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THE ROLE OF MIRCO-RNA, COMPLEMENT PROTEINS, AND IMMUNE CELLS IN BOVINE LUTEAL FUNCTION AND RESCUE

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ABSTRACT

The corpus luteum (CL) is a transient endocrine tissue on the ovary that secretes the hormone progesterone that is essential for the maintenance of pregnancy in mammals. Functional maintenance of the CL during the estrous cycle is important for the continued secretion of progesterone to allow for the establishment of pregnancy. Luteal rescue in the cow takes place between days 17-20 of the estrous cycle. If luteal rescue does not occur during early pregnancy, the CL regresses and the expectant pregnancy will be lost, which can lead to major financial losses for farmers. The interplay between different molecular mechanisms, including the role of micro-RNA (miRNA) and the role of different immune components, by which the CL is maintained are not fully understood. Understanding these mechanisms and how they aid in maintaining the CL could help elucidate answers to prevent early pregnancy loss in dairy cows. The objectives of this study were to 1) Understand if let-7, a miRNA, can modulate mitochondrial respiration and progesterone production in the functional CL; 2) To identify a phenotype of resident immune cells in the CL exposed to uterine infused interferon-tau (IFNT) and/or pregnancy specific protein B (PSPB) using a transcriptomic profiling approach; and 3) To characterize the expression and the role of complement components in the CL.

In the first study, the abundance of let-7a and let-7b were characterized in isolated mitochondria from midcycle luteal cells. Neither let-7a nor let-7b were expressed in isolated luteal mitochondria except in one animal. Additionally, neither let-7a nor let-7b abundance changed over 5 days of culture, or in response to luteinizing hormone (LH) or prostaglandin F2a (PGF2A). However, when luteal cells were transfected with a let-7b mimic, mitochondrial respiration markers decreased including basal respiration and proton leak. Production of production and spare capacity tended to decrease. There was no change in mitochondrial respiration in response to let-7a. The let-7b mimic also decreased progesterone

production from luteal cells on day 5 and 7 of culture, while let-7a mimic increased progesterone production on day 3 of culture. The abundance of EGR1 and PGRMC1, both targets of let-7a and let-7b, were also assessed via flow cytometry after transfection with the let-7 mimics and inhibitors. Interestingly, the let-7a inhibitor increased the percentage of EGR1 positive cells and the mean fluorescence intensity (MFI) of EGR1. There was no effect of let-7b on EGR1. Both the let-7a mimic and inhibitor increased the MFI of PGRMC1 with no change on the percentage of PGRMC1 positive luteal cells. The let-7b mimic and inhibitor also increased the MFI of PGRMC1, and the inhibitor increased the percentage of PGRMC1 positive cells.

In the second chapter, first experiment evaluated whether treatment of luteal cells with luteotropic hormones could alter T cell activation. This experiment measured the abundance of T cell activation molecules CD3e, IL2 inducible T cell kinase (ITK), LCK proto-oncogene (LCK), and protein tyrosine phosphatase non-receptor type 7 (PTPN7) were measured in T cells cocultured with luteal cells previously treated with interferon-tau (IFNT), prostaglandin E2 (PGE2), and a combination treatment. Both CD3e and LCK mRNA was less in T cells that had been cocultured with PGE2-treated luteal cells. PTPN7 mRNA tended to be less in T cells exposed to luteal cells treated with IFNT, PGE2, and a combination treatment, while ITK mRNA did not change. The second experiment was a transcriptomic profiling study comparing luteal tissue and resident immune cells isolated from CL exposed to uterine infusions of IFNT and/or PSPB for either 3-days or 6-days. The purpose was to evaluate if PSPB could alter the transcriptome of the CL and/or resident immune cells, and if a phenotype of resident immune cells could be elucidated from this experimental model of pregnancy. There were only 3 differentially abundant transcripts found in resident immune cells from the 6-day IFNT and 6-day IFNT+PSPB infusion treatments, while there were 5 differentially abundant transcripts identified in luteal tissue from the same treatment. The upregulated pathways associated with transcripts

with Q < 0.15 in resident immune cells were eNOS signaling and the complement system, while IL17 signaling, coagulation, and iNOS signaling were the upregulated pathways in luteal tissue. In the 3-day IFNT and 6-day IFNT infusion comparison, 8 differentially abundant transcripts were identified in resident immune cells and 56 differentially abundant transcripts were identified in luteal tissue. The upregulated pathways associated with transcripts with Q < 0.15 in resident immune cells were ephrin A signaling, sphingosine-1-phosphate signaling, and IL15 production, while ILK signaling and IL8 signaling were upregulated pathways in luteal tissue. Lastly, there were 83 differentially abundant transcripts identified in resident immune cells from the 3-day BSA and 3-day IFNT infusion treatments and 117 differentially abundant transcripts identified in luteal tissue from the 3-day infusions. Interferon signaling and antiviral response pathways were upregulated pathways associated with the differentially abundant transcripts in both resident immune cells and luteal tissue.

In the third study, the abundance of complement C3 and C4 were characterized across the estrous cycle and during early pregnancy and an attempt was made to determine a functional role of C3 in the CL. The assessment of C3 and C4 abundance during luteal regression found that C3 and C4 did not change at any time point after an *in vivo* injection of PGF2A, but there was a tendency for *C3* to increase 24 hours after injection compared to the 0 hour control. There was also no change in abundance of C3 and C4 during acquisition of luteolytic capacity, however, *C3* tended to be less abundant in the day 6 CL compared to the day 4 CL. During early pregnancy, on day 17, there was a 2-fold numerical decrease in C3 between the cyclic and pregnant CL, and no change in C3 between the CL of the cycle and pregnancy on day 18. While there was no change in *C3* on day 17, there was an increase in *C3* in the CL of pregnancy compared to the cycle on day 18. C4 abundance did not change between the CL of the cycle and pregnancy on day 17, but there was a 5-fold numerical increase in C4 abundance on day 18 of

pregnancy compared to the cycle. There were no changes in *C4* abundance on day 17 or day 18. In response to various luteotropic and luteolytic hormones, C3 abundance per cell (MFI) decreased with IFNT+PGE2 treatment and PGF2A+PGE2 treatment compared to individual treatments with these hormones. However, *C3* decreased in response to PGF2A compared to control, and the effect of PGF2A remained when PGE2 was added to the treatment. *C4* abundance increased in response to IFNT compared to control, and remained increased when PGF2A was added to the treatment. There was a tendency for *C4* abundance to increase with IFNT+PGE2. Lastly, the effect of C3 on luteal cell induced T cell proliferation was tested by knocking down C3 in both luteal cells and T cells. There was no change in T cell proliferation after C3 knockdown.

In summary, several mechanisms involving miRNA and immune components were investigated to understand how the CL can remain functional both during the estrous cycle and during the period of maternal recognition of pregnancy. One major finding was let-7b does reduce mitochondrial respiration and progesterone production in luteal cells. This finding is important and suggests critical roles for miRNA in facilitating luteal function. Additionally, the identification of differentially abundant transcripts in the CL in response to uterine infused PSPB is a novel finding that suggests PSPB does alter the transcriptome of the CL by altering several immune signaling pathways during early pregnancy. Along with these findings, the characterization of the expression of complement C3 and C4, suggest these proteins might play a role in the CL of pregnancy, but unfortunately the exact role was unable to be characterized in this study. These findings provide a framework for future directions of luteal research to further elucidate the role of the immune system in the CL of early pregnancy as well as further understanding how miRNA might facilitate the continued function of the CL.

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"There is actually no such thing as failure. You either get the success you want or the lesson you needed." ~James Wedmore

It is probably fitting I found this quote on one of my favorite dog training blogs. It quickly became one of my favorite quotes to apply not only to dog training and life, but also to the reality of the scientific process. It is because of the support of a great number of people I was able to sort through my "failures" and prevail to finish my PhD.

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Chapter 1

Literature Review

The development of the corpus luteum

The estrous cycle

In mammals, the female reproductive system operates in a cyclical manner. In species with certain periods of receptivity for mating, such as cattle, swine, and sheep, the female reproductive cycle is called the estrous cycle. The short window of time during the cycle when the female is receptive for mating (usually around the time of ovulation) is called estrus. In addition, some species like horses, have seasonally directed reproductive cycles meaning these animals only breed during certain times of the year to ensure their young are born during a time with ample resources for rearing young. Humans and some primates, however, do not have estrous cycles and rather their cycles are called menstrual cycles. These monthly cycles are characterized by the shedding of the endometrium of the uterus each month in preparation for the next menstrual cycle.

The bovine estrous cycle is roughly 21 days in length on average. The estrous cycle is regulated by a feedback loop also known as the hypothalamic-pituitary-ovarian axis. Neurons within the hypothalamus release a hormone called gonadotropin releasing hormone (GnRH). The released GnRH is carried to the anterior pituitary gland by the portal blood vessels which stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the gonadotropes. FSH and LH are then carried through the systemic circulation to the ovary itself where they both act to stimulate the production of hormones such as estradiol and the development of a dominant follicle. The concentration of estradiol secreted by the dominant follicle increases over the course of the follicular phase, or the phase during the development of

the dominant follicle. Eventually, the concentration of estradiol reaches a certain threshold and instead of inducing negative feedback on the hypothalamus, estradiol induces a positive feedback. In addition to estradiol sensitizing the gonadoptropes in the anterior pituitary to GnRH, estradiol also increases the amplitude of LH and FSH release from the anterior pituitary. Eventually the amplitude of LH secreted reaches its peak and a large concentration of LH is released. This is called the LH surge. FSH does surge as well, but to a much lesser extent. The LH surge causes ovulation of the oocyte and the luteinization of the remaining follicle to form the corpus luteum (CL; luteinization to be discussed in more detail later).

Ovulation of the oocyte leads to a decline in estradiol production while progesterone concentrations steadily increase with the development of the corpus luteum. Like estradiol, LH and FSH concentrations also decline immediately following ovulation. As the CL develops and matures, progesterone concentrations continue to rise and sustain negative feedback on the hypothalamus and pituitary gland. If a pregnancy does not occur, the corpus luteum regresses (this process will be discussed in more detail later) which results in a decline in progesterone concentrations releasing the negative feedback on the hypothalamus and anterior pituitary and allows for the estrous cycle to resume again with the ovulation of the next oocyte. (Summarized from Senger 2013)

Luteinization

After the surge of LH and subsequent ovulation, the remaining cells in the ovulated follicle rapidly undergo changes in response to stimulation from LH to allow for the development of the corpus luteum. One result of the LH surge is the breakdown of the basement membrane, the structure separating the thecal and granulosal cells, allowing for the intermingling of these cell types in the ruminant CL (Farin et al., 1986). Several other major processes occur to allow for the development of the corpus luteum, including an increase in steroidogenic enzymes for the

production of progesterone, angiogenesis, hypertrophy and proliferation of certain cell types (Robinson and Woad, 2017).

The switch from estradiol to progesterone production is one of the hallmarks of luteinization in ruminants because the primary role of the corpus luteum is to produce the hormone progesterone. Although discussed in more detail in the next section, the binding of LH to the LH receptor (LHR) ultimately leads to the upregulation of transcription factors such as GATA4, SF-1, and Sp1 to increase production of proteins involved in progesterone production including steroidogenic acute regulatory protein (StAR/StARD1), cytochrome P450 side chain cleavage enzyme (CYP11A1), and 3-beta-hydroxysteroid dehydrogenase (HSD3B) (LaVoie and King, 2009 rev). Aromatase (CYP19A1) is the enzyme responsible for converting testosterone to estradiol in granulosal cells. It declines in abundance during luteinization in the cow (Hickey et al. 1990). However, the CL of other species, such as in the rat and human, have the ability to produce estradiol with retention of CYP19A1 during luteinization (Sasano et al., 1989; Hickey et al., 1990).

The CL is a very vascular tissue, and during luteinization, the development of the intricate network of blood vessels is critical for the development and survival of the CL. The origin of these developing vessels is from “vascular initiation points” within the preovulatory follicles that provide the framework for the developing vessels (Robinson et al., 2009 rev). However, there are several mechanisms by which angiogenesis is stimulated in the ovulatory follicle.

The LH surge increases the abundance of two important angiogenic factors, vascular endothelial growth factor A (VEGFA) and fibroblast growth factor-2 (FGF2) (Robinson et al., 2007). In fact, inhibition of VEGFA with an antibody or receptor antagonist in the primate follicle prevented luteal vascular development and reduced progesterone secretion (Fraser et al., 2000; Wulff et al., 2001; Hazzard et al., 2002). Inhibition of both VEGFA and FGF2 in the

bovine CL also suppresses luteal development and progesterone production (Yamashita et al., 2008). In the developing bovine CL, VEGFA protein is mostly located in the steroidogenic cells (Berisha et al., 2000), while FGF2 localization is primarily within endothelial cells prior to the LH surge and within the steroidogenic cells shortly after the LH surge (Berisha et al., 2006). Prostaglandins may also facilitate angiogenesis during ovulation. Prostaglandin E2 concentrations in the follicle increase just before ovulation and is necessary for successful ovulation in primates, cows, horses, and rodents (Kim and Duffy, 2016rev). Stimulation of two PGE2 receptors, PTGER1 and PTGER2, induces angiogenesis in the granulosal layer in primates (Trau et al., 2015). Hypoxia is a driver of angiogenesis in tumor models through the transcription factor hypoxia inducible factor 1 (HIF1), which upon its activation can upregulate VEGFA. HIF1A, one of the subunits of HIF1, is upregulated in the developing bovine CL along with VEGFA (Nishimura et al., 2009). Additionally, hypoxic conditions (3% O_2) *in vitro* also increased both HIF1A and VEGFA protein abundance in bovine luteal cells, suggesting hypoxia might be involved in angiogenesis in the CL (Nishimura et al., 2009).

Luteal development is also characterized by its rapid growth. The tissue mass of a CL can increase 20-fold over a twelve-day period in ewes, and in cattle the tissue mass can double within a 60-70 hour period (Jablonka-Shariff et al., 1993; Reynolds et al., 1994; Zheng et al., 1994). This rapid growth is mostly attributed to hyperplasia, or proliferation of cells, due to an 8-fold increase in DNA content (a measure of cell proliferation) during luteal development (Zheng et al., 1994). However, cells can also hypertrophy during this phase as well. Endothelial cells are the most proliferative cells in the CL during luteal development (Christenson and Stouffer, 1996). Due to the rapid angiogenesis upon luteinization, the increased proliferation of endothelial cells results in a 15-fold increase in volume of endothelial cells in the functional bovine CL (Robinson et al., 2006; Hojo et al., 2009). The luteal steroidogenic cells do not proliferate to the same extent as the endothelial cells. In the primate CL, there were no cells noted that co-stained for HSD3B and Ki-

67, a marker of cell proliferation, during luteal development (Christenson and Stouffer, 1996). A similar finding was noted in the ovine CL where the majority of the proliferating cells on days 2-12 of the estrous cycle were not the steroidogenic cells (Jablonka-Shariff et al., 1996). The same was true in the bovine CL where the most proliferative cells were small nonsteroidogenic cells, including endothelial cells (Zheng et al., 1994). Maalouf et al. (2016) performed a microRNA (miRNA) profiling study comparing miRNA abundance in day 4 and midcycle (day 10-12) CL and found day 4 CL had an increased abundance of miRNA regulating carbohydrate metabolism, while midcycle CL had an increase in abundance of miRNA regulating the cell cycle and apoptosis signaling. This suggests that miRNA may also play a role in regulating luteal development. Overall, rapid angiogenesis and increased cell proliferation upon luteinization leads to the development of the CL, a tissue that grows at a rate faster than any known tumor (Reynolds et al. 2000 rev).

Acquisition of luteolytic capacity

During early luteal development in the ruminant CL, the developing CL will not regress in response to a luteolytic dose of PGF2A (Henricks et al., 1974; Tsai et al., 1998). Despite luteolysis not occurring, there are effects of PGF2A within the early bovine CL (day 4) such as a decrease in *3BHSD* and *PTGS2* (Tsai and Wiltbank, 1998). After day 6 of the estrous cycle, the CL responds to a luteolytic dose of PGF2A with a decline in progesterone production and luteal regression (Henricks et al., 1974). This process is known as the acquisition of luteolytic capacity (Tsai and Wiltbank, 1998). The exact mechanisms of how the CL acquires this capacity still remain unclear.

Mondal et al. (2011) performed a transcriptomic profiling study comparing the bovine CL on day 4, prior to acquisition of luteolytic capacity, and day 11, after acquisition of luteolytic capacity. Additionally, PGF2A injections were administered and CL collected at 0, 4, and 24 hours after PGF2A injection on both days. Interestingly, there were some similarities in

transcripts 4 hours after PGF2A between D4 and D11 CL. Therefore, the day 4 CL does respond to PGF2A, but the response does not result in luteolysis.

Initially it was hypothesized that the resistance of the early CL to regress in response to PGF2A was because of a difference in PGF2A receptors (PTGFR) expression. However, it was determined that PTGFR were present in the bovine CL and there was no difference in receptor concentration or affinity between the day 2, 4, 6, or 10 CL (Wiltbank et al., 1995). In fact, within 2 days after ovulation, high affinity PTGFR were present in the bovine CL (Wiltbank et al., 1995). Thus, the number of PTGFR is not the reason for the acquisition of luteolytic capacity. The acquisition of luteolytic capacity has also been studied in porcine corpora lutea in which the CL does not under go luteolysis in response to PGF2A before day 9 (Diaz et al., 2000). The regulation of steroid hormone receptors in response to PGF2A in the pig was examined as a possible mechanism for the acquisition of luteolytic capacity. The day 9 CL had a greater abundance of nuclear progesterone receptor while the day 17 had a greater abundance of the estrogen receptor-β and upregulation of estradiol biosynthesis (Diaz and Wiltbank, 2004). This suggests the regulation of these receptors and estradiol biosynthesis might facilitate the acquisition of luteolytic capacity in the pig.

The steroidogenic cells may not be the only cells involved with the acquisition of luteolytic capacity. The CL is a heterogeneous tissue and it is possible that cells such as endothelial cells could contribute to this process. Endothelin-1 (EDN1) is a protein produced by the endothelial cells to regulate vascular tone. The complex vasculature in the CL would indicate that EDN1 might play an important role in luteal function. EDN1 abundance is greatest during luteal regression (Girsh et al., 1996b), and was proposed to be one of the regulators of luteolytic capacity. EDN1 is not present in the bovine CL on day 4 of the cycle (Levy et al., 2000), however, EDN1 is not likely responsible for mediating the effects of PGF2A in the developing CL because EDN1 inhibits progesterone synthesis in both the day 4 and day 10 bovine CL

(Choudhary et al., 2005). Overall, the exact mechanism for how the CL acquires its luteolytic capacity remains to be elucidated.

The functional corpus luteum

Cell types in the corpus luteum

The corpus luteum is a heterogeneous tissue comprised of several cell types including steroidogenic cells (large and small), endothelial cells, pericytes immune cells, and fibroblasts. The interaction between these cells is important for the overall function of the CL.

Luteal steroidogenic cells

Large luteal steroidogenic cells (LLC) arise from granulosal cells, while the small luteal steroidogenic cells (SLC) arise from thecal cells (Alila et al., 1984). The LLC comprise roughly 40% of the luteal volume while SLC comprise about 27% of the luteal volume in the bovine CL (O'Shea et al., 1989). On a cell number basis, LLC comprise only 3.5% of the total cells in the bovine CL while the SLC comprise roughly 26% of the total cells (O'Shea et al., 1989). SLC are between 12-22 microns in size, while LLC are about 25-50 microns (O'Shea et al., 1979; Fitz et al., 1982; Chegini et al., 1985; O'Shea et al., 1989). The importance of the LLC and SLC is their ability to produce progesterone, however the regulation of progesterone production between these two cell types is actually quite different. SLC continuously produce low concentrations of progesterone, while LLC continuously produce high concentrations of progesterone (Rodgers et al., 1982; Fitz et al., 1982; Hoyer et al., 1985; Weber et al., 1987). Upon LH stimulation, SLC have enhanced production of progesterone while LLC are not very responsive to LH stimulation (Hoyer et al., 1985; Hansel et al.,1987). Throughout the estrous cycle the steroidogenic cells undergo hypertrophy and hyperplasia. Generally, SLC continue to proliferate and undergo hypertrophy throughout the estrous cycle while LLC undergo hypertrophy with limited proliferation (Rao et al., 1978; Farin et al., 1986; Murphy et al., 2000). In the bovine CL of pregnancy, SLC accounted for roughly 85% of the total cell population while large luteal cells

accounted for 8-12% (Chegini et al., 1985). Small LC may further differentiate into LLC, as evidenced by Alila et al. (1984) where LLC from CL collected in the estrous cycle or during pregnancy bound an anti-thecal antibody (SLC indicator). This suggests the possibility of SLC to differentiate into LLC.

Endothelial cells

Endothelial cells (EC) are the cells lining the capillaries within the CL. The CL has a vast network of capillaries (as described previously during luteal development) and because of this, EC are the most numerous cell type in the CL comprising about 52% of all the cells in the bovine CL (O'Shea et al., 1989). During luteinization, the rapid increase in angiogenesis results in increased proliferation of EC resulting in a 15-fold increase in volume of EC in the functional bovine CL (Robinson et al., 2006; Hojo et al., 2009). Additionally, the greater density of luteal blood vessels in the developing CL (Hojo et al., 2009) and high rate of metabolism in the CL allows for greater blood flow through the CL compared to other tissues like the uterus or adrenal gland (Wiltbank et al., 1988). One of the primary roles of endothelial cells is the regulation of certain molecules including nutrients, hormones, and even immune cells that can pass through the lining of the capillary to either enter or exit a tissue. This role is critically important in the CL.

The ability of endothelial cells to regulate the passage of substrates and immune cells through the capillaries in the CL depends on their cell-to-cell contacts, presence of various cell membrane proteins, and certain factors that can induce vascular permeability. The endothelial cells lining the capillaries are linked together by interendothelial junctions. Adherens junctions can occur between these cells when a cleft forms between two cells' membranes and the protein VE-cadherin acts as a "bridge" between the two membranes (Boron and Boulpaep, 2017). Tight junctions, where the membranes of both cells appear to fuse, can also form these junctions and are held together by a group of proteins called claudins. The permeability of endothelial cells to allow nutrients and hormones into the luteal tissue is highly regulated by vascular endothelial

growth factor A (VEGFA). Treatment of human granulosal-endothelial cell cocultures with human chorionic gonadotropin (hCG) induced a VEGFA-dependent down regulation of VE-cadherin and claudin 5 abundance, which is associated with vascular permeability (Herr et al., 2013).

However, for immune cells (leukocytes) to migrate into a tissue, specific proteins must be expressed on the surface of endothelial cells and leukocytes to mediate this process. Very simply, an immune cell within the lumen of a capillary will roll across on the luminal side of an endothelial cell, adhere to a recognition protein on the surface of the endothelial cell, and then extravasate through the endothelium. L-selectin and P-selectin are two proteins expressed on leukocytes and endothelial cells, respectively, and play a role in the binding of a leukocyte to an endothelial cell. Both of these proteins have been found to be expressed on leukocytes and endothelial cells, respectively, in the bovine CL during early development suggesting these proteins play a role in mediating immune cell (eosinophil) invasion during early luteal development (Rohm et al., 2002). During luteal regression, endothelial cells are also thought to be the mediators of immune cell recruitment through the production of chemokine monocyte chemoattractant protein 1 (CCL2; Townson, 2006). Interestingly, endothelial cells also have the ability to induce proliferation of T lymphocytes *in vitro* and induce T lymphocyte adhesion to luteal endothelial cells enhanced by low concentrations of progesterone or prostaglandin E2 and F2A (Walusimbi et al., 2017).

Immune cells

The presence of immune cells in the bovine CL has been documented since Lobel and Levy's work in 1968 in which they documented the presence of leukocytes in the CL throughout the luteal phase. The types of leukocytes present in the bovine CL include macrophages, T cells, eosinophils, neutrophils, dendritic cells, and low numbers of B cells (Spanel-Borowski et al., 1997; Penny et al., 1999, Bauer et al., 2001; Townson et al., 2002; Poole and Pate, 2012). The

importance of these leukocytes and their relevance to luteal function has been a primary research area in the realm of luteal biology.

Alila and Hansel (1984) induced systemic lymphopenia in cyclic heifers to determine if immunosuppression alters luteal function. The immunosuppressed heifers had lower progesterone plasma concentrations compared to controls, suggesting leukocytes might facilitate luteal function. The facilitation of luteal function by immune cells is also supported by the variation of different immune cell types across the estrous cycle. Generally, the greatest number of immune cells occurs during luteal development and luteal regression.

Monocytes are a type of immune cell derived from stem cells in the bone marrow that travel through the systemic circulation and can replenish resident macrophages, be recruited to sites of inflammation, and differentiate into macrophages and dendritic cells within a tissue. Monocytes and macrophages are the most abundant immune cell type in the CL with the greatest abundance toward the end of the estrous cycle (Bagavandoss et al., 1988; Penny et al., 1999; Townson et al., 2002). There is an increase in monocyte chemoattractant protein-1 (CCL2), now known as C-C motif chemokine ligand 2, a protein that recruits monocytes and macrophages into a tissue at the time of luteal regression (Townson et al., 2002; Townson, 2006). Additionally, inflammatory cytokines, such as tumor necrosis factor alpha (TNF), interferon gamma (IFNG), and IL1B, which are involved in luteal regression, also aid in the signaling and function of these cells in the CL (Okuda and Sakumoto, 2003 rev.; Neuvians et al., 2004). However, Penny et al. (1999) also demonstrated a large abundance of monocytes and macrophages during luteal development (days 1-5), which suggests these cells might facilitate vascular development. In fact, Turner et al. (2011) found that ablation of macrophages in a mouse model led to increased hemorrhage in luteal and thecal layers of tissue, decreased endothelial cell abundance, increased erythrocyte abundance, and follicular atresia. Using a macrophage (CD11b+)-depleted mouse model Care et al. (2013) demonstrated that the luteal vascular network was disrupted from a lack

of expression of vascular endothelial growth factor, resulting in lack of luteal development. Thus, monocytes and macrophages are important facilitators of vascular growth during luteal development.

Polymorphonuclear leukocytes are also present in the CL. These include eosinophils and neutrophils. Both eosinophils and neutrophils are found within the bovine CL during luteal development along with increased abundance of IL8 (CXCL8), a neutrophil chemoattractant (Reibiger and Spanel-Borowski, 2000; Rohm et al., 2002; Jiemtaweeboon et al., 2011). The number of these cells in the CL generally tends to decline over the course of the estrous cycle (Penny et al., 1999; Reibiger and Spanel-Borowski, 2000; Jiemtaweeboon et al., 2011). Treatment with IL8 (CXCL8) *in vitro*, and supernatant from activated neutrophils, stimulated neutrophil migration in the early CL, as well as the formation of capillary-like structures from luteal endothelial cells, suggesting neutrophils may mediate angiogenesis during luteal development (Jiemtaweeboon et al., 2011). Neutrophils may play a role in luteolysis; the acute inflammatory response during luteal regression leads to the recruitment of many immune cells, such as neutrophils. Neutrophils increase in the bovine CL within 5 minutes after a PGF2A injection (Shirasuna et al., 2012).

Although the abundance and perceived functions of these different immune cells within the CL can vary, immune cells within the CL remain important for proper luteal development, function, and regression.

Steroidogenesis and mitochondrial function in the CL

The primary role of the corpus luteum is to produce the steroid hormone progesterone, the hormone required to maintain a pregnancy in mammals. The enzymes and proteins responsible for this process must be upregulated upon luteinization.

Steroidogenesis begins with an unesterified cholesterol molecule (27-carbon molecule). The source of cholesterol in the CL may come from low density lipoproteins (LDL) or high

density lipoproteins (HDL) in the plasma, acetate precursors in the cell, or intracellular stores of cholesterol esters. Lipoproteins are the main source of cholesterol for steroiodogenesis (Pate and Condon, 1982). Lipoproteins enter the cell via receptor mediated endocytosis. The receptors for LDL and HDL are LDLR and scavenger receptor type B class 1 (SR-B1), respectively. Abundance of both receptors have been found to increase in response to gonadotropins in both rat and porcine CL (Lopez et al., 1999; Sekar and Veldhuis, 2004). Cholesterol from these lipoproteins, from the cell membrane, or liberated from lipid stores in the cell via hormone sensitive lipase (HSL) can then be directed to the mitochondria for the first step in steroidogenesis.

The rate limiting step of steroidogenesis is the transport of cholesterol across the mitochondrial membrane. The mechanism for how cholesterol is transported across the mitochondrial membrane is not fully understood, but it does involve steroidogenic acute regulatory protein (StAR/STARD1), which is located on the outer mitochondrial membrane and facilitates the transfer of cholesterol across the mitochondrial membrane (Clark et al., 1994; Bose et al., 2002). This step is the rate-limiting step in the steroidogenic pathway. Once cholesterol has been transported into the mitochondrial matrix, the first cleavage of cholesterol occurs by an enzyme called the P450 side chain cleavage enzyme (CYP11A1). CYP11A1 cleaves cholesterol into pregnenolone, a 21-carbon molecule (Halkerston et al., 1961). Pregnenolone then exits the mitochondria and undergoes conversion to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B) (Samuels et al., 1951).

Three proteins that are upregulated during luteinization are STARD1, CYP11A1, and HSD3B (King and LaVoie, 2012 rev), whereas aromatase (CYP19A1) declines in abundance (Hickey et al., 1990). Luteinizing hormone (LH) binds to the LH receptor present on granulosal and thecal cells in the follicle and induces an increase in the second messenger, cyclic AMP (cAMP; Davis et al., 1986). The LH receptor is a Gs protein-coupled receptor and, upon its

activation, releases the alpha subunit of the G protein-coupled receptor to activate the enzyme adenylate cyclase (AC). AC converts ATP to cyclic AMP. Cyclic AMP activates protein kinase A (PKA), which in turn, activates the transcription factor, cyclic AMP response-element-binding protein (CREB). Activated CREB is thought to be the one of the major regulators of genes involved in steroidogenesis in the follicle (Mukherjee et al., 1996). However, other transcription factors, such as GATA4, SF-1 (NR5A1), and SP1 are important in the upregulation of STARD1, CYP11A1, and HSD3B in the CL (LaVoie and King, 2009 rev).

Just as the production of progesterone can be increased upon luteinization, progesterone production can be decreased during luteolysis. AMP-activated protein kinase (AMPK) is a master regulator of cell metabolism and is also involved in the regulation of steroidogenesis in the CL. The activation of PKA by LH in luteal steroidogenic cells inhibits the activation of AMPK by reducing phosphorylation on the Thr-172 residue (associated with AMPK activation) and increasing Ser-485 phosphorylation (associated with AMPK inhibition; Hou et al., 2010). The inactivated AMPK allows for the activation of hormone sensitive lipase (HSL), an enzyme that is responsible for the lipolysis of lipid droplets and release of cholesterol. During luteolysis, signaling of PGF2A through its receptor activates AMPK (Bowdridge et al., 2015), which in turn inhibits HSL, reducing the amount of available cholesterol. Activated AMPK also downregulates STARD1, CYP11A1, and HSD3B, another mechanism by which steroidogenesis might be regulated by AMPK (Tosca et al., 2007).

Luteolysis and Luteal Rescue

Luteolysis in ruminants

In the absence of a pregnancy, the CL regresses to allow for the next estrous cycle to begin. The uterus is necessary for regression as the absence of the uterus prolongs the life of the CL (Loeb, 1923; Wiltbank and Casida, 1956; Moor et al., 1970). The factor released from the uterus that induces luteolysis in the ruminant is PGF2A (McCracken et al., 1970). However,

experiments transplanting the ovary to the neck of sheep, moving the ovary away from its association with the uterine horn to the abdomen, or separating the ovarian artery from the utero-ovarian vein suggests the effect of the PGF2A is local and requires the ovary to be in close proximity to the uterus for PGF2A to elicit its effect via an ovarian artery, utero-ovarian vein countercurrent mechanism (Baird et al., 1968; Moor et al., 1970 Barrett et al., 1971; McCracken et al., 1972). Once uterine PGF2A reaches the CL it results in both the functional and structural demise of the CL.

The functional regression of the CL is defined as the decline in progesterone production. PGF2A induces a loss of LH receptors, thus a subsequent loss in LH stimulation and progesterone production (Grinwich et al., 1976; Diekman et al., 1978; Spicer et al., 1981; Guy et al., 1995). *In vitro* and *in vivo* studies have demonstrated that PGF2A can inhibit LH-stimulated progesterone production (Pate and Condon, 1984; Pate and Nephew, 1988). PGF2A can inhibit lipoprotein induced progesterone synthesis *in vitro* (Pate and Nephew, 1988; Pate and Condon, 1989), but the ability of PGF2A to inhibit the use of lipoproteins for steroidogenesis appears to be after cholesterol transport to the mitochondria and before side chain cleavage by CYP11A1 (Grusenmeyer and Pate, 1992). Signaling of PGF2A through its receptor can activate AMPK and inhibit HSL to reduce the amount of available cholesterol (Bowdridge et al., 2015). PGF2A signaling and activated AMPK have also been shown to downregulate both protein and mRNA of STARD1 and HSD3B, and protein of CYP11A1(Tsai et al., 2001; Tosca et al., 2007). Reduced *HSD3B* expression after PGF2A stimulation, however, is not concomitant with reduced HSD3B activity, and thus might not be responsible for the decline in progesterone during PGF2A induced luteolysis (Juengel et al., 1998). Other cell types in addition to steroidogenic cells can also contribute to the functional demise of the CL during regression.

Like the steroidogenic cells in the CL, endothelial cells also express the PGF2A receptor, PTGFR (Mamluk et al., 1998). Endothelial cells secrete endothelin-1 (EDN1) in response to

treatment with PGF2A and treatment with EDN1 decreases progesterone production from dispersed luteal cells within 2 hours (Girsh et al., 1996a). The potential role of endothelial cells might explain how *in vitro* stimulation of luteal steroidogenic cells with PGF2A does not decrease progesterone production alone, but inhibits the LH-stimulated progesterone production (Pate and Condon, 1984). When endothelial cells are cocultured with steroidogenic cells and these cells are treated with PGF2A, EDN1 abundance is increased and progesterone production declines, suggesting this might be one mechanism by which endothelial cells function during luteal regression (Girsh et al., 1995; Girsh et al., 1996a; Girsh et al., 1996b).

Endothelial cells also participate in the structural demise of the CL during luteal regression. The destabilization of the vasculature in the CL is one such target. The proangiogenic factors, VEGFA and FGF2, are downregulated during luteolysis, whereas antiangiogenic factors and inhibitors of FGF2, such as thrombospondin 1 and 2 and pentraxin 3 (*PTX3*), are upregulated in response to PGF2A (Zalman et al., 2012). The extracellular matrix (ECM) also undergoes extensive remodeling as a result of luteal regression. Matrix metalloproteases (MMP) and plasminogen activators are responsible for the breakdown of basement membranes and tissue remodeling. Both factors are upregulated in the bovine CL after an *in vivo* injection of PGF2A (Smith et al., 1999; Kleim et al., 2007). The loss of the ECM during luteal regression can also result in cell death. Apoptosis is one of the main mechanisms of cell death induced by PGF2A. PGF2A induces apoptosis through the intrinsic pathway by increasing the abundance of the apoptosis regulator Bcl-2 like protein 4 (BAX) resulting in cytochrome C release from the mitochondria and caspase-9 and -3 activity (Yadav et al., 2005).

Luteal rescue in ruminants

Maternal recognition of pregnancy is the process by which the maternal reproductive system recognizes a conceptus, resulting in physiological changes to accommodate the conceptus, including the rescue of the CL. The maternal recognition of pregnancy signal varies by species. In

the ruminant, the signal is a conceptus-derived protein called, interferon tau (IFNT; Godkin et al., 1982), whereas in the human it is human chorionic gonadotropin (hCG). This portion of the review will discuss luteal rescue in ruminants.

Toward the end of the estrous cycle and around the time of luteolysis, PGF2A is released from the uterus in a pulsatile manner (Zarco et al., 1988). However, during pregnancy the release of PGF2A from the uterus is secreted in a more constant fashion (Peterson et al., 1976), but the CL is resistant to PGF2A-induced luteolysis (Silvia and Niswender, 1984). The mechanisms of this resistance have not been fully elucidated. It has been demonstrated that the activity of 15-hydroxyprostaglandin dehydrogenase (PGDH), the enzyme that converts PGF2A to its metabolite PGFM, is greater in the CL of pregnancy on day 13 in the ewe compared to day 13 of the cycle, which is one possible mechanism for luteal resistance during pregnancy (Silva et al., 2000). The abundance of PGF2A receptors (PTGFR) on the CL of pregnancy was thought to be a mechanism by which luteal resistance occurs. However, in pregnant ewes the number of PTGFR during early pregnancy was greater than cyclic animals on day 15, indicating the number of PTGFR is not the reason for luteal resistance (Wiepz et al., 1992). Another prostaglandin, PGE2, is a luteotropic molecule and its concentrations increase during pregnancy. PGE2 synthesis in the endometrium increases during early pregnancy and is transported to the ovary via the utero-ovarian vasculature (Lee et al., 2012). PGE2 delivered to the ovary during pregnancy is thought to increase the intraluteal production of PGE2 and signaling through the PGE2 receptors to facilitate luteal rescue (Lee et al., 2012). However, it is important to note the number of PGE2 receptors in the CL of early pregnancy does not increase (Wiepz et al., 1992).

Signaling through IFNT may also alter how the CL responds to pulses of PGF2A. IFNT in cattle can be detected in the trophectoderm of a developing conceptus as early as day 14-15 of pregnancy (Farin et al., 1990). IFNT secreted by the conceptus has local effects on the uterine environment by decreasing both estrogen and oxytocin receptors, which are thought to disrupt the

pulsatile release of PGF2A from the uterus (Spencer et al., 1995). Additionally, IFNT signaling can also upregulate interferon-stimulated genes (ISG), which may play a role in luteal resistance. One of the most notable ISG is *ISG15*, which is upregulated in the bovine endometrium during pregnancy and localized to the glandular epithelium (Austin et al., 2004). Transcriptomic analysis of the ovine CL during early pregnancy demonstrated that *ISG15* and *MX1* were greater in the CL of pregnancy and were also increased in luteal cells treated with IFNT *in vitro* (Antoniazzi et al., 2013). *PTX3*, an activator of the classical complement cascade, IL-6, VEGF, and LHR were in greater abundance in the CL of pregnancy compared to the cycle (Antoniazzi et al., 2013). Therefore, IFNT alters gene expression in the CL during luteal rescue. In fact, recent transcriptomic and proteomic profiling studies of the bovine CL support this. Hughes et al. (2020) collected CL from cattle on days 14, 17, 20, and 23 of pregnancy and found 1157 mRNA and 27 miRNA that were differentially abundant and 29 proteins that tended to change. Consistent with luteal rescue, the mRNA that increased were associated with interferon signaling and DNA repair and those that decreased were associated with luteolytic pathways. Hughes et al. (2019) compared the CL on day 17 of pregnancy to the CL on day 17 of the cycle and found 140 differentially abundant mRNA and 24 differentially abundant proteins. The pathways associated with these differentially abundant mRNA in the CL of pregnancy included extracellular matrix remodeling, vascular stability, and T cell receptor signaling.

The role of immune cells in the CL of pregnancy is not well-established. As discussed previously, IFNT upregulates the abundance of *ISG15* both in the uterus and the CL of pregnancy (Austin et al., 2004; Antoniazzi et al., 2013). The abundance of *ISG15* is also increased in peripheral immune cells (both PBL and PBMC) in pregnant cows on days 18-20 of pregnancy (Gifford et al., 2007; Green et al., 2010). Within the CL, IFNT can stimulate expression of IL8, a cytokine that promotes neutrophil migration *in vitro* and this finding is consistent with the increased abundance of IL8 and neutrophils in the bovine CL on day 16 of pregnancy (Shirasuna

et al., 2015). Although there is information about peripheral immune cells and luteal-resident neutrophils during pregnancy in the cow, little is known about the abundance of luteal-resident T cells during pregnancy.

The abundance of T cells in the CL of pregnancy in other species may provide information as to what occurs in the bovine CL. PBMCs collected from women during early pregnancy and co-cultured with luteal steroidogenic cells induced progesterone production to a concentration that matched that of cells treated with hCG (Hashii et al., 1998). In the same study, luteal cells treated with IL10 and IL4, both anti-inflammatory cytokines, found that progesterone production by luteal cells was enhanced (Hashii et al., 1998). The equine CL of early pregnancy has few CD4+ and CD8+ T cells (Lawler et al., 1999). Throughout gestation, the canine CL did not have a change in the number of CD4+ cells and TNF, a proinflammatory cytokine, also did not change over the course of gestation in the canine CL (Nowaczyk et al., 2017). One study by Magata et al. (2012) evaluated different immunotolerogenic factors in the bovine CL during pregnancy. Prostaglandin E synthase, the enzyme that generates PGE2, Foxp3, a transcription factor associated with T regulatory cells, and TGFB1 an antiinflammatory cytokine, were upregulated in the CL of pregnancy (Magata et al., 2012). Interestingly, *TNF* was also upregulated in the CL of pregnancy (Magata et al., 2012). The number of γδ+ T cells in the CL of pregnancy was not different compared to the cycle on day 18, but there were more CD8αβ+ T cells and γδ+CD8αβ+ T cells (Poole and Pate, 2012). The transcriptomic and proteomic profiling study by Hughes et al. (2019) found that T cell activation signaling, IL2 signaling, and TGFβ signaling were all associated with the differentially abundant mRNA in the bovine CL on day 17 of pregnancy. Molecules involved in the T cell receptor signaling cascade, including CD3e, ITK, PTPN7, and LCK were found to be in greater abundance in the CL of pregnancy compared to the cycle and were confirmed via qPCR (Hughes et al., 2019). Based on these findings, T cell

activation and signaling might be important in maintaining the CL during early pregnancy, but the phenotype of T cells in the bovine CL at this time remains unknown.

T lymphocytes in the corpus luteum

T lymphocyte development and function

Lymphoid progenitor cells arise in the bone marrow and eventually migrate to the thymus. Lymphoid progenitor cells lack expression of the T cell receptor (TCR), CD4, and CD8, and are called double negative (DN) thymocytes. These DN thymocytes undergo four stages of development. It is during these stages when the DN thymocytes acquire the TCR through the interaction with thymic cells that secrete thymic factors to stimulate the genes RAG1 and RAG2, which encode components of the TCR. The TCR is traditionally composed of two chains, the TCR α-chain and the TCR β-chain. Upon the DN thymocyte's completion of the fourth stage of development, the cell now expresses a complete αβ TCR as well as the two co-receptor molecules, CD4 and CD8 and is referred to as a double positive (DP) thymocyte.

The DP cell now under goes a selection process through its interaction with cortical epithelial cells in the thymus. These cortical epithelial cells express major histocompatibility complex (MHC) class I and class II molecules containing self-peptides. The TCR positive thymocytes that recognize MHC class I molecules become CD8-committed T cells, while those that recognize MHC class II molecules become CD4-committed T cells. This process is called positive selection. Additionally, this developing DP thymocyte can also undergo negative selection, which is the process by which the TCR interacts with the self-peptide associated with the MHC molecule. If the TCR recognizes the self-peptide, this DP cell will then undergo Fas-mediated apoptosis because this could ultimately lead to destruction of host cells by T cells (e.g. autoimmune diseases). T cells can also undergo apoptosis if the cell fails to properly recognize MHC class I or II and this is called death by neglect. The newly developed CD4+ cells are referred to as a T helper cell and the CD8+ cells are referred to as T cytotoxic cells. These cells

are ready to migrate out of the thymus and into the periphery. It is important to note that T cells can also express another type of TCR besides the αβ TCR. The other type of TCR is a γδ TCR and this cell type will be discussed in more detail below (Summarized from Germain, 2002 rev and Takahama, 2006 rev).

Naïve CD4+ T cells can differentiate into various T cell phenotypes based upon interaction with an antigen presenting cell (APC) and stimulation from various cytokines. There are three classical APC: macrophages, dendritic cells, and B cells. The first step in differentiation of naïve CD4+ T cells, or Th0, is the interaction of the TCR and co-receptor, CD4, with the antigen-MHC II complex. Upon this interaction, cytokines released from APCs can direct the differentiation of the Th0 cell.

IL12 is the main cytokine that initiates the differentiation of Th0 cells into T helper 1 (Th1) cells (Trinchieri et al., 2003), whereas IL4 and IL2 are the main cytokines that drive T helper 2 (Th2) development (Luckheeram et al., 2012). Several transcription factors are involved in Th1 differentiation, but T-bet plays the largest role in this differentiation because it induces the production of interferon-γ, the most notable cytokine produced by Th1 cells. T-bet can also suppress the development of both Th2 and Th17 cells (Lugo-Villarino et al., 2003; Lazarevic et al., 2011). Th1 cells can also produce TNF and IL2 (Del Prete, 1992). The primary function of Th1 cells is to eliminate intracellular pathogens and activating phagocytic cells like macrophages (Del Prete, 1992; Luckheeram et al., 2012). Th2 cell induction is in response to IL2 and IL4 and is mediated by the transcription factor GATA3 (Zhu et al., 2006). GATA3 mediates the differentiation of Th2 cells by suppressing Th1 cell development and promoting Th2 cytokine production including IL4, IL5, and IL13 (Zhu et al., 2006; Luckheeram et al., 2012). The main functions of Th2 cells are to support B cell function, induce an immune response against extracellular pathogens (humoral immunity), and involvement in allergic reactions (Del Prete, 1992; Zhu et al., 2006; Luckheeram et al., 2012). There is a third type of T helper cell, Th17 cells.

Th17 cell differentiation is mediated by TGFB1 and also needs the presence of IL6 (Veldhoen et al., 2006). The transcription factor that regulates the differentiation process in Th17 cells is retinoic acid receptor-related orphan receptor gamma-T (RORγT) (Luckheeram et al., 2012). IL17, a proinflammatory cytokine, is the cytokine produced by Th17 cells. Th17 cells act by destroying extracellular pathogens and fungi (Veldhoen et al., 2006; Luckheeram et al., 2012).

