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ROLE OF LUTEAL CELL-DERIVED EXOSOMES IN COMMUNICATION WITH IMMUNE
CELLS

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by
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Abstract

The corpus luteum (CL) is a vital organ for the production of progesterone (P_4) which maintains pregnancy in ruminants. Immune cells infiltrate the CL and increase in number during luteal regression. These immune cells communicate with luteal cells to either sustain the CL for maintenance of pregnancy or promote regression of the CL, resulting in progression to the next estrous cycle. Luteal cell communication with T lymphocytes (T cells) can program the T cell responses, and luteal cells from fully functional CL can activate T cells through paracrine mediators. The mediators of this communication are not well understood. To investigate these interactions, we hypothesized that luteal cells secrete extracellular vesicles, such as exosomes, and that these exosomes serve as a means of communication from luteal steroidogenic cells to resident immune cells. Exosomes are 15-100 nm vesicles released from cells that contain protein, miRNA, and mRNA, which can later function of target cells. Currently, there is no research on the role of exosomes in the CL or the interaction between luteal cell-derived exosomes and the immune system. Thus, the objective of the first study was to determine an optimal method for the isolation of exosomes from luteal cell-conditioned media. Luteal cells were isolated and cultured for 48 hours. Media was collected, concentrated and exosomes were isolated by three methods: ultracentrifugation and two commercial exosome isolation reagents. Analysis of exosomes using electron microscopy revealed the expected cup-shaped morphology. Concentration of exosomes isolated from regressing luteal cell-conditioned media was less than from midcycle luteal cell-conditioned media.

An experiment was conducted to determine if luteal cell-derived exosomes affected T cell function by interacting with surface markers on T cells or internalization. To determine if T cells can internalize luteal cell-derived exosomes, luteal cell-derived exosomes were co-cultured with

T cells and analyzed using FlowSight imaging cytometry and confocal microscopy. This experiment revealed that the majority of T cells internalized exosomes.

Because T cells internalize exosomes, a study was conducted to determine the effect of exosomes on T cell function. A preliminary dose-response experiment revealed an increase in proliferation when increasing numbers of exosomes were added. The maximum T cell proliferation achieved was 23%. T cells were also collected and their phenotype was determined by quantitative polymerase chain reaction (qPCR) for expression of TH1 (T helper cell 1) and TH2 (T helper cell 2) mRNA expression. Midcycle luteal cell-derived exosomes increased TNF and decreased IL10 mRNA expression while regressing luteal cell-derived exosomes decreased TNF, IFNG, IL4 and IL10 mRNA expression.

Finally, an experiment was conducted to determine if luteal-derived exosomes affect monocyte differentiation and if so, determine the characteristics of the macrophages that arise. Midcycle or regressing luteal cell-derived exosomes were added to PBMCs along with GM-CSF or M-CSF and mRNA expression for macrophage cell type-specific genes was determined. Midcycle luteal cell-derived exosomes drove monocyte differentiation into macrophages that were characterized by highly significant expression of TNF, IL1B, NOS2, CD36, IL10 and TGF β while regressing luteal cell-derived exosomes induced greater expression of TNF, NOS2, CCL24 and IL10.

In summary, bovine luteal cells from both physiological states released exosomes into culture media with luteal cells from regressing CL releasing fewer exosomes than midcycle luteal cells. Exosomes were internalized into T cells, and midcycle luteal cell-derived exosomes induced T cell proliferation. Also, midcycle luteal cell-derived exosomes were able induce a

TH1 phenotype while regressing exosomes down regulated all T cell markers measured. Finally, midcycle and regressing luteal cell-derived exosomes drove monocyte differentiation that was dependent on both prior priming toward M1 or M2 and on source of the exosomes. In conclusion, bovine luteal cells release exosomes that may facilitate communication with immune cells and provide an alternative method to direct cell-cell contact by which luteal cells communicate with immune cells to control the luteal microenvironment.

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Chapter 1: Review of Literature Introduction

Reproductive efficiency has declined over the past fifty years (Lopez-Gatius, 2003) and although early embryonic loss is difficult to measure, it is one of the primary contributors to reduced conception rates (Diskin et al., 2006). Reduced fertility leads to a greater number of days open, longer calving intervals, and more services before a successful pregnancy can be established (Gröhn and Rajala-Schultz, 2000). Inskeep and Dailey (2005) have estimated that 57% of reproductive loss in dairy cattle occurs before the onset of placentation. During this key period in early pregnancy, conceptus signaling is essential for rescuing the corpus luteum (CL) from regression and maintaining pregnancy. The CL, which secretes large amounts of progesterone (P₄), is essential for the establishment and continuation of pregnancy in all mammals. Development of the early CL requires extensive tissue remodeling and angiogenesis to achieve a functional CL. In the absence of pregnancy, the CL regresses in response to uterine-derived prostaglandin F₂ alpha (PGF_{2α}). In ruminants, if successful fertilization has occurred, the elongating embryo secretes interferon tau (IFNT), which rescues the CL from regression (Knickerbocker et al., 1986). Immune cells are present in the CL throughout its lifespan and play an important role in regulating luteal function.

Immune cells infiltrate the CL, but the role of immune cells in the fully functional CL has not been elucidated. Luteal cell communication with T lymphocytes (T cells), can program the T cell responses (Walusimbi and Pate, 2014), and luteal cells from fully functional CL can activate T cells through paracrine mediators (Petroff et al., 1999). We hypothesized that luteal cells secrete extracellular vesicles (EVs), such as exosomes, and that these exosomes serve as a means of communication from luteal steroidogenic cells to resident immune cells. Exosomes have been studied in many species during various physiological states, including events related to

reproduction, but the role of exosomes in the CL has not been investigated. The objective of this study was to isolate bovine luteal cell-derived exosomes, determine the effects of exosomes on T cells and determine if luteal cell-derived exosomes affect macrophage polarization. The long term goal of this project was to determine the interaction between luteal cells and immune cells and how these interactions may modulate the CL during critical events such as maintenance and regression.

The Estrous Cycle

Ruminants have estrous cycles that are characterized by relatively short periods of sexual receptivity with long luteal phases. Cattle have an estrous cycle of approximately 21 days and are polyestrous, meaning they are in heat many times a year. The estrous cycle can be divided into two distinct phases that are named after the dominant structures present on the ovary: the follicular phase (proestrus and estrus) and the luteal phase (metestrus and diestrus). The follicular phase begins with the regression of the CL and ends with ovulation (Dieleman et al., 1983). This phase is short and comprises about 20% of the estrous cycle. During this phase the primary reproductive steroid is estrogen (E_2), which is produced by the growing, dominant follicle. The luteal phase begins after ovulation and ends with the regression of the CL. This phase is much longer and comprises about 80% of the estrous cycle. During this period, the dominant ovarian structure is the CL and the primary reproductive hormone is P_4 . Follicles continue to grow and regress during this period but they do not reach dominance because of the inhibitory action of P_4 .

Follicular phase

The follicular phase, which includes proestrus and estrus, is characterized by increasing frequency of pulses of gonadotropin releasing hormone (GnRH) from the hypothalamus followed by secretion of follicle stimulation hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Moenter et al., 1991). During proestrus the stimulated follicle matures and increasing amounts of E_2 are secreted from the dominant follicle. The follicular phase begins with the regression of the CL (Short, 1961). This drop in P_4 causes FSH and LH to increase in response to GnRH. The increase in FSH and LH stimulates increased production of E_2 by ovarian follicles (Moore et al., 1969).

Proestrus lasts from 2 to 5 days and is characterized by a major transition from a period of P_4 dominance to a period of E_2 dominance. During this period, follicles are recruited for ovulation and the female reproductive system prepares for the onset of estrus and mating. The initial recruitment of follicles is dependent on increasing concentrations of FSH (Dobson, 1978). Follicles produce E_2 and inhibin that suppresses FSH secretion from the anterior lobe of the pituitary. When estrogen concentration is sufficiently high, it signals the surge center (preovulatory center of the hypothalamus) to release large quantities of GnRH, which stimulates the secretion of LH. This surge of LH is about 10 times greater than tonic LH concentrations (Mondal et al., 2006).

Although the follicular phase comprises only about 20% of the estrous cycle, the process of follicular growth and atresia occurs continuously throughout the estrous cycle. Antral follicles of various sizes develop in response to tonic concentrations of LH and FSH (Dobson, 1978). These antral follicles are always present and vary in size from small, medium, or large.

During follicular development, LH binds to LH-specific membrane receptors located on the cells of the theca interna of the developing follicle. The binding of LH to its receptors activates a cascade of events involving the conversion of cholesterol to testosterone. Testosterone then diffuses out of the cells of the theca interna and enters the granulosa cells. The granulosa cells contain receptors for FSH. When FSH binds to its receptor it causes the conversion of testosterone to E_2 . This two-cell, two-gonadotropin pathway continues to function until concentrations of estrogen increase to a threshold that induces the preovulatory LH surge. In the early 1960's, the "Two-Cell" theory was first suggested by R.V. Short (1961). In 1976, Moor expanded this theory by utilizing a culture system to show that theca cells were the source of follicular androgens and interactions with the granulosa cells were necessary for the production of estrogens.

The maturation of antral follicles occurs in four phases: recruitment, selection, dominance, and atresia. Recruitment is the phase of follicular development in which a cohort of small antral follicles begins to grow and produce E_2 (Hirshfield, 1991). Some of the recruited follicles undergo atresia (degeneration) while the other follicles that do not degenerate are selected. In the cow, only a single follicle is selected to become dominant. This selected follicle produces increasing amounts of E_2 as well as inhibin (McCullagh, 1932; Jong and Sharpe, 1976). Inhibin is a protein hormone produced by antral follicle that selectively inhibits the release of FSH from the anterior pituitary (L' Hermite et al., 1972) while LH continues to rise. This inhibition of FSH results in inhibition of smaller follicles, assuring selection of a single follicle to ovulate. Inhibin was initially described by McCullagh in 1932 but it was difficult to isolate. Direct evidence of inhibin did not appear until the 1970's (Vaze et al., 1979) and it was not isolated until 1985 by Roberson et al.

Luteal phase

The luteal phase is dominated by the CL and lasts from the time of ovulation until luteolysis. The luteal phase includes metestrus and diestrus. When peripheral estradiol concentrations reach a threshold, neurons in the preoptic area are triggered to release a surge of GnRH, which results in a high-amplitude surge release of LH leading to ovulation of the dominant follicle (reviewed by Richards et al., 2002). For a follicle to ovulate, concentrations of progesterone must be low ($<1\text{ng/ml}$; Ireland and Roche, 1982) allowing for increased frequency of LH pulses. Ovulation involves the remodeling of the ovarian surface, which results in the release of the oocyte into the oviduct to be fertilized. The process of ovulation resembles a localized inflammatory response with an increase in vascularity, swelling, migration of immune cells into preovulatory follicles, and tissue reorganization (Espey, 1980). In cattle, ovulation occurs approximately 24 hours after the LH surge (Dieleman et al., 1983).

During ovulation, the walls of the follicle collapse and create folds which cause the theca and granulosa cells to mix (Corner, 1919). After ovulation the theca interna and granulosa cells of the follicle undergo a dramatic transformation. The basement membrane that separated the granulosa and theca cells degenerates and the two cell types intermingle and undergo luteinization in response to LH, becoming the large and small luteal cells, respectively (Stocco et al., 2007). Granulosa cells develop into large luteal cells and theca interna cells develop into small luteal cells (Donaldson and Hansel, 1965). Large luteal cells measure 20-70 μm and secrete P_4 . Small luteal cells measure less than 20 μm and secrete P_4 and androgens (Weber et al., 1987).

Corpus Luteum

When the dominant follicle ruptures at ovulation, blood vessels within the follicular wall rupture, resulting in a bloody clot-like appearance called the corpus hemorrhagicum (CH). The CH can be observed from the time of ovulation until about day 3 of the estrous cycle. About days 3-5, the CH transforms into the CL, which will increase in size and produce increasing amounts of P₄. The corpus luteum is one of the most rapidly growing tissues in the body (Reynolds et al., 1994) and in 1906, Loeb suggested that the CL closely resembles a 'transitory tumor' with the rate of luteal growth equal to that of the fastest growing tumors.

The CL is a transient endocrine organ that consists of endothelial cells (EC), large and small steroidogenic cells, fibroblasts, smooth muscle cells and immune cells (O'Shea et al., 1989). The CL undergoes three physiological states: development (luteinization), maintenance (luteotropism), and regression (luteolysis). Development of the early CL requires extensive tissue remodeling, rapid angiogenesis and reprogramming of gene and protein expression to achieve a functional CL. The CL produces P₄ during the maintenance state, which has numerous functions including suppression of ovulation, maintenance of pregnancy and suppressive effects on immune cells (Spencer and Bazer, 2004). In the absence of pregnancy, the CL will rapidly regress due to pulsatile secretion of PGF_{2α} from the uterus. This rapid regression involves the loss of steroidogenic capacity by luteal steroidogenic cells. 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)

The principal hormone that stimulates P₄ production by the small luteal cells is LH (Niswender and Nett, 1988). Most of the LH receptors are located on small luteal cells.

Luteinizing hormone binds to the LH receptor which activates protein kinase (PKA). Activation of PKA increases synthesis of StAR (steroid acute regulatory protein; Sugawara et al., 1995), the activity of cytochrome P450_{scc} and 3 β -HSD (3- β -hydroxysteroid dehydrogenase) which increases P₄ secretion (Niswender et al., 2000). Progesterone (pregn-4-ene-3,20-dione) is a steroid hormone essential for pregnancy in ruminants. Cholesterol undergoes oxidation to produce 20, 22-dihydroxycholesterol (Niswender et al., 2000). This product is further oxidized to produce pregnenolone. This reaction is catalyzed by cytochrome P450_{scc} (CYP11A1). Next, pregnenolone is converted to progesterone by oxidizing the 3-hydroxyl group to a ketone group. This reaction is catalyzed by 3-beta-hydroxysteroid dehydrogenase/delta-deltaisomerase (Dewick, 2002).

Large luteal cells are responsible for 80% of the total P₄ production by the CL (Niswender et al., 1985). Receptors for growth hormone are located primarily on large luteal cells (Lucy et al., 1993). Growth hormone (GH) was shown to stimulate P₄ and oxytocin secretion by bovine luteal cells in vitro (Liebermann and Schams, 1994) and to support luteal development in vivo (Lucy et al., 1994; Juengel et al., 1997).

Luteolysis in ruminants and other domestic animals is caused by pulsatile release of PGF_{2 α} from the uterus (McCracken, 1971). Prostaglandin F_{2 α} secretion by the bovine CL is regulated by many factors such as interleukin 1 beta (IL1B) (Pate and Townson, 1994), TNF (Sakumoto et al., 2000), and growth factors FGF2 and IGF1 (Schams et al., 1995). Luteolysis defines the end of the luteal phase (days 17-19) when the CL loses its functional integrity and drastically decreases in size. During the late luteal phase, if the pregnancy recognition signal is not present, PGF_{2 α} is released from the uterus and causes luteal regression and P₄ concentrations

decline rapidly. A regressed CL will become a corpus albicans (CA), which appears as a white scar-like structure.

Immune cells in the CL

The immune system is involved in optimal function of the reproductive system, including events related to ovulation and maintenance of function. Immune cells and their cytokine products have powerful local effects within body tissues. The immune system is known to regulate ovarian function (Bukovsky et al., 1977) and leucocytes, present in ovarian tissue, modulate the immune system via local secretion of cytokines (Petroff et al., 1999).

Macrophages have a wide range of functions including phagocytosis, antigen presentation, and secretion of diverse cytokines, growth factors, and tissue-remodeling agents (Gordon, 2007). Macrophages exhibit a high level of phenotypic plasticity and are involved in diverse physiological processes, including the innate immune system (Wynn and Barron, 2010), defense against external pathogens (Galli et al., 2011), removal of apoptotic cells (Gordon and Taylor, 2005) and angiogenesis (Biswas and Mantovani, 2010). Monocytes and macrophages belong to the myeloid lineage of leucocytes. Monocytes originate from bone marrow progenitor cells and travel through the circulation to specific tissues where they differentiate into macrophages depending on the tissue microenvironment (Mooser and Edwards, 2008). There are considerable differences and adaptabilities in macrophage phenotypes, and their differentiation and function within tissues is influenced by local environmental signals (Sunderkotter et al., 1994).

T lymphocytes (T cells) are a type of white blood cell that plays a role in cell-mediated immunity. T cells mature in the thymus and are characterized by their distinct function. T helper cells (CD4⁺) assist other white blood cells in immunological processes such as the activation of cytotoxic T cells (CD8⁺) and macrophages and recognize their targets by binding to MHC class II molecules (Mosmann and Coffman, 1989). Cytotoxic T cells are involved in destroying virus-infected cells and they recognize their targets by binding to MHC class I molecules. T regulatory cells (T regs) maintain tolerance to self-antigens and generally suppress or down-regulate induction and proliferation of effector T cells. T reg cells have been demonstrated in several animal model systems and in humans to be essential for suppressing destructive alloantigenic immunity in pregnancy (Aluvihare et al., 2004).

Lobel and Levy (1968) first described the presence of white blood cells in the bovine corpus luteum. They observed that lymphocytes were present in the connective tissue surrounding the luteal vasculature on day 14 of the estrous cycle and infiltrated the luteal cells on days 15–17. In the developing bovine CL granulocytes 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). Macrophages and monocytes, on the other hand, are fewer during luteal development, but are greater in number in midcycle and regressing CL (Penny et al., 1999; Lawler et al., 1999; Townson et al., 2002).

Espey (1980) compared mammalian ovulation to a localized inflammatory reaction, because of the similarities in production of the inflammatory mediators. The process of ovulation causes leakage in the vasculature, recruitment of leukocytes and production of prostaglandins (Salmon and Higgs, 1987). Prostaglandin F_{2α} induces intracellular events such as activation of phospholipase C (PLC), protein kinase C (PKC), and prostaglandin production by prostaglandin

endoperoxide synthase (PGS) that contribute to luteal regression. The preovulatory follicle expresses increased PGE₂, interleukin 8, monocyte chemoattractant protein-1 (Arici et al., 1997), macrophage migration inhibitory factor (Wada et al., 1999), platelet endothelial cell adhesion molecule (PECAM-1) and intercellular adhesion molecule-1 (ICAM-1; Oakley et al., 2010), which are thought to facilitate leukocyte infiltration. Pro-inflammatory cytokines such as TNF, IL-1B, and IFNG are involved in bovine luteolysis (Neuvians et al., 2004; Korzekwa et al., 2008).

Endothelial cells from the bovine CL express type1 TNF receptors (Okuda et al., 1999) and there is evidence that the combination of TNF and IFNG is cytotoxic (Petroff et al., 2001; Taniguchi et al., 2002). Sakumoto et al., (2000) showed that TNF concentrations increase during the late-luteal phase (days 13–18) of the bovine estrous cycle. Neuvians et al., (2004) showed that TNF, IFNG, IL1B, NOS2 and FGF2 were upregulated during induced luteolysis in cattle. Monocyte chemoattractant protein-1 (MCP1) is a chemokine produced in the bovine CL during regression (Townson et al., 2002) that has the potential to facilitate attachment and migration of immune cells (monocytes, macrophages and T-lymphocytes) from the bloodstream into the CL. The results from these studies showed that cytokines are involved not only in structural, but also in functional luteolysis.

Leukocytes are present in the CL of many species including rodents (Paavola, 1979), cows (Lobel and Levy, 1968), sheep (Cavender and Murdoch, 1988), horses (Lawler et al., 1999), pigs (Hehnke et al., 1994), and primates (Adams and Hertig, 1969). In functional CL, 25% of the T lymphocytes were T helper cells (CD4+), 45% were cytotoxic T cells (CD8+), and 30% were gamma delta ($\gamma\delta$) T cells, with no change in these proportions during luteolysis (Poole and Pate, 2012). There are a greater number of immune cells in the late luteal phase and

regressing CL (Bukovsky et al., 1995; Best et al., 1996), which are hypothesized to assist in luteolysis. A study by Petroff et al. (1997) demonstrated that luteal cells isolated from regressing CL induced greater T cell activation than midcycle luteal cells.

Macrophages are the most abundant immune cells in the ovary. In the ovary, macrophages accumulate in the theca layer of developing follicles, and there is evidence that macrophages play a functional role in follicle growth, ovulation, and vascular homeostasis (Turner et al., 2014). Mice that have a mutation in the gene for colony stimulating factor have fewer macrophages after the induction of ovulation (Watanabe et al., 1997). After ovulation, macrophages migrate into the developing corpus luteum in mouse (Cohen et al., 1997), rat (Brannstrom et al., 1994), cow (Penny et al., 1999), pig (Standaert et al., 1991) and human ovaries (Brannstrom et al., 1994). Macrophage infiltration influences the steroidogenic function of luteal cells by the secretion of various cytokines, as well as remodeling after luteal regression by phagocytosis (Kirsh et al., 1981). Ducann et al., (1998) showed that luteal macrophages increased in number throughout the luteal phase of women to a maximum in the late-luteal phase. Luteal rescue with HCG was correlated with a significant reduction in the number of macrophages when compared with those of the late-luteal phase. Inflammatory cytokines, T cells and macrophages increase during regression of the CL (Penny et al., 1999). During late luteal regression, 70% of all proliferating cells in the bovine CL are CD14⁺ macrophages (Bauer et al., 2001).

Shirasuna and Miyamoto (unpublished observation, reviewed in 2013) investigated the expression of M1 (classically activated, pro-inflammatory) and M2 macrophages (alternatively activated, anti-inflammatory) in the CL by expression of CD40 (marker of M1 macrophages) and CD163 (marker of M2 macrophages). They showed that CD163⁺ macrophages were

predominant in developing CL, whereas the CD40⁺ macrophages were predominant in regressing CL. Although this study suggests that M2 macrophages are predominant in the midcycle CL and M1 macrophages are predominant in the regressing CL, this study did not evaluate any other macrophage markers. Interestingly, medium conditioned by culture of early CL upregulated CD163 mRNA expression in peripheral blood mononuclear cells, consistent with M2 macrophage polarization. Conversely, medium conditioned by culture of regressing CL stimulated CD40 mRNA expression, consistent with M1 macrophage polarization (Shirasuna and Miyamoto, 2013).

Care et al., (2013) investigated the role of macrophages in the CL of pregnancy. They showed that macrophage depletion in mice after conception caused an arrest of embryo implantation associated with diminished plasma P₄ and poor uterine receptivity. Implantation failure was reduced by administration of bone marrow-derived CD11b⁺F4/80⁺ monocytes/macrophages. This supports an essential role for macrophages in the continuation of pregnancy by proper regulation of P₄. In CD11b⁺ cell-depleted mice the CL were abnormal, with elevated prostaglandin-endoperoxide synthase 2 (Ptgs2), hypoxia-inducible factor 1-alpha (Hif1a) and other inflammation and apoptosis genes with reduced expression of the steroidogenic genes, Star, Cyp11a1, and Hsd3b (Care et al., 2013). Progesterone treatment restored the capacity for pregnancy in CD11b⁺ cell-depleted mice. This study illustrates the importance of macrophages for survival of the CL and maintenance of pregnancy.

Immune cells are present in the CL throughout its lifespan and play an important role in directing luteal function. Pro-inflammatory cytokines, which are secreted by immune cells, are involved in luteolysis. Luteal cells isolated from regressing CL are greater stimulators of T cell activation than midcycle luteal cells. Macrophages also play an important role in luteal function

and are the most abundant immune cell in the regressing CL. Immune cells and luteal cells are known to communicate through direct cell-to-cell contact but communication through secretory factors is still not fully understood.

Exosomes

Exosome are cup-shaped, cell-secreted membranous vesicles of endocytic origin ranging in size from 30 to 100 nm in diameter (Mathivanan et al., 2010). Exosomes became of interest for immunologists in 1996 when they were proposed to play a role in antigen presentation. In 1996, it was shown that Epstein-Barr virus-transformed B lymphocytes secreted exosomes by fusion of multivesicular bodies with the plasma membrane. Exosomes secreted by these cells harbor MHC class II dimers bound to antigenic peptides, molecules essential for the adaptive immune response (Raposo et al., 2013). Various analyses of T cell activation by exosomes have shown that exosome-borne MHC-peptide complexes can directly bind to their cognate T cell receptor and activate primed CD4⁺ and CD8⁺ T cells (Bobbri et al., 2011). The outcome of T cell activation depends on the physiological state of the cells that secrete them. For example, exosomes secreted by mature dendritic cells are more efficient activators of T cells in vitro than those of immature dendritic cells (Bobbri et al., 2011).

Exosomes play an important role in intercellular communication and possess numerous specific immune functions. Upon contact, exosomes transfer molecules that can reprogram their recipient cells. Exosomes carry genetic material such as miRNA and mRNA, and thus serve as a vehicle for genetic messages (Raposo et al., 2013). These vesicles can bind to cells through receptor-ligand interactions such as antigen presentation or fusion with the target-cell membrane.

By fusion, exosomes can deliver exosomal surface proteins and cytoplasm to the recipient cell (Bobrie et al., 2011). Exosomes were originally thought to be exocytosed vesicles from the plasma membrane originating from the endocytic pathway. Trams et al. (1981) described exosomes as microvesicles derived from domains of the plasma membrane released from normal and neoplastic cells and having enzymatic activity outside the cell. It was proposed that these vesicles might play an important role in physiologic functions.

All exosomes have a lipid bilayer membrane, similar density (1.10-1.21 g/ml), similar protein composition, and contain mRNAs and miRNA that can carry tissue or cell specific material (Keller et al., 2006). Exosomes originate from a variety of cell types and have autocrine, paracrine, or endocrine actions (Johnstone et al., 2005). Their functions are diverse and related to the physiological functions of the donor cells (reviewed by Keller et al., 2006). Exosomes are released from many different types of cells, including dendritic cells, T cells, mast cells, tumor cells, mesenchymal stem cells, neural cells, platelets, hepatocytes, and endothelial cells (Ludwig and Giebel, 2011). Exosomes have also been detected in a variety of fluids, including serum, plasma, saliva, breast milk, bronchial lavage fluid, pleural effusions, urine, ocular fluids, semen, amniotic fluids, and synovial fluid (Simpson et al., 2009).

There are many characteristics that allow for the purification and identification of exosomes. Specific proteins appear to be enriched in exosomes, which are utilized for various functions in the cell. Loading peptides into MHC requires chaperone proteins, present in exosomes, such as heat shock protein 70 and 90 (HSP 70 and HSP 90). ExoCarta, a compendium for proteins and RNA molecules identified in exosomes, shows that 89% of proteomic studies reported HSP 70, 68% reported ALIX, 37% reported TAG101, and 37% reported clathrin in exosomes independent of the cellular origin. Proteins that are enriched in exosomes include

integrins ($\alpha 3$, $\alpha 4$, αM , αL , $\beta 1$, and $\beta 2$) and tetraspanins (CD63, CD9, CD37, CD53, CD81 and CD82; Fèvrier and Raposo, 2004). Tetraspanins associate with other tetraspanins, integrins, growth factor receptors, and MCH II proteins forming protein rafts (Boucheix and Rubinstein, 2001). Tetraspanins play a role in the organization of large molecular complexes and membrane subdomains that may be important for an alternative route of exosome formation without protein ubiquitination. In addition to proteins, exosomes are enriched in lipids such as ceramide and sphingolipids, which promote membrane budding (Trajkovik et al., 2008). Rab proteins play a role in docking, membrane fusion, and vesicle formation in exosomes (Mathivanan et al., 2010). Annexins also play a role in membrane trafficking and fusion. Futter and White (2007) proposed that annexins play a role in clathrin-dependent internalization leading to multi vesicular vesicle (MVB) formation. The high level of regulation in the formation, content and secretion of exosomes indicates the importance of exosome biogenesis.

It recently became clear that currently used protocols for exosome purification actually purify different subtypes of extracellular vesicles. Alberts et al., (2012) showed that different types of extracellular vesicles are present within sucrose gradients ranging from 1.11 and 1.19 g/ml. Extracellular vesicles in the exosome pellet display similar equilibrium densities but different kinetics to reach this density, and thus are likely compositionally different. Kowal et al., (2014) confirmed these results by showing that the vesicles equilibrating at lower density fractions (1.11g/ml) were less dependent on RAB27A for their secretion than those floating at 1.14g/ml. These results show that not all exosomes are the same and that they can be composed of different proteins, mRNA, and miRNA which give them different kinetics.

Multivesicular Body Biogenesis

Multivesicular bodies (MVBs) originate in early endosomes whose primary role is to sort proteins and lipids for degradation in lysosomes or transfer them to direct cell function to the recipient cell. During MVB formation, early endosomes sequester specific proteins and lipids into intraluminal vesicles (ILVs), which pinch into the endosome to form MVBs (Piper and Katzmann, 2007). Many ILVs located within MVBs are shuttled to lysosomes for degradation (Trajkovic et al., 2008). Some ILVs are responsible for recycling or secretion of proteins such as CD163 and MHC II. As MVBs are formed, proteins, lipids, mRNA, and miRNA gather into ILVs (György et al., 2011).

