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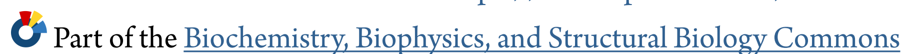
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Recruitment of monocytes to the pre-ovulatory ovary

By

Alex Whitaker

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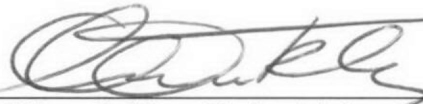

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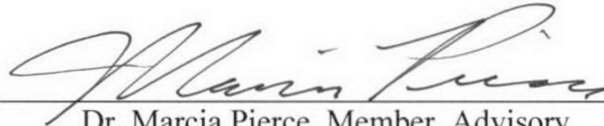

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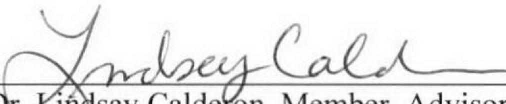

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Recruitment of monocytes to the pre-ovulatory ovary

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Bachelor of Science
Eastern Kentucky University
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Submitted to the Faculty of the Graduate School of
Eastern Kentucky University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
May, 2016

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DEDICATION

This thesis is dedicated to my parents
Steve and Debby Whitaker
for their unwavering encouragement.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Oliver R. Oakley, for his support, guidance, and help regarding the completion of this project. If not for the many demonstrations of experimental techniques, reassurance when experiments failed, and stimulating ideas and background knowledge, this research may not have been finished. In addition, I would like to thank the other committee members, Dr. Marcia Pierce and Lindsey Calderon for comments and assistance with writing. I would also like to give thanks to the various undergraduates who assisted with my projects so they could gain research experience. Research reported in this publication was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 5P20GM103436-13 and a Bridges to the Doctorate Program (R25) under grant number R25GM102776.

The Recruitment of monocytes to the pre-ovulatory ovary

Alex Whitaker

Ovulation is a recurring biological process that involves inflammatory reactions that degrade and restructure tissue. It requires the chemotaxis of leukocytes to the ovary to help regulate and promote these events. Hormonal signals and chemokines that are released during the peri-ovulatory period initiate the release of specific leukocyte populations, possibly from the spleen, that infiltrate the ovary and facilitate the release of oocytes from ovarian follicles. These studies utilized a super-ovulation protocol to initiate ovulation in immature balb/c and cd1 mice. Ovaries were collected at several times post human chorionic gonadotropin (hCG) injection and analyzed by multi-color flow cytometry. Oviducts were collected from mice 20 hours after hCG administration and cumulous oocyte complexes were collected, counted and averaged to determine ovulation rate. Immunohistochemistry was used to determine the location of specific cell types in the ovary. We identified a specific population of monocytes, deemed ly6c^{high} monocytes, which infiltrate the ovary at a specific time. Immediately before ovulation occurs, ly6c^{high} monocytes are found in increased numbers in the ovaries, possibly to promote inflammation and follicular rupture. After ovulation, the numbers of these monocytes decreases rapidly, likely because once they enter the tissues they differentiate into either M1 macrophages, to further promote inflammation, or M2 macrophages to stimulate corpus luteum development. Studies have shown that animals deficient of these subsets of monocytes and macrophages have reduced rates of ovulation. Several other leukocyte populations were identified infiltrating the ovary, suggesting multiple leukocyte subsets are involved in ovulation. In this regard, in the ovary, the

ly6c^{high}monocytes may function as a source of M1 and M2 macrophages that initiate the inflammatory process and facilitate the rest of the reproductive process.

Keywords: flow cytometry, neutrophil, Ly6c^{high} monocyte, macrophage, ovulation

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Chapter 1

Introduction

Ovulation

In many women, ovulation is a naturally occurring, monthly inflammatory process that results in the release of an oocyte for possible fertilization. Ovulation is brought about by many physiological changes, such as fluctuations in hormonal levels and cell signaling[1]. These changes cause different types and numbers of white blood cells to migrate to the ovaries to ensure ovulation occurs successfully[2]. Specifically, the types of white blood cells found in follicular fluid include granulocytes, lymphocytes, monocytes and macrophages[2]. It has also been reported that macrophages, neutrophils, eosinophils and mast cells are recruited to the theca layer of follicles[3,4]. These cells are recruited to very specific areas in the ovaries, namely directly to the follicle at the boundary between the theca and granulosa cells. This infiltration, at precise points during the ovulatory process, leads to a weakening of the follicular wall and follicular rupture. Subsequently, the leukocytes are also critical for corpus luteum formation and luteolysis[1,5,6]. In regards to ovulation, many of these inflammatory leukocytes can be found near mature follicles, releasing products such as matrix metalloproteinases and proteolytic enzymes that will help weaken the follicular wall, allowing oocyte release, and helping to reorganize tissue[6,7].

Without these regular alterations in physiology, the recruitment of white blood cells is hindered, and ovulation either has a reduced success rate or does not occur, resulting in reduced fertility and reproductive diseases such as polycystic ovarian syndrome (PCOS)[8]. A genetic defect in any key component in the formation or

recruitment of leukocytes can result in reduced ovulatory success. For example, mice that have decreased levels of colony-stimulating factor-1, and therefore decreased macrophage numbers, do not ovulate normally[3,9]. Mice that have malfunctioning macrophages also have abnormal ovulation rates[3,9]. When these specific white blood cell populations are absent, the success of ovulation can be reduced by as much as 50 percent[7,9]. Specific populations of white blood cells are necessary for ovulation to occur, as they release cytokines and enzymes that help promote the inflammation at the ovarian follicles so that they will rupture and release the oocyte, or egg cell[1]. After follicular rupture, other white blood cells promote tissue repair and clean up the process of ovulation can restart and the corpus luteum can form and be maintained[6]. The corpus luteum is important in maintaining pregnancy via progesterone secretion. These processes are able to occur because white blood cells, including monocytes and macrophages, produce cytokines, which can recruit more white blood cells to this natural site of inflammation as well as promote further inflammation by accumulating in large quantities[7]. One of the key roles of the ovaries is steroidogenesis; the recruitment of leukocytes to the ovary may also facilitate steroidogenesis in ovarian cells such as granulosa-lutein cells[2].

Leukocytes have been implicated as a major factor in ovulation for many reasons. One reason to support their role is that a hindered or abnormal ovulation process appears when leukocyte numbers are decreased or non-functioning. Many genetic defects and immune diseases can result in incorrectly functioning white blood cells, or can cause reduced numbers or their complete absence. In these instances, fertility issues often arise. For example, in splenectomized mice and in osteopetrotic mice that cannot produce

macrophage colony-stimulating factor-1 (MCF-1), there are reduced numbers of leukocytes and macrophages, respectively, and in both cases, pregnancy rates and litter sizes are reduced[3,6,7,9]. Similarly, a mutation in the microftalmia locus causes macrophages to malfunction, resulting in lowered ovulation success[3]. In individuals that have normal leukocyte numbers and activity, their leukocyte activity or recruitment can be blocked by various mechanisms. For instance, by using antibodies against neutrophils, or by blocking the chemokine interleukin-8, neutrophils can be stopped from reaching the ovary or working properly, causing ovulation to become aberrant[3]. Indomethacin, which is a non-steroidal anti-inflammatory (NSAID) drug, has also been shown to inhibit ovulation rates by hindering prostaglandin pathways and causing abnormal follicle rupture[3]. Furthermore, upon being provided with adequate numbers of normally functioning leukocytes, ovulatory rates seem to be restored. In instances where leukocytes are abnormal through a genetic defect, the patient treated with a bone marrow transplantation demonstrated recovered ovulation rates[3,9]. Lastly, leukocytes have been implicated in playing a role in ovulation merely by their presence as well as their products. Macrophages, monocytes, mast cells, eosinophils, and neutrophils have all been found in ovarian tissue[3]. Many products of leukocytes have been observed in ovaries during and after ovulation, including growth factors, cytokines, and chemokines, which help recruit leukocytes to the site and promote the inflammatory process of ovulation[2,10]. In fact, chemokine gene transcription has been demonstrated to be at its highest in the ovary before ovulation takes place, indicating leukocytes are needed for ovulation and likely for corpus luteum formation as well[6].

The specific populations of white blood cells entering the ovaries, how they function and how they are involved in causing ovulation has yet to be fully elucidated. The exact source of these cells has also not been confirmed. However, it is clear that they are integral to ovarian function including ovulation, corpus luteum formation, and corpus luteum degradation. Once the specific populations of leukocytes involved in successful ovulation together with the signals recruiting them to the ovary can be identified, we will be able to better treat patients suffering from ovarian reproductive problems.

Estrogen and Ovulation

Estrogen is one of the most influential hormones in the female body. Estrogen occurs in three forms; estrone, estradiol, and estriol. This hormone helps regulate a multitude of physiological processes, especially those relating to female reproduction. Estradiol is the most potent of the three estrogen forms and predominates throughout the fertile years of a woman's life. Estrone predominates after menopause in women, while estriol is the major form found during pregnancy. Estrogen has an anti-inflammatory effect and can regulate the expression of many cell adhesion molecules on the endothelium[11]. Specifically, estrogen decreases expression of adhesion molecules on the vascular endothelium, causing it to regulate the number of leukocytes that are able to migrate (extravasate) into the tissue[11]. Estrogen is also capable of suppressing key transcription factors, such as NF-κβ. The NF-κβ pathway is involved in regulating a multitude of immune cell genes and cytokines, as well as lowering chemokine production of GRO3 (CXCL3), ultimately reducing recruitment of polymorphonuclear cells, such as neutrophils[11]. The anti-inflammatory nature of estrogen is highlighted by the

exacerbation of inflammatory diseases seen in women once they become post-menopausal and circulating levels of estrogen are decreased[11]. The drop in estrogen levels before ovulation and the subsequent ovarian influx of leukocytes is likely a critical signal allowing for leukocyte recruitment.

There are several hormones involved in the process of ovulation, the levels of which fluctuate throughout the cycle. Estrogen, particularly estradiol, is one of the most predominant hormones influencing ovulation. During the follicular phase of ovulation, estrogen levels continuously rise until ovulation occurs. After ovulation, estrogen levels drop sharply, and the levels of another important hormone, progesterone, rise greatly. After ovulation occurs, the ruptured follicle forms a corpus luteum, which secretes high levels of progesterone. If pregnancy occurs, then progesterone levels will remain high, but levels will drop if no implantation occurs, allowing corpus luteum regression and menstruation to occur. Progesterone plays a role in a variety of reproductive events, and makes the uterus a hospitable environment for implantation and zygote development by making the tissue extremely vascular. There are also two gonadotropic hormones involved in the process of ovulation. These include follicle stimulating hormone (FSH), which promotes the growth of immature ovarian follicles, and luteinizing hormone (LH), which triggers ovulation and promotes the growth of the corpus luteum[6]. The levels of both FSH and LH remain low until immediately before ovulation. At this point both hormone concentrations rapidly rise, with a slight increase in FSH levels and a much larger spike in LH levels. FSH is necessary to trigger folliculogenesis while LH plays a more significant role in causing ovulation to occur and corpus luteum develop.

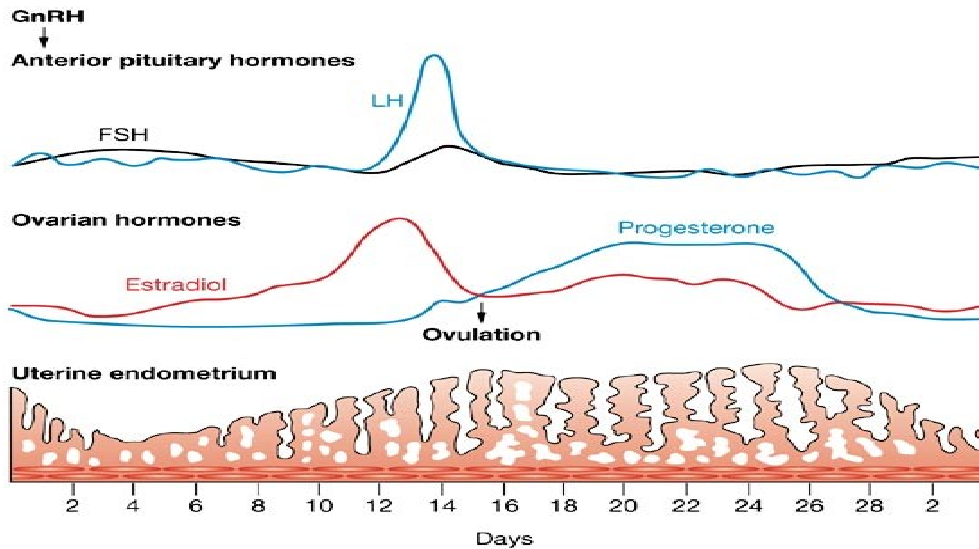

Figure 1. Hormone fluctuation in menstrual cycle.

