Influence of Parturition and Diets Enriched in n-3 or n-6 Polyunsaturated Fatty Acids on Immune Response of Dairy Cows During the Transition Period*

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ABSTRACT

The objectives of this study were to evaluate the functional properties of immunocompetent cells in dairy cows fed diets enriched in n-3 or n-6 polyunsaturated fatty acids during the transition period. Six weeks before calving, 21 primiparous and 27 multiparous pregnant Holstein dairy cows were randomly allotted to 1 of 3 dietary fat treatments: calcium salts of palm oil (Megalac), micronized soybeans, or whole flaxseed, which are, respectively, rich in saturated, n-6, or n-3 fatty acids. On wk 6 and 3 before parturition, cows received a subcutaneous injection of ovalbumin to measure the antibody response in colostrum and serum. Colostrum samples were collected at the first milking after calving, and blood samples were taken 6, 3, and 1 wk before the expected calving date and 1, 3, and 6 wk after calving. Blood mononuclear cells were cultured to evaluate the proliferative response to concanavalin A and the in vitro productions of interferon-γ, tumor necrosis factor-α, nitric oxide, and prostaglandin E$_2$. The serum antibody response to ovalbumin was unaffected by dietary fatty acids, but the response was lower in primiparous cows than in multiparous cows. A significant diet × parity interaction indicated that colostral antibody level against ovalbumin was significantly higher in multiparous cows fed soybeans than in those fed flaxseed or Megalac; there was no difference among treatments for primiparous cows. The lymphocyte response to concanavalin A was lower in cows fed soybeans than in those receiving flaxseed or Megalac when the cells were incubated with autologous serum. The proliferative response of mononuclear cells incubated with autologous serum was suppressed in the 1st wk after calving in both primiparous and multiparous cows, and multiparous cows showed a higher response than primiparous cows throughout the experiment. There was a significant interaction between parity and diet as a result of a greater production of interferon-γ by mononuclear cells incubated with autologous serum in multiparous cows than in primiparous cows fed flaxseed; there was no difference among cows fed the other diets. Interferon-γ production was reduced around calving while the inverse was observed for productions of nitric oxide and tumor necrosis factor-α. Productions of nitric oxide, prostaglandin E$_2$, and tumor necrosis factor-γ were greater in primiparous cows than in multiparous cows. In conclusion, functional properties of lymphocytes and monocyte/macrophage lineage of dairy cows during the transition period are modulated by parturition and the composition of polyunsaturated fatty acids in the diet.
(**Key words:** dairy cow, immune response, polyunsaturated fatty acids)

Abbreviation key: AS = autologous serum, **ConA** = concanavalin A, **FBS** = fetal bovine serum, **FLA** = whole flaxseed, **IFN-γ** = interferon–γ, **LPS** = lipopolysaccharide, **MEG** = Megalac, **OVA** = ovalbumin, **PBMC** = peripheral blood mononuclear cells, **PG** = prostaglandin, **PGE$_2$** = prostaglandin E$_2$, **PUFA** = polyunsaturated fatty acids, **RPMI** = Roswell Park Memorial Institute, **SOY** = micronized soybeans, **TNF-α** = tumor necrosis factor-α.

INTRODUCTION

Animals protect themselves against microbes by different mechanisms that are innate or adaptive. Both innate and adaptive immunity are activated and regulated through the production of several mediators, which include cytokines and eicosanoids. Research with animal models has identified nutritional approaches that may modulate the production of key factors involved in the activation and regulation of innate and adaptive immunity. Among these approaches, n-3 and n-6 polyunsaturated fatty acids (**PUFA**) have been shown to be important modulators of immune reactions (Calder et al., 2002). Dietary fats rich in n-3 or n-6

PUFA modulate the inflammatory responses in experimental animal models (Pomposelli et al., 1989) and in clinical trials (Stenson et al., 1992). In mice fed an enriched n-3 PUFA diet, inflammatory reactions were reduced, and different types of antibody response to antigenic stimulations were developed compared with mice fed an n-6 enriched diet (Albers et al., 2002). Mechanisms involved in the regulation of immune response are not yet completely understood, but there is evidence that PUFA influence cellular communication and activation through the synthesis of prostaglandins (**PG**), tumor necrosis factor-α (**TNF-α**), interferon-γ (**IFN-γ**), and other factors such as nitric oxide (Calder et al., 2002).

Linoleic acid (18:2n6) can be converted through enzymatic reactions into arachidonic acid, which is the precursor of the pro-inflammatory mediators, prostaglandin E_2 (**PGE$_2$**) and leukotriene B_4. The same elongase and desaturase can also convert the α-linolenic acid (18:3n3), an n-3 fatty acid, to eicosapentaenoic acid (eicosapentaenoic acid: 20:5n3). Eicosapentaenoic acid is the precursor for the synthesis of PGE_3 and leukotriene B_5 that cause less severe inflammatory reactions than PGE_2 and leukotriene B_4 (Yaqoob and Calder, 1995). These lipid mediators act as paracrine and autocrine regulators through a family of transmembrane receptors found in many cells, including immune cells, and induce pro-inflammatory cytokines such as TNF-α, interleukin-1, and interleukin-6 (James et al., 2000).

During the perinatal period, several immune functions such as lymphocyte response to mitogens and production of antibodies have been shown to be depressed in dairy cows (Kehrli et al., 1989). Development of new feeding strategy in which fatty acid composition of the diet may influence the regulation of immune response through the production of less inflammatory factors could contribute to attenuate the immunosuppressive effect caused by parturition and could improve recovery of immune functions involved in defense against pathogens. The objectives of this study were to evaluate the functional properties of immunocompetent cells in dairy cows fed diets enriched in n-3 or n-6 PUFA during the transition period. This knowledge will contribute to improve animal health based on the relationships between nutrition and immune functions.

MATERIALS AND METHODS

Animals, Diets, and Immunization

The experiment was conducted at the Dairy and Swine Research and Development Center, Lennoxville, QC, from November 1999 to February 2001 using 27 multiparous and 21 primiparous Holstein cows. Cows were blocked within parity for similar expected calving dates; there were 9 blocks of multiparous cows and 7 blocks of primiparous cows. The experiment was carried out from 6 wk before the expected calving date to wk 6 of lactation. Cows were housed in tie stalls and were fed individually. Cows within blocks were assigned randomly to 1 of 3 treatments. The 3 experimental diets consisted of fat supplements based on either whole flaxseed (**FLA**), calcium salts of palm oil, Megalac (**MEG**), or micronized soybeans (**SOY**), and they were designed at the beginning of the experiment to yield similar CP and ether extract concentrations. Pre-calving diets (Table 1) were formulated to meet requirements for cows that averaged 675 kg of BW. After calving, diets (Table 2) were formulated to meet requirements for cows that averaged 625 kg of BW and produced 40 kg/d of milk with 3.85% fat (NRC, 1989). Feed consumption was recorded daily. Diets were fed twice daily for 10% orts. Samples of each experimental diet were taken weekly, frozen, and composited on a 4-wk basis. Composited samples were mixed thoroughly and subsampled for chemical analyses according to the methods already used by Petit (2002). On wk 6 before parturition, all cows were injected subcutaneously with 2 mg of ovalbumin (**OVA**) grade V (Sigma, Oakville, ON, Canada) in incomplete Freund's adjuvant (Gibco BRL, Toronto, ON, Canada). A second injection was given on wk 3 before parturition. The animal protocols of the experiment were approved by an animal care committee at our institution.

Blood Sampling for Peripheral Blood Mononuclear Cells and Monocytes Isolation

Blood samples were taken on wk 6, 3, and 1 before the expected calving date and on wk 1, 3, and 6 after calving. Six tubes of blood from the jugular vein were drawn into 10-mL K_3EDTA-vacuum tubes (Becton Dickinson and Cie, Rutherford, NJ) for leukocyte isolation. At the same time, one blood sample was taken into a vacuum tube with no additive for preparation of heat-inactivated (56°C for 30 min) autologous serum (**AS**).