Some proteins are more important in MVB formation, loading and transport than others (Gruenberg and Stenmark, 2004). A very important protein, annexin-II, is required for proper separation of newly formed MVBs from early endosomes (Mayran et al., 2003). Other proteins are important in MVB formation such as Tsg101, which assists MVB and ILV budding (Doyotte et al., 2005), endosomal coatamer protein (COP), ARF1 and the small GTPase, RAB7, which are thought to have roles in endosomal organization and protein export (Gruenberg and Stenmark, 2004). Multivesicular bodies release exosomes from the plasma membrane by fusion of the MVB membrane with the plasma membrane of the cell (Piper and Katzmann, 2007). Exosomes are formed from internalized endocytic vesicles and are a population of ILVs that are secreted into the extracellular space.

Exosome formation begins with initiation of the endocytotic pathway as proteins and lipids are taken in at the cell surface by endocytosis (Maxfield and McGraw, 2004). Once internalized, the endocytotic vesicles are transported to early endosomes in which the mildly acidic pH causes an uncoupling of housekeeping receptors from their ligands to provide transport

to be either recycled back to the plasma membrane or continue through the endocytotic pathway to multivesicular bodies (MVBs) and late endosomes (Maxfield and McGraw, 2004).

Exosomes originate from internal MVBs. Formation of the internal vesicles of MVBs requires endosomal sorting complexes required for transport (ESCRT) proteins (ESCRT-I, ESCRT-II, and ESCRT-III), tetraspanins, and the lipid lysobisphosphatidic acid (LBPA) (Babst, 2005). Endosomal sorting complexes bind to mono-ubiquitinated endosomal/cargo proteins to allow sorting and concentration of these proteins into ILVs.

A few studies have addressed the function of ESCRT proteins in the biogenesis and secretion of exosomes. Tamai et al. (2010) reported that Hrs promotes exosome secretion by dendritic cells while others did not find any role for TSG 101, Alix, or VSP4 (proteins part of the ESCRT complex) in exosomal secretion by oligodendroglial cells or of Vsp4B in release of exosomes by direct budding from the plasma membrane (Trajkovic et al., 2008). Bobrie et al. (2011) showed the inhibition of Vps4B increases secretion of exosomes and soluble proteins by MHC class II- expressing HeLa cells. Depletion of TSG101 and ESCRT-1 component also leads to reduced exosome secretion in HeLa-CIYA (Colombo et al., 2013) and MCF-7 tumor cells (Baietti et al., 2012).

To address function, attempts to inhibit the secretion of exosomes have been performed. Chemical inhibitors of various molecules (inhibitors of sphingomyelinase, of Na^+/H^+ and Na^+/Ca^+ channels or of H^+ pump) have been reported to decrease exosome secretion in model cell lines (reviewed by Marleau, 2012). These inhibitors act on molecules with pleiotropic functions within the cell and induce major nonexosomal related changes in the cells (Bobrie et al., 2011).

Exosomes in Reproduction

Exosomes are very important in cell-to-cell communication; therefore, scientists have begun to study their role in reproduction. For example a recent study by Corrigan et al. (2014) showed that secondary cells, found in the accessory sex glands of *Drosophila melanogaster*, secrete exosomes and, after mating, these exosomes fuse with sperm and interact with female reproductive tract epithelia. This study demonstrated that the release of exosomes was required to inhibit female re-mating behavior, suggesting that exosomes are downstream effectors of bone morphogenetic proteins (BMP) signaling. A similar study performed in mice, showed that the tetraspanin CD9, expressed on exosomes, played an important role in gamete fusion, because CD9-deficient oocytes are unable to fuse with sperm (Kaji et al., 2000). Miyado et al. (2008) showed that transgenic mouse oocytes transferred CD9 to the sperm head via vesicles described as 'exosome-like'. These vesicles were consistent with the size and shape of exosomes but no other verification was performed.

During the early stages of the human reproductive process, the ovarian follicle, seminal fluid, endometrium, embryo and trophoblast cells are all possible sources of EVs that have the potential to modulate maternal immune function locally (Tannetta et al., 2014). During later pregnancy, Redman et al., (2007) showed that the syncytiotrophoblast (STB) of the placenta is the primary source of EVs and the STB releases EVs directly into the maternal blood constituting a major signaling mechanism between fetus and mother.

Ovarian follicular fluid EVs were first described in the horse. This study identified miRNAs within follicular fluid EVs, identical to those in the somatic cells (granulosa and cumulus; Silveira et al., 2012). It is known that proper cell-to-cell communication within the ovarian follicle is critical for the growth and maturation of a healthy oocyte. Cell communication

within the follicle involves many signaling molecules and is affected by maternal age (Matzuk et al., 2002). This study also showed that miRNA present in exosomes from ovarian follicular fluid varied with the age of the mare, and a number of different miRNA were detected in young versus old mare follicular fluid. Matzuk demonstrated a possible function of exosomes in cell communication within the ovarian follicle.

Exosomes also have the potential to direct immune cell function. For example, exosomes isolated from the serum of pregnant women suppressed the expression of CD3 and JAK3 in T cells, which are important in T-cell signaling (Taylor et al., 2006). A difference in CD3 and JAK3 protein was detected between women delivering at term and those delivering preterm. This study suggested that exosomes may be involved in immune cell responses at the time of delivery. Marker analysis suggested that the release of FasL-containing exosomes may be a mechanism that the placenta uses to promote a state of immune privilege (Taylor et al., 2006).

Myxovirus resistance 1 (MX1), which is an antiviral protein that is strongly upregulated during pregnancy partially colocalized with exosomal protein CD63, demonstrating that MX1 can be packaged in exosomes (Racicot et al., 2012). MX1 was also found to be housed as cargo within exosomes and when inhibitors of MX1 were used, reduced numbers of exosomes were seen which suggests that MX1 is involved in exosome formation and is not merely cargo. Together, these are just a few studies that highlight the importance of exosomes in cell communication and the regulation of the reproductive system during crucial events, such as pregnancy and mating. Therefore, it was essential to determine the role of exosomes in the CL during two very critical time points: maintenance and regression.

SUMMARY

Premature regression of the CL is a major contributor to decreased reproductive efficiency in cows. The loss of the CL results in loss of P₄ and therefore loss of the embryo. Induction of luteal regression by PGF_{2α} results in an increased number of immune cells in the CL and requires proper communication between luteal cells and immune cells. Exosomes, which house miRNA, mRNA, lipids and proteins, are utilized in cell-to-cell communication. The objectives of this study were to isolate bovine luteal cell-derived exosomes and determine the effect of exosomes on T cells and macrophages during luteal maintenance and regression. The long term goal of this project was to determine the effect that luteal cell-derived exosomes have on immune cells and how that may modulate the CL during critical events such as maintenance and regression.

CHAPTER 2: Examining the Role of Bovine Luteal Cell-derived Exosomes on T Cell Function

ABSTRACT

Midcycle luteal cells induce T lymphocyte (T cell) proliferation via secretory factors. The hypothesis to be addressed is that exosomes (carrying various cell-to-cell communication factors) are secreted by luteal cells, which influence function of the corpus luteum (CL) by programming T cell functions. Although T cells in the CL exhibit only limited proliferation, proliferation assays are used in vitro as a means to measure activation.

In this study, corpora lutea were collected from cyclic cows and luteal cells were isolated by enzymatic digestion. Luteal cells were then cultured, counted, and medium was collected. Luteal conditioned media was then concentrated and exosomes were isolated by ultracentrifugation. Exosomes were purified by floating on a sucrose gradient and verified for size and morphology by transmission electron microscopy and quantified using NanoSight Tracking Analysis (NTA). The number of luteal cells counted at the time of media collection and exosomes isolated were compared to determine if the number of exosomes secreted differs depending on the physiological state of the CL. It was determined that midcycle luteal cells secrete a greater number of exosomes than luteal cells derived from regressing CL.

To determine if T cells internalize exosomes, resident T cells were labeled with antibodies against CD3 and exosomes were loaded with carboxyfluorescein succinimidyl ester (CFSE). T cells were cultured with or without exosomes and samples were analyzed by imaging flow cytometry. The majority of the T cells had internalized exosomes.

In a preliminary experiment, increasing number of exosomes were added to CFSE-labeled T cells and proliferation was assessed by flow cytometry. This experiment showed that exosomes derived from midcycle and regressing luteal cells induced T cell proliferation. Finally, exosomes collected from 3 different midcycle CL were isolated and added to CFSE-labeled T cells. After 72 hours, proliferation was measured by flow cytometry. Exosomes from midcycle luteal cells induced T cell proliferation. T cells were also collected and mRNA abundance for T cell specific markers was measured by quantitative polymerase chain reaction (qPCR). Results showed that midcycle luteal cell-derived exosomes induced upregulation of TNF mRNA while regressing luteal cell-derived exosomes down regulated all mRNA analyzed. These studies show that luteal cell-derived exosomes are taken up by T cells and cause activation. Also, midcycle and regressing luteal cell-derived exosomes have differential effects on T cell phenotype.

INTRODUCTION

The CL is essential for the production of progesterone (P_4) and maintenance of pregnancy in ruminants. There are three critical time points in the life of the CL: development, maintenance, and regression. The immune system is essential for optimal function of the reproductive system including events related to ovulation and maintenance of luteal function (Bobrie et al., 2011). Furthermore, the type of communication between luteal cells and immune cells changes with functional status of the CL. Brzezicka and Pate (2013) demonstrated that luteal cells from midcycle CL activate T cells regardless of cell-cell contact, which shows that the signaling involves secreted molecules. Luteal cells obtained from regressing CL required cell-cell contact

to fully activate T cells. From these studies, it was hypothesized that luteal cell-derived exosomes could be a signaling mechanism involved in T cell proliferation.

Exosomes are cell-secreted membranous vesicles of endocytic origin ranging in size from 30 to 100 nm in diameter and homogeneous with respect to their cup-shaped morphology (Mathivanan et al., 2010). Exosomes play an important role in intercellular communication and possess numerous specific immune functions. Upon contact, exosomes transfer molecules that can reprogram the recipient cells. Various analyses of T cell activation by exosomes showed that exosomes with MHC complexes can bind to their cognate T cell receptor and activate primed CD4⁺ and CD8⁺ T cells (Bobrie et al., 2011). The outcome of T cell activation depends on the physiological state of the cells that secrete the exosomes. For example, exosomes secreted by mature dendritic cells are more efficient activators of T cells in vitro than those of immature dendritic cells (Bobrie et al., 2011).

Exosomes have been isolated and purified from numerous cells and tissue from many species. This information can be found at ExoCarta (www.ExoCarta.org). Protein, mRNA, miRNA, and lipids contained in exosomes are recorded in this database. Exosomes have not been isolated from the CL and there is very little known about exosomes in the cow. Current knowledge about exosomes and their role in the immune system provide evidence that luteal cell-derived exosomes may interact with T cells. Parolini et al. (2009) showed direct evidence that human tumor exosomes fuse with a target cell, demonstrating that exosomes can release their internal contents into the cytosol of a recipient cell. Wubbolts et al. (2003) and Lamparski et al. (2002) showed that dendritic cell and B-cell-derived exosomes contain high quantities of MHC-I, MHC-II and CD86, allowing for potent activation of T cells via antigen presentation. Because exosomes were first discovered as vesicles released from reticulocytes (Johnstone et al.,

1987), there are many studies investigating the immunological effects of exosomes. These effects vary depending on the physiological state of the cells that secrete them.

While it is established that exosomes are found in many biological fluids and cell types, it was uncertain if bovine luteal cell-derived exosomes have the potential to direct immune cell function. With little to no knowledge of the function of exosomes in the bovine CL, the objective of this project was to determine the function of exosomes and if this response changes depending on the physiological state of the CL. We hypothesize that midcycle luteal cell-derived exosomes induce T cell proliferation and that midcycle luteal cell-derived exosomes induce a TH1 phenotype while regressing luteal cell-derived exosomes induce a TH2 phenotype.

MATERIALS AND METHODS

Reagents and Antibodies

Carboxyfluorescein succinimidyl ester (CFSE) and concanavalinA (ConA) were purchased from Sigma-Aldrich (St. Louis, MO). AIMV medium, Hams F12 medium, and bovine calf serum used for plating cells were purchased from Life Technologies (Grand Island, NY, USA). Insulin, transferrin, and selenium were obtained from VWR (Philadelphia, PA) while gentamycin was purchased from LifeTechnologies (Grand Island, NY, USA). Anti-mouse immunoglobulin G2 α (IgG2 α) magnetic beads were purchased from Miltenyi Biotech (Auburn, CA). Antibodies against bovine antigens, CD3 (MM1A), CD2 (MUC2A) and $\gamma\delta$ (GB21A), were purchased from VMRD, Inc. (Pullman, WA).

Corpora Lutea Collection and Luteal Cell Culture

Corpora lutea were collected transvaginally from normally cyclic cows on Days 9–12 after estrus (Day 0) and 8 hours after a luteolytic dose of prostaglandin F2 alpha (PGF_{2α}; 25 mg Lutalyse) administered on Day 9 or 10 after estrus. The protocols used for tissue collection were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #44196).

Luteal cells were isolated from functional (Days 9–12) and regressing (8hr after PGF_{2α} administration) CL following the procedure described by Pate (1993). Cell viability and number were determined by hemocytometer and trypan blue dye exclusion (Life Technologies, Grand Island, NY, USA). Cell culture was performed in T75 flasks (Fisher Scientific, Waltham, MA USA) which were coated with Hams F12 medium + 10% bovine calf serum for 30 minutes at 37°C. Flasks were washed twice with Hams F12 medium containing gentamycin (10 µg/mL). Luteal cells were seeded at 15x10⁶ luteal cells per T75 flask and were maintained in Hams F12 medium supplemented with insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL; ITS), gentamycin (10 µg/mL) and LH (10 ng/mL). Cells were allowed to adhere for 24 hours. After adhesion, medium were discarded, cells were washed twice with 5 mL Hams F12 medium supplemented with gentamycin (10 µg/mL), and fresh medium was added. Media were collected 24 hours later into 50mL conical tubes and stored at -20°C for future isolation of exosomes. Before flasks were discarded, two sections per flask were counted by use of an ocular micrometer grid to calculate average cell number per flask. This number was used to compare the number of exosomes released during each physiological stage of the CL.

Exosome Isolation

To determine the best isolation method, three techniques were tested: (1) ultracentrifugation, (2) LifeTechnologies Exosome Isolation Reagent (Invitrogen; Grand Island, NY) and (3) 101Bio Exosome Isolation Reagent (101Bio, Palo Alto, CA). For each trial, 4 mL of luteal cell-conditioned media was processed, all obtained from the same flask of luteal cells.

To test the ultracentrifugation technique, the media was centrifuged at 300xg for 5 minutes, 1200xg for 20 minutes and 10,000xg for 30 minutes to remove cellular debris. The supernatants were centrifuged at 110,000xg for 24 hours in an ultracentrifuge (Beckman, L8-70M) using a 50-57i rotor. Supernatant was decanted and saved as “exosome-depleted media”. The pellet was resuspended in 80 μ L 1X TEN (0.1M NaCl, 0.01M Trizma base, 1mM EDTA).

To test the Life Technologies Exosome Isolation Reagent, 2 mL of reagent was added to 4mL of media, vortexed, and incubated overnight at 4°C. After incubation, the sample was centrifuged at 10,000xg for 1 hour at 4°C. The supernatant was discarded and the invisible pellet was resuspended in 80 μ L of 1X TEN (all procedures done per manufacturers protocol).

To test the 101Bio Exosome Isolation Reagent, solutions A, B, and C (unknown composition) were combined in order and vortexed. This mixture was added to 4 mL of luteal-cell conditioned culture media, vortexed for 30 seconds, and incubated at 4°C for 1 hour. Two layers were present after incubation. The top layer, which was the color of the cell culture medium, was aspirated and discarded. No middle layer was present. The bottom white layer was transferred to a fresh 1.5 mL tube and centrifuged for 3 minutes, 1,000xg at 4°C. The resulting supernatant was aspirated and allowed to air dry for 5 minutes. The remaining pellet was resuspended in 80 μ L of 1X TEN, transferred to a PureExo spin column and centrifuged for 5 minutes at 5,000xg at 4°C. The filtrate containing exosomes was collected (all procedures done

per manufacturers protocol). Protein concentration of all samples was determined using a Pierce BCA Protein Assay Kit (Thermoscientific, Pittsburg, PA USA) with a standard curve made with bovine serum albumin.

For all subsequent experiments, exosomes were obtained via ultracentrifugation. Media were thawed and concentrated 20-fold using Amicon Ultra-15 10K Centrifugal Filter Devices (Millipore, Billerica, MA) to remove excess water. The concentrated media were spun at four centrifugation steps using an ultra-centrifuge (Beckman, L8-70M): 300xg for 5 minutes, 1200xg for 20 minutes and 10,000xg for 30 minutes two times to remove large cellular debris from the medium, leaving the low density exosomes in the supernatant. The supernatants were centrifuged at 110,000xg for 24 hours and the pellets containing the exosomes were resuspended in 500 μ L 1X TEN buffer.

Exosome Purification

The resuspended pellet (in 500 μ L 1X TEN) was mixed with 1.5 mL of 80% sucrose in 1X TEN. Two milliliters of 50% sucrose in 1X TEN were then carefully layered on top, followed by a layer of 0.6 mL of 10% sucrose in 1X TEN. The sample was then ultra-centrifuged (Beckman, L8-70M) at 110,000xg for 24 hours at 4°C in a SW55Ti rotor. Following ultracentrifugation, each gradient was aliquoted into four 1.125 mL fractions into 1.5 mL tubes, using an Auto Densi-Flow Density Gradient Fractionator (Labconco, Kansas City, MO). The top fraction, containing the low-density exosomes, was diluted in sterile 1X TEN and concentrated using Amicon Ultra-4 10K Centrifugal Filter Units (Millipore, Billerica, MA) to a final volume of 150 μ L. This step helps to sterilize the sample for cell culture, dilute out the sucrose, and concentrate the sample to a working volume.

Exosome Verification

After isolation, purification and concentration, exosomes were prepared for imaging on a transmission electron microscope (TEM). Briefly, 5 μ L of sample was added to copper hexagonal mesh grids (Electron Microscopy Science, Ft. Washington, PA). The sample was allowed to settle on the grid for three minutes. After settling, excess sample was removed by wicking and 5 μ L of uranyl acetate was added to the grid to stain the background. After 30 seconds, excess uranyl acetate was removed by wicking. The sample grids were then analyzed using a JEM 1200 EXII transmission electron microscope (The Pennsylvania State University, PA). Pictures were captured using a TEM high resolution camera model F224 (Tietz, Gauting, Germany). Exosomes were identified based on size (less than 100 nm) and a rounded, cup-shaped morphology typical of exosomes.

Exosome Quantification

Three hundred microliters of the purified samples were transferred into the sample chamber of a Nanosight LM10 analysis unit with a 635 nm laser (Malvern Instruments, USA). The particles in the laser beam undergo Brownian motion and videos of these particle movements were recorded. Samples were recovered by aspirating out of the chamber. The Nanosight Tracking Analysis (NTA) 2.3 software was used to analyze each video and determine the particle concentration and the size distribution of the particles. Three videos of 30 seconds duration were recorded for each sample at appropriate dilutions with a shutter speed setting of 1500 (exposure time 30 milliseconds) and camera gain of 560. The detection threshold was set at 6 and at least 1000 tracks were analyzed for each video. Concentration was provided in particles per milliliter.

T Cell Isolation from the Corpus Luteum

T lymphocytes were isolated from functional (Days 10–12) CL following the procedure described by Poole and Pate (2012). Briefly, tissue was dissociated with collagenase, the supernatant collected, and dissociated cells were washed three times in 1X PBS at 300xg for 10 minutes at 4°C. The cells were then incubated with antibodies directed against bovine CD2 (mouse anti-bovine CD2 IgG2a; MUC2a) and delta proteins (gamma delta; GB21A) for 30 minutes followed by three washes in 1X PBS at 300xg for 10 minutes at 4°C. Anti-mouse immunoglobulin G_{2a} magnetic beads were added and incubated for 30 minutes. Total T cells were immunomagnetically isolated using an AutoMacs cell separator.

Exosome CFSE label and uptake

Exosomes were labeled with 7.5 uM CFSE for 30 minutes at 37°C. A high concentration of CFSE must be used to ensure labeling of such small vesicles. Labeled exosomes were centrifuged overnight at 110,000xg with 5% exosome-depleted fetal bovine serum (FBS) to bind and remove any noninternalized CFSE. Fetal bovine serum contains large amounts of exosomes so it was essential to use exosome-depleted FBS to avoid exosome contamination. The exosome pellet was collected the following morning and resuspended in 200 µL of 1X TEN. Total T cells isolated from the CL were counted using a hemocytometer and plated in a 96 well plate (1.5x10⁵/well). CFSE-labeled exosomes, 20x10⁶ in 20 µL 1X TEN, were added to each well containing T cells in a final volume of 200 µL. T cells and exosomes were incubated with gentle shaking at 37°C for 4 hours. After incubation, cells were collected from the wells and washed three times with PBS-EDTA at 300xg for 10 minutes to remove any exosomes that were not internalized. The cell pellet was resuspended in 200 µL of PBS-EDTA and incubated with 1µg

of anti-CD3 (mouse monoclonal anti-CD3 IgG1; MM1A) for 30 minutes on ice. After incubation, cells were washed three times with PBS-EDTA at 300xg for 10 minutes to remove any unbound primary antibody. Following the washes, the cell pellet was resuspended in 200 μ L of PBS-EDTA + 5% goat serum and incubated with 1 μ g anti-CD3⁺-CF405S (mouse anti-bovine CD3 IgG1; MM1A, VMRD, Inc. Pullman, WA) for 30 minutes on ice, gently mixing every 10 minutes. After incubation, cells were washed three times to remove any unbound secondary antibody. The first wash contained PBS-EDTA + 5% goat serum, the second wash contained PBS-EDTA + 2.5% goat serum and the third wash was in PBS-EDTA. The cell pellet was then fixed in 1 mL of 1% paraformaldehyde (PFA) on ice for 20 minutes followed by three washes in PBS-EDTA at 300xg for 10 minutes. Cells were resuspended in PBS+ 2% FBS, the tube was covered with aluminum foil to keep light out and stored at 4°C overnight. Fixed cells were analyzed on an Amnis FlowSight (USA, Seattle, WA) the following morning. IDEAS software was used to analyze data. A scatter plot was constructed and a double positive gate was set. This gate was set using the IDEAS software where each point on the scatter plot represents a picture. Each point was selected and pictures were examined to determine double positive cells. This software then calculated a percentage of double positive cells based on the set gate.

This experiment was repeated using the same protocol as described above with a few minor changes to enhance the picture quality. These changes included: T cells labeled with goat anti-mouse IgG Alex Fluor 555 antibody (ab150114; Abcam, Cambridge, MA, USA) and imaging using an Olympus Fluoview 300 using an Olympus IX70 inverted microscope.

T Cell Isolation

For cell culture, blood was collected from the jugular vein of cows into a sterilized bottle containing 0.5M EDTA. The protocols used for blood collection were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #44196). Blood was transported back to the lab on ice. Blood was aliquoted into sterile 16x125mm borosilicate glass tubes, covered with parafilm and centrifuged at 300xg at 4°C for 15 minutes. Buffy coats were collected, and slowly pipetted into sterile 16x125mm borosilicate glass tubes containing Ficoll-Paque Plus (GE Health Care, USA). Tubes were covered with parafilm and centrifuged at 660xg at 25°C for 30 minutes. The dense mononuclear band was collected and placed into 50 mL centrifuge tubes containing 15 mL cold PBS-EDTA. Tubes were centrifuged at 660xg for 10 minutes at 4°C. Cells were washed twice by resuspending cells in cold PBS-EDTA and separating at 300xg for 10 minutes at 4°C. Anti-CD2 and anti-gamma delta antibodies were used to isolate total T cells from peripheral blood mononuclear cells by positive selection using an autoMACS Pro Separator (Miltenyi Biotech) as described by Ndiaye et al. (2007).

T Cell Proliferation Assay

This experiment was conducted to determine the effect of exosomes isolated from luteal cell-conditioned media from a functional CL on activation of peripheral T cells. Gamma delta and CD2 positive T cells were harvested and labeled with CFSE (1.75 μ M) in AIMV medium by incubating at 37°C for 15 minutes followed by incubating at room temperature for another 10 minutes. The cells were washed three times at 300xg for 10 min with Hams F12 medium containing 5% exosome-depleted FBS to bind any non-internalized CFSE.

Exosomes were added to 1.5×10^5 CFSE-labeled T cells in three amounts: 1×10^6 , 2×10^6 , and 5×10^6 . Due to the small quantity of exosomes isolated from the regressing luteal cell conditioned media, only 2 concentrations of exosomes (1×10^6 and 2×10^6) were used. Non-labeled T cells and CFSE-labeled T cells without exosomes were used as negative controls. Cells were incubated at 37°C for 72 hours. Proliferation was measured by flow cytometry and FlowJo V10 was used to analyze the data. Non-proliferating cells were gated using the CFSE-labeled, non-stimulated T cells.

After the preliminary experiment was analyzed, the experiment was repeated with the largest possible concentration of exosomes (15×10^6). Briefly, T cells were labeled with $1.5 \mu\text{M}$ CFSE for 15 minutes at $37^\circ\text{C} + 5\% \text{CO}_2$ followed by a 10 minute incubation at room temperature. Labeled T cells were washed three times at $300 \times g$ in Hams F12 medium containing 5% exosome-depleted FBS which was used to bind any non-internalized CFSE. The T cell pellet was resuspended in $200 \mu\text{L}$ of AIM5 medium and 1.5×10^6 T cells were added per well to a 96 well plate. Exosomes isolated from three different midcycle CL were added at 20×10^6 exosomes per well. Each treatment was run in duplicate. Positive controls for proliferation included concanavalinA (ConA), luteal cells, and luteal-conditioned medium. Non-labeled T cells and CFSE-labeled T cells without exosomes were used as negative controls.

Cells were incubated at 37°C for 48 hours. Proliferation was measured by flow cytometry and FlowJo V10 was used to analyze the data. Non-proliferating cells were gated using the CFSE-labeled, non-stimulated T cells.

T Cell Phenotype

Midcycle (20×10^6) and regressing (15×10^6) luteal cell-derived exosomes were added to 2×10^6 T cells per well in a 12 well plate. T cells and exosomes were cultured in Aim V medium + ITS and gentamycin for 72 hours at $37^\circ\text{C} + 5\% \text{CO}_2$. After 72 hours, media was collected and T cells were pelleted by centrifuging at $300 \times g$ for 10 minutes at 4°C . The T cell pellets were frozen in 1.5 mL centrifuge tubes at -20°C for further RNA isolation.

RNA Isolation and Quantitative PCR

Total cellular RNA was extracted by adding 1 mL TRIZOL (Life Technologies) to each 1.5 mL centrifuge tube and proceeding with the manufactures protocol. Concentration of total cellular RNA was determined using Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Extracted RNA was stored at -80°C until cDNA synthesis. RNA ($1 \mu\text{g}$) was reverse transcribed. Complementary deoxyribonucleic acid (cDNA) synthesis was performed using DyNAmo cDNA Synthesis Kit (Thermo Scientific; Pittsburgh, PA, USA) as per the manufacture's protocol.

Quantitative PCR (qPCR) was performed on 4 genes and a reference gene used as an internal control (bovine ribosomal protein L19; RPL19), which was determined to not be affected by treatment. Primer sequences are shown in Table 2.1. The reaction was designed for a total reaction volume of $20 \mu\text{L}$. The template cDNA was added to each reaction in $5.0 \mu\text{L}$. The Bioline SensiMix SYBR No ROX Kit (Taunton, MA, USA) was used. The following conditions were used: one step of denaturing at 95°C for 5 minutes followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing for 45 seconds (specific annealing temperatures are recorded in Table 2.1), and extension at 72°C for 60 seconds; this was followed by one step of extra

elongation at 72°C for 5 minutes. For each gene, a standard curve was prepared from purified cDNA PCR product and used to determine steady-state concentrations of respective mRNAs in duplicate wells for each sample.

The PCR amplification products exhibited a single melting curve. Amplicons were electrophoretically separated on 1.5% agarose gels and visualized with ethidium bromide. For initial validation, the band matching the size of the expected cDNA fragment for each gene was excised and purified using the QIAquick Gel Extraction Kit (Qiagen Sciences) for sequence confirmation. Additional PCR product was precipitated and used to make the standard curve. All standard curves used in this experiment had slopes between -3.2 to -3.4 and efficiencies between 95%-110%.

