

Natural killer cell populations and cytotoxic activity in pigs fed mother's milk, formula, or formula supplemented with bovine lactoferrin

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BACKGROUND: Natural killer (NK) cells are components of the innate immune defense system, and their levels differ between breast and formula-fed (FF) infants. Lactoferrin (Lf) modulates NK cell cytotoxicity *ex vivo*. We hypothesized that dietary bovine Lf (bLf) would increase NK cell populations and cytotoxicity.

METHODS: Piglets were sow-reared (SR), FF, or 1 g/l bLf-fed (LF) for 21 d. NK cells (CD3⁺CD4⁺CD8⁺) in blood (peripheral blood mononuclear cells (PBMCs)), spleen, and mesenteric lymph node (MLN) were determined by flow cytometry. PBMC NK cells were tested for cytotoxic activity against target K562 cells *ex vivo* in the presence of media (unstimulated), interleukin-2, or bLf. NK cell mRNA expression was determined by reverse transcription-quantitative PCR.

RESULTS: SR and LF piglets had more NK cells in MLN ($P = 0.0097$) and spleen ($P = 0.0980$) than FF piglets. In PBMCs, SR piglets had more NK cells than FF piglets ($P = 0.0072$); LF piglets were intermediate and not different from FF or SR piglets. NK cell intelectin-2 mRNA expression was 2.5-fold higher ($P = 0.0095$) in LF than SR or FF piglets. NK cells in SR piglets exhibited greater ($P < 0.0001$) cytotoxic activity than those in LF or FF piglets, which was supported by greater perforin mRNA expression.

CONCLUSION: Dietary bLf increased blood NK cell populations and NK Lf receptor expression but not NK cell cytotoxicity.

Neonates face a dramatic transition at birth as they move from the protected intrauterine environment to one full of foreign antigens (1). Owing to limited prenatal exposure to antigens, neonates initially rely on their innate immune system for protection. Natural killer (NK) cells are large, granular lymphocytes that protect the body against infections through their ability to kill target cells as well as secrete inflammatory cytokines that further induce themselves or neighboring immune cells to mount a protective response. This mechanism of immune defense is critical for protecting the neonate before the development of pathogen-specific cytotoxic T lymphocytes.

NK cell populations are higher in the blood of breast-fed infants than in formula-fed (FF) infants (2,3), leading to

the hypothesis that human breast milk components influence the development of NK cells. Lactoferrin (Lf) is a multifunctional glycoprotein found in high concentrations in human breast milk (~2.1 g/l) (4,5), but at 20-fold lower levels (100 mg/l) in bovine milk (6) and infant formula (7). Lf has been shown to increase the cytotoxic activity of NK cells isolated from adult human blood and subsequently stimulated with Lf *ex vivo* (8). In addition, Lf also increased target cell sensitivity to NK cell lysis via the activation of the Fas signaling pathway for apoptosis (9). Taken together, these findings support a potential role for milk-borne Lf in mediating NK cell cytotoxicity.

Therefore, the goal of this study was to investigate the effect of dietary Lf on NK cell populations and *ex vivo* NK cell cytotoxicity in the preclinical piglet model. Piglets were selected as they are more similar than neonatal rodents to human infants in terms of anatomy, physiology, immunology, and metabolism (10–12). In addition, piglets can be reared independently from their mother and thrive on bovine milk-based formula (10). Furthermore, the major populations of immune cells in pigs are similar to those of humans (13).

We hypothesized that NK cell populations and cytotoxicity would differ between piglets fed bovine Lf (bLf)-supplemented formula and those fed unsupplemented formula, as well as between piglets fed mother's milk and those fed unsupplemented formula, and that these parameters would be similar between Lf-fed (LF) and sow-reared (SR) piglets.

RESULTS

NK Cell Population Size

NK cells isolated from blood, mesenteric lymph node (MLN), and spleen were stained with NK cell markers and analyzed by flow cytometry. In MLN, SR and LF animals had a greater percentage of NK cells than FF animals ($P = 0.0097$). The NK cell population size in peripheral blood mononuclear cells (PBMCs) was larger in SR as compared with FF piglets ($P = 0.0072$), whereas LF piglets were intermediate and not significantly different from either group (Table 1). Similar to MLN, the splenic NK cell population tended ($P = 0.0980$) to be larger

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Table 1. NK cell populations (CD3⁺CD4⁺CD8⁺ events as a percentage of total CD3⁺ events) in MLN, PBMCs, and spleen of 21-d-old SR, FF, or LF piglets