CD4+ T cells can also differentiate into regulatory T cells (Treg), however, the specific subtype depends upon the site at which these cells are primed. Inducible Tregs (iTreg) develop in the peripheral lymphoid organs, while natural Tregs are developed in the thymus and released as fully functional Tregs (Luckheeram et al., 2012). TGFB1 is responsible for the differentiation of Treg cells (Chen et al., 2003). The difference between iTreg and nTreg is, iTregs develop from naïve CD4+CD25- cells in response to TGFB1 (Chen et al., 2003). The major transcription factor involved in this differentiation is forkhead box P3 (Foxp3) and because of this Treg cells are often referred to as CD4+CD25+Foxp3+ cells (Chen et al., 2003). The function of Treg cells, as their name states, is to maintain immunologic tolerance and suppress inflammatory responses. The cytokines produced by Treg cells include the antiinflammatory cytokines IL10 and TGFB1 (Luckheeram et al., 2012).

Another subtype of T cells are T cells that express the γ and δ chains of the TCR. The γ and δ chains of the TCR were discovered in 1987 in the mouse (Born et al., 1987). The γ and δ chains are relatively well-conserved across many species (Hayday, 2000). In fact, γδ T cells have been found in circulation in ruminants and occur at a relatively high frequency compared to the human or mouse (Hein and Mackay, 1991). In young ruminants, γδ T cells represent up to 60% of the peripheral T cell population with this percentage declining as the animal ages, suggesting these cells may play a role in defense against invading pathogens (Hein and Mackay, 1991). The exact role of γδ T cells is still being investigated, but they are thought to act as a bridge between

the innate and adaptive immune system. γδ T cells are different from the αβ T cells because they are not MHC-restricted and can recognize unprocessed and nonpeptide antigens. Interestingly, γδ T cells recognize and respond to pathogen-associated molecular patterns (PAMPs) which is one possible mechanism for linking the innate and adaptive immune system (Hedges et al., 2005). One unique feature that may also affect their function is that γδ T cells can also express workshop cluster 1 (WC1), a cysteine rich scavenger receptor, and its various isoforms (WC1.1, WC1.2 and WC1.3; Mackay et al., 1989; Wijngaard et al., 1992; McGill et al., 2013). WC1+ γδ T cells are known to produce both inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (CSF2), IFNG, and TNF, as well as anti-inflammatory cytokines such as IL10 and TGFB1, depending upon the WC isoform expressed (McGill et al., 2013; Guerra-Maupome et al., 2019).

T lymphocytes in the CL

The CL contains a diverse population of T cells including T helper (CD4+), cytotoxic (CD8+), and γδ T cells. The type of T cells residing in the CL varies depending on the stage of the estrous cycle, and thus the luteal microenvironment.

During the time between luteal development and the functional midcycle CL, the number of CD4+ and CD8+ cells does not change (Penny et al., 1999). Interestingly, γδ T cells comprise roughly 30% of the total T cells in the functional bovine CL, while CD4+ and CD8+ cells comprise 25% and 45%, respectively (Poole and Pate, 2012). There is a greater number of CD8+ cells compared to the number of CD4+ cells between luteal development and the midcycle functional CL (Penny et al., 1999; Townson et al., 2002). Despite the relatively low abundance of CD4+ cells in the functional CL, there is a greater abundance of Treg (Foxp3+) cells (Poole and Pate, 2012). Within the CD8+ population of cells, there is a greater number of CD8αβ+ T cells than CD8αα+ T cells in the functional midcycle CL (Poole and Pate, 2012).

Luteal cells have the ability to elicit T cell proliferation *in vitro* (Petroff et al., 1997). Luteal cells from a midcycle CL have been shown to induce proliferation of γδ+WC- T cells and increased IL10 expression, suggesting a more immunotolerant environment in the midcycle CL (Walusimbi and Pate, 2014). Progesterone may be one factor initiating the immunosuppressive environment as progesterone can induce IL10 and Th2 cytokine (IL3 and IL4) production in the presence of a progesterone blocking factor in the mouse (Szekeres-Bartho and Wegmann, 1996). Additionally, progesterone induces a dose dependent inhibition of luteal cell-stimulated T cell proliferation *in vitro* (Cannon et al., 2003). However, T cells lack a nuclear progesterone receptor, so the effect of progesterone is not mediated through this receptor (Cannon et al., 2003). Instead, the effect of progesterone on T cells might be mediated through membrane progesterone receptors, such as PGRMC1, which has greater mRNA abundance in the CL on day 11 of the cycle compared to day 18 (Ndiaye et al., 2012).

During luteolysis, the number of CD4+ and CD8+ cells in the CL increases (Penny et al., 1999; Bauer et al., 2001). However, the proportion of resident CD4+, CD8+, and γδ T cells does not change compared to the functional CL (Poole and Pate, 2012). During luteolysis, the ability of luteal cells to stimulate proliferation of T cells does change. Luteal cells exposed to a luteolytic dose of PGF2A stimulate greater proliferation of T cells (Petroff et al., 1997; Cannon et al., 2003). Luteal cells from regressing CL also stimulate the proliferation of γδ+WC+ T cells to a greater extent than midcycle CL (Walusimbi and Pate, 2014). The number of Foxp3+ Tregs in the regressing CL is also lower compared to the midcycle CL (Poole and Pate, 2012). In regard to the CD8+ population of cells, the regressing CL had a greater number of CD8αα+ T cells and γδ+CD8αα+ T cells (Poole and Pate, 2012). Together, this suggests that during luteal regression, the decline in Treg cells allows luteolysis to occur, while the increase in CD8αα+ and

γδ+CD8αα+ T cells act to regulate the inflammatory response and prevent overstimulation of autoreactive T cells.

MHC proteins are another factor that can impact T cell function in the CL. MHC, as described above, is a cell surface protein that T cells use to recognize an antigen during the adaptive immune response. MHC class I is present on all cell types and acts as "self-antigen" to prevent host cells from being destroyed by the adaptive immune system. MHC class II proteins are present on APCs and when loaded with peptides from a pathogen, they direct T cell effector function. Fairchild et al. (1989) was the first to report both MHC class I and class II were present on luteal cells, and treatment with IFNG increased abundance of both classes of MHC on luteal cells *in vitro*. Both large and small luteal cells express MHC class II and its abundance is increased around the time of luteal regression (day 18) (Benyo et al., 1991). When compared to the CL on day 18 of the cycle, MHC class II expression is lower in the CL on day 18 of pregnancy (Benyo et al., 1991). The increased abundance of MHC class II molecules in the regressing CL has been found to enhance the ability of luteal cells from regressing CL to stimulate T cell proliferation (Petroff et al., 1997). When luteal cells were treated with staphylococcal enterotoxin (SEB) to aid in T cell proliferation, the luteal cells from regressing CL treated with SEB had enhanced T cell proliferation while when treated with anti-MHC antibody this response was attenuated (Petroff et al., 1997). Luteal cells express both MHC class I and class II (Fairchild et al., 1989), but in regressing CL it appears that MHC class II can inhibit T cell proliferation *in vitro*. However, Davis and Pate (2007) found using anti-MHC class II antibodies did not inhibit T cell proliferation *in vitro*. It should be noted that in this study the regressing CL collected from animals given a PGF2A injection did not result in a decrease in progesterone, so these cells might have behaved more like midcycle CL and not a regressing CL, thus no attenuation in T cell proliferation. Interestingly, these results demonstrated that that γδ T cells can be stimulated to proliferate by luteal cells. The question still remains about how luteal

cells act as APC since they express both MHC class I and II. It is unlikely that the luteal cells are presenting foreign peptides through its MHC class II molecule to stimulate T cells. Nonimmune cells within the CL, including both endothelial cells and steroidogenic cells, do express the MHC class II antigen processing components (Benyo et al., 1991; Cannon et al., 2007), suggesting these cells can act as APC.

The Complement System

Activation and regulation of complement

The complement system, or complement cascade, is an important component of the innate immune system. The complement cascade is comprised of over 30 proteins and is one of the first lines of defense against an invading pathogen. It is best known for its role in developing a hole in an invading pathogen's membrane by forming a "membrane attack complex" (MAC). There are three pathways by which the complement cascade can be activated in response to an invading pathogen: the classical, alternative, and mannan-binding lectin pathways. While the complement cascade is notoriously known for its complicated nomenclature, all three pathways of activation follow similar trends and converge on one central protein, C3. The complement system is also involved in a variety of other pathways and functions outside of its traditional role in destroying invading pathogens including T cell differentiation, coagulation, regulation of the adaptive immune system, and debris clearance.

The classical pathway for the activation of complement was the first pathway of activation discovered. This pathway requires an antibody to be present on the invading pathogen to activate the cascade. In the event that a pathogen is able to invade a host, the immune system is "alerted." If the pathogen has been previously recognized by the adaptive immune system, antibodies produced by B cells will be released and mark the pathogen for destruction. This antibody can be recognized by the first protein in the complement cascade, C1q. C1q is a globular

protein containing 6 globular heads, which are held together by long collagen-like tails. C1q is part of the C1 complex that contains two other proteins, C1s and C1r.

When C1q recognizes the antibody on the invading pathogen, it binds to the pathogen and this binding induces a conformational change in the C1q protein. This conformational change induces the activation of C1r which then cleaves C1s to generate an active serine protease. The now activated C1s cleaves the next two proteins in the complement cascade, C4 and C2. C4 is cleaved first into two fragments C4a and C4b. C4b binds to the pathogen's surface and binds to C2, which allows for the cleavage of C2 into C2a and C2b by C1s. Now, the cleavage products of C4 and C2, C4b and C2b respectively, complex together and remain on the pathogen's surface to act as the C3 convertase. This C3 convertase converts the next protein in the cascade, C3, into C3a and C3b. The cleavage of C3 is the converging point of all of the activation pathways. C3a acts as an anaphylatoxin, meaning C3a induces an inflammatory response, while C3b binds to the pathogen's surface.

The next protein to be cleaved in the cascade is C5. However, in order for C5 to be cleaved the C5 convertase has to be present. The C5 convertase in the classical pathway is the complexing of C3b with the C3 convertase (C4bC2b). The complexing of these proteins leads to the cleavage of C5 into two cleavage products C5a and C5b. Once C5 has been cleaved, there is the rapid accumulation of the remaining complement components in the cascade to form the MAC. C5b binds to the pathogen's surface and recruits C6 through C9. The recruitment of C6 through C9 leads to the formation of the MAC. The MAC creates a pore in the pathogen's membrane and allows the contents within the pathogen to leak out ultimately destroying the pathogen.

The alternative pathway is perhaps the most common way complement is activated and does not require an antibody to be present for its activation. This pathways starts with C3 and is characterized by the spontaneous cleavage of C3. C3 is an abundant protein in the systemic

circulation and it can undergo spontaneous hydrolysis to form C3(H_2O). The resulting C3(H_2O) can bind to another circulating protein, Factor B. Once bound, Factor B within this complex can be cleaved by another circulating protein Factor D, resulting in two fragments known as Ba and Bb. Bb remains complexed with C3(H_2O) forming C3(H_2O)Bb, the C3 convertase of the alternative pathway. This C3 convertase can act in a feed forward system of cleaving more and more circulating C3 into C3a and C3b. The resulting cleavage product, C3b, can bind to the surface of pathogens and initiate the complement cascade in the same manner as the classical pathway.

The third and final pathway is the mannan-binding lectin pathway. This pathway proceeds in a very similar to the classical pathway except instead of requiring an antibody on the surface of pathogen, the mannan-binding lectin protein (similar in structure to the C1 complex) binds to mannose sugar residues on the surface of the pathogen. The binding of mannan-binding lectin on the surface of a pathogen initiates a conformational change in the protein, similar to that of C1q, activating MASP-1 and MASP-2. Like C1r and C1s in the classical pathway, MASP-1 and MASP-2 cleave C2 and C4 upon activation leading to the formation of the C3 convertase (C4bC2b). The mannan-binding lectin pathway then proceeds in the same fashion as the classical pathway.

The activation of all three of these pathways also has to be regulated to prevent excessive complement activation and possible destruction of host cells. This regulation occurs by a number of proteins, which are ubiquitously expressed on most cell types, and act at different levels of the complement cascade to prevent the damage of host cells. The classical cascade can be regulated at the first step in the cascade by C1 inhibitor (C1INH). This protein acts by binding to C1s and C1r and displacing them from C1q to prevent the activation of C1s and C1r and the subsequent cleavage of C2 and C4. Additionally, the last step in the cascade, the formation of the MAC, can be inhibited by the cell membrane protein, CD59 (protectin). CD59 prevents C9 from interacting

with C5b, C6, C7, and C8 complex, thus preventing the formation of the MAC. The most regulated step in the cascade, however, is the formation of a C3 convertase and cleavage of C3 because this step is the converging point of all three pathways. C4b-binding protein (C4BP) is one protein which regulates the classical pathway by preventing the formation of the C3 convertase. C4BP binds to C4b on the surface of a pathogen to prevent C4b from interacting with C2b to form the C3 convertase.

In the alternative pathway, the proteins involved in preventing the formation of the C3 convertase include decay accelerating factor (DAF; CD55), complement receptor 1 (CR1), membrane cofactor of proteolysis (MCP; CD46), and factor H. Each of these proteins binds to C3b on the host cell's surface and displaces Bb (this was the cleavage product of Factor B) from interacting with C3b to prevent the formation of the C3 convertase, C3bBb, and preventing the cascade from continuing. Additionally, factor I can inactivate C3b by cleaving it and forming iC3b which can be further cleaved to the fully inactivated form, C3dg. Factor I is an active regulator of the classical pathway. CR1, factor H, and MCP can act as cofactors to allow factor I to inactivate C3b and prevent the formation of a C3 convertase.

Inadequate regulation of complement activation and/or the absence of certain complement proteins can have serious consequences for the host animal. Although rare, there are certain genetic mutations which can result in the absence both complement cascade proteins as well as the regulatory proteins. For example, paroxysmal nocturnal hemoglobinuria is a life-threatening condition characterized by the lysis of red blood cells by complement in an uncontrolled manner. While this condition is very rare (1-5 people affected per million people), it results from a somatic mutation in hematopoietic stem cells in the PIGA gene, the gene encoding part of the GPI anchor which anchors both CD59 and CD55 to the cell membrane. Without part of this anchor, CD59 and CD55 are unable to function properly and complement activation

occurs at an uncontrolled rate on the surface of red blood cells, thus lysing these cells (Summarized from Janeway et al. 2001 and Sompayrac 2012)

Role of complement in the adaptive immune system

Although complement is best known for its role in the innate immune system, complement has also been found to modulate functions of the adaptive immune system. The link between complement and the adaptive immune system was made with the discovery of complement receptors on lymphocytes (Lay and Nussenzieg, 1968). Experiments conducted in the 1970s by Pepys (1972) demonstrated that C3-depleted mice were unable to mount a large antibody response, demonstrating a possible link between C3 and the adaptive immune system. It was also around this time that Bianco et al. (1970) observed a certain proportion of lymphocytes in both mouse and humans, which they referred to as complement receptor lymphocytes (CRL), were able to bind sheep erythrocytes sensitized with an antibody and complement (EAC) when C3 bound to the lymphocyte first. However, if anti-C3 antibodies were present it prevented the interaction between EAC and the CRL indicating C3 was the complement component mediating this interaction (Eden et al., 1971). The type of lymphocyte that was termed "complement receptor lymphocytes" was determined to be B cells because these CRL had immunoglobulin (IgG) determinants on their cell membrane and were not derived from the thymus (they lacked the theta antigen) (Eden et al., 1971). Additionally, human B cells were also found to have the ability to regulate complement activation. B cells treated with β1H globulin (later named Factor H), which binds to the cell membrane, induced the release of the C3 inactivator, C3bINA (later named Factor I) (Lambris et al., 1980). The release of C3bINA cleaved C3b into C3bi, the inactivated form of C3b, which was able to bind to complement receptor 2 (CR2) on the B cell membrane. These early studies demonstrated that complement components could bind to the membranes of lymphocytes indicating complement could mediate lymphocyte function, including other cell types, but further research was warranted

Since this time, complement components have been found to mediate T cell immunity. T cells have the ability produce complement components including C3 and C5 (Strainic et al., 2008), contain complement regulators CD46 (MCP), CD55 (DAF), and CD59 (Heeger et al., 2005; Christmas et al., 2006; Pavlov et al., 2008), and have complement receptors including complement receptor 1 (CR1) (Torok et al., 2015), C3a receptor (C3aR) and C5a receptor (C5aR).

The ability of T cells to produce C3 and express C3aR intracellularly is vital for human T cell homeostatic survival (C5 and C5aR to a lesser extent). Resting human $CD4^+$ T cells contain stores of C3 and cathepsin L (CTSL), a C3 cleaving protein, and C3 is cleaved continuously in these resting cells (Liszewski et al., 2013). Similar to the C3 convertase, CTSL cleaves C3 into C3a and C3b. Once cleaved, C3a can bind to its receptor, C3aR, expressed on lysosomes, to sustain mTOR activation for cell survival (Liszewski et al., 2013). If this process of intracellular C3 production is inhibited in naïve T cells, the T cell activation intermediate that inhibits apoptosis, phosphorylated AKT, is reduced and the viability of these cells is significantly reduced (Strainic et al., 2008).

Not only is T cell survival dependent on complement, but the effectiveness of a T cell response is dependent on complement. C3 deficient mice are highly susceptible to a variety of infections, including influenza (Kopf et al., 2002). This observation in the knockout mouse is consistent with the first known naturally occurring C3 deficient animal, the C3-deficient line of Brittanys (canine). These animals were highly susceptible to a variety of infections, both bacterial and viral in origin (Blum et al., 1985). C3-deficient mice exposed to influenza virus had reduced virus-specific $CD4^+$ and $CD8^+$ T cell recruitment to the lung and T-helper cell-dependent antibody response was also reduced in these animals, demonstrating that C3 is critical for regulating T cell responses (Kopf et al., 2002).

The interaction of T cells with antigen presenting cells (APCs) also induces complement production by both cell types and can alter T cell effector function. Upon the interaction of T cells and APCs, C3a and C5a secretion is induced from both cell types (Heeger et al., 2005 and Strainic et al., 2008). Additionally, C3aR and C5aR on the surface of both T cells and APCs are increased upon this interaction (Strainic et al., 2008). When the locally produced C3a and C5a binds to their respective receptors on the T cell it induces T cell proliferation and differentiation (Montz et al., 1990; Strainic et al., 2008; Kwan et al., 2013). If the interaction between C3a and C5a and their respective receptors is absent or inhibited, MHC class II and co-stimulatory abundance is reduced on APCs (Strainic et al., 2008) and Treg cell development is induced (Kwan et al., 2013). Consistent with this finding, C3 deficient APCs also have decreased MHC class II abundance (Peng et al., 2006). Costimulation during the interaction between T cells and APCs has been shown to transiently decrease the abundance of DAF (CD55), one of the complement inhibitors (Heeger et al., 2005). Additionally, in mice that lack DAF expression on both APCs and T cells there is increased T cell proliferation upon interaction, suggesting the downregulation of DAF might be important for proper T cell immunity (Heeger et al., 2005).

The differentiation of T cells also appears to be linked to complement signaling. Without signaling through the C3aR or C5aR upon T cell activation, $CD4^+$ cells are induced to become Treg cells (Strainic et al., 2013; Kwan et al., 2013). The absence of C3aR or C5aR signaling in mice deficient in both C3aR and C5aR or wild-type mice treated with pharmaceutical antagonists of these receptors induced a greater percentage of $Foxp3^+$ regulatory T cells after T cells were activated with CD3 and CD28 antibodies and IL2 compared to their respective controls (Strainic et al., 2013). Treg development ($CD4^+CD25^+FOXP3^+$) can also be induced in anti-CD3-activated $CD4^+$ T cells in the presence of IL2 if both CD46 (MCP) and the complement receptor 1 (CR1/CD35) are ligated (Kemper et al., 2003; Torok et al., 2015). These anti-CD3 activated $CD4^+$ T cells with CD46 ligation are able to produce IL10 and TGFB1 (Kemper et al., 2003). The

ability of Tregs to function is also dependent upon complement signaling. Natural (thymus derived) $CD4^+$ $FOXP3^+$ T cells express C3aR and C5aR. If signaling via C3a and C5a occurs through their respective receptors these cells lose their immunosuppressive ability through the downregulation of Foxp3.. However, if signaling through C3aR and C5aR is inhibited there is enhanced suppressive capacity of the natural Treg cells both *in vitro* and *in vivo* (Kwan et al., 2013). Further supporting this claim, induced regulatory T cells (iTreg) are also developed when C3aR and C5aR signaling is inhibited in $CD4^+$ due to autoinductive signaling from transforming growth factor-β1 (TGFB1) that prevents C3a and C5a production from these cells which results in increased Foxp3 expression and production of IL10 (Strainic et al., 2013). Overall, the ability of C3a and C5a to signal through their respective receptors appears to be one pathway that regulates the number of Treg cells both in the thymus and in the periphery.

Role of complement in reproductive tissues

While the exact role of the complement system in reproductive tissues remains a rather understudied area of complement biology, early research suggests complement is not only present in reproductive tissues, but also might play an important role for successful reproduction. There is evidence that both male and female reproductive tracts, both fluids and tissues, contain complement components, some of which appear to be regulated hormonally. However, relevant to this current body of research, the abundance and function of complement components in the CL remains unknown.

Both sperm and seminal plasma in the male reproductive tract contain components of the complement system. In seminal plasma, C3, C4, factor I, factor H, and C4BP protein have been found either through immunoblotting or ELISA (Vanderpuye et al., 1992). However, the abundance of these proteins is much lower than that found in plasma. Spermatozoa express complement regulators including CD46, CD55, and CD59 to some capacity depending upon the stage of development and exposure to the female reproductive tract (Valentovicova et al., 2005).

For example, CD46 (in humans) is present in spermatozoa that have undergone the acrosome reaction, but not in spermatozoa with intact acrosomes (Anderson et al., 1989). CD55 (in guinea pigs) and CD59 (in humans) are highly expressed both in the acrosome and over the entire surface of the cell in epididymal spermatozoa (He et al., 2000; Rooney et al., 2009). The presence of these complement regulators is thought to protect the spermatozoa from antisperm antibodies and complement mediated death in the female reproductive tract. In fact, if CD59 is blocked using an anti-CD59 antibody, sperm cells were more susceptible to complement-mediated death in the female reproductive tract (Rooney et al., 2009).

In the female reproductive tract, complement components are present in both in the ovary and the uterus, including the trophoblast of a developing embryo. In the human uterus, both C3 and C4 have been found in endometrial fluid (Vanderpuye et al., 1992). C3 protein is hormonally regulated in the uterus and is upregulated in the rat uterus in response to estradiol and is produced by uterine epithelial cells (Sundstrom et al., 1989). If progesterone is given simultaneously, it suppresses the stimulatory effects of estradiol and C3 mRNA abundance is not upregulated. However, the inhibitory effects of progesterone on C3 protein and mRNA are reversed if RU-486, a progesterone receptor antagonist, is used (Hasty et al., 1992). These data suggests progesterone might mediate the rate of C3 mRNA turnover in the rat uterus.

During follicular development, complement components are present in both follicular fluid and on the oocyte itself. The follicular fluid from both humans and cattle contain active complement components (Fahmi et al., 1985; Perricone et al., 1990). The amount of active complement (measured via sheep red blood cell (RBC) lysis assay) in follicular fluid from cattle was found to be the greatest during estrus and lysed RBCs at a rate 20-fold that of complement from serum (Fahmi et al., 1985). Human follicular fluid also contains a significantly higher concentration of complement anaphylatoxins (activated complement; C3a, C4a, C5a) compared to human plasma, as well as most of the complement proteins of the classical and alternative

pathway (Perricone et al., 1990; Yoo et al., 2013). The source of complement proteins in the follicular fluid is granulosal cells (Yoo et al., 2013). Gene chip analysis of mRNA from granulosal cells from human patients undergoing IVF treatments were compared to proteomic analysis via liquid-chromatography/mass spectrometry (LC/MS) and western blots of the follicular fluid from the same follicle. The mRNA present in the granulosal cells were the same proteins found in the follicular fluid, suggesting granulosal cells might be the source of follicular complement proteins (Yoo et al., 2013). Within the follicle, the maturing oocyte does express CD55 on its plasma membrane (He et al., 2000). Microarray analysis of primordial follicles transitioning to primary follicles in the rat indicated mRNA associated with the complement cascade to be upregulated, suggesting complement may play a role in follicular development, but further research is warranted (Nilsoon et al., 2010). Further supporting the claim that complement may mediate follicular development, is the altered abundance of complement components in the follicles of women with cystic ovaries (Perricone et al., 2000; Ambekar et al., 2015). Women with hereditary angioedema (HAE), a disorder with recurrent swelling in the extremities and face, while not infertile, often have cystic ovaries similar to women with polycystic ovarian syndrome (PCOS). Both women with HAE and PCOS have reduced abundance of complement components of the classical cascade including C1 and the C1 inhibitor in the follicular fluid (Perricone et al., 2000; Ambekar et al., 2015). Women being treated for HAE with the drug Danzol showed a marked increase in C1 inhibitor and improvement in clinical symptoms, suggesting a possible correlation between complement and follicular development.

While complement components are found in the ovary, the abundance of complement components in the CL is not known. Several transcriptomic and proteomic profiling studies have found complement components to be present, but the abundance and function of these proteins have not been classified (Kfir et al., 2018; Hughes et al., 2019). Early complement research investigating complement regulators in the human ovary, found, through in-situ hybridization and

immunostaining, that human C4b-binidng protein (C4BP) was present in the regressing CL and corpus albicans, but not the follicle or functional CL (Criado-Garcia et al., 1999). Pathway analysis from a genomic profiling study comparing the developing (day 4) and the functional CL (day 11) found, of the 318 genes differentially expressed in the CL on day 11, there was higher mRNA abundance of genes related to complement activation (Kfir et al., 2018). Proteomic profiling of the CL on day 17 of pregnancy compared to day 17 of the cycle found C3 to be less abundant in the CL of pregnancy compared to the CL of the cycle (Hughes et al., 2019). Transcriptomic profiling of the same tissues revealed CD55 to be in greater abundance in the CL of pregnancy compared to the cycle (Hughes et al., 2019). Interestingly, C4 was present in the media from cocultures of midcycle luteal steroidogenic cells with autologous T cells (Brzezicka et al., unpublished). The limited studies that have identified complement components in the CL suggests complement might be important for ensuring the optimal function of the CL.

The role of miRNA in the corpus luteum

miRNA and their function

miRNA were first discovered in 1993 in *C. elegans* when two small transcripts were found to bind to the 3' UTR (untranslated region) on lin-14 mRNA and downregulated LIN14 protein abundance. These transcripts were also found to be essential for larval development. Eventually, these small transcripts were found to be lin-4, the first miRNA (Lee et al., 1993). The other notable "first" miRNA discovered in *C. elegans* was Let-7 (Reinhart et al., 2000). Let-7 was found to be a 21-nucleotide transcript that regulated several critical genes for the larval development of *C. elegans*. Since this discovery there is a greater understanding of how miRNA arise and their regulation of mRNA.

miRNA are transcribed from the noncoding regions of DNA by RNA polymerase II (in the nucleus), giving rise to a pri-miRNA. The pri-miRNA is unstable, and undergoes cleavage by Drosha or DiGeorge syndrome chromosomal 8 region (DGCR8), resulting in pre-miRNA (Han et

al., 2006). The pre-miRNA is more stable because it has a hairpin loop structure. This hairpin loop is important because the 2 nucleotide overhang is recognized by exportin 5, a protein in the membrane of the nucleus that transports the pre-miRNA from the nucleus into the cytosol upon GTP hydrolysis (Han et al., 2006). Once in the cytosol, the pre-miRNA undergoes splicing by Dicer. Dicer produces a double stranded mature miRNA. The thermodynamically stable strand remains and the other strand degrades, resulting in a single stranded, mature miRNA (Macgregor-Das and Das, 2018). The mature miRNA then associates with argonaute-2 (AGO2) and the RNA-induced silencing complex (RISC) is formed, capped by the protein GW-182. The mature miRNA is then able to target a specific mRNA based upon its sequence. Canonically, miRNA bind to the 3'UTR on a target mRNA and the RISC either prevents translation or degrades the mRNA, preventing protein production.

One of the first studies of miRNA in the corpus luteum found that Dicer1 deficient mice were infertile because of luteal insufficiency due to poor vascular development (Otsuka et al., 2008). The poor vascular development was found to be because of a lack of miR-17-5p and Let-7b. When these miRNA were injected into the ovary, luteal angiogenesis was restored (Otsuka et al., 2008). miR-34a has been shown to support luteal formation and function by increasing progesterone concentrations and inhibiting luteal cell proliferation (Maalouf et al., 2016). Inhibition of miR-96 in human luteinized granulosa cells was found to increase apoptosis and decrease progesterone production suggesting this miRNA might also regulate luteal function (Mohammed et al., 2017). These studies suggest miRNA could be critical for luteal function. Several miRNA profiling studies have supported this claim. Maalouf et al. (2016) evaluated the importance of miRNA during luteal development by comparing the miRNA profile in CL on day 4 and day 11 of the estrous cycle. Interestingly, there was a greater number of miRNA expressed on day 11 compared to day 4. The pathway analysis of miRNA from the midcycle CL suggested these miRNA regulate cell cycle and apoptosis, while the day 4 miRNA regulated carbohydrate

metabolism, both of which are consistent with these stages of development (Maalouf et al., 2016). Maalouf et al. (2014) found 15 differentially abundant miRNA between the CL of pregnancy and the cycle on day 17. These miRNA were predicted to regulate genes in pathways such as immune function and apoptosis. miRNA profiling across early pregnancy (days 14, 17, 20, 23) in the cow found the 27 miRNA that changed were associated with fatty acid degradation and steroidogenesis (Hughes et al., 2020). A recent study analyzing miRNA clusters in the porcine CL of pregnancy found the miR-99b cluster (miR-99b, miR-125a, miR-Let7e) can sustain progesterone production and can be regulated by PGE2 (Przygrodzka et al., 2020).

The most abundant miRNA in the CL is let-7a-5p (Pate and Hughes, 2018). In fact, the Let-7 family comprises about 87% of the miRNA in the CL, however their role in the CL remains unknown (Maalouf et al., 2014). Current work in our laboratory found through a pulldown assay that both Let-7a-5p and Let-7b target genes from the mitochondrial genome (Baddela et al., unpublished). Targeting of mitochondrial genes by miRNA is a new mechanism that has been explored fairly recently in cardiovascular physiology. miRNA have been found in the mitochondria in a few species including the mouse (Bian et al., 2010), rat (Kren et al., 2009; Barrey et al., 2011), and the human (Mercer et al., 2011; Sripada et al., 2012a). The miRNA in the mitochondria are often referred to colloquially as “mitomiRs.” Among the different species in which miRNA were identified in the mitochondria, several Let7 family members are common (Sripada et al., 2012b). The major question is how miRNA are transported into the mitochondria and this answer remains unknown. Initially it was thought miRNA contained a seed sequence to direct miRNA to the mitochondria because miR-29b has a 3’ terminal motif and is translocated to the nucleus (Hwang et al., 2007). However, this theory was refuted by Das et al. (2017) when they found miR-181c, which is known to localize to the mitochondria in mouse cardiomyocytes, had the same 3’ terminal motif as miR-181a which localized to the nucleus. Several other theories have been hypothesized including translocation of Ago2 and the miRNA together, translocation

of Ago2 and miRNA separately after the removal of GW182 (the cap on RISC), phosphorylation of Ago2 to facilitate translocation, or the translocation of Ago2 with miRNA facilitated by PNPase (Macgregor-Das and Das, 2018).

There is also continuing debate about whether the mitochondria can generate its own miRNA. The mitochondrial genome is a double stranded circular molecule that encodes for only 13 proteins for oxidative phosphorylation, 22 tRNAs, and 2 rRNAs (Smits et al., 2010). Several papers have suggested the miRNA present in the mitochondria may align to the mitochondrial genome at the tRNA loci (Barrey et al., 2010; Sripada et al., 2012). Several human miRNA (miR-1974, miR-1977, and miR-1978) all mapped to the mitochondrial genome at tRNA and rRNA genes (Bandiera et al., 2011). Additionally, two mitochondrial tRNA (tRNALys and tRNAMet) were found to immunoprecipitate with Ago2 (Manataki and Mourelatos, 2005; Beitzinger et al., 2007). However, one component that has yet to be found within the mitochondria is miRNA processing machinery.

The role of mitomiRs is still unknown. Currently, only a handful of studies have demonstrated that mitomiRs target mitochondrial genes that led to a change in mitochondrial function. The best example of this is the study by Das et al. (2012b) where they demonstrated miR-181c targets mt-CO1 (a mitochondrial gene, cytochrome c oxidase I) in rat cardiomyocytes and increases both mitochondrial respiration and reactive oxygen species (ROS) production. Several other miRNA have been implicated in mediating ROS production. In the Bandiera et al. (2013) review, they summarize that miR-145, miR-23b, and miR-210 can have an antioxidant effect on the mitochondria while miR-335, miR-34a, miR-338, miR-210, and miR-181c all have a prooxidant effect. In regard to luteal function, further work will need to be done to determine if miRNA, particularly members of the Let-7 family can target mitochondrial genes and impact mitochondrial function. If this is true, this could have interesting implications on luteal function.

Overall, the CL is an important endocrine tissue that is vital for the maintenance of pregnancy in mammals. While much is known about the role of resident immune cells in the functional and regressing CL, little is known about the phenotype of immune cells in the CL of pregnancy, and if other immune components such as complement proteins might facilitate the function of immune cells in the CL. Understanding how the immune system within the CL facilitates luteal rescue and maintenance might offer future solutions for improving fertility in dairy cows. Additionally, the area of miRNA research is a relatively new area of luteal biology research. There are no studies to date that have examined the role of miRNA in mediating mitochondrial function in the bovine CL. The possibility of miRNA regulating mitochondrial function in the CL is an exciting and novel area of research that could have great impacts on improving luteal function in dairy cows.

Overall hypotheses

1. Let-7, a miRNA, is able to alter mitochondrial function in bovine luteal cells.
2. Pregnancy induces changes in resident immune cells and complement components that support the survival of the CL.

Objectives

1. Determine if let-7, a miRNA, is present in luteal cell mitochondria can modulate mitochondrial respiration and progesterone production in the functional CL.
2. To identify a phenotype and functions of resident immune cells in the CL exposed to uterine infused interferon-tau (IFNT) and/or pregnancy specific protein B (PSPB) using a transcriptomic profiling approach.

3. To characterize the expression and the function of complement components in the CL.

Chapter 2

Let-7 modulates mitochondrial function and progesterone production in bovine luteal cells

Previous work in our laboratory demonstrated that the let-7 miRNA family comprised nearly 87% of all the miRNA in the CL (Maalouf et al. 2014). Within this family of miRNA, let-7a and let-7b were selected for a biotin-based pulldown assay to determine mRNA targets of these miRNA (unpublished work). Interestingly, not only did let-7a and let-7b pulldown similar nuclear gene targets, but also mitochondrial gene targets (unpublished work). In fact, both let-7a and let-7b pulled down all of the genes that encode for electron transport chain (ETC) proteins on the heavy (H) strand of the mitochondrial DNA.

The possibility of nuclear genome-derived miRNA not only entering the mitochondria, but having some functional effect was debated for some time. However, there is evidence that this phenomenon is in fact true, and nuclear genome-derived miRNA can be translocated into the mitochondria and have a functional effect (Bian et al., 2010; Das et al., 2012; Sripada et al., 2012a; Zhang et al., 2014; Li et al., 2016; Li et al., 2019). Sripada et al. (2012a) purified mitochondria from both HeLa and HEK cells and demonstrated that 1539 known miRNA were associated with mitochondrial fractions (Sripada et al., 2012a). Alignment of these sRNA to the human mitochondrial genome found that 4 miRNAs, hsa-miR-4461, hsa-miR-4463, hsa-miR-4484, and hsa-miR-4485 mapped to positions on the mitochondrial genome associated with two ETC protein subunits, ND4L and ND5, as well as 16 ribosomal RNA genes, suggesting miRNA can target the mitochondrial genome (Sripada et al., 2012a). Bian et al. (2010) demonstrated using a miRNA microarray assay that purified mitochondria from mouse liver contained a unique population of highly expressed miRNA that was different compared to whole mouse liver tissue. They also confirmed the microarray results using quantitative real-time polymerase chain reaction (qRT-PCR) and several miRNA, including miR-705, miR-202-5p, and miR-134 were greater in

the mitochondrial fraction compared to whole tissue (Bian et al., 2010). In a functional study, Das et al. (2012) detected miR-181c in the mitochondria isolated from rat cardiomyocytes using qRT-PCR. When miR-181c was overexpressed in these cells it downregulated cytochrome c oxidase subunit 1 (mt-CO1) protein and increased oxygen consumption and the production of reactive oxygen species from the mitochondria (Das et al., 2012; Das et al., 2014). Another miRNA, miR-1, also decreased the expression of mt-CO1 protein as well as ND1 in C2C12 mouse myoblast cells (Zhang et al., 2014), and three other miRNA, miR-21, let-7b-5p, and miR-92a-2-5p targeted mitochondrial gene cytochrome b (mt-Cytb) and upregulated its expression (Li et al., 2016; Li et al., 2019).

Despite the evidence that miRNA are found in the mitochondria and can exert a functional effect, the mechanism by which miRNA are directed to and translocated into the mitochondria is still not well understood. Macgregor-Das and Das (2018) summarize four proposed mechanisms by which miRNA might be translocated into the mitochondria: 1) Argonaute 2 (Ago2), an important protein in the RNA-induced silencing complex (RISC), translocates with associated miRNA through translocases in the outer mitochondrial membrane (TOM) into the mitochondria, 2) Ago2 and the miRNA enter the mitochondria separately, 3) Ago2 undergoes posttranslational modification (i.e. phosphorylation) which might help direct the Ago2-miRNA complex into the mitochondria, and 4) the Ago2-miRNA complex translocates through outer mitochondrial pores with the help of polynucleotide phosphorylase (PNPASE). Initially, it was thought a miRNA-seed sequence might direct the subcellular localization of miRNA into the mitochondria. Hwang et al. (2007) found that human miR-29b localizes to the nucleus and it has a unique 3’-terminal motif (AGUGUU) compared to miR-29a and miR-29c. miR-29a and miR-29c did not localize to the nucleus, so it was thought this motif might direct miR-29b to the mitochondria (Hwang et al., 2007). However, miR-181c and miR-181a share the same 3’-terminal motif, but only miR-181c localizes to the mitochondria, suggesting this might

not be the mechanism by which all miRNA are directed to the mitochondria (Das et al., 2017). It is well understood that Ago2 can be found in the mitochondria (Kren et al., 2009; Bandiera et al., 2011; Barrey et al. 2011; Bian et al., 2010; Das et al. 2012; Zhang et al. 2014), however as the proposed mechanisms above suggest, it seems as if Ago2 might play a role in directing miRNA to the mitochondria, but it is unclear if Ago2 enters the mitochondria associated with a miRNA, or if they enter the mitochondria separately. Bandiera et al. (2011) predicted a mitochondrial localization sequence in the N-terminus of Ago2, however, when this sequence was tagged with GFP it was not detected in the mitochondria (Zhang et al. 2014). Yet, Ago2 does bind to specific transcripts from the mitochondrial genome, however, the cap that holds the RISC together, GW182, is not found in the mitochondria (Zhang et al. 2014). Thus without the traditional RISC machinery, the mechanism by which the Ago2:miRNA complex acts to modulate transcripts in the mitochondria might different from than in the cytosol. Posttranslational modifications might help facilitate Ago2 subcellular localization because phosphorylation of Ago2 at serine-387 promotes localization to processing bodies (P bodies), which could be one mechanism by which the Ago2:miRNA complex is delivered to the mitochondria (Zeng et al. 2008). If miRNA enter the mitochondria alone, without association with Ago2, PNPASE might facilitate this transport because PNPASE plays an important role in shuttling nuclear-derived mRNAs into the mitochondria (Wang et al., 2010). Without PNPASE, mitochondrial RNA processing is disrupted and ATP production is decreased along with impaired mitochondrial morphology (Wang et al., 2010).

There is limited evidence that the mitochondria could make their own miRNA. The only evidence of mitochondria making their own miRNA was a genomics study that mapped three human miRNA with a perfect match to the mitochondrial genome on two mitochondrial transfer RNA genes (Bandiera et al., 2011). However, the proteins that are necessary for miRNA processing, such as Dicer, have not been found in isolated mitochondrial fractions (Das et al.,

2012). Thus, more work needs to be completed to elucidate if miRNA can be derived from the mitochondrial genome.

The evidence that miRNA can translocate into the mitochondria and exert functional effects led to the hypothesis that let-7 targets mitochondrial genes and can modulate mitochondrial function in the CL. The objectives of this study were to identify if let-7 is found in the mitochondria isolated from luteal steroidogenic cells, and to determine if let-7 can modulate mitochondrial respiration and progesterone production.

Materials and Methods

Tissue Collection

The procedures performed for tissue collection were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Cyclic Holstein dairy cows were observed for signs of estrus. The day of standing estrus was considered day 0 of the cycle. Corpora lutea were collected via colpotomy between days 10 and 12 of the estrous cycle. The tissue was then dissociated and cultured as previously described (Pate, 1993).

Mitochondrial Isolation and Mitochondrial Purity

Immediately following the dissociation of the CL, mitochondria were isolated from the luteal steroidogenic cells using the Mitochondria Isolation Kit (Thermo). Following the instructions of the kit, 20 x 10^7 luteal cells were used per isolation. Mitochondria isolated for downstream protein analysis were resuspended in 2% CHAPS Tris Base lysis buffer (2g of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.303g of Tris Base, 0.88g of NaCl into 100 mL, pH = 7.2), while mitochondria isolated for downstream RNA and/or miRNA analysis were resuspended in the Exiqon cell lysis buffer (miRCURY, Exiqon). The cytosolic

fraction and remaining cells were also kept for downstream applications. Mitochondrial purity was assessed via western blot and quantitative polymerase chain reaction (qPCR).

Protein concentrations from the mitochondrial fraction, cytosolic fraction, and whole cell lysates were assessed using a Bradford assay (BioRad). PROTEAN TGX Precast Gels (BioRad) were used for protein separation, with 30 μg of protein from each fraction loaded per lane. The proteins were then transferred using an iBlot dry blotting system to a polyvinylidene fluoride (PVDF) membrane (Invitrogen). After transfer, the gel was stained with Coomassie Blue Stain to ensure proper protein transfer. The membrane was blocked using 5% nonfat dry milk in TBST. The purity of the mitochondrial isolation was assessed using a previously validated rabbit anti-cytochrome C primary antibody (AbCam) at a 1:1000 dilution as a mitochondrial marker. Cytosolic (endoplasmic reticulum) contamination was assessed using a previously validated mouse anti-calnexin primary antibody (AbCam) at a 1:2000 dilution. The blot was incubated overnight at 4°C. The following day, after washing the blot with TBST, the blot was incubated with a donkey anti-rabbit IgG horseradish peroxidase secondary antibody (GE Healthcare) and a sheep anti-mouse IgG horseradish peroxidase secondary antibody (GE Healthcare) both at a 1:10,000 dilution for two hours at room temperature. The blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (BioRad) and were imaged using a ChemiDoc XRS System (BioRad).

The purity of the mitochondrial isolation was also assessed using quantitative PCR. *RPL19* and *ACTB* were used as cytosolic gene markers, and these primers had been previously validated in the lab. The mitochondrial gene marker used was mt-COI and the primers were validated prior to this experiment. RNA was isolated using the miRCURY kit (Exiqon) and was treated with DNase (Promega) to eliminate any gDNA contamination. cDNA was synthesized using the AzuraQuant kit (Azura Genomics), and qPCR was performed using AzuraQuant green fast qPCR mix LoRox (Azura Genomics).

miRNA quantitative PCR in isolated mitochondria and cultured luteal cells

Total RNA (including miRNA) from the mitochondria was isolated using the miRCURY RNA isolation kit (Exiqon). Total RNA from cultured luteal cells was isolated using the Qiagen's RNeasy RNA isolation kit using supplementary protocol #1 for purification of miRNA. cDNA was synthesized using the miScript II RT Kit (Qiagen). The HiSpec Buffer was used for cDNA synthesis for quantifying mature miRNA. qPCR for let-7a and let-7b were performed using the miScript PCR Sybr Green Kit (Qiagen). The forward primer for let-7a and let-7b were previously developed and validated in this lab (unpublished). U6 was used as a reference gene.

Let-7 transfection and Seahorse assay

Freshly isolated luteal cells were cultured in a Seahorse XF 24-well plate (Agilent) with 80,000 cells per well with Ham's F12 medium supplemented with insulin-transferrin-selenium (ITS; 5 μg/mL each of insulin and transferrin, and selenium 5 ng/mL) and 10% newborn calf serum. This cell number was validated as the optimal cell number prior to the start of this experiment. On day 1 of culture, the cells were washed and fresh medium containing ITS and 10% serum (no gentamicin added) was added to the cells. The miRNA mimic and inhibitor (Ambion) were mixed with TRANS-IT TKO transfection reagent (Mirus Bio) and added at a final concentration of 500 nM per well. After 18 hours (day 2 of culture), the transfection reagent was removed and fresh medium containing ITS and gentamicin was added to the cells. The transfection efficiency was assessed using Siglo Green (Horizon) and the transfection efficiency was 85.9%. The Seahorse XFe24 sensor cartridge was incubated with 1 mL per well of Seahorse XF calibrant overnight in a non-CO_2 incubator. On day 3 of culture, 500 μl of filtered Seahorse Base Medium (Agilent; pH 7.4, supplemented with 10 mM glucose, 1 mM pyruvate, 2 mM L-glutamine) was added to each well. The Mitochondrial Stress Test Kit (Agilent) was the assay performed using the Seahorse XFe24 analyzer (Agilent). The Mitochondrial Stress Test

drugs were prepped and added at the following concentrations to each port on the Seahorse sensor cartridge:

- Port A: Final well concentration equaled 1.12 μM of oligomycin.
- Port B: Final well concentration equaled 2.2 μM of trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP).
- Port C: Final well concentration equaled 1.12 μM of Rotenone/Antimycin A (R/AA).
- Port D: 75 μl of Seahorse Base Medium

Prior to the start of the assay, the cells were incubated in a non-CO_2 incubator for at least 1 hour. After the Mitochondrial Stress Test was run, cells were counted in three different areas per well on the 20X objective for an estimate of cell number.

