Title: Longitudinal characterization of bovine monocyte-derived dendritic cells from mid-gestation into subsequent lactation reveals nadir in phenotypic maturation and macrophage-like cytokine profile in late gestation

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Abstract

Changes in monocyte and dendritic cell populations during bovine pregnancy and lactation remain poorly described despite the key roles these cells play in immune tolerance and activation. Using a prospective longitudinal study, we characterized CD14⁺ monocyte-derived dendritic cell (moDC) differentiation and maturation and captured monocyte composition dynamics from mid-gestation through calving and into the subsequent lactation in dairy cows (n=7). First, we measured absolute counts of classical (CD14⁺CD16⁻, cM), intermediate (CD14⁺CD16⁺, intM), and nonclassical (CD14⁻CD16⁺, ncM) monocytes in the blood and determined proportions of individual subsets within the total monocyte population. We found the proportion of cM decreased and intM increased significantly by early lactation, whereas there was a nadir in the proportion of ncM in late gestation, two weeks prepartum. Monocyte composition appears to be regulated in pregnancy, possibly to limit the proportion of highly inflammatory monocytes i.e. intM. Ultimately, we found that moDC differentiated from CD14⁺ monocytes isolated in the early dry period of late gestation had impaired *E. coli*-induced maturation, with nadirs in upregulation of CD80 and MHC II, and downregulation of CD14. The moDC from late gestation also had altered cytokine profiles with greatest production of pro-inflammatory IL-1 β and anti-inflammatory IL-10. These data suggest monocytes in late gestation, in contrast to other stages of pregnancy and lactation, differentiate and mature into moDC less capable of eliciting strong T cell activation, and have macrophage-like cytokine profiles. These results provide insight into maternal immune modulation and elucidate potential immune changes necessary to facilitate bovine pregnancy.

Keywords

Bovine; pregnancy; monocyte-derived dendritic cells; monocytes

Abbreviations

cM (classical monocyte), DC (dendritic cell), DIM (days in milk), ED (early dry period), EL (early lactation), intM (intermediate monocytes), LD (late dry period), Mφ (macrophage), MG (mid-gestation), moDC (monocyte-derived dendritic cells), MOI (multiplicity of infection), ncM (nonclassical monocyte), PBMC (peripheral blood mononuclear cells), PC (post-calving), T helper 1 (Th1), T helper 17 (Th17)

1. Introduction

During pregnancy the maternal immune system is presented with the challenge of needing to concurrently tolerate the growth of a semi-allogenic fetus and respond to invading pathogens. Highly inflammatory, T helper 1 (Th1) and T helper 17 (Th17)-type responses in pregnancy have been shown to be detrimental to the health of the fetus, thus, the maternal immune system must be carefully regulated (Rosbottom et al., 2008, Betz, 2012, Krishnan et al., 2013). Maternal immune regulation is dynamic; dairy cattle show varying responses to infectious agents depending upon their stage of pregnancy and lactation (Innes, 2007, Rosbottom et al., 2008, Quesnell et al., 2012, Sipka et al., 2013). Previous work found cows in the third trimester of pregnancy, particularly during the non-lactating period of late gestation (known as the ‘dry period’), had a heightened susceptibility to persistent infections caused by mastitis- and abortion-causing pathogens including *E. coli* and *N. caninum* with hindered pro-inflammatory, Th1-type responses relative to the postpartum period and earlier stages of gestation (Williams et al., 2000, Green et al., 2002, Anderson, 2007, Rosbottom et al., 2008, Quesnell et al., 2012). Monocytes and monocyte derived cells play a major role in maternal immune regulation throughout pregnancy across multiple species; these cells are involved at all stages of pregnancy including implantation, maintenance of pregnancy, and parturition and show dynamic regulation by stage of gestation (Oliveira et al., 2012, Gomez-Lopez et al., 2014, Leno-Duran et al., 2014, Schumacher et al., 2014). Though previous work on dairy cattle indicated that certain immune cell populations have altered function in pregnancy, this area of research remains largely underdeveloped (Lamote et al., 2004, Lamote et al., 2006, Maeda et al., 2013, Pomeroy et al., 2015). We lack an understanding of underlying mechanisms in immune tolerance, gestational

stage-dependent changes in maternal immune function, and how this relates to overall health of the dam and fetus.

