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MICROVASCULAR ENDOTHELIAL CELLS OF THE BOVINE CORPUS LUTEUM: A COMPARATIVE STUDY OF THE ESTROUS CYCLE AND PREGNANCY

BY

Jessica A. Cherry
B.S., University of New Hampshire, 2003

THESIS

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Master of Science
in
Animal Science

September, 2006

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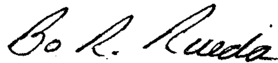

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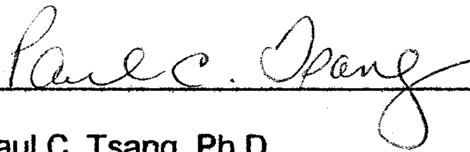

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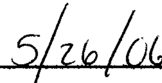

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ABSTRACT

MICROVASCULAR ENDOTHELIAL CELLS OF THE BOVINE CORPUS LUTEUM: A COMPARATIVE STUDY OF THE ESTROUS CYCLE AND PREGNANCY

by

Jessica A. Cherry

University of New Hampshire, September, 2006

Endothelial cells derived from the corpus luteum (CLENDOs) exhibit a diverse array of characteristics that seemingly complement their wide-ranging properties in luteal function and fate. Reproductive status of the animal (i.e., non-pregnant vs. pregnant) may contribute to the phenotypic/physiologic diversity of microvascular endothelial cells, but information in this regard is limited. Here, structural and functional attributes of CLENDOs derived from the estrous cycle (Days 9-12) were compared with those of pregnancy (Day 60). Initially, lectin binding properties were examined in the cells. Using fluorescent-tagged lectins for *Bandeiraea simplicifolia* I or concanavalin A, flow cytometric analysis indicated that lectin-binding on CLENDOs of the estrous cycle did not differ from those of pregnancy ($P > 0.05$). Next, the CLENDOs were exposed to tumor necrosis factor alpha (TNF; 50 ng/ml) and/or interferon gamma (IFNG; 30 ng/ml) to assess cytokine-induced intracellular signaling [inhibitor kappa B alpha (IκBα)

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degradation and induction of interferon regulatory factor 1 (IRF-1)], production of cytokines [TNF and monocyte chemoattractant protein 1 (CCL2)], and cell death. Western blot analysis revealed that both types of CLENDOs exhibited a rapid decrease in IκBα ($P < 0.05$), and an equally rapid increase in IRF-1 ($P < 0.05$), in response to cytokine treatment that was not influenced by reproductive status ($P > 0.05$). IFNG stimulated production of TNF by the CLENDOs ($P < 0.05$), revealing that CLENDOs are a source of TNF, regardless of reproductive status ($P > 0.05$). Based on these findings, the ability of TNF to mediate IFNG-induced effects was assessed using etanercept, a TNF antagonist. Etanercept inhibited direct actions of TNF with respect to CCL2 secretion and IRF-1 induction ($P < 0.05$), but did not alter IFNG effects ($P > 0.05$). Lastly, we determined by measuring the incidence of cell death that CLENDOs of the estrous cycle are similarly susceptible to TNF- and TNF + IFNG-induced death as CLENDOs of pregnancy. These results indicate that several attributes of CLENDOs from the estrous cycle are retained by those of early pregnancy, including lectin binding properties, activation of specific cytokine-initiated intracellular signals, and the instigation of cytokine-induced inflammatory events.

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CHAPTER I

LITERATURE REVIEW

Introduction

As the world's population exceeds 6.5 billion people, with estimates reaching 10 billion by the year 2050, the demand for food products grows ever greater [1]. In 2005, the estimated world consumption of beef and dairy products based upon some of the most populous countries (China, European Union, India, Russia, and the United States) was 50,000 metric tons and 85,000 metric tons, respectively [2, 3]. From this perspective, it would seem essential to increase reproductive rates in cattle to meet growing demands for beef and dairy products. Unfortunately, fertility rates are currently abysmal in cattle. Conception rates in lactating cows are approximately 35-45% [4]. Embryo mortality rates stand at 30-40% [5]. One of the most important aspects of early pregnancy is the development and maintenance of a functional corpus luteum (CL), a structure within the ovary that secretes the 'hormone of pregnancy', progesterone. Understanding the cellular and molecular characteristics of CL formation, function, and regression within the estrous cycle and throughout pregnancy are key components to improving fertility in cattle, thus optimizing beef and dairy production to feed the growing world population.

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Estrous Cycle

The bovine estrous cycle is approximately 21 days and is dominated and controlled by the CL. The estrous cycle is characterized by four distinct phases: estrus, metestrus, diestrus, and proestrus. Estrus is the phase during which the cow is sexually receptive to the bull. More importantly, it is at the end of this phase that ovulation or the release of the oocyte for fertilization occurs [6]. The mature, tertiary, or Graafian follicle is the structure that contains the oocyte. The follicle is a fluid-filled structure consisting primarily of two cell types, theca and granulosa cells [7]. The main function of the follicle, along with supporting the growth and maturation of the oocyte, is to produce estradiol. Estradiol is produced through the joint efforts of both the theca and granulosa cells, which carry out this process in what is known as the two-cell theory of estradiol production [8, 9]. Theca cells compose the outside of the follicle (theca externa) and contain the vasculature (theca interna). Granulosa cells constitute the inside cavity of the follicle and are separated from the theca cells and vasculature by a basement membrane [7]. Granulosa cells also form a mass surrounding the oocyte called the cumulus oophorus, which anchors it to the granulosa cell layer within the follicle [10]. As ovulation occurs (approximately 10 hours after the end of estrus – day 0), the oocyte is expelled from the follicle and travels into the oviduct to await fertilization [6]. With ovulation, the basement membrane separating the theca and granulosa layers of the follicle begins to disintegrate. This leads to an invasion of capillaries and new vessel formation extending from the theca interna [11]. Also, under the stimulation of luteinizing hormone (LH),

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theca and granulosa cells undergo a process known as luteinization, in which the cells transform into small and large luteal cells, respectively [12]. As a result of all these events, the remnants of the follicle transform into a different structure, both physically and functionally, the CL [6, 7].

At the conclusion of estrus, metestrus begins (days 1-4). This phase is characterized by the beginning of CL dominance and relatively low circulating levels of progesterone and estradiol. It is during this time that the CL matures and gradually increases its progesterone production. Estradiol production stems from an initial wave of follicle development, but these follicles eventually die by atresia due to the prevailing concentrations of progesterone present [6].

The next 10-15 days of the estrous cycle are described as the diestrus phase (days 5-18) and can be thought of as the classic period of the luteal phase when progesterone production is at its maximum. During diestrus, a second wave of follicular development may occur, but the follicles quickly meet the same atretic fate as those of the first wave. At approximately day 17, the CL undergoes the process of luteal regression if pregnancy does not occur. This is classically characterized and attributed to the uterine release of the luteolytic agent prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [6].

Once the process of luteal regression is initiated, proestrus begins (days 18-20). At this time, a final wave of follicular growth occurs, which eventually results in the dominance of a Graafian ovulatory follicle due to declining concentrations of progesterone. Concomitantly, increasing estradiol from the dominant follicle signals to the hypothalamus and pituitary gland in the brain.

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This triggers increased gonadotropin releasing hormone (GnRH) secretion, which leads to a release of LH and follicle stimulating hormone (FSH). A surge in LH secretion occurs approximately 12 hours after the peak in estradiol. Together, these effects result in the onset of estrus, ovulation (10 hours after the end of estrus), and the commencement of CL formation, function, and regression for the subsequent estrous cycle [6].

Corpus Luteum

The CL is a transient endocrine tissue that forms within the ovary from the remnants of the ovulated follicle. The primary responsibility of the CL is to maintain pregnancy, which was first described by Frankel in 1903 who discovered that removal of the CL resulted in abortion in rabbits [13, 14]. At the time, Frankel did not know the identity or nature of the substance responsible for maintaining pregnancy. However, 30 years later Allen and Corner described an extract from the CL that maintained the pregnancy of ovariectomized rabbits [14, 15]. The component isolated, subsequently called progesterone, was crystallized by Allen and Wintersteiner in 1934 [14, 16].

Progesterone is a steroid produced and secreted by the CL. It is primarily the 'hormone of pregnancy' that influences uterine function, but also has other biological targets and effects. Working in concert with estradiol, progesterone prepares the genital tract, uterus, and cervix to establish and sustain a pregnancy. Progesterone affects the mucosal lining of the genital tract, influencing the transport of the oocyte or fertilized ovum in the oviduct, prepares

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the uterus for implantation of the embryo and subsequent pregnancy, and increases the viscosity of cervical mucus, thereby providing a barrier between the uterus and the outside environment [14].

The CL consists primarily of four distinct cell types: large steroidogenic cells, small steroidogenic cells, endothelial cells, and fibroblasts. Steroidogenic cells make up approximately 30% of the tissue, while the most abundant cell type of the CL is the endothelial cell population, comprising approximately 50% of the midcycle tissue. Lastly, fibroblasts constitute approximately 10% of the total cell population, with immune cells and other unidentified cell types encompassing the remaining 10% [17].

As evidenced from the total numbers of endothelial cells, the CL is a highly vascularized tissue. The tissue is characterized by having a relatively high rate of blood flow (~30 mL/min x g of tissue) compared to other tissues (e.g. ~3 mL/min x g of tissue for the adrenal gland). Also, 65-95% of total ovarian blood flow is directed toward the CL during the midcycle phase [18]. The dense capillary network constitutes the communication system between the CL and the rest of the body; delivering hormones, nutrients, and substrates for steroidogenic capacity, and distributing progesterone to its target tissues [18, 19]. The vasculature is essential for the formation, maintenance, and regression of the CL, with the opposing processes of angiogenesis and angioregression, respectively, in the forefront.

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Angiogenesis and Corpus Luteum Formation

Angiogenesis is defined as the generation of new blood vessels through the sprouting (migration and proliferation) of existing blood vessels [20]. Endothelial cells are mainly responsible for angiogenesis. They must initiate a morphogenetic cascade in which extracellular matrix is degraded and in which cells migrate toward an angiogenic stimulus, proliferate, and organize into a network of new capillaries [21]. The process of angiogenesis occurs rarely in adults. It is primarily restricted to pathological conditions such as tumor growth and metastasis, and physiological conditions such as wound healing, inflammation, and processes pertaining to the female reproductive system (e.g., placentation) [22-24].

In the female reproductive system, angiogenesis occurs in the uterus and placenta during pregnancy, in addition to the mammary glands. Also, a hormonally-regulated, cyclic form of angiogenesis occurs in the uterus and ovaries throughout each estrous cycle [20]. In particular, the angiogenic process in the ovary is of paramount importance to angiogenesis during the formation of the CL. The relatively brief lifespan of the CL provides a useful model for understanding the natural angiogenic life cycle (formation, maintenance, and regression), important not only for reproduction, but also in terms of understanding angiogenesis in tumor growth and regression [24].

Angiogenesis in the ovary is primarily controlled by angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiopoeitins (Ang 1 and Ang 2). VEGF affects vasodilation,

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capillary permeability, and stimulates endothelial cell proliferation. The first angiogenic cytokine identified in the ovary was bFGF [25], which is a potent endothelial cell mitogen. Ang 1 and Ang 2 are important growth factors in the formation of new blood vessels [23, 26].

The angiogenic process in the follicle precedes angiogenesis in the CL, or luteal angiogenesis. Ovarian follicles consist primarily of four stages of growth: primordial, primary, secondary, and tertiary or Graafian. Primordial and primary follicles generally have no vascular network of their own and therefore are not associated with any aspects of angiogenesis. As follicles develop an antrum (secondary and Graafian follicles), a capillary network is established. There is a direct connection between growth and differentiation of these follicles and an increased density of blood vessels within the theca cell layers. The vascular network is restricted to the theca interna and does not penetrate the basement membrane into the granulosa cell layers. The granulosa layers of the Graafian follicle, therefore, are avascular. Nutrients and metabolites, including hormones, are exchanged via diffusion [19, 23, 24].

At ovulation, the basement membrane separating the granulosa and theca layers disintegrates, permitting the invasion of migrating endothelial cells. This process, called luteinization, is partially dependent upon angiogenesis. The CL undergoes massive growth in a matter of days and is accompanied by an equally massive neovascularization. In sheep, for example, new vessel formation in the CL is detected within 36 hours of ovulation [27] and more than 50% of the proliferating cells in the vascular compartment are endothelial cells [28]. The rich

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vascular network of the CL is established to support the dynamic endocrine function of the gland [23, 24]. Inadequate vascular formation may play a role in ovarian cystic diseases and reduced luteal function [23].

Corpus Luteum Maintenance

The maintenance of the vasculature is of major importance to the midcycle CL. Rate of endothelial cell proliferation decreases at this time compared to the forming CL, but there is still extensive angiogenesis [23, 29]. Steroidogenic capacity of the CL is at a maximum, with the vasculature maintaining an intimate relationship with the steroidogenic cells to ensure progesterone synthesis and secretion. In fact, each steroidogenic cell is in direct contact with at least two or more neighboring capillaries during this period of time [24].

Angioregression and Corpus Luteum Regression

At the onset of luteal regression, a decline in serum progesterone begins, followed by a rapid decrease in luteal weight [30]. This rapid loss of tissue is accompanied by a reduction of blood flow and loss of vasculature, a process known as angioregression, which results in the sharp decrease in numbers of endothelial cells. Angioregression is a process usually associated with tissue involution, such as scar formation during wound healing. According to Modlich et al. (1996) [21], there are two putative mechanisms by which angioregression occurs in the CL of the estrous cycle. The first is the loss of endothelial cell tight junctions and subsequent detachment of cells from the basement membrane of

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the microvasculature. In the guinea pig, early signs of endothelial cell degeneration within the CL include loss of tight junctions, protrusion of cells into the capillary lumen, and formation of adherens junctions with endothelial cells on the opposite side of the lumen [31]. Following detachment of the endothelial cells, apoptosis and subsequent removal of the apoptotic bodies from the circulation occurs. The second mechanism of angioregression is characterized by contraction and occlusion of arterioles and small arteries through proliferation of smooth muscle cells surrounding the vessels. Little is known about the endocrine events that lead to this mechanism of regression, but vessel occlusion suggests that contraction and limited perfusion of the CL may predispose it to endothelial cell detachment and deletion [21].

Another aspect of luteal regression is the role of the uterus. The uterus has a prominent role in regulating the lifespan of the CL and initiating the process of luteal regression in most mammals. Hysterectomy prolongs the lifespan of the CL in the cow [32], and luteal structure and function are normally maintained for the duration of a pregnancy after removal of the uterus [33]. $PGF_{2\alpha}$ is the hormone from the uterus that influences luteal lifespan [34]. Endometrial tissue concentrations of $PGF_{2\alpha}$ are maximal during luteal regression. But the strongest evidence that $PGF_{2\alpha}$ is involved in luteal regression stems from the fact that treating animals with prostaglandin synthesis inhibitors, such as indomethacin, blocks spontaneous luteal regression [14]. Also, immunizing cattle to $PGF_{2\alpha}$ prevents spontaneous luteal regression [14]. The luteolytic actions of $PGF_{2\alpha}$ differ among species and between *in vivo* and *in vitro* models. Proposed

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mechanisms of $PGF_{2\alpha}$ action include a large and rapid decline in luteal blood flow due to its vasoconstrictive effects, reduced LH receptor expression, and direct cytotoxic effects, to name a few [14].

Although the role of $PGF_{2\alpha}$ in luteal regression is important, it cannot account for all aspects of luteal regression. For instance, $PGF_{2\alpha}$ *in vitro* does not inhibit basal progesterone production by steroidogenic luteal cells, leading one to believe that other cell types or substances contribute to luteal regression [35]. One widely-recognized aspect of luteal regression in most species is the accumulation of immune cells and the onset of immune-mediated events that degrade tissue. At the time of luteal regression, T lymphocytes and macrophages increase in the CL of the bovine [36, 37], equine [38], murine [39], leporine [40], porcine [41], and human [42, 43]. An increase in major histocompatibility complex (MHC) class II expression on bovine [44], ovine [45], and human [42] steroidogenic cells also suggests immune-mediated mechanisms are in place. T lymphocytes and macrophages are important in the production of cytokines, and MHC class II molecule expression is essential to activating and targeting the immune response.

Activated T lymphocytes and macrophages in the CL produce cytokines, particularly interferon gamma (IFNG) and tumor necrosis factor alpha (TNF), respectively. IFNG and TNF mRNA expression is detectable in the CL throughout the estrous cycle, with TNF levels rising slightly during luteal regression [46]. The properties of IFNG and TNF will be discussed further in later sections, but in terms of luteal regression, they are anti-steroidogenic,

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apoptotic, and increase the expression of MHC molecules in steroidogenic luteal cells [47].

Another immunological component of luteal regression is the attraction and recruitment of immune cells through chemokines, which facilitate adhesion, activation, and chemotaxis of leukocytes. Of particular interest is monocyte chemoattractant protein 1 or chemokine ligand 2 (CCL2), a chemokine produced by endothelial cells of the CL [48], and known for its recruitment of monocytes, basophils, natural killer cells, and T lymphocytes [49]. CCL2 expression has been documented in the bovine [50, 51], murine [39, 52], and ovine [53, 54] CL. In the bovine CL, CCL2 expression (mRNA and protein) increases during the latter half of the estrous cycle (days 12-18) and is accompanied by an accumulation of immune cells, indicating CCL2 has a role in immune cell recruitment prior to and during luteal regression [51].

Another molecule implicated in luteal regression is the vasoconstrictive peptide, endothelin 1 (EDN1). EDN1 is a 21-amino acid peptide produced and secreted by endothelial cells [55]. It temporarily inhibits progesterone production by the CL in the murine [56], ovine [57, 58], bovine [55], and human [59]. EDN1 has become a major focus in luteal regression based on its potential role in $PGF_{2\alpha}$-induced luteal regression. During the early luteal phase, when the CL does not respond to $PGF_{2\alpha}$, EDN1 has no effect on progesterone secretion [60]. But, during the midcycle and late cycle CL, administration of $PGF_{2\alpha}$ in conjunction with EDN1 results in reduced plasma progesterone concentrations, which are reversed by an EDN1 antagonist [55, 58, 61]. These results indicate

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that EDN1 mediates luteolytic effects of $PGF_{2\alpha}$ from a functional standpoint [62]. However, there is no documentation that EDN1 alters the structural integrity of the CL [63].

