. 1A). In contrast, diet did not affect the IgA concentrations in Asc contents, but total Asc IgA concentrations were higher on day 14 than day 7 (P < 0.0001, Fig. 1B).

**T-cell populations**

T helper cells decreased, cytotoxic T cells increased, and the CD4 to CD8 T-cell ratio decreased in PBMC, MLN, spleen, and IPP as piglets aged (Table 1). Dietary bLF did not affect T-cell populations (Supplemental Fig. 3A–C). Neither time nor diet affected the proportion of double positive CD4+CD8+ (a mature effector memory cell population in pigs) within PBMC, MLN, spleen, or IPP (Supplemental Fig. 3D).

**Other immune cell populations**

Immune cell populations were affected by age but not by dietary bLF. The NK (CD3+CD4+CD8+) cell populations in PBMC, MLN, and spleen increased, whereas PBMC and IPP B-cell populations decreased with age (Table 2). Total major histocompatibility complex II+ cell populations decreased in PBMC, MLN, and IPP, but increased in spleen, with age (Table 2). Polymorphonuclear cells in the MLN and IPP decreased with age (Table 2). Dietary bLF did not affect immune cell populations (Supplemental Fig. 4A–D).

**Cytokine production**

**PHA-induced.** Splenocytes from 7-d-old piglets fed 1300 mg LF/(kg BW · d) (LF3) produced more IFN-γ than cells from the spleen of Ctrl piglets (Fig. 2). IFN-γ production by cells isolated from the MLNs of Ctrl and LF3 piglets was similar, and dietary LF did not affect IL-6 or IL-10 production in response to PHA stimulation of cells isolated from MLNs or spleen (Supplemental Fig. 5).

**LPS-induced.** Dietary, but not ex vivo, bLF altered cytokine production by spleen and MLN mononuclear cells. Ex vivo bLF did not affect cellular cytokine production in response to LPS stimulation (Figs 3 and 4). This was true whether the LPS and bLF were pre-incubated for 1 hour prior to addition to cell culture (PreLPS + LF) or were added to cell culture simultaneously (LPS + LF). Dietary LF substantially affected cytokine production by MLNs (Fig. 3) and spleen (Fig. 4) cells from 7-d-old piglets. MLN cells from LF1 piglets produced more IFN-γ than those isolated from Ctrl piglets (Fig. 3A). MLN cells from LF1 piglets produced more IL-6 and more IL-10 than those isolated from Ctrl piglets (Fig. 3B). LF3 piglet MLN cells tended to produce more IL-6 (P = 0.05) and did produce more IL-10 than cells isolated from Ctrl piglets (Fig. 3B, C). LF3 piglet MLN cells tended to produce more IL-6 (P = 0.05) and did produce more IL-10 than those isolated from Ctrl piglets (Fig. 3B, C). MLN production of TNF-α was not affected by dietary bLF (Fig. 3D). Splenocytes from either LF1 or LF3 piglets produced more IFN-γ than those from Ctrl piglets (Fig. 4A). IL-6 production by splenocytes was not affected by dietary LF (Fig. 4B). Only splenocytes from LF3 piglets produced more IL-10 or TNF-α than Ctrl piglets (Fig. 4C, D).

**Discussion**

The key finding from this study was an improvement in the immune response of colostrum-deprived neonatal piglets fed high doses of bLF, including in systemic and mucosal immune cells capable of producing higher quantities of cytokines than in piglets fed formula containing lower quantities of bLF. Although no changes in immune cell population sizes were observed, the trend toward (P = 0.07) increased serum IgG concentrations, substantially enhanced cytokine production, and lower mortality observed in piglets fed bLF support a role for LF in the initiation of protective immune responses in these immunologically challenged neonates.
In previous studies, bLF was shown to increase animal growth (30–34). In our study, dietary bLF did not affect the growth of colostrum-deprived piglets. This is likely because bLF was incorporated into the diets at the time of formulation, resulting in diets that were isocaloric and isonitrogenous, whereas those found in a normal room, i.e., piglets were not maintained in a specific pathogen-free environment. All piglets began the workup cluster.