Bovine monocytes have only recently been classified into three major subsets, classical (cM), intermediate (intM), and nonclassical (ncM), based on CD14 and CD16 expression analogous to their human counterparts (Hussen et al., 2013, Hussen et al., 2014, Corripio-Miyar et al., 2015, Hussen et al., 2016). Unlike CD14⁻ ncM, both CD14⁺ subsets, cM and intM, have been shown to be highly responsive to LPS stimulation (Hussen et al., 2013, Hussen et al., 2014). Changes in monocyte subset composition have been shown to influence disease susceptibility and pregnancy outcome in species such as mouse and human, but these changes have not been characterized in cattle (Al-ofi et al., 2012, Melgert et al., 2012, Devevre et al., 2015, Tang et al., 2015). These monocyte populations circulating in the periphery migrate into tissues either during inflammation or steady-state conditions where they may differentiate into macrophages (M ϕ) or dendritic cells (DC).

Across multiple species, DC are shown to be regulated in pregnancy and play a crucial role in generating maternal immune tolerance toward fetal antigens (Bachy et al., 2008, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Pomeroy et al., 2015). Recently we found key differences in monocyte derived DC (moDC) phenotype and cytokine production in a cross-sectional study comparing moDC from late gestation cows to moDC from non-pregnant cows in early lactation. We found moDC had limited Th1-type responses and were likely to have a poor ability to activate T cells during late gestation (Pomeroy et al., 2015).

Here we aim to expand upon our previous work and capture the dynamics of monocyte subsets, and the function and phenotype of respective moDC populations through a prospective longitudinal study in cows from mid-gestation through calving into the subsequent lactation. The

objective of this study was to analyze differentiation and maturation with UV-killed *E. coli*-stimulation of moDC, derived from blood CD14+ across pregnancy and into early lactation. We investigated phenotype and function analyzing surface marker expression (CD14, CD40, CD80, MHC II) and cytokine production (IL-1 β , TNF α , IL-10) following *E. coli* stimulation. Furthermore we measured monocyte subset composition (cM, intM, ncM) in blood at the designated time points to describe the composition of monocyte subsets in blood as a baseline and further understand the implications of starting monocyte population on moDC differentiation and maturation.

2. Materials and methods

2.1. Animals

Seven Holstein–Friesian cows, in the second trimester of pregnancy between 90-110 days carrying calf (‘mid-gestation’, MG, 90-110dcc/P-190-170d) as determined from insemination date were selected from the Cornell University Veterinary College Teaching Dairy Barn. No disease (i.e. metabolic, reproductive, mastitis) was recorded within 30 days prior to the time of first sampling for all enrolled animals. Peripheral blood was collected by jugular venipuncture from enrolled animals into 250ml vacuum bottles containing EDTA and 10 ml vacutainer glass serum tubes with no additive (Becton Dickinson, Franklin Lakes, NJ). Each enrolled animal was sampled repeatedly throughout the remainder of pregnancy and into the subsequent lactation as depicted in figure 1. Blood was collected at MG, early dry period within the first week since dry-off ~60-53 days prior to expected calving date (‘early dry’, ED, 1-7d dry/P-60-53d), late dry period ~2 weeks prior to expected calving date (‘late dry’, LD, 43d dry/P-14d), 4-6 days following parturition (‘post-calving’, PC, P+4-6d), and the final collection ~45-55 days following parturition before breeding (‘early lactation’, EL, P+45-55d) (Fig. 1). Disease was

monitored daily and recorded in DairyComp® by farm management; metritis cases were diagnosed within the first two weeks postpartum by abnormal vaginal discharge and foul odor of the discharge. All animal procedures were approved by the Cornell Institutional Animal Care and Use Committee (project number: 2007-0110).

