REGULATION OF THE RECRUITMENT AND FUNCTION OF T LYMPHOCYTES BY ENDOTHELIAL AND STEROIDOGENIC CELLS

A Dissertation in
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by
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ABSTRACT

The corpus luteum (CL) secretes progesterone, which is required for the establishment and maintenance of pregnancy in mammals. Therefore, understanding the mechanisms that regulate luteal function may facilitate the development of new strategies to improve reproductive efficiency in dairy cows. Luteal function is regulated by immune cell mediators, but what is not clearly understood is 1) how the recruitment of immune cells into the CL is regulated and 2) how luteal cells communicate with and affect the function of particular subsets of T lymphocytes. To understand how the process immune cell recruitment into the CL is regulated, a protocol was developed to isolate endothelial cells from the CL. Endothelial cells were enriched from dissociated luteal cells by sequential filtration through microsieves of 50, 15 and 10 µm. About 90% of the enriched endothelial cells bound BS-1 lectin and the majority were immunoreactive for von Willebrand factor VIII. To determine if endothelial cells activated autologous T lymphocytes, endothelial cells were cocultured with autologous T lymphocytes. Endothelial cells activated autologous T lymphocytes by inducing proliferation and increasing the proportion of IL2Rα+ T lymphocytes compared to T lymphocytes cultured alone. To determine if prostaglandins (PGs) modulate the recruitment of T lymphocytes into the CL, endothelial cells were treated with progesterone (0, 5, 1, 5, 10 and 20 µM), PGE₂ (0.002, 0.02 or 0.2 µM) and PGF₂α (0.002, 0.02 or 0.2 µM) and exposed to either PBS or TNFα (50 ng/mL) and IL1β (50 ng/mL). Calcein-AM labeled T lymphocytes were then incubated with endothelial cells and the number of adhered T lymphocytes was determined by fluorescence. T lymphocyte adhesion to endothelial cells was affected by
the concentration of progesterone (cubic response; P<0.05) in both cytokine treated and
untreated endothelial cells. Increasing the concentration of PGE$_2$ and PGF$_{2\alpha}$ from 0 to
100 ng/mL linearly increased and decreased, respectively, the binding of T lymphocytes
on endothelial cells and there was a significant interaction of the prostaglandin treatment
and cytokine treatment (P<0.05). To determine the effect of luteal prostaglandins on
bovine T lymphocytes, the expression of receptors for PGF$_{2\alpha}$ (PTGFR), PGE$_2$ (PTGER2
and 4), and PGI$_2$ (PTGIR) in bovine T lymphocytes was examined. T lymphocytes
expressed transcripts for PTGIR, PTGER2 and 4, but not PTGFR. Luteal cells treated
with either prostaglandin H2 synthase inhibitor (indomethacin) or phospholipaseA2
inhibitor (PLA2X) to inhibit endogenous prostaglandins were cocultured with T
lymphocytes. Inhibiting endogenous prostaglandins had no effect on luteal cell-induced T
lymphocyte proliferation (P>0.05). In addition to the effects of prostaglandins on T
lymphocytes, acute (Ca$^{2+}$ influx and Zap70 phosphorylation) and chronic (cytokine gene
expression, interleukin 2 receptor $\alpha$ expression and cell cycle distribution) effects of
progesterone on T lymphocytes were examined. Progesterone increased Ca$^{2+}$ influx,
interleukin 2 (IL2) gene expression, but decreased the activity of Zap70 and the
proportion of T lymphocytes in the S-phase of the cell cycle in a dose-dependent manner
(P<0.05). Progesterone had no effect on IL2 receptor $\alpha$ protein (P>0.05). Inhibition of the
synthesis of steroids including progesterone in luteal cells using P450 side chain cleavage
enzyme inhibitor, aminoglutethimide (AG) decreased the proportion of $\gamma$δ$^+$IL10$^+$ T
lymphocytes (P<0.05). To determine if the microenvironment within the CL modulates
the function of resident T lymphocytes, luteal cells from either functional or regressing
CL were cultured with $\gamma$δ$^+$ T lymphocytes. The proportion and type of $\gamma$δ$^+$ T lymphocyte
induced to proliferate, and the cytokines produced were assessed by flow cytometry.

Luteal cells of midcycle significantly induced proliferation of the γδ+ WC1− subset (P<0.05) while luteal cells of regressing CL induced proliferation of the γδ+ WC1+ subset (P<0.05). In addition, midcycle luteal cells increased γδ+IL10+ and γδ+GATA-3+ and reduced γδ+IFNγ+ T lymphocytes (P<0.05), but had no effect on the proportion of IL4+, TNFα+ and T-bet+ γδ+ T lymphocytes. Coculture of γδ+ T lymphocytes with luteal cells from regressing CL had no effect on the proportion of γδ+ T lymphocytes expressing each cytokine. To test the hypothesis that midcycle luteal cells induce hyporesponsiveness in γδ+ T lymphocytes, γδ+ T lymphocytes were cultured alone or with midcycle luteal cells. γδ+ T lymphocytes were harvested and restimulated with concanavalin A (ConA).

Exposure of γδ+ T lymphocytes to luteal cells did not alter the mitogenic response of γδ+ T lymphocytes to ConA. To determine the mechanism by which luteal cells stimulate γδ+ T lymphocytes, the expression of activated leukocyte cell adhesion molecule (ALCAM/CD166) which is a ligand for CD6 that is mainly expressed on γδ+WC1− T lymphocytes was examined in luteal tissue and its effect on the proliferation of γδ+ T lymphocytes was examined. The steady-state concentration of ALCAM mRNA was inversely proportional to the expression of ALCAM protein across the luteal phase.

ALCAM was expressed by steroidogenic cells and noncapillary endothelial cells, and its expression on luteal cells was increased by LH, IFNγ and PMA (P<0.05), but not PGF₂α.

Inhibition of CD6-ALCAM interactions by anti-CD6 antibodies tended (P=0.06) to reduce luteal cell-induced γδ+ T lymphocyte proliferation, but siRNA-mediated suppression of ALCAM did not decrease luteal cell-induced γδ+ T lymphocyte proliferation (P>0.05). In conclusion, PGE₂, PGF₂α and progesterone potentially
modulate T lymphocyte recruitment into the CL. Within the CL, the function of T lymphocytes is modulated by the microenvironment. The high concentration of progesterone within the functional CL may potentially suppress the function of resident T lymphocytes. In addition, luteal cells in a functional CL selectively decrease the synthesis IFNγ in γδ⁺ T lymphocytes and induce the expansion of γδ⁺WC1⁻, but upon the induction of luteal regression, luteal cells induce the expansion of γδ⁺WC1⁺ T lymphocytes, which are associated with inflammation.
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Chapter 1

Literature review

Introduction

Reproductive efficiency in high producing dairy cows has declined over the last fifty years (Butler, 2000; Lucy, 2001; López-Gatius, 2003) and yet profitability of dairy operations depends on the cow’s reproductive efficiency. Reproductive inefficiency is reflected by the frequency of pregnancy losses, of which greater than 40% occur after successful fertilization in early pregnancy (Diskin et al., 2006). It has been estimated that each of these losses costs dairy farmers approximately $555 (De Vries et al., 2006). The reduction in reproductive efficiency in high producing dairy cows is associated with advances made in genetic, nutritional and management practices over the last five decades that have resulted in tremendous gains in milk production (Butler, 2000).

Lactation in high producing cows creates a state of negative energy balance postpartum, which delays normal ovulation (Butler, 1981). This conditions, coupled with a high incidence of reproductive disorders and inactive ovaries (López-Gatius, 2003) have a negative impact on reproduction. The high metabolic rate has been suggested to clear reproductive hormones at a faster rate (Sangsritavong et al., 2002; Rinehart et al., 2009) while low energy balance has been suggested to interfere with the reproductive axis at the level of the hypothalamus (Butler, 2000; Walsh et al., 2011). Environmental factors such as heat stress and inadequate nutrition further compound the problem (López-Gatius et
al., 2003; Walsh et al., 2011). In addition to environmental factors, within-animal factors such as low progesterone concentration prior to and after fertilization may contribute to pregnancy failure (Garverick and Smith, 1986; Inskeep, 2004; López-Gatius et al., 2004; Sàbat et al., 2008). Indeed, supplementation of pregnant animals with progesterone improved pregnancy rates (Larson et al. 2007; Sàbat et al., 2008). Contrary to these reports, others have not found any positive effects of supplemental progesterone on pregnancy rates (Arndt et al., 2009). The effects of progesterone supplementation on pregnancy rates are variable (Lonergan, 2011, rev.), but supplementation early after ovulation results in better pregnancy rates (Johnson, 1958; Eduvie and Seguin, 1982; Robinson et al., 1989; Mann et al., 2006). In cows, where treatment with exogenous progesterone resulted in higher pregnancy rates, luteal dysfunction is part of the problem and therefore, understanding the mechanisms that regulate luteal function may lead to identifying methods to improve reproductive efficiency.

The CL is an ovarian endocrine gland that secretes progesterone, a hormone that regulates the duration of the estrous cycle and is responsible for the establishment and maintenance of pregnancy in mammals. In the absence of pregnancy, the CL regresses in response to uterine-derived PGF$_{2\alpha}$. Luteolysis can be induced by administration of exogenous PGF$_{2\alpha}$ (Inskeep, 1973; McCracken et al., 1972). In the event of successful fertilization in ruminants, the elongating embryo secretes IFN-tau, a signal that rescues the CL from regression by altering the secretion pattern of PGF$_{2\alpha}$ pulses (Knickerbocker et al., 1986; Imakawa et al., 1987; Kazemi et al., 1988; Helmer et al., 1989; Roberts et al., 1990).
Events surrounding the genesis, demise and perhaps rescue of the CL (pregnancy) have been shown to involve changes in immune system mediators (Penny et al., 1999; Lawler et al., 1999; Townson et al., 2002; Wu et al., 2004, rev.). These immune mediators such as cytokines affect luteal function (Pate, 1995, rev.; Liptak et al., 2005). The fate of the CL at critical stages during the estrous cycle and pregnancy may be decided by the way luteal cells interact with immune cells present within the CL. Immune cells are present in developing, fully functional (before regression), regressing, and early pregnancy CL (Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002). Although the role of leukocytes in the CL at particular stages is not clearly understood, some subsets of leukocytes and associated inflammatory mediators (cytokines) are upregulated during luteal regression (Penny et al., 1999; Townson et al., 2002) and thus may play a significant role in facilitating luteal demise (Paavola, 1979; Pate, 1995; Pate and Keyes, 2001, revs.). It is hypothesized that the unique local environment that exists at different stages of the CL instructs the resident immune cells to deliver luteotropic or luteolytic support. If immune cells are important players in the physiology of CL, it is important to determine which subsets have an impact on luteal function and decipher how they communicate with steroidogenic and nonsteroidogenic cells. The objectives of this study were to determine the factors that modulate the recruitment of immune cells into the corpus luteum, and to examine the functional interactions between luteal cells and T lymphocytes.
Formation of the corpus luteum

The process that transforms a preovulatory follicle into a CL involves a complex series of events. The process is initiated by pulses of pituitary luteinizing hormone (LH) induced by hypothalamic gonadotropin releasing hormone (GnRH) in response to a positive feedback of estradiol on the hypothalamus (Knobil, 1988). Luteinizing hormone signals through the luteinizing hormone receptor (LHR) present on thecal and granulosal cells and initiates events that culminate in ovulation (Amsterdam et al., 1975). The biochemical processes that occur prior to ovulation include the synthesis of paracrine factors that lead to the expansion of cumulus cells and oocyte resumption of meiosis, breakdown of the basement membrane, recruitment of immune cells, induction of the follicle wall rupture, and differentiation of granulosal and thecal cells into luteal cells. The signal from LH is transmitted via the Gs protein/adenylate cyclase/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway (Marsh, 1970), which targets the cyclic AMP response binding element (CREB) and other transcription factors that drive transcription of gene products that allow ovulation and cellular differentiation to occur. Downstream effectors of LH signaling include epidermal growth factor receptor (EGFR) ligands (Park et al., 2004; Woods and Johnson, 2007), prostaglandins (Tsafiri et al., 1972, Armstrong, 1972; Murdoch et al., 1981; Hedin et al., 1987; Espy et al., 1989; Filion et al., 2001), and enzymes such as membrane metalloproteinases (MMP) and plasminogen activator (PA), which degrade extracellular matrix (Beers et al., 1975; Canipari and Strickland 1985; Murdoch et al., 1986; Butler et al., 1991; Brännström et al., 1988; Curry and Osteen 2003; Lind et al., 2006) and steroidogenic enzymes (Conley
et al., 1995). Differentiation of granulosal and thecal cells involves increased responsiveness to gonadotropins and elevated steroidogenic capacity (Makris et al., 1983; Dieleman and Blankenstein, 1985; Conley et al., 1995). In the cow, the capacity to synthesize progesterone increases while that of estradiol decreases (Conley et al., 1995; Spitschak and Vanselow, 2012).

**The role of the immune system in formation of the corpus luteum**

The hypothesis that the process of ovulation is an inflammatory-like process was first proposed in detail by Espy (1980). Over the years evidence supporting this hypothesis has been generated in both vivo and in vitro experimental systems. The process of ovulation, triggered by the LH surge and is accompanied by leakage in the vasculature, accumulation of leukocytes, synthesis of prostaglandins and the proteolytic cascade of enzymes; these are the hallmarks of inflammation (Salmon and Higgs, 1987). Indeed, the process of ovulation was inhibited by anti-inflammatory drugs such as indomethacin (Armstrong, 1972; Tsafiriri et al., 1972; Espey et al., 1989; Murdoch, 1989) and could be restored by prostaaglandins (Gaytán et al., 2002). Prostaglandins are present at inflammatory sites (William and Morley, 1973), but in this particular case they may be involved in changes in vascular dynamics such as, vascular dilation and permeability (Murdoch, 1986; Hirasawa et al., 1986; Abisogun et al., 1988). Changes in vascular dynamics during ovulation are associated with increased blood flow and create a leaky vasculature that allows for recruitment of immune cells at sites of inflammation (Brännström, 1998). Neutrophils and eosinophils, monocyte/macrophages, mast cells
and basophils migrate out of the vascular compartment before ovulation, after the initiation of the LH surge in sheep (Cavender and Murdoch, 1988).

The increase in prostaglandins, especially PGE₂, at inflammatory sites correlates with the abundance of leukocytes (Salmon et al., 1983) and leukocytes have been detected in the theca of the preovulatory follicle. In the preovulatory follicle, increased PGE₂, interleukin 8 (Arici et al., 1996; Połec’ et al., 2008), monocyte chemoattractant protein-1 (Arici et al., 1997), macrophage migration inhibitory factor (Wada et al., 1999), thymus expressed chemokine (Zhou et al., 2009), P-selectin (Bonello et al., 2004; Sayasith et al., 2005), platelet endothelial cell adhesion molecule (PECAM-1) and intercellular adhesion molecule-1 (ICAM-1; Oakley et al., 2010) may promote leukocyte infiltration. Neutrophils, eosinophils and macrophages are recruited to the preovulatory follicle (Brännström et al., 1993a; Murdoch and McCormick, 1993; Gaytan et al., 2003), presumably from the spleen (Oakley et al., 2010; Hedin, 2010).

The presence of leukocytes at the time of ovulation increases the rate of ovulation in the rat and mouse (Hellberg et al., 1991; Zhou et al., 1999). Furthermore, antibody-mediated depletion of neutrophils, as well as drug-induced depletion of macrophages, reduces ovulation in the rat (Brännström et al., 1995a; van der Hoek et al., 2000). Recently, depletion of eosinophils using dexamethasone reduced progesterone production in midcycle bovine CL, probably due to disrupted angiogenesis (Kliem et al., 2012). The effects of immune cells on ovulation are likely mediated by leukocyte-derived cytokines (Brännström 1993b; Brännström et al., 1994a; 1994b;1995b; Adashi, 1998; Fedorcsák et al., 2007) that may directly or indirectly regulate the process of ovulation. In general, in a preovulatory follicle, leukocytes are proposed to induce

**Development and function of the corpus luteum**

The CL is short-lived with three distinct stages in every estrous cycle i.e, luteinization, luteotropism and luteolysis. The lifespan of the CL determines the duration of the estrous cycle, which ranges between 17-24 days in cows. In the event of fertilization, the embryo secretes IFNtau, which extends the life of the CL through gestation (Knickerbocker et al., 1986). The CL is required for at least 200 days to maintain pregnancy (Estergreen et al., 1967). Two major cell types derived from the ovulatory follicle, steroidogenic (theca and granulosa cells) and nonsteroidogenic cells (endothelial cells and fibroblasts), constitute the ovarian endocrine CL.

**Steroidogenic cells**

Following ovulation, theca and granulosa cells undergo morphological, biochemical and functional changes to give rise to small and large steroidogenic cells, respectively (Corner et al., 1919; Priedkalns et al., 1968; O’Shea et al., 1980; Alila and Hansel, 1984). Although the two cell types seem to be nonoverlapping populations, in the bovine CL, small luteal cells may develop into large luteal cells (Alila and Hansel, 1984). Large luteal cells account for 3.5-4% of luteal cells in a mature CL and occupy up to 40%
in volume (O’Shea et al., 1989; Rodgers et al., 1984; Sangha et al., 2002). The proportion of small luteal cells is approximately 26.7% and these occupy 27.7% of all cells in a mature CL (O’Shea et al., 1989). These two cell types differ greatly in their size among other characteristics. The size of large luteal cells ranges from 18 μM-50 μM while the size of small luteal cells range from 12-22 μM (Fitz et al., 1982; Rodgers et al., 1984; Chegini et al., 1984; Schwall et al., 1986; Weber et al., 1987; O’Shea et al., 1989). These sizes are similar to those reported in CL of rats (Smith et al., 1989) and humans (Lei et al., 1991). Steroidogenic cells undergo proliferation (hyperplasia) and increase in size (hypertrophy) as the cycle progresses. Large luteal cells mainly undergo hypertrophy while small luteal cells undergo proliferation (Farin et al., 1986; Schwall et al., 1986; O’Shea et al., 1986; Watson and Sertich, 1990; Smith et al., 1994). The growth of small and large luteal cells is very conspicuous in the early CL (Spitschak and Vanselow, 2012). Zheng et al. (1994) showed that the majority of the proliferating cells were small cells, including nonsteroidogenic cells, and that this occurred in the developing (days 1-4) and mature CL (days 5-10). Proliferation substantially declines in late phase (days 18-21). Large luteal cells also possess a higher cytoplasmic/nuclear ratio with cytoplasm containing more abundant mitochondria, lipid droplets, dense granules and lysosomes compared to small luteal cells (Chegini et al., 1984). Other morphological changes that occur before and after ovulation in domestic species are detailed by Smith et al. (1994).

Differences in steroidogenic function between small and large luteal cells have been well-documented in a variety of species (Wiltbank, 1994). Basal progesterone production is greater in large compared to small luteal cells (Fitz et al., 1982; Rodgers et
al., 1983; Alila et al., 1988). Large luteal cells, unlike small luteal cells, are less responsive to LH stimulation due to the presence of fewer LH receptors (Weber et al., 1987). Progesterone synthesis in large luteal cells is partly explained by the high expression of the hydroxysteroid dehydrogenase β1 (HSD3B1) gene (Spitschak and Vanselow, 2012). Small luteal cells have numerous LH receptors and upon stimulation with LH, progesterone synthesis increases 3 to 4-fold (Fitz et al., 1982; Rodgers et al., 1983). In the pig and cow, small and large luteal cells cooperatively produce progesterone (Lemon and Mauléon, 1982; Rodgers et al., 1985; Del Vecchio et al., 1994; Niswender et al., 1994).

In the cow, ovarian vein plasma progesterone increases from 0.056 μg/mL on day 1 (day 0 = estrus) to about 1.25 μg/mL on day 8 and to about 1.80 μg/mL on days 14 and 15 of the estrous cycle (Dobrowolski et al., 1968). In the absence of a viable embryo, the CL is destined for regression. This process is initiated by uterine PGF$_2\alpha$ and marked by a decline in progesterone and cell death due to apoptosis (Zheng et al., 1994).

**Nonsteroidogenic cells**

The breakdown of the periovulatory follicle basement membrane decompartmentalizes the structure of the follicle. During this process, immune cells infiltrate the CL and endothelial cells from the theca migrate, proliferate and form new capillaries from existing blood vessels (angiogenesis). Both immune cells and endothelial cells contribute to the development of the CL (Shirasuna et al., 2012; rev.).
Endothelial cells

A network of capillaries in the developing CL is derived from the thecal vasculature through the process of angiogenesis (Augustin et al., 1995) and is supported by luteal intrinsic factors. The angiogenic capacity of follicles and the CL was first reported by Gospodarowicz and Thakral (1978) and later the angiogenic factors, mainly fibroblast growth factors (FGF) and vascular endothelial growth factors (VEGF), were reported in ovine and bovine (Grazul-Bilska et al., 1991; Grazul-Bilska et al., 1992a; Zheng et al., 1993; Neuvians et al., 2004) as well as human (Suzuki et al., 1998) CL. These two factors are responsible for formation of the vast capillary network (Woad et al., 2009), but also critical for regulating luteal function (Yamashita et al., 2008).

Before ovulation, the vasculature of the ovulatory follicle is restricted to the thecal layer, but upon induction of the LH surge, thecal endothelial cells undergo a process known as angiogenesis. Angiogenesis is a cascade of events that include migration, proliferation and formation of new blood vessels from pre-existing blood vessels (Cavender and Murdoch, 1988). Also, there is evidence to suggest that some of the new blood vessels are generated as a result of the bone marrow-derived progenitor cells in a process referred to as neovascularization (Kizuka et al., 2012). The proliferation of endothelial cells in the CL is high and has been compared to that of solid tumors (Neeman et al., 1997). In the early luteal phase of ovine (Rodger et al., 1997) and human CL (Christenson and Stouffer, 1996), more than 50% and up to 85%, respectively, of proliferating cells are endothelial cells. The number of blood vessels per unit area and the size of the lumen increase (Cavender and Murdoch, 1988) and most steroidogenic cells
are in contact with a capillary in a mature CL. The changes in luteal vasculature throughout the bovine estrous cycle follow the secretory function of the CL (Zheng et al., 1993). These authors demonstrated that vascular density is high in developing and mature CL (days 1-4 and 5-17), but significantly declines during the late stage (days 18-21). In the late stage, capillaries are almost absent, but the large microvessels are still present. In the event of luteal regression, endothelial cells are targeted first compared to other cells (Farin et al., 1986; Sawyer et al., 1990) mainly due to apoptosis (O’Shea et al., 1977). The disruption of endothelial integrity during regression is partly mediated by transforming growth factor beta (TGFB; Maroni and Davis, 2011).

The volume of vasculature present at the different stages of the estrous cycle is controlled by angiogenic and antiangiogenic factors produced by steroidogenic cells (Reynolds and Redmer, 1998 rev.; Reynolds et al., 2000 rev.; Plendl, 2000 rev.; Robinson et al., 2009 rev.). In ovine (Grazul-Bilska et al., 1991) bovine (Grazul-Bilska et al., 1992b; Zheng et al., 1993; Nevians et al., 2004) human (Wulff et al., 2000) and porcine (Boonyaprakob et al., 2003) CL, the major angiogenic factors have been found to be fibroblast growth factor (FGF) and vascular endothelial growth factor (VGEF).

The proportion of endothelial cells in a mature CL of bovine (Rodgers et al., 1984; O’Shea et al., 1989), ovine (O’shea et al., 1986), human (Lei et al., 1991) and guinea pig (Azmi and O’Shea, 1984) CL is greater than 50%. This extensive vasculature delivers oxygen, nutrients, gonadotropins and lipoprotein-derived cholesterol, required for steroidogenic function of the CL (O'Shaughnessy and Wathes, 1985; Pate and Condon, 1982; Pate and Condon, 1989). The CL has a high rate of metabolism and there is a higher rate of blood flow to the CL compared to the ovarian stroma and reproductive
organs, such as the uterus and vagina (Bruce and Moore, 1976; Wiltbank et al., 1988; Zheng et al., 1993). By regulating blood flow, the vasculature is directly involved in not only maintaining luteal function, but also facilitating luteal regression (Girsh et al., 1995; Azmi et al., 1982; Miyamoto et al., 2005).

In addition to having a supportive role in steroidogenesis, luteal endothelial cells may be actively involved in the recruitment of immune cells in the CL (Townson, 2006). The process of recruiting immune cells into tissues has been widely studied. It is a stepwise process, in which immune cells roll, stop, attach/adhere and extravasate through the endothelium. This interaction is mediated by adhesion molecules present on immune cells that recognize counterparts on endothelial cells (Springer, 1994; 1995 rev). Generally, the expression of adhesion molecules on luteal endothelial cells and the mechanisms that regulate recruitment of immune cells in the CL have not received a lot of attention.

**Immune cells**

Leukocytes are present in CL of a number of species including rodent (Pavoola, 1979; Bagavandoss et al., 1988; Brännström et al., 1994c; McCormack et al., 1998; Komatsu et al., 2003), bovine (Lobel and Levy, 1968; Spanel-Borowski et al., 1997; Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002; Davis and Pate, 2007), ovine (Murdoch et al., 1988), equine (Lawler et al., 1999), porcine (Hehnke et al., 1994) and primate (Adams and Hertig 1969; Wang et al., 1992). The numbers and types of immune cells present in the CL change relative to the stage of the estrous cycle and the
physiological state of the animal (Penny et al., 1999; Duncan et al., 1998; Lawler et al., 1999; Towson et al., 2002). Early in the developing CL, granulocytes (neutrophils and eosinophils) are abundant, but they are barely detectable in advanced stages of luteal development and regression (Priedkalns et al., 1968; Reibiger and Spanel-Borowski, 2000; Rohm et al., 2002; Jiemtaweboon et al., 2011). Macrophages/monocytes, on the other hand, are fewer during stages of development and early pregnancy, but are higher in midcycle and regressing CL (Penny et al. 1999; Lawler et al., 1999; Townson et al., 2002). Various subsets of T lymphocytes are also present in the CL prior to the onset of luteolysis (Spanel-Borowski et al., 1997; Lawler et al., 1999; Penny et al., 1999; Townson et al., 2002; Poole and Pate, 2012) and stay elevated during luteal regression (Penny et al., 1999; Bauer et al., 2001). Recently, neutrophils have been shown to infiltrate the bovine CL as early as 5 minutes after PGF$_{2\alpha}$ administration (Shirasuna et al., 2012). The increase in immune cells prior to luteal regression as well as the presence of cytokines that inhibit steroidogenesis (Nothnick and Pate, 1990; Fairchild and Pate, 1991; Benyo and Pate, 1992; Townson and Pate, 1996) support arguments that immune cells potentiate luteolysis. Cytokines such as interferon γ (IFNγ) and tumor necrosis factor α (TNFα) inhibit progesterone production and are cytotoxic to luteal cells (Fairchild and Pate, 1991; Benyo and Pate, 1992; Fukuoka et al., 1992, Wang et al., 1992). Conversely, the presence of T lymphocytes in the CL prior to the onset of luteal regression (Penny et al., 1999), suggests these cells may perform different functions in a functional CL. In vitro studies have shown that T lymphocyte-derived cytokines, IL4 and IL10 augment progesterone production from luteal cells (Hughes et al., 1990; 1991; Emi et al., 1991; Hashii et al., 1998).
**T lymphocytes**

T lymphocytes are broadly defined by the expression of an antigen receptor, the T cell receptor (TCR), acquired by developing thymocytes in the thymus (Snodgrass et al., 1985). Lymphoid progenitor cells, which originate from the bone marrow in adult animals, colonize the thymus and interact with thymic stromal cells to mature and differentiate into two types of lymphocytes with two distinct heterodimeric TCRs; αβ⁺ and γδ⁺ (Anderson et al., 1996 rev.; Takahama, 2006 rev.;). Before mature αβ T lymphocytes exit the thymus, they go through a rigorous selection process to select those that recognize self-major histocompatibility complex I and II (MHC I and II), but eliminate those that would react to self-antigens (Kruisbeek et al., 1981; 1983; Maruic-Galesic et al., 1988; Takahama, 2006, rev.). During the maturation process, T lymphocytes that express the αβ TCR also express surface proteins (CD4 and CD8) that uniquely divide these cells into two nonoverlapping populations: CD4⁺ and CD8⁺ αβ⁺ T lymphocytes. These surface glycoproteins are important for thymocyte development of αβ⁺ T lymphocytes and act as co-stimulatory molecules in mature T cells during T lymphocyte activation (Fung-Leung et al., 1991; Leahy, 1995).

The molecular, biochemical or hormonal signals that lead to the commitment of lymphoid progenitor cells to either αβ or γδ lineage are still being studied. Both αβ⁺ and γδ⁺ cells are present in blood, but also populate and circulate between lymphoid and nonlymphoid tissues. Their characteristics have been described in birds, rodents, humans and cattle, among other species (Glick, 1979; Sharma, 1991, rev.; Zhu and Paul, 2008; Wilson et al., 1996). Current understanding of the functions of αβ⁺ and γδ⁺ lymphocytes
has mostly come from rodents and humans, but livestock species and birds continue to shape the knowledge of how T lymphocytes function. Whereas the knowledge of how $\alpha\beta^+$ T lymphocytes function is abundant, knowledge about the function $\gamma\delta^+$ lymphocytes is still evolving.

In addition to their primary purpose of protecting the body against foreign pathogens, $\alpha\beta^+$ or $\gamma\delta^+$ T lymphocytes and or their products clearly modulate the function of reproductive organs and certainly the outcome of reproductive processes such as pregnancy (Erlebacher et al., 2004). Interestingly, steroids produced by reproductive organs also have a pronounced effect on T lymphocyte development (Kong et al., 2002) and function (Szekeres-Bartho et al., 1983; Low and Hansen, 1988; Burton and Kehrli, 1996; Feinberg et al., 1992; Bouman et al., 2005).

**$\alpha\beta$ (CD4 helper and CD8 cytotoxic) T lymphocytes**

$\text{CD}4^+$ T lymphocytes are helper cells that produce cytokines, which activate and support functions of B cells, $\text{CD}8^+$ lymphocytes, and macrophages. The T cell receptor of $\text{CD}4^+$ and $\text{CD}8^+$ T lymphocytes recognizes antigens in the context of MHCII and MHCI respectively, (Meuer et al., 1982; Dialynas et al., 1983, rev.; Swain, 1983, rev.). Upon recognition of MHC-antigen complexes, $\text{CD}4^+$ T lymphocytes differentiate into subpopulations with specific functional characteristics based on the cytokine profile they secrete. Th0 are the precursors of Th1 and Th2 and secrete both Th1 and Th2 cytokines. Th1 secrete IFN$\gamma$ and interleukin (IL)-2 and a greater amount of TNF$\alpha$ compared to Th2, while Th2 cells secrete IL4, IL5, IL9, IL10 and IL13; Th3 secrete TGFB, but not IL2,
IFNγ, IL10 or IL4 (Mosmann and Coffman, 1989, rev.; Mosmann and Sad, 1996, rev.). It was also established that Th1 CD4+ T lymphocytes are involved in cell mediated immunity while Th2 were involved in humoral immunity (Mosmann and Coffman, 1989, rev.). In addition to the classical Th1 and Th2, CD4+ T lymphocytes also generate Th17 (Bettelli et al., 2007) and regulatory T lymphocytes (Vigouroux et al., 2004; Abraham et al., 2008). Differentiation of each of these CD4+ T lymphocyte subsets is driven by a specific transcription factor in addition to a specific cytokine environment; Th1, Th2, and Th17 require T-bet (Szabo et al., 2000; Szabo et al., 2002; Lovett-Racke and Rocchini, 2004), GATA-3 (Zheng and Flavell, 1997) and the orphan nuclear receptor RORγt (Ivanov et al., 2006, rev.) transcriptional factors, respectively. Differentiation of Th1, Th2 and Th17 also requires the presence of IL12, TGFβ and IL-6 cytokines, respectively (Miyaura and Iwata, 2002; Chakir et al., 2003; Mus et al., 2010). The development of one T cell subset antagonizes the development of another. For instance, cytokines secreted by Th1 are antagonistic to Th2 development (Gajewski and Fitch, 1988; Grogan et al., 2001; Zhu et al., 2006; Usui et al., 2006). Also, interleukin 23 (IL23), which promotes the development of Th17 lymphocytes, inhibits T-bet and Foxp3, which are transcription factors required for differentiation of Th1 and regulatory T lymphocytes (Mus et al., 2010). It is clear that in addition to the nature of the antigenic signal received by precursor cells, the environment in which the precursor cell is stimulated determines its fate and effector functions (Swain et al., 1991; Kelso et al., 199; Miyaura and Iwata, 2002).

Similar to human T lymphocytes, T lymphocytes in cattle are identified by orthologs of CD2, CD4 and CD8 (Baldwin et al., 1986; Ellis et al., 1986; Howard et al.,
CD4\textsuperscript{+} cells constitute 30\% (Baldwin, 1986), while CD8\textsuperscript{+} T cells account for about 20\% of PBMCs (Ellis et al., 1986). These populations significantly vary depending on the age of the animal (Ayoub and Yang, 1996).

\textit{γδ\textsuperscript{+} (gamma delta) T lymphocytes}

A second T cell receptor was first discovered in mice lymphocytes (Brenner et al., 1986) and later in humans (Ioannides et al., 1987). These lymphocytes do not express the αβ TCR chains, but express γ and δ chains. The bovine orthologs of human γ and δ chains were cloned and γδ\textsuperscript{+} T lymphocytes identified in cattle (Takeuchi et al., 1992).

Gamma delta T lymphocytes utilize their TCR in a different way than αβ\textsuperscript{+} T cells. Their recognition of antigens is not restricted to MHC-bound antigens (Morita et al., 1995; Schild et al., 1994; Weintraub et al., 1994).