Let-7 transfection and measuring progesterone concentrations

Luteal cells were transfected with let-7 mimics and inhibitors as described above. Media was collected from the transfected luteal cells on day 3, 5, and 7 of culture. Progesterone secretion in response to let-7 transfection was measured via an ELISA as previously described (Petroff et al. 1997 with modifications described in Hughes et al. 2020).

Let-7 transfection and abundance of EGR1 and PGRMC1

Luteal cells were cultured and transfected with let-7 mimics and inhibitors as described above. Transfection efficiency was assessed and observed to be 83%. The luteal cells were collected on day 7 of culture using trypsin. The cells were fixed using 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. The cells were stained using 3.75 μg/mL of either mouse anti-EGR1 primary antibody (AbCam, ab55160) or rabbit anti-PGRMC1 (AbCam, ab224054).

Isotype controls for EGR1 and PGRMC1, mouse IgG isotype control (AbCam) and rabbit IgG isotype control (AbCam, ab172730), were used respectively. The primary antibody and isotype control staining was incubated in 20% goat serum with PBS + EDTA for 30 minutes on ice. The secondary antibodies used were goat anti-mouse IgG: AlexaFluor 488 (AbCam) and goat anti-rabbit IgG: AlexaFluor 488 (AbCam, ab150077) for EGR1 and PGRMC1, respectively. The fluorescence was read using the Guava EasyCyte flow cytometer and counting 30,000 events. FlowJo (version 10.6.2) was used to gate the luteal cell size (~7 μm or greater) using size reference beads (ThermoFisher) and to calculate the abundance of EGR1 and PGRMC1 positive cells as well as mean fluorescence intensity of these proteins in this cell population.

Statistical Analysis

The statistical analyses were performed using either the mixed model of SAS 9.4 (Statistical Analysis System Institute) or Minitab 19 (Minitab, Inc). For let-7 abundance in cultured luteal cells, the model fitted the effects of day of culture, treatment, and day of culture*treatment interaction. U6, the reference gene, was used as a covariate in the model. An ANOVA was used to determine treatment differences in mitochondrial respiration, progesterone production, and EGR1 and PGRMC1 abundance. Animal was included as an effect in the model and a Tukey post-hoc test was used to determine the difference between the scrambled control and mimic/inhibitor. The data are represented as least squares means ± pooled SEM. Differences were considered significant when $p < 0.05$, and a tendency when $0.05 < p < 0.1$.

Results

The aim of this experiment was to determine if let-7 miRNA are present in isolated mitochondria of luteal cells, and if let-7 modulates mitochondrial function by measuring mitochondrial respiration and progesterone production in let-7-transfected luteal cells.

The mature sequences of let-7a and let-7b and the two-nucleotide difference between the two sequences are highlighted in Figure 1. The isolation and assessment of

mitochondrial purity was determined via western blot and qPCR (Figure 2). The mitochondrial marker, cytochrome c, was enriched in the mitochondrial fraction compared to the cytosolic fraction (Figure 2a). Calnexin, an endoplasmic reticulum (ER) protein, was also detected in the mitochondrial fraction (Figure 2a). The cytosolic gene markers RPL19 and ACTB were not detected in either the cytosolic fraction or the mitochondrial fraction (Figure 2b). The mitochondrial gene marker, mt-CO1, was detected in the mitochondrial fraction at a low abundance and was detected in the cell fraction at a very high abundance (Figure 2c). mt-CO1 was not detected in the cytosolic fraction (Figure 2c).

Neither let-7a nor let-7b were detected in the isolated mitochondrial fraction via qPCR, however both miRNA were detected in luteal cells (Figure 3a). Both let-7a and let-7b were detected at low abundance in the mitochondrial fraction of one animal (Figure 3b). In cultured luteal cells, both let-7a and let-7b were detected, but did not change over the course of five days in culture (Figure 4). Let-7a and let-7b abundance in luteal cells also did not change in response to treatment with LH or PGF2A (Figure 4).

The mitochondrial stress test demonstrated that the let-7b mimic decreased several parameters of mitochondrial function, while both the let-7a mimic and inhibitor had no effect on mitochondrial function. Basal respiration, proton leak, ATP production, and spare capacity all decreased in response to the let-7b mimic compared to control (Figure 5), while nonmitochondrial oxygen consumption and maximal respiration did not change (data not shown). There were no changes in these measurements in cells treated with the let-7b inhibitor (data not shown).

Progesterone production by luteal cells on D3 of culture was greater in cells transfected with the let-7a mimic compared to control (Figure 6a), but there was no change on day 5 or 7 of culture (data not shown). There was no effect of the let-7a inhibitor on progesterone production (Figure 6b). The let-7b mimic decreased progesterone production on both day 5 and day 7 of culture (Figure 7), with no change in progesterone concentration on day 3 of culture

(data not shown). There was no effect of the let-7b inhibitor on progesterone production (Figure 7a and 7b).

The abundance of EGR1 and PGRMC1, targets of both let-7a and let-7b, were assessed via flow cytometry. Neither the percentage of EGR1 positive cells nor the mean fluorescence intensity (MFI) changed after transfection with the let-7a mimic (data not shown), however, there was an increase in the percentage of EGR1 positive cells and MFI in response to the let-7a inhibitor (Figure 8). There were no changes in the percentage of EGR1 positive cells or MFI of cells transfected with the let-7b mimic or inhibitor (data not shown). Both the let-7a mimic and let-7a inhibitor increased MFI of cells expressing PGRMC1 (Figure 9a), but did not affect the percentage of PGRMC1 positive cells (Figure 9b). The let-7b mimic increased MFI of PGRMC1 positive cells, whereas the let-7b inhibitor increased both the percentage and MFI of cells expressing PGRMC1 (Figure 10)

Discussion

In the first study to demonstrate that miRNAs could be detected in the mitochondria, 15 nuclear-encoded miRNAs were identified in isolated mitochondria from the liver of adult rats with a miRNA microarray (Kren et al. 2009). Kren et al. (2009) confirmed the presence of mature miRNA in the mitochondria via qPCR, but only in relatively low abundance. A predicted target of miR-130a, a miRNA found in the mitochondria, included mitochondrial encoded cytochrome c oxidase III (mt-CO3) (Kren et al. 2009), suggesting a possible role of miRNA regulating mitochondrial genome derived transcripts. Subsequently, 169 potential targets of miRNA in the mitochondrial genome with 80 significant potential targets were found in an *in silico* analysis (Barrey et al., 2011). Similarly, four miRNA identified from isolated sRNAs from human mitochondria, aligned to four mitochondrial genes (Sripada et al., 2012a). Interestingly, let-7b had several potential mitochondrial gene targets including ATP6, ATP8, COX2, and ND5,

all encoding subunits of proteins involved with the electron transport chain. Let-7b was confirmed to be localized in the mitochondria isolated from human myotubes via *in situ* hybridization (Barrey et al., 2011). The alignment of the let-7b sequence and localization to the mitochondria also suggested that miRNA could regulate mitochondrial mRNA (Barrey et al., 2011).

Since these early studies, there is now evidence that miRNA can regulate mitochondrial mRNA, and thus mitochondrial function. Overexpression of miR-181c in rat cardiomyocytes can localize to the mitochondria and decrease cytochrome c oxidase (mt-CO1) protein abundance, which results in the increased expression of cytochrome c oxidase subunit 2 (mt-CO2) protein expression and remodeling of cytochrome c oxidase (complex IV; Das et al., 2012). The remodeling of the electron transport chain protein complex IV as a result of miR-181c overexpression, leads to altered mitochondrial function including an increased oxygen consumption rate and increased reactive oxygen species generation (Das et al., 2014). In addition to miRNA decreasing protein expression in the mitochondria, miR-1 has been found to decrease the expression of ND1 and mt-CO1 proteins in C2C12 mouse myoblast cells (Zhang et al. 2014). Three other miRNA, miR-21, let-7b-5p, and miR-92a-2-5p, can also increase translation of one mitochondrial gene target, cytochrome b in both rat (miR-21) and mouse (let-7b-5p and miR-92a-2-5p) cardiomyocytes (Li et al., 2016; Li et al., 2019).

The present study demonstrated for the first time that two members of the let-7 miRNA family, let-7a and let-7b, do have a functional role in downregulating mitochondrial functional in bovine luteal cells. The first objective was to determine if let-7 miRNA could be detected in isolated mitochondria from bovine luteal steroidogenic cells and assess the purity of this fraction using western blot and qPCR. Both of these methods are standard practice in assessing mitochondrial purity (Bian et al. 2010, Das et al. 2012, Das et al. 2014, Wang et al. 2015, Li et al. 2019). The initial attempts at isolating mitochondria from luteal steroidogenic cells demonstrated

mitochondria were present in the mitochondrial fraction along with endoplasmic reticulum (ER) contamination. Although the mitochondrial fraction was enriched in cytochrome c (mitochondrial marker) compared to the cytosolic fraction, due to the intimate interaction between the mitochondria and the ER there was still ER contamination as seen by the overexposed Calnexin band in the mitochondrial fraction. Perhaps additional centrifugation steps as described in Das et al. 2014, or a magnetic bead capture protocol (Li et al. 2019) might have yielded more pure mitochondria.

The qPCR results were insufficient for determining mitochondrial purity. The cytosolic gene markers, RPL19 and ACTB, were not present in the mitochondrial fraction, suggesting relatively pure mitochondria. However, these transcripts were also not detected in the cytosolic fractions, as would be expected. It is possible that the mitochondrial isolation process using the BioRad Mitchondria Isolation kit yielded less RNA from the cytosolic fraction than the cellular or mitochondrial fraction. Perhaps not enough RNA was present to be quantified, or the RNA contained within the cytosol was degraded, or the buffers from the isolation kit did not allow for further downstream applications. The purity of mitochondria as assessed by qPCR was demonstrated in previous studies, but the BioRad Mitochondria Isolation kit was not used in any of those studies (Bian et al. 2010, Das et al. 2012, Das et al. 2014, Wang et al. 2015, Li et al. 2019).

Unfortunately, let-7a and let-7b were not detected in the isolated mitochondria using qPCR. Both let-7a and let-7b were detected in the luteal cells, which was expected given previous results that let-7 miRNA are abundant in luteal tissue (Maalouf et al. 2014). The lack of let-7a and let-7b in the isolated mitochondria was likely due to lack of sensitivity of the assay. Let-7 miRNAs have been found in the mitochondria of both human and mouse cells (Bian et al. 2010, Barrey et al. 2011, Sripada et al 2012, and Li et al. 2019). Several of these studies have quantified miRNA from the mitochondria using qPCR (Bian et al. 2010, Das et al. 2012, Li et al.

2016, and Li et al. 2019). In this particular assay, let-7a and let-7b were only detected in the mitochondria at a very low abundance for one animal (Figure 3b). The studies that have quantified miRNA in the mitochondria also seem to detect relatively low abundance of these transcripts (Kren et al. 2009). Often these values are reported as fold-change relative to the abundance of miRNA in the cytosol or relative to 5s rRNA, an rRNA found in both the cytosol and mitochondria (Das et al. 2012, Zhang et al. 2014, Wang et al. 2015, and Li et al. 2016). Given the functional data observed in this study and other studies that have demonstrated that both let-7a and let-7b are present in the mitochondria (Bian et al. 2010, Barrey, et al. 2011, Sripada, et al 2012, and Li et al. 2019), it could be concluded that let-7a and let-7b do translocate into the mitochondria and have a functional effect, however, our assay was not sensitive enough to detect these miRNA.

Both let-7a and let-7b remained abundant in whole luteal cells over five days in culture. Neither let-7a nor let-7b abundance changed over the five days of culture, or with treatment of LH or PGF2A. This suggests the stability and perhaps constitutive production of these miRNA. These data support previous data in our lab that the let-7 family of miRNA are highly abundant and relatively stable in luteal tissue regardless of the status of the animal (Maalouf et al. 2014).

The abundance of let-7 miRNA in luteal tissue might suggest it would be difficult to tease apart and observe specific effects of an individual let-7. However, decreased mitochondrial function and steroidogenesis in luteal cells in response to let-7b was observed, and both let-7a and let-7b increased the abundance of their cytosolic mRNA targets. Mitochondrial respiration was measured and basal respiration decreased while ATP production and spare capacity tended to decrease in response to the let-7b mimic compared to the scrambled control, suggesting possible damage to the integrity of the ETC (Hill et al., 2012). Generally, regulation of the electron transport chain and oxidative phosphorylation is dependent on substrate availability (primarily the ratio between ADP and ATP abundance), and not by hormonal or allosteric

regulation. Simply stated, this means the transfer of electrons through the electron transport chain slows down when there is increased ATP while rate of transfer of electrons increases when there is increased concentrations of ADP. Let-7b tended to decrease ATP production, which in conjunction with decreased basal respiration, proton leak, and a tendency for reduced spare capacity, indicates that let-7b was able to slow down the rate of electron transport through the ETC, and thus reduce mitochondrial respiration. However, exact mechanism by which this occurs is not known. Several groups have transfected cells with miRNA and found a decrease in the abundance of ETC protein subunits including miR-181c which decreased complex IV subunits (Das et al., 2012; Das et al., 2014) and let-7a which decreased ND4, a subunit of complex I (Sharma et al., 2021). Both miR-181c and let-7a decreased mitochondrial respiration, and the authors hypothesized this decrease was a result from altered activity by the complexes because of the miRNA altering the abundance of the ETC protein subunits (Das et al., 2012; Das et al., 2014; Sharma et al., 2021). Unfortunately, the abundance of ETC protein subunits were not measured in response to let-7 transfection in the current study, so it is not known if the same type of mechanism is occurring with let-7.

Despite the studies above that suggest the ETC might become unstable as a result of miRNA regulation, decreased proton leak, as observed with let-7b, is generally associated with mitochondrial membrane integrity, stability of the ETC, and decreased uncoupling of protein activity (Hill et al. 2012). Perhaps, in an effort to preserve mitochondrial function, the remodeling of the ETC in response to let-7b does not completely destroy the integrity of the ETC, but rather slows down the rate of respiration to prevent overaccumulation of ROS that result from high rates of mitochondrial respiration. This would be in alignment with the idea that through some unknown mechanism, possibly by regulating mitochondrial gene targets, let-7b is able to slow the rate of electron transport through the ETC. The let-7 inhibitors in this experiment did not affect mitochondrial function (or progesterone production), which is similar to other studies completed

in our lab using miRNA inhibitors (Maalouf et al. 2016; Hughes et al. 2020). It is possible the sequence of the inhibitors was off by a nucleotide (or two), or had cross-reactivity with other similar miRNA resulting in no observed effect on mitochondrial function or progesterone production (Robertson et al., 2010).

Progesterone production was increased on day 3 of culture by let-7a, but was decreased on day 5 and 7 by let-7b. The regulation of progesterone by let-7 in the ovary has been demonstrated before. Both let-7b and let-7c decreased progesterone production from cultured human granulosal cells (Sirotkin et al., 2009), which is consistent with the results from this study. While the exact mechanism by which let-7a and let-7b altered progesterone production in the current study was not assessed, let-7b decreases *STAR* in MLTC-1 cells (mouse Leydig cell line; Men et al., 2017). This might be one mechanism by which let-7b decreased progesterone production in bovine luteal cells and it would be an interesting follow up study. Other steroidogenic enzymes, CYP11A1 and HSD3B, are not predicted targets of let-7b (or let-7a), so unless they are regulated indirectly by let-7b, regulation of STAR by let-7b is one of the most probable mechanisms by which let-7b regulated progesterone production. Interestingly, miRbase (www.mirbase.org), a database that uses miRNA sequences and annotation to predict miRNA targets, predicts that let-7a would also target STAR. However, let-7a increased progesterone production on day 3 of culture. It seems unlikely if let-7a targets STAR there would be an increase in progesterone production. However, ATP supply and steroidogenesis are linked and this might be one way this is the mechanism by which let-7a regulated progesterone production differently than let-7b. An early experiment performed using Leydig tumor cells, found that STAR function requires both hydrolysis of ATP and an electrochemical gradient to function (King and Stocco, 1996). Interestingly, in both primary and tumor Leydig cells cholesterol transport into the mitochondria is linked to mitochondrial ATP availability (Midzak et al., 2011). ATP synthesis was critical for steroidogenesis in both primary and tumor Leydig cells, and

disruption of the membrane potential and inhibition of the electron transport chain resulted in decreased testosterone production and ATP synthesis in primary Leydig cells (Midzak et al., 2011). These findings suggest that since let-7a did not alter ATP production, or any other measure of mitochondrial function, progesterone production should not have been altered. While let-7a increased progesterone production on day 3 of culture, perhaps there enough substrate available (ATP) to increase progesterone production for the short term, and subsequently did not alter progesterone production on day 5 and 7 of culture. Let-7b, however, decreased progesterone production and also decreased proton leak and basal respiration and tended to decrease ATP production and spare capacity. Perhaps in addition to possibility decreasing STAR, the reduction in ATP production and basal respiration combined resulted in the decreased progesterone production.

The opposite effect of progesterone production by let-7a on day 3 compared to let-7b on day 5 and 7 of culture might suggest a compensatory balance mechanism between let-7a and let-7b in regulating progesterone production in the CL. The functional CL of the cow produces large concentrations of progesterone at the cost of increased production of ROS (Sawada and Carlson, 1996). Increased ROS can be detrimental to progesterone production by inhibiting several enzymes/pathways of progesterone synthesis including the inhibition of CYP11A1, 3BHSD, and even the transport of cholesterol into the mitochondria (Sugino, 2006). Compensatory mechanisms exist in the CL to mitigate damage from the large amount of ROS including high concentrations of antioxidants, such as copper-zinc superoxide dismutase (SOD1) in the cytosol and manganese superoxide dismutase (SOD2) in the mitochondria during the middle of the estrous cycle (Sugino, 2006). However, it has also been demonstrated that miRNA might also have the ability to not only regulate steroidogenesis, but regulate ROS as well. Previous work done in our laboratory has demonstrated that the knockdown of DROSHA, an important enzyme in miRNA processing, decreased progesterone production from bovine luteal cells (Maalouf et al.

2016a). As stated above, miRNA also alter progesterone secretion from cultured human granulosal cells (Sirotkin et al. 2009). The increase in progesterone production from let-7a in this study could result in large amounts of ROS production. ROS have been demonstrated to activate the NFkB pathway which can lead to the upregulation of Lin28 (a well-known Let-7 regulator; Shyh-Chang et al. 2013) and downregulate the transcription of let-7, including let-7a (Iliopoulos et al. 2009, Markopoulos et al. 2018, Babu and Tay 2019). In contrast, the inhibition of NFkB results in the increased abundance of let-7a (Iliopoulos et al. 2009). ROS can also act directly on miRNA processing enzymes like Dicer, as both treatment with ROS and knockdown of Dicer can lead to the downregulation of miRNA. Thus, miRNA might drive their own regulation.

This experiment did not examine any mechanistic function by which let-7 might be acting to regulate mitochondrial function or progesterone production. Measuring the abundance of steroidogenic enzymes, ETC protein subunits, and ROS might provide more mechanistic insight.

The paradox of the results from this study was that let-7b reduced mitochondrial function, but both the mimic and inhibitor increased the abundance of its nuclear genome target, progesterone receptor membrane component 1 (PGRMC1). Let-7a mimic and inhibitor also increased the abundance of PGRMC1, and the inhibitor tended to increase the abundance of early growth response 1 (EGR1). It was expected that both let-7a and let-7b would decrease the abundance of EGR1 and PGRMC1, as both genes were predicted targets and pulled down in a previous assay in our lab. Although the electron transport chain (ETC) proteins were not directly measured, it is interesting that mitochondrial function while cytosolic mRNA targets increased in response to let-7b. There is evidence that miRNA can act differently and regulate targets in an opposite manner in different cellular compartments including the mitochondria and the cytosol. The most well-described is miR-1, which was found to enhance the translation of mitochondrial transcripts mt-ND1 and mt-CO1 in undifferentiated C2C12 cells (mouse myoblasts) (Zhang et al.,

2014). Although miR-1 enhanced the abundance of mitochondrial-derived proteins, miR-1 decreased the abundance of the cytoplasmic protein targets, HDAC4 and ELL2, demonstrating that miRNA might have differing effects in the mitochondria compared to the cytosol (Zhang et al., 2014). The differing cellular compartments could be one reason for the difference in action between mitochondrial and cytosolic gene targets of let-7a and let-7b

As discussed above, both PGRMC1 and EGR1 are nuclear genome targets of let-7a and let-7b. Of the nuclear genome targets of let-7a and let-7b, PGRMC1 and EGR1 were selected due to their relevance in luteal biology and to determine the effect of let-7 on nuclear encoded genes. PGRMC1 is involved in many important functions in luteal tissue, including cholesterol metabolism (Rohe et al., 2009) and steroidogenesis (Hughes et al., 2007). EGR1 is a transcription factor that is involved in luteal regression by increasing the expression of genes involved with luteal regression, including transforming growth factor beta 1 (TGFB1), in response to prostaglandin F2A (Hou et al. 2008). In this study, EGR1 expression did not change with either mimic, however, PGRMC1 expression increased with both mimics. EGR1 expression tended to increase with the let-7a inhibitor, while PGRMC1 expression increased with both inhibitors. It was unusual in this study to see an effect of the inhibitor because previous studies from our lab using miRNA mimic and inhibitors did not show any effect of the inhibitors (Maalouf et al. 2016; Hughes et al. 2020). More unusual was to see the same effect of the mimic and inhibitor increasing PGRMC1 expression. It would be expected that the let-7 mimic would downregulate PGRMC1 protein expression, while the let-7 inhibitor would increase PGRMC1 expression. miRNA inhibitors are known to bind directly to specific miRNA of interest within the argonaute 2 complex to prevent the miRNA of interest from interacting with its target mRNA, resulting in increased translation of the target mRNA (Hogan et al. 2014). However, why the miRNA mimics increased PGRMC1 abundance is more peculiar. There is evidence that miRNA, including let-7, can induce target gene translation during cellular quiescence, but inhibit target gene translation

during cell proliferation (Vasudevan et al., 2007). The same holds true in the absence of growth factors where miRNA activate translation and increase protein expression of their target mRNA (Vasudevan et al., 2007). Additionally, if miRNA are injected directly into the nucleus, translation of the target mRNA is upregulated, but not if the miRNA are injected into the cytoplasm (Truesdell et al., 2012). Perhaps it is possible that either the stage of the cell cycle, or *in vitro* culture conditions induced PGRMC1 expression by the let-7 mimics. There is substantial evidence that another let-7 family member, let-7i, does regulate PGRMC1 abundance (Wendler et al. 2010; Nguyen et al. 2018), however there is a two nucleotide difference in the sequence of mature bta-let7i, and this difference might be enough that neither let-7a nor let-7b functionally target PGRMC1.

Interestingly, while EGR1 is a target of both let-7a and let-7b from our pulldown assay, several miRNA target predictor programs, including TargetScan and miRDB, do not predict EGR1 to be a target of let-7a or let-7b based on its sequence. The present study indicates that neither let-7a nor let-7b target EGR1 functionally as neither mimic changed EGR1 abundance. Interestingly, the knockdown of EGR1, a transcription factor, increases the abundance of several let-7 miRNA, including let-7b (Gaeta et al. 2017). It is possible that if let-7 miRNA did target EGR1, let-7 could act within a negative feedback loop to regulate its own abundance. Alternatively, these mimics could have acted “off-target” on other regulators of PGRMC1 and EGR1 to cause the increased expression.

Conclusion

The novel findings from this study demonstrated that let-7 can modulate mitochondrial function and progesterone production in the bovine CL. These findings add to the current body of literature that miRNA can mitochondrial function as well as demonstrate a new regulator of progesterone production from luteal cells. While the exact mechanism by which let-7 alters mitochondrial function and progesterone production is not known and was not evaluated in this

study, it is interesting that let-7 can regulate two important mitochondrial processes at once and appear to be regulated outside of metabolic demand and hormone signaling.

(A) bta-let7a-5p sequence

ugagguaguagguuguauaguu

(B) bta-let7b sequence

ugagguaguagguugugugguu

Figure 2-1. The mature sequences of the let-7 miRNA used in this study, bta-let7a-5p and bta-let7b. A) the mature sequence of bta-let7a-5p, and B) the mature sequence of bta-let7b. The nucleotides highlighted in red demonstrate the 2 nucleotide difference between the two sequences.

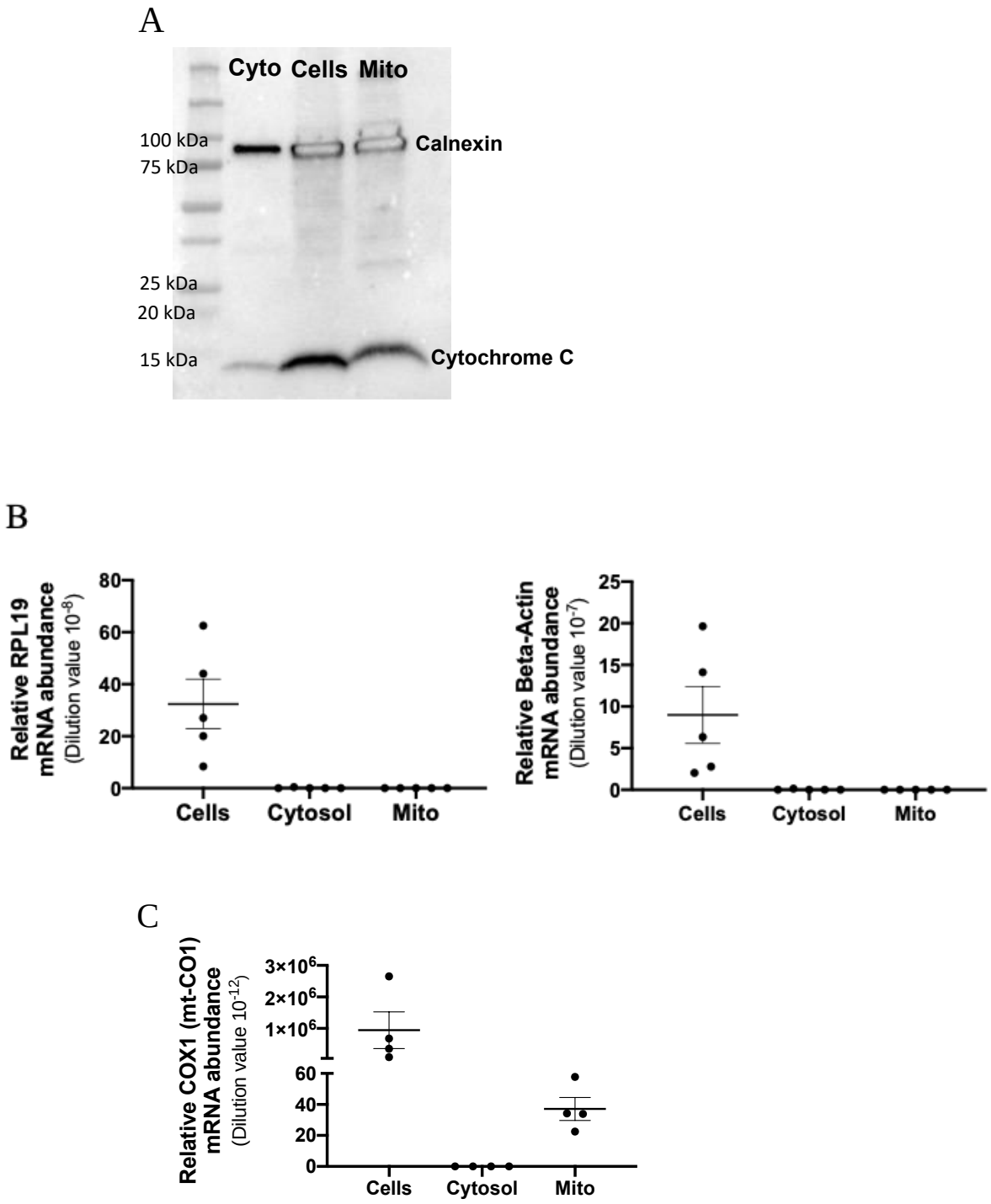


Figure 2-2. Assessment of the purity of mitochondria isolated from luteal cells. A) a representative western blot with calnexin (67kDa) as an ER membrane marker and cytochrome c

(12 kDa) as a mitochondrial marker. The cytosolic (cyto) and mitochondrial (mito) fractions along with whole cell lysates are represented. B) mRNA for the cytosolic gene markers, RPL19 and ACTB, in cells, cytosolic, and mitochondrial (mito) fractions. C) mRNA for the mitochondrial gene marker, mt-CO1, in cells, cytosolic, and mitochondrial (mito) fractions.

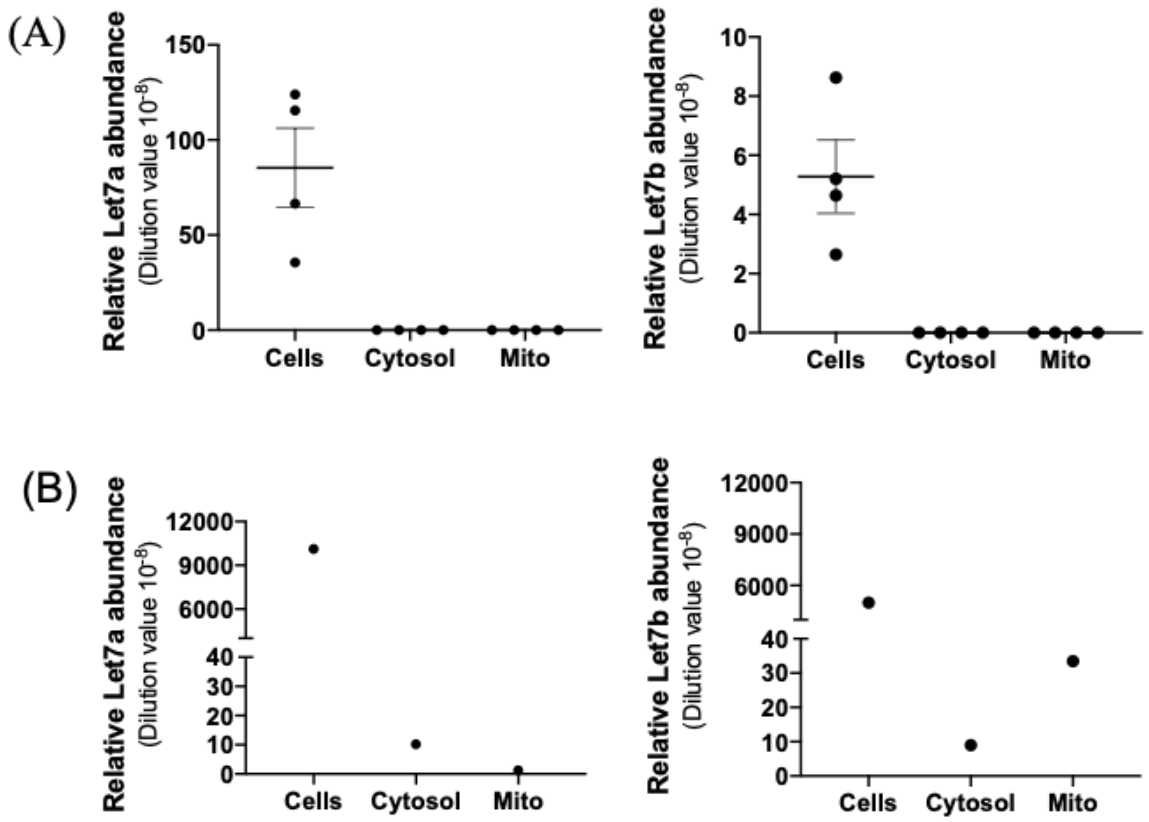


Figure 2-3. Let-7a and let-7b abundance in isolated mitochondria. A) Let-7a and let-7b abundance in cells, cytosolic, and mitochondrial (mito) fractions. B) Let-7a and let-7b in the mitochondria from one animal.

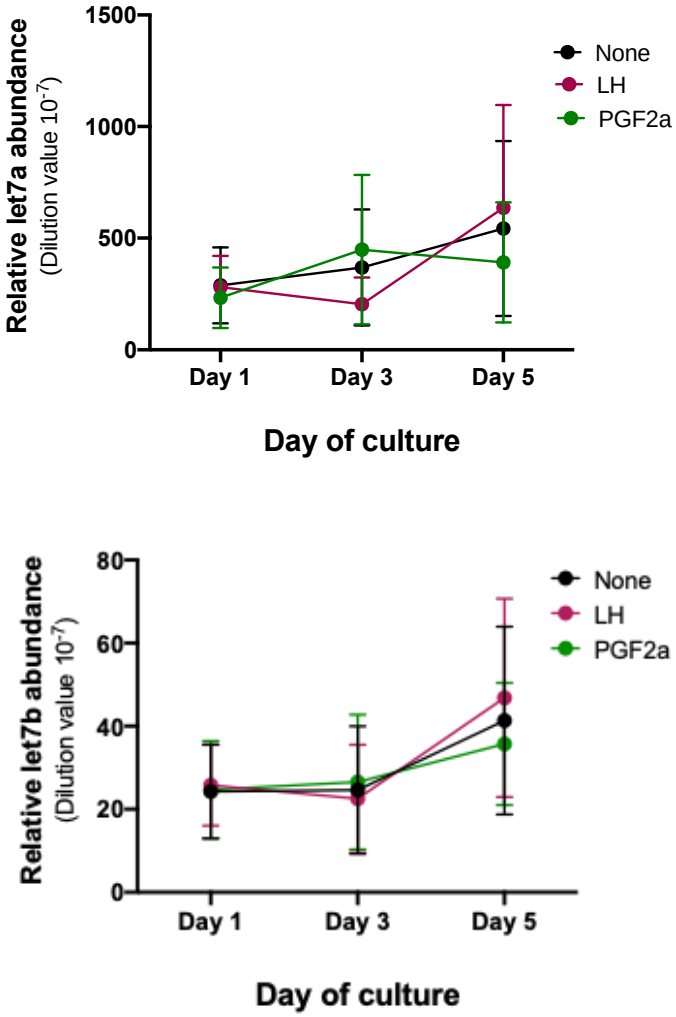


Figure 2-4. The abundance of let-7a and let-7b over five days in culture and in response to LH or PGF2A.

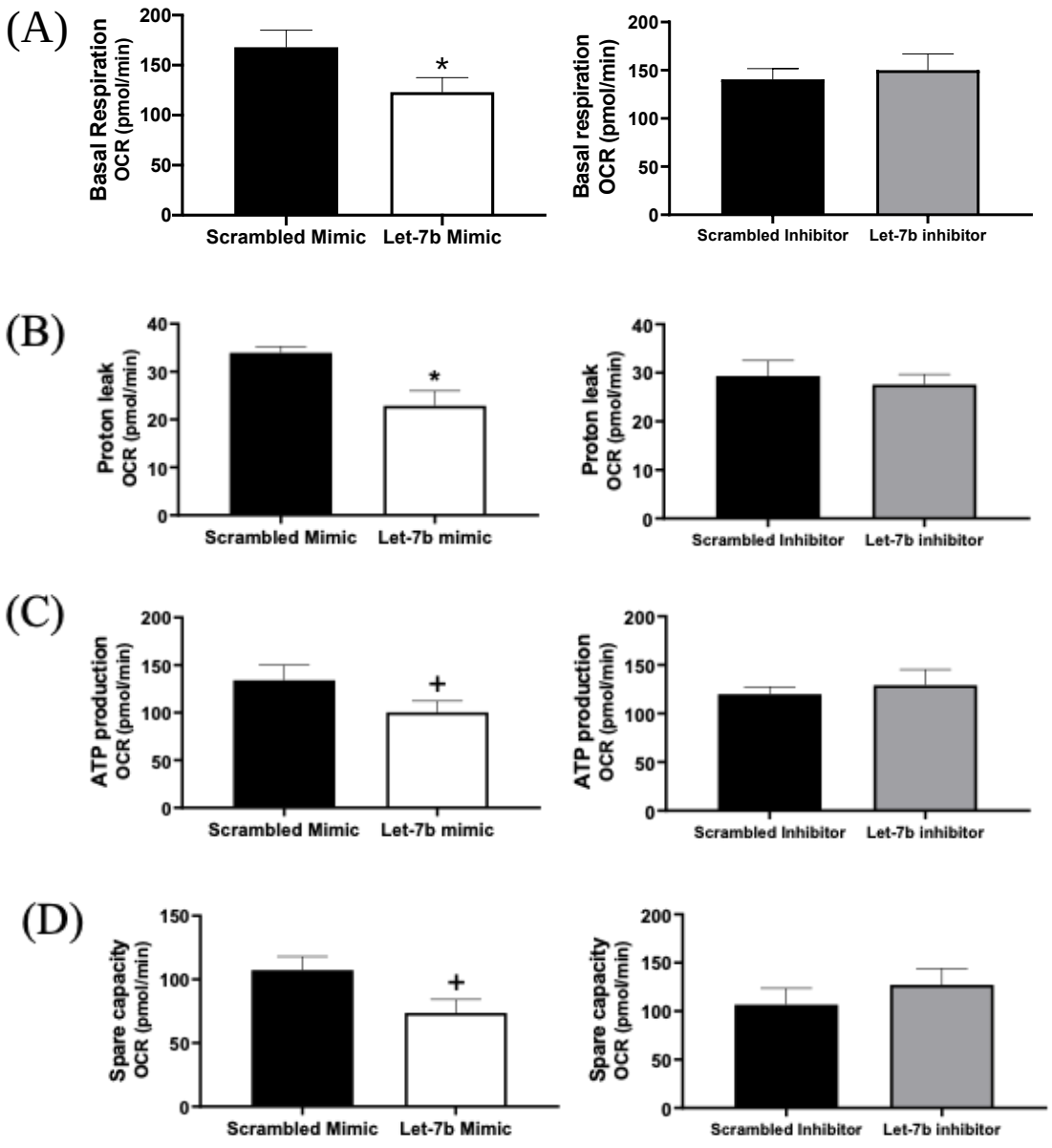


Figure 2-5. The effect of let-7b on mitochondrial function in luteal cells. A) Basal respiration in response to let-7b mimic (left) and inhibitor (right). B) Proton leak in response to let-7b mimic (left) and inhibitor (right). C) ATP production in response to let-7b mimic (left) and inhibitor (right). D) Spare capacity in response to let-7b mimic (left) and inhibitor (right). * indicates $P < 0.05$, and + indicates $0.05 < P < 0.1$. OCR = oxygen consumption rate

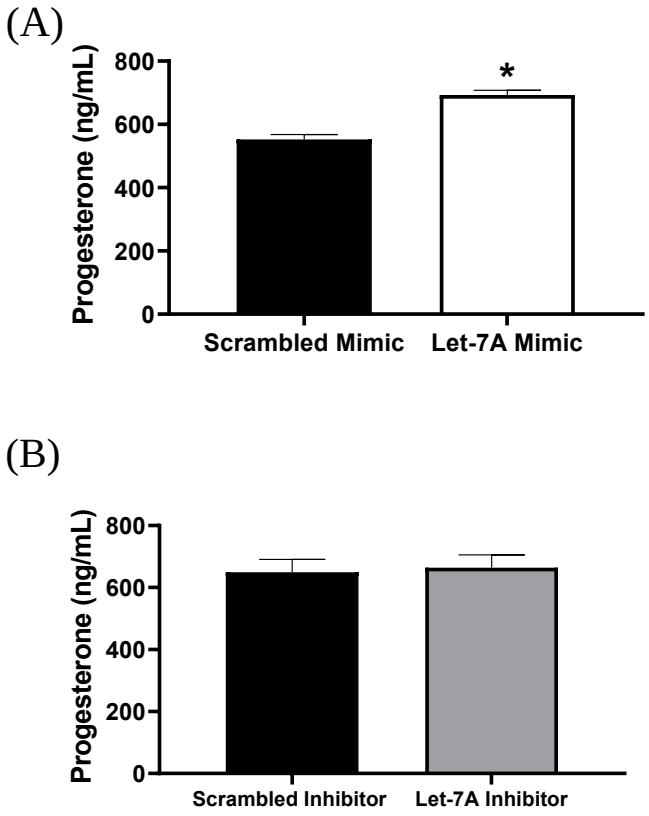


Figure 2-6. The effect of let-7a on progesterone production by luteal cells on day 3 of culture. A) Effect of let-7a on progesterone on day 3 of culture, B) Effect of the let-7a inhibitor on progesterone production. * indicates $P < 0.05$.

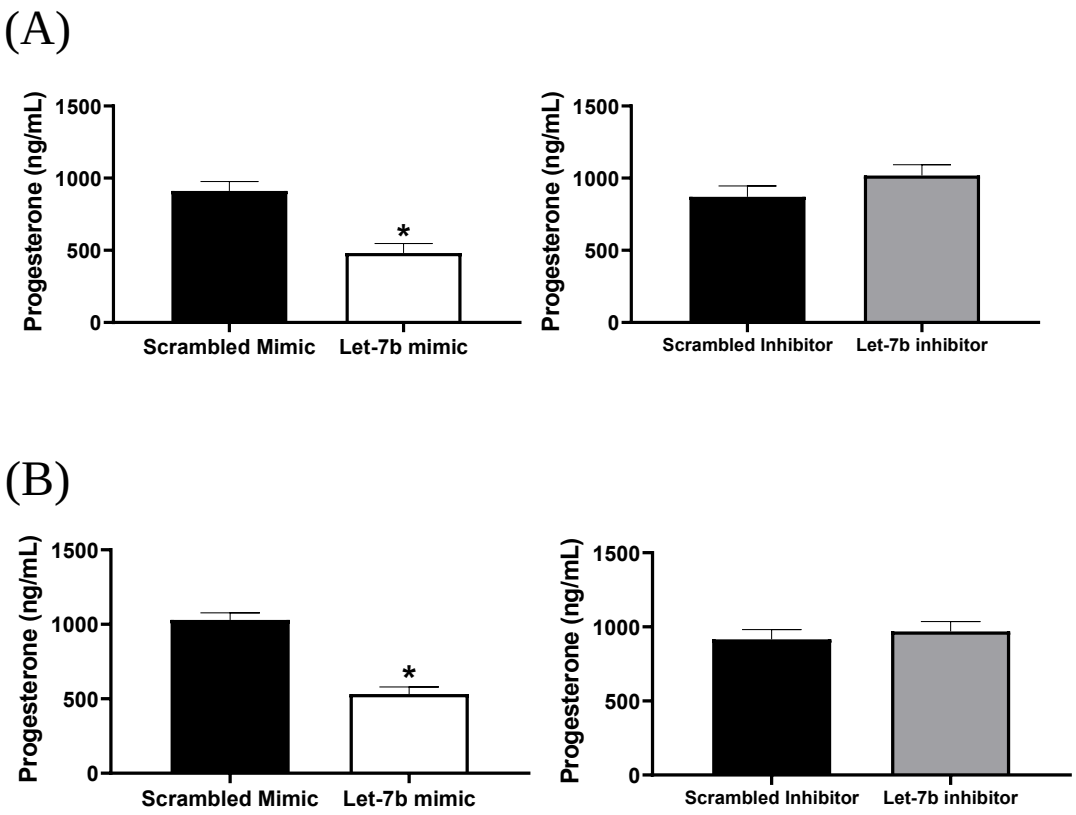


Figure 2-7. The effect of let-7b on progesterone production on day 5 and 7 of culture. A) Progesterone production in response to let-7b mimic (left) and let-7b inhibitor (right) on day 5 of culture. B) Progesterone production in response to let-7b mimic (left) and let-7b inhibitor (right) on day 7 of culture. * indicates $P < 0.05$

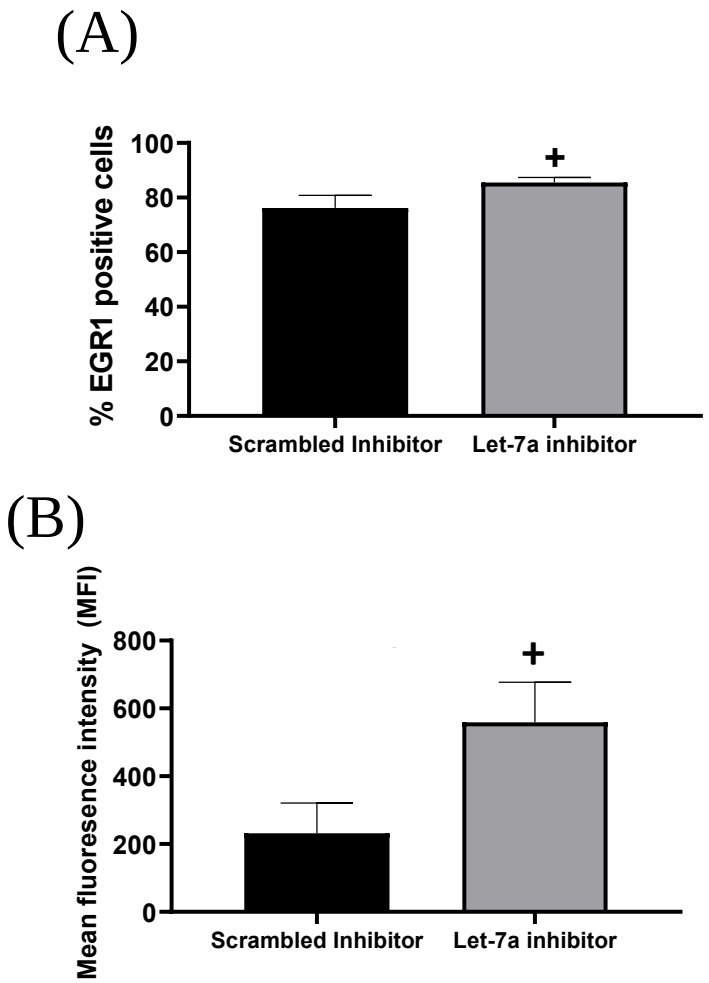


Figure 2-8. The effect of let-7a inhibitor on EGR1. A) The percentage of EGR1 positive cells in response to let-7a inhibitor. B) The mean fluorescence intensity of EGR1 on luteal cells in response to let-7a inhibitor. + indicates $0.05 < P < 0.1$

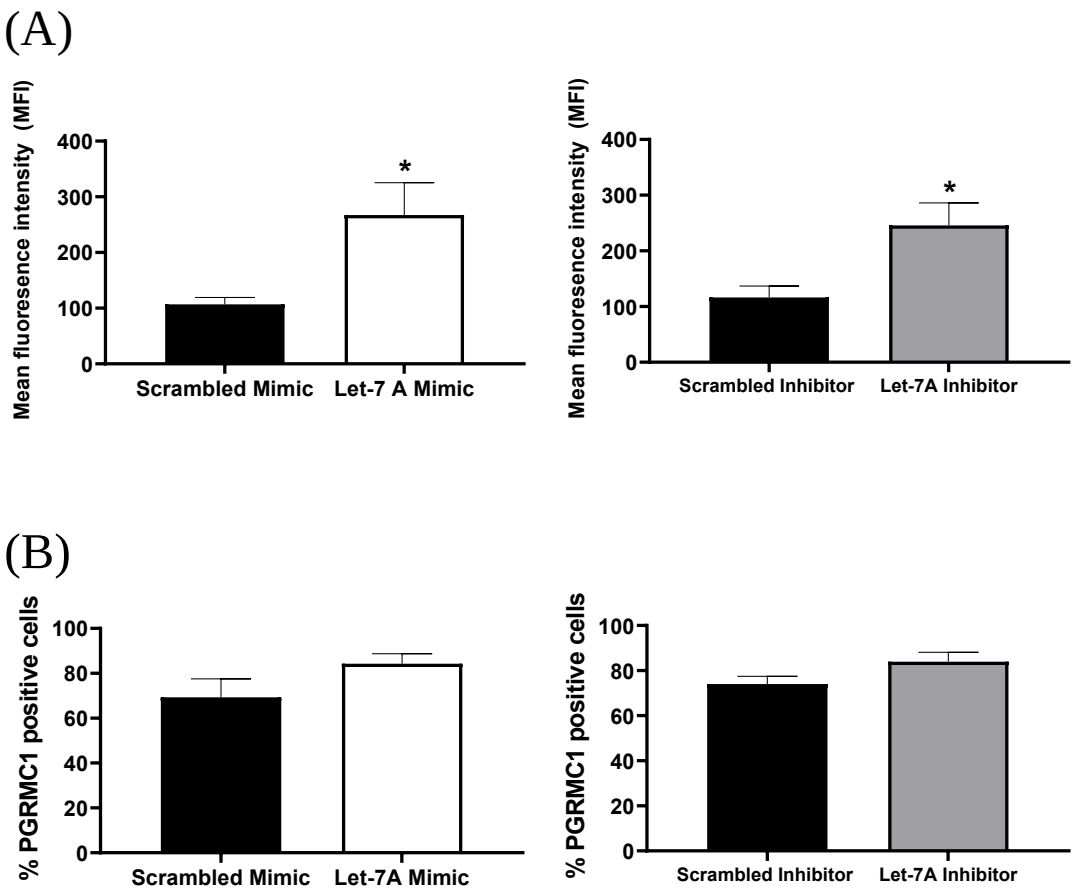


Figure 2-9. The effect of let-7a mimic and inhibitor on PGRMC1 abundance. A) The effect of let-7a mimic (left) and let-7 inhibitor (right) on MFI of PGRMC1. B) The effect of let-7a mimic (left) and let-7a inhibitor (right) on percentage of PGRMC1 positive cells. * denotes $P < 0.05$.