FIGURE 1

2.2. Generation of peripheral blood CD14+ moDC

Monocyte derived DC were generated from CD14+ monocytes as described previously by Pomeroy et al. (2015); CD14+ positive selection was preformed based on the use of UV irradiated *E. coli* ECC-Z stimulation to induce moDC maturation in conjunction with previous work from Hussen et al. (2013) and (2014) indicating CD14+ cM and intM are the monocyte subsets responsive to LPS stimulation. In brief, peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation. The PBMC were incubated with anti-human CD14 antibodies conjugated with paramagnetic microbeads (Miltenyi Biotech, Inc., Auburn, CA) in MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA), transferred to MACS LS magnetic separation column, and after repeatedly washing the column with MACS buffer the magnetically labeled cells were collected (Miltenyi Biotech, Inc.). Purity was determined by flow cytometry and was shown to be >90%. Monocytes were re-suspended in differentiation media (phenol-free complete RPMI with l-glutamine, supplemented with 10% autologous serum, 20 ng/ml recombinant bovine IL-4 and, 20 ng/ml recombinant bovine GM-CSF both from Kingfisher Biotech), plated in 6-well tissue culture plates at 1.5×10^6 cells in 3 ml differentiation media per well, and cultured for 5 days (37 °C, 5% CO₂) in presence of recombinant cytokines to generate immature moDC; at 2-3 d cultures were given 1ml/well of fresh differentiation media.

2.3. Inactivation of *E. coli* ECC-Z

E. coli ECC-Z was inactivated as described by Pomeroy et al. (2015). In brief, frozen stock of the previously characterized *E. coli* ECC-Z strain known to cause mild persistent clinical mastitis (Dogan et al., 2006; Lippolis et al., 2014) was grown to log phase in LB liquid. Bacteria were pelleted and diluted in PBS. Bacterial suspension was plated on agar plates prior to inactivation to determine concentration of stock solution. The bacterial suspension was inactivated using UV irradiation in the laboratory of Dr. Randy W. Worobo (Department of Food Science and Technology, Cornell University). The UV irradiated *E. coli* suspension failed to produce colonies on LB agar plates. Aliquots of UV irradiated *E. coli* were resuspended in sterile 10% glycerol/PBS and stored at -80°C .

2.4. Maturation of moDC with UV irradiated *E. coli*

Maturation of moDC was induced by bacterial stimulation through the addition of UV irradiated *E. coli* to immature moDC cultures in an MOI (multiplicity of infection) of 10 for 24 h based on previous work (Cavatorta et al., 2009, Pomeroy et al., 2015). Cultures of moDC without bacterial stimulation served as media controls. Cells were harvested after 24 h *E. coli* stimulation for phenotypic analysis by flow cytometry. Cell culture supernatants were collected at this time and stored at -80°C until further cytokine analysis by ELISA.

2.5. Flow cytometry

2.5.1. Monocyte subset staining

Composition of monocyte subsets in PBMC was determined using a similar staining protocol as described by Hussen et al. (2013). To identify monocyte subsets isolated PBMC were analyzed using directly labeled mouse monoclonal antibodies against human CD16:FITC (MCA5665F, AbD Serotec, Raleigh, NC), mouse anti-human CD14:RPE (MCA1568PE, AbD

Serotec), and CD172a (Kingfisher Biotech) conjugated with Alexa Fluor[®] 647 dye using monoclonal antibody labeling kit (Life Technologies). Discrimination between live and dead cells was done by adding propidium iodide (PI, 1 µg/ml, Sigma Aldrich). A minimum of 75,000 events gated on viable cells were recorded for each sample. Due to issues with reagent availability, PBMC from MG were isolated and stored in freezing media (8mM HEPES, 9% DMSO, 45% HI FBS, antibiotic-antimitotic in phenol-free RPMI, +L-glut), slowly frozen at -80 °C, and transferred to liquid nitrogen until monocyte subset staining could be conducted. No differences were found in monocyte populations between frozen and fresh samples (data not shown). Absolute counts of monocyte subsets were estimated based on PBMC counts isolated per volume of blood.

