Microvascular Endothelial Cells of the Bovine Corpus Luteum: A Comparative Examination of the Estrous Cycle and Pregnancy

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Abstract. Endothelial cells derived from the corpus luteum (CLENDOs) exhibit diverse characteristics presumably serving their wide-ranging roles in luteal function and fate. Here, several attributes of CLENDOs derived from cows at midcycle (days 9–12 of the estrous cycle) were compared with CLENDOs from early pregnancy (day 60 of pregnancy). Flow cytometric analysis of cells fluorescently-tagged with the lectins Bandeiraea simplicifolia (BS-1) and Concanavalin A (ConA) indicated that CLENDOs of midcycle CL do not differ from those of pregnancy. Mean fluorescence intensity for BS-1 was 15 ± 1 and 23 ± 7 fluorescent units for midcycle CLENDOs and CLENDOs of pregnancy, respectively (P>0.05). For ConA, mean fluorescence was 25 ± 2 and 26 ± 1 fluorescent units, respectively (P>0.05). The CLENDOs were also exposed to cytokines to assess differences in activation of nuclear factor kappa B signaling (NF-κB), induction of the transcription factor interferon regulatory factor 1 (IRF1), cytokine production, and cytokine-induced cell death. In response to TNF, for instance, both types of CLENDOs exhibited a rapid, 5-fold decrease in NF-κB inhibitor alpha (NFKBIA) protein expression (P<0.05), and a 4-fold increase in IRF1 expression (P<0.05), that did not differ with phenotype (P>0.05). Similarly, both types of CLENDOs produced tumor necrosis factor alpha and chemokine ligand 2 in response to IFNG stimulation (P<0.05) that did not differ with phenotype (P>0.05). Lastly, extended exposure of CLENDOs of midcycle CL to cytokines induced cell death (~50% cell death vs. control) similar to the incidence of cell death seen previously in CLENDOs of early pregnancy. The results indicate that several physical and functional characteristics of CLENDOs of midcycle CL are retained through early pregnancy, including lectin-binding properties, sensitivity to cytokines, and the activation of cytokine-initiated intracellular signals.

Key words: Bovine, Corpus luteum, Cytokine, Endothelial cell, Lectin

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helial cells might differ physically and functionally between CL of the estrous cycle and pregnancy. The objective of the current study was to determine whether reproductive status (i.e., non-pregnant vs. pregnant) influences the diversity of microvascular endothelial cells derived from bovine CL (CLENDOS). Lectin binding properties (i.e., binding of Bandeiraea simplicifolia and Concanavalin A) were compared between CLENDOs of the estrous cycle and pregnancy as the basis of physical attributes. Effects of the cytokines tumor necrosis factor alpha (TNF) and interferon gamma (IFNG) were assessed as measures of functional characteristics; specifically, cytokine-induced intracellular signaling [NF-kB inhibitor alpha (NFKBIA) and interferon regulatory factor 1 (IRF1)], cytokine production [TNF and chemokine ligand 2 (CCL2)] and cell death were evaluated.

Materials and Methods

Reagents

Plastic culture vessels were purchased from Corning (Corning, NY, USA) and Becton-Dickinson (Franklin Lakes, NJ, USA). NUNC-Immuno MaxiSorp 96-well ELISA plates were purchased from Nalge Nunc (Rochester, NY, USA). Microvascular endothelial cell medium (EGM-2MV) was obtained from Cambrex Bioscience (Walkersville, MD, USA). Minimum Essential Medium Eagle (MEM), Hanks’ Balanced Salts (HBSS), fetal bovine serum (FBS), 0.25% trypsin/0.2% EDTA, FITC-labeled BS-1 and ConA, α-D-galactose, α-D-glucose, monoclonal anti-β-actin, and protease and phosphatase I and II inhibitor cocktails were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gentamicin was obtained from Invitrogen (Carlsbad, CA, USA). HRP-linked anti-rabbit IgG was purchased from Cell Signaling Technology (Danvers, MA, USA). Interferon regulatory factor 1 (IRF1, C-20) and NF-kB inhibitor alpha (NFKBIA, C-21) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence reagent kit (ECL) was obtained from Amersham Biosciences (Piscataway, NJ, USA). Recombinant murine TNF was purchased from United States Biological (Swampscott, MA, USA). Recombinant bovine IFNG and bovine TNF screening sets (Pierce Biotechnology, Rockford, IL, USA) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human monocyte chemoattractant protein 1/chemokine ligand 2 (CCL2) DuoSet ELISA Development System was from R&D Systems (Minneapolis, MN, USA). All remaining reagents and materials were purchased from Sigma, VWR International (West Chester, PA, USA) or Thermo Fisher Scientific.