Collectively, vasoactive peptides, such as $PGF_{2\alpha}$ and EDN1, and components of the immune system, including T lymphocytes, macrophages, cytokines, and chemokines, contribute to luteal regression. Further study is required to fully elucidate the signals affecting endothelial cell function within the CL during angioregression and subsequent luteal regression.

Corpus Luteum of Pregnancy

Gestation length for a cow is approximately 280 days. The CL is required to maintain the pregnancy for the first 200 days, as removal of the CL before this time results in termination of the pregnancy [64]. In the ovine CL, the morphological characteristics of steroidogenic cells of pregnancy are similar to those of the midcycle CL. The ability of steroidogenic cells to produce progesterone is maintained, but declines slowly as gestation progresses. Signs of cell death in isolated areas appear shortly before parturition. The structural and functional changes observed in the CL of pregnancy during regression are similar to those of the estrous cycle, with the exception that elimination of dead and dying cells occurs over a longer period of time [65].

Mechanistically, the maintenance of the CL of pregnancy is not fully understood. Maternal recognition of pregnancy through the secretion of interferon tau and inhibition of uterine $PGF_{2\alpha}$ secretion are among the

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mechanisms which must occur in order to maintain pregnancy [14]. In cattle, luteolytic effects of $PGF_{2\alpha}$ are blocked by an endometrial prostaglandin inhibitor or a factor produced by the embryo, bovine trophoblast protein-1 complex, also known as embryonic interferon or interferon tau [30, 66, 67]. Additionally, preventing an influx of immune cells and the actions of cytokines within the CL of early pregnancy appear to be essential. High concentrations of progesterone are immunosuppressive and inhibit lymphocyte proliferation and function [47] and MHC expression on luteal cells [68]. Nevertheless, in-depth knowledge of the signals and intracellular mechanisms involved in maternal recognition of pregnancy and the maintenance of the CL during pregnancy are not fully understood and therefore require further study.

Endothelial Cells of the Bovine Corpus Luteum

Endothelial cells composing the microvasculature of the bovine CL play a significant role in the formation, function, and regression of the CL. Angiogenesis is essential to the development of the gland; an extensive vascular network must be formed in order for the tissue to survive, as an inadequate vasculature is associated with reduced luteal function [23]. Maintenance and stability of the vasculature is paramount to the endocrine function of the gland, importing nutrients, substrates, and hormones, and exporting progesterone to its target tissues [18]. The endothelial cell component of the CL is the first to undergo cell death during luteal regression, thereby eliminating the blood supply to the tissue and dictating its fate [31]. The CL is a transient gland, but it is also one of the

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most highly vascularized tissues in the body, with endothelial cells composing approximately 50% of the total number of cells [17]. Given the crucial roles of endothelial cells within the CL, it is important to understand them from both morphologic and physiologic standpoints.

There are four distinct types of microvascular endothelial cells of the bovine corpus luteum (CLENDOs). Originally, five types were characterized, but the fifth was later attributed to be of immature granulosa cell origin, secreting progesterone and being unresponsive to LH stimulation [69]. The remaining four types of CLENDOs are characterized based upon morphology, function, surface molecule expression, and cytokeratin 8, 9, and 18 expression [70-75]. All cell types maintain contact-inhibited growth and unique morphologic characteristics in long-term cultures. Also, all express angiotensin converting enzyme (ACE) and internalize acetylated low-density lipoprotein (acLDL), which are hallmarks of endothelial cells [70].

Type 1 CLENDOs are isomorphic cells having a cobblestone appearance in confluent cultures [70]. They express factor VIII-related antigen and ACE, both of which are localized to the perinuclear area, and they exhibit moderate uptake of acLDL. The surface of type 1 cells contains many microvilli and globular protrusions in the central area of the apical side of the cell. They also express two families of adhesion molecules, neuronal cell adhesion molecule (NCAM-140) and epithelial cadherin (E-CAD). These cells stain positively for cytokeratin 18 (CK18) [71, 74].

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Type 2 CLENDOs are polymorphic cells with numerous nuclei and lacking prominent cobblestone morphology [70]. They are similar to type 1 cells in that they express factor VIII-related antigen and ACE, and exhibit moderate acLDL uptake. Similar to type 1 cells, type 2 cells are NCAM-140 positive, but do not express CAD molecules. These cells are also CK18 positive [71, 74].

Type 3 CLENDOs are spindle-shaped cells containing at least one prominent intracellular vacuole [70]. They have a smooth cell surface with processes at the poles, and express factor VIII-related antigen and ACE throughout the cytoplasm. Unlike type 1 and 2 CLENDOs, type 3 cells readily uptake acLDL and store it in lysosomes. They do not express NCAM or CAD, and they do not contain CK18 filaments [71, 74].

Type 4 CLENDOs are predominantly round cells with no intracellular vacuoles [70]. They have short filipodia radiating around the cell margins. Similar to type 3 cells, type 4 cells express factor VIII-related antigen and ACE throughout the cytoplasm, acLDL uptake is abundant in lysosomes, they do not express NCAM or CAD, and they are CK18 negative [71, 74].

Expression of the intermediate filament CK18 may be one of the more important distinctions among the unique classifications of endothelial cell types. The cytokeratin 18 positive (CK18+) phenotype is rare within CLENDOs of the bovine CL [70, 76, 77]. Morphologically and functionally, CK18+ CLENDOs are different from the more abundant cytokeratin 18 negative (CK18-) cells, but appear morphologically similar to CK18+ endothelial cells of the bovine aorta [76]. Factor VIII expression is 5-fold lower in CK18+ CLENDOs than CK18-

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CLENDOs, and acLDL uptake is 10-fold lower. Functionally, CK18+ and CK18- CLENDOs differ in cytokine production in response to TNF stimulation. TNF-induced granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed only by CK18- CLENDOs, and TNF-induced 'regulated on activation, normal T cell expressed and secreted' (RANTES) mRNA levels are 200 times higher in CK18- CLENDOs than CK18+ CLENDOs [75]. Conversely, CK18+ CLENDOs express mRNA for prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ receptors, while CK18- CLENDOs do not [78].

From the aspects of angiogenesis and angioregression, expression of angiopoeitins (Ang 1 and Ang 2) among the different CLENDO types is interesting. Both CK18+ and CK18- CLENDOs do not contain Ang 1 mRNA, and only CK18- contain Ang 2 mRNA [79]. Ang 1 and Ang 2 mRNA are present in the developing and functional CL [26]. Wulff et al. (2000) localized Ang 1 expression in steroidogenic and endothelial cells throughout the human CL [80], but its localization within the bovine CL has not been reported. Although its source is unknown, Ang 1 stimulates sprouting and maturation of blood vessels *in vitro*, while Ang 2 is a competitive inhibitor, binding to the same tyrosine kinase with immunoglobulin and epidermal growth factor homology domains (Tie-2 receptor), but not activating the intracellular signals of angiogenesis. Also, levels of Ang 2 are elevated during luteal regression, suggesting that Ang 2 has a role in angioregression [26, 63].

The endothelium is a major target for cytokines in most tissues. Endothelial cells serve as part of the barrier or 'gatekeeper' between the blood

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stream and the tissues, determining the traffic and action of cells and molecules within the body [81]. As previously mentioned, there are large numbers of endothelial cells in the CL and thus endothelial-immune cell interactions are reasoned to be important in luteal function. Treatment of CLENDOs with the cytokine IFNG alters their morphology, which might lead to important functional alterations. For instance, type 1-4 cells become flattened and enlarged upon IFNG treatment [63]. Type 3 cells form large, lipid-filled vacuoles, possibly indicating the onset of senescence [73]. IFNG also elevates CAD levels in type 1 cells, and disturbs actin structure and reduces fibronectin content in type 1, 3, and 4 cells [72]. As a result, IFNG could lead to an anti-proliferative effect, which is observed in type 1-4 CLENDOs [73]. Also, stimulation of CLENDOs with TNF, IFNG, or TNF + IFNG results in increased secretion of CCL2, suggesting a role for immune-mediated mechanisms in luteal function [48]. Similarly, placement of CLENDOs in co-culture with activated immune cells also results in increased CCL2 secretion [82].

In summary, the four types of CLENDOs within the bovine CL are morphologically and physiologically unique, but contribute to the formation, function, and regression of the microvasculature. Important distinctions based upon CK18 expression, $PGF_{2\alpha}$ receptor presence, and response to cytokines reveal that further study is required to determine the physiologic significance of these cell types during different phases of CL function, both during the estrous cycle and pregnancy.

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Interferon Gamma

Interferons were first characterized in 1957 by Isaacs and Lindenmann as substances that interfere with viral replication [83]. Their antiviral capabilities led to their discovery, but it is now known that interferons are one of the main classes of cytokines naturally produced in the body with far-reaching implications beyond the fight against disease. Specifically, interferons influence the overall function of the immune system and therefore the body as a whole.

There are two major groups of interferons, type I and type II, which are classified primarily by structural homology and receptor binding specificity. Type I interferons are separated into two main types, IFNα and IFNβ [84], but also include IFNδ, IFNε, IFNκ, IFNτ, and IFNω [85]. IFNα is a family of related proteins primarily produced by leukocytes, and IFNβ is a single protein produced mainly by fibroblasts. The type I interferons are widely expressed and largely induced in response to viral infections [84, 86]. In contrast, IFNγ (IFNG) is the only type II interferon. It is called an interferon solely on the merits of its antiviral effects, but it is a completely different molecule from the other interferons both in structure and action [87].

IFNG was originally referred to as 'immune interferon' or 'immune-induced interferon' because it is produced by lymphocytes following antigenic stimulation; whereas 'classical interferons' are molecules produced by leukocytes or non-lymphoid cells [88, 89]. As the old nomenclature describes, IFNG is primarily activated by immune and inflammatory stimulation. It was thought to be produced solely by T lymphocytes through MHC class I and II antigenic

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stimulation and by natural killer cells through TNF and macrophage cofactor stimulation. Recently however, professional antigen presenting cells such as monocytes/macrophages have also been identified as sources of IFNG [90, 91].

IFNG elicits major effects on cells of the immune system including regulating MHC class I and II expression on mononuclear phagocytes, endothelial cells, and epithelial cells. IFNG also activates and regulates the differentiation and function of monocytes/macrophages (hence IFNG once being known as 'macrophage-activating factor' [92]), and regulates B cell functions such as immunoglobulin production and isotype/class switching [84, 90, 91]. IFNG has a major role in inflammatory response, most notably through its synergistic action with TNF. The synergistic actions of IFNG/TNF will be discussed in detail later, but briefly, IFNG increases the expression of TNF receptors on the surface of cells [93, 94], and enhances TNF production by macrophages in an endotoxin (lipopolysaccharide)-induced inflammatory response [90].

The wide variety of IFNG actions is perhaps due to the relatively ubiquitous expression of IFNG receptor throughout the body [90, 95]. The IFNG receptor (IFNGR) is a transmembrane, multi-chain structure composed of two ligand binding units, IFNGR1, and two signal transduction units, IFNGR2 [84, 91] (Fig. 1). The extracellular domain of the receptor is the ligand binding site, while the intracellular domain functions in signal transduction. IFNGR1 is constitutively expressed and plays the critical role in receptor-binding recognition. In contrast, IFNGR2 has the major role of signal transduction and is the limiting factor in

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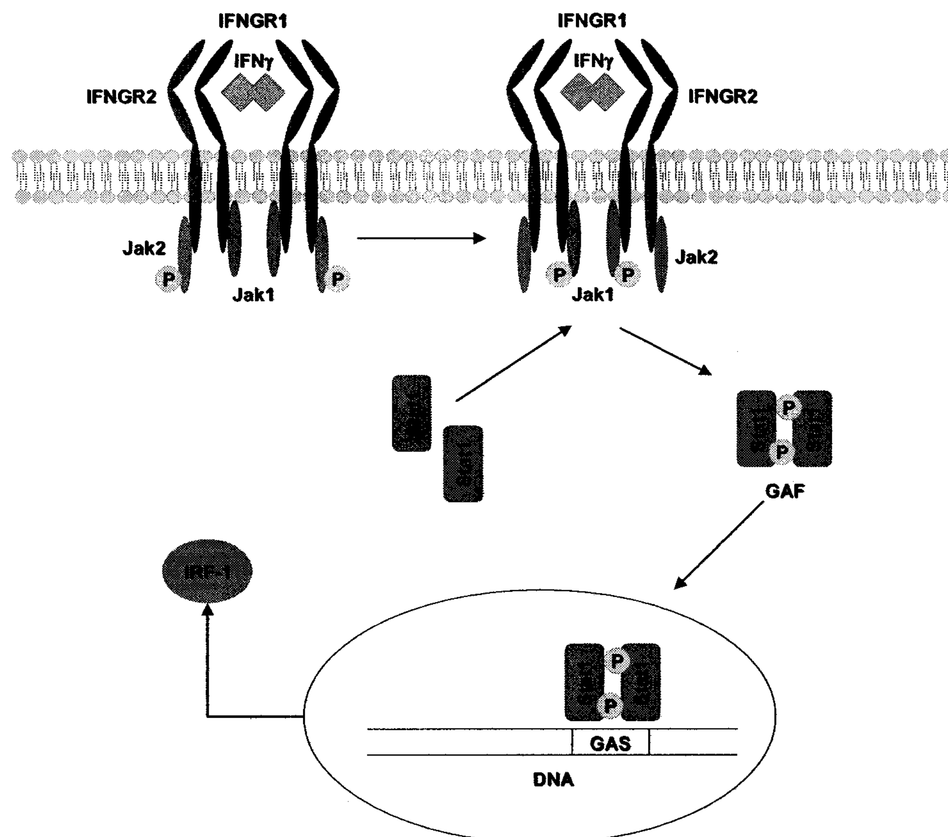
IFNG action. It is also constitutively expressed, but at low levels and under tight regulation [84]. Upon dimerization of the IFNG ligand subunits [96], subsequent binding to IFNGR1 occurs, resulting in IFNGR1 and IFNGR2 association [84]. The IFNG receptor alone is incapable of inducing an intracellular signal. Therefore, it associates with Janus tyrosine kinases (enzymes named for the Roman God Janus who had two faces). The Janus kinases (Jaks) have two faces/sites, one a binding site to associate with the IFNG receptor and the other a catalytic site with tyrosine kinase phosphorylation capabilities [97, 98]. In particular, IFNGR1 associates with Jak1 and IFNGR2 with Jak2 [84] (Fig. 1). These Jaks, in turn, interact with signal transducers and activators of transcription (Stat) signaling cascades.

Figure 1. IFNG signaling pathway.



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When the IFNGR1 and IFNGR2 subunits interact, Jak1 and Jak2 are activated by Jak2 autophosphorylation and subsequent transphosphorylation of Jak1 [91] (Fig. 1). The activation of Jak1 attracts a particular Stat, Stat1, from the cytoplasm to associate with the receptor complex [99] (Fig. 1). Upon association, Stat1 is phosphorylated and homodimerizes [100] to become gamma interferon activated factor (GAF) [101] and translocates to the nucleus (Fig. 1). In the nucleus, GAF targets gamma interferon activated site (GAS) elements on DNA to initiate or suppress transcription of IFNG-regulated genes (Fig. 1), such as the transcription factor interferon regulator factor 1 (IRF-1) [85].

IRF-1 is a member of an ever growing family of transcription factors known as interferon regulatory factors (IRFs). It was first characterized as a regulator of virus-inducible enhancer-like elements of the human IFNβ gene [102]. IRF-1 is expressed at low levels in unstimulated cells but is greatly induced upon stimulation with cytokines, such as IFNG and TNF [103]. IRF-1 activation is important in the development of T lymphocyte subsets (CD4+ and CD8+) [104] and is capable of inducing typical IFNG functions, such as increasing MHC expression [103].

The most crucial action of IRF-1 is its ability to activate transcription and interact with transcription factors, such as nuclear factor kappa B (NF-κB). IRF-1 as a transcription factor controls the expression of many different target genes, including cyclooxygenase 2 [105], an enzyme responsible for prostaglandin production in inflammation, and caspases 1 and 7, which regulate apoptosis [96].

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The role of IRF-1 in apoptosis can be either pro- or anti-apoptotic. IRF-1 activation alone does not lead to cell death unless accompanied by the actions of certain cytokines (fibroblast-associated (Fas) ligand and TNF + IFNG) or as result of a pathologic condition [103]. The pro-apoptotic actions of IRF-1, particularly induced by treatment of cells with TNF + IFNG, are of interest in the present study. Furthermore, IFNG-activated IRF-1 inhibits TNF-induced NF-κB activation, which is a protective, anti-apoptotic pathway [106]. Conversely, IRF-1 is thought to contribute to cell survival in certain physiologic situations [103].

In the bovine CL, IFNG mRNA is detectable at all stages of the estrous cycle [46]. Penny and co-workers (1999) reported the presence of IFNG mRNA around the time of luteal regression, which coincided with an infiltration of T lymphocytes, the primary source of IFNG [36]. In addition, IFNG mRNA declines during late diestrus (day 18) and at 1h post $PGF_{2\alpha}$-induced luteal regression [46]. Currently, there are no protein or absolute quantitative mRNA data regarding IFNG expression in the bovine CL.

Despite the relative lack of information available about IFNG expression, there are several postulated roles of IFNG in the bovine ovary. IFNG enhances Fas ligand (FasL)-induced killing of granulosa, theca [107], and luteal cells [108]. IFNG augments the expression of MHC class II molecules on bovine steroidogenic luteal cells [109] and microvascular endothelial cells of the CL (type 2 in particular) [110]. Along with promoting immune response mechanisms, IFNG enhances the production of $PGF_{2\alpha}$ by bovine luteal cells and inhibits LH-stimulated progesterone production [111].

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IFNG signaling in bovine luteal cells is transduced by the Jak-Stat pathway, but does not alter NF-κB expression. Stat1, but not IκBα, protein levels are significantly increased following IFNG treatment, indicating signaling through the Jak-Stat, but not NF-κB pathway. Also, IFNG influences the transcription and translation of IRF-1 [112].

Tumor Necrosis Factor Alpha

TNF was first described as an endotoxin-induced factor produced by macrophages [113] that causes necrosis of tumors [114]. For several years, the focus of TNF research centered on cancer biology [115]. Since the time of its characterization however, TNF is now known to regulate immune responses (inflammatory and apoptotic pathways), cellular proliferation and differentiation, tumorigenesis, and viral replication [116, 117]. Additionally, as TNF has a major role in regulating immune cell activity, it is also involved in pathological conditions, including autoimmune disorders, such as rheumatoid arthritis, irritable bowel syndrome [118], and psoriasis [119].