2.5.2. Monocyte-derived dendritic cell staining

Expression of surface markers on moDC was measured with flow cytometry as described previously by Pomeroy et al. (2015). In brief, moDC were blocked with 10% normal goat serum (Life Technologies), subsequently incubated with primary unlabeled monoclonal mouse antibodies against bovine MHC II DR orthologue (TH4B), CD14 (MM61A), CD40 (ILA158A), and CD80 (ILA159) (Kingfisher Biotech), and then indirectly labeled with secondary Alexa Fluor[®] 488 conjugated monoclonal goat antibodies against mouse IgG₁ or IgG_{2α} (Life Technologies). Secondary controls were of moDC stained only with Alexa Fluor[®] 488 conjugated secondary antibodies. No differences were found in baseline autofluorescence as determined by consistent MFI of secondary controls across samples (data not shown). Labeled cells were stained with LIVE/DEAD[®] Fixable Red (Life Technologies) to exclude dead cells during analyses, and then fixed with 4% paraformaldehyde. A minimum of 10,000 events gated on viable cells were recorded for each sample. All measurements were taken with a

FACSCalibur (Becton Dickinson). All flow cytometry data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

2.6. Measurement of IL-10, TNF α , and IL-1 β by ELISA

Supernatant levels of bovine IL-10 were measured using ELISA as described in Pomeroy et al. (2015). In brief, Thermo Cliniplate EB flat-bottom 96-well plates (ThermoFisher, Vantaa, Finland) were coated with capture monoclonal mouse antibody against bovine IL-10 (1 μ g/ml; CC320; AbDSerotec) in 0.05 M carbonate buffer (pH 9.6). Plates were washed then blocked with 2% fish skin gelatin (Sea Block buffer, ThermoFisher) in PBS. Following blocking supernatant samples were added. Known concentrations of recombinant bovine IL-10 (Kingfisher Biotech) were assayed simultaneously to generate a standard curve from which sample concentrations were determined. Following sample and standard incubation, plates were washed, and biotinylated detection monoclonal mouse antibody against bovine IL-10 (1 μ g/ml; CC320; AbD Serotec) was added. All samples and standards were assayed in duplicate.

Supernatant levels of TNF α and IL-1 β were measured according to using commercially available kits according to the manufacturer (bovine TNF α DuoSet® ELISA, DY2279, R&D Systems, Minneapolis, MN; bovine IL-1 β ELISA, Thermo Scientific, Rockford IL).

2.7. Statistical analysis

All data were analyzed using the SAS v 9.4 statistical analysis program (SAS Institute Inc., Cary, NC). Initially, data were graphed and evaluated for outliers. Outliers were inspected and reanalyzed where necessary. Significant changes in monocyte subset composition in blood, moDC surface marker expression, and moDC cytokine production over time were determined using polynomial regression (SAS, PROC MIXED); linear models adjusted for repeated measures within cow and accounted for between subject factors. Regression coefficients were

evaluated for statistical significance. If data did not fit a linear polynomial model, data were analyzed using multiple comparison tests with repeated measures ANOVA; a Tukey for post hoc tests was used. For all analyses a type I error of ≤ 0.05 was used.

3. Results

All cows completed all samplings and carried their pregnancies to full term without any recorded or observed clinical disease or other complications during pregnancy. After calving, 2 cows developed metritis.