\textbf{Antigen recognition of γδ\textsuperscript{+} T lymphocytes}

Unlike αβ\textsuperscript{+} T cells, the mechanisms by which γδ\textsuperscript{+} T lymphocytes recognize antigen and are stimulated by antigen presenting cells are still elusive, but some important findings have been made (Ribot et al., 2011). Murine γδTCR recognize nonclassical major histocompatibility molecule T22 via a conserved motif (W... EGYEL) present in the γδTCR complementary-determining region 3δ (CDR3δ) loop and T22 (Bonneville et al., 1989; Sandstrom et al., 2012). This recognition does not involve peptides (Weintraub et al., 1994). Human γδ\textsuperscript{+} T lymphocytes of the Vδ1 TCR phenotype
have also been shown to recognize nonpolymorphic molecules such as CD1 (Spada et al., 2000). Morita et al. (1995) demonstrated that human \(V_\gamma 2V_\delta 2^+\) T lymphocytes directly recognize bacterial nonpeptide prenylpyrophosphate antigens in a manner that does not require antigen uptake, MHC class I or II expression and or CD1 (a, b or c). In these experiments, fixed accessory cells also presented prenylpyrophosphate antigens to \(V_\gamma 2V_\delta 2^+\) T lymphocytes. In tumor cells, where the mevalonate pathway has been blocked, leading to the accumulation of isoprenylpyrophosphate (IPP), \(V_\gamma 9V_\delta 2^+\) cells induce killing of tumor cells (Li et al., 2009). In contrast, there is evidence to suggest that, similar to \(\alpha \beta^+\) T lymphocytes, some subsets of \(\gamma \delta^+\) T lymphocytes recognize alloantigens via MHC II (Flament et al., 1994). Indeed, proliferation of resting human \(\gamma \delta\) T lymphocytes was inhibited when allogeneic dendritic cells (DCs) were treated with antibodies to HLA-DR (Takamizawa et al., 1995). In addition, these authors demonstrated that the response to DCs was largely mediated by CD28, a costimulatory receptor for CD80 ligand. Indeed, Ribot et al. (2012) have demonstrated that CD27+ and CD27− murine \(\gamma \delta^+\) T lymphocytes express CD28 and that signaling through CD28 by B7 molecules, is important for IL2-mediated survival and proliferation.

Another subset of human \(\gamma \delta^+\) T lymphocytes that populates the intestine, \(V_\delta 1^+\), recognizes stress-induced major histocompatibility complex (MHC) class I–related chains A and B (MICA and MICB) on intestinal epithelial cells (Groh et al., 1998). Major histocompatibility complex class I–related chains deliver two signals to \(V_\delta 1^+\): activation of the TCR and costimulation through a natural killer cell activating ligand, NKG2D (Wu et al., 2002). These signals result in expansion of \(V_\delta 1^+\) \(\gamma \delta^+\) T lymphocytes (Qi et al., 2003). Murine \(\gamma \delta^+\) T lymphocytes that reside in the skin also referred to as DETCs,
produce keratinocyte growth factor and are important in wound repair (Jameson et al., 2002). Bovine γδ+ T lymphocytes also recognize protein and nonprotein antigens (Welsh et al., 2002). However, it has not yet established if any of the antigens that are recognized by murine and human γδ+ T lymphocyte subsets are expressed by cultured luteal cells.

γδ+ T lymphocytes in cattle

Compared to primates or rodents, ruminants have a higher proportion of γδ+ T lymphocytes in the peripheral blood (Itohara et al., 1989, Hein and Mackay, 1991). In young calves, γδ+ T lymphocytes constitute up to 60% of peripheral blood lymphocytes (Wyatt et al., 1994). In ruminants and pigs, γδ+ T lymphocytes are generally identified by the expression of workshop cluster 1 molecule (WC1), which is a transmembrane glycoprotein that belongs to the scavenger receptor cysteine-rich family of proteins including CD5 and CD6 (Mackay et al., 1989; Clevers et al., 1990; Wijngaard, et al., 1992; Morrison and Davis, 1991; Carr et al., 1994). Workshop Cluster 1 appears during thymic development (Mackay et al., 1989) and is a coreceptor on γδ+ T lymphocytes that is involved in regulating cell cycle progression and proliferation (Takamatsu et al., 1997; Hanby-Flarida et al., 1996a; Rogers et al., 2005a).

Anti-WC1 antibodies (IL-A29), but not anti-CD5 or anti-δTCR, augment proliferation of bovine γδ+ T lymphocytes in an autologous mixed leukocyte reaction (AMLR) (Hanby-Flarida et al., 1996a; Baldwin et al., 2000). These reports indicate that signaling through WC1 may deliver a γδ+ T lymphocyte activation signal. Indeed, the amino acid sequence of the intracytoplasmic tail contains both potential immunoreceptor
tyrosine-based activation motifs (ITAM) and a Src homology binding motif (SH-2) (Wijnagaard et al., 1992; Rogers et al., 2005a). Contrary to the stimulatory effect of anti-WC1 antibodies in bovine γδ⁺ T lymphocytes, anti-WC1 antibody (SC-29) inhibits IL2 synthesis and arrests IL2-dependent ovine S-59 γδ⁺ T lymphocytes in G0/G1 phase of the cell cycle (Takamatsu et al., 1997). It was later demonstrated that this effect was a result of activation of tyrosine phosphates that led to dephosphorylation of mitogen activated protein kinase (MAPK) extracellular regulated kinase 2 (Erk2) (Kirkham et al., 1997). The SC-29 antibody also induces a decrease in the gene expression of the transcription factor E2F (Kirkham et al., 1998). In these studies, the cell cycle arrest could be reversed by removing the antibody or by stimulating γδ⁺ T lymphocytes with anti-CD3 antibodies and Concanavalin A (ConA) mitogen.

Phenotypic and related functional characteristics categorize γδ⁺ T lymphocytes into two major populations: CD2⁺ CD8⁻γδTCR⁺WC1⁺ and CD2⁺CD8⁺γδTCR⁺WC1⁻ (Clevers, et al., 1990; Machugh et al., 1997). The former are more abundant in circulation; the latter localize in tissues such as the spleen (Machugh et al., 1997; Wilson et al., 1999). Subpopulations within the γδ⁺WC1⁺ have also been defined based on the antigenic diversity of the WC1 protein (O’Keeffe et al., 1994; Herzig and Baldwin, 2009). Thus, specific antibodies that recognize WC1 isoforms, WC1.1, 1.2 and 1.3 have been used to differentiate the functional properties of these subsets (Wijnagaard et al., 1994; Rogers et al., 2005a; b).

The functional diversity of γδ⁺ T lymphocytes in cattle is increased by the expression of 13 WC1 genes in the bovine genome and WC1 variants generated by alternative splicing (Herzig and Baldwin, 2009). Gene expression profiling and functional
studies have demonstrated that both of these subsets have effector as well as regulatory roles (Baldwin et al., 2000; Pollock and Welsh, 2002; Hoek et al., 2009). Gamma delta T cells synthesize both Th2 (IL4 and 10) and Th1 cytokines (TNFα and IFNγ) that are important in adaptive immunity (Ferrick et al., 1995; Wen et al., 1998; Baldwin et al., 2000; Baldwin et al., 2002; Tanaka et al., 2008; Johnson et al., 2008; Hoek et al., 2009; Ashour and Niederkorn, 2006), but also possess characteristics of innate immunity (Collins et al., 1998; Hedges et al., 2005). Gamma delta T lymphocytes have innate abilities to recognize pathogen-associated molecular patterns (Kerns et al., 2009; Martin et al., 2009) and respond to auto-antigens (Okragley et al., 1996; Sathiyaseelan et al., 2002). Gamma delta T lymphocytes also express class II MHC molecules and process and present antigens (Collins et al., 1998; Price and Hope, 2009).

The γδ+ T lymphocytes present in circulation and at mucosal surfaces may be very important in responding to foreign antigens (Ferrick et al., 1995; Kennedy et al., 2002), while those present in peripheral nonlymphoid tissues may be involved in tissue homeostatic mechanisms (Jameson and Havran, 2007; rev.). In the CL, the majority of the resident γδ+ T lymphocytes are of the γδTCR+WC1− phenotype (Poole and Pate, 2012). The role of these cells in luteal function is not clear, but their function may depend on the microenvironment present within the CL at different physiological stages (Poole and Pate, 2012).
Significance of immune cells in luteal function

T lymphocytes

In vitro studies have been very instrumental for deciphering the mechanisms by which immune cells modulate the function of luteal cells; cytokines produced by T lymphocytes likely facilitate luteolysis (Pate, 1994, rev.; Pate and Townson, 1994; Pate and Keyes, 2001, rev.). Conversely, T lymphocytes have been shown to enhance progesterone production in human (Emi et al., 1991), pig (Hughes et al., 1990) and rat (Hughes et al., 1991) granulosa cells. In addition, there is evidence that antiinflammatory cytokines such as IL10 and IL4 and an unidentified low molecular weight protein of T lymphocyte origin stimulate progesterone production (Hashii et al., 1998; Emi et al., 1991). When autologous porcine T lymphocytes obtained from blood were co-cultured with granulosa cells, there was no effect on progesterone production (Maier and Chew, 1990). In addition, depletion of both macrophages and lymphocytes did not alter progesterone secretion and did not extend the duration of pseudopregnancy in the rabbit (Seiner et al., 1992). In contrast, T lymphocytes may have a role in luteal regression and thus affect the length of the estrous cycle. In the rat, where luteal regression is dependent on prolactin, Kuranaga et al. (1999) showed that CD3\(^+\)FasL\(^+\) cells were localized to areas where apoptosis was observed in luteal cells and that the expression of FasL was increased by prolactin. Removal of CD3\(^+\) T lymphocytes expressing Fas ligand inhibited apoptosis in luteal cell cultures (Kuranaga et al., 2000).

General immunosuppressants have been used to demonstrate the effect of immune cells on luteal function. Dexamethasone-treated rats with intact uteri exhibited prolonged
estrous cycles, but this response was not observed when hysterectomized rats were treated with PGF$_{2\alpha}$ (Wang et al., 1993). In cattle, Alila and Hansel (1984) reported that lymphopenia reduced progesterone production by decreasing pituitary LH, but no effect on estrous cycle length was observed. In heifers treated with betamethasone, the length of the estrous cycle increased by 10 days (Kanchev et al., 1976), but it was not clear if betamethasone reduced the number of immune cells in the CL.

**Macrophages/monocytes**

The role of macrophages in ovarian physiology has been reviewed by Wu et al. (2004). Macrophages facilitate processes such as ovulation, luteinization, vascularization, steroid production and apoptosis. Macrophage ablation in the CD11b-diphtheria toxin receptor (DTR) mouse model disrupts angiogenesis that might subsequently affect luteal function (Turner et al., 2011). The effects of macrophages on progesterone production by luteal cells have been demonstrated in human and rodents to be both inhibitory and stimulatory. Peritoneal macrophages decrease basal and agonist-induced progesterone production in rat granulosal cells (Shakil and Whitehead 1994; Whitehead and Lacey, 1996). In addition, when human luteal cells are depleted of macrophages, there is an increase in progesterone production, and removal of macrophages decreases the amount of IL-1β (Castro et al., 1998). On the contrary, peritoneal macrophages increase progesterone production in human (Halme et al., 1985) porcine (Maier and Chew, 1990) and mouse (Kirsh et al., 1983) granulosal-luteal cells. Deletion of macrophages has provided a greater understanding of their contribution to luteal function. Mice deficient in
colony stimulating factor-1(CSF-1) have prolonged estrous cycles compared to the wild type (Cohen et al., 1997). Colony stimulating factor-1 is responsible for the recruitment, proliferation and differentiation of monocytes into macrophages. Similarly, conditional ablation of macrophages using diphtheria toxin results in endothelial depletion in the mouse CL and loss of tissue integrity (Turner et al., 2011).

Neutrophils and eosinophils

Neutrophils and eosinophils also affect luteal function. In the rat, activated neutrophils inhibit LH-stimulated progesterone production (Pepperell et al., 1992). In the mouse, antibody-mediated depletion of neutrophils disrupts the estrous cycle by increasing the length of diestrus (Sasaki et al., 2009; Sasaki et al., 2011). In the sheep, depletion of eosinophils results in low progesterone production, but has no effect on PGF$_2$α induced luteal regression (Murdoch and Steadman, 1991). However, supernatants of activated bovine neutrophils induce capillary-like structures in endothelial cells, and therefore neutrophils may also play a role in luteal angiogenesis (Jiemtaweboon et al., 2011).

The origin of immune cells recruited to the ovary

Two studies by Narai et al. (1995) and Endo and Kanayama (1998) demonstrated that splenectomy increased the length of pseudopregnancy in rabbits. These studies indicate that the spleen may be the source of immune cells recruited to the ovary. Only recently
has the hypothesis been tested that immune cells recruited to the ovary are from the spleen. Oakley et al. (2010) demonstrated that splenectomy decreased the number of macrophage/granulocytes and lymphocytes in the ovary, and there was a strong inverse relationship between leukocyte numbers in the spleen and ovary. Also, reduction of circulating lymphocytes in heifers by antilymphocyte serum, without affecting other blood cells, resulted in reduced luteinizing hormone (LH) pulses and decreased serum progesterone (Alila and Hansel, 1984). This observation suggests that T lymphocytes that affect luteal function may originate from peripheral blood. In the bovine CL, double immunostaining of leukocytes and proliferating cells (Ki67+) showed that 20% and 70% of proliferating cells in a mature and regressing CL were leukocytes (Bauer et al., 2001). The majority of the proliferating leukocytes were CD14+. This suggests that the population of immune cells may contribute to the increased numbers observed during luteal regression.

**Recruitment of immune cells in the corpus luteum**

Leukocyte-endothelial interactions are mediated by ligands on immune cells such as L-selectin (CD62L), P-selectin ligand (PSGL-1), E-selectin ligand (ESL-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), very late antigen-4 (VLA-4, CD49d/CD29), leukocyte function-associated antigen-1 (LFA-1, CD11b/CD18), and macrophage antigen-1(Mac-1, CD11a/CD18), and corresponding counter ligands/receptors on endothelial cells such as L-selectin ligand, P-selectin (CD62P), E-selectin (CD62E), CD31, vascular adhesion molecule-1 (VCAM-1/CD106), and
intercellular adhesion molecule-1 (ICAM-1/CD54) (Ulbrich et al. 2003, rev.). In addition to adhesion molecules, cytokines with chemotactic properties (chemokines) facilitate the process of immune cell migration into tissues by providing cues about the position or site to which immune cells are directed (Viola and Luster, 2008). The mechanisms that regulate the recruitment of immune cells into the corpus luteum are not well understood, but certainly involve changes in endothelial cell adhesion molecules and chemokines (Rohm et al., 2002; Townson and Liptak, 2003).

Adhesion molecules

Neutrophils, eosinophils, monocyte/macrophages, mast cells and basophils migrate out of the vascular compartment before ovulation, but after the initiation of the LH surge (Cavender and Murdoch, 1988). Eosinophils contribute 90% of all cells in developing bovine CL and their recruitment is at least positively correlated with the expression of P-selectin (Reibiger and Spanel-Borowski, 2000; Rohm et al., 2002). Neutrophils, on the other hand, have been shown to increase during PGF$_{2\alpha}$-induced luteolysis and their recruitment is partly explained by the increase in the surface localization of P-selectin on endothelial cells (Shirasuna et al., 2012). Surprisingly, in this study, PGF$_{2\alpha}$ decreased ICAM-1 and E-selectin, but had no effect on VCAM gene expression. In the rat CL, ICAM-1 expression is associated with the number of monocyte/macrophages infiltrating into the CL during regression (Olson and Townson, 2000). Oakley et al. (2010) demonstrated that the recruitment of immune cells to the periovulatory rat follicle correlated with the expression of VCAM-1 and ICAM-1 and
therefore, these molecules may facilitate recruitment of T lymphocytes. The temporal protein expression of ICAM-1, VCAM-1 and E-selectin has not been studied in the CL. Furthermore, the effect of paracrine factors such as steroids and prostaglandins that modulate adhesion of immune cells to endothelium have not been studied in the CL.

**Chemokines**

Chemokines, also referred to as chemoattractants, are cytokines that activate and regulate trafficking of immune cells into tissues or to sites of inflammation and injury (Rossi and Zlotnik, 2000, rev.). Chemokines are present in the CL and are implicated in the recruitment of leukocytes (Townson and Liptak, 2003). Recruitment of macrophages, neutrophils and lymphocytes into the developing CL is associated with the expression of MCP-1, IL-8, Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES/CCL5) and thymus-expressed chemokine (TECK/CCL25) (Arici et al., 1997; Machelon et al., 2000; Wu et al., 2007; Zhao et al., 2001; Jiemtaweeboon et al., 2011). During luteal regression, increased expression of MCP-1 (Tsai et al., 1997; Haworth, et al., 1998; Townson et al., 1996; Senturk et al., 1999; Townson et al., 2002; Nagaosa et al., 2002) and IL8 (Shirasuna et al., 2012) are associated with increased number of macrophages and neutrophils, respectively. Luo et al. (2011) demonstrated that the expression of IL8, MCP-1, CCL8, and CCL4 genes was increased by PGF$_{2\alpha}$ in the pig. It is suggested that the increase in chemokines during the late stage of the CL, or after PGF$_{2\alpha}$-induced regression, augments immune cell recruitment. Both interleukin 8 and MCP-1 are secreted by endothelial cells (Townson et al, 2002; Senturk et al., 1999), but
there is no evidence that PGF$_{2\alpha}$ and/or progesterone directly affect the expression of these chemokines (Cavicchio et al., 2002). Also, because recruitment of macrophages and T lymphocytes occurs prior to the onset of luteal regression, the factors that regulate this recruitment are not known.

**Regression of the corpus luteum**

A considerable amount of research has been done to understand the mechanisms that control the lifespan of the CL, and luteal regression in particular (Niswender et al., 2000; Schams and Berisha, 2004; Skarzynski et al., 2008; Miyamoto et al., 2009, Shirasuna et al., 2012; Pate and Keyes, 2001). The events leading to demise of the CL are complex, but are initiated by uterine PGF$_{2\alpha}$ and involve changes in blood flow, vasoactive factors and immune cell-mediated mechanisms (Nett et al., 1976; Miyamoto and Shirasuna, 2009, rev.; Shirasuna et al., 2012). Ultimately luteal structural demise is mediated by apoptotic mechanisms (Carambula et al., 2003).

Luteal regression in cattle and other species is initiated by luteolytic pulses of uterine PGF$_{2\alpha}$ (Schramm et al., 1983) or exogenous administration of PGF$_{2\alpha}$ (McCracken et al., 1972; Hansen et al., 1987; McCracken et al., 1999, rev.). In the livestock industry, PGF$_{2\alpha}$ is commonly used in protocols to synchronize the estrous cycle and/or ovulation (Hearnshaw et al., 1974; Pursely et al., 1995). Induction of luteal regression can be achieved by a single dose of about 25 mg (McCracken et al., 1972) in cattle. The CL is developmentally sensitive to exogenous PGF$_{2\alpha}$ in a number of species. In cattle, the CL is sensitive to PGF$_{2\alpha}$ after day 5 (Henricks et al., 1974; Tsai et al., 1998). In the pig and
monkey (Summers et al., 1985), regression occurs only after day 13 and 8, respectively (Diehl and Day, 1974; Diaz et al., 2000).

The process of luteal regression has been categorized into functional and structural regression (Schams and Berisha, 2004). Functional regression refers to the decline in progesterone synthesis. Structural regression follows and culminates in cell death and tissue disintegration, a process completed within 3-4 days after onset.

Prostaglandin (PG) F$_{2\alpha}$ inhibits LH-stimulated progesterone synthesis in vivo and in vitro (Hearn and Webley, 1987; Pate and Condon, 1984; Pate and Nephew, 1988). Prostaglandin F$_{2\alpha}$ reduces the expression of LH receptors (Reynolds et al., 1981; Mamluk et al., 1998; Guy et al. 1995; Smith et al., 1996), steroidogenic acute regulatory protein (StAR) (Juengel et al., 1995; Pescador et al., 1996), 3βHSD (3β-hydroxysteroid dehydrogenase), prostaglandin F$_{2\alpha}$ (FP) receptors (Tsai et al., 1998; Tsai et al., 2001), and P450 side chain cleavage(P450scc), but increases intracellular calcium and prostaglandin G/H synthase-2 mRNA (Davis et al., 1987; Tsai et al., 1998; Tsai et al., 2001). In species such as the rat, PGF$_{2\alpha}$ increases 20α-hydroxysteroid dehydrogenase, which metabolizes progesterone (Brambaifa and Schillinger, 1984; Stocco et al., 2000). PGF$_{2\alpha}$ also inhibits endothelial cell support by directly decreasing messenger RNA for VEGF and indirectly decreasing FGF2 via increased pentraxin 3 (PTX3) and thrombospondin 1 and 2, genes (Zalman et al., 2012). In addition, PGF$_{2\alpha}$ induces a decrease in blood flow (Miyamoto et al., 2005) and an increase in the expression of endothelin-1 (ET-1), which contributes to luteal regression (Apa et al., 1998; Levy et al., 2000; Choudhary et al., 2005).

Whereas PGF$_{2\alpha}$ induces a decline in progesterone secretion in vivo, the effects of PGF$_{2\alpha}$ on progesterone production in cultured luteal cells are ambivalent. PGF$_{2\alpha}$ was
found to increase (Korzekwa et al., 2008a) and decrease (Henderson and McNatty, 1975; Pate and Condon, 1984) progesterone production in bovine luteal cells. This discrepancy is a result of the concentration of PGF$_{2\alpha}$ and LH used in these studies (Benhaim et al., 1987 Benhaim et al., 1990). Using 10 ng/mL of PGF$_{2\alpha}$ inhibits LH-stimulated progesterone production while 1 μM augments progesterone production, similar to LH (Korzekwa et al., 2008a). Recently, Korzekwa et al. (2008a) demonstrated that antisteroidogenic effects of PGF$_{2\alpha}$ were observed when steroidogenic cells were cocultured with endothelial cells and lymphocytes. These studies support earlier observations that cytokines of immune cell origin have profound effects on luteal cell prostaglandin production, progesterone synthesis and viability (Fairchild and Pate, 1991; Benyo and Pate, 1992; Pate, 1995, rev.; Petroff et al., 2001).

**Immune system-mediated mechanisms of luteal regression**

Since the receptors for PGF$_{2\alpha}$ are abundant on large luteal cells, PGF$_{2\alpha}$ action on large luteal cells may induce a factor that inhibits LH-induced adenylate cyclase activity in small luteal cells (Silvia et al., 1984). The factors induced by PGF$_{2\alpha}$ inhibit LH-induced, as well as basal, progesterone secretion. Although PGF$_{2\alpha}$ inhibits LH-induced progesterone production in vitro, PGF$_{2\alpha}$ alone has no effect on basal progesterone production (Henderson and McNatty, 1975; Pate and Condon, 1984) and there is no evidence of cell death. These observations led to alternative hypotheses to identify agents that inhibit basal progesterone synthesis and induce cell death.
It is widely accepted that cell death during luteal regression is mediated by apoptotic mechanisms (Yadav et al., 2005), but PGF$_{2\alpha}$ does not directly induce apoptosis in luteal cells. During spontaneous luteal regression, indicators of apoptosis such as formation of oligonucleosomes and DNA fragmentation are observed in the CL bovine (Juengel et al., 1993) and other species. Taniguchi et al. (2002) showed that in late stage CL (days 19-21), the steady state message for Fas increased, suggesting that apoptosis may be Fas-mediated. Evidence for the role of the Fas-Fas ligand system in luteal regression was shown in the rat (Kuranaga et al., 2000). These authors demonstrated that CD3$^+$ cells, but not steroidogenic cells, expressed FasL, and the removal of CD3$^+$ cells inhibited apoptosis induced by the luteolytic signal. In addition, immune mediators are believed to be involved in luteal regression. Culture of luteal cells with IFN$_\gamma$ increases the expression of Fas mRNA and induced cell death (Taniguchi et al., 2002). The effects of IFN$_\gamma$ are potentiated by TNF$_\alpha$ (Petroff et al., 2001; Taniguchi et al., 2002).

Proinflammatory cytokines TNF$_\alpha$, IL-1$\beta$ and IFN$_\gamma$ are present throughout the estrous cycle in the bovine CL and are upregulated during luteal regression (Petroff et al., 1999; Neuvians et al., 2004). TNF$_\alpha$ is synthesized by luteal cells (Sakamuto et al., 2011), but also secreted by macrophages and T lymphocytes. The receptors for IFN$_\gamma$ and TNF$_\alpha$ are expressed in small and large bovine luteal steroidogenic and endothelial cells (Okuda et al., 1999; Friedman et al., 2000; Sakamoto et al., 2000; Korzekwa et al., 2008b; Sakamoto et al., 2011). Both cytokines have cytotoxic effects on luteal cells. TNF$_\alpha$ induces apoptosis in endothelial cells (Friedman et al., 2000) and when combined with IFN$_\gamma$, they both decrease progesterone synthesis and induce apoptosis in cultured bovine luteal cells (Fairchild and Pate, 1991; Benyo and Pate, 1992; Pate, 1995, rev.; Petroff et al., 2001;
Korzekwa et al., 2008). Both cytokines also induce prostaglandin synthesis in vitro and in vivo (Sakamuto et al., 2000; Petroff et al., 2001; Korzekwa et al., 2008b). Collectively, these data support the hypothesis that immune cells may be involved in luteal regression. Because of the rapid recruitment of immune cells into the CL, luteal regression, like ovulation, is often described as an inflammatory-like process.

**Mediators of interactions between immune cells and luteal cells**

**Cell Surface mediators**

Luteal cells and their precursors express proteins that are characteristic of myeloid cells and thus may establish communication with lymphoid cells. In the cow, three populations of luteal cells, small, large dense and very large dense were found to express MHCII (Fairchild and Pate, 1989; Benyo et al., 1991; Petroff et al., 1997; Cannon et al., 2003; Cannon et al., 2007b) and elements of the antigen processing machinery (Cannon et al., 2006). Similarly, human granulosa-lutein cells also express moderate levels of MHCII during the midluteal phase and early pregnancy, but the expression is elevated during the late luteal phase (Fujiwara et al., 1993; Bukovsky et al., 1995). In addition, granulosa-lutein cells express, CD86 and CD14 (Bukovsky et al., 1995; Herath et al., 2007), ICAM-1/CD54 (Viganò et al., 1997) and leukocyte function-associated antigen 3 (LFA3) (Fujiwara et al., 1993). Leukocyte function-associated antigen-3 and ICAM-1 interact with CD2 and LFA-1/CD18, respectively and are critical costimulatory molecules for sufficient T lymphocyte activation (Makgoba et al., 1989). Cluster of
differentiation 4, CD86 and CD14 (lipopolysaccharide receptor) proteins are mainly expressed on CD4$^+$ T cells and macrophage/monocytes, respectively.

Because luteal cells express MHCII and other ligands for which receptors exist on T lymphocytes, they could have functions similar to antigen presenting cells (APCs). In vitro, blocking ICAM-1 partially inhibited interaction between human granulosal cells and T lymphocytes (Viganò et al., 1997). Also, blocking costimulatory molecules CD80 and CD86 in bovine luteal cells diminishes luteal cell-induced T cell proliferation (Cannon et al., 2007a). Interestingly when T lymphocytes are cocultured with luteal cells, γδ$^+$ T lymphocytes proliferate (Davis and Pate, 2007). The repertoire of potential γδ$^+$ T lymphocyte ligands that are expressed by luteal cells may include surface molecules such as CD1, homologues of MICA/B, peptide or nonpeptide antigens or other unidentified soluble factors.

It is not clear how resident γδ$^+$ T lymphocytes in the CL interact with luteal cells to influence luteal function. Luteal cells could interact with γδ$^+$ T lymphocytes in a manner that is similar to keratinocytes (Singer et al., 1997) or thymic epithelial cells (Pourquié et al., 1990; Corbel, 1992a). These cells express ligands such as activated leukocyte adhesion molecule (ALCAM/CD166), which is also expressed on activated leukocytes. It is proposed that ALCAM is expressed on luteal cells and mediates the interaction between γδ$^+$ T lymphocytes and luteal cells. Activated leukocyte adhesion molecule is a 100-105 kDa transmembrane glycoprotein that belongs to the immunoglobulin superfamily of cell adhesion molecules (Bowen et al., 1995). It is a highly conserved glycoprotein that was first identified, characterized and named SC1 (Tanaka and Obata, 1984), BEN (Pourquié et al., 1990) and DM-GRASP (Burns et al.
Activated leukocyte cell adhesion molecule orthologues to SC1/BEN/DMGRASP have been found in zebrafish (Kanki et al., 1994), rodents (Prince et al., 1992; Kanki et al., 1994), humans (Bowen et al., 1995) and cows (Konno et al., 2001). BEN/SC1/DMAGRAP/ALCAM/ is expressed in central and peripheral neural tissue (Tanaka and Obata, 1984; Pourquié et al., 1990; Konno et al., 2001), thymus and Bursa epithelial cells (Pourquié et al., 1990; Corbel, 1992a), hematopoietic progenitor cells (Uchida et al., 1997), lung (Uchida et al., 1997), reproductive tissues (Uchida et al., 1997; Fujiwara et al., 2003; Adriaenssens et al., 2010, 2011; Kim et al., 2011), activated leukocytes (Corbel, 1992b; Bowen et al., 1995), endothelial cells (Degen et al., 1998; Ohneda et al., 2001; Masedunskas et al., 2006), keratinocytes (Singer et al., 1997; Sanders et al., 2011) and metastasizing melanoma cells (Degen et al., 1998; van Kampen, et al., 2000). Its exclusive expression only on activated leukocytes was the basis for its name in humans (Bowen et al., 1995). Bowen and others also demonstrated that CD6, a scavenger receptor with cysteine rich domains (SRCR), highly expressed in T lymphocytes, is a receptor for ALCAM ligand. In bovine tissues, Konno et al. (2001) utilized human CD6 to identify and localize ALCAM. Similar to BEN, ALCAM is involved in cell-cell adhesion via heterotypic (ALCAM/CD6) and homotypic binding (ALCAM/ALCAM) (Corbel et al., 1996; Debnardo and Chang, 1996; Hassan et al., 2004). Heterotypic interactions are 100-fold stronger than homotypic interactions (Hassan et al., 2004).

The wide range of tissues and cell types in which ALCAM is expressed suggests that it may be involved in many biological processes. During embryonic hematopoietic cell differentiation (Ohneda et al., 2001), T cell activation (Hassan et al., 2004; Gimferrer
et al., 2004; Kato et al., 2006; Zimmerman et al., 2006) and germ cell development (Ohbo et al., 2003), ALCAM is transiently expressed. This suggests that ALCAM is strictly regulated in normal cells. During development, ALCAM is expressed at specific time frames and is restricted to certain tissues and cells (Tanaka and Obata, 1984; Uchida et al, 1997). In individual cells, ALCAM is present on the plasma membrane, but can be internalized by endocytosis following ubiquitination (Thelen et al., 2008). Binding of antibodies to ALCAM also triggers internalization (Piazza et al., 2005). ALCAM protein is also in plasma (Hatta et al., 2009). Whereas membrane-bound ALCAM may enhance angiogenesis, presence of its soluble form (sALCAM) inhibits angiogenesis as well as metastasis, presumably through homotypic interactions (Ikeda and Quertermous, 2004; van Kilsdonk et al., 2008). The two isoforms are a result of alternative splicing (Ikeda and Quertermous, 2004), but also current evidence shows that ALCAM is enzymatically cleaved by ADAM 17/TACE (Bech-Serra et al., 2006; Micciché et al., 2011). In addition, the protein kinase C activator, phorbol myristate acetate (PMA), and ionomycin can also induce ALCAM shedding (Micciché et al., 2011).

Expression of the ALCAM gene is regulated by methylation as well as activation by the transcription factor, nuclear factor-κB (King et al., 2010; Wang et al., 2011). In addition to transcriptional regulation, ALCAM is posttranscriptionally regulated by microRNA (Wang et al., 2011; Jin et al., 2011). Inflammatory mediators such as IFNγ and TNFα that activate the nuclear factor-κB (NFκB) pathway also increase ALCAM expression (Saifullah et al., 2004; Abidi et al., 2006; Singer et al., 1997; Cayrol et al., 2008). During inflammation, ALCAM is upregulated on endothelial cells and activated leukocytes (Levesque et al., 1998; Cayrol et al., 2008).
The expression of ALCAM is high in tissues and cells involved in rapid growth such as cancer cells (Swart, 2002, rev.). Progression of several aggressive cancers (melanoma, prostate cancer, breast cancer, colorectal carcinoma, bladder cancer, and esophageal squamous cell carcinoma are associated with ALCAM expression (Ofori-Acuquah and King, 2008). Thus, ALCAM has become a prognostic maker for most cancers (Mezzzanica et al., 2008; Ishigami et al., 2011). In normal cells, ALCAM has been shown to regulate processes such as axonogenesis (Burns et al., 1991; Karagogeos et al., 1997) and axon orientation (Avci et al., 2004), angiogenesis (Ohneda et al., 2001), recruitment and activation of immune cells (Masedunskas et al., 2006; Nummer et al., 2007; Cayrol et al., 2008; Lee and Imhof, 2008, rev.; Guerraty et al., 2011; Hassan et al., 2004; Kato et al., 2006), inhibiting apoptosis and melanoma metastasis (Swart et al., 2002) and migration of tumor cells (King et al., 2010; Wang et al., 2011). In humans, it is used as a marker for mesenchymal stem cells (Alsalameh et al., 2004) as well as cancer stem cells (Levin et al., 2010).

In reproductive tissues, ALCAM is expressed very early in both female and male mouse gonads during fetal development (Ohbo et al., 2003). In adult mouse reproductive tissues, ALCAM protein has been detected in the cumulus-oocyte complex (Hernandez-Gonzalez et al., 2006), blastocyst, luminal and glandular endometrial epithelial cells (Fujiwara et al., 2003; Kim et al., 2011). ALCAM is upregulated predominantly in cumulus cells after induction of ovulation in mouse and human ovaries and its expression is positively related with oocyte competence (Adriaenssens et al., 2010, 2011; Wathlet et al., 2011). In mouse cumulus-oocyte complexes (COCs), expression of ALCAM is upregulated following hCG (human chorionic gonadotropin) administration prior to
ovulation (Hernandez-Gonzalez et al., 2006). During ovulation, selective upregulation of ALCAM occurs in cumulus but not granulosal cells (Hernandez-Gonzalez et al., 2006). However, it is not clear whether ALCAM is also expressed in thecal cells. It is hypothesized that ALCAM is expressed on luteal cells and that it is involved in mediating the interaction between luteal cells and $\gamma\delta^+$ T lymphocytes.