Some piglets died during the study, which is to be expected because they were colostrum-deprived and not maintained in a specific pathogen-free facility. Thus, the time period between birth and gavage with sow serum, albeit short, left piglets vulnerable to potential infection. Serum IgG concentrations of birth and gavage with sow serum, albeit short, left piglets vulnerable to potential infection. Serum IgG concentrations of piglets in this study were lower than those in piglets consuming maternal colostrum and milk (35) and were about half (36) to one-fourth the amounts (37) previously reported for colostrum-deprived piglets. These discrepancies are likely due to the specific pool of sow serum used to transfer passive immunity to the piglets in this study. Low serum Ig concentrations likely contributed to mortality. A recent study suggests that serum IgG concentrations below 10 mg/mL increase piglet mortality (29).

Unlike piglets, human infants receive maternal Ig through placental transfer (38,39), with preterm infants receiving less maternal Ig (39,40). As a result, colostrum-deprived human infants are less vulnerable at birth than colostrum-deprived piglets. However, it is well recognized that even term infants are somewhat immunocompromised because of their inexperienced and underdeveloped immune systems (41–43). Additionally, the similarity of our environmental conditions to those in the real world improves the translation of this research to humans. Thus, the increased risk of piglet mortality is justified because the conditions that increase mortality improve the applicability of the research to human infants.

Several additional aspects also suggest these results would apply to humans. Piglets are outbred mammals known to be excellent models for human gastrointestinal and immune development (44–46). Specifically, T-, B-, and NK-cell development observed in the current study was similar to what is observed in human immune development (2,47–49) and what was previously observed in pigs (25). These outbred piglets were maintained in an environment containing a wide variety of microbes typical of those found in a normal room, i.e., piglets were not maintained in a specific pathogen-free environment. All piglets began the

### TABLE 1

| T-cell populations in the PBMCs, MLNs, spleen, and IPPs of 7- and 14-d-old piglets1 |
|-------------------------------|-------------------|-------------------|-----------------|-----------------|------------------|
|                               | Day 7             | Day 14            | P value         |
| T-helper cells, CD3+CD4+CD8–   |                   |                   |                 |
| PBMC                          | 77.3 ± 1.2        | 69.5 ± 1.6        | 0.0011          |
| MLN                           | 80.8 ± 0.8        | 76.8 ± 1.1        | 0.004           |
| Spleen                        | 80.4 ± 1.8        | 73.6 ± 1.7        | 0.013           |
| IPP                           | 90.9 ± 1.3        | 79.7 ± 2.5        | 0.0001          |
| Cytotoxic T-cells, CD3+CD4+CD8–|  |                   |                 |
| PBMC                          | 5.8 ± 0.5         | 9.8 ± 0.7         | <0.0001         |
| MLN                           | 4.5 ± 0.7         | 8.0 ± 0.8         | 0.0002          |
| Spleen                        | 4.8 ± 0.8         | 6.1 ± 0.6         | 0.006           |
| IPP                           | 1.5 ± 0.4         | 1.8 ± 0.3         | 0.003           |
| Helper/cytotoxic ratio        |                   |                   |                 |
| PBMC                          | 19.2 ± 2.2        | 9.1 ± 1.1         | <0.0001         |
| MLN                           | 40.7 ± 5.3        | 17.1 ± 4.3        | 0.0016          |
| Spleen                        | 46.9 ± 6.2        | 20.2 ± 3.4        | 0.0007          |
| IPP                           | 314 ± 44          | 97.3 ± 15.9       | 0.002           |
| Mature effector memory T-cells, CD3+CD4+CD8+| | | |
| PBMC                          | 5.7 ± 0.4         | 5.7 ± 0.6         | 0.89            |
| MLN                           | 11 ± 1            | 11 ± 1            | 0.71            |
| Spleen                        | 5.9 ± 0.4         | 5.9 ± 0.3         | 0.96            |
| IPP                           | 2.3 ± 0.2         | 3.6 ± 0.5         | 0.14            |

1 T-cell populations expressed as % total CD3+ events. Values are means ± SEMs, n = 34–38. CD, cluster determinant; IPP, ileal Peyer’s patch; MLN, mesenteric lymph node; PBMC, peripheral blood mononuclear cell.