Peripheral blood mononuclear cells (**PBMC**) were isolated from whole blood according to a method described by Carlson and Kaneko (1973) with some modifications. Briefly, blood samples were layered on Ficoll-Hypaque Plus (Amersham Pharmacia, Montreal, QC, Canada), and PBMC were collected at the interface after centrifugation (400 × *g* for 40 min) and washed twice with Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (wash solution; Gibco BRL, Toronto, ON, Canada). Finally, PBMC were resuspended in Roswell Park Memorial Institute (**RPMI**) 1640 medium (Gibco BRL) supplemented with 12 m*M* HEPES, 2 m*M* glutamine, 23

Table 1. Ingredient and chemical composition of the pre-calving diets (DM basis, except DM).[1,2]

Item	FLA	MEG	SOY	SEM
Ingredient				
Corn silage	33.1	33.7	33.2	
Grass silage	24.0	24.4	24.3	
Megalac	0	2.7	0	
Micronized soybean[3]	0	0	9.4	
Whole flaxseed	5.9	0	0	
Protein supplement[4]	5.1	7.1	0	
Barley	6.2	6.2	6.3	
Hay	24.8	25.0	25.1	
Soybean oil	0	0	0.7	
Mineral and vitamin premix[5]	0.9	0.9	1.0	
Chemical				
DM, %	42.5	39.9	41.0	3.0
CP, %	14.1	15.5	14.4	0.7
Ether extract, % of DM	4.8[a]	3.8[b]	4.9[a]	0.2
NDF, % of DM	43.0	43.3	45.0	0.7
ADF, % of DM	26.5	27.4	27.2	0.7
NE_L,[6] Mcal/kg	1.60	1.60	1.59	
Fatty acids, % of total fatty acids				
C12:0	0[b]	0.8[a]	0[b]	0.1
C14:0	0[c]	1.4[a]	0.6[b]	0.1
C16:0	12.3[c]	27.3[a]	14.6[b]	0.3
C16:1	0[c]	0.44[a]	0.32[b]	0.02
C18:0	2.7[b]	3.1[a]	3.0[ab]	0.1
C18:1	17.5[b]	22.7[a]	20.3[ab]	0.9
C18:2	31.7[b]	31.2[b]	47.7[a]	1.5
C18:3	35.8[a]	13.1[b]	13.5[b]	2.1

[a–c]Means within a row with a different superscript differ at $P < 0.05$.

[1]Least square means with pooled standard error of the mean (SEM); mean of 6 monthly samples that were prepared by compositing weekly samples.

[2]FLA = Fat supplement based on whole flaxseed, MEG = fat supplement based on Megalac, and SOY = fat supplement based on micronized soybeans.

[3]MICRO-SOYA Elite, 40% CP (Semences Prograin Inc. St-Césaire, QC, Canada).

[4]Contained 52.6% CP, 8.5% ADF, and 13.5% NDF.

[5]Contained 3.0% Ca, 12.0% P, 8.0% Mg, 6.8% Na, 10.5% Cl, 1.5% K, 2.0% S, 40 mg/kg of Se, 7700 mg/kg of Zn, 200 mg/kg of I, 120 mg/kg of Co, 6500 mg/kg of Mn, 4450 mg/kg of Fe, 1600 mg/kg of Cu, 800,000 IU/kg of vitamin A, 245,000 IU/kg of vitamin D, and 7500 IU/kg of vitamin E.

[6]Calculated using published values of feed ingredients (NRC, 1989).

mM sodium bicarbonate, 28 uM 2-mercapto-ethanol, and 1% antibiotic-antimycotic solution (Gibco BRL). The number of viable cells was determined by trypan blue exclusion using a hemacytometer.

Monocytes were isolated by adherence as described by Wahl and Smith (1992) with some modifications. The PBMC were layered on tissue plate previously treated with heat-inactivated fetal bovine serum (**FBS**; Gibco BRL) for 1 h at 37°C. Monocytes were allowed to adhere for 1 h at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were removed, and adherent cells (monocytes) were rinsed with warm Hank's balanced salt solution and then incubated in RPMI 1640 containing 0.01M EDTA for 20 min at 37°C in 5% CO_2 atmosphere. The plate was shaken, and the monocytes were removed, washed twice in Hank's balanced salt solution, and suspended in RPMI 1640 for PGE_2 and TNF-α production or in Dulbecco's modified Eagle medium F-12 (Sigma) for nitric oxide production. The number of via-

ble monocytes was determined by trypan blue exclusion using a hemacytometer. The purity of monocytes was 70%, and the verification of purity of monocytes was done by flow cytometry.

Proliferative Response of Peripheral Blood Mononuclear Cells

Isolated PBMC were diluted at 2.5×10^6 cells/mL and were plated in triplicate into 96-well flat-bottom microtiter plates (Becton Dickinson and Cie) to determine the proliferative response to concanavalin A (**ConA**; Sigma). Lymphocyte blastogenenis assays were performed as described by Schultz and Adams (1978) with some modifications. Briefly, 50 μL of each PBMC suspension were mixed with 100 μL of RPMI 1640 supplemented with ConA and either 5% AS or 5% FBS (8.3 $\times 10^5$ cells/mL in the final culture). In culture supplemented with AS, final concentrations of 0.125, 0.5, and

Table 2. Ingredient and chemical composition of the post-calving diets (DM basis, except DM).[1,2]

Item	FLA	MEG	SOY	SEM
Ingredient				
Corn silage	16.9	19.7	17.6	
Grass silage	21.0	23.8	21.6	
Megalac	0	4.7	0	
Micronized soybean[3]	0	0	20.3	
Whole flaxseed	9.7	0	0	
Protein supplement[4]	2.2	4.7	0	
Corn grain	37.0	33.0	37.6	
Soybean meal	10.3	12.0	0	
Mineral and vitamin premix	2.9[5]	2.1[6]	2.9[5]	
Chemical				
DM, %	45.7	42.8	44.3	1.8
CP, %	15.4	15.5	15.3	0.2
Ether extract, % of DM	5.7[a]	4.7[b]	5.8[a]	0.2
NDF, % of DM	35.5	37.3	37.8	0.9
ADF, % of DM	20.5	21.4	20.9	0.8
NE_L,[7] Mcal/kg	1.72	1.72	1.72	
Fatty acids, % of total fatty acids				
C12:0	0[b]	0.9[a]	0[b]	0.1
C14:0	0[c]	1.8[a]	0.5[b]	0.1
C16:0	10.1[b]	33.2[a]	13.0[b]	1.3
C16:1	0[c]	0.32[a]	0.24[b]	0.01
C18:0	2.6[b]	3.3[a]	3.1[a]	0.1
C18:1	16.4[c]	27.3[a]	23.2[b]	0.4
C18:2	27.1[b]	25.7[b]	47.8[a]	1.6
C18:3	43.7[a]	7.3[b]	12.3[b]	1.5

[a-c]Means within a row with a different superscript differ at $P < 0.05$.

[1]Least square means with pooled standard error of the mean (SEM); mean of nine monthly samples that were prepared by compositing weekly samples.

[2]FLA = fat supplement based on whole flaxseed, MEG = fat supplement based on Megalac, and SOY = fat supplement based on micronized soybeans.

[3]MICRO-SOYA Elite, 40% CP (Semences Prograin Inc. St-Césaire, QC, Canada).

[4]Contained 52.6% CP, 8.5% ADF, and 13.5% NDF.