**Soluble mediators**

**Prostaglandins**

Prostaglandins and other products of arachidonate are synthesized in CL of cows (Milvae and Hansel, 1983; Hayashi et al., 2003; Weems et al., 2012), humans (Bogan et al., 2008), pigs (Waclawik et al., 2008) and sheep (Silva et al., 2000). During the estrous cycle, both PGE$_2$ and PGF$_{2\alpha}$ are synthesized, but the ratio of PGE$_2$:PGF$_{2\alpha}$ is higher in early compared to regressing CL (Milvae and Hansel, 1983; Hayashi et al., 2003; Arosh et al., 2004; Waclawik et al., 2008). In most species, prostaglandins regulate the estrous cycle by controlling lifespan of the CL (Inskeep, 1973). Indeed, indomethacin administered in the uterus inhibits development and function of the CL (Milvae and Hansel, 1983). Autocrine and paracrine actions of PGE$_2$ and PGF$_{2\alpha}$ are important in regulating bovine luteal function, specifically progesterone synthesis (Milvae and Hansel, 1983; Pate and Condon, 1984; Pate and Condon, 1989; Arosh et al., 2004). These two prostaglandins have opposing effects on luteal function (Akinlosotu et al., 1988; McCracken et al., 1999). In vivo studies in the cow have demonstrated that prostaglandin
E₂ is luteotropic (Gimenez and Henricks, 1983) while PGF₂α is luteolytic (Inskeep, 1973). Thus, there is selective synthesis, transport and signaling of PGE₂ during development and significant elevation of PGF₂α during luteolysis (Arosh et al., 2004). Because there is strong evidence to support the role of prostaglandins in immune cell function, prostaglandins synthesized in the CL may potentially regulate recruitment and function of immune cells.

Prostaglandins were originally found in inflammatory exudates and their injection into dermal tissue reproduced cardinal signs of inflammation (Flower, 1977). In the ocular inflammation model, inhibitors of cyclooxygenase alleviated clinical signs of inflammation and reduced leukocyte migration (Yamauchi et al., 1979). There are, however, contradictory reports about the effect of prostaglandins on migration of leukocytes. PGE₂, E₁ and F₂α were found to induce random migration of polymorphonuclear cells (PMNs); PGF₂α induced a concentration-dependent migration of PMNs (Shibuya, 1976). Prostaglandin F₂α, but not PGE₁ or E₂, enhanced casein-induced chemotaxis of PMNs (Diaz-Perez et al., 1976). Conversely, PGE₂ and PGF₂α either inhibited random migration of PMNs (Kalmár and Gergely, 1983) or had no effect on migration or chemotaxis (Bray and Franco, 1978).

Prostaglandins of the E series are known for their immunosuppressive effects on T lymphocytes (Goodwin et al., 1977; Chouaib et al., 1985). Whereas PGE₂ suppresses phytohemagglutinin (PHA)- and interleukin 2 (IL2)-dependent bovine and ovine T cell proliferation, PGF₂α has no effect on T cell proliferation (Low and Hansen, 1988). In addition, PGE₂ downregulates IL2 and granulocyte macrophage colony stimulating factor (GM-CSF) gene expression in bovine lymphocytes (Emond et al., 1998). Furthermore,
PGE₂ inhibits the secretion of IFNγ-induced interleukin 12 (IL12) in macrophages (Tineke et al., 1995; Iwasaki et al., 2003).

Prostaglandins influence endothelial-leukocyte interactions. When endothelial monolayers or granulocytes are incubated with PGE₂ or PGF₂α, the migration index of granulocytes increases and this increase is diminished when the endothelium is treated with aspirin (Beesely et al., 1979). Prostaglandin E₂, ibuprofen and indomethacin (inhibitors of PGH synthase) also inhibit bradykinin-induced bovine aortic endothelial cell (BAECs) monolayer permeability to albumin (Farmer et al., 2001). Because bradykinin mainly augments paracellular transport, these data support the inhibitory action of PGE₂ on paracellular transport through the endothelium. In human pulmonary artery endothelial cells (HPAECs), PGE₂ strengthens the endothelial cell barrier by increasing adherens junctions (VE-cadherin) and peripheral F-actin (Birukova et al., 2007). Mesri et al. (1996) established that the mechanism by which PGE₂ inhibits transendothelial migration of activated T lymphocytes does not involve adhesion molecules. There is evidence that prostaglandins also regulate expression of adhesion molecules. PGE₂ inhibits expression of ICAM-1 protein in human mixed leukocyte reaction via EP₂ and EP₄ receptors (Morichika et al., 2003). Prostaglandin E₂ also decreases TNFα-induced ICAM-1 expression on human gingival fibroblasts (Noguchi et al., 1999). Contrary to this finding, PGE₂ induces expression of ICAM-1 on cultured human umbilical vein endothelial cells (HUVECs) (Winkler et al., 1997).

Other prostaglandins of the E-series also modulate adhesion molecules on endothelial cells. Prostaglandin E₁ inhibits T cell adhesion to endothelial cells by selective inhibition of TNFα-induced ICAM-1 (Weiss et al., 2006). Also, PGE₁ inhibits
infiltration of macrophages in glomerulonephritis (Cattell et al., 1990). In vitro, PGE\textsubscript{1} inhibited both leukocyte adherence and transendothelial migration through suppression of LFA-1 (CD11a/CD18) in human lung vascular endothelial cells (HLVECs). Contrary to these data, pretreatment of both HUVEC monolayers and polymorphonuclear leukocytes (PMNL) with TNF\textalpha, resulted in increased leukocyte transmigration (Hofbauer et al., 2000). Less information is available about the effects of prostaglandins of the D and F series. Prostaglandin D\textsubscript{2} (PGD\textsubscript{2}) attenuated VCAM-1 as well as MCP-1 expression in HUVECs treated with IL-1\beta (Negoro et al., 2005). Administration of PGF\textsubscript{2\alpha} to pseudopregnant rats increased leukocyte adhesion to luteal venules (Minegishi et al., 2002). In the rat, luteolytic prolactin increased ICAM-1 expression and number of monocytes/macrophages in the CL (Olson and Townson, 2000). Because the luteolytic factor in the bovine CL is PGF\textsubscript{2\alpha}, it is hypothesized that it will have similar activity.

**Sex steroids**

Progesterone and estradiol regulate trafficking of immune cells by modulating permeability characteristics and adhesion molecule expression of the endothelium. Estradiol modulates paracellular permeability of human endothelial cells by disrupting and uncoupling adherens junctions from \(\alpha\)-catenin (Cho et al., 1999; Groten et al., 2005). This increases endothelial monolayer permeability. These effects are not induced by progesterone and are blocked by ER antagonist ICI182780 (Groten et al., 2005). In addition to the effects on adherens junctions, estradiol reduces transendothelial electrical resistance of tight junctions and affects gene expression of occludin, leading to increased
paracellular permeability (Cho et al., 1999; Ye et al., 2003). These effects are dependent on the concentration and time of exposure (Ye et al., 2003).

Estradiol also enhances adhesion of both PMNs and activated peripheral blood mononuclear cells (PBMCs) to TNFα-stimulated HUVECs via increased expression of ICAM-1, VCAM-1 and E-selectin (Cid et al., 1994; Winkler et al., 1997). However, estradiol decreased VCAM-1 gene expression and subsequent adhesion of U937 monocytoid cells in lipopolysaccharide (LPS)-stimulated human endothelial cells (Simoncini et al., 2000). Treatment of HUVECs with estradiol also reduces V-cadherin mRNA and endothelial barrier properties (Fujimoto et al., 1998). Overall, the effects of estradiol on expression of adhesion molecules and adhesion of immune cells are positive and negative, dependent on the cell types used and the agents used in activating endothelial cells.

The role of progesterone in endothelial-leukocyte interactions also varies depending on the cell types and experimental conditions. Progesterone has been shown to have no effect on adhesion molecule expression in HUVECs (Winkler et al., 1997). Contrary to this finding, Aziz and Wakefield (1996) demonstrated that progesterone suppresses IL-1α-induced E-selectin expression. In addition, progesterone also decreases the expression of ICAM-1 and VCAM-1 in LPS activated human endothelial cells (Simoncini et al., 2004). Also, progesterone decreases TNFα-induced expression of VCAM-1 protein and mRNA in HUVECs (Otsuki et al., 2001) in a concentration-dependent manner. In this study, the decrease in VCAM-1 was not reversed by either RU486 or ZK98299 (progesterone receptor antagonists). Furthermore, Koh (2002, rev.), reported that both progesterone and methoxyprogesterone acetate (MPA) suppressed
soluble forms of VCAM-1, E-selectin and ICAM-1 and MCP-1 in healthy postmenopausal women.

Progesterone has an inhibitory effect on leukocyte adhesion to endothelial cells in vitro (Simoncini et al., 2004). In addition, injection of progesterone antagonist, ZK 137316, in ovine endometrium, inhibited progesterone-induced recruitment of eosinophils expressing MCP-1 and MCP-2 (Asselin et al., 2001). Conversely, Cid et al. (1994) reported that progesterone enhanced adhesion of PMNs to TNFα-activated HUVECs.

In addition to regulating adhesion and trafficking of immune cells, progesterone also affects the function of immune cells and T lymphocytes in particular. Progesterone suppresses T lymphocyte function (Szekereres et al. 1981), but the mechanisms of how progesterone achieves this are still being explored. Relative to estradiol, Szekeres and others noted that progesterone could inhibit the cytotoxic activity of T lymphocytes toward human embryonic fibroblasts. Progesterone delivers its inhibitory activity by directly binding to T lymphocytes (Szekeres-Bartho et al., 1983). When progesterone binds to T lymphocytes, it induces progesterone-induced blocking factor (PIBF), which inhibits prostaglandin synthesis and acts on CD8+ cells to induce a suppressor phenotype (Szekeres-Bartho et al., 1985; Szekeres-Bartho et al., 1989; Szekeres-Bartho et al., 1989b). Polger et al., 1999 demonstrated that 100% of γδ+ T lymphocytes of pregnancy expressed the nuclear progesterone receptor (PGR). Although the expression of PGR may be induced in human T lymphocytes during pregnancy (Szekeres-Bartho et al., 1990), the expression of PGR in human T lymphocytes is controversial. Bovine T lymphocytes from peripheral blood do not express PGR (Ndiaye et al., 2012). The action of progesterone on
immune cells is suggested to be mediated by membrane progesterone receptors (Schust et al., 1996; Ehring et al., 1998; Cannon et al., 2003; Dosiou et al., 2008; Giannoni et al., 2011; Ndiaye et al., 2012).

Szekeres-Bartho and Wegmann (1996) demonstrated that PIBF mediated progesterone actions by altering the balance of Th1/Th2 cytokines in both CD8+ and CD4+ splenocytes. When both these cell types were activated in the presence of PIBF, they secreted more IL3, IL4 and IL10 compared to controls. This effect of progesterone was also demonstrated to occur in antigen-specific CD4+ T cells and Th1 differentiated double positive thymocytes (Piccinni et al., 1995; Miyaura and Iwata, 2002). In addition, progesterone has also been shown to induce a Th2 cytokine profile in splenocytes (Yates et al., 2010) and IL10 synthesis in T regulatory (Mao et al., 2010) and dendritic cells (Xu et al., 2011) in mice.

In sheep, progesterone prevents xenograft and allograft rejection (Pauda et al., 2005; Majewski and Hansen, 2002). Long term treatment of progesterone also affects either migration or proliferation of T lymphocytes in the endometrium (Gottshall and Hansen, 1992). It is also suggested that progesterone acts indirectly on immune cells in the endometrium (Wang et al., 1988). Progesterone stimulates the secretion of uterine milk protein (UTMP), which has immunosuppressive effects on T lymphocytes (Skopets and Hansen, 1993; Liu and Hansen, 1995; Hansen, 1998). Progesterone also inhibits PHA-induced proliferation of T lymphocytes in humans (Stites et al., 1983), cows, sheep (Low and Hansen, 1988) and buffalo (Pampori and Pandita, 2012). Blocking the progesterone receptor with RU486 does not inhibit the antiproliferative effects of
progesterone (Monterroso and Hansen 1993) and thus, the immunosuppressive effects of progesterone may be mediated by nongenomic mechanisms (Ehring et al., 1998).

**Summary**

An inverse relationship between milk yield and embryonic loss/conception rate in high producing dairy cattle has been observed for over 50 years. If conception rates continue to decline, the cost of milk production will increase and the profitability of dairy farms will be substantially reduced. In high producing cows, greater than 40% of pregnancies are lost within the first 5 weeks after insemination, a period within which the function of the CL is absolutely required. Although a number of hypotheses have been generated to explain the reduction in conception rates, there is evidence to suggest that at least in some cows, luteal dysfunction (insufficient progesterone) contributes to the observed low pregnancy rates. Indeed, progesterone supplementation in these animals improves the success of pregnancy.

Luteal function is regulated by immune cell mediators. Cytokines such as IL1β, IFNγ and TNFα inhibit gonadotropin-induced progesterone synthesis, and increase PGF$_{2\alpha}$ synthesis in luteal cells. Furthermore, IFNγ and TNFα are cytotoxic to luteal cells in vitro and thus may facilitate PGF$_{2\alpha}$-induced demise of the CL. In contrast, IL4 and IL10 support progesterone synthesis by luteal cells. Immune cells are suggested to facilitate luteal regression, but immune cells, including T lymphocytes and cytokines, are present in the CL prior to the onset of luteal regression. This indicates that the activities of
immune cells present in a functional CL must be regulated not to induce premature luteal regression.

The role T lymphocytes play in a functional CL and the T lymphocyte subset involved in facilitating luteal regression remains unclear. Before these questions are answered, there is a need to understand the mechanisms that regulate the recruitment of immune cells into the CL. Current evidence indicates that progesterone and prostaglandins regulate adhesion and migration of immune cells. Therefore, luteal prostaglandins and progesterone may be involved in the recruitment of immune cells into the CL. The interactions between resident immune cells and luteal cells and the mediators of these interactions are not fully known. Luteal cells synthesize soluble factors such as prostaglandins and progesterone, and express cell surface-associated molecules such as MHC II, LFA 3, ICAM-1, and CD14. These factors may be important in regulating T lymphocyte processes such as cytokine synthesis. T lymphocytes present in the CL include major (CD4+, CD8+ and γδ+) and minor (γδ+WC1+ and γδ+WC1−) subsets. To identify cellular targets for improving reproductive efficiency, there is need to define what subset is detrimental to or supportive of luteal function. Addressing the areas above could lead to identification of new strategies to improve fertility in dairy cattle and other species.
Overall hypothesis

The activity of T lymphocytes recruited to the CL is modulated by the local luteal environment to ensure optimal luteal function

Objectives

1. Isolate endothelial cells and determine their interaction with T lymphocytes
2. Determine if prostaglandins regulate the recruitment of T lymphocytes into the CL
3. Determine if luteal prostaglandins modulate T lymphocyte function
4. Examine the mechanisms by which progesterone regulates T lymphocyte function
5. Evaluate functional changes in γδ+ T lymphocytes exposed to luteal cells from a functional and regressing CL
6. Identify the mechanisms by which luteal cells activate T lymphocytes
Chapter 2

Isolation of endothelial cells from the bovine corpus luteum and their functional interactions with T lymphocytes

Introduction

The CL is a transient endocrine gland with a heterogeneous population of cells that can be distinguished by functional or morphological characteristics (Fields and Fields, 1996). Endothelial cells are specifically pivotal to the rapid development and regression of the CL. They form a capillary network that not only delivers nutrients to luteal tissue, but also facilitates the endocrine function of the CL. Endothelial cells also act as a conduit for the recruitment of leukocytes, which contribute to luteal function and remodeling.

Endothelial cells are the most numerous cells in a mature bovine CL and are estimated to be about 50% of all luteal cells (O’Shea et al., 1989). The size of endothelial cells ranges from 8-10 µm (Lei et al., 1991; Fields and Fields, 1996). It is less than that of small and large steroidogenic cells that range from 15-23 µm and 23-50 µm, respectively (Chegini et al., 1991; Weber et al., 1987; Lei et al., 1991). Thus, size is a good characteristic to separate endothelial cells from steroidogenic cells.

In the recent past research has focused on isolating, identifying and characterizing endothelial cells from the bovine CL (Spanel-Borowski and van der Bosch, 1993; Plendl et al., 1996a; Klipper et al., 2004), but there is still a need to develop efficient, reliable
and quick methods of isolation of pure endothelial cells to understand their transcriptome, proteome and interactions with parenchymal and immune cells, and their overall function in luteal physiology.

Over the years, a variety of methods have been used to isolate endothelial cells from enzymatically dispersed cells. Microvascular endothelial cells were isolated from bovine adrenal gland and CL by filtration (Folkman et al., 1979) and percoll density gradient centrifugation, respectively (Spanel-Borowski and van der Bosch, 1993). The methods used to purify isolated endothelial cells include cloning (Folkman et al., 1979), culture in endothelial cell growth promoting media, use of antibodies (Dong et al., 1997; Baldysiak-Figiel et al., 2004), lectins ligated to magnetic beads (MACS; Drake and Loke, 1991; Christenson and Stouffer, 1996; Klipper et al., 2004) and fluorescence activated cell sorting (FACS; Voyta et al., 1984; van Beijnum et al., 2008). These methods take advantage of the specific antigens/molecules expressed on endothelial cells to extract endothelial cells from complex mixtures of cell populations. Although these methods yield highly purified populations, they are limited by availability and specificity of antibodies, especially those directed toward bovine antigens. Secondly, these methods are limited by lower yield and viability of cells, relatively longer time and require expensive equipment and reagents. The limited number of cells isolated is insufficient to establish in vitro cell cultures without subculture and extended passaging. Immortalized luteal endothelial cell lines have also been developed to generate an unlimited supply of endothelial cells (Korzekwa et al., 2011). It is still relevant to develop better methods that maximize the number of cells recovered and minimize the number of contaminating cells.
for the purposes of descriptive and functional studies using primary cells rather than cell lines.

One of the functional roles of endothelial cells is to regulate recruitment of leukocytes into the CL (Rohm et al., 1990). During the process of recruitment, endothelial cells interact with leukocytes via adhesion molecules such as platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), P-selectin (CD62P), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) that have complementary ligands on leukocytes (Newman et al., 1990; Bochner et al., 1991; Walter and Issekutz, 1997; Woodfin et al., 2007). In addition, endothelial cells also express class II MHC molecules (MCH II) and costimulatory ligands such as lymphocyte function-associated antigen 3 (LFA3/CD58), CD40 and CD80 that interact with receptors on T lymphocytes (Yellin et al., 1995; Karmann et al., 1996; Cannon et al., 2007a; 2007b). This interaction may lead to changes in the function of leukocytes as observed in the case of the interaction of endothelial cells with allogeneic leukocytes (Ashida et al., 1981). Alternatively, endothelial cells may affect the function of leukocytes by secreting cytokines (Nilsen et al., 1998). Leukocytes modulate the steroidogenic function of luteal cells (Castro et al., 1998) and are suggested to play a role in luteal regression (Pate, 1995, rev.; Bukovský et al., 1995). Leukocytes are present in the CL, and their numbers change throughout the estrous cycle. For instance, T lymphocytes are more abundant in midcycle and regressing CL compared to early CL (Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002). The objectives of the present study were to develop a protocol for isolating endothelial cells and determine if luteal endothelial cells influenced the function of autologous T lymphocytes.
Results

**Characteristics of enriched endothelial cells**

Cell number and viability of endothelial cells recovered from the CD31 positive fraction or filtrate are shown in Figure 2-2. More endothelial cells were recovered using filtration compared to immunomagnetic separation (P<0.05). Similarly, viability was greater in endothelial cells recovered from the filtrate compared to the positively selected fraction (P<0.05). Hence, filtration was a more efficient method of endothelial cell isolation.

In freshly dissociated cell preparations, endothelial cells were observed to be smaller than the steroidogenic cells, and often appeared in clusters as shown by black arrowheads (Fig. 2-3A). The steroidogenic cells appear darker (white arrowheads) due to abundance of intracellular granules (Fig. 2-3A). The size of enriched endothelial cells is about 10 µm in diameter (Fig. 2-3B). Enriched endothelial cells were grown on 24-well culture plates and attachment was not observed until day 3. Enriched endothelial cells in culture exhibited triangular, elongated and cobblestone morphology, and were confluent on day 7-9 (Fig. 2-3C, D and Fig. 2-4A, B). Confluent endothelial cells, beginning on days 14-17, occasionally formed enclosed spaces described as lumen-like structures (Fig. 2-4C and D) and organized to form strings of connected cells characteristic of spontaneous angiogenesis (Fig. 2-5).

Cells recovered from the filtrate were analyzed by flow cytometry to determine their size (forward scatter) and intracellular complexity (side scatter) characteristics (Fig. 2-6). Larger, more complex cells in region 3 (R3) were eliminated by filtration (Fig. 2-
6A). The cells in region 3 are likely to be steroidogenic cells. Endothelial cells are likely to be in regions R1 and R3, although the majority of cells in R1 may be erythrocytes. To determine the purity of the isolated endothelial cells, lectin binding was performed on enriched endothelial cells. Nearly 90% of enriched endothelial cells were BS-1 lectin positive, and this proportion was significantly higher than that in cells recovered from the retentate (Fig. 2-6B). In addition to determining the presence of endothelial cells in the filtrate, presence of contaminating steroidogenic cells was detected by steady state concentration of StAR mRNA. Lower concentrations of StAR mRNA (P=0.06) were detected in the filtrate compared to mixed luteal cells (Fig. 2-6C).

To further characterize the endothelial cells, the cells recovered from the filtrate were cultured and labeled with vWF and BS-1 lectin-rhodamine. Most, but not all, of the cells from the filtrate were vWF positive (Fig. 2-7). In addition, almost all cells were stained by BS-1 lectin (Fig. 2-8)

**Effect of endothelial cells on T lymphocytes**

The effects of endothelial cells and steroidogenic cells on activation and proliferation of T lymphocytes are shown in Figure 2-9 (A, B). Coculture of T lymphocytes with endothelial cells significantly increased the proportion of CD25⁺ T lymphocytes similar to PMA-Ionomycin (P<0.05), but there was no effect of steroidogenic cells on CD25 expression (P>0.05). In contrast, steroidogenic cells induced a higher proportion of T lymphocytes to proliferate compared to endothelial cells (P<0.05). T lymphocytes were exposed to luteal endothelial cells and their phenotype
analyzed (Fig. 2-9 C, D). Endothelial cells did not alter the proportion of either CD8αα⁺
and CD8αβ⁺ or γδ⁺WC1⁺ and γδ⁺WC1⁻ T lymphocyte subsets (P>0.05).

**Discussion**

Using sequential filtration, endothelial cells were isolated from the CL and their
effect on the function of autologous T lymphocytes determined. Endothelial cells account
for 46-48% (Rodgers et al., 1984) 52.3% (O’Shea et al., 1989), and 61% (Azmi and
Bongso, 1985) of cells in sheep, cow and goat CL, respectively. They are smaller in
average size (10.8 µm) compared to small luteal cells (17.2 µm) and large luteal cells
(38.4 µm). Whereas the range in size of small luteal cells may overlap with the size of
endothelial cells, the bulk of these cells are well over 12 µm. In addition, the size of
fibroblasts is over 12 µm (Fields and Fields, 1996; Weber et al., 1987; Lei et al., 1991).
Thus, separation by filtration as demonstrated by Plendl et al. (1996a) in the isolation of
endothelial cells from a variety of vascular beds in the pig can be applied to the CL. In
the present study, luteal cells were filtered through a series of microsieve sizes, from 50
to 10 µm. The method used in this study clearly eliminated larger cells with internal
complexity as shown in Figure 2-6. The 50 µm sieve was used to eliminate undigested
tissue while the 15 µm and 10 µm sieves eliminated large luteal cells and also reduced
the bulk of small luteal cells.

The yield and viability obtained by filtration in this study is far greater than that
obtained by immunomagnetic separation (Fig. 2-2). The low yield in the latter method
could potentially result from low specificity to bovine antigens, because the antibody
used was developed against sheep antigens. Secondly, the stability of CD31 molecules on endothelial cells during the enzymatic dissociation has not been described for luteal endothelial cells. The low viability of endothelial cells isolated by immunomagnetic separation could be explained by the time required to complete the isolation process. Approximately 2 hours was required to complete immunomagnetic separation following enzymatic digestion of luteal tissue compared to approximately 30-45 minutes when filtration was used.

The filtration method was optimal for maximizing recovery of endothelial cells. Using supernatants collected from sequential washes of mixed luteal cells as a source of endothelial cells does not recover endothelial cells that aggregate and sediment with the larger steroidogenic cells. Single cell suspensions obtained after collagenase digestion often contain strings or clumps of small cells that may be held together by unbroken intercellular junctions. These form groups of small cells that segregate with the larger cells and consequently are eliminated from the final filtrate. This explains the presence of BS-1 lectin positive cells in the mixed cell fraction. Recovery of endothelial cells would likely be increased by ensuring that a single cell suspension is obtained from the tissue digestion procedure.

To study the biology of luteal endothelial cells, the isolated primary cells must be free of contaminating cells. In the current study, approximately 90% of the freshly isolated cells in the filtrate bound BS-1 lectin. Immunofluorescence staining of cells from passage 1 showed that almost all cells were bound by BS-1 lectin, indicating that in fresh cells, the BS-1 negative cells in the enriched endothelial cells were nonadherent cells or were eliminated during culture. Similarly, the majority of the cultured, nonpassaged cells were
immunoreactive for vWF. Both vWF and BS-1 lectin have been shown to specifically bind to bovine endothelial cells (Tuori et al., 1994; Plendl et al., 1996b; Herrman et al., 1996; Meidan et al., 2005). The presence of StAR mRNA in the enriched endothelial cells indicates the presence steroidogenic cells, especially small luteal cells, which are less or equal to 10 µm in diameter. Because nearly 90% of the enriched endothelial cells bound BS-1 lectin and were immunoreactive for VWF, which is specific for endothelial cells, the proportion of steroidogenic cells in the enriched endothelial cells is minimal. Taken together, the filtration method enriched endothelial cells with high purity.

The filtration method used in this study was expected to yield endothelial cells that reflect the heterogeneity of endothelial cells from the CL (Spanel-Borowski and van der Bosch, 1993; Davis et al., 2003; rev.). Spanel-Borowski and van der Bosch (1990) described isomorphic epithelioid, polymorphic epithelioid, spindle-shaped, round, and phase-dense phenotypes of endothelial cells from the CL. In the present study, spindle-shaped, round and cobblestone were observed in culture. Because the various types of luteal endothelial cells may have sizes larger than 10 µm, the filtration method may not have captured all of the endothelial subtypes. Also, the tendency to aggregate and the fact that growth and morphological characteristics in vitro may be dependent on the type of growth factors in the media (Stolz and Jacobson, 1991) may explain why all of the subtypes of endothelial cells were not observed in culture. The heterogeneity of endothelial cells in the CL presents a big challenge in understanding the biology of this vascular bed. Whereas FACS and other methods that rely on expression of specific surface proteins may yield consistent results, they may not capture all four subtypes of endothelial cells described (Spanel-Borowski and van der Bosch, 1993). The specific
proteins targeted for isolation may not be expressed or at least expressed uniformly on all the subsets. Thus, the filtration method may be more reliable.

The understanding of endothelial cell function has evolved from just maintaining the integrity of blood vessels to active participation in recruitment and function of immune cells (Pober and Sessa, 2007, rev). Human endothelial cells from umbilical vein, pulmonary artery and ovarian vein act as accessory cells to the mitogenic response of T lymphocytes in vitro (Ashida et al., 1981) and have been suggested to play a role in transplantation rejection (Hughes et al., 1990; Savage et al., 1993; Rollins et al., 1994). The interaction of endothelial cells with heterologous T lymphocytes occurs via MHC II and costimulatory molecules such as LFA3, which interact with the T cell receptor and CD2, respectively on T lymphocytes (Dustin et al., 1996; Karmann et al., 1996; Murakami et al., 1999). Alloactivation has been shown to generate regulatory T cells (Tregs) and Th17 cells (Bedke et al, 2010; Taflin et al., 2011), but the generation of Tregs is independent of the TCR and dependent on LFA3 (Taflin et al., 2011). This indicates that endothelial cells expressing LFA3 can induce Tregs from autologous T lymphocytes. To date no studies have shown that endothelial cells activate autologous T lymphocytes, except for a study in cattle that shows that endothelial cells from cows infected with *Cowdria ruminantium* present antigen to autologous T lymphocytes (Mwangi et al., 1998). In the present study, luteal endothelial cells induced IL2Rα and proliferation of autologous T lymphocytes. However, it was not determined if the IL2Rα expression was increased on Tregs. The activation and proliferative response is likely to be mediated by cell surface associated molecules expressed by endothelial cells. Although the expression of LFA3 on luteal endothelial cells has not been studied, luteal endothelial cells express
MHC II-DRα protein and CD80 mRNA (Cannon et al., 2007a; 2007b). These and other molecules could be responsible for the activation of T lymphocytes. Therefore, endothelial cells may modulate the activity of migrating T lymphocytes.

**Conclusion**

This study demonstrates that isolating endothelial cells by filtration is an inexpensive and efficient method of recovering a large number of relatively pure endothelial cells from the CL. The activated and induction of proliferation of autologous T lymphocytes by luteal endothelial cells is novel and may potentially lead to a new area of research.
Figure 2-1

Collagenase dispersed luteal cells

50µm sieve

dispersed luteal cells

A) Supernatant

B) 15µm sieve

10µm sieve

Small cells<10µm

Figure 2-1 Schematic illustration of the filtration process used to enrich endothelial cells from dispersed luteal cells. A) Dispersed luteal cells were serially centrifuged at 200xg, 128xg, 72xg and 32xg for 10 minutes at 4°C each time. B) Supernatants were combined and centrifuged at 300xg and the resulting cell pellet was resuspended and filtered through 15 and 10 µm sieves.
Figure 2.2. Yield and viability of isolated endothelial cells. A) The number of cells recovered after filtration and immunomagnetic isolation. B) Viability of cells isolated using filtration and immunomagnetic separation. Different letters indicate significance (P<0.05).
Figure 2-3. Characteristics of fresh and cultured endothelial cells.
A) Freshly dissociated cells showing endothelial cells (arrow), small steroidogenic (dashed arrow head) and large steroidogenic cells (solid arrow head). B) The size of enriched endothelial cells (~10 µm). C) Morphology of cultured enriched endothelial cells at day 4. D) Confluent enriched endothelial cells (day 9).
Figure 2-4. Morphology and organization of cultured enriched endothelial cells in culture at 2 weeks. A) Cells with narrow elongated shape. B) Cells with broad flattened morphologies. C) Small enclosed structures in day 7 cultures (lumen-like) and D) small and large lumen-like structures at day 14 of culture.
Figure 2-5. Spontaneous formation of capillary-like structures in endothelial cell cultures at 2 weeks. A and B) Low magnification. C and D) High magnification.
Figure 2-6. Endothelial cell characteristics. A) Side and forward scatter characteristics of mixed luteal cells and enriched endothelial cells. Regions (R1, R2 and R3) indicate distinct populations of cells based on size (forward scatter) and intracellular complexity (side scatter). B) The proportion of BS-1 binding in enriched endothelial cells. C) Steady-state abundance of StAR transcripts in enriched endothelial cells and cells recovered from the retentate. Means with different letters are significantly different (P<0.05).
Figure 2-7. Von Willebrand factor VIII staining in enriched cultured cells. Cells stained with IgG were used as controls (Bottom panels). Mag X100 (top panels), MagX400 (Middle panel).
Figure 2-8. **BS-1 lectin staining** in enriched cultured endothelial cells. Unstained cells were used as controls (Bottom panels). Mag X100 (top panels), MagX400 (Middle panel).
Figure 2-9. Effect of endothelial cells on T lymphocyte function. Comparison of the proportion of T lymphocytes expressing IL2R in response to endothelia or steroidogenic cells (A). Comparison of the proportion T lymphocytes of proliferating in response to endothelial or steroidogenic cells (B). Effect of endothelial cells on CD8+ (C) and γδ+ (D) T lymphocyte phenotypes. Means with different letters are significantly different (P<0.05).
Materials and Methods

Reagents and antibodies

Reagents used for polymerase chain reaction (PCR) were: TRIZol® reagent (Invitrogen), iScript™ cDNA synthesis kit (Bio-Rad), Power SYBR® Green PCR master mix (Applied Biosystems), and RNase-free DNaseI (Ambion), QIAquick gel extraction kit (QIAGen). Reagents used for cell culture were: Ham’s F-12 nutrient mixture and AIMV cell culture media (Gibco, Invitrogen), carboxyfluorescein succinimidyl ester (CFSE; Sigma), insulin transferrin and selenium (ITS; VWR), bovine serum albumin (BSA; Sigma), phorbol 12-myristate 13-acetate (PMA) and ionomycin calcium salt (Sigma), EBM-2 and EGM-2 SingleQuot kit Suppl. & growth factors (Lonza). Antibodies used for the isolation of T lymphocytes and endothelial cells were: CD2 (MUC2A; VMRD), anti-sheep CD31 (AbD Serotec), anti-mouse-IgG2a, anti-mouse IgG1 and IgG2a+b magnetic beads (Miltenyi). Antibodies used for T lymphocyte phenotyping were: δTCR-N12 (CACT61A; VMRD), CD8α (BAQ111A; VMRD), CD8β (BAT82A; VMRD), and WC1 (IL-A29; VMRD), anti-mouse IgM:PE (STAR81F; AbD Serotec), anti-mouse IgG1:PE (STAR81PE; AbD Serotec).
**CL dissociation**

Corpora lutea were collected transvaginally from cyclic Holstein cows on days 10-12 of the estrous cycle. The procedure was approved by the Institutional Animal Care and Use Committee of Pennsylvania State University (IACUC # 27578). The CL were immediately placed in ice cold Ham’s F-12 medium containing gentamycin (20 µg/mL) and 0.5% BSA. The process of tissue dissociation followed procedures detailed by Pate (1993). Briefly, luteal tissue was weighed, sliced, minced and incubated in Ham’s F-12 medium with collagenase (2000 U/g tissue) at 37°C for 2 hours to disperse luteal cells. Medium containing dispersed cells was filtered through a 50 µm pore size nylon microsieve (BioDesign Inc.) supported by a plastic sieve holder. The filtrate was serially centrifuged at 200xg, 128xg, 72xg and 32xg, 10 minutes each time at 4°C, to pellet luteal steroidogenic cells. The supernatant after each centrifugation was collected in 50 mL polystyrene tubes (always kept on ice) and used for endothelial cell isolation by size exclusion. Isolation of endothelial cells by magnetic separation was applied on both the pellet and cells recovered from the supernatant.