Statistical Analyses and Data Analysis

Results for quantity of exosomes released from luteal cells are presented as mean \pm SEM. Quantity of secreted exosomes and T cell proliferation data were analyzed using MIXED procedure of SAS with orthogonal comparisons and bonferroni's correction using SAS (Ver. 9.3; SAS Institute). T cell phenotype qPCR data was obtained by concentration values from the standard curve and analyzed by t-test using SAS (Ver. 9.3; SAS Institute). Differences in gene expression were deemed significant when the P-value was <0.05 . The data are presented as mean \pm SEM.

RESULTS

Exosome Isolation, Morphology, and Quantity

Exosomes were isolated using three different methods: ultracentrifugation, LifeTechnologies Exosome Isolation Reagent and 101Bio Exosome Isolation Reagent. Protein quantity was measured by Pierce Bicinchoninic acid (BCA) Protein Assay Kit. The ultracentrifugation method, LifeTechnologies Reagent, and 101Bio Reagent generated 9.52 μg , 9.23 μg , and 8.18 μg protein respectively (Figure 2.1). From this experiment it was determined that yield was similar among the three methods, with ultracentrifugation perhaps generating the greatest amount of protein. Samples were then analyzed using transmission electron microscopy (TEM) and evaluated size and shape (Figure 2.2). It was determined that the samples were not pure exosomes based on the various sizes of particles.

Next, midcycle and regressing luteal cell-derived exosomes were isolated by ultracentrifugation and purified using a sucrose gradient. These samples were then analyzed using TEM (Figure 2.3). Samples were evaluated for size and shape. Samples showed vesicles consistent with exosomes that exhibited a cup-shaped morphology and a diameter of 10-100nm. Some vesicles that were larger, 100-200nm, were seen but a majority, over 85%, were 100 nm or below. Based on this, exosomes were determined to be present in both samples along with some microvesicles (MV). Also, NanoSight analysis confirms particles ranging from 30-200 nm with most particles around 100nm (Figure 2.4A). No differences in exosome morphology were seen between the samples (midcycle and regressing).

It was of interest to determine if the quantity of secreted exosomes was dependent on the physiological state of the CL. This information was collected using NanoSight technology where multiple videos were collected on each sample. Using NanoSight Tracking Analysis of exosomes

collected from multiple midcycle and regressing CL, it was determined that midcycle luteal cells secreted more exosomes than regressing luteal cells ($p < 0.05$; Figure 2.4B). Also, D14 luteal cells secreted fewer exosomes than midcycle (D9-12) luteal cells.

Exosome Uptake by T Cells

Exosomes isolated from midcycle luteal cells were co-cultured with T cells and after 4 hours exosome uptake was measured by using an Amnis FlowSight (Figure 2.5). All data from this run was compiled and analyzed using IDEAS software. Using this software, a scatterplot of double positive cells was obtained. From this plot, it appears that over 90% of T cells had exosomes internalized.

Images obtained from the FlowSight were of low resolution and the CF405S fluorescence was dim. Therefore, this experiment was repeated and images were obtained using an Olympus Fluoview 300 which provided brighter fluorescence and higher quality images, respectively. Pictures obtained show CD3 labeled T cells with internalized exosomes (Figure 2.6). Some T cells had more exosomes internalized than others.

Effect of Exosomes on T Cell Proliferation & Phenotype

Midcycle or regressing luteal cell-derived exosomes were added to T cells in a dose dependent manner and proliferation was measured. Exosomes from both midcycle and regressing luteal cells stimulated T cell proliferation in a dose dependent manner (Figure 2.7). This experiment was repeated using the highest possible number of midcycle exosomes (15×10^6). Midcycle luteal cell-derived exosome samples induced T cell proliferation ($p < 0.05$, Figure 2.8).

This experiment was repeated using midcycle or regressing luteal cell-derived exosomes. Midcycle luteal cell-derived exosomes (20×10^6) and regressing luteal cell-derived exosomes (15×10^6) were added to T cells using the same conditions as described above. T cells that were co-cultured with midcycle luteal cell-derived exosomes exhibited increased TNF mRNA abundance ($p < 0.05$), reduced IL10 ($p < 0.05$) and no change in IL4 and IFNG mRNA expression, compared to controls (Figure 2.9). T cells co-cultured with regressing luteal cell-derived exosomes exhibited reduced TNF, IFNG, IL4 and IL10 mRNA abundance, compared to controls ($p < 0.05$, Figure 2.10).

DISCUSSION

In this study, exosomes were isolated and purified from bovine corpora lutea collected during luteal maintenance and regression. Currently, there is no information about luteal cell-derived exosomes and very little information about the role of exosomes in the cow. The interaction of bovine luteal cell-derived exosomes with T cells provides another mechanism by which luteal cells may communicate with resident T cells. It has become clear that exosomes have the potential to play a vital role in regulating the immune system and new research has suggested techniques to control exosomal (or exosome-mediated) communication between cells (reviewed by Azmi et al., 2013).

The first experiment was to determine if bovine luteal cells release exosomes. To accomplish this, bovine luteal cells were cultured and media was collected. To determine the best isolation method for bovine luteal cell-derived exosomes, three commonly used isolation techniques were utilized to obtain exosomes: ultracentrifugation, Life Technologies Exosome

Isolation Reagent, and 101Bio Exosome Isolation Reagent. Because the same amount of luteal-conditioned medium from the same animal was analyzed, direct comparisons of total exosomal protein yield could be made. Ultracentrifugation seemed to be the best method for the most total protein recovery, but all three methods resulted in similar protein yields. They et al. (2006) showed that ultracentrifugation followed by a sucrose gradient is the best method to isolate exosomes from biological fluids. The Life Technologies Reagent and 101Bio Reagent literature state that their kits isolate purified exosomes, which could account for the lower protein yield. This theory was tested by viewing each sample using transmission electron microscopy. Each of the isolation methods yielded particles of varying sizes and shapes, confirming that the exosomes obtained from each method were not homogeneous. Further purification by floatation on a sucrose gradient was necessary to obtain more pure fractions of exosomes, regardless of isolation method. It was concluded that to ensure sample purity, floating on a sucrose gradient was necessary which was also determined by Webber and Clayton (2013).

Traditional exosome isolation techniques were utilized, which involves ultracentrifugation, purification using a sucrose gradient, and verification by TEM (reviewed by Vlassov et al., 2012). Ultracentrifugation is the most cost effective and most widely used method. The TEM revealed 10-200 nm particles with a cup-shaped morphology, consistent with that of exosomes and MV. This determined that the samples had exosomes with some MV and that exosomes could be successfully isolated from luteal cell culture media.

The quantity of exosomes was determined by use of the NanoSight instrument and NanoSight Tracking Analysis software (NTA). Greater concentrations of exosomes were observed in midcycle luteal cell-derived samples compared to exosomes collected from regressing luteal cells. This suggests that midcycle luteal cells secrete more exosomes and this

event may be needed to assist in luteal maintenance. During luteal maintenance, maximal amounts of progesterone are secreted and the increasing amounts of exosomes may be utilized to maintain this function. For this study, exosomes were isolated from regressing luteal cells that were obtained by administering $\text{PGF}_{2\alpha}$ and collecting the CL 8 hours later. If luteolysis begins between 30 minutes to 2 hours following PG administration, (Acosta and Miyamoto, 2004), then collecting the CL for this study 8 hours after PG injection may be too far into luteal regression and luteal cells may not need to release exosomes at this time because immune cells have already infiltrated the CL, the CL has already begun apoptosis and P_4 has already begun to decrease. This could explain the reduction of exosomes. Additionally, previous studies in our lab (Brzezicka and Pate, 2013) showed that midcycle luteal cells induced activation of T cells with or without contact, while regressing (8 hour PPG) luteal cells required contact with T cells to provide activation. Results from this study may explain the previous findings because regressing luteal cells secrete fewer exosomes and this may be the reason that regressing luteal cells need contact to activate T cells. Additionally, studies show that there are larger numbers of exosomes isolated from tumor cells (Andre et al., 2002; Taylor et al., 2008; Rosell et al., 2009) which could indicate a potential biomarker for disease. The cargo in exosomes, proteins, mRNA, miRNA and lipids may be different in midcycle and regressing luteal cell-derived exosomes, which could also be a reason why midcycle luteal cells can signal to T cells through secretory factors while regressing luteal cells cannot. Further studies are being conducted to determine the content of these exosomes. It was also observed that D14 luteal cells released fewer exosomes than midcycle (D9-12) luteal cells. There is no data published which suggests that D14 CL are functionally different than D12 CL which is why this observation is interesting.

We tested the hypothesis that bovine luteal cell-derived exosomes interact with resident immune cells, specifically T cells, by internalization of exosomes, resulting in activation of the T cells. It is well known that exosomes interact with T cells. Abusamra et al. (2005) demonstrated that human prostate tumor-derived exosomes suppress T cell proliferation and cause apoptosis in CD8⁺ T cells. When T cells were cultured with midcycle luteal cell-derived exosomes, we found a majority of the T cells had internalized exosomes. This indicates that T cells can take up exosomes and that luteal cell-derived exosomes have the potential to direct T cells by releasing their contents inside the T cell.

T cell proliferation assays are a way to measure T cell activation. It was unclear how many exosomes were needed to stimulate T cell proliferation so a single dose response experiment was conducted. Exosomes from both midcycle and regressing luteal cells stimulated T cell proliferation in a concentration dependent manner. Although midcycle and regressing luteal exosomes induced similar T cell proliferation, we know from the first study that regressing luteal cells secrete about half the number of exosomes as midcycle luteal cells. Therefore, adding the same number of exosomes and comparing the effects would be an inappropriate comparison. Therefore for this reason, comparisons can only be made within an individual physiological state, in this case midcycle or regression.

The T cell proliferation experiment was repeated using three midcycle luteal cell-derived exosome samples. Regressing luteal cell-derived exosomes were not utilized in this experiment because of exceptionally low yields. As above midcycle exosome stimulated a modest percentage of the T cells to proliferate. Therefore, not only are bovine luteal cell-derived exosomes internalized by T cells, but they are able to activate T cells, as assessed by T cell proliferation.

Additionally, these experiments also determined the phenotype of T cells when exposed to midcycle or regressing luteal cell-derived exosomes. Interestingly, regressing luteal cell-derived exosomes decreased mRNA abundance for TNF, IFNG, IL4 and IL10. These results support the theory that regressing luteal cell-derived exosomes may down regulate T cell responses. Regressing luteal cell-derived exosomes may be utilized to resolve inflammation in the regressing CL, turn off cytokine responses, and regulate luteolysis. Exosomes displaying immunosuppressive effects on T cells are present in milk and colostrums (Admyre et al., 2007). Poole and Pate (2012) showed the loss of regulatory T cells in the regressing CL shown by the down regulation of FoxP3⁺ T cells.

Midcycle luteal cell-derived exosomes upregulated the expression of TNF and down regulated IL10 mRNA consistent with a TH1 response. Reyes et al. (2005) showed that cytokines such as IL1B, IL6 and TNF are essential in ovarian cycle regulation and play an important function during growth and development of ovarian follicles. Hasan et al. (2002) noted that an exaggerated inflammatory response and elevated cytokine concentrations in mouse ovaries, mediated by TH1 cells, were compatible with normal ovarian cyclicity, ovulation, and fertility.

In conclusion, exosomes can be isolated from midcycle and regressing luteal cell-conditioned media with fewer exosomes isolated from regressing luteal cell conditioned media. Additionally, luteal cell-derived exosomes can be internalized into T cells, induce proliferation, and direct T cell responses through induction of a specific phenotype. These results are consistent with the hypothesis that exosomes could serve as a mechanism for communication from luteal steroidogenic cells to T cells.

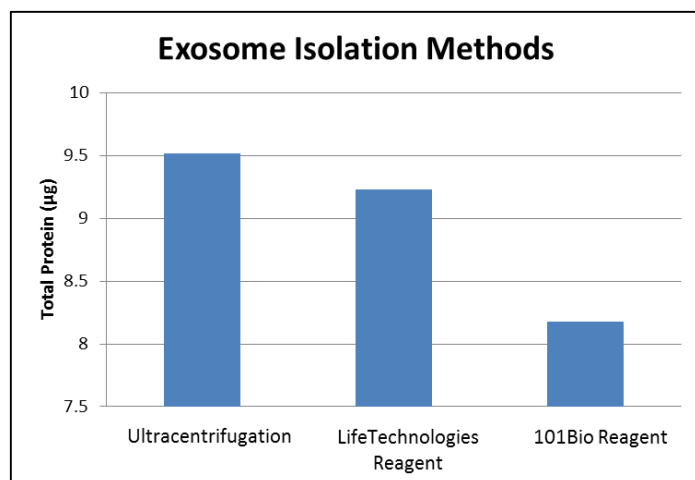


Figure 2.1: **Exosomal protein yield from three exosome isolation methods.** BCA assay was used to measure total protein.

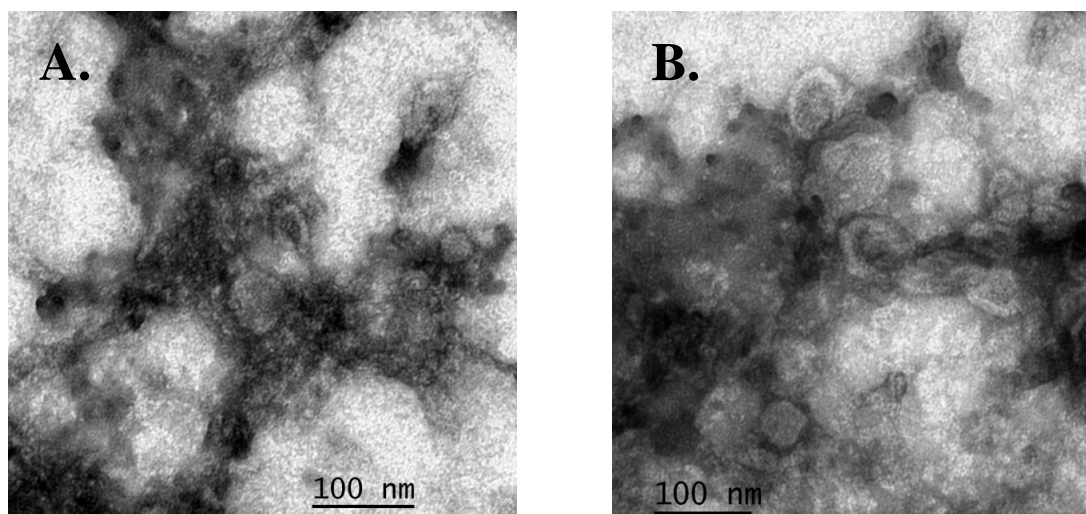


Figure 2.2: **Electron microscopy of luteal cell-derived exosomes.** (A) Exosomes isolated by LifeTechnologies Exosome Isolation Reagent. (B) Exosomes isolated from 101Bio Reagent. Exosome-like structures are seen mixed with larger, clumpy proteins.

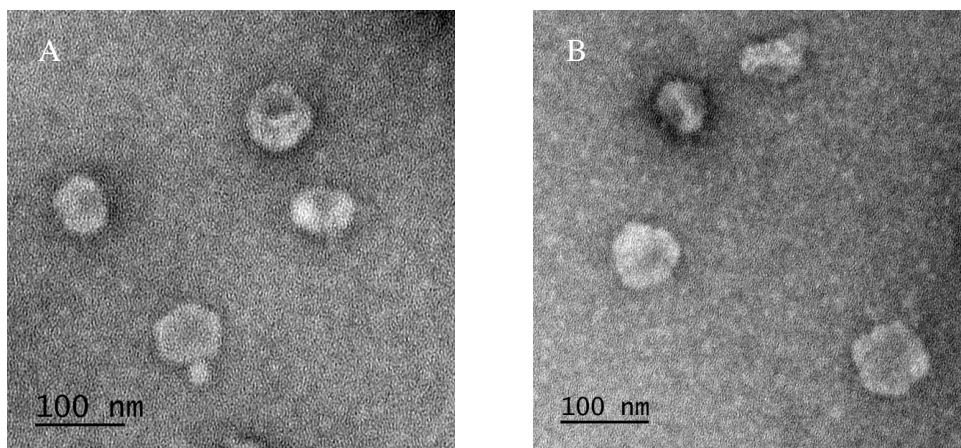


Figure 2.3: **Electron microscopy of luteal cell-derived exosomes.** (A) Exosomes isolated from midcycle luteal cells. (B) Exosomes isolated from regressing luteal cells. Cup-shaped morphology and 10-100 nm size are consistent with exosomes. Both exosome samples were isolated by ultracentrifugation followed by flotation on a sucrose gradient.

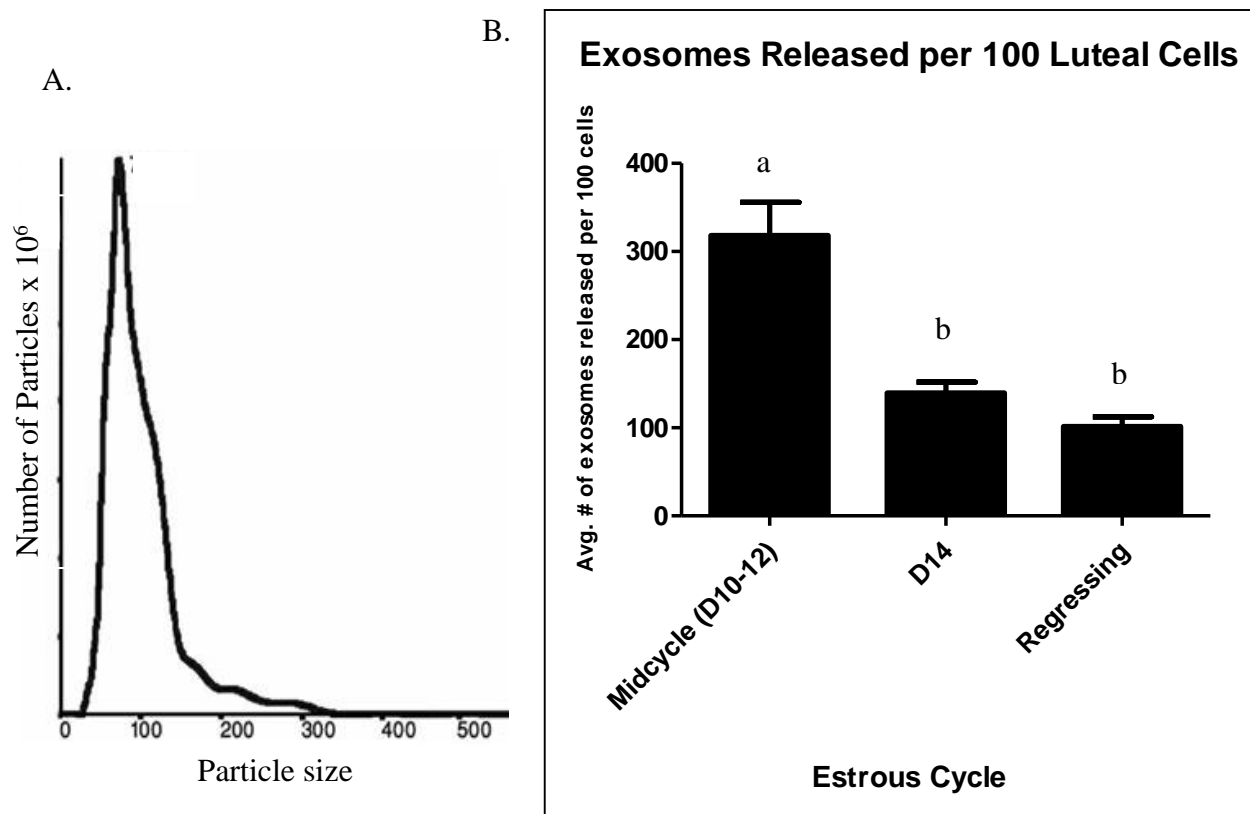


Figure 2.4: **Effect of estrous cycle on exosome secretion by luteal cells.** (A) NanoSight representative graph of particle size. (B) Quantity of secreted exosomes was determined by NanoSight and NTA. Bars with different letters indicate significance ($P < 0.05$; $n = 5$)

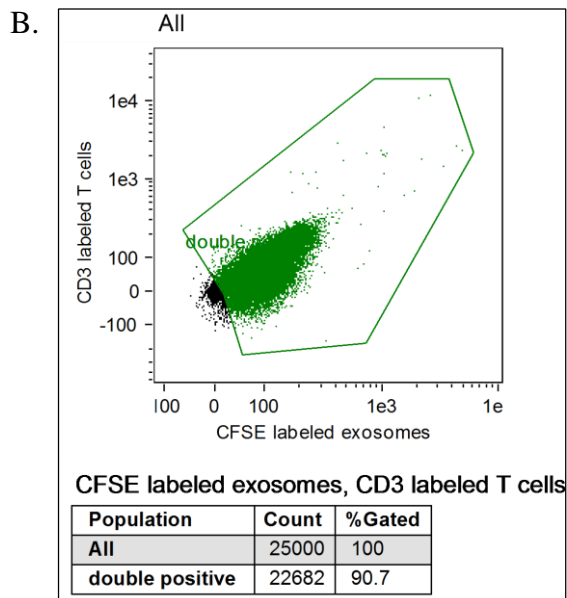
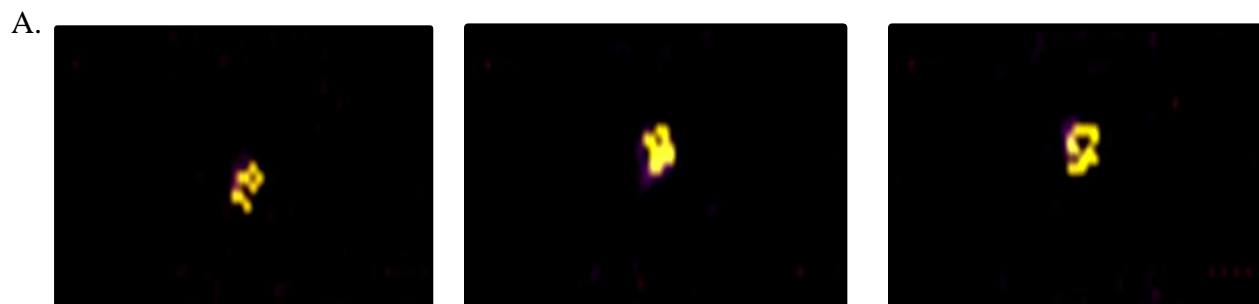


Figure 2.5: **Exosome uptake by T cells.** (A) Representative images of CD3⁺ T cells labeled with Anti CD3⁺ CF405S (purple). Midcycle luteal cell derived exosomes labeled with CFSE (yellow). Images obtained on an Amnis FlowSight. (B) Scatter plot of CFSE- labeled exosomes and CD3 labeled T cell fluorescence. The graph is gated on double positive cells showing over 90% of CFSE exosomes co-localized with CD3 T cells.

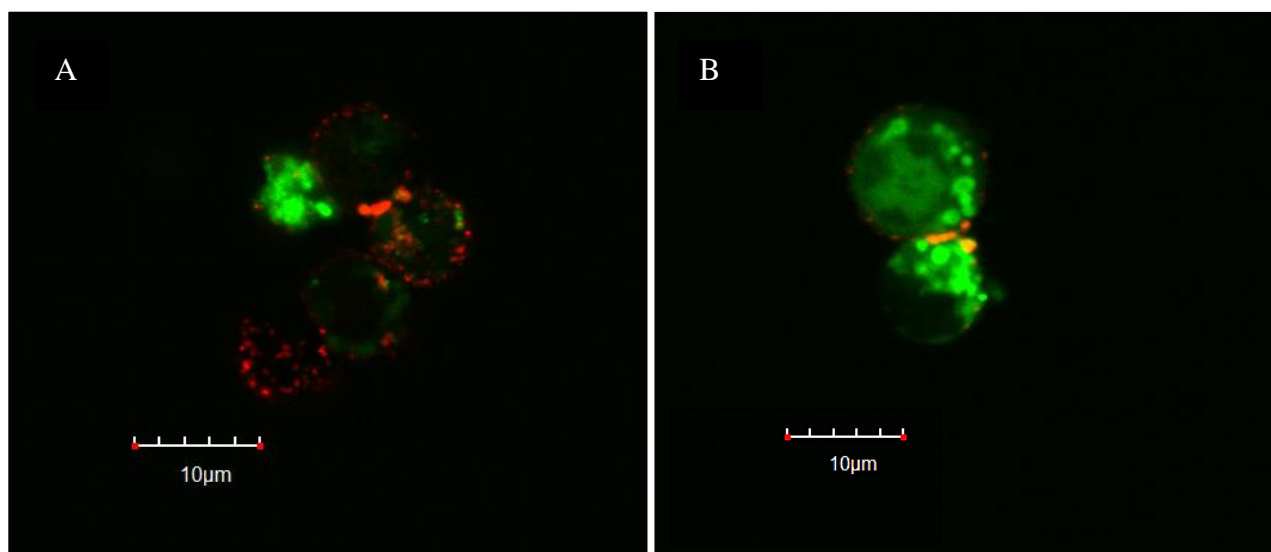


Figure 2.6: **Exosome uptake by T cells.** (A & B) Representative images of CD3⁺ T cells labeled with Anti CD3⁺ Alexa Fluor 555 (red). Midcycle luteal cell-derived exosomes labeled with CFSE (green).

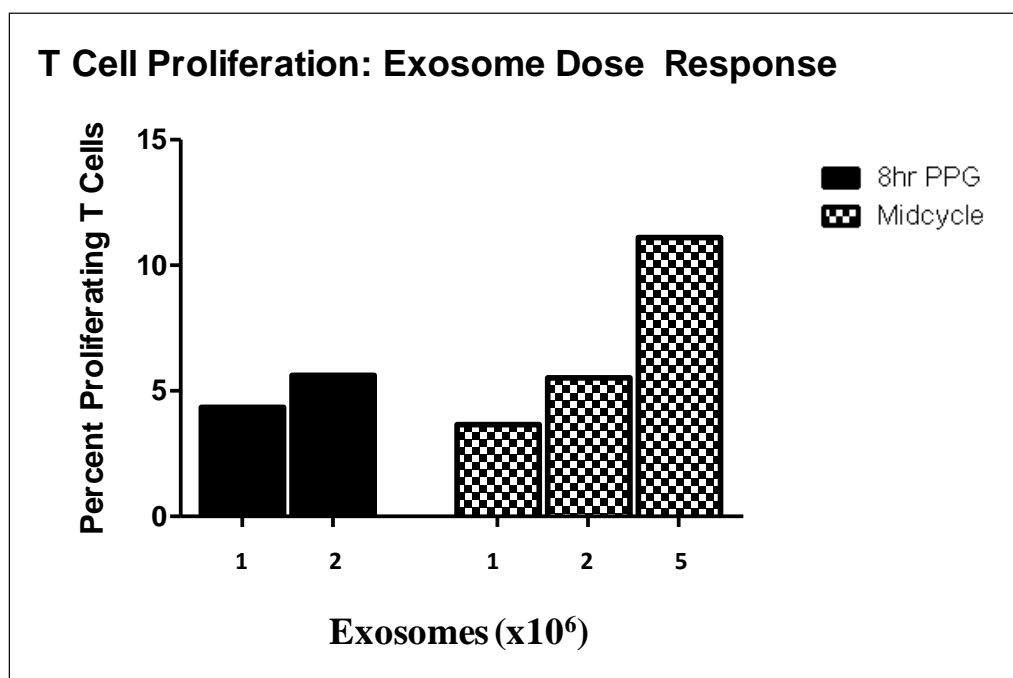


Figure 2.7: **Dose dependent effect of exosomes on T cell proliferation.** Increasing numbers of exosomes from midcycle and regressing luteal cells were added to T cells and T cell proliferation was measured (n=1).