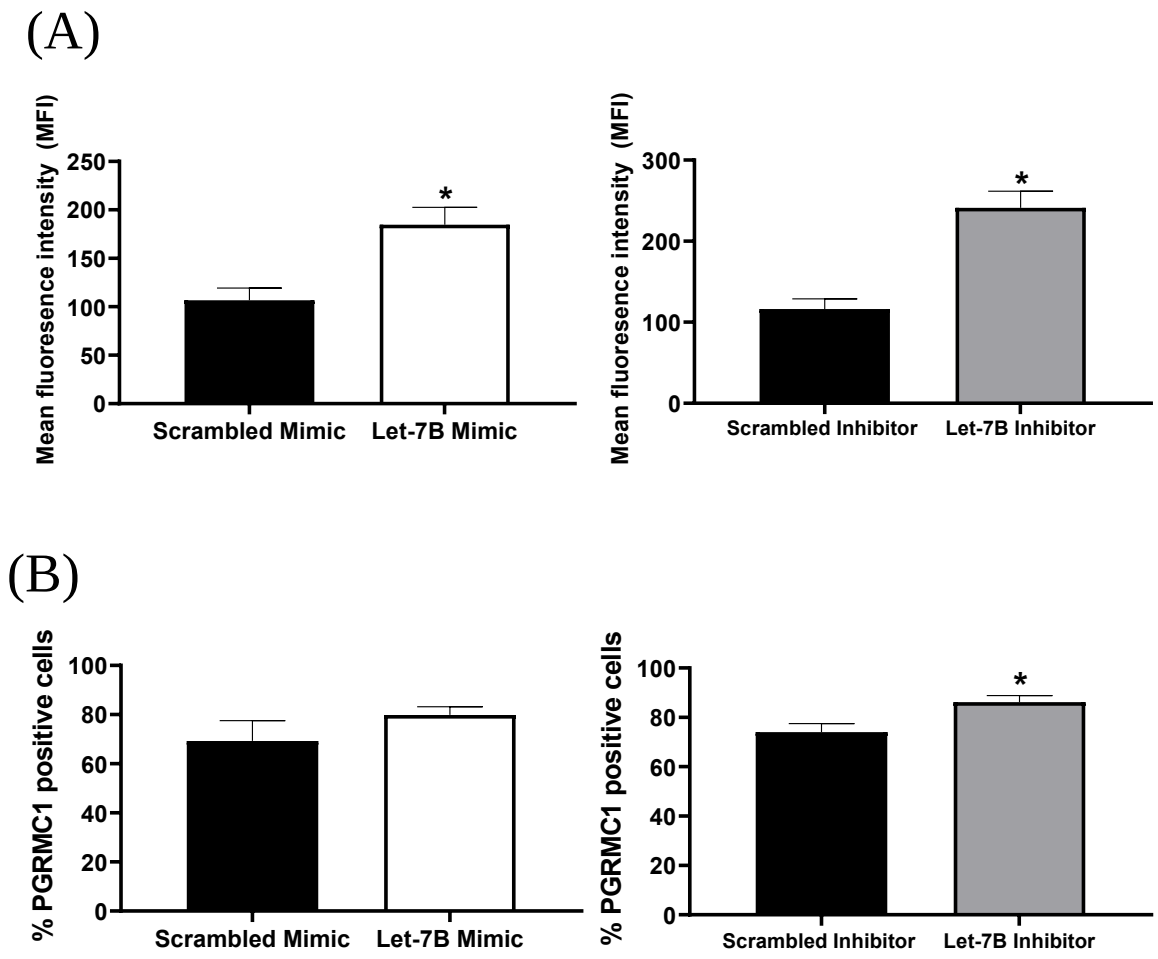


Figure 2-10. The effect of let-7b mimic and inhibitor on PGRMC1 abundance. A) The effect of let-7b mimic (left) and let-7b inhibitor (right) on the MFI of PGRMC1. B) The effect of let-7b mimic (left) and let-7b inhibitor (right) on the percentage of PGRMC1 positive cells. * denotes $P < 0.05$

Chapter 3

Uterine infused interferon tau and pregnancy associated glycoproteins upregulate immune cell signaling and structural stabilization pathways in bovine luteal tissue and resident immune cells

Introduction

The presence of immune cells in the corpus luteum has been known since Lobel and Levy's work in 1968 when they identified macrophages and lymphocytes in the CL throughout the estrous cycle. The characterization of immune cells in the CL during luteal regression is well established, but less is known about immune cells, specifically T cells, in the CL of early pregnancy. T cell proportions in the CL are generally about 25% $CD4^+$ T cells, 45% $CD8^+$ T cells, and 30% $\gamma\delta^+$ T cells (Poole and Pate, 2012). These proportions do not change upon induction of luteolysis (Poole and Pate, 2012). Regulatory T cells (Tregs, $Foxp3^+$) are in greater abundance in the functional CL compared to the regressing CL (Poole and Pate, 2012). In the bovine CL of early pregnancy (day 18), there is a greater proportion of $\gamma\delta^+CD8^+$ T cells, an anti-inflammatory type of T cell (Poole and Pate, 2012). There is some evidence that the proportion of $Foxp3^+$ cells is greater in the CL of pregnancy compared to the peripheral blood (Walusimbi et al., unpublished). Other immunotolerance markers including Foxp3 and PGE synthase (PGES) mRNA are greater in the CL of pregnancy than in persistent CL (Magata et al., 2012). Hughes et al. (2019) performed a transcriptomic profiling study comparing day 17 CL of pregnancy and of the cycle. T cell activation and signaling and IL2 signaling were pathways upregulated in the CL of pregnancy, suggesting the importance of T cells in the CL of pregnancy. The findings from Hughes et al. (2019) initiated the first experiment of this chapter which aimed to understand if the

T cell activation molecules identified by Hughes et al. (2019) would be altered in T cells cocultured with luteal cells that had been primed by hormones associated with early pregnancy.

However, signaling by hormones to the CL is more complicated than just a few hormones acting on one cell type. The current transcriptomic and proteomic profiling studies that have examined the CL of early pregnancy have demonstrated that there are changes that occur in the CL of pregnancy, likely as a result from exposure to IFNT produced by the conceptus (Hughes et al., 2019, Hughes et al., 2020; Mezera et al., 2021). However, the conceptus also produces another type of hormone called pregnancy associated glycoproteins (PAGs). PAGs are a family of proteins that have a similar structure to pepsin and are related to aspartic proteinases (Green et al., 2000). It is now known that PAGs, including PAG1, also known as pregnancy-specific protein B (PSPB), can be detected in maternal sera as early as day 15 of pregnancy via radioimmunoassay (Sasser et al., 1986), but generally the concentration of PAGs in the blood rapidly increases within the first 4 weeks after artificial insemination and peaks just prior to calving (Green et al., 2005) and can continue to be detected for 80-100 days after calving due to their long half-life (Zoli et al., 1992). However, studies trying to determine the role of PAGs in the CL have been very limited. Del Vecchio et al. (1996) found when bovine luteal cells were treated with PSPB *in vitro,* the production of PGE2, a luteotropic hormone, by luteal cells was increased. This finding was also supported by Weems et al. (1998), except PSPB increased PGE2 production by luteal cells in a time and dose dependent manner. Wallace et al. (2015) postulated a possible role for PAGs in cattle during pregnancy and suggested that PAGs might have an immunomodulatory and luteotropic role, which would all be beneficial for maintaining the CL of pregnancy. These speculations do make sense if PAGs induce PGE2 production from the CL, as PGE2 is a luteotropic hormone and is also has “anti-inflammatory” properties, as it can inhibit the proliferation of T cells (Goodwin et al., 1977; Low and Hansen, 1988). To address some of these speculations, the second experiment of this chapter was an “RNA sequencing fishing expedition”

to understand what transcriptomic changes PAGs induce in the CL as well as broadly characterize the phenotype of $CD45^+$ cells in the CL in response to IFNT and/or PAGs. Both IFNT and/or PAGS were infused into the uterus between days 14-20 of the cycle to mimic the time of early pregnancy and would also demonstrate that if any changes were observed in the CL, the uterine-infused hormones were able to reach the CL and induce changes. In conclusion, the aim of these experiments was to elucidate a further understanding for the role of immune cells in the CL of pregnancy and address a new area of luteal biology by understanding the role of PAGs signaling in the CL.

Materials and Methods

Tissue and blood collection

The procedures performed for tissue collection were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

T cell experiment

Cyclic Holstein dairy cows were observed for signs of estrus. The day of standing estrus was considered day 0 of the cycle. Corpora lutea were collected via colpotomy between days 10 and 12 of the estrous cycle. The tissue was then dissociated and cultured as previously described (Pate, 1993). Two days later, blood was collected from the same animal through venipuncture of the jugular vein. The blood was used for peripheral T cell isolation, as described below.

Infusion Study

The infusion study used cyclic Holstein dairy heifers. Heifers were synchronized using a luteolytic dose of prostaglandin F2alpha (PGF2A, Lutalyse,25 mg; Pfizer) and observed for signs of estrus. On day 14 after observation of a standing heat, an intrauterine two-way Foley catheter was inserted through the cervix and into the uterus where it remained for the duration of the infusion period. The infusion treatments were as follows: 1) Saline + 200 μg/ml BSA in 20 ml

from day 14 to day 16, 2) Saline + 200 μg/ml BSA + 200 μg IFNT in 20 ml from day 14 to 16, 3) Treatment 2 from day 14 to day 16 + Treatment 2 from day 17 to day 19, and 4) Treatment 2 from day 14 to 16 + 100 μg PSPB from day 17 to 19. Corpora lutea were collected via colpotomy either on Day 17 (3 day infusion) or Day 20 (6 day infusion) of the estrous cycle depending on treatment group. One quarter of the CL was flash frozen in liquid nitrogen for RNA isolation. The remaining tissue was then dissociated and resident immune cells were isolated as described below.

Methods for T cell experiment

Luteal cell culture and T cell coculture

CL were dissociated and isolated luteal cells were cultured as previously described (Pate, 1993). After plating luteal cells, cells were treated with PGE2 (10 ng/ml), IFNT (1 ng/ml), and a combination treatment. On day 1 of culture, medium was replaced and cells were treated again with PGE2, IFNT, and a combination treatment. T cells were isolated from peripheral blood mononuclear cells (PBMCs) as previously described in Poole and Pate (2012). Cell number and viability was determined by counting cells stained with trypan blue (Thermo Fisher) on a hemocytometer. After isolation, T cells (CD2/γδ positive selection) were added to washed luteal cells on day 2 of culture and cultured in AIMV media. T cells were collected at 12, 24, and 48 hours after addition to luteal cells. T cells were frozen in TRIZOL for future RNA isolation.

RNA Isolation and quantitative PCR

RNA was isolated using TRIZOL protocol. RNA quality was assessed on an Experion (BioRad) and had an RNA quality index (RQI) greater than 7. RNA underwent a DNase (Promega) procedure to eliminate any gDNA contamination. cDNA was synthesized using the AzuraQuant kit (Azura Genomics), and qPCR was performed using AzuraQuant green fast qPCR mix LoRox (Azura Genomics). GAPDH was used as a reference gene and not change with treatment.

Statistical Analysis

The statistical analyses were performed using the mixed model of SAS 9.4 (Statistical Analysis System Institute). An ANOVA was used to determine differences in mRNA among treatments. Animal was included as an effect in the model and GAPDH, the reference gene, was used as a covariate in the model. A Dunnett's post-hoc test was used to compare each treatment to the vehicle control. The data are represented as least squares means ± pooled SEM. Differences were considered significant when $p < 0.05$, and a tendency when $0.05 < p < 0.1$.

Methods for infusion study

Isolation of resident immune cells

CL were dissociated and resident immune cells were isolated as described in Poole and Pate (2012). However, an anti-CD45 antibody (WSU) was used to isolate resident immune cells (T cells, macrophages, monocytes, neutrophils) and cells were positively sorted using an AutoMACS (Miltenyi). Purity of resident immune cells was assessed by culturing cells for 24 hours and counting the number of steroidogenic cells in each well. There was less than <1% luteal cell contamination.

RNA Isolation and transcriptomic analysis

Total RNA was isolated from both tissue and isolated resident immune cells using the mirVana RNA Isolation Kit (Thermo Fisher). RNA quality and concentration was assessed using an Agilent Bioanalyzer (RIN > 7) and BioRad's Experion (RQI > 7). Prior to sequencing, samples were treated with DNase and concentrated using RNA Clean and Concentrator (Zymo). RNA samples were shipped on dry ice to Beijing Genomics Institute (BGI; Shenzhen, Guangdong, China) for Next-Generation Sequencing (NGS). RNA quality assessed at BGI using Agilent's Bioanalyzer and all samples had a RIN > 8 and RNA quality deemed good for generating a library and downstream sequencing. The DNBSEQ platform was used for sequencing the library. High quality reads were then filtered from the entire set of raw reads

generated and aligned to the reference genome. HISAT/Bowtie2 was used to align the clean reads to the *Bos taurus* assembly 5.0.1. Differential abundance was assessed using DESeq2. All fold changes are reported as log2 fold changes. A $Q < 0.05$ was considered significant, and $0.05 < Q < 0.01$ was considered a tendency. Pathway analysis of differentially abundant and/or transcripts that tended to differ was completed using Ingenuity Pathway Analysis (IPA; Qiagen).

Results

T cell activation

The mRNA encoding for the T cell receptor activation molecules CD3e, IL2 inducible T cell kinase (ITK), LCK proto-oncogene (LCK), and protein tyrosine phosphatase non-receptor type 7 (PTPN7) were measured in T cells cocultured with luteal cells that were previously treated with IFNT, PGE2, and a combination treatment (Figure 1). The abundance of CD3e and LCK mRNA was less in T cells that had been cocultured with PGE2-treated luteal cells compared to control (Figure 2a and 2c), while ITK mRNA did not change (Figure 2b). The abundance of PTPN7 tended to be less in T cells exposed to luteal cells treated with IFNT, PGE2, and a combination treatment compared to control (Figure 2d).

Infusion study RNA sequencing (RNAseq)

Both whole luteal tissue and resident immune cells (RIC) isolated from luteal tissue were collected from heifers that had either 3-day uterine infusions (BSA or IFNT), or 6-day uterine infusions (IFNT or IFNT+PSPB). RNA isolated from both luteal tissue and RIC was sent for RNA sequencing analysis.

There was an average of 66.32 million total clean reads per sample with an average of 85.29% of reads uniquely mapping to the reference genome, and an average of 60.12% of reads aligning to protein coding regions in the genome for the RNA isolated from resident immune cells. There was an average of 65.78 million total clean reads per sample with an average of

77.44% of reads uniquely mapping to the reference genome, and an average of 57.78% of reads aligning to protein coding regions in the genome for the RNA isolated from whole luteal tissue.

6-day IFNT compared to 6-day IFNT+PSPB

A total of 18,230 transcripts were identified in the RIC 6-day infusion samples. Of the 18,230 transcripts, only 3 were differentially abundant ($Q < 0.05$ and log2 fold change > 1). Two transcripts, LOC786348 and CR2, were in greater abundance in response to the 6-day IFNT+PSPB infusion compared to the 6-day IFNT infusion, while one transcript, 107131165, was less abundant in response to the 6-day IFNT+PSPB infusion (Figure 3 and Table 1). Ten transcripts tended to differ in abundance ($0.05 < Q < 0.15$ and log2 fold change > 1), while two transcripts tended to differ in abundance, but had a log2 fold change less than 1 (Figure 3 and Table 1). The differentially abundant transcripts and transcripts that tended to differ are shown graphically in Figure 4. Ingenuity Pathway Analysis (IPA; Qiagen) was performed for all transcripts with a $Q < 0.15$. There were 11 pathways associated with these transcripts including eNOS signaling and complement system signaling in the top five pathways identified (Figure 5). Pathway analysis of the 100 most abundant transcripts in resident immune cells from the 6-day IFNT+PSPB infusion found EIF2 signaling, regulation of eIF4 signaling, mTOR signaling, and NRF2-mediated oxidative stress response to be the top upregulated pathways associated with these transcripts (Figure 6). These top 100 most abundant transcripts are listed in supplementary table 1.

A total of 17,935 transcripts were identified in the luteal tissue from the 6-day infusion samples. Of the 17,935 transcripts, 5 were considered differentially abundant ($Q < 0.05$), but only 2 transcripts, MAP1LC3C and ARMH4, had a log2 fold change greater than 1 or less than -1 (Figure 7 and Table 2). There were an additional 15 transcripts that tended to differ between treatments (Table 2). Of the 15 transcripts that tended to differ, 8 transcripts had a log2 fold

change greater than 1 or less than -1, while 7 transcripts had a log2 fold change between 0 and 1, or between 0 and -1 (Table 2). IPA analysis for all transcripts with a Q < 0.15 found 17 pathways associated with these transcripts including several pathways involved with IL17 signaling, coagulation, and iNOS signaling (Figure 8).

3-day IFNT compared to 6-day IFNT

A total of 18,176 transcripts were identified in resident immune cells from the 3-day and 6-day IFNT infusion samples. Of the 18,176 identified transcripts, 8 were considered differentially abundant (Q < 0.05), and 6 of these transcripts had a log2 fold change greater than 1 or less than -1 (Figure 9 and Table 3). There were an additional 32 transcripts that tended to differ (Q < 0.15) between the 3-day and 6-day IFNT infusions (Supplementary Table 2). IPA analysis found 13 pathways associated with all transcripts with a Q < 0.15 including ephrin A signaling, sphingosine-1-phosphate signaling, and IL15 production in the top 5 pathways associated with these transcripts (Figure 10).

A total of 17,746 transcripts were identified in luteal tissue collected from heifers that received the 3-day and 6-day IFNT infusions. Of the 17,746 transcripts identified, 56 transcripts were considered differentially abundant (Q < 0.05) and 28 transcripts had a log2 fold change either greater than 1 or less than -1 (Figure 11). There were an additional 80 transcripts that tended to differ (0.05 < Q < 0.15) between treatments (Figure 11). IPA analysis identified 40 pathways associated with transcripts with Q < 0.15, however, only the top 13 pathways are displayed in Figure 11. ILK signaling and IL8 signaling are 3 pathways of interest within the top 13 pathways identified (Figure 12).

3-day BSA compared to 3-day IFNT

A total of 18,077 transcripts were identified in resident immune cells from the 3-day BSA and 3-day IFNT infusion samples. Of the 18,077 identified transcripts, there were 83

differentially abundant transcripts (Figure 13). There were 77 transcripts in greater abundance in response to the 3-day IFNT infusion compared to the 3-day BSA infusion (Supplementary table 3), and 6 transcripts that were less abundant in response to the 3-day IFNT infusion compared to the 3-day BSA infusion. These 6 transcripts were C-X-C motif chemokine ligand 14 (CXCL14), protein disulfide isomerase family A member 5 (PDIA5), monoamine oxidase A (MAOA), high mobility group box 3 (HMGB3), signaling receptor and transporter of retinol (STRA6), and CD9 molecule (CD9). As expected, one of the top pathways upregulated in response to the 3-day IFNT infusion was interferon signaling (Figure 14). Several pathways involved in antiviral responses were also upregulated.

A total of 17,731 transcripts were identified in luteal tissue collected from heifers receiving the 3-day BSA and 3-day IFNT uterine infusions. Of the 17,731 identified transcripts in luteal tissue, 117 were differentially abundant (Figure 15). There were 87 transcripts in greater abundance in response to the 3-day IFNT infusion and 30 were less abundant in response to the 3-day IFNT infusion compared to the 3-day BSA infusion (Supplementary table 4). Similar to the findings in RIC, IPA analysis found interferon signaling and antiviral responses to be some of the upregulated pathways in luteal tissue in response to the 3-day IFNT infusion (Figure 16).

Discussion

The functional role of T cells and other resident immune cells in the CL of pregnancy is still an understudied area of luteal biology. Additionally, there are still many questions about understanding how the conceptus-derived hormones, IFNT and PAGs, influence the physiology of the CL of pregnancy. To date, there have been very limited studies investigating the role of PAGs in luteal biology. This project aimed to elucidate a better understanding of T cells and other resident immune cells in CL of early pregnancy, and how various hormones associated with pregnancy might influence the function of these cells.

Hughes et al. (2019) demonstrated in a transcriptomic profiling study that T cell activation and signaling and IL2 signaling were pathways upregulated in the CL of pregnancy on day 17 compared to the cyclic CL on day 17. Additionally, several molecules involved in the T cell receptor (TCR) signaling cascade were found to have differentially abundant mRNA. These molecules were greater in the CL of pregnancy in the transcriptomic profiling study and were confirmed to be greater via quantitative PCR (Hughes et al., 2019). These molecules included CD3e, IL2 inducible T cell kinase (ITK), LCK proto-oncogene (LCK), and protein tyrosine phosphatase non-receptor type 7 (PTPN7). An attempt was made to mimic pregnancy *in vitro* to see if PGE2, IFNT, and/or a combination treatment could alter how luteal cells activate T cells and change the abundance of T cell activation molecules identified in Hughes et al. (2019). Interestingly, PGE2 treatment of luteal cells decreased CD3e and LCK mRNA in cocultured T cells, while PTPN7 tended to decrease in response to luteal cells treated with PGE2, IFNT, and the combination treatment. While it was hypothesized that T cell activation would be stimulated, and thus these T cell activation molecules would have increased in abundance, it is interesting that the luteal cells treated with these hormones decreased the abundance of these mRNA in cocultured T cells. It is certainly possible that the window of T cell activation was missed, as these mRNA were measured after 12 hours of coculture. However, it is also possible that these T cells reached a steady state, or an anergic state. Anergy is a hyporesponsive state that a T cell enters following incomplete T cell activation, typically defined by the lack of costimulation, and is generally considered a tolerance mechanism (Schwartz, 2003). The lowered abundance of mRNA for these T cell activation molecules might suggest these T cells are not going to be activated again, and thus the downregulation of the mRNA. While PGE2 is a known inhibitor of T cell proliferation (Goodwin et al., 1977) and this effect has been demonstrated in bovine T cells (Low and Hansen, 1988), PGE2 can also inhibit $CD4^+$ T cell activation by inhibiting LCK (Chemnitz et al., 2006). It is important to note that the T cells in this experiment were not

exposed to the PGE2 treatment directly, only the luteal cells were, and PGE2 was able to alter the luteal cells in way that was able to mediate T cell activation. PGE2 decreases the abundance of MHC class II in mouse macrophages (Mittal et al., 2001). Interestingly, MHC class II is expressed by luteal cells but expression is less in the CL of pregnancy compared to the CL of the cycle (Benyo et al., 1991). Luteal cells also express the costimulatory molecules, CD80 and CD86, and blocking these molecules with an antibody decreases luteal cell-stimulated T cell proliferation (Cannon and Pate, 2001). In human monocytes, PGE2 decreases CD80 and CD86 expression (Morichika et al., 2003). It would be interesting for a future study to determine if PGE2 decreases MHC class II, CD80, and CD86 on luteal cells, and if this is the mechanism by which T cell activation is decreased, and if these cells enter an anergic state. Additionally, the ability of luteal cells from the CL of pregnancy to stimulate T cell proliferation has not been studied. Although this experiment attempted to mimic pregnancy by treating luteal cells with hormones of pregnancy, using luteal cells isolated from the CL of pregnancy would be the best way to determine if the CL of pregnancy alters T cell proliferation and activation. This experiment also only measured mRNA, which could be seen as a limitation. Measuring the protein would have been the best methodology, especially because a couple of these molecules (ITK and LCK) are phosphorylated when activated. Measuring the protein phosphorylation would have given a more accurate depiction of T cell activation. Measuring protein via western blot was attempted, but, unfortunately, obtaining enough protein to quantify these proteins via western blot was not achieved.

Overall, the abundance of the T cell activation molecules, CD3e and LCK, decreased while PTPN7 tended to decrease in response to luteal cells treated with PGE2. This might be to induce a more immunotolerogenic environment in the CL of pregnancy and reduce T cell immunity. Supporting these findings, the transcriptomic profiling study mimicked pregnancy *in vivo* by infusing IFNT and/or PAGs into the uterus followed by examination of the

transcriptomic changes in the CL. The pathways upregulated in response to IFNT and/or PAGs also seem to support an immunotolerogenic environment.

Several questions about the critical window of early pregnancy were addressed in the second experiment including 1) what transcripts and/or pathways do pregnancy associated glycoproteins (PAG; will be referred to as PSPB from now on) alter in both whole luteal tissue and in luteal-resident immune cells, and 2) what is the phenotype of resident immune cells in the CL of early pregnancy. The 6-day IFNT infusion compared to the 6-day IFNT+PSPB infusion was designed to determine potential effects of PSPB on luteal tissue and resident immune cells. Interestingly, there were only 3 differentially abundant transcripts that changed in resident immune cells (RIC) isolated from CL exposed to the PSPB infusion. One of the differentially abundant transcripts that was greater in RIC from the 6-day IFNT+PSPB treatment was C3d receptor 2 (CR2), a complement protein receptor. This is the first report that PSPB can regulate complement-associated molecules. The traditional role of CR2 (CD21) is to bind the inactive cleavage product of C3, C3dg, and act within a receptor complex on the surface of B cells to activate B cells and trigger antibody production (Janeway et al., 2001). Although there are essentially none, or very few, B cells in the CL (Penny et al., 1999), CR2 is also known to be expressed on human T cells, although in lower abundance than on B cells (Fischer et al., 1991). It is possible the abundance of CR2 mRNA detected in this study originated from T cells. The role of CR2 on T cells may be to help with the adhesion of T cells to other cells to facilitate cell-cell interactions (Levy et al., 1992). Unfortunately, no studies to date have evaluated CR2 in the CL or any other reproductive tissue, so the abundance and functional role of CR2 in the female reproductive tract remains unknown. Interestingly, other complement components, including C3, C4, and CD55, have repeatedly been reported to change in abundance in various transcriptomic and proteomic profiling studies of the CL of pregnancy (Hughes et al., 2019, Hughes et al., 2020;

Mezera et al., 2021), suggesting complement components may play a role in the CL of pregnancy. The next chapter will expand upon the topic of complement in the CL.

The other transcript that was increased in RIC as a result of PSPB exposure was LOC786348, Serpin B4-like. Although this locus was an unannotated transcript within the bovine genome, it is most likely associated with Serpin B4-like. Serpins are a group of serine protease inhibitors that have a wide range of functions including roles in immunomodulation and coagulation (Law et al., 2006). While serpins are most notably known in reproductive biology for their role in serving as an immunomodulatory molecule to prevent fetal rejection in the ruminant uterus (Padua and Hansen, 2010), serpins have also been implicated in the CL of pregnancy. For example, serpin family E member 1 (SERPINE1) expression is induced in response to PGF2A and is known for its role during luteal regression to promote apoptosis, matrix remodeling, and vascular instability (Farberov and Meidan, 2016). However, SERPINE1 is also regulated by IFNT and decreases in response to IFNT in isolated luteal endothelial cells (Basavaraja et al., 2017). Additionally, while SERPINE1 expression normally increases in response to PGF2A in luteal tissue, the presence of IFNT inhibits the PGF2A-induced increase, suggesting SERPINE1 is downregulated in the CL of pregnancy to promote tissue stability (Basavaraja et al., 2017). Consistent with this finding, transcriptomic and proteomic profiling of the CL of pregnancy revealed that serpin family A member 5 (SERPINA5) mRNA and serpin family C member 1 (SERPINC1) protein were less abundant in the CL of pregnancy compared to the CL of the cycle (Hughes et al., 2019). Hughes et al. (2019) concluded that the decrease in the abundance of serpins may help stabilize the vasculature in the CL of pregnancy. Another serpin, serpin family G member 1 (SERPING1), is an inhibitor of the complement cascade and was found to increase between days 14 through 23 of early pregnancy (Hughes et al., 2020), which is consistent with the increase in abundance of another complement inhibitor, CD55, in the CL of pregnancy (Hughes et al., 2019; Hughes et al., 2020). However, keeping in mind this likely serpin molecule

was found in RIC, serpins do appear to play a role in the immune response and T cell immunity (Law et al., 2006; Bots and Medema et al., 2008), so perhaps serpins play a similar role in modulating immune cell response in the CL like they do in the uterus. Interestingly, certain serpins seem to increase (like serpin B4-like) and others decrease during in the CL of pregnancy. Since this locus for this particular transcript was unannotated, more information is needed to understand which specific serpins (if any) are expressed by specific immune cell populations in the CL and how these molecules might be directing immune cell function in the CL of pregnancy. Treating specific immune cell populations such as T cells and macrophages with IFNT and PSPB *in vitro* and measuring the abundance of various serpins including serpin B4, SERPINE1, and SERPING1 might offer further insight into how serpins are directing immune cell function in the CL of pregnancy.

Although there were only 3 differentially abundant transcripts found in RIC from the 6-day infusion treatments, pathway analysis was performed with 15 transcripts that had a $Q < 0.15$. One limitation to this analysis is the Ingenuity Pathway Analysis (IPA; Qiagen) software recommends a minimum of 100 differentially abundant transcripts for the best pathway analysis results. Despite this, the current pathway analysis is at least a starting point for understanding important pathways upregulated by PAGs in RIC. Two of the top pathways identified include eNOS signaling and complement signaling. Complement signaling and its role within T cells will be discussed in more detail in the next chapter, however, it is important to point out that complement signaling continues to be an important pathway identified in profiling studies of the CL of pregnancy (Hughes et al., 2019, Hughes et al., 2020, Mezera et al., 2021). Endothelial nitric oxide synthase (eNOS; NOS3), is an enzyme expressed in endothelial cells and is responsible for the production of nitric oxide (NO) to increase vascular dilation and permeability (Duran et al., 2010). eNOS can also be expressed in other cell types, including T cells (Kleinert and Forstermann, 2007). The bovine CL expresses both eNOS and inducible nitric oxide synthase

(iNOS), the latter is normally expressed by macrophages (Skarzynski et al., 2003). In the bovine CL, eNOS is expressed in capillaries in the periphery of the CL from the middle to late estrous cycle (Shirasuna et al., 2010), and is most abundant in the late CL (days 14-17; Skarzynski et al. 2003). eNOS and NO produced by the CL during the late estrous cycle play an important role in luteolysis by aiding in a transient increase in blood flow to the CL during luteal regression (Shirasuna et al., 2010) and inhibiting luteal steroidogenesis (Skarzynski and Okuda, 2000). Interestingly, if NO production is inhibited the luteolytic function of PGF2A is blocked (Skarzynksi et al., 2003). However, understanding the role of eNOS and NO signaling in the CL of pregnancy is less understood. NO is known to increase the production of PGE2 by both bovine luteal cells (Skarzynski and Okuda et al., 2000) and equine luteal cells (Ferrerira-Dias et al., 2011), which could be one potential role of eNOS and NO signaling in the CL of pregnancy. Additionally, transcriptomic profiling of the CL of pregnancy indicated that angiogenesis, VEGF signaling, and coagulation were all pathways upregulated in the CL of pregnancy compared to the CL of the cycle and are associated with the functional roles of eNOS (Hughes et al., 2019). Consistent with this finding, in an analysis of temporal changes in luteal mRNA during early pregnancy, a cluster of differentially abundant mRNA that peaked on day 17 were associated with NO and reactive oxygen species production by macrophages (Hughes et al., 2020). Perhaps, eNOS and NO production is important during days 14-17 for improving the structural integrity of the CL for continued maintenance, but begins to decline after day 20 so as not to compromise the CL. However, this is all speculation because no studies have examined eNOS signaling in any resident immune cell population in the CL. Evaluating eNOS expression in specific resident immune cell populations from the CL of pregnancy would be the first step to understanding the importance of eNOS in immune cells of the CL. Additionally, knocking down eNOS in immune cells and coculturing with luteal endothelial cells might be an interesting assay to determine if endothelial cell migration (angiogenesis) is altered in anyway.

In an attempt to characterize the phenotype of resident immune cells during early pregnancy, the top 100 most abundant transcripts identified in resident immune cells from the 6-day IFNT+PSPB treatment were analyzed using pathway analysis. The purpose was to determine if there were specific pathways that might provide insight into whether these immune cells are more "anti-inflammatory" or "proinflammatory." EIF2 signaling, eIF4 signaling, mTOR signaling, and NRF2-mediated oxidative stress response were 4 of the 5 upregulated pathways associated with the 100 most abundant transcripts found in resident immune cells. These pathways are not very specific to one phenotype of immune cells, and if anything these pathways are quite broad. However, all of these pathways have a common theme, regulation of transcription and translation. EIF2 and eIF4 part of a group of factors known as eukaryotic translation initiation factors (eIF). These proteins play an important role in initiating the start of mRNA translation (Hao et al., 2020). eIFs are regulated by mTOR, mammalian target of rapamycin (Hao et al., 2020). mTOR is a serine/threonine kinase known for its role in integrating environmental cues to cellular function. mTOR is stimulated by a variety of factors including growth factors and amino acids, and can also "sense" energy levels in cells to link metabolic changes and cellular function, including altering the differentiation of T cells (Powell et al., 2012). mTOR signaling occurs through two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Interestingly, depending upon which complex signaling occurs through, can alter T cell differentiation (Powell et al., 2012). Signaling through mTORC1 promotes mRNA translation by releasing the inhibition on eIF-4e as well as upregulation of genes involved with glucose metabolism, an important source of energy for activated T cells (Powell et al., 2012). Signaling through mTORC2, however, promotes cell survival, metabolism, and proliferation as well as promotion of Th2 differentiation (Powell et al., 2012). Intracellular complement signaling, which is necessary for T cell homeostasis, stimulates mTOR signaling and promotes T cell survival (Liszewski et al., 2013) while inhibition of mTORC1 promotes T cell

anergy. Exactly how mTOR signaling might direct effector function of resident immune cells from the CL of pregnancy is not known, but understanding whether these immune cells are signaling through mTORC1 and/or mTORC2 might provide more insight into the phenotype of these cells. Immune cell activation possibly mediated mTOR signaling might also tempered by the NRF2-mediated oxidative stress response pathway. Nuclear factor erythroid derived 2-related factor-2 (NRF2) is a transcription factor that regulates the production of antioxidant and other cellular protectant proteins to protect the cell from inflammatory stressors. NRF2 can also inhibit the secretion of proinflammatory cytokines (Kim and Surh, 2009). It would seem likely that this pathway would help to promote a more anti-inflammatory environment, however, it is hard to conclude if these immune cells actually have an anti-inflammatory phenotype. One reason is because this study examined a population of resident immune cells ($CD45^{+}$) isolated from the CL that included many different cell types including T cells, macrophages, monocytes, and neutrophils. Due to the heterogeneous mixture of cell types, it is not clear if all of these cell types share these pathways, or if there are competing roles of these immune cells given their diverse canonical functions.

In the luteal tissue collected from the 6-day treatments, there were 5 differentially abundant transcripts, but only 2 had a log2 fold change greater than 1 (or less than -1). These 2 differentially abundant transcripts are microtubule associated protein 1 light chain 3 gamma (MAP1LC3C) and armadillo-like helical domain containing 4 (ARMH4). MAP1LC3C was decreased in response to the 6-day IFNT+PSPB infusion compared to the 6-day IFNT infusion. MAP1LC3C belongs to a family of genes often associated with autophagy. Two genes within this family, MAP1LC3A and MAP1LC3B, were increased in the regressing CL compared to a midcycle CL (Aboelenain et al., 2015). It would make sense to decrease the abundance of transcripts within this family during early pregnancy to maintain the integrity of the CL. Unfortunately ARMH4 has not been studied in the CL of any species to date and its function

elsewhere is not well understood. Pathway analysis was performed using 20 transcripts with Q < 0.15. Some of the top pathways identified in luteal tissue to be upregulated in response to the 6-day IFNT+PSPB infusion were prothrombin activation, IL17 signaling, coagulation, and iNOS signaling. Some of these pathways are consistent with previous studies of the CL of pregnancy. Hughes et al. (2020) found thrombin signaling (2 pathways), IL17 signaling (3 pathways), and NO and ROS by macrophages were some of the top pathways with differentially abundant mRNA between days 14-23 of early pregnancy. Prothrombin and coagulation pathways were also identified in day 17 CL of pregnancy (Hughes et al., 2019). IL17 is a cytokine produced by Th17 cells that is associated with proinflammatory states as it recruits and activates neutrophils and promotes the production of other proinflammatory mediators (Polese et al., 2014). Although the environment within the CL of pregnancy is hypothesized to be more of an anti-inflammatory environment, very little is known about Th17 cells in the CL. In human peripheral blood, Th17 cell numbers appear to be lower or at least stable in in pregnant women compared to nonpregnant women (Santer-Nanan et al., 2009; Nakashima et al., 2010). Most studies examining the abundance of Th17 cells both in the periphery and the uterus during pregnancy focused on pathologies of pregnancy or recurrent miscarriages, in which case Th17 cells are found to be more abundant (Polese et al., 2014). Several studies reviewed by Polese et al. (2014) found progesterone shifts T cell differentiation toward Treg development and away from Th17 differentiation. Interestingly, IFNT decreases IL17 in the mouse intestinal tract (Ren et al., 2016). The suppression of Th17 cell differentiation and IL17 secretion by progesterone and IFNT, respectively, suggests a similar mechanism might occur in the CL of pregnancy. However, 5 pathways associated with IL17 signaling were upregulated in response to the 6-day IFNT+PSPB, suggesting PAGs may upregulate these pathways associated with IL17 signaling. Also, there were 3 pathways associated with IL17 signaling identified in the CL of pregnancy by Hughes et al. (2020). It seems perplexing that a cytokine associated with proinflammatory

pathways would be upregulated in the CL of pregnancy. There is, however, a fairly high degree of plasticity between Treg cells and Th17 cells (Kleinewietfeld and Hafler, 2013). TGFB and IL2 are necessary to induce a Treg ($Foxp3^{+}$) phenotype, while TGFB and IL21, IL6, or IL1B induce a Th17 phenotype. However, Tregs can acquire a Th17-like phenotype in response to IL1B and IL6 and still remain suppressive in function (Kleinewietfeld and Hafler, 2013). Th17 cells can also take on an anti-inflammatory state in the presence of IL4 (Kleinewietfeld and Hafler, 2013). Perhaps the plasticity between these cell types is what is occurring in luteal tissue during early pregnancy and PAGs might be the regulator. However, further studies will need to be conducted to understand more about Th17 cells in the CL, and the putative role of PAGs.

Similarly to IL17, iNOS (NOS2) is an enzyme typically associated with inflammatory states. iNOS, or inducible nitric oxide synthase, is typically "induced" in a wide range of cells by cytokines and other signaling molecules to produce large amounts of nitric oxide during an immune response. As with eNOS signaling, although overproduction of NO would be detrimental to luteal tissue, controlled production of NO by iNOS also allows for vasculature remodeling and PGE2 production (Lechner et al., 2005), which may be supportive in the CL of pregnancy.

Overall, the comparison between 6-day IFNT and 6-day IFNT+PSPB suggests that PAGs may be involved in immunomodulation in the CL. Further studies would need to be conducted to understand the role of PAGs in the CL, but this is the first study to demonstrate uterine-derived PAGs can induce observable transcriptional changes in the CL.

Eighty-three transcripts were found to be different in the RIC of the 3-day IFNT group compared to the 3-day BSA group. Most of the transcripts that were in greater abundance in the 3-day IFNT infusion treatment were interferon-stimulated genes (ISG), which demonstrates that IFNT infused into the uterus reached the CL and was able to induce transcriptional changes in the RIC. The main pathways associated with the differentially abundant transcripts were those involved in interferon signaling and other antiviral signaling pathways, which is unsurprising

given the number of ISGs that were differentially abundant. In the luteal tissue, there were 117 differentially abundant transcripts. The majority of the different transcripts were also ISGs. Similar to the RIC, interferon signaling and other antiviral signaling pathways were upregulated in response to IFNT, providing the "proof of concept" that IFNT infused into the uterus did evoke transcriptomic changes in luteal tissue.

The 3-day IFNT infusion compared to the 6-day IFNT infusion aimed to address short versus long exposure to IFNT in early pregnancy. In the RIC there were only 8 differentially abundant transcripts with an additional 32 transcripts that tended to differ. The pathways that were identified to be upregulated in the 6-day IFNT infusion treatment compared to the 3-day IFNT infusion treatment were quite different than those identified in the 3-day infusion comparisons. Ephrin A signaling, sphingosine-1-phosphate signaling, and IL15 production were in the top 5 pathways identified. Although these weren't direct comparisons, there is a clear shift from upregulation of interferon signaling and antiviral pathways in the 3-day infusions compared to pathways associated with tissue homeostasis, cell proliferation, and T cell homeostasis when exposed to a uterine infusion of IFNT for 6-days, all of which would be beneficial to luteal survival during early pregnancy. Ephrin A signaling was one pathway upregulated in the 6-day IFNT infusion compared to the 3-day IFNT infusion in RIC. Ephrins are ligands of the Eph receptor (erythropoietin-producing hepatocellular carcinoma) and the interaction of these molecules are involved in a variety of processes including embryonic development and tissue homeostasis (Darling and Lamb, 2019). Limited research has been conducted to understand ephrin signaling in the CL. However, in the mouse ovary, ephrin-A5 is required for a complete LH response and subsequent ovulation as ephrin-A5 knockout mice ovulate fewer oocytes compared to controls (Buensuceso et al., 2016). In the CL another ephrin, ephrin B1, is increased immediately following ovulation in luteinizing human granulosal cells (Egawa et al., 2003). It is speculated that ephrin B1 might play a role in endothelial cell migration and tissue remodeling as

ephrins are known to play a role in both processes (Egawa et al., 2003). There is evidence that ephrin receptors and signaling play a role in activating immune cells and facilitating immune cell migration (Darling and Lamb, 2019). Both ephrin A and ephrin B receptors and ligands are expressed in monocytes, macrophages, and T cells and facilitate cell migration, cell-cell contact, T cell activation, and TCR signaling (Darling and Lamb, 2019). As discussed previously, Hughes et al. (2019) found that T cell activation and signaling was increased in the CL of pregnancy and perhaps ephrin is one mechanism by which this happens. Sphingosine-1-phosphate (S1P) signaling is another pathway associated with cell proliferation, migration, and survival as well as angiogenesis and immune responses (Becker et al., 2011; Aoki et al., 2016) and was upregulated in RIC from the 6-day IFNT infusion. S1P is a bioactive sphingolipid that is produced by the phosphorylation of sphingosine by sphingosine kinases and acts through its G protein-coupled receptor, S1P receptors 1-5 (Aoki et al., 2016). In the ovary, S1P is associated with follicular fluid high density lipoproteins and induces migration of primary luteinized human granulosal cells, suggesting S1P might play a role in luteal development (Becker et al., 2011). In the rat CL, S1P blocked PGF2A induced apoptosis of luteal cells and increased blood vessel density (Hernandez et al., 2009). S1P also improved vascular integrity in the ovary of rat models for ovarian hyperstimulation syndrome (Di Pietro et al., 2017). S1P might one mechanism by which the CL of pregnancy evades regression from uterine pulses of PGF2A. However, the study of S1P in immune cells of the CL has not been studied. S1P is known to be expressed in inflamed tissues and helps keep T cells at the site of inflammation (Aoki et al., 2016). Additionally, S1P receptor 1 (S1PR1) receptor signaling in T cells can induce Treg development (Aoki et al., 2016). These might be potential pathways of interest for studying the role of S1P signaling in RIC of the CL. Lastly, IL15 signaling was also an upregulated pathway in RIC after the 6-day IFNT infusion compared to the 3-day IFNT infusion. IL15 is considered to be a proinflammatory cytokine because it stimulates the production of memory T cells and promotes the survival of $CD8^+$ T cells

(Brincks and Woodland, 2010; Pilipow et al., 2015). More relevant to the CL, IL15 is involved in the development and activation of $\gamma\delta^+$ T cells (Pilipow et al., 2015). Poole and Pate (2012) reported a greater number of $\gamma\delta^+$ CD8$\alpha\beta^+$ T cells, an anti-inflammatory cell type, to be present in the CL of early pregnancy. It would be interesting to determine if IL15 might be responsible for the increased number of this cell type in the CL of pregnancy.