3.1. Monocyte population dynamics show increasing intM and ncM, and decreasing cM composition postpartum relative to late gestation

Greatest proportion (mean=31.7%, SD=13.1%) and absolute count (mean= 7.591×10^5 cells/ml, SD= 2.671×10^5 cells/ml) of total monocyte population occurred PC (Fig. 2A, Table 1). Monocyte subset composition is shown in figure 1B. The proportion of cM had a significant time-dependent quadratic relationship in which lowest values occurred in EL, with a plateau occurring in late gestation; cM proportions over pregnancy (MG, ED, LD) and PC were relatively consistent (Fig. 2C). There was no significant difference detected in absolute counts of cM across pregnancy or subsequent lactation (Table 1). The proportion of intM had a significant time-dependent linear relationship in which lowest values occurred in MG, slightly increasing as pregnancy progressed into the subsequent lactation and reached highest proportions at EL (Fig. 2D). There was a significant increase in absolute counts of intM from LD to PC ($p=0.05$); cows which acquired metritis ($n=2$) were outliers with low intM counts at PC (0.44591×10^5 cells/ml, 0.44639×10^5 cells/ml) (Table 1). The absolute counts of cM and intM used in moDC culture were estimated based on proportions measured in PBMC; cultures from EL had highest intM

counts and lowest cM counts compared to all time points (Fig. 3). The proportion of ncM had a significant time-dependent quadratic relationship in which it decreased from MG until a nadir occurred in LD and then subsequently increased into EL (Fig. 2E). In conjunction, there was a significant increase in absolute counts of ncM at PC (ED v. PC (p=0.05) LD v. PC (p=0.008), EL v PC (p=0.03); cows which acquired metritis (n=2) were outliers with low ncM counts at PC (0.29045×10^5 cells/ml, 0.19966×10^5 cells/ml) (Table 1).

FIGURE 2

TABLE 1

FIGURE 3

3.2. *E. coli*-stimulated moDC have hindered MHC II and CD80 upregulation, and high CD14 expression in early dry period of late gestation

As shown in figure 4, moDC had greater phenotypic maturation following calving (PC, EL) compared to stages of late gestation (ED, LD). The expression of CD14 as determined by MFI had a significant time-dependent cubic relationship in which maximum expression occurred in ED, and minimum expression at PC, with higher levels of CD14 expression across pregnancy (MG, ED, LD) relative to non-pregnant stages (PC, EL) (Fig. 4 A&B). The MFI of CD14 expression in *E. coli*-stimulated moDC shows least variation during PC and EL (Fig. 4 A&B). All moDC upregulated co-stimulatory molecules CD40 and CD80 following *E. coli* stimulation, however, percent CD80+ *E. coli*-stimulated moDC had a significant time-dependent cubic relationship over the sampling period which indicated a nadir in CD80+ cells at ED and a plateau with maximum CD80+ cells at PC into EL (Fig. 4 A&C, Table 1S). There were no significant changes in CD40 expression; this marker was constitutently expressed throughout pregnancy into the subsequent lactation at comparable levels (Fig. 4A, data not shown). We found upregulation

of MHC II expression as determined by delta MFI (MFI *E. coli*-stimulated moDC– MFI unstimulated moDC) had a significant time-dependent quadratic relationship indicating a nadir at ED, maximum in EL (Fig. 4 A&D).

FIGURE 4

3.3. moDC have greatest production of IL-1 β and IL-10 in late gestation

Concentrations of IL-1 β following *E. coli* stimulation had a significant time-dependent cubic relationship over the sampling period which indicated a transient peak in production at ED and higher levels at LD relative to MG, PC, and EL (Fig. 5 A&D). We found moDC from all stages were capable of upregulating TNF α production in response to *E. coli*,-induced maturation; there was no significant time-dependent relationship (Fig. 5 B&E). No significant production of IL-1 β or TNF α was detected in the supernatant from unstimulated moDC. Concentrations of IL-10 following *E. coli* stimulation had a significant time-dependent quadratic relationship which indicated maximum concentrations at LD (Fig. 5 C&F). Interleukin 10 was detected a varying levels in supernatant from unstimulated moDC across the sampling period so delta IL-10 (*E. coli*-stimulated moDC–unstimulated moDC) was used to compare IL-10 upregulation following *E. coli* stimulation (Fig. 5 C&G). Upregulation of IL-10 had a significant time-dependent cubic relationship which indicated maximum upregulation at ED and a nadir at PC (Fig. 5 C&G).