TNF is a cytokine produced primarily by macrophages, but also by B and T lymphocytes, mast cells, fibroblasts, endothelial cells, and neuronal tissue. It is a 26-kDa transmembrane protein cleaved by the metalloprotease TNF-alpha-converting enzyme (TACE) to a 17-kDa soluble form. TNF is arranged in stable homotrimers and is active in membrane-bound or soluble forms [117, 120].

TNF exerts its biological functions via the TNF receptor superfamily, particularly TNF-receptor 1 (TNF-R1) and TNF-receptor 2 (TNF-R2). Membrane-

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bound TNF readily activates both receptors, while soluble TNF predominantly signals through TNF-R1. TNF-R1 is constitutively expressed in most tissues, while TNF-R2 is highly regulated and restricted to cells of the immune system. Both receptors contain a pre-ligand-binding assembly domain (PLAD) that results in receptor trimerization upon activation by TNF [120].

The intracellular domains of the TNF receptors are different and of functional importance. TNF-R1 contains a protein-protein interaction domain referred to as a death domain (DD) that is crucial to the death-inducing activity of the receptor [120]. Upon binding of TNF, an inhibitory protein called the silencer of death domain (SODD) is released [117] (Fig. 2). The DD can then recruit other proteins containing DD, such as TNF receptor-associated death domain (TRADD). TRADD in turn attracts Fas-associated death domain (FADD), which initiates signaling events through caspase activation that lead to apoptosis [121] (Fig. 2).

TRADD also attracts TNF-R-associated factor 2 (TRAF2), which can lead to anti-apoptotic pathways. TRAF2 binding to TRADD results in the production of the anti-apoptotic protein cellular inhibitor of apoptosis protein-1 (cIAP-1) (Fig. 2). Also, TRAF2 attracts NF-κB-inducing kinase (NIK) and receptor interacting protein (RIP), which separately attract inhibitor of κB kinase (IKK), activating NF-κB (Fig. 2). Unlike TNF-R1, TNF-R2 does not signal through a DD. Instead, it directly recruits TRAF2, and signals through the NF-κB pathway, or induces the expression of membrane-bound TNF which subsequently activates TNF-R1 [117, 121].

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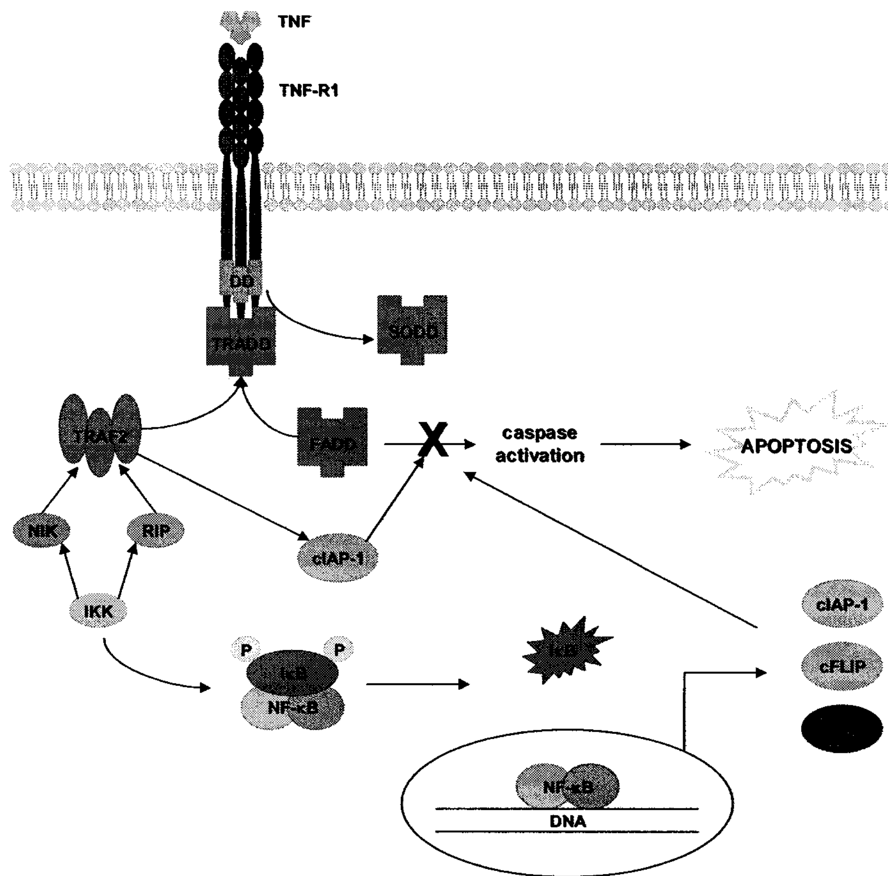
Figure 2. TNF signaling pathway.



As mentioned, a commonality between TNF-R1 and TNF-R2 is their ability to induce NF-κB activation [122]. NF-κB is a transcription factor located in the cytoplasm associated with an inhibitor complex, inhibitor κB (IκB), in an unactivated state. Upon activation of IKK, IκB is phosphorylated and relinquishes NF-κB, which then translocates to the nucleus and induces gene expression (Fig. 2). NF-κB activation increases expression of inflammatory proteins [123], and interestingly, anti-apoptotic molecules such as cIAP-1 [121], cellular FLICE-like inhibitory protein (cFLIP), and B-cell leukemia/lymphoma 2 (Bcl-2) [124] (Fig. 2). The latter products of NF-κB activation lead to an interesting paradigm of TNF receptor signaling, particularly TNF-R1. Ligand

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binding can lead to both pro-apoptotic and anti-apoptotic pathways via activation of DD-associated proteins. Also, the absence or inhibition of NF-κB activity results in increased cellular susceptibility to TNF-induced apoptosis, whereas activation of NF-κB protects against it [121]. These pro- and anti-apoptotic actions of TNF are of particular interest in the current study.

In the CL, the presence and localization of TNF and its receptors have been extensively studied in numerous species. In the rabbit, lipopolysaccharide-induced TNF production in the regressing CL is elevated, but also present in the early CL (day 5) [125]. There are large numbers of macrophages in the regressing CL of the rabbit, suggesting that macrophages are the source of TNF. But TNF secretion by the early CL and the scarcity of macrophages indicate that other cell types within the rabbit CL produce TNF [125]. Recent studies describing TNF secretion in the porcine CL have further added to the confusion of the cellular sources of TNF within the CL.

TNF protein is present in the porcine CL during the estrous cycle and pregnancy with no difference between the two physiologic states [126]. According to Hehnke-Vagnoni and co-workers (1995) [126], TNF is localized to the endothelial cells of functional CL, while macrophages are rarely seen and not associated with TNF staining. No TNF was detected in CL undergoing functional luteal regression [126]. In contrast, Zhao and co-workers (1998) [127] report that macrophages are the major source of TNF in the porcine CL. TNF is co-localized to cells that also stain positively as macrophages, whereas endothelial cells consistently lacked TNF staining. A bioassay of the individual cell

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populations incubated with lipopolysaccharide similarly revealed macrophages as the major source of TNF secretion in the porcine CL [127]. In the pig, TNF receptors are present and functional within the CL throughout the estrous cycle and early pregnancy. Receptor concentration increases in the late luteal stage of the estrous cycle (day 15) compared to the CL of pregnancy on the same day, suggesting a role for TNF in luteal regression [128]. TNF also induces apoptosis of porcine luteal cells [129]. TNF receptors are localized to small luteal cells (small steroidogenic and endothelial cells) of porcine CL [130], implying a possible autocrine action of TNF on endothelial cells of the CL.

In the bovine CL, TNF protein increases around luteal regression, days 13-18 of the bovine estrous cycle, but there is no difference in TNF mRNA expression throughout the estrous cycle [131]. Intraluteal TNF concentration increases after progesterone begins to decline, but there is no increase in systemic concentrations of TNF [132]. TNF-R1 mRNA levels are greatest in the early luteal phase (days 3-7) [131]. In CLENDOs, TNF receptor protein expression is seen in type 3 CK18- CLENDOs of the estrous cycle having two binding affinities (low and high) for TNF. TNF-R1 mRNA is expressed by the CLENDOs, but the levels are less than the CL as a whole [133].

In the bovine CL of pregnancy, TNF mRNA is present in equal amounts throughout gestation, but TNF receptor protein expression is higher during the early and late stages of pregnancy as compared to the middle of pregnancy [134].

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TNF has a variety of effects on the CL. *In vitro*, TNF alone and in combination with IFNG increases $PGF_{2\alpha}$ synthesis by bovine luteal cells [135]. TNF does not affect basal progesterone production but does suppress LH-stimulated progesterone production. *In vivo*, low concentrations of TNF promote luteal regression and shorten the estrous cycle, whereas high concentrations activate CL function and prolong the estrous cycle in cattle [136]. A low dose of TNF (1 μg) stimulates $PGF_{2\alpha}$ production but does not affect progesterone production. Conversely, a high dose of TNF (10 μg) stimulates progesterone and the luteotrophic PGE_2 [136]. These divergent effects of TNF highlight its potential role in regression of the CL, suggesting different concentrations could stimulate either pro- or anti-apoptotic pathways.

TNF promotes cell proliferation in rat ovarian theca-interstitial cells [137], yet induces apoptotic cell death in bovine CLENDOs [138, 139]. TNF-R1 mRNA expression is higher in endothelial cells of the CL than steroidogenic cells, and TNF induces time- and dose-dependent death in endothelial cells [138]. It is hypothesized that progesterone within the CL regulates cell survival in relation to TNF-induced death. Physiologic levels of progesterone prevent TNF-induced death of CLENDOs [138], and steroidogenic cells, which produce progesterone, are consistently protected against TNF-induced death [138]. Other luteolytic actions of TNF include increasing endometrial production of $PGF_{2\alpha}$ [140], enhancing MHC class I expression on bovine luteal cells [135], and inhibiting luteotrophic estradiol production in the porcine CL [141].

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With regard to the CL of pregnancy, TNF plays similar roles in its regression as mentioned for the CL of the estrous cycle, but TNF also potentially contributes to the maintenance of the CL of pregnancy. *In vitro*, TNF stimulates PGE_2 and $PGF_{2\alpha}$ production, which increase progesterone output by luteal cells. The output of PGE_2 and $PGF_{2\alpha}$ by the CL at day 200 of gestation is greater than at day 14 of the estrous cycle, and progesterone production from day 200 is increased by PGE_2 but not LH. Also, there are higher numbers of PGE_2 receptors in pregnant versus nonpregnant ewes [142], and large luteal cells are known to produce progesterone in response to PGE_2 [143]. Therefore, TNF stimulates the production of PGE_2 and $PGF_{2\alpha}$ by the CL of pregnancy, and indirectly increases its progesterone production [144].

Synergy of Tumor Necrosis Factor Alpha and Interferon Gamma

TNF and IFNG are two cytokines that influence the actions of each other. The synergistic action of TNF and IFNG was first documented as a cytotoxic effect in which the two cytokines in combination were more potent than either cytokine alone [145]. This synergy of TNF and IFNG to induce cell death has been documented for rat hepatocytes [146], mouse luteal cells [147], and bovine luteal cells [135, 148]. Originally, the synergistic action of TNF and IFNG was attributed to the regulation of TNF receptors by IFNG. That is, IFNG induces the synthesis of TNF receptors, resulting in an upregulation of both types of TNF receptors (TNF-R1 and TNF-R2) on cells [94]. The enhancement of TNF receptors, and thus TNF binding, was thought to be the mechanism for the

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cytotoxic effect [93, 149, 150]. However, others contend that IFNα and IFNβ do not have a significant effect on TNF receptors, but nonetheless enhance the cytotoxic activity of TNF [151]. Alternatively, intracellular signaling might account for the synergistic action of TNF and IFNG.

Intracellular signaling has a prominent role in regulating the effects of cytokines. Stat1 activation by IFNG and NF-κB activation by TNF are necessary for the induction of genes potentially important for mediating their cooperative activities, such as IRF-1. TNF and IFNG independently and synergistically promote the expression of IRF-1 [152]. IRF-1 inhibits NF-κB-mediated activation of cell survival signals through modulation of NF-κB transcriptional activity [106]. Therefore, IRF-1 induction by TNF and IFNG alone or synergistically might prevent activation of the anti-apoptotic NF-κB pathway, leading to cell death/apoptosis (Fig. 3).

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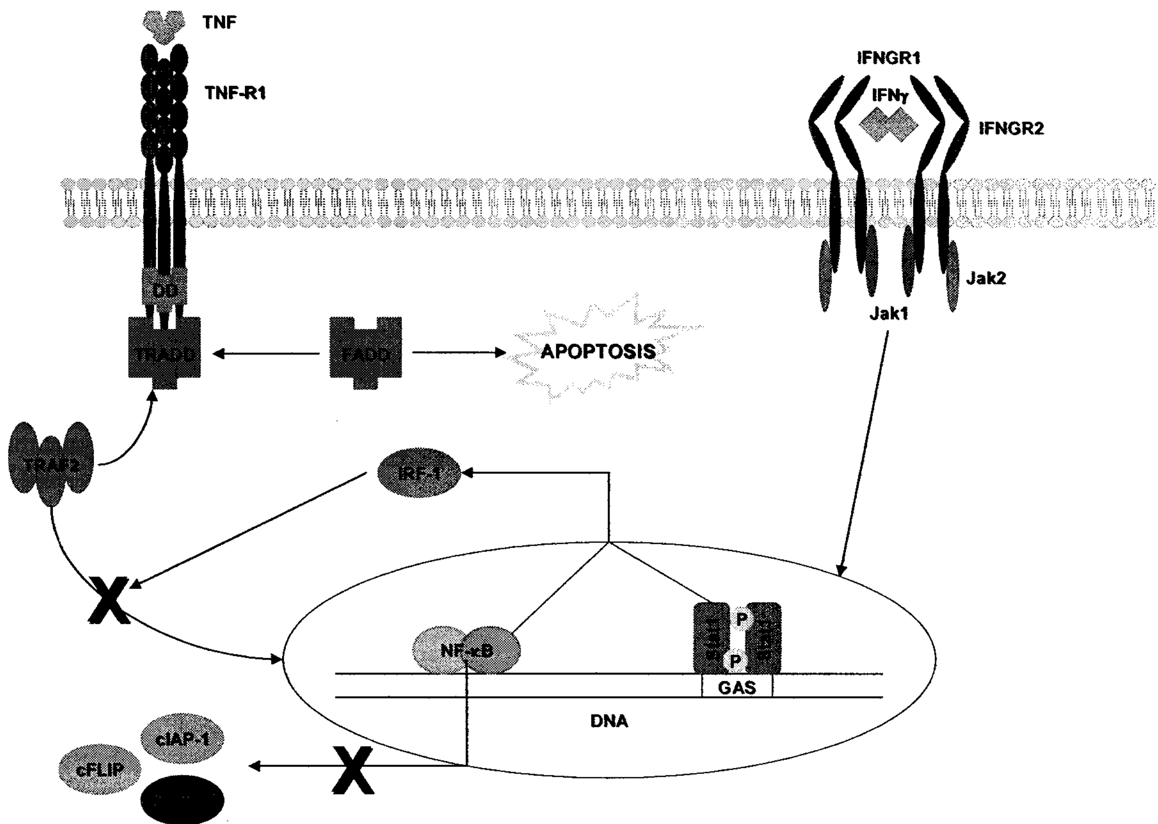
Figure 3. Synergistic death effect of TNF and IFNG.



Other mechanisms by which TNF and IFNG promote death and destruction of tissues include augmenting Fas-mediated apoptosis and enhancing elements of an immune response. In the ovary, TNF and IFNG potentiate Fas-mediated apoptosis of granulosa and luteal cells [108, 153, 154], increase MHC class I [155] and intercellular adhesion molecule I (ICAM-1) expression [156], and enhance the secretion of CCL2 [48, 82, 157].

Lectins

Lectins are a class of non-immunologic proteins and glycoproteins that bind specifically to carbohydrates. They were originally referred to as hemagglutinins or phytoagglutinins because they originate from plant extracts

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and have the ability to agglutinate erythrocytes [158]. Interestingly, ricin, the deadly toxin occasionally used by extremist groups in terrorist plots, was the first isolate from a plant, the castor bean (*Ricinus communis*), to show properties of hemagglutination. 'Lectin' comes from the Latin term *legere*, meaning to pick out or choose. The name lectin was chosen due to the blood-type specific hemagglutination exhibited by some of the extracts from plants [159]. For example, the extract from *Griffonia simplicifolia* (also known as *Bandeiraea simplicifolia*) has almost exclusive specificity for blood type B [160]. The name lectin now encompasses any non-immune origin, sugar-specific agglutinin, regardless of source (plant, animal, microbial) and blood type specificity [158]. Lectins from sources other than plants, such as animals, viruses, bacteria, protozoa, etc., are rarely used in the more common applications of lectins [161], which include precipitating carbohydrate-containing macromolecules, and agglutinating plant and animal cells along with bacteria and yeast [162]. Because lectins bind carbohydrate moieties on cell surfaces, they can be inhibited by sugars for which they are specific. For example, concanavalin A binds to D-mannose/D-glucose [163, 164]. Placing D-glucose in solution with concanavalin A inhibits binding of concanavalin A to cell surfaces.

Concanavalin A (ConA), isolated from the jack bean (*Canavalia ensiformis*) [165], is the most extensively studied lectin [163]. It serves as a structural probe for carbohydrates in solution and on cell surfaces. It is also a lymphocyte mitogen, anticancer agent, reagent for differentiating normal from malignant cells, and model for antibody-antigen reactions [158, 164]. ConA is an

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exclusively protein lectin. It is one of the few lectins not covalently bound to a carbohydrate (glycoprotein), making it similar to wheat germ agglutinin (WGA), another protein lectin [164].

Bandeiraea simplicifolia I (BS-I), also known as *Griffonia simplicifolia* I, isolated from *Bandeiraea simplicifolia* seeds, is a type B blood specific hemagglutinin [166]. *Bandeiraea simplicifolia* seeds yield two lectins with different sugar-binding specificity, BS-I binds D-galactose, while BS-II binds 2-acetamido-2-deoxy-D-galactose. Unlike ConA, BS-I is a glycoprotein, binding specifically to α-D-galactose [164].