Cell culture

Aliquots of purified CLENDOS of midcycle (days 9–12 of the estrous cycle; day 0 = day of ovulation) and early pregnancy (day 60), isolated using methods previously described [17], were used in all experiments. As in the previous paper [17], purified microvascular endothelial cell populations derived from bovine CL were generated commercially by Cambrex Biosciences (BioWhittaker, Walkersville, MD, USA). The cells were cultured in EGM-2-MV with 3% FBS, growth factors [recombinant long R insulin-like growth factor-1, recombinant human fibroblast growth factor-B, recombinant human vascular endothelial growth factor, recombinant human epidermal growth factor, hydrocortisone, ascorbic acid, *Note: exact concentrations of growth factors are proprietary information, antibiotic (30 μg/ml gentamicin sulfate), and anti-fungal (15 ng/ml amphotericin-B)] in a 37 C humidified incubator of 95% air and 5% CO2. Bovine pulmonary arterial endothelial cells (BPAECs; American Type Culture Collection (ATCC); Manassas, VA) were cultured in MEM with 10% FBS and 20 μg/ml gentamicin in a 37 C humidified incubator of 95% air and 5% CO2.

Lectin binding experiments

CLENDOS of midcycle (passage 5) and early pregnancy (passage 5) were seeded at a density of 30,000 cells/ml in T25 flasks. At confluence, cells were removed from each flask by enzymatic treatment using HBSS modified with 2 mM EDTA and 0.25% trypsin with 0.2% EDTA. The cell suspensions were spun at 200 × g for 10 min by centrifugation, counted using trypan blue dye exclusion, and concentrated at 400,000 cells/ml. One ml of the cell suspension was placed into five 12 × 75 mm polystyrene round bottom test tubes and centrifuged at 250 × g for 10 min. Cell pellets were resuspended and incubated with 100 μl PBS (control) or FITC-labeled Bandeiraea simplicifolia (BS-1) and Concanavalin A (ConA) at a concentration of 2.5 μg/ml for 1 h at 4 °C. The lectins BS-1 and ConA have been used routinely to isolate endothelial cells and to assess their functional characteristics within the CL [18–23], including at least one study in which lectin binding was compared between luteal microvascular endothelial cells of the estrous cycle and pregnancy [24]. Additional controls included samples containing the competing sugar to BS-1 and ConA (200 mM α-D-galactose and α-D-glucose, respectively) [24]. After the 1 h incubation, cells were rinsed with HBSS modified with 2 mM EDTA and centrifuged for 10 min at 250 × g. Cell pellets were then fixed by resuspension in 375 μl PBS and 125 μl 4% paraformaldehyde. The fixed cells were placed on 12 × 75 mm cell strainer (35 μm) capped polystyrene round bottom test tubes and centrifuged for 5 min at 200 × g in preparation for flow cytometric analysis. The cells were analyzed using a four-color, dual-laser FACSCalibur machine (BD Biosciences, Palo Alto, CA, USA) and data were collected using Cellquest (BD Biosciences). Mean fluorescence lectin binding was calculated using WinMDI software (Joseph Trotter, Scripps Research Institute). BPAECs (200,000 cells/ml) were used as a positive control of BS-1 and ConA binding for comparative purposes [25, 26].