**Isolation of luteal endothelial cells by filtration**

The process of isolating endothelial cells by filtration is summarized by the schematic in Figure 1. The supernatant obtained by serial centrifugation of mixed luteal cells was further centrifuged at 300xg to pellet smaller cells. The pellet was resuspended in 10 mL of phosphate buffered saline-2 mM EDTA (PBS-EDTA) and gently agitated for
5-10 minutes to allow separation of cell aggregates, then filtered sequentially through 15
and 10 µm pore size nylon microsieves into a glass beaker placed on ice. The filtrate was
centrifuged at 300xg and the supernatant discarded. Five to ten mL of cold, freshly
prepared red blood cell lysis buffer (0.14 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, PH
7.4) was added to the pellet for 5 minutes. The cells were subsequently diluted with
excess endothelial cell culture medium (EBM2 medium plus EGM™-2 SingleQuot) and
centrifuged at 300xg. The pellet was resuspended in endothelial cell culture medium and
the number and viability of cells recovered was determined by flow cytometry (EasyCyte
Plus, Millipore) after labeling with Viacount dye (Guava Viacount, Millipore). Forward
and side scatter characteristics of cells obtained from the enriched endothelial cells and
mixed luteal cells were compared.

**Isolation of endothelial cells by magnetic separation**

Mixed luteal cells were incubated with anti-CD31 primary antibody (10 µg/mL)
for 30 minutes at 4°C. After incubation, the cells were washed 3X with PBS-EDTA at
250xg. The supernatant was discarded and the pellet incubated with mouse anti-sheep
IgG2a magnetic beads in 1.8 mL of PBS-EDTA for 30 minutes. CD31⁺ cells were
obtained by performing two rounds of positive selection using autoMACS™ Pro
Separator (Miltenyi, Germany). The number and viability were determined by flow
cytometry after labeling with Viacount dye.
Characterization of endothelial cells isolated by filtration

Size and internal complexity

The physical characteristics of enriched endothelial cells relative to mixed luteal cells were evaluated by forward (FS) and side scatter (SCC) properties, and the proportion of endothelial cells in the enriched fraction was determined by BS-1 lectin binding. A portion of enriched endothelial cells recovered after filtration through the 10 µm nylon microsieve was labeled with BS-1 lectin-FITC (Sigma) to determine the percentage of endothelial cells in the filtrate. Fresh cells (1x10^6) were incubated with 2 µg/mL of BS-1 lectin-FITC for 30 minutes. The cells were centrifuged three times at 300xg and resupended in PBS-EDTA before flow cytometric analysis. Cells not labeled with BS-1 lectin-FITC were used as a control.

Immunostaining

Cell monolayers obtained from the enriched endothelial cell fractions were examined for expression of vWF and their ability to bind BS-1 lectin. Endothelial cells cultured in 24-well plates were fixed with 3% paraformaldehyde for 5-7 minutes at 4°C and washed 2X with PBS. The fixed cells were then treated with 0.5% Triton-X in 3% paraformaldehyde for 3-5 minutes at 4°C, washed again 3X with PBS and incubated with anti-human vWF (4 µg; Dako) overnight at 4°C. After incubation, the cells were
incubated with PBS+5% goat serum to block nonspecific binding and washed once with PBS. Secondary antibodies (rabbit anti-human IgG) were added and cells were incubated for 1 hour at 4°C and subsequently washed 3X with PBS. To stain the nuclei, 500 μL of DAPI (3 μg/mL) were added to all wells and incubated for 5-6 minutes at 4°C. The monolayers were washed 3X with PBS. Staining for DAPI and vWF was visualized using an inverted fluorescence microscope (Axiovert, Zeiss, Germany). In addition to staining cells grown in cell culture plates, endothelial cells were also cultured on glass coverslips, fixed with paraformaldehyde and stained with either BS-1-rhodamine conjugate (2 μg; Sigma) or anti-human VWF. Unstained cells were used as control for BS-1. After incubation with primary and secondary antibodies, the coverslips were mounted on glass slides with ProLong® Gold anti-fade reagent (Invitrogen). Images of stained cells were acquired using a DP71 color camera mounted on a BX51 Olympus fluorescence microscope.

**RNA isolation and PCR**

The presence of contaminating steroidogenic cells in the endothelial cell fraction was determined by detection of steroidogenic acute regulatory protein (StAR) messenger RNA abundance using PCR. Freshly isolated cells from the enriched endothelial cells and mixed luteal cells fractions were frozen at -80°C and later thawed. Total RNA was extracted using TRIzol reagent. The concentration of RNA was estimated using spectrophotometry (NanoDrop® ND-1000; NanoDrop Technologies, Inc.). Total RNA was treated with RNase-free DNase1 to eliminate genomic DNA contamination.
Oligonucleotide primers (forward: 5’-GCATCCTCAAAGACCAGGAG-3’; reverse: 5’-CTTGACGTTGGGATTCCACT-3’) were designed from bovine StAR mRNA sequence (NM_174189.2) and amplified a 194bp cDNA transcript. To validate the primers, the product was separated on a 1.5% agarose gel, visualized by ethidium bromide, cut and extracted using QiAquick Gel Extraction Kit. The product was submitted for sequencing. Ribosomal protein 19 (RPL19) was used as a housekeeping gene. The primers (forward: 5’-ATCGATCGCCACATGTATCA-3’; reverse: 5’-GCGTGCTTCCTTGGTCTTAG-3’) based on bovine RPL19 sequence (NM_001040516) were used to amplify a 196bp fragment. The primers were validated by sequencing the PCR product. Total RNA (2 µg) was reverse transcribed using the iScript cDNA synthesis kit following the manufacturer protocol. Hot start qPCR (7500 Fast Real-Time PCR System, Applied Biosystems) was used to detect the amount of mRNA for StAR using Power SYBR® Green PCR master mix for a total of 35 cycles. The conditions were as follows: denaturing: 95°C for 30 seconds; annealing: 60°C for 45 seconds; extension 72°C: for 60 seconds; extra elongation, 72°C for 5 minutes. A control sample in which the mRNA was not reverse transcribed was included as a negative control. The quantity of mRNA was evaluated in duplicate wells for each sample from three biological replicates. A standard curve generated from the purified product was used to determine the relative amount of original mRNA present in the sample.
Isolation of T lymphocytes

T lymphocytes were isolated from peripheral blood mononuclear cells (PBMCs) by positive selection using autoMACS Pro Separator (Miltenyi Biotech, Germany) as described by Ndiaye et al. (2008). Briefly, a leukocyte rich layer was obtained after centrifugation of blood. PBMCs were isolated from the leukocyte layer by centrifugation over a ficoll density gradient. T lymphocytes were separated by positive selection from PBMCs with anti-CD2 and anti-γδ antibodies using autoMACS™ Pro Separator. Purity, evaluated as percentage of cells positive for CD3, was (>95 %). Cell numbers were determined as described above.

T lymphocyte activation and proliferation

T lymphocytes (12.5 x10^6/mL) were labeled with carboxyfluorescein succinimidyl ester (CFSE; 1.75 µM) in AIMV medium and incubated at 37°C for 15 minutes and incubated at room temperature for another 10 minutes. The cells were diluted 5-fold with medium containing 10% fetal calf serum and washed three times at 300xg for 10 minutes. To determine if endothelial cells activated T lymphocytes, endothelial cells isolated by the filtration method were seeded at 1x10^6 cells/ well in 24-well plates and then cocultured with T lymphocytes (5x10^5/well). Also, isolated steroidogenic cells (5x10^5) were cultured with T lymphocytes (5x10^5/well). After 72 hours of culture, T lymphocytes were harvested, labeled with anti-CD25 antibodies (1 µg/200 µL) and the proportion of stained cells was analyzed by flow cytometry. In another experiment, endothelial or
steroidogenic cells were cultured with CFSE-labeled T lymphocytes (5x10^5/well) for 72 hours. Proliferation of T lymphocytes was assayed by flow cytometry.

**T lymphocyte staining and phenotype analysis**

To determine if endothelial cells altered the phenotype of T lymphocytes, endothelial cells were cultured with T lymphocytes (1x10^6) for 72 hours in 24-well plates. The T lymphocytes were then harvested and stained with antibodies against CD8α, CD8β, δTCR and WC1 following the protocol described by Poole and Pate (2012). Briefly, cells resuspended in 200 µL were incubated with primary antibodies on ice for 30 minutes. The cells were then washed 3X with PBS-EDTA containing 5% normal goat serum (NGS). The pellet was resuspended in 200 µL of PBS-EDTA-5% NGS and appropriate secondary antibodies were added. The cells were incubated for an additional 30 minutes on ice and subsequently washed 3X with PBS-EDTA-5% NGS. The final pellet was resuspended in 1 mL of PBS-EDTA. The proportion of stained cells was determined by flow cytometry.

**Statistical analyses**

Data were analyzed by Proc mixed procedures of SAS (Statistical analysis system Inc. version 9.3). Tukey-Kramer multiple comparisons post-test was used to separate means generated from proliferation data. Data generated from qPCR was subjected to
analysis of co-variance, using the housekeeping gene as a covariate. In all cases, statistical significance was considered when the P value was <0.05.
Chapter 3

The role of prostaglandins in mediating luteal cell-T lymphocyte interactions

Introduction

Prostaglandins are lipid mediators that belong to a class of molecules referred to as eicosanoids. Prostaglandins are synthesized from arachidonic acid, a polyunsaturated fatty acid, which is derived from plasma membrane phospholipids (Ricciotti and FitzGerald, 2011, rev.). Enzymatic oxidation of arachidonic acid by prostaglandin H synthase (PGH synthase) yields endoperoxide (PGH2), an intermediate product that is converted to prostaglandins F$_{2\alpha}$, E$_2$, D$_2$ and I$_2$ and Thromboxane A2 (TXA2) by specific isomerases (Smith and Lands, 1972; Brock et al., 1999; Simmons et al., 2004, rev.). Prostaglandins F$_{2\alpha}$, E$_2$, D$_2$ and I$_2$ regulate physiological and/or pathological processes in cells of vascular, nervous, immune and reproductive systems (Miller, 2008).

In the reproductive system, prostaglandins regulate processes such as ovulation (Gaytán et al., 2006), implantation (Kennedy, 1977; Holmes and Gordashko, 1980; Achache et al., 2010), luteal function (Lewis and Warren, 1977; Arosh et al., 2004) and parturition (Karim, 1971; Olson, 2003). Prostaglandins are synthesized by murine and bovine embryos (Kennedy, 1977; Holmes and Gordashko, 1980; Ulbrich et al., 2009), sheep, cow, rabbit, rat and guinea pig endometria (Land et al., 1976; Lytton and Poyser, 1982; Poyser, 1984; Brown and Poyser, 1985; Shirasuna et al., 2004) and CL of cattle.
(Hayashi et al., 2003; Shirasuna et al., 2004; Atli et al., 2012) and other animals (Wiltbank and Ottobre, 2003).

Prostaglandins produced locally in the CL and those that originate from the uterus (McCracken, 1972; Stefánczyk-Krzymowska et al., 2005) regulate both the function and lifespan of the CL in bovine (Milvae and Hansel, 1983; Milvae, 1986; Hayashi et al., 2003; Weems et al., 2012), human (Bogan et al., 2008), porcine (Waclawik et al., 2008), ovine (Silva et al., 2000), and rodent (Bioti, 1999, rev) species. Prostaglandins E₂ and I₂ are luteotropic whereas PGF₂α is luteolytic (Heath et al., 1983; Milvae and Hansel, 1983; Pate and Condon, 1984; Milvae, 1986; Miyamoto et al., 1993).

Synthesis of luteal prostaglandins in the bovine CL is regulated by cytokines such as IL1β (Nothnick and Pate, 1990; Townson and Pate, 1994), IFNγ (Fairchild and Pate, 1989; 1991), and TNFα (Benyo and Pate, 1992; Galvão et al., 2012). These cytokines are expressed in the CL throughout the estrous cycle (Petroff et al., 1999), and they inhibit luteinizing hormone-induced progesterone synthesis and induce cytotoxicity in luteal cells (Fairchild and Pate, 1991; Petroff et al., 2001; Cannon et al., 2006). These data indicate that luteal prostaglandins are regulated by cytokines, however, it is not known if luteal prostaglandins regulate the function of immune cells.

Immune cells are present in CL of murine (Pavoola, 1979; Bagavandoss et al., 1988; Brännström et al., 1994b; McCormack et al., 1998; Komatsu et al., 2003), bovine (Lobel and Levy, 1968; Spanel-Borowski et al., 1997; Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002; Davis and Pate, 2007), ovine (Murdoch et al., 1988), equine (Lawler et al., 1999), porcine (Hehnke et al., 1994) and primate (Adam and Hertig, 1969; Wang et al., 1992) species. The numbers of immune cells in the CL change throughout
the estrous cycle. For instance, T lymphocytes are more abundant in midcycle and regressing CL compared to early CL (Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002). Although immune cells are present in the CL prior to the onset of luteal regression (Poole and Pate, 2012), regression is associated with increased populations of monocyte/macrophages (Penny et al., 1999), neutrophils (Shirasuna et al., 2012), T lymphocytes (Townson et al., 2002), and increased expression of cytokines (Neuvian et al., 1994; Kliem et al., 2009). These events are suggested to facilitate luteal demise. The increase in immune cells during luteal regression is partly due to influx of immune cells (Bauer et al., 2001) and is associated with increased expression of chemokines/chemoattractants such as MCP-1 (Townson et al., 1996; Penny et al., 1998; Townson et al., 2002; Tsai et al., 2001), Interleukin 8 (IL8) and CXCL1 (Jiemtaweeboon et al., 2011) observed after PGF$_{2\alpha}$ administration.

Prostaglandins are associated with recruitment of leukocytes at sites of inflammation (Flower, 1977), and in the presence of inhibitors of PGH synthase, there is reduced leukocyte migration and alleviation of clinical signs of inflammation (Yamauchi et al., 1979). The increase in prostaglandins, especially PGE$_2$, at inflammatory sites correlates with abundance of immune cells (Salmon et al., 1983). Thus, PGE$_2$ is suggested to be involved in immune cell recruitment. Also, prostaglandins PGE$_2$, E$_1$ and PGF$_{2\alpha}$ have been found to regulate migration of polymorphonuclear cells (PMNs) (Shibuya, 1976). In the bovine CL, PGF$_{2\alpha}$ is suggested to recruit neutrophils by enhancing the expression of P-selectin and interleukin 8 (IL-8) and neutrophil adhesion to endothelial cells (Shirasuna et al., 2012). The mechanisms involved in the recruitment of immune cells prior to the onset of regression are currently not known. The process of
immune cell recruitment is a multi-step process, which involves rolling, arrest, firm adhesion and transmigration (Ley et al., 1996). Prostaglandins may be involved in regulating leukocyte recruitment at any of these steps. In the present study, an in vitro static assay was used to determine if prostaglandins regulate adhesion of T lymphocytes to endothelial cells.

In addition to the suggested role in recruiting immune cells to sites of inflammation, PGE$_2$, E$_1$, D$_2$ and I$_2$ directly act on immune cells (Virgolini et al., 1992; Gerlo et al., 2004). For example, prostaglandins of the E series suppress T lymphocyte proliferation (Goodwin et al., 1977; Chouaib et al., 1985; Low and Hansen, 1988; Emond et al., 1998). Prostaglandin I$_2$ also suppresses the production of proinflammatory cytokines and expression of costimulatory molecules on antigen presenting cells (Zhou et al., 2007; Wang et al., 2011). It is therefore likely that in addition to their classical effects on steroidogenesis, prostaglandins may regulate the interaction between luteal cells and resident leukocytes. It was hypothesized that PGF$_{2\alpha}$, PGE$_2$ and PGI$_2$ regulated adhesion of T lymphocytes to endothelial cells. It was also hypothesized that prostaglandins of luteal origin regulate the proliferative response of T lymphocytes to luteal cells.
Results

Expression of prostaglandin receptors on T lymphocytes

Regulation of T lymphocyte function by prostaglandins requires that receptors for prostaglandins are expressed by T lymphocytes. The message (mRNA) for PTGER2 and 4, and PTGIR, but not PTGFR was present in bovine T lymphocytes (Fig. 3-1).

Effect of prostaglandins on luteal cell-induced T lymphocyte proliferation

Bovine T lymphocytes expressed prostaglandin receptors (Fig 3-1), but there was no direct effect of PGE2, PGI2, and indomethacin on T lymphocyte proliferation (Fig. 3-2). Inhibiting endogenous prostaglandin synthesis with PLA2X or indomethacin had no effect on luteal cell-induced T lymphocyte proliferation (P>0.05; Fig. 3-3). Also, the replacement of prostaglandins PGE2, PGI2 and PGF2α, alone or in combination did not significantly alter luteal cell-induced proliferation of total T lymphocytes (Fig. 3-4) or T lymphocyte subsets (Fig. 3-5).

Effect of prostaglandins on the adhesion of T lymphocytes to luteal endothelial cells

To examine if PGF2α, PGE2 or PGI2 regulate recruitment of T lymphocytes into the CL, adhesion of T lymphocytes to luteal endothelial cells was examined. Increasing the concentration of PGE2 from 0 to 100 ng/mL linearly increased the binding of T lymphocytes to endothelial cells that were not treated with cytokines compared to those
treated with cytokines (P<0.05; Fig 3-6A). In addition, there was a significant interaction between PGE$_2$ and cytokine treatments (P<0.05). Prostacyclin (PGI$_2$) had no effect on adhesion of T lymphocytes to luteal endothelial cells (P>0.05; Fig 3-6B). There was a significant interaction between PGF$_{2\alpha}$ and cytokine treatments (Fig. 3-6C). Increasing the concentration of PGF$_{2\alpha}$ linearly increased the binding of T lymphocytes to endothelial cells not treated with cytokines (P<0.05). Conversely, increasing concentrations of PGF$_{2\alpha}$ decreased T lymphocyte adhesion in endothelial cells treated with cytokines in a relationship described by a quadratic equation (P<0.05).

**Discussion**

Prostaglandins are critical in regulating reproductive processes, and are synthesized by reproductive organs such as the ovary and the uterus. They are pivotal in deciding the fate of the CL during the estrous cycle and early pregnancy in a number of species (McCracken, 1972; Pratt et al., 1977; Arosh et al., 2004; Allen, 2005). The immune system also plays a role in determining the success of pregnancy at the level of the uterus (Lala et al., 1988; Parhar et al., 1989), but also in the normal function and demise of the CL (McCracken, 1972; Heath et al., 1983; Milvae and Hansel, 1983; Pate and Condon, 1984; Milvae, 1986; Miyamoto et al., 1993).

Prostaglandins regulate functions of immune cells, and their effects are mediated by multiple receptors that recognize specific subtypes of prostaglandins. Thus, prostaglandins have pleotropic effects on immune cell functions (Ricciotti and FitzGerald, 2011, rev.). Because immune cells are present in the CL and can affect luteal
function, it was hypothesized that luteal prostaglandins regulate the recruitment and function of immune cells in the CL. The process of recruitment of immune cells in the CL is poorly understood, but there is evidence that PGF$_{2\alpha}$ may be involved in the recruitment of immune cells during luteal regression (Shirasuna et al., 2012). In the present study, PGF$_{2\alpha}$ and PGE$_{2}$ influenced the adhesion of T lymphocytes to luteal endothelial cells. The effects of PGF$_{2\alpha}$ on T lymphocyte binding were dependent upon the presence of cytokines, indicating that PGF$_{2\alpha}$ may differentially modulate the recruitment of T lymphocytes in a functional and regressing CL. Whereas PGF$_{2\alpha}$ may enhance T lymphocyte recruitment before luteal regression, it may prevent T lymphocyte recruitment in the presence of inflammatory mediators, especially during luteal regression. Prostaglandin F$_{2\alpha}$ may increase T lymphocyte adhesion by increasing the expression of chemokines (Haworth et al., 1998; Luo et al., 2011; Sales et al., 2009) and adhesion molecules such as, P-selectin (Shirasuna et al., 2012) on endothelial cells. Prostaglandin F$_{2\alpha}$ has no chemotactic activity, but increases the recruitment of other immune cells such as, eosinophils (Murdoch, 1987) and polymorphonuclear cells (PMNs; Shirasuna et al., 2012; de Menezes et al., 2005).

To date, there is no report of either PGE$_{2}$ or PGI$_{2}$ being involved in recruitment of immune cells in the CL. Cyclooxygenase products promote adhesion of granulocytes to endothelial cells (Pearson et al., 1979; Gimbrone et al., 1984; Weiss et al., 1998). Prostaglandin E$_{2}$ inhibits lymphocyte binding (To and Schrieber, 1990; Mesri et al., 1996) and transmigration (Oppenheimer-Marks, 1994; Mesri et al., 1996) across endothelial cells, but also enhances Formyl-Methionyl-Leucyl-Phenyalanine (FMLP)-induced adhesion of leukocytes in vivo (Tromp et al., 2000). In the present study,
increasing concentrations of PGE₂ linearly increased adhesion of T lymphocyte binding to endothelial cells not treated with cytokines, and there was a significant interaction between PGE₂ and cytokines. This suggests that the effects of PGE₂ on T lymphocyte binding to endothelial cells may be different in the presence and absence of inflammatory conditions. Prostaglandin E₂ may enhance T lymphocyte adhesion by increasing adhesion molecules such as ICAM-1 (Winkler et al., 1997) on endothelial cells. Combining observations from the effects of PGF₂α and PGE₂ on T lymphocyte adhesion indicates that PGF₂α and PGE₂ may modulate recruitment of T lymphocytes into the CL before luteal regression.

When T lymphocytes are recruited to the CL, their functions may be modulated by luteal prostaglandins to promote luteal survival or facilitate luteal demise. The present study demonstrated that bovine T lymphocytes expressed receptors for PGE₂ and PGI₂, but not PGF₂α. Prostaglandin E₂ receptors, EP2 and EP4 are also present on human T lymphocytes (Gerlo et al, 2004). Also, the IP receptor is present on murine T lymphocytes (Jaffar et al., 2002; Nakajima et al., 2010). The absence of PTGFR transcripts in T lymphocytes shown in this study is supported by the lack of PTGFR protein in bovine PBMCs (Shirasuna et al., 2012). This indicates that bovine T lymphocytes may not directly respond to PGF₂α via PTGFR. Indeed, T lymphocyte proliferation induced by luteal cells was not affected by PGF₂α. Similar to the finding in this study, prostaglandin F₂α had no effect on T lymphocyte activation and proliferation of human T lymphocytes (Chouaib et al., 1985), and pre-incubation of peripheral blood leukocytes (PBLs) with 100 ng/mL PGF₂α did not affect IL2 synthesis or mitogen-induced proliferation of bovine T lymphocytes (Emond et al., 1998). PGF₂α may,
however, indirectly affect T lymphocyte proliferation by its action on luteal cells (Cannon et al., 2003).

Although PGE$_2$ has inhibitory effects on T lymphocytes (Chouaib et al., 1985; Chouaib et al., 1987; Emond et al., 1998; Low and Hansen, 1998), inhibition of endogenous prostaglandins and (or) replacement of PGE$_2$ had no effect on luteal cell-induced T lymphocyte proliferation. Because bovine T lymphocytes express receptors for PGE$_2$, that lack of effect of PGE$_2$ on luteal cell-induced T lymphocyte proliferation indicates that activating signals from luteal cells may override the inhibitory effect of PGE$_2$. Human T lymphocytes were shown to respond to PGE$_2$ via EP2 and EP4 receptors by augmenting cAMP, decreasing Ca$^{2+}$ influx, IL2R$\alpha$ binding, IL2 synthesis and ultimately inhibiting proliferation (Chouaib et al., 1985; Chouaib et al., 1987; Edmond et al., 1998; Gerlo et al., 2004). Similar to PGE$_2$, there is evidence to support the inhibitory action of PGI$_2$ on T lymphocyte proliferation (Gordon et al., 1979) and other immune cells, such as dendritic cells (Kuo et al, 2012). Prostaglandin I$_2$ has been shown to promote Th1 responses (Nakajima et al., 2010) and differentiation of Th17 cells (Zhou et al., 2012). In the current study, PGI$_2$ had no effect on luteal-cell induced T cell proliferation. Although prostaglandins had no effect on luteal cell-induced T cell proliferation, luteal prostaglandins may be involved in regulating T lymphocyte functions other than proliferation, which were not evaluated in this study.
Conclusion

This study demonstrated that bovine T lymphocytes expressed receptors for PGE$_2$ and PGI$_2$, but not PGF$_{2\alpha}$, but none of the prostaglandins tested demonstrated inhibitory or stimulatory effects on luteal cell-induced T lymphocyte proliferation. Prostaglandin E$_2$ and PGF$_{2\alpha}$ may, however, regulate recruitment of T lymphocytes into the CL.
Figure 3-1. Gene expression of prostaglandin receptors in T lymphocytes. TC= T cells, LC= luteal cells, NRT= no reverse transcriptase, RPL19= internal control, PTGFR=prostaglandin F$_{2\alpha}$ receptor, PTGER2 and PTGER4=prostaglandin E$_2$ receptors and PTGIR=prostacyclin receptor.
Figure 3-2. Effect of prostaglandins and indomethacin on T cell proliferation. T lymphocytes were treated with indomethacin (Indo) and either PGE$_2$ (A), PGI$_2$ (B) or PGF$_{2\alpha}$ (C) at 1, 10 or 100 ng/mL. Untreated T lymphocytes were used as control (n=4).
Figure 3-3. Effect of inhibition of endogenous prostaglandin synthesis on luteal cell-induced T cell proliferation. Prostaglandin synthesis was inhibited using phospholipase A2 inhibitor (PLA2X; A) or indomethacin (Indo; B). Untreated T lymphocytes were used as controls (n=3).
Figure 3-4. Effect of prostaglandin replacement on luteal cell-induced T cell proliferation. Proliferation of T lymphocytes induced by indomethacin treated luteal cells in presence of PG E₂ (A), PGI₂ (B) and PGF₂α (C), and a combination of the prostaglandins (D). Untreated T lymphocytes were used as a control. Four independent experiments were performed for each of the prostaglandins whereas three experiments were conducted for the combinations. Different letters indicate significant differences (P<0.05).
Figure 3-5. Effect of prostaglandins on luteal cell-induced proliferation of CD4+ CD8+ and γδ+ T lymphocyte. Proliferation of T lymphocytes induced by luteal cells treated with indomethacin (Indo) in the presence of 10 or 100 ng/mL of either PGE₂ (A) or PGI₂ (B). Untreated T lymphocytes were used as controls (n=3).
Figure 3-6

**Figure 3-6. Effect of prostaglandins on adhesion of T lymphocytes to luteal endothelial cells.** A) Fluorescence of T lymphocytes adhered to luteal endothelial cells pretreated with A) 1, 10 or 100 ng/mL of PGE$_2$, B) PGI$_2$ and C) PGF$_2\alpha$. Broken lines indicate endothelial cells treated with IL1β (50 ng/mL) and TNFα (50 ng/mL) while solid lines indicate endothelial cells treated with PBS 6 hours before addition of T lymphocytes (n=3). †=significant linear response (P<0.05), Φ= quadratic response (P=0.09).
Materials and methods

Prostaglandin receptor gene expression

To determine if bovine T lymphocytes express PTGFR, PTGE2R, PTGER4 and PTGI2R receptors, RNA was extracted from freshly isolated T lymphocytes using reagents and procedures described in Chapter 2. Primers for PTGFR, PTGER2, PTGER4 and PTGIR were designed based on the following Genbank accession numbers; PTGFR (NM_181025.2), PTGER2 (NM_174588.2), PTGER4 (NM_174589), PTGIR (NM_001015622.2). The primers used were: PTGFR-265bp (forward: 5’-CACAGACAAGGCAGGTCTCA-3’; reverse: 5’-CTCCACAACAGCGTCTGGTA-3’), PTGER2-278bp (forward-5’-AATGCGTTCAGTCCTCTGCT-3’; reverse-5’-TGCAAGAAGTGCTTGTCAG-3’), PTGER4-173bp (forward: 5’-TCTCTGGTGTCATCTCTG-3’; reverse: 5’-GTCTTCCGCAAGGAGATGTA-3’), PTGIR-278bp (forward: 5’-GGCACGGCGAGGAGGTTG-3’; reverse: 5’-TCCGAGAGTCGCTTGGCCA-3’). The conditions for PCR and controls are similar to those described in Chapter 2.

Proliferation assays

Three experiments were conducted to determine if prostaglandins modulated luteal cell-induced T cell proliferation. The first experiment was designed to test the
effect of blocking endogenous prostaglandin production on luteal cell-induced T cell proliferation. In this experiment, total T lymphocytes (12.5 x10^6/mL) were labeled with CFSE (1.75 µM) in AIMV medium, incubated at 37°C for 15 minutes and incubated at room temperature for another 10 minutes. The cells were diluted 5 fold with medium containing 10% fetal calf serum and washed three times at 300xg for 10 minutes. CFSE-labeled (5x10^5/well) T lymphocytes were then cocultured with luteal cells (5x10^5/well) pre-treated with indomethacin (28 μg/mL) or PLA2X (1.75 μg/mL) in 24-well plates. Luteal cells were washed before T lymphocytes were added. In the second experiment, the hypothesis that prostaglandins and indomethacin induce T lymphocyte proliferation was tested. T lymphocytes labeled with CFSE were treated separately with indomethacin, or 1, 10 and 100 ng/mL of PGE_2, PGF_{2α} and PGI_2. In the third experiment, exogenous PGE_2, PGF_{2α} and PGI_2 at 1, 10 and 100 ng/mL were added to indomethacin-treated luteal cell-T lymphocyte cocultures. Lastly, the effect of exogenous PGE_2 and PGI_2 (10 and 100 ng/mL) on luteal cell-induced proliferation of CD4^+ , CD8^+ and γδ^+ T lymphocytes, in the absence of endogenous prostaglandins, was examined. In all the experiments, T lymphocytes were added following the addition of prostaglandins. CFSE-labeled T lymphocytes cultured alone were used as a control. Also, proliferation was assayed by flow cytometry after 72 hours of culture.

**T lymphocyte adhesion assay**

Endothelial cells isolated from the CL were seeded (1x10^6 cells/well) in 96-well plates in EBM2 medium. At confluence, the cells were treated with prostaglandins E_2
(0.002, 0.02 or 0.2 \mu M), I_2 (0.002, 0.02 or 0.2 \mu M) and F_{2\alpha} (0.002, 0.02 or 0.2 \mu M),
followed by TNF\alpha (50 ng/mL) and IL-1\beta (50 ng/mL) or PBS for the last 6 hours of a 21-hour incubation period. Vehicle control wells were included for each treatment. The T lymphocyte adhesion assay was conducted under static conditions. T lymphocytes (5x10^5) in 100 \mu L of RPMI, labeled with Calcein-AM (Vybrant\textsuperscript{TM} cell adhesion assay kit) at a final concentration of 5 \mu M, were added to endothelial monolayers and incubated for 30 minutes at 37\degree C. Nonadhered T lymphocytes were gently removed by washing with PBS (4X). The number of T cells adhered to the endothelial monolayer was determined by fluorescence on a plate reader (VictoR\textsuperscript{TM}; PerkinElmer) for 1 second at 485/535 nm. The data are presented as fluorescence units as a percentage of control.

**Statistical analyses**

Data generated from proliferation assays were analyzed by Proc mixed procedures of SAS using the individual animal as a random factor. Where appropriate, Tukey-Kramer multiple comparisons post-test was used to adjust for multiple comparisons. Data from adhesion assays was also subjected to orthogonal polynomial regression analysis to determine the response in T lymphocyte binding to increasing concentrations of hormone treatments. In all cases, statistical significance was considered when the P value was <0.05.
Chapter 4

Effect of progesterone on bovine T lymphocyte function

Introduction

Progesterone (P₄) is a steroid hormone produced by the corpus luteum (CL). Progesterone is important for regulating the duration of the estrous cycle, and is also responsible for the establishment and maintenance of pregnancy in mammals. In addition to its effects on reproductive tissues, P₄ modulates the function of the immune system. Whereas P₄ actions are classically mediated by the nuclear receptors (PGR) in reproductive and other tissues (Spencer and Bazer, 1995; Gava et al., 2004; Salvetti et al., 2007; Okumu et al., 2010), the expression of nuclear progesterone receptors in immune cells is restricted. In particular, PGR is not detectable bovine (Cannon et al., 2003) T lymphocytes and its expression in human T lymphocytes is still controversial (Szekeres-Bartho et al., 1990; Mansour et al., 1994; Chiu et al., 1996; Polger et al., 1999; Bamberger et al., 1999; Zhao et al., 2001; Ulziibat et al., 2006). The effects of P₄ in T lymphocytes cells are proposed to be mediated by membrane progesterone receptors (Schust et al., 1996; Ehring et al., 1998; Dosiou et al., 2008; Giannoni et al., 2011; Ndiaye et al., 2012). Membrane progesterone receptors (mPRs) mediate P₄ actions in other cell types, such as the oocyte (Zhu et al., 2003a; 2003b) and sperm (Jacob et al., 1998), and induce nongenomic responses, such as Ca²⁺ influx, in T lymphocytes (Ehring...
et al., 1998; Dosiou et al., 2008). Nongenomic responses occur very rapidly and are independent of gene transcription.

In addition to the observed rapid responses, \( P_4 \) actions modulate sustained responses that alter immune cell migration, proliferation and cytokine production. For instance, \( P_4 \) has been shown to have immunosuppressive effects (Stites et al., 1983; Cannon et al., 2003) and this property of \( P_4 \) is important for survival of the fetal allograft during pregnancy. Progesterone acts directly on T lymphocytes to induce progesterone-induced blocking factor (PIBF), which inhibits prostaglandin synthesis and acts on CD8\(^+\) cells to induce a suppressor phenotype (Szekeres-Bartho et al., 1983; Szekeres-Bartho et al., 1985; Szekeres-Bartho et al., 1989a; 1989b). Progesterone-induced blocking factor shifts the balance of cytokines toward a Th2 cytokine profile in both CD8\(^+\) and CD4\(^+\) splenocytes (Szekeres-Bartho and Wegmann, 1996). When both these cell types were activated in the presence of PIBF, they secreted IL3, IL4 and IL10. This effect of \( P_4 \) also occurs in antigen-specific CD4\(^+\) T cells and Th1 differentiated double-positive thymocytes (Piccinni et al., 1995; Miyaura and Iwata, 2002). In addition, \( P_4 \) has also been shown to induce a Th2 cytokine profile in splenocytes (Yates et al., 2010) and IL10 synthesis in mouse T regulatory and dendritic cells (Mao et al., 2010; Xu et al., 2011). In sheep, \( P_4 \) prevents xenograft and allograft rejection (Pauda et al., 2005; Majewski and Hansen, 2002), and when animals are chronically treated with \( P_4 \), it affects migration and (or) proliferation of T lymphocytes in the endometrium (Gottshall and Hansen, 1992). It is suggested that the action of \( P_4 \) on immune cells in the endometrium are a result of an indirect action (Wang et al., 1998). Progesterone stimulates the secretion of uterine milk protein (UTMP), which has immunosuppressive effects on T lymphocytes (Skopets and
Hansen, 1993; Liu and Hansen, 1995; Hansen, 1998). Progesterone also inhibits proliferation of human, bovine and ovine T lymphocytes (Stites et al., 1983; Low and Hansen, 1988; Pampori and Pandita, 2012), and blocking the progesterone receptor with RU486 does not inhibit the antiproliferative effects of progesterone (Monterroso and Hansen, 1993).