Tissue	Dietary treatments			Model P value
	SR	FF	LF	
PBMC	20.7 ± 13.4*	8.9 ± 3.0 [†]	11.7 ± 5.0* [†]	0.0072
MLN	6.5 ± 2.5*	3.6 ± 1.8 [†]	7.0 ± 3.2*	0.0097
Spleen	12.2 ± 5.4	6.8 ± 5.2	11.7 ± 6.4	0.0980

Data are expressed as mean ± SD ($n = 9-13$ per group). Within a row, different superscripts (*,†) indicate significant differences at $P \leq 0.05$.

FF, formula-fed; LF, bovine lactoferrin-fed; MLN, mesenteric lymph node; NK cell, natural killer cell; PBMCs, peripheral blood mononuclear cells; SR, sow-reared.

in SR than in FF animals, with LF intermediate between SR and FF.

Intelectin-2 mRNA Expression in NK cells Isolated From PBMCs

Expression of the Lf receptor (LfR) intelectin-2 (ITLN-2) was measured by reverse transcription-quantitative PCR in NK cells isolated from PBMCs. ITLN-2 mRNA expression was 2.5-fold higher ($P = 0.0095$) in NK cells from LF piglets than in NK cells from SR or FF animals (Figure 1).

NK Cell Cytotoxicity

NK cells isolated from PBMCs of SR animals, whether unstimulated or stimulated with interleukin-2 (IL-2) or bLf, exhibited greater ($P < 0.0001$) cytotoxic activity than those isolated from LF or FF piglets (Figure 2). As expected, IL-2 stimulated NK cell activity in all of the diet groups with an average NK cell cytotoxicity of 40.5 ± 13.7 , 22.1 ± 9.1 , and $10.2 \pm 3.5\%$ for SR, FF, and LF animals, respectively. IL-2-stimulated cytotoxicity was significantly higher ($P < 0.0001$) than bLf-stimulated cytotoxicity (24.2 ± 18.4 , 4.6 ± 5.8 , and $3.4 \pm 4.4\%$) or unstimulated cytotoxicity (25.1 ± 20.8 , 4.9 ± 4.3 , and $3.2 \pm 2.5\%$). NK cells from FF piglets appeared to respond more strongly to IL-2 stimulation than those from LF animals. A subanalysis of NK cell cytotoxicity for FF and LF animals showed an interaction between stimulation and diet ($P = 0.0013$). In this model, the cytotoxic activity of NK cells from FF animals was significantly greater ($P < 0.0001$) than that from LF animals after IL-2 stimulation. However, FF and LF NK cell cytotoxicity was similar when cells were stimulated with bLf or left unstimulated.

NK Cell-Associated Gene Expression in NK cells Isolated From PBMCs

In an effort to determine possible molecular mechanisms that could contribute to the decreased cytotoxic activity of NK cells from FF and LF animals, reverse transcription-quantitative PCR was performed on RNA extracted from unstimulated peripheral blood CD3⁺CD4⁺CD8⁺ NK cells (Figure 3). The mRNA abundance of perforin, an effector molecule for NK cells, was three- and sixfold lower in NK cells from FF and LF piglets, respectively, than in those from SR piglets ($P = 0.0038$, Figure 3a). In addition, the expression of NK group 2 member D (NKG2D) receptor, an activating receptor found on NK cells and known as killer cell lectin-like receptor subfamily K

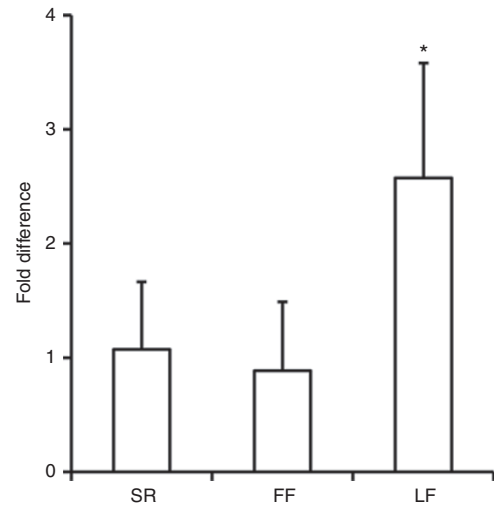


Figure 1. Intelectin-2 mRNA abundance was higher in natural killer cells of 21-d-old bLf-fed (LF, $n = 3$) piglets than in those of sow-reared (SR, $n = 7$) or formula-fed (FF, $n = 6$) animals. Normalized values for intelectin-2 were calculated by dividing the target quantity mean by the quantity mean of the reference gene (peptidylprolyl isomerase A). Fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for FF pigs. Data are expressed as mean ± SD of the fold difference relative to FF animals. * $P \leq 0.05$. bLf, bovine lactoferrin.