### TABLE 2

| Other immune cell populations in the tissues of 7- and 14-d-old piglets2 |
|----------------|-------------------|-------------------|
|                 | Day 7             | Day 14            | P value         |
| NK cells3, CD3+CD4+CD8+|                  |                   |                 |
| PBMC            | 4.2 ± 0.8         | 8.8 ± 1.3         | 0.0120          |
| MLN             | 1.1 ± 0.2         | 2.1 ± 0.3         | 0.0002          |
| Spleen          | 3.5 ± 0.9         | 3.9 ± 0.4         | 0.0015          |
| IPP             | 0.6 ± 0.1         | 0.5 ± 0.1         | 0.42            |
| B-cells3, CD21+MHCII+|                 |                   |                 |
| PBMC            | 13.0 ± 1.2        | 9.2 ± 0.9         | 0.0020          |
| MLN             | 32.8 ± 2.1        | 29.8 ± 2.2        | 0.33            |
| Spleen          | 21.7 ± 1.7        | 23.0 ± 1.4        | 0.53            |
| IPP             | 77.3 ± 3.2        | 65.4 ± 2.4        | 0.0050          |
| MHCII+ cells3   |                   |                   |                 |
| PBMC            | 70.7 ± 3.1        | 58.1 ± 3.0        | 0.0048          |
| MLN             | 40.5 ± 2.0        | 35.3 ± 2.2        | 0.09            |
| Spleen          | 41.1 ± 2.9        | 49.6 ± 2.7        | 0.0389          |
| IPP             | 77.4 ± 3.2        | 65.4 ± 2.4        | 0.0086          |
| PMN, PM, SWC8+CD172α+CD163–|        |                   |                 |
| MLN             | 40.1 ± 3.4        | 27.5 ± 2.3        | 0.0040          |
| IPP             | 54.8 ± 4.5        | 28.5 ± 3.1        | <0.0001         |

1 Values are means ± SEMs, n = 34–38. CD, cluster determinant; IPP, ileal Peyer’s patch; MHC, major histocompatibility complex; MLN, mesenteric lymph node; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear cell; SWC, swine workshop cluster.

2 Expressed as % CD3–events.

3 Expressed as % lymphocytes.

4 Expressed as % CD163–events.
study with normalized passive immunity achieved through oral gavage with pooled sow serum. The decreased mortality in piglets fed BLF in the current study has been reported in human infants. In a clinical study of preterm (~30 wk), very-low-birthweight (<1500 g) infants, infants supplemented with BLF (100 mg/d) had decreased incidence of blood-borne bacterial, viral, and fungal infections, reduced incidence of late-onset sepsis, and less mortality (50–52). Lastly, the amino acid sequences of human (53) and porcine (54) LF are 69% and 72.6% homologous to BLF, respectively, indicating that BLF could potentially be efficacious in both species. Thus, the results of our study are potentially translational in nature.

LF is relatively resistant to digestion and passes through the gastrointestinal tract intact (55). Piglets can absorb macromolecules up to 3 days after birth because of general gut leakiness (56,57). Although serum concentrations of macromolecules absorbed through a leaky gut typically decrease with age, piglets fed BLF had detectable serum BLF. Because clearance of BLF from circulation and dietary intake of BLF prior to sampling was not carefully controlled, the importance of measurable BLF in the serum of BLF-fed piglets is somewhat ambiguous. However, LF has been detected in the urine and feces of breastfed infants (58,59). In addition, LF has been shown to be transported into systemic circulation from the intestinal epithelium in mice (60), rats (61), pigs (23), and calves (62). Intestinal LF receptors (LFRs) have been characterized in the pig and are expressed on the brush-border membrane (22). LFRs are homologous to a protein called intelectin that is expressed in mice, cows, humans, and pigs (63,64). A LFR for human fetal intestinal brush-border membranes was previously identified (65). The presence of LFRs on intestinal surfaces as well as the presence of BLF in the serum of piglets fed BLF suggests that BLF may be absorbed by a receptor-mediated mechanism and can therefore exert systemic as well as local effects.