[5]Contained 8.8% Ca, 12.0% P, 4.5% Mg, 14.5% Na, 2.1% S, 1.5% K, 14 mg/kg of Se, 2415 mg/kg of Zn, 65 mg/kg of I, 35 mg/kg of Co, 2179 mg/kg of Mn, 533 mg/kg of Cu, 2255 mg/kg of Fe, 380,400 IU/kg of vitamin A, 77,700 IU/kg of vitamin D, and 1800 IU/kg of vitamin E.

[6]Contained 9.0% Mg, 20.5% Na, 5.0% S, 4.2% K, 31.5% Cl, 40 mg/kg of Se, 7500 mg/kg of Zn, 200 mg/kg of I, 120 mg/kg of Co, 6450 mg/kg of Mn, 1600 mg/kg of Cu, 270 mg/kg of Fe, 800,000 IU/kg of vitamin A, 245,000 IU/kg of vitamin D, and 3250 IU/kg of vitamin E.

[7]Calculated using published values of feed ingredients (NRC, 1989).

1 μg/mL (optimal concentration) of ConA were used to activate the cells, whereas 0.06, 0.125, and 0.5 μg/mL (optimal concentration) of ConA were used for cells incubated with FBS. These concentrations were established from preliminary assays that were performed to determine sub-optimal and optimal concentrations of ConA. Controls without ConA were always included for each sample. Plates were incubated at 37°C in 5% CO_2 air for 72 h, and 50 μL of 5-bromo-2-deoxyuridine solution were added to the cells as described in the 5-bromo-2-deoxyuridine assay kit (Roche Diagnostic, Laval, QC, Canada). The cells were then incubated for another 16 h. The quantification of cell proliferation was based on the measurement of 5-bromo-2-deoxyuridine incorporation during DNA synthesis using an anti-5-bromo-2-deoxyuridine conjugate with peroxydase as described in the kit. Absorbance values, which directly correlated with the amount of DNA synthesis and the proliferative response of PBMC, were read on a Spectra Max 250 ELISA reader (Molecular Devices, Sunnyvale, CA) at 370 nm (reference wavelength: 492 nm). The values were expressed as optical density units.

Production of IFN-γ

For analysis of IFN-γ in supernatants, PBMC were adjusted at 1×10^7 cells/mL and were cultured in RPMI 1640 supplemented with ConA (5 μg/mL) and either 5% AS or FBS in 24-well microplates (Corning, New York, MA) for 24 h at 37°C in a 5% CO_2 air (Ishikawa et al., 1994). Culture supernatants were collected after centrifugation and stored at –80°C until assayed. Bovine IFN-γ was measured using an ELISA kit (Veterinary Infectious Diseases Organization, Saskatoon, SK, Canada). The assay was performed as previously described (Baca-Estrada et al., 1995). Briefly, MaxiSorp-

plates (96-well flat-bottom; Canadian Life, Toronto, ON, Canada) were coated with capture antibody (1/4000; Mab 2-2-1) at 4°C for 18 h in carbonate buffer (pH 9.6). After each incubation, the plates were washed 6 times with Tris-buffered saline plus 0.05% Tween 20. Two-fold serial dilutions of recombinant bovine IFN-γ (rBoIFN-γ; Lot# BoG015124) were prepared and used as standard. Samples and standard were diluted in Tris-buffered saline plus 0.05% Tween 20 plus 0.5% gelatine and incubated for 2 h at room temperature. Detection antibody (1/5000; lot # 92–131), biotinylated goat anti-rabbit IgG (H+L) (1/10,000; Sigma), and strep-tavidin-alkaline phosphatase (1/3000; Cederlane Laboratories Ltd, Hornby, ON, Canada) were diluted in Tris-buffered saline plus 0.05% Tween 20 and 0.5% gelatine and incubated for 1 h at room temperature. P-nitrophenyl phosphate alkaline phosphatase substrate (Sigma) was added in each well for color development, and plates were read on a Spectra Max 250 ELISA reader at 405 nm (reference wavelength: 490 nm) after 30 min. The IFN-γ concentration was expressed in pg/mL. Intra- and interassay coefficients of variation were 8 and 10%, respectively.

Production of PGE$_2$ and TNF-α

Concentrations of PGE$_2$ and TNF-α were measured in supernatants of activated monocytes. Briefly, 1×10^6 cells/mL were primed with 100 U/mL of recombinant bovine IFN-γ (generously supplied by Novartis, Basel, Switzerland) in RPMI 1640 supplemented with 5% FBS or AS and incubated for 18 h in 24-well microplates at 37°C in 5% CO$_2$ air. After this incubation period, the plates were centrifuged, and the cells were resuspended in Hank's balanced salt solution without Ca^{2+} and Mg^{2+}, washed 2 more times, and finally resuspended in fresh RPMI-1640 supplemented with 5% AS or FBS and 1 μg/mL lipopolysaccharide (**LPS**) (*Escherichia coli* 055:B5; Sigma). The cells were then incubated for an additional 6 h. Culture supernatants were collected after centrifugation and stored at –80°C until assayed for PGE$_2$ and TNF-α. Prostaglandin E$_2$ was measured by radioimmunoassay as described previously (Jaffe and Behrman, 1974). An anti-PGE$_2$-BSA antibody (ICN Biomedicals Inc., Aurora, OH) was used in the radioimmunoassay. Intra- and interassay coefficients of variation were 8 and 12%, respectively.

An ELISA kit provided by the Veterinary Infectious Diseases Organization was used to measure TNF-α. The assay was performed according to the protocol developed at Veterinary Infectious Diseases Organization and described previously in the IFN-γ production section with some modifications. Briefly, capture antibody (1D 11–13) was diluted 1/1000 in carbonate-coating

buffer. Detection antibody (1/1500; Pool-88) was diluted in Tris-buffered saline plus 0.05% Tween 20 and 0.5% gelatine. Internal standards consisting of serially diluted rBoTNF-α (Lot# NCBOT1024751/36) were prepared in Tris-buffered saline plus 0.05% Tween 20 and 0.5% gelatine as samples. Plates were read at 405 nm (reference wavelength: 495 nm) until 30 min. Concentrations of TNF-α were expressed in ng/mL. Intra- and interassay coefficients of variation were 5 and 10%, respectively.

Production of Nitric Oxide

Production of nitric oxide was evaluated by measuring its more stable metabolites, nitrites, and nitrates (Boulanger et al., 2001). Monocytes (1×10^6 cells/mL) were primed 18 h with rBoIFN-γ 100 U/mL at 37°C in 5% CO$_2$ air, in Dulbecco's modified Eagle medium F-12 without serum because it contains nitrates that interfere with the assay. The pellet was washed twice, resuspended in Dulbecco's modified Eagle medium F-12, and stimulated with 1 μg/mL LPS for another 52 h. Supernatants were collected and stored at –20°C until assayed. Nitrites plus nitrates concentration was measured according to the procedure based on Greiss reaction described by Boulanger et al. (2001).

Determination of Plasma Progesterone

Plasma on wk 1, 3, and wk 6 after calving were separated from peripheral blood by centrifugation and stored frozen (–20°C) until time of assay. Plasma concentrations of progesterone were measured by radioimmunoassay as described by Guilbault et al. (1988). Intra- and interassay coefficients of variation were 8 and 10%, respectively.

Determination of Plasma Fatty Acids

Concentrations of fatty acids in plasma on wk 2 before calving, at calving, and on wk 6 after calving were measured after their extraction using the procedures outlined by Delbecchi et al. (2001), and fatty acids methyl esters were prepared as described by Folch et al. (1957). Methyl ester profiles of fatty acids were measured by GLC on a Hewlett-Packard 6890 chromatograph (Hewlett-Packard Ltd., Montreal, QC, Canada) with a G1315A autosampler equipped with a flame ionization detector and a split-splitless injector as described by Delbecchi et al. (2001).