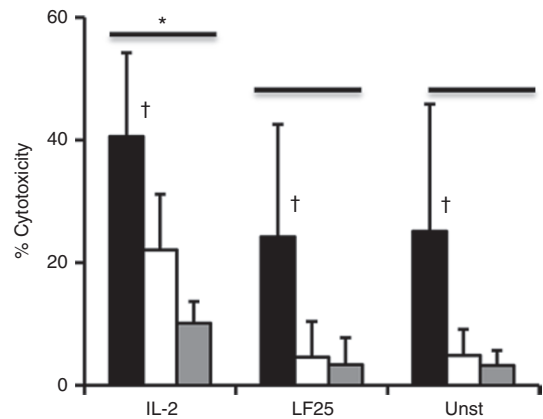


Figure 2. Cytotoxicity of natural killer (NK) cells isolated from 21-d-old sow-reared (SR; $n = 11$; black), formula-fed (FF; $n = 14$; white), and bLf-fed (LF; $n = 11$; gray) piglets. Peripheral blood mononuclear cell (PBMC) NK cells were either unstimulated (Unst) or stimulated with interleukin-2 (IL-2) (20 ng/ml) or Lf (25 μ g/ml) and incubated for 48 h with DiOC₁₈-labeled K562 target cells at 10:1 effect-to-target cell ratio. Propidium iodide (PI) was added at the end of incubation to label dead cells. Cytotoxicity is reported as killed target (PI⁺DiOC₁₈⁺) cells as a percent of total target (DiOC₁₈⁺) cells. Data are expressed as mean ± SD. Proc generalized linear model (GLM) full model, $P < 0.0001$; treatment, $P < 0.0001$; diet, $P < 0.0001$; and treatment × diet interaction, $P = 0.3969$. Different superscripts indicate significant differences at $P \leq 0.05$. *Indicates differences between stimulants; †indicates significant differences between diets. bLf, bovine lactoferrin.

member 1 (KLRK1), was 3.5-fold lower on NK cells from FF animals than on those from SR animals, whereas the NKG2D receptor gene expression in NK cells of LF animals was intermediate and not significantly different from either group (Figure 3b).

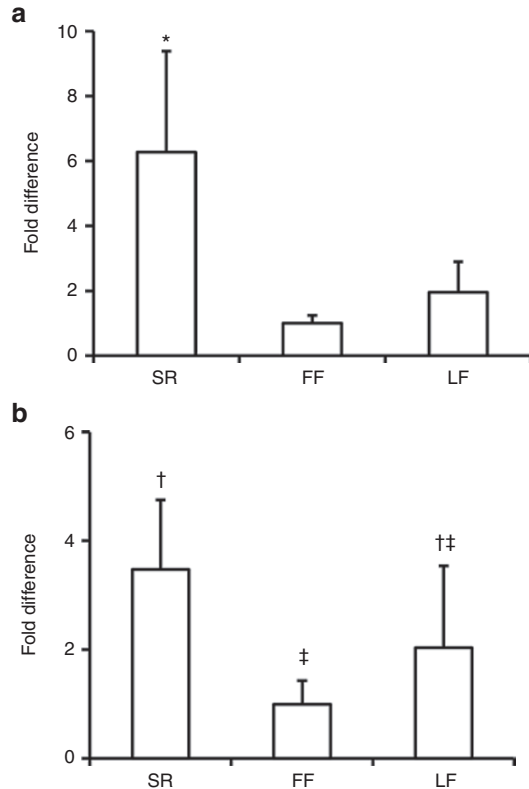


Figure 3. Perforin (a) and NK group 2 member D (NKG2D) receptor (b) mRNA abundance in natural killer cells isolated from 21-d-old sow-reared (SR, $n = 7$), formula-fed (FF, $n = 4-5$), and bLf-fed (LF, $n = 3$) piglets. Normalized values for the target genes were calculated by dividing the target quantity mean by the quantity mean of the reference gene. Fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for FF pigs. Data are expressed as mean \pm SD. Different superscripts (*, †, ‡) indicate significant differences at $P \leq 0.05$. bLf, bovine lactoferrin.