Lectins are classically used for cell agglutination purposes. They primarily interact with carbohydrate moieties on the cell surface in the form of glyco-proteins and glycolipids. However, lectins can also interact with glycoproteins and glycolipids within organelles such as chloroplasts and mitochondria, if the cell has been permeabilized. Additionally, lectins have been used as membrane structural probes, determining the localization and function of carbohydrates on the plasma membrane or intracellular membranes [163]. Of relevance to the present study is their ability to be labeled, with either radioisotopes or fluorophores, to quantitatively assess lectin binding sites on cells. Also of significance to the present study is the use of lectins coupled to certain media, such as magnetic beads, to purify specific cell populations.

In reproductive physiology, lectins are used to purify mixed cell populations. In particular, the lectin BS-I has been used to isolate CLENDOs from a mixed population of cells from the bovine CL containing small and large

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steroidogenic cells, fibroblasts, pericytes, immune cells, and endothelial cells [62, 167-171]. CLENDOs bind the lectins BS-I and ConA, among others. BS-I is a constitutive endothelial cell marker throughout the developmental stages of the CL, while ConA exhibits a broad binding expression pattern throughout the ovary [20]. But other authors have indicated that BS-I binding differs between CLENDO phenotypes and CLENDOs of the estrous cycle versus CLENDOs of pregnancy [172]. These conflicting data indicate further study of lectin expression in CLENDOs is required.

Also of interest to the present study is lectin expression with regard to angiogenesis. Particularly, endothelial phenotype, with respect to lectin expression, is thought to change in migrating versus contact-inhibited cultures of bovine aortic endothelial cells [173]. As the CL is a tissue that undergoes angiogenesis, maintenance of the vasculature, and angioregression, changing lectin expression throughout the course of the estrous cycle and pregnancy could be of significance to the overall processes of luteal formation, function, and regression.

Objectives and Hypotheses

To our knowledge, there are few studies that directly compare CL of the estrous cycle versus pregnancy, and even fewer that assess the luteal vasculature. Therefore, the current study set out to compare structural and functional attributes of CLENDOs of the estrous cycle with those of pregnancy to determine if reproductive status contributes to the diversity of microvascular

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endothelial cells. The parameters assessed were lectin binding, IκBα degradation, IRF-1 expression, TNF and CCL2 production and secretion, and apoptosis. The current hypotheses are as follows: 1) There is no difference in lectin expression between CLENDOs of the estrous cycle and pregnancy. 2) There is no difference in TNF-induced IκBα degradation between CLENDOs of the estrous cycle and pregnancy. 3) There is no difference in TNF- and IFNG-induced IRF-1 expression between CLENDOs of the estrous cycle and pregnancy. 4) CLENDOs are a source of TNF. 5) There is no difference in IFNG-induced TNF secretion between CLENDOs of estrous cycle and pregnancy. 6) There is no difference in cytokine-induced production of CCL2 between CLENDOs of the estrous cycle and pregnancy. 7) TNF and TNF + IFNG are cytotoxic to CLENDOs of the estrous cycle and pregnancy, while IFNG alone is not.

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CHAPTER II

MICROVASCULAR ENDOTHELIAL CELLS OF THE BOVINE CORPUS LUTEUM: A COMPARATIVE STUDY OF THE ESTROUS CYCLE AND PREGNANCY

Introduction

The corpus luteum (CL) is a temporary endocrine gland that forms within the ovary following ovulation and contributes to estrous/menstrual cycle regularity and the establishment and maintenance of early pregnancy [14]. Aside from its transient nature, an intriguing aspect of the CL is its adaptable lifespan. In domestic livestock, the CL remains functional for as few as 15-18 days to as many as 200-300 days depending upon species and reproductive status of the animal [174]. In the bovine for instance, the CL develops, functions, and begins to regress within 17-18 days of the estrous cycle, or it can have a functional lifespan of more than 200 days during pregnancy [64]. Similarly, the functional lifespan of ovine CL varies from 15-16 days during the estrous cycle to approximately 50 days during pregnancy [175]. In fact, in most placental mammals the continuation of luteal function extends well beyond the estrous/menstrual period and is required for the establishment and maintenance of pregnancy for a significant portion of the first trimester [14]. Thus, plasticity of the CL would appear to be critical to its function: alterations in cellular

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composition and hormonal responsiveness permit adaptations to reproductive status (i.e., estrous cycle → pregnancy).

Historically, relatively few studies have directly assessed attributes of the CL of the estrous cycle versus the CL of pregnancy. Those that have compared the two physiologic states have primarily focused on the composition and physiology of the steroidogenic cell populations. For example, in the ovine, corpora lutea of the estrous cycle and of pregnancy are of similar size, weight, and cellular composition [65, 175, 176]. Steroidogenic cells in both types of corpora lutea comprise about 60% of the tissue [65], and have comparable volume density, cytoplasmic to nuclear ratio, cell number/mm^3, and cell volume [175]. Yet the "small" steroidogenic cells of corpora lutea of pregnant ewes are larger, and both the "small" and "large" cells are less responsive to luteinizing hormone (LH) stimulation than the comparable steroidogenic cells of corpora lutea from nonpregnant ewes [176]. A similar distinction in LH response is also observed in steroidogenic cell populations of bovine corpora lutea [177]. Progesterone concentration in corpora lutea of pregnant ewes is greater than in corpora lutea of non-pregnant ewes, even though no differences exist in luteal weight or circulating concentrations of progesterone [176]. In the bovine, however, higher progesterone concentrations exist in the milk of pregnant versus nonpregnant cows [178].

From the standpoint of luteal regression, a few studies have compared key components of the process in corpora lutea under different physiological states (nonpregnant vs. pregnant), such as prostaglandin receptor expression

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and immune function. Wiepz and colleagues (1992) indicate that while ovine corpora lutea possess 7-10-fold more receptors for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) than prostaglandin E_2 (PGE_2), regardless of reproductive status, there are generally more of both types of receptors in corpora lutea of pregnant than nonpregnant ewes [142]. $PGF_{2\alpha}$ is a known luteolytic agent [34], while PGE_2 is luteotrophic [136], delays luteal regression, and suppresses $PGF_{2\alpha}$ actions [14]. Therefore, it appears that continuation of the CL into pregnancy in the ewe is not due to downregulation of prostaglandin receptors, thereby rendering the CL unresponsive to the luteolytic $PGF_{2\alpha}$. Instead, other mechanisms must contribute to luteal regression and be suppressed or inactive for the CL to maintain its structure and function throughout pregnancy. One such possibility is the role of the immune system in luteal regression. In the ovine, bovine, and equine, expression of major histocompatibility (MHC) class II molecules is diminished in corpora lutea of pregnant animals compared to cyclic animals [38, 44, 45]. This suggests that mechanisms of immune suppression exist within the CL of pregnancy to sustain its function. Consistent with these observations, luteal regression at the end of pregnancy in the ovine is reported to be similar to that of the estrous cycle (e.g., characterized by endothelial cell loss and immune cell infiltration [175]), but occurs over an extended period of time (i.e., several weeks [179]) in the postpartum ewe [65].

The aforementioned studies focus almost exclusively on the steroidogenic cell population of the CL, but the CL is comprised not only of “small” and “large” steroidogenic cells, but also endothelial cells, fibroblasts, and immune cells [17].

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Endothelial cells make up about 50% of the midcycle CL [17] and are vital in the formation, function, and regression of the tissue. Angiogenesis is essential to the development of the gland, as an inadequate vasculature is associated with reduced luteal function [23]. Stability of the vasculature is paramount to the endocrine function of the gland, importing nutrients, substrates, and hormones, and conveying progesterone to its target tissues [18]. Endothelial cells of the CL are among the first cells to undergo apoptosis during luteal regression, thereby eliminating the blood supply to the tissue and dictating its fate [31]. Since endothelial cells are the first cell type to be eliminated during regression of the CL, their maintenance in the CL of pregnancy would lead one to believe that microvascular endothelial cells of the CL (CLENDOs) of the estrous cycle and of pregnancy differ. Potential physical and functional differences in CLENDOs could help explain how the CL of the estrous cycle has a defined lifespan of 17-18 days, whereas the CL of pregnancy is maintained for as many as 200 days.

To our knowledge, there is currently only one study in which bovine CLENDOs of the estrous cycle and pregnancy have been directly compared. Plendl et al. (1996) reported different expression of surface carbohydrates (lectins) based on reproductive status and cellular morphology in bovine CLENDOs [172]. Specifically, CLENDOs of pregnancy of the cobblestone phenotype (versus arcuate) have significant binding capacity for the lectins *Bandeiraea simplicifolia* I (BS-I), wheat germ agglutinin (WGA), and concanavalin A (ConA) compared to the estrous cycle [172]. Also, spontaneous angiogenic activities, such as cellular migration in band-like structures and formation of ring

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like-structures *in vitro*, are observed exclusively in CLENDOs of pregnancy [172]. These results indicate that cellular recognition markers, such as lectins, differ between CLENDOs of pregnancy and the estrous cycle. Physical differences such as these infer different functions of these cells within the microvasculature, particularly with regard to angiogenic capabilities.

Considering the relatively few studies which have directly compared CL of the estrous cycle versus pregnancy, and even fewer studies assessing the diversity of the luteal vasculature, the current study determined if reproductive status contributes to the diversity of luteal microvascular endothelial cells. Lectin binding was compared between CLENDOs of the estrous cycle and pregnancy as a basis of physical/morphological attributes. Responsiveness to the cytokines tumor necrosis factor alpha (TNF) and interferon gamma (IFNG) were assessed as functional attributes to determine if immune mechanisms are similar between CLENDOs of the estrous cycle and pregnancy. Specifically, intracellular signaling, cytokine production, and cell death were evaluated.

Materials & Methods

Reagents

Plastic culture vessels were purchased from Corning, Inc. (Corning, NY) and Becton-Dickinson (Franklin Lakes, NJ). NUNC-Immuno MaxiSorp 96-well ELISA plates were purchased from Nalge Nunc (Rochester, NY). Microvascular endothelial cell medium (EGM-2MV) was obtained from Cambrex Bioscience (Walkersville, MD). Minimum Essential Medium Eagle (MEM), Hanks' Balanced

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Salts (HBSS), fetal bovine serum (FBS), 0.25% trypsin/0.2% EDTA, FITC-labeled BS-I and ConA, α-D-galactose, α-D-glucose, monoclonal anti-β-actin, and protease and phosphatase I & II inhibitor cocktails were purchased from Sigma (St. Louis, MO). Gentamicin was obtained from Invitrogen (Carlsbad, CA). HRP-linked anti-rabbit IgG was purchased from Cell Signaling Technology (Danvers, MA). Interferon regulatory factor 1 (IRF-1) and inhibitor kappa B alpha (IκBα) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence reagent kit (ECL) was from Amersham Biosciences (Piscataway, NJ). Recombinant murine TNF was purchased from United States Biological (Swampscott, MA). Recombinant bovine IFNG and bovine TNF screening sets were purchased from Pierce Biotechnology (Rockford, IL). Human monocyte chemoattractant protein 1 (CCL2) DuoSet ELISA Development System was from R&D Systems (Minneapolis, MN). APO-BrdU Assay was purchased from Phoenix Flow Systems Inc. (San Diego, CA). Etanercept was a gift from Dr. Bo R. Rueda. All remaining reagents and materials were purchased from Sigma, VWR International, Inc. (West Chester, PA), or Fisher Scientific (Pittsburg, PA).

Cell Culture

Aliquots of purified CLENDOs of midcycle (day 12) and early pregnancy (day 60), isolated as previously described [48], were used in all experiments. 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,

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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 antifungal (15 ng/ml amphotericin-B)] in a 37°C humidified incubator of 95% air and 5% CO_2. Bovine pulmonary arterial endothelial cells (BPAECs) were cultured in MEM with 10% FBS and 20 μg/ml gentamicin in a 37°C humidified incubator of 95% air and 5% CO_2.

Lectin Binding Experiments

CLENDOs of midcycle (passage 5) and early pregnancy (passage 5) were seeded at a density of 30,000 cells/ml in T_{25} 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 x 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 x 75 mm polystyrene round bottom test tubes and centrifuged at 250 x g for 10 min. Cell pellets were resuspended and incubated with 100 μl PBS (control) or FITC-labeled BS-I or ConA at a concentration of 2.5 μg/ml for 1 h at 4°C. Additional controls included samples containing the competing sugar to BS-I and ConA (200 mM α-D-galactose and α-D-glucose, respectively) [172]. After the 1 h incubation, cells were rinsed with HBSS modified with 2 mM EDTA and centrifuged for 10 min at 250 x g. Cell pellets were then fixed by resuspension in 375 μl PBS and 125 μl 4% paraformaldehyde (1% final concentration). The fixed

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cells were placed on 12 x 75 mm cell strainer (35 μm) capped polystyrene round bottom test tubes and centrifuged for 5 min at 200 x g in preparation for flow cytometric analysis. The cells were analyzed using a four-color, dual-laser FACSCalibur machine (BD Biosciences, Palo Alto, CA) and data were collected using Cellquest (BD Biosciences, Palo Alto, CA). 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 for comparative purposes [180, 181].

Cytokine-stimulated Intracellular Signaling Experiments

TNF-induced Degradation of IκBα

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, basal medium (EBM-2) for 2 h. CLENDO cultures were exposed in duplicate to vehicle (control) or 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, 1% Triton X-100) containing protease and phosphatase I & II inhibitor cocktails. The cell lysates were sonicated for 5 sec and concentrated to 30-40 μg protein. IκBα degradation was quantified by western blot analysis using an IκBα (C-21) primary antibody.

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Cytokine-induced IRF-1

CLENDOs of midcycle (passage 5) and early pregnancy (passage 7) were cultured in 6-well plates to confluence. Prior to cytokine treatment, fresh medium was applied to the cells. CLENDO cultures were exposed in duplicate to vehicle (control), TNF (50 ng/ml), TNF + etanercept (10 μg/ml, 30 min pre-treatment), IFNG (30 ng/ml), IFNG + etanercept, TNF + IFNG, or TNF + IFNG + etanercept for 90 min or 48 h. 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. IRF-1 was quantified by western blot analysis using an IRF-1 (C-20) primary antibody.

Western Blot Analysis

Protein samples (30-40 μg) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred (75 V for 2 h) to polyvinylidine 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, 0.05% Tween-20) at room temperature for 1 h. Membranes were probed with primary antibodies IκBα (C-21) or IRF-1 (C-20) used at dilutions of 1:1000 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 1:2000 dilution 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

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then exposed for 1-10 min using the Kodak Image Station 440 to determine band intensities.. β-actin (1:5000) 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 medium was applied to the cells. EC were exposed in duplicate to vehicle (control) or IFNG (30 ng/ml or 300 ng/ml) for 24 or 48 h. Upon completion of the time points, conditioned medium was collected and TNF in the conditioned medium 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 medium was applied to the cells. CLENDO cultures were exposed in duplicate to vehicle (control), TNF (50 ng/ml), TNF + etanercept (10 μg/ml, 30 min pre-treatment), IFNG (30 ng/ml), IFNG + etanercept, TNF + IFNG, or TNF + IFNG + etanercept for 48 h. Upon termination of the experiments, conditioned medium was collected. Secretion of CCL2 into culture medium was measured using ELISA.

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Immunoassays

TNF

TNF secretion from CLENDOs was measured using a commercially available sandwich ELISA specific for bovine TNF (Pierce Biotechnology, Inc.). 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 pg/ml to 2500 pg/ml. The detection limit of the assay was 29.13 pg/ml 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 (R&D Systems). Use of this assay kit for the measurement of bovine CCL2 has previously been validated [182], and verified [48, 82]. 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 15.6 pg/ml to 1000 pg/ml. The detection limit of the assay was 1.84 pg/ml 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

CLENDOs of midcycle (passage 5) and early pregnancy (passage 7) were seeded at a density of 30,000 cells/ml in 6-well plates and cultured to confluence.

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Along with fresh medium, EC were exposed to vehicle (control), TNF (50 ng/ml), IFNG (30 ng/ml), or TNF + IFNG for 48 h. For qualitative analysis, numbers of attached cells in 3 fields of view per well were counted. For quantitative analysis, cells were removed after 48 h by brief enzymatic treatment using HBSS modified with 2 mM EDTA and 0.25% trypsin with 0.2% EDTA. The cell suspensions were spun at 200 x g for 10 min by centrifugation, and the supernatant was removed using the pour and dab method. The cell pellets were resuspended in 500 μl HBSS and placed on 12 x 75 mm cell strainer (35 μm) capped polystyrene round bottom test tubes and centrifuged for 5 min at 200 x g. The strained cells were fixed using 500 μl of 2% paraformaldehyde (1% final concentration). The cells were fixed in the dark at 4°C for 45 min. After fixation, the cells were spun down at 200 x g for 10 min, supernatant removed, and then taken through two PBS washes. After the washes, the cell pellets were resuspended in 1 ml cold 70% ethanol and stored at -20°C. Quantification of apoptotic cells was determined using a modified terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assay, APO-BrdU, according to the manufacturer's instructions. Briefly, PRB-1 antibody (anti-BrdU) conjugated to Alexafluor 647 quantified breaks in cellular DNA, and propidium iodide staining measured DNA content. Cells of the human lymphoma line, HL-60, supplied by the manufacturer, were used as positive and negative controls, in which cells were either untreated (negative) or treated to induce apoptosis (positive). Cell death was measured using a four-color, dual-laser FACSCalibur machine. Data were collected using Cellquest and analyzed using WinMDI software.

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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) or Systat (Point Richmond, CA) 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

Lectin Binding of CLENDOs

Results of previous studies indicate lectin binding differs among endothelial cells of the bovine CL depending upon reproductive status and phenotype [172]. The present experiments were conducted to further assess lectin binding in CLENDO cultures derived from CL of the estrous cycle and pregnancy (Figs. 4 & 5). Flow cytometric analysis of FITC-labeled lectins revealed comparable BS-I (Fig. 4) and ConA (Fig. 5) binding of CLENDOs of the estrous cycle and pregnancy. Specificity of lectin binding was demonstrated in all cases except one (BS-I on CLENDOs of pregnancy) by inhibition with the appropriate competing sugar ($P < 0.05$, Figs. 4 & 5).

