Immune Response of Dairy Cows Fed Diets Enriched in n-3 or n-6 Polyunsaturated Fatty Acids 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 one of three 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 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 \textit{in vitro} productions of interferon-\(\gamma\), tumor necrosis factor-\(\alpha\), 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 than in multiparous cows. A significant diet x parity interaction indicated that colostral antibody level against ovalbumin was significantly higher in multiparous cows fed soybeans than in those fed flaxseed or Megalac while 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 first 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-\(\gamma\) by mononuclear cells incubated with autologous serum in multiparous than in primiparous cows fed flaxseed while there was no difference among cows fed the other diets. Interferon-\(\gamma\) production was reduced around calving while the inverse was observed for productions of nitric oxide and tumor necrosis factor-\(\alpha\). Productions of nitric oxide, prostaglandin E\(_2\), and tumor necrosis
factor-α were greater in primiparous than in multiparous cows. In conclusion, functional properties of lymphocytes and monocyte/macrophage lineage of dairy cows during the transition period is modulated by parturition and the composition of polyunsaturated fatty acids in the diet.

(Key words: dairy cow, immune response, polyunsaturated fatty acids)

Abbreviation key: AA = arachidonic acid, AS = autologous serum, ConA = concanavalin A, BrdU = 5-bromo-2-deoxyuridine, EPA = eicosapentaenoic acid, FA = fatty acids, FBS = fetal bovine serum, FLA = whole flaxseed, IFN-γ = interferon–γ, IL-1 = interleukin-1, IL-6 = interleukin-6, LPS = lipopolysaccharide, MEG = Megalac, NO = nitric oxide, PBMC = peripheral blood mononuclear cells, P4 = progesterone, PG = prostaglandin, PUFA = polyunsaturated fatty acids, 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; Miles and Calder, 1998). Dietary fats rich in n-3 or n-6 PUFA modulate the inflammatory responses in experimental animal models (Pomposelli et al., 1989; Watanabe et al., 1993) and in clinical trials (Stenson et al., 1992; Kremer, 2000). In mice fed enriched n-3 PUFA diet, inflammatory reactions are reduced and different types of antibody response to antigenic stimulations are developed compared to mice fed n-6 enriched diet (Albers et al., 2002; Lin et al., 2000). 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 (NO) (Miles and Calder, 1998; Calder et al., 2002).

Linoleic acid (18:2n6), an n-6 FA, can be converted through enzymatic reactions in arachidonic acid, which is the precursor of the pro-inflammatory mediators, PGE₂ and leukotriene B₄. The same elongase and desaturase can also convert the α-linolenic acid (18:3n3), an n-3 FA, to eicosapentaenoic acid (EPA: 20:5n3), the precursor for the synthesis of PGE₃ and leukotriene B₅ (LTB₅), that leads to less severe inflammatory reactions than PGE₂ and LTB₄ (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 (IL-1) and interleukin-6 (IL-6) (James et al., 2000; Miles and Calder, 1998).

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; Ropstad, 1989; Goff, 1996; Mallard et al., 1998). Development of new feeding strategies in which fatty acids (FA) composition of the diet may prevent immunosuppression after calving should contribute to reduce the incidence of infection and disease. 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 Centre, 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 and there were 9 blocks of multiparous and 7 blocks of primiparous cows. The experiment was carried out from 6 wk before the expected calving date up to wk 6 of lactation. Cows were housed in tie stalls and fed individually. Cows within blocks were assigned randomly to one of three treatments. The three experimental diets (Table 1) consisted of fat supplements (Table 2) 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 were formulated to meet requirements for cows that averaged 625 kg of BW. After calving, diets 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 week 3 before parturition.