FIGURE 5

4. Discussion

In non-bovine species, monocytes and DC are known to be key drivers of maternal immune tolerance and are differentially regulated to accommodate unique needs at each stage of pregnancy; similar dynamic changes have not been characterized in cattle (Blois et al., 2007, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Schumacher et al., 2014).

In this longitudinal study, we identified unique profiles in monocyte composition and moDC maturation at specific stages of gestation and subsequent lactation that suggest greatest alterations to these cell populations occurs over the dry period in late gestation.

Peripheral monocyte composition experienced dynamic changes dependent on the stage of pregnancy and lactation (Fig. 2&3). Within the total monocyte population we observed the greatest proportion of intM and lowest proportion of cM at early lactation 45-55 d following calving where all previous stages had relatively little change (Fig. 2 C&D, Fig. 3). Cows also experienced a nadir in the proportion of ncM 2 wk prior to calving, suggesting monocyte composition may relate to changes associated with preparation for labor in addition to pregnancy maintenance. Both intM and ncM have been associated with a pro-inflammatory response during various inflammatory conditions in humans, including preeclampsia in which affected pregnant women have significantly higher levels of both subsets indicating monocyte populations must be regulated to maintain a healthy pregnancy (Al-ofi et al., 2012, Melgert et al., 2012, Devevre et al., 2015, Tang et al., 2015). Bovine intM and cM share functional homology to their human counterparts; intM are highly pro-inflammatory and more mature than cM (Hussen et al., 2013, Hussen et al., 2014, Corripio-Miyar et al., 2015, Hussen et al., 2016). Minimizing inflammatory and allogenic responses throughout pregnancy by limiting the available reservoir of intM may be one possible mechanism in maternal immune tolerance, but without functional data we cannot confirm this.

Like monocyte composition, moDC maturation had dynamic, stage dependent changes over pregnancy and lactation. In line with our previous work, we observed the lowest levels of CD14 expression and greatest upregulation of MHC II expression following *E. coli* stimulation in moDC from non-pregnant stages of lactation compared to the early dry period which had the

highest and lowest, respectively (Fig. 3, Table 1S) (Pomeroy et al. 2015). Additionally, there was a significant nadir in the percentage of CD80+ moDC following maturation in the early dry period, with highest levels plateauing following calving carrying into early lactation (Fig. 3 C, Table 1S). High CD14 expression indicates moDC from the early dry period are phenotypically less mature in contrast to those from the post-calving period and early lactation. The nadir in co-stimulatory molecule CD80 and antigen-presentation molecule MHC II upregulation in the early dry period further indicates these moDC had hindered maturation which likely coincides with weaker T cell activation; the maximum upregulation of both these markers was observed in early lactation. Like in other species, bovine moDC differentiation and maturation may be influenced by the initial monocyte composition (Sanchez-Torres et al. 2001; Randolph et al. 2002). The increase in high MHC II expressing intM and decrease in cM at early lactation may relate to the peak in moDC MHC II upregulation observed at this time point (Fig. 3, Fig. 4D) (Hussen et al 2013). However, regression models of the dynamic, stage-dependent changes in markers of moDC maturation were not identical in shape to those of the starting monocyte composition suggesting other pregnancy factors also play a role in moDC maturation.

In addition to phenotypic changes, moDC cytokine production following maturation was also dependent on stage of pregnancy and lactation. Dendritic cells may secrete and upregulate of mRNA expression of IL-10 and IL-1 β depending upon the stimulant, though typically to a lesser extent when compared to M ϕ (Norimatsu et al., 2003, Langelaar et al., 2005, Heller et al., 2012). The greatest upregulation of both pro- and anti-inflammatory cytokines, IL-1 β and IL-10, by moDC was observed in the early dry period (Fig. 5). Our findings pertaining to IL-10 production go in line with our previous cross sectional study in which moDC from late gestation dry cows had greater upregulation of IL-10 following *E. coli*-stimulation compared to those from early