In luteal tissue a similar shift in pathways occurred with longer IFNT exposure. Instead of interferon signaling and antiviral pathways, as observed with the 3-day infusions, ILK signaling and IL8 signaling were the top pathways of interest identified in luteal tissue from the 6-day IFNT infusion treatment. Integrin-linked kinase (ILK) is a scaffold protein that connects the actin cytoskeleton with the cytoplasmic tail of β subunits of integrins (Yen et al., 2014). ILK aids in cell migration, survival, and even angiogenesis (Yen et al., 2014). In the CL, ILK signaling has not been extensively studied. Talbott et al. (2017) found ILK signaling to be upregulated in CL 1 hour post-PGF2A injection, which might suggest ILK signaling might initiate remodeling or restructuring of the CL as it prepares to undergo luteal regression. The same restructuring along with cell survival promoted by ILK signaling might be one effect of longer term IFNT exposure to support the CL during early pregnancy. Unlike ILK signaling pathways, IL8 signaling has been studied in the CL due to its primary role in recruiting and activating neutrophils (Polec et al., 2009; Jiemtaweeboon et al., 2011). IL8 is important in ovulation as the loss or inhibition of IL8 prevents the recruitment of neutrophils to the ovary and results in inhibited ovulation in rabbits (Ujioka et al., 1998). During luteal development, neutrophils are recruited to the developing bovine CL by IL8 and appear to stimulate angiogenesis (Jiemtaweeboon et al., 2011). IL8 mRNA also increases in the bovine CL in response to *in vivo* administration of PGF2A and IL8 increased migration of neutrophils *in vitro*, suggesting a role in immune cell recruitment to the CL during luteal regression (Talbott et al., 2014). Shirasuna et al. (2015) found an increased number of neutrophils in the bovine CL of

pregnancy and IL8 was greater compared to the CL of nonpregnant animals. Additionally, *in vitro* IFNT directly increased IL8 mRNA in luteal cells, and IFNT-activated neutrophils, along with IL8, increased progesterone production from luteal cells (Shirasuna et al., 2015). IFNT also promotes lymphoangiogenesis in the bovine CL of early pregnancy (Nitta et al., 2011) and has been shown to downregulate luteolytic genes in luteal endothelial cells and promote their survival (Basavaraja et al., 2017). Overall, IL8 signaling in the CL appears to be upregulated by IFNT exposure and might be important for the recruitment of neutrophils and vascular remodeling for stability of the CL of pregnancy. This would be an interesting pathway to study further for understanding the mechanisms of luteal rescue and maintenance of the CL of pregnancy.

Conclusion

Although there is a lot of work to be done to understand the functional role of immune cells in the CL of pregnancy and understanding how PSPB influences the state of this tissue, this study demonstrated that PSPB can alter the transcriptome of both resident immune cells and luteal tissue. It is clear that PSPB supports pathways that promote immunomodulation and structural remodeling to support sustained luteal function during pregnancy. Unfortunately, the phenotype of resident immune cells in the experimental model of pregnancy did not yield very clear results, but possibly supports immune cell activation and inhibition of inflammatory responses. However, follow up functional experiments will be needed to support these claims.

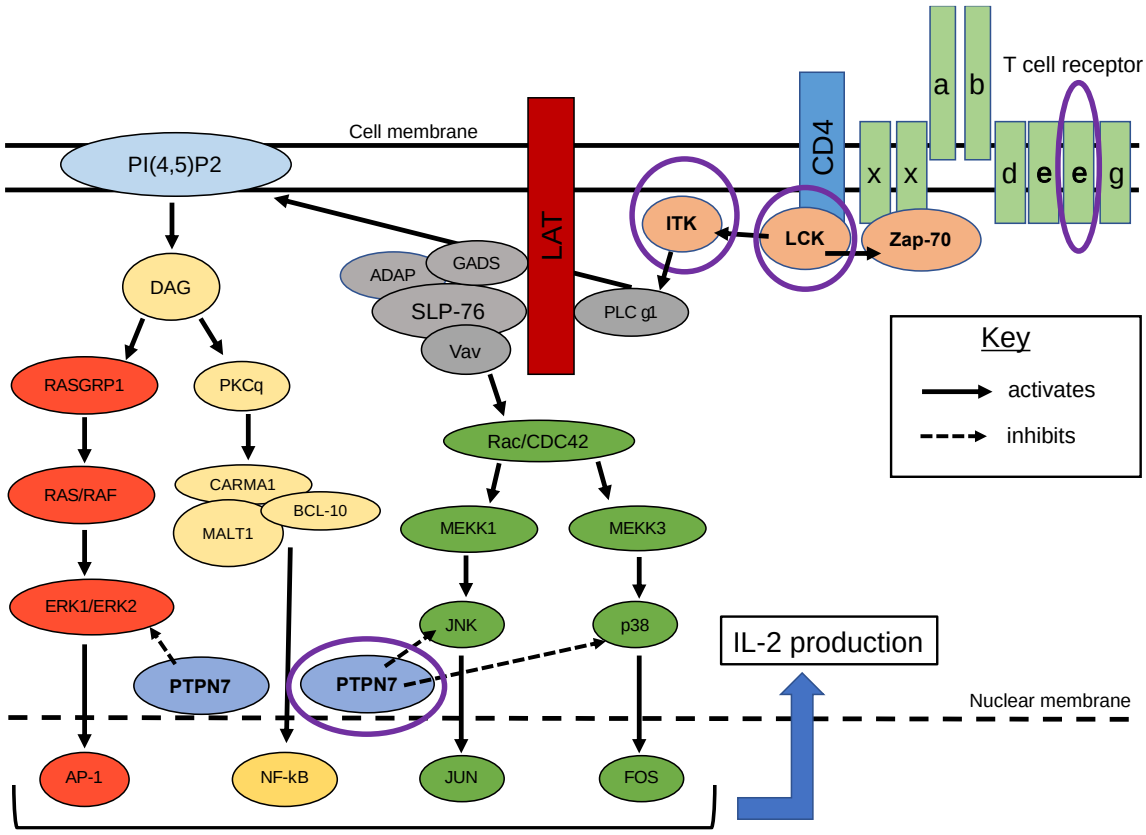


Figure 3-1. A schematic of T cell receptor (TCR) signaling upon T cell activation. Molecules circled in purple were evaluated in T cells cocultured with luteal cells treated with IFNT, PGE2, and a combination treatment.

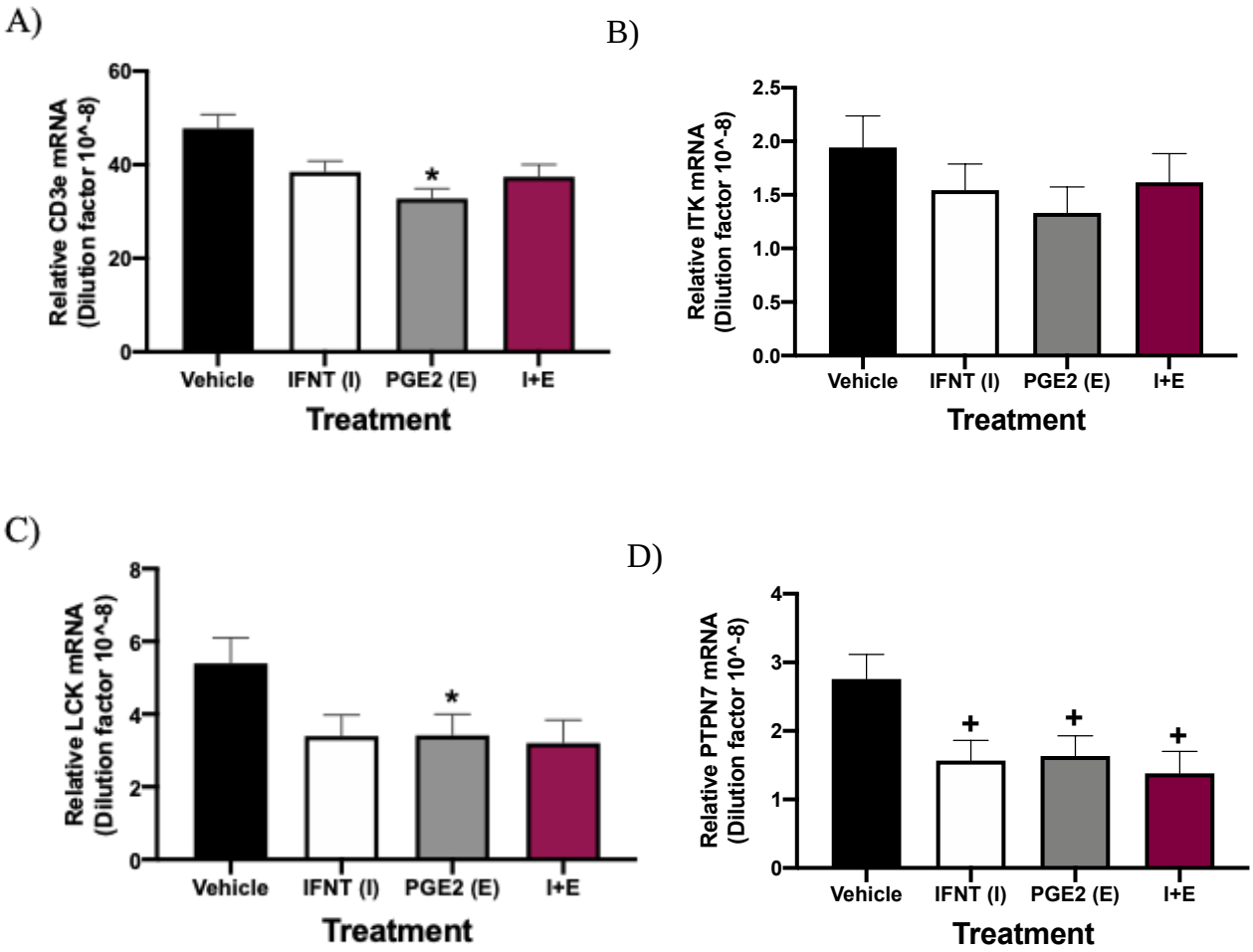


Figure 3-2. T cell receptor activation molecules in T cells cocultured with pre-treated luteal cells. A) CD3e mRNA, B) ITK mRNA, C) LCK mRNA, and D) PTPN7 mRNA. * denotes a statistical significance with $p < 0.05$ and + denotes a statistical tendency $0.05 < p < 0.1$

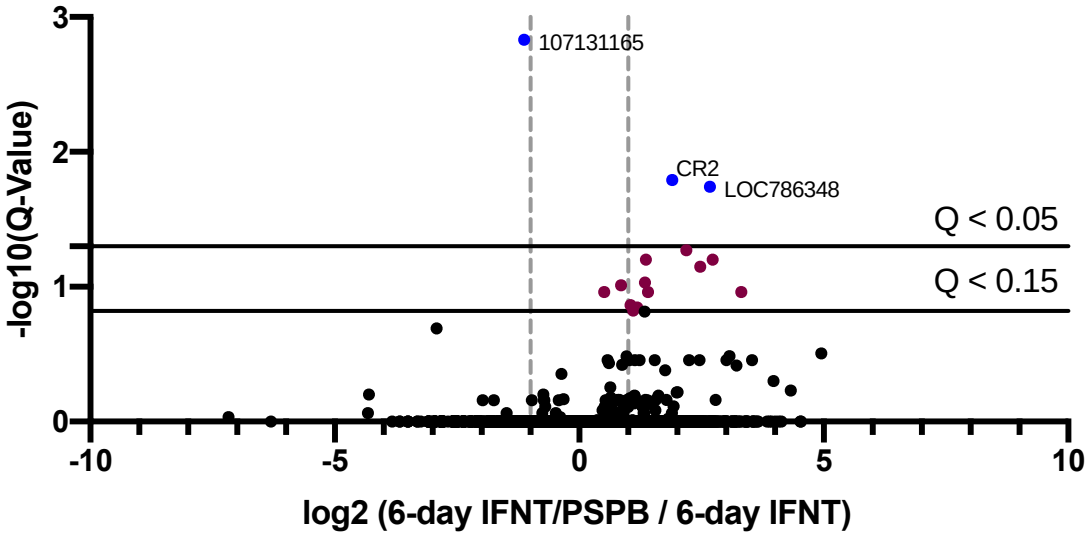


Figure 3-3. Volcano plot of transcripts from resident immune cells isolated from CL exposed to 6-day uterine infusion treatments. The blue points represent differentially abundant mRNA (Q < 0.05 and Log2 fold change ≥ 1 or ≤ -1) from immune cells isolated from CL exposed to 6 day uterine infusion treatments. The maroon points represent mRNA that tended to differ (0.05 < Q < 0.15 and Log2 fold change ≥ 1 or ≤ -1) between treatments from immune cells isolated from CL exposed to 6 day uterine infusion treatments. The solid black lines indicate thresholds for the Q-value, either less than 0.05 or less than 0.15. The grey dotted line indicates the log2 fold change threshold of either 1 (upregulated in 6-day IFNT+PSBP) or -1 (downregulated in 6-day IFNT+PSPB).

Gene Name	Q-value	Log2 Fold Change
Zinc finger protein 665-like (107131165)	0.00148	-1.13
C3d receptor 2 (CR2)	0.02	1.9
Serpin B4-like (LOC786348)	0.02	2.67
Heat shock protein family B member 8 (HSPB8)	0.0542	2.19
SH3 and cysteine rich domain 2 (STAC2)	0.0635	1.36
Protein kinase AMP-activated non-catalytic subunit gamma 3 (PRKAG3)	0.0635	2.72
Amine oxidase copper containing 1 (AOC1)	0.0711	2.47
Glycoprotein nmb (GPNMB)	0.0927	1.34
Protein phosphatase 1 regulatory inhibitor subunit 1b (PPP1R1B)	0.109	3.31
Complement C1q like 1 (C1QL1)	0.109	1.41
Calcium homeostasis modulator family member 2 (CALHM2)	0.137	1.04
Cyclic nucleotide gated channel subunit alpha 3 (CNGA3)	0.143	1.18
Neurexin 2 (NRXN2)	0.149	1.10
GRB2 associated regulator of MAPK1 subtype 2 (GAREM2)	0.0979	0.854
Rho GTPase activating protein 1 (ARHGAP1)	0.109	0.508

Table 3-1. Table of transcripts (Q < 0.15) from resident immune cells isolated from CL exposed to 6-day infusions. The 3 transcripts at the top of the table, highlighted in light grey, were considered differentially abundant with Q < 0.05 and a log2 fold change greater than 1 or less than -1. The transcripts in the middle of the table, highlighted in a darker grey, tended to differ between 6-day infusion treatments with 0.05 < Q < 0.15 and log2 fold change greater than 1 or less than -1. The last 2 transcripts at the bottom of the table, highlighted in dark grey, tended

to differ between 6-day infusion treatments, but had a log2 fold change less than 1. Log2 fold change of 0 indicates no change between 6-day infusions. A positive number for log2 fold change indicates greater abundance in the 6-day IFNT+PSPB infusion compared to 6-day IFNT infusion, while a negative log2 fold change indicates less abundance in the 6-day IFNT+PSPB infusion compared to the 6-day IFNT infusion.

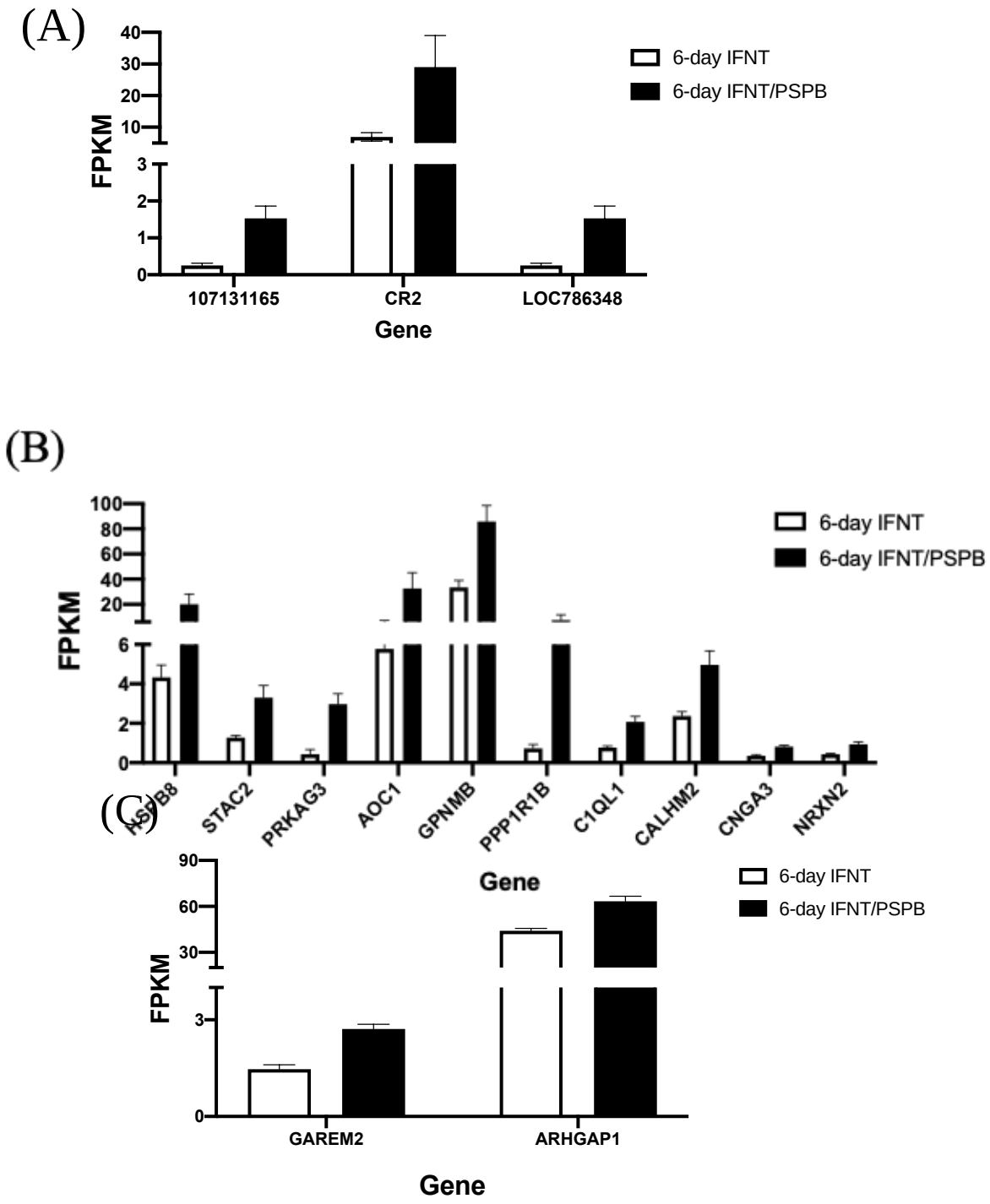


Figure 3-4. A graphical representation of fragments per kilobase million (FPKM) for each transcript with Q < 0.15 from resident immune cells (6-day infusions). A) FPKM of

transcripts with Q < 0.05 and log2 fold change greater than 1 or less than -1, B) FPKM of transcripts with 0.05 < Q < 0.15 and log2 fold change greater than 1 or less than -1. C) FPKM of transcripts with 0.05 < Q < 0.15 and log2 fold change between 0 and 1.

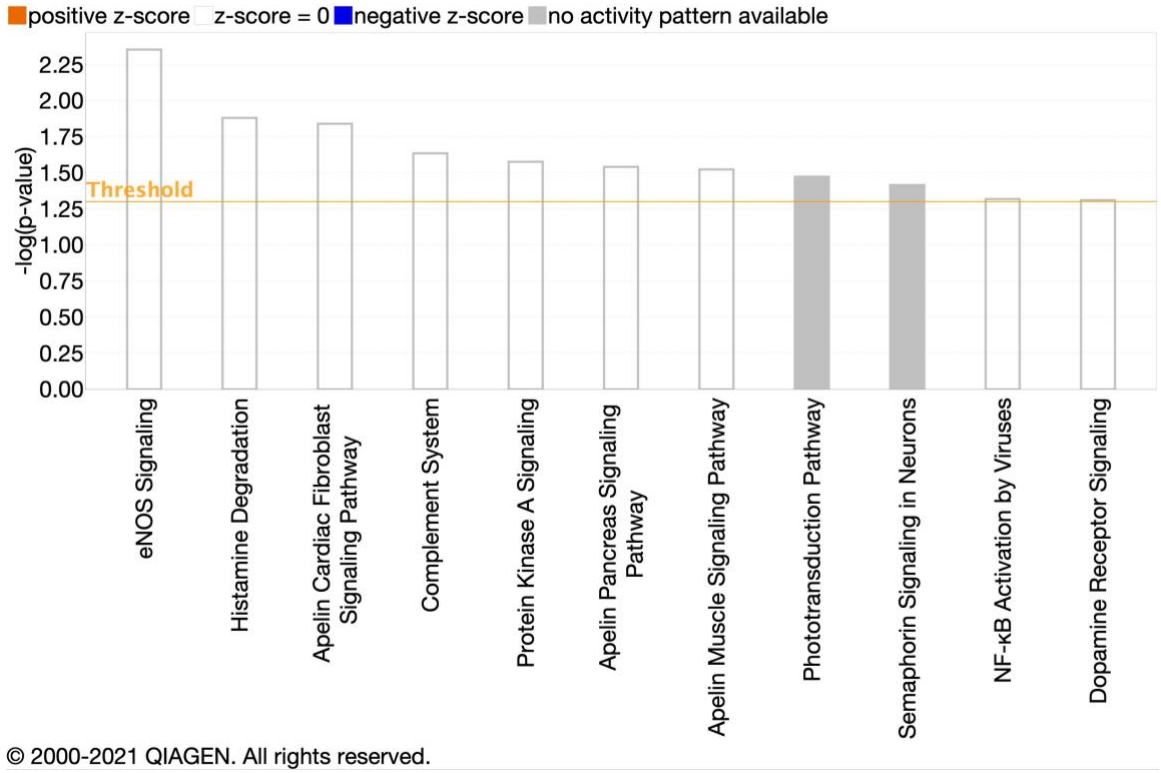


Figure 3-5. Ingenuity pathway analysis (IPA; Qiagen) of all transcripts from resident immune cells with Q < 0.15 (6-day infusions). The top 11 pathways IPA associated with transcripts with Q < 0.15 from resident immune cells isolated from CL exposed to 6-day uterine infusions.

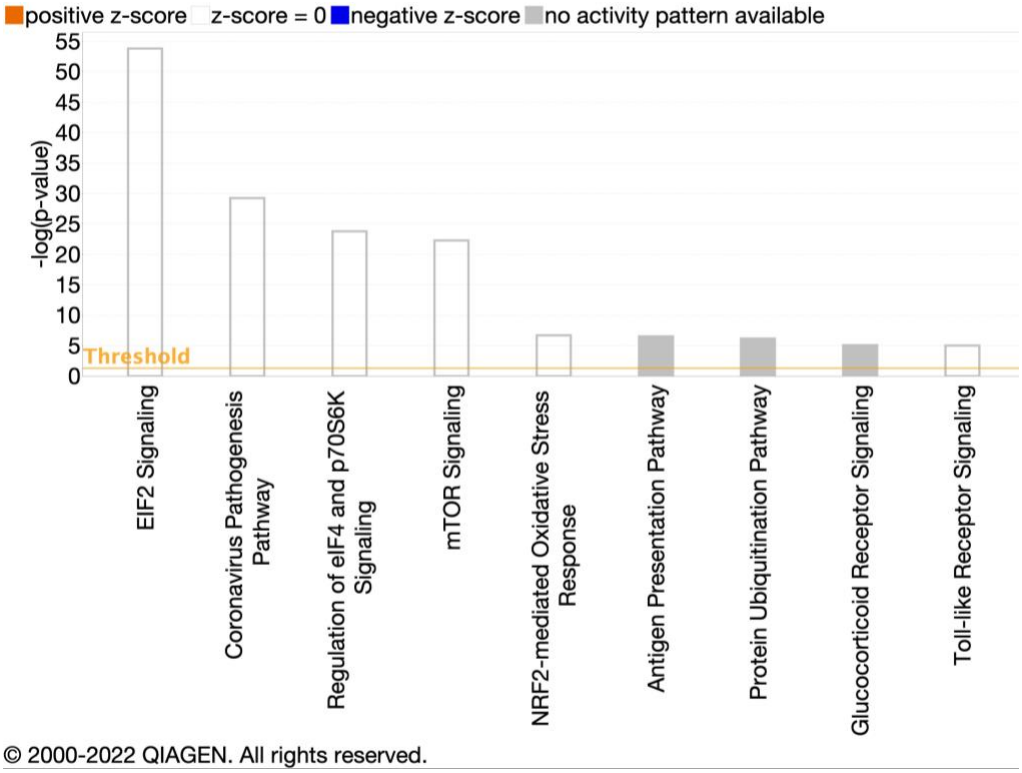


Figure 3-6. Pathway analysis of the top 100 most abundant transcripts in resident immune cells from 6-day IFNT+PSPB infusion. The top 9 pathways associated with the 100 most abundant transcripts in resident immune cells from the 6-day IFNT+PSPB infusion.

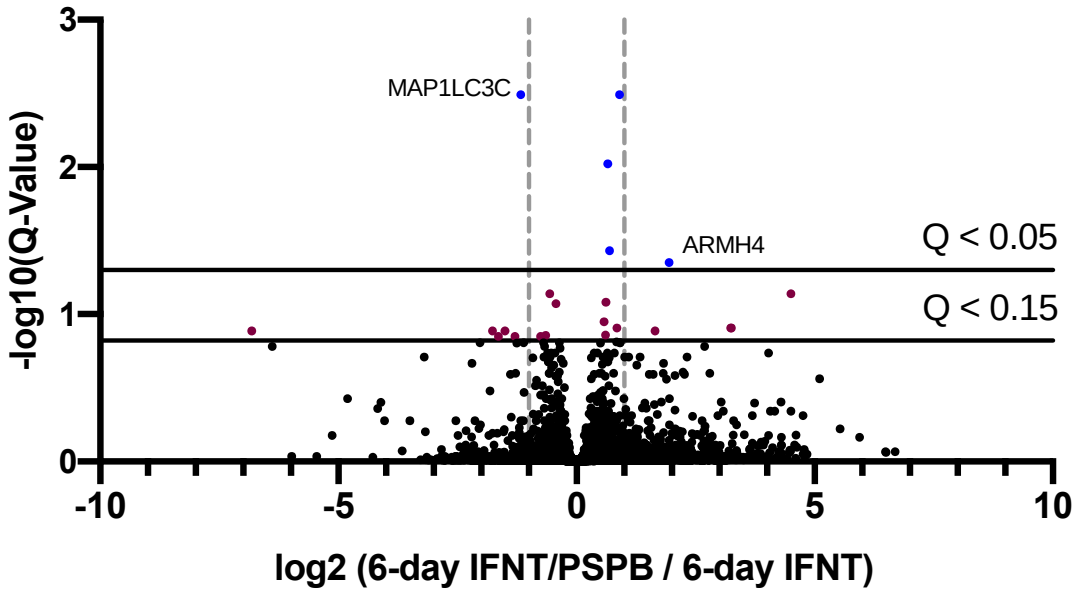


Figure 3-7. Volcano plot of transcripts from luteal tissue exposed to 6-day uterine infusion treatments. The blue points represent differentially abundant mRNA (Q < 0.05 and Log2 fold change ≥ 1 or ≤ -1) from luteal tissue exposed to 6 day uterine infusion treatments. The maroon points represent mRNA that tended to differ (0.05 < Q < 0.15 and Log2 fold change ≥ 1 or ≤ -1) between treatments from luteal tissue exposed to 6 day uterine infusion treatments. The solid black lines indicate thresholds for the Q-value, either less than 0.05 or less than 0.15. The grey dotted line indicates the log2 fold change threshold of either 1 (upregulated in 6-day IFNT+PSBP) or -1 (downregulated in 6-day IFNT+PSPB).

Gene Name	Q-value	Log2 Fold Change
Microtubule associated protein 1 light chain 3 gamma (MAP1LC3C)	0.0032	-1.17
Armadillo-like helical domain containing 4 (ARMH4)	0.044	1.94
Cyclic nucleotide gated channel alpha 3 (CNGA3)	0.0032	0.899
Zinc finger protein 665-like (101902574)	0.0095	0.658
Family with sequence similarity 214 member A (FAM214A)	0.037	0.69
PHD finger protein 21B (PHF21B)	0.12	3.23
Cytochrome P450 family 4 subfamily F member 2 (CYP4F2)	0.12	3.25
Epiregulin (EREG)	0.13	-1.50
C-C motif chemokine ligand 2 (CCL2)	0.13	-1.77
Early growth response 4 (EGR4)	0.13	-6.82
RNA binding motif protein 20 (RBM20)	0.13	1.65
Regulator of calcineurin 1 (RCAN1)	0.14	-1.63
CUB domain containing protein 1 (CDCP1)	0.14	-1.30
RNA polymerase II subunit F (POLR3F)	0.08	0.61
Solute carrier family 41 member 1 (SLC41A1)	0.08	-0.43
Bone morphogenetic protein receptor type 1A (BMPR1A)	0.11	0.58
Melanocyte inducing transcription factor (MITF)	0.12	0.85
Deoxyribonuclease 1 (DNASE1)	0.14	0.61
Thrombomodulin (THBD)	0.14	-0.65
Interferon regulatory factor 1 (IRF1)	0.14	-0.75

Table 3-2. Table of transcripts (Q < 0.15) from luteal tissue exposed to 6-day uterine infusions. The top 2 transcripts, highlighted in light grey, were differentially abundant transcripts (Q < 0.05) with a log2 fold change greater than 1 or less than -1. The next 3 transcripts,

highlighted in a darker grey, were also differentially abundant transcripts, but with a log2 fold change between 0 and 1. The transcripts highlighted in dark grey tended to differ between treatments ($0.05 < Q < 0.15$) with a log2 fold change greater than 1 or less than -1. The transcripts highlighted in white also tended to differ between treatments ($0.05 < Q < 0.15$), but did not have a log2 fold change greater than 1 or less -1. Log2 fold change of 0 indicates no change between 6-day infusions. A positive number for log2 fold change indicates greater abundance in the 6-day IFNT+PSPB infusion compared to 6-day IFNT infusion, while a negative log2 fold change indicates less abundance in the 6-day IFNT+PSPB infusion compared to the 6-day IFNT infusion.

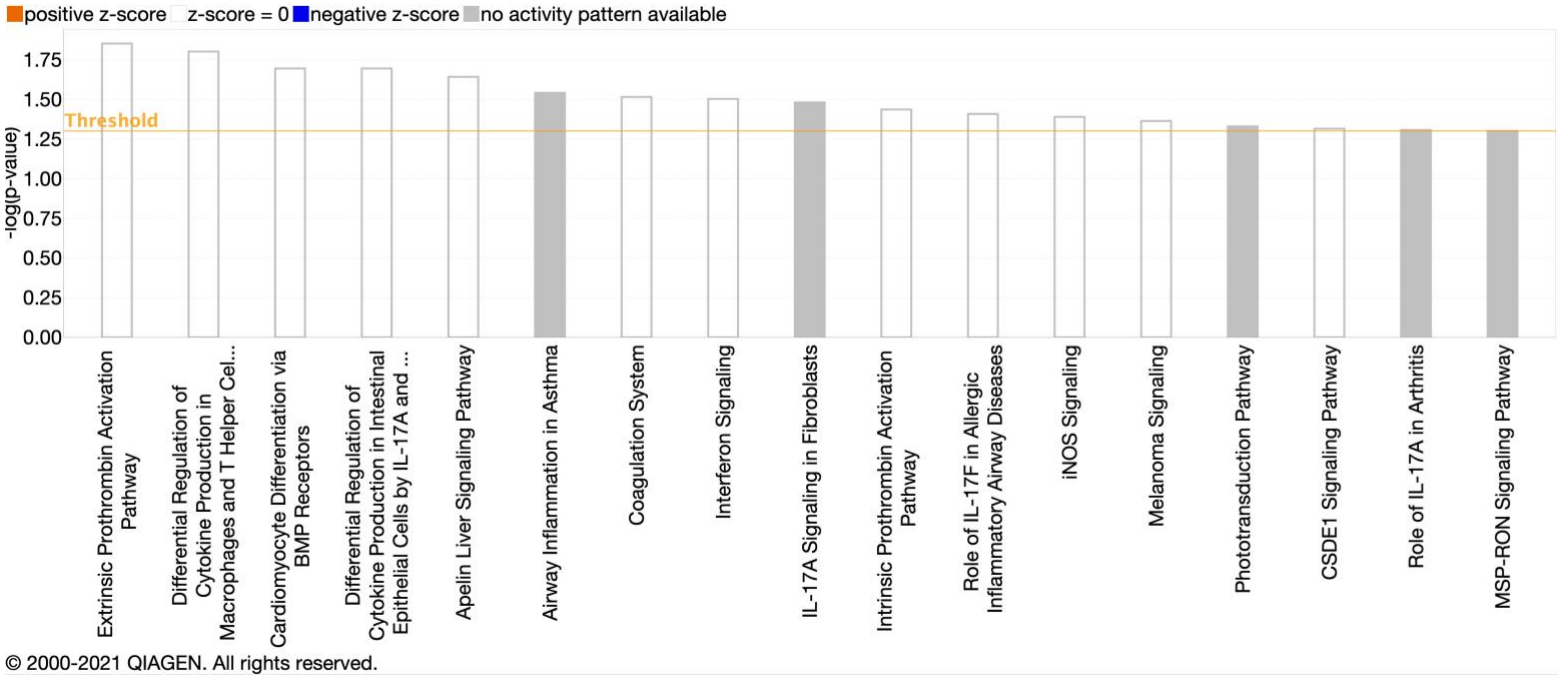


Figure 3-8. Ingenuity pathway analysis (IPA; Qiagen) of all transcripts from luteal tissue with Q < 0.15 (6-day infusions). The top pathways IPA associated with transcripts with Q < 0.15 from luteal tissue exposed to 6-day uterine infusions.

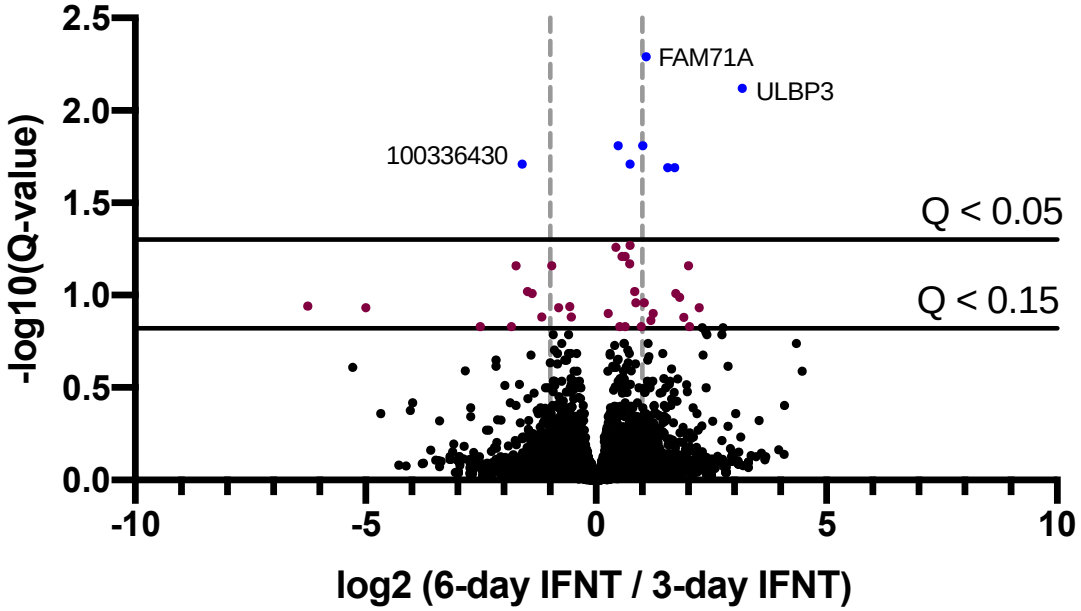


Figure 3-9. Volcano plot of transcripts from resident immune cells exposed to 3-day and 6-day IFNT uterine infusion treatments. The blue points represent differentially abundant mRNA ($Q < 0.05$) from immune cells isolated from CL exposed to 3-day and 6-day IFNT uterine infusion treatments. The maroon points represent mRNA that tended to differ ($0.05 < Q < 0.15$) between the 3-day and 6-day treatments from resident immune cells. The solid black lines indicate thresholds for the Q-value, either less than 0.05 or less than 0.15. The grey dotted line indicates the log2 fold change threshold of either 1 (upregulated in 6-day IFNT) or -1 (downregulated in 6-day IFNT).

Gene Name	Q-value	Log2 Fold Change
Family with sequence similarity 71 member A (FAM71A)	0.00512	1.08
UL16 binding protein 3 (ULBP3)	0.00762	3.17
Rho Family GTPase 1 (RND1)	0.0154	1.01
Pre-B lymphocyte gene 2 (100336430/VPREB2)	0.0195	-1.61
UL16 binding protein 3 like (LOC100850276)	0.0205	1.70
Leucine rich repeat containing 4 (LRRC4)	0.0205	1.55
STE20 Like Kinase (SLK)	0.0154	0.48
EPH Receptor A2 (EPHA2)	0.0195	0.73

Table 3-3. Table of transcripts (Q < 0.05) from resident immune cells exposed to 3-day and 6-day IFNT uterine infusions. The top 6 transcripts, highlighted in light grey, were differentially abundant transcripts (Q < 0.05) with a log2 fold change greater than 1 or less than -1. The last 2 transcripts, highlighted in a darker grey, were also differentially abundant transcripts, but with a log2 fold change between 0 and 1. Log2 fold change of 0 indicates no change between 3-day and 6-day IFNT infusions. A positive number for log2 fold change indicates greater abundance in the 6-day IFNT infusion compared to 3-day IFNT infusion, while a negative log2 fold change indicates less abundance in the 6-day IFNT infusion compared to the 3-day IFNT infusion.

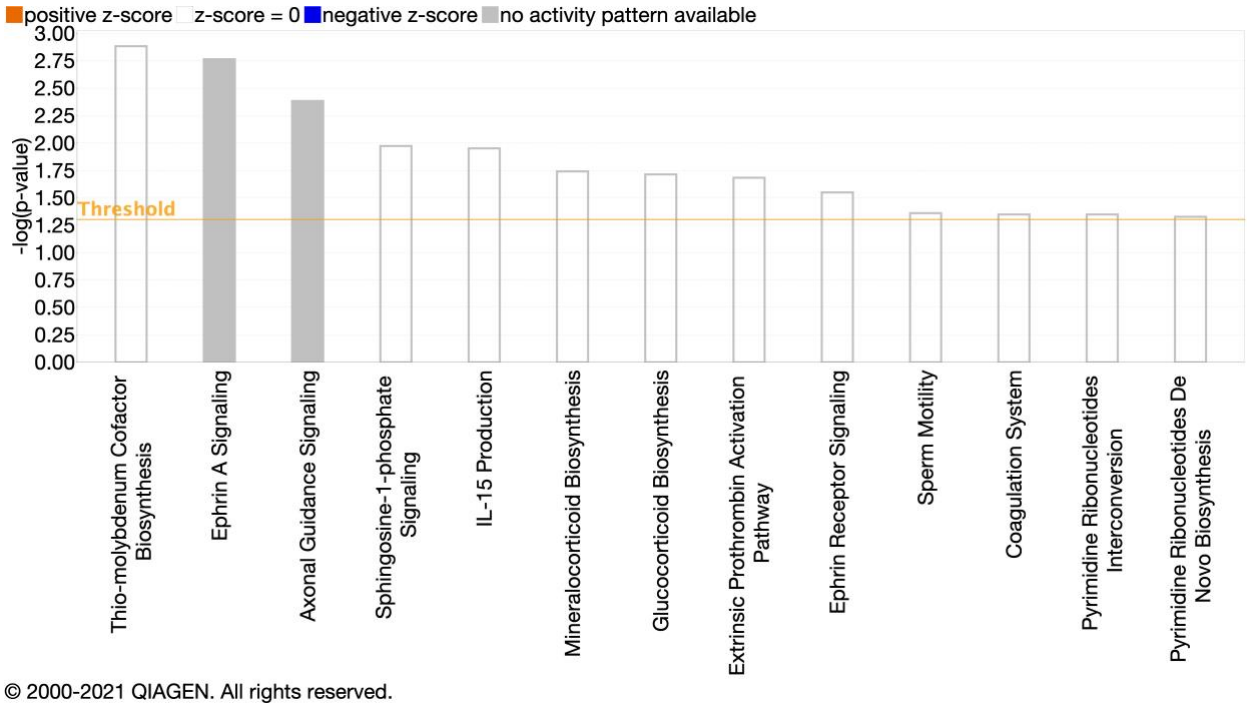


Figure 3-10. Ingenuity pathway analysis (IPA; Qiagen) of all transcripts from resident immune cells with Q < 0.15 (3-day and 6-day IFNT infusions). The top pathways IPA associated with transcripts with Q < 0.15 from resident immune exposed to 3-day and 6-day uterine IFNT infusions.

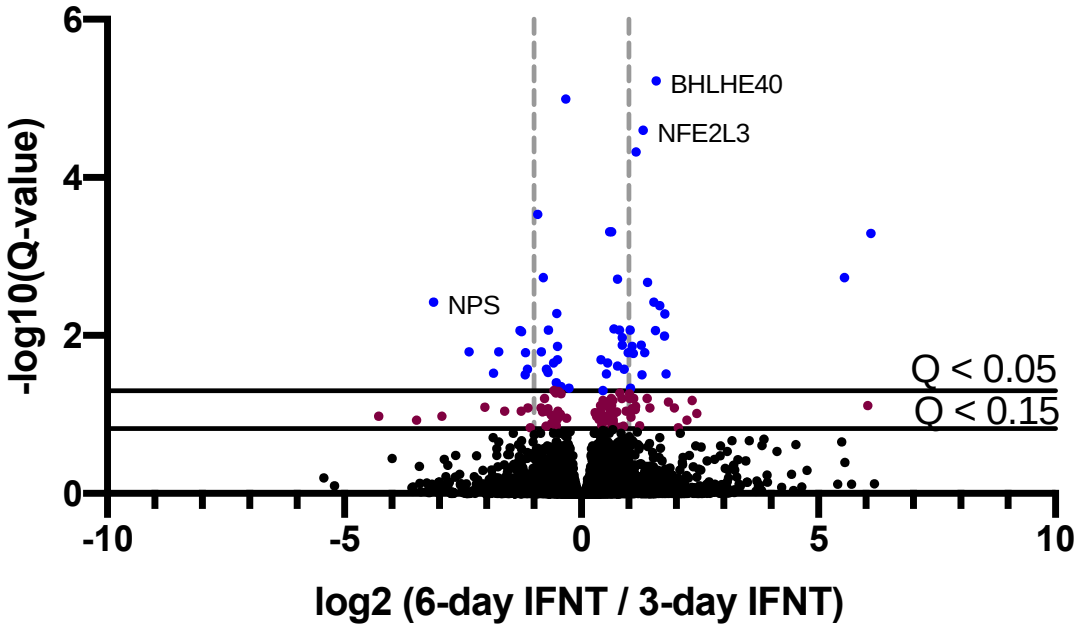


Figure 3-11. Volcano plot of transcripts from luteal tissue exposed to 3-day and 6-day IFNT uterine infusion treatments. The blue points represent differentially abundant mRNA (Q < 0.05) from luteal tissue exposed to 3-day and 6-day IFNT uterine infusion treatments. The maroon points represent mRNA that tended to differ (0.05 < Q < 0.15) between the 3-day and 6-day treatments in luteal tissue. The solid black lines indicate thresholds for the Q-value, either less than 0.05 or less than 0.15. The grey dotted line indicates the log2 fold change threshold of either 1 (upregulated in 6-day IFNT) or -1 (downregulated in 6-day IFNT).