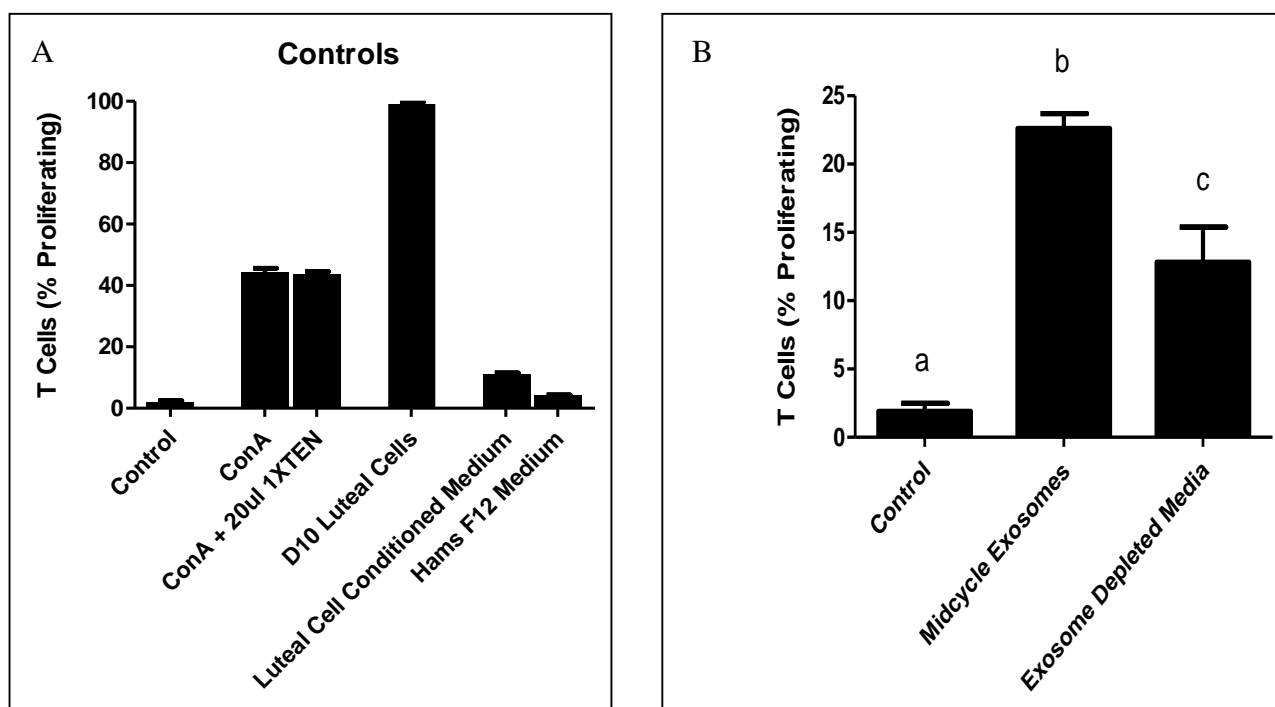


Figure 2.8: **Effect of luteal cell-derived exosomes on T cell proliferation.** A) Positive and negative controls for T cell proliferation experiment. B) Effect of exosomes isolated from 3 individual midcycle CL (R1271, R1273, R1274) on T cell proliferation. Values with different letters indicate significance, ($P < 0.05$; $n = 3$)

Gene	Sequence 5'-3'	Annealing	Accession No.
IL4	F: GCTGAACATCCCACAACGA R: TCGTCTTGGCTTCATTCACA	60°C	EU276069.1
IL10	F:CTGACAGCAGCTGTATCCACTTG R: GTGCAGTTGGTCCTTCATTTGA	60°C	NM_174088.1
TNF	F: AGGGAAGAGTCCCCAGGTG R: CCCC GGAGAGTTGATGTCG	60°C	XM_006041931.1
IFNG	F:ACTCCGGCCTAACTCTCTCCC R:AGGCCACCCTTAGCTACAT	60°C	NM_174086.1
RPL19	F: ATCGATCGCCACATGTATCA R: GCGTGCTTCCTTGGTCTTAG	60°C	NM_001040516.1

Table 2.1: **Primer Information.** Gene name (column 1), primer sequence (5'-3'; column 2), annealing temperature (column 3), and Genbank Accession number (column 4) for primer sets used for quantitative polymerase chain reaction assays.

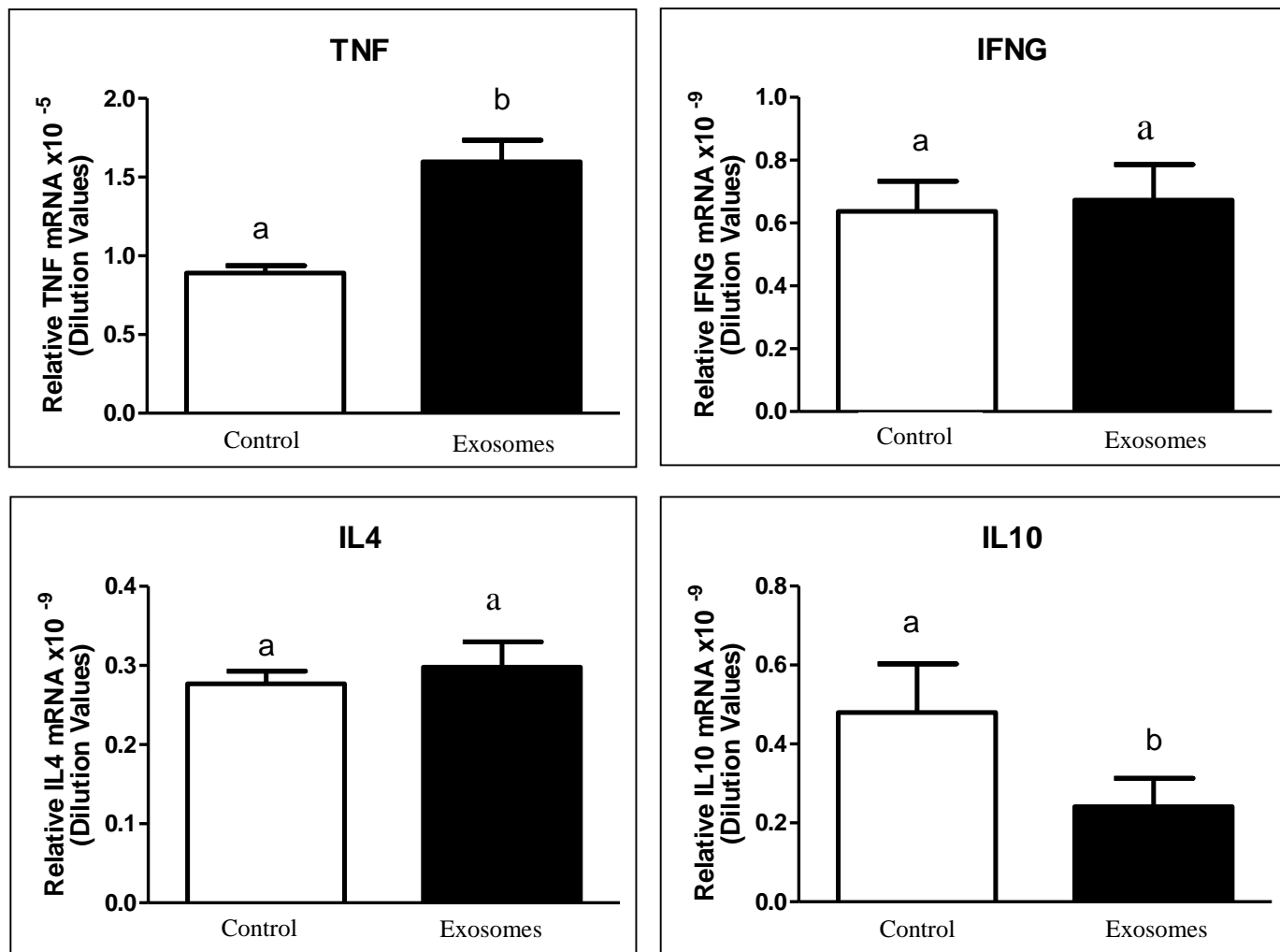


Figure 2.9: Quantitative PCR (qPCR) analysis of TNF, IFNG, IL4 and IL10 mRNA abundance in bovine T cells treated with midcycle luteal cell-derived exosomes. Bars with different letters indicate significance (P<0.05; n=2)

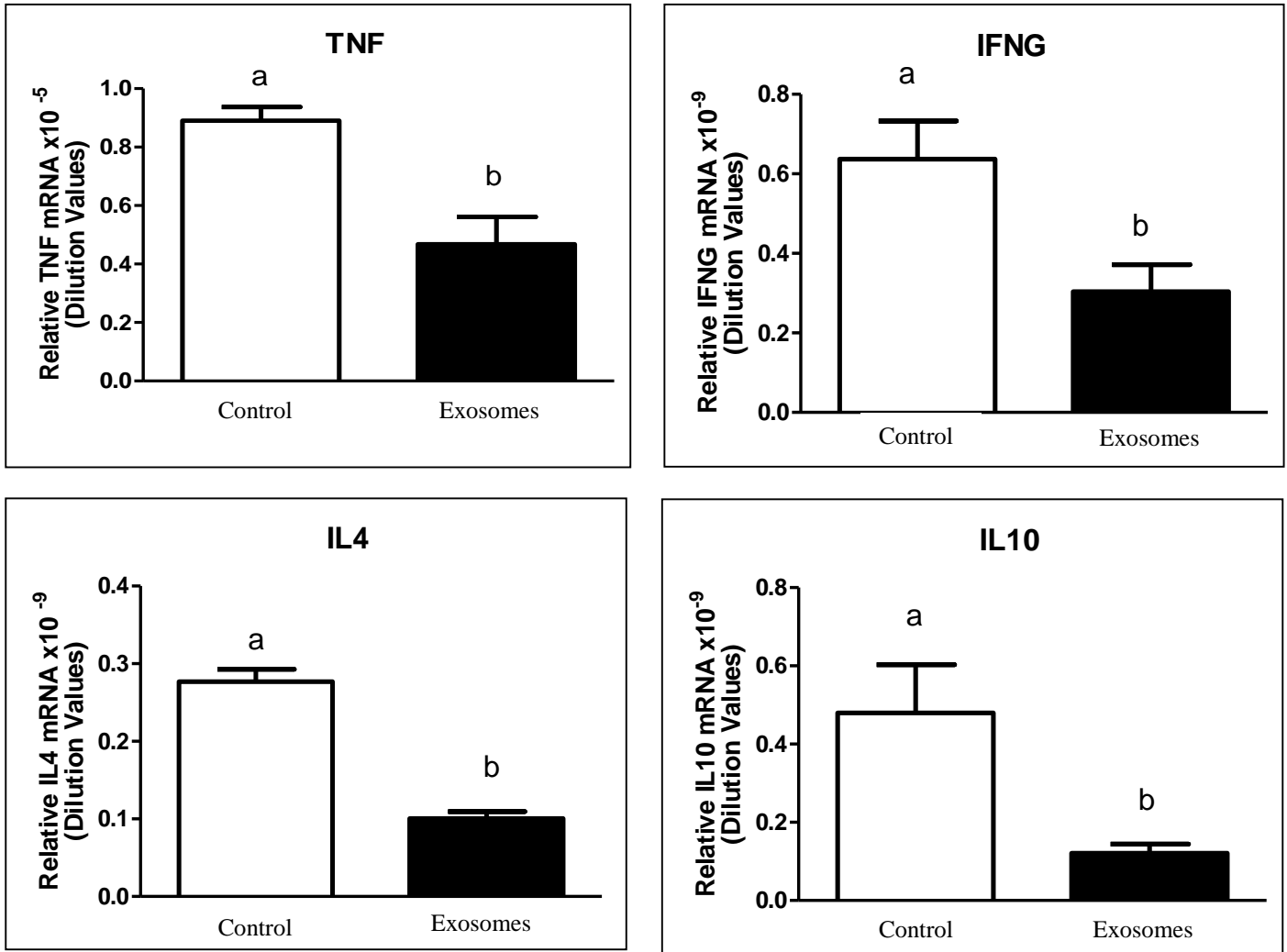


Figure 2.10: Quantitative PCR (qPCR) analysis of TNF, IFNG, IL4 and IL10 mRNA expression in bovine T cells treated with regressing luteal cell-derived exosomes. Bars with different letters indicate significance ($P < 0.05$; $n=2$).

Chapter 3: Effect of Luteal Cell-Derived Exosomes on Macrophage Polarization

ABSTRACT

Monocytes differentiate into macrophages and dendritic cells to elicit an immune response. Macrophages are broadly classified as either M1 or M2 based on expression of proteins. There is limited information on protein expression to classify bovine macrophages. The objectives of this project were to: 1. Determine an optimal serum-free culture method for bovine monocytes, 2. Differentiate bovine monocytes into M1 or M2 macrophages in vitro, 3. Determine proteins that characterize M1 and M2 macrophages in the cow, and 4. Determine if luteal cell-derived exosomes influence macrophage polarization and phenotype.

Media, cell number and time in culture were tested for optimum conditions. Monocytes were treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) + interferon gamma (IFN γ) + lipopolysaccharide (LPS) or macrophage colony-stimulating factor (M-CSF) + interleukin 4 (IL4) or M-CSF + interleukin 10 (IL10) or M-CSF + IL10 + interleukin 13 (IL13). Expression of cell genes indicative of macrophage polarization was determined using quantitative polymerase chain reaction (qPCR), including tumor necrosis factor (TNF), indoleamine-pyrrole 2,3-dioxygenase (IDO), interleukin 1-beta (IL1B), inducible nitric oxide synthase (NOS2), mannose receptor 1 (MRC1), kruppel like growth factor 4 (KLF4), chemokine C-C motif ligand 24 (CCL24), cluster of differentiation 36 (CD36), cluster of differentiation 163 (CD163), interferon regulatory factor 4 (IRF4), interleukin 10 (IL10), and transforming growth factor beta (TGFB).

The culture medium that best supported the attachment, growth and viability of bovine monocytes was Xivo-10 media supplemented with insulin, transferrin, selenium and gentamycin. Macrophages treated with IL4 exhibited a giant cell morphology, as described in other species. Treatment with MCSF + IL10 or MCSF + IL10 + IL13 resulted in greater steady state concentration of CD163, IL10 and CD36 mRNA respectively, indicative of M2 macrophages. Treatment with GMCSF + IFNG + LPS resulted in greater concentrations of TNF, IL1B, IDO and NOS2 mRNAs, indicative of M1 macrophages. Some genes such as IRF4, TGFB, CCL24, KLF4 and MRC1 were highly expressed in both M1 and M2 differentiated macrophages.

The final hypothesis to be addressed was that exosomes are secreted by luteal cells and influence the function of the CL by directing macrophage polarization toward an M1 or M2 phenotype. Exosomes were isolated from midcycle or regressing luteal cell-conditioned media by ultracentrifugation, purified and quantified. Macrophages were primed with either GMCSF or MCSF and midcycle or regressing exosomes were added. Cells were collected and expression of cell type-specific genes was determined using qPCR. The mRNAs quantified were TNF, IL1B, IDO, and NOS2 to identify M1 macrophages, and IL10, CD36, and CD163 to identify M2 macrophages. Transforming growth factor beta and CCL24 mRNA expression was also determined. From the literature, it is known that TGFB has immune-regulatory functions (Edin et al., 2012) and CCL24 is strongly chemotactic for resting T cells (Patel et al., 1997). Midcycle luteal cell-derived exosomes stimulated expression of IL1B and NOS2 in GMCSF- and MCSF-primed macrophages and stimulated TGFB, CD36, IL10 and TNF mRNA expression in MCSF-primed macrophages. Regressing luteal cell-derived exosomes stimulated expression of TNF, NOS2 and IL10 in GMCSF-primed macrophages and stimulated CCL24 mRNA expression in MCSF-primed macrophages.

Midcycle and regressing luteal cell-derived exosomes affected monocyte differentiation and macrophage polarization. Exosomes induced genes characteristic of both M1 and M2 macrophages. This programming could help modulate the luteal function during critical time points.

INTRODUCTION

Macrophages and T lymphocytes (T cells) are present in the CL, particularly at the time of luteolysis (Pate and Keyes, 2001), and are thought to play a role in regulating the physiological state of the CL. The luteal microenvironment is postulated to determine the immune cell response and eventually the fate of the CL (Poole and Pate, 2012). Macrophages are broadly classified as either M1 (classically activated macrophages) or M2 (alternatively activated macrophages) based on expression of proteins. The M1 macrophages are generally considered to inhibit cell proliferation and cause tissue damage. The M2 macrophages promote cell proliferation and tissue repair (Mills, 2001). M2 macrophages can be clustered into at least 3 subgroups (M2a, M2b, M2c). These subtypes have various functions including regulation of immunity, tissue repair, and wound healing (Murray, 2011a).

M1 macrophages are typically identified by their production of nitric oxide (NO), which inhibits cell proliferation (Hibbs et al., 1987), while M2 macrophages produce ornithine, which promotes proliferation and repair through production of polyamines and collagen (Mills, 2001). M2 is the “default” program present in resident macrophages. While markers for M1 and M2 macrophages have been extensively identified in humans and mice, specific markers have not been described for bovine macrophages.

Many studies using human and mouse cells include serum in the culture medium in which macrophages were differentiated. Serum is obtained by lysing platelets, which contain large amounts of TGF β . Transforming growth factor beta is a potent inhibitor of M1 activity and NOS2 production, thereby promoting M2 activity (Vodovotz, 1993; Mills, 2001; Morris, 2009). Serum contains over 500 pg/mL of TGF β which is similar to the concentration found in wounds and is a sufficient amount to inhibit macrophage NOS2 production. Therefore, it was desirable to determine if bovine monocytes could be maintained in a serum-free culture system.

Numerous studies have demonstrated that exosomes play a role in cell-to-cell communication. Bhatnagar et al. (2007) showed that macrophages infected with intracellular pathogens release exosomes that contain pathogen associated molecular patterns (PAMPs) and can stimulate pro-inflammatory responses in uninfected macrophages. Yang et al. (2011) observed shuttling of fluorescently-labeled exogenous miRNAs from IL4 activated macrophages to breast cancer cells without direct cell-to-cell contact. Further experimentation revealed that this shuttling was via exosomes.

The focus of this experiment was on communication between midcycle or regressing luteal cell-derived exosomes and macrophages. Because macrophages are one of the most abundant immune cells in the regressing CL (Bagavandoss et al., 1988; Best et al., 1996; Takaya et al., 1997; Penny et al., 1999; Bauer et al., 2001) it was essential to determine the role exosomes might play in regulating macrophage function. The hypothesis tested in this experiment was that exosomes are secreted from bovine luteal cells and that they can direct macrophage polarization and influence macrophage phenotype.

MATERIALS AND METHODS

For *Luteal Cell Isolation and Culture, Exosome Isolation, Purification, and Quantification, RNA Isolation, cDNA synthesis, and Quantitative PCR* methods, please refer to Chapter 2 Materials and Methods.

Quantitative PCR

For all qPCR experiments, RPL19 (bovine ribosomal protein L19; RPL19) was used as an internal control, which was determined to not be affected by treatment.

Collection of Blood and PBMC isolation

For cell culture, blood was collected from the jugular vein of Holstein dairy cows into a sterilized bottle containing 0.5 M EDTA. The protocols used for blood collection were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #44196). Blood was transported back to the lab on ice. Blood was aliquoted into sterile 16x125mm borosilicate glass tubes, covered with parafilm and centrifuged at 300xg at 4°C for 15 minutes. Buffy coats were collected, and slowly pipetted into sterile 16x125mm borosilicate glass tubes containing Ficoll-Paque Plus (GE Health Care, USA). Tubes were covered with parafilm and centrifuged at 660xg for 30 minutes at 25°C. The dense mononuclear band was collected and placed into 50 mL centrifuge tubes containing 15 mL cold PBS-EDTA. Tubes were centrifuged at 660xg for 10 minutes at 4°C. Cells were washed twice by resuspending cells in cold PBS-EDTA centrifuged at 300xg for 10 minutes at 4°C.

PBMC Cell Culture

PBMC were isolated, as described above, and cell viability was determined by flow cytometry (GuavaEasyCyte Plus; Millipore, Billerica, MA) using Guava Viacount dye (Millipore). Flasks (T25; Sigma-Aldrich, St. Louis, MO), were coated with HamsF12 medium (LifeTechnologies) + 10% newborn calf serum (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 30°C in an atmosphere of 5% CO₂. Flasks were washed twice with HamsF12 media + gentamycin (10 µg/mL; Gentamycin Reagent Solution; Invitrogen Corporation). PBMC were seeded at 28x10⁶ cells per T25 flask. To determine an optimal time for monocyte adhesion, two different time periods were tested: 24 hours and 48 hours. To determine a media that supported the growth and viability of PBMC, three different media were tested: RPMI medium supplemented with insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 µg/mL; ITS), and gentamycin (10 µg/mL), RPMI medium + 5% FBS supplemented with insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 µg/mL; ITS, VWR, Philadelphia, PA), and gentamycin (10 µg/mL), and X-Vivo10 medium supplemented with insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 µg/mL; ITS), and gentamycin (10 µg/mL).

Based on the results obtained from the trial above, for all subsequent experiments, monocytes were allowed to adhere for 48 hours after which they were washed twice with HamsF12 media + gentamycin (10 µg/mL) and maintained in X-Vivo10 media supplemented with insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 µg/mL; ITS), and gentamycin (10 µg/mL).

Cytokine Treatments

All cytokines used in this experiment were purchased from Kingfisher Biotech, INC (St. Paul, MN, USA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

<u>Cytokine</u>	<u>Concentration</u>
Recombinant equine GMCSF	100 ng/mL
IFNG	50 ng/mL
LPS	10 ng/mL
Recombinant mouse MCSF	100 ng/mL
Recombinant bovine IL4	10 ng/mL
Recombinant bovine IL10	10 ng/mL
Recombinant bovine IL13	10 ng/mL

Table 3.1: Cytokines concentrations used in all macrophage culture experiments.

M1/M2 in Vitro Differentiation

After adhesion, macrophages were activated toward either a classical pathway (M1 macrophages) with GMCSF or an alternative pathway (M2 macrophages) with MCSF.

Macrophages were then differentiated into M1 macrophages by treating with GMCSF + IFNG + LPS or into M2 macrophages by treating with MCSF + IL10, MCSF + IL4, or MCSF + IL10 + IL13.

Day 0	Day 2	Day 3	Day 4	Expected Cell Type
Seed 28x10 ⁶ PBMC	No treatment	No treatment	Collect	Control monocytes
Seed 28x10 ⁶ PBMC	400 ng GMCSF	400 ng GMCSF	Collect	Activated to become M1
Seed 28x10 ⁶ PBMC	400 ng GMCSF	400 ng GMCSF + 200 ng IFNG + 40 ng LPS	Collect	M1
Seed 28x10 ⁶ PBMC	400 ng MCSF	400 ng MCSF	Collect	Activated to become M2
Seed 28x10 ⁶ PBMC	400 ng MCSF + 40 ng IL4	400 ng MCSF + 40 ng IL4	Collect	M2a
Seed 28x10 ⁶ PBMC	400 ng MCSF + 40 ng IL10 + 40 ng IL13	400 ng MCSF + 40 ng IL10 + 40 ng IL13	Collect	M2b
Seed 28x10 ⁶ PBMC	400 ng MCSF + 40 ng IL10	400 ng MCSF + 40 ng IL10	Collect	M2c

Table 3.2: Protocol used for the in vitro differentiation of bovine monocytes to M1 or M2 macrophages.

Exosome/Monocyte Culture

PMBC were isolated and seeded at 10×10^6 per well of a 12 well plate. Once monocytes attached, cells were washed twice with HamsF12 media + gentamycin and maintained in X-Vivo10 media supplemented with ITS and gentamycin.

Exosomes from midcycle (n=4) and regressing (n=3) luteal cells were isolated as described in chapter 2 and 20×10^6 midcycle and 15×10^6 regressing exosomes were added to monocytes along with GMCSF or MCSF for 72 hours at $30^\circ\text{C} + 5\% \text{CO}_2$. Because less regressing luteal cell-derived exosomes could be isolated, different numbers of exosomes were added. After incubation, media were collected and stored in glass vials at -20°C . Cells were then collected by adding 1 mL of TRIZOL reagent to each T25 flask. Finally, RNA isolation, cDNA synthesis, and qPCR were performed, as described in Chapter 2. Abundance of mRNA was measured by qPCR for TNF, IL1B, IDO, and NOS2 to identify M1 macrophages, and IL10, CD36, and CD163 to identify M2 macrophages (primer information in table 3.3). Also, CCL24, TGFB, CD11b and CD11c mRNA expression was analyzed.

In an additional experiment, PMBC were isolated and seeded at 10×10^6 per well in a 12 well plate. Once monocytes attached, cells were washed twice with HamsF12 media + gentamycin and maintained in X-Vivo10 media supplemented with ITS and gentamycin. Next, midcycle (n=1) and regressing (n=1) exosomes were isolated, as described in chapter 2, and 20×10^6 midcycle and 15×10^6 regressing exosomes were added to unprimed monocytes for 72 hours at $30^\circ\text{C} + 5\% \text{CO}_2$. It was of interest to determine if luteal cell derived exosomes could have the same responses on macrophages without priming agents. Quantitative polymerase chain reaction was performed for TNF, NOS2, IL1B, IDO, CD163, CD36, IL10, CCL24, TGFB, CD11b and CD11c (primer information in table 3.3).

Statistical Analyses and Data Analysis

Results for macrophage differentiation are presented as standard error mean (SEM) of relative mRNA expression. Messenger RNA expression values were obtained from a standard curve. Differences were determined by ANOVA with a Bonferroni post-test. Comparisons were made between each cytokine treatment (GMCSF + IFNG + LPS or MCSF+ IL4, MCSF+ IL10 and MCSF+IL10 + IL13) and its control (GMCSF or MCSF only). Differences in gene expression were deemed statistically significant when the P-value was <0.05 and are indicated with an asterisk.

Results for mRNA abundance in macrophages treated with midcycle or regressing luteal cell-derived exosomes are presented as SEM of fold change. Fold change was calculated and controls were set as a fold change of 1. Treatment comparisons were made by t-test using SAS (Ver. 9.3; SAS Institute). Differences in gene expression were deemed statistically significant when the P-value was <0.05 and are indicated with an asterisk.

Statistical tests could not be performed on macrophages treated with midcycle or regressing luteal cell-derived exosomes without priming agents because this experiment was only completed one time. Only observations can be reported until this experiment is repeated.

RESULTS

Bovine PBMC Culture

Under suitable conditions, monocytes will adhere to a serum coated flask. This process is essential for their survival in culture. The time required for monocyte adhesion was tested at 24 and 48 hours. After 24 hours, it was observed that many cells were still floating in the media and if the flask was washed, only about 30% confluency was observed. After 48 hours, it was observed that almost all of the monocytes had adhered and if washed, the flask appeared to be about 80-90% confluent. It was determined that for confluency, a minimum of 48 hours was needed to allow monocytes to adhere to the flask and survive. To determine a medium that supported the growth and viability of monocytes, three different media were tested. The culture medium that best supported the attachment, growth and viability of monocytes was X-Vivo10 media supplemented with ITS and gentamycin (Figure 3.1C).

M1/M2 Macrophage Morphology

M1 and M2 macrophages exhibited different morphology in culture. M1 macrophages (Figure 3.2A) exhibited a more spiny appearance while M2 macrophages exhibited a more rounded and compact appearance (Figure 3.2B). The morphology of all M2 macrophages was similar, however, macrophages treated with IL4 exhibited a giant cell morphology (Figures 3.3A-D).

M1 and M2 Macrophage Markers

Treatment with GMCSF + IFNG + LPS resulted in greater concentration of TNF (Figure 3.4), NOS2 (Figure 3.5), IL1B (Figure 3.6), and IDO (Figure 3.7) mRNAs, indicative of M1 macrophages. Macrophages treated with MCSF + IL10 exhibited in greater steady state

concentration of CD163 (Figure 3.8) and IL10 (Figure 3.9) mRNA compared to other treatments, indicative of M2 macrophages. Treatment with MCSF + IL10 + IL13 resulted in greater steady state concentration of CD36 (Figure 3.10). Some genes such as CCL24 (Figure 3.11), MRC1 (Figure 3.12), KLF 4 (Figure 3.13), IRF4 (Figure 3.14), and TGFB (Figure 3.15) were highly expressed in both M1 and M2 macrophages.

Effects of Exosomes on Macrophage Differentiation

Midcycle luteal cell-derived exosomes drove monocyte differentiation into macrophages that were characterized by high expression of NOS2 (Figure 3.16) and IL1B (Figure 3.17) mRNA in GMCSF- and MCSF-primed macrophages and high expression of IL10 (Figure 3.18), CD36 (Figure 3.19), TGFB (Figure 3.20) and TNF (Figure 3.21) in MCSF-primed macrophages ($p < 0.05$). No change in mRNA expression was determined in IDO (Figure 3.22), CD163 (Figure 3.23) or CCL24 (Figure 3.24). Regressing luteal cell-derived exosomes induced high expression of TNF (Figure 3.27), NOS2 (Figure 3.28) and IL10 (Figure 3.29) mRNA in GMCSF-primed macrophages and high expression of CCL24 (Figure 3.30) mRNA in MCSF-primed macrophages ($p < 0.05$). A decrease in TGFB mRNA expression was discovered in GMCSF-primed macrophages ($p < 0.05$; Figure 3.31). No changes in IL1B (Figure 3.32), IDO (Figure 3.33), CD163 (Figure 3.34) and CD36 (Figure 3.35) mRNA expression were seen.