DISCUSSION

In the infant, NK cells contribute to early protective responses against a variety of infections before pathogen-specific cytotoxic T lymphocytes are developed (14). NK cells also secrete inflammatory cytokines to induce the activation of neighboring cells or the adaptive immune cells (15). Therefore, the neonatal innate immune system plays a crucial role in both protecting the neonate from pathogenic infection and shaping the adaptive immune system for further protection. Diet influences NK cell population size and cytotoxic activity in neonates. Previous studies have shown that breast-fed infants had a higher percentage of NK cells in PBMCs than did FF infants (2,3). In the current study, NK cell population size in the blood and tissues of SR pigs was also larger than that of FF pigs. In addition to the larger population size, NK cells from SR animals also exhibited higher NK cell cytotoxicity than FF animals. This was true whether the cells were unstimulated or stimulated (by IL-2). These results demonstrate an effect of sow milk on innate immune development.

The influence of early feeding on NK cell production may be attributed to components found in human breast milk (16). For example, Lf, an iron-binding glycoprotein found in breast

milk, has been suggested to play an important role in modulating the innate immune system (17). In the current study, pigs that were fed formula containing 1 g/l bLf exhibited an NK cell population size more similar to SR pigs than to FF pigs. Because we observed effects of dietary bLf on NK cell population size, we assessed mRNA expression of bLf receptor on this subpopulation of cells. ITLN-2 has been identified as the LfR in many cell types (18). Cells with higher ITLN-2 expression would be more likely to respond when exposed to bLf. We found that LF piglets had 2.5-fold higher NK cell ITLN-2 mRNA expression than SR or FF piglets, suggesting that oral supplementation of bLf may increase expression of LfR. We hypothesized that this increase in expression of LfRs would enable NK cells isolated from LF piglets to exhibit greater NK cell cytotoxicity than NK cells from either FF or SR piglets after bLf stimulation.

However, bLf stimulation did not affect NK cell activity in LF animals. Regardless of dietary treatment, *ex vivo* stimulation of NK cells with 25 μ g/ml of bLf resulted in similar cytotoxic activity as that observed from unstimulated NK cells. Our results contradict the findings of Horwitz (19), Kuhara (20), and Shimizu (21), who showed that *ex vivo* stimulation of NK cells with bLf increased NK cell cytotoxic activity. These studies examining the effect of bLf on NK cell activity were either conducted in mice (20), contained accessory cells with NK cells, allowing for assistance from other immune cells (19,21), or involved subjects (mice) who were immunologically challenged with a hapten or virus (19,20). Therefore, the lack of effect of bLf on NK cell activity may be due to the condition of our samples. In our study, NK cells were isolated from healthy, unchallenged, neonatal animals. In addition, Lf may not be effective in enhancing NK cell activity without assistance from other immune cells or a viral/hapten stimulus. Stronger immunological challenge may enhance NK cell response to bLf stimulation. For the most part, bLf decreased activation of NK cell cytotoxic activity after IL-2 stimulation. This suggests that bLf may play a role in immune regulation. The mechanism of this immunoregulatory effect of bLf *in vivo* is unknown. We also found that NK cells from SR or FF animals responded more strongly to IL-2 stimulation than those from LF animals. This could be due to Lf's ability to reduce surface expression of IL-2 receptors on T helper 1 (T_H1)-related inflammatory cells (22). Lf inhibits proliferation of T_H1 cell lines but not T_H2 cell lines through altered expression of the receptor for the autocrine growth factor IL-2, but not of the receptor for IL-4 (22). In addition, Crouch (23) and colleagues demonstrated that Lf can alter IL-2 production in mononuclear cells, which may indirectly relate to the reduction of NK cell activity. To summarize, neither oral supplementation nor *ex vivo* stimulation with bLf affects NK cell cytotoxicity in neonatal pigs.