T lymphocytes are present in CL of cows (Lobel and Levy, 1968; Spanel-Borowski et al., 1997; Penny et al., 1999; Bauer et al., 2001; Townson et al., 2002; Davis and Pate, 2007). However, the knowledge of how the functions of resident T lymphocytes are modulated by the local environment is scarce. Previously, Cannon et al. (2003) demonstrated that progesterone from bovine luteal cells inhibited T lymphocyte proliferation, but the mechanism by which $P_4$ suppressed T cell proliferation is not known. Therefore, the objective of this study was to determine if T lymphocyte recruitment, cell cycle progression, cytokine gene expression, intracellular calcium, T cell receptor (TCR) and IL2 receptor signaling are regulated by $P_4$.

**Results**

**T lymphocyte recruitment**

T lymphocytes infiltrate the CL when $P_4$ production is at its peak and lymphocyte numbers are sustained throughout luteal regression. To test the hypothesis that $P_4$ is involved in the process of T lymphocyte recruitment, adhesion of T lymphocytes to luteal endothelial cells was determined after endothelial cells were pretreated with $P_4$. In
addition, endothelial cells were treated with either PBS or a combination of IL1-β and TNFα. T lymphocyte binding to endothelial cells varied as the concentration of P₄ increased (Fig.4-1). There was a cubic response (P<0.05; R²>0.9) in T lymphocyte binding to increasing concentrations of P₄ in both cytokine-treated and nontreated endothelial cells.

**TCR signaling and intracellular calcium**

To test the hypothesis that progesterone regulates TCR signaling, T lymphocytes were incubated with various concentrations of P₄ and the phosphorylation status of Zap70, a tyrosine kinase downstream of TCR signaling, was determined (Fig. 4-2). Progesterone at 5 and 10 µM decreased the phosphorylation status of Zap70 in the absence and presence of anti-CD3 antibodies (Fig. 4-2 A, B). In addition to Zap70 phosphorylation, P₄ induced a rapid increase in intracellular calcium in T lymphocytes. There was a significant increase in calcium influx in response to increasing concentrations of P₄ (P<0.05) as illustrated by a greater mean fluorescence intensity (Fig. 4-3 A, B). The response in calcium influx at 20 or 50 µM of P₄ was comparable to that induced by ionomycin (positive control).

**Cytokine profile, ILRα expression and cell cycle distribution**

Progesterone increased the steady-state concentration of IL2 mRNA in a dose-dependent manner (Fig 4-4). In addition, P₄ tended to increase IFNγ (P=0.06) at 0.16µM.
There was no effect of P₄ on either IL2Rα expression or the proportion of IL2Rα⁺ T lymphocytes induced by PMA and ionomycin (Fig. 4-5).

The effect of P₄ on cell cycle is shown in Figure 6. There was a dose-dependent effect of P₄ on the cell cycle at 48 hours. Progesterone (50 μM) significantly decreased the proportion of cells in the S-phase and increased the proportion of cells in G0/G1-phase (P<0.05).

**Discussion**

Lymphocytes are recruited into the CL during midcycle, when P₄ secretion is high, and their numbers are further elevated when progesterone synthesis declines (Bauer and Spanel-Borowski, 2001, Penny et al., 1999, Townson et al., 2002). However, it is not known if P₄ regulates the recruitment of T lymphocytes into the CL. In the present study, it was hypothesized that P₄ modulated T lymphocyte adhesion to endothelial cells. The results demonstrated that the receptivity of endothelial cells to lymphocytes is modulated by the concentration of P₄ and the effect is independent of cytokine treatment. Moderate concentrations of P₄ may be permissive whereas high concentrations may be inhibitory to lymphocyte binding to endothelial cells. Progesterone concentrations of 0.003-0.02 μM promoted adhesion of PBMCs to endothelial cells (Cid et al., 1994) whereas 0.01 μM of progesterone inhibited adhesiveness of leukocytes to endothelial cells (Simoncini et al., 2004). Additionally, P₄ promoted recruitment of eosinophils to the uterus (Asselin et al., 2001). It is proposed that high concentrations of P₄ may partially block lymphocyte recruitment by altering the expression of adhesion molecules. Progesterone inhibited the
expression of VCAM-1 and ICAM-1 in HUVECs (Otsuki et al., 2001; Piercy et al., 2002; Simoncini et al., 2004). The results support the hypothesis that moderate concentrations of P₄ may be permissive while high concentrations may be inhibitory to lymphocyte binding on endothelial cells.

When T lymphocytes are recruited into the CL, their functions may be modulated by P₄, as previously shown by Cannon et al. (2003); progesterone inhibited luteal cell-induced T lymphocyte proliferation, but the mechanism by which P₄ suppressed proliferation of T lymphocytes was not elucidated. In the current study, high concentrations of P₄ affected cell cycle progression by arresting cells in G0/G1-phase and inhibiting progression of cells to the S-phase. Also, P₄ dose-dependently decreased the phosphorylation of Zap70, but increased intracellular calcium. These effects of P₄ are likely to be mediated by mPRs because PGR is not detectable in T lymphocytes (Schust et al., 1996; Ehring et al., 1998; Dosiou et al., 2008; Giannoni et al., 2011; Ndiaye et al., 2012). Progesterone inhibits growth of several cell types via nongenomic actions. Sager et al. (2003) demonstrated that P₄ inhibited proliferation of uterine cervical cell lines, C-41, C33A and Me-180 via nongenomic mechanisms. Antiprogestins such as, mifepristone, onapristone and ZK-112993, that inhibit PGR did not block the antiproliferative effects of P₄. Also, blocking the P₄ receptor with RU486 did not inhibit the antiproliferative effects of P₄ in bovine and ovine lymphocytes (Monterroso and Hansen 1993). Additionally, incubation of C-41 cells with 31.8 μM P₄ arrested the cells in G0/G1 phase and induced apoptosis and necrosis (Bertelsen et al., 2004). This inhibitory response was different from that observed to be mediated by PGR (Skildum et
Therefore, P₄ may suppress T lymphocyte proliferation by inhibiting cell cycle progression.

No studies have implicated P₄ in regulating T cell receptor signaling. Intracellular calcium and activation of Zap70 are critical for TCR-mediated T lymphocyte activation. Tyrosine phosphorylation at position 319 is critical for TCR signaling, and its inhibition impairs T cell stimulation via the TCR. Zeta-associated protein kinase bridges extracellular TCR signals to downstream intracellular signaling proteins such as phospholipase C (PLCγ1) and Ras (Dosio et al., 2008; Williams et al., 1999). This augments the TCR signal by phosphorylation of other adaptor proteins such as Linker for T cell activation (LAT), which, in turn, activates PLC (Zhang et al., 1998; Laethem et al., 2001). Phospholipase C hydrolyses of PI (4, 5) P₂ to IP₃, which results in release of intracellular calcium from the endoplasmic reticulum (Abraham and Weiss, 2004). These events culminate in the activation of genes that regulate cytokine production in T cells. The progesterone-induced decrease in Zap70 activity shown in this study is similar to that observed when Jurkat cells were exposed to low concentrations (1 µM) of dexamethasone (Laethem et al., 2001), but opposite to those obtained at high concentrations of dexamethasone (10 µM; Barts et al., 2006). The results in this study support the hypothesis that P₄ may suppress very early events of TCR signaling.

Chronic effects of P₄ on T lymphocytes include changes in cytokine synthesis (Szekeres-Bartho and Wegmann 1996). It was hypothesized that P₄ would inhibit the Th1 cytokines, IL2 and IFNγ, and IL2Rα expression. The results showed that treatment of T lymphocytes with P₄ resulted in a dose-dependent increase in the steady-state concentration of IL2 mRNA. However, there was no effect on the expression of IL2Rα.
protein. Progesterone had no direct effect on IL2 or IFNγ in murine and human T lymphocytes (Mannel et al., 1990; Szekeres-Bartho et al., 1989). These two cytokines have adverse effects on ovarian function. Specifically, IL2 inhibited gonadotropin-induced P₄ production in bovine granulosal cells (Raja et al., 1995) and human granulosal-lutein cells (Wang et al., 1991). Also, IFNγ has been shown to have cytotoxic and inhibitory effects on gonadotropin-induced P₄ production (Fairchild and Pate, 1991; Benyo and Pate, 1992; Pate, 1995, rev.; Petroff et al., 2001). However, the increase in IL2 by P₄ may be beneficial in P₄-induced immune suppression. Interleukin 2 is essential for survival, activation, maintenance and suppressive activity of Tregs (de la Rosa et al., 2004; Yates et al., 2007).

In summary, the effects of P₄ on T lymphocyte function are dependent on the concentration. Progesterone rapidly increased intracellular Ca²⁺, but decreased the activity of Zap70. Chronically, P₄ influenced the adhesion of T lymphocytes to endothelial cells, inhibited the progression of T lymphocytes to the S-phase, and induced the expression of IL2, but not IL2Rα or IFNγ. Taken together, progesterone may regulate the recruitment and function of T lymphocytes within the CL.
Figure 4-1. The effect of $P_4$ on T lymphocyte adhesion to luteal endothelial cells. T lymphocytes were incubated with endothelial cells pretreated with progesterone and either vehicle (solid line) or IL-1$\beta$ and TNF$\alpha$ (50 ng/mL each; broken line). ‡ = (cubic response $P<0.05$; $n=3$).
**Figure 4.2**

The effect of $P_4$ on Zap70 phosphorylation. Representative western blots of three independent experiments showing P-ZAP70 in T lymphocytes cultured for 18 hours and then incubated with $P_4$ (0.5-20 μM) in the absence (A) and presence of anti-CD3 (B) for 5 minutes at 37°C. Total Zap70 was used as control (CTRL).
Figure 4-3. The effect of P₄ on intracellular calcium. A) Representative graph showing Ca²⁺ influx (ratio of Fluo-3/Fura red) in T lymphocytes treated with P₄ (0.5-20 μM) for a total of 180 seconds. The arrow indicates the point at which P₄ was added. B) Mean fluorescence intensity associated with the ratio of Fluo-3/Fura red. Means with different letters are significantly different (P<0.05; n=3). The progesterone vehicle, ethanol (0 μM P₄) served as a negative control while ionomycin was used as a positive control.
Figure 4-4. The effect of P4 cytokine gene expression. T lymphocytes were treated with P4 (0-1.59 µM) for 48 hours. A) Steady-state abundance of IL2R mRNA. B) Steady-state abundance of IFNγ mRNA. Vehicle (ethanol) served as a negative control. Different letters indicate significant differences (P<0.05; A: n=4, B: n=3). £ indicates (P=0.06).
Figure 4-5. The effect of P4 on PMA-ionomycin induced IL2Rα expression. Representative dot plots from three independent experiments showing IL2Rα+ T lymphocytes after treatment with P4 (1-20 μM) for 72 hours in the presence of PMA-ionomycin. Vehicle (Ethanol) served as a negative control.
Figure 4-6. The effect of P₄ on cell cycle. Cell cycle distribution in T lymphocytes treated with P₄ (0.05-50 μM) in the presence of PMA-ionomycin for 48 hours (n=4). The asterisk (*) indicates significant differences from cells treated with PMA-ionomycin alone (P<0.05).
Materials and methods

Reagents and antibodies

Reagents used for western blot analyses were: CelLytic Mammalian Tissue Lysis/Extraction reagent (Sigma), Restore™ PLUS western blot stripping buffer (Thermo Scientific), SuperSignal West Femto Chemiluminescent Substrate and BCA™ protein assay kit (Thermo Scientific), phosphatase inhibitor cocktail 2 (Sigma). Antibodies used for western analyses were: rabbit mAb #3165-Zap70, rabbit mAb #2717-P-Zap70 (tyr319)/Syk (Tyr 352) (Cell Signaling Technologies, Inc.) and anti-rabbit IgG-horseradish peroxidase conjugate (#NA9340V, GE Healthcare). Antibodies for flow cytometry were: IL2Rα (CACT116A; VMRD), mouse IgG1:PE (STAR32PE; AbD Serotec). Reagents used for intracellular calcium analysis were: progesterone (P₄; Sigma), Fluo-3 and Fura-red (Molecular probes, Invitrogen). Reagents used for cell cycle analysis were: paraformaldehyde (PFA; Sigma), RNaseA (Sigma) and propidium iodide (PI; Sigma). Reagents used for the cell adhesion assay were: Vybrant™ cell adhesion assay kit (Molecular probes, Invitrogen).

Cell cycle analysis

T lymphocytes (1x10⁶) were cultured in AIMV in 24-well plates and treated with PMA (10 ng/mL) and ionomycin (500 ng/mL) in the presence of P₄ at 0.05, 0.5, 5, 10, 20
and 50 μM for 12 and 48 hours at 37°C and 5% CO₂. After culture, the cells were transferred to 1.5 mL tubes and centrifuged at 200xg for 8 minutes. The supernatant was decanted and the pellet was washed in 1 mL of cold 1XPBS, at 200xg at 4°C. After removing the supernatant, the pellet was resuspended in 300 μL of 1XPBS and mixed once on a voltex machine. Ice cold ethanol was then added to the cell suspension drop-wise while mixing on a voltex machine. The cells were then incubated for 1 hour at 4°C. After the incubation, the cells were centrifuged at 200xg for 10 minutes to remove the ethanol. The supernatant was decanted and the pellet was washed with 1 mL of 1XPBS at 200xg. The pellet was resuspended in 250 μL of 0.1% triton-X-PBS containing 0.3 mg/mL RNaseA and 10 μg/mL propidium iodide (PI). The cells were mixed and then incubated for 1 hour at 37°C. The cells were then transferred to a 96-well plate and fluorescence read using a flow cytometer (Guava EasyCyte).

**Intracellular calcium**

T lymphocytes (20 x10⁶/mL) were labeled with 8 μg/mL of Fluo-3 and 10 μg/mL of Fura-Red in Ringer’s solution (160 mM NaCl, 4.5mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, pH 7.4). T lymphocytes were then incubated for 25 minutes at 37°C and centrifuged at 250xg for 6 minutes to remove the excess dye. The cells were then resuspended in 1mL of Ringer’s solution and aliquoted into tubes to a final concentration of 2.85 x10⁶ cells/mL. Cells were treated with 0, 0.5, 5, 10, 20 and 50 μM of P₄. Ionomycin (2 μg/ml) was used as a positive control and 0.3% ethanol in Ringers
solution was used as negative control. The cells were analyzed on a Coulter-XL-MCL flow cytometer for 180 seconds immediately following the addition of P₄.

**IL2 receptor expression**

Purified T lymphocytes were cultured and treated with increasing concentrations of P₄ (1.6, 5 and 20 μM) with or without PMA-ionomycin (10 ng/mL-1μg/mL) for 72 hours at 37°C. Cells treated with or without vehicle were used as controls. T lymphocytes were then harvested, washed with PBS containing PBS-EDTA (PBS-EDTA) at 250xg at 4°C and the pellet was resuspended in 200 μL of PBS-EDTA. Anti-IL2Rα primary antibody (1 μg) was added to the cells and then incubated on ice for 30 minutes. Harvested cells were washed with PBS-EDTA before staining. After incubation of the primary antibody, the cells were washed 3X at 250xg each time and the resulting pellet was resuspended in 200 μL of PBS-EDTA containing 10% NGS. Anti-mouse IgG1:PE secondary antibody was added and the cells incubated for 30 minutes at 4°C. The cells were again washed three times with PBS-EDTA containing 10% NGS and resuspended in PBS-EDTA. Expression of IL2Rα was analyzed by flow cytometry (Guava EasyCyte).

**Cytokine gene expression**

To determine the effect of progesterone on IL2 and IFNγ cytokine gene expression, 2 x10⁶ T lymphocytes were cultured in AIMV medium in 24-well plates and treated with P₄ (0.03, 0.16 and 1.59 μM) for 48 hours at 37°C. At the end of the culture
period, medium was removed and the cells were washed with PBS. Total RNA was extracted, the concentration determined, and the steady-state abundance of IFNγ and IL-2 determined using qPCR following conditions as described previously. The primers used were: IFNγ (forward: 5’-ACTCCGGCCTAACTCTCTCCC-3’; reverse: 5’-AGGCCCACCCTTAGCTACAT-3’), IL2 (forward-5’-GTGAAGTCATTGCTGCTGGA-3’; reverse-5’-GGTTCAGGTTTTTGCTTGGA-3’).

The primers were designed based on the following Genbank accession numbers; IFNγ (NM_174086), IL2 (NM_180997). Each primer was validated following procedures described previously in Chapter 2. Also, the controls used are similar to those described in Chapter 2.

**Zap70 phosphorylation**

Two experiments were conducted to determine the effect of P₄ on Zap70 phosphorylation. In the first experiment, T lymphocytes (2x10⁶) were cultured for 18 hours in serum free RPMI medium, then treated with P₄ (5, 10 and 20 µM) for 5 minutes.

In the second experiment, 24-well culture plates were coated with 10 µg/mL of anti-CD3 (MM1A; VMRD) antibody overnight at 4°C. The plates were washed twice with RPMI medium containing 10% fetal calf serum (FCS) to remove unbound antibody before T lymphocytes were added. T lymphocytes treated with P₄ at 0.5, 5, 10 and 20 µM for 5 minutes were added to the wells and incubated for another 5 minutes. The cells were immediately removed at the end of the incubation and placed on ice. Protein was extracted using CelLytic™ reagent and the cell lysates were stored at -80°C. Protein
concentration was determined using the BCA™ protein assay kit and Zap70 and P-Zap70 (Tyr 319) were visualized by western blot analysis. Untreated cells served as a control.

**Western blot analysis**

Proteins were separated under reducing conditions on a 10% SDS-PAGE gel for 2 hours at 120V before they were transferred onto polyvinyl difluoride (PVDF) membranes in a wet transfer system for 2 hours at 400 mA. After protein transfer, the membrane was immediately placed in 5% nonfat milk (NFM) and incubated for 1 hour at room temperature to block nonspecific binding. To detect phospho-Zap70 (P-Zap70), the membrane was incubated with rabbit anti-human P-Zap70 primary antibody diluted in 2% NFM (1:1000) overnight at 4°C. The membrane was washed 3X (10 minute each time) with Tris-buffered saline and 0.05% Tween 20 (TBST) and further incubated with a secondary antibody (anti-rabbit IgG1-HRP [1:10000]) for 2 hours at room temperature. The membrane was washed 3X (10 minutes each time) before adding the HRP substrate (SuperSignal West Femto Chemiluminescent Substrate). After incubating for 5 minutes, excess substrate was removed and images were acquired using a CHEMI DOC XRS imager (Bio-Rad). After detection of P-Zap70, the membrane was stripped using Restore™ PLUS western blot stripping buffer for 10 minutes, blocked with 5% NFM and probed with rabbit anti-Zap70 primary antibody (1:1000) overnight at 4°C. Following washing with TBST, the membrane was incubated with anti-rabbit-HRP secondary antibody in 2% NFM. Images were acquired as described above.
**T lymphocyte adhesion assay**

Endothelial cells isolated from the CL were seeded (1x10^6 cells/well) in 96-well plates in EBM2 medium. Once monolayers were established, they were treated with P4 (0.5, 1, 5, 10 and 20 µM), followed by exposure to TNFα (50 ng/mL), IL-1β (50 ng/mL) or PBS for the last 6 hours of a 21-hour incubation period. Vehicle control wells were included for each treatment. The T lymphocyte adhesion assay was conducted under static conditions. T lymphocytes (5x10^5) in 100 µL of RPMI, labeled with Calcein-AM (Vybrant™ cell adhesion assay kit) at a final concentration of 5 µM were added to endothelial monolayers and incubated for 30 minutes at 37°C. Nonadhered T lymphocytes were gently removed by washing with PBS (4X). The number of T lymphocytes adhered to the endothelial monolayer was determined by fluorescence on a plate reader (VictoR™; PerkinElmer) for 1 second at 485/535 nm. The data are presented as fluorescence units.

**Statistical analyses**

Data were analyzed by Proc Mixed procedures of SAS (SAS Institute Inc., Cary, NC, USA, 2011) using the individual animal as a random factor. Where appropriate, Tukey-Kramer multiple comparison post-test was used to separate means. Data generated from qPCR was subjected to analysis of covariance, using the housekeeping gene as a covariate. Data from adhesion assays was also subjected to orthogonal polynomial regression analysis to determine the response in T lymphocyte binding to increasing
concentrations of hormone treatments. In all cases, statistical significance was considered when the P value was <0.05.
Chapter 5

Midcycle luteal cells induce an antiinflammatory phenotype of gamma delta T lymphocytes

Introduction

Understanding the mechanisms that regulate luteal function may facilitate the development of new strategies to improve reproductive efficiency in dairy cows. T lymphocytes are present in the CL prior to the onset of luteolysis (Spanel-Borowski et al., 1997; Lawler et al., 1999; Penny et al., 1999; Townson et al., 2002; Poole and Pate, 2012) and remain elevated during luteal regression (Penny et al., 1999; Bauer et al., 2001). The increase in immune cells and cytokines that inhibit steroidogenesis during luteal regression (Nothnick and Pate, 1990; Fairchild and Pate, 1991; Benyo and Pate, 1992; Townson and Pate, 1996) supports the argument that immune cells potentiate luteolysis. In contrast, the presence of T lymphocytes in the CL prior to the onset of luteal regression (Penny et al., 1999) indicates that these cells may support normal luteal function. In vitro studies have shown that T lymphocyte-derived cytokines, IL4 and IL10 augment progesterone production in luteal cells (Hughes et al., 1990; Hughes et al., 1991; Emi et al., 1991; Hashii et al., 1998) while IFNγ and TNFα inhibit progesterone production (Fairchild and Pate, 1991; Benyo and Pate, 1992; Fukuoka et al., 1992, Wang et al., 1992). These observations indicate that immune cells may be involved in normal luteal function as well as regression and that their functions may be dependent on the
stage of the CL. Because different subsets of T lymphocytes exist in the CL, there is need
to identify the subsets involved in luteal function and regression. Recently, Davis and
Pate (2007) demonstrated that coculture of T lymphocytes with bovine luteal cells
induced IL10 and IFNγ production and proliferation of gamma delta (γδ)⁺ T
lymphocytes, but it is not known if both IL10 and IFNγ are synthesized by the
proliferating γδ⁺ T lymphocytes.

T lymphocyte subsets present in the bovine CL include helper CD4⁺, cytotoxic
(CD8⁺) and γδ⁺ T lymphocytes (Penny et al., 1999; Townson et al., 2002; Davis and Pate,
2007; Poole and Pate, 2012). Gamma delta T lymphocytes represent 20-70% of
circulating T lymphocytes in young ruminants (Mackay et al., 1989) and are also present
in the CL of cows (Davis and Pate, 2007, Poole and Pate, 2012). In cattle, γδ⁺ T
lymphocytes are generally identified by the expression of workshop cluster 1 (WC1),
which belongs to the scavenger receptor cystein-rich family of proteins (Mackay et al.,
Phenotypic and related functional characteristics categorize γδ T lymphocytes into two
major populations, that is, CD2⁻CD8⁻γδTCR⁺WC1⁺ and CD2⁺CD8⁺γδTCR⁺WC1⁻
(Clevers, et al., 1990; Machugh et al., 1997). The former are more abundant in circulation
while the latter localize in tissues such as the spleen (Machugh et al., 1997; Wilson et al.,
1999). Subpopulations within the γδ⁺WC1⁺ have been also defined based on the
molecular diversity of the WC1 protein (O’keeffe et al., 1994; Herzig and Baldwin,
2009). Thus, specific antibodies that recognize WC1 isoforms, WC1.1, 1.2 and 1.3 have
been used to delineate the functional properties of these subsets (Wijngaard et al., 1994;
Rodgers et al., 2005a; 2005b). The diversity of γδ⁺ T lymphocytes based on the
expression of WC1, or the different WC1 isoforms, and localization to specific sites, reflects the functional plasticity of these cells. Gene expression profile and functional studies have demonstrated that both of these subsets have effector as well as regulatory roles (Baldwin et al., 2000; Pollock and Welsh, 2002; Hoek et al., 2009). Gamma delta T cells of bovine and other species synthesize both Th2 (IL4 and 10) and Th1 cytokines (TNFα and IFNγ) that are important in adaptive immunity (Ferrick et al., 1995; Wen et al., 1998; Baldwin et al., 2000; Baldwin et al., 2002; Tanaka et al., 2008; Johnson et al., 2008; Hoek et al., 2009), but also possess characteristics of innate immunity (Collins et al., 1998; Hedges et al., 2005). The γδ+ T lymphocytes present in circulation and at mucosal surfaces may be very important in responding to foreign antigens (Ferrick et al., 1995 Kennedy et al., 2002), while those present in peripheral nonlymphoid tissues may be involved in tissue homeostasis. In the CL, the majority of the resident γδ+ T lymphocytes are of γδ+WC1− phenotype and less than 10% constitute γδ+WC1+ (Poole and Pate, 2012). The role of these cells in luteal function is not clear, but their function may dependent on the microenvironment present within the CL at different physiological stages (Poole and Pate, 2012). In the present study, it was proposed that luteal cells from midcycle CL would induce hyporesponsiveness and an antiinflammatory cytokine profile in γδ+ T lymphocytes, and stimulate expansion of γδ+ T lymphocyte subset with anti-inflammatory phenotype.
Results

Proportions of WC1 subsets of $\gamma\delta^+$ T lymphocytes in the CL and peripheral blood

Gamma delta T lymphocytes may be divided into those that express WC1 and those that do not. Those that express WC1 include WC1.1+, WC1.2+ and WC1.1+/1.2+, among additional WC molecules that have only recently been identified. All three populations of $\gamma\delta^+$WC1+ cells were found in both peripheral blood and the CL (Fig 5-1A). In all cases the WC1.1+/1.2+ population represented the smallest subset (Fig. 5-1A, B). The proportions of WC1.1+ and WC1.2+ in the CL were similar and they were both numerically higher than WC1.1+/1.2+.

When $\gamma\delta^+$ T lymphocytes isolated from blood were cultured with luteal cells isolated from midcycle CL, there was no change in the proportion of $\gamma\delta^+$WC1.1+ and $\gamma\delta^+$WC1.2+ T lymphocytes, but there was a 63% reduction in WC1.1+/WC1.2+ (Fig. 5-2 B). The proportion of $\gamma\delta^+$ lymphocytes that expressed CD8α+ or CD8α+WC1+ was reduced 41 and 53%, respectively, by exposure to luteal cells (Fig. 5-2 B). In addition to the phenotypic changes, midcycle luteal cells also induced greater proliferation of a subset of $\gamma\delta^+$ T lymphocytes that expressed few or none of the WC1 molecules (Fig. 5-3). In comparison to midcycle luteal cells, cells isolated from CL exposed to PGF$_{2\alpha}$ for 8 hours induced greater proliferation of $\gamma\delta^+$WC1+ compared to $\gamma\delta^+$ WC1– T lymphocytes (Fig. 5-4A, B). It was observed that there was a reduction in the proportion of $\gamma\delta^+$CD8α+ T lymphocytes independent of the state of luteal cells, but this reduction was not accounted for by proliferation (Fig. 5-5). Nonetheless, luteal cells exposed to PGF$_{2\alpha}$ induced a greater proliferative response.
To determine if luteal cells also induced specific changes in the cytokine profile of γδ+ and CD4+ T lymphocytes, Th1 and Th2 cytokine profiles were analyzed in T lymphocytes after exposure to luteal cells isolated from midcycle or regressing CL (Fig. 5-6 and 5-7). Exposure of γδ+ T lymphocytes to midcycle luteal cells increased the proportion of γδ+IL10+ and γδ+GATA-3+ cells (P<0.05) and GATA-3+ (P<0.05), but decreased the proportion of γδ+IFNγ+ cells (P<0.05). Alternatively, there was an increase in both CD4+IL10+ and CD4+IFNγ+ cells in response to midcycle luteal cells (P<0.05), but changes in associated transcription factors were not apparent. No changes were observed in Th1 and Th2 cytokines and associated transcription factors in γδ+ T lymphocytes exposed to regressing luteal cells. Similarly, there was no effect on CD4 T cells except a decline in TNFα (P<0.05).

Analysis of IL10-producing subsets of γδ+ T lymphocytes revealed that γδ+WC1+ and γδ+WC1− cells were capable of producing IL10 (Fig. 5-8A). There was, however, no significant effect of luteal cells on IL10 in γδ+ WC1− T lymphocytes (P>0.05). Luteal cells induced a greater response in IL10 in WC1+ compared to WC1− cells (P<0.05). The increase in IL10 in γδ+ T lymphocytes in response to luteal cells was inhibited when luteal cells were treated with aminoglutethimide, a cytochrome P450scc inhibitor (Fig. 5-8B). Lastly, the hypothesis that luteal cells from midcycle CL induced hyporesponsiveness in γδ+ T lymphocytes was tested. Gamma delta T lymphocytes exposed to luteal cells responded to ConA stimulation as well as those that were not exposed to luteal cells (P>0.05). A similar response was observed in CD4+ and CD8+ cells.
**Discussion**

In the CL, it is proposed that $\gamma\delta^+$ T lymphocytes may be involved in tissue homeostasis. Therefore, the current study identified functional changes induced in $\gamma\delta^+$ T lymphocytes exposed to luteal cells from either functional or regressing CL (after administration of PGF$_{2\alpha}$ in vivo). First, the subsets of $\gamma\delta^+$ T lymphocytes expressing different WC1 isoforms were identified in the peripheral circulation and within the CL. Similar to reports by others (Wijngaard et al., 1994; Wyatt et al., 1994; Machugh et al., 1997; Blumerman et al., 2006), the present study showed that the majority of $\gamma\delta^+$ T lymphocytes in peripheral blood are WC1$^+$ while those in tissues are WC1$^-$ and that a minor population of $\gamma\delta^+$WC1$^+$ T lymphocytes co-express both WC1.1 and 1.2 isoforms. The expression of different isoforms of WC1 molecules increases the functional diversity of $\gamma\delta^+$ T lymphocytes.

It has been previously shown that $\gamma\delta^+$ T lymphocytes are present in the CL and that they proliferate in response to luteal cells in vitro (Davis and Pate, 2007). In the present study, the question of whether luteal cells induce expansion of a specific subset of $\gamma\delta^+$ T lymphocytes was addressed. It was observed that coculture of $\gamma\delta^+$ T lymphocytes with midcycle luteal cells tended to reduce the proportions of $\gamma\delta^+$CD8$\alpha^+$, $\gamma\delta^+$ WC1$^+$ and $\gamma\delta^+$WC1.1$^+$/1.2$^+$ T lymphocytes, but did not alter the proportions of either $\gamma\delta^+$WC1.1$^+$ or $\gamma\delta^+$WC1.2$^+$ T lymphocytes. The $\gamma\delta^+$WC1.1$^+$/1.2$^+$ T lymphocytes represent a minor population that possesses unique biochemical characteristics relative to WC1.1 and WC1.2 (Wijngaard et al., 1994; Rogers et al., 2006). It is not clear if this subset represents a functionally unique population. The reduction in $\gamma\delta^+$CD8$\alpha^+$ T lymphocytes was consistently observed, even when $\gamma\delta^+$ T lymphocytes were exposed to regressing
luteal cells (Fig.6). Rather than the depletion of the $\gamma\delta^+$CD8$\alpha^+$ population, it is proposed that luteal cells specifically downregulate the expression of CD8 molecules. The CD8$^+$ coreceptor has been reported to be expressed on WC1$^-$, but not WC1$^+$ (Clevers, et al., 1990; Machugh et al., 1997). In contrast, the present study showed that CD8 is expressed on a small population of $\gamma\delta^+$WC1$^+$ T lymphocytes. Additional experiments are needed to verify the existence of this population of cells.

In the present study, the proliferative response of $\gamma\delta^+$WC1$^-$ T lymphocytes was higher in the presence of luteal cells from midcycle and lower in the presence of luteal cells from regressing CL. In contrast, the response of $\gamma\delta^+$WC1$^+$ T lymphocytes was higher in the presence of luteal cells from regressing compared to midcycle CL. Although there was no difference in the proportion of WC1$^+$ cells between midcycle and regressing CL (Poole and Pate, 2012), it is possible that the WC1$^+$ population increases as luteal regression progresses. The data in the present study indicate that WC1$^+$ and WC1$^-$ $\gamma\delta$ T cells could respond to different signals. While the signals that stimulate WC1$^-$ cells are present in functional CL, those that stimulate WC1$^+$ cells may be triggered by PGF$_{2\alpha}$ and become available during luteal regression. It is also possible that the environment provided by luteal cells from a functional CL is inhibitory or insufficient to promote expansion of any of the WC1 subsets, WC1.1, 1.2 or 1.3. Blumerman et al. (2006) demonstrated that $\gamma\delta^+$WC1$^+$ and $\gamma\delta^+$WC1$^-$ T lymphocytes use different combinations of the TCR and therefore could respond to different signals and consequently perform different functions in the CL. Gene expression analysis and functional studies suggest that in cattle, $\gamma\delta^+$ T lymphocytes that express WC1 are inflammatory, whereas those that do not express WC1 are regulatory (Kennedy et al., 2002; Hedges et al., 2003; Meissner
et al., 2003). Therefore, midcycle luteal cells support expansion of γδ+ T lymphocytes with a regulatory phenotype while exposure of luteal cells to PGF$_{2\alpha}$ supports the expansion of γδ+ T lymphocytes with an inflammatory phenotype. Although the γδ+WC1+ are not induced to proliferate by a functional CL, they are stimulated to synthesize IL10. Gamma delta T lymphocytes that express the WC1 coreceptor express regulatory cytokines such as IL10 and TGFβ and have suppressive functions in vitro (Rodgers et al., 2005; Hoek et al., 2009). Interleukin 10 is a regulatory cytokine that inhibits proinflammatory cytokine synthesis (Moore et al., 2001, rev.). The ability to express regulatory and inflammatory cytokines shows that γδ+WC1+ T lymphocytes are functionally pleiotropic. This plasticity may be a function of the local environment in which these cells are stimulated. The majority of the γδ+WC1+ cells are found in the peripheral circulation, but a small proportion of these cells is also found in tissue such as the CL (Poole and Pate, 2012). These cells may play a regulatory role in the CL through the synthesis of IL10. In the present study, both γδ+WC1− and γδ+ WC1+ T lymphocytes expressed IL10. The proportion of WC1+IL10+, but not WC1 IL10+ cells increased in response to midcycle luteal cells. The synthesis of IL10 in γδ+ T lymphocytes was found to be dependent on steroid production. Because progesterone is the principal steroid secreted by luteal cells, progesterone may be responsible for IL10 synthesis. Progesterone has been shown to induce a Th2 cytokine profile in splenocytes (Yates et al., 2010), T lymphocytes (Miayura and Iwata, 2002), T regulatory cells (Mao et al., 2010) and dendritic cells (Xu et al., 2011). In addition, progesterone, through the induction of progesterone-induced blocking factor (PIBF), induces IL10 synthesis and other Th2 associated cytokines in murine γδ+ T lymphocytes (Szekeres-Bartho and Wegmann,
In the bovine CL, IL10 steady-state mRNA is higher at midcycle (peak of progesterone production) compared to regressing CL (Poole and Pate, unpublished). In addition, Sanders et al. (2008) have shown that IL10 and IL4 cytokines increase in the peripheral circulation in midcycle compared to luteal regression. Both IL10 and IL4 increase progesterone production from luteal cells (Hashii et al., 1998). Therefore, progesterone present in midcycle CL may induce IL10 in resident γδ\(^+\) and CD4\(^+\) T lymphocytes, which may be involved in regulating the activities of macrophage/monocytes and αβ\(^+\) T lymphocytes that are recruited to the CL prior to the onset of luteal regression and may also enhance luteal steroidogenesis.