Cytokine-stimulated intracellular signaling experiments

TNF-induced degradation of NFKBIA

CLENDOS of midcycle (passage 6) and early pregnancy (passage 6) were seeded at a density of 30,000 cells/ml and cultured in 6-well plates to confluence (1–2 weeks). Prior to cytokine treatment, cells were equilibrated in pre-warmed, gassed, and serum-free basal medium (EBM-2) for 2 h. CLENDO cultures were exposed in duplicate to vehicle (control) and TNF (50 ng/ml) for 15 min. After cytokine treatment, the cells were placed on wet ice to terminate the experiment. The conditioned medium was removed and the cells rinsed with cold PBS and then lysed using a lysis buffer (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1
mM EGTA and 1% Triton X-100) containing protease and phosphatase I and II inhibitor cocktails. The cell lysates were sonicated for 5 sec and then centrifuged at 10,000 × g for 10 min. NF-κB inhibitor alpha (NFKBIA) degradation was quantified by western blot analysis using an NFKBIA (C-21) primary antibody.

Cytokine-induced IRF1
CLENDOs of midcycle (passage 5) and early pregnancy (passage 7) were cultured in 6-well plates to confluence. Prior to cytokine treatment, fresh serum-free medium was applied to the cells. CLENDO cultures were exposed in duplicate to vehicle (control), TNF (50 ng/ml), and IFNG (30 ng/ml) for 90 min. After cytokine treatment, the cells were placed on wet ice to terminate the experiment. Conditioned medium was removed and the cells were rinsed, lysed, and sonicated as described above. IRF1 was quantified by western blot analysis using an IRF1 (C-20) primary antibody.

Western blot analysis
Protein samples (40 μg) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred (75 V for 2 h) to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked with 5% fat-free milk in TBST (Tris-buffered saline Tween-20: 50 mM Tris HCl pH 7.5, 0.15 M NaCl and 0.05% Tween-20) at room temperature for 1 h. Membranes were probed with primary antibodies to NFKBIA (C-21) or IRF1 (C-20), each used at a dilution of 1:1,000 with 5% fat-free milk in TBST overnight at 4 °C. Four 5 min washes with TBST were performed prior to membrane incubation with an anti-rabbit peroxidase conjugated IgG used at a 1:2,000 dilution (TBST with 5% fat-free milk) for 1 h. Following four washes with TBST, bound antibody was detected using ECL detection reagents according to the manufacturer’s instructions. The blots were then exposed for 1–10 min using the Kodak Image Station 440 to determine band intensities. Blots were re-probed with β-actin (1:5,000) antibodies and the intensity of β-actin was used as an internal control to normalize the data.

IFNG-induced TNF secretion
CLENDOs of midcycle (passage 6) and early pregnancy (passage 7) were seeded at a density of 30,000 cells/ml in 24-well plates and grown to confluence. Prior to cytokine treatment, fresh serum-free medium was applied to the cells. CLENDOs were exposed in duplicate to vehicle (control) and IFNG (30 ng/ml) for 24 or 48 h. Upon completion of the time-points, conditioned medium was collected and TNF was measured using an enzyme-linked immunosorbent assay (ELISA).

Cytokine-induced CCL2 secretion
CLENDOs of midcycle (passage 5) and early pregnancy (passage 7) were cultured in 6-well plates to confluence. Prior to cytokine treatment, fresh serum-free medium was applied to the cells. CLENDO cultures were exposed in duplicate to vehicle (control), TNF (50 ng/ml), IFNG (30 ng/ml), and TNF + IFNG for 48 h. Upon termination of the experiments, conditioned medium was collected. Secretion of CCL2 into culture medium was measured using a commercially available sandwich ELISA specific for bovine TNF.