Verification of Lectin Binding

Lectin binding of BS-I and ConA was also assessed in BPAECs as a positive control (Fig. 6) [180, 181]. Binding of ConA was greater than BS-I in these macrovascular endothelial cells ($P < 0.05$). As above, specificity of lectin binding

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for both BS-I and ConA was demonstrated by displacement with α-D-galactose and α-D-glucose, respectively ($P < 0.05$, Fig. 6).

TNF Induces Degradation of IκBα

Cultures of CLENDOs exposed to TNF had substantial degradation of IκBα ($P < 0.05$), but there was no difference in IκBα degradation attributed to reproductive status ($P > 0.05$, Fig. 7).

TNF and IFNG Induce IRF-1 Expression

In cultures of CLENDOs, TNF induced a robust increase in IRF-1 ($P < 0.05$, Fig. 8) as did IFNG ($P < 0.05$, Fig. 9). There was no difference in cytokine-induced IRF-1 expression, however, attributed to reproductive status ($P > 0.05$, Figs. 8 & 9).

IFNG Induces TNF Secretion by CLENDOs

Conditioned medium from the cultures revealed a dose-dependent increase in TNF secretion by CLENDOs of the estrous cycle and pregnancy in response to IFNG stimulation ($P < 0.05$, Fig. 10). However, there was no difference in TNF production by CLENDOs attributed to reproductive status ($P > 0.05$, Fig. 10).

IFNG and TNF Induce CCL2 Secretion by CLENDOs

Conditioned medium from the cultures also revealed an increase in CCL2 secretion by CLENDOs of the estrous cycle and pregnancy in response to TNF

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and IFNG stimulation alone and in combination ($P < 0.05$, Fig. 11). Again, however, there was no difference in CCL2 production by CLENDOs based upon reproductive status ($P > 0.05$, Fig. 11).

Etanercept Inhibits CCL2 Production and IRF-1 Expression Induced by TNF, but not IFNG

Etanercept is a synthetic, soluble TNF receptor that functions as a TNF antagonist [183]. In cultures of CLENDOs, etanercept diminished TNF-induced CCL2 production by CLENDOs ($P < 0.05$), but had no effect on IFNG- or TNF + IFNG-induced CCL2 secretion ($P > 0.05$, Fig. 11). Reproductive status of the cow did not alter CLENDO responsiveness ($P > 0.05$, Fig. 11). Etanercept also inhibited IRF-1 expression by TNF and TNF + IFNG following a 90 min treatment period ($P < 0.05$, Fig. 12b), but had no effect on IFNG-induced IRF-1 expression ($P > 0.05$, Fig. 12b). There was no difference in etanercept inhibition of IRF-1 in CLENDOs attributed to reproductive status at 90 min ($P > 0.05$, Fig. 12b). However, etanercept failed to inhibit cytokine-induced IRF-1 expression following a 48 h treatment period (Fig. 12d), and IRF-1 expression was greater in CLENDOs of the estrous cycle ($P < 0.05$, Fig. 12d).

Cytotoxic Effect of TNF on CLENDOs of the Estrous Cycle

The cytokines TNF and TNF + IFNG are cytotoxic to CLENDOs of pregnancy [139]. In the current study, cytokines had a similar effect on CLENDOs of the estrous cycle. That is, IFNG alone failed to induce cell death, but TNF and TNF

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+ IFNG induced death in approximately 30% and 10% of the cells based upon visual observation (Fig. 13) and quantification of APO-BrdU labeling by flow cytometric analysis (Fig. 15), respectively.

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Figure 4. BS-I lectin binding of CLENDOs of the estrous cycle and pregnancy as determined by flow cytometric analysis.

Figure 4a.

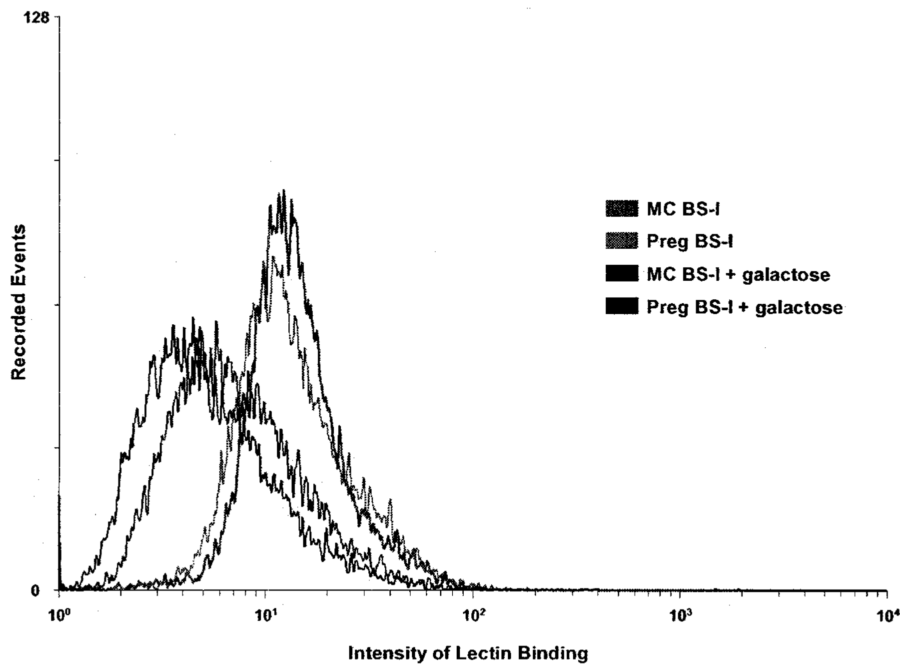
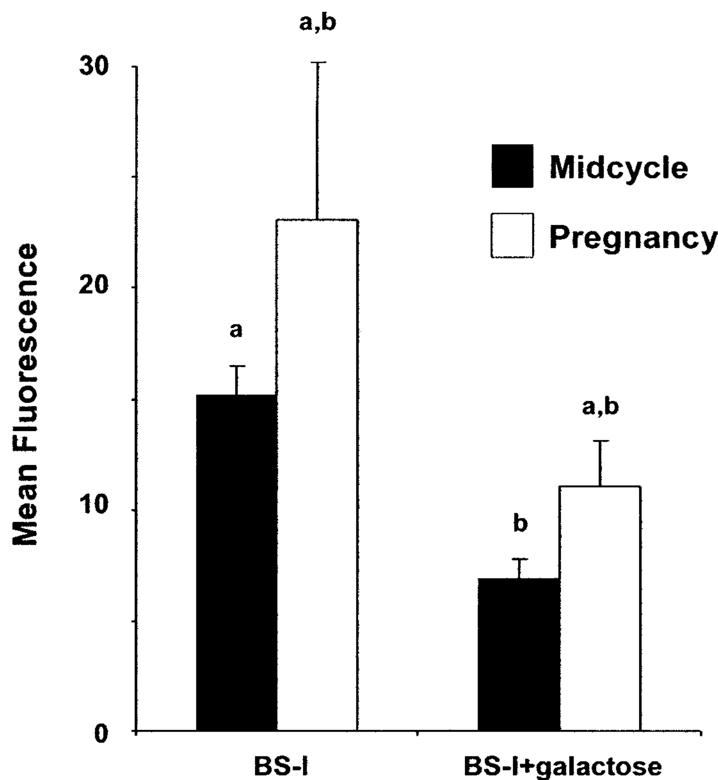


Figure 4b.



Representative flow cytometric histogram of BS-I binding in CLENDOs (Fig. 4a). A right shift along the x-axis indicates increased lectin binding of FITC-labeled BS-I. Addition of the competing sugar, α-D-galactose, displaced lectin binding, yielding a shift equivalent to controls. The geometric mean fluorescence of the histograms was extrapolated and is presented as bars $\pm$ SEM, n=3 experiments (Fig. 4b). BS-I binding was increased, but comparable in CLENDOs of midcycle CL and CL of pregnancy ($P > 0.05$). Different letters denote significant differences in lectin binding among the treatments and cell types ($P < 0.05$).

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Figure 5. ConA lectin binding of CLENDOs of the estrous cycle and pregnancy as determined by flow cytometric analysis.

Figure 5a.

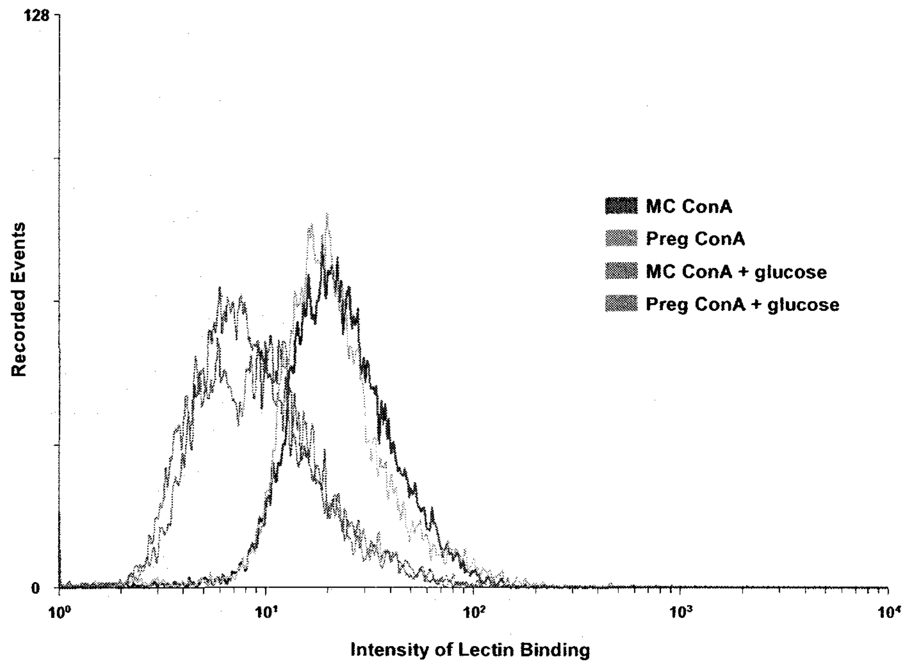
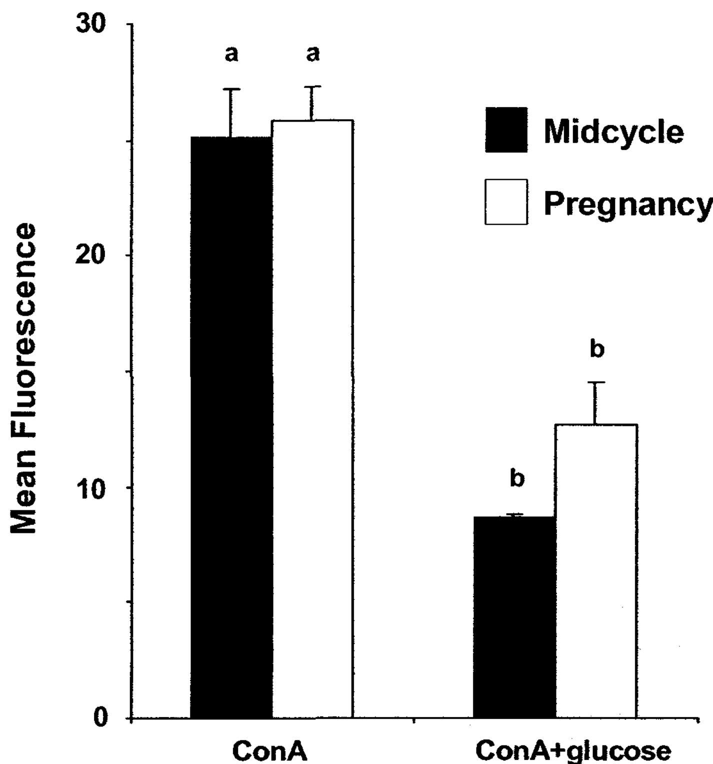


Figure 5b.



Representative flow cytometric histogram of ConA binding in CLENDOs (Fig. 5a). A right shift along the x-axis indicates increased lectin binding of FITC-labeled ConA. Addition of the competing sugar, α-D-glucose, displaced lectin binding, yielding a shift equivalent to controls. The geometric mean fluorescence of the histograms was extrapolated and is presented as bars ± SEM, n=3 experiments (Fig. 5b). ConA binding was increased, but comparable in CLENDOs of midcycle CL and CL of pregnancy ($P > 0.05$). Different letters denote significant differences in lectin binding among the treatments and cell types ($P < 0.05$).

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Figure 6. Lectin binding of BPAECs as a positive control for lectin binding of CLENDOs.

Figure 6a.

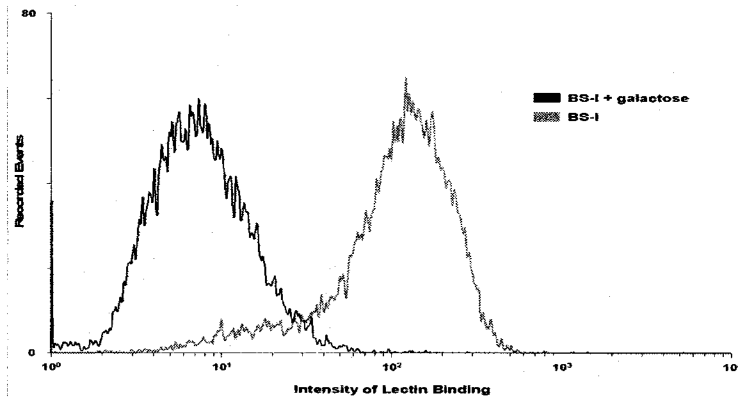
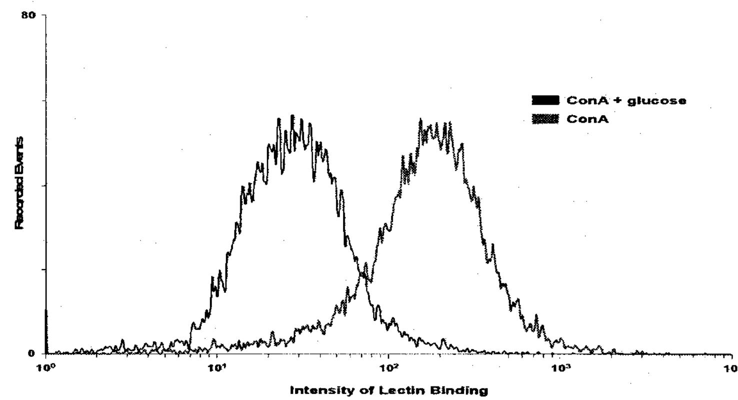


Figure 6b.



Representative flow cytometric histogram of BS-I (Fig. 6a) and ConA (Fig. 6b) binding in BPAECs. Similar characteristics of lectin binding and displacement were observed in macrovascular endothelial cells. Bars represent mean fluorescence ± SEM, n=3 experiments (Fig. 6c). Different letters denote significant differences in lectin binding among the treatments and cell types ($P < 0.05$).

Figure 6c.

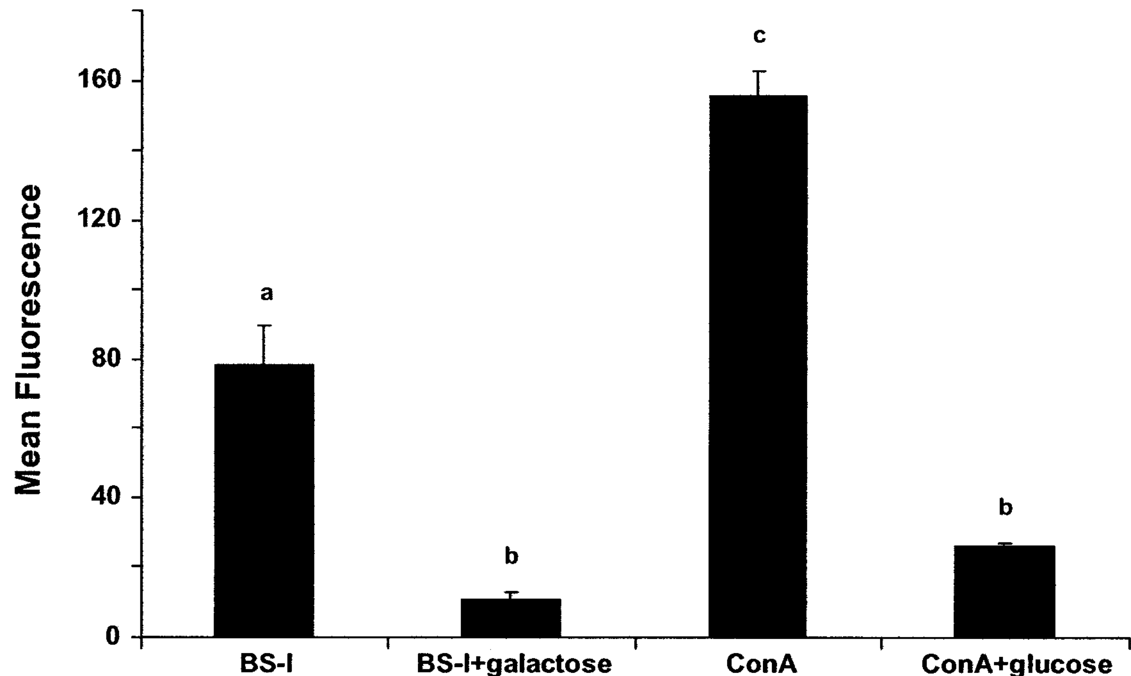


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Figure 7. TNF-induced IκBα degradation in CLENDOs of the estrous cycle and pregnancy.

Figure 7a.

