Title: Impact of *in vitro* treatments of physiological levels of estradiol and progesterone observed in pregnancy on bovine monocyte-derived dendritic cell differentiation and maturation

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Abstract

The specific factors which regulate differentiation and maturation of dendritic cells in bovine pregnancy remain unclear. We evaluated the influence of physiologically relevant *in vitro* treatments of progesterone (PG) and estradiol (E2) observed in late pregnancy on the differentiation and maturation of CD14⁺ monocyte-derived dendritic cell (moDC) from non-pregnant, lactating dairy cows (n=7). We found that moDC differentiated in the presence of both E2 and PG had impaired *E. coli*-induced phenotypic maturation, specifically a significant reduction in CD80 and MHC II expression. Contrary to our previous work characterizing moDC from late gestating dairy cattle, we did not observe an increase in CD14 expression relative to the untreated control; this increase was only observed in the current data in the dexamethasone-treated moDC. The moDC treated with a combination of both E2 and PG had significantly greater upregulation of anti-inflammatory cytokine IL-10 relative to the untreated control, but TNF α production was not suppressed; only dexamethasone-treated moDC showed abrogated TNF α production. These data suggest moDC may be regulated by E2 and PG to hinder phenotypic maturation and regulate inflammatory responses. Pregnancy-associated hormone profiles appear to be involved in the generation of maternal immune tolerance in pregnancy. These hormone-facilitated changes to moDC in pregnancy may also impede optimal immune responses to both invading pathogens and routine vaccinations administered in late gestation through limited antigen presentation and increased anti-inflammatory cytokine production. These results provide insight into maternal immune modulation and elucidate potential immune changes necessary to facilitate bovine pregnancy.

Keywords

Bovine; monocyte-derived dendritic cell; estradiol; progesterone

Abbreviations

DC (dendritic cell), Dex (dexamethasone), E2 (estradiol), iDC (immature moDC), M ϕ (macrophage), mDC (mature moDC), moDC (monocyte-derived dendritic cells), MOI (multiplicity of infection), PBMC (peripheral blood mononuclear cells), PG (progesterone), T helper 1 (Th1), T helper 17 (Th17)

1. Introduction

During pregnancy the maternal immune system is regulated by both maternal and fetal factors to generate tolerance toward the semi-allogenic fetus (Nahum et al., 2004, Schumacher et al., 2014, Fair, 2015). Across multiple species dendritic cells (DC) are known to play a crucial role in generating maternal immune tolerance toward fetal antigens by preventing the development of highly inflammatory, fetal antigen-specific responses (Zenclussen et al., 2005, Kahn and Baltimore, 2010, Petroff, 2011). Our previous work indicated that these cells are regulated in bovine late gestation (Bachy et al., 2008, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Pomeroy et al., 2015). We found previously that relative to non-pregnant cows, monocyte-derived DC (moDC) from cows in late gestation had hindered phenotypic maturation and altered cytokine secretion in response to *E. coli* stimulation and thus were likely to have a poor ability to activate T cells during late gestation. We also showed that these moDC from cows in late gestation showed heightened IL-10 production and hindered MHC II upregulation (Pomeroy et al., 2015). Hormone profiles unique to late gestation may affect the ability of DC to prime and activate T cells during this stage of pregnancy.

Multiple factors in pregnancy have been shown to regulate various maternal immune cell populations; these cells may be regulated differently depending on the stage of pregnancy in order to meet the specific demands for successful implantation, maintenance of pregnancy, and preparation for parturition (Bauersachs and Wolf, 2013, Gomez-Lopez et al., 2014, Robertson and Moldenhauer, 2014). In murine and human models, the ability of DC to differentiate and mature can be altered by pregnancy-associated hormones, including estradiol (E2) and progesterone (PG), both of which are highly upregulated in pregnancy (Lamote et al., 2004, Segerer et al., 2009, Xu et al., 2011, Cordeau et al., 2012, Lasarte et al., 2013, Schumacher et al.,