OVA Antibody Detection

Blood samples were taken on wk 6, 4, 3, 2, and 1 before calving and on wk 1 after calving for OVA anti-

Table 3. Dry matter intake and fatty acid composition of plasma of transition cows fed a total mixed diet containing whole flaxseed (FLA), Megalac (MEG), or micronized soybeans (SOY).[1]

	Diet			
	FLA	MEG	SOY	SEM
DMI, kg/d	16.6[a]	14.7[b]	16.1[ab]	0.6
Fatty acid, % of total fatty acids				
C14:0	1.4[a]	1.3[ab]	1.1[b]	0.1
C16:0	14.3[b]	17.9[ab]	14.0[b]	0.5
C16:1	1.9[a]	1.7[a]	1.4[b]	0.1
C18:0	17.0[a]	14.3[b]	15.2[ab]	0.7
C18:1t9	0.8[b]	0.9a[b]	1.1[a]	0.1
C18:1c9	12.4[a]	13.8[a]	9.7[b]	0.6
C18:1c11	0.59[a]	0.59[a]	0.40[b]	0.04
C18:2	35.3[b]	41.2[a]	45.0[a]	1.6
C18:3n3	7.6[a]	3.8[b]	4.0[b]	0.3
C20:3n6	1.5	1.5	1.4	0.1
C20:4	1.5	1.7	1.5	0.1
C20:5n3	0.6[a]	0.3[b]	0.3[b]	0.1
omega-6/omega-3	4.9[b]	11.1[a]	11.4[a]	0.4

[a,b]Means within a row with different superscripts differ at $P < 0.05$.

[1]Least square means with pooled standard error of the mean (SEM).

bodies detection. Serum was separated from coagulated peripheral blood by centrifugation and stored at $-20°C$ until time of assay. Colostrum was collected at first milking, centrifuged for 15 min at $300 \times g$ to remove the fat, and stored ($-20°C$) until assayed. Antibody to OVA was detected by indirect ELISA and quantified based on optical density measurements according to the procedure described by Lessard et al. (2003) with some modifications. Briefly, MaxiSorp-plates (96-well flat-bottom) were coated with OVA grade V (2.5 $\mu g/mL$; Sigma) in carbonate buffer (pH 9.6) at 4°C for 18 h. After 5 washings with Tris-buffered saline plus 0.05% Tween 20, the plates were blocked with Tris-buffered saline plus 0.05% Tween 20 for 2 h at 37°C. Two-fold serial dilutions (1/1000 down to 1/128,000) of positive cow serum were prepared and used as standard (relative range: 1000 to 7.8 U). Cow serum (1/2000) and colostrum (1/1000) were diluted in Tris-buffered saline plus 0.05% Tween 20 and incubated with agitation for 2 h at room temperature. Five washing with Tris-buffered saline plus 0.05% Tween 20 were done to remove excess unbound antibodies, and anti-OVA were detected with rabbit anti-bovine IgG (whole molecule)-alkaline phosphatase conjugate (1/10,000 working dilution in Tris-buffered saline plus 0.05% Tween 20; Sigma). The plates were incubated with agitation for another 2 h at room temperature and washed 5 times with Tris-buffered saline plus 0.05% Tween 20. The P-nitrophenyl phosphate alkaline phosphatase substrate was added to each well for color development, and plates were read on a Spectra Max 250 ELISA reader at 405 nm (reference wavelength: 630 nm) after 30 min. Pooled cow serum was chosen as an interassay standard. Intra-

and interassay coefficients of variation were 5 and 12%, respectively.

Statistical Analysis

Data were assessed for normality, and most data were transformed prior to statistical analysis (IFN-γ, PGE$_2$, OVA, progesterone, and TNF-α). Data on nitric oxide production and on proliferative response of lymphocytes are presented as stimulated minus non-stimulated values. The data were analyzed using the general linear model procedure of the SAS (SAS, 2000). Tukey multiple-comparison test was applied to separate means. The statistical models included the following main effects and interactions: block, diet, week (week relative to calving), parity, diet by week, diet by parity, and parity by week. The diet effect was calculated with the error cow (diet). Probability values > 0.10 were considered nonsignificant. All data in figures and tables are presented as least squares means ± SEM. In this study, all multiparous cows that were used calved during the winter, whereas the primiparous cows calved the following fall. Therefore, both parity and season effects were confounded in our statistical model. However, based on previous studies, parity number more likely affects immune parameters than season (Gilbert et al., 1993; Mehrzad et al., 2002). For this reason, parity effect was used as the main effect in our statistical model and in the interpretation of results.

RESULTS

Intake of DM and Fatty Acids in Plasma

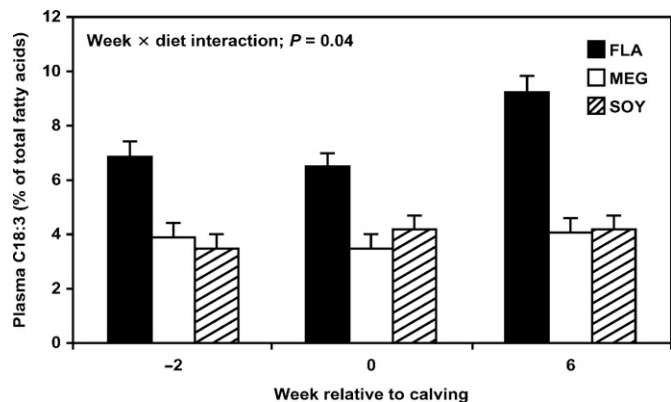Intake of DM in the first 6 wk postcalving, expressed in kg/d, was significantly lower for cows fed MEG than

Figure 1. Plasma C18:3 concentration (% of total fatty acids) on wk −2, 0, and 6 relative to calving in dairy cows fed flaxseed (FLA), micronized soybeans (SOY), or Megalac (MEG). Each bar represents least square means ± SEM (n = 16).

for cows fed FLA, and DMI of cows fed SOY was similar to that of cows fed either MEG or FLA (Table 3). Concentration of C18:3 in plasma was affected by dietary treatment. A significant interaction between diet and week (P = 0.04; Figure 1) showed that concentration of plasma C18:3 changed over time for cows fed FLA while it remained similar for those fed MEG or SOY, which would result from the greater C18:3 concentration in the FLA-based diet than in those based on MEG or SOY (Table 2). Relative amount of eicosapentaenoic acid (C20:5n-3) and n-6 to n-3 ratio were, respectively, higher and lower for cows fed FLA than for cows fed MEG or SOY.

Plasma Progesterone Concentration After Calving

There was no interaction between diet and week and between parity and week for plasma progesterone concentration in the early post-calving period, and diet and parity had no effect (data not shown). Plasma progesterone concentration increased significantly from wk 1 to 6 after calving in all cows regardless of dietary treatments. On average, plasma progesterone concentration ranged between 48.75 pg/mL (CI = [31.78, 78.09]) and 1000 pg/mL (CI = [635.92, 1572.53]) from wk 1 to 6. CI = confidence interval.