In an additional experiment, exosomes were added to T cells without priming agents, MCSF or GMCSF. Although direct comparisons cannot be made because these were run in separate experiments, it appears that exosomes induced much lower expression of all mRNAs quantified in both midcycle and regressing samples when the priming agent was not added. Midcycle luteal cell-derived exosomes appeared to induce increases in NOS2, IL10 and TGFB,

decreases in CD163, and no change in TNF, IL1B, IDO, or CD36 mRNA expression compared to untreated (control) macrophages (see figures 3.25 and 3.26). Regressing luteal cell-derived exosomes appeared to increase NOS2, CD163 and CD36 mRNA expression while no changes were seen in TNF, IL1B, IDO, IL10, or TGF β mRNA expression (see figures 3.36 and 3.37).

To determine the macrophage/dendritic cell phenotype of the differentiated cells, cluster of differentiation 11B (CD11b) and CD11c mRNA expression was determined in all of the samples used for the above experiments. Cluster of differentiation 11B mRNA expression was present in all samples, midcycle and regressing although, CD11C expression appeared higher in samples from midcycle (Figure 3.38) than regressing (Figure 3.39).

DISCUSSION

Monocytes cultured in RPMI medium appeared unhealthy and did not survive in culture. Monocytes cultured in RPMI medium + 5% FBS serum survived but the goal was to determine an optimal serum-free culture so that medium components were defined and consistent. Also, serum contains TGF β , which is a potent inhibitor of M1 activity and NOS2 production and therefore a promoter of M2 activity and ornithine production (Vodovotz 1993; Mills 2001; Morris 2009). X-Vivo medium allowed for optimal survival of bovine monocytes in a defined environment, therefore, this medium was utilized in all subsequent monocyte culture experiments.

M1 and M2 macrophages had different morphology with M1 treated macrophages having more projections making them appear more elongated and M2 treated macrophages with a more round appearance. This same morphology has been described by Leavy (2013). Macrophages

treated with IL4 exhibited a giant cell morphology that occurs during macrophage fusion, as described in hamsters (Ptak et al., 1970), human (Hassan et al., 1989), mouse (Tanaka et al., 1989) and bovine (Nickerson & Sordillo 1985). McNally and Anderson (2011) investigated the role of human macrophage fusion factors IL4, IL13, and α -tocopherol and revealed the role of mannose receptor in macrophage fusion. While we were not able to detect an increase in MRC1 mRNA in IL4 treated monocytes, giant cells still formed. This means that an alternative mechanism is involved in macrophage fusion. Yoshihara et al. (2004) observed giant cells only when bovine monocytes were cultured with GMCSF plus conditioned media from concanavalin A stimulated monocytes.

GMCSF has traditionally been used to prime macrophages toward an M1 phenotype and MCSF toward an M2 phenotype. Previous studies, suggested that human macrophages primed with MCSF cannot mature into M1 macrophages (Fleetwood et al., 2007; Fleetwood et al., 2009). It was speculated that this is due to the failure of MCSF-primed macrophages to secrete IL12 in response to LPS stimulation (Verreck et al., 2004). A recent study showed that both MCSF- and GMCSF-polarized macrophages treated with cytokines IFNG and LPS to induce an M1 phenotype have similar expression of M1 markers (Jacuin et al., 2013). This study suggests that MCSF and GMCSF prime macrophages in a similar manner, and human MCSF macrophages stimulated by IFNG and LPS can polarize toward M1. The current understanding is that the change from MCSF macrophages to M2 macrophages needs to be reconsidered and that the pro-M2 word seems more appropriate for unstimulated MCSF macrophages (Jacuin et al., 2013). The studies performed in this experiment did not determine if MCSF can be used to prime bovine monocytes toward an M1 phenotype or if GMCSF can prime toward an M2 phenotype. It

would be interesting to determine if bovine monocytes are able to express similar M1 or M2 mRNA markers when MCSF or GMCSF is used to prime them.

In macrophages treated with GMCSF + IFNG + LPS to induce an M1 phenotype, expression of TNF, IL1B, IDO and NOS2 mRNA expression was stimulated. These results are consistent with extensive reports from other species, such as humans (Mantovani & Locati, 2009) and mice (Hongwei et al., 2012). M1 macrophage markers seem to be fairly conserved between species.

Treatment with MCSF + IL10 resulted in greater steady state concentration of CD163 mRNA, indicative of an M2c phenotype (Mantovani et al., 2004). Treatment with MCSF + IL10 + IL13 resulted in greater steady state concentration of CD36 mRNA, indicative of an M2a phenotype (Mantovani et al., 2004). Additionally, treatment with MCSF and IL10 induced greater steady state concentration of IL10 mRNA. Gene expression indicative of M2 macrophages was more difficult to define than that of M1 macrophages. This may be due to the diversity of M2 macrophages, called M2a, M2b, and M2c, which express somewhat different genes and proteins. M2a (induced by treatment with IL4 or IL13) and M2b (induced by stimulation with immune complexes such as TLR or IL1R ligands) macrophages exert immunoregulatory functions and drive Th2 responses, whereas M2c cells (generated by treatment with IL10) play a predominant role in the suppression of immune responses, tissue remodeling, and matrix deposition (Mantovani et al., 2004).

In contrast to previous observations in mouse macrophages (Sato et al., 2010; Chartouni et al., 2010) or in human monocytes (Lehtonen et al., 2005), we did not identify IRF4 as a specific M2 macrophage marker. Also, contrary to human studies where KLF4 was upregulated in M2 macrophages (Liao et al., 2011), KLF4 was upregulated in both M1 and M2 bovine macrophages

demonstrating it is not a reliable M2 marker in bovine macrophages. Additionally, Chroneos and Sheppard (1995) showed that M2 human alveolar macrophages express MRC1, which was not found to be true in the current study. In bovine macrophages, both GMCSF + IFNG + LPS (M1)- and MCSF + IL10 (M2)-treated macrophages expressed high concentrations of MRC1. Moreover, we reinforced the notion that the cow is distinct with respect to expression of M2 markers of macrophage polarization compared to mice and humans. This information is vital to correctly classifying macrophages in the cow.

It was proposed that midcycle and regressing luteal cell-derived exosomes have the potential to induce macrophage polarization and the physiological state from which the exosomes were derived may influence gene expression by macrophages. We hypothesized that midcycle luteal cell-derived exosomes induce greater expression of M2 macrophage markers and regressing luteal cell-derived exosomes induce greater expression of M1 macrophage markers.

Midcycle luteal cell-derived induced macrophages that were characterized by highly significant expression of NOS2 and IL1B mRNA in GMCSF- and MCSF-primed macrophages, significant expression of IL10, CD36 and TGFB in MCSF-primed macrophages, significant expression of TNF mRNA in MCSF-primed macrophages and no change in IDO, CD163 or CCL24 mRNA expression. Interestingly, pro-inflammatory cytokines such as NOS2 and IL1B mRNA expression was substantially increased when macrophages were treated with midcycle exosomes. This was not what we had expected but, the expression of pro- and anti-inflammatory markers may be needed to regulate immune cell infiltration and maintenance of the CL during the midcycle luteal phase. Cluster of differentiation 36 is an anti-inflammatory marker and TGFB and IL10 are thought to have regulatory functions therefore, this balance between M1 and M2 macrophages may be critical for survival of the CL.

Regressing luteal cell-derived exosomes exosome induced high expression of TNF, NOS2, and IL10 mRNA in GMCSF-primed macrophages, significant expression of CCL24 mRNA expression in MCSF-primed macrophages. Additionally, a significant decrease in TGFB mRNA expression in GMCSF-primed macrophages and no changes in IL1B, IDO, CD163 and CD36 mRNA expression were seen. Macrophages display plasticity and have the ability to change their physiology in response to their environment. Interleukin 10 expression along with pro-inflammatory genes may be explained by Mosser and Edwards (2008) who suggested a new grouping of macrophage populations: host defense, wound healing and immune regulation. This new grouping allows for the recognition that macrophages in tissues must be regulated and expression of one type of macrophage would not be expected. Additionally Murry and Wynn (2011b) proposed that the three basic macrophage populations can blend in various “shades” of activation and macrophage polarization should not be considered as two distinct outcomes, such as pro- and anti-inflammatory. These data continue to support the theory that a balance between the macrophage populations may be critical for modulating the complex luteal microenvironment.

To determine if the monocytes from these experiments differentiated into macrophages or dendritic cells when stimulated with GMSCF or MCSF, CD11b and CD11c mRNA expression was determined. Cluster of differentiation 11B is most highly expressed on macrophages while CD11c is most highly expressed on dendritic cells, although there is some overlap in expression. Cluster of differentiation 11B was greater than CD11c in all samples. Interestingly, CD11c mRNA expression was greater in cells treated with midcycle exosomes compared to those treated with regressing exosomes. Although, higher CD11c expression was

seen in the fresh and no treatment samples used in that experiment which suggests that there may have been more dendritic cells from the isolation and plating.

In an additional experiment, it was of interest to determine if the priming agents, GMCSF and MCSF, were essential for exosomes to affect macrophage polarization. Although direct comparisons cannot be made because these samples were run in separate experiments from the primed macrophages, it appears that exosomes from both midcycle and regressing luteal cells induced much lower expression of all mRNA when the priming agents were not added compared to when priming agents had been added. Midcycle exosomes appeared to induce NOS2, CD36, IL10 and TGFB mRNA, reduce CD163, CCL24 and IL1B mRNA, and had no effect on TNF or IDO mRNA expression. Regressing luteal cell-derived exosomes appeared to induce NOS2, IDO, CCL24, CD163 and CD36 mRNA expression and had no effect TNF, IL1B, TGFB, or IL10 mRNA expression. Although this experiment was only performed one time and will need to be repeated, it was interesting that monocytes need GMCSF or MCSF for exosomes to induce highly significant changes in mRNA expression. Additionally, it was interesting that exosomes have the capacity to induce changes in mRNA without any additional priming agent. These experiments also show that exosomes are most likely not delivering the mRNA but rather inducing their expression in the macrophages. We can hypothesize this because the same numbers of exosomes were added to macrophages without priming agents and mRNA expression decreased substantially from when priming agents were added.

It would be of interest to determine the effects of cytokines (IFNG + LPS, IL4, IL10, and IL13) on monocytes without priming agents. It is not known if these cytokines can drive bovine monocyte differentiation into M1 or M2 macrophages without priming. The priming agents may be essential to drive monocytes differentiation to macrophages. When monocytes from

circulation are brought into tissues, they receive many signals from the immune system, such as local cytokines, to direct their function and phenotype. In culture, these signals are absent and priming agents may be needed to help mature monocytes to macrophages.

From these studies we can conclude that both midcycle and regressing luteal cell-derived exosomes can induce macrophage polarization. Although we cannot directly compare the effects of midcycle to regressing luteal cell-derived exosomes effects on macrophages, midcycle luteal cell-derived exosomes were able to induce very high expression of M1 macrophage markers, even higher expression than GM-CSF + IFN- γ + LPS treated macrophages. This shows that midcycle exosomes are very potent stimulators of macrophage polarization, which may explain why midcycle luteal cells do not need contact to stimulate T cells and why more exosomes are released from midcycle luteal cells than regressing. Although we do not know for sure if the exosomes are delivering these mRNA or inducing the expression, it is still very interesting that exosomes have the potential to direct macrophage function. Further studies will need to be conducted to determine the contents of these exosomes, which will help clarify their function.

Gene	Sequence 5'-3'	Annealing	Accession No.
CD36	F: CTCTGTGTCTAAGCTACATCTC R: AGTGGTAACCAGTTGGAAGTC	60°C	NM_001278621.1
CCL24	F: ACCAGCTTACCAACAGGAGC R: CGGGCTTAGATGAAGGTGCT	60°C	NM_001046596.2
IRF4	F: AACTACATGATCCCGCCCCA R: TCGGCAGACCTTATGCTTGG	60°C	NM_001206162.1
TNF	F: AGAGGGAAGAGTCCCCAGGTG R: CCCCGGAGAGTTGATGTCTG	60°C	XM_006041931.1
IL1B	F: GCAACCGTACCTGAACCCAT R: CCATCTCCCATGGAACCGAG	60°C	XM_006056023.1
NOS2	F: AGAGACGGGGAGATCGGAAA R: TACCGGAACGTGTTGGTGAG	60°C	XM_005963683.1
KLF4	F: TCCCACCGCTCCATTAC R: ATGAGAACTCTTCGTGTAGG	60°C	NM_001105385
CD163	F: CCACTTCCGGTCCCTTCACTC R: GCACACAGTTCCTCCACTCTT	58°C	NM_001163413
MRC1	F: TCGAGTTGAGCCACTTCA R: TCTTTCACCAGAGGGATCAC	55°C	XM_003582925
IDO	F: GGGCCCATGACTTATGAGAA R: GAGGCAGCTGCTATTTCCAC	60°C	NM_001101866
IL10	F: CTGACAGCAGCTGTATCCACTTG R: GTGCAGTTGGTCCTTCATTTGA	60°C	NM_174088.1
TGFB	F: CCGAACCTGTGTTGCTCTCT R: TGAGGTAGCGCCAGGAATTG	60°C	NM_001166068.1
CD11b	F: CTCTTGCAATTTGCTGCCAAC R: CGAGAAGCAGAGGCCATTTG	60°C	NM_001039957
CD11c	F: ACCTCCAGCCTATGTTGGCA R: GAGGTCATCCTGGCAGACGT	60°C	XM_010819391.1
RPL19	F: ATCGATCGCCACATGTATCA R: GCGTGCTTCCTTGGTCTTAG	60°C	NM_001040516.1

Table 3.3: **Primer information.** Gene name (column 1), primer sequence (5'-3'; column 2), annealing temperature (column 3), and Genbank Accession number (column 4) for primer sets used for quantitative polymerase chain reaction assays.

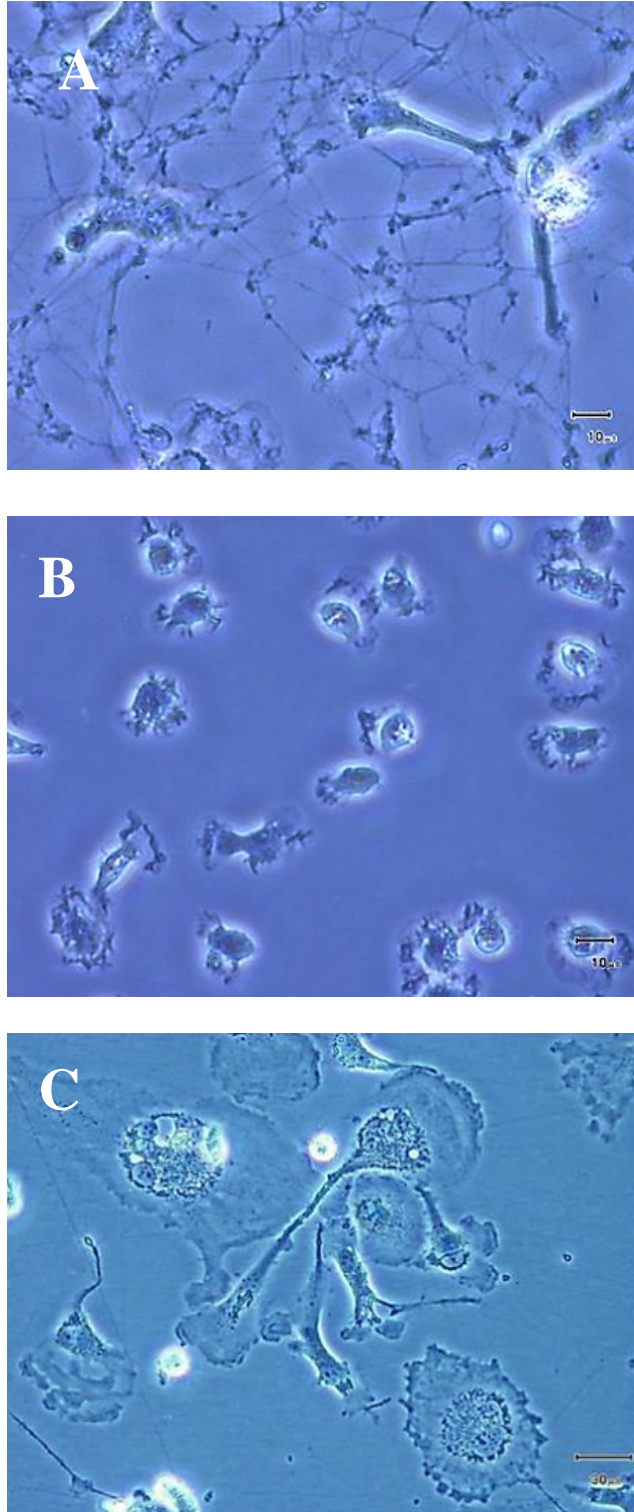


Figure 3.1: PBMC after 72 hours of culture in (A) RPMI, 40X magnification (B) RPMI + 5% FBS, 40X magnification (C) X-VIVO10, 20X magnification.

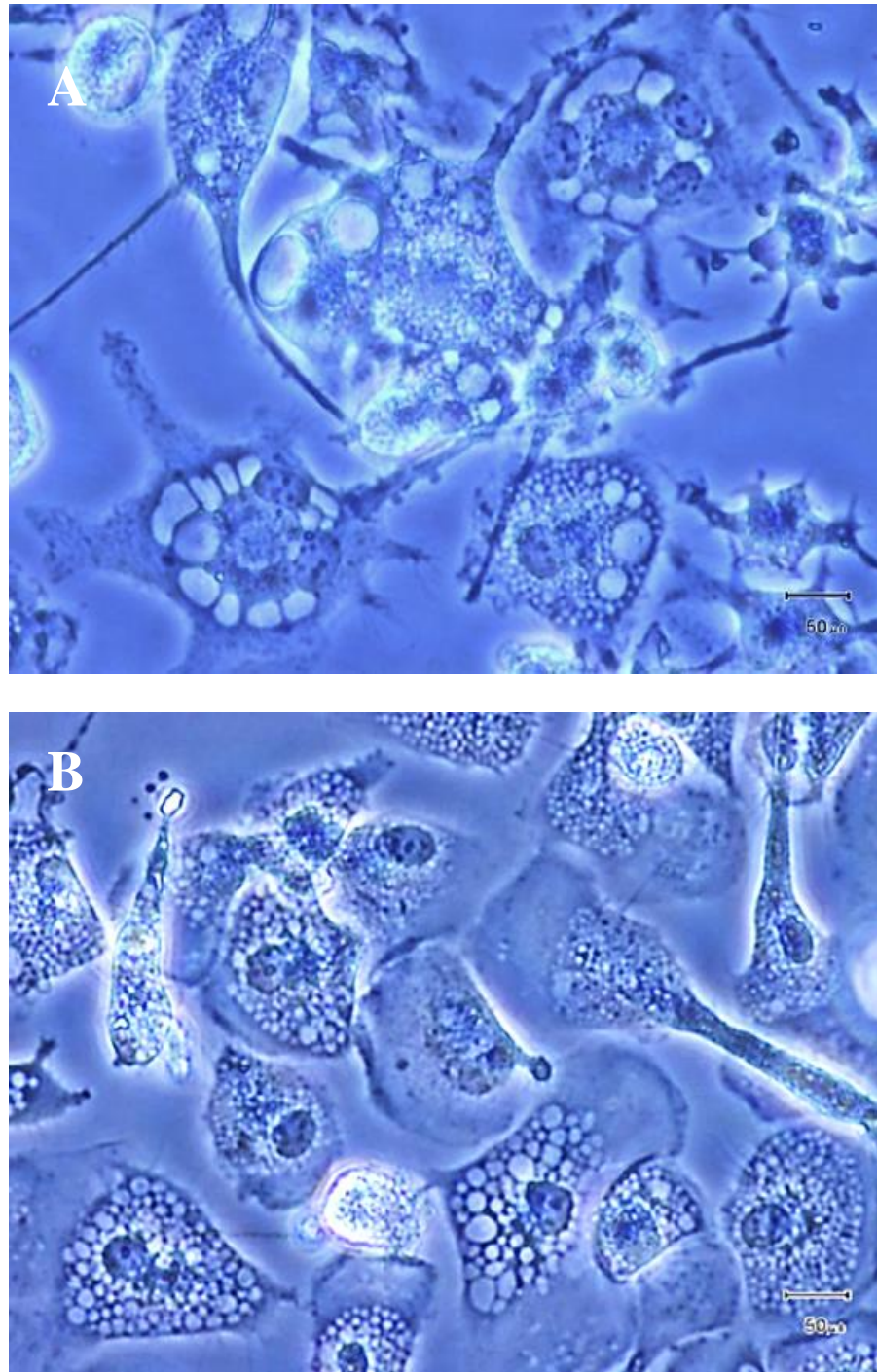
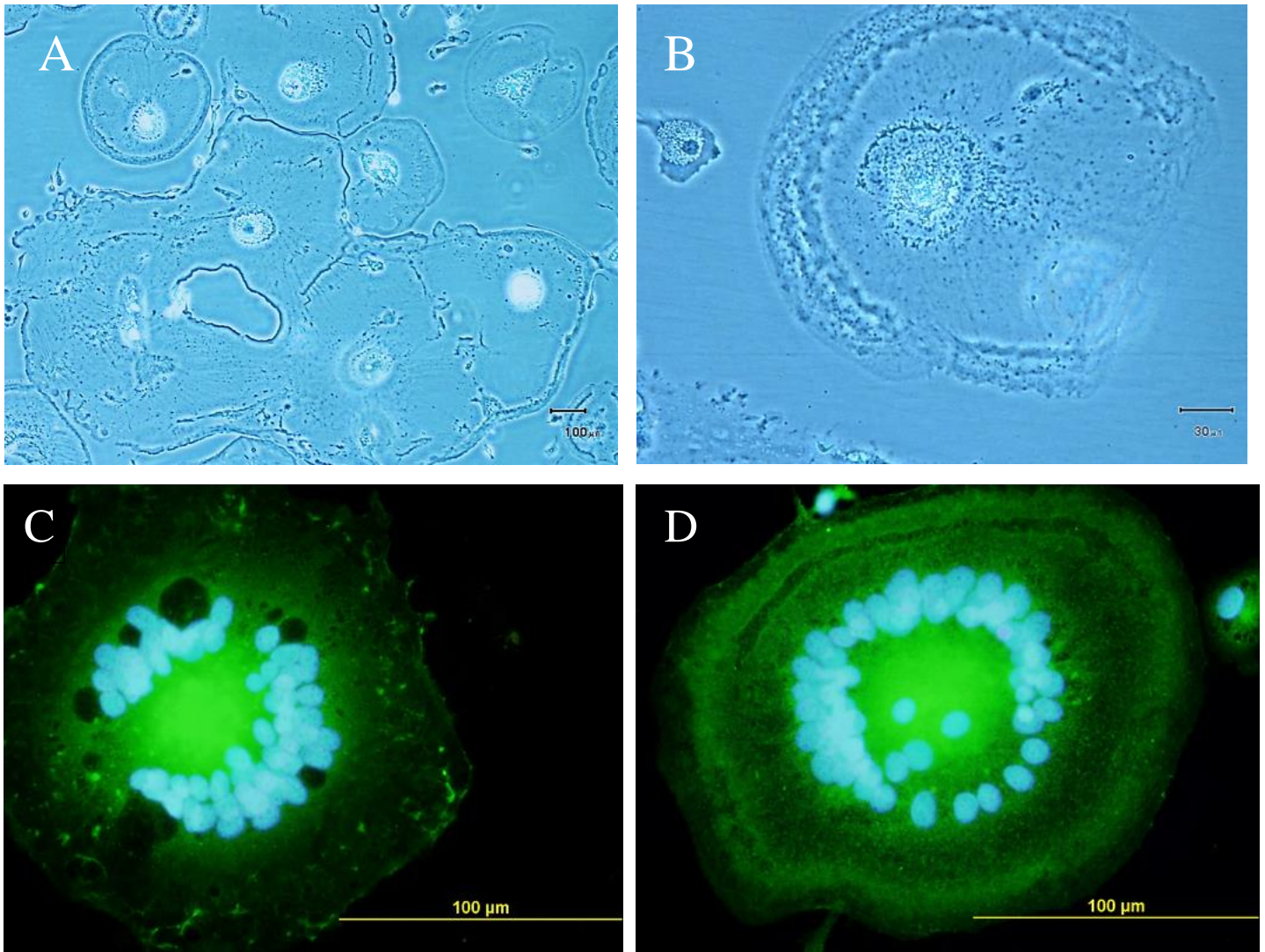


Figure 3.2: Macrophages after 5 days in culture treated with (A) GMCSF (100 ng/mL) + LPS (10 ng/mL) + IFNG, 10X (50 ng/mL) (B) MCSF (100 ng/mL) + IL10 (10 ng/mL), 10X magnification.



Figures 3.3: (A & B) Macrophages cultured for 5 days treated with MCSF (100 ng/mL) + IL4 (10 ng/mL; A: 4x, B: 20X). Cells undergo fusion and exhibit a giant cell morphology. (C & D) Macrophages cultured for 5 days treated with MCSF (100 ng/mL) + IL4 (10 ng/mL; A & B: 4X). Giant cells stained with phalloidin (green) and dapi (blue). These cells exhibit a multinucleated giant cell morphology.