lactation cows (Pomeroy et al., 2015). As previously mentioned, moDC can be regulated by steroid hormones (Papenfuss et al., 2011, Xu et al., 2011, Lasarte et al., 2013). Poili et al. (2006) found estradiol enhanced production of IL-1 β in both human uterine macrophages and blood monocytes. Dairy cattle increasing circulating levels of estradiol in late gestation which greatly surpass levels observed at estrus; autologous serum used to culture moDC containing these circulating factors in addition to the monocytes prior *in vivo* exposure may be related to the rise in IL-1 β production we observed in the dry period (Echternkamp and Hansel, 1973, Smith et al., 1973). In non-bovine species, IL-1 β and IL-10 are key cytokines involved in priming the uterus for labor and preventing preterm labor in late gestation, respectively (Sato et al., 2001, Robertson et al., 2006, Boro et al., 2014). In conjunction with our previous work (Pomeroy et al., 2015) showing reduced moDC IL-12 production in late gestation cows, these increased levels of IL-1 β and IL-10 with unsuppressed TNF α levels are more indicative of M ϕ cytokine profile rather than that of DC (Werling et al., 2004, Denis and Buddle, 2008). This preferential *in vitro* differentiation of monocytes into M ϕ -like cells in late gestation despite the addition of exogenous GM-CSF and IL-4 to promote DC differentiation may relate to previous work describing the accumulation of M ϕ in the mammary gland during involution within the early dry period and in the endometrium approaching calving (Sordillo and Streicher, 2002, Oliveira and Hansen, 2008, Oliveira and Hansen, 2009).

4.1. Conclusion

This study demonstrates there are distinct, dynamic changes in monocytes and respective monocyte derived cells by stage of gestation and lactation in dairy cattle. The data indicate that cows experience a reduction in intM and ncM during pregnancy, and a nadir in phenotypic moDC maturation concurrent with a M ϕ -like cytokine profile in the early dry period of late

gestation. The reduction in monocytes capable of generating stronger inflammatory responses in conjunction with hindered DC maturation likely promotes and maintains a viable, full term pregnancy by preventing the development of conceptus-derived antigen-specific responses. Future research should address specific roles and function of monocyte subsets and respective derived cells in pregnancy maintenance and preparation for labor as it relates to the dynamic changes we observed.

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Tables

Table 1. Changes in absolute counts of monocyte subsets from peripheral blood over pregnancy and lactation. Mean with standard deviation of all enrolled animals (n=7, missing data at MG (n=5) and ED (n=6)). Bold indicates time points that had one or more significant differences from other time points (p<0.05).

	Mid-gestation	Early dry	Late dry	Post-calving	Early lactation
Total monocyte population (CD127a+) 10 ⁵ cells/ml	4.97534 (±1.06231)	5.15939 (±1.34377)	4.76751 (±1.33409)	7.5881 (±2.67147)	4.99155 (±1.79158)
Classical monocytes (CD172a+CD14+CD16-) 10 ⁵ cells/ml	2.79034 (±0.99817)	3.63731 (±0.90306)	3.646 (±0.68536)	3.6753 (±3.22558)	3.09059 (±1.08718)
Intermediate monocytes (CD172a+CD14+CD16+) 10 ⁵ cells/ml	1.04582 (±1.62374)	0.57665 (±0.26718)	0.74694 (±0.59223)	2.58210 (±2.24307)	1.06926 (±0.51890)
Nonclassical monocytes (CD172a+CD14-CD16+) 10 ⁵ cells/ml	0.83274 (±1.14193)	0.61384 (±0.68549)	0.17373 (±0.13838)	2.8976 (±2.71907)	0.60789 (±0.58731)

Figure Legends

Figure 1. Blood sampling timeframe over pregnancy and lactation. Sample time points starting in mid-gestation (MG) at 90-110d carrying calf (dcc)/approximately 190-170 d prior to parturition (P) estimated by insemination date and final blood samples taken in subsequent lactation in early lactation (EL) at 45-55d after parturition. The dry period begins at approximately 60d prior to the expected calving date. The ‘-’ signifies prior to, and ‘+’ signifies after parturition. Abbreviations: parturition (P), days carrying calf (dcc), days dry (d dry), mid-gestation (MG), early dry (ED), late dry (LD), post-calving (PC), early lactation (EL).