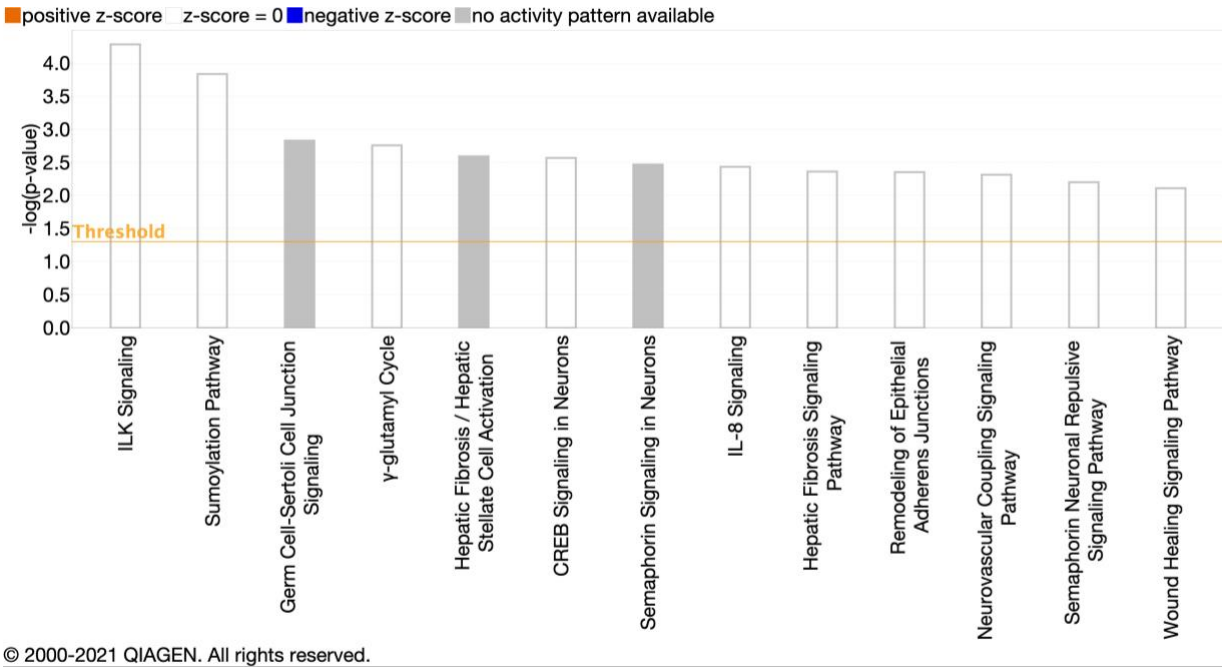


Figure 3-12. Ingenuity pathway analysis (IPA; Qiagen) of all transcripts from luteal tissue with Q < 0.15 (3-day and 6-day IFNT infusions). The top pathways IPA associated with transcripts with Q < 0.15 from luteal tissue exposed to 3-day and 6-day uterine IFNT infusions.

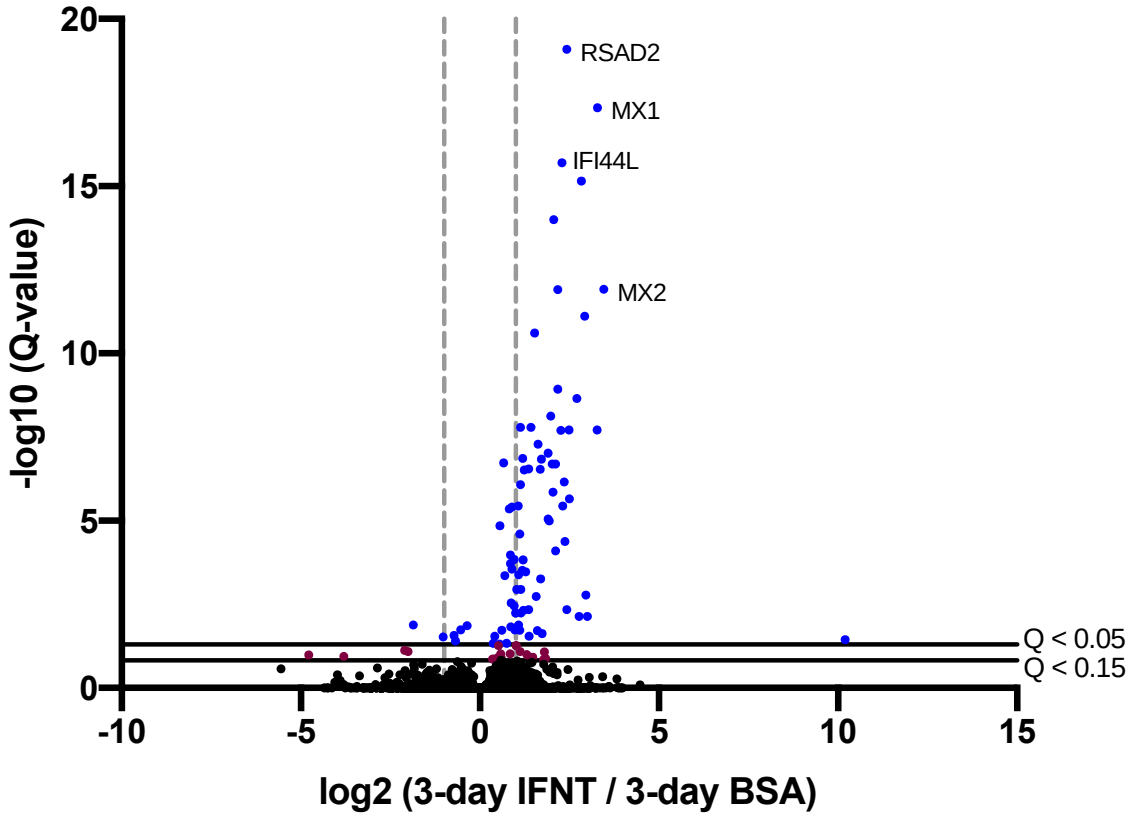


Figure 3-13. Volcano plot of transcripts from resident immune cells exposed to 3-day uterine infusion treatments. The blue points represent differentially abundant mRNA (Q < 0.05) from resident immune cells isolated from CL exposed to 3-day uterine infusion treatments. The maroon points represent mRNA that tended to differ (0.05 < Q < 0.15) between the 3-day treatments from resident immune cells. The solid black lines indicate thresholds for the Q-value, either less than 0.05 or less than 0.15. The grey dotted line indicates the log2 fold change threshold of either 1 (upregulated in 3-day IFNT) or -1 (downregulated in 3-day IFNT).

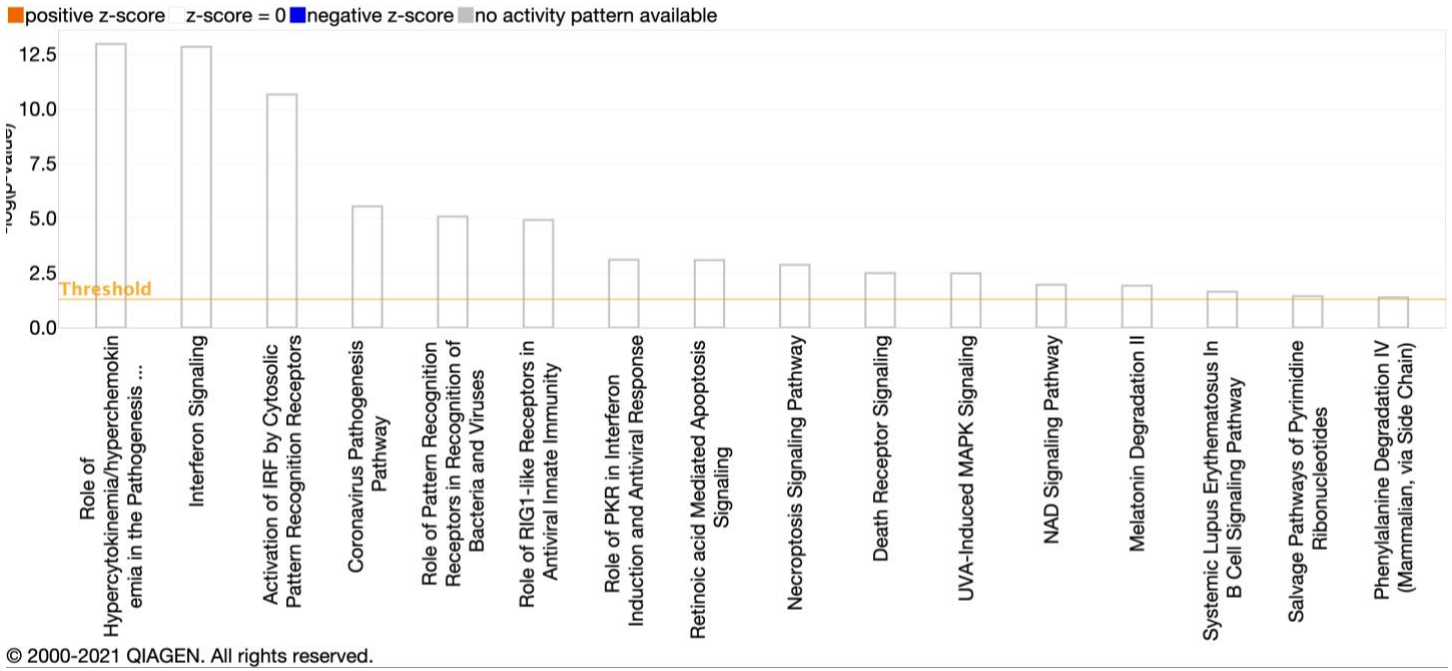


Figure 3-14. Ingenuity pathway analysis (IPA; Qiagen) of differentially abundant transcripts from resident immune cells exposed to 3-day uterine infusion treatments. The top pathways associated with differentially abundant transcripts ($Q < 0.05$) from resident immune cells exposed to 3-day uterine infusions.

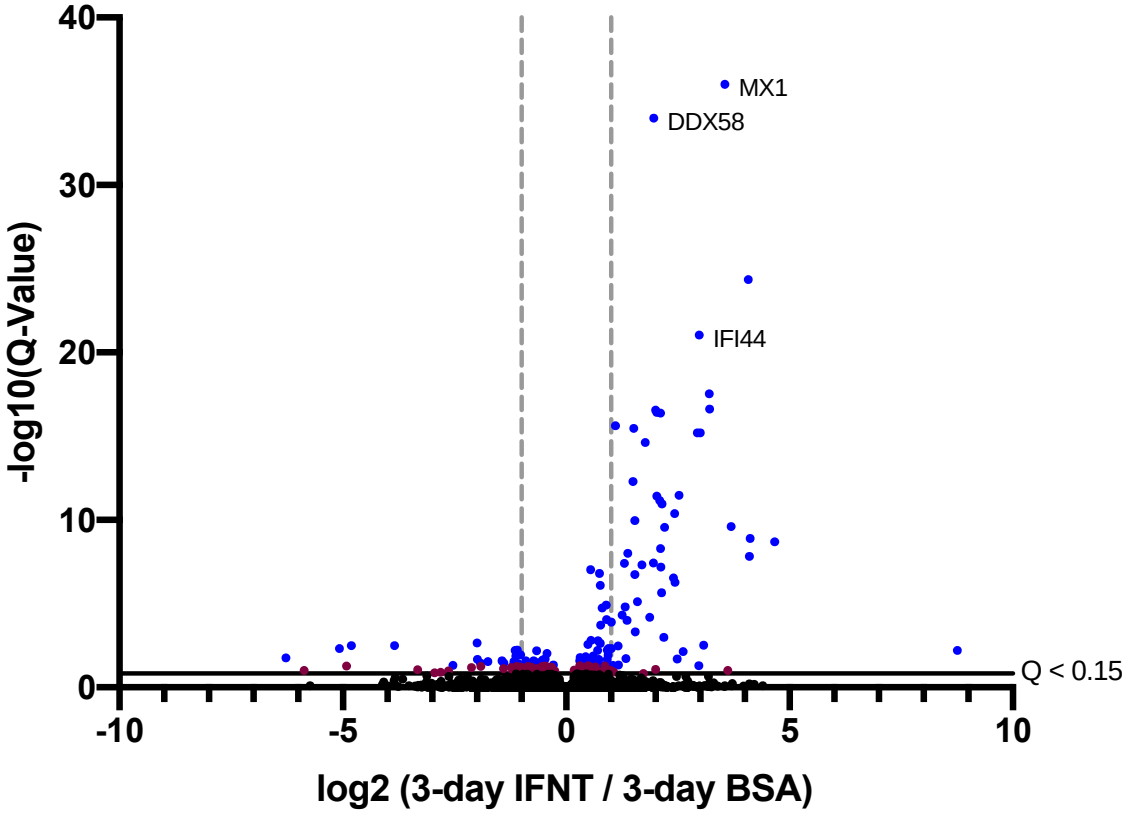


Figure 3-15. Volcano plot of transcripts from luteal tissue exposed to 3-day uterine infusion treatments. The blue points represent differentially abundant mRNA (Q < 0.05) from luteal tissue exposed to 3-day uterine infusion treatments. The maroon points represent mRNA that tended to differ (0.05 < Q < 0.15) between the 3-day treatments from luteal tissue. The solid black line indicate thresholds for the Q-value of 0.15. The grey dotted line indicates the log2 fold change threshold of either 1 (upregulated in 3-day IFNT) or -1 (downregulated in 3-day IFNT).

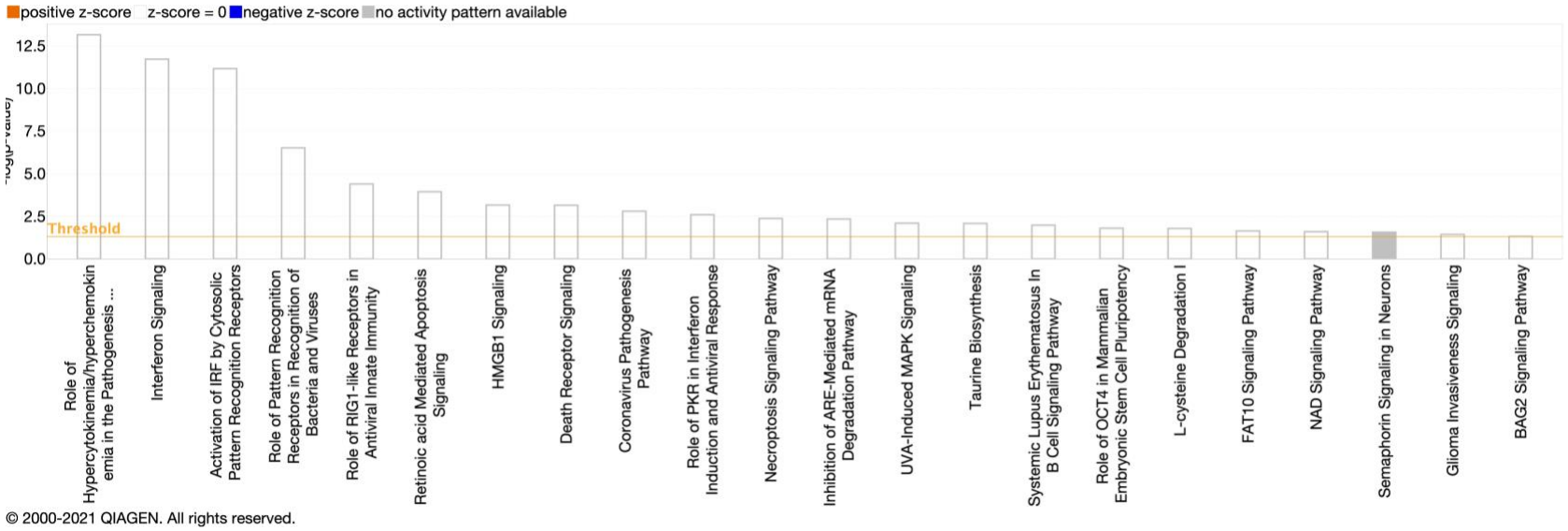


Figure 3-16. Ingenuity pathway analysis (IPA; Qiagen) of differentially abundant transcripts from luteal tissue exposed to 3-day uterine infusion treatments. The top pathways associated with differentially abundant transcripts ($Q < 0.05$) from luteal tissue exposed to 3-day uterine infusions.

Gene	Average FPKM	Q-value
'CD74'	8771.9625	0.999964012
'HSPA1A'	6997.94	0.999964012
'LOC783680'	5110.5575	0.999964012
'BOLA'	4890.02	0.999964012
'TPT1'	3906.87	0.999964012
'TMSB10'	3690.4775	0.999964012
'TIMP2'	3627.475	0.999964012
'TMSB4X'	3420.2775	0.999964012
'FTH1'	3418.4675	0.999964012
'UBC'	3152.5025	0.999964012
'UBB'	3111.0925	0.999964012
'RPLP1'	3093.9675	0.999964012
'IFITM3'	3024.3875	0.999964012
'UBA52'	2975.92	0.999964012
'RPS2'	2832.82	0.999964012
'ACTB'	2709.59	0.999964012
'HSPA8'	2527.5325	0.999964012
'ZFP36'	2426.7925	0.999964012
'RPL10'	2353.5375	0.999964012
'RPL23A'	2341.85	0.999964012
'JSP.1'	2290.22	0.999964012
'RPS11'	2279.24	0.999964012
'BOLA-DRA'	2264.465	0.999964012
'CCL4'	2263.5125	0.999964012
'RPS27'	2197.365	0.999964012
'RPL13A'	2006.1125	0.999964012
'RPL37'	1997.435	0.999964012
'RPLP0'	1930.335	0.999964012
'PFN1'	1913.765	0.999964012
'FTL'	1886.23	0.999964012
'C1QB'	1850.7225	0.999964012
'MGP'	1844.4125	0.999964012
'LOC104968634'	1843.8	0.999964012
'EEF1A1'	1764.79	0.999964012
'JUNB'	1731.19	0.999964012
'CCL5'	1643.3625	0.999964012
'RPL31'	1617.0475	0.999964012
'HSD3B1'	1593.9025	0.999964012
'CST3'	1589.145	0.999964012
'GSTA1'	1562.1625	0.999964012
'RPS15'	1491.455	0.999964012
'RPS16'	1481.5625	0.999964012

'DNAJB1'	1478.9275	0.999964012
'RPL27A'	1471.7675	0.999964012
'ACTG1'	1463.75	0.999964012
'BRB'	1456.025	0.999964012
'RPSA'	1399.045	0.999964012
'RPL18A'	1375.505	0.999964012
'RPL18'	1365.2825	0.999964012
'RPL32'	1346.8525	0.999964012
'RPS24'	1332.1625	0.999964012
'RPS29'	1328.8325	0.999964012
'RPS15A'	1325.445	0.999964012
'RPS5'	1323.395	0.999964012
'RPS8'	1307.315	0.999964012
'RACK1'	1290.4275	0.999964012
'RPS7'	1286.22	0.999964012
'RPS17'	1277.7925	0.999964012
'RPL17'	1258.4275	0.999964012
'B2M'	1254.245	0.999964012
'LOC100848815'	1245.1475	0.999964012
'SAT1'	1206.28	0.999964012
'RPS25'	1203.42	0.999964012
'SPARC'	1191.795	0.999964012
'HSP90AA1'	1173.84	0.999964012
'RPS10'	1172.0825	0.999964012
'JUN'	1146.9875	0.999964012
'RPL23'	1136.465	0.999964012
'STAR'	1104.96	0.999964012
'C1QC'	1094.6475	0.999964012
'RPS3A'	1083.06	0.999964012
'RPS20'	1081.84	0.999964012
'PSAP'	1071.155	0.999964012
'RPS18'	1069.4925	0.999964012
'FOS'	1067.57	0.999964012
'RPL12'	1063.9125	0.999964012
'SRGN'	1057.7275	0.999964012
'IFITM3'	1052.5725	0.999964012
'LAPTM5'	1050.9575	0.999964012
'RPS19'	1037.485	0.999964012
'BTG1'	1032.5325	0.999964012
'CFL1'	1030.17	0.999964012
'PTMA'	1026.23	0.999964012
'HSPB1'	1025.5225	0.999964012
'VIM'	1023.85	0.999964012

'RPS12'	1018.85	0.999964012
'BLA-DQB'	1018.5725	0.999964012
'IFITM1'	1001.81	0.999964012
'SH3BGRL3'	967.7025	0.999964012
'GNAS'	951.9175	0.999964012
'GAPDH'	951.13	0.999964012
'RPL29'	945.9075	0.999964012
'ITM2B'	943.4125	0.999964012
'RPL11'	938.9625	0.999964012
'RPS13'	925.3125	0.999964012
'FCER1G'	924.01	0.999964012
'SLC25A6'	923.2875	0.999964012
'IFI6'	920.505	0.999964012
'IFITM2'	906.0675	0.999964012
'EGR1'	899.2825	0.999964012

Supplementary Table 3-1. List of top 100 most abundant transcripts in resident immune cells from the 6-day IFNT+PSPB infusion.

Gene	log2 (6-day-IFNT / 3-day-IFNT)	P-value	Q-value
'MOC	0.735367072	2.97E-05	0.053855538
'UTP6'	0.425867335	3.35E-05	0.055339728
'IER3'	0.630720141	4.11E-05	0.062261398
'LOC1	0.561644677	4.46E-05	0.062349925
'CORO	0.732625292	5.17E-05	0.06704933
'CEP29	2.004092444	6.43E-05	0.06984457
'EPHA	-1.73400008	6.54E-05	0.06984457
'RMI2'	-0.962428006	6.38E-05	0.06984457
'ADA	0.83534607	9.98E-05	0.095419552
'STAC	-1.485935331	9.52E-05	0.095419552
'C1QL	-1.38713567	1.12E-04	0.097014695
'GRHL	1.726738891	1.11E-04	0.097014695
'CYP2	1.815836559	1.25E-04	0.102830194
'10713	1.043832989	1.40E-04	0.109913663
'EMP1'	0.86150396	1.45E-04	0.109913663
'LOC5	-6.250920261	1.58E-04	0.114892525
'ZNF57	-0.57374887	1.65E-04	0.115073848
'KRT8'	2.235973477	1.86E-04	0.116728814
'LOC5	-4.992126092	1.86E-04	0.116728814
'S1PR4	-0.810673021	1.74E-04	0.116728814
'DDX3	0.263391358	2.14E-04	0.125298828
'C2CD	1.240935345	2.11E-04	0.125298828
'MAD1	-0.536752404	2.35E-04	0.131076662
'BOLA	-1.177809424	2.38E-04	0.131076662
'EGR4'	1.903829045	2.46E-04	0.131496098
'F3'	1.190220673	2.64E-04	0.13671695
'CD163	-1.837700687	3.16E-04	0.147753922
'TTYH	0.627636818	3.01E-04	0.147753922
'CD274	0.979900041	3.01E-04	0.147753922
'MAPK	0.5122977	3.29E-04	0.147753922
'KCNK	2.029081895	3.30E-04	0.147753922
'COL6	-2.515295207	3.34E-04	0.147753922

Supplementary Table 3-2. Transcripts with a tendency to differ in resident immune cells from 3-day IFNT and 6-day IFNT infusions. A total of 32 transcripts tended ($0.05 < Q < 0.15$) to differ in resident immune cells between 3-day and 6-day IFNT infusions.

Gene	log2 (3-day-IFNT / 3-day-BSA)	P-value	Q-value
'RSAD2'	2.427392522	4.47E-24	8.07E-20
'MX1'	3.284083703	5.05E-22	4.56E-18
'IFI44L'	2.291147932	3.33E-20	2.01E-16
'LOC100139670	2.834256543	1.56E-19	7.05E-16
'HERC6'	2.057000968	2.74E-18	9.91E-15
'MX2'	3.46150295	4.02E-16	1.21E-12
'DDX58'	2.178218376	4.83E-16	1.25E-12
'OAS1Y'	2.929255066	3.38E-15	7.63E-12
'ZNFX1'	1.526372694	1.22E-14	2.44E-11
'SIGLEC1'	2.177637314	6.43E-13	1.16E-09
'OAS1Z'	2.705291076	1.34E-12	2.21E-09
'USP18'	1.977593467	4.95E-12	7.45E-09
'HERC5'	1.427979753	1.16E-11	1.61E-08
'PARP12'	1.138245985	1.26E-11	1.62E-08
'OAS2'	3.274438258	1.65E-11	1.96E-08
'CMPK2'	2.494366505	1.74E-11	1.96E-08
'IFI44'	2.26315038	1.88E-11	1.99E-08
'EIF2AK2'	1.627470676	5.12E-11	5.14E-08
'DHX58'	1.907434478	9.96E-11	9.46E-08
'ADAR'	1.198258972	1.53E-10	1.38E-07
'LOC509283'	1.714057344	1.68E-10	1.45E-07
'CMTR1'	0.66141684	2.29E-10	1.88E-07
'IFIT3'	2.115571759	2.66E-10	2.00E-07
'IFITM2'	2.023969601	2.63E-10	2.00E-07
'TRANK1'	1.364291797	3.89E-10	2.81E-07
'XAF1'	1.689867079	4.17E-10	2.89E-07
'EPSTI1'	1.236731057	4.52E-10	3.02E-07
'ZBP1'	2.350597026	1.08E-09	6.94E-07
'IFIT5'	1.130074112	1.34E-09	8.35E-07
'IFITM3'	2.039260806	2.34E-09	1.41E-06
'LOC514978'	2.496227105	3.80E-09	2.21E-06
'IFITM3'	2.316518764	6.33E-09	3.57E-06
'PNPT1'	1.066961206	6.63E-09	3.63E-06
'PLSCR2'	0.890975362	7.27E-09	3.86E-06
'CMTR2'	0.821234827	8.57E-09	4.42E-06
'ISG15'	1.904048835	1.79E-08	8.96E-06
'IFITM1'	1.941196702	2.05E-08	1.00E-05
'PSMF1'	0.55676819	3.00E-08	1.43E-05
'PARP10'	1.111587489	5.41E-08	2.51E-05
'IFI6'	2.373353612	9.33E-08	4.21E-05
'RTP4'	2.11569762	1.79E-07	7.87E-05
'ZCCHC2'	0.848815401	2.45E-07	1.05E-04
'PML'	0.953927527	3.46E-07	1.45E-04
'LOC618737'	1.209167255	3.55E-07	1.46E-04

'UBE2L6'	0.857226042	4.80E-07	1.93E-04
'TDRD7'	0.892084522	7.08E-07	2.78E-04
'IRF7'	1.181984378	7.90E-07	3.04E-04
'SAMD9'	1.27538784	8.91E-07	3.35E-04
'IRF9'	1.076824373	1.13E-06	4.16E-04
'USP25'	0.696333106	1.21E-06	4.37E-04
'UBA7'	1.694876137	1.54E-06	5.44E-04
'IFI16'	1.030733167	3.33E-06	0.001133336
'PARP14'	1.140759239	3.30E-06	0.001133336
'OAS1X'	2.962054547	4.92E-06	0.001646034
'MGC126945'	1.575375686	5.57E-06	0.001827359
'IFIH1'	0.876213031	8.79E-06	0.002833729
'LOC615899'	0.956169381	1.10E-05	0.003486383
'IFI27'	2.423962479	1.47E-05	0.004486549
'SPIB'	1.359780959	1.45E-05	0.004486549
'REC8'	1.219191002	1.61E-05	0.004831668
'CD79A'	1.141979405	1.99E-05	0.005785002
'CGAS'	0.998143091	1.97E-05	0.005785002
'CLEC4F'	2.776088506	2.49E-05	0.007128637
'FCRL3'	3.004859229	2.53E-05	0.007128637
'CXCL14'	**-1.859431474**	**4.73E-05**	**0.013153827**
'DTX3L'	1.078623073	4.82E-05	0.013177125
'PDIA5'	**-0.355843542**	**5.08E-05**	**0.013688231**
'LOC100848575'	0.863432235	5.60E-05	0.014866009
'HSH2D'	0.977024069	6.95E-05	0.018175258
'MAOA'	**-0.534510973**	**7.11E-05**	**0.018352277**
'LY6E'	1.109059422	7.44E-05	0.018658465
'TRIM56'	0.612866872	7.36E-05	0.018658465
'LOC618409'	1.60561022	7.73E-05	0.019111862
'LY6L'	1.742948122	9.59E-05	0.023393283
'HMGB3'	**-0.719749118**	**1.12E-04**	**0.026858018**
'STX1A'	1.373589612	1.19E-04	0.028214506
'ELMO2'	0.417470669	1.21E-04	0.028470657
'STRA6'	**-1.030427544**	**1.29E-04**	**0.029964419**
'PRSS2'	10.19284391	1.55E-04	0.035379706
'CD9'	**-0.680465619**	**1.74E-04**	**0.039191146**
'RNF114'	0.375778038	2.08E-04	0.045822016
'CPNE9'	0.749879465	2.08E-04	0.045822016
'RIPK3'	0.554645688	2.25E-04	0.049040755

Supplementary Table 3-3. Differentially abundant transcripts in resident immune cells from 3-day BSA and IFNT infusions. A total of 83 transcripts were differentially abundant (Q < 0.05) in resident immune cells. There were 77 transcripts that were in greater abundance in the 3-

day IFNT infusion compared to the 3-day BSA infusion. There were 6 transcripts that were in lower abundance in the 3-day IFNT infusion compared to the 3-day BSA infusion (bold font)

Gene	log2 (3-dayIFNT / 3-day BSA)	P-value	Q-value
'MX1'	3.548939844	5.36E-41	9.49E-37
'DDX58'	1.964860078	1.17E-38	1.04E-34
'LOC100139670'	4.072621902	7.64E-29	4.51E-25
'IFI44'	2.974272824	2.05E-25	9.07E-22
'OAS1Y'	3.203579623	8.19E-22	2.90E-18
'RSAD2'	3.207609989	7.85E-21	2.32E-17
'CMPK2'	2.001874757	1.10E-20	2.78E-17
'SLFN11'	2.023987433	1.67E-20	3.71E-17
'DHX58'	2.114805821	2.10E-20	4.14E-17
'LOC100299281'	1.100163952	1.35E-19	2.39E-16
'IFIT5'	1.508590047	2.08E-19	3.34E-16
'LOC100298356'	2.932255603	4.28E-19	6.27E-16
'USP18'	3.004934113	4.60E-19	6.27E-16
'IFI16'	1.7686448	1.90E-18	2.41E-15
'PARP12'	1.496842916	4.39E-16	5.18E-13
'IFI44L'	2.529581357	3.09E-15	3.43E-12
'IFITM3'	2.023645333	3.61E-15	3.76E-12
'IFITM2'	2.090507221	7.15E-15	7.04E-12
'IFITM1'	2.146595532	1.15E-14	1.07E-11
'IFITM3'	2.42674889	4.59E-14	4.07E-11
'ABHD1'	1.537621006	1.33E-13	1.12E-10
'OAS2'	3.695778527	3.09E-13	2.48E-10
'SIGLEC1'	2.199589501	3.65E-13	2.81E-10
'ISG15'	4.115115528	1.70E-12	1.25E-09
'LOC514978'	4.66431793	2.90E-12	2.06E-09
'HERC6'	2.113354111	7.35E-12	5.01E-09

'HERC5'	1.37991372	1.50E-11	9.86E-09
'MX2'	4.101580597	2.31E-11	1.46E-08
'IRF7'	1.950421179	6.13E-11	3.74E-08
'EIF2AK2'	1.304715403	6.68E-11	3.94E-08
'ZNFX1'	1.691955495	8.58E-11	4.90E-08
'RTP4'	2.115693139	1.15E-10	6.37E-08
'CMTR1'	0.548656955	1.73E-10	9.27E-08
'ADAR'	0.748808142	2.93E-10	1.52E-07
'EPSTI1'	1.532861875	3.64E-10	1.84E-07
'LOC618737'	2.40053034	5.79E-10	2.85E-07
'IFIT3'	2.431319255	1.14E-09	5.44E-07
'CMTR2'	0.76013354	1.74E-09	8.12E-07
'XAF1'	2.133684286	4.79E-09	2.17E-06
'IFIH1'	1.596307951	1.76E-08	7.77E-06
'PLSCR2'	0.895107814	2.78E-08	1.20E-05
'PARP10'	1.322534757	3.71E-08	1.56E-05
'PNPT1'	0.806267713	4.34E-08	1.79E-05
'DTX3L'	1.255488262	1.20E-07	4.83E-05
'LOC786258'	1.8661632	1.63E-07	6.42E-05
'TRIM34'	0.90755763	2.29E-07	8.83E-05
'KCNN2'	1.363720866	2.65E-07	9.97E-05
'LGALS9'	1.007874148	3.35E-07	1.24E-04
'GEMIN2'	0.77159918	5.28E-07	1.91E-04
'CGAS'	1.547388746	1.35E-06	4.77E-04
'IFIT2'	2.18914514	3.02E-06	0.001049917
'RND3'	0.551314537	4.60E-06	0.001567151
'PSMF1'	0.70987487	4.78E-06	0.00159873
'BACH2'	**-1.994398276**	**6.75E-06**	**0.002215563**

'TNFSF10'	0.764524542	7.37E-06	0.002374535
'CHMP5'	0.488491379	8.60E-06	0.002721705
'OAS1Z'	3.075555432	9.77E-06	0.003035754
'107131217'	**-3.845320127**	**1.04E-05**	**0.003158626**
'LOC783508'	**-4.810551228**	**1.05E-05**	**0.003158626**
'TIMD4'	1.160195487	1.12E-05	0.003305199
'CCDC114'	0.945679516	1.61E-05	0.004663237
'IRF9'	1.015800471	1.64E-05	0.004693808
'ULBP3'	**-5.076023174**	**1.71E-05**	**0.004808583**
'TREX1'	0.923472846	2.12E-05	0.005863903
'BTG2'	**-1.095917312**	**2.17E-05**	**0.005905703**
'PRSS2'	8.751269663	2.27E-05	0.006094537
'TRANK1'	0.705068413	2.31E-05	0.006094537
'NKPD1'	**-1.148529714**	**2.34E-05**	**0.006096174**
'ARID4A'	**-0.659859232**	**2.49E-05**	**0.006399672**
'ZBP1'	2.615554976	2.89E-05	0.007305369
'RAPGEF1'	**-0.432750701**	**3.72E-05**	**0.009284322**
'SP110'	0.933008585	4.74E-05	0.011492509
'NR6A1'	**-1.019277289**	**4.69E-05**	**0.011492509**
'KLF2'	0.602367856	5.60E-05	0.013415711
'KSR1'	**-1.130571685**	**5.75E-05**	**0.013581725**
'ANKMY2'	0.434760291	6.29E-05	0.014662236
'VTA1'	0.309583162	7.18E-05	0.016508202
'RAET1L'	**-6.277317529**	**7.49E-05**	**0.017011621**
'XRN2'	0.574562504	7.92E-05	0.017756948
'SAMD9'	1.339742919	8.54E-05	0.01891554
'TRMT44'	**-0.47084168**	**9.16E-05**	**0.020043787**
'IFI27'	2.487536544	9.40E-05	0.020304904

'AMMECR1'	0.917227087	9.76E-05	0.020839721
'CDO1'	0.724596816	1.00E-04	0.020903391
'LRRC4'	**-1.988431189**	**1.00E-04**	**0.020903391**
'LOC541220'	**-0.512728938**	**1.07E-04**	**0.022050055**
'LOC785630'	**-0.538057654**	**1.21E-04**	**0.02469664**
'MNT'	**-1.437990981**	**1.27E-04**	**0.025604971**
'ANKRD23'	**-0.729171053**	**1.36E-04**	**0.026458557**
'ANXA6'	0.304981568	1.35E-04	0.026458557
'KIAA0319'	**-0.903360551**	**1.33E-04**	**0.026458557**
'TNFSF9'	**-1.756112446**	**1.48E-04**	**0.028333997**
'B3GALT1'	**-1.170889003**	**1.49E-04**	**0.028333997**
'CLGN'	**-0.554358188**	**1.61E-04**	**0.03027296**
'TDRD7'	0.646477205	1.74E-04	0.031973169
'TRIM56'	0.41849517	1.75E-04	0.031973169
'SESN1'	0.522134008	1.73E-04	0.031973169
'TTYH3'	**-0.684404688**	**1.77E-04**	**0.032007305**
'KAT2A'	0.332071892	1.88E-04	0.03368859
'RUNX1'	**-1.919005511**	**1.96E-04**	**0.034638683**
'ST3GAL1'	**-1.393595294**	**2.00E-04**	**0.03512471**
'CLEC3B'	0.415479553	2.11E-04	0.036636806
'RBM43'	0.710553501	2.20E-04	0.037782949
'C21H14orf132'	**-1.079429431**	**2.22E-04**	**0.037782949**
'GRINA'	0.428681097	2.51E-04	0.042280444
'NT5DC3'	**-0.737970443**	**2.64E-04**	**0.044200769**
'LOC511936'	1.168725738	2.74E-04	0.045025106
'MGA'	**-0.285237885**	**2.73E-04**	**0.045025106**
'MUC19'	**-2.533750914**	**2.89E-04**	**0.047022209**
'PSMD5'	0.391389036	2.94E-04	0.04733229

'PDE4D'	**-1.04080795**	**2.97E-04**	**0.04733229**
'RHOBTB1'	0.477927394	3.05E-04	0.048230895
'PARP14'	1.04160478	3.11E-04	0.048821629
'ATP8B4'	0.955337043	3.21E-04	0.049457045
'DUSP22'	**-1.186291348**	**3.18E-04**	**0.049457045**
'PML'	0.824420515	3.28E-04	0.049939048
'IFI6'	2.964368821	3.30E-04	0.049939048

Supplementary Table 3-4. Differentially abundant transcripts in luteal tissue from 3-day BSA and IFNT infusions. A total of 117 transcripts were differentially abundant ($Q < 0.05$) in luteal tissue. There were 87 transcripts that were in greater abundance in the 3-day IFNT infusion compared to the 3-day BSA infusion. There were 30 transcripts that were in lower abundance in the 3-day IFNT infusion compared to the 3-day BSA infusion (bold font).

Chapter 4

Complement components may play a role in the bovine corpus luteum of early pregnancy

Introduction

The complement system, or complement cascade, is an important component of the innate immune system and is comprised of over 30 proteins. While traditionally the complement system is most known for forming a membrane attack complex (MAC) in the membrane of an invading pathogen, other functional roles of complement including roles in T cell differentiation (Kemper et al., 2003; Heeger et al., 2005; Strainic et al., 2008, Strainic et al., 2013, Kwan et al., 2013; Torok et al., 2015), metabolism (Hess and Kemper 2016), and regulation of the adaptive immune system (Ricklin et al., 2016) are gaining notoriety for other functional roles of these important proteins.

The abundance, expression pattern, and functional role of complement in the CL is not known. Complement components exist in the female reproductive tract, including in the uterus in both rats and humans (Sundstrom et al., 1989; Hasty et al., 1992; Vanderpuye et al., 1992; Hasty et al., 1992), the follicle of humans, cows, and rats (Fahmi et al., 1985; He et al. 2000 Perricone et al., 1990; Nilsson et al., 2010; Yoo et al., 2013; Ambekar et al., 2015), and the corpus albicans of humans (Criado-Garcia et al., 1999). Depending on the time of the estrous/menstrual cycle, follicular fluid contains complement components, often at concentrations greater than circulating plasma concentrations (Perricone et al., 1990; Yoo et al., 2013). The source of these complement components has been suggested to come from granulosal cells (Yoo et al., 2013). Nearly all of the complement components of both the classical and alternative pathway except C5 and C6 have been identified in human follicular fluid via liquid

chromatography/mass spectrometry (LC/MS) and mRNA of the same complement components was present in human granulosal cells (Yoo et al., 2013). Yoo et al. (2013) concluded that complement proteins found in the follicular fluid are derived from granulosal cells, and this would also suggest that luteal steroidogenic cells are also capable of producing complement proteins.

Interestingly, several transcriptomic and proteomic profiling studies have found complement components to be present in the CL. Pathway analysis of 318 upregulated genes in the functional CL (day 11) compared to the developing CL (day 4) found that complement activation was one major pathway upregulated in the functional CL (Kfir et al., 2018). The genes associated with the complement pathway in this study included subunits of complement C1 protein (*C1QA, C1QB, C1S*), complement C4 (*C4A*), and complement factor H (*CFH*). Proteomic profiling of the CL of early pregnancy (day 17) found complement C3 to be less abundant in the CL of pregnancy compared to the cycle (Hughes et al., 2019). Transcriptomic analysis of the same tissues found *CD55* to be in greater abundance in the CL of pregnancy compared to the cycle (Hughes et al., 2019). In a similar transcriptomic profiling study, *CD55* was also found to increase in luteal tissue from day 14 of pregnancy to day 23, along with two other negative regulators of the complement system, *CFH* and serpin family G member 1 (*SERPING1*; Hughes et al. 2020). Several other complement components of significance identified in this study include a subunit of C1, *C1QBA*, C5a receptor (*C5AR1*), *C7*, and C4 binding protein (*C4BPA*; Hughes et al., 2020). The many complement components identified in the study of early pregnancy support the hypothesis that complement plays a functional role in the CL of early pregnancy. Supporting this claim is another transcriptomic analysis comparing CL from the first month of pregnancy (day 20) to cyclic animals. The key difference of that study is that the CL were not exposed to uterine PGF2A pulses from the uterus. An over-representation pathway analysis found that IGF availability was one pathway associated with altered genes ($Q < 0.10$) in the Reactome database

(Mezera et al., 2021). One gene associated with this pathway was complement C4, *C4A*. Interestingly, C4 was present in the media from cocultures of midcycle luteal steroidogenic cells with autologous T cells (Brzezicka et al., unpublished).

While complement proteins are present in the CL, the abundance of these proteins and their functional role in the CL are unknown. The aim of this study was to characterize the abundance of C3 and C4 across the lifespan of the CL and elucidate a function for complement in the CL. The specific objectives of this study were to 1) characterize the abundance of C3 and C4 across the life cycle of the CL including during early pregnancy, 2) elucidate if luteotropic and luteolytic hormones regulate C3 and C4 abundance, and 3) determine if there is a functional role of C3 in luteal cell-T cell interactions.

Materials and Methods

Tissue Collection

The procedures performed for tissue collection were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Cyclic Holstein dairy cows were observed for signs of estrus. The day of standing estrus was considered day 0 of the cycle. The CL collected for the luteal regression study were removed by colpotomy on estrous day 10-11 at 0, 0.5, 1, 2, 4, 8, 12, or 24 hours after a luteolytic dose (25 mg) of Lutalyse (Pfizer). The CL collected for the acquisition of luteolytic capacity (day 4 and 6) experiment were collected from cows that had been synchronized for estrus using a controlled internal drug release device (CIDR; Zoetis). The CIDR was inserted and remained in place for six days, after which a luteolytic dose (25 mg) of Lutalyse was given, and CIDRs were removed the following day. About two days after cows showed signs of heat and a large preovulatory follicle was observed via ultrasound, an ovulatory dose of GnRH (100 μg Factrel; Zoetis) was administered. The day 4 CL were collected from the ovary after slaughter, while the day 6 CL were collected via

colpotomy. The CL from the early pregnancy experiment were collected as described by Hughes et al. (2019) for the day 17 CL and Benyo et al. (1991) for the day 18 CL. CL collected for the cell culture experiments were collected via colpotomy between days 10 and 12 of the estrous cycle.

Luteal cell culture

CL were dissociated and isolated luteal cells were cultured as previously described (Pate, 1993). On day 1 of culture, cells were treated with PGF2A (10 ng/ml), PGE2 (10 ng/ml), IFNT (1 ng/ml for mRNA experiment; 10 ng/ml for flow cytometry experiment), and a combination of treatments for 24 hours. On day 2 of culture, cells collected for mRNA analysis were collected in miRcury kit lysis buffer for RNA isolation (described below). Cells collected for flow cytometry were collected using trypsin and stained as described in Chapter 2.

Isolation of peripheral T lymphocytes

T lymphocytes were isolated from peripheral blood mononuclear cells (PBMCs) as previously described in Poole and Pate (2012). Purity of T lymphocytes was evaluated as percentage of CD3 positive cells and was > 85%. Cell number and viability was determined by counting cells stained with Trypan Blue (Thermo Fisher) on a hemocytometer.

Western Blot Analysis

Tissue was homogenized in either a urea lysis buffer (7 M urea, 2 M thiourea, 5 mM dithithreitol, 2% weight/ volume CHAPS) for the luteal regression tissue samples, or 2% CHAPS Tris Base lysis buffer (2g of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.303g of Tris Base, 0.88g of NaCl into 100 mL, pH = 7.2) for all remaining tissue samples. Protein concentrations from the homogenized tissue were assessed using a Bradford assay (BioRad). PROTEAN TGX Precast Gels (BioRad) or Mini-PROTEAN TGX Precast Stain-Free Gels (BioRad) were used for protein separation, with 30 μg of protein from each fraction loaded per lane. The proteins were then transferred using an iBlot dry blotting

system to a polyvinylidene fluoride (PVDF) membrane (Invitrogen). After transfer, the gel was stained with Coomassie Blue Stain to ensure proper protein transfer, or an image of the stain-free gel total protein was used to assess transfer quality. The membrane was blocked using 5% nonfat dry milk in TBST. The blots were incubated with either an anti-C3 antibody (AbCam) at a 1:2000 dilution, or an anti-C4 antibody (Acris) at a 1:1000 dilution overnight at 4 °C. The following day, after washing the blot with TBST, the blot was incubated with either a donkey anti-rabbit IgG horseradish peroxidase secondary antibody (GE Healthcare) for C3 blots, or a sheep anti-mouse IgG horseradish peroxidase secondary antibody (GE Healthcare) for the C4 blots, both at a 1:10,000 dilution for two hours at room temperature. The blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (BioRad) and were imaged using a ChemiDoc XRS System (BioRad). If GAPDH was used as a loading control, the blots were stripped and re-probed for GAPDH using anti-GAPDH (Thermo Fisher) primary antibody at a 1:20,000 dilution and sheep anti-mouse IgG horseradish peroxidase secondary antibody (GE Healthcare). The C3, C4, and GAPDH bands as well as total protein were quantified using BioRad Image Lab 6.0 software.