2014). The relationship of these two hormones, E2 and PG, appears to be complex and dependent on context and concentration. The majority of studies show immunosuppressive effects of both E2 and PG on DC differentiation and maturation i.e. reduction in CD80 and MHC II, enhanced anti-inflammatory cytokine production with concurrent suppressed pro-inflammatory cytokine responses, and T cell stimulatory capacity (Hughes et al., 2008, Segerer et al., 2009, Jones et al., 2010, Xu et al., 2011, Lasarte et al., 2013). Both E2 and PG are relatively high in bovine late gestation relative to other stages of lactation, and in the case of E2, this hormone drastically increases as cows approach calving in the third trimester of pregnancy (Hunter et al., 1970, Echternkamp and Hansel, 1973, Smith et al., 1973). Previous work in dairy cattle showed that *in vitro* treatments of PG had an effect on lymphocyte transcription factors which may promote T regulatory cell formation, and E2 and PG influence the function of neutrophils. However despite the importance of DC in maternal immune regulation across other species, we lack an understanding of the impact of these hormones on DC (Lamote et al., 2004, Lamote et al., 2006, Maeda et al., 2013).

Here we aim to expand upon our previous work and study the effects of two major pregnancy hormones on moDC differentiation and maturation to further understand what factors may regulate changes observed in late gestation. The objective of this study was to analyze differentiation and maturation of bovine moDC, derived from blood CD14⁺ monocytes with exogenous *in vitro* treatments of physiological levels of E2 and PG found in late gestation. We investigated phenotype and function analyzing surface marker expression (CD14, CD40, CD80, MHC II) and cytokine production (TNF α , IL-10) following *E. coli* stimulation.

2. Materials and methods

2.1. Animals

Seven Holstein–Friesian cows were included in the study. These cows were either in their second or third lactation, were greater than 30 days in milk (DIM) and had not been bred. Cows were selected from the Cornell University Veterinary College Teaching Dairy Barn. No disease (i.e. metabolic, reproductive, mastitis) was observed 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. All animal procedures were approved by the Cornell Institutional Animal Care and Use Committee (project number: 2007-0110).

2.2. Generation of peripheral blood CD14+ moDC

Monocyte-derived DC were generated from CD14+ monocytes as described previously by Pomeroy et al. (2015). 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% heat-inactivated charcoal/dextran treated FBS (lot# J13137, Atlanta Biologicals), 20 ng/ml recombinant bovine IL-4 and, 20 ng/ml recombinant bovine GM-CSF both from Kingfisher Biotech), plated in 24-well tissue culture plates at $5-7 \times 10^5$ cells in 1 ml differentiation media per well containing hormone treatments, 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 0.5ml/well of

fresh differentiation media containing the respective treatments. Monocytes were treated with 65 pg/ml of E2 (E2758, Sigma Aldrich), 10 ng/ml of PG (P8783, Sigma Aldrich), a combination of both E2 and PG, or 50ng/ml of dexamethasone (DEX) (D4902, Sigma Aldrich); an untreated control was cultured in differentiation media with no addition of exogenous hormones.

Dexamethasone was used as a positive control to show suppression of moDC differentiation and maturation (van Kooten and Gelderman, 2011). Estradiol and PG treatments were selected based on range of circulating levels measured in plasma and serum documented in the literature and measurements determined by radioimmunoassay (radioimmunoassay performed by the Endocrinology Laboratory in the Animal Health Diagnostic Center/ New York State Veterinary Diagnostic Laboratory accredited by the American Association of Veterinary Laboratory Diagnosticians) from cows in 60-30 days carrying calf (data not shown) (Hunter et al., 1970, Smith et al., 1973).

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 broth (Merck Millipore, Billerica, MA, USA). 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 re-suspended 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 (Pomeroy et al., 2015). Cultures of moDC without bacterial stimulation served as media controls. Cell culture supernatants were collected after 24 h *E. coli* stimulation and stored at -80°C until further cytokine analysis by ELISA. Cells were also harvested at this time for phenotypic analysis by flow cytometry using Accutase™ Solution according to the manufacturer's instructions (Merck Millipore).