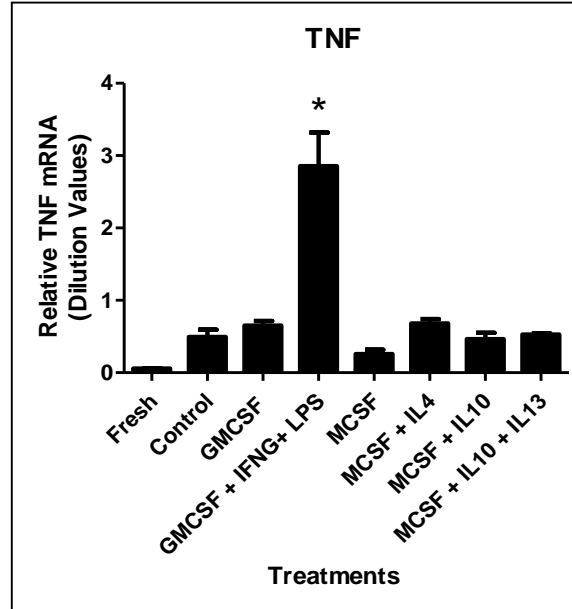


Figure 3.4: TNF mRNA expression in bovine differentiated macrophages (n=3)

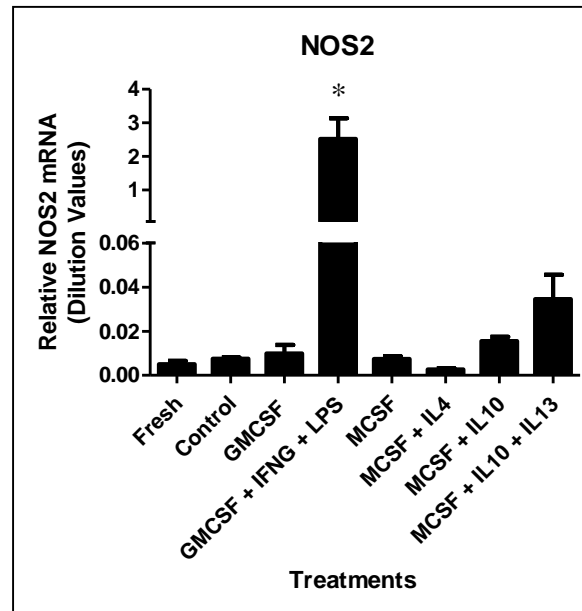


Figure 3.5: NOS2 mRNA expression in bovine differentiated macrophages (n=3)

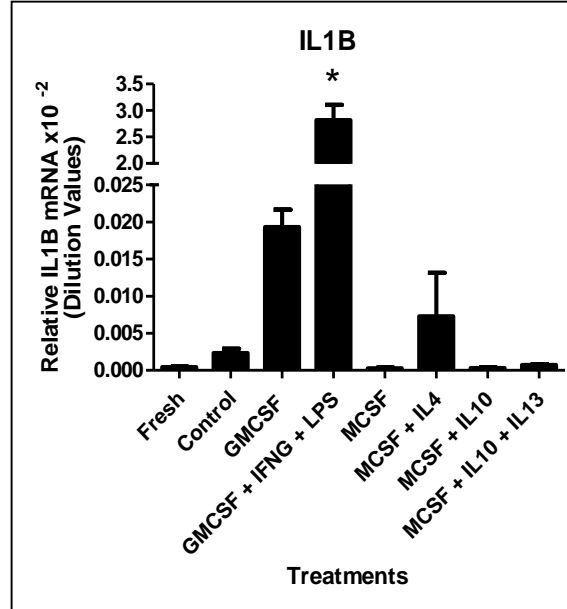


Figure 3.6: IL1B mRNA expression in bovine differentiated macrophages (n=3)

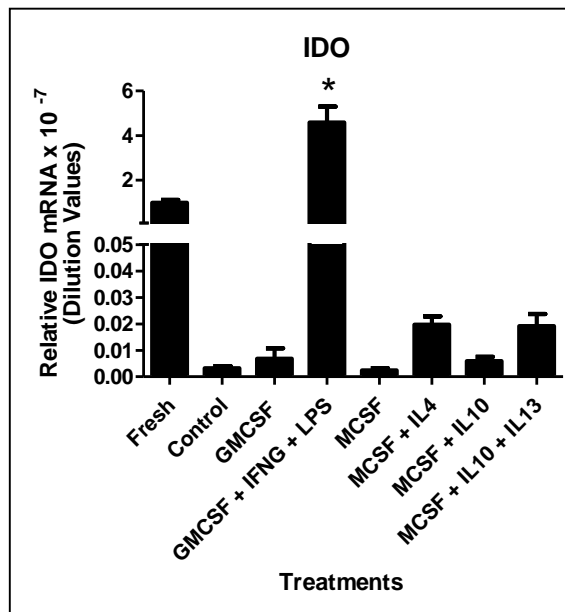


Figure 3.7: IDO mRNA expression in bovine differentiated macrophages (n=3)

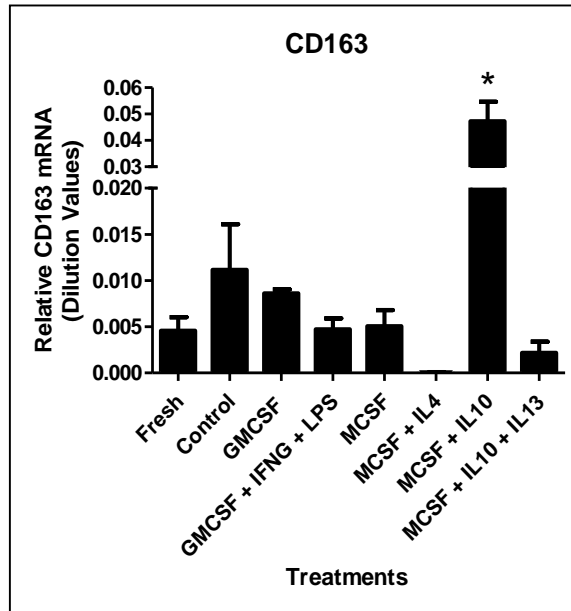


Figure 3.8: CD163 mRNA expression in bovine differentiated macrophages (n=3)

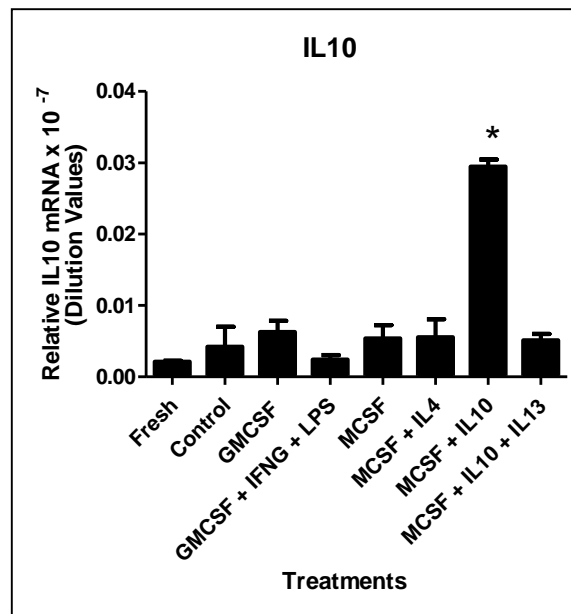


Figure 3.9: IL10 mRNA expression in bovine differentiated macrophages (n=3)

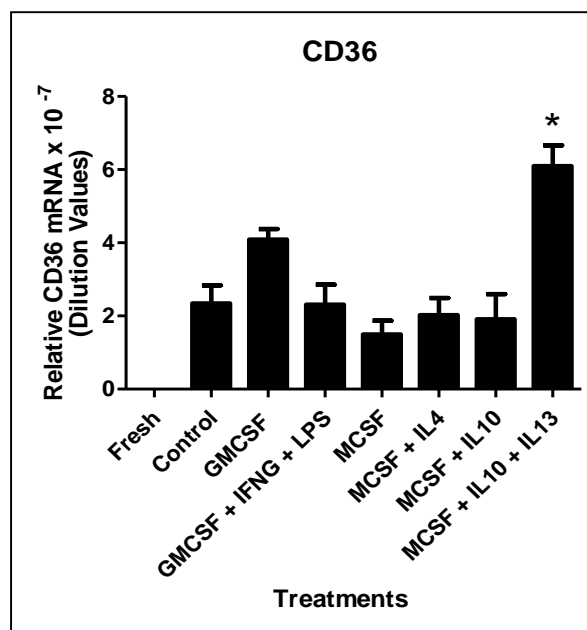


Figure 3.10: CD36 mRNA expression in bovine differentiated macrophages (n=3)

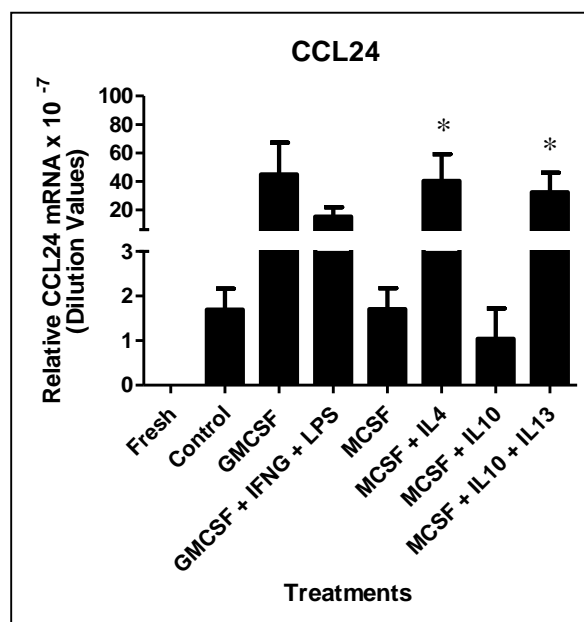


Figure 3.11: CCL24 mRNA expression in bovine differentiated macrophages (n=3)

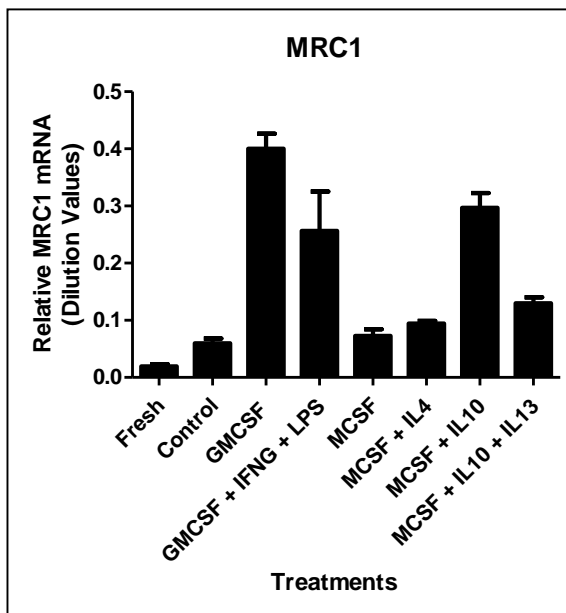


Figure 3.12: MRC1 mRNA expression in bovine differentiated macrophages (n=3)

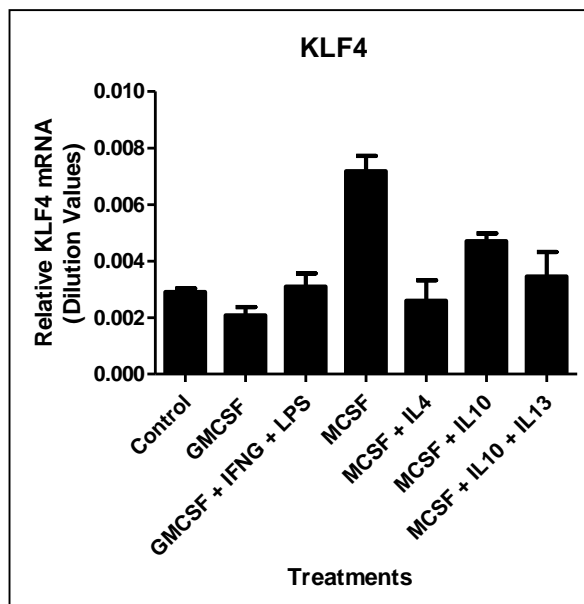


Figure 3.13: KLF4 mRNA expression in bovine differentiated macrophages (n=3)

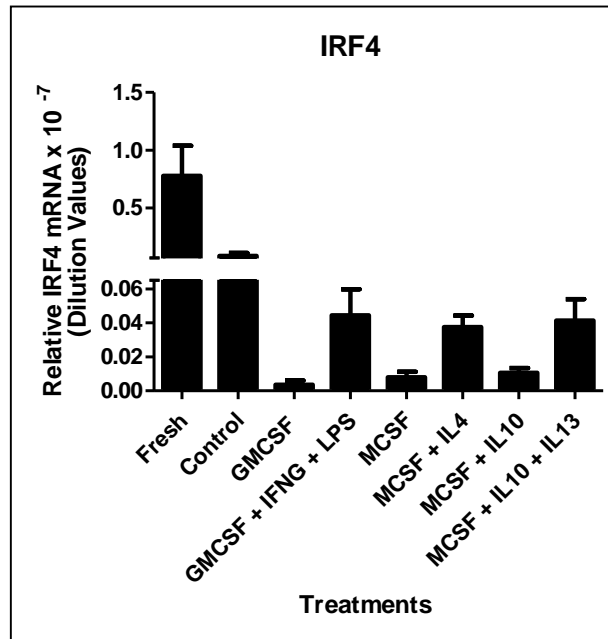


Figure 3.14: IRF4 mRNA expression in bovine differentiated macrophages (n=3)

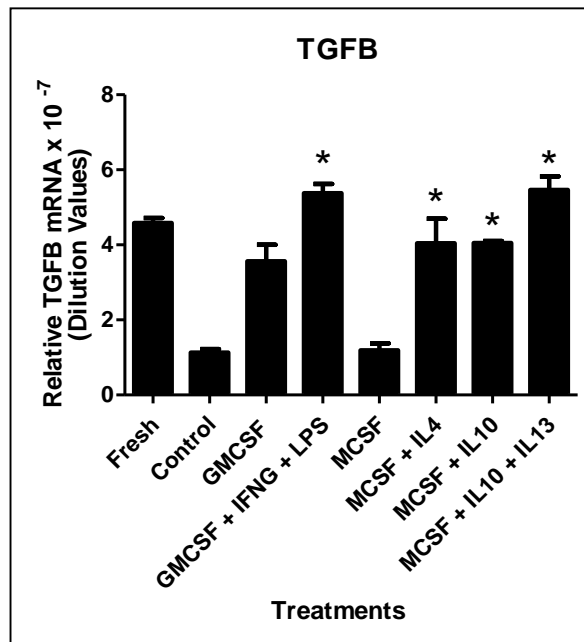


Figure 3.15: TGFβ mRNA expression in bovine differentiated macrophages (n=3)

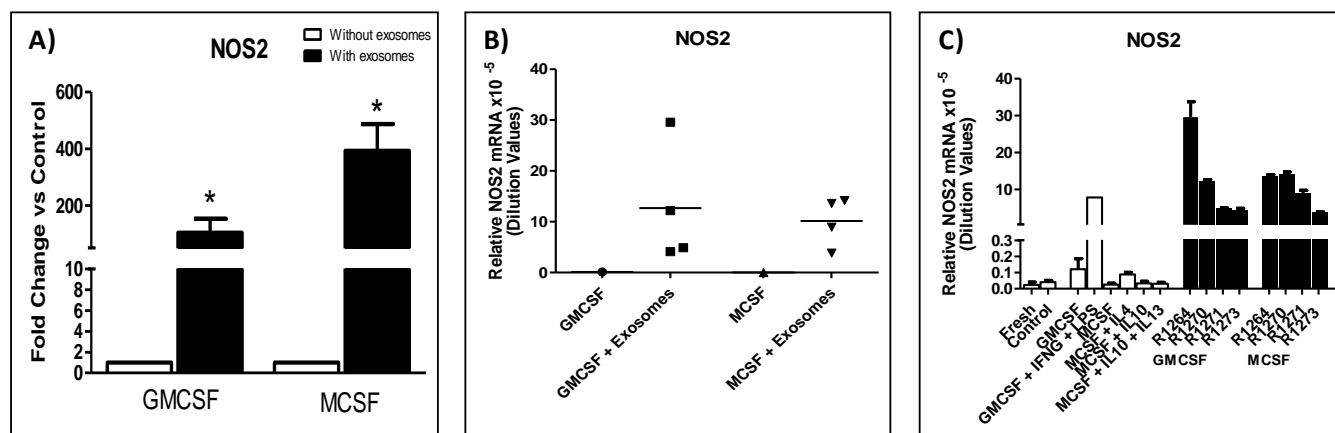


Figure 3.16: NOS2 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

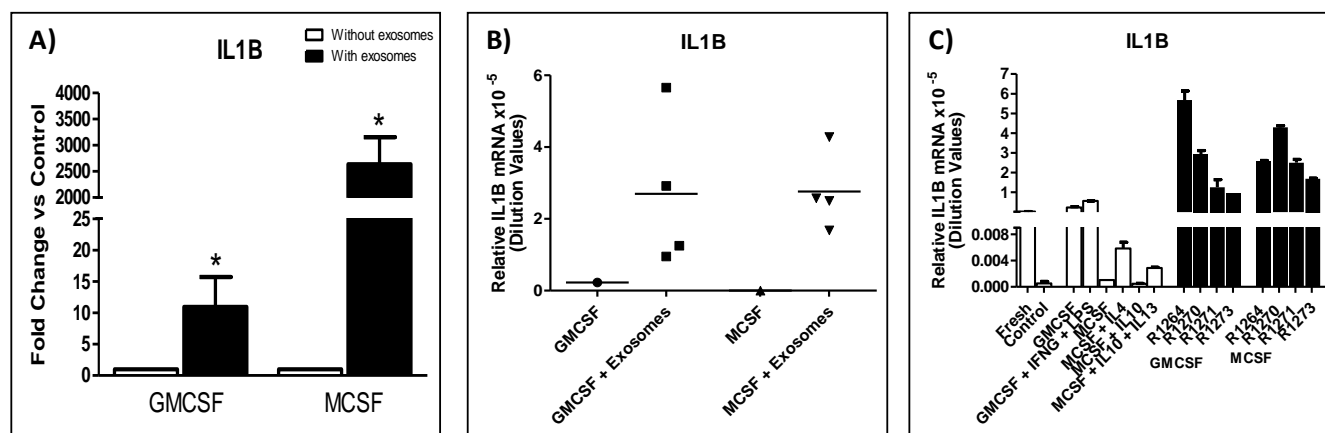


Figure 3.17: IL1B fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

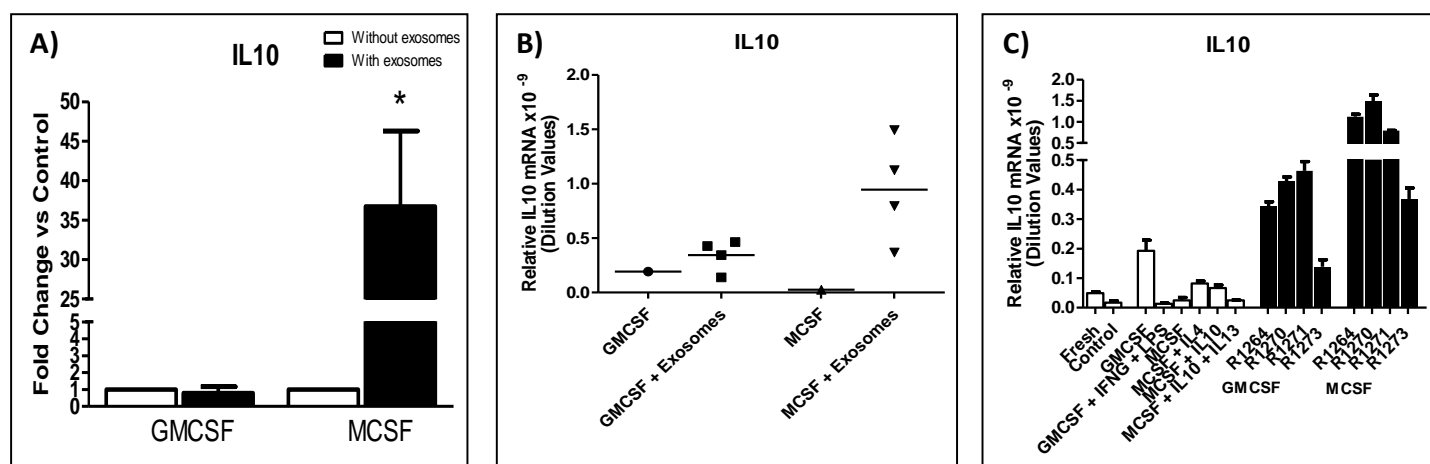


Figure 3.18: IL10 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

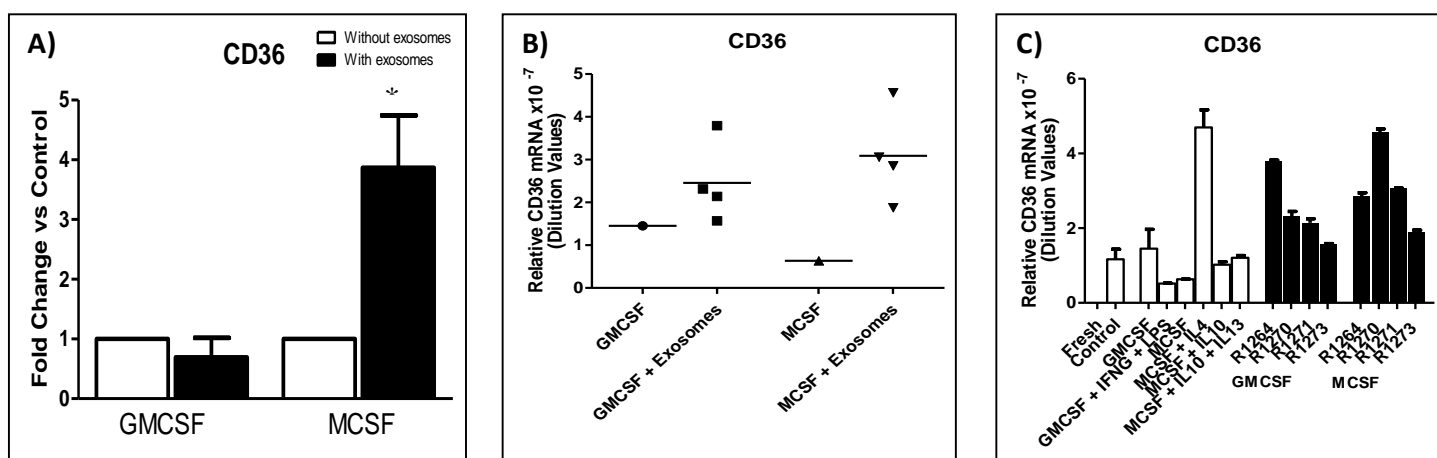


Figure 3.19: CD36 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

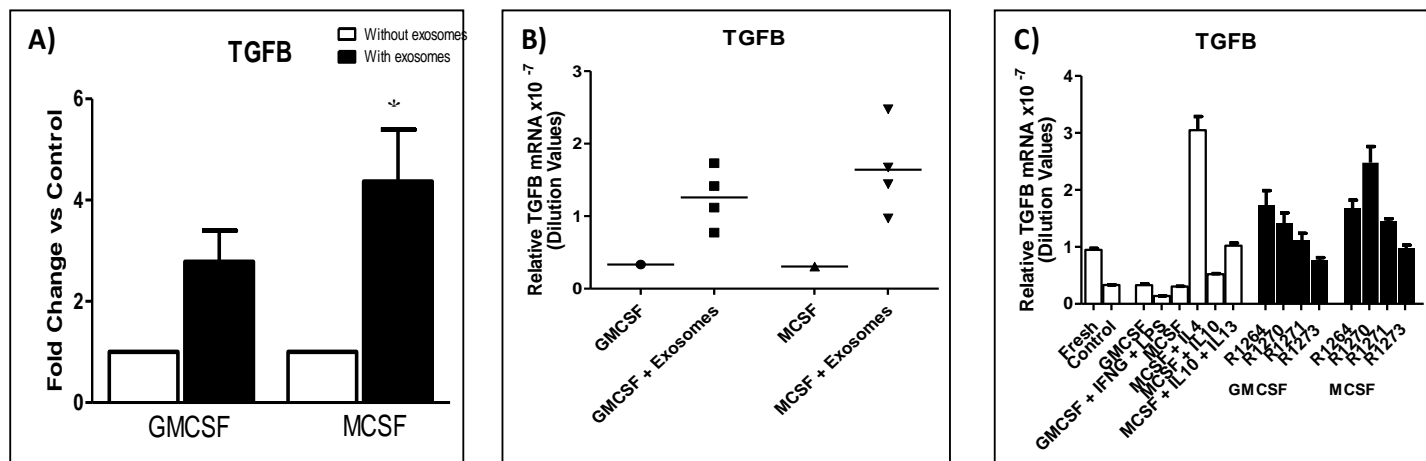


Figure 3.20: TGFβ fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

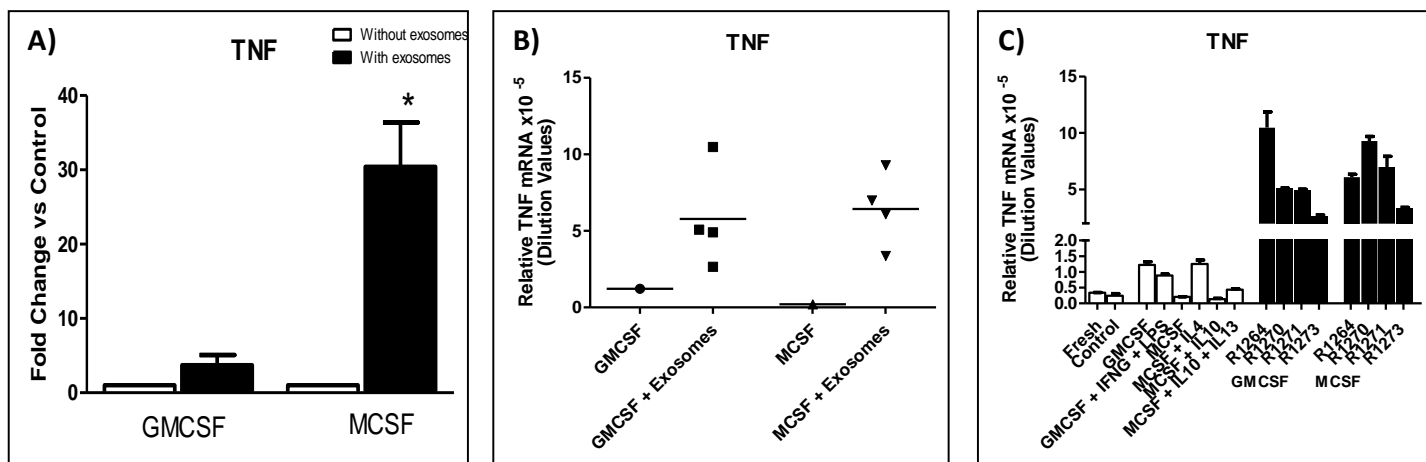


Figure 3.21: TNF fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

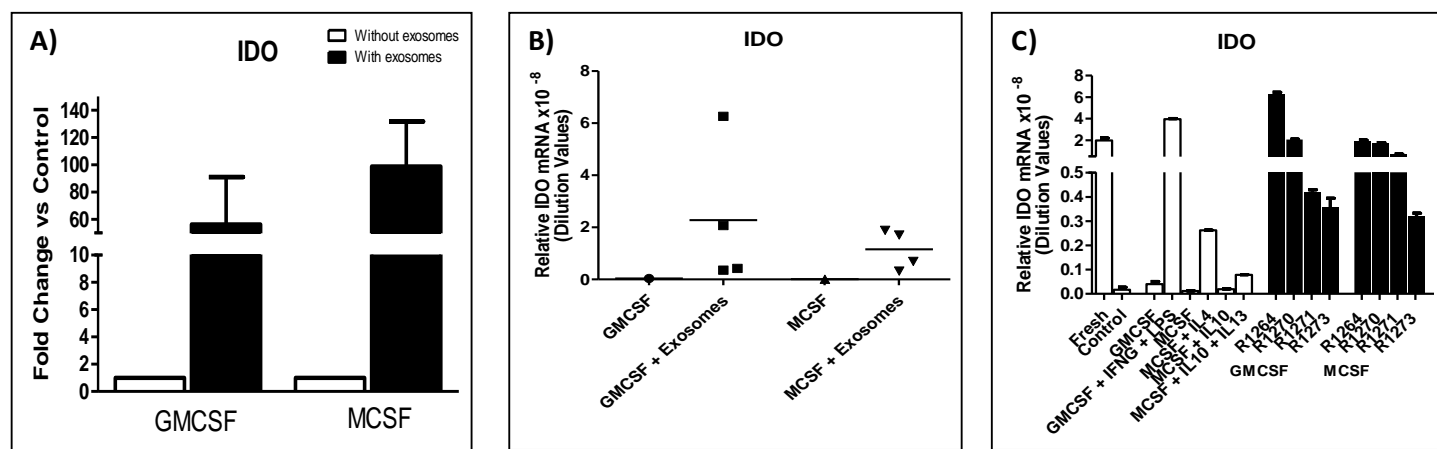


Figure 3.22: IDO fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

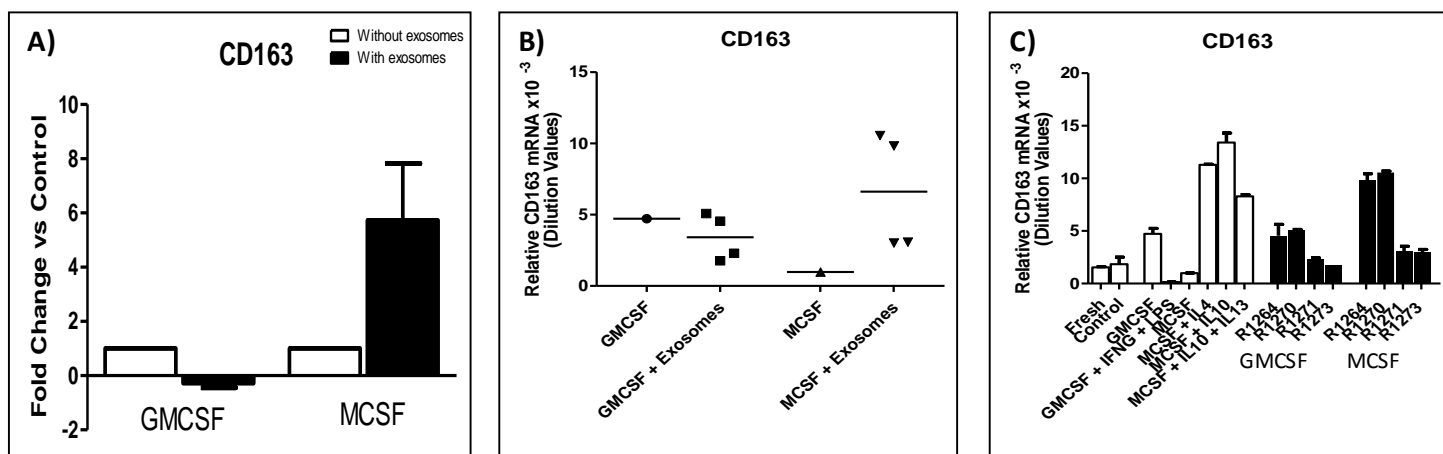


Figure 3.23: CD163 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

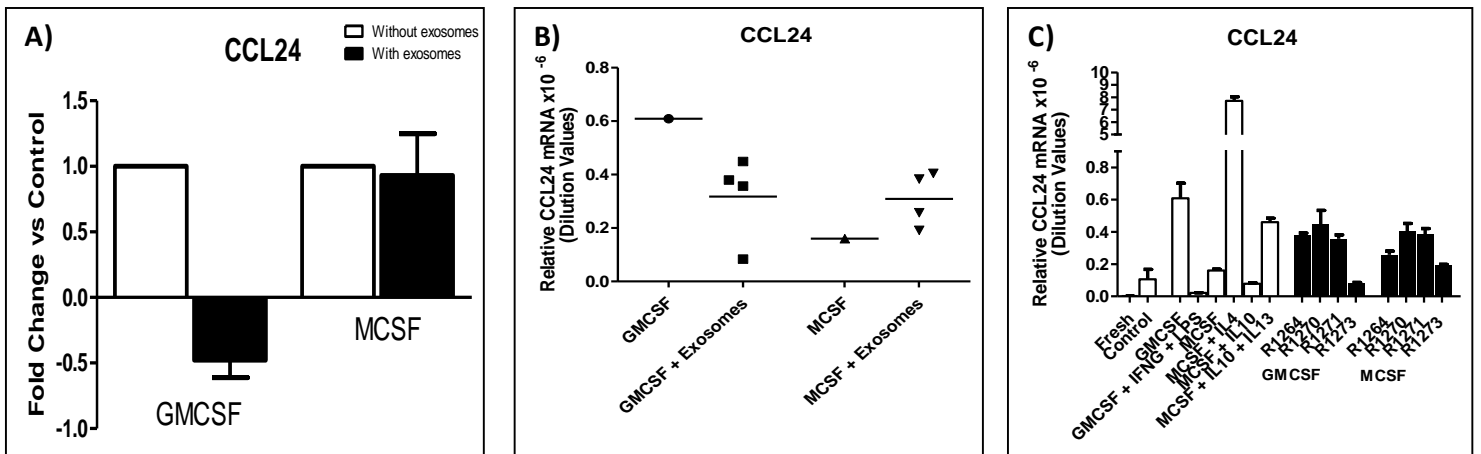


Figure 3.24: CCL24 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with midcycle luteal cell-derived exosomes (A) derived from 4 different CL (R1264, 1270, 1271, 1273; n=4; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