RNA Isolation and quantitative PCR

Total RNA was isolated from tissue and cultured cells using the miRcury kit (Exiqon) and subsequently underwent preparation using DNase (Promega) to eliminate any gDNA contamination. cDNA was synthesized using the AzuraQuant kit (Azura Genomics), and qPCR was performed using AzuraQuant green fast qPCR mix LoRox (Azura Genomics). *RPL19* was used as a reference gene for all tissues and cultured cells and was not altered by treatment or status of the animal.

GapmeR transfection and T lymphocyte proliferation

Luteal cells were seeded with 100,000 cells per well on a 96-well plate. On day 1 of luteal cell culture, GapmeRs (Qiagen) were added to luteal cells at a final concentration of 1 μM. After 48 hours (day 3 of culture), Ham's F12 culture medium was changed and luteal cells

transfected with the FAM-labeled scrambled control GapmeR were collected with trypsin and transfection efficiency was assessed via flow cytometry (transfection efficiency ~84%). On day 1 of T lymphocyte culture, T lymphocytes were placed in a 24-well plate with 500,000 cells per well in AIMV medium (Gibco). GapmeRs were added to T lymphocytes at a final concentration of 500 nM. After 48 hours (day 3 of culture), T lymphocytes were collected and stained with carboxyfluorescein succinimidyl ester (CFSE; 1.75 μM; Thermo Fisher) as per the manufacturer's protocol. The T cells transfected with the FAM-labeled scrambled control GapmeR were used to assess transfection efficiency via flow cytometry (transfection efficiency ~84%). The CFSE-stained T lymphocytes were added to the corresponding treated luteal cells (32,000 cells/well) on day 5 of luteal cell culture and cocultured in AIMV medium. After 72 hours of culture, proliferation of T lymphocytes was assessed using flow cytometry.

Statistical analysis

The statistical analyses were performed using the mixed model of SAS 9.4 (Statistical Analysis System Institute). An ANOVA was used to determine differences in protein and mRNA abundance between treatment and/or animal status. If multiple membranes were used for the same experiment, membrane was included in the model as a block. For cell culture experiments where the same animal received all treatments, animal was included in the model as a block. For western blot analysis either GAPDH or total protein was used as a covariate in the model. For mRNA analysis, *RPL19* was the reference gene used as a covariate in the model. A Dunnett's post-hoc test was used for the luteal regression experiment and mRNA abundance in treated cultured cells. A Tukey post-hoc test was used for all other experiments. The data are represented as least squares means ± pooled SEM. Differences were considered significant when $p < 0.05$, and a tendency when $0.05 < p < 0.1$.

Results

The aim of this study was to determine the abundance of complement C3 and C4 across the life cycle of the CL, including during early pregnancy, as well as to elucidate a functional role of complement in the bovine CL.

Complement during luteal regression

The abundance of C3 and C4 protein and mRNA during luteal regression was assessed in luteal tissue after an *in vivo* injection of PGF2A, using western blot and qPCR, respectively. Both C3 and C4 abundance did not change at any time after the PGF2A injection compared to control (C3, Figure 1; C4, Figure 2). There was a tendency for *C3* to increase 24 hours after injection compared to the 0 hour control (Figure 3a). There was no change in *C4* after the PGF2A injection (Figure 3b).

Complement during acquisition of luteolytic capacity

Neither C3 nor C4 changed in the CL between day 4 and day 6 (Figure 4, 5). There was a tendency for *C3* to be less abundant in the day 6 CL compared to the day 4 CL (Figure 6a), however, there was no change in *C4* (Figure 6b).

Complement in CL from early pregnancy

C3 was not different in the CL of the cycle compared to the CL of pregnancy on either day 17 or 18 (Figure7a,b), however, it is worth noting that C3 in the CL of pregnancy was numerically half of that in the CL of the cycle. C4 was not different in CL of the cycle and pregnancy on days 17 or 18 (Figure 8a,b). However, C4 abundance in the day 18 CL of pregnancy was numerically 5-fold greater compared to the CL of the cycle (Figure 8b). *C3* did not differ on day 17 of pregnancy compared to cyclic controls, however, there was greater *C* on day 18 of pregnancy compared to cyclic controls (Figure 9a and 9b). *C4* was not different on day 17 or day 18 of pregnancy compared to cyclic controls (Figure 9c and 9d).

Complement regulation in the CL by luteotropic and luteolytic hormones

C3 protein after treatment with IFNT, PGE2, PGF2A, and combination treatments in cultured luteal cells was assessed via flow cytometry. There was a main effect of treatment for both the percentage of C3 positive luteal cells and mean fluorescence intensity (MFI) after treatment with IFNT, PGE2, and a combination treatment (Figure 10a and 10b). The percentage of C3 positive cells and MFI decreased with the combination treatment compared to the individual treatments (Figure 10a and 10b). There were no changes in the percentage of C3 positive luteal cells nor MFI of C3 after treatment with IFNT, PGF2A, or a combination treatment (Figure 10c and 10d). There was also no change in the percentage of C3 positive cells after treatment with PGE2, PGF2A, or a combination treatment (Figure 10e), but MFI of C3 decreased with the PGE2 and PGF2A combination treatment compared to the individual treatments (Figure 10f).

C3 in cultured luteal cells decreased in response to PGF2A compared to control (Figure 11a). Addition of PGE2 with PGF2A did not reverse the effect of PGF2A (Figure 11b). When IFNT was added to the treatment with PGF2A, *C3* was not different from control (Figure 11c). A combination treatment of IFNT and PGE2 also did not change *C3* (Figure 11d). *C4* increased in response to treatment with IFNT alone compared to control (Figure 12a). Neither PGF2A, PGE2 or the combination of these had any effect on *C4* (Figure 12b), nor did they alter the stimulatory effect of IFNT (Figure 12c, d).

C3 knockdown and T cell proliferation

GapmeRs (Qiagen), locked nucleic acid antisense oligonucleotides, were used to knockdown C3 in both luteal cells and T cells. C3 was effectively knocked down in luteal cells on day 5 of culture compared to scrambled control (Figure 13a), while C3 was effectively knocked down in T cells on day 3 of culture compared to scrambled control (Figure 13b). After transfection with GapmeRs and coculture with transfected luteal cells for 72 hours, T cell

proliferation was measured via flow cytometry. T cell proliferation did not change after knockdown of C3 (Figure 14).

Discussion

Although the abundance and function of complement in the CL is unknown, it is clear that complement proteins are present in the bovine CL and discovering the functional role of these proteins in the CL would help answer the question that the proteomic and transcriptomic profiling studies to date have been unable to answer. C3 and C4 were chosen as complement components of interest because C3 was identified in the proteomic profiling study by Hughes et al. (2019) to be less abundant in the CL of pregnancy. Additionally, C4 was present in culture media collected from T cell-luteal cell cocultures with luteal cells isolated from functional CL. Primers and antibodies were successfully validated for these molecules. Although CD55 was increased in abundance in the CL of pregnancy in the transcriptomic profiling study by Hughes et al. (2019) and was a complement component of interest to study, we were unable to validate an antibody or primers for CD55. .

This study is the first to profile C3 and C4 in the CL during critical times, including luteal regression, acquisition of luteolytic capacity, and early pregnancy. Previous transcriptomic and proteomic studies demonstrated that complement components are present in the functional CL (Kfir et al., 2018) and during early pregnancy (Hughes et al., 2019, Hughes et al., 2020, Mezera et al., 2021), however, these studies utilized whole tissue. Due to the vascular nature of the CL, it is possible that complement proteins from circulation accounted for the complement detected in these studies. Yoo et al. (2013) reported that human granulosal cells can produce complement proteins and are the source of complement proteins found in follicular fluid, so it could be assumed that luteal steroidogenic cells, derived from granulosal and thecal cells, would also have the capability to produce complement proteins. In fact, the present study confirmed that luteal steroidogenic cells have the capability to make C3 and C4.

In trying to elucidate a functional role of C3 and C4 in the CL, these molecules were measured in luteal tissue across the lifespan of the CL. In the luteal regression time course experiment, there was a tendency of *C3* to increase 24 hours after a PGF2A injection (Figure 3a), however, C3 remained unchanged. (Figure 1). The mean fluorescence intensity (MFI) of C3 and the percentage of C3 positive cells also did not change in cultured luteal cells in response to PGF2A (Figure 10c and 10d). It is possible that 24 hours after an *in vivo* injection of PGF2A is the initiation for increasing the transcription of C3 to prepare for luteal regression by increasing C3 protein abundance. Increasing C3 abundance could be helpful for the luteolysis pathway to occur. Upon activation of the complement cascade, C3 is cleaved into two fragments, C3a and C3b. C3a acts as an anaphylatoxin, meaning it can help recruit leukocytes through chemotaxis as well as mediate local inflammation and vascular permeability (Ricklin et al., 2016; Reis et al., 2019). Although it was not studied in this experiment, C5a is another anaphylatoxin that acts very similarly to C3a and it is a key activator of neutrophils (Reis et al., 2019). Recruitment of macrophages, monocytes and neutrophils (Bagavandoss et al. 1988; Penny et al., 1999; Townson et al., 2002; Shirasuna et al., 2012) at the end of the estrous cycle and destabilization of the vasculature in the CL because of exposure to PGF2A (Zalman et al. 2012) are pathways important to luteolysis, and are similar to how C3 exerts a functional effect. Recruitment of immune cells and destabilization of the vasculature might be possible explanations for why *C3* tended to increase 24 hours after a PGF2A injection. However, further experimentation evaluating C3 abundance in a further regressed CL would be needed to support this claim.

C4b-binding protein (C4BP) was detected in the human regressing CL and corpus albicans by *in situ* hybridization and immunostaining, but was not detected in the follicle or functional CL (Criado-Garcia et al., 1999). C4BP is an inhibitor of the classical and lectin binding complement pathways. It acts by binding the protein C4b, a component of the C3 convertase, and preventing the formation of the C3 convertase, thus preventing the cleavage of C3 and the continuation of

the complement cascade. Although C4BP was not evaluated in this study, perhaps if C4BP is also abundant in the regressing bovine CL it could help mediate the activation of C3 and prevent overactivation of complement during luteal regression. However, although *C3* tended to increase in luteal tissue 24-hours after an *in vivo* PGF2A injection, it decreased in response to PGF2A in cultured luteal cells. In luteal tissue, it is possible that a different cell type(s) responds to PGF2A and accounts for the overall tendency of *C3* to increase in luteal tissue. Endothelial cells and immune cells, including T cells, B cells, neutrophils, and macrophages, all secrete and express complement components on their cell surface (Ricklin et al. 2016; Lubbers et al., 2017), so it is difficult to elucidate which cell type is producing *C3* from whole tissue. Additionally, it is well established that PGF2A also acts differently on luteal cells *in vitro* compared to *in vivo.* PGF2A does not decrease basal progesterone production in cultured luteal cells, but does inhibit LH-stimulated progesterone production (Pate and Condon 1984). Perhaps differences in the *in vitro* and *in vivo* model systems account for *C3* abundance. Unfortunately, the effect of PGF2A on C3 in bovine immune cells or endothelial cells *in vitro* (or *in vivo*) has not been evaluated, The only evidence of PGF2A altering complement abundance in either cell type is in human monocytes. PGF2A does not alter the C2 in human monocytes (Lappin and Whaley 1982), however more work needs to be completed to understand how PGF2A alters C3. C4 protein and mRNA did not change at any time after the PGF2A injection, suggesting C4 is not one of the complement proteins that plays an important during luteal regression.

Days 4 and 6 of the estrous cycle were chosen as a critical time to evaluate because this is the period of acquisition of luteolytic capacity. Prior to day 4, the CL will not regress in response to a luteolytic dose of PGF2A, but after day 6 the CL will regress. It was important to determine if C3 and/or C4 might be mediators of this phenomenon. C3 and C4 did not change during acquisition of luteolytic capacity, however, *C3* tended to decrease in day 6 CL compared to day 4 CL. The tendency for decreased *C3* in day 6 CL could be the initiation for ultimately decreasing the

abundance of *C3* in the functional CL. Poole and Pate (2012) demonstrated that the microenvironment of the functional CL supports a more "anti-inflammatory" environment due to the increased number of regulatory T cells and *TGFB1* in the functional CL. This type of microenvironment would be consistent with decreased *C3* after the acquisition of luteolytic capacity because complement activation, in the traditional sense, is more conducive to a proinflammatory environment.

C3 was less abundant in the day 17 CL of pregnancy compared to the cycle in a proteomic profiling study completed by Hughes et al. (2019). While there was no difference in this study, C3 was numerically 2-fold less in the day 17 CL of pregnancy compared to the day 17 cyclic CL, which supports the findings of the proteomic profiling study by Hughes et al. (2019), but C3 was not different in the day 18 CL. *C3* did not change on day 17, but was increased in the day 18 CL of pregnancy. One limitation of measuring C3 in tissue is the heterogeneous nature of the CL. The variety of cell types in the CL and the vascular nature of the CL might create a "perfect storm" for trying to elucidate the expression pattern of a protein that is present in systemic circulation. Complement proteins are produced by hepatocytes in the liver and C3 is one of the most abundant circulating complement proteins in the blood with a circulating concentration of about 1.2 mg/ml (Ricklin et al., 2016). Also, although *C3* was greater on day 18 of pregnancy, it does not mean this *C3* was translated. In fact, the C3 western blot data would suggest the greater amount of *C3* on day 18 was not translated as there was no difference in the protein between cyclic and pregnant CL. One reason this mRNA might not be translated is due to the prevention of translation by miRNA, and this might be one explanation for these discrepancies between mRNA and protein expression on both day 17 and 18 of pregnancy. Although miRNA regulation of C3 was not assessed, there were was 1 differentially abundant miRNA, miR-2389, from the miRNA profiling study by Maalouf et al. (2014) that was predicted to target C3. It would be an interesting follow up study to determine if this miRNA (or others) are responsible for the

discrepancies in mRNA and protein in the CL of pregnancy. Additionally, instead of using western blotting and qPCR to assess C3 abundance in luteal tissue, perhaps *in situ* hybridization and/or immunostaining should have been used.

C3 was measured in cultured luteal steroidogenic cells after treatment with the luteotropic hormones of pregnancy, IFNT and PGE2. The percentage of C3 positive luteal cells and concentration of C3 per cells decreased with the combination treatment of IFNT and PGE2 compared to the individual treatments. The concentration of C3 per cell also decreased with the combination treatment of PGF2A and PGE2 compared to individual treatments, which is consistent with the *C3* data. Although neither PGE2 nor PGF2A alone decreased C3, the decreased *C3* in response to PGF2A (alone and with PGE2) and decreased C3 when PGE2 was combined with PGF2A or IFNT provides evidence that prostaglandins regulate complement. PGE2 upregulates CD55, a complement inhibitor, in colorectal cancer and in the endometrium in humans (Holla et al., 2005; Richards et al., 2019), and PGE2 downregulates C2 in human monocytes (Lappin and Whaley 1982). PGF2A does not alter C2 in human monocytes (Lappin and Whaley 1982). Therefore, prostaglandins do regulate the abundance of complement components, however in luteal cells, it is the combination of prostaglandins (PGE2 and PGF2A) and IFNT that regulate the C3. The regulation of C3 by these hormones could also be cell type dependent. An important future direction would be to measure C3 in T cells and macrophages, relatively abundant immune cells in the CL, after treatment with PGE2, PGF2A, and IFNT and determine if C3 differs compared to luteal cells.

IFNT increased *C4* in cultured luteal cells, but was not regulated by PGF2A nor PGE2. Unfortunately, C4 protein could not be evaluated because a suitable antibody for flow cytometry could not be found, and the abundance of C4 protein was too low in cultured luteal cells to be detected via western blot. In human synovium, C4 protein increased after treatment with interferon-alpha (IFNA1) and interferon-gamma (IFNG) for 7 days (Collins et al. 1996). Both *C3*

and *C4* mRNA were also increased in response to IFNG in hepatocytes (Mitchell et al., 1996). Unfortunately, little has been done to evaluate the abundance of C4 in reproductive tissues and its responsiveness to various hormones. C4 has been detected in the cervical mucus in some women during the luteal phase, in endometrial fluid (Vanderpuye et al., 1992), and in follicular fluid (Yoo et al., 2013). To our knowledge, the current experiment is the first to report that C4 is increased in response to IFNT.

The variability of results and difficulty finding suitable antibodies that recognized the bovine protein made elucidating the function of complement in the bovine CL a challenging task. Based upon the expression data and previous proteomic/transcriptomic profiling studies from our lab, complement proteins changed during early pregnancy in the bovine CL and this life stage of the CL might be the one where complement has functional effects. The transcriptomic profiling study by Hughes et al. (2019), which used the same tissues from pregnancy (day 17) as this current study, found that T cell activation and signaling/IL2 signaling and TGFB signaling were pathways upregulated during the period of early pregnancy known as maternal recognition of pregnancy. Complement is important for T cell signaling. Complement proteins play an important role in homeostatic functions of T cells as well as differentiation of T cells (Dunkelberger and Song et al. 2010; Lubbers et al., 2017). T cells can produce both C3 and C5 and express the C3a receptor (C3aR) and C5a receptor (C5aR; Strainic et al., 2008). The intracellular production and cleavage of C3 (and C5 to a lesser extent) is critical for T cell homeostatic survival (Strainic et al., 2008; Liszewski et al., 2013). C3 is continuously cleaved in resting $CD4^+$ T cells by cathepsin L (CTSL), and the cleavage product C3a can bind to its receptor C3aR on lysosomes, which ultimately activates mTOR for cell survival (Liszewski et al., 2013). If intracellular production of C3 is inhibited, the viability of T cells is significantly reduced (Strainic et al., 2008). C3 (and C5) is also important during T cell and antigen presenting cell (APC) interactions. During T cell and APC interactions, C3a and C5a secretion is induced from both cell types (Heeger et al., 2005;

Strainic et al., 2008) and the abundance of C3aR and C5aR on the surface of both cell types is increased (Strainic et al., 2008). When locally produced C3a and C5a bind to their respective receptors it induces T cell proliferation and differentiation (Montz et al., 1990; Strainic et al., 2008; Kwan et al., 2013). If the interaction between C3a and C5a and their receptors is inhibited or absent, MHC class II proteins are reduced on APCs (Strainic et al., 2008) and regulatory T cell (Treg; Foxp3$^+$) development is induced (Kwan et al., 2013; Strainic et al., 2013). Treg function also depends on complement signaling because the lack of signaling through C3aR and C5aR in Tregs enhances the suppressive capacity of Treg cells both *in vitro* and *in vivo* (Kwan et al., 2013). In the CL of pregnancy, there is a greater number of Foxp3$^+$ T cells compared to peripheral blood (Poole et al. unpublished) and a greater number of CD8$\alpha\beta^+$ T cells and $\gamma\delta^+$ CD8$\alpha\beta^+$ T cells.

We hypothesized that knockdown of C3 in cocultured luteal and T cells would reduce C3 signaling and result in a decrease in T cell proliferation and an increase in the number of Tregs (Foxp3$^+$). The purpose of knocking down C3 was to mimic the lower abundance of C3 in the CL of pregnancy, and to determine if this is a possible mechanism of function for C3 during early pregnancy. However, T cell proliferation did not change, and unfortunately, Tregs were unable to be detected and a change could not be reported (data not shown). The robust literature about the importance of C3 signaling (or lack of signaling) in T cell proliferation and differentiation to Tregs indicates this mechanism would likely occur within the CL. The lack of differences in this study could be a result of experimental design, or C3 is not involved in mediating T cell and luteal cell interactions in the CL. Interestingly, previous work in this laboratory demonstrated that when alpha-2-macroglobulin (A2M), a protein in the same family as complement that contains structural similarities to C3 (Janssen et al., 2005), was depleted from luteal-conditioned media, proliferation of $\gamma\delta^+$ T cells was reduced compared to cells exposed to nondepleted luteal-conditioned media (Brzezicka, 2013). These findings suggest the possibility of C3 acting in the

manner that was hypothesized, but the experimental design was not conducive for observation of differences. Previous validation demonstrated C3 was effectively knocked down using GapmeRs in luteal cells on day 5 of culture and day 3 of culture in T cells. GapmeRs were chosen as the method for knocking down C3 because of the high specificity of GapmeRs, transfection reagent was not needed, which spared the viability of transfected cells, and because bovine cells used in this experiment limited the use of complement C3 inhibitors. The C3 inhibitors on the market today are designed to work against human C3 which shares only 76% homology to bovine C3, and were not likely to be effective in bovine cells. GapmeR transfected T cells were added to C3 GapmeR transfected luteal cells when C3 knockdown was found to be maximal and cocultured for 72 hours. The purpose was to expose T cells to luteal cells when C3 was very low in both cell types. However, perhaps the time in culture after addition of the T cells to the luteal cells was too long and C3 rebounded, and thus the effects were not observed. The half-life of C3 in plasma is roughly 72 hours (Ekdahl et al., 2019), and the half-life of *C3* has been estimated to be roughly 1.8 hours in hepatocytes (Mitchell et al., 1996), however the half-life of C3 protein in cells. The rate at which cells produce C3, especially in this T cell-luteal cell coculture system, is unknown. Measuring C3 in both luteal and T cells after coculture would be an ideal follow up experiment to determine if either cell type stimulates the other to produce C3 and impeded the effect of C3 knock down. Additionally, adding the GapmeR-treated T cells to the GapmeR-treated luteal cells earlier in culture and measuring T cell proliferation on the day of culture with the greatest knockdown might have been a better approach. Although C4 showed promise in terms of playing a role in the CL of pregnancy as it was regulated by IFNT, it is not known if C4 plays a role in T cell immunity, so it was not the protein of choice to target for this experiment.

Conclusion

The findings from this study are the first to report the abundance of complement components, C3 and C4, across the lifespan of the bovine CL and the first to attempt to determine

a functional role of complement in the CL. This study confirmed that luteal steroidogenic cells do produce C3 and C4. This is also the first report that IFNT, the hormone responsible for maternal recognition of pregnancy, increased C4, and that PGF2A decreased C3. PGE2 plus IFNT also decreased C3. It is suggested that complement might be involved in maintenance of the CL of early pregnancy. The interaction between T cells and luteal steroidogenic cells was hypothesized to be one mechanism that might be affected by the decrease in C3 abundance in the CL of pregnancy. However, C3 did not alter T cell proliferation in the T cell-luteal cell coculture system. Although T cell proliferation was not altered by knockdown of C3 and this may not be the mechanism of action for C3 in the CL, C3 almost certainly has a role to play in the CL and future work will be needed to determine its function.

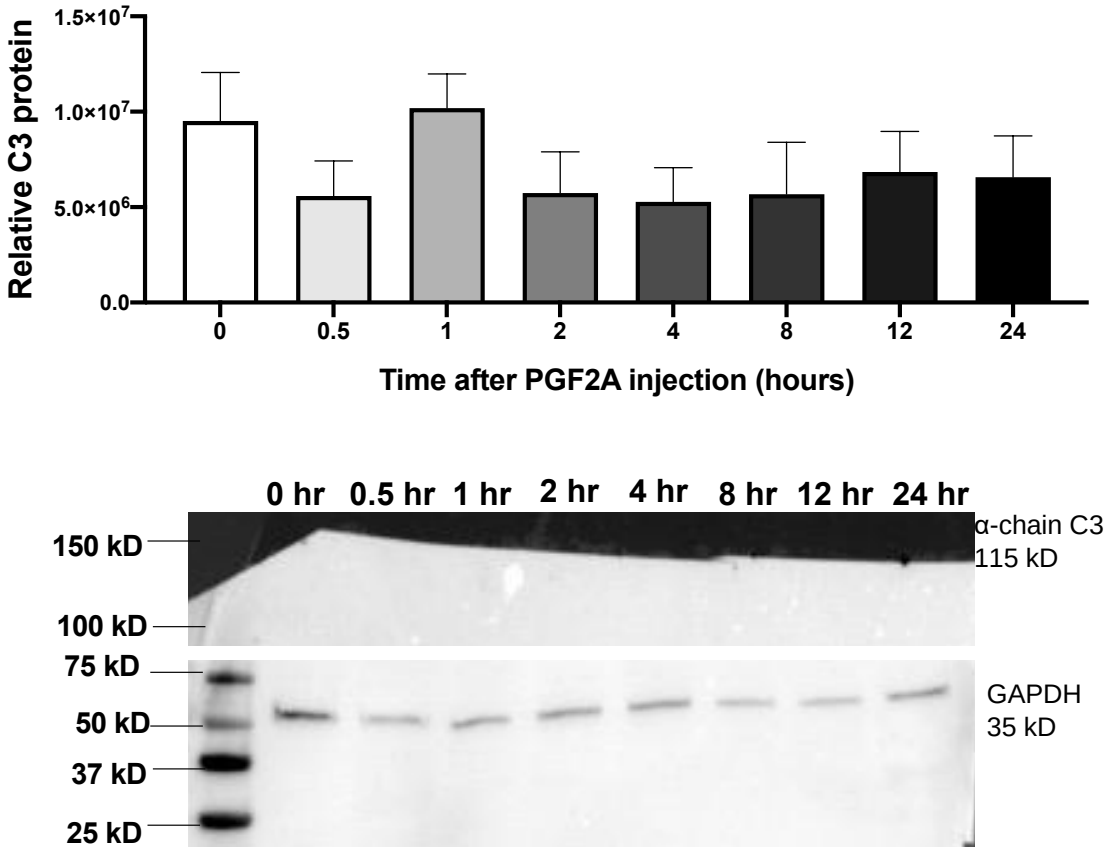


Figure 4-1. Time course study of relative C3 protein after *in vivo* PGF2A injection. Relative C3 protein in luteal tissue after PGF2A injection (n=4) and representative western blot of C3 protein after PGF2A injection. GAPDH was used as a loading control.

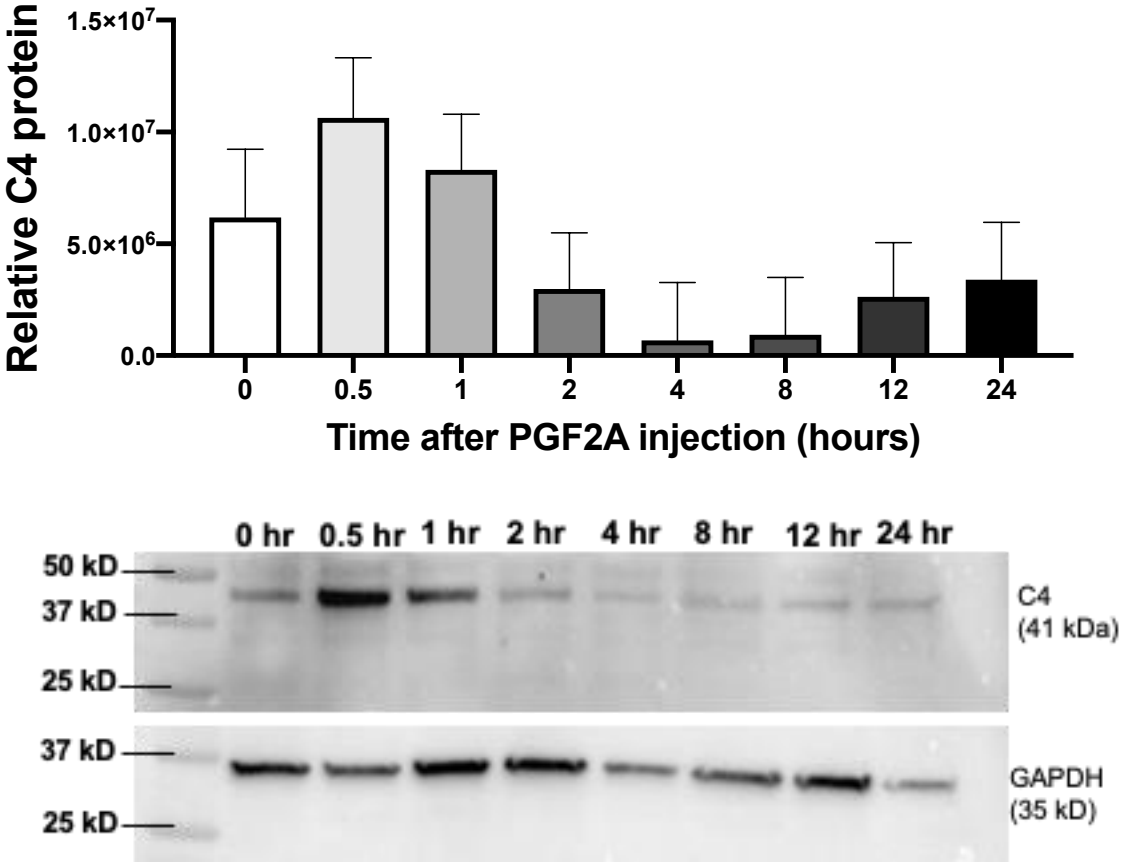
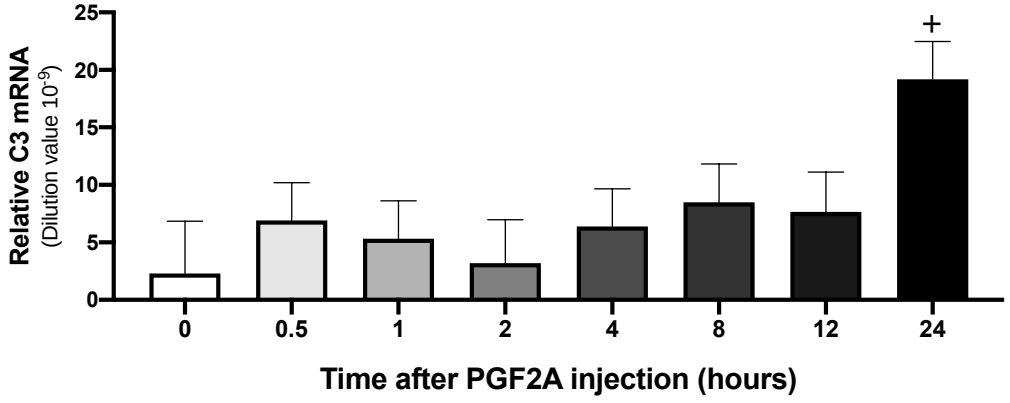


Figure 4-2. Time course study of relative C4 protein after *in vivo* PGF2A injection. Relative C4 protein abundance in luteal tissue after PGF2A injection (n=4) and representative western blot of C4 protein after PGF2A injection. GAPDH was used as a loading control.

(A)



(B)

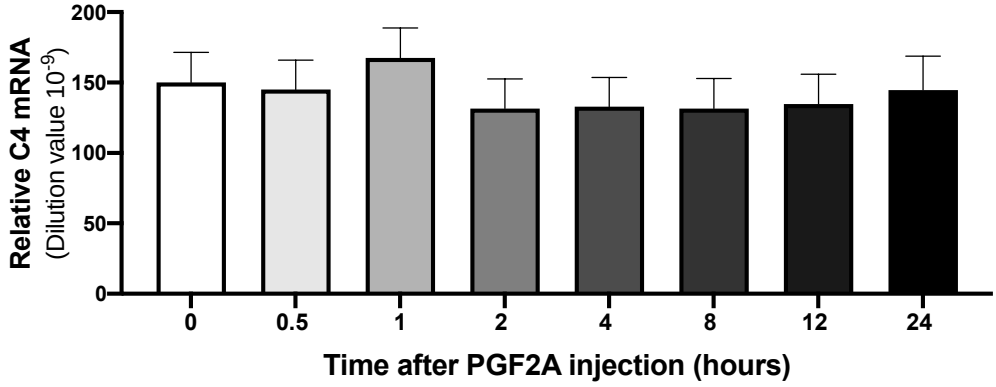


Figure 4-3. Time course study of C3 and C4 mRNA in luteal tissue after *in vivo* PGF2A injection. A) C3 mRNA after PGF2A injection (n=4), B) C4 mRNA after PGF2A injection (n=4); + indicates a statistical tendency $0.05 < p < 0.1$

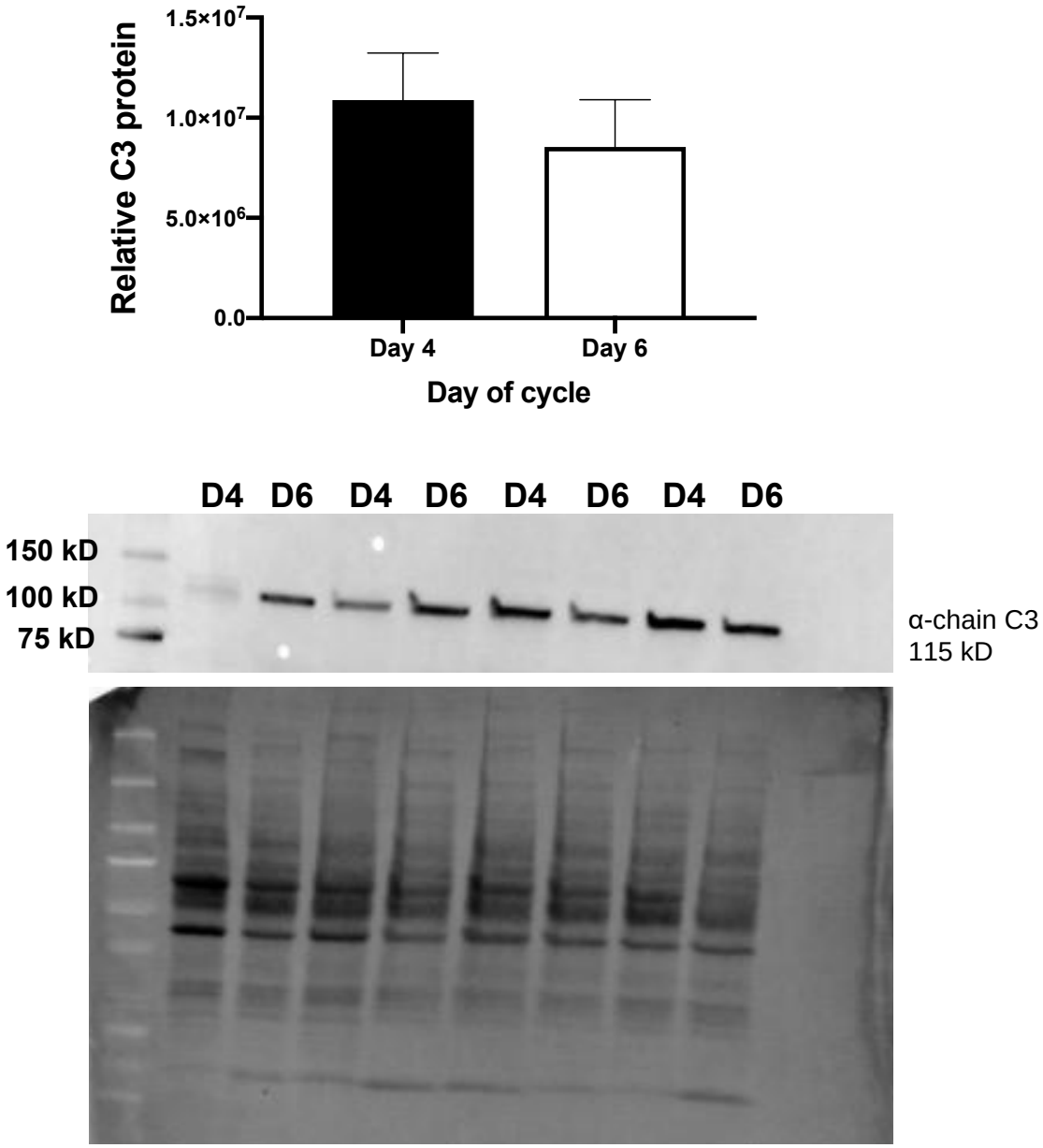


Figure 4-4. C3 protein in luteal tissue during acquisition of luteolytic capacity. Relative C3 protein in luteal tissue on day 4 and 6 of the estrous cycle (n=4) and western blot image of C3 protein in day 4 and 6 luteal tissue. The stain free membrane image below the blot represents total protein for the blot above.

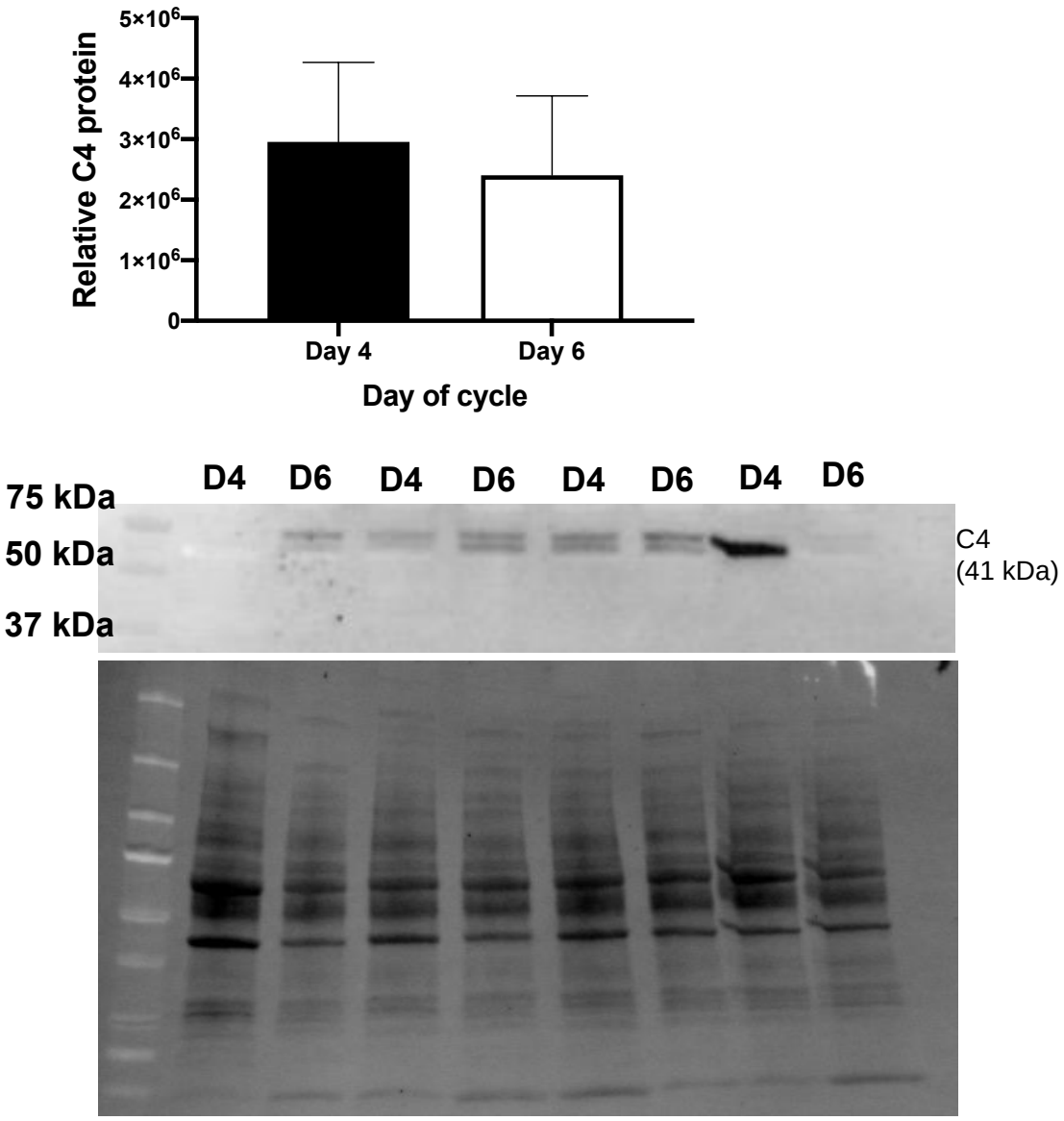
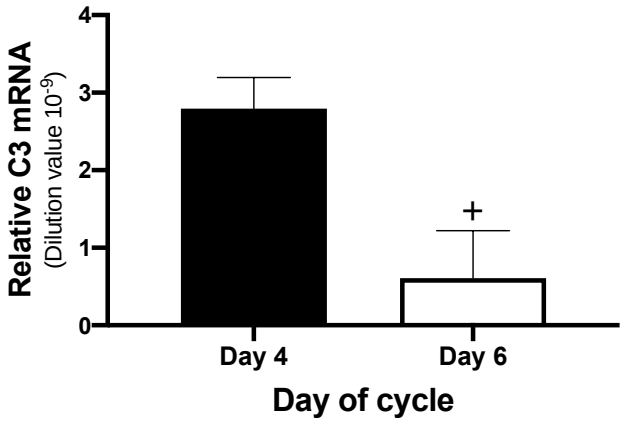


Figure 4-5. Relative C4 protein in luteal tissue during acquisition of luteolytic capacity. A) C4 protein in luteal tissue on day 4 and 6 of the estrous cycle (n=3) and western blot image of C4 in day 4 and 6 CL. Note: The first D4 sample on the left and last D6 sample on the right were excluded as no C4 protein was detected. The stain free membrane image below the blot represents total protein for the blot above.

(A)



(B)

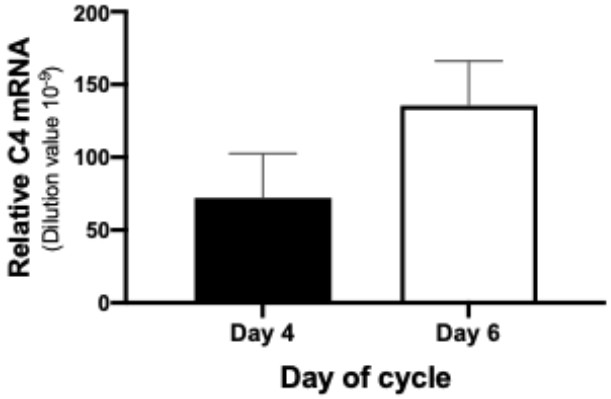


Figure 4-6. C3 and C4 mRNA in luteal tissue during acquisition of luteolytic capacity. A) C3 mRNA in day 4 and day 6 CL (n=4), B) C4 mRNA in day 4 and day 6 CL (n=4); + indicates a statistical tendency $0.05 < p < 0.1$

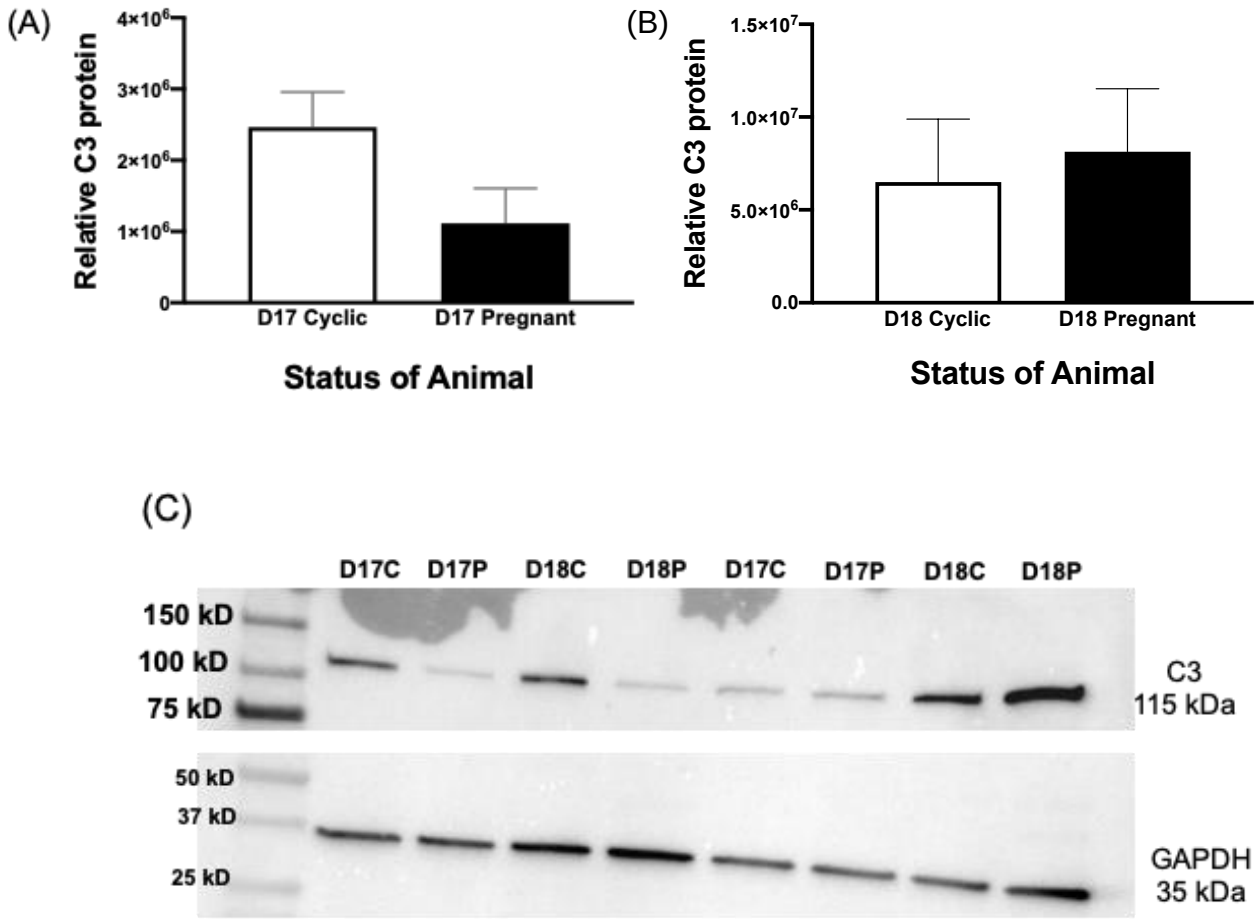


Figure 4-7. Relative C3 protein in luteal tissue from day 17 and 18 of pregnancy. A) C3 protein in day 17 CL of cyclic and pregnant animals (n=4), B) C3 protein in day 18 CL of cyclic and pregnant animals (n=4), C) A representative western blot of C3 protein from day 17 and day 18 CL of both cyclic and pregnant animals.