Blood Sampling for Peripheral Blood Mononuclear Cells and Monocytes Isolation

Blood samples were taken 6, 3, and 1 wk before the expected calving date and 1, 3, and 6 wk after calving. Blood from the jugular vein was drawn into K₃EDTA-vacuum tube (Becton Dickinson and Cie,
Rutherford, NJ, USA) for leucocytes isolation. At the same time, one blood sample was taken into a vacuum tube with no additive for preparation of heat-inactivated (56°C, 30 min) autologous serum (AS). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient separation. Briefly, blood samples were layered on Ficoll-Hypaque Plus (Amersham Pharmacia, Montreal, QC, Canada) and PBMC were collected at the interface after centrifugation (400 x g for 40 min) and washed twice with Hank’s balanced salt solution (HBSS) without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (wash solution; Gibco BRL, Toronto, ON, Canada). Finally, PBMC were resuspended in RPMI 1640 medium (Gibco BRL) supplemented with 12 mM HEPES, 2 mM glutamine, 23 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 adheration. The cells were layered on tissue plate previously treated with fetal bovine serum (FBS; Gibco BRL) for 1 h at 37°C in a 5% CO\textsubscript{2} atmosphere. Non-adherent cells were removed, and adherent cells (monocytes) were rinsed with warm HBSS and removed from plate by incubating in RPMI 1640 containing 0.01M EDTA for 20 min at 37°C in 5% CO\textsubscript{2} atmosphere. The plate was shaken and the monocytes were removed. Then, they were washed twice in HBSS and suspended in RPMI 1640 for PGE\textsubscript{2} and TNF-\textalpha production or in Dulbecco’s modified Eagle medium F-12 (DMEM-F12; Sigma) for NO production.

**Proliferative Response of Peripheral Blood Mononuclear Cells**

Isolated PBMC were diluted at 2.5 x 10\textsuperscript{6} cells/ml and were plated in triplicate into 96-well flat-bottom microtiter plates (Becton Dickinson and Cie) to determine proliferative response to concanavalin A (ConA; Sigma). Briefly, 50 ul of each PBMC suspension were mixed with 100 ul of RPMI 1640 supplemented with ConA and either 5% AS or 5% FBS. In culture supplemented with AS, final concentrations of 0.125, 0.5, and 1 ug/ml (optimal concentration) of ConA were used to activate the cells
whereas 0.06, 0.125, and 0.5 ug/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% CO2 air for 72 h and 50 ul of 5-bromo-2-deoxyuridine (BrdU) solution was added to the cells as described in the BrdU 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 BrdU incorporation during DNA synthesis using an anti-BrdU 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 (OD) units.

**Production of Interferon-γ**

For analysis of bovine interferon-γ (IFN-γ) in supernatants, PBMC were adjusted at 1 x 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, NY, MA) for 24 h at 37°C in a 5% CO2 air. 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 [VIDO], 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; lot #2-2-1 Mab) 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 (PBST). 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 phosphate buffered saline solution with 0.05% Tween 20 and 0.5% gelatine (PBST-g) and incubated for 2
Production of Prostaglandin E<sub>2</sub> and Tumor Necrosis Factor-<alpha>

Concentrations of PGE<sub>2</sub> and TNF-α were measured in supernatants of activated monocytes. Briefly, 1 x 10<sup>6</sup> cells/ml were primed with 100 U/ml rBoIFN-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<sub>2</sub> air. After this incubation period, the plates were centrifuged and the cells were resuspended in HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup>, washed two 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<sub>2</sub> and TNF-α. Prostaglandin E<sub>2</sub> was measured by radioimmunoassay (RIA) as described previously (Jaffe and Behrman, 1974). An anti-PGE<sub>2</sub>-BSA antibody (ICN Biomedicals Inc., Aurora, OH) was used in the RIA. Intra- and inter-assay coefficients of variation were 8 and 12%, respectively.

An ELISA kit provided by the VIDO was used to measure TNF-α. The assay was performed according to the protocol developed at VIDO and described above in IFN-γ production section with some modifications. Briefly, capture antibody (1D11-13) was diluted 1/1000 in carbonate-coating buffer. Detection antibody (1/1500; Pool-88) was diluted in PBST-g. Internal standards consisting of serially
diluted rBoTNF-α (Lot# NCBOT1024751/36) were prepared in PBST-g 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 inter-assay coefficients of variation were 5% and 10%, respectively.