The general increase in cytokine production by mucosal and systemic immune cells isolated from piglets fed dietary BLF indicates a priming of the immune system. These specific cytokines were analyzed to measure cytokines important in early (TNF-α, IL-6, IL-10) and adaptive (IL-12p40, IFN-γ, IL-4) immune responses using available porcine-specific reagents. The highest dietary dose of BLF, 1300 mg/kg BW, affected cytokine production by systemic immune cells. Both the 367-mg and 1300-mg doses of BLF affected cytokine production by mucosal immune cells. IL-10 production was increased by both MLNs and spleen cells from piglets fed high doses of dietary BLF. IL-10 was increased under both unstimulated and LPS-stimulated conditions. This indicates a general priming of the IL-10 response in piglets fed BLF. Because IL-10 is a key mediator of intestinal homeostasis as well as immune regulation and is expressed by both innate and adaptive immune cells (66), increased production of this cytokine...
potentially limits inflammation. To some extent, the increase in IL-10 production could be in response to increased inflammatory cytokines. When isolated from LF3 piglets, spleen, but not MLN, cells increased IFN-γ and TNF-α production, and MLN, but not spleen, cells tended (P = 0.05) to increase IL-6 production, demonstrating organ-specific effects of dietary bLF treatment. Strong TNF-α and IL-6 production indicates a robust innate immune response by the cells from piglets fed bLF compared with those fed the Ctrl diet. The improved IFN-γ response indicates that dietary bLF may be most important in T-helper-1 type adaptive immune responses and thus is important in protection from infection (67). Because timing is highly important in an immune response, these cytokine results are likely a result of analysis of 72-h supernatants, and different results may have been observed if additional time points were analyzed. One implication of these results is that dietary bLF results in cells better prepared to respond to challenge (IL-6, TNF-α, IFN-γ) and better prepared to recover from that response (IL-10).

There are several possible explanations for the effects of LF on cytokine response. Intact bLF absorbed into the bloodstream via the gastrointestinal tract may have direct effects on tissue resident cells via receptor-mediated mechanisms or via indirect uptake of LF. LFRs are present on immune cells such as T cells, B cells, NK cells, macrophages, and dendritic cells (15–17). Additionally, nonspecific LF uptake by T cells of the lamina propria has been demonstrated using pig intestinal explant tissue (68). A second possibility is that bLF exerts its influence through the education of immune cells in the microenvironment of the gastrointestinal tract. These gut immune cells then migrate via the lymphatic system to the MLNs and eventually to the spleen (69,70). In fact, the immune system may be primed by bLF via a combination of these mechanisms.

Piglets were not challenged with a specific immune stimulant in vivo. Thus, the effects of bLF on the immune response observed in this study may not be applicable to neonates facing specific types of infections or other immune challenges. Importantly, based on the measured parameters, dietary bLF did not trigger an immune response to itself. This is supported by the low amounts of cytokine production in response to bLF stimulation. When combined with the lack of effect on growth and overall well-being, these results indicate that bLF is likely safe for oral consumption at the amounts tested in this study.

In summary, human milk is the ideal form of nutrition for human infants. However, 60–90% of infants worldwide consume infant formula sometime during the first year of life (71). Hence, bovine milk–based formulas should attempt to mimic the biologic actions of breast milk. These formulas currently provide a limited amount of bLF. Based on the effects of bLF demonstrated herein, and in previous studies (51,72), inclusion of bLF in infant formulas should be considered. In addition, this study identified potential immune end points for use in clinical intervention trials. There remains a need for future investigations to determine the effects of dietary administration of bLF on specific cell types, at specific times of infant development, and in neonates exposed to a variety of specific immune stimuli.

Acknowledgments
The authors thank Barbara Pilas and Ben Montez in the Roy J. Carver Biotechnology Center Flow Cytometry Core at the University of Illinois for their guidance. The authors thank Marcia Monaco, Shelly Hester, Jill Shunk, Kilia Liu, Casey Radlowski, Emily Radlowski, Min Li, Mei Wang, and Krystal Woo for their technical expertise. The authors thank Cuvi Yi for diet bLF analysis. S.S.C., N.C., and S.M.D. designed the research. S.S.C. and E.A.R. conducted the research. S.S.C. analyzed the data and wrote the paper. S.M.D. is responsible for the final content. All authors read and approved the final manuscript.