To investigate potential mechanisms for improved NK cell cytotoxicity in animals fed mother's milk, NK cell gene expression was analyzed. Perforin and NKG2D receptors are the two genes we chose to analyze. These molecules are directly related to NK cell lytic function. Perforin is a membrane-disrupting protein and an essential enabler of granzyme-mediated

apoptosis in target cells (24). Perforin deficiency impairs NK cell activity in perforin-knockout mice (25). Our data showed that perforin mRNA expression in NK cells of FF or LF piglets was sixfold or threefold lower, respectively, than that of SR animals. Expression did not differ between LF and FF piglets. The NKG2D receptors are killer cell immunoglobulin-like receptors that are expressed on the surface of NK cells and mediate NK cell cytotoxicity (26) through costimulation (27). Therefore, high expression of NKG2D receptor in NK cells implies that these cells have higher tendency to be activated (28). The mRNA expression of NKG2D receptor was 3.5-fold higher in NK cells of SR animals than FF animals. The mRNA expression of NKG2D receptors in NK cells of LF animals was intermediate between those of SR and FF animals, suggesting a positive effect of bLf. These results suggest that increased perforin and NKG2D receptor expression in NK cells from SR pigs may be driving the increased cytotoxic activity of those cells. However, because these data were collected from a limited number of animals and exhibited wide variability, these results must be confirmed by measurements of protein expression and with a larger number of samples in each dietary treatment group.

The increased cytotoxicity of NK cells from SR piglets suggests a potential mechanism whereby milk improves the neonatal innate immune response. Greater NK cell cytotoxicity was supported by higher mRNA expression of perforin and NKG2D in NK cells from SR animals. It remains to be determined whether it is a specific component of sow milk or another aspect of sow-rearing (e.g., contact with the sow and littermates) that is responsible for the upregulated NK cell activity and gene expression. As mRNA expression is not a direct measurement of perforin protein, future studies should include intracellular staining of perforin and cell surface staining of NKG2D to measure the protein content in NK cells of SR and FF piglets before and after stimulation. In addition, interferon- γ production by NK cells in the different diet groups would provide information about the activation status of NK cells. Although several subpopulations of NK cells have been described in mice and humans, these have not yet been well characterized in pigs. Measurement of parameters such as interferon- γ production would advance our understanding of the effect of diet on the development of NK cells and the relevance of the isolated porcine NK cell population (CD3⁻CD4⁻CD8⁺) used in this study to the CD56^{low}CD16⁺ NK cell population in humans, which exhibits cytotoxic activity and interferon- γ production.

Future experiments evaluating several functions of NK cells will clarify whether NK cells from FF piglets have reduced effector function, poor response to stimulation, or actively repressed cytotoxicity. For instance, measurement of granzyme, perforin, or interferon- γ production in response to receptor cross-linking or application of monoclonal antibodies to NKG2D would determine whether the cellular responses to stimulation differ. The expression of these proteins could be measured by flow cytometry, enzyme-linked immunosorbent spot assay, or enzyme-linked immunosorbent assay.

Measurement of calcium flux after such treatment would also determine whether NK cells from piglets in the various treatment groups were equally able to respond to stimulation through signal transduction pathways. Decreased expression of cell surface Fas, a mediator of apoptosis, would indicate cells of reduced effector capability. Knowledge of the specific stage of NK cell function that is affected by neonatal diet could enable the design of appropriate interventions. Therefore, on the basis of the observations reported herein, this line of research should be pursued.

Overall, diet had a significant impact on NK cell population and activity. Piglets that were fed mother's milk had a larger NK cell population size in blood and immune tissues and greater cytotoxic activity (PBMcs) as compared with piglets that were FF. However, the overall effects of bLf on NK cell development in this study were inconclusive. Dietary bLf supplementation did not increase NK cell cytotoxicity; however, the fact that LF piglets have a greater number of peripheral blood NK cells with increased expression of LfR could potentially improve immune protection of the neonate. In addition, supplementation for longer than 21 d or higher doses of bLf may be more effective in enhancing the innate immune response in these animals because the dose used in this study (1 g/l) is at the lower end of what is present in human breast milk (~2.1 g/l). Therefore, future studies are needed to determine whether the immune regulatory function of bLf on NK cell activity is dependent upon a certain set of conditions, i.e., pathogenic challenge, age, or species. As NK cells are an important first line of defense against infection, understanding the factors that increase their number and improve their killing ability may enable us to better protect FF infants from infection through dietary means.

METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO), unless indicated otherwise.

bLf

Powdered bLf (98% purity) was obtained from DMV International (FrieslandCampina, The Netherlands). Total iron content was 120 mg/kg powder, which would represent 11.9% saturation if all iron was bound by bLf. bLf was reconstituted in double-distilled, deionized water to a concentration of 100 g/l. During formula preparation, 10 ml of this solution was added to 1 l of formula for a final concentration of 1 g/l bLf.