Luteal cells may induce expansion of γδ\(^+\) or αβ T lymphocytes, but limit the response to further stimulation. This hypothesis was tested in the present study by stimulating CD4\(^+\), CD8\(^+\) and γδ\(^+\) with ConA after the T lymphocytes were cocultured with midcycle luteal cells. The response of either γδ\(^+\) or αβ\(^+\) T lymphocytes to ConA was not affected by prior exposure to luteal cells. This implied that the interactions that occur between luteal cells and T lymphocytes in a functional CL do not induce anergy.

In addition to the effects on proliferation, γδ\(^+\) T lymphocyte effector functions were regulated by luteal cells. It was hypothesized that luteal cells from a functional CL would induce a Th2 cytokine profile and hyporesponsiveness of γδ\(^+\) T lymphocytes. Luteal cells from midcycle induced IL10 and the associated transcription factor GATA-3, but reduced IFNγ without a reduction in T-bet in γδ\(^+\) T lymphocytes. Whereas these results are consistent with a Th2 cytokine profile, midcycle luteal cells also induced IFNγ and IL10 synthesis in CD4\(^+\) T lymphocytes without any changes in T-bet\(^+\) or GATA-3\(^+\) cells. The induction of IL10 in both γδ\(^+\) and CD4\(^+\) T lymphocytes may be intended to
balance IFNγ from CD4⁺ T lymphocytes. These results are consistent with those of Davis and Pate (2007), who reported an increase in both IFNγ and IL10 when mixed T lymphocytes were cocultured with luteal cells. The lack of induction of all cytokines by luteal cells from regressing CL was unexpected. However, CD4⁺ T lymphocytes synthesized higher amounts of IFNγ and TNFα relative to γδ⁺ T lymphocytes in response to stimulation with PMA and ionomycin. These results reveal the complex nature and specificity of interactions between luteal cells and T lymphocytes. Each of these cell types may be responding to specific signals present in the CL. The different signals may help create a balance in the cytokine profile rather than a shift to an environment of predominantly Th1 or Th2 cytokines.

**Conclusion**

The environment within a functional CL does not inactivate resident T lymphocytes, but rather maintains quiescence by inducing the expansion of γδ⁺ T lymphocytes with an antiinflammatory phenotype and by differentially regulating cytokine synthesis from γδ⁺ and αβ⁺ T lymphocytes.
Figure 5-1. Abundance of γδ+WC1.1+, γδ+WC1.2+, γδ+WC1.1/1.2+ population in blood and CL. A) Representative dot plots showing the proportion of γδ+WC1.1+, γδ+WC1.2+, γδ+WC1.1/1.2+ in blood (PTC) and CL (RTC). B) The proportion of γδ+WC1.1+, γδ+WC1.2+, γδ+WC1.1/1.2+ in the CL (n=4).
Figure 5-2

(A) Representative dot plots showing the proportion of γδ+ WC1.1+, γδ+ WC1.2+ and γδ+ CD8+ when exposed to luteal cells (GDLC) or cultured alone (GD). B) The proportion of γδ+ WC1.1+, γδ+ WC1.2+ and γδ+ WC1+ and γδ+ CD8+ when exposed to luteal cells (n=3).

Figure 5-2. Effect of midcycle luteal cells on γδ T lymphocyte functional phenotypes. A) Representative dot plots showing the proportion of γδ+WC1.1+, γδ+WC1.2+ and γδ+WC1+ and γδ+CD8+ when exposed to luteal cells (GDLC) or cultured alone (GD). B) The proportion of γδ+WC1.1+, γδ+WC1.2+ and γδ+WC1+ and γδ+CD8+ when exposed to luteal cells (n=3).
Figure 5-3. Effect of midcycle luteal cells on proliferation $\gamma \delta^+ T$ lymphocytes. Representative dot plots of experiments showing the effect of exposure of $\gamma \delta^+ T$ lymphocytes to luteal cells on the proliferation of WC1.1$^+$ (n=5), WC1.2$^+$ (n=5) and WC1.3$^+$ subsets (n=3).
Figure 5-4. Comparison of proliferation of $\gamma\delta^{+}$WC$1^+\gamma\delta^{+}$WC$1^-$ T lymphocytes induced by luteal cells isolated from either midcycle or regressing CL. A) Representative dot plots showing the effect of exposure of $\gamma\delta^+$ T lymphocytes to luteal cells on the proliferation of WC$1^+$, WC$1^-$ subsets. B) Graph showing the percentage of $\gamma\delta^+WC1^+$, $\gamma\delta^+WC1^-$ T lymphocytes (n=3). Different letters indicate significant differences (P<0.05).
Figure 5-5. Comparison of the proliferation of γδ+CD8+ T lymphocytes induced by luteal cells isolated from either midcycle or regressing CL. The dot plots are representative of three independent experiments.
Figure 5-6. Effect of midcycle luteal cells on cytokine synthesis and transcription factor expression in T lymphocytes. CD4⁺ or γδ⁺ cultured in the presence or absence of luteal cells and subsequently stimulated with PMA-Io (n=3-4). The top panel shows the proportion of cells positive for Th2 cytokines (IL10, IL4) and the associated transcription factor GATA-3. The bottom panel shows the proportion of cells positive for Th1 cytokines (TNFα, IFNγ) and the associated transcription factor T-bet. Different letters indicate significant differences (P<0.05).
Figure 5-7. Effect of regressing luteal cells on cytokine synthesis and transcription factor expression T lymphocytes. CD4⁺ or γδ⁺ cultured in the presence or absence of luteal cells and subsequently stimulated with PMA-Io (n=3-4). The top panel shows the proportion of cells positive for Th2 cytokines (IL10, IL4) and the associated transcription factor GATA-3. The bottom panel shows the proportion of cells positive for Th1 cytokines (TNFα, IFNγ) and the associated transcription factor T-bet. Different letters indicate significant differences (P<0.05).
Figure 5-8. IL10 synthesis in γδ+ T lymphocytes. A) Proportion of IL10+ among WC1+ and WC1- (n=3), WC1.1+, WC1.2+ and WC1.3+ γδ+ T lymphocytes (n=5). B) The proportion of IL10+ cells and associated fluorescence intensity (bottom graph) induced by aminoglutethimide (AG) treated luteal cells (n=4).
Figure 5-9. Proliferative response of $\gamma^\delta^+$, CD4$^+$ and CD8$^+$ in response to ConA after exposure to luteal cells (n=4). C= T lymphocytes cultured alone, LC= Luteal cells.
Materials and methods

Reagents and antibodies

Reagents used for cell culture were: Brefeldin A (BFA) and Concanavalin A (Sigma). Antibodies used for flow cytometric analysis were: CD4 (ILA-11; VMRD), CD8α (CACT80C; VMRD), CD8α (BAQ11A; VMRD), δTCR-N12 (CACT61A; VMRD), WC1 (IL-A29; VMRD), WC1.1 (BAG25A; VMRD), WC1.2 (CACTB32A; VMRD), WC1.3 (CACT21A; VMRD), IL10 (MCA111B; AbD Serotech), IFNg (MCA1783; AbD Serotech), IL4 (MCA1963; AbD Serotech), TNFa:Biotin (MCA2335B; AbD Serotech), IgG1 negative control (MCA928; AbD Serotech), IgM negative control (MCA2063; AbD Serotech), IgG1-FITC (STAR132F; AbD Serotech), IgG1-RPE (SATR132PE; AbD Serotech), IgM-FITC (102002; AbD Serotech), IgM-RPE (102009; AbD Serotech), Streptavidin FITC (STAR2B; AbD Serotech), anti-Rabbit IgG FITC (STAR34; AbD Serotech). Other antibodies were: Anti-human GATA-3 (Sigma) and anti-human T-bet (clone 16H4L5; Invitrogen) and rabbit IgG negative control (Thermo Scientific).

Cell preparation and phenotype analysis

Purified γδ+ T lymphocytes were either cultured alone or cocultured with luteal cells for 72 hours. Gamma delta T lymphocytes were harvested and dual labeled with
antibodies against WC1.1 and WC1.2 or WC1 and CD8α primary antibodies in 200 μL of PBS-EDTA and incubated on ice for 30 minutes. The cells were washed 3X with PBS-EDTA containing 5% NGS and subsequently incubated with appropriate secondary antibodies for 30 minutes on ice. The cells were again washed 3X with PBS-EDTA containing 5% NGS and resuspended in PBS-EDTA to a concentration of 500 cells/μL and analyzed by flow cytometry. Unlabeled cells, appropriate isotype, and positive controls were used for gating.

**Cell preparation and intracellular staining**

Gamma delta or CD4+ T lymphocytes were cultured alone or exposed to luteal cells from either functional (days 10-12) or regressing CL (8 hours post PGF2α administration) to determine if luteal cells differentially regulated cytokine synthesis. In a parallel experiment luteal cells from functional CL were treated with aminoglutethimide (50 μg/mL) before γδ+ T lymphocytes were added to determine if IL10 production in γδ+ T lymphocytes was regulated by progesterone synthesized by luteal cells in culture. After 72 hours of coculture, the T lymphocytes were harvested and stimulated with ConA (40 μg/mL) overnight, followed by PMA (10 ng/mL) and ionomycin (1 μg/mL) for 6 hours. Brefeldin A (10 μg/mL) was added for the last 4 hours. The cells were fixed in 4% paraformaldehyde for 20 minutes at RT, washed and resuspended in permeabilization buffer (0.1 % saponin, 0.1 % sodium azide and 5% goat serum in PBS) for 15 minutes at RT. Primary antibodies to IL4, IL10, IFNγ, TNFα, GATA-3 (GATA-binding protein 3), T-bet (T-box expressed in T cells) and appropriate isotype controls were added to tubes
containing cells and incubated on ice for 1 hour. After incubation, cells were washed three times at 300xg with wash buffer (0.1% sodium azide and 5% goat serum in PBS) and the cells were again incubated with appropriate secondary antibodies on ice for 30 minutes. After incubation, the cells were washed three times at 300xg and resuspended in PBS and analyzed by flow cytometry.

**Proliferation assay**

Two experiments were conducted to determine the effect of luteal cells isolated from a functional CL on responses in \( \gamma \delta^+ \) T lymphocytes. First, an experiment was designed to test the hypothesis that luteal cells from midcycle CL induced hypo-responsiveness in \( \gamma \delta^+ \) T lymphocytes. In this experiment, \( \gamma \delta^+ \) T lymphocytes (5x10^5/well) were either cultured alone or with luteal cells (1.5x10^5/well) in 24-well plates for 72 hours in AIMV medium supplemented with ITS. \( \gamma \delta^+ \) T lymphocytes were then harvested, labeled with CFSE (1.75 µM) in AIMV medium and incubated at 37°C for 15 minutes and incubated at room temperature (RT) for another 10 minutes. The cells were diluted 5 fold with medium containing 10% FBS and washed three times at 300xg for 10 minutes. The cells were then cultured in AIMV medium containing 5% FBS and treated with ConA (40 µg/mL) for an additional 72 hours. Proliferation was assayed by flow cytometry. The second experiment was conducted to determine if luteal cells induced proliferation of a specific subset of \( \gamma \delta^+ \) T lymphocytes. In this experiment, CFSE labeled \( \gamma \delta^+ \) T lymphocytes were either cultured alone or with luteal cells for 72 hours in AIMV medium containing insulin (5 µg/mL) transferrin (5 ng/mL) selenium (5 µg/mL) and
gentamycin (20 µg/mL). To determine if luteal cells induced proliferation of a specific subset of γδ T cells, the CFSE-labeled γδ T lymphoctes harvested from coculture were labeled with antibodies to WC1, WC1.1, WC1.2 or WC1.3. Appropriate secondary antibodies were used to identify the labeled cells.

**Statistical analysis**

To determine the effect of luteal cells on changes in γδ+ T lymphocyte phenotypes and proliferating subsets, Th1 and Th2 cytokines and associated transcription factors, the data were subjected to a paired t-test. Statistical significance was considered when the P value was less than 0.05.
Chapter 6

Expression and regulation of bovine activated leukocyte cell adhesion molecule in the corpus luteum

Introduction

Activated leukocyte adhesion molecule (ALCAM/CD166) is a 100-105 kDa transmembrane glycoprotein that belongs to the immunoglobulin superfamily of cell adhesion molecules and is expressed on activated human leukocytes (Bowen et al., 1995; Corbel, 1992b). It is also expressed by other cell types such as neurons (Tanaka and Obata, 1984; Pourquié et al., 1990; Konno et al., 2001), thymic and bursa epithelial cells (Pourquié et al., 1990; Corbel, 1992), hematopoietic progenitor cells (Uchida et al., 1997), endometrial epithelial and cumulus cells (Fujiwara et al., 2003; Adriaenssens et al., 2010, 2011; Kim et al., 2011), endothelial cells (Degen et al., 1998; Ohneda et al., 2001; Masedunskas et al., 2006), keratinocytes (Singer et al., 1997; Sanders et al., 2011), metastasizing melanoma cells (Degen et al., 1998; van Kempen, et al., 2000) and on both human mesenchymal (Alsalameh et al., 2004) and cancer stem cells (Levin et al., 2010). It is highly conserved and ALCAM orthologs (SC1, BEN, DM-GRASP) are found in quail and chicken (Tanaka and Obata, 1984; Pourquié et al., 1990; Burns et al. 1991), zebra fish (Kanki et al., 1994), rodents (Prince et al., 1992; Kanki et al., 1994), human (Bowen et al., 1995) and cattle (Konno et al., 2001). Bowen et al. (1996) demonstrated that ALCAM interacts with CD6, a scavenger receptor with cysteine-rich domains.
(SRCR), which is highly expressed on mature T lymphocytes. This interaction is important for optimal T lymphocyte activation (Hassan et al., 2004; Hassan et al., 2006; Kato et al., 2006). In addition to forming strong ALCAM/CD6 heterotypic cell-cell interactions, ALCAM also forms weaker homotypic ALCAM/ALCAM interactions (Corbel et al., 1996; Debnardo and Chang, 1996; Hassan et al., 2004). The heterotypic interactions are 100-fold stronger than homotypic interactions (Hassan et al., 2004).

ALCAM is highly expressed in tissues and cells involved in rapid growth such as cancer cells (Swart, 2002). Progression of several aggressive cancers of the skin, prostate, breast, colon and rectum, bladder, and esophageal squamous cells is associated with ALCAM expression (Ofori-Acuah and King, 2008). Thus, ALCAM has become a prognostic maker for most cancers (Mezzanzanica et al., 2008; Ishigami et al., 2011). Activated leukocyte cell adhesion molecule is transiently expressed during embryonic hematopoietic cell differentiation (Ohneda et al., 2001), T cell activation (Hassan et al., 2004; Gimferrer et al., 2004; Kato et al., 2006; Zimmerman et al., 2006) and germ cell development (Ohbo et al., 2003). In addition, ALCAM expressed on endothelial cells enhances angiogenesis in vitro (Ikeda and Quertermous, 2004). During inflammation, ALCAM is upregulated on endothelial cells and activated leukocytes (Levesque et al., 1998; Cayrol et al., 2008) and is involved in the activation and recruitment of immune cells to sites of inflammation (Masedunskas et al., 2006; Nummer et al., 2007; Cayrol et al., 2008; Lee and Imhof, 2008; Guerraty et al., 2011; Hassan et al., 2004; Kato et al., 2006). Also, ALCAM has been shown to regulate processes such as axonogenesis (Burns et al., 1991; Karagogeos et al., 1997), axon orientation (Avci et al., 2004), apoptosis,
metastasis (Swart et al., 2002) and migration of tumor cells (King et al., 2010; Wang et al., 2011).

In reproductive tissues, ALCAM is expressed during fetal development in female mouse ovary and testis (Ohbo et al., 2003). In adult reproductive tissues, ALCAM protein has been detected on the ovarian surface epithelium (Mezzanzanica et al., 2008), cumulus-oocyte complex (Hernandez-Gonzalez et al., 2006), blastocyst, and on luminal and glandular endometrial epithelial cells (Fujiwara et al., 2003; Kim et al., 2011). During ovulation, ALCAM is selectively upregulated on cumulus, but not mural, granulosa cells (Hernandez-Gonzalez et al., 2006). It is not clear if ALCAM is also expressed in theca cells.

Immune cells are present in the CL throughout the estrous cycle, and are proposed to mediate processes that culminate in luteal regression (Pate, 1995, rev.; Pate and Keyes, 2001, rev.; Pate et al., 2010, rev.), but the mechanisms through which immune cells communicate with luteal cells are not fully understood. Bovine luteal cells in vitro activate T lymphocytes to proliferate (Petroff et al., 1997, Cannon et al., 2003) and γδ+ T lymphocytes are more responsive than αβ+ T lymphocytes (Davis and Pate, 2007). Bovine γδ+ T lymphocytes that do not express WC1 (γδ+WC1−) express CD6, the receptor for ALCAM (Tuo et al., 1999). In the present study it was hypothesized that luteal cells expressed ALCAM and that ALCAM mediated luteal cell-induced γδ+ T lymphocyte proliferation.
Results

Gene and protein expression of ALCAM

The presence of mRNA corresponding to the bovine ALCAM gene was detected in the luteal tissue (Fig. 6-1A) and confirmed by sequencing. The steady-state abundance of ALCAM mRNA was higher (P<0.05) in the early compared to midcycle and late CL (Fig. 6-1B). In addition, CL collected 12 hours after administration of PGF$_{2\alpha}$ at midcycle had higher (P<0.05) steady-state abundance of ALCAM mRNA when compared to midcycle (Fig. 6-1C). The expression of ALCAM protein during the estrous cycle was higher at days 10-12 and day 18 compared to day 4 (P<0.05). During the course of PGF$_{2\alpha}$-induced luteal regression, the decline in ALCAM protein was not observed until 12 hours (Fig. 6-2B). The expression of ALCAM in the CL of early pregnancy (day 17) was lower (P<0.05) compared to that at day 17 of the estrous cycle (Fig. 6-2C).

Compared to glycosylated rhALCAM (120 kDa), the molecular weight of ALCAM detected in luteal tissue and cell lysates was approximately 65 kDa (Fig 6-2 D). Detection of ALCAM in other cell types revealed that bovine PBMCs and the human monocyte cell line (THP-1) also expressed the 65 kDa protein, but only the human granulosa cell tumor cell line (KGN) showed a 100 kDa protein (Fig. 6-3).

To further determine the expression of ALCAM during the estrous cycle, and its cellular localization, frozen luteal tissue sections were stained with anti-ALCAM and BS-1 lectin (endothelial cells). Similar to results obtained by western blot analysis, fewer ALCAM$^+$ cells were present in tissue sections collected on day 4 and during luteal regression (12 hours pPGF$_{2\alpha}$) compared to those collected on day 10 (Fig. 6-4). Activated
leukocyte cell adhesion molecule was expressed on steroidogenic cells, but not on the majority of endothelial cells (Fig. 6-5). Occasionally, ALCAM was detected on the luminal side of blood vessels (Fig. 6-5, bottom panel).

The proportion of luteal cells expressing ALCAM was determined in freshly isolated and cultured luteal cells (Fig. 6-6A, B). There was no difference in the proportion of ALCAM\(^+\) cells or intensity of ALCAM expression between fresh and cultured cells (P>0.05). The proportions of ALCAM\(^+\) cells and ALCAM expression were similar in freshly isolated cells from midcycle and regressing CL (Fig. 6-7A, B). To determine other cell types expressing ALCAM, dispersed luteal cells from regressing CL were stained with anti-CD18 and anti-CD14, which identify all immune cells and monocyte/macrophages, respectively (Fig 6-8A, B), and BS-1 lectin (Fig. 6-8B). Activated leuckocyte cell adhesion molecule was expressed on immune cells, the majority of which were monocyte/macrophages. Endothelial cells did not express ALCAM.

**Regulation of ALCAM expression on luteal cells**

To determine the paracrine factors that regulate ALCAM expression, luteal cells were treated with LH, PGF\(_{2\alpha}\), IFN\(\gamma\) and PMA (Fig. 6-9A, B). All treatments except PGF\(_{2\alpha}\) increased the proportion of ALCAM\(^+\) cells, but only LH increased the intensity of ALCAM expression (Fig. 6-9A).
Effect of inhibiting ALCAM-CD6 interaction on γδ⁺ T lymphocyte proliferation

First, the expression of CD6 on γδ⁺ T lymphocytes was determined by flow cytometry. About 12% of γδ⁺ T lymphocytes expressed CD6 (Fig. 6-10). The γδ⁺ T lymphocytes that expressed CD6 were predominantly WC1⁻ and only about 5-7% of WC1⁺ cells co-expressed CD6 (Fig. 6-10). Second, to determine if ALCAM⁺ cells induced proliferation of γδ⁺ T lymphocytes, isolated ALCAM⁺, ALCAM⁻ and mixed luteal cells were cocultured with γδ⁺ T lymphocytes (Fig. 6-11A). Both ALCAM⁻ and ALCAM⁺ cells induced γδ⁺ T lymphocyte proliferation to the same extent as mixed luteal cells (P>0.05). Third, luteal cells were incubated with anti-ALCAM antibodies (to inhibit ALCAM-CD6 interactions) before they were cocultured with γδ⁺ T lymphocytes. Incubating luteal cells with anti-ALCAM antibodies had no effect on luteal cell-induced proliferation of γδ⁺ T lymphocytes (Fig. 6-11B). Fourth, γδ⁺ T lymphocytes were incubated with anti-CD6 antibody and rhALCAM to inhibit the interaction between γδ⁺ T lymphocytes and luteal cells. Anti-CD6 antibody tended to reduce luteal cell-induced γδ⁺ T lymphocyte proliferation (Fig. 11C), and rhALCAM reduced γδ⁺ T lymphocyte proliferation by about 10% (P<0.05; Fig. 6-11D). Fifth, ALCAM expression was suppressed using siRNA specific for bovine ALCAM. Luteal cells were transfected with siRNA (Fig. 6-12A), with a transfection efficiency of approximately 69% (Fig 6-12B). The expression of ALCAM was significantly diminished (Fig 6-13A, B). Luteal cells in which ALCAM was suppressed were cocultured with γδ⁺ T lymphocytes and proliferation determined. Suppression of ALCAM expression did not affect the proliferative response of either γδ⁺ or CD2⁺γδ⁺ T lymphocytes (Fig. 6-13C, D).
Discussion

This is the first report to show that ALCAM is expressed in the CL of any species. Bovine ALCAM has been partially cloned (Rodgers et al., 2002) and its expression in tissues examined using anti-human antibody clone 3A6 (Konno et al., 2001). In the present study, the expression of ALCAM mRNA was inversely proportional to protein during the estrous cycle. This relationship indicates that ALCAM is posttranscriptionally regulated. One possibility is that in the early CL, the protein is selectively targeted by protein degradation mechanisms. Activated leukocyte cell adhesion molecule undergoes ubiquitin-mediated degradation (Thelen et al., 2008). Another possibility is that translation of ALCAM mRNA is under inhibitory control. Mechanisms that may impair the translation of ALCAM mRNA include microRNA (miRNA). In the human, it has been established that ALCAM is suppressed by microRNAs 9, 192 and 215 (Wang et al., 2011; Jin et al., 2011). Expression of bomiR-542-3p, which targets ALCAM, has been confirmed to be present in the bovine ovary (Hossain et al., 2009). Activated leukocyte adhesion molecule is involved in cell-cell interaction and therefore, inhibiting its translation in the developing CL may be necessary for tissue remodeling and cell kinetics in the developing CL. ALCAM protein is elevated in the midcycle compared to the early CL, and stays elevated in late CL and during the early stages of luteal regression. In a mature CL, lower concentrations of ALCAM mRNA may be due to efficient translation into protein by a mechanism supported by LH. Thelen et al. (2012) established that ERK (externally regulated kinase) and TOR (target of rapamycin) signaling regulate ALCAM translation via the 3’ UTR (untranslated region) in neurons.
The form of ALCAM protein identified in luteal cells (~ 65 kDa) was different from that reported in other species. The size of ALCAM protein is ~100-105 kDa in humans (Patel et al., 1995), 95-100 kDa in chickens (Pourquié, et al., 1990), and 120 kDa in the rat (Bowen et al., 1997). The 65 kDa corresponds to the ALCAM precursor protein. Posttranslational glycosylation of the precursor protein accounts for the observed high molecular weight in other species (Bowen et al., 1995) and other forms of ALCAM observed in different tissue of the same species (Pourquié, et al., 1990; Denzinger et al., 1999). It remains to be confirmed if bovine ALCAM is not glycosylated.

Activated leukocyte cell adhesion molecule in the CL was expressed on steroidogenic cells, leukocytes and only rarely on endothelial cells. The expression of ALCAM on luteal cells was maintained in cultured cells and its expression was increased by LH, IFNγ and PMA, but not PGF$_{2\alpha}$. Luteinizing hormone may be responsible for the increased expression of ALCAM at midcycle and late luteal phases. The action of LH may be to regulate mechanisms that enhance ALCAM protein translation. Similar to FSH, the stimulation of ALCAM by an LH-mediated increase in ALCAM expression may be mediated by MAPK (mitogen activated protein kinase) rather than PKA (protein kinase A) signaling (Adriaenssens et al. 2011). Treatment of cells with IFNγ increased ALCAM expression. These results are in agreement with those of Singer et al. (1997), who showed that IFNγ increased ALCAM expression on keratinocytes. IFNγ presumably acts through its receptors on luteal cells (Suter et al., 2001; Ma et al., 2011). The source of IFNγ in the CL is likely to be resident lymphocytes and thus, the presence of activated lymphocytes in the midcycle and late stage CL might affect expression of ALCAM on luteal cells.
Leukocytes, including macrophages, are present in the bovine CL (Bauer et al., 2001; Townson et al., 2002). In the present study, a proportion of ALCAM$^+$ cells expressed CD18 and CD14 surface proteins, which are characteristic of myeloid cells (Spanel-Borowski and Ricken, 1997). Thus, leukocytes may partly explain the observed decline in the abundance of ALCAM protein in the CL in early pregnancy. The number of class II MHC positive cells, which may be leukocytes, declined in early pregnancy (Benyo et al., 1991). Because ALCAM is involved in homotypic and heterotypic interactions (Bowen et al., 1995; Hassan et al., 2004), the ALCAM expressed on leukocytes resident in the CL may homotypically interact with steroidogenic and endothelial cells.

ALCAM expressed on steroidogenic cells may heterotypically interact with resident T lymphocytes through the CD6 receptor. This notion is derived from the observations of Singer et al. (1997), who demonstrated that keratinocytes expressed ALCAM and interacted with resident skin lymphocytes via CD6. Keratinocytes are antigen presenting (APC)-like cells to αβ$^+$ and γδ$^+$ T lymphocytes (Havran et al., 1991; Komori et al., 2012). All mature αβ$^+$ and γδ$^+$ T lymphocytes that do not express the workshop cluster 1 molecule express CD6. CD6 belongs to a family of SRCR receptors and is involved in the activation of T lymphocytes (Bott et al., 1993; Gimferrer et al., 2004; Hassan et al., 2006). Interaction of CD6 with ALCAM is important for T lymphocyte activation (Kato et al., 2006; Zimmerman et al., 2008). In the present study, the hypothesis that luteal cell-induced γδ$^+$ T lymphocyte proliferation was dependent on ALCAM was tested. Blocking ALCAM on mixed luteal cells with anti-ALCAM antibodies had no effect on luteal cell-induced proliferation of γδ$^+$ T lymphocytes. This
observation could be attributed to incomplete blocking of all ALCAM molecules. Whereas ALCAM\(^+\) cells could not be successfully depleted from luteal cells, isolated ALCAM\(^+\) cells induced \(\gamma\delta\)\(^+\) T lymphocyte proliferation as well as mixed luteal cells. These results indicated that luteal cells expressing CD166 are capable of inducing proliferation of \(\gamma\delta\)\(^+\) T lymphocytes. Furthermore, blocking CD6 with anti-CD6 and rhLCAM reduced proliferation of \(\gamma\delta\)\(^+\) T lymphocytes. However, siRNA-mediated suppression of ALCAM was unable to inhibit luteal cell-induced T lymphocyte proliferation.

**Conclusion**

This is the first report to show that ALCAM is expressed in the CL. It is predominantly expressed in steroidogenic cells and its abundance changes relative to the physiological state of the CL. The expression of ALCAM was regulated by both luteotropic and luteolytic factors. Finally, although ALCAM may play a limited role in the activation \(\gamma\delta\)\(^+\) T lymphocytes, it may play a role in luteal function.
Figure 6-1

A) Representative ethidium bromide stained agarose gel showing the 360bp amplicon. Spleen tissue was used as a negative control. B) Comparison of steady-state mRNA in luteal tissue collected at early (day 5), midcycle (10-12) and late stages (day 18). C) Comparison of steady-state mRNA between midcycle and regressing CL (12 hours pPGF<sub>2α</sub>). At least three animals were used at each time. Different letters indicate significant differences (P<0.05).
Figure 2. ALCAM protein expression in the CL. A. Western blot and densitometry data showing ALCAM in tissue collected at day 4, days 10-12 and day 18 (n=3). B. Western blot and densitometry data showing ALCAM in tissue collected at days 10-12, and 1, 8 and 12 hours pPGF$_{2\alpha}$ (n=3). C. Western blot and densitometry data showing ALCAM in tissue collected at day 17 of the estrous cycle and day 17 of pregnancy (n=3). Different letters are significantly different (P<0.05). D. Comparison of bovine ALCAM from luteal cell lysates and recombinant human ALCAM (rhALCAM).
Figure 6-3

**Figure 6-3. Comparison of ALCAM expression in luteal cells and cell lines.** THP-1: Human acute monocytic leukemia cell line, BPBMCs: Bovine peripheral blood mononuclear cells, Hela: Human cervical cancer cell line, OGE: Ovine grandular epithelial cell line, KGN: Human granulosa cell line, LC: Bovine luteal cells.
Figure 6-4. ALCAM expression in luteal tissue collected at day 4, 10-12 of the estrous cycle, and 12 hours pgf2α. The photomicrographs are representative of results from three independent experiments. Scale bars represent 20 µm (Mag=400X). The inset in the middle panel shows ALCAM at 1000X magnification. The inset in the left panel shows staining with control IgG.
**Figure 6-5. Localization of ALCAM in luteal tissue.** Representative photomicrographs of three independent experiments showing ALCAM (Red) and BS1-lectin (green) staining in luteal tissue of midcycle. ALCAM is expressed in small (arrow) and large (asterisk) luteal cells. Scale bars represent 20 µm (Mag=400X). The insets in the top left and right panels shows staining with control IgG.
Figure 6-6. ALCAM expression on dispersed luteal cells. A) Proportion of ALCAM$^+$ cells and associated mean fluorescence intensity in fresh and cultured cells. Different letters indicate significant differences (P<0.05). B) The dot plots show ALCAM in fresh cell, and are representative of results obtained from three independent experiments.
Figure 6-7. Comparison of ALCAM expression in midcycle and regressing CL. A) Proportion of ALCAM$^+$ cells and associated mean fluorescence intensity of ALCAM in luteal cells isolated from midcycle and regressing CL (n=4). B) Representative dot plots of stained cells.
Figure 6-8. ALCAM expression on nonsteroidogenic cells. A) Representative dot plots of three independent experiments showing the proportions of ALCAM+/CD18+ and ALCAM+/CD14+ cells. B) Photomicrographs showing ALCAM and BS-1 lectin (top), ALCAM and CD18 (middle) and ALCAM and CD14 (bottom). Scale bars represent 10 µm (Mag=1000X) and 20 µm (Mag=2000X).
Figure 6-9. **Regulation of ALCAM in luteal cells.** A) The proportion of ALCAM+ cells and associated mean fluorescence intensity of ALCAM in response to LH (10 ng/mL), PGF$_{2\alpha}$ (10 ng/mL), IFN$\gamma$ (50 ng/mL) and PMA (1 $\mu$g/mL). Control= untreated cells. B) Representative dot plots associated
Figure 6-10. CD6 expression on $\gamma^\delta$ T lymphocytes isolated from peripheral blood. The dot plots are representative of results obtained from three independent experiments.
Figure 6-11

Effect of blocking ALCAM and CD6 on luteal cell-induced γδ⁺ T lymphocyte proliferation. A) Stimulation of γδ⁺ T lymphocytes by ALCAM⁺ luteal cells (n=3). B) Proliferation of γδ⁺ T lymphocytes induced by luteal cells that were preincubated with anti-ALCAM (5-30 μg/mL). C) Proliferation of γδ⁺ T lymphocytes pre-incubated with anti-CD6 (10 μg/mL; n=4). D) Proliferation of γδ⁺ T lymphocytes pre-incubated with rhALCAM (10 μg/mL; n=4). Different letters indicate significant differences (P<0.05).
Figure 6-12. Transfection of luteal cells with siRNA. A) Representative photomicrographs of luteal cells transfected with control siRNA conjugated to Cy3 (C-siRNA-CY3). Top panels (a & b) indicate fluorescence and bright field images of transfected cells. Bottom panels indicate fluorescence and bright field images of untransfected cells. B) Representative dot plot showing transfection efficiency.
Figure 6-13. Effect of ALCAM silencing on \(\gamma\delta^+\) T lymphocyte proliferation. A) Mean fluorescence intensity of ALCAM in cells transfected with ALCAM-siRNA. Untransfected and luteal cells transfected with control siRNA (C-siRNA) were used as controls. B) Representative histogram showing ALCAM fluorescence intensity in control and transfected luteal cells. Proliferation of \(\gamma\delta^+\) T lymphocytes (C: n=4) and T lymphocytes (D: n=3) induced by luteal cells transfected with ALCAM-siRNA. Different letters indicate significant differences (P<0.05)
Materials and methods

Reagents and antibodies

Antibodies used for cell culture were: CD3 (MM1A; VMRD) and IL2Rα (CACT116A; VMRD). Antibodies used for immunostaining were: LAT (rabbit mAb #9166; Cell Signaling Technologies Inc.), Lck (rabbit mAb # 2787; Cell Signaling Technologies Inc.) and anti-rabbit Ig-FITC (STAR34B; AbD Serotech), anti-mouse IgG2a:FITC (Southern Biotechnology Associates) and anti-mouse IgG1 alexa fluor® 546 (Invitrogen)

T lymphocyte proliferation assay

To determine if paraformaldehyde-fixed luteal cells stimulated T lymphocytes to proliferate, 20x10^6 T lymphocytes were loaded with CFSE at a final concentration of 1.75 µM in AIMV medium. The cells were incubated for 15 minutes at 37°C followed by incubation at room temperature (RT) for 10 minutes. Excess CFSE was removed by washing the cells with AIMV medium containing 10 % serum 3X at 250xg for 10 minutes each time. The pellet was reconstituted in 500 µL AIMV medium supplemented with ITS. T lymphocytes labeled with CFSE (5x10^5) were either cultured alone or
cocultured with luteal cells (5x10^5) treated with either paraformaldehyde (0.06, 0.1, 1 and 4%) at RT for 15 minutes or with medium in a 24-well cell culture plate.