Immunoassays
TNF: TNF secretion from CLENDOs was measured using a commercially available sandwich ELISA specific for bovine TNF. All samples were run in duplicate in 96-well plates at a volume of 100 μl conditioned medium/well. A standard curve was created using recombinant bovine TNF at concentrations ranging from 39 to 2,500 pg. The detection limit of the assay was 29 pg and the mean interassay coefficient of variation was 7%. Results are expressed as pg of TNF per ml of conditioned medium.

CCL2: CCL2 secretion from CLENDOs was measured using a commercially available sandwich ELISA specific for human CCL2. Use of this assay kit for the measurement of bovine CCL2 has previously been validated [27], and verified [17, 28]. All samples were run in duplicate in 96-well plates at a volume of 100 μl conditioned medium/well. A standard curve was created using recombinant human CCL2 at concentrations ranging from 16 to 1,000 pg. The detection limit of the assay was 2 pg and the mean interassay coefficient of variation was 6%. Results are expressed as pg of CCL2 per ml of conditioned medium.

Cytokine-induced cell death
Cytokine-induced death of CLENDOs of early pregnancy has been reported previously by our laboratories [29]. In the current experiment, cytokine-induced death of CLENDOs of the estrous cycle was assessed in a similar manner. Briefly, CLENDOs of midcycle (passage 5) were seeded at a density of 30,000 cells/ml in 6-well plates and cultured to confluence. Along with fresh serum-free medium, the cells were exposed to vehicle (control), TNF (50 ng/ml), IFNG (30 ng/ml), and TNF + IFNG for 48 h. After 48 h the number of attached cells in each culture well was counted in random microscopic fields (minimum of 3 fields per well) and averaged for the duplicate wells. The percentage of viable cells (i.e., attached cells) was calculated against the control cultures and compared across treatments. The work was repeated three times (n=3 separate experiments) using a fresh stock of CLENDOs of midcycle for each experiment.

Statistical analyses
Results were analyzed by two-sample t-test, paired t-test or by ANOVA using the general linear model procedure of Minitab (State College, PA, USA) or Systat (Point Richmond, CA, USA) followed by a Tukey’s multiple comparison test. The results are expressed as mean ± SEM and represent three independent experiments. A P value of less than 0.05 was considered significant.

Results
CLENDOs bind BS-1 and ConA
The binding properties of lectins have been used to isolate endothelial cells and assess their functional characteristics [18–23]. In this study, lectin binding was assessed in CLENDOs from bovine CL of midcycle and early pregnancy, but did not differ (Figs. 1 and 2). Flow cytometric analysis of FITC-labeled lectins revealed comparable binding of BS-1 (Fig. 1) and ConA (Fig. 2)
No differences solely attributable to reproductive status were evident. The specificity of lectin binding was demonstrated by displacement of binding with the appropriate competing sugar (P<0.05); α-D-galactose for BS-1 (Fig. 1) and α-D-glucose for ConA (Fig. 2).

**verification of lectin binding**

Lectin binding of BS-1 and ConA was also assessed in BPAECs as a positive control (Fig. 3) [25, 26]. It was immediately evident that binding of ConA was greater than BS-1 in these cells of macrovascular origin (P<0.05). As above, specificity of the lectin binding for both BS-1 and ConA was verified by displacement with α-D-galactose and α-D-glucose, respectively (P<0.05, Fig. 3).

**Cytokines induce NFKBIA degradation and IRF1 expression in CLENDOs**

The responsiveness to cytokines can be monitored by their ability to stimulate intracellular signaling pathways like NFκB and the induction of cytokine responsive transcription factors like IRF1 [30]. Acute exposure of the CLENDOs to TNF (15 min) induced a 5-fold decrease in NFKBIA (P<0.05) in both cell types, indicating that TNF stimulates NFκB signaling (Fig. 4). But there was no effect of reproductive status on NFKBIA degradation in response to TNF (P>0.05, Fig. 4). Extended exposure of the CLENDOs to either TNF or IFNG (90 min) triggered a robust increase (4-fold and 14-fold, respectively) in IRF1 protein expression (P<0.05, Figs. 5 and 6, respectively). There was no difference in cytokine-induced IRF1 expression, however, due to reproductive status (P<0.05, Figs. 5 and 6).
Cytokines induce TNF and CCL2 secretion by CLENDOs