Production of Nitric Oxide

Production of NO was evaluated by measuring its more stable metabolites, nitrites and nitrates (NOx). Monocytes (1X10^6 cells/ml) were primed 18 h with rBoIFN-γ 100 U/ml at 37°C in 5% CO2 air, in DMEM-F12 without serum because it contains nitrates that interfere with the assay. The pellet was washed twice, resuspended in DMEM-F12 and stimulated with 1 ug/ml LPS for another 52 h. Supernatants were collected and stored at –20°C until assayed. NOx 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 (P4) were measured by RIA as described by Guilbault et al. (1988). Intra- and inter-assay coefficients of variation were 8% and 10%, respectively.

Determination of Plasma Fatty Acids

Concentrations of FA in plasma on wk 1, 3, and wk 6 after calving were measured after their extraction using the procedures outlined by Delbecchi et al. (2001) and the preparation of serum FA methyl esters as described by Folch et al. (1957). Methyl ester profiles of FA were measured by GLC on a Hewlett-Packard 6890 chromatograph (Hewlett-Packard Ltd, Montreal, QC, Canada) with a G1315A
autosampler equipped with a flame ionisation detector and a split-splitless injector as described by Delbecchi et al. (2001).

Ovalbumin Antibody Detection

Serum of wk 6, 4, 3, 2, and 1 before calving and wk 1 after calving 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 x g to remove the fat and stored (-20°C) until assayed. Antibody to ovalbumin (OVA) was detected by indirect ELISA and quantified based on OD measurements. Briefly, MaxiSorp-plates (96-well flat-bottom) were coated with OVA grade V (2.5 ug/ml; Sigma) in carbonate buffer (pH 9.6) at 4°C for 18 h. After five washings with PBST, the plates were blocked with PBST for 2 h at 37°C. Two-fold serial dilutions 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 PBST and incubated with agitation for 2 h at room temperature. Five washing with PBST 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 PBST; Sigma). The plates were incubated with agitation for another 2 h at room temperature and washed five times with PBST. The P-nitrophenyl phosphate alkaline phosphatase substrate was added to each well for colour development and plates were read on a Spectra Max 250 ELISA reader at 405 nm (reference wavelength: 630 nm) after 30 min. Pool of cow serum was chosen to standardize the interassay. Intra- and inter-assay 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, PGE₂, OVA, P₄ and TNF-α). Data on NO production and on proliferative response of lymphocytes are presented as stimulated minus non-stimulated values. The data were analysed using the general linear model procedure of the SAS (SAS, 2000). Tukey-Kramer multiple-comparison test was applied to separate means. The statistical models included the following main effects and interactions: 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 greater than 0.10 were considered non significant. All data are presented as lsmeans ± SEM. In this study, all multiparous cows that were used calved during the winter whereas the primiparous cows calved the following fall. Although both parity and season effects were confounded, we referred to parity effect to describe differences between primiparous and multiparous cows based on previous studies indicating that parity number affects more likely than season immune parameters (Gilbert et al., 1993; Lacetera et al., 2002; Mehrzad et al., 2002).

RESULTS

Fatty Acids in Serum

Concentration of C₁₈:₃ in blood was affected by dietary treatment. A significant interaction between diet and week (P = 0.04; data not shown) showed that concentration of plasma C₁₈:₃ increased significantly in cows fed FLA while it remained similar for those fed MEG or SOY, which would result from the greater C₁₈:₃ concentration in FLA based diet than in those based on MEG or SOY (Table 3). Relative amount of EPA (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 P₄ concentration in the early post-calving period and diet and parity had no effect. Plasma P₄ concentration increased significantly from wk 1 to 6 after calving in all cows regardless of dietary treatments. On average, plasma P₄ concentration ranged between 48.75 pg/ml (IC=[31.78, 78.09]) and 1000 pg/ml (IC=[635.92, 1572.53]) from wk 1 to 6.

Ovalbumin Antibody Production in Serum during Transition

Antibody production against OVA in primiparous (Figure 1A) and multiparous (Figure 1B) 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 an increase of IgG level after the 1ˢᵗ and 2ⁿᵈ injection of OVA. Moreover, the level of antibody against OVA in serum was significantly higher in multiparous than in primiparous cows, which averaged 69.18 OD units (CI=[56.39, 84.88]) and 48.75 OD units (CI=[38.14, 55.13]), respectively.