There are many hormones involved in the process of ovulation, which include the ovarian hormones estradiol and progesterone, and the gonadotropins, follicle stimulating hormone and luteinizing hormone. Day 0 through approximately 15 correlates to the follicular phase, when the follicle grows and endometrium thickens, and day 15 through 28 corresponds to the secretory phase, at which corpus luteum formation occurs. Ovulation occurs in between these two phases, which is stimulated by the LH surge[12]. *Source*: Therapy Insight: preserving fertility in cyclophosphamide-treated patients with rheumatic disease. Dooley, M. A. & Nair, R. *Nature clinical practice. Rheumatology* 4, 250-257, doi:10.1038/ncprheum0770 (2008).

Follicle Development

In the ovary, there are many follicles that contain an oocyte which will go through many changes involving growth and maturation, ovulation, corpus luteum development and regression[6]. Follicles first assemble, then develop and mature, and finally ovulate; each process is a part of folliculogenesis[13]. First, primordial follicles form and are completely developed at birth, and constitute the reserve a female will have throughout her life until they are all used, resulting in menopause[14]. At rest, these primordial follicles are small and do not develop further; however when the primordial follicles leave the reserve they will continue to mature and develop[14]. Primordial follicles will grow in size and become structurally more complex as they develop into mature follicles.

There are varying sizes of follicles that can be grouped into three distinct classes based on their size and complexity. Primary follicles are the smallest and consist of a growing oocyte surrounded by a layer of cuboidal granulosa cells, which begin to express FSH receptors[14]. The zona pelucida begins to form at this stage, which surrounds the oocyte. These continue to develop and grow and can then be classified as secondary follicles, which consists of two to ten layers of granulosa cells surrounding a fully grown oocyte inside the zona pelucida, and a basal lamina surrounding the granulosa cells. Theca cells also develop outside of the basal lamina at this stage of folliculogenesis, which is where blood vessels begin to form. These blood vessels will bring the hormones necessary for further development to the follicle. When the follicle begins to show a cavity, or antrum, at one side of the oocyte in the granulosa cells, it is termed a tertiary follicle. When a follicle is completely mature, there is follicular fluid in the antrum. The last step of folliculogenesis entails ovulation, at which point the follicle ruptures and releases the oocyte for fertilization[13].

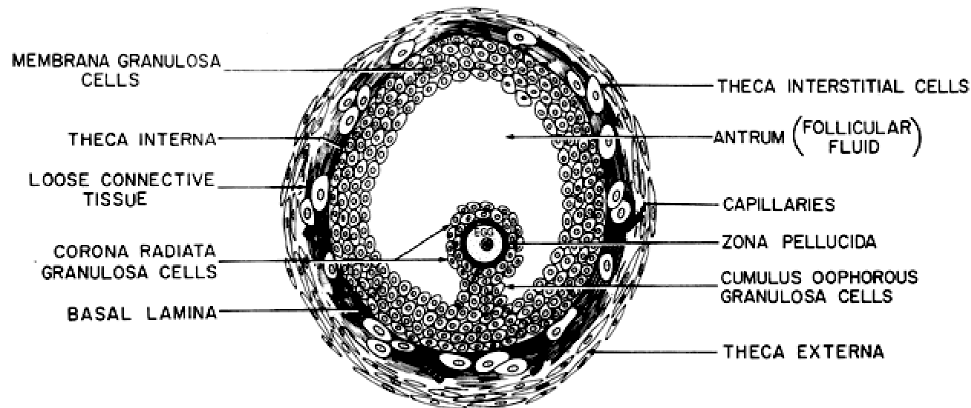

Figure 2. An ovarian Graafian follicle.

A mature ovarian follicle with an oocyte in the center. Granulosa cells surround the oocyte. A basement membrane separates granulosa cells from theca cells. (From Erickson GF: The ovary: Basic principles and concepts. *Source*: Felig P, Baxter JD, Broadus AE, Froman LA, (eds): *Endocrinology and Metabolism.* 3rd ed. New York: McGraw-Hill, 1987.

Gonadotropins and hormones play a large role in directing the development of follicles to become preovulatory. FSH is the primary hormone stimulating follicles to start growing[15]. Follicle growth begins when FSH reaches the ovary, which is detected by receptors on granulosa cells. FSH causes granulosa cells to rapidly divide and to express the enzyme aromatase, that cleaves testosterone into estradiol[15]. LH is the second gonadotropin that plays an important role in ovulation. Theca cells, on the outer layer of the follicle, respond to LH and will begin to produce testosterone[15]. Testosterone diffuses through the basement membrane to the granulosa cells on the inner layer of the follicle, convert it to estradiol through the enzyme aromatase[15]. This is all initiated by the rush of LH reaching the ovary in the middle of the ovulation cycle[15]. LH also determines which follicle is the most mature or dominant because it is detected to a greater degree by the follicle with more LH receptors and fewer FSH receptors[15]. It temporarily stops all other follicles from continuing to mature. The mid-cycle surge of LH also promotes

granulosa cells to become luteinized after ovulation[6,15]. The effects of both important gonadotropins can be mimicked by using pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), which mimic FSH and LH respectively. These two compounds are readily available to use in laboratory settings for reproductive research.

Mature follicles that are not selected for ovulation will undergo atresia, meaning most cells in the atretic follicle will go through apoptosis. The selected follicle will continue to develop through ovulation, which occurs after the follicle ruptures from a variety of proteases degrade the basal membrane[2]. In mice, multiple follicles will go through this process instead of the typical single follicle ovulating in humans[1]. This breakdown releases the oocyte, along with cumulous cells which are differentiated from granulosa cells. These cumulous cells are mucous-like and will protect the oocyte and help carry it down the oviduct, fallopian tube in humans. The leftover follicular cells will develop into the corpus luteum, which will produce progesterone[6,16,17]. If the oocyte does not get fertilized and no pregnancy occurs, then the corpus luteum will degrade, stop synthesizing progesterone, and the uterine lining will degrade and return to normal[15]. If pregnancy does occur, the corpus luteum is responsible for continued progesterone production, which keeps the uterine wall thick and full of blood vessels for zygote development[16].

The ovarian tissue, specifically the theca and granulosa cells, are believed to be derived from the immune system. Both cell types, especially theca cells, are capable of secreting interleukin-8, which is a major recruitment factor for leukocytes[10]. Monocytes are able to induce this secretion in ovarian tissue[10]. Corpus luteum formation and

degradation, like ovulation, is also highly associated with immune cell changes[16]. Granulosa-lutein cells have been implicated as a source of chemokine release, which is necessary for attracting leukocytes to help with corpus luteum remodeling[2]. One study was able to show an increase in interleukin-8 production in cultures of granulosa lutein cells and leukocytes compared to cultures of only granulosa lutein cells[1,2]. The interaction between ovarian cells and leukocytes seems to be effective at optimizing production of chemokines and hormones[1]. The regulation of chemokine production in ovarian cells is thought to involve stimuli such as hCG, tumor necrosis factor-alpha, and interleukin-1[2]. The changes in chemokine expression are also cyclic, indicating leukocyte infiltration is necessary at precise times in the estrus cycle[1]. Neutrophils are an important inflammatory cell that have been found in ovarian tissue during ovulation, specifically around the thecal layer of follicles[2]. Neutrophils are abundant during corpus luteal formation, while monocytes and macrophages seem to dominate during corpus luteal degradation[1,16].

Reproductive Diseases

Polycystic ovary syndrome (PCOS) is the most common reproductive endocrine disorder among women, affecting an estimated one in five women of reproductive age in the United States[18,19]. The disease often presents with enlarged ovaries that have cysts on them, limiting function and estrogen production, ultimately resulting in hormonal imbalance. The hormonal imbalance causes the manifestation of symptoms such as excessive acne, balding or hair thinning, amenorrhea or irregular menstruation, facial hair growth, insulin resistance and obesity[20]. PCOS can also lead to hypertension, diabetes, and a metabolic syndrome[19]. This disease is often associated with reproductive

challenges and infertility. One chemokine, monocyte chemoattractant protein-1, has been identified in follicular fluid and serum in women with PCOS in increased levels compared to healthy women[19]. Increased serum levels of this particular chemokine may be promoting the insulin resistance and obesity that is seen in PCOS women[19]. This suggests that specific amounts of chemokines, cytokines and leukocytes are required in the reproductive tissue of healthy women ONLY at precise times, with quantities outside that threshold resulting in reproductive irregularities and diseases.

Endometriosis is another major reproductive disorder, characterized by the abnormal ectopic growth of endometrial tissue outside the uterine cavity[21,22]. This disease has been associated with decreased function of T cells and natural killer cells, and increased activation and infiltration of macrophages[21,22].

Spleen

The spleen is a secondary lymphoid organ which functions to filter blood, removing old red blood cells and monitoring for and removing abnormal or pathogenic particles via its resident population of immune cells[23,24]. Monocytes and macrophages, among other leukocytes, can be found in the spleen[24]. The spleen has recently been identified as a reservoir for leukocytes, acting as an immediate source of white blood cells when tissue becomes damaged or inflamed[23]. This is in contrast to the bone marrow, which is a much slower acting source of leukocytes for damaged tissue. In particular, ly6c^{high} and ly6c^{low} monocytes have been found in the red pulp of the spleen, and the ly6c^{high} monocytes in particular have been proven to be specifically recruited from the spleen to sites of tissue injury[23]. One study found that, after inducing ischemia

of the myocardium, there was a decrease in ly6c monocyte numbers in the spleen, an increase in their numbers in the blood, and no change in bone marrow numbers, indicating the monocytes left the spleen and entered circulation to reach the site of injury[23]. However, this same study showed that neutrophil numbers in the spleen did not change[23].

The immune system plays an important role in the reproductive system, as an abnormal immune system is associated with various fertility complications, such as preeclampsia, intrauterine growth restriction, and recurring abortions[24]. There has been some indication that the spleen is the source of leukocyte donation for the ovary, as splenectomized mice have reduced ovulation rates compared to both mice with intact spleens or mice that have been reconstituted with splenocytes[8]. Furthermore, women who have had splenectomies and become pregnant endured more hardships than women who had a spleen, including the increased need for caesarian section and fertility treatment, and a higher risk for preterm delivery[24]. It has also been demonstrated that an inverse correlation between leukocyte numbers in the spleen and in the ovary exists during ovulation[7]. This suggests that the spleen may play a role in the reproductive system, and may also act as a source of leukocytes for normal, physiological inflammatory processes, such as ovulation. The spleen is likely supplying leukocytes to reproductive tissues, such as the ovary, oviduct, and uterus, to facilitate a rapid and timely ovulation. Furthermore, bone marrow transplants have been shown to be effective at restoring ovulation rates in rodents with defective white blood cells[3]. Sources of white blood cells, like the spleen or bone marrow, may both be capable of restoring ovulatory function.