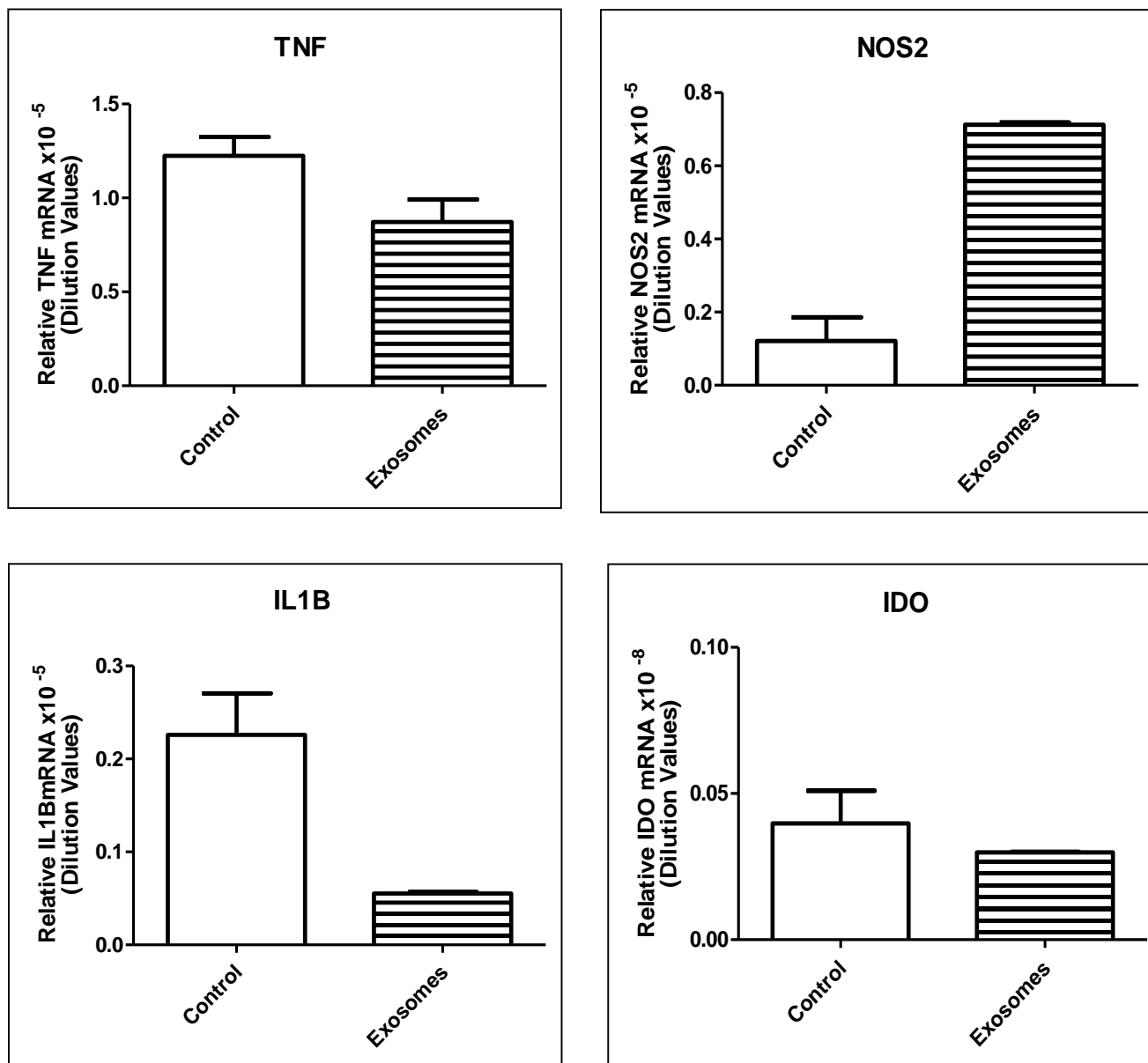


Figure 3.25: TNF, NOS2, IL1B and IDO mRNA expression in bovine macrophages co-cultured with or without midcycle luteal derived exosomes without priming agents (n=1)

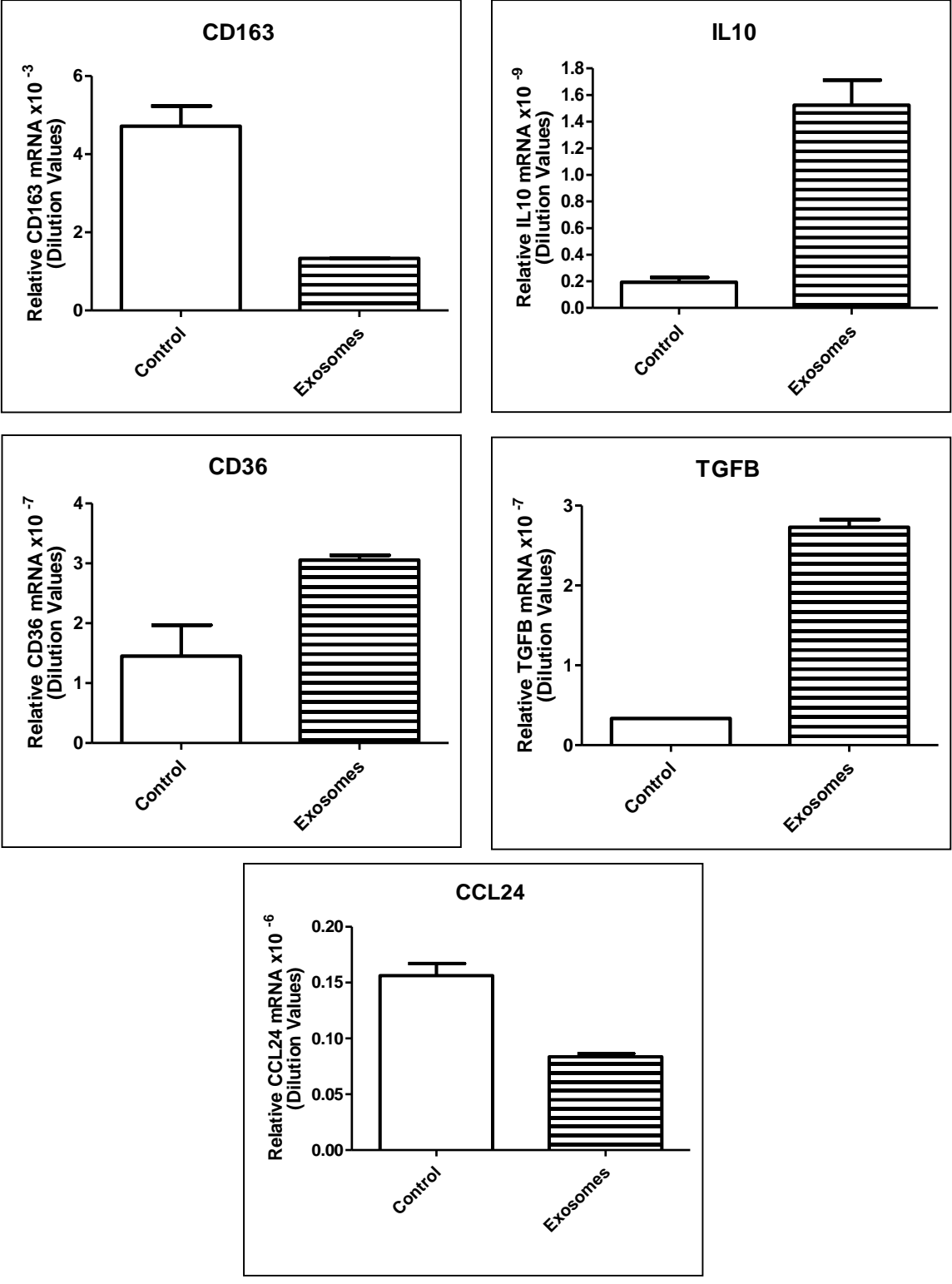


Figure 3.26: CD163, IL10, CD36, TGFB, and CCL24 mRNA expression in bovine macrophages co-cultured with or without midcycle luteal derived exosomes without priming agents (n=1)

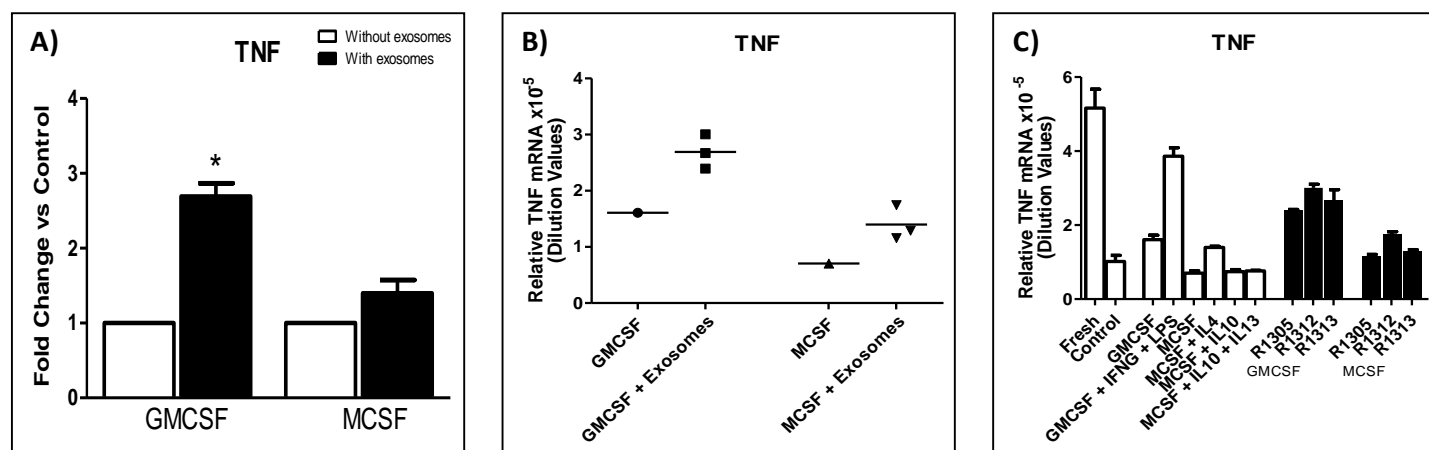


Figure 3.27: TNF fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

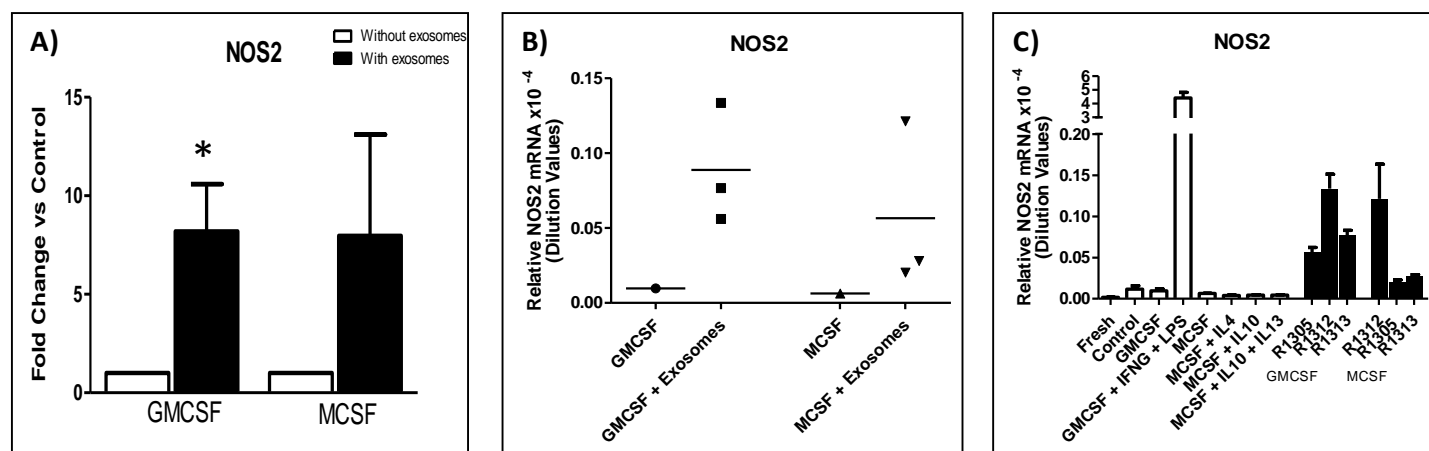


Figure 3.28: NOS2 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

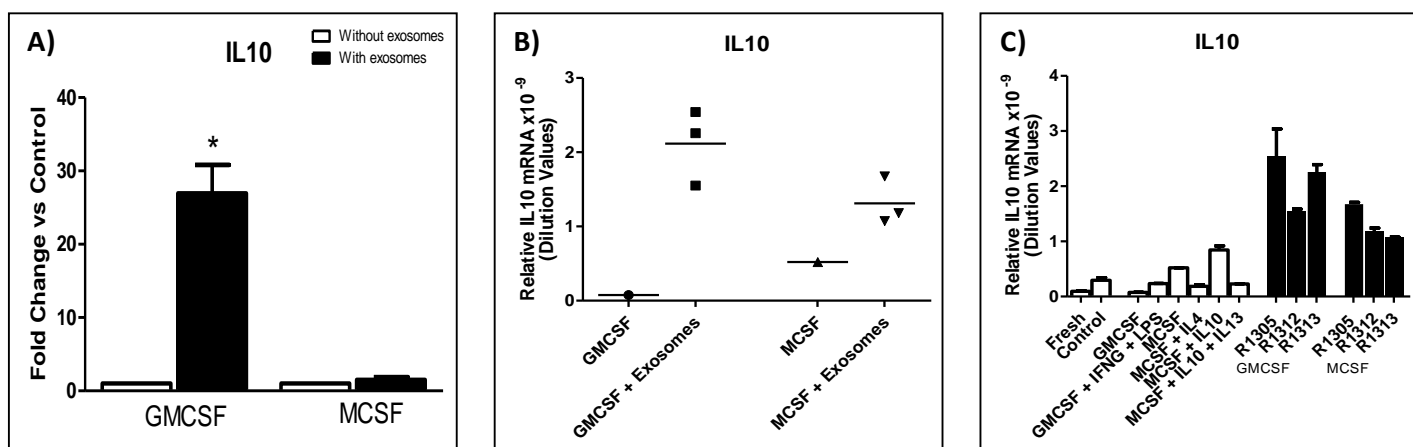


Figure 3.29: IL10 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

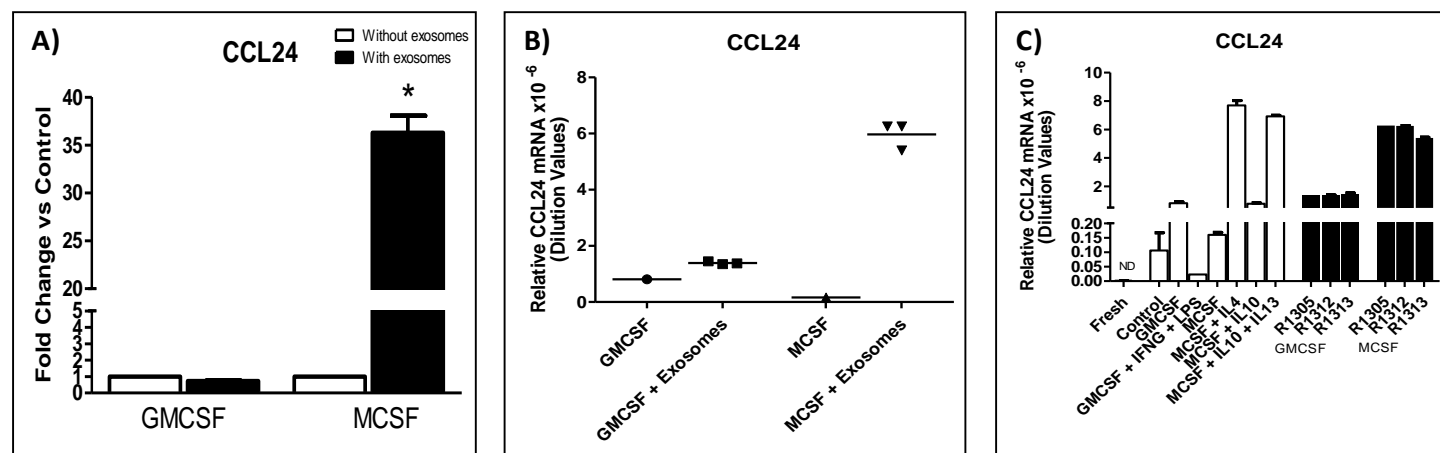


Figure 3.30: CCL24 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

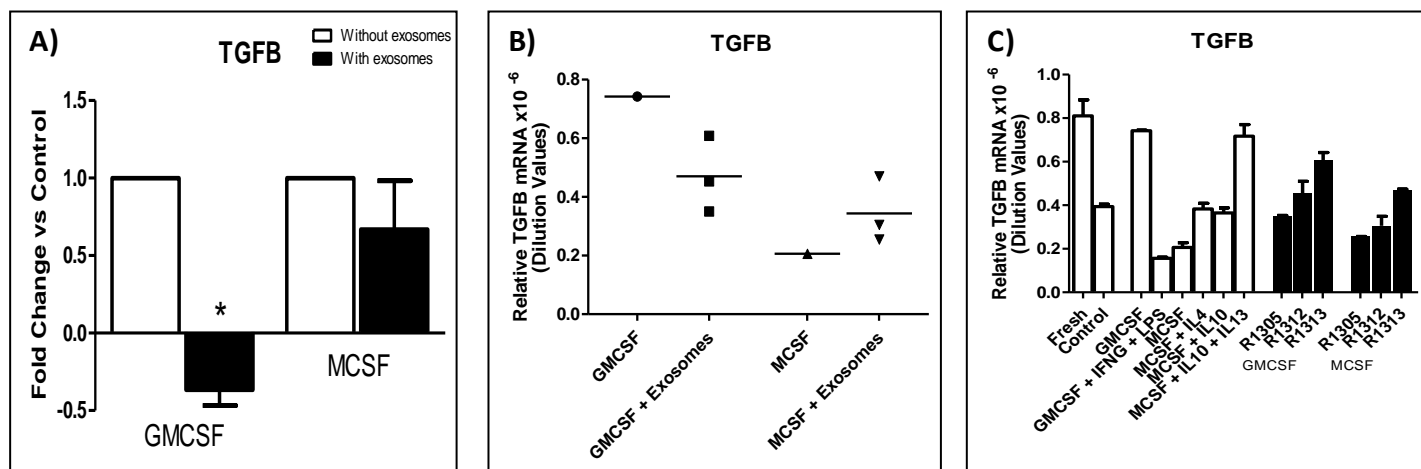


Figure 3.31: TGFβ fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

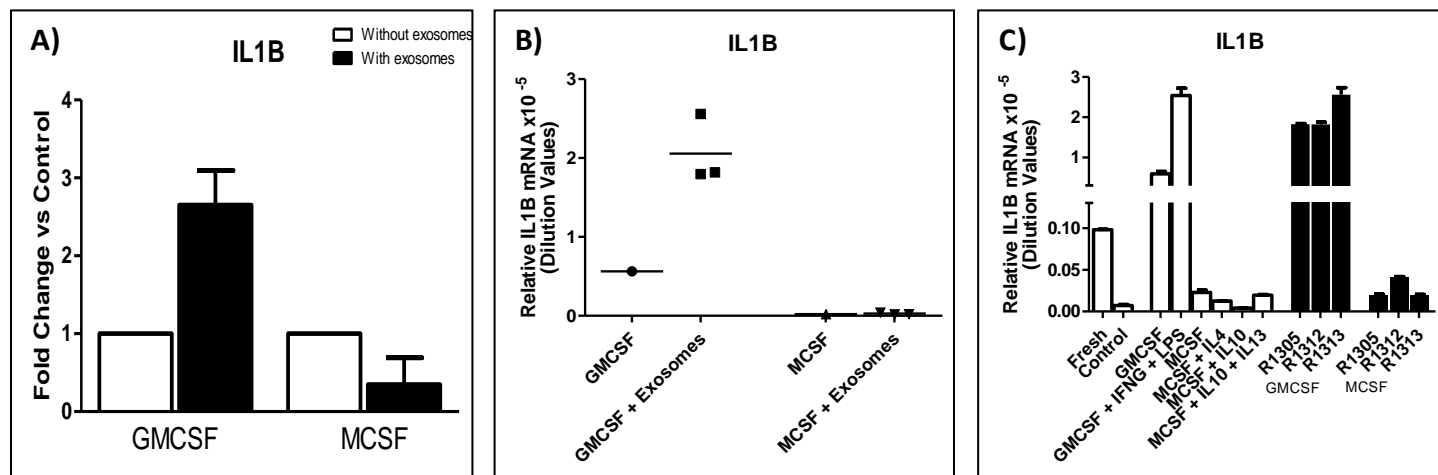


Figure 3.32: IL1β fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

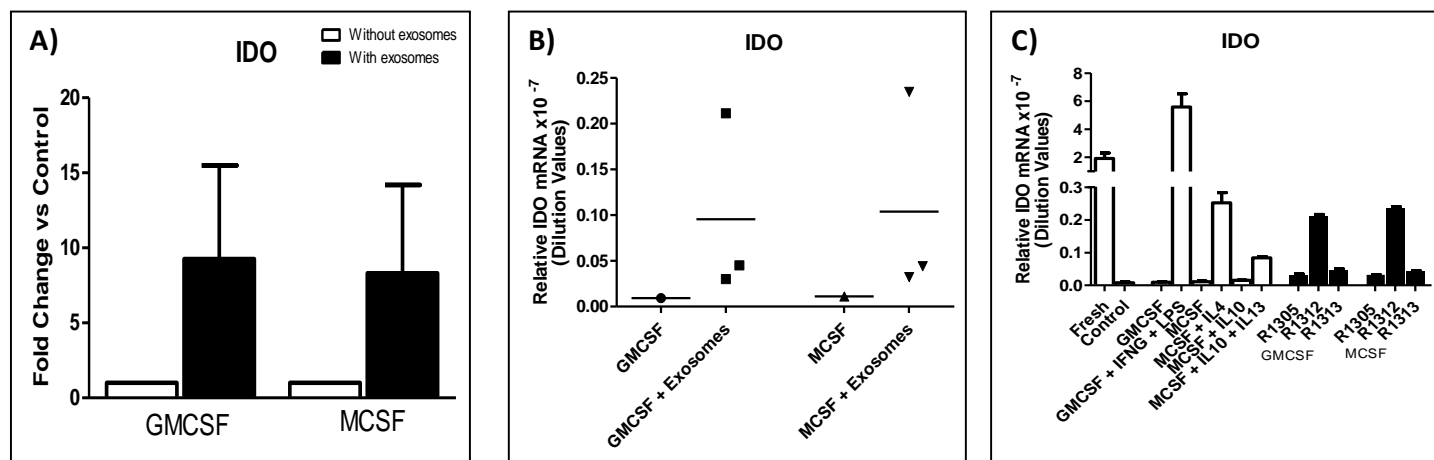


Figure 3.33: IDO fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

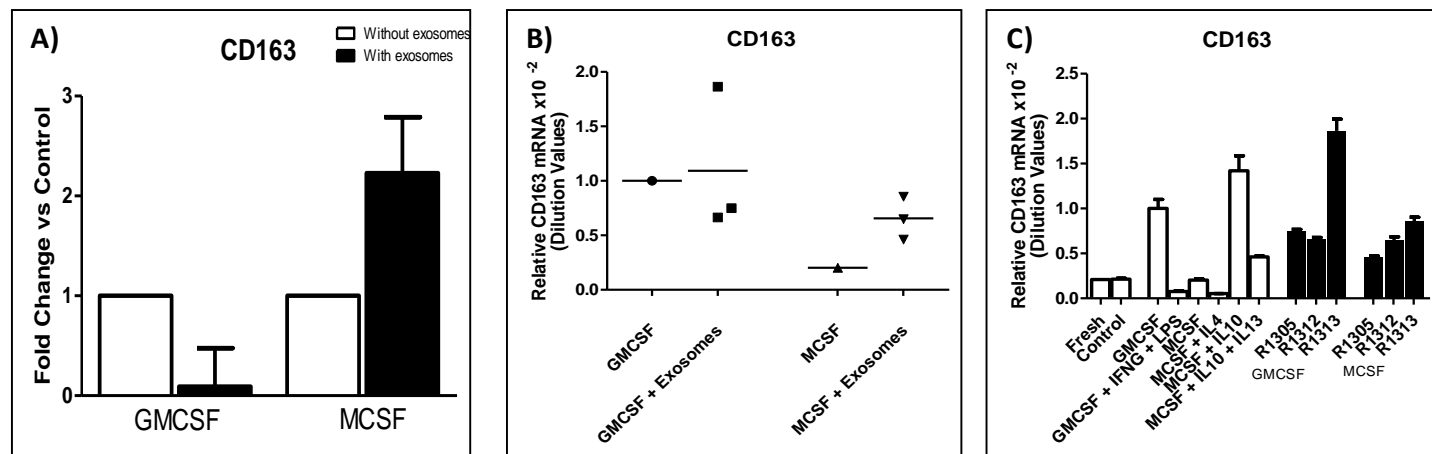


Figure 3.34: CD163 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

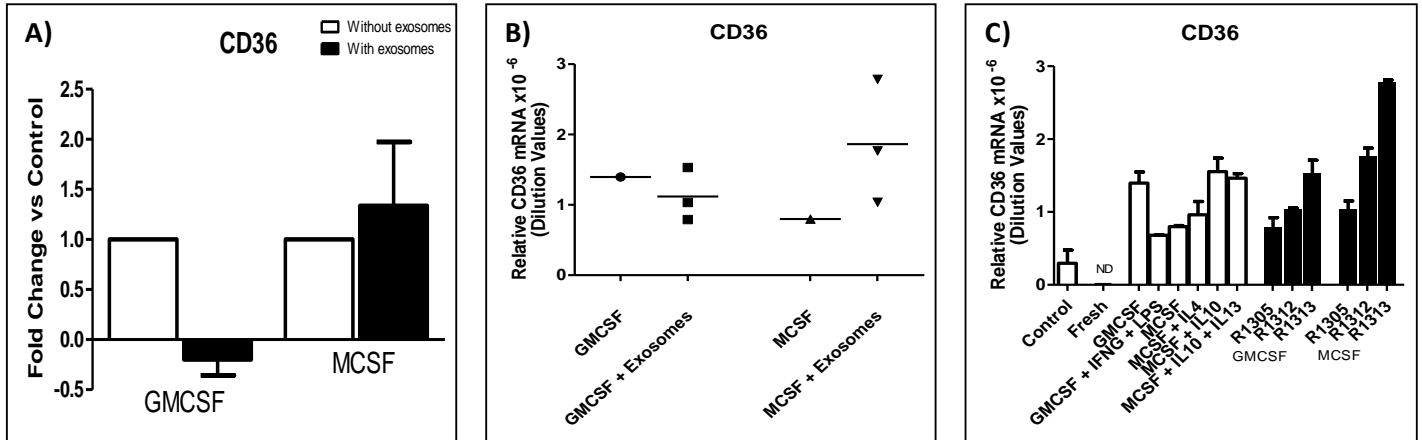


Figure 3.35: CD36 fold change in bovine GMCSF- or MCSF-primed macrophages incubated with regressing luteal cell-derived exosomes (A) derived from 3 different CL (R1305, 1312, 1313; n=3; p<0.05, T-test) (B) Dot plot of relative mRNA expression (C) Additional controls and individual samples.