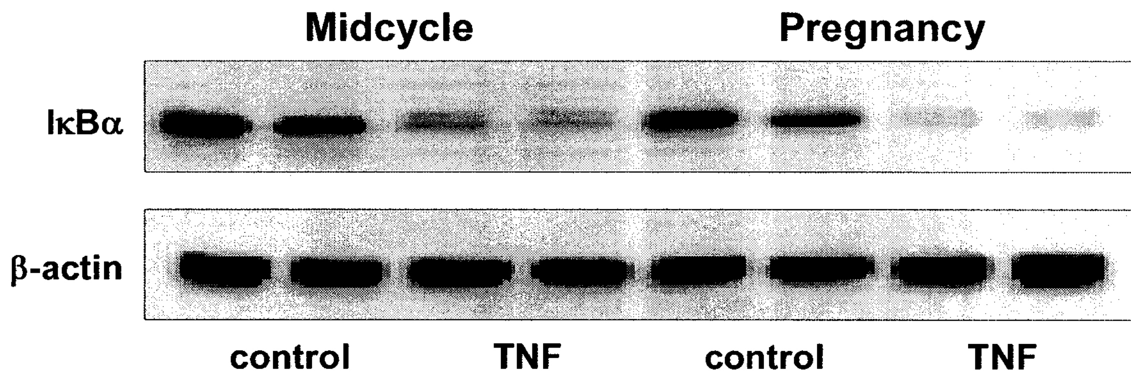
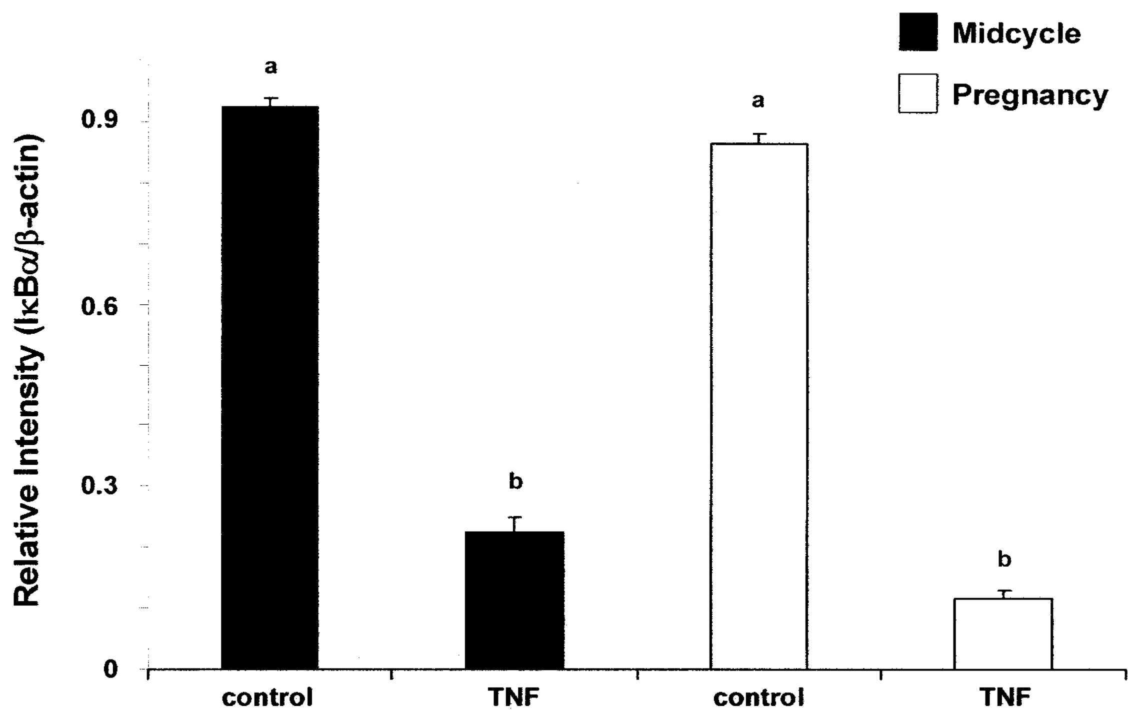


Figure 7b.



Representative western blot (Fig. 7a) and bar graph (Fig. 7b) depicting TNF-induced IκBα degradation in CLENDOs. Within 15 minutes of exposure to TNF, CLENDOs exhibited a 5-fold decrease in IκBα ($P < 0.05$). 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 IκBα normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in IκBα degradation among the treatments and cell types ($P < 0.05$).

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Figure 8. TNF-induced IRF-1 expression in CLENDOs of the estrous cycle and pregnancy.

Figure 8a.

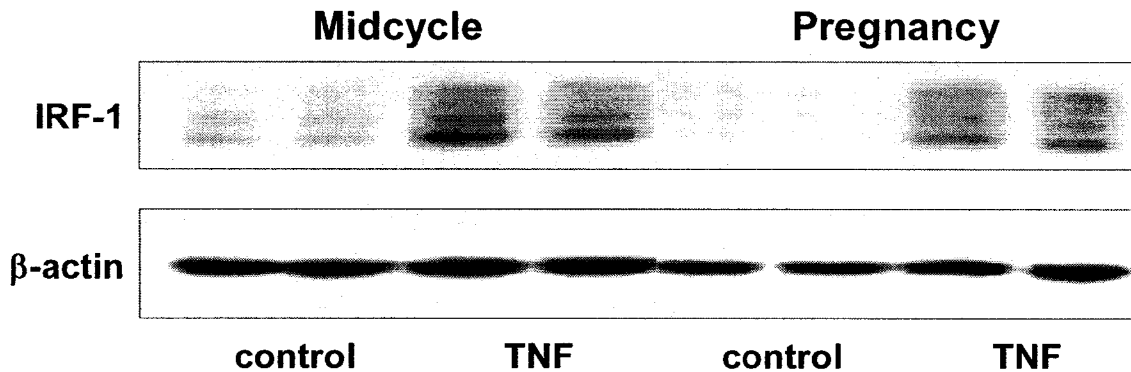
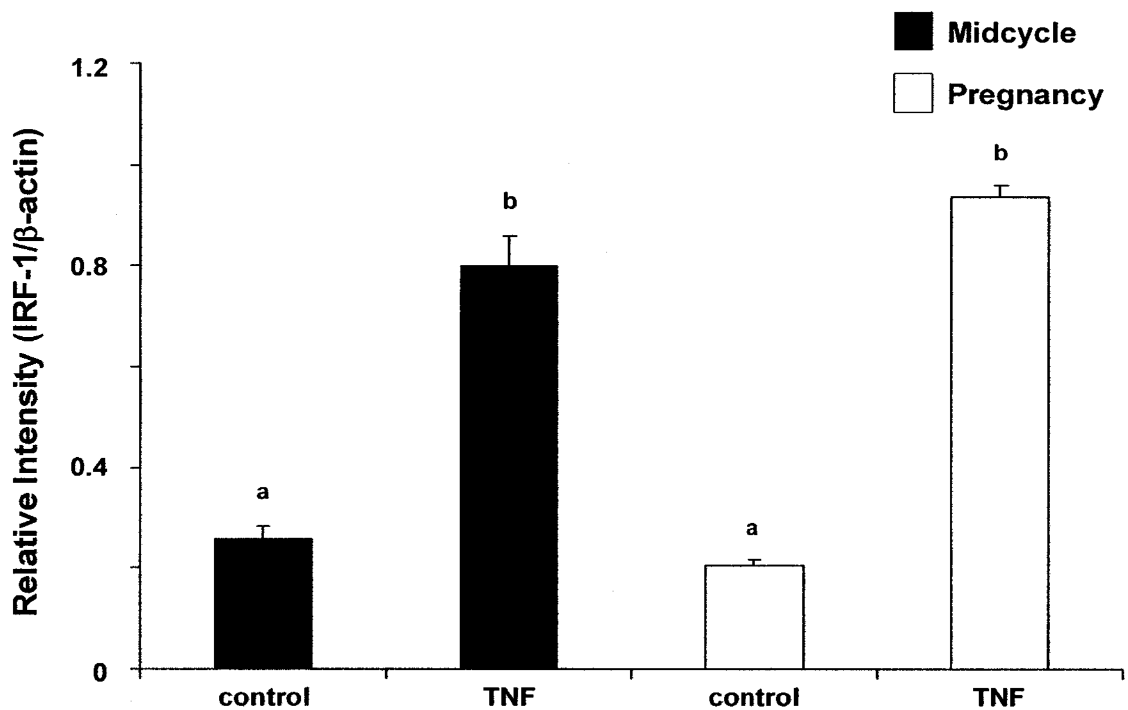


Figure 8b.



Representative western blot (Fig. 8a) and bar graph (Fig. 8b) depicting TNF-induced IRF-1 expression in CLENDOs. Within 90 minutes of exposure to TNF, CLENDOs exhibited a 4-fold increase in IRF-1 expression ($P < 0.05$). 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 IRF-1 normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in IRF-1 expression among the treatments and cell types ($P < 0.05$).

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Figure 9. IFNG-induced IRF-1 expression in CLENDOs of the estrous cycle and pregnancy.

Figure 9a.

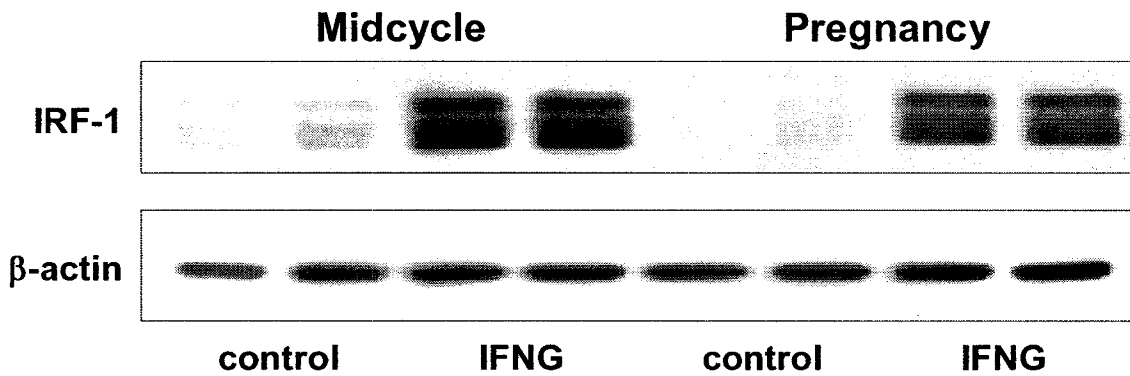
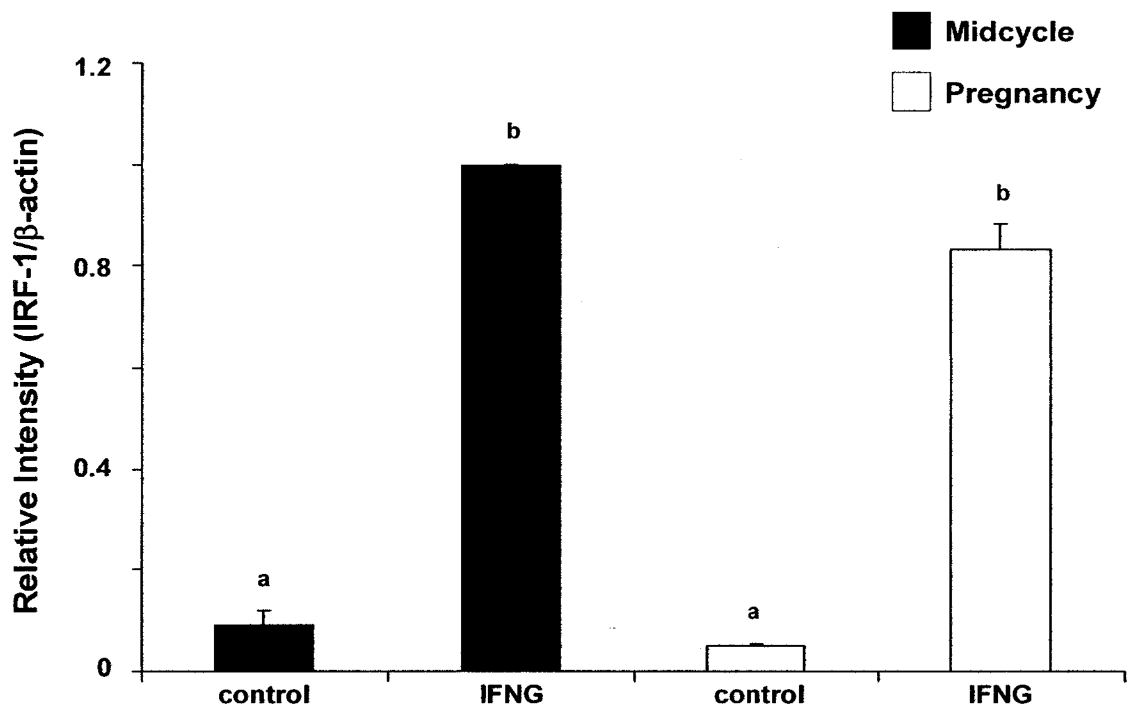


Figure 9b.



Representative western blot (Fig. 9a) and bar graph (Fig. 9b) depicting IFNG-induced IRF-1 expression in CLENDOs. Within 90 minutes of exposure to IFNG, CLENDOs exhibited a 14-fold increase in IRF-1 expression ($P < 0.05$). 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 IRF-1 normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in IRF-1 expression among the treatments and cell types ($P < 0.05$).

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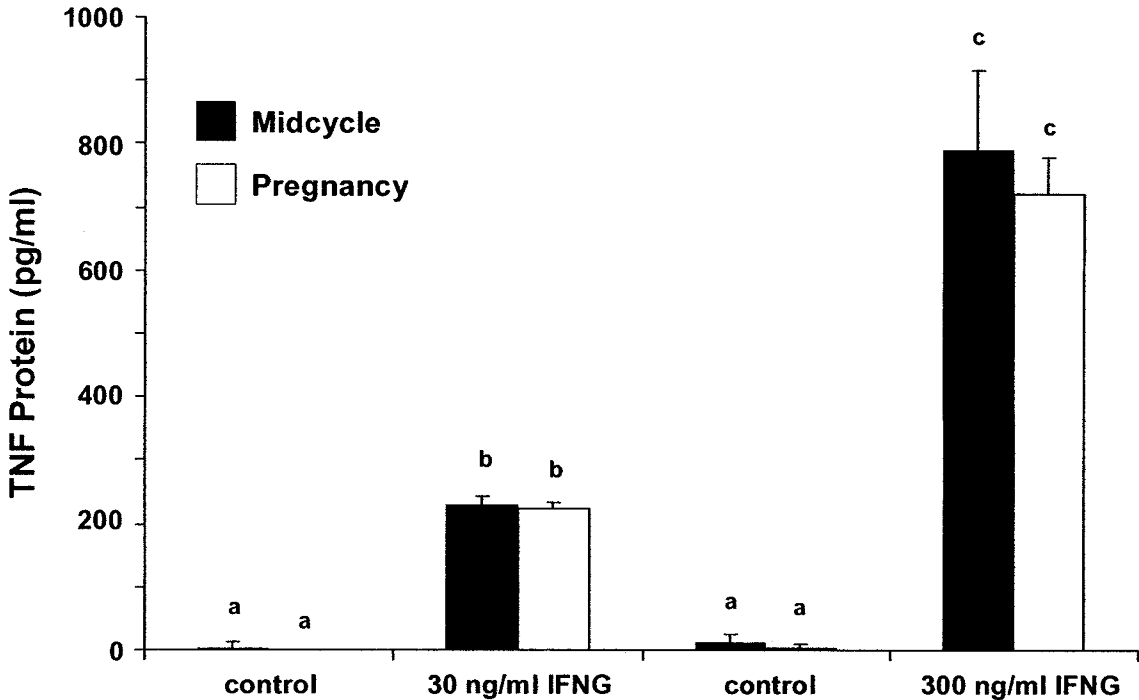
Figure 10. IFNG-induced TNF secretion by CLENDOs of the estrous cycle and pregnancy.



TNF secretion by CLENDOs of the estrous cycle and pregnancy in culture over a 48 h treatment period as determined by ELISA of conditioned culture medium. Response to IFNG at 30 ng/ml and 300 ng/ml is shown. Overall response to IFNG did not differ between CLENDOs of the estrous cycle and pregnancy ($P > 0.05$). Bars represent mean protein secretion ± SEM (n=3 experiments). Different letters denote differences in TNF protein secretion among the treatments and cell types ($P < 0.05$).

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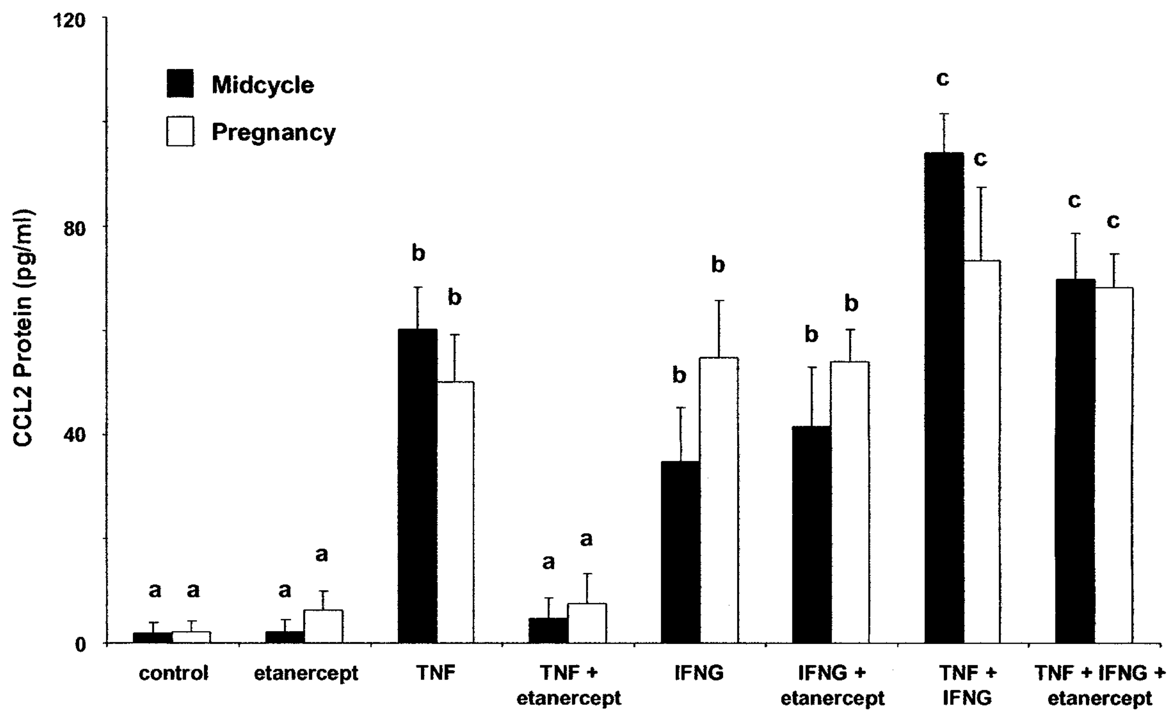
Figure 11. Cytokine-induced CCL2 secretion by CLENDOs of the estrous cycle and pregnancy.