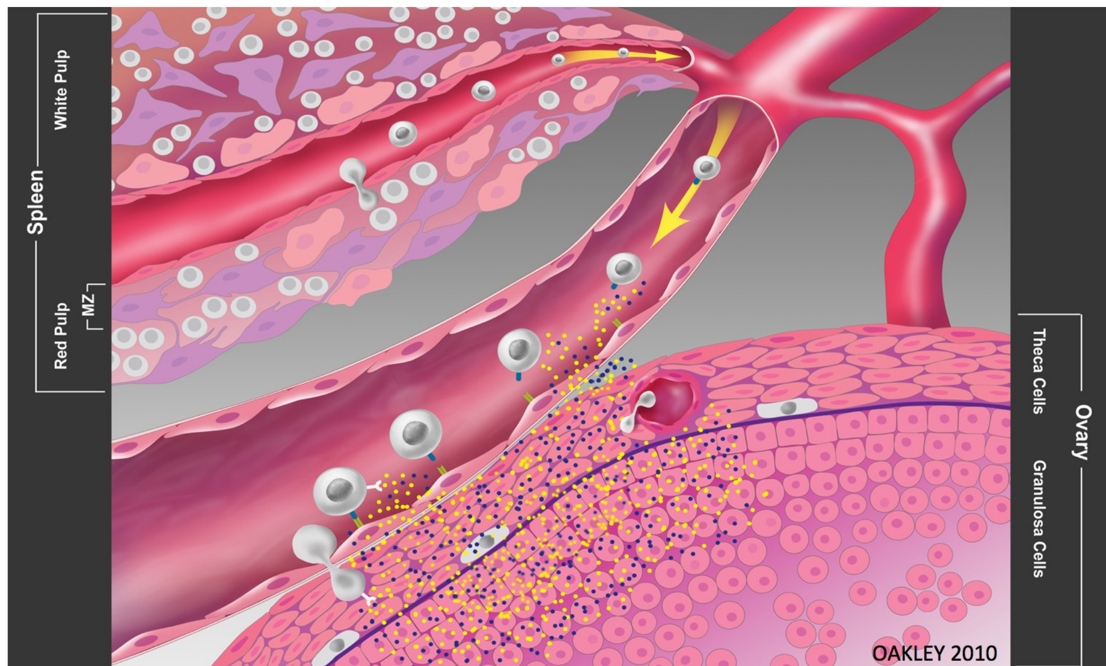

Figure 3. Splenic leukocyte recruitment to ovary.

The spleen may act as a reservoir for leukocytes, releasing them in response to chemokines released by ovarian cells. Following ovarian infiltration, the leukocytes migrate to a mature follicle, and begin to release cytokines and chemokines to help break down the follicle membrane so that ovulation can occur. *Source:* Pituitary-ovary-spleen axis in ovulation. Oakley OR, Frazer ML, and Ko C. (2011). Trends in endocrinology and metabolism: TEM 22, pp.345-352.

Ly6c^{high} Monocyte

Monocytes are innate white blood cells that arise in the bone marrow and travel through the vasculature to inflamed or infected tissue sites, where they eventually differentiate into macrophages or dendritic cells[25-29]. They are typically some of the first leukocytes to arrive at inflammatory sites[4,28]. Peripheral inflammation is typically a primary signal causing increased production of monocytes[27]. In the vasculature, monocytes interact with cell adhesion molecules, such as the vascular cell adhesion molecule (VCAM-1) and the intercellular adhesion molecule (ICAM-1), expressed by the endothelium which help slow the monocytes so that they can pass through the endothelium to reach the inflamed tissue and differentiate into macrophages[30].

Monocytes are characterized by the expression of CD11b, CD115 in mice, CD14 and CD16 in humans, and lack the surface markers expressed by B, T, natural killer, and dendritic cells, which include B220, CD3, NK1.1, MHCII (IA), and CD90.2[25,27]. They are also distinguishable from their differentiated forms, macrophage and dendritic cells, by their lack of F4/80 glycoprotein and CD11c expression[23]. There are two predominant subsets of monocytes, which are the ly6c^{high} and ly6c^{low} monocyte, named by the variable levels of expression of ly6c (lymphocyte antigen 6c)[23]. These two subsets are functionally distinct and express different surface markers[23,28]. The ly6c^{high} monocyte is inflammatory while ly6c^{low} monocyte is involved in the resolution of inflammation[23,25,28,31].

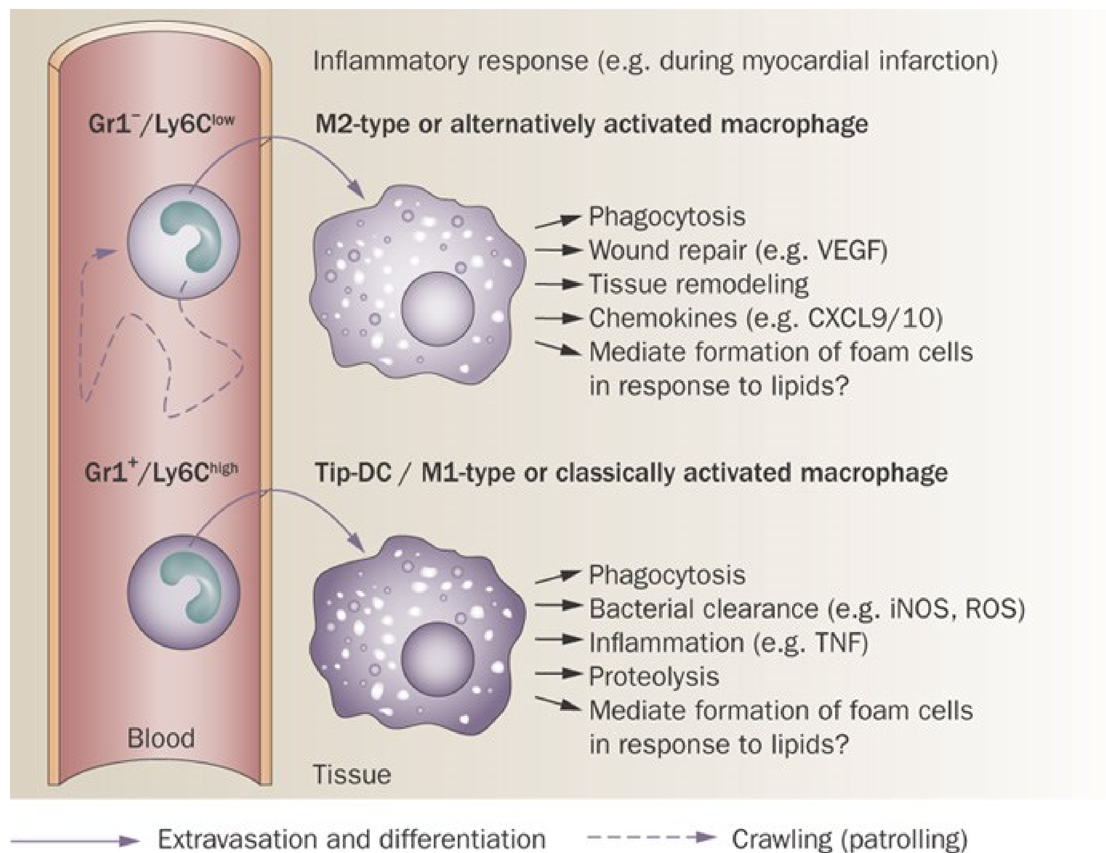

Figure 4. Two functionally distinct subsets of monocytes.

The ly6c^{high} monocyte can differentiate into an inflammatory M1-like macrophage upon tissue infiltration. It can also patrol the vasculature, decreasing ly6c expression to become a ly6c^{low} monocyte. The ly6c^{low} monocyte can differentiate into an M1-like macrophage upon tissue infiltration, which functions primarily to resolve inflammation and repair tissue.[32] *Source*: Monocytes in atherosclerosis: subsets and functions. Woollard, K. J. & Geissmann, F. *Nature reviews. Cardiology* 7, 77-86, doi:10.1038/nrcardio.2009.228 (2010).

The ly6c^{high} monocytes are short-lived cells involved in inflammatory processes and typically migrate to infected or inflamed tissue[25,27,28,31,33]. Ly6c^{high} populations are a transitory subset because they quickly begin to lower expression of ly6c after entering the circulation and tissues[27,28]. These inflammatory monocytes are typically one of the first immune cells to arrive at a site of injury[34]. They are larger than their ly6c^{low} counterpart[23] and express the chemokine receptor CX_3CR1 in low levels, but express high levels of Ly6c (Gr-1) and the chemokine CCR2[25,26,28,31,33]. In humans, this same monocyte is classified as CX_3CR1$^{low}CD14^{high}$ for surface receptor expression[31]. CCR2 is the major chemokine receptor utilized by this monocyte to infiltrate inflamed tissues and cross the endothelium[26,31,35]. Blocking CCR2 activity results in drastically reduced monocyte numbers[31]. Similarly, knocking out the gene for CCR2 (CCR2$^{-/-}$) in mice also causes drastically decreased recruitment of monocytes and macrophages to tissue[36]. Ly6c^{high} monocytes also express chemokine receptors CCR7 and CCR8, and migrate towards chemokines such as CCL2, CCL7, and CCL8[31]. These chemokines were previously named to reflect their function as monocyte chemoattractant proteins; for instance, CCL2 was previously referred to as monocyte chemoattractant protein-1 (MCP-1)[5]. These ligands are produced by endothelial tissue, helping recruit leukocytes to specific tissues; in the pre-ovulatory ovary, CCL2 is produced by stroma cells near the follicle, recruiting macrophages to this specific site[1]. They are also produced by fibroblasts, smooth muscle cells, and other monocytes, macrophages, and

lymphocytes[19,29]. CCL2 production is often increased in response to inflammatory stimuli such as TNF-α, interferon-gamma (IFN-γ), and lipopolysaccharide, which is a product of pathogens[29]. In addition, it escalates an inflammatory process by stimulating adhesion molecule and cytokine production[35]. Another molecule present on the ly6c^{high} monocyte is L-selectin, or CD62L, that facilitates the monocyte homing to inflamed tissues[27]. This subset of monocytes can differentiate into ly6c^{low} monocytes simply by decreasing expression of ly6c or develop into an M1 macrophage, the classical pro-inflammatory macrophage[26,27,30,33]. Since it can differentiate into a ly6c^{low} monocyte, it can further differentiate into wound repair M2 type macrophages[34]. Though most monocytes differentiate into macrophages upon exiting the bloodstream, this transition is not always immediate[34]. These monocytes can also exhibit regulatory functions on other white blood cells, such as neutrophils, and stop them from becoming activated by producing prostaglandin E2 (PGE_2) and interleukin-10[37]. One study was able to show that in the presence of commensal bacteria, ly6c^{high} were able to take on a regulatory role in addition to their inflammatory role, to limit collateral damage from other inflammatory leukocytes, especially neutrophils[37].

In the ovary, these monocytes would be found before ovulation occurs, as they promote the inflammation and swelling of the ovarian follicles. The follicles will eventually rupture and release oocytes for fertilization. They may also be involved in the process of corpus luteum degradation, which would be highly inflammatory. Monocytes may also be playing a regulatory role on the ovarian cells, causing granulosa-lutein cells to secrete interleukin-8 and MCP-1, which would prompt the recruitment of more leukocytes[6,10]. Furthermore, they may be able to regulate other inflammatory cells, like

neutrophils, and prevent them from releasing damaging products too early or in too high a concentration[37].

Ly6c^{low} Monocyte

The ly6c^{low} monocytes are non-inflammatory; they promote wound healing, tissue repair and remodeling[23,33]. These monocytes are also capable of promoting angiogenesis and fibrosis in wounds[34]. The ly6c^{low} monocytes are also better at processing apoptotic cells than their counterpart, which helps them in repairing tissue[33]. These monocytes express the chemokine receptor CX_3CR1 in high levels, but express low levels of ly6c and the chemokine CCR2[25-27,31,33]. This monocyte in humans can be characterized as having CX_3CR1$^{high}CD14^{low}CD16^{high}$ expression[31]. The ly6c^{low} monocytes are recruited to tissues where there is an excess of CX_3CL1, also known as fractalkine, that is expressed on endothelial tissue and smooth muscle cells[5,25-27,35,38]. Fractalkine is able to cause monocyte adhesion and facilitate chemotaxis[25,38]. Fractalkine is also specialized in that it is able to be cleaved from its membrane-bound form into a soluble product that can accumulate to further promote chemotaxis of monocytes[35,38,39]. This particular chemokine binds to the G-coupled protein receptor CX_3CR1, which is expressed by monocytes and macrophages[38]. Fractalkine increases when responding to hCG, resulting in increased production of progesterone by granulosa cells[1]. These monocytes are longer-lived cells and can become residential macrophages in tissues, especially those that are noninflamed[25-27,31,33]. This subset of monocytes has been described as an intermediate between ly6c^{high} monocytes and ly6c^{low} macrophages, as ly6c^{high} monocytes gradually reduce ly6c expression[27,31,34]. They can also differentiate

into the alternatively activated M2 macrophage, that promotes tissue repair and remodeling[30].