Ovalbumin 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 2). 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 Concanavalin A during Transition

Isolated PBMC from cows fed SOY showed a lower proliferative response than isolated PBMC from cows fed FLA or MEG when the cells were stimulated with sub-optimal concentration of ConA and incubated with AS (Figure 3A). 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 ug/ml) and supplemented with AS was significantly higher on wk 6 post calving than on wk 6 before calving and on wk 1 after calving (Figure 3B). Moreover, the proliferative response of lymphocyte was significantly higher in multiparous 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 ug/ml) when FBS was added to cultures (Figure 3C). Similar results were obtained at 0.06 ug/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 ug/ml of ConA (data not shown).

In Vitro Productions of Interferon-γ, Tumor Necrosis Factor-α, Prostaglandin E₂, and Nitric Oxide

There was a significant interaction between parity and diet for IFN-γ production by ConA-activated PBMC when AS was added to cell cultures (Figure 4A). In vitro production of IFN-γ was significantly higher in multiparous cows fed FLA compared to primiparous cows fed FLA. Moreover, the production of
IFN-γ by PBMC incubated with AS was significantly reduced on wk 1 after calving compared to those measured on wk 3 before calving and on wk 3 and 6 post calving (Figure 4B). There was no interaction between main effects for IFN-γ production of ConA-activated PBMC when FBS was added to cell cultures (Figure 4C). However, in vitro production of IFN-γ was significantly impaired on wk 1 after calving compared to 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 5A) or FBS (Figure 5B) was greater in primiparous than multiparous cows. Moreover, the production of TNF-α by LPS-activated monocytes incubated with AS was greater on wk 3 and 6 post calving than that measured on wk 6 before calving (Figure 5A). Similarly, increased production of TNF-α by monocytes was observed after calving compared to wk 6 before parturition (Figure 5B) when the cells were incubated in presence of FBS.

PGE₂ production of LPS-activated monocytes incubated with either AS or FBS was similar among diets and weeks. However, in vitro production of PGE₂ was significantly higher in primiparous than in multiparous cows when LPS-activated monocytes were incubated either with AS (Figure 6A) or FBS (Figure 6B). There was no interaction between main effects for in vitro production of NO by activated monocytes. Production of NO was not affected by dietary treatments but production was greater in primiparous than in multiparous cows (Figure 7). In vitro production of NO was significantly greater on wk 1 after calving than on wk 6 before calving.
DISCUSSION

In this study, the influence of parturition and diet containing n-3 and n-6 fatty acids was evaluated on the immune response of primiparous and multiparous cows throughout the transition period. Results showed that both parturition and dietary PUFA affect 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 primiparous and multiparous cows when the cells were incubated with AS. However, when the cells were incubated with FBS, proliferation was suppressed in multiparous cows only. These results are in accordance with previous reports indicating that the transitional period from parturition to lactation is immunosuppressive and a reduction in the lymphocyte response to mitogenic stimulation occurs in the first week postpartum (Wells et al., 1977; Kehrli et al., 1989; Lessard et al., 2003). However, our results would indicate that serum factors present in cow serum contribute to suppress the proliferative response after calving in primiparous and multiparous cows although the proliferative response of PBMC isolated from multiparous cows was impaired independently of the source of serum used in culture when cells were incubated with FBS as shown by the significant parity by week interaction. The effects of parity on the lymphocyte response to mitogenic stimulation due to autologous cow serum and FBS corroborate those previously reported by Kashiwazaki (1985) and suggest that immuno-suppression after calving was more severe in multiparous than in primiparous cows. The difference between multiparous and primiparous cows on the lymphocyte proliferation supports the results showing that neutrophil functions of multiparous cows (more than 3 parities) are more profoundly impaired than those of younger cows (Gilbert et al. 1993; Mehrzad et al. 2002).

The effect of diet on the proliferative response of PBMC isolated from cows fed SOY and stimulated with sub-optimal concentration of ConA was reduced compared to 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 is likely due to factors that are present in cow serum. Feeding cows with FLA, SOY or MEG
affected composition of blood FA. Blood concentrations of n-3 FA increased in cows fed FLA, which
resulted in a marked reduction in the n-6 to n-3 FA ratio in cows fed FLA compared to 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 FA ratio three times lower than that of cows fed either MEG or SOY. Changes in
composition of serum FA 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 FA 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-kappa 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 FA metabolism (Abayasekara and Wathes, 1999; Houdijk et al., 2001; 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 cow fed FLA. This
result contrasts with those reporting that feeding oils rich in alpha-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 FA on immune response (Calder et al., 2002). For instance, supplementation of healthy human diet with linseed oil providing 2 g C18:3n-3 per day did not affect lymphocyte proliferation and production of TNF-α, IL-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 five days after calving, lymphocyte response to ConA was impaired in cows fed FLA compared to 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 first wk postpartum in the previous experiment (Lessard et al., 2003) while cows started to receive the experimental diets six weeks before the expected day of parturition in the present experiment; feeding FA for more a longer period could then result in different effects on the lymphocyte response of the transition cow.

In order to characterize the dietary and parturition effects on immunity of primiparous and multiparous cows, production of IFN-γ by PBMC and TNF-α, PGE₂, and NO by enriched monocyte cultures were determined. These factors play 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; Yang et al., 1997). 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 are capable of producing TNF-α (Sordillo et al. 1995), whereas CD8+ suppressor lymphocytes appear to be activated and contribute to increase the expression of IL-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 NO production. These results suggest that the functional properties of monocytes/macrophages lineage were up-regulated 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 to 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 NO 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 NO synthetase (Khair-El-Din et al., 1996). However, a number of studies has also failed to demonstrate an effect of n-3 PUFA on pro-inflammatory cytokines such as TNF-α and IL-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.

PBMC isolated from primiparous cows fed 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 human and by Wallace et al. (2001) in rodents who showed a decrease in the production of IFN-γ by lymphocytes after inclusion of fish oil in 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 FA 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 PGE₂, TNF-α, and NO 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 differences between primiparous and multiparous cows for immune cells functions may be related to increased risk of developing several postpartum complications due 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 both primiparous and 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 SOY diet compared to that of primiparous and multiparous cows that were on 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 FA 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 interferon-γ and an increased production of tumor-necrosis factor-α and nitric oxide after parturition. Enrichment of the diet with n-3 or n-6 polyunsaturated fatty acids during the periparturient period had limited effects on cellular immune functions. However, lymphocyte proliferative response was reduced in cows fed n-6 polyunsaturated fatty acids enriched diet compared to cows that received the n-3 polyunsaturated fatty acids 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 tumor-necrosis factor-α, prostaglandin E2, and nitric oxide were impaired compared to those of primiparous cows. However, lymphocyte function as measured by production of IFN-γ was increased in multiparous cows fed n-3 polyunsaturated fatty acids enriched diet and impaired in primiparous cows fed the same dietary treatment. In conclusion, functional properties of lymphocytes and monocyte/macrophage lineage of dairy cows during the transition period is modulated by parturition and the composition of polyunsaturated fatty acids 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

The authors would like to thank Liette Veilleux, Sylvie Dallaire, and Marie Dupuis for their excellent technical assistance, Steve Methot for statistics analysis, and the dairy barn staff for animal care and data collection. They are grateful to Veterinary Infectious Diseases Organization (VIDO) (Saskatoon, SK, Canada) for generously supplying bovine IFN-γ and TNF-α ELISA kit. They also are grateful to Novartis (Basel, Switzerland) for generously supplying of the recombinant bovine IFN-γ.

REFERENCES


Figure 1. 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 week -3 (arrow) before parturition (n = 6 and n = 8, for primiparous and multiparous cows, respectively).

Figure 2. Level of antibody against ovalbumin in colostrum of multiparous and primiparous dairy cows fed flaxseed (FLA), Megalac (MEG) or micronized soybeans (SOY). Each bar represents the Lsmeans ± SEM (n = 6 and n = 8, for primiparous and multiparous cows, respectively).

Figure 3. 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 (0.06ug/ml) and supplemented with 5% of autologous serum (AS) (n = 7 and n = 9, for primiparous and multiparous cows, respectively). Each bar represents the means ± SEM.

The week effect on proliferative response of PBMC; cells were activated with ConA (0.125ug/ml) and supplemented with 5% of (B) AS or (C) foetal bovine serum (FBS) (n = 7 and n = 9, for primiparous and multiparous cows, respectively). Incorporation of BrdU was determined by ELISA and quantified based on optical density measurement.

Figure 4. 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 RPMI 1640 supplemented with 5 ug/ml of concanavalin A and 5 % of autologous serum (AS). The
week effect on IFN-γ production of PBMC activated with ConA (5 ug/ml) and supplemented with 5% of (B) AS or (C) foetal bovine serum (FBS) for 24 h (n = 7 and n = 9, for primiparous and multiparous cows, respectively).

**Figure 5.** In vitro production of tumor necrosis factor-alpha (TNF-α) by activated monocytes in transition dairy cows. Cells were cultured in RPMI 1640, primed 18 hours with 100 U/ml of recombinant bovine interferon-γ and stimulated with 1 ug/ml of lipopolysaccharide and 5 % of (A) autologous serum (AS) or (B) foetal bovine serum (FBS) for 6 h (n = 7 and n = 9, for primiparous and multiparous cows, respectively).

**Figure 6.** In vitro production of prostaglandin (PGE₂) by activated monocytes in transition dairy cows. Cells were cultured in RPMI 1640, primed 18 hours with 100 U/ml of recombinant bovine interferon-γ and stimulated with 1 ug/ml of lipopolysaccharide and 5 % of (A) autologous serum (AS) or (B) foetal bovine serum (FBS) for 6 h (n = 7 and n = 9, for primiparous and multiparous cows, respectively).

**Figure 7.** In vitro production of nitrite plus nitrate (NOx) by activated monocytes in transition dairy cows. Cells were cultured in DMEM-F12, primed 18 hours with 100 U/ml of recombinant bovine interferon-γ and stimulated with 1 ug/ml of lipopolysaccharide for 52 h (n = 7 and n = 9, for primiparous and multiparous cows, respectively).
Table 1. Ingredient and chemical composition of experimental diets (DM basis except DM)\(^1,2\)

<table>
<thead>
<tr>
<th>Diets</th>
<th>FLA</th>
<th>MEG</th>
<th>SOY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn silage</td>
<td>13.1</td>
<td>19.5</td>
<td>14.5</td>
</tr>
<tr>
<td>Grass silage</td>
<td>29.0</td>
<td>43.3</td>
<td>32.3</td>
</tr>
<tr>
<td>Megalac(^3)</td>
<td>0</td>
<td>3.8</td>
<td>0</td>
</tr>
<tr>
<td>Micronized soybean(^4)</td>
<td>0</td>
<td>0</td>
<td>17.7</td>
</tr>
<tr>
<td>Whole flaxseed</td>
<td>10.4</td>
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<td>0</td>
</tr>
<tr>
<td>Soybean meal</td>
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<td>0</td>
</tr>
<tr>
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<tr>
<td>Barley</td>
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<td>18.9</td>
<td>32.0</td>
</tr>
<tr>
<td>Mineral and vitamin premix</td>
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<td>3.8(^5)</td>
<td>3.5(^5,6)</td>
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<td></td>
</tr>
<tr>
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<td>NEL, Mcal/kg(^7)</td>
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<td>1.73</td>
<td>1.69</td>
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<tr>
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<td>19.6</td>
<td>17.9</td>
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<tr>
<td>TCA insoluble N, % of N</td>
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<tr>
<td>Ether extract, % of DM</td>
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<tr>
<td>NDF, % of DM</td>
<td>37.8</td>
<td>37.6</td>
<td>38.8</td>
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<tr>
<td>ADF, % of DM</td>
<td>22.2</td>
<td>23.1</td>
<td>21.7</td>
</tr>
<tr>
<td>RUP, % of N(^7)</td>
<td>35</td>
<td>33</td>
<td>39</td>
</tr>
<tr>
<td>NFC, % of DM(^7)</td>
<td>37.3</td>
<td>30.3</td>
<td>35.6</td>
</tr>
<tr>
<td>Fatty acids, % of total fatty acids</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>0</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
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<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>C16:0</td>
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<td>14.4</td>
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<td>0</td>
</tr>
<tr>
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<td>2.7</td>
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</tr>
<tr>
<td>C18:3</td>
<td>43.9</td>
<td>8.3</td>
<td>13.0</td>
</tr>
</tbody>
</table>

\(^1\) Fat supplement based on FLA= whole flaxseed, MEG = Megalac, and SOY= micronized soybeans.
\(^2\) Lsmean of eighteen fortnightly and nine monthly samples that were prepared by compositing weekly samples.
\(^3\) Megalac calcium salts of palm oil, Church and Dwight Co., Inc., Princeton, NJ;
\(^4\) MICRO-SOYA Elite 40% CP, Semences Prograin Inc. St-Césaire, QC J0L 1T0, Canada.
\(^5\) Contained 10.0% Ca, 4.3% P, 4.3% Mg, 13.8% Na, 1.8% S, 1.4% K, 7.5% NaCl, 1760 mg/kg of Zn, 54 mg/kg of I, 32 mg/kg of Co, 1914 mg/kg of Mn, 436 mg/kg of Cu, 2154 mg/kg of Fe, 15 mg/kg of Se, 197,200 IU/kg of vitamin A, 66,600 IU/kg of vitamin D, and 1030 IU/kg of vitamin E.
\(^6\) 250 g of calcium carbonate (38.0% Ca) was fed per cow per day.
\(^7\) Calculated using published values of feed ingredients (NRC, 1989).
<table>
<thead>
<tr>
<th></th>
<th>Diets&lt;sup&gt;1&lt;/sup&gt;</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>FLA</td>
<td>MEG</td>
<td>SOY</td>
</tr>
<tr>
<td>CP, % of DM</td>
<td>25.2</td>
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<tr>
<td>Ether extract, %</td>
<td>31.4</td>
<td>76.4</td>
<td>24.4</td>
</tr>
<tr>
<td>ADF, % of DM</td>
<td>21.7</td>
<td>0</td>
<td>5.6</td>
</tr>
<tr>
<td>NDF, % of DM</td>
<td>34.3</td>
<td>0</td>
<td>ND&lt;sup&gt;2&lt;/sup&gt;</td>
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Fatty acids, % of total fatty acids

<table>
<thead>
<tr>
<th></th>
<th>FLA</th>
<th>MEG</th>
<th>SOY</th>
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<tr>
<td>C10:0</td>
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<td>0.6</td>
<td>0</td>
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<tr>
<td>C12:0</td>
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<td>1.6</td>
<td>0</td>
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<tr>
<td>C14:0</td>
<td>0</td>
<td>6.2</td>
<td>0</td>
</tr>
<tr>
<td>C14:1</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>C16:0</td>
<td>5.2</td>
<td>47.1</td>
<td>11.9</td>
</tr>
<tr>
<td>C16:1</td>
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<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>C18:0</td>
<td>3.4</td>
<td>3.5</td>
<td>3.3</td>
</tr>
<tr>
<td>C18:1</td>
<td>18.5</td>
<td>32.3</td>
<td>20.8</td>
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<td>16.1</td>
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<td>56.9</td>
</tr>
<tr>
<td>C18:3</td>
<td>56.8</td>
<td>0.3</td>
<td>7.1</td>
</tr>
</tbody>
</table>

<sup>1</sup>Fat supplement based on FLA = whole flaxseed, MEG = Megalac, and SOY = micronized soybeans.

<sup>2</sup>ND = not determined.
Table 3. Fatty acid composition of plasma of transition cows fed a total mixed diet containing whole flaxseed (FLA), Megalac (MEG) or micronized soybeans (SOY)\textsuperscript{1}

<table>
<thead>
<tr>
<th>Fatty acid, % of total fatty acids</th>
<th>FLA</th>
<th>MEG</th>
<th>SOY</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>1.4\textsuperscript{a}</td>
<td>1.3\textsuperscript{ab}</td>
<td>1.1\textsuperscript{b}</td>
<td>0.1</td>
</tr>
<tr>
<td>C16:0</td>
<td>14.3\textsuperscript{b}</td>
<td>17.9\textsuperscript{ab}</td>
<td>14.0\textsuperscript{b}</td>
<td>0.5</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.9\textsuperscript{a}</td>
<td>1.7\textsuperscript{a}</td>
<td>1.4\textsuperscript{b}</td>
<td>0.1</td>
</tr>
<tr>
<td>C18:0</td>
<td>17.0\textsuperscript{a}</td>
<td>14.3\textsuperscript{b}</td>
<td>15.2\textsuperscript{ab}</td>
<td>0.7</td>
</tr>
<tr>
<td>C18:1\textsuperscript{t9}</td>
<td>0.8\textsuperscript{b}</td>
<td>0.9\textsuperscript{a}</td>
<td>1.1\textsuperscript{a}</td>
<td>0.1</td>
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<tr>
<td>C18:1\textsuperscript{c9}</td>
<td>12.4\textsuperscript{a}</td>
<td>13.8\textsuperscript{a}</td>
<td>9.7\textsuperscript{b}</td>
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<tr>
<td>C18:1\textsuperscript{c11}</td>
<td>0.59\textsuperscript{a}</td>
<td>0.59\textsuperscript{a}</td>
<td>0.40\textsuperscript{b}</td>
<td>0.04</td>
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<tr>
<td>C18:2</td>
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<td>41.2\textsuperscript{a}</td>
<td>45.0\textsuperscript{a}</td>
<td>1.6</td>
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<tr>
<td>C18:3\textsuperscript{n3}</td>
<td>7.6\textsuperscript{a}</td>
<td>3.8\textsuperscript{b}</td>
<td>4.0\textsuperscript{b}</td>
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<tr>
<td>C20:3\textsuperscript{n6}</td>
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<td>1.5</td>
<td>1.4</td>
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</tr>
<tr>
<td>C20:4</td>
<td>1.5</td>
<td>1.7</td>
<td>1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>C20:5\textsuperscript{n3}</td>
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<td>0.3\textsuperscript{b}</td>
<td>0.3\textsuperscript{b}</td>
<td>0.1</td>
</tr>
<tr>
<td>omega-6/omega-3</td>
<td>4.9\textsuperscript{b}</td>
<td>11.1\textsuperscript{a}</td>
<td>11.4\textsuperscript{a}</td>
<td>0.4</td>
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</tbody>
</table>

\textsuperscript{1}Least squares means with pooled standard error (SE).
\textsuperscript{a,b}Means within diet with a different subscript differs at $P < 0.05$. 

1Least squares means with pooled standard error (SE).
\textsuperscript{a,b}Means within diet with a different subscript differs at $P < 0.05$. 

Fig. 1A

**OVA antibody response (Units)**

- FLA
- MEG
- SOY

**Week relative to calving**

**Week effect: \( P < 0.001 \)**
Fig. 1B

Week effect: $P < 0.001$

- FLA
- MEG
- SOY

Week relative to calving

OVA antibody response (Units)
Fig. 2

Parity x diet interaction: $P = 0.01$
Parity effect: $P = 0.001$
Diet effect: $P = 0.005$

Fig. 3A
Parity effect: $P = 0.001$
Week effect: $P = 0.04$

Fig. 3B
Fig. 3C

Parity x week interaction: $P = 0.08$
Parity x diet interaction: $P = 0.09$

Fig. 4A
Parity effect: $P = 0.02$
Week effect: $P = 0.001$
Fig. 4C

Week effect: $P = 0.005$

- Primiparous FBS
- Multiparous FBS
Fig. 5A

Parity effect: $P < 0.001$
Week effect: $P = 0.02$

Primiparous AS
Multiparous AS

Week relative to calving

TNF-α (ng/ml)
Fig. 5B

Parity effect: $P < 0.001$
Week effect: $P = 0.001$

Primiparous FBS
Multiparous FBS
Parity effect: $P = 0.01$

Fig. 6A

Week relative to calving
Parity effect: $P = 0.004$

**Fig. 6B**
Fig. 7

Parity effect: $P = 0.03$

Week effect: $P = 0.03$