2.5. Flow cytometry

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 IgG_{2α} or Alexa Fluor® 647 conjugated monoclonal goat antibodies against mouse IgG₁ (Life Technologies). Secondary controls were of moDC stained only with conjugated secondary antibodies. No differences were found in baseline autofluorescence as determined by consistent MFI of secondary controls across samples (data not shown). Discrimination between live and dead cells was done by adding propidium iodide (PI, 1 µg/ml, Sigma Aldrich). 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 and TNFα 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 µg/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 µg/ml; CC320; AbD Serotec) was added. All samples and standards were assayed in duplicate.

Supernatant levels of TNFα were measured according to using a commercially available kit according to the manufacturer (bovine TNFα DuoSet® ELISA, DY2279, R&D Systems, Minneapolis, MN).

2.7. Statistical analysis

All data were analyzed using the GraphPad Prism 5 statistical analysis program (GraphPad Software, Inc., La Jolla, CA USA). Initially, data were graphed and evaluated for outliers. Outliers were inspected and reanalyzed where necessary. Significant changes in moDC surface marker expression, and moDC cytokine production between untreated control, was determined by testing whether the fold change over untreated control was significantly different from 1 i.e. no fold change from untreated control, using a non-parametric, Wilcoxon-ranked signed test. A type I error of ≤ 0.05 was used.

3. Results

As determined by percent of cells negative for propidium iodide, cell viability was not impacted by hormone treatment; no differences were detected between treatment groups and untreated controls in either unstimulated, immature DC (iDC) or *E. coli*-stimulated mature DC (mDC) cultures (Supplementary data, Fig. 1S).

3.1. *In vitro* E2 and PG treatments hinder MHC II and CD80 expression

Few changes in surface marker expression were detected following differentiation in unstimulated iDC populations. The iDC treated with E2 showed a moderate decrease in CD40 and CD80 following the differentiation period relative to untreated control iDC (median fold change=0.918, $p=0.0313$; 0.5526, $p=0.0156$ respectively), however, following maturation with UV-killed *E. coli* CD40 expression increased across treatments and E2 treated mDC reached comparable expression levels as untreated controls. Dexamethasone treatment significantly increased CD14 expression (3.77, $p=0.156$; 3.1, $p=3.13$), decreased CD40 expression (0.584, $p=0.0156$; 0.604, $p=0.0156$), and decreased MHC II expression (0.157, $p=0.0156$; 0.0864, $p=0.0156$) in both iDC and mDC. However, dexamethasone had no significant impact on CD80 expression relative to the untreated control (Fig. 1 a-i). Treatments with E2, PG, and combination of E2 and PG hindered the increase in expression of MHC II following maturation (0.8702, $p=0.0156$; 0.793 $p=0.0156$; 0.825, $p=0.0156$). Similarly, hindered MHC II expression by PG could be observed prior to *E. coli*-stimulation in iDC (0.847, $p=0.0313$) (Fig. 1 a,e,i). In addition to MHC II expression, unlike dexamethasone, E2 and PG treatment significantly decreased expression of CD80 in mDC at comparative levels (0.8055, $p=0.0156$; 0.8076, $p=0.0313$) and the combination of E2 and PG had a similar trend which approached significance (0.8731, $p=0.0938$). Reduced CD80 expression by E2 treatment could be observed prior to stimulation in

iDC and the combination of E2 and PG had a similar trend that also approached statistical significance (0.5526, $p=0.0156$; 0.6667, $p=0.0781$) (Fig. 1 a,d,h).

FIGURE 1

3.2. Combined treatment of E2 and PG enhances IL-10 upregulation following UV-killed *E. coli*-stimulation

Interleukin 10 was detected at varying levels in supernatant from unstimulated moDC. Therefore, delta IL-10 (*E. coli*-stimulated mDC–unstimulated iDC) was used to compare IL-10 upregulation following *E. coli* stimulation (Fig. 2 a). Upregulation of IL-10 following UV-killed *E. coli*-stimulation was enhanced in moDC treated with a combination of both E2 and PG relative to untreated controls (1.258, $p=0.0156$). However individual treatments of either E2 or PG alone did not enhance IL-10 upregulation (Fig. 2 a). Interestingly, dexamethasone treatment did not enhance IL-10 production (Fig. 2 a).