In the ovary, these monocytes would appear after ovulation has occurred, indicating they could repair the ruptured follicle tissue. This monocyte may also be involved in corpus luteal formation, which is needed upon successful fertilization of an oocyte[1]. Corpus luteal formation involves a tremendous amount of tissue remodeling and vascularization, suggesting these monocytes may play a role in the task.

M1 Macrophage

Monocytes that enter tissue will eventually differentiate into macrophages, especially if they come into contact with M-CSF[27]. Macrophages are cells that are involved in the innate immune system, and can help destroy pathogens, remove apoptotic cells, and promote angiogenesis[40]. Monocyte differentiation is dependent on the tissue environment they enter and whether they encounter inflammatory or anti-inflammatory cytokines, chemokines, or other products. Monocytes can be either classically or alternatively activated, causing them to turn into either a more damaging M1 macrophage or a wound-healing M2 macrophage, respectively. Macrophages have been implicated in playing a role in ovulation, with a variety of studies finding the presence of macrophages in ovarian tissue during ovulatory events[3]. Macrophages have also been identified as the most abundant type of leukocyte in the ovary[40]. Macrophages can promote angiogenesis and are involved in maintaining the vasculature of the corpus luteum[40].

M1-like macrophages appear after monocytes encounter lipopolysaccharide, interferon-gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), which are all

inflammatory signals[30,40]. The M1 macrophages will continue to promote inflammatory processes and proteolysis of tissue by secreting such cytokines as IL-6 and IL-12, as well as TNF-α and IFN- γ [30,34,40]. They will also release CCL2, CCL7, and CCL8, which will recruit more ly6c^{high} monocytes to the inflamed tissue[30]. M1-macrophages are powerful effector cells; they destroy pathogens and apoptotic cells, as well as promote the progression of the inflammatory process[40]. M1-like macrophages have been identified in the regressing corpus luteum, likely cleaning up dead or dying cells[1,40]. Inflammatory products, such as TNF-α, also increase during luteolysis, helping to promote apoptosis of luteal cells while recruiting more leukocytes[1].

M2 Macrophage

The ly6c^{low} monocytes develop into the alternatively activated M2-like macrophages, that are responsible for cleaning up and repairing damaged tissue, as well as remodeling it. These macrophages are often called wound healing macrophages. M2-like macrophages are more likely to appear in environments filled with anti-inflammatory products, such as interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-13 (IL-13)[40]. They can produce a variety of anti-inflammatory mediators, such as tumor growth factor-beta (TGF-β)[34] and vascular endothelial growth factor (VEGF), that promotes angiogenesis[40]. These macrophages are characterized by low expression of ly6c and high expression of F4/80 and MHC class II, as well as the expression of CD206 (mannose receptor), Mer tyrosine kinase (MerTK), and FC receptors CD23 and CD64[34]. M2-like macrophages are also anti-inflammatory and can induce apoptosis in other immune cells, such as neutrophils, to reduce overall inflammatory reactions [34]. They stimulate $CD4^{+}$ T helper 2 cells and regulatory cells, which along with the M2 macrophage, are better able

to regulate immune processes and help tissue repair[40]. M2-like macrophages have been found in the developing corpus luteum in high levels, suggestive of an involvement in promoting angiogenesis[40]. These cells may also help regulate immune responses in the ovary during these complex processes in a homeostatic manner[1].

Neutrophils

Neutrophils are polymorphonuclear granulocytes and are the most abundant of circulating leukocytes[40]. They are short lived cells that are part of the innate immune system, responding early to inflammatory stimuli[40]. Granulocyte colony stimulating factor promotes the proliferation of neutrophils[40]. Neutrophils produce very toxic and damaging products, such as reactive oxygen species (ROS), proteases, superoxides, and cytokines, that both clear pathogens and destroy tissue[17,37]. They also contain many proteolytic enzymes, such as collagenases and elastases, that are used to degrade extracellular matrix to facilitate extravasation into tissues[5,17]. These granulocytic leukocytes are both attracted to and activated by Interleukin-8[4,5]. Another potent factor causing neutrophil attraction is growth-regulated oncogene-α[4,5]. Both of these chemoattractants can be categorized into the CXC subfamily of chemokines[4,5].

Neutrophils have been shown to appear in ovarian follicles before ovulation takes place, suggesting they play a role in the inflammatory process leading to ovulation[3,4,6]. Specifically, they have been found accumulate in the theca layers of preovulatory ovaries[3,4]. They have also been found around follicles that rupture abnormally[3]. In support of this idea, administering neutralizing antibodies against neutrophils reduces ovulation rates in rats[3,40]. A similar effect occurs when antibodies are given against IL-8,

which inhibits neutrophils from entering tissue, resulting in decreased ovulation rate[3]. Neutrophils may be modulated in the ovulation process by ly6c^{high} monocytes that produce PG$E_2$37. PGE_2 is able to regulate smooth muscle cells, endothelial cells and white blood cells and their production of cytokines, chemokines, and expression of adhesion molecules[41]. This regulation of activity might help to keep the neutrophils from weakening follicular walls until optimal conditions are met and oocytes are ready to burst from the follicle. Neutrophils may also play a role after ovulation in corpus luteum development by promoting angiogenesis[1,40].

Chemotaxis

Chemotaxis is the movement of a cell from one area to another in response to a specific chemical gradient or cue, and is typically a major process involved in immune responses[6,42]. This process can both attract and repel specific cells, based on the cue provided[6]. Leukocytes are constantly monitoring the body for immune disturbances by circulating through the blood stream until they are attracted to a particular site in response to chemokines, which are soluble proteins that promote chemotaxis[6]. In many instances, chemokines, which are small, low molecular weight cytokines with chemoattractant properties, are responsible for chemotaxis, as well as leukocyte activation[5,10,36,43]. Chemokines are structurally very similar and are divided into four subfamilies based on the location of their cysteine residues, which are C, CC, CXC, and CX_3C[4,5,10,21,42,43]. Because of their comparable structures, many chemokines share similar functions, making them redundant. Chemokines are typically inflammatory as they can activate leukocytes and effectively recruit leukocytes to sub-endothelial tissues[42,44]. Chemokines are produced and released by endothelial cells and stromal cells, that assist leukocytes in

determining where they need to go, and also by leukocytes themselves, self-promoting the number of leukocytes recruited[6]. Some important leukocytes recruited to tissue via chemokine gradients include monocytes, macrophages and neutrophils, which are often the first immune cells to appear at inflammatory sites. Chemokines in the CC family tend to recruit monocytes and lymphocytes, while neutrophils respond primarily to the CXC subfamily[5,21]. Several chemokines have been found in and around follicles in the ovary and have thus been implicated in ovarian function[5]. Chemokine regulation in the ovary is complex and specific, allowing for specific leukocytes to be recruited to precise locations in the ovary at optimal times to cause ovulation and the following reproductive processes to occur efficiently[5].

Monocyte chemoattractant proteins 1, 2, and 3 (MCP-1, MCP-2, MCP-3), also called CCL2, CCL8, and CCL7, respectively, are some of the primary chemokines responsible for the chemotaxis of monocytes[36,42,45,46]. These chemokines are categorized into the CC subfamily (or beta family) and have approximately 70% shared homologous structure[36,42,44,45]. CCL2 can be produced by a variety of cells, including endometrial stromal cells and mesothelial cells, in response to a variety of triggers, such as estrogen[21]. CCL2 is involved in follicle development, ovulation, steroidogenesis, and corpus luteum formation, which suggests that hormones may be acting as catalysts to increase production of CCL2[19]. They also are produced in response to inflammatory signals, such as cytokines[29]. The monocyte chemoattractant proteins bind to G-protein coupled receptors (GPCRs), resulting in various signaling cascades[5,36,42,45,46]. The chemokine receptor CCR2 is an example of one of these GPCRs on monocytes, which is expressed in abundance and is the predominant receptor allowing for monocyte recruitment, only

binding specifically to monocyte chemoattractant proteins[29,36,42,44,46,47]. There are two isoforms of CCR2, named alpha and beta, in humans, but only one form in mice[36]. Knockout of the CCR2 gene results in greatly decreased monocyte and macrophage recruitment to tissue, showing just how important this receptor is for monocyte recruitment[36]. CCL2 has been identified in the pre-ovulatory ovary, as well as indicated as a likely player in corpus luteum formation, as it has angiogenic abilities[5]. It is also one of the major chemokines involved in corpus luteum degradation, and is considered an indicator of luteolysis[5]. The presence of CCL2 in such high levels in the ovary at each of these reproductive stages suggests the necessity of monocyte involvement.

Neutrophils and other leukocytes are both drawn toward and activated by the chemokine interleukin-8 (IL-8)[2,4-6,45]. Interleukin-8 is classified as a CXC (alpha family) chemokine[2,4-6,10]. This chemokine has been found in both human follicular fluid and granulosa-lutein cells in pre-ovulatory follicles, indicating the presence of neutrophils during ovulation[1,10]. Furthermore, IL-8 is secreted by theca cells and granulosa cells, as well as leukocytes, indicating there is a necessity for white blood cells in the ovary[2,6,10]. The level of IL-8 secreted increases as follicles grow, with the highest amounts produced by dominant follicles[10]. IL-8 may also be a regulator of steroid synthesis in the ovary and initiate ovulatory events[10]. Another likely chemoattractant for neutrophils are the collagen-like peptides that result from tissue degradation of the basement membrane in ovarian follicles[3,17]. Growth regulated oncogenes (GRO) are also members of the CXC family; they are primarily chemotactic for neutrophils but can also be potent activators of them as well[3-5,45]. They bind to the CXCR2 receptor, while IL-8 can bind to both the CXCR1 and CXCR2 receptors[45]. Growth related oncogenes are more chemotactically

potent than interleukin-8[4]. The expression of GRO has been found in the pre-ovulatory ovary of rats[3]. Both IL-8 and GRO-α have been found in high concentrations in human follicular fluid[4]. They have also been determined to be expressed on preovulatory follicles[5]. TNF-α, which is an inflammatory product itself, has been identified as a signal that causes increased production of both GRO-α and IL-8 in granulosa lutein cells[4,5]. Estrogen, which can modulate many physiological processes, has been demonstrated as being capable of reducing GRO3 (CXCL3), thereby reducing neutrophil recruitment[11]. Another modulator of GRO expression is hCG, which is able to increase GRO expression[5]. It may also be that different mechanisms of recruitment are enacted to draw neutrophils to specific locations. In one study where indomethacin was used to inhibit neutrophil infiltration into the ovary, it was shown that neutrophils were blocked from entering the medulla but still were able to reach the follicle, suggesting multiple mechanisms of recruitment[3]. GRO and IL-8 are capable of promoting angiogenesis, and therefore have a possible role in corpus luteum development[5,6]. Furthermore, these two chemokines have been found in the pre-ovulatory ovary, suggesting that neutrophils are a major class of white blood cells stimulating reproductive processes such as ovulation[5].

There are also adhesion molecules on endothelial tissue which alter in expression to recruit immune cells through extravasation. Expression of adhesion molecules on endothelial tissue are necessary for leukocyte recruitment; they work by slowing down circulating leukocytes and helping them adhere to endothelial tissue, and then by drawing them through the endothelium into tissues that require them. These molecules include platelet-endothelial cell adhesion molecule -1 (PECAM-1, CD31), intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin, all of

which play a role in stimulating leukocyte recruitment from circulation into tissue. PECAM-1, through intracellular signaling, can induce activation in other inflammatory genes, like ICAM-1, to promote the inflammatory process[7]. ICAM-1 is able to help leukocytes slow down and adhere to endothelial tissue, so that PECAM-1 can be more effective at drawing them into tissue from circulation[7]. Both PECAM-1 and ICAM-1 have increased expression prior to ovulation, indicating leukocytes are actively recruited to the ovary[7].