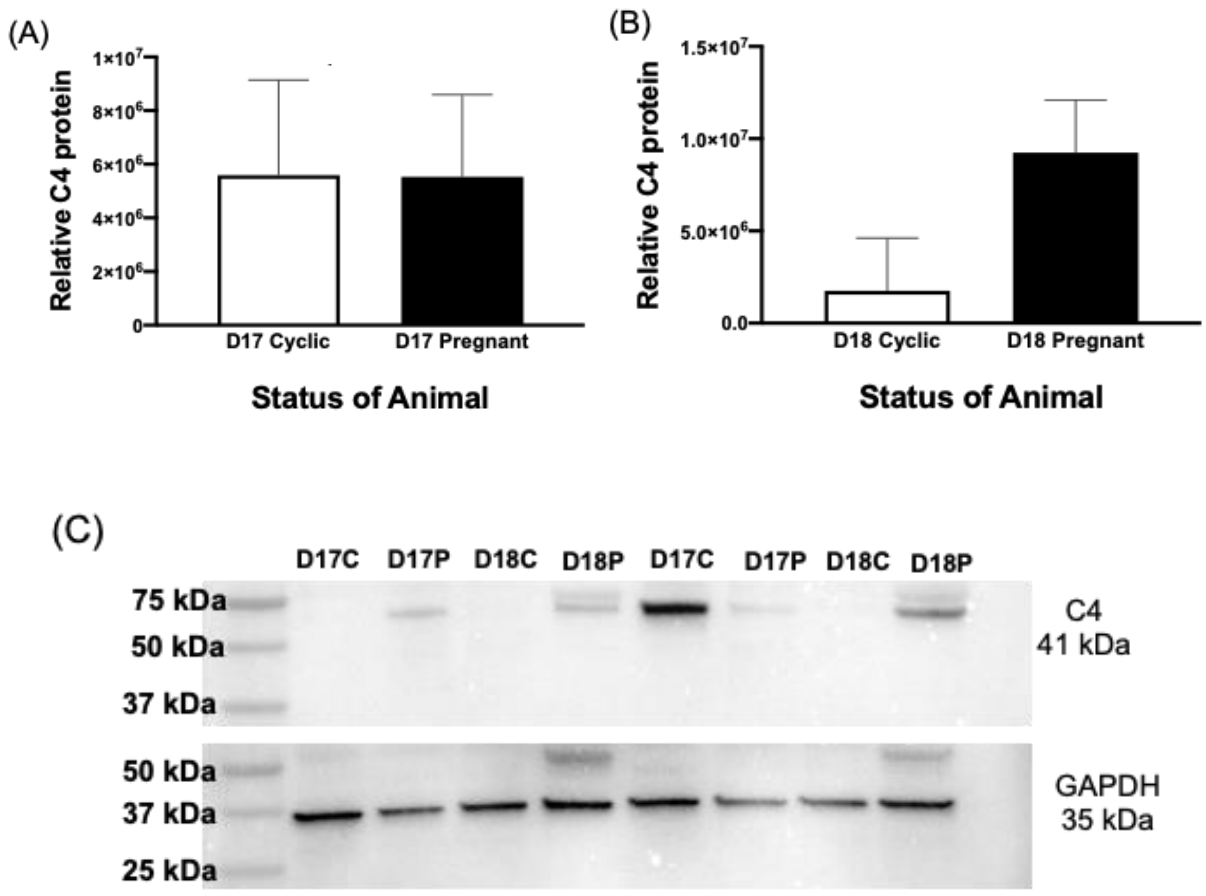


Figure 4-8. Relative C4 protein in luteal tissue from day 17 and 18 of pregnancy. A) C4 protein in day 17 CL of cyclic and pregnant animals (n=4), B) C4 protein in day 18 CL of cyclic and pregnant animals (n=4), C) A representative western blot of C4 protein from day 17 and day 18 CL of both cyclic and pregnant animals.

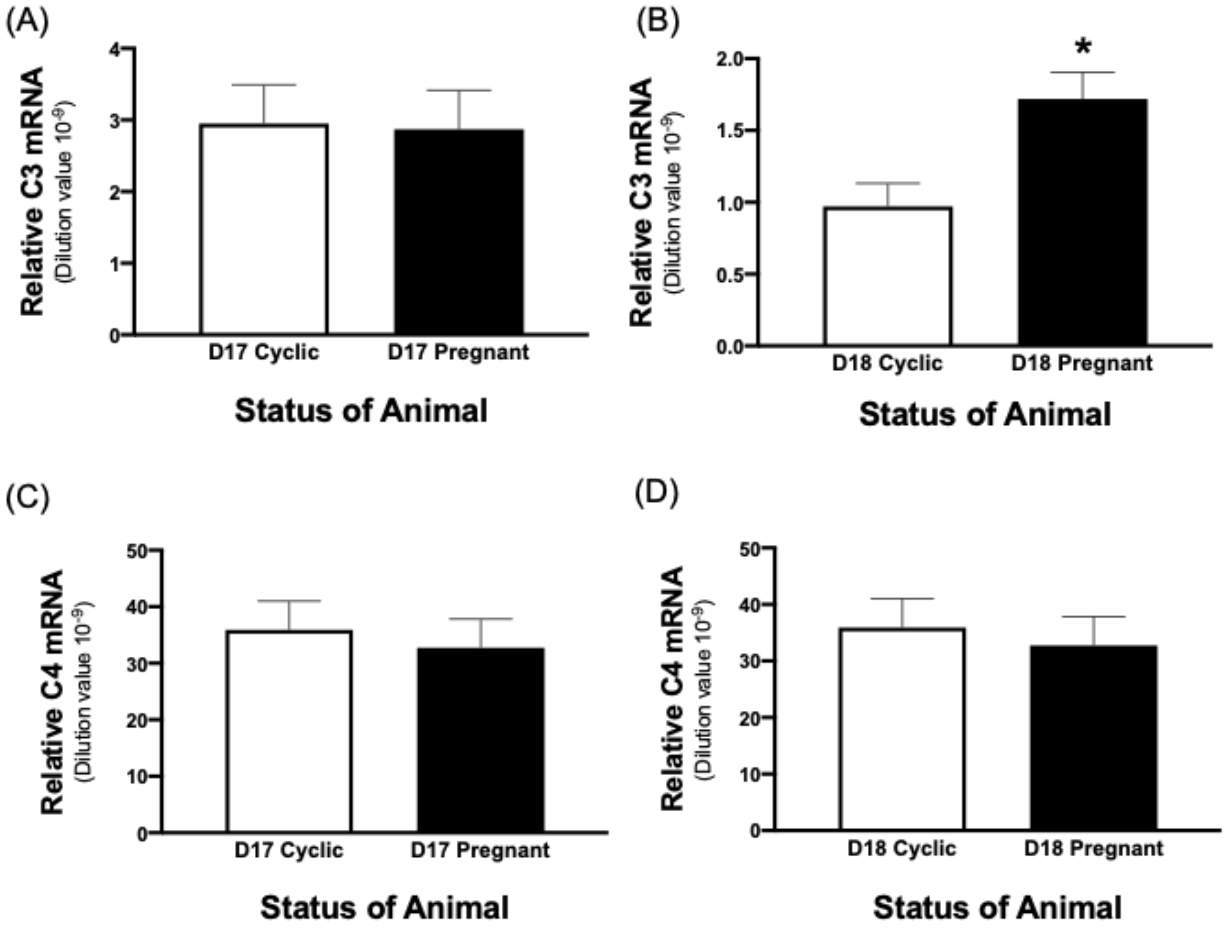


Figure 4-9. Relative C3 and C4 mRNA in luteal tissue from day 17 and day 18 of pregnancy. A) C3 mRNA on day 17 (n=6)**,** B) C3 mRNA on day 18 (n=4 cyclic, n=3 pregnant), C) C4 mRNA on day 17 (n=6), D) C4 mRNA on day 18 (n=4 cyclic, n=3 pregnant); * indicates statistical significance $p < 0.05$

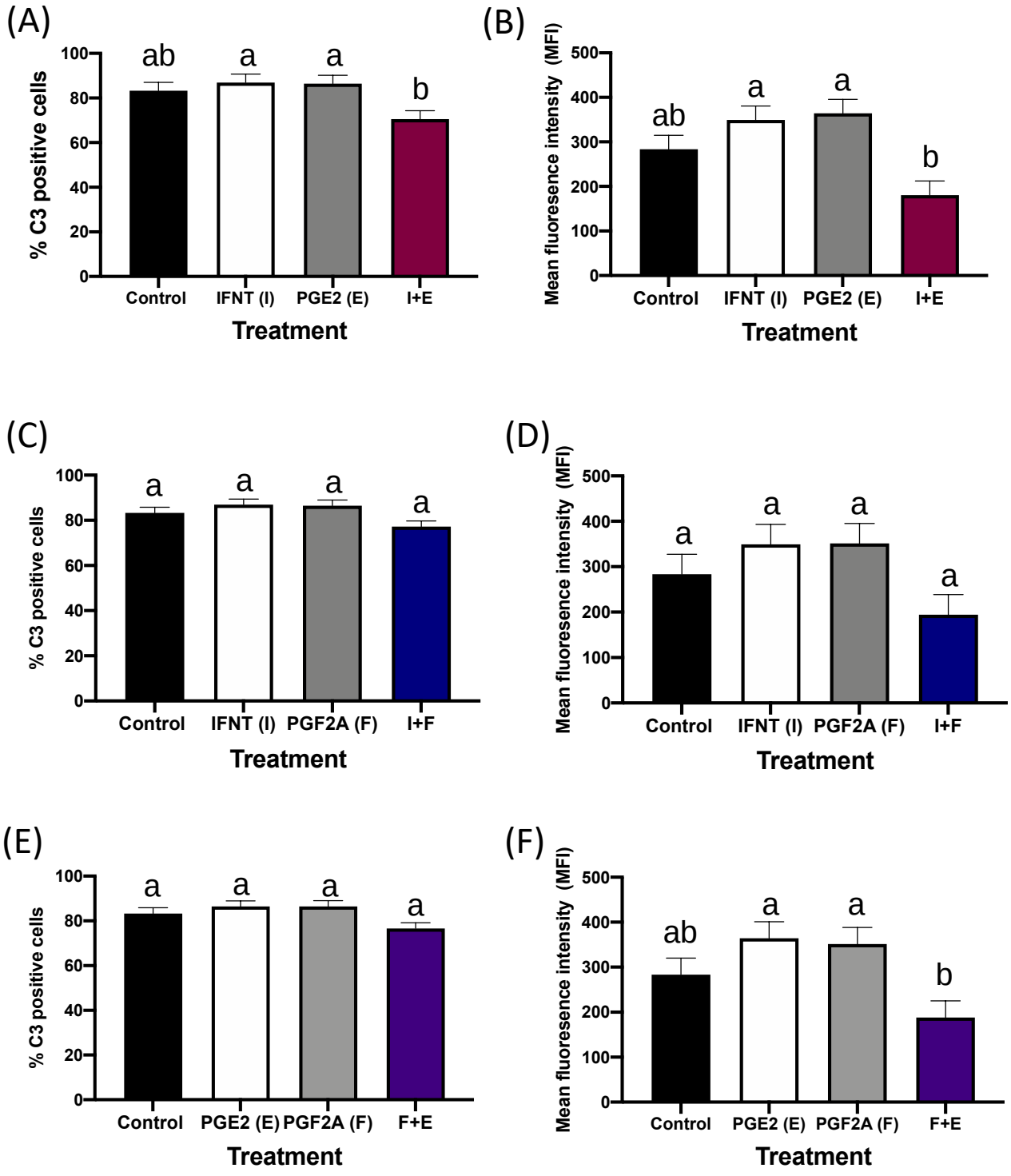


Figure 4-10. Abundance of C3 protein and percentage of C3 positive luteal cells after treatment with IFNT, PGE2, and PGF2A. A) Percentage of C3 positive cells after treatment

with IFNT and PGE2, B) Mean fluorescence intensity (MFI) of C3 in luteal cells treated with IFNT and PGE2. C) Percentage of C3 positive cells after treatment with IFNT and PGF2A, D) MFI of C3 in luteal cells treated with IFNT and PGF2A, E) Percentage of C3 positive cells after treatment with PGE2 and PGF2A, F) MFI of C3 in luteal cells treated with IFNT and PGF2A; n=5; different letters denotes statistical significance with $p < 0.05$

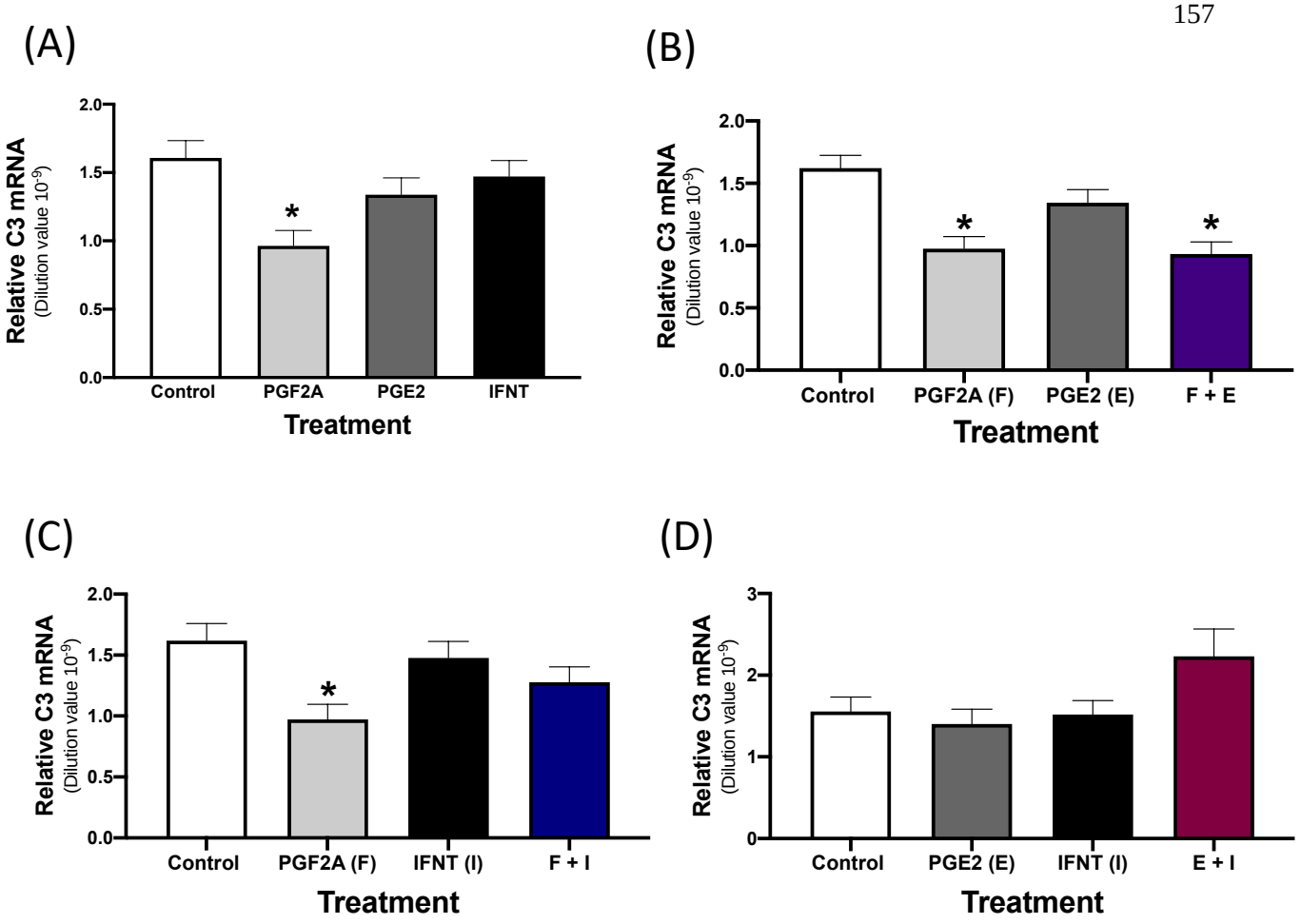


Figure 4-11. Relative C3 mRNA in luteal cells treated with PGF2A, PGE2, IFNT, and combination treatments. A) C3 mRNA after treatment with PGF2A, PGE2, IFNT, B) C3 mRNA after treatment with PGF2, PGE2, and a combination treatment, C) C3 mRNA after treatment with PGF2A, IFNT and a combination treatment, D) C3 mRNA after treatment with PGE2, IFNT, and a combination treatment; n=4; * denotes statistical significance compared to control with $p < 0.05$

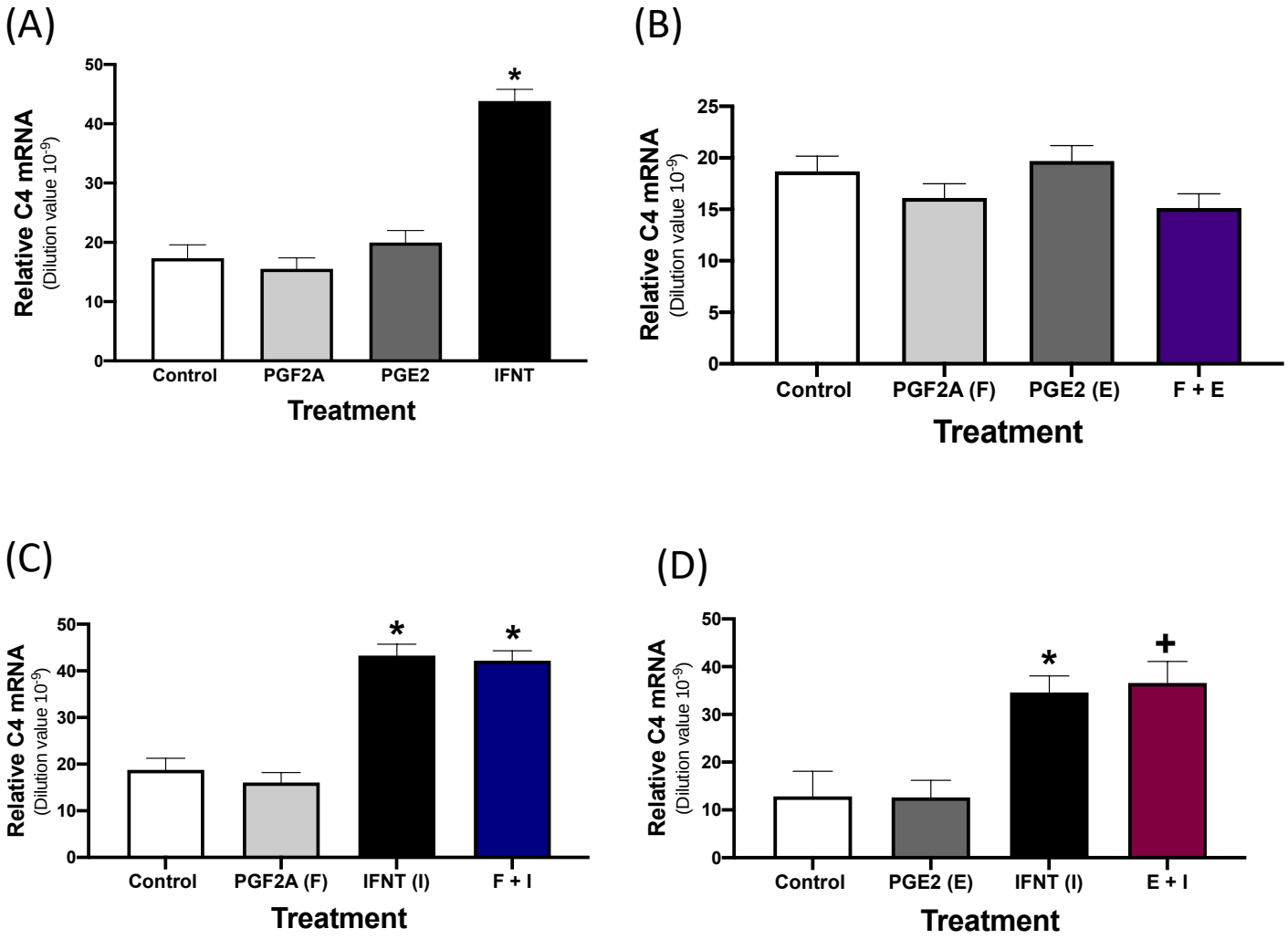


Figure 4-12. Relative C4 mRNA in luteal cells treated with PGF2A, PGE2, IFNT, and combination treatments. A) C4 mRNA after treatment with PGF2A, PGE2, IFNT, B) C4 mRNA after treatment with PGF2, PGE2, and a combination treatment, C) C4 mRNA after treatment with PGF2A, IFNT and a combination treatment, D) C4 mRNA after treatment with PGE2, IFNT, and a combination treatment; n=4; * denotes statistical significance compared to control with $p < 0.05$; + denotes a statistical tendency compared to control with $0.05 < p < 0.1$

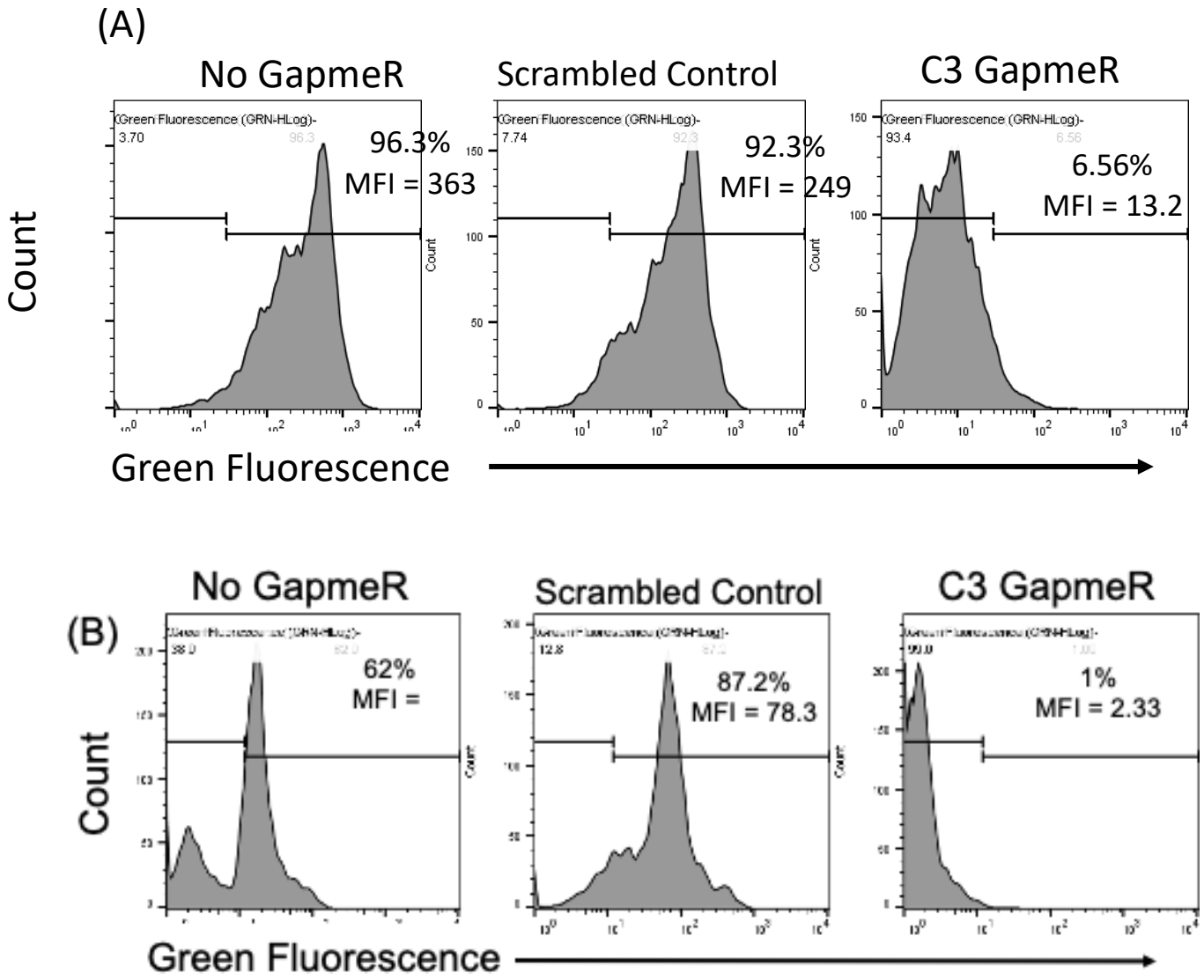


Figure 4-13. Effectiveness of GapmeR knockdown in cultured luteal cells and T cells. A) Representative histograms showing the effectiveness of the C3 knockdown by C3 GapmeR in luteal cells on day 5 of culture. B) Representative histograms showing the effectiveness of the C3 knockdown by C3 GapmeR in T cells on day 3 of culture.

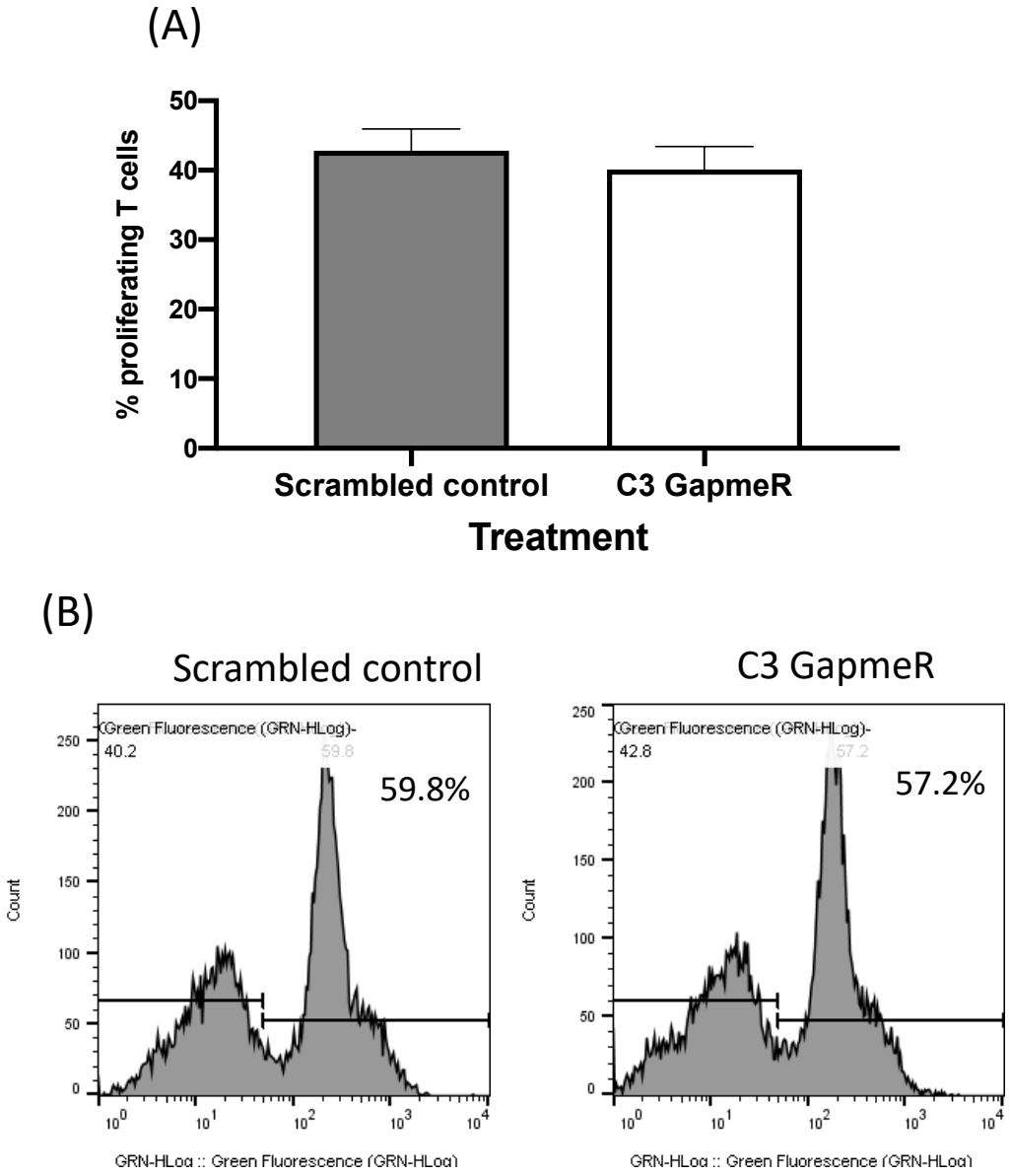
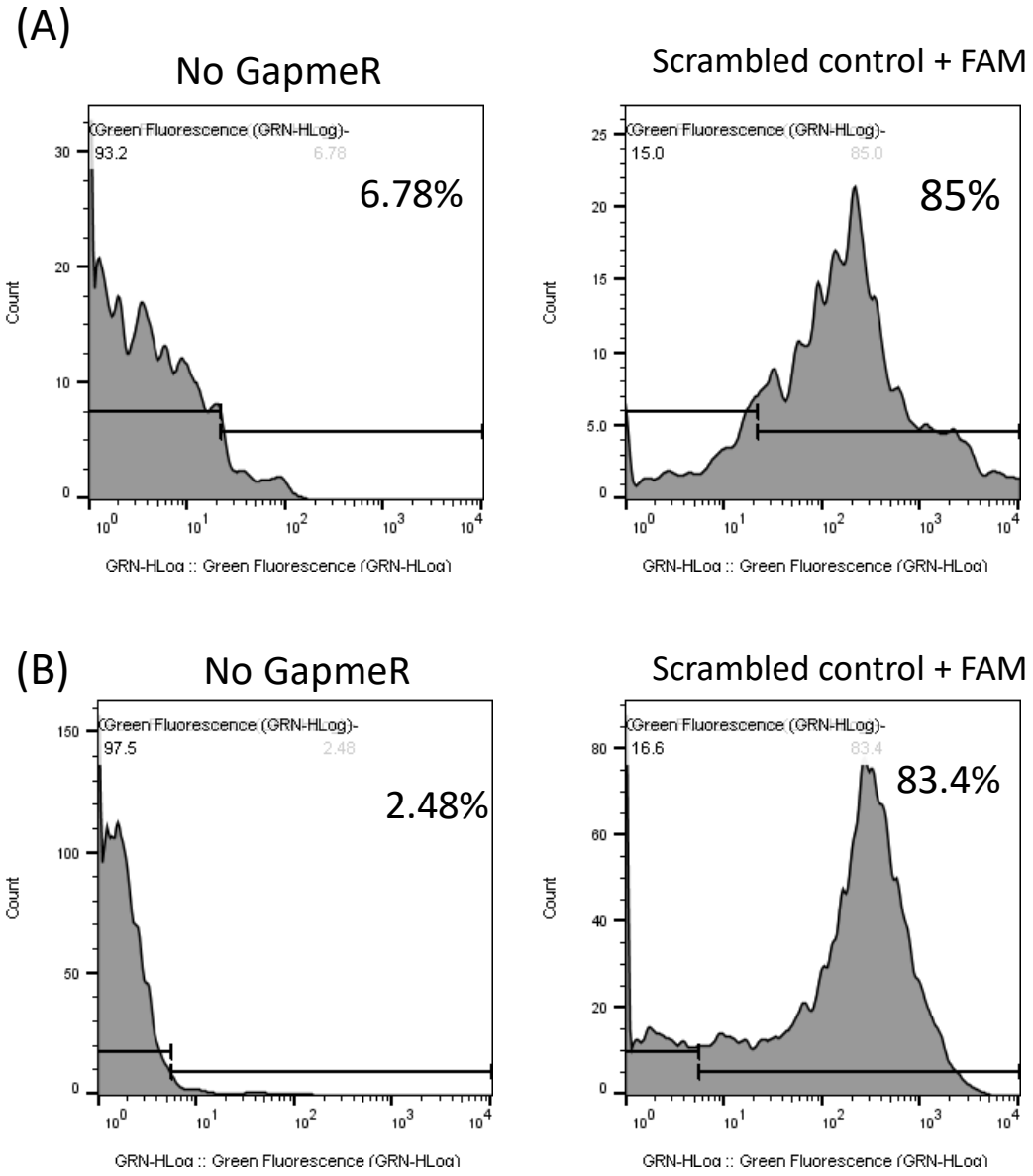


Figure 4-14. T cell proliferation after C3 knockdown in both luteal cell and T cell and 72 hours of coculture. A) T cell proliferation after C3 knockdown (n=4). B) Representative histogram plots of T cell proliferation after 72 coculture.



Supplementary Figure 4-1: Transfection efficiency of GapmeRs in luteal cells and T cells.

A) Representative histograms showing the transfection efficiency of GapmeRs in luteal cells. B) Representative histograms showing the transfection efficiency of GapmeRs in T cells.

Chapter 5

Summary and Conclusion

The successful establishment and maintenance of pregnancy in mammals requires a functional corpus luteum (CL). The CL is a diverse tissue with many different cell types including T cells and other resident immune cells that contribute to its overall function. Additionally, other signaling molecules such as micro-RNA (miRNA) and other immune components, such as complement, also appear to contribute to the function of the CL, but less is known about how these molecules contribute to overall luteal function. Understanding how these cell types and signaling molecules might support a functional CL could lead to important discoveries to improve reproductive success in many species including the dairy cow. This project investigated several mechanisms by which the functional CL could be supported. The first chapter aimed to determine if let-7, a miRNA, is present in the mitochondria of luteal cells and if it could alter mitochondrial function and progesterone production. The second chapter aimed to determine if 1) uterine PSPB signals to the CL alters the transcriptome of either whole luteal tissue and/or resident immune cells, and 2) if a general phenotype of resident immune cells from the CL of early pregnancy could be elucidated. Lastly, the third chapter aimed to characterize the expression of complement proteins, C3 and C4, in the CL and determine a functional role of C3.

Chapter 2 conclusion

The let-7 and mitochondria study was designed to answer the question of whether the miRNA, let-7, was present in the mitochondria isolated from bovine luteal cells, and if it could alter mitochondrial function and/or progesterone production in the CL.

Despite an attempt to determine the presence of let-7 in mitochondria isolated from bovine luteal cells, neither let-7a nor let-7b were detected in the mitochondria. This is one

weakness of this chapter as these data do not provide clear evidence that let-7 is within the mitochondria carrying out its function. It was concluded that there were issues with the methodology, either in the mitochondria isolation process, or the miRNA qPCR and not because let-7 is not present in the mitochondria. It would be beneficial in the future to attempt different methodology, such as using a new mitochondrial isolation technique, to have a definitive answer about whether or not let-7 is present in the mitochondria of bovine luteal cells. Evidence from our pulldown assay and the literature (Das et al., 2012; Das et al., 2014; Sharma et al., 2021) suggested miRNA can target mitochondrial mRNA, and our lab was curious to see if let-7 could alter mitochondrial function and/or progesterone production despite not being able to detect its presence in the mitochondria.

The main findings were that let-7b decreased basal respiration and proton leak and tended to decrease ATP production and spare capacity, while let-7a had no impact on mitochondrial respiration. Additionally, let-7b also decreased progesterone production by luteal cells on day 5 and 7 of culture, while let-7a increased progesterone production on day 3 of culture. These findings are very novel given luteal tissue is a highly metabolically active tissue requiring mitochondrial respiration for ATP production for both fuel for the tissue as well as for production of progesterone, the primary function of this tissue. While these data answered the main question about whether let-7 could alter mitochondrial function and progesterone production, the mechanism by which let-7 regulates these processes is still unknown. Other studies have demonstrated miRNA can target and reduce expression of ETC protein subunits encoded by the mitochondrial genome, and they hypothesize the reduction in expression of these subunits by miRNA can alter the function of the ETC proteins in a manner that can reduce mitochondrial function (Das et al., 2012; Das et al., 2014; Sharma et al., 2021). However, the exact mechanism by which miRNA regulate mitochondrial respiration is still under investigation. One weakness of this experiment is the abundance of mitochondrial gene targets were not measured as a result of

let-7 transfection. Nuclear gene targets of let-7, EGR1 and PGRMC1, were assessed. However, the results were ambiguous as the let-7 mimics unexpectedly increased the abundance of PGRMC1 and EGR1 (let-7a only) and the inhibitors expectedly increased the abundance of these proteins. While this is not indicative of what the mimics and inhibitors might be doing in the mitochondria, these results suggest either 1) the mimics acted "off target" on another regulator of PGRMC1 and EGR1, 2) let-7 can increase translation of its targets, or 3) let-7 acts differently in different subcellular locations. Regardless of the nuclear gene target results, had the mitochondrial gene targets been assessed, this study would be more robust and provide evidence about the mechanism by which let-7 might be acting to regulate mitochondrial function.

Additionally, the mechanism by which let-7 regulated progesterone production is also unknown and was not assessed in the current study. It seems mostly likely that let-7 would regulate progesterone production by either targeting steroidogenic enzymes, or by regulating mitochondrial respiration to reduce the amount of ATP necessary for steroidogenesis. Let-7b is known to decrease *STAR* in mouse Leydig cells (Men et al., 2017), and *STAR* is a predicted target of let-7a, but neither CYP11A1 nor HSD3B are predicted targets of let-7. In addition to the necessary enzymes, steroidogenesis also requires ATP. If ATP is reduced by disruption of the mitochondrial membrane potential, or inhibition of the electron transport chain, steroidogenesis decreases (Men et al., 2017). Let-7b tended to decrease ATP production, which in conjunction with the downregulation of STAR, is one likely mechanism by which let-7 can regulate progesterone production by luteal cells. In order to strengthen the current study, confirming the regulation of STAR by let-7 in luteal cells in conjunction with the mitochondrial function data would provide evidence for how let-7 alters steroidogenesis.

Overall, these findings are the first to demonstrate that let-7 can regulate mitochondrial respiration and steroidogenesis in bovine luteal cells. While the mechanisms by which let-7 acts to regulate these pathways still remains unknown, this study does provide information about a

new regulator of progesterone production and mitochondrial respiration in luteal cells.

Chapter 3 conclusion

Overall, the main findings from the uterine infusion study provided evidence that uterine PSPB does signal to the CL and regulates a small number of transcripts in both resident immune cells and whole luteal tissue. PSPB differentially regulated only 3 transcripts in resident immune cells and only 5 transcripts in whole luteal tissue compared to control. Individually, the two transcripts that were identified and discussed in resident immune cells, CR2 and serpin B4-like, both play roles in immunomodulation and might help with immune cell adhesion and promote structural integrity of the CL. Pathway analysis also support these conclusions as complement signaling and eNOS signaling were pathways by which these immune cells might be acting to support structural integrity and maintenance of the CL. However, these pathways need to be further evaluated because one weakness of this analysis is that less than 100 genes were used for pathway analysis, meaning these pathways might only reflect on a few transcripts included in the analysis, and not accurately reflect mechanisms by which these immune cells as a whole might be acting.

The same holds true with the luteal tissue analysis. There were 5 differentially abundant transcripts in response to the PSPB, but only 2 had a log2fold change that met criteria. One transcript was MAP1LC3C, which decreased in response to PSPB. MAP1LC3C is a protein associated with autophagy and its family of proteins are known to increase during luteal regression (Aboelenain et al., 2015), so it makes sense why PSPB would down regulate the abundance of this transcript in order to maintain the integrity of the CL. Pathway analysis found prothrombin signaling, IL17 signaling, and iNOS signaling to be upregulated in luteal tissue in response to PSPB. These same pathways that were upregulated by PSPB in luteal tissue were also found to be upregulated in a transcriptomic profiling study of early pregnancy (Hughes et al.,

2020), confirming these pathways are important for luteal function during pregnancy. However, exactly how these pathways are functioning within the CL of pregnancy is not known. It seems probable that these pro-inflammatory pathways might be important for activating an immune response that could lead to the remodeling of the CL for continued longevity during pregnancy. IL17 is produced by Th17 cells and is primarily known for recruiting and activating neutrophils. IL17 signaling could promote the recruitment of neutrophils to the CL and induce angiogenesis as neutrophils are known to promote angiogenesis in the developing CL (Jiemtaweeboon et al., 2011). Assessing the abundance of neutrophils in the CL of pregnancy and how they might promote angiogenesis in response to IL17 would be an interesting future direction. Additionally, IL17 is produced by Th17 cells and there is some plasticity between Th17 cells and Treg cells. Our lab has previously demonstrated there is a greater proportion of $\gamma\delta^{+}CD8^{+}$ T cells, an antiinflammatory type of T cell, in the CL of pregnancy on day 18, suggesting a more immunotolerant phenotype of T cells in the CL of pregnancy (Poole and Pate, 2012). However, determining the phenotypes of T cells in the CL of pregnancy would help elucidate the function of IL17 signaling.

In fact, determining the phenotype of resident immune cells in the CL of pregnancy was assessed in this experimental model of pregnancy. However, because the immune cells ($CD45^{+}$) isolated from the CL included many cell types including T cells, macrophages, monocytes, and neutrophils, elucidating the phenotype of these cells was difficult and a limitation to determining a true phenotype. In order to best assess the phenotype of immune cells using a transcriptomic approach, it would have been best practice to isolate each immune cell type and perform transcriptomic profiling separately for a more pure assessment. The top 100 most abundant transcripts from resident immune cells exposed to the IFNT+PSPB infusion were selected and underwent pathway analysis in hopes of understanding what these immune cells might be doing in the CL of pregnancy. The pathways identified were ones involved with the regulation of

transcription and translation as well as mTOR signaling, which can either promote immune cell activation or inhibit it depending on environmental cues. Additionally, NRF2 signaling was another upregulated pathway that promotes the transcription of antioxidant and inhibit proinflammatory cytokine production. Follow up experiments, such as treating $CD45^+$ cells (or examine each cell type individually) with IFNT and PSPB and to determine whether mTOR signaling is occurring through mTORC1 and/or mTORC2 would offer more insight into the phenotype of these cells because mTOR signaling through these different complexes can change immune cell differentiation. It would also be interesting to measure the abundance of NRF2 in resident immune cells from the CL of pregnancy and determine the milieu of cytokines to determine if NRF2 might suppresses proinflammatory cytokines.

While the main focus of the experiment was about the results PSPB signaling, the 3-day uterine infusions (3-day BSA compared to 3-day IFNT) proved to be a "proof of concept" comparison, as IFNT infused into the uterus was able to induce transcriptomic changes in the CL. Interestingly, when the 3-day infusion pathways were compared to the pathways identified in the 3-day IFNT and 6-day IFNT comparison, there was a clear shift from an upregulation of interferon signaling and antiviral pathways to pathways associated with tissue homeostasis, cell proliferation, and T cell homeostasis. This suggests prolonged IFNT exposure during early pregnancy might be beneficial for promoting transcriptomic changes to support sustained luteal function during pregnancy.

Overall, this study is the first to report on the transcriptomic changes in the CL and resident immune cells as a result of uterine PSPB signaling to the CL. PSPB aids in promoting the immunomodulation effects that are both pro- and anti-inflammatory pathways. These pathways all suggest they might be necessary for structural remodeling and maintaining the integrity of the CL during pregnancy. Unfortunately, the phenotype of resident immune cells

remains unclear from these data, however, immune cell activation and suppression of inflammatory pathways supported by these data might be important in the CL of pregnancy.

Chapter 4 conclusion

The complement project aimed to determine the expression of C3 and C4 across the life span of the CL and determine a functional role of C3. The main life stage of interest was the CL pregnancy because previous data from our lab found differentially abundant complement components in the CL of pregnancy (Hughes et al., 2019; Hughes et al., 2020). C3 and C4 expression was assessed during luteal regression and acquisition of luteolytic capacity (between day 4 and 6) because these are important time points in luteal biology where the CL either dies off (regression), or the CL acquires the ability to respond to a luteolytic dose of PGF2A. However, neither of these life stages showed remarkable results. In the CL of pregnancy, C3 had a 2-fold numerical decrease on day 17 with no change in *C3*, while there was no change in C3 on day 18, but an increase in *C3* on day 18 of pregnancy. C4 had no changes in the CL of pregnancy on day 17, but a 5-fold numerical increase in the CL of pregnancy on day 18 with no changes in *C4.* Hormonal regulation supported these findings as C3 decreased in response to both IFNT+PGE2 and PGF2A+PGE2, suggesting the combination of hormones during pregnancy is necessary to regulate C3. *C4* increased in response to IFNT, and when IFNT was combined with PGE2 and PGF2A, consistent with the western blot findings of pregnancy. The complement expression data, in addition to the hormonal regulation data suggested that pregnancy might be the critical time point where complement plays a role.

It was hypothesized reduced C3 abundance (as observed in the CL of pregnancy) could result in decreased T cell proliferation and increase the proportion of $Foxp3^{+}$ T cells. Complement plays an active role in T cell function, differentiation, and homeostasis (Dunkelberger and Song et al. 2010; Lubbers et al., 2017), and T cell activation and signaling

were important upregulated pathways in the CL of pregnancy (Hughes et al., 2019). However, when C3 was knocked down using GapmeRs in both luteal and T cells, there was no change in luteal cell-induced T cell proliferation and the number of $Foxp3^+$ T cells were unable to be measured. While there were no changes observed in this experiment, it might just mean this is not the pathway by which C3 exerts a functional role. It is unknown if C3 alters steroidogenesis, so measuring progesterone production from culture media after *in vitro* knock down of C3 in luteal cells would be an easy pathway to assess with relevant importance to luteal biology. Unfortunately, one weakness of this paper is the variability by which complement components are expressed and the difficulty in finding reagents, like antibodies, suitable for the bovine model. Ideally, this study would have also evaluated complement inhibitors like CD55, which was found to be increased in the CL of pregnancy on day 17 (Hughes et al., 2019; Hughes et al., 2020), however proper antibodies and primers were unable to be found to measure the abundance of this molecule. Perhaps the wrong complement protein was chosen for study and it is actually the inhibition of C3 by CD55 in the CL of pregnancy that promotes an immunotolerant microenvironment to support sustained luteal function. However, unless proper reagents can be found, this might be a difficult to experiment to perform using a bovine model.

While the role of complement in the CL still remains unknown, these data are the first to characterize the expression and hormonal regulation of C3 and C4 in the bovine CL. Both these data and other profiling studies (Hughes et al., 2019; Hughes et al., 2020; Mezera et al., 2021) suggest the CL of pregnancy is the life stage of importance to complement function, but future studies are necessary to elucidate its functional role.

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Publications and abstracts

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