To determine if blocking the TCR and Interleukin 2 receptor (IL2Rα/CD25) would inhibit luteal cell-induced T lymphocyte proliferation, the effect of plate-bound and soluble anti-CD3 on T lymphocyte proliferation was first determined. Twenty four-well cell culture plates were coated with anti-CD3 antibodies (10 µg/mL) in PBS and incubated overnight at 4°C. The coated wells were washed twice with RPMI medium containing 10% FCS before CFSE-labeled T lymphocytes were added. Soluble anti-CD3 was added to T lymphocytes in wells that were not coated with anti-CD3 antibodies. In addition, CFSE-labeled T lymphocytes were separately pre-incubated with anti-CD3 and anti-IL2Rα antibodies and then cocultured with luteal cells.

To determine the effect of immunosuppressive drugs on luteal cell-induced T lymphocyte proliferation, CFSE-labeled T lymphocytes were separately pretreated with forskolin (5 µM), dbcAMP (100 µM), CsA (0.5 µg/mL) and FK506 (1 µM) for 30 minutes at 37°C and washed 2X with medium before they were either treated with ConA or cocultured with luteal cells. In all experiments, T lymphocytes not treated with any compound or not exposed to luteal cells were used as a negative control and proliferation was assayed by flow cytometry after 72 hours in culture.

**Zap70 phosphorylation**

Luteal cells (5x10^5) were cultured in 24-well plates overnight and washed with warm medium to remove nonattached cells. T lymphocytes (2x10^6/well) in 0.5 mL of
RPMI medium were incubated with luteal cells at 37°C and harvested after 5, 10, 30 and 60 min. The harvested T lymphocytes were immediately chilled on ice, followed by centrifugation and lysis using CelLytic Mammalian Tissue Lysis/Extraction reagent. Phosphatase inhibitors were added and the lysates were stored at -80°C until western blot analyses.

**Immunostaining**

To examine if T lymphocytes interacted with luteal cells via the T cell receptor, luteal cells were cultured in 35mm μ-dishes (ibiTreat, Ibidi) or on glass slides. The cells were washed with warm medium to remove unattached cells. T lymphocytes were then added and incubated with luteal cells for 2-2½ hours. The wells or glass to which T cells were added were gently washed to remove unbound T lymphocytes and then fixed with 4% PFA at RT for 15 minutes. The cells were subsequently labeled with primary antibodies against class II MHC (DRα) and CD3 for 30 minutes at 4°C. After a series of washes, the cells were incubated with secondary antibodies, anti-mouse IgG2a-FITC and anti-mouse IgG1-alexa fluoro 546 for an additional 30 minutes. Unbound secondary antibody was washed off and the cells were stained with DAPI for 5 minutes. Images were captured using a DP71 color camera mounted on a BX51 Olympus fluorescence microscope. In addition, high resolution 3D images were captured using Applied Precision (DV Elite imaging system) microscope.

Luteal cells (5x10⁵) were cultured in 24-well plates overnight and washed with warm medium to remove unattached cells. T lymphocytes (2x10⁶/well) in 0.5 mL of
RPMI medium were incubated with luteal cells at 37°C and harvested after 10 and 30 minutes. The harvested T lymphocytes were immediately chilled on ice, centrifuged at 4°C and fixed with 4% PFA. The cells were washed twice and incubated in PBS containing 0.5% Triton-X and subsequently stained with primary antibodies (1:100) against LAT and Lck for 30 minutes on ice. The cells were subsequently washed three times with PBS-EDTA containing 5% NGS (NGS) at 200xg. The resulting pellet was resuspended in PBS-EDTA containing 5% NGS and sheep anti-rabbit secondary antibody was added and incubated for 30 minutes on ice. After incubation, the cells were washed three times at 200xg with PBS-EDTA containing 5% NGS. The pellet was resuspended in 20 μL. A drop of cells was placed on a slide and mounting medium containing DAPI was added. Images of stained cells were obtained using a fluorescent microscope.

**Statistical analyses**

For all experiments in which proliferation was performed the data were analyzed using one-way analysis of variance (ANOVA) and when the F test was significant, multiple means were compared and P values were adjusted using the Tukey-Kramer method. In addition, T tests were performed where appropriate. Means were considered significantly different when the p value was less than 0.05. The data are presented as means±SEM.
Chapter 7

Preliminary findings on the mechanisms by which luteal cells induce T lymphocyte activation

Introduction

Luteal cells isolated from both regressing and functional corpora lutea induce proliferation of autologous T cells in vitro (Petroff et al., 1997; Cannon et al., 2003; Davis and Pate, 2007). In addition to proliferation, coculture of luteal cells with autologous T lymphocytes increases both IL10 and IFNγ (Davis and Pate, 2007). These observations suggest that there are functional interactions between luteal cells and T lymphocytes. Because both IL10 and IFNγ have been shown to regulate steroidogenesis in vitro (Fairchild and Pate, 1991; Benyo and Pate, 1992; Pate, 1995, rev.; Hashii et al., 1998; Petroff et al., 2001; Korzekwa et al., 2008), the interaction between luteal cells and autologous T lymphocytes may be reciprocal. The molecular nature of this interaction has not been investigated, but there is evidence to suggest that this interaction may be similar to that observed between antigen-presenting cells (APC) and T lymphocytes (Monks et al., 1998). To support the idea that luteal cells may communicate with T lymphocytes via the TCR, Benyo et al. (1991) and Petroff et al. (1997) demonstrated that bovine luteal cells expressed class II MHC molecules (MHC II) and that luteal cells treated with staphylococcal enterotoxin B (SEB) elicited proliferation of T lymphocytes. In addition, MHC II associated antigen processing components (Cannon et al., 2006) and
costimulatory molecules CD80 and CD86 were expressed in luteal tissue (Cannon et al., 2007a). In addition, anti-CD80 and CD86 markedly inhibited luteal cell-induced T lymphocyte proliferation. Class II MHC, CD86 and CD14, intercellular adhesion molecule-1 (ICAM-1/CD54) and leukocyte function-associated antigen 3 (LFA3) are also expressed in human granulosa-lutein cells (Fujiwara et al., 1993; Bukovsky et al., 1995; Viganò et al., 1997; Herath et al., 2007). Leukocyte function-associated antigen-3 and ICAM-1 interact with CD2 and LFA-1/CD18, respectively and are critical costimulatory molecules for sufficient T lymphocyte activation (Makgoba et al., 1989). Whereas the activation of T lymphocytes via MHCII and costimulatory molecules is restricted to CD4⁺ T lymphocytes, MHC nonrestricted γδ⁺ T lymphocytes have been shown to be the most responsive to luteal cell stimulation (Davis and Pate, 2007). This indicates that luteal cells express other molecules that stimulate γδ⁺ T lymphocytes.

Engagement of the TCR with MHC-antigen complex and subsequent formation of the immunological synapse (IS) initiates a series of early intracellular events that ultimately lead to the transcription of relevant genes (Palacios and Weiss, 2004). Early TCR signaling results in recruitment of a Src tyrosine kinase, Lck, which phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) present in the CD3 and ζ chains present in the TCR, providing a docking site for Zeta-chain-associated protein kinase 70 (Zap70). Zap70 phosphorylates downstream adaptor proteins such as linker of activated T cells (LAT), which in turn phosphorylates SPL-76 adaptor protein (Palacios and Weiss, 2004).

The objectives of this study were to determine if luteal cell-induced T lymphocyte proliferation is mediated by cell surface-associated molecules, and if luteal cells stimulate
T lymphocytes via the TCR. In addition, the effect of immunosuppressive compounds on luteal cell-induced T lymphocyte proliferation was evaluated.

**Results**

Luteal cells were fixed with PFA and then cocultured with T lymphocytes (Fig. 7-1A). Luteal cells fixed with 0.06, 0.1 and 1% PFA stimulated proliferation of T lymphocytes comparable to the control (unfixed luteal cells; P>0.05). Fixing luteal cells with 4% PFA significantly diminished luteal cell-induced T cell proliferation compared to the control (P<0.05). Proliferation of T lymphocytes by fixed luteal cells was also assessed when T lymphocytes were separated from fixed luteal cells by membrane inserts (Fig. 7-1B). Coculture of T lymphocytes with luteal cells the presence of inserts significantly inhibited T lymphocyte proliferation (P<0.05) compared to cocultures without inserts. This result indicated that T lymphocytes proliferated in response to cell-surface associated molecules. To determine if T lymphocytes established contact with luteal cells via the T cell receptor, luteal cells incubated with T lymphocytes were stained with antibodies against CD3 and MHC II (Fig. 7-2). Luteal cells expressed MHC II (Fig. 7-2 b), and formed conjugates with T lymphocytes (Fig. 7-2f). However, there was no interaction of MHC II on luteal cells and CD3 on T lymphocytes. Occasionally, CD3 on T lymphocytes conjugated to luteal cells was seen to cluster at the point of contact (Fig 7-2c, h). The majority of T lymphocytes that formed conjugates with luteal cells showed uniform distribution of CD3 (Fig. 7-2g, i). To test the hypothesis that luteal cells activated T lymphocytes via CD3, the ability of anti-CD3 antibodies to stimulate T
lymphocyte proliferation was assessed (Fig 7-3A). Plate-bound, but not soluble anti-CD3 antibodies induced T lymphocyte proliferation (P<0.05). Also, proliferation of T lymphocytes preincubated with anti-CD3 was assessed in luteal cell-T lymphocyte cocultures (Fig 7-3B). Preincubation of T lymphocytes with anti-CD3 did not inhibit luteal cell-induced T lymphocyte proliferation. Additionally, T lymphocytes were incubated with luteal cells for a short period (0-60 minutes) and the activity of Zap70 (Fig. 7-3 C), and distribution of LAT and Lck (Fig. 7-4) were determined. Incubation of T lymphocytes with luteal cells did not increase the phosphorylation of Zap70 and neither did it alter the distribution of Src tyrosine kinase, Lck or adaptor protein LAT. Finally, T lymphocytes were incubated with compounds that suppress specific and nonspecific T lymphocyte proliferation in vivo and in vitro (Fig 7-5). Cyclosporine A, FK506, dbcAMP did not suppress T lymphocyte proliferation induced by either conA or luteal cells (P>0.05). Surprisingly, forskolin inhibited luteal cell-induced T cell proliferation.

**Discussion**

The focus of this study was to understand the mechanism by which luteal cells stimulate T lymphocytes. In the first experiment, luteal cells were fixed with PFA to limit secretion of soluble molecules. Luteal cells fixed with PFA only stimulated T lymphocytes when the two cells types were not separated by a cell culture insert. This suggested that luteal cell surface-associated molecules were involved in activating T lymphocytes. The nature of these molecules is still not known, but because luteal cells have characteristics similar to APCs (Benyo et al., 1991, Fujiwara et al., 1993, Cannon et
al., 2006; 2007a), it was proposed that the interaction between luteal cells and T lymphocytes was mediated by the TCR. The results showed that very few T lymphocytes bound to luteal cells exhibited CD3 clusters at the point of contact with luteal cells. Because most of the cells did not show CD3 aggregation, it indicated that most of the interactions between luteal cells and T lymphocytes occurred via other molecules. Alternative mechanisms of T activation/costimulation have been reviewed by Kohlmeier and Benedict (2003). Leukocyte function associated antigen-1 (LFA-1), intercellular adhesion molecule 1 (ICAM-1) and LFA-3 are other molecules that provide stimulation to T lymphocytes. These are expressed by luteal cells and may be essential in supporting luteal cell-induced T lymphocyte activation (Fujiwara et al., 1993; Viganò et al., 1997). Although these molecules on their own may not induce sufficient signals for full T lymphocyte activation (Makgoba et al., 1989), they may support unknown ligands expressed by luteal cells. It has been shown that in vitro, blocking ICAM-1 partially inhibits the interaction between human granulosa cells and T lymphocytes (Viganò et al., 1997). Also, blocking costimulatory molecules CD80 and CD86 in bovine luteal cells markedly diminishes luteal cell-induced T lymphocyte proliferation (Cannon et al., 2007a).

Signaling associated with the activation of the TCR after TCR-MHC/antigen interaction involves autophosphorylation and recruitment of Lck to the immunological synapse followed by phosphorylation of the TCR complex-associated protein zeta, CD3ζ (Palacios and Weiss, 2004). Phosphorylation of ITAMs on ζ chains recruits Zap70 (van Oers et al. 1994; Isakov et al., 1995; Steinberg et al., 2003) and subsequently activates Zap70, which then phosphorylates downstream adaptor protein LAT (Palacios and Weiss,
2004; Wang et al., 2010). Activation of Zap70 occurs by phosphorylation at sites that include Tyr 319 (Pelosi et al., 1999). Zeta-chain-associated protein kinase 70 augments the TCR signal by phosphorylation of other adaptor proteins such as LAT, which in turn activates Phospholipase C (Zhang et al., 1998; Williams et al., 1999; Laethem et al., 2001). Phospholipase C (PLC) hydrolyses PI (4, 5) P2 to IP3, which results in release of intracellular calcium from the endoplasmic reticulum (Abraham and Weiss, 2004).

Aggregation of CD3 at points of contact is often shown at the immunological synapse in APC/antigen-TC interactions (Monks et al., 1998), but the current results showed that most LC-TC (luteal cell-T lymphocyte) conjugates did not stain positively for CD3. In addition to the lack of aggregation of CD3 in most luteal cell-T lymphocyte (LC-TC) contacts, T lymphocytes pre-incubated anti-CD3 proliferated in response to luteal cells. Also, incubation of T lymphocytes with luteal cells did not alter phosphorylation of Zap70 and had no effect on surface distribution of Lck and LAT. These findings lead to the suggestion that the interaction of luteal cells with T lymphocytes is independent of the TCR and that luteal cell-induced activation of T lymphocytes in the coculture system may not involve antigen presentation. However, it was noted that T lymphocytes not incubated with luteal cells contained phosphorylated Zap70. This could have masked any changes induced by luteal cells. The presence of phosphorylated Zap70 in rested cells could have arisen from the antibody-mediated separation of T lymphocytes. Therefore, future experiments will focus on using T lymphocytes isolated without use of antibodies.

T cell receptor signaling results in activation of proteins downstream of TCR, and activation of transcription factors such as, NFκB, Fos/Jun and nuclear factor of activated T cells (NFAT). These transcription factors are activated by a calcium-dependent
phosphatase, calcineurin (Blackwell and Christman, 1997; Macián et al., 2001; Hogan et al., 2003). This pathway is inhibited by immunosuppressive drugs such as, CsA and FK506, and cAMP and forskolin (Lui et al., 1991; Sigal and Dumont, 1992; Zenke et al., 1993; Tsurata et al., 1995; Kawamura et al., 1995; Jimenez et al., 2001). Furthermore, CsA and FK506 inhibit proliferation of αβ+ and γδ+ T lymphocytes stimulated via αβTCR and δTCR (Chen et al., 1996). Therefore, it was proposed that these compounds would inhibit luteal cell-induced T lymphocyte proliferation. T lymphocytes pretreated with CsA, FK506, cAMP and forskolin were cocultured with luteal cells or stimulated with ConA. The results showed that CsA and FK506 were unable to inhibit both ConA and luteal cell-induced T lymphocyte proliferation. The lack of inhibition of ConA stimulated proliferation by CSA and FK506 is inconsistent with studies that have demonstrated decreased cytokine production and proliferation on immune cells (Palacios, 1981; Gergely and Aszalos, 1990; Han et al., 1995; Kuga et al., 2008). It was therefore, impossible to draw any conclusions from the effects of CsA and FK506 on luteal cell-induced T lymphocyte proliferation.

Cyclic AMP, forskolin and compounds such as cholera toxin and prostaglandin E2, which increase cAMP in T lymphocytes, suppress human T lymphocyte proliferation (Stobo et al., 1979; Muñoz et al., 1990). Activation of the cAMP pathway results in inhibition of Th1, but not Th2 cytokines (Muñoz et al., 1990). Elevation of cAMP in T lymphocytes blocks the synthesis of Th1 cytokines by inhibiting activation and binding of NFAT and NFκB (Tsurata et al., 1995; Jimenez et al., 2001). In the present study, forskolin, but not dbcAMP, partially inhibited luteal cell-induced T lymphocyte proliferation. The explanation for differential effects of forskolin and cyclic AMP on
luteal cell-induced T lymphocyte proliferation is that, forskolin provided a more sustained signal compared to dbcAMP. In addition, dbcAMP may be subjected to degradation by endogenous phosphodiesterases.

**Conclusion**

Paraformaldehyde-fixed luteal cells induced proliferation of T lymphocytes, indicating that luteal cell surface-associated molecules may be involved in T lymphocyte activation. However, the activation of T lymphocytes may not occur via the TCR. Forskolin inhibited luteal cell-induced T lymphocyte proliferation, indicating that inhibition of cAMP signaling may be one of the mechanisms by which luteal cells activate T lymphocytes.
Figure 7-1. Effect of paraformaldehyde (PFA) on luteal cell-induced T lymphocyte proliferation. A) Luteal cells treated with PFA (0.06-4%; Cont= T lymphocytes cultured alone; n=4) Untreated luteal cells (black bars) were used as a control. B) T lymphocytes cocultured with luteal cells in the presence and absence of 0.4 µm inserts (I; n=3). Representative photomicrographs at the bottom of the graphs show luteal cells treated with (right panel) and without (middle panel) PFA. MagX400. Different letters indicate significant differences (P<0.05).
Figure 7-2. CD3 (red) and MHC II (green) staining in luteal cell-T lymphocytes conjugates. Representative 2D (a, b, c, d, e & f) and 3D photomicrographs (g, h, & i) of 3 independent experiments (a, b, c, d, e & f, Mag X200; g, h, & i, Mag X1000). IgG controls are shown in d & e. bright field image of cells in c are shown in f. The inset in c shows CD3 clustering at the interface between a T lymphocyte and luteal cell. TC= T lymphocytes, LC= luteal cell.
Figure 7-3. Activation of the TCR and Zap70 phosphorylation. A) Stimulation of T lymphocytes treated with plate-bound (Pb) and soluble (Solu) anti-CD3 antibodies (n=3). B) T lymphocytes preincubated with 10 μg/mL of anti-CD3 or anti-CD5 cocultured with luteal cells (n=4). C) Representative blot showing Zap70 phosphorylation in T lymphocytes incubated with luteal cells for 5, 10, 30 and 60 minutes. T lymphocytes not treated with either luteal cells or antibodies were used as controls (cont). Different letter indicate significant differences (P<0.05).
Figure 7-4. LCK and LAT in T lymphocytes cocultured with luteal cells. Representative photomicrographs showing the cellular distribution of LCK (a, b & c) and LAT (d, e & f) in T lymphocytes before and after incubation with luteal cells for 10 or 30 minutes (n=3). MagX400.
**Figure 7-5.** The effect of immune suppressive agents on luteal cell-induced T lymphocyte proliferation. A) ConA stimulation of T lymphocytes pretreated with either dbcAMP (100 µM), or forskolin (5 µM; n=2). B) ConA stimulation of T lymphocytes preincubated with either CsA (0.5 µg/ML) or FK506 (1 µM; n=2). C) Luteal cell stimulation of T lymphocytes pretreated with either dbcAMP (100 µM), or forskolin (5 µM; n=4). D) Luteal cell stimulation of T lymphocytes pretreated with and with either CsA (0.5 µg/ML) or FK506 (1 µM; n=3).
Materials and methods

Reagents and antibodies

Antibodies used for cell culture were: CD3 (MM1A; VMRD) and IL2Rα (CACT116A; VMRD). Antibodies used for immunostaining were: LAT (rabbit mAb #9166; Cell Signaling Technologies Inc.), Lck (rabbit mAb # 2787; Cell Signaling Technologies Inc.), anti-rabbit Ig-FITC (STAR34B; AbD Serotech), anti-mouse IgG2a:FITC (Southern Biotechnology Associates) and anti-mouse IgG1 Alexa Fluor® 546 (Invitrogen)

T lymphocyte proliferation assay

To determine if paraformaldehyde-fixed luteal cells stimulated T lymphocytes to proliferate, 20x10^6 T lymphocytes were loaded with CFSE at a final concentration of 1.75 µM in AIMV medium. The cells were incubated for 15 minutes at 37°C followed by incubation at room temperature (RT) for 10 minutes. Excess CFSE was removed by washing the cells with AIMV medium containing 10 % serum 3X at 250xg for 10 minutes each time. The pellet was reconstituted in 500 µL AIMV medium supplemented with ITS. T lymphocytes labeled with CFSE (5x10^5) were either cultured alone or
cocultured with luteal cells (5x10^5) treated with either paraformaldehyde (0.06, 0.1, 1 and 4%) at RT for 15 minutes or with medium in a 24-well cell culture plate.

To determine if blocking the TCR and Interleukin 2 receptor (IL2Rα/CD25) would inhibit luteal cell-induced T lymphocyte proliferation, the effect of plate-bound and soluble anti-CD3 on T lymphocyte proliferation was first determined. Twenty four-well cell culture plates were coated with anti-CD3 antibodies (10 µg/mL) in PBS and incubated overnight at 4°C. The coated wells were washed twice with RPMI medium containing 10% FCS before CFSE-labeled T lymphocytes were added. Soluble anti-CD3 was added to T lymphocytes in wells that were not coated with anti-CD3 antibodies. In addition, CFSE-labeled T lymphocytes were separately pre-incubated with anti-CD3 and anti-IL2Rα antibodies and then cocultured with luteal cells.

To determine the effect of immunosuppressive drugs on luteal cell-induced T lymphocyte proliferation, CFSE-labeled T lymphocytes were separately pretreated with forskolin (5 µM), dbcAMP (100 µM), CsA (0.5 µg/mL) and FK506 (1 µM) for 30 minutes at 37°C and washed 2X with medium before they were either treated with ConA or cocultured with luteal cells. In all experiments T lymphocytes not treated with any compound or not exposed to luteal cells were used as a negative control and proliferation was assayed by flow cytometry after 72 hours in culture.
Zap70 phosphorylation

Luteal cells (5x10^5) were cultured in 24-well plates overnight and washed with warm medium (37°C) to remove nonattached cells. T lymphocytes (2x10^6/well) in 0.5 mL of RPMI medium were incubated with luteal cells at 37°C and harvested after 5, 10, 30 and 60 min. The harvested T lymphocytes were immediately chilled on ice, followed by centrifugation and lysis using CelLytic Mammalian Tissue Lysis/Extraction reagent. Phosphatase inhibitors were added and the lysates were stored at -80°C until western blot analyses were performed.

Immunostaining

To examine if T lymphocytes interacted with luteal cells via the T cell receptor, luteal cells were cultured in 35mm µ-dishes (ibiTreat, Ibidi) or on glass slides. The cells were washed with warm medium to remove unattached cells. T lymphocytes were then added and incubated with luteal cells for 2-2½ hours. The wells or glass slides to which T cells were added were gently washed to remove unbound T lymphocytes and then fixed with 4% PFA at RT for 15 minutes. The cells were subsequently labeled with primary antibodies against class II MHC (DRα) and CD3 for 30 minutes at 4°C. After a series of washes, the cells were incubated with secondary antibodies, anti-mouse IgG2a-FITC and anti-mouse IgG1 alexa fluoro 546 for an additional 30 minutes. Unbound secondary antibody was washed off and the cells were stained with DAPI for 5 minutes. Images were captured using a DP71 color camera mounted on a BX51 Olympus fluorescence
microscope. In addition, high resolution 3D images were captured using Applied Precision (DV Elite imaging system) microscope.

Luteal cells (5x10^5) were cultured in 24-well plates overnight and washed with warm medium to remove unattached cells. T lymphocytes (2x10^6/well) in 0.5 mL of RPMI medium were incubated with luteal cells at 37°C and harvested after 10 and 30 minutes. The harvested T lymphocytes were immediately chilled on ice, centrifuged at 4°C and fixed with 4% PFA. The cells were washed twice and incubated in PBS containing 0.5% Triton-X and subsequently stained with primary antibodies (1:100) against LAT and Lck for 30 minutes on ice. The cells were subsequently washed three times with PBS-EDTA containing 5% NGS (NGS) at 200xg. The resulting pellet was resuspended in PBS-EDTA containing 5% NGS and sheep anti-rabbit secondary antibody was added and incubated for 30 minutes on ice. After incubation, the cells were washed three times at 200xg with PBS-EDTA containing 5% NGS. The pellet was resuspended in 20 µL. A drop of cells was placed on a slide and mounting medium containing DAPI was added. Images of stained cells were obtained using a fluorescent microscope.

**Statistical analyses**

For all experiments where proliferation was performed the data were analyzed using one-way analysis of variance (ANOVA) and where the F test was significant, multiple means were compared the Tukey-Kramer method was used to separate means. In addition, T tests were performed where appropriate. Means were considered
significantly different when the p value was less than 0.05. The data are presented as means±SEM.
Summary

Addressing the problem of declining fertility in dairy cattle hinges on deciphering the complex mechanisms that regulate processes in the reproductive organs, including the CL. A functional CL is absolutely required for the establishment and maintenance of pregnancy in mammals and therefore, it is important to understand the mechanisms that ensure optimal luteal function. Immune cell mediators modulate luteal function. Therefore, identifying T lymphocyte subsets that negatively or positively affect luteal function provides cellular targets for developing strategies to improve reproductive efficiency in dairy cattle.

Cytokines such as IL1β, IFNγ and TNFα inhibit gonadotropin-induced progesterone synthesis and increase PGF$_{2α}$ synthesis in luteal cells. Furthermore, IFNγ and TNFα are cytotoxic to luteal cells and thus may facilitate PGF$_{2α}$-induced demise of the CL. Although immune cells are suggested to facilitate luteal regression, immune cells including T lymphocytes and cytokines are present in a functional CL prior to the onset of luteal regression, suggesting that the activities of immune cells present in a functional CL may be modulated not to induce premature luteal regression. Therefore, the focus of the studies herein was to understand how immune cells are recruited to the CL and how their functions are modulated by luteal cells.

Endothelial cells form a physical barrier between tissues and circulating immune cells. The immune cells must cross the endothelial cell barrier to access their targets in tissues. There are temporal changes in immune cells during the estrous cycle, suggesting
that recruitment of immune cells in the CL is regulated. Endothelial cells were isolated from CL, characterized and used to study their interactions with T lymphocytes. The purity of enriched endothelial cells was about 90%. Luteal endothelial cells induced IL2Rα expression and proliferation of T lymphocytes. Activation of autologous T lymphocytes by endothelial cells may occur during transmigration of T lymphocytes across the endothelium. Luteal endothelial cells may, therefore, regulate the activity of T lymphocytes that are recruited into the CL. The increased expression of the IL2Rα is likely to enhance survival for T lymphocytes migrating through the endothelium. It will be important in the future to 1) determine the cell type activated, 2) determine whether the activating signals are derived from soluble factors and (or) cell surface-associated molecules, and if similar responses are induced by endothelial cells from regressing CL.

The mechanisms that regulate the recruitment of T lymphocytes in the CL are not fully known. In Chapter 3 it was established that PGE2 and PGF2α linearly increased and decreased, respectively, the adhesion of T lymphocytes to endothelial cells in a concentration-dependent manner and this response was dependent on whether the endothelial cells had been treated with TNFα and IL1β. These results imply that luteal prostaglandins may be involved in the recruitment of T lymphocytes to the CL by modulating endothelial cell function. The recruitment of T lymphocytes in the CL may follow the pattern of luteal prostaglandin secretion. Increasing T lymphocyte adhesion in endothelial cells not treated with cytokines indicates that PGE2 and PGF2α may facilitate recruitment of T lymphocytes in a functional CL. Although PGF2α-induced luteal regression is associated with enhanced recruitment of T lymphocytes, PGF2α may be involved in resolving inflammation by inhibiting the adhesion of T lymphocytes to
endothelial cells during luteal regression. In the future, it will be important to determine if
prostaglandins regulate the expression of adhesion molecules on endothelial cells.

Resident immune cells are suggested to functionally interact with luteal cells, but
the mechanisms by which luteal cells interact with T lymphocytes are not fully known.
The luteal environment is composed of both cells and soluble factors, and progesterone is
one such factor that is known to regulate immune cell function. In chapter 4, the
mechanisms by which progesterone affects T lymphocyte function were assessed.

Progesterone is an immunosuppressive hormone that affects all immune cells, including
T lymphocytes. The suppressive effects of progesterone include suppression of T
lymphocyte proliferation, enhancing a Th2 cytokine profile with suppressive cytokines (IL10) and proteins such as PIBF, inhibiting sustained calcium influx and NFAT (transcription factor)- driven cytokine gene expression. In the present study, progesterone increased the gene expression of IL2 and intracellular calcium, but decreased Zap70 phosphorylation and the proportion of cells entering the S-phase of the cell cycle.

Progesterone had no effect on IL2Rα. Progesterone may transiently increase intracellular Ca^{2+}, which results in the activation of NFAT leading to the transcription of IL2. Interleukin 2 may be important for maintaining survival of IL2Rα{T} T lymphocytes. Although progesterone may maintain cell survival, inhibitory effects on cell cycle progression prevent T lymphocytes from proliferating. Furthermore, the reduction of Zap70 phosphorylation implies that progesterone may interfere with TCR signaling during MHC-antigen-induced T lymphocyte activation. Progesterone present in the CL may also inhibit the function of resident T lymphocytes by inducing suppressive cytokines such as IL10. Indeed, blocking steroid synthesis in luteal cells decreased the
number of γδ^+IL10^+ T lymphocytes. In addition to the direct effects of progesterone on T lymphocytes, progesterone modulated T lymphocyte adhesion to luteal endothelial cells. The effects of progesterone were concentration-dependent. The low concentrations (1-5 µM) tended to increase whereas the high concentrations (10-20 µM) tended to inhibit T lymphocyte adhesion. It is proposed that in a functional CL, progesterone inhibits T lymphocyte recruitment, but in a regressing CL, low progesterone concentrations provide a permissive environment for T lymphocyte recruitment. This may explain why an increased number of T lymphocytes are observed in the CL during luteal regression. Future experiments will focus on understanding the mechanisms involved such as, modulation of adhesion molecule expression. The actions of progesterone assessed in all the studies were dependent on the concentration. The high concentrations of progesterone present in a functional CL of the estrous cycle or pregnancy may suppress the local immune response without affecting other tissues.

Previously, coculture of luteal cells with T lymphocytes stimulated proliferation of γδ^+ T lymphocytes, and increased the secretion of IL10 and IFNγ (Davis and Pate, 2007). The characteristics of the responding γδ^+ T lymphocytes or the subset of T lymphocyte secreting the cytokines were not determined. In the present study, the subsets of proliferating γδ^+ T lymphocytes in response to luteal cells from functional and regressing CL were examined. Luteal cells from midcycle induced proliferation of γδ^+WC1^− and increased IL10, but decreased IFNγ synthesis. Although γδ^+WC1^+ were not induced to proliferate by midcycle luteal cells, they were stimulated to synthesize IL10. Midcycle luteal cells also increased the synthesis of both IL10 and IFNγ in CD4^+ T lymphocytes. In contrast to midcycle luteal cells, luteal cells from regressing CL induced
proliferation of $\gamma\delta^+\text{WC1}^-$ and did not affect the synthesis of IL10 or IFN$\gamma$ in either $\gamma\delta^+$ or CD4$^+$ T lymphocytes. These findings indicate that luteal cells in a functional CL program the $\gamma\delta^+$ T lymphocytes to play a regulatory role. Interleukin10 may be necessary to inhibit the activation of other immune cells present in the CL, which would otherwise be activated by IFN$\gamma$. It is noteworthy that $\gamma\delta^+$ and CD4$^+$ cells differentially responded to midcycle luteal cells. Also, the synthesis of IFN$\gamma$ was highest in CD4$^+$ T lymphocytes, suggesting that activated CD4$^+$ lymphocytes may be detrimental to luteal function. Future experiments will be designed to determine if the IL10-producing $\gamma\delta^+$ T lymphocyte directly inhibit IFN$\gamma$ synthesis in CD4$^+$ T lymphocytes, and also determine the response of luteal cells to T lymphocytes in the coculture system.

The specific activation of the $\gamma\delta^+\text{WC1}^-$ subset of $\gamma\delta^+$ T lymphocyte by luteal cells from a functional CL lead to the hypothesis that midcycle luteal cells expressed ligands that were recognized by the $\gamma\delta^+\text{WC1}^-$ cells. The $\gamma\delta^+\text{WC1}^-$ cells express a costimulatory molecule CD6, which is a receptor for ALCAM/CD166. Activated leukocyte cell adhesion molecule was expressed on the surface of midcycle luteal cells. Blocking the interaction between CD166 and CD6 using anti-CD6 and recombinant human ALCAM decreased luteal cell-induced $\gamma\delta^+$ T lymphocyte proliferation by 10%. However, the suppression of ALCAM using siRNA did not alter the proliferative response of $\gamma\delta^+$ T lymphocytes. Because the $\gamma\delta^+\text{WC1}^-$ cells are fewer, suppression of CD166 expression was insufficient to limit their activation. Alternatively, additional costimulatory molecules may compensate for the reduction of CD166. The effectiveness of both recombinant ALCAM and anti-CD6 was probably due to saturated ligation of surface CD166. However, this needs to be tested by titrating recombinant ALCAM or anti-CD6
antibodies. Identifying ligands that are recognized by $\gamma\delta^+$ T lymphocytes is a nascent area of research and therefore luteal cells may provide a suitable model cell. It is proposed that a shotgun proteomic approach may be a way to screen luteal cell membranes for $\gamma\delta^+$ T lymphocyte ligands.