Fractalkine, or CX_3CL1, is a chemokine that falls into the CX_3C family; however it is different from other chemokines because it is the only member in this class[43]. Fractalkine is expressed on the membrane of endothelial cells and smooth muscle cells, signaling leukocytes to adhere to specific tissue[35,38]. This adherence helps leukocytes lose velocity in the bloodstream and stick to vessel walls, facilitating migration into the tissues. Fractalkine expression varies depending on the environment; inflammatory stimuli such as LPS decrease expression, while factors such as IL-10, have the alternative effect of increasing expression[38]. Furthermore, fractalkine is unique compared to other chemokines as it can also be cleaved by TNF-α-converting enzyme, which leaves behind soluble fractalkine[39,43]. This soluble form can aggregate in large quantities and act as a chemoattractant for leukocytes as well as smooth muscle cells[43]. Any cell expressing CX_3CR1, the receptor for CX_3CL1, is capable of adhering to or migrating towards this ligand[38]. In addition to these functions, fractalkine can also induce proliferation of smooth muscle cells by acting on epidermal growth factor receptor[39,43]. Epiregulin, the ligand for epidermal growth factor receptor, seems to be regulated by the fractalkine ligand, causing increased production of it, which causes cell proliferation[43]. Fractalkine,

along with CCL2 and IL-8, are some of the few chemokines that are capable of inducing smooth muscle cell proliferation[43]. Furthermore, epiregulin can be induced by angiogenic factors such as angiotensin II and thrombin, suggesting that cells involved in angiogenesis would be drawn towards epiregulin, as well as fractalkine[43]. In the ovary, each of these factors may play a role in corpus luteum formation that involves the rapid revascularization of tissue.

Hormone fluctuations in the ovary may be playing a key role in regulating expression of many of these chemokines in ovarian cells, as chemokine expression seems to be cyclic[1,19]. Luteinizing hormone may be one of the most influential hormones in altering chemokine expression in a timely fashion[3]. CCL2 levels have been correlated to LH levels[19]. Estrogen may also be involved in regulating time dependent expression of various cytokines and chemokines[7]. For example, following lowered levels of estradiol, macrophage numbers increase for luteal regression[1].

The reproductive system is complex; many cellular signals are required to recruit the cells that play pivotal roles in coordinating ovulation and corpus luteum formation. These signals, which include both hormones and chemokines, are precisely regulated to recruit white blood cells, such as monocytes and macrophages. These white blood cells then facilitate dynamic changes in ovarian tissue by promoting both inflammation and tissue repair, making follicular growth, ovulation, and corpus luteum formation possible. Gaining insight as to which cells and signals are involved in the reproductive system, as well as the complex interaction between them, will ultimately lead to a better understanding of fertility and fertility abnormalities.

Chapter 2

Material & Methods

Animal Handling and Hormone Injections

Female cd1 and balb/c mice were obtained from Charles River (Roanoke, IL) at approximately three weeks of age and allowed to acclimate for one week. Mice were then stimulated to ovulate via intraperitoneal injection using a super ovulation protocol (SP). This included using 10 IU of pregnant mare serum gonadotropin (PMSG)(G4877; Sigma, St. Louis, MO) followed 48 hrs later by 10 IU of human chorionic gonadotropin (hCG)(CG10; Sigma). Mice were euthanized via carbon dioxide asphyxiation followed by cervical dislocation at the following time points post hormone injection: 48 hr post PMSG, 6 hr post hCG, 12 hr post hCG, 20 hr post hCG, and 24 hr post hCG. All animal procedures were approved by Eastern Kentucky University's Animal Care and Use Committee.

Tissue Isolation and Flow Cytometry

Spleens and ovaries were collected from all mice and processed accordingly. Cell counts were performed by running 40μl of a 1:50 dilution of spleen and 1:10 dilution of ovarian cells on the Accuri C6 flow cytometer. Single cell suspensions of approximately $1x10^6$ cells were then stained with antibodies specific to monocytes using a panel described in Table 1 and with antibodies specific to macrophages and other white blood cells using a panel described in Table 2. Other panels were used to find M2-like macrophages (Table 3) and generalized white blood cells (Table 4). Cells were washed once with PBA and then with PBS. Cells were resuspended in 50ul PBS and analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Monocytes and macrophages were determined as a lineage$^-$ (all PE conjugated antibodies) and

$CD11b^{+}$ population (Fig. 1). Ly-6c high monocytes were determined as the $f4/80^{-}CD11c^{-}Ia^{-}$ and $Ly-6c^{+}$ population (Fig. 1). Ly-6c low monocytes were determined as the $f4/80^{-}CD11c^{-}Ia^{-}$ and $Ly-6c^{-}$ population (Fig. 1). Macrophages were determined as the $f4/80^{+}CD11c^{+}Ia^{+}$ and $Ly-6c^{-}$ population (Fig. 1). Data was compensated on the BD Accuri software. Data were analyzed and plotted using SigmaPlot version 11.0 (Systat Software, Inc., Chicago, IL). On graphs, error bars represent one SEM around the average of data per group. One-way ANOVA (Tukey test) was used to determine statistical significance among groups. $P < 0.05$ was considered significant.

Table 1. Antibodies and concentration used when creating cell staining mixture for finding macrophages, Ly6c^{high} and Ly6c^{low} monocytes.

Fluorochrome	Antibody	Company	Cat. No.	Dilution	Amount (ul)
PBA	-	-	-	-	680
FITC	Ly-6c	BD Biosciences	553104	1:20	34
APC-Cy7	CD11b	BD Biosciences	557657	1:20	34
PE	CD90.2	BD Biosciences	553005	1:20	34
PE	B220	BD Biosciences	553090	1:20	34

Table 1 (continued).

Fluorochrome	Antibody	Company	Cat. No.	Dilution	Amount (ul)
PE	CD49b	BD Biosciences	558759	1:20	34
PE	NK1.1	BD Biosciences	557391	1:20	34
PE	LY-6g	BD Biosciences	561104	1:20	34
APC	f4/80	Biolegend	13116	1:20	34
APC	CD11c	BD Biosciences	561119	1:20	34
APC	Ia	BD Biosciences	562823	1:20	34

Notes: All antibodies are mixed into PBA. 20ul of the mixture was added to each cell suspension and allowed to stain for a minimum of 20 minutes at 4°C. Amounts listed account for staining 30 samples. Monocyte panel was designed based off of a previous study[23]. The Accuri C6 was set to collect 50,000 events. Laser settings were set to two red and two blue lasers. FL1 lens was 533/30, FL 2 lens was 585/40, FL 3 lens was 780/60, and FL4 lens was 675/25.

Table 2. M1-like macrophage panel.

Fluorochrome	Antibody	Company	Cat. No.	Dilution	Amount (ul)
PBA	-	-	-	-	680
Alexa488	CD197	Biolegend	120110	1:20	34
PE	F4/80	Biolegend	123110	1:20	34

Table 2 (continued).

Fluorochrome	Antibody	Company	Cat. No.	Dilution	Amount (ul)
APC-Cy7	CD3	BD Biosciences	560590	1:20	34
Alexa647	Ia	BD Biosciences	562367	1:20	34

Notes: Antibodies and concentration used when creating cell staining mixture for M1 macrophages. All antibodies were mixed into PBA. 20ul of the mixture was added to each cell suspension to stain for 20 minutes at 4°C. Amounts account for the staining of 30 samples. The Accuri C6 was set to collect 50,000 events. Laser settings were set to three red and one blue lasers. FL1 lens was 533/30, FL 2 lens was 585/40, FL 3 lens was 670LP, and FL4 lens was 675/25.

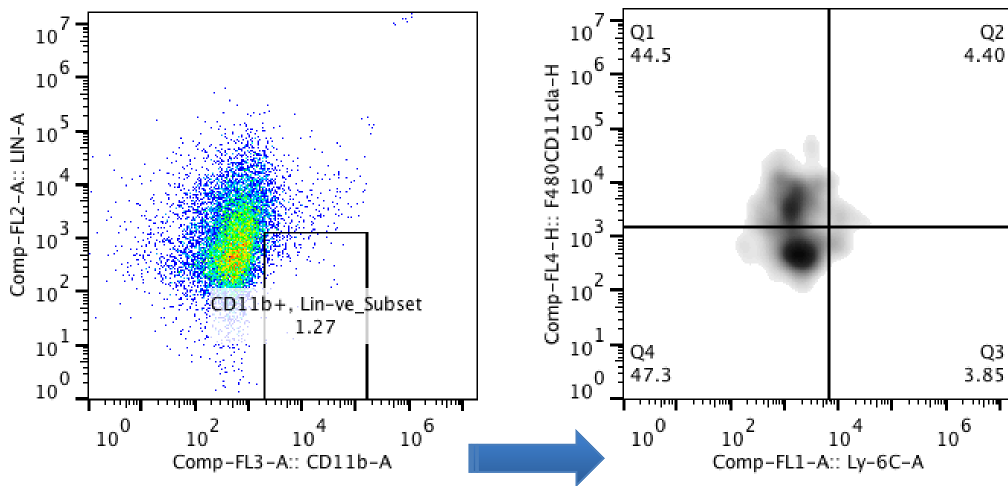

Figure 5. Identification of monocyte and macrophage populations out of all leukocyte populations.

The graph on the left identifies all monocytes and macrophages as lineage negative and cd11b positive cells. The graph on the right specifically identifies ly-6c high and ly-6c low monocyte subsets and macrophages as positive for F4/80, cd11c, and IA within the first population identified (cd11b positive, lineage negative cells).

Additional Antibody Panels

Table 3. M2-like macrophage panel.

Fluorochrome	Antibody	Company	Cat. No.	Dilution	Amount (ul)
PBA	-	-	-	-	680
FITC	CD23	BD Biosciences	553138	1:20	34
PE	F4/80	Biolegend	123110	1:20	34
PE-Cy7	CD206	BD Biosciences	141720	1:20	34
APC	CD11c	BD Biosciences	550261	1:20	34
-	FC Block	BD Biosciences	553142	-	10

Notes: Antibodies and concentration used when creating cell staining mixture for M2-like macrophages. All antibodies were mixed into PBA. FC Block was added to cell suspensions for 20 minutes before the antibody mixture was added. 20ul of the mixture was added to each cell suspension to stain for 20 minutes at 4°C. Amounts account for the staining of 30 samples. The Accuri C6 was set to collect 50,000 events. Laser settings were set to three red and one blue lasers. FL1 lens was 533/30, FL 2 lens was 585/40, FL 3 lens was 670LP, and FL4 lens was 675/25.

Table 4. General white blood cell panel.

Fluorochrome	Antibody	Company	Cat. No.	Dilution	Amount (ul)
PBA	-	-	-	-	680

Table 4 (continued).

Fluorochrome	Antibody	Company	Cat. No.	Dilution	Amount (ul)
FITC	CD3	Ancell	703-040	1:20	34
PE	F4/80	Biolegend	123110	1:20	34
PercpCy5.5	Ly6g	BD Biosciences	560602	1:20	34
APC	CD11c	BD Biosciences	550261	1:20	34

Notes: Antibodies and concentration used when creating cell staining mixture for general white blood cell populations. All antibodies were mixed into PBA. 20ul of the mixture was added to each cell suspension to stain for 20 minutes at 4°C. Amounts account for the staining of 30 samples. The Accuri C6 was set to collect 50,000 events. Laser settings were set to three red and one blue lasers. FL1 lens was 533/30, FL 2 lens was 585/40, FL 3 lens was 670LP, and FL4 lens was 675/25.

Spleen Processing

Following euthanasia at the specified times, spleens were harvested and processed by pushing them through 70µm filters and washed with PBS by centrifugation at 300xg for five minutes at 4ºC. To reduce erythrocyte numbers, 3ml of 1x ACK was added for one minute, after which 5ml of PBS was added to reduce ACK activity. Cells were then washed twice with PBS by centrifugation. Cells were resuspended in 2ml of PBS and cell counts were performed on the single cell suspensions at a 1:50 dilution on the BD ACCURI. Protocol was borrowed from and utilized as previously described[48].

Ovary Processing

Following euthanasia of mice at the specified times, both ovaries were harvested and fatty tissue was carefully removed. Ovaries were then minced into approximately 20

pieces and placed into 15ml tubes. To remove remaining fatty tissue pieces, 10ml PBS was added to the tube, and all but 2ml of solution was removed from tube and disposed. Ovarian cells were allowed to settle 30 minutes before collagenase DNase solution (CDS) was added to break down collagen. Tubes containing CDS and ovary cells were then incubated for 30 minutes at 37ºC. Mixtures were then dissolved further by using a syringe with an 18G needle and filtered through a 70µm filter into a new 15ml tube. Cells were then centrifuged at 300xg for five minutes at 4ºC, supernatant was dumped, and cells were resuspended in 2ml HBSS. Cell counts were performed on the single cell suspensions at a 1:10 dilution on the BD ACCURI. Protocol was borrowed from and utilized as previously described[7].