CCL2 secretion by CLENDOs of the estrous cycle and pregnancy in culture over a 48 h treatment period as determined by ELISA of conditioned culture medium. Responses to the cytokines TNF and IFNG, and the TNF antagonist, etanercept, are shown. Overall response to cytokines did not differ between CLENDOs of the estrous cycle and pregnancy ($P > 0.05$). Bars represent mean protein secretion $\pm$ SEM (n=3 experiments). Different letters denote differences in CCL2 protein secretion among the treatments and cell types ($P < 0.05$).

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Figure 12. Effect of etanercept on cytokine-induced IRF-1 expression in CLENDOs of the estrous cycle versus pregnancy.

Figure 12a.

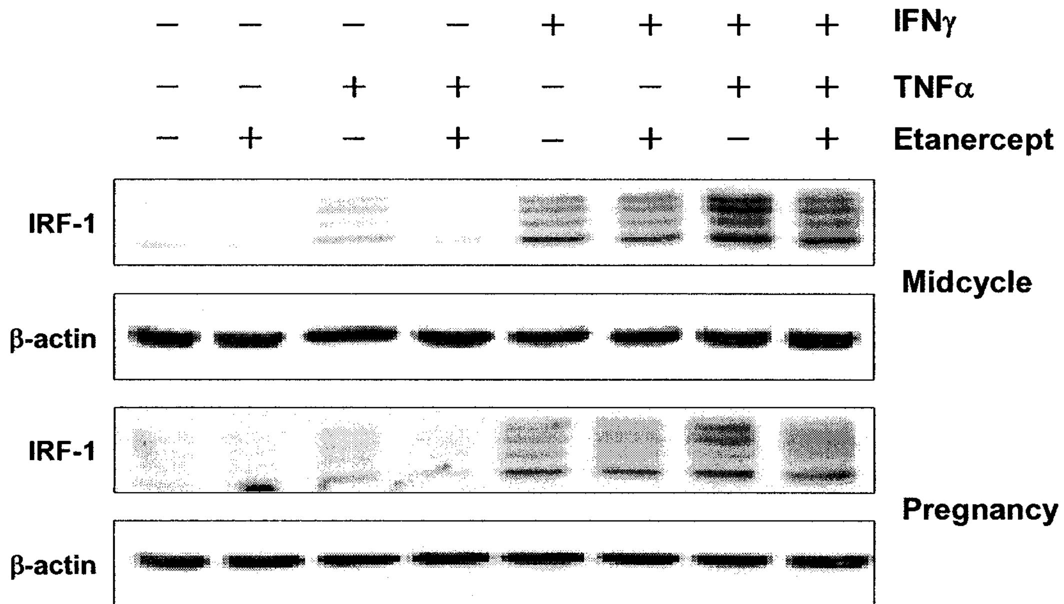
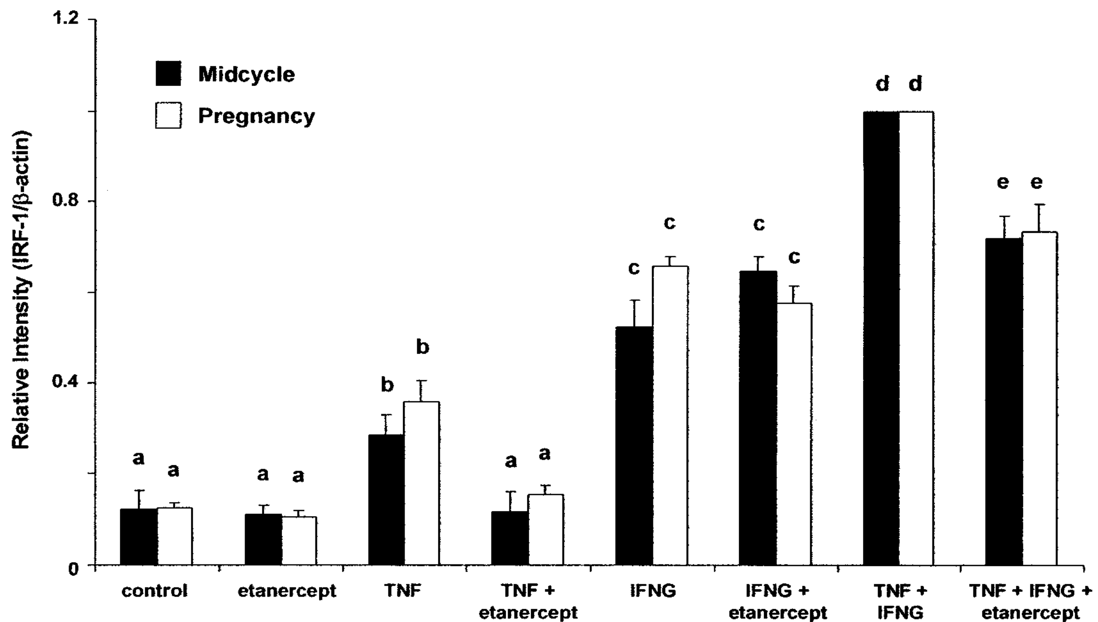


Figure 12b.



Representative western blot (Fig. 12a) and bar graph (Fig. 12b) depicting cytokine-induced IRF-1 expression in CLENDOs of the estrous cycle and pregnancy over a 90 min treatment period. Responses to the cytokines TNF and IFNG, and the TNF antagonist, etanercept, are shown. Overall response to cytokines did not differ between CLENDOs of the estrous cycle and pregnancy ($P > 0.05$). Bars represent relative intensity of IRF-1 normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in IRF-1 expression among the treatments and cell types ($P < 0.05$).

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Figure 12c.

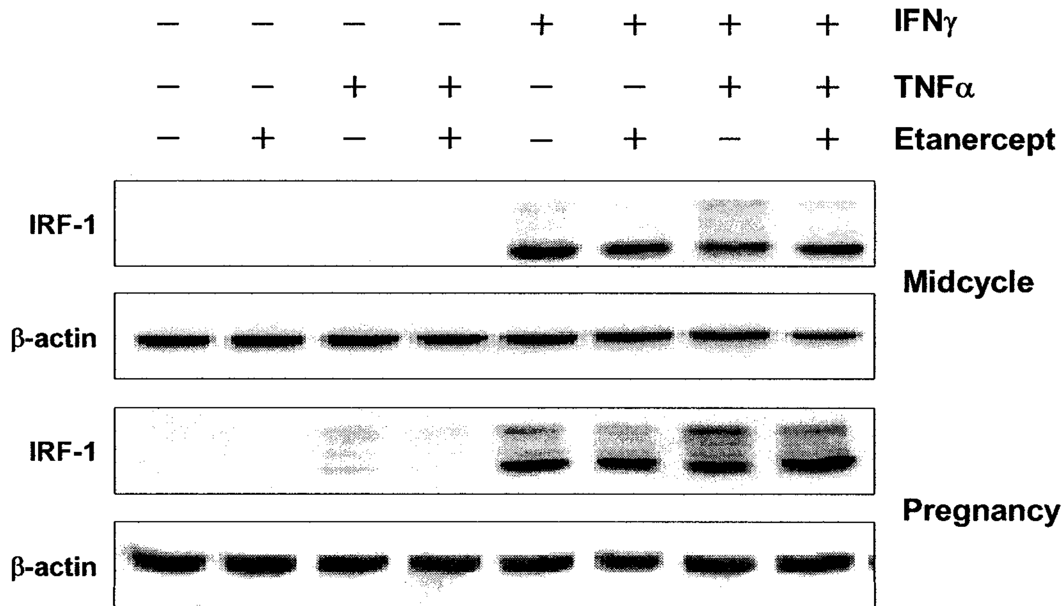
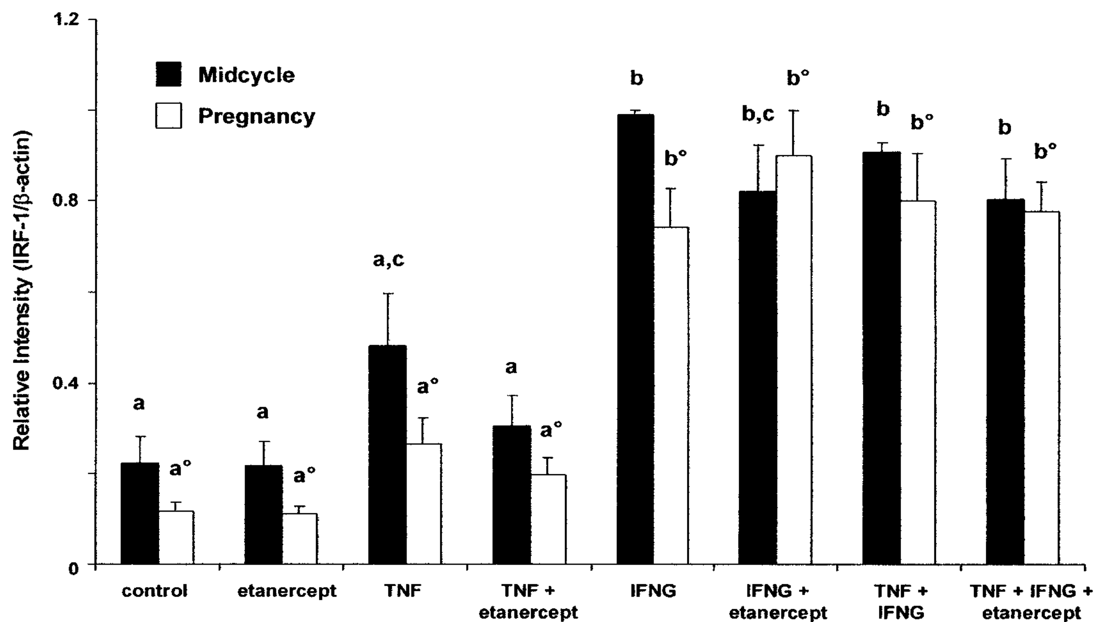


Figure 12d.



Representative western blot (Fig. 12c) and bar graph (Fig. 12d) depicting cytokine-induced IRF-1 expression in CLENDOs of the estrous cycle and pregnancy over a 48 h treatment period. Responses to the cytokines TNF and IFNG, and the TNF antagonist, etanercept, are shown. Overall response to cytokines differed between CLENDOs of the estrous cycle and pregnancy ($P < 0.05$). Bars represent relative intensity of IRF-1 normalized to β-actin ± SEM (n=3 experiments). Different letters denote significant differences in IRF-1 expression among the treatments and cell types ($P < 0.05$).

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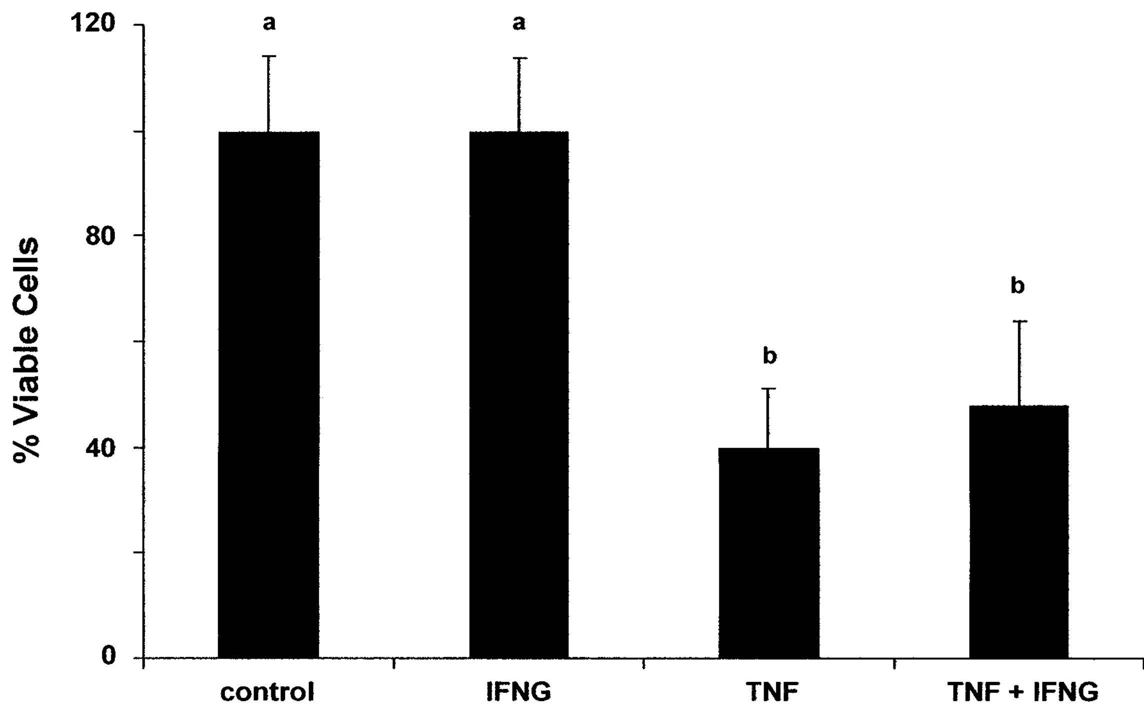
Figure 13. Qualitative cytokine-induced cell death in CLENDOs of the estrous cycle.



Cytokine-induced cell death in CLENDOs of the estrous cycle over a 48 h treatment period. Responses to the cytokines TNF and IFNG are shown. IFNG failed to induce cell death in CLENDOs from midcycle CL. Conversely, TNF and TNF + IFNG resulted in approximately 60% death ($P < 0.05$). Bars represent percentage of viable cells $\pm$ SEM (n=3 experiments). Different letters denote differences in cell death among the treatment groups ($P < 0.05$).

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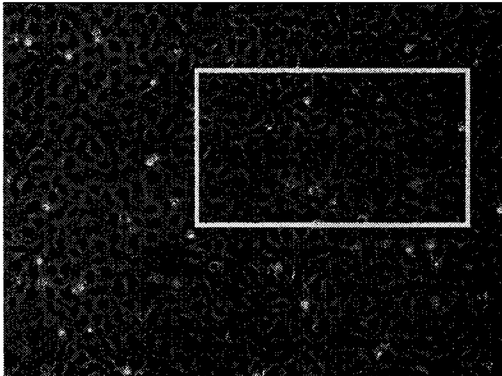
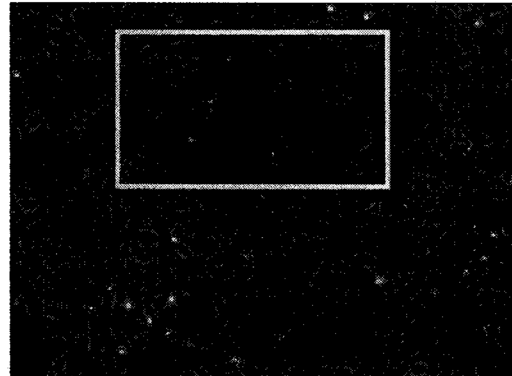
Figure 14. Cytokine-induced cell death visual observations.

Figure 14a.



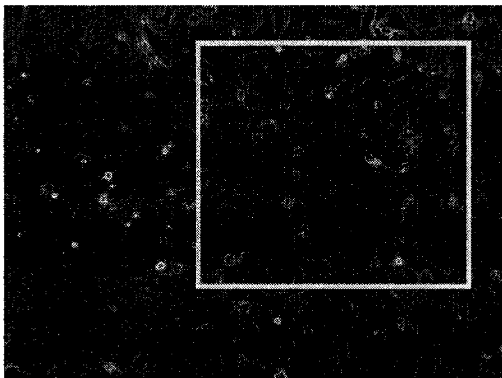
Control

Figure 14b.



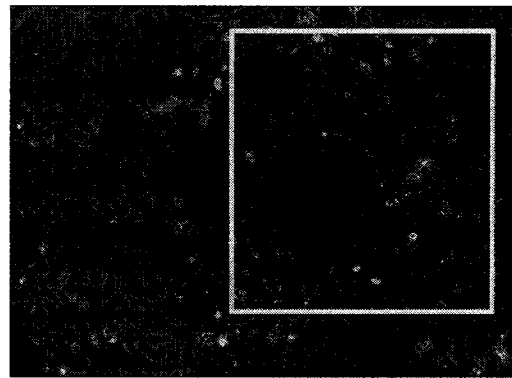
IFNG

Figure 14c.



TNF

Figure 14d.



TNF + IFNG

Representative phase-contrast photomicrographs (25x magnification) of midcycle CLENDO cultures following 48 h of the indicated treatments (Fig. 14). Yellow boxes highlight areas affected by the corresponding treatments. Control cells display cobblestone morphology, with the monolayer intact (Fig. 14a). IFNG-treated cells display large, flattened, senescent-like cells between areas of intact monolayer (Fig. 14b). TNF treatment resulted in characteristic signs of death, including the obvious reduction in attached cells (Fig. 14c). Finally, TNF + IFNG treatment resulted in similar effects seen with TNF alone, with fewer cells attached to the cultureware (Fig. 14d).

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Figure 15. Quantitative cytokine-induced cell death in CLENDOs of the estrous cycle.

Figure 15a.