OVA Antibody Production in Serum During Transition

Antibody production against OVA in primiparous (Figure 2A) and multiparous (Figure 2B) cows was not affected by composition of n-3 and n-6 PUFA in the diet. There was a significant week effect in the antibody response to OVA in both groups of cows as a result of
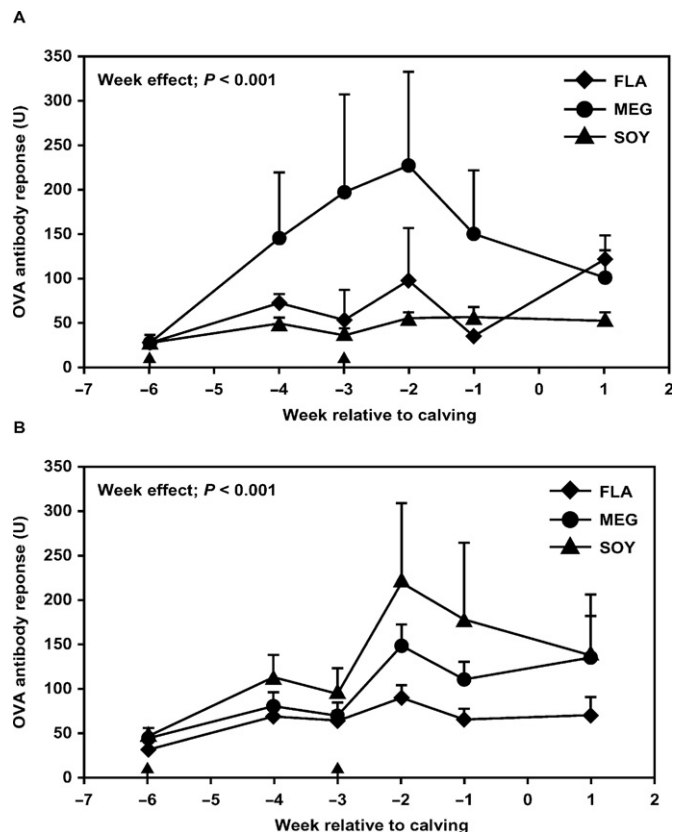

Figure 2. Antibody response against ovalbumin (OVA) in A) primiparous and B) multiparous dairy cows fed flaxseed (FLA), Megalac (MEG), or micronized soybeans (SOY). Cows were injected with OVA on week −6 and −3 (arrows) before parturition (n = 6 and 8 for primiparous and multiparous cows, respectively). Data are represented as least square means ± SEM.

an increase of IgG level after the first and second injection of OVA. Moreover, the level of antibody against OVA in serum was significantly higher in multiparous cows than in primiparous cows, which averaged 69.18 optical density units (CI = [56.39, 84.88]) and 48.75 optical density units (CI = [38.14, 55.13]), respectively.

OVA Antibody Level in Colostrum

There was a significant interaction between parity and diet for the level of antibody against OVA in the colostrum of cows (Figure 3). The level of antibody against OVA was significantly higher in the colostrum of multiparous cows fed SOY than in the colostrum of those fed MEG or FLA, whereas there was no difference among diets between primiparous cows.

Lymphocyte Proliferation to ConA During Transition

Isolated PBMC from cows fed SOY showed a lower proliferative response than isolated PBMC from cows
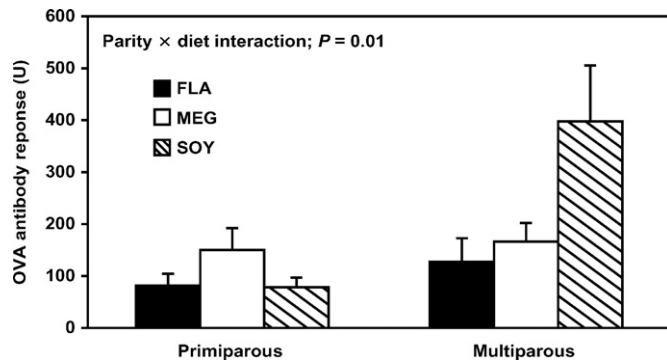
Figure 3. Level of antibody against ovalbumin (OVA) in colostrum of multiparous and primiparous dairy cows fed flaxseed (FLA), Megalac (MEG), or micronized soybeans (SOY). Each bar represents least square means ± SEM (n = 6 and 8 for primiparous and multiparous cows, respectively).

fed FLA or MEG when the cells were stimulated with sub-optimal concentration of ConA and incubated with AS (Figure 4A). Moreover, the proliferative response of multiparous PBMC stimulated with sub-optimal concentration of ConA and supplemented with AS was significantly higher than the proliferative response of primiparous PBMC. There were no interactions between diet and week, parity and diet, or parity and week on lymphocyte responses to ConA stimulations in AS-supplemented cultures. However, there was a significant week effect, which would indicate that the proliferative response of PBMC stimulated with ConA (0.125 μg/mL) and supplemented with AS was significantly higher on wk 6 postcalving than on wk 6 before calving and on wk 1 after calving (Figure 4B). Moreover, the proliferative response of lymphocyte was significantly higher in multiparous cows than in primiparous cows. Similar week and parity effects were observed when PBMC were stimulated with other concentrations of ConA and incubated in presence of either AS or FBS (data not shown).

There was a significant parity by week interaction in the PBMC proliferative response to ConA (0.125 μg/mL) when FBS was added to cultures (Figure 4C). Similar results were obtained at 0.06 μg/mL of ConA in cultures (data not shown). These results would suggest that the proliferative response of multiparous PBMC was lower on wk 1 before calving than on wk 3 before and after calving, while there was no difference among weeks in primiparous cows. No interaction between parity and week was observed when PBMC were stimulated with 0.5 μg/mL of ConA (data not shown).

In Vitro Productions of IFN-γ, TNF-α, PGE$_2$, and Nitric Oxide

There was a significant interaction between parity and diet for IFN-γ production by ConA-activated PBMC
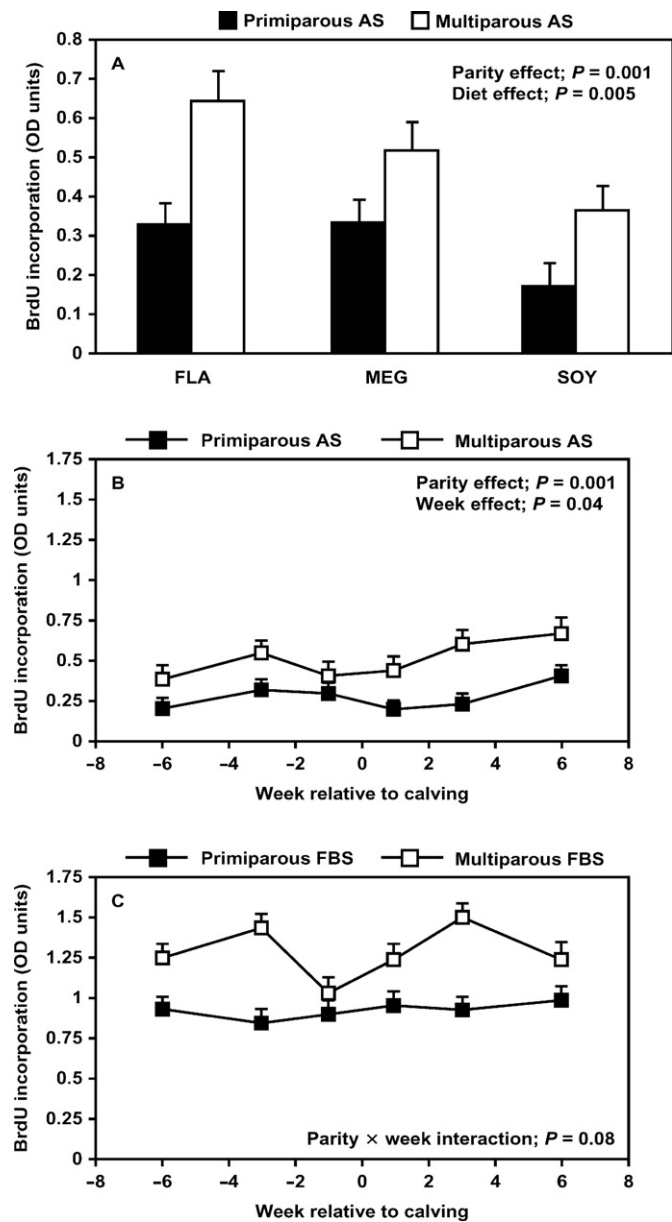