Figure 2. Changes in monocyte subset proportions from peripheral blood over pregnancy and lactation. The mean of the proportion (A) of CD172a+ cells in peripheral blood mononuclear cells (PBMC). Gating strategy on CD172a+ population used to determine relative proportion and absolute counts of individual subsets (B). The mean of the proportion of classical monocytes (cM) (C), intermediate monocytes (intM) (D), and nonclassical monocytes (ncM) (E) within total monocyte population (cM+intM+ncM). (n=7, missing data at MG (n=5) and ED (n=6)). Error bars represent SEM. Significant changes (minimum and maximums) were determined using polynomial regression; only significant fits as indicated with triangles and solid black lines were depicted. Terms of the regression models were considered significant if $p < 0.05$ when tested against the null hypothesis that the coefficients of the terms were zero. All regression models correct for repeated measures.

Figure 3. Changes in CD14+ monocyte subset composition in culture isolated from peripheral blood over pregnancy and lactation. Black bars represent the means of cM counts estimated in culture and white bars represent the means of the intM counts estimated in culture. Error bases represent SEM. Counts of individual subsets were estimated by using proportion of intM and cM in blood and total number of monocytes plated. Significant differences indicated by * $p < 0.05$, ** $p < 0.01$

Figure 4. Phenotypic maturation induced by 24hr stimulation with UV irradiated *E. coli* ECC-Z stimulation in moDC generated from CD14+ monocytes over pregnancy into lactation. Representative histograms of moDC expression of CD14, CD40, CD80, and MHC II from one cow from MG to EL; dashed line represents secondary, negative staining control,

dotted line represents stained, unstimulated moDC, and solid line represents stained, *E. coli*-stimulated moDC (A) . Dynamics of relative expression as determined by geometric mean fluorescence intensity (MFI) of CD14 (B) in *E. coli*-stimulated, moDC. Dynamics of *E. coli*-stimulated moDC population positive for CD80 (C). Delta MFI of MHC II (D) in moDC to investigate MHC II upregulation in response to *E. coli* stimulation Circles represent the mean (n=7) at each time point (B-D). Error bars represent SEM. Significant changes (minimum and maximums) were determined using polynomial regression; only significant fits as indicated with triangles and solid black lines were are depicted. Terms of the regression models were considered significant if $p < 0.05$ when tested against the null hypothesis that the coefficients of these terms were zero. All regression models correct for repeated measures.

Figure 5. Cytokine production following 24hr stimulation with UV irradiated *E. coli* ECC-Z in moDC generated from CD14+ monocytes over pregnancy into lactation. IL-1 β (A,D), TNF α (B,E), and IL-10 (C,F,G) concentration (pg/ml/10⁶ unstimulated, iDC) in cell-free supernatant harvested 24hr following UV irradiated *E. coli* ECC-Z stimulation as determined by sandwich ELISA. Upregulation of cytokines determined by comparing unstimulated, iDC to *E. coli* ECC-Z, mDC (A, B, C). White bars represent unstimulated, immature moDC (media control), and black bars represent *E. coli* ECC-Z-stimulated moDC (A, B, C). Bars represent mean (n=7) with error bars representing SD. Significant differences were determined by repeated measures ANOVA and Tukey post-test and are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Significant changes (minimum and maximums) were determined using polynomial regression for (D-G); only significant fits as indicated with triangles and solid black lines were are depicted. Terms of the regression models were considered significant if $p < 0.05$ when tested against the

null hypothesis that the coefficients of the terms were zero. All regression models correct for repeated measures.