Cumulous Oocyte Complex Counts

Oviducts were collected from 20 hr post hCG mice in order to count cumulous oocyte complexes (COCs). The oviducts were isolated and a small cut was made in the ampulla to allow the COCs to exit the oviduct. The COCs were then counted under a microscope. The average number found in one set (n=3) of hCG 20hr mice was determined to be the ovulation rate.

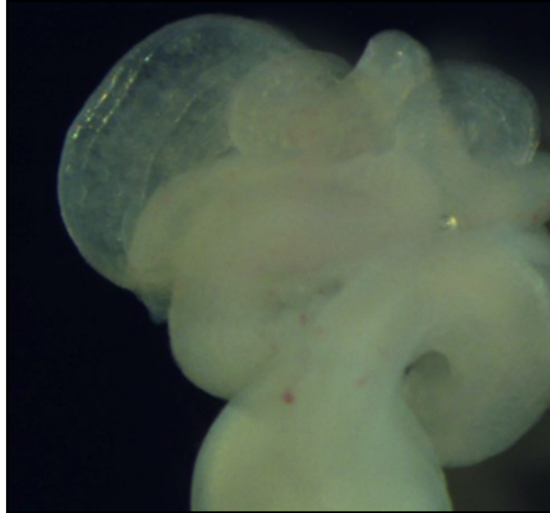

Figure 6. Oviduct from a mouse.

The oviduct, after being isolated from the ovary. The ampulla is distinct from the rest of the oviduct and it usually translucent. Most cumulous-oocyte complexes reside in the ampulla. Photo courtesy of Dr. Phil Bridges.

Tissue embedding

Ovaries and spleen were collected from mice and formalin fixed for three hours, then transferred to PBS. Tissues were dehydrated through a series of hour-long graded ethanol baths. They were then transferred into two baths of room temperature Histoclear II for an hour each and one bath of 55°C Histoclear II for 45 minutes for clearing. The tissues were then placed into melted (55°C) paraffin to prepare for embedding. Tissues were then embedded onto cassettes using disposable molds and a paraffin dispenser and were allowed to dry overnight. Tissues were cut 7µm thick using a microtome. Slides were allowed to dry overnight at 38°C.

Immunohistochemistry

Slides were then put in three 5 minute baths of Histoclear II to deparaffin, followed by a graded series of ethanol baths for 2 minutes each to rehydrate the tissue.

Tissues underwent antigen retrieval (specific to each primary antibody), which included warming antigen Retrievagen A (BD Biosciences, Cat. No. 550524) or B (BD Biosciences, Cat. No. 550527) in a microwave for 5 minutes to approximately 192°F and then heating the slides in the solution for up to 20 minutes (Table 5). Slides were allowed to cool for about 5 minutes. Tissue was circled with a pap pen. Bloxall (Vector, Cat. No. SP-6000) was placed on all tissues for ten minutes to block endogenous peroxidase binding. Slides were rinsed with Tris Buffered Saline (TBS) with Tween and then 10% Normal Goat Serum Block (Vector, Cat. No. S-1000) was applied to all tissue for 5 minutes. Normal serum was dumped off and primary antibody was applied (Table 5). Primary antibody was rinsed off using TBS with Tween and then Goat anti-Rabbit (Vector, Cat. No. BA-1000) at a 1:500 dilution was applied for 15 minutes. Slides were again rinsed with TBS with Tween and then Elite ABC Reagent (Vector, Cat. No. PK-7100) was added for 15 minutes. This solution was rinsed off using TBS with Tween and then Cardassian DAB, (Biocare Medical, Cat. No. DBC859H), a chromagen substrate, was added onto the tissue for 5 minutes or until color appeared. The chromagen substrate was thoroughly rinsed off with TBS with Tween. Slides were then counterstained for 15 seconds with Hemotoxylin. Slides were then rinsed for two minutes with 95% ethanol, two washes of 100% ethanol for two minutes each, and two washed of Histoclear II for two minutes each. Slides were dried and a coverslip was placed over the tissue using Permount. Slides were allowed to dry overnight and examined under microscope for positive colored cells. Protocol borrowed from previous studies[49].

Table 5. Primary antibodies used in immunohistochemistry along with antigen retrieval specifics and incubation times.

Primary Antibody	Company	Cat. No.	Retrievagen	Dilution	Incubation
B220/ CD45R	BD Biosciences	557390	B, 5 min	1:100	4°C, overnight
CD30	BD Biosciences	553824	A, 10 min	1:100	4°C, overnight
Anti-PE-CAM1	R & D Systems	af3628	A, 10 min	1:100	4°C, overnight
Anti-neutrophil elastase antibody	Abcam	ab68672	A, 20 min	1:2000	Room temperature, 2 hours
CD45	Abcam	ab10558	A, 10 min	1:100	4°C, overnight

Notes: Primary antibody dilutions are also included. B220 was used to identify B cells, CD30 was used to identify activated B and T cells, anti-PE-CAM was used to identify blood vessels and endothelial cells, CD45 was used to identify white blood cells in general, and anti-elastase was used to identify functional neutrophils.

Chapter 3

Results

Leukocyte infiltration of ovary

Leukocyte infiltration into the ovary was counted by collecting total events by flow cytometry. Cells were only counted if they were larger than red blood cells. A significant increase was found in the group hCG 6hr compared to the control group, showing the increase in leukocyte infiltration prior to ovulation.

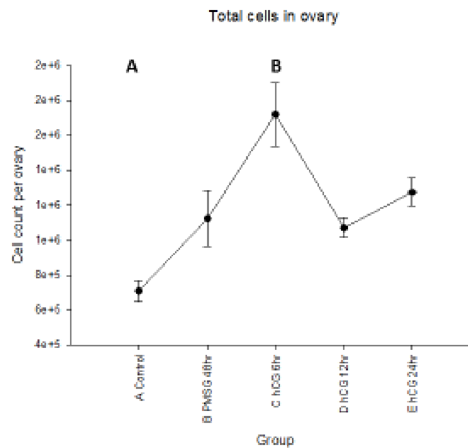

Figure 7. Total cell count in ovaries during ovulation.

The number of leukocytes in the ovary increases during ovulation as compared to immature control mice. The group hCG 6hr was significantly increased compared to the control. Different letters show significant difference. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

Ly6c^{high} Monocytes

Ly6c^{high} monocytes that were stained and counted by flow cytometry showed a significant increase in percentages and absolute numbers for group hCG 6hr compared to all other groups. For absolute numbers, hCG 12hr was also significantly increased compared to the control group.

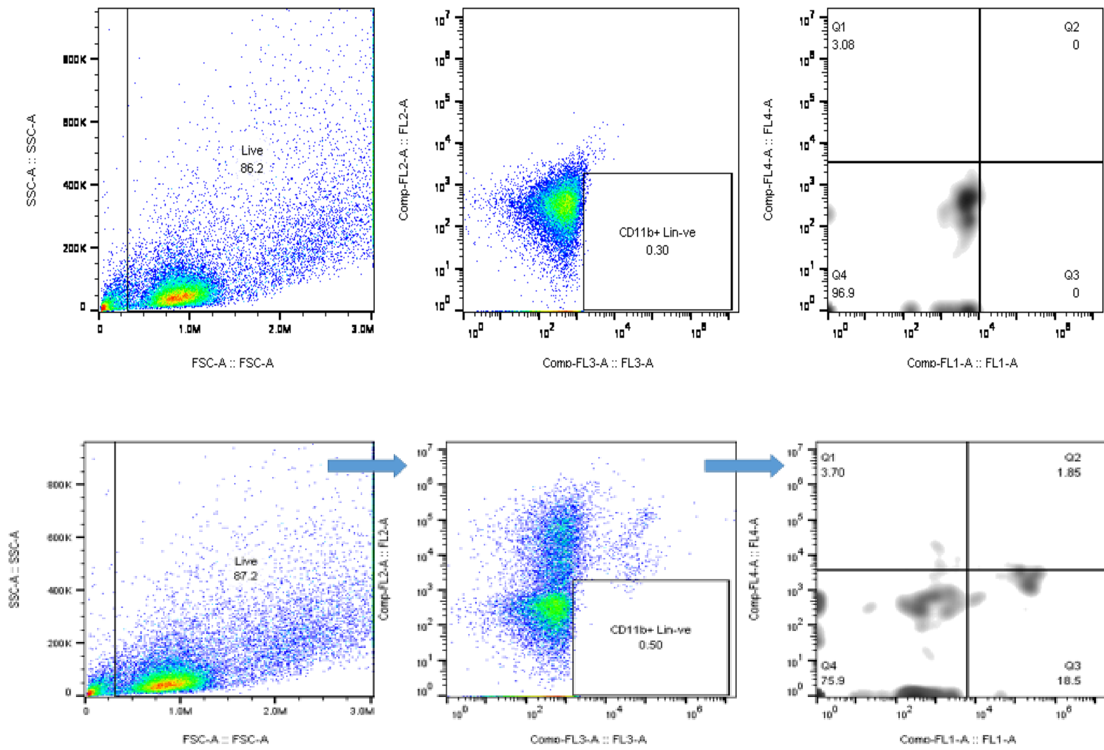

Figure 8. Gating strategy for ly6c high and low monocytes.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. CD11b+ and lineage negative cells were then selected and narrowed into either ly6c high or low monocytes. These cells were further defined by lack of expression of IA, F480, and CD11c. Ly6$\boldsymbol{c}^{\boldsymbol{high}}$ monocytes fell into the lower right quadrant while Ly6$\boldsymbol{c}^{\boldsymbol{low}}$ monocytes fell into the lower left quadrant.

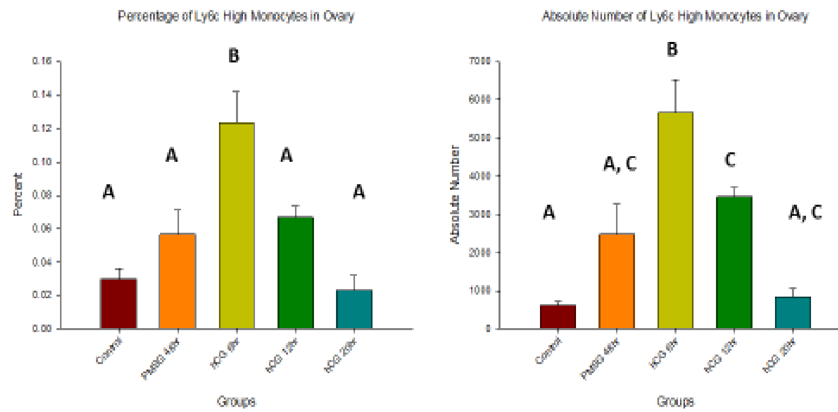

Figure 9. Percentage (left) and absolute number (right) of ly6c^{high} monocytes in the ovary at different time points during ovulation.

Demonstrates the influx of ly6$\boldsymbol{c}^{\boldsymbol{high}}$ monocytes during ovulation, specifically before ovulation occurs at hCG 6hr. Group hCG 6hr is significantly increased compared to all other groups in both percentage and absolute numbers. Group hCG 12hr is also increased compared to control for absolute numbers. Different letters show significant difference. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

Ly6c^{low} Monocytes

The gating strategy for ly6c^{low} monocytes was the same as depicted in figure 8. Ly6c^{low} monocytes showed a delayed increase in absolute numbers, appearing after ovulation at the time point hCG 20hr. A significant increase was found in group hCG 20hr compared to groups control, PMSG 48hr, and hCG 12hr.

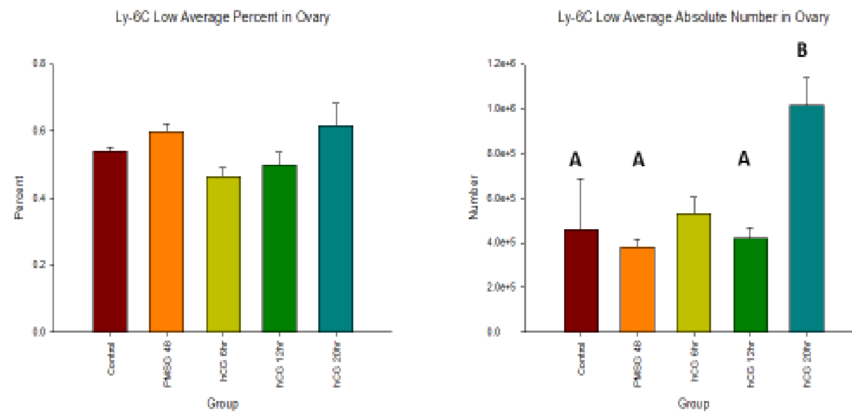

Figure 10. Percentage (left) and absolute number (right) of ly6c^{low} monocytes in the ovary during ovulation.