Figure 4. Proliferative response of peripheral blood mononuclear cells (PBMC) to concanavalin A (ConA) in transition dairy cows fed flaxseed (FLA), Megalac (MEG), or micronized soybeans (SOY). A) Effect of dietary fatty acids on proliferative response of PBMC. Cells were activated with suboptimal concentration of ConA (0.06 μg/mL) and supplemented with 5% of autologous serum (AS) (n = 7 and 9 for primiparous and multiparous cows, respectively). Each bar represents the least square means ± SEM. Week effect on proliferative response of PBMC: cells were activated with ConA (0.125 μg/mL) and supplemented with 5% of (B) AS or (C) fetal bovine serum (FBS) (n = 7 and 9 for primiparous and multiparous cows, respectively). Incorporation of 5-bromo-2-deoxyuridine was determined by ELISA and quantified based on optical density measurement. Data are represented as least square means ± SEM.
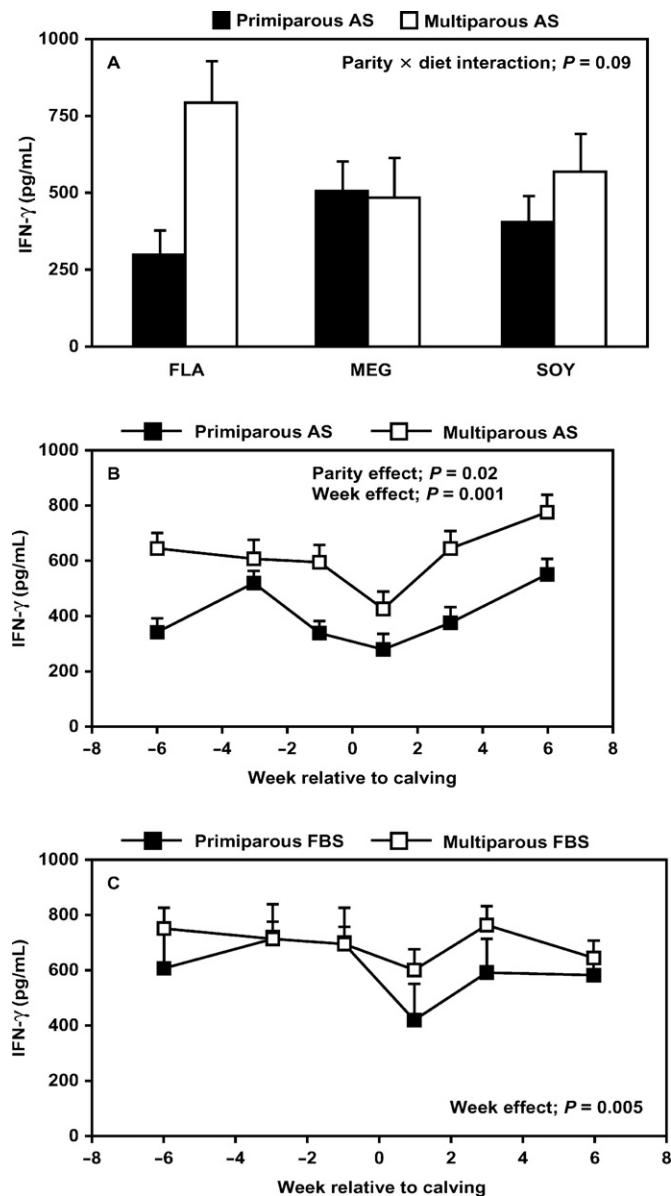
Figure 5. In vitro production of interferon-γ (IFN-γ) by activated peripheral blood mononuclear cells (PBMC) in transition dairy cows. A) Effect of dietary fatty acids on IFN-γ production: cells were cultured in Roswell Park Memorial Institute 1640 supplemented with 5 μg/mL of concanavalin A and 5% of autologous serum (AS). Week effect on IFN-γ production of PBMC: cells were activated with ConA (5 μg/mL) and supplemented with 5% of (B) AS or (C) fetal bovine serum (FBS) for 24 h (n = 7 and 9 for primiparous and multiparous cows, respectively). Data are represented as least square means ± SEM.

when AS was added to cell cultures (Figure 5A). In vitro production of IFN-γ was significantly higher in multiparous cows fed FLA than in primiparous cows fed FLA. Moreover, the production of IFN-γ by PBMC incubated with AS was significantly reduced on wk 1 after calving compared with those measured on wk 3 before calving and on wk 3 and 6 postcalving (Figure
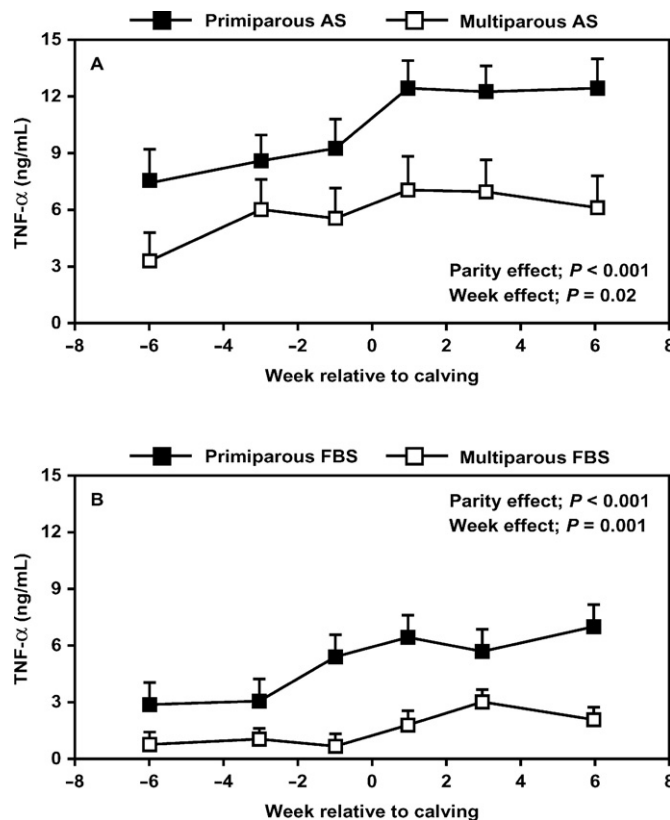

Figure 6. In vitro production of tumor necrosis factor-α (TNF-α) by activated monocytes in transition dairy cows. Cells were cultured in Roswell Park Memorial Institute 1640 primed 18 h with 100 U/mL of recombinant bovine interferon-γ and stimulated with 1 μg/mL of lipopolysaccharide and 5% of (A) autologous serum (AS) or (B) fetal bovine serum (FBS) for 6 h (n = 7 and 9 for primiparous and multiparous cows, respectively). Data are represented as least square means ± SEM.

5B). There was no interaction between main effects for IFN-γ production of ConA-activated PBMC when FBS was added to cell cultures (Figure 5C). However, in vitro production of IFN-γ was significantly impaired on wk 1 after calving compared with that measured on wk 6 and 3 before and after calving.