Cytokines and chemokines have been implicated in luteal regression, but little is known about their production by endothelial cells of the corpus luteum. The production of TNF by CLENDOs increased in a concentration dependent manner in response to IFNG stimulation (P<0.05, Fig. 7). However, there was no difference in TNF production attributed to reproductive status (P>0.05, Fig. 7). The conditioned medium from these cultures also revealed an increase in CCL2 concentration in response to TNF and IFNG stimulation (P<0.05, Fig. 8), but again the responses were not influenced by reproductive status (P>0.05, Fig. 8).

Cytotoxic effect of TNF on CLENDOs of the estrous cycle

The cytokines TNF and TNF + IFNG are cytotoxic to CLENDOs of early pregnancy, reducing cell viability by approximately 30 and 50%, respectively [29]. In the current study, exposure of CLENDOs of midcycle CL to TNF or TNF + IFNG resulted in a similar degree of cell death after 48 h (~50% cell death for TNF and TNF + IFNG, respectively, compared to controls; P<0.05). CLENDOs of midcycle did not differ from CLENDOs of pregnancy with respect to the effect of TNF (P>0.05). Bars represent relative intensity of NFKBIA normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in NFKBIA degradation among the treatments and cell types (P<0.05).

Discussion

Here we have determined that microvascular endothelial cells derived from bovine corpora lutea (CLENDOs) of the estrous cycle and pregnancy have similar properties for lectin binding and responsiveness to the cytokines TNF and IFNG. Specifically, the extent of BS-1 and ConA binding was comparable in CLENDOs of both stages of CL. TNF induction of NFKBIA degradation, IRF1 protein expression, and cell death were also similar in CLENDOs of the estrous cycle and pregnancy. IFNG stimulated IRF1 produc-
Fig. 5. TNF-induced IRF1 expression in CLENDOs of the estrous cycle and pregnancy. CLENDOs of midcycle CL and pregnancy were treated for 90 min with control media or TNF (50 ng/ml). A representative western blot (Fig. 5A) and a bar graph (Fig. 5B) depicting average changes in TNF-induced IRF1 expression in CLENDOs are shown. CLENDOs of midcycle did not differ from CLENDOs of pregnancy with respect to the effect of TNF (P>0.05). Bars represent relative intensity of IRF1 normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in IRF1 expression among the treatments and cell types (P<0.05).

Fig. 6. IFNG-induced IRF1 expression in CLENDOs of the estrous cycle and pregnancy. CLENDOs of midcycle CL and pregnancy were treated for 90 min with control media or IFNG (30 ng/ml). A representative western blot (Fig. 6A) and a bar graph (Fig. 6B) depicting average changes in IFNG-induced IRF1 expression in CLENDOs are shown. CLENDOs of midcycle did not differ from CLENDOs of pregnancy with respect to the effect of IFNG (P>0.05). Bars represent relative intensity of IRF1 normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in IRF1 expression among the treatments and cell types (P<0.05).

Fig. 7. IFNG-induced TNF production by CLENDOs of the estrous cycle and pregnancy. TNF production by CLENDOs of midcycle CL and pregnancy in culture over a 48 h treatment period as determined by ELISA of conditioned culture medium. The average response to IFNG at 30 ng/ml and 300 ng/ml is shown. Bars represent mean protein concentration ± SEM (n=3 experiments). Different letters denote differences in TNF protein concentration among the treatments and cell types (P<0.05).