No TNF α was detected in the supernatant from unstimulated iDC across treatments thus only concentrations of TNF α produced from *E. coli*-stimulated mDC were compared to untreated control mDC. We found mDC treated with E2 or PG or the combination of E2 and PG were capable of upregulating TNF α and there was no significant increase or decrease relative to the untreated control. There was a trend in moDC treated with both E2 and PG towards an increase in TNF α production (1.363, $p=0.0781$) (Fig. 2 b). Dexamethasone treated moDC had abrogated TNF α production, and the majority of samples were below limits of detection resulting in a drastic decrease relative to untreated control mDC (0, $p=0.0147$) (Fig. 2 b).

4. Discussion

Dendritic cells are known to be key drivers of maternal immune tolerance and are regulated to accommodate unique needs at each stage of pregnancy by multiple pregnancy-associated

factors; factors which regulate these cells in bovine pregnancy have yet to be determined (Blois et al., 2007, Della Bella et al., 2011, Negishi et al., 2012, Leno-Duran et al., 2014, Schumacher et al., 2014, Pomeroy et al., 2015). In this study, we analyzed the effect of *in vitro* exposure to physiological levels of E2 and PG found in late gestation on aspects of moDC phenotype and cytokine production from non-pregnant dairy cows. Our findings suggest levels of E2 and PG in late gestation regulate DC to promote tolerance through inhibition of phenotypic maturation and increase in the production of anti-inflammatory cytokine IL-10.

We were able to recapitulate some aspects of hindered phenotypic maturation observed in late gestation in moDC from non-pregnant lactating cows using *in vitro* treatments of E2 and PG at levels typically found in circulation during late gestation. We also observed some differences following the differentiation period, in unstimulated iDC in E2 or PG treated cultures relative to untreated controls where treated cells had a reduced expression of co-stimulatory markers (CD40, CD80) or MHC II, respectively (Fig. 1 a-e). Following maturation induced by UV-killed *E. coli* stimulation, the moDC treated with these pregnancy-steroid hormones had reduced expression of CD80 and MHC II (Fig. 1 a, f-i). These results agree with our previous work characterizing moDC from cows in late gestation without addition of exogenous hormones; specifically we had observed hindered MHC II expression following *E. coli* stimulation in moDC from non-lactating cows in late gestation (Pomeroy et al. 2015). Though dexamethasone suppressed MHC II like E2 and PG it did so to a much greater extent. Interestingly, unlike treatments of E2 and PG, dexamethasone treatment did not suppress CD80 expression, but rather it suppressed CD40 expression and further greatly enhanced CD14 expression even following maturation. This difference in pregnancy steroid hormones and dexamethasone effect suggests that pathways which hinder maturation in moDC likely differ between these hormones (Fig. 1)

(Yoshimura et al., 2001, Franchimont, 2004). The decrease in co-stimulatory molecule CD80 and antigen-presentation molecule MHC II upregulation in E2 and PG treated moDC indicates that pregnancy steroid hormones may play a role in modulating the immune response specifically in DC and may relate to weaker T cell activation in late gestation.

In addition to phenotypic changes, moDC cytokine production was influenced by *in vitro* hormone treatment. The upregulation of anti-inflammatory cytokine, IL-10 following maturation was relatively higher in moDC treated with both E2 and PG however numerically the data suggest PG is likely the main driver in these changes (Fig. 2 a). Our findings pertaining to IL-10 production go in line with our previous work 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). In non-bovine species, IL-10 is a key cytokine involved in preventing preterm labor in late gestation, and maintaining tolerances (Sato et al., 2001, Robertson et al., 2006, Boro et al., 2014). Pro-inflammatory cytokine, TNF α , was not significantly altered with E2 or PG treatment, though there was a trend with treatments including PG suggesting it may elevate production of TNF α . In contrast to E2 and PG, dexamethasone treatment abrogated TNF α production (Fig. 2 b). These cytokine data suggests again that the pathway in which dexamethasone suppresses moDC maturation is likely different from E2 and PG because dexamethasone suppressed pro-inflammatory TNF α while no significant changes were found with regards to IL-10 production, but the combination of E2 and PG enhanced IL-10 production but did not suppress TNF α production (Yoshimura et al., 2001, Franchimont, 2004). The observed upregulation of TNF α may be necessary at various stages of pregnancy, and argument that is similar for certain inflammatory cytokines and what is observed in other mammalian species, (Monzon-Bordonaba et al., 2002, Okuda and Sakumoto, 2003, Fair, 2015).