Demonstrate the appearance of ly6c^{low} monocytes post ovulation. Group hCG 20hr is significantly increased compared to groups control, PMSG 48hr, and hCG 12hr in absolute numbers. Different letters show significant difference. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

CD11c+ Dendritic Cells

No significant difference was found for CD11c positive cells, nor was any trend noticed. CD11c positive cells did not seem to be entering the ovary.

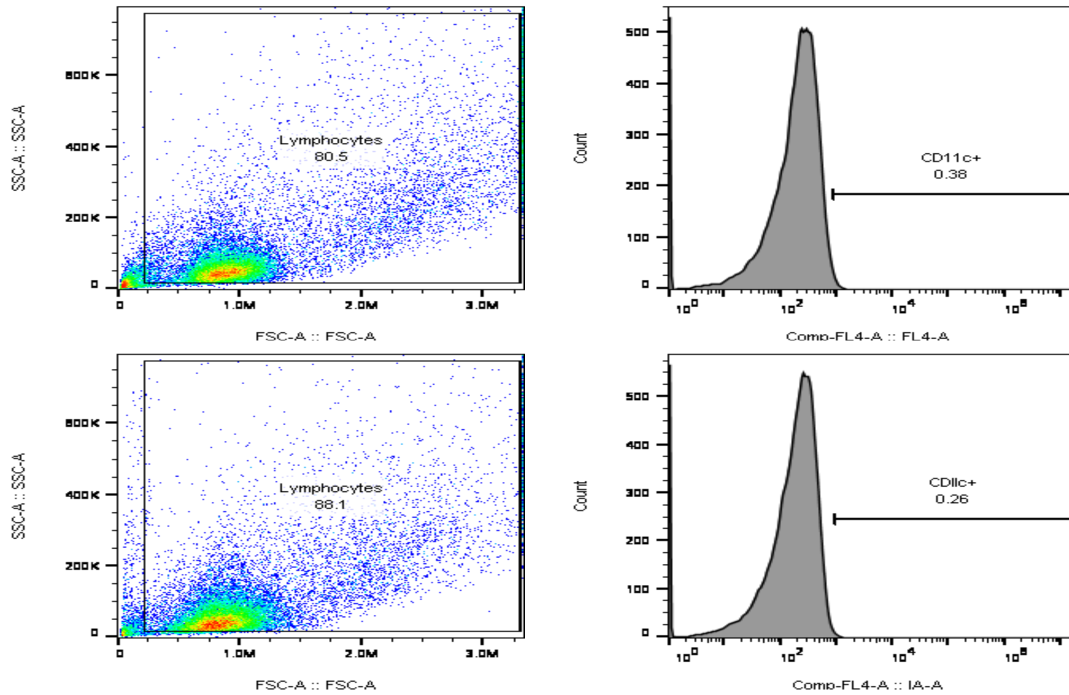

Figure 11. Gating strategy for CD11c positive cells.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. A histogram was then created showing all CD11c positive cells.

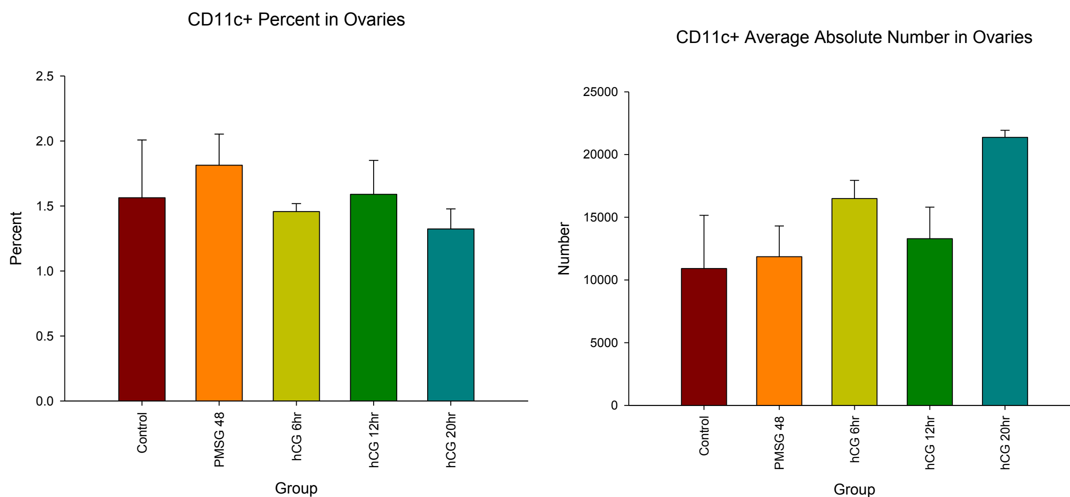

Figure 12. Percentage (left) and absolute number (right) of CD11c+ staining cells in ovary during ovulation.

Results do not show much of a trend, indicating static behavior for dendritic cells. No significant difference between groups was found. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

CD206+ M2-like Macrophages

No significant difference was found for CD206 positive cells, nor was any trend noticed. CD206 positive cells did not seem to be entering the ovary.

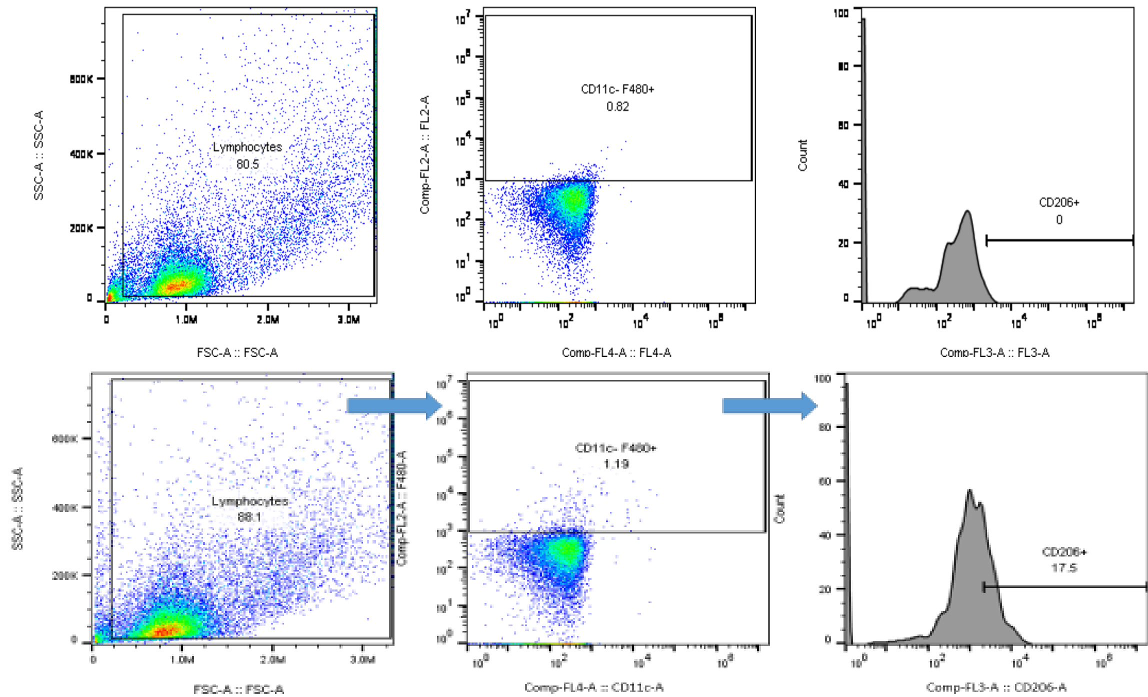

Figure 13. Gating strategy for CD206 positive cells.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. Next F480+ cells were selected, and further defined on a histogram as CD206 positive cells.

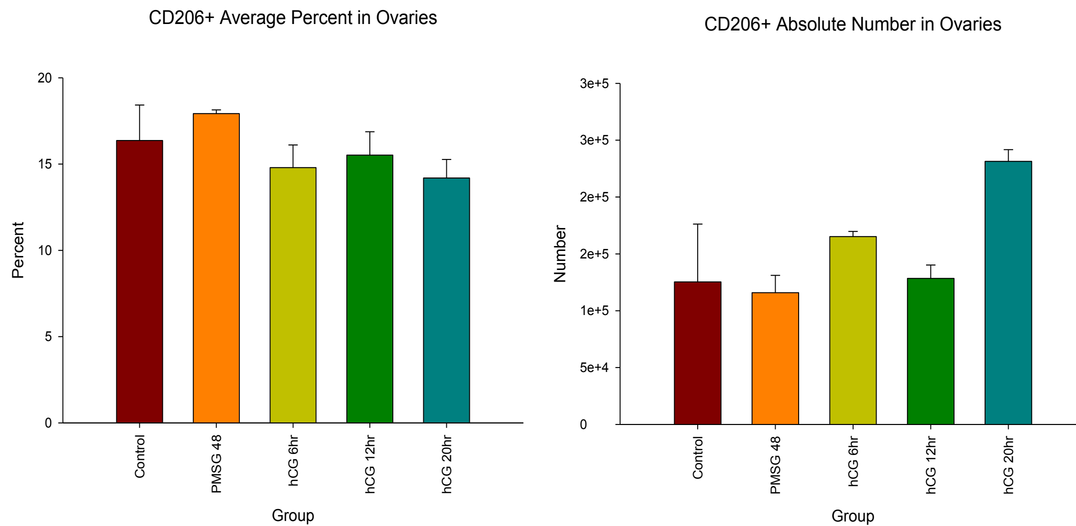

Figure 14. Percentage (left) and absolute number (right) of CD206+ cells in ovary during ovulation.

Results do not indicate any real trend. An increase in CD206 cells may be occurring at hCG 20hr as absolute numbers doubled. No significant difference was found between groups. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

CD197+/IA+ M1-like Macrophages

No significant difference was found for CD197/Ia positive cells, nor was any trend noticed. CD197/Ia positive cells did not seem to be entering the ovary.

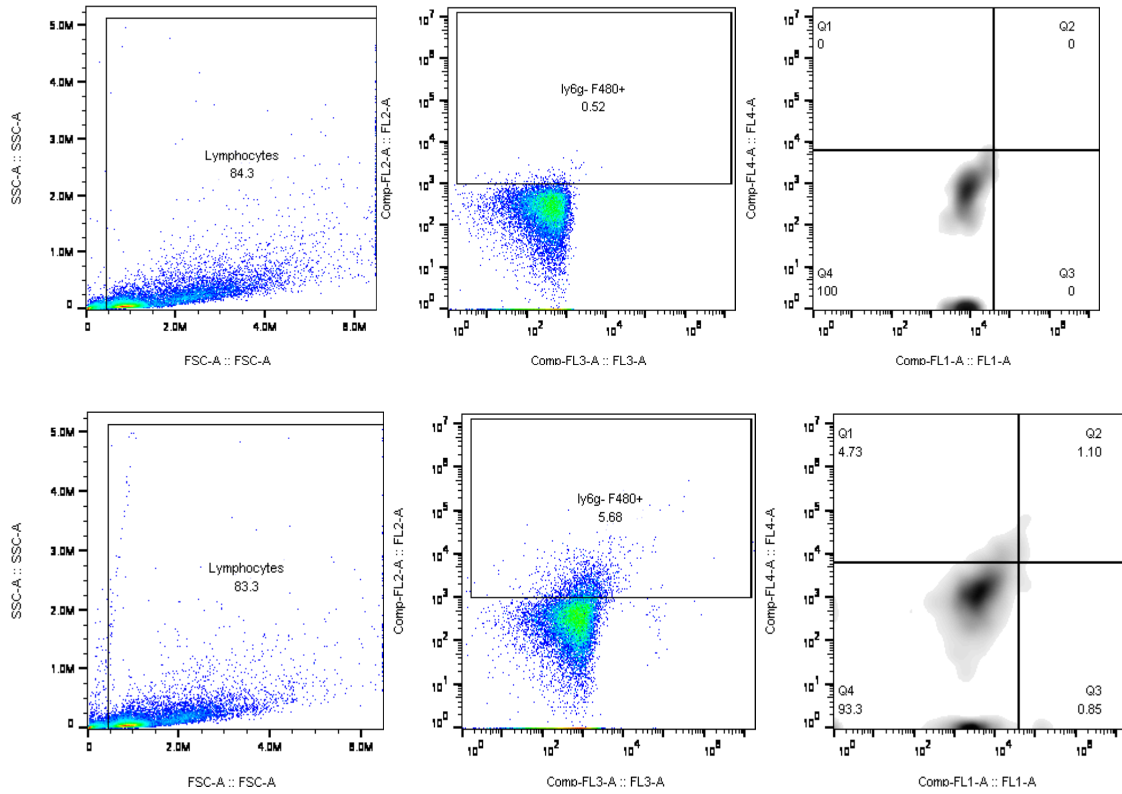

Figure 15. Gating strategy for M1-like macrophages.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. Next F480+ cells were selected, and further defined as being double positive for Ia and CD197. These cells fell into the upper right quadrant.