Fig. 8. Cytokine-induced CCL2 secretion by CLENDOs of the estrous cycle and pregnancy. CCL2 secretion by CLENDOs of midcycle CL and pregnancy in culture over a 48 h treatment period as determined by ELISA of conditioned culture medium. The responses to the cytokines TNF and IFNG are shown. Bars represent mean protein concentration ± SEM (n=3 experiments). Different letters denote differences in CCL2 protein concentration among the treatments and cell types (P<0.05).
might be best validated by other approaches to ensure the isolation of pure populations of endothelial cells (e.g., CD31 and von Willebrand factor immunostaining; acetylated LDL uptake) and rule out the presence of contaminating cell types in the preparation. Physiologically, cell surface expression of carbohydrates influences endothelial cell targeting, migration and angiogenic capability [23].

Microvascular endothelial cells are a major target of cytokines during immune processes, particularly inflammation, in most tissues. Knowing that large numbers of microvascular endothelial cells comprise the CL [33], endothelial cells and their responsiveness to cytokines are reasoned to be important in the process of luteal regression. Here we have discovered two intracellular signaling pathways responsive to TNF and IFNG in CLENDOs. Nuclear factor kappa B (NF-κB) activation, via NFKBIA degradation, and the transcription factor IRF1 were induced equally in CLENDOs of the estrous cycle and pregnancy. Others have noted similar signaling events of TNF and IFNG in bovine steroidogenic cells [30], but this is the first report to our knowledge investigating these aspects of cytokine signaling in bovine CLENDOs.

Activation of the NF-κB signaling pathway stimulates the production of proinflammatory mediators, including TNF and CCL2 [34, 35]. In the current study, TNF-induced degradation of NFKBIA supports the concept that NF-κB activation occurs in both types of CLENDOs. Hence, proinflammatory pathways within the microvascular cells of CL, complementing those of luteal steroidogenic cells, can be activated during either the estrous cycle or pregnancy. Certainly the observation that CLENDOs produce TNF (current study) and CCL2 (current study [17, 28]) is consistent with the idea that microvascular endothelial cells of the CL contribute to immune-mediated aspects of luteal regression.

TNF-induced expression of IRF1 further validates the concept of NF-κB activation, as IRF1 induction is mediated by NF-κB [36]. IRF1 expression is also provoked by signal transducers and activators of transcription (STAT). IFNG, which stimulates the Janus kinase (JAK)-STAT pathway, is a potent inducer of IRF1 [37]. Here, CLENDOs of the estrous cycle and of pregnancy were equally responsive to IFNG stimulation, as evidenced by increased IRF1 expression. IRF1 regulates the transcription of IFNG-responsive genes, such as MHC expression [38], and thus may upregulate the expression of MHC class II molecules on IFNG-treated, bovine luteal steroidogenic cells [39] and CLENDOs [40] during luteal regression.

One culminating effect of cytokine action in CLENDOs is the occurrence of cell death. Cytokine-induced apoptotic death of CLENDOs of pregnancy has been previously documented [29]. In the present study, TNF-induced death of CLENDOs of the estrous cycle was similar to that reported by Pru and co-workers for CLENDOs of pregnancy [29]. However, unlike the previous study, the combination of TNF + IFNG did not enhance the incidence of death. Instead, IFNG provoked a morphological change in select cells, which resulted in enlarged, “senescent-like” cells and little or no cell death. Of note is the fact that this morphological change also occurred with IFNG treatment alone; but similar to CLENDOs of pregnancy [29], did not induce cell death. IFNG-induced phenotypic changes of CLENDOs have also been reported by others [41, 42]. We suggest IFNG negatively influences CLENDOs of the estrous cycle (triggers cell senescence?) but does not augment cell

Fig. 9. Cytokine-induced cell death in CLENDOs of the estrous cycle. Representative phase-contrast photomicrographs (25 x magnification) of CLENDOs of midcycle CL after 48 h of culture with the indicated treatments. Control cells exhibited primarily cobblestone morphology with the monolayer intact (A). TNF treatment resulted in cell death, characterized by fewer attached cells (B). IFNG-treated cultures contained cells with a large, flattened, senescent-like appearance (denoted by arrows) between areas of intact monolayer (C). The combination of TNF + IFNG treatment resulted in effects similar to TNF alone, with fewer cells attached, but also resulted in several large, flattened, senescent-like cells (denoted by arrows; D).
death. Instead IFNG may diminish the metabolic activity of these cells, thus provoking some of the cells to become cytostatic, yet avoiding cell death. An example of this would be autophagy.