It may also be possible that this pro-inflammatory cytokine is regulated by other factors in pregnancy and not by E2 or PG. Candidates for regulation of pro-inflammatory cytokines were suggested to be metabolic metabolites such as adiponectin (Kabara et al., 2014).

This study demonstrates the effects of pregnancy-associated factors, E2 and PG present at elevated levels simulating bovine late gestation, on moDC and reveals that these steroid hormones may be modulators of maternal immune response in late pregnancy. Previous work in other species generally found these hormones to hinder maturation and alter cytokine profile of DC generally minimizing T cell activation and highly inflammatory responses; for example, Xu et al. (2011) found *in vitro* treatments of PG diminishes the expression of maturation markers CD80 and MHC II, and elevated IL-10 while concurrently reducing IL-12 production, (Butts et al., 2008, Hughes et al., 2008, Jones et al., 2010, Xu et al., 2011). In non-bovine species, these hormones have been shown to inhibit the NF- κ B pathway in multiple ways including, the suppression of translocation via I κ B α or interfere with NF- κ B transcriptional activity. Suppression of the NF- κ B pathway negatively impacts effective antigen presentation by DC (Yoshimura et al., 2001, Davies et al., 2004, Lasarte et al., 2013). Diminished DC maturation generated by E2 and PG likely promotes and maintains a viable, full term pregnancy by preventing the development of conceptus-derived antigen-specific responses (Arck et al., 2007). In addition to the effects on DC, both E2 and PG have been shown to regulate other bovine immune cells including neutrophil phenotype and function and T cell transcription factors indicating these hormones play a larger role in maternal immune regulation and work in concert to generate tolerance (Lamote et al., 2004, Lamote et al., 2006, Maeda et al., 2013). Future research should investigate further functional aspects modulated by these hormones, determine if this relates to changes observed *in vivo*, and understand the pathways which are regulated by E2

and PR signaling. In addition to a more in-depth analysis of the effects of E2 and PG on bovine moDC, other pregnancy-associated factors should also be addressed; unlike humans and rodents, dairy cattle experience drastic metabolic changes over the course of pregnancy and lactation that are likely to impact moDC phenotype and function (Sordillo et al., 2009, Rulle et al., 2012, Sim et al., 2016).

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Figure Legends

Figure 1. MoDC phenotypic differentiation and maturation induced by 24h stimulation with UV IR *E. coli* ECC-Z. Representative histograms of moDC expression of CD14, CD40, CD80, and MHC II; dashed line represents secondary, negative staining control, dotted line represents stained, unstimulated iDC, and solid line represents stained, *E. coli*-stimulated mDC (a). Fold changes over untreated, control in relative expression of CD14 (b,f), CD40 (c,g), CD80 (d,h), and MHC II (e,i) as determined by geometric mean fluorescence intensity (MFI) in unstimulated, immature moDC (b-e) and UV IR *E. coli*-stimulated moDC (f-i). Solid black bars represent median (n=7). Error bars represent interquartile range. Significant differences were determined using a Wilcoxon-ranked signed test comparing medians to hypothetical value of 1. * indicates significant of $p < 0.05$.

Figure 2. MoDC IL-10 and TNF α production following maturation induced by 24h stimulation with UV IR *E. coli* ECC-Z. Fold changes over untreated, control in upregulation of IL-10 (a); upregulation of IL-10 i.e. Δ IL-10 was defined as the difference in concentration of IL-10 in cell-free, cell culture supernatant between UV IR *E. coli*-stimulated moDC and unstimulated, immature moDC. Fold change over untreated, control in TNF α production in cell culture supernatant from UV IR *E. coli*-stimulated moDC (b). Solid black bars represent median (n=7). Error bars represent interquartile range. Significant differences were determined using a

Wilcoxon-ranked signed test comparing medians to hypothetical value of 1. * indicates significant of $p < 0.05$.

Figure 1

Figure 2