Dietary Treatments and Animal Protocol

All animal care and experimental procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (29) and were approved by the Institutional Animal Care and Use Committee at the University of Illinois (Urbana, IL). Pregnant sows ($n = 8$) were purchased from Midwest Research Swine (Gibson, MN) and transported to the animal facilities within the Edward R. Madigan Building. Upon arrival, sows were vaccinated with LitterGuard LT-C (Pfizer Animal Health, Exton, PA), Respire (Pfizer Animal Health), and Rhinogen BPE (Intervet, Millsboro, DE) and placed on gestation diet enriched with antibiotic (BMD, Montréal, Québec, Canada). Sows received a booster vaccination 2 wk before farrowing. Beginning on approximately day 111 of gestation, sows were monitored for farrowing and, with the exception of the SR ($n = 13$) piglets, animals were removed from the sow 4 h

following vaginal delivery. Piglets were randomized by litter into three diet groups. Piglets in the formula group (FF; $n = 11$) were fed a non-medicated sow milk replacer formula (Advance Baby Pig Liquiwean; Milk Specialties Global Animal Nutrition, Carpentersville, IL) by pump 22 times daily at a volume of 360 ml/kg body weight per day. Piglets in the Lf group (LF; $n = 10$) received the same formula at the same rate with the addition of 1g/l bLf. FF and LF piglets were individually housed in environmentally controlled rooms (25 °C) in specially designed cages that held three piglets separated by Plexiglas partitions. The study was conducted in three replicates. Samples from a subset of animals were used for certain analyses. When this is the case, the specific number of animals is indicated in the results.

Sample Collection

On day 21 postpartum, piglets were sedated with intramuscular injection of Telazol (7 mg/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA) and blood was collected by intracardiac puncture into heparin-containing vacuum tubes (BD Biosciences, San Jose, CA) for PBMC isolation. Piglets were then euthanized by an intracardiac injection of sodium pentobarbital (72 mg/kg body weight, Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI). After euthanasia, spleen and MLN samples were collected for total cell isolation.

PBMC Isolation

Blood was diluted 2:1 with RPMI-1640 (Invitrogen, Gibco, Grand Island, NY), layered onto Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) and spun at 400g for 40 min at 20 °C. PBMCs were collected from the gradient interface. Red blood cells were lysed using lysis buffer (0.15 mol/l NH₄Cl, 10 mmol/l KHCO₃, and 0.1 mmol/l Na₂EDTA). PBMCs were then washed three times in wash buffer composed of Hank's buffered salt solution (no Ca²⁺, no Mg²⁺ (HBSS; Invitrogen, Gibco) supplemented with 2% BSA, 1 mmol/l Na₂EDTA, 50 µg/ml gentamicin (Invitrogen, Gibco), 1,000 U/ml penicillin, and 100 µg/ml streptomycin). PBMCs were suspended in complete RPMI-1640 (10% FBS, 2 mmol/l glutamine, 50 µg/ml gentamicin, 1 mmol/l sodium pyruvate, 20 mmol/l HEPES (Invitrogen, Gibco), 1,000 U/ml penicillin, and 100 µg/ml streptomycin). The number of viable cells was assessed by counting after staining with trypan blue (Invitrogen, Gibco). Cells were then phenotyped and quantified by flow cytometry or used for NK cell isolation as described below.

Total Cell Isolation From Spleen and MLN

Spleen and MLN samples were collected and stored on ice in collection buffer containing antibiotics until processing. Tissues were washed three times (PBS (Invitrogen, Gibco), 50 µg/ml gentamicin, 1,000 U/ml penicillin, and 100 µg/ml streptomycin). Washed tissues were placed in C-tubes (Miltenyi Biotec, Auburn, CA) with 10 ml Hank's buffered salt solution and chopped using a Gentle MACS (Miltenyi Biotec). The resulting cell solutions were strained through a 100 µm (BD Falcon, San Jose, CA) followed by a 40 µm cell strainer (BD Falcon). After lysing the red blood cells, the isolated cells were washed three times in wash buffer and suspended in complete RPMI-1640. The number of viable cells was assessed by counting after staining with trypan blue (Invitrogen, Gibco). Cells were then phenotyped by flow cytometry as described below.