Dietary treatments had no effect on TNF-α production by enriched monocyte cultures activated with LPS and incubated with either AS or FBS. No interactions between diet and week and between parity and week were observed for TNF-α production by monocytes for all culture conditions. However, on average, the production of TNF-α by enriched monocyte cultures stimulated with LPS and incubated with AS (Figure 6A) or FBS (Figure 6B) was greater in primiparous cows than in multiparous cows. Moreover, the production of TNF-α by LPS-activated monocytes incubated with AS was greater on wk 3 and 6 postcalving than that measured on wk 6 before calving (Figure 6A). Similarly, increased
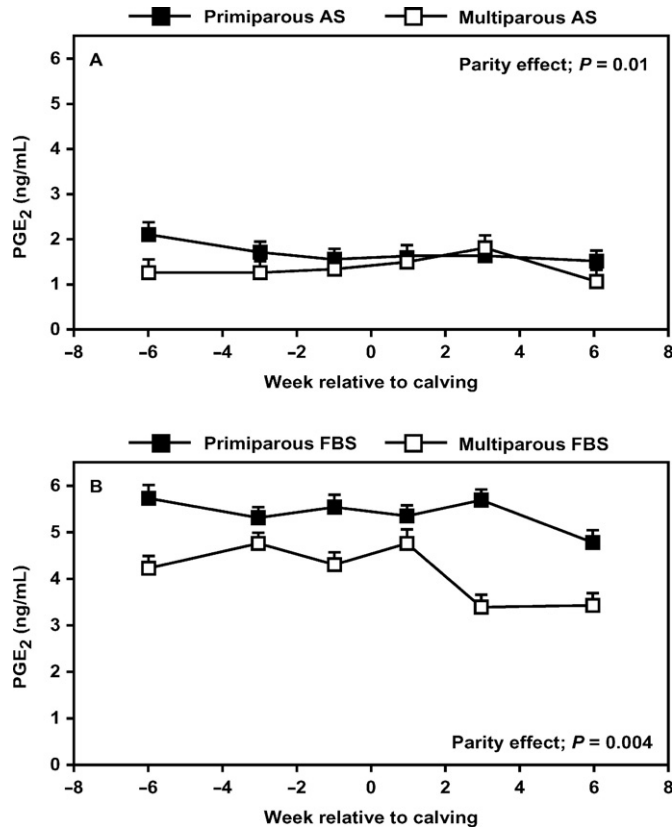
Figure 8. In vitro production of nitrite plus nitrate (NOx) by activated monocytes in transition dairy cows. Cells were cultured in Dulbecco's modified Eagle medium F-12 primed 18 h with 100 U/mL of recombinant bovine interferon-γ and stimulated with 1 μg/mL of lipopolysaccharide for 52 h (n = 7 and 9 for primiparous and multiparous cows, respectively). Data are represented as least square means ± SEM.

Figure 7. In vitro production of prostaglandin E$_2$ (PGE$_2$) by activated monocytes in transition dairy cows. Cells were cultured in Roswell Park Memorial Institute 1640 primed 18 h with 100 U/mL of recombinant bovine interferon-γ and stimulated with 1 μg/mL of lipopolysaccharide and 5% of (A) autologous serum (AS) or (B) fetal bovine serum (FBS) for 6 h (n = 7 and 9 for primiparous and multiparous cows, respectively). Data are represented as least square means ± SEM.

DISCUSSION

In this study, primiparous and multiparous dairy cows were fed diets enriched in n-3 or n-6 fatty acids during the transition period to evaluate the influence of dietary PUFA and parturition on humoral and cellular immunity. Results showed that parturition affects the functional immune properties of PBMC of cows during the transition period. A marked decrease of PBMC lymphocyte response to ConA mitogenic stimulation was observed around parturition and during early lactation in both primiparous and multiparous cows. These results are in accordance with previous reports indicating that the transitional period from parturition to lactation is immunosupressive and a reduction in the lymphocyte response to mitogenic stimulation occurs in the 1st wk postpartum (Kehrli et al., 1989; Lessard et al., 2003). Our results also indicated that factors present in cow serum contribute to reduce the proliferative response after calving in primiparous and multiparous cows. In multiparous cows, the presence of serum factors did not seem to be required to suppress proliferation, as the proliferative response of PBMC was also impaired when lymphocytes were incubated with FBS. These results suggest that the functional properties of PBMC from multiparous cows were more affected or sensitive to the influence of parturition than those of PBMC from primiparous cows. These results are in agreement with previous results that showed a more severe impairment of neutrophil functions in multiparous cows (>3 parities) than in younger cows (Gilbert et al., 1993; Mehrzad et al., 2002) and a suppressive effect of parturition on lymphocyte response to mitogenic stimulation (Kashiwazaki et al., 1985).

production of TNF-α by monocytes was observed after calving compared with wk 6 before parturition (Figure 6B) when the cells were incubated in the presence of FBS.

The PGE$_2$ production of LPS-activated monocytes incubated with either AS or FBS was similar among diets and weeks. However, in vitro production of PGE$_2$ was significantly higher in primiparous cows than in multiparous cows when LPS-activated monocytes were incubated either with AS (Figure 7A) or FBS (Figure 7B). There was no interaction between main effects for in vitro production of nitric oxide by activated monocytes. Production of nitric oxide was not affected by dietary treatments, but production was greater in primiparous cows than in multiparous cows (Figure 8). In vitro production of nitric oxide was significantly greater on wk 1 after calving than on wk 6 before calving.
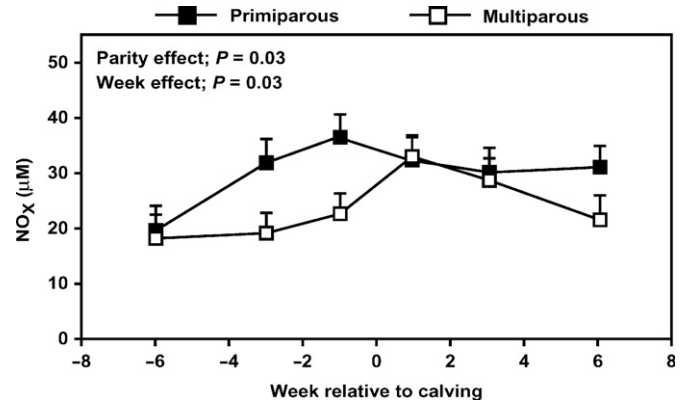
The proliferative response of PBMC isolated from cows fed SOY and stimulated with sub-optimal concentration of ConA was reduced compared with that of PBMC from cows fed MEG or FLA when the cells were incubated with AS. No similar diet effects were observed when the cells were incubated with FBS, indicating that impaired lymphocyte proliferative response in cows fed SOY was likely due to factors that are present in cow serum. Feeding cows with FLA, SOY, or MEG affected composition of plasma fatty acids. Plasma concentrations of n-3 fatty acids increased in cows fed FLA, which resulted in a marked reduction in the n-6 to n-3 fatty acids ratio in cows fed FLA compared with those fed SOY or MEG as previously reported by Lessard et al. (2003). Similarly, Petit (2002) reported that dairy cows fed FLA have a n-6 to n-3 fatty acids ratio that is 3 times lower than that of cows fed either MEG or SOY. Changes in composition of plasma fatty acids may be responsible for the dietary effect on lymphocyte response to mitogens. Differences in blood composition of n-3 and n-6 PUFA may influence production of mediators such as leukotrienes and prostaglandins, which are known to be involved in the regulation of cytokine production and consequently in the response of immune cells to stimuli. Fatty acids are precursors of PG, and n-3 and n-6 fatty acids lead, respectively, to the synthesis of series 3 and series 2 PG (Yaqoob and Calder, 1995). Moreover, many effects mediated by PUFA on immune cells appear to be exerted in an eicosanoid-independent manner. There is now evidence that n-3 and n-6 PUFA affect immune cell functions by regulating the expression of key genes encoding for molecules involved in the signal transduction pathway such as nuclear transcription factor-κ B and peroxisome proliferator-activated receptors (Calder et al., 2002). The regulation of these metabolic pathways may also be affected by parturition and early lactation, as physiological status has been shown to influence hormonal response, nutrient use, and fatty acids metabolism (Abayasekara and Wathes, 1999; Holtenius et al., 2003). Further studies are needed to determine the influence of parturition and early lactation on the metabolism of PUFA and their effects on regulation of immune cells functions.