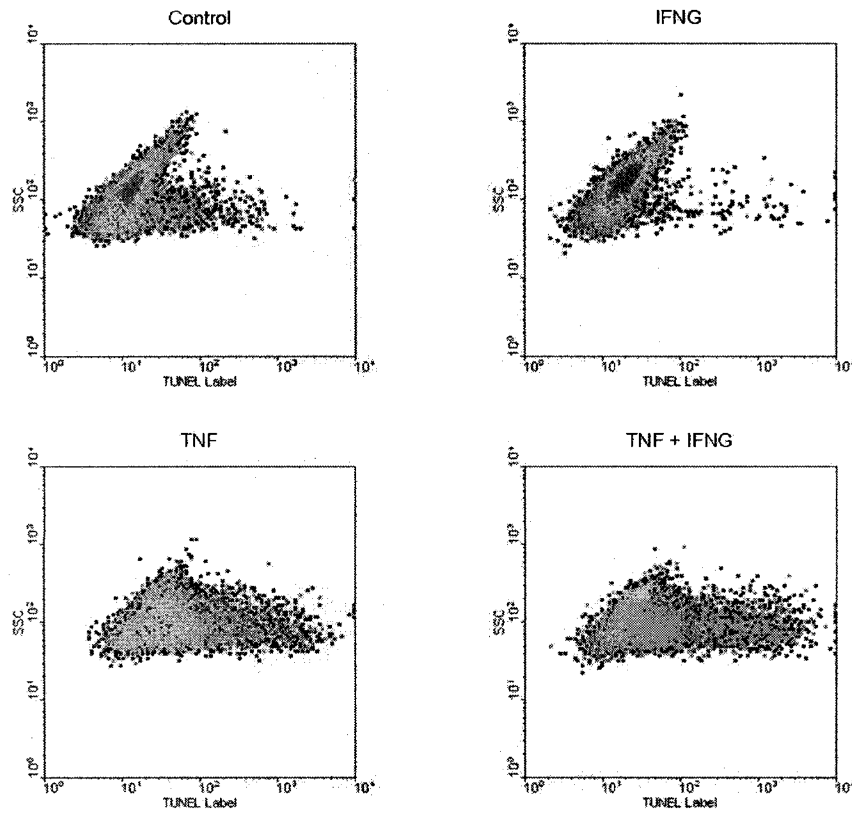
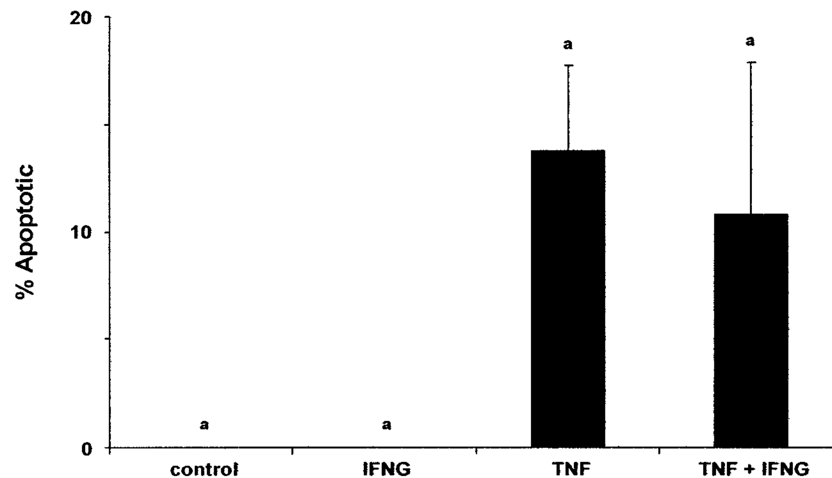


Figure 15b.



Cytokine-induced cell death in CLENDOs of the estrous cycle over a 48 h treatment period. Responses to the cytokines TNF and IFNG are shown as representative flow cytometric density plots of TUNEL labeling in CLENDOs (Fig. 15a). A shift in the cell population to the right indicates increased TUNEL label/apoptosis. The percentage of apoptotic cells was extrapolated and is represented as bars $\pm$ SEM, n=3 experiments (Fig. 15b). IFNG failed to induce cell death, but TNF and TNF + IFNG resulted in approximately 15% and 10% death, respectively. However, there was no difference in cell death among the treatment groups ($P > 0.05$).

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Discussion

In the present study, structural and functional characteristics of CLENDOs of the estrous cycle and pregnancy were compared to elucidate the influence of reproductive status on the diversity of the luteal microvasculature. We determined CLENDOs of the estrous cycle and pregnancy have similar properties of lectin binding and responsiveness to the cytokines TNF and IFNG. Specifically, the extent of BS-I and ConA binding was comparable in CLENDOs of the estrous cycle and pregnancy. TNF induction of IκBα degradation, IRF-1 production, and cell death were likewise similar in CLENDOs of the estrous cycle and pregnancy. IFNG stimulated IRF-1 production and, in combination with TNF, induced apoptosis in CLENDOs of the estrous cycle and pregnancy. In addition, these studies provide the first convincing evidence that CLENDOs are a source of TNF production, but this phenomenon was not influenced by reproductive status of the cow. Collectively, these results indicate that microvascular endothelial cells of the bovine CL do not change morphologically or functionally throughout the estrous cycle and early pregnancy. These findings are significant because previous studies have reported or alluded to physiological distinctions between the CL of the estrous cycle and of pregnancy.

Binding of the lectins BS-I and ConA were detected by flow cytometric analysis of CLENDOs of the estrous cycle and pregnancy and revealed relatively equal mean fluorescence intensities. Determining lectin binding properties of these cell types is important because BS-I is an accepted endothelial cell marker which has been used extensively in magnetic bead isolation of CLENDOs for *in*

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vitro experimentation [62, 167, 168, 170, 171]. Lectin binding to endothelial cells, however, is species, age, organ, and vascular bed specific [173]. BS-I is considered a constitutive endothelial cell marker throughout the developmental stages of the corpus luteum [20], but others indicate that BS-I expression is more ubiquitous, with BS-I binding found in an array of bovine cell types including epithelial cells, endothelial cells, smooth muscle cells, thymocytes, and fibroblasts [184]. These observations indicate BS-I potentially binds other cell types within the CL and not exclusively endothelial cells, as previously suggested [173]. Thus, magnetic bead isolation of CLENDOs based upon BS-I binding might not be an ideal approach for obtaining pure cultures of CLENDOs, as it has the potential to introduce other, contaminating cell types into the culture.

In one previous study, Plendl et al. (1996) reported differences in lectin binding between CLENDOs of the estrous cycle and pregnancy and among CLENDO phenotypes [172]. Two distinct cell types derived from the estrous cycle and pregnancy were described: cobblestone and arcuate morphology. Using flow cytometric analysis, the authors found BS-I binding to both cobblestone and arcuate CLENDOs of pregnancy upregulated (~2-fold) as compared to the same cell types of the estrous cycle. Furthermore, BS-I binding to cobblestone endothelial cells is approximately 3-fold greater than arcuate endothelial cells from CL of pregnancy. In contrast, binding of BS-I to the cobblestone cell type in CL of the estrous cycle is approximately 1.5-fold less than the arcuate phenotype [172]. Collectively, these results indicate differences in lectin binding based on reproductive status and phenotype. In the current

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study, we found no difference in lectin binding due to reproductive status. The disparity between our results and those of Plendl and co-workers (1996) might be due to differences in experimental methods. For instance, the cell types utilized in the current investigation were both cobblestone and arcuate morphology, and presumably constitute the predominant phenotypes of endothelial cells within the bovine CL. Plendl et al. (1996) used specific phenotypes as previously described by Spanel-Borowski and van der Bosch (1990) [70]. If specific morphologic phenotypes have indeed higher lectin binding than others as Plendl et al. (1996) suggest, then a mixture of the predominant phenotypes might mask substantial differences in lectin binding for a particular cell type. Further quantitative analysis of lectin binding will be needed to clarify specificity in CLENDOs, not only because of the emerging use of lectins in magnetic bead isolation, but also because of their role in cell-cell interactions. Hyperglycosylation or upregulation of lectin binding on endothelial cells has emerged as a mechanism of cell migration in bovine aortic endothelial cells [173]. Migrating endothelial cells precede proliferation of endothelial cells during angiogenesis. Upregulation of lectin binding is considered important in these cell-cell interactions, particularly with other endothelial cells or immune cells, especially in the context of inflammatory processes [20, 173].

BPAECs were used in the current experiments as a positive control for BS-I and ConA binding, as documented by others [180, 181]. The BPAECs display higher mean fluorescence intensity than CLENDOs. The difference in lectin binding could be due to innate differences in the macrovasculature versus

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microvasculature, or a difference in cell numbers used in the experiments. The BPAECs were analyzed for lectin binding at a concentration of ~200,000 cells/ml, whereas the CLENDOs were analyzed at ~400,000 cells/ml. Considering the same concentration of FITC-labeled lectin was used for each cell type, the BPAECs could have been oversaturated with FITC-labeled lectin, revealing a higher binding capacity. Additionally, ConA binding was higher than BS-I in BPAECs. This is not unexpected, as there is documentation alluding to a higher binding capacity for ConA compared to BS-I in BPAECs, based upon lectin affinity chromatography [180].

The endothelium is a major target for cytokines in most tissues and is pivotal to immune processes, particularly inflammation. Endothelial cells serve as part of the barrier between the blood stream and the tissues, determining the traffic and action of cells and molecules within the body [81]. Knowing that large numbers of endothelial cells compose the CL [17], endothelial cells and their responsiveness to cytokines are reasoned to be important in luteal regression. Here, intracellular signaling pathways of CLENDOs induced in response to TNF and IFNG stimulation were investigated. Nuclear factor kappa B (NF-κB) activation, via IκBα degradation, and IRF-1 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 [112], but this is the first study, to our knowledge, investigating these aspects of cytokine signaling in bovine CLENDOs.

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Activation of the NF-κB signaling pathway stimulates the production of proinflammatory mediators, such as TNF and CCL2 [123, 185], possibly contributing to immune mediated events during luteal regression. In the current study, TNF-induced IκBα degradation indicates NF-κB activation in both cell types, revealing that proinflammatory pathways are equally inducible during the estrous cycle and pregnancy. Likewise, TNF-induced IRF-1 expression further validates NF-κB activation in both cell types, as IRF-1 induction is mediated by NF-κB [96]. IRF-1 expression is also mediated by signal transducers and activators of transcription (Stat). Therefore, IFNG, which signals through the Janus kinase (Jak)-Stat pathway, is a potent inducer of IRF-1 [84]. In the current study, CLENDOs of the estrous cycle and pregnancy equally expressed IRF-1 following stimulation with IFNG. IRF-1 regulates the transcription of IFNG-responsive genes, such as MHC expression [103]. In fact, IFNG induces the expression of MHC class II molecules on bovine luteal steroidogenic cells [109] and CLENDOs [110]. Also, IFNG increases the expression of TNF receptors on the surface of cells [93, 94]. The role of IRF-1 in these actions of IFNG is not known, but based on our observations and those of Suter and co-workers (2001) [112], IFNG and/or TNF activation of IRF-1 might induce these effects in the CL, thus contributing to immune-mediated events during luteal regression.

One culminating effect of cytokines in CLENDOs is the onset of apoptosis. Apoptotic death in CLENDOs of pregnancy induced by TNF alone and in combination with IFNG has been documented [139]. An incidence of 30% death and 50% death was reported following TNF and TNF + IFNG treatment,

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respectively [139]. In the present study, cytokine-induced apoptosis of CLENDOs of the estrous cycle was measured both qualitatively and quantitatively. Our visual observations were similar to those reported by Pru and co-workers (2003) [139] for CLENDOs of pregnancy in that TNF resulted in significant death (~60%). However, TNF + IFNG did not increase the incidence of death. Instead, IFNG provoked a morphological change in the cells, with little to no death, and more enlarged, senescent-like cells. Similar observations following IFNG treatment have been reported by others [72, 73]. The combination of a morphological change in the cells along with the appearance of senescent-like cells indicates that IFNG may have an overall negative influence on CLENDOs without resulting in apoptosis. IFNG could possibly alter the metabolic activity of the cells, particularly decreasing it, resulting in a cytostatic culture. This would explain the anti-proliferative nature of the cells and the lack of death.

Quantitative analysis of cytokine-induced death by TUNEL assay and flow cytometric analysis yielded results of less cell death than our visual observations. TNF induced approximately 15% death, while TNF + IFNG induced approximately 10% death. TUNEL staining was used to identify strand breaks in DNA, the final step in the apoptotic process. Cells undergoing apoptosis (i.e. increased caspase production) but not to the point of DNA degradation, would not be detected by the TUNEL assay. Thus, our cytometric results are a conservative measure of cell death as compared to the more subjective measure of visual assessment. Moreover, flow cytometric analysis of TUNEL staining

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eliminates any subjective bias of visual counting of TUNEL-positive dead cells. Given these considerations, the conservative estimate of cytokine-induced apoptosis of CLENDOs in the current study is not surprising.

Understanding the mechanisms of TNF receptor signaling could be key to understanding the apoptotic actions of TNF and TNF + IFNG on CLENDOs. TNF, via TNF receptor 1, signals through both pro- and anti-apoptotic pathways [121]. Ligand binding induces the activation of death domain associated proteins, such as caspases, while also inducing the activation of NF-κB-associated anti-apoptotic proteins such as cellular FLICE-like inhibitory protein (cFLIP), B-cell leukemia/lymphoma 2 (Bcl-2), and cellular inhibitor of apoptosis protein-1 (cIAP-1) [121]. Inhibition of NF-κB activity by proteins such as IRF-1 leads to increased cellular susceptibility to TNF-induced apoptosis [121]. TNF and IFNG independently and synergistically promote the expression of IRF-1 [152]. IRF-1 inhibits NF-κB mediated activation of cell survival signals through modulation of NF-κB transcriptional activity [106]. Therefore, IRF-1 induction by TNF and IFNG could inhibit the anti-apoptotic NF-κB pathway, resulting in cell death/apoptosis. In the present study, TNF and IFNG alone and in combination induced IRF-1 expression in CLENDOs, but there was no effect attributable to reproductive status. The ability of TNF and IFNG to activate signaling pathways in CLENDOs of the estrous cycle and pregnancy that result in IRF-1 expression could explain the noted increase in death seen with TNF + IFNG as compared to TNF alone by Pru and co-workers (2003) [139]. With TNF alone, both pro- and anti-apoptotic pathways become activated, resulting in some limited cell death.

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Conversely, with TNF + IFNG, the pro-apoptotic pathway prevails. The synergistic increase in IRF-1 expression decreases the activation of the anti-apoptotic NF-κB pathway, thereby inhibiting production of anti-apoptotic proteins, and increasing the extent of cell death. Measuring the expression of anti-apoptotic proteins under similar cytokine treatments could provide insight into this paradigm.

The cellular source(s) of TNF within the CL has been debated for a number of years. Hehnke-Vagnoni and co-workers (1995) localized TNF to endothelial cells of the porcine CL, stating that macrophages are rarely evident and not associated with TNF production [126]. In contrast, Zhao and co-workers (1998) identified macrophages as the major source of TNF within the porcine CL, finding that endothelial cells consistently lacked TNF staining [127]. For both studies, the basis of TNF assessment utilized immunohistochemistry and/or TNF bioassay. Neither study measured TNF production by quantitative immunoassay using purified cell preparations. The present study used a bovine TNF ELISA to determine TNF production by CLENDOs of the estrous cycle and pregnancy. Under the stimulation of IFNG, both cell types dose-dependently produced an equivalent amount of TNF. This study is the first to quantify TNF production by endothelial cells of the bovine CL. The implications of this production include the possible autocrine effect TNF may have on CLENDOs to influence cellular signaling and apoptosis. TNF receptors are localized to small luteal cells (small steroidogenic and endothelial cells) of the CL [130, 133] indicating that endothelial cells could produce TNF under IFNG stimulation and respond to TNF

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in an autocrine manner. These possibilities raise the question of whether or not some actions of IFNG are mediated through TNF pathways. We assessed this possibility using a TNF antagonist, the synthetic soluble TNF receptor, etanercept [183].

Cytokine-induced CCL2 production by CLENDOs of pregnancy has been previously documented by our laboratory [48, 82]. We have now determined that cytokine-induced CCL2 production also occurs in CLENDOs of the estrous cycle, and thus, is not affected by reproductive status. Etanercept did not affect IFNG-induced CCL2 production, suggesting that IFNG-induced production of CCL2 is not via an autocrine effect of TNF. Consistent with the results seen for CCL2, etanercept also did not alter IFNG-induced IRF-1 expression. The efficacy and specificity of etanercept was demonstrated by its ability to inhibit TNF-induced CCL2 production and IRF-1 expression. Together, these results indicate the actions of IFNG are not mediated through TNF pathways. However, the ability of etanercept to prevent TNF-induced signaling has important experimental implications for luteal regression. Future *in vivo* work, such as treating cows with etanercept, could be conducted to determine the effect of TNF inhibition during natural luteal regression.

Another interesting finding of the etanercept experiments was the outcome observed following treatment exposure time. Following a 90 min treatment period, etanercept inhibited TNF-induced IRF-1 expression, and there was no difference in IRF-1 expression due to reproductive status of the cow. However, following a 48 h treatment period, there was no significant inhibition of TNF-

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induced IRF-1 expression, and IRF-1 expression was greater in CLENDOs of the estrous cycle. Suter et al. (2001), using CLENDOs of pregnancy, did not detect TNF-induced IRF-1 expression at 24 or 48 h [112]. In contrast, not only was TNF-induced IRF-1 detected after 48 h in the current study, but there was an overall increase in IRF-1 expression due to reproductive status, with a trend toward greater IRF-1 expression in CLENDOs of the estrous cycle. It appears CLENDOs of the estrous cycle respond more readily to cytokines over an extended exposure period than CLENDOs of pregnancy. The reason for this is unknown, but perhaps CLENDOs of pregnancy establish mechanisms to tolerate systemic levels of cytokines and immune events associated with implantation and pregnancy.

The current study compared structural and functional characteristics of CLENDOs of the estrous cycle and CLENDOs of pregnancy. Our results indicate luteal microvascular endothelial cells do not drastically change, structurally or functionally, as the cells transition from the estrous cycle into pregnancy. However, initial disparities in the response of CLENDOs of the estrous cycle versus pregnancy regarding TNF- and TNF + IFNG-induced apoptosis could indicate potential differences in the susceptibility of these cells to mechanisms of apoptosis. Such mechanisms have important implications in understanding the adaptable existence of the luteal microvasculature, and also in comprehending the basic processes of angiogenesis and angioregression. These observations are significant not only to reproduction and fertility, but also to our understanding of angiogenesis and angioregression for cancer research.

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APPENDIX

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LAST NAME	Townson	**FIRST NAME**	David
DEPT	Animal and Nutritional Sciences (UBANSC)	**NEXT REVIEW DATE**	2/27/2005
OFF-CAMPUS ADDRESS (if applicable)	Kendall Hall	**IACUC #**	020201
		REVIEW LEVEL	C
		DATE OF NOTICE	2/26/2004

PROJECT TITLE Role of endothelial cell-immune cell interactions in PGF-induced luteolysis

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a project report with regard to the involvement of animals before that date. If your project is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your project is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation. If you have questions or concerns about your project or this approval, please feel free to contact the Regulatory Compliance Office at 862-2003 or 862-3536.

Please note: Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. *Participation is mandatory* for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

Please refer to the IACUC # above in all correspondence related to this project. The IACUC wishes you success with your research.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File

ORIG APP'L 2/27/2002

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