Isolation of NK Cells and Phenotypic Identification for Purity

NK cells were prepared from the PBMCs by negative selection with magnetic-assisted cell separation. Briefly, an antibody cocktail containing mouse immunoglobulin G1 anti-pig CD172a (clone 74-22-15; Southern Biotech, Birmingham, AL), anti-pig CD21 (clone BB6-11C9.6; Southern Biotech), and anti-pig CD3 (clone PPT3; Southern Biotech) was prepared and mixed with 1×10^6 cells/ml. Samples were incubated on ice for 30 min. After centrifugation, cells were washed with autoMACS rinsing solution/10% BSA (Miltenyi Biotec). Then, the cells were incubated with anti-mouse immunoglobulin G1 MicroBeads (Miltenyi Biotec) on ice for 15 min and applied to LD columns (Miltenyi Biotec). Undesired cells (CD3⁺CD21⁺CD172a⁺) were labeled with the antibodies and subsequently with the magnetic microbeads, which attached to the column. The desired cells (CD3⁻CD21⁻CD172a⁻) flowed through the

column and were collected in 15 ml conical tubes. The flow-through cells were stained with anti-CD3:PECy5 (Southern Biotech), anti-CD21:PE (Southern Biotech), and anti-CD172a:FITC (Southern Biotech) to determine the effectiveness of isolation. These cells were also stained with NK cell markers, anti-CD3:PECy5, anti-CD4:FITC (clone 74-12-4; Southern Biotech), and anti-CD8:PE (clone 76-2-11; Southern Biotech) to determine the percentage of flow-through cells that were NK cells. The flow-through cell population was $92.5 \pm 5.8\%$ CD3⁻CD21⁻CD172a⁻ and $80.7 \pm 13.4\%$ CD3⁻CD4⁻CD8⁺ NK cells (Supplementary Figure S1 online) (30). Of the total PBMCs, diet tended to have an effect on NK cell yield as $32 \pm 13\%$ in SR, $22.6 \pm 15.3\%$ in FF, and $20.8 \pm 9.8\%$ in LF animals were CD3⁻CD4⁻CD8⁺ NK cells ($P = 0.07$). Flow staining is described below. The enriched NK cell fraction was used in the cytotoxicity assay, which is described below.

NK Cell Cytotoxicity Assay by Flow Cytometry

A flow cytometry-based assay (31) was used to determine the lytic activity of NK cells. Briefly, K562 tumor cells, a human erythroleukemia cell line (CCL-243; ATCC, Manassas, VA), were used as the target cells. These non-adherent cells were grown in 75 cm² cell culture flasks and cell density was adjusted to 2×10^5 cells/ml with complete growth medium (ATCC-formulated Iscove's Modified Dulbecco's Medium and 10% FBS; ATCC). The K562 cells were pre-labeled with DiOC₁₈ (Invitrogen, Gibco), a lipophilic carbocyanine membrane dye for identification of total target cells. Ten microliters of DiOC₁₈ stock solution (3 mmol/l) was added to each 1×10^6 cells/ml of phosphate-buffered saline and incubated for 20 min at 37 °C. The cells were washed twice with phosphate-buffered saline and resuspended in complete RPMI-1640. Meanwhile, 1×10^5 effector cells (NK cells) were placed in round-bottom 96-well plates and pre-stimulated with 25 µg/ml of bLf or 20 ng/ml of IL-2 (recombinant human IL-2; Invitrogen, Gibco), or 10 µl of complete RPMI (unstimulated) for 2 h at 37 °C, after which DiOC₁₈ pre-labeled target cells were added at a 10:1 effector-to-target cell ratio and further incubated for 48 h at 37 °C. Propidium iodide (PI; Invitrogen Gibco) was then added to the cell mixture and incubated for 30 min at 37 °C to identify killed cells. Immediately after incubation, cytotoxicity was measured by flow cytometry (LSR II; BD Biosciences). The relative percentage of live and dead target cells was determined using FlowJo software (Version 7.0; TreeStar, Ashland, OR). PI⁺DiOC₁₈⁺ cells were identified as killed/dead target cells. PI⁻DiOC₁₈⁺ cells were identified as live target cells. Cytotoxicity was reported as killed target (PI⁺DiOC₁₈⁺, R1) cells as a percentage of total target (DiOC₁₈⁺, R2) cells using spontaneous target cell death as a normalizer. Percentage of cytotoxicity was calculated by the formula (R1/R2 in the test - R1/R2 of spontaneous cell death) $\times 100$. Spontaneous target cell death was determined by incubation of DiOC₁₈-labeled K562 target cells in RPMI alone, followed by PI staining after harvest.