In the present study, lymphocyte proliferative response was not impaired in cows fed FLA. This result contrasts with those reporting that feeding oils rich in α-linolenic acid (n-3 PUFA) decreased lymphocyte proliferation in rats (Albers et al., 2002; Calder et al., 2002). However, controversy exists in the literature concerning the effects of n-6 and n-3 fatty acids on immune response (Calder et al., 2002). For instance, supplementation of healthy human diet with linseed oil providing 2 g C18:3n-3/d did not affect lymphocyte proliferation

and production of TNF-α, interleukin-6, and IFN-γ by PBMC (Thies et al., 2001a, b). Discrepancies among experiments could be due to difference between species, amount and source of n-3 PUFA added to diets, and physiological status. The present results contrast also with those previously reported in a study using similar diets (Lessard et al., 2003), where it was observed that 5 d after calving, lymphocyte response to ConA was impaired in cows fed FLA compared with those fed MEG or SOY. Cows began to receive the experimental diets after calving and were fully fed these diets only by the end of the 1st wk postpartum in the previous experiment (Lessard et al., 2003); in the present experiment cows started to receive the experimental diets 6 wk before the expected day of parturition. Feeding fatty acids for a longer period could then result in different effects on the lymphocyte response of the transition cow.

To characterize the dietary and parturition effects on immunity of primiparous and multiparous cows, production of IFN-γ by PBMC and TNF-α, PGE$_2$, and nitric oxide by enriched monocyte cultures were determined. These factors play an important role in the regulation of both natural and acquired immunity. Previous studies showed that populations of leukocytes in blood change in periparturient cows compared with cows in midlactation, indicating that the percentage of T cells declines while there is a concomitant increase in granulocytes and monocytes (Sordillo et al., 1995). In the present study, productions of IFN-γ and TNF-α were, respectively, impaired and increased in periparturient cows. These results corroborate those previously reported by Ishikawa et al. (1994), Sordillo et al. (1995), and Shafer-Weaver and Sordillo (1997). These results are consistent with the observation that during the periparturient period, a greater proportion of monocytes is capable of producing TNF-α (Sordillo et al., 1995), whereas CD8+ suppressor lymphocytes appear to be activated and contribute to the increase in the expression of interleukin-4 mRNA and to reduce IFN-γ mRNA levels of PBMC isolated from postpartum cows (Shafer-Weaver and Sordillo, 1997). Activation of monocytes was also characterized by an increase in nitric oxide production. These results suggest that the functional properties of monocytes/macrophages lineage were upregulated during the parturition period, while those related to cell-mediated immunity were temporarily suppressed. These effects were observed independently of the source of serum (FBS or AS) used in cell culture. However, PGE$_2$ production by LPS-activated monocytes was not affected by parturition. This result suggests that the activation status of different cell populations after calving may as well be considered as an explanation of the modulation of the immune response after parturition.

However, they contrast with results obtained in a previous study (Lessard et al., 2003) in which a decrease in PGE_2 production by LPS-activated PBMC was observed after parturition. The difference could be due to experimental dietary treatments, cell culture conditions, and parity number of cows, which differed in both studies. In the present experiment, experimental diets were given to cows 6 wk before calving, whereas in the previous study, cows started to receive dietary treatments after calving. Moreover, activation of cells with LPS was performed on enriched monocyte cultures that were primed with IFN-γ, while LPS was added to non-activated PMBC in the previous work.

Dietary treatments had no effect on PGE_2, TNF-α, and nitric oxide productions by LPS-activated monocytes isolated from cows during the transition period. These results contrast with those previously reporting that n-3 PUFA decrease production of pro-inflammatory cytokines and PGE_2 by monocytes and lower level of mRNA for inducible nitric oxide synthetase (Khair-El-Din et al., 1996). However, a number of studies have also failed to demonstrate an effect of n-3 PUFA on proinflammatory cytokines such as TNF-α and interleukin-1 (Calder, 2001) and on production of IFN-γ (Yaqoob et al., 2000). Species differences and metabolic and hormonal changes that occur after calving and in early lactation may account for different dietary effects observed in this study.

The PBMC isolated from primiparous cows fed the FLA diet produced less IFN-γ than those isolated from multiparous cows fed FLA, and PBMC isolated from both primiparous and multiparous cows fed MEG or SOY responded similarly. The decrease in IFN-γ production by PBMC isolated from primiparous cows fed FLA diet are consistent with the results reported by Gallai et al. (1995) in humans and by Wallace et al. (2001) in rodents; those researchers showed a decrease in the production of IFN-γ by lymphocytes after inclusion of fish oil in the diet. However, an inverse effect was observed in multiparous cows. Overall, results showed that functional properties of PBMC in multiparous cows during the periparturient period differ from those observed in primiparous cows, and further studies are needed to characterize the influence of dietary fatty acids on immunity of cows during the transition period.

There were important differences between primiparous and multiparous cows, indicating that ConA or LPS-activated immune cells from primiparous cows produced less IFN-γ and more PGE_2, TNF-α, and nitric oxide than multiparous cows. Although the parity effect was confounded with the season effect, these results support previous studies showing that parity number influences immune response through the depression of neutrophil functions (Gilbert et al., 1993). These differ-

ences between primiparous and multiparous cows for immune cell functions may be related to increased risk of developing several postpartum complications attributable to infectious causes in cows with greater parity number (Curtis et al., 1985; Gröhn et al., 1990).

Feeding precalving diets enriched in n-3 or n-6 PUFA to dairy cows did not affect the antibody response to OVA in either primiparous or multiparous cows. These results corroborate those previously reported in dogs (Wander et al., 1997), rats (Fritsche et al., 1992), and laying hens (Sijben et al., 2002), indicating that n-3 PUFA have no effect on antibody response. Interestingly, antibody transfer was increased in multiparous cows fed the SOY diet compared with that of primiparous and multiparous cows that were fed other dietary treatments. One possible explanation for this effect is that PUFA dietary content may influence secretory function of mammary epithelial cells in multiparous cows. Previous results strongly suggest that n-6 PUFA composition of mammary epithelial cells play an important role in the regulation of secretory activity and that in vitro addition of C20:4 (n-6) to mammary fragments from lipid-depleted rats increases synthesis and secretion of caseins and intracellular transport of prolactine (Ollivier-Bousquet et al., 1997). Therefore, dietary fats may affect composition of fatty acids in mammary epithelial cells and, therefore, modulate the transfer of blood plasma proteins such as immunoglobulins by transcytosis (Burgoyne and Duncan, 1998).

CONCLUSION

In this study, we showed that cellular immune functions of dairy cows are modulated during the transition period as shown by a marked decrease in lymphocyte proliferative response to mitogenic stimulation and production of IFN-γ and an increased production of TNF-α and nitric oxide after parturition. Enrichment of the diet with n-3 or n-6 PUFA during the periparturient period had limited effects on cellular immune functions. However, lymphocyte proliferative response was reduced in cows fed n-6 PUFA-enriched diet compared with cows that received the n-3 PUFA diet throughout the transition period. Results also showed that activated immune cells isolated from primiparous and multiparous cows during the transition period respond differently. In multiparous cows, monocyte functions as measured by productions of TNF-α, PGE_2, and nitric oxide were impaired compared with those of primiparous cows. However, lymphocyte function as measured by production of IFN-γ was increased in multiparous cows fed n-3 PUFA-enriched diet and impaired in primiparous cows fed the same dietary treatment. In conclusion, functional properties of lymphocytes and mono-

cyte/macrophage lineage of dairy cows during the transition period is modulated by parturition and the composition of PUFA in the diet. A better understanding of the influence of parturition and specific dietary fatty acids on immune functions is required to develop nutritional strategies that will improve cow's health after calving and during early lactation.

ACKNOWLEDGMENTS

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