The cellular source(s) of TNF within the CL has been debated for a number of years. For instance with the porcine CL, one study localized TNF to endothelial cells [43]; whereas another [44] identified macrophages as the major source of TNF. Neither study, however, directly measured TNF production using purified cell preparations and a quantitative immunoassay. In the current study, a bovine TNF ELISA was used to measure TNF concentrations in CLENDO-conditioned medium of the estrous cycle and pregnancy. Following IFNG stimulation, both cell types produced an equivalent amount of TNF in a dose-dependent manner. This study is the first to our knowledge to report TNF production by endothelial cells of the bovine CL, the implications of which include a possible autocrine effect of TNF on CLENDOs to influence cellular signaling and apoptosis. Considering TNF receptors are localized to small luteal cells (i.e., small steroidogenic cells and endothelial cells) of the CL [45, 46], endothelial cells might produce TNF under IFNG stimulation and respond to TNF in an autocrine manner. These possibilities raise the question of whether or not some actions of IFNG are mediated through TNF pathways, warranting further examination.

Our findings challenge speculation that distinctions exist between microvascular endothelial cells comprising the CL of the estrous cycle versus the CL of pregnancy, particularly with respect to lectin binding. It appears that only one other study has critically compared carbohydrate expression by these cell types. In that study, Plendl and coworkers [24] reported greater lectin binding for CLENDOs of pregnancy than the estrous cycle, and that lectin binding differed among morphological phenotypes. The discrepancy between our results and those of Plendl and co-workers might derive from differences in experimental approach. Specifically, the morphology of the cell types used in the current study was both cobblestone and arcuate in culture (results not shown), but no attempt was made to isolate or distinguish the two morphologies prior to lectin-binding experiments or flow cytometric analysis. Instead, the two morphologies were identified as the predominant phenotypes for both CL of the estrous cycle and pregnancy. In contrast, Plendl and coworkers used selective culture techniques to isolate and analyze the cobblestone-shaped from arcuate-shaped cells as previously described [47]. Thus, if specific morphologic phenotypes indeed have higher lectin binding characteristics than other phenotypes as Plendl et al. suggest [24], then combining the two predominant phenotypes without accounting for the actual or relative proportion of each (as in the current study) could mask potential differences in lectin binding. We suggest that further quantitative analysis of lectin binding be conducted to clarify these issues, especially considering that lectins are used widely for magnetic bead isolation of endothelial cell populations, but also because of the recognized role of carbohydrate expression (and thus, lectin binding) in cell-cell interactions.

In conclusion, the current study directly compared physical and functional attributes of CLENDOs of the estrous cycle and CLENDOs of pregnancy. Here the focus of the study was directed primarily toward cytokine responsiveness, as a prelude to immune-mediated aspects of luteal regression. The results indicate luteal microvascular endothelial cells do not change drastically as the corpus luteum transitions from the estrous cycle into early pregnancy. We acknowledge, however, that disparities may exist in the responsiveness of these cells to angiogenic stimuli (e.g., VEGF) or other signals not examined in the current investigation. Other researchers, for instance, have shown hormonal production and gene expression by CLENDOs are altered simply by passage of the cells [19, 48]. Such disparities have important implications in understanding the adaptable existence of the luteal microvasculature, but also in comprehending the basic processes of angiogenesis and angioregression. These observations are important not only to reproduction and fertility, but also to our understanding of angiogenesis and angioregression in cancer research.

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