Phenotypic Identification of Mononuclear Cells From Blood and Total Cells From MLN and Spleen

The phenotypes of NK cell populations from peripheral blood, MLN, and spleen were monitored by flow cytometry using a panel of fluorescently labeled monoclonal antibodies. NK cells were identified using mouse anti-pig CD3:PECy5 (Southern Biotech), mouse anti-pig CD4:FITC (Southern Biotech), and mouse anti-pig CD8:PE (Southern Biotech). All staining procedures took place on ice and extra care was taken to prevent unnecessary light exposure. Briefly, one million cells per well were blocked with a mixture of 5 µg/ml unlabeled anti-CD16 (Clone G7; AbD Serotec, Raleigh, NC) and 5% mouse serum (Southern Biotech) to prevent nonspecific binding of antibodies to cells. Anti-CD3, anti-CD4, and anti-CD8 antibodies were added to the wells, incubated for 15 min, centrifuged, and then the supernatant was aspirated. Cells were washed twice with staining buffer (PBS, no Ca²⁺, no Mg²⁺, 0.1% sodium azide, and 1% BSA (Invitrogen, Gibco)), and then fixed with 2% paraformaldehyde. Staining was assessed using an LSRII flow cytometer (BD Biosciences). The relative percentage of NK cell populations was determined using FlowJo software (version 7.0, FlowJo; TreeStar). NK cells were identified as CD3⁻CD4⁻CD8⁺ events (30) and are expressed as a percentage of CD3⁻ events.

NK Cell Gene Expression

Total RNA was extracted and purified from NK cells isolated from PBMCs with the TRIzol reagent (Invitrogen, Grand Island, NY) following the manufacturer's protocol. RNA was quantified by spectrophotometry using a Nanodrop 1000 (Thermo Scientific, Rockford, IL) at 260 nm. A total of 10 million NK cells were used for the extraction and yielded ~800–1,000 ng/μl of RNA. RNA concentration was adjusted to 0.25 μg/ml using RNase free water (Invitrogen), and RNA quality was analyzed using a Bioanalyzer (model 2100; Agilent Technologies, Santa Clara, CA) in the W.M. Keck Center at the University of Illinois. All samples had an RNA integrity number >6.

Reverse transcription was performed using an Eppendorf Thermal Cycler (Fisher Scientific, Pittsburgh, PA). Each reaction contained 3 μg of total RNA and 10 μl of mixture from High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) involving 100 mmol/l deoxyribonucleotide triphosphate (dNTP), 10X RT Buffer, 10X RT Random Primers, MultiScribe Reverse Transcriptase, RNase inhibitor, and Diethylpyrocarbonate (DEPC)-treated water (Invitrogen) in a final volume of 20 μl.

Quantitative real-time PCR was conducted for ITLN-2 (Ss03374218_m1), KLRK1 (NKG2DR; Ss03394782_g1), and perforin (Ss03373694_m1), and peptidylprolyl isomerase A (Ss03392377_m1) using Taqman PCR expression assays (Applied Biosystems). Samples were submitted to a total of 40 amplification cycles per run and fluorescence intensity was detected using a Taqman ABI 7900 machine (Applied Biosystems). Peptidylprolyl isomerase A was used as a reference gene (31). Results were expressed using the relative standard curve method. Briefly, serial dilutions (1:5 to 1:15,625) from a stock of pooled porcine NK cell cDNA were made and run on each plate. Each sample was run in triplicate with primers to assess the target gene and peptidylprolyl isomerase A. Normalized values for each target were calculated by dividing the target quantity mean by the peptidylprolyl isomerase A quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the normalized calibrator sample (in this case the average of FF group). All samples that were tested for statistical differences were run on the same plate.

Statistical Analysis

Data were analyzed using the Proc generalized linear model within SAS (Version 9.2; SAS Institute, Cary, NC). The model for NK cell population and gene expression analyses contained diet as the main effect. The model for NK cell cytotoxicity contained the effect of diet, treatment, and diet × treatment interaction. Normal distribution of the data was confirmed by the normality test within SAS and a Shapiro–Wilk number ≥0.75 indicated that values were normally distributed. Data are reported as mean ± SD. Comparisons with $P < 0.05$ were considered significant and comparisons with $P < 0.10$ were considered trends.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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