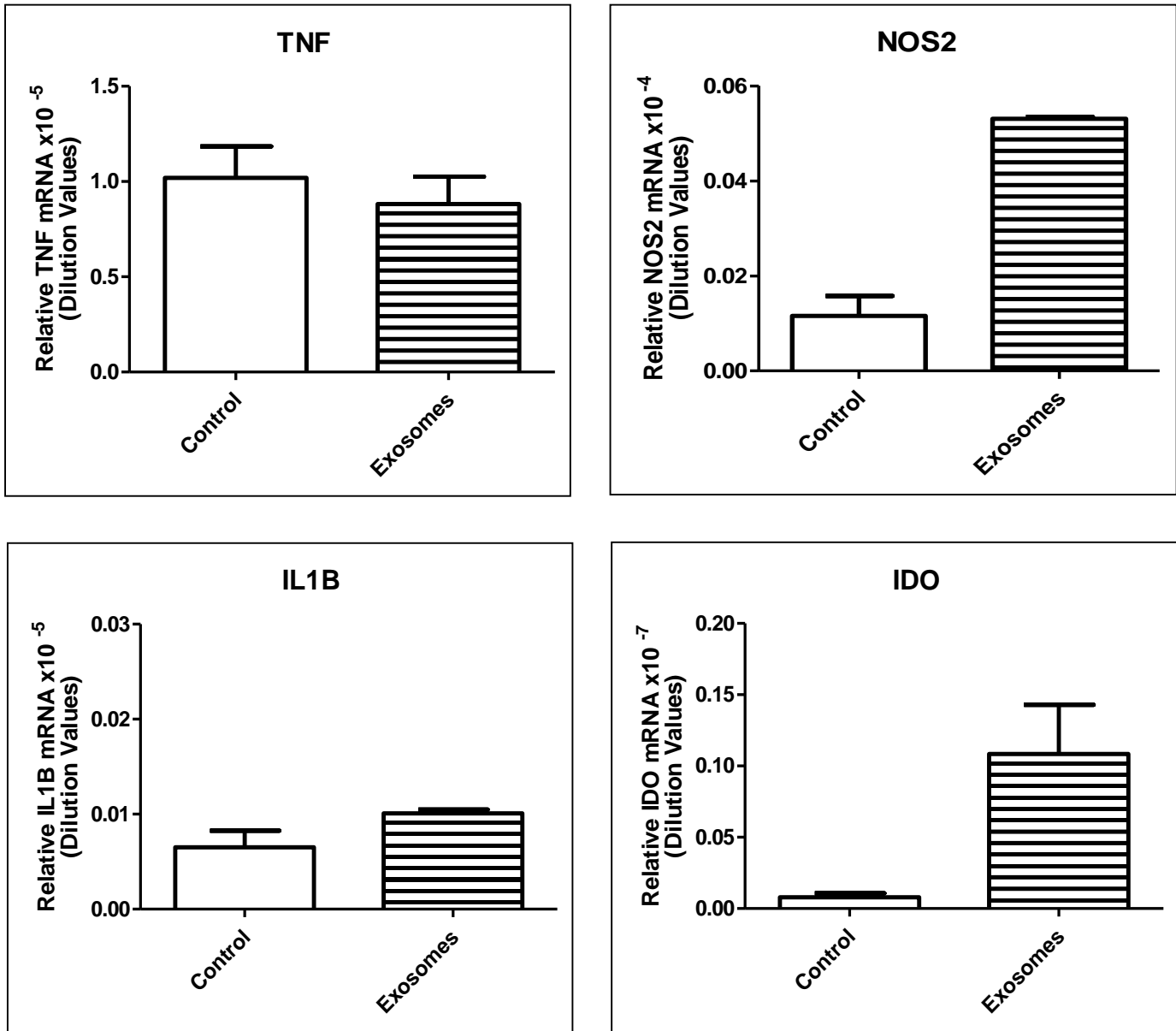


Figure 3.36: TNF, NOS2, IL1B and IDO mRNA expression in bovine macrophages co-cultured with or without regressing luteal cell-derived exosomes (n=1)

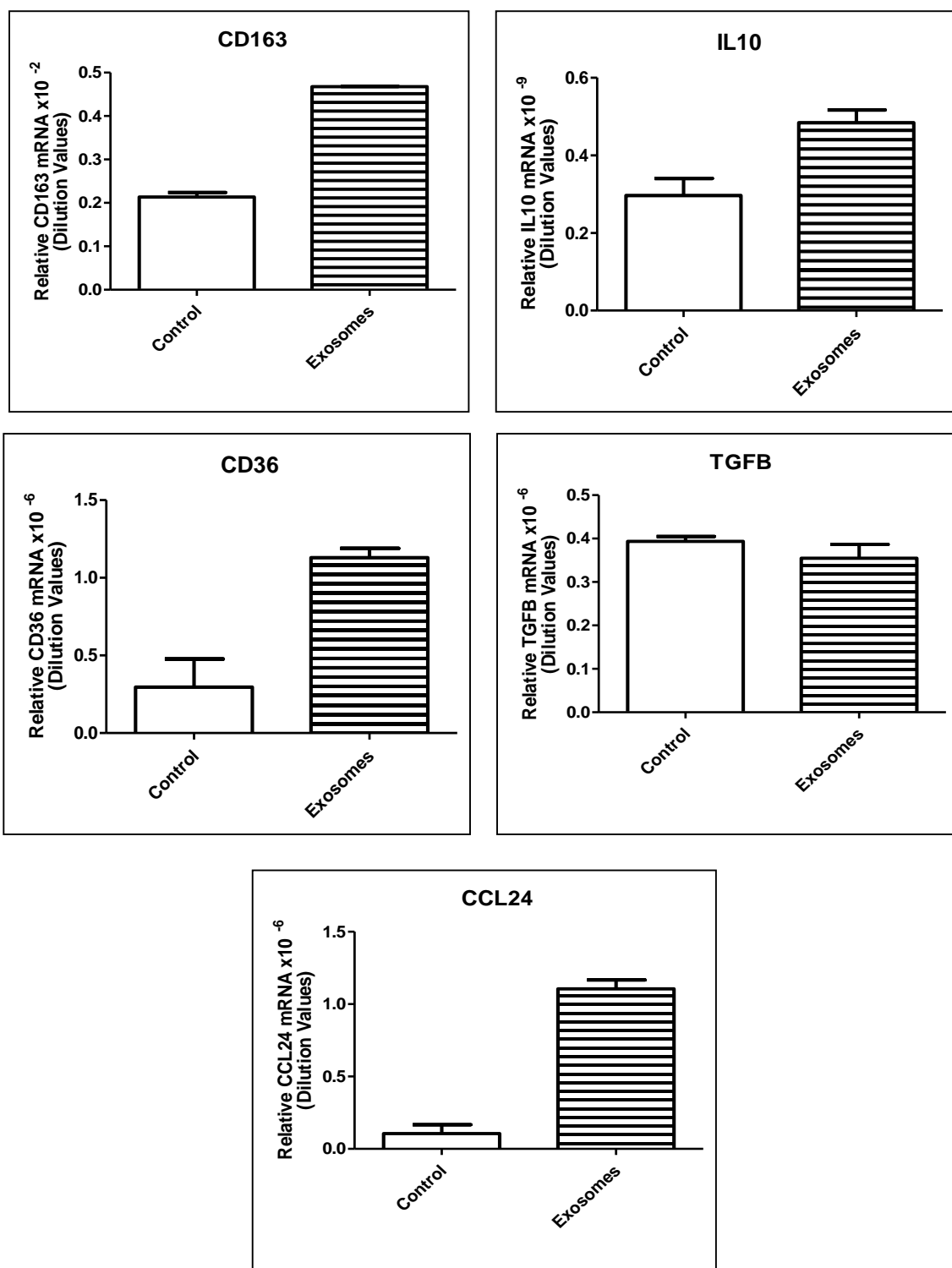


Figure 3.37: CD163, IL10, CD36, TGFB, and CCL24 mRNA expression in bovine macrophages co-cultured with or without regressing luteal cell-derived exosomes (n=1)

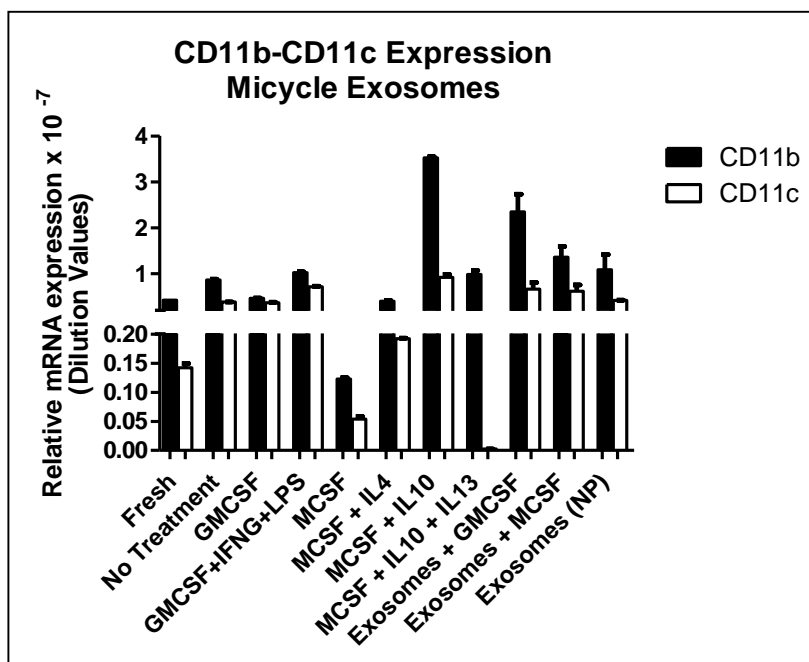


Figure 3.38: Quantitative PCR (qPCR) analysis of CD11b and CD11c mRNA expression in bovine macrophages when co-cultured with midcycle luteal cell-derived exosomes. These samples were used in the experiments in Chapter 3.

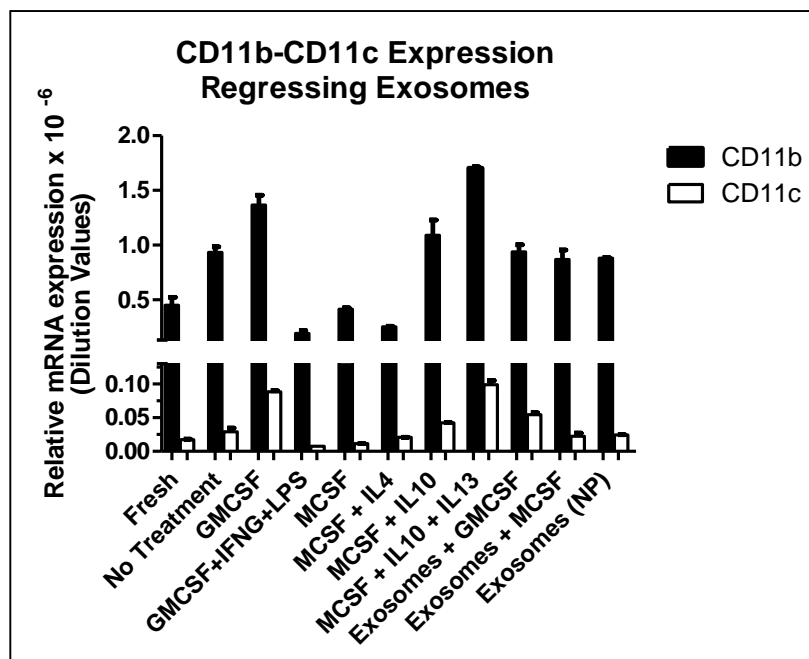


Figure 3.39: Quantitative PCR (qPCR) analysis of CD11b and CD11c mRNA expression in bovine macrophages when co-cultured with regressing luteal cell-derived exosomes. These samples were used in the experiments in Chapter 3.

Chapter 4: Discussion and Summary

Fertility has declined over the past fifty years (Lopez-Gatius, 2003) with many pregnancy losses occurring shortly after fertilization (Diskin et al., 2006). Inskeep and Dailey (2005) estimated that 73% of reproductive loss in dairy cattle is due to early embryonic loss, with most losses occurring before the onset of placentation. During this key period in early pregnancy, conceptus signaling is essential for rescuing the corpus luteum (CL) from regression and maintaining pregnancy. The CL secretes large amounts of progesterone (P_4) and is essential for the establishment and continuation of pregnancy in all mammals. In ruminants, if successful fertilization occurs, the embryo secretes interferon tau (IFNT), which rescues the CL from regression (Knickerbocker et al., 1986). In the absence of pregnancy, the CL regresses in response to prostaglandin F2 alpha ($PGF_{2\alpha}$). The progression of luteolysis is dependent upon activation of immune responses (Atli et al., 2012).

Luteal cells can program resident immune cells and luteal cells from fully functional CL can activate T cells through paracrine mediators but regressing luteal cells cannot (Brzezicka and Pate, 2013). Luteal cells and resident immune cells interact to regulate homeostasis within the tissue during periods of tissue remodeling. We hypothesized that luteal cells secrete extracellular vesicles, such as exosomes, and that these exosomes serve as a means of communication from luteal steroidogenic cells to resident immune cells. Exosomes have been studied in many species during various physiological states, including events related to reproduction, but the role of exosomes in the CL has not been investigated.

Current research with exosomes is focused on the role of exosomes in the regulation of cancer and the use of exosomes in immunotherapy. A Phase I clinical trial involving the

administration of dendritic cell-derived exosomes loaded with MAGE3 epitopes has been started in France (Institut Gustave Roussy and Institut Curie) in patients who have MAGE3-expressing metastatic melanoma tumors. Exosome research is a fairly new field and continues to grow as the potential for the use of exosomes as biomarkers of chronic diseases increases. Although exosome research is focused around cancer research, studies have been conducted to determine their role in reproduction. For example, a recent study showed that secondary cell-derived exosomes of the *Drosophila* accessory sex glands interact with epithelial cells in the female reproductive tract and induce changes in female remating behavior (Corrigan et al., 2014). More related to this project, exosomes were isolated from equine ovarian follicular fluid and exosomal miRNA and protein were compared in follicular fluid from young versus old mares. This study showed that miRNA present in exosomes from ovarian follicular fluid varied with the age of the mare. Although no functional studies were completed, this study shows that exosomes from ovarian follicular fluid have cargo that can be used in cell-to-cell communication.

In Chapter 2, the aim was to isolate bovine luteal cell-derived exosomes from culture media and investigate their role in luteal cell-T cell communication. Results indicated that bovine luteal cell-derived exosomes could be isolated from midcycle and regressing luteal cell culture media and that more exosomes were secreted from midcycle than regressing luteal cells. Previous studies in the lab (Brzezicka and Pate, 2013) showed that midcycle luteal cells were able to induce activation of T cells with or without contact while regressing luteal cells required contact with T cells to provide full activation. Thus, luteal cells from fully functional CL secrete molecules that activate T cells in a paracrine manner. During luteal regression, the secretion of these molecules is apparently limited. This hypothesis was further verified from these studies which showed that regressing luteal cells secrete fewer exosomes and this may be the reason that

regressing luteal cells need contact to activate T cells. Further evidence supports this hypothesis because midcycle luteal cell-derived exosomes were internalized by T cells and activate T cells. This experiment shows that luteal cell-derived exosomes have the potential to direct T cell responses.

Chapter 2 also included information to determine the phenotype of T cells when exposed to midcycle or regressing luteal cell-derived exosomes. Interestingly, regressing luteal cell-derived exosomes induced a down regulation of mRNA expression for TNF, IFNG, IL4 and IL10. These data support the hypothesis that regressing luteal cell-derived exosomes down regulate T cell responses. Regressing luteal cell-derived exosomes may be utilized to resolve inflammation in the regressing CL, control cytokine responses, and regulate luteolysis. Walusimbi and Pate (2014) found that luteal cells from regressing CL induced a slight decline in the proportion of CD4⁺ T cells synthesizing TNF, but no effects on other cytokines. Further studies will be needed to determine the effects of exosomes isolated from various times during luteal regression on T cells. This may help determine the role of exosomes in early luteal regression when immune cell infiltration is highest and P₄ begins to decline.

Midcycle luteal cell-derived exosomes upregulated mRNA expression of TNF and downregulated IL4 and IL10 in T cells, which indicates an upregulation of a TH1 response. Hasan et al. (2002) noted that elevated cytokines in mouse ovaries, mediated by TH1 cells, were compatible with normal ovarian cyclicity, ovulation, and fertility. However, Walusimbi and Pate (2014) showed that when $\gamma\delta^+$ T cells were cocultured with luteal cells from midcycle CL, there was an increase in IL10⁺ cells and a decrease in IFNG⁺ cells, suggesting a TH2 response. Although the data from these studies does not agree with previous data in the lab, the role of luteal cell-derived exosomes in T cell communication is still very new and further studies will

need to be completed. From these studies it can be hypothesized that midcycle luteal cell-derived exosomes may induce a TH1 inflammatory response associated with normal ovarian function, while other communication factors may be inducing a TH2 response.

Before the focus of Chapter 3 could be addressed, it was vital to determine bovine M1 and M2 specific macrophage markers. While M1 and M2 macrophage markers have been extensively studied in mice and humans, specific markers for the cow were known. Bovine monocytes were differentiated into M1 or M2 macrophages with cytokines. Once differentiated, macrophages were collected and qPCR was performed. Results show that M1-directed macrophages exhibited greater mRNA expression of NOS2, IL1B, TNF and IDO while M2-directed macrophages had greater mRNA expression of CD36, CD163 and IL10. Other markers, such as IRF4, CCL24, MRC1, TGFB, and KLF4, were measured but these markers were upregulated in both M1 and M2 macrophages. Once specific M1 and M2 macrophage markers were determined, the effect of exosomes on macrophage polarization could be determined.

The focus of Chapter 3 was to determine the effect of midcycle and regressing luteal cell-derived exosomes on macrophage polarization. Monocytes were co-cultured with regressing or midcycle luteal cell-derived exosomes along with priming agents, GM-CSF or M-CSF. Priming agents such as GM-CSF and M-CSF are thought to assist with macrophage maturation and may be needed for exosomes to stimulate macrophages. The mechanisms of colony stimulating factors are quite distinct with different tissue distribution, different structures, and different receptors with M-CSF receptor being the tyrosine kinase, c-Fms (Sherr et al., 1985) and the GM-CSF receptor comprising α (ligand binding) and β (signaling) subunits (Metcalf, 1991). Granulocyte-macrophage colony stimulating factor signals through JAK2 and STAT5 and stimulates the expression of STAT5 target genes while M-CSF signals through Ras and MAP-K (Lehtonen et

al., 2002). Granulocyte- macrophage colony stimulating factor treatment leads to the formation of M1 macrophages whereas MCSF treatment leads to the formation of M2 macrophages, in human monocytes (Verreck et al., 2006).

Results from this study indicate that midcycle luteal cell-derived exosomes induced NOS2 and IL1B mRNA in GMCSF- and MCSF-primed macrophages, significant expression of IL10, CD36, TGFB and TNF mRNA in MCSF-primed macrophages and no change in IDO, CD163 or CCL24 mRNA expression. Results from this study suggest that macrophages require specific priming for midcycle luteal cell-derived exosomes to induce mRNA expression of these genes. This specific priming may be essential to controlling the luteal microenvironment which suggests that if macrophages are primed towards a certain phenotype, exosomes would either be able to induce macrophage specific genes or not.

Regressing luteal cell-derived exosomes exhibited greater mRNA concentration of TNF, NOS2 and IL10 mRNA in GMCSF-primed macrophages, significant expression of CCL24 mRNA expression in MCSF-primed macrophages, significant decrease in TGFB mRNA expression in GMCSF-primed macrophages and no change in IL1B, IDO, CD163 and CD36 mRNA expression. It is interesting that in order for regressing luteal cell-derived exosomes to induce M1 or M2 macrophage genes, it seems that the macrophages need to be educated or primed correctly. It would be interesting to determine the expression of GMCSF and MCSF during midcycle and regression in the bovine CL. This may help to determine which genes are more likely to be induced by exosomes in the tissue during midcycle and regression.

Tumor necrosis factor alpha is most known to have a pro-inflammatory function but its likely role in the CL is to stimulate prostaglandin synthesis and promote apoptosis. Sakumoto et al. (2011) found that transcripts for TNF-RI and TNF-RII were expressed in bovine CL

throughout the estrous cycle. The abundance of TNF-RII transcripts was increased at the regressed luteal stage compared to other stages, whereas TNF-RI transcript abundance did not significantly change. It is interesting that both midcycle and regressing luteal-cell derived exosomes induced TNF mRNA expression. It would be interesting to determine if exosomes can affect the expression of TNF transcripts which may explain why TNF mRNA is increased in macrophages when treated with midcycle and regressing luteal cell-derived exosomes. It is also interesting that midcycle luteal cell-derived exosomes were only able to induce TNF mRNA in MCSF-primed macrophages while regressing luteal cell-derived exosomes were only able to induce TNF mRNA in GMCSF-primed macrophages. This suggests that if macrophages are primed towards a classical pathway, midcycle luteal cell-derived exosomes could not induce TNF mRNA but regressing luteal cell-derived exosomes could. This provides control within the luteal microenvironment. Also, it is interesting to keep in mind that IL10 mRNA expression was induced in macrophages when treated with midcycle or regressing luteal-cell derived exosomes which may imply a regulatory function. Future studies will need to be done to determine the content of the exosomes which may help enhance our knowledge of their role in immune function.

An additional preliminary study was conducted to determine the role of exosomes in macrophage polarization when priming agents were not added. Nitric oxide synthase, IL10, CD36, and TGF β mRNA expression appeared to increase, CD163, CCL24, and IL1 β mRNA expression appeared to decrease, and no changes in IDO or TNF were observed in macrophages that were stimulated with midcycle luteal cell-derived exosomes in the absence of priming agents. Although NOS2 is a strong pro-inflammatory gene (MacMicking et al., 1997), IL10 and TGF β are known to have regulatory functions that may dampen immune responses (Edin et al.,

2012) and CD36 has a role in homeostasis and phagocytosis (Fadok et al., 1998). Additionally, TGFB mRNA expression was higher in resident T cells from midcycle compared to regressing CL (Walusimbi & Pate, 2014). This balance of regulatory, anti-inflammatory, and pro-inflammatory gene expression in macrophages may be essential to regulate the CL.

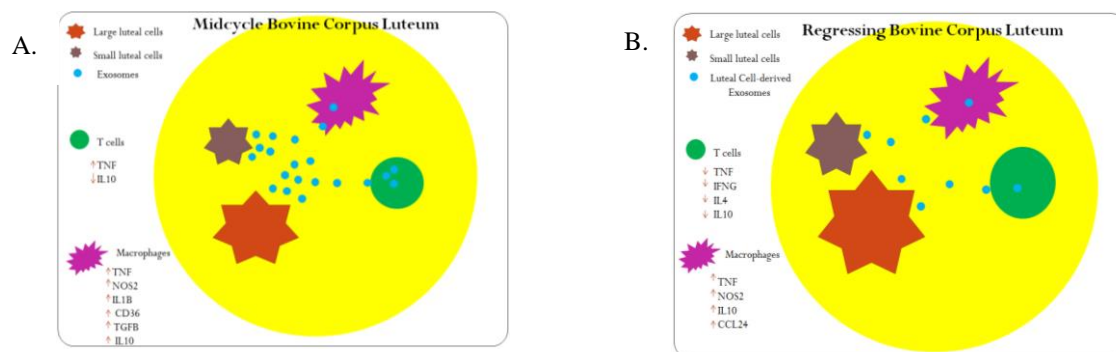
Regressing luteal cell-derived exosomes appeared to increase mRNA expression of CD36, CD163, IDO, CCL24 and NOS2, with no changes in IL1B, TNF, TGFB and IL10 mRNA expression. It is interesting that regulatory gene mRNA expression, such as TGFB and IL10, appeared unchanged when macrophages were treated with regressing luteal cell-derived exosomes but increased when treated with midcycle luteal-cell derived exosomes. This may indicate the significance of their expression and regulatory function in the midcycle CL. Additionally, IDO appeared to increase when macrophages were treated with regressing luteal cell-derived exosomes but not with midcycle luteal cell-derived exosomes. Indoleamine-pyrrole 2,3-dioxygenase expression is induced by interferon gamma (IFNG) which is increased during luteal regression (Fairchild & Pate, 1991). The role of IDO may explain why it is important for regressing luteal cell-derived exosomes to induce IDO expression in macrophages.

The results from this study share many similarities to the previous study when priming agents were added but some differences were seen such as when macrophages were treated with midcycle luteal cell-derived exosomes TNF mRNA expression did not appear to change and IL1B, CD163 and CCL24 appeared to decrease when no priming was added compared to when priming agents were added. It is interesting that TNF mRNA expression was only increased when macrophages, treated with midcycle luteal cell-derived exosomes, were primed with MCSF and TNF was unchanged without priming agents. This further supports the theory that macrophages need to be correctly primed to induce certain phenotypes. In regressing luteal cell-

derived exosomes, TNF and TGFB mRNA appeared not to change and IDO, CD163 and CD36 mRNA appeared to increase in macrophages when no priming agents were added compared to when priming agents were added. Even though this experiment was only performed one time and statistics cannot be computed, this experiment may aid in understanding the role of exosomes in macrophage polarization. This study should be repeated to confirm these results.

These are the first studies to investigate the role of exosomes in the bovine corpus luteum. These studies also explain an alternative way bovine luteal steroidogenic cells communicate with immune cells other than by direct cell-to-cell contact. Little is known about exosomes in reproduction. Luteal cells were not known to secrete exosomes until these experiments and more studies will need to be completed to determine the content of exosomes isolated from different physiological states of the CL such as development, maintenance, regression, and pregnancy.

Model Diagrams:



- (A) Midcycle bovine corpus luteum: Midcycle luteal cells secrete more exosomes which can be internalized into T cells, induce T cell proliferation and increase TNF and decrease IL10 mRNA expression. Midcycle luteal cell-derived exosomes can also induce TNF, NOS2, IL1B, CD36, TGFB and IL10 mRNA expression in primed macrophages.
- (B) Regressing bovine corpus luteum: Regressing luteal cells secrete less exosomes which can induce a decrease in TNF, IFNG, IL4 and IL10 mRNA expression in T cells. Regressing luteal cell-derived exosomes can also induce TNF, NOS2, IL10 and CCL24 mRNA expression in primed macrophages.

Proposed future studies:

One very essential future study would be to optimize a protocol for exosome isolation from luteal cell-conditioned media. From these studies, we were only able to isolate about two to three exosomes per midcycle luteal cell and one exosome per regressing luteal cell. We believe this may be explained by the extensive handling and processing of the samples by media concentration and purification. Recently, new products for exosome isolation from cell culture media have been developed and may be essential to recover large numbers of exosomes for subsequent experiments.

Another important experiment would be to collect exosomes at various times during luteal regression, eg. 1, 2, 3, and 4 hours after administration of $\text{PGF}_{2\alpha}$. We know that structural regression of the CL begins 2 hours after administration of $\text{PGF}_{2\alpha}$. In these experiments, luteal cells collected 8 hours post $\text{PGF}_{2\alpha}$ injection were used to follow up previous experiments done in the lab (Brzezicka and Pate, 2013). This time point may be too far into luteal regression to fully compare the role of exosomes during various physiological states of the CL.

Additionally, it would be of interest to determine the ratio of exosomes released from midcycle and regressing luteal cells so that exosomes could be added to cultures based on the actual concentration you would expect to find in the CL. Also, it may be of interest to relate this number to the number of target cells used in each experiment. For instance, it would be interesting to add exosomes at various ratios to the target cell. This would allow for results from midcycle and regressing luteal cell-derived exosomes to be directly compared. In these experiments we used the highest number of exosomes possible, not expecting to see the profound effects we did.

Furthermore, studies need to be conducted to determine the components of these exosomes. Proteins, mRNA and miRNA can be determined by liquid chromatography- mass spectrometry (LC-MS), RNA sequence, and deep sequencing, respectively. It would be interesting to determine if the content of exosomes changes at critical times in the lifespan of the CL, such as at midcycle, regression, and pregnancy recognition.

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