Modulation of cytokine gene expression and secretion during the periparturient period in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Johne’s disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is estimated to infect more than 22% of US dairy herds. Periods of immunosuppression may contribute to the transition from the subclinical to the clinical stage of infection. Understanding the effects of stressors such as parturition on the escalation of disease may provide information that will help to manage JD. The objective of this study was to characterize cytokine gene expression and secretion in periparturient dairy cows naturally infected with MAP. Blood was collected from the jugular vein of healthy noninfected, and subclinically and clinically infected dairy cows for 3 weeks pre-calving to 4 weeks post-calving. Real-time PCR was performed to evaluate the expression of the following cytokine genes by peripheral blood mononuclear cells: IFN-γ, TNF-α, IL-12p35, IL-10, TGF-β, and IL-4. To assess the effects of parturient immunosuppression on cytokine gene expression, RT-PCR data were analyzed by using 2^{-ΔΔCt} values calibrated to ΔCt value at +1 day relative to calving for each animal. Overall, cytokine gene expression was not influenced by infection status of the cows in this study. However, significant effects in cytokine gene expression were noted across sampling days within the periparturient period. Expression of IFN-γ by NS and ConA-stimulated PBMCs declined at calving compared with prepartum values in both control and infected cows. Similarly, a decline in expression of IL-4 and IL-10 was observed for cells isolated from subclinically infected cows after stimulation with ConA. ConA-stimulated PBMCs isolated from infected cows secreted higher concentrations of IFN-γ compared with the controls. A significant decline in IFN-γ secretion was noted for MPS-stimulated cells for clinical cows from day 21 to day 1. Stimulating cells with MPS resulted in greater secretion of IL-10 by infected cows during the postpartum period. A trend was also observed for higher TGF-β secretion by NS PBMCs isolated from clinical cows in the postpartum period. Cells isolated from clinically infected cows and stimulated with MPS secreted higher levels of nitric oxide throughout the periparturient period when compared to control or subclinically infected cows. These data suggest that parturition is a very dynamic time period for host immunity, with potential for altered immunity to hinder the ability of dairy cows to thwart infectious diseases.

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1. Introduction

Johne’s disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is estimated to infect more than 22% of US dairy herds and cost the US dairy industry $250 million annually (Ott et al., 1999).
Dairy cows generally become infected as neonates through the fecal–oral route. Infected animals may remain in the subclinical, or asymptomatic, stage of the disease for several years following initial exposure of the bacteria (Larsen et al., 1975). During this stage, the majority of animals shed a negligible amount of bacteria, and granulomas begin to form in the small intestine. Stressors, such as parturition, may induce the transition from the subclinical to a more clinical stage of disease characterized by consistent fecal shedding of the bacteria, intermittent but persistent diarrhea, progressive weight loss, and an eventual death. Many questions still exist as to what triggers the progression of the disease.

Although it is anecdotal information, cows with paratuberculosis may advance to the clinical stage of the disease during the weeks following parturition. Research on what prompts the progression of disease during this time period is lacking. The transition period, defined as 3 weeks prior to and the 3 weeks following parturition, represents a time of physiological stress for the dairy cow (Goff and Horst, 1997). The immunosuppression experienced by the healthy periparturient cow has been well characterized. The week immediately following calving is marked by a decrease in lymphocyte proliferation (Kehrli et al., 1989b; Meglia et al., 2005) and the ability of neutrophils to migrate and phagocytize (Kehrli et al., 1989a; Lee and Kehrli, 1998). At calving, there is an increase in the activity of CD8+ lymphocytes compared with those from mid- to late-lactation animals (Shafer-Weaver and Sordillo, 1997). Endocrine and metabolic changes (Goff and Horst, 1997), in combination with immune suppression, contribute to the susceptibility of the cow to both metabolic and infectious diseases. The dramatic changes in estrogen and progesterone in the periparturient period potentially play a key role in immune suppression. In periparturient dairy cows, progesterone concentrations decline rapidly at parturition and remain low during the initial weeks of lactation (Radcliff et al., 2003). Progesterone has the ability to up-regulate Th2 cell activity and contributes to natural suppression of cell-mediated immunity (Miyaura and Iwata, 2002). Plasma estrogen concentrations increase dramatically 2 weeks prior to calving and then decline rapidly at calving (Radcliff et al., 2003). Previous work has shown that estradiol enhances Th1 development in vivo (Maret et al., 2003). The decline in progesterone and estradiol followed by decreased immune function may contribute to the progression of JD from the subclinical to the clinical state after calving.