**Impact**

The protocol developed for enriching endothelial cells can be used in other species and therefore, will advance research that is aimed at understanding the biology of endothelial cells. Activation of autologous T lymphocytes by luteal endothelial cells is a characteristic that has not been described before. Therefore, this may be a new area of research.

It is now clear that luteal cells selectively modulate the function T lymphocyte subsets present in the CL. Using the example of $\gamma\delta^+$ T lymphocytes; luteal cells elicit responses in specific T lymphocytes depending on the physiological state of the CL. The expansion of $\gamma\delta^+$WC1$^-$ population by luteal cells from a functional CL identifies this population as a target for further studies on immune cells involved in the rescue of the CL from regression.

The detection of ALCAM on luteal cells was a key finding. First, because ALCAM was highly expressed in mature CL and very low in developing CL indicates that it may be an important regulator of luteal function. Second, ALCAM is expressed on a variety of cancer cells. Therefore, understanding the processes in which it is involved in luteal cells may provide valuable information for cancer research.
Zap70 was identified as a novel target for progesterone. Decreasing the activity of Zap70 indicates that progesterone may impair TCR activation. This mechanism may be used to control the activity of T lymphocytes toward paternal antigens during pregnancy.
The recruitment of T lymphocytes in a functional CL is enhanced by prostaglandins E\textsubscript{2} and F\textsubscript{2α}, but this process is modulated by progesterone. The function of T lymphocytes within the CL is further modulated by luteal cells. Luteal cells (LC) decrease the synthesis of steroidogenic inhibitory cytokine, IFN\textgamma, and induce the synthesis of the luteotropic cytokine, IL10. Interleukin 10 may suppress the activation of other immune cells within the functional CL to avoid premature luteal regression, but also support progesterone synthesis. Luteal cells of a functional CL also selectively induce the expansion of γδ\textsuperscript{+}WC1\textsuperscript{−} cells which may help maintain luteal function. In addition to modulating the recruitment and activation of T lymphocytes, progesterone may play a role in the induction of IL10 synthesis in γδ T cells. Upon the initiation of luteal regression by PGF\textsubscript{2α}, progesterone synthesis declines and the number of lymphocytes recruited the CL increases. Luteal cells of the regressing CL selectively induce the expansion of γδ\textsuperscript{+}WC1\textsuperscript{+} cells, which may facilitate luteolysis.
References


Adashi EY. The potential role of interleukin-1 in the ovulatory process: an evolving hypothesis. Mol Cell Endocrinol 1998;140:77-81


Adriaenssens T, Wathlet S, Segers I, Verheyen G, Devos A, Van der Elst J, Coucke W, Devroey P, Smitz J. Cumulus cell gene expression is associated with oocyte...
developmental quality and influenced by patient and treatment characteristics. Hum Reprod 2010;25:1259-1270

Akinlosotu BA, Diehl JR, Giminez T. Prostaglandin E2 counteracts the effects of PGF2α in indomethacin treated cycling gilts. Prostaglandins 1988;35:81-93

Alila HW, Corradino RA, Hansel W A comparison of the effects of cyclooxygenase prostanoids on progesterone production by small and large bovine luteal cells. Prostaglandins 1988;36:259-270

Alila HW, Hansel W. Origin of Different Cell Types in the Bovine Corpus Luteum as Characterized by Specific Monoclonal Antibodies. Biol Reprod 1984;31;1015-1025


Ashour HM, Niederkorn JY. γδ T Cells promote anterior chamber-associated immune deviation and immune privilege through their production of IL-10. J Immunol 2006;177:8331-8337


Ayoub IA, Yang TJ. Age-dependant changes in peripheral blood lymphocyte subpopulations in cattle: a longitudinal study. Develop Comp Immunol 1996;20:353-363


Azmi TI, Bongso TA. The ultrastructure of the corpus luteum of the goat. Pertanika 1985;8:215–222

Azmi TI, O'Shea JD, Bruce NW, Rodgers RJ. Morphometry of the functional and regressing corpus luteum of the guinea pig. Anat Rec 1984;210:33-40

Azmi TI, O'Shea JD, Lee CS Rodgers RJ. Effects of a synthetic prostaglandin analogue, cloprostenol, on the corpus luteum of the guinea pig. Prostaglandins. 1982;24:519-526


Benyo DF, Pate JL. Tumor necrosis factor-alpha alters bovine luteal cell synthetic capacity and viability. Endocrinology 1992;130:854-60.


Blumerman SL, Herzig CT, Rogers AN, Telfer JC, Baldwin CL. Differential TCR gene usage between WC1- and WC1+ ruminant gammadelta T cell subpopulations including those responding to bacterial antigen. Immunogenetics 2006;58:680-692


Bogan RL, Murphy MJ, Stouffer RL, Hennebold JD. Prostaglandin synthesis, metabolism, and signaling potential in the Rhesus Macaque corpus luteum throughout the luteal phase of the menstrual cycle. Endocrinology 2008;149:5861-5871
Boiti C. A review of luteolytic and luteotrophic effects of prostaglandins in the corpus luteum of pseudopregnant rabbits: some in vivo and in vitro insights. World Rabbit Sci 1999;7:221-228


Brännström M, Norman RJ. Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. Hum Reprod 1993b;8:1762-1775

Brännström M, Pascoe V, Norman RJ, McClure N. Localization of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. Fertil Steril 1994b;61:488-495


Brännström M, Woessner Jr JF, Koos RD, Sear CH, LeMaire WJ. Inhibitors of mammalian tissue collagenase and metalloproteinases suppress ovulation in the perfused rat ovary. Endocrinology 1988;122:1715-1721


Brown CG, Poyser NL. Further studies on prostaglandin and thromboxane production by the rat uterus during the oestrous cycle. J Reprod Fertil. 1985;73:391-399

Bruce NW, Moor RM. Capillary blood flow to ovarian follicles, stroma and corpora lutea of anaesthetized sheep. J Reprod Fertil 1976;46:299-304


Cannon MJ, Davis JS, Pate JL. Presence and regulation of messenger ribonucleic acids encoding components of the class II major histocompatibility complex-associated antigen processing pathway in the bovine corpus luteum. Reproduction 2006;131:689-698
Cannon MJ, Davis JS, Pate JL. The class II major histocompatibility complex molecule BoLA-DR is expressed by endothelial cells of the bovine corpus luteum. Reproduction 2007b;133:991-1003

Cannon MJ, Petroff MG, Pate JL. Effects of prostaglandin F2alpha and progesterone on the ability of bovine luteal cells to stimulate T lymphocyte proliferation. Biol Reprod 2003;69:695-700


Carr, MM, Howard CJ, Sopp P, Manser JM, Parsons KR. Expression on porcine γδ lymphocytes of a phylogenetically conserved surface antigen previously restricted in expression to ruminant γδ T lymphocytes. Immunol 1994;81:36-40


Cavicchio VA, Pru JK, Davis BS, Davis JS, Rueda BR, Townson DH: Secretion of monocyte chemoattractant protein-1 by endothelial cells of the bovine corpus luteum: regulation by cytokines but not prostaglandin F2alpha. Endocrinology 2002;143:3582-3589.


Chegini N, Ramani N, Rao Ch V. Morphological and biochemical characterization of small and large bovine luteal cells during pregnancy. Mol Cell Endocrinol 1984;37:89-112


Choudhary E, Sen A, Inskeep EK, Flores JA. Developmental sensitivity of the bovine corpus luteum to prostaglandin F2α (PGF2α) and Endothelial-1 (ET-1): is ET-1 a mediator of the luteolytic actions of PGF2α or a tonic inhibitor of progesterone secretion? Biol Reprod 2005;72:633-642

Christenson LK, Stouffer RL. Isolation and culture of microvascular endothelial cells from the primate corpus luteum. Biol Reprod 1996;55:1397-1404

Cid MC, Kleinman HK, Grant DS, Schnaper HW, Fauci AS, Hoffman GS. Estradiol enhances leukocyte binding to tumor necrosis factor (TNF)-stimulated endothelial cells via an increase in TNF-induced adhesion molecules E-selectin, intercellular adhesion molecule type 1, and vascular cell adhesion molecule type 1. J Clin Invest 1994;93:17-25


Corner GC. On the origin of the corpus luteum of the sow from both granulosa and theca interna. Am J Anat 1919;26:116-183

Curry Jr TE, Osteen KG. The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endocr Rev 2003;24:428-465


Davis JS, Rueda BR, Spanel-Borowski K. Microvascular endothelial cells of the corpus luteum. Reprod Biol and Endocrinol 2003;1:89-104

Davis JS, Weakland LL, Weiland DA, Farese RV, West LA. Prostaglandin F2a stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis and mobilizes intracellular Ca2+ in bovine luteal cells. Proc Natl Acad Sci USA 1987;84:3728-3732

Davis TL, Pate JL. Bovine luteal cells stimulate proliferation of major histocompatibility nonrestricted gamma delta T cells. Biol Reprod 2007;77:914-922

Davis WC, Ellis JA, Machugh ND, Baldwin CL. Bovine pan T-cell monoclonal antibodies reactive with a molecule similar to CD2. Immunol 1988;63:165-167

de Menezes GB, Pimenta dos Reis WG, Moreira Santos JM, Gama Duarte ID, Nogueira de Francisci, J. Inhibition of prostaglandin F2a by selective cyclooxygenase 2 inhibitors accounts for reduced rat leukocyte migration. Inflammation 2005;29:163-169


Dieleman SJ, Blankenstein DM. Progesterone-synthesizing ability of preovulatory follicles of cows relative to the peak of LH. J Reprod Fert 1985;75:609-615

Diskin MG, Murphy JJ, Sreenan JM. Embryo survival in dairy cows managed under pastoral conditions. Anim Reprod Sci 2006;96:297-311


Dong QG, Bernasconi S, Lostaglio S, DeCalmanovici RW, Martin-Padura I, Breviario F, Garlanda C, Ramponi S, Mantovani A, Vecchi A. General strategy for isolation of endothelial cells from murine tissues characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. Arterioscl Throm Vas 1997;17:1599-1604


Eduvie LO, Seguin BE. Corpus luteum function and pregnancy rate in lactating dairy cows given human chorionic gonadotropin at middiestrus. Theriogenology 1982;17:415-422


Ellis JA, Baldwin CL, Machugh N.D. Characterization by a monoclonal antibody and functional analysis of a subset of bovine T lymphocytes that express BoT8, a molecule analogous to human CD8. Immunol 1986;58:351-358


Farin CE, Moeller CL, Sawyer HR, Gamboni F, Niswender GD. Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. Biol Reprod 1986;35:1299-1308


Fields MA, Fields PJ. Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. Theriogenology 1996;45:1295-1325


Flower RJ. The role of prostaglandins in inflammatory reactions. Naunyn Schmiedebergs Arch Pharmacol 1977;297:S77-S79


Girsh E, Greber Y, Meidan R. Luteotrophic and luteolytic interactions between bovine small and large luteal-like cells and endothelial cells. Biol Reprod 1995;52:954-962

Glick B. The Avian Immune System. Avian Diseases, 1979;23:282-289


Gospodarowicza Denis, Thakral KK. Production of a corpus luteum angiogenic factor responsible for proliferation of capillaries and neovascularization of the corpus luteum Proc. Natl Acad Sci USA 1978;7:847-851

Gottshall SL, Hansen PJ. Regulation of leucocyte subpopulations in the sheep endometrium by progesterone. Immunology 1992;76:636-641


Grazul-Bilska AT, Redmer DA, Killilea SD, Kraft KC, Reynolds LP. Production of mitogenic factor(s) by ovine corpora lutea throughout the estrous cycle. Endocrinology 1992;130:3625-3632


Guy MK, Juengel JL, Tandeski TR, Niswender GD. Steady-state concentrations of mRNA encoding the receptor for luteinizing hormone during the estrous cycle and following prostaglandin F2a treatment of ewes. Endocrine 1995;3:585-589


Haniby-Flarida MD, Transk OJ, Yang TJ, Baldwin CL. Modulation of WC1, a lineage-specific cell surface molecule of γ/δ T cells, augments cellular proliferation. Immunol 1996;88:116-123


Hedin L. Invaders from the spleen: an unexpected origin of the leukocytes participating in ovulation. Endocrinology 2010;151:4096-4099

Hein WR, Mackay CR. Prominence of the γδ T cells in the ruminant immune system. Immunol Today 1991;12:30-34

Hellberg P, Thomsen P, Janson PO, Brännström M. Leukocytesupplementation increases the luteinizing hormone-induced ovulation rate in the in vitro-perfused rat ovary. Biol Reprod 1991; 44:791-797


Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, Bryant CE, Sheldon IM. Ovarian follicular cells have innate capabilities that modulate their endocrine function. Reproduction 2007;134:683-693


Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 2003;17:2205-2232


Howard CJ, Parsons KR, Jones BV, Sopp S, Pocock DH. Two monoclonal antibodies (CC17, CC29) recognizing an antigen (Bo5) on bovine T lymphocytes, analogous to human CD5. Vet Immunol Immunopathol 1988;19:127-139


Hughes FM, Pringle CM, Gorospoe, WC. Production of progestin-stimulatory factor (s) by enriched populations of rat T- and B-lymphocytes. Biol Reprod 1991;44:922-926


Itohara S, Nakanishi N, Kanagawa O, Kubo R, Tonegawa S. Monoclonal antibodies specific to native murine T-cell receptor gamma delta: analysis of gamma delta T cells during thymic ontogeny and in peripheral lymphoid organs. Proc Natl Acad Sci USA 1989;86:5094-5098


Jacob A, Hurley I, Mandel FS, Hershlag A, Cooper GW, Benoff S. Human sperm non-nuclear progesterone receptor expression is a novel marker for fertilization outcome. Mol Hum Reprod 1998;4:533-542


Johnson, WC, Bastos, RG, Davis, WC, Goff, WL. Bovine WC1-γβT cells incubated with IL-15 express the natural cytotoxicity receptor CD335 (NKp46) and produce IFN-γ in response to exogenous IL-12 and IL-18. Develop Comp Immunol 2008;32:1002-1010

Jones LS, Ottobre JS, Pate JL. Progesterone regulation of luteinizing hormone receptors on cultured bovine luteal cells. Mol Cell Endocrinol 1992;85:33-39


Kalmár L, Gergely P. Effect of prostaglandins on polymorphonuclear leukocyte motility. Immunopharmacology 1983;6:167-175


Kazemi M, Malathy PV, Keisler DH, Roberts RM. Ovine trophoblast protein-1 and boving trophoblast protein -1 are present as specific components of uterine flushings of pregnant ewes and cows. Biol Reprod 1988;39:457-463

Kelso A. Educating T cells: early events in the differentiation and commitment of cytokine-producing CD4+ and CD8+ T cells. Springer Semin Immunopathol 1999;21:231-248


Kirkham PA, Takamatsu HH, Parkhouse RM. Growth arrest of gammadelta T cells induced by monoclonal antibody against WC1 correlates with activation of multiple tyrosine phosphatases and dephosphorylation of MAP kinase erk2. Eur J Immunol 1997;27:717-725


Komori HK, Witherden DA., Kelly R, Sendaydiego K, Jameson JM, Teyton L, and Havran WL. Cutting Edge: Dendritic epidermal γδ T cell ligands are rapidly and locally expressed by keratinocytes following cutaneous wounding. J Immunol 2012;188:2972-2976


Levin TG, Powell AE, Davies PS, Silk AD, Dismuke AD, Anderson EC, Swain JR, Wong MH. Characterization of the intestinal cancer stem cell marker CD166 in the human and mouse gastrointestinal tract. Gastroenterol 2010;139:2072-2082


Lobel BL, Levy E. Enzymatic correlates of development, secretory function and regression of follicles and corpora lutea in the bovine ovary. II. Formation, development and involution of corpora lutea. Acta Endocrinol 1968;59(Suppl. 132):5-63


Lucy MC. Reproductive loss in high producing dairy cattle: where will it end? J Dairy Sci 2001;84:1277-1293

Luo W, Diaz FJ, Wiltbank MC. Induction of mRNA for chemokines and chemokine receptors by prostaglandin F2α is dependent upon stage of the porcine corpus luteum and intraluteal progesterone. Endocrinol 2011;152:2797-2805


Machelon V, Emilie D. Production of ovarian cytkines and their role in ovulation in mammalian ovary. Eur Cytokine Netw 1998;8:137-143

Machelon V, Nomé F, Emilie D. Regulated on activation normal T expressed and secreted chemokine is induced by tumor necrosis factor-α in granulosa cells from human pre-ovulatory follicle. J Clin Endocrinol Metab 2000;85:417-424


Maier R, Chew BP. Effects of monocytes and lymphocytes on progesterone secretion by granulosa cells in the pig. Theriogenology 1990; 33:1045-1056


Makris A, Olsen D, Ryan KJ. Significance of the Δ5 and Δ4 steroidogenic pathways in the hamster preovulatory follicle. Steroids 1983;42:641-651

Mamluk R, Chen D-B, Greber Y, Davis JS, Meidan R. Characterization of messenger ribonucleic acid expression for Prostaglandin F2α and luteinizing hormone receptors in various bovine luteal cell types. Biol Reprod 1998;58:849-856

Mann GE, Fray MD, Lamming GE. Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. Vet J 2006;171:500-503


Mansour I, Reznikoff-Etievant MF, Netter A. No evidence for the expression of the progesterone receptor on peripheral blood lymphocytes during pregnancy. Hum Reprod 1994;9:1546-1549


Maroni D, Davis JS. TGFβ1 disrupts the angiogenic potential of microvascular endothelial cells of the corpus luteum. J Cell Sci 2011;124:2501-2510


Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing γδ T cells selectively expand in response to pathogen products and environmental signals. Immunity 2009;31:321-330


Matalka KZ. The effect of estradiol, but not progesterone, on the production of cytokines in stimulated whole blood, is concentration-dependent. Neuro Endocrinol Lett. 2003;24:185-191


McCracken J. Prostaglandin F2α and corpus luteum regression. Ann NY Acad Sci 1971;180:456-469


Mesri M, Liversidge J, Forrester JV. Prostaglandin E2 and monoclonal antibody to lymphocyte function-associated antigen-1 differentially inhibit migration of T lymphocytes across microvascular retinol endothelial cells in the rat. Immunology 1996;88:471-447


Milvae RA, Hansel W. Prostacyclin, prostaglandin F2a and progesterone production by bovine luteal cells during the estrous cycle. Biol Reprod 1983;29:1063-1068


Morichika T, Takahashi HK, Iwagaki H, Yagi T, Saito S, Kubo S, Yoshino T, Akagi T, Mori S, Nishibori M, Tanaka N. Effect of prostaglandin E2 on intercellular adhesion molecules-1 and B7 expression in mixed lymphocyte reaction. Transplantation 2003;75:2100-2105


Morrison WI, Davis WC. Differentiation antigens expressed predominantly on CD4-CD8-T lymphocytes (WC1, C2). Vet Immunol Immunopathol 1991;27:71-76

Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Ann Rev Immunol 1989;7:145-173

Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;17:138-146

Murakami K, Ma W, Fuleihan R and Pober JS. J Human endothelial cells augment early CD40L expression in activated CD4+ cells through LFA-3-mediated stabilization of mRNA. Immunology 1999;163:2667-2673


Murdoch WJ. Effect of a steroidal (prednisolone) and nonsteroidal (indomethacin) anti-inflammatory agent on ovulation and follicular accumulation of prostaglandin F2α in sheep. Prostaglandins 1989;37:331-334


Mwangi DM, Mahan SM, Nyanjui JK, Taracha ELN, and Mckeever DJ. Immunization of cattle by infection with cowdria ruminantium elicits T lymphocytes that recognize
autologous, infected endothelial cells and monocytes. Infect Immun 1998; 1855-1860


Nagaosa K., Shiratsuchi A, Nakanishi Y. Determination of cell type specificity and estrous cycle dependency of monocyte chemoattractant protein-1 expression in corpora lutea of normally cycling rats in relation to apoptosis and monocyte/macrophage accumulation. Biol Reprod 2002;67:1502-1508


Ndiaye K, Poole DH, Pate JL. Expression and regulation of functional oxytocin receptors in bovine T lymphocytes. Biol Reprod 2008;78:786-793


Nilsen EM, Johansen FE, Jahnsen FL, Lundin KE, Scholz T, Brandtzaeg P, Haraldsen G.


Nothnick WB, Pate JL. Interleukin-1 beta is a potent stimulator of prostaglandin synthesis in bovine luteal cells. Biol Reprod 1990;43:898-903


O'Keeffe MA, Metcalfe SA, Glew MD, Bowden T, McInnes S, Kimpton WG, Cahill RN, Heine WR, Walker, ID. Lymph node homing cells biologically enriched for gamma delta T cells express multiple genes from the T19 repertoire. Int Immunol 1994;6:1687-1697


Olson KK, Anderson LE, Wiltbank MC, Townson DH. Actions of prostaglandin F2α and prolactin on intercellular adhesion molecule-1 expression and monocyte/macrophage accumulation in the rat corpus luteum. Biol Reprod 2001;64:890-897

Olson KK, Anderson LE, Wiltbank MC, Townson DH. Actions of prostaglandin F2α and prolactin on intercellular adhesion molecule-1 expression and monocyte/macrophage accumulation in the rat corpus luteum. Biol Reprod 2001;64:890-897


O'Shea JD, Nightingale MG, Chamley WA. Changes in small blood vessels during cyclical luteal regression in sheep. Biol Reprod 1977;17:162-177


O'Shea JD, Rodgers RJ, Wright PJ. Cellular composition of the sheep corpus luteum in the mid- and late luteal phases of the oestrous cycle. J Reprod Fertil 1986;76:685-691

O'Shea, JD, Cran, DG, Hay, MF. Fate of the theca interna following ovulation in the ewe. Cell Tissue Res 1980;210:305-319


Palacios EH, Oncogene WA. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene 2004;23:7990-8000


Pate JL, Condon WA. Effects of prostaglandin F2$\alpha$ on agonist-induced progesterone production in cultured bovine luteal cells. Biol Reprod 1984;31:427-435


Pate JL, Condon WA. Regulation of steroid and cholesterol synthesis by prostaglandin F2$\alpha$ and lipoproteins in bovine luteal cells. J Reprod Fert 1989;87:439-446

Pate JL, Keyes PK. Immune cells in the corpus luteum: friends or foes? Reprod 2001;122:665-676

Pate JL, Nephew,KP. Effects of in vivo administration of prostaglandin F2$\alpha$ on lipoprotein utilization in cultured bovine luteal cells. Biol Reprod 1988;38:568-576


Pate JL. Intercellular communication in the bovine corpus luteum. Theriogenology 1996;45, 1381-1397

Pate JL. Isolation and culture of fully differentiated bovine luteal cells. Meth Toxicol 1993;3B:360-370


Pepperell JR, Wolcott K, Behrman HR. Effects of neutrophils in rat luteal cells. Investigations concerning the relationship of ovarian eosinophilia to ovulation and luteal function in the sheep. Endocrinology 1992;130:1001-1008


Pollock JM, Welsh MD. The WC1â– gd T-cell population in cattle: a possible role in resistance to intracellular infection. Vet Immunol Immunopath 2002;89:105-114

Poole DH, Pate JL. Luteal microenvironment directs resident T lymphocyte function in cows. Biol Reprod 2012;86:29


Price SJ, Hope JC. Enhanced secretion of interferon-gamma by bovine gammadelta T cells induced by coculture with Mycobacterium bovis-infected dendritic cells: evidence for reciprocal activating signals. Immunology 2009;126:201-208

Priedkalns J, Weber AF, Zemjanis R. Qualitative and quantitative morphological studies of the cells of the membrana granulosa, theca interna and corpus luteum of the bovine ovary. Zeitschrift ffor Zellforschung 1968;85:501-520

Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 1995;44:915-923


Reynolds LP, Redmer DA. Expression of the angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in the ovary. J Anim Sci 1998;76:1671-1681


Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol 2011;31:986-1000

Rincón M, Flavell RA, Davis RJ. Signal transduction by MAP kinases in T lymphocytes. Oncogene 2001;20:2490-2497


Rodger FE, Young FM, Fraser HM, Illingworth PJ. Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic, gonadotrophin rescue, in the human corpus luteum. Hum Reprod 1997;12:1723-1729


Rodgers RJ, O'Shea JD, Findlay JK. Progesterone production in vitro by small and large ovine luteal cells. J Reprod Fert 1983;69:113-124


Rodgers RJ, O'Shea JD, Findlay JK. Do small and large luteal cells of the sheep interact in the production of progesterone? J Reprod Fertil 1985;75:85-94
Rogers AN, VanBuren DG, Hedblom EE, Tilahun ME, Telfer JC, Baldwin CL. Function of ruminant gammadelta T cells is defined by WC1.1 or WC1.2 isoform expression. Vet Immunol Immunopathol 2005b;108:211-217

Rogers AN, Vanburen DG, Hedblom EE, Tilahun ME, Telfer JC, Baldwin CL. Gammadelta T cell function varies with the expressed WC1 coreceptor. J Immunol 2005a;174:3386-3393


Savage CO, Hughes CC, McIntyre BW, Picard JK, Pober JS. Humand CD4+ T cells proliferate to HLA-DR+ allogeneic vascular endothelium: Identification of accessory interactions. Transplantation 1993;56:128-134

Sawyer HR, Niswender KD, Braden TD, Niswender GD. Nuclear changes in the ovine luteal cells in response to PGF2a. Dom Anim Endocrinol 1990;7:229-238


Schust DJ, Anderson DJ, Hill JA. Progesterone-induced immunosuppression is not mediated through the human progesterone receptor. Hum Reprod 1996;11:980-985


Shibuya E, Masuda K, Izawa Y. Prostaglandin E1, F2 and F2a inhibit migration of polymorphonuclear in the rabbit in a non-chemotactic directional fashion. Prostaglandins 1976;12:165-174


Skarzynski DJ, Ferreira dias G, Okuda K. Regulation of luteal function and corpus luteum regression in cows: hormonal control, immune mechanisms and intercellular communication. Reprod Dom Anim 2008;43:57-65


Smith CJ, Greer TB, Banks TW, Sridaran R. The response of large and small luteal cells from the pregnant rat to substrates and secretagogues. Biol Reprod 1989;41:1123-1132

Smith GW, Gentry PC, Roberts RM, Smith MF. Ontogeny and regulation of luteinizing hormone receptor messenger ribonucleic acid within the ovine corpus luteum. Biol Reprod 1996;54:76-83


Spanel-Borowski K, van der Bosch J. Different phenotypes of cultured microvessel endothelial cells obtained from bovine corpus luteum. Study by light microscopy and by scanning electron microscopy (SEM). J Cell Science 1993;106:879-890


Spitschak M, Vanselow J. Bovine large luteal cells show increasing de novo DNA methylation of the main ovarian CYP191A promoter P2. Gen Comp Endocrinol 2012;178:37-45


Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. Science, 2002;295:338-342


Takamatsu HH, Kirkham PA, Parkhouse RM. A gamma delta T cell specific surface receptor (WC1) signaling G0/G1 cell cycle arrest. Eur J Immunol 1997;27:105-110


Tanaka H, Obata K. Developmental changes in unique cell surface antigens of chick embryo spinal motoneurons and ganglion cells. Devel Biol 1984;106:26-37


Townson DH, Pate JL. Regulation of prostaglandin synthesis by interleukin-1 beta in cultured bovine luteal cells. Biol Reprod 1994;51:480-485


lymphocytes and their possible roles in normal and tubal pregnancy oviducts.
Human Reprod 2006;21:2281-2289


van Oers NS, Killeen N, Weiss A. ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR zeta in murine thymocytes and lymph node T cells. Immunity 1994;1:675-685


Watson ED, Sertich PL. Secretion of prostaglandins and progesterone by cells from corpora lutea of mares. J Reprod Fert 1990;88:223-229


Wen L, Barber DF, Pao W, Wong SF, Owen MJ, Hayday A. Primary gamma delta T cell clones can be phenotypically and functionally as Th1/Th2 Cells and illustrate the association of CD4 with Th2 differentiation. J Immunol 1998;160:1965-1974

Whitehead S, Lacey M. Inhibitory effect of peritoneal macrophages on progesterone release from cocultured rat granulosa cells is reversed by dexamethasone: evidence for an action independent of nitric oxide and distal to cyclic adenosine 3’5’-monophosphate generation. Biol Reprod 1996;54:1317-1325


Wijngaard PLJ, Metzelaar MJ, Machugh ND, Morrison WI, Clevers HC. Molecular characterization of the WC1 antigen expressed specifically on bovine CD4-CDS-γδ T lymphocytes. J Immunol 1992;10:3273-3277


Wilson E, Aydintug MK, Jutila MA. A circulating bovine γδ T cell subset, which is found in large numbers in the spleen, accumulates inefficiently in an artificial site of inflammation: correlation with lack of expression of E-selectin ligands and L-selectin. J Immunol 1999;162:4914-4919


Woods DC, Johnson AL. Protein kinase C activity mediates LH-induced ErbB/Erk signaling in differentiated hen granulose cells. Reproduction 2007;133:733-741


Wu R, Van der Hoek KH, Ryan NK, Norman RJ, Robker RL. Macrophage contributions to ovarian function. Hum Reprod Update 2004;10:119-133


Zhao XJ, McKerr G, Dong Z, Higgins CA, Carson J, Yang ZQ, Hannigan BM. Expression of oestrogen and progesterone receptors by mast cells alone, but not lymphocytes, macrophages or other immune cells in human upper airways. Thorax 2001;56:205-211


Zheng W-P, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell, 1997;89:587-596


Zhu Y, Bond J, Thomas P. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. PNAS 2003a;100:2237-2242
Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. PNAS 2003b;100:2231-2236

Appendix

Endothelial cells isolation protocol

Materials

A. Equipment
   1. Centrifuge
   2. Flow cytometer
   3. Heater/water bath
   4. Hood/Sterile environment

A. Lab supplies
   1. Micro sieves 50, 15 and 10 μm (BioDesign Inc. of New York)
   2. Sieve holders (BioDesign Inc. of New York)
   3. 50 mL polypropylene conical tubes (Fisher)
   4. 10 mL pipette
   5. 100-200 mL Beakers
   6. Graduated spinner flask

B. Biological reagents
   1. Hams-F-12 media (Invitrogen)
   2. BS-1 lectin (Sigma)
   3. Anti-vWF antibodies (Dako)
   4. Ficoll-Paque plus (GE Healthcare)

C. Chemicals and solutions
   1. Tissue dissociation media
      a) 200 mL Hams-F12 media (Invitrogen)
      b) 1g Bovine serum albumin (Sigma)
      c) 400 μL Gentamycin (10 mg/mL) (Sigma)
   2. Washing media
      a) 200 mL hams-F12 media
      b) 1g Gentamycin (10 mg/mL)
3. 1xPBS+2mM EDTA, PH=7.4
   a) 8g NaCl
   b) 0.2g KCl
   c) 1.44g Na₂HPO₄
   d) 0.24g KH₂PO₄
   e) 4 mL 2mM EDTA
   f) Milli Q water-IL

4. Red blood cell lysis buffer (PH 7.4)
   a) 0.14 M NH₄Cl,
   b) 10 mM KHCO₃,
   c) 0.1 mM EDTA,

Procedure
Luteal tissue dissociation

Enzymatic dissociation of luteal tissue follows the procedures described by Pate (1993). Briefly, Luteal tissue was weighed, sliced, minced and incubated in Ham’s F-12 media with collagenase at 37°C for 2 hrs to disperse luteal cells. Medium containing dispersed cells was filtered through a 50 µm pore size nylon microsieve supported by a plastic sieve holder. The filtrate was serially centrifuged at 200xg, 128xg, 72xg and 32xg for 10 min at 4°C each time to pellet luteal steroidogenic cells (LSCs). The supernatant after each centrifugation round was collected in 50 mL polypropylene tubes (always kept on ice) and used for endothelial cell isolation by size exclusion.

Filtration

1. Collect of supernatants obtained from washing luteal cells
2. Centrifuge at 200xg and collect pellets.
3. Resuspend pellet in PBS-EDTA in a 50 mL polypropylene tube and vigorously shake to yield a single cell suspension
4. Place a 15 µm microsieve on to a sieve holder and create a conical shape by pressing inward with a sterile pipette.
5. Place the sieve holder on top of a beaker (to collect the filtrate) and wet it with a small volume of PBS-EDTA.

6. Carefully pour the cell suspension in the 50 mL polypropylene tube onto the sieve and gently swirl the sieve holder to ensure that all the cell suspension goes through the sieve. Allowing the sieve to touch the ends of the beaker helps increase the rate of flow through.

7. Using a similar set up (d-f), but with 10 μm microsieve, filter the cell suspension once again.

8. Transfer the filtrate to 50 mL polypropylene tubes and centrifuge at 250xg.

9. To remove red blood cells add freshly prepared red blood cell lysis buffer and incubate on ice for 5 minutes. Add an equal volume of washing media at the end of the incubation and centrifuge at 250xg. Also, a ficoll density gradient can be used to remove red blood cells. In this case, layer 4 mL of filtrate onto 3 mL of ficoll density gradient and centrifuge at 650xg at room temperature (RT) for 30 minutes.

10. Count cells using a haemocytometer or flow cytometer (Guava viaaccount dye).

   Note: The number of endothelial cells recovered can be increased by the procedures to the luteal cell pellet (A) since aggregates of endothelial cells sediment with larger cells.
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2008-2012  Graduate Assistant, Pennsylvania State University
2008-2010  Teaching Assistant, Faculty of Agriculture, Makerere University
2007-2008  Graduate Research Associate, The Ohio State University
2005-2007  Graduate Research Associate, The Ohio State University
2004-2005  Lecturer, Faculty of Agriculture Busoga University


Publications
Pate JL and Walusimbi S. Gamma-delta T cell subsets differentially respond to bovine luteal cells. Abstract 2012, SSR, August 12th-15th, State College, PA, USA.
Walusimbi S & Pate JL. Luteal cells establish contact with T lymphocytes via the T cell receptor and induce deletion of γδ+ T cells with a proinflammatory phenotype. Abstract 2011, SRR, August 1st-4th, Portland, Oregon, USA.