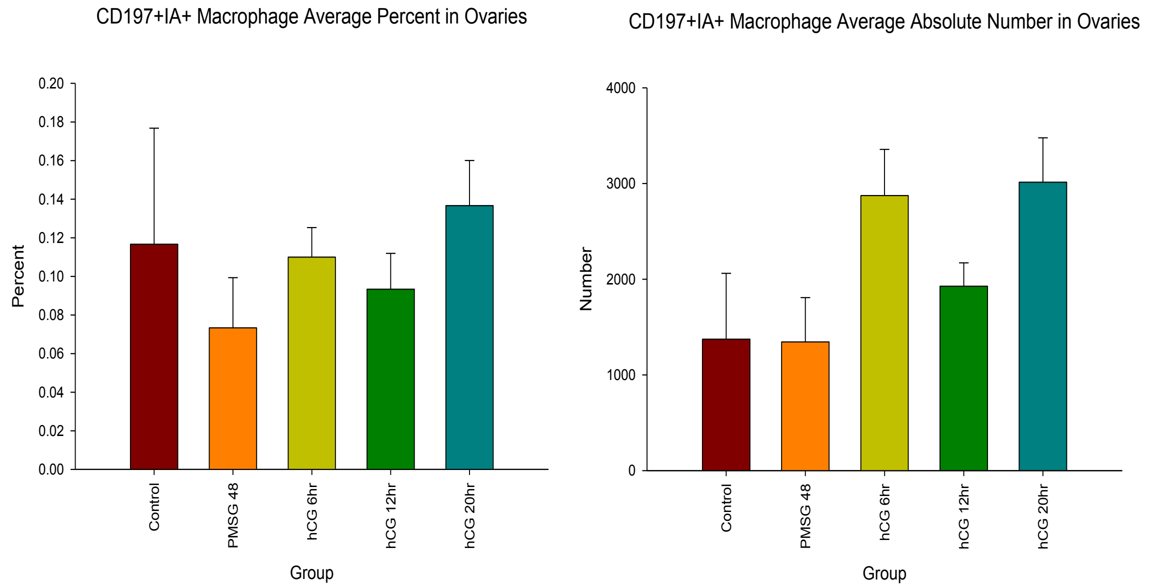

Figure 16. Percentage (left) and absolute number (right) of CD197+/IA+ M1-like Macrophages in the ovary during ovulation.

Results show an increase in M1-like macrophage absolute numbers during ovulation compared to the control group. No significant difference was found between groups. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

Ly6g+ Neutrophils

No significant difference was found for Ly6g positive cells, although an increasing trend was noticed in neutrophil numbers. Ly6g positive cells are likely entering the ovary throughout ovulation.

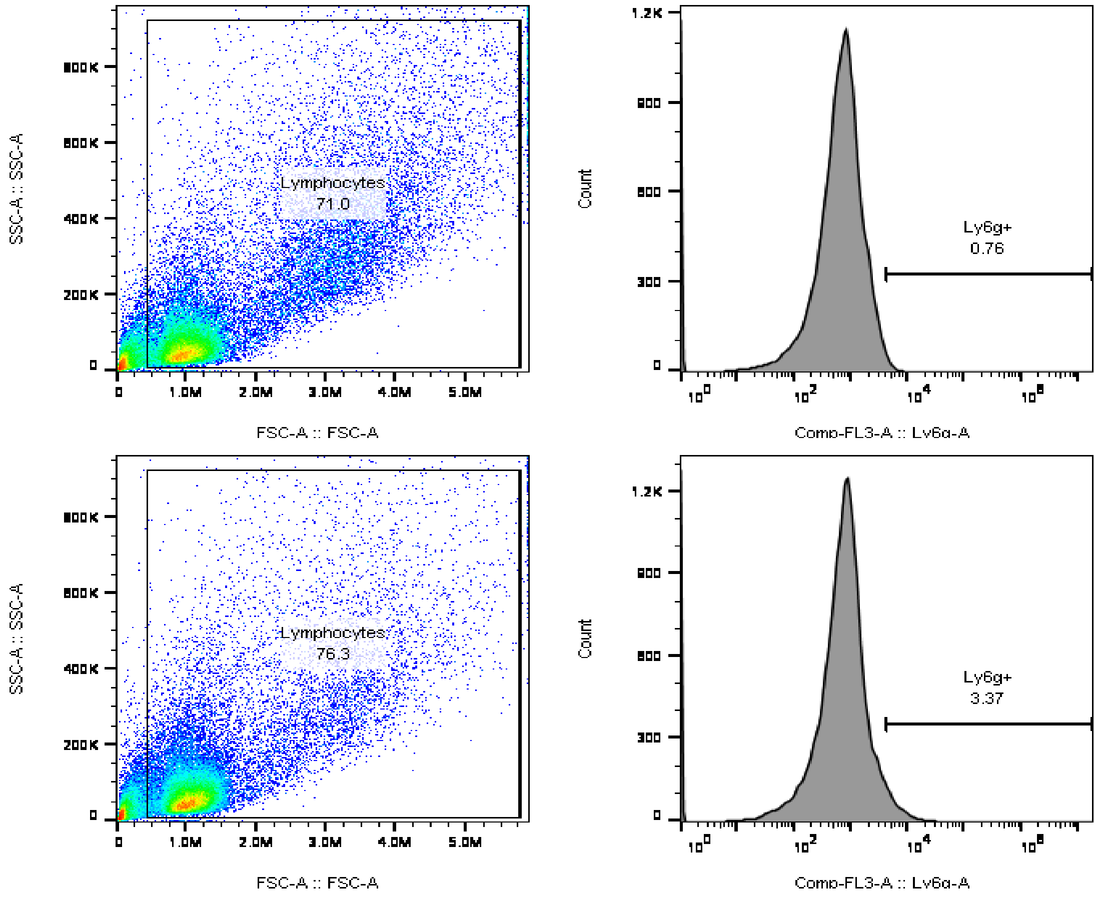

Figure 17. Gating strategy for ly6g+ cells.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. A histogram was then created showing all ly6g positive cells.

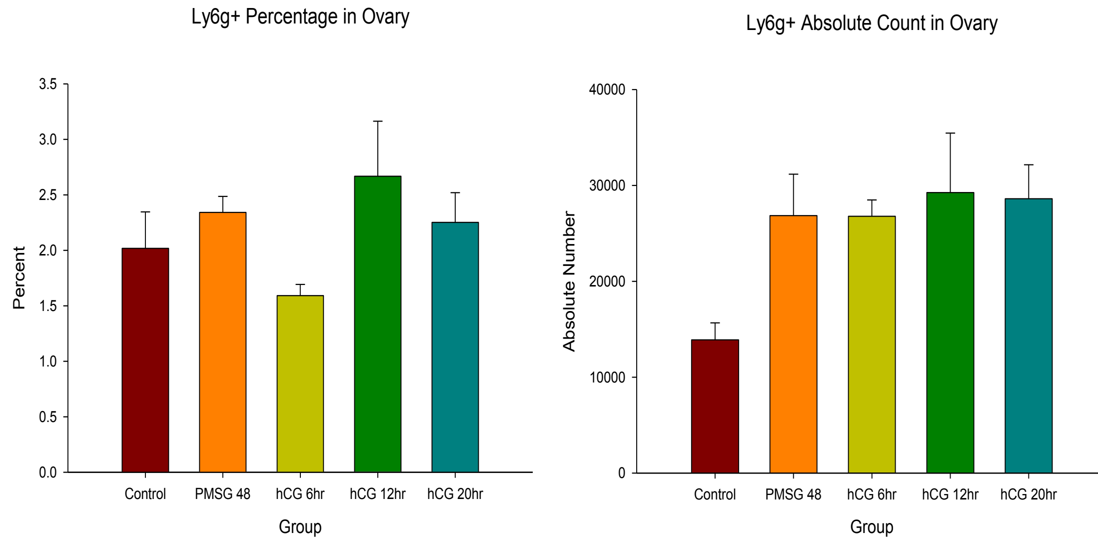

Figure 18. Percentage (left) and absolute number (right) of Ly6g+ neutrophils in the ovary during ovulation.

Results indicate a trend showing an increase in neutrophil absolute numbers during ovulation compared to the control group. No significant difference was found between groups. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

CD3+ T Cells

No significant difference was found for CD3 positive cells, nor was any trend noticed. CD3 positive cells did not seem to be entering the ovary.

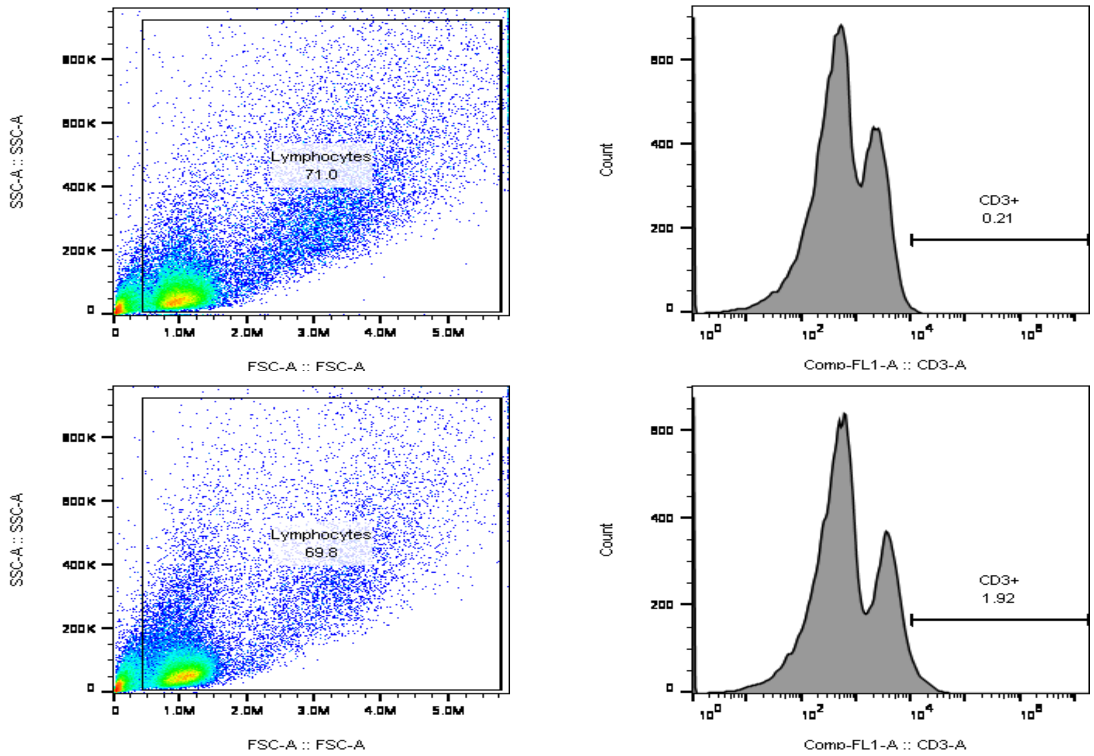

Figure 19. Gating strategy for CD3+ cells.