The transition from the subclinical to the clinical stage of infection is characterized by a shift from cell-mediated (Th1) immunity to a Th2 response. Classical proinflammatory Th1 cytokines include IFN-γ, IL-12, and TNF-α (Mosmann et al., 1986; Roach et al., 2002). Expression of IL-12 promotes Th1 lymphocytes by inducing the secretion of IFN-γ (Bontkes et al., 2005) and subsequently enhancing the formation of granulomas (Smith et al., 1997). Th2 cytokines are predominantly considered to be suppressive and anti-inflammatory. Interleukin-4, IL-10, and TGF-β are capable of inhibiting Th1 cytokines, suppressing T-cell functions, and deactivating macrophages (Ho et al., 1992; Mullins et al., 2001).

A recent study demonstrated that nonstimulated PBMCs isolated from nonpregnant, nonlactating dairy cows with subclinical MAP infection had higher IFN-γ expression than did PBMCs from clinically infected cows or noninfected healthy controls (Coussens et al., 2004). Interestingly, in the same study, expression of IL-12 for infected animals was decreased compared to the controls. Recent data also demonstrated an increase in IFN-γ expression in nonpregnant, nonlactating subclinically infected cows (Khalifeh and Stabel, 2004b) and an upregulation of TGF-β and IL-10 in clinical cows naturally infected with MAP (Khalifeh and Stabel, 2004a). These studies provide further support for the paradigm of a Th1 response in early stages of JD with a switch to Th2-mediated responses as disease progresses.

To date, limited research is available characterizing host immunity in periparturient dairy cows infected with MAP or the potential impact of periparturient immunosuppression. Therefore, the objective of this study was to characterize cytokine gene expression and secretion in dairy cows naturally infected with MAP during the periparturient period as compared with the healthy control cows.

2. Materials and methods

2.1. Animals

Twenty-one multiparous Holstein cows and two primiparous Holstein cows were grouped according to the infection status. These three groups consisted of 5 noninfected healthy cows, 14 cows naturally infected with MAP, but asymptomatic, and 4 naturally infected cows with clinical Johne’s disease. The two primiparous cows were both in the subclinically infected group. The stage of infection was determined by fecal shedding of MAP, IFN-γ secretion, and specific antibody response to MAP. Infection was monitored by bacteriologic culture for the fecal shedding of MAP by standard
methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/tube (BBL™ Herrold’s egg yolk agar slants with mycobactin J, amphotericin, nalidixic acid, and vancomycin; Becton Dickinson and Co., Sparks, MD) and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/tube and were asymptomatic. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3–5-year period and had been purchased from herds with no recent history of Johne’s disease. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for MAP and IFN-γ) performed during that period. Whereas subclinically infected cows had a high antigen-specific IFN-γ responses and negligible Ab titers and clinically infected cows had low to moderate antigen-specific IFN-γ responses and high Ab titers. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center (NADC), Ames, IA).

2.2. Blood collection, culture conditions, and sample collection

Blood was collected from the jugular vein in 2× acid–citrate–dextrose (ACD; 1:10). For each animal, blood was collected at −21 days, −14 days, −7 days, +1 day, +7 days, +14 days, +21 days, and +28 days relative to calving. Peripheral blood mononuclear cells were isolated from theuffy coat fractions of peripheral blood. PBMCs were resuspended in RPMI-1640 containing 10% fetal calf serum (Atlanta Biologics, Atlanta, GA), 100 U of penicillin G (Gibco, Grand Island, NY) with 10% fetal calf serum blood. PBMCs were resuspended in RPMI-1640 isolated from the buffy coat fractions of peripheral to calving. Peripheral blood mononuclear cells were used in the experiments.

2.3. Bacteria

*M. avium* subsp. *paratuberculosis* strain K-10 (NADC) was grown in Middlebrook 7H9 broth (pH 6.0) supplemented with mycobactin J (2 mg/L; Allied Monitor, Fayette, MO) and oleic acid–albumin–dextrose complex (Becton Dickinson Microbiology, San Jose, CA). The bacteria were harvested, washed three times with PBS (137 mM sodium chloride, 10 mM phosphate, and 2.7 mM potassium chloride, pH 7.4), and sonicated on ice for 10 min. After incubating at RT for 10 min, the bacteria was sonicated for an additional 10 min and then centrifuged at 2500 × g for 20 min. Supernatant was removed and absorbance was read at 540 nm. Bacterial stocks (10⁹ mL⁻¹) were frozen in PBS at −80 °C until used in the experiments.

2.4. RNA extraction and RT-PCR

Peripheral blood mononuclear cells from each of the sampling time points were resuspended in RPMI-1640 (Gibco, Grand Island, NY) containing 10% fetal calf serum. PBMCs from each cow at each sampling time point were split into two aliquots: one aliquot that was cultured with medium alone (NS) and one that was stimulated with ConA (10 μg/mL). Cells were cultured in 75-cm² flasks at a concentration of 1 × 10⁷ PBMCs per flask at 39 °C in 5% CO₂ in a humidified atmosphere for 24 h. RNA was extracted from NS and ConA-stimulated PBMCs by using the standard protocol for Trizol Reagent (Invitrogen Life Technologies Corp., Carlsbad, CA). All RNA samples were purified by using the RNeasy® Mini Kit Protocol for RNA Cleanup (Qiagen, Valencia, CA). Samples were treated with TURBO DNA-free (Ambion, Austin, TX). The quantity of total RNA was determined by UV spectrophotometry. RNA samples were frozen at −80 °C until converted to cDNA.

Real-time RT-PCR was performed by using an Applied Biosystems 7500 DNA sequence detection system (PerkinElmer Corp., Foster City, CA). Total RNA extracted from both NS and ConA-stimulated cells was converted to first strand cDNA. Briefly, 2 μg of total RNA was added to 12-μL reaction mixture consisting of 10 mM oligo(dT)12–18 primer (Invitrogen, Carlsbad, CA) and RNase-free water. The reaction mixture was incubated at 70 °C for 5 min and then quickly chilled on ice to 20 °C. To the reaction mixture, 4 μL of 5 × first strand buffer (Invitrogen, Carlsbad, CA), 2 μL of 10 mM dNTP mix (Invitrogen, Carlsbad, CA), 1 μL of 0.1 M dithiothreitol (Invitrogen, Carlsbad CA), and 2 U SuperScript™ RNase H-reverse transcriptase (Invitrogen, Carlsbad CA), were added for a total volume of 20 μL. The reaction mixture was incubated at 42 °C for 1 h, heated to 70 °C for 15 min, and then cooled to 37 °C. Two units of DNase-free RNase H (Invitrogen, Carlsbad, CA) were added to the mixture that then was incubated at 37 °C for 20 min to remove the original RNA template. The RNase H was inactivated by heating the reaction mixture at 70 °C for 10 min. All cDNA samples were stored at −80 °C until RT-PCR analyses were performed.

For RT-PCR analysis, SYBR Green PCR master mixture (PerkinElmer Corp., Foster City, CA), template cDNA, and gene-specific primers for IFN-γ, TNF-α, IL-12p35, IL-4, IL-10, TGF-β, IGF-1, and β-actin were combined in a 20-μL reaction mixture. Primer sequences are listed in Table 1. All reactions were performed in triplicate. The β-actin gene was used as the control for the calculation of dCt. RT-PCR data were analyzed by using the 2^(-ΔΔCt) method as described previously (Livak and Schmittgen, 2001). The mean +1 days relative to calving dCt value within the treatment was used as the reference expression point.

2.5. Measurement of IFN-γ, IL-10, TGF-β, and nitric oxide production in cell culture supernatants by ELISA

Bovine IFN-γ was measured by using the Bovigam test kit (Biocor Animal Health, Omaha, NE) as described by the manufacturer. The minimal sample concentration of IFN-γ that could be detected by using the kit was 80 pg/mL. Bovine IL-10 was quantified by coating MaxiSorp microtiter plates (Nunc, Rochester, NY) with mouse anti-bovine IL-10 in coating buffer (15 mM sodium carbonate, 34 mM sodium bicarbonate, pH 9.6) (100 μL/well at 2 μg/mL) (MCA2110, Serotec, Raleigh, NC) overnight at RT. Plates were washed five times with PBS containing 1% Tween 80 (washing buffer). The samples and serial twofold dilutions of bovine IL-10 standard (0.3125–20 ng/mL) (generous gift from Dr. Jayne Hope, Compton, UK) were added to duplicate wells and incubated at RT for 1 h. Plates were then washed five times with washing buffer before incubating with the detection antibody, mouse anti- bovine IL-10:biotin (MCA2111B, Serotec, Raleigh, NC). Plates were washed five times with washing buffer, 100 μL of avidin–HRP conjugate (diluted 1:800) (PharMingen, San Diego, CA) was added to each well, and the plates were incubated for 45 min at RT. After another wash cycle, plates were incubated with substrate solution (40 mM 2,2′-azino-diethyl-benzthiozoline-6-sulfonic acid (ABTS)) in citrate buffer (50 mM, pH 4.0) and H2O2 (30% solution as 1:30 dilution). Color development was quantified after 30 min by measuring absorbance at 405 nm with a Wallac Victor 1420 multilabel counter ELISA plate reader (PerkinElmer, Gaithersburg, MD). The minimal sample concentration of IL-10 that could be detected by using the kit was 0.3125 units.

Bovine TGF-β was quantified using the Quantikine® Human TGF-β1 Immunoassay kit as described by the manufacturer and by using standards supplied (R&D Systems, Inc., Minneapolis, MN). Significant concentrations of latent TGF-β1 are found in bovine cell culture supernatants. Latent TGF-β was activated by the addition of 1N HCl followed by 10 min of incubation. The samples were neutralized by the addition of 1.2N NaOH/0.5 M HEPES. The concentration determined by the standard curve was multiplied by the dilution factor 1.4 to account for the activation procedure. Because of the high cost of the kit and large

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Sequence</th>
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<td>R</td>
<td>AGGCCGGCTTTGCACAT</td>
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<td>R</td>
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F, forward; R, reverse.

number of samples in this study, only NS and MPS-stimulated cell culture supernatants were evaluated at −14 days, −7 days, +1 day, +7 days, +14 days, and +21 days for this assay. The minimal sample concentration of TGF-β that could be detected by using the kit was 31.2 pg/mL.

To determine the amount of nitric oxide (NO) produced, the stable oxidation product, nitrite, was quantitated. For this analysis, 24 h cell culture supernatants from NS, ConA, and MPS-stimulated PBMCs were evaluated at −21 days, −14 days, −7 days, +1 day, +7 days, +14 days, +21 days, and +28 days. The samples and serial twofold dilutions of sodium nitrate standard (0.028–18 μM) were added in duplicate to a 96-well round bottom plate (Corning, Corning, NY). The culture supernatant (100 μL) was mixed with 100 μL of Greiss reagent (0.5% sulfanilamide; Sigma Chemical Co., St. Louis, MO) in 2.5% phosphoric acid (Mallinckrodt Chemical Co., St. Louis, MO) and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, MO). The mixture was allowed to incubate at RT for 10 min, and color development was measured at 505 nm. The concentration of nitrite in the supernatant was quantified by comparison with absorbance values of sodium nitrite standards within a linear curve fit. The minimal sample concentration of NO that could be detected using the kit was 0.28 μM.

2.6. Statistical analysis

RT-PCR data were analyzed by using the 2−(ΔΔCt) method as previously described (Livak and Schmittgen, 2001). β-Actin was used as the reference gene, and the dCt value at +1 day for each animal was used as the reference expression point. Outliers were determined by the SAS/STAT PROC RobustReg software. To evaluate the appropriateness of β-actin as a reference gene, the 2−(ΔCt) method was used as previously described (Schmittgen and Zakrajsek, 2000). This test indicated that β-actin would serve as a suitable reference for healthy and MAP-infected animals.

Several two-factor repeated measures analyses of variance were performed (one for each stimulation level-gene combination) comparing fold expression of two treatments through time. Levene’s homogeneity of variance test was performed on data to determine transformation necessity. All analyses were performed on transformed data where necessary, but raw data means are presented for ease of interpretation. If a significant F-test value from the ANOVA was obtained at P ≤ 0.05, differences of least squares means were used as the pairwise multiple comparison test for determining day or infection group × day differences. Means differed if P < 0.05 and tended to differ if 0.05 ≤ P ≤ 0.15. Analyses were performed using PROC MIXED in SAS® PC Windows version 9.1.3 software (SAS Institute, Cary, NC).

3. Results

3.1. Effect of infection status on cytokine gene expression

In this study, +1 day was used as the reference expression point in the analyses of the gene expression data. Due to sampling error, three of the four clinical cows did not have useable RNA samples for the +1 day time point. Because of the nature of the periparturient study, we were not able to retrospectively isolate additional PBMCs from the blood of these cows at +1 day for RNA extraction. In the 2−(ΔΔCt) analysis, it is critical to have the dCt for the reference expression point day. On the basis of these results, we were not able to evaluate the cytokine gene expression data for the clinical cows in this study. All gene expression data presented herein contrasts results between healthy noninfected control cows and subclinically infected cows.

Regardless, there were no significant effects due to infection status on the expression of cytokines, IFN-γ, IL-12p35, TNF-α, IL-4, IL-10, and TGF-β during the period extending from 3 weeks prior to calving to 4 weeks post-calving (Figs. 1A and 2). There was also no effect of infection group on expression of IGF-1 from NS or ConA-stimulated PBMCs (Fig. 3), however, a strong trend was present demonstrating higher expression of IGF-1 for NS PBMCs isolated from subclinically infected cows compared with that of control cows in the prepartum period.

3.2. Effect of parturition on cytokine gene expression

There was a significant effect of parturition on IFN-γ expression in NS (P < 0.05) and ConA-stimulated (P < 0.01) PBMCs (Fig. 1A and B). For both groups, expression declined beginning on −14 days through calving. Expression in NS PBMCs did not recover during the postpartum period. However, IFN-γ expression by ConA-stimulated PBMCs was significantly (P < 0.01) greater by +28 days compared with −7 days (Fig. 1B).

In the NS PBMCs, expression of TNF-α declined for both groups of cows from −7 days to +1 day and rebounded by +7 days (P < 0.01) (Fig. 1C). Despite the lack of an overall effect of parturition on ConA-
Fig. 1. Expression of Th1 cytokines, IFN-γ and TNF-α, by peripheral blood mononuclear cells from healthy control cows (●) and cows naturally infected with Mycobacterium avium subsp. paratuberculosis (■). (A) IFN-γ expression by nonstimulated (NS) PBMCs (parturition effect, \( P < 0.05 \)). (B) IFN-γ expression by ConA-stimulated PBMCs (parturition effect, \( P < 0.001 \)). (C) TNF-α expression by NS PBMCs (parturition effect, \( P < 0.01 \)). (D) TNF-α expression by ConA-stimulated PBMCs (parturition effect, \( P < 0.05 \)). Significant differences on a given day relative to day 1 are represented by asterisks (\( P < 0.05 \)).

Fig. 2. Expression of Th2 cytokines, IL-10 and IL-4, by peripheral blood mononuclear cells from healthy control cows (●) and cows naturally infected with Mycobacterium avium subsp. paratuberculosis (■). (A) IL-10 expression by nonstimulated (NS) PBMCs (parturition effect, \( P < 0.05 \)). (B) IL-10 expression by ConA-stimulated PBMCs. (C) IL-4 expression by NS PBMCs. (D) IL-4 expression by ConA-stimulated PBMCs. Significant differences on a given day relative to day 1 are represented by asterisks (\( P < 0.05 \)).

Concentrations for subclinical and control cows
/C6
2.18 before and 4 weeks after calving, regardless of infection group.

Expression of both cytokines remained relatively stable during the 3 weeks after calving, but control cows showed an increase in IL-10 expression during the postpartum period. Stimulating the PBMCs with ConA did not result in overall effects of parturition on IL-10 mRNA expression (Fig. 2B); however, there was a decline in expression between -21 days and -7 days in subclinical cows (P < 0.05). There was no effect of parturition on IL-4 expression by NS PBMCs (Fig. 2C). A linear decrease in IL-4 expression was noted for ConA-stimulated PBMCs isolated from subclinically infected cows between -21 days and +1 day (P < 0.05) (Fig. 2D).

For both infection groups, there was a significant increase in IGF-1 expression at parturition followed by a sharp decline post-calving (P < 0.001) (Fig. 3).

3.3. IFN-γ, IL-10, TGF-β, and nitric oxide secretion

Overall, throughout the sampling period, production of IFN-γ by ConA-stimulated PBMCs tended to be greater in subclinical (14.36 ± 1.6 ng/mL; P < 0.06) and clinical (14.28 ± 2.7; P < 0.12) cows than the controls (8.30 ± 2.2) (Fig. 4A). There was an overall effect of parturition and an interaction of infection group and parturition (P < 0.0001), but this effect was most likely the result of extremely high concentration of ConA-stimulated IFN-γ noted on -21 days for the clinical cows. By -14 days, this value was within the range of the average values noted for subclinical and control cows. A significant (P < 0.05) increase in ConA-stimulated IFN-γ secretion was observed for subclinically infected cows on day +21 and day +28 post-calving as compared to other groups. When PBMCs were stimulated with MPS, there was a tendency (P < 0.11) for clinical cows to secrete more IFN-γ compared with the controls (Fig. 4B). A significant (P < 0.001) decline in MPS-stimulated IFN-γ secretion was observed for clinical cows from -21 days (2.18 ± 0.8 ng/mL) to +1 day (0.75 ± 0.2 ng/mL). Concentrations for subclinical and control cows remained relatively stable throughout the sampling period.

Overall, secretion of IL-10 by NS and ConA-stimulated PBMCs was not affected by the infection group (Fig. 5). There was a spike in IL-10 secretion in the prepartum period by NS PBMCs from subclinical cows (Fig. 5A). There was a spike in IL-10 secretion by ConA-stimulated PBMCs from subclinical cows between 14 days, this value was within the range of the average values noted for subclinical and control cows. A significant (P < 0.05) increase in ConA-stimulated IFN-γ noted on -21 days for the clinical cows. By -14 days, this value was within the range of the average values noted for subclinical and control cows. A significant (P < 0.05) increase in ConA-stimulated IFN-γ secretion was observed for subclinically infected cows on day +21 and day +28 post-calving as compared to other groups. When PBMCs were stimulated with MPS, there was a tendency (P < 0.11) for clinical cows to secrete more IFN-γ compared with the controls (Fig. 4B). A significant (P < 0.001) decline in MPS-stimulated IFN-γ secretion was observed for clinical cows from -21 days (2.18 ± 0.8 ng/mL) to +1 day (0.75 ± 0.2 ng/mL). Concentrations for subclinical and control cows remained relatively stable throughout the sampling period.

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cows with secretion increasing sharply from −21 days to −14 days and then rapidly declining to +1 day (P < 0.01) (Fig. 5A). Clinical cows followed a similar pattern with secretion increasing sharply from −14 days to −7 days and then declining to a nadir of 0.307 U at +1 day. This resulted in an overall interaction of infection group and parturition (P < 0.001). When PBMCs were stimulated with ConA, there was no longer an effect of parturition (data not shown). For the MPS-stimulated PBMCs, the days were combined into three periods: −21 days, −14 days, and −7 days = precalving; +1 day = calving; +7 days, +14 days, +21 days = post-calving. Also, because of similar trends for both infected groups, subclinical and clinical cows were combined into one infected group. Secretion of IL-10 tended (P < 0.09) to be greater for infected cows compared with that of control cows at calving and during the postpartum period (Fig. 5B). Interestingly, stimulating the PBMCs with MPS resulted in a 7.7-fold, 9.7-fold, and 12.0-fold increase in secretion for control, subclinical, and clinical cows, respectively, compared with secretion from NS PBMCs.

Overall, parturition did not seem to have an effect on TGF-β production by PBMC’s stimulated with medium only (NS) (Fig. 5C). However, at +7 days, subclinical cows had significantly lower TGF-β secretion compared with control cows (P < 0.05; 868.5 ± 79.3 pg/mL vs. 1187.3 ± 77.9 pg/mL) and clinical cows (P < 0.01; 868.5 ± 71.1 pg/mL vs. 1305.6 ± 98.9 pg/mL) at +7 days. In addition, an overall trend for higher TGF-β secretion by NS PBMC isolated from clinical cows was noted in the postpartum period. There was no overall effect of cow infection status on concentrations of TGF-β when PBMCs were stimulated with MPS, except at +1 day when clinical cows had significantly (P < 0.05) increased TGF-β secretion compared with that of subclinical and control cows (Fig. 5D).

Nitric oxide production by NS PBMCs was not affected by either infection group or parturition (data not shown). In the MPS-stimulated cells, the clinical cows (0.75 ± 0.2 μM) tended to have greater NO production compared with that of the control (0.31 ± 0.15 μM; P < 0.10) and the subclinical cows (0.42 ± 0.09 μM; P < 0.15), and an overall infection group × parturition interaction was noted (P < 0.10) (Fig. 6B). Similar effects were observed for ConA-stimulated cells, but the effects were not significant (Fig. 6A).

3.4. Serum progesterone, 17β-estradiol, and IGF-1

For all cows sampled, regardless of infection status, progesterone concentrations declined as parturition
approached (Fig. 7A). This decline resulted in a strong
day effect \((P < 0.001)\). At \(-21\) days, subclinical cows
showed significantly \((P < 0.05)\) elevated progesterone
compared with control cows. During the sampling days
after calving, concentrations of serum progesterone
decreased and reached values lower than that that could
be detected by using the kit \((0.2 \text{ ng/mL})\). Estradiol
concentrations remained fairly constant for control and
clinically infected cows until \(-7\) days (Fig. 7B). At day
\(-7\), estradiol concentrations began to decline and by
day \(+7\) reached a nadir of \(10 \text{ pg/mL}\). This decline was
characteristic for all cows, regardless of infection status,
resulting in an overall day effect \((P < 0.001)\). At \(-7\)
days, estradiol spiked for subclinically infected cows
with concentrations greater than both control
\((P < 0.001)\) and clinical \((P < 0.001)\) cows and declined
thereafter.

In general, for healthy control cows and infected
cows, IGF-1 concentrations declined between \(-14\) days
and \(+1\) day and then began to recover after parturition
(Fig. 7C). The decline in the weeks prior to parturition
and subsequent recovery of IGF-1 serum concentrations
within the first few weeks after parturition resulted in an
overall day effect \((P < 0.0001)\). There was no overall
effect of MAP infection on serum IGF-1 concentrations.

4. Discussion

Johne’s disease has a devastating impact on the
health of the dairy cow, and this disease has had
cumulative economic impact on the dairy producer.
Most animals will encounter MAP during the first year
of life and will remain in the subclinical, or asympto-
matic, stage of the disease for several years. The
transition from subclinical to clinical state coincides
with the differentiation of naïve CD4+ T-cells from a Th1 to a Th2 immune response. Limited research exists that focuses on the role that parturition plays, if any, in the progression of JD from the subclinical to clinical state. To the best of our knowledge, this study is the first to document changes in cytokine gene expression and secretion in dairy cows naturally infected with MAP throughout the periparturient period.

Even the healthy dairy cow experiences increased challenges to the immune system during the periparturient period. Increases in both metabolic and infectious disease are observed during this time (Goff and Horst, 1997). Throughout mid- to late-lactation, cytokine profiles mirror a Th1 immune response. However, as transition to lactation occurs, there is a shift in the CD4+ population supporting a Th2 response (Shafer-Weaver et al., 1999). The immune system is further compromised by decreases in both neutrophil and lymphocyte function that is caused by parturition (Kehrli et al., 1989a,b).

To better understand the interaction of parturition and MAP infection, we evaluated the ability of cytokines to modulate progression of JD during the periparturient period by first examining the expression of the Th1 cytokines. In the current study, MAP infection did not have an effect on the gene expression of IFN-γ, TNF-α, or IL-12. These results are in contrast to previous work in our laboratory that concluded that subclinical JD cows have greater IFN-γ expression and secretion in the immediate postpartum period compared with subclinical cows (Stabel, 2000; Khalifeh and Stabel, 2004a,b). However, NS PBMCs from subclinical cows did tend to secrete more IFN-γ compared with clinical and control cows in the postpartum period. Interferon-γ is important for initiating the innate immune response to intracellular bacteria and it is one of the first cytokines to be activated in subclinical MAP-infected dairy cows (Sweeney et al., 2002). Together, IFN-γ and TNF-α promote the protective formation of granulomas (Roach et al., 2002), a critical step in controlling MAP infection. Both IFN-γ and TNF-α expression by NS PBMCs declined as parturition approached, which is in agreement with previous observations suggesting a decline in Th1 cytokines at parturition (Shafer-Weaver et al., 1999).

Interestingly, IL-12p35 expression was not affected by infection or parturition. This is in contrast to Coussens et al. (2004) who showed an effect of MAP infection on the Th1 cytokines, IL-4 and IL-10, and the Th3 cytokine, TGF-β. The transition from the Th1 to Th2 represents a switch from cell-mediated to antibody-mediated immunity. In the current study, there was no effect of parturition or MAP infection on gene expression of the Th2 or Th3 cytokines. Recent data from our laboratory suggested that there was an upregulation of TGF-β and IL-10 expression in clinical cows naturally infected with MAP (Khalifeh and Stabel, 2004a). TGF-β has been shown to act synergistically with IL-10 in repressing macrophage activation (Mullins et al., 2001). Despite minimal differences in TGF-β expression, NS and MPS-stimulated PBMCs isolated from clinical cows secreted more TGF-β during the immediate postpartum period compared with subclinical and control cows. MPS-stimulated PBMCs from infected cows secreted more IL-10 than did the control cows at calving and during the postpartum period. The increase in IL-10 secretion observed during the postpartum period for infected cows may result in the down-regulation of both IL-10 and macrophage activation (Sweeney et al., 2002). The Th2 cytokine, IL-4, functions to promote Th1 cell suppression. Others have reported upregulation of IL-4 gene expression during the immediate postpartum period but this effect was not observed in the present study (Shafer-Weaver and Sordillo, 1997; Shafer-Weaver et al., 1999).

It is of interest to note that the cytokine profiles for the cows in our study typically followed the established Th1/Th2 dichotomy observed in periparturient dairy cows. We did not, however, observe an effect of MAP infection on cytokine expression. Both the innate and adaptive immune responses are lowest during the periparturient period (Mallard et al., 1998). To date, there is not a complete characterization of the consequences of this immunosuppression. There exists a possibility that the immunosuppression at calving was severe enough to essentially override the traditional Th1/Th2 paradigm observed in MAP-infected animals. This may explain why we did not observe differences in cytokine expression between infection groups.

Hormonal fluctuations during the periparturient period contribute to the typical immunosuppression observed at calving. The polarization of a specific Th response may be influenced by the hormonal status of the animal. On the basis of their potential role during this critical time period, we evaluated IGF-1, progesterone, and 17β-estradiol concentrations in the peripheral blood. All the cows exhibited typical hormonal
responses observed around parturition (Radcliff et al., 2003). Despite our hypothesis, MAP infection did not appear to affect blood hormone concentrations.

One major hypothesis of the current study was that subclinical cows would transition to the clinical stage of the disease during the 4 weeks postpartum. However, not one of the 14 subclinical cows on the study seemed to make the transition to the clinical stage of the disease during the 4 weeks postpartum. This conclusion was based on diagnostic measurements such as fecal shedding of the bacteria, antigen-specific IFN-γ responses, and ELISA titers for the entire sampling period. Further studies are needed to determine if the cytokine profile of JD cows is altered later in lactation than the +28 days observed in the current study.

5. Conclusions

Results of this study indicate that parturition modulates mRNA expression of IFN-γ, TNF-α, IL-10, and IGF-1 in dairy cows. Infection with MAP did not significantly alter cytokine gene expression compared with that of control cows, but secretion of IFN-γ, IL-10, and TGF-β were up-regulated by infection in the postpartum period. The periparturient period did not alter the transition from subclinical to clinical infection status in cows with paratuberculosis, but the period of observation was relatively brief. A more protracted period of study would likely yield more significant effects. In addition, the highly dynamic nature of the immediate periparturient period may have precluded distinct observations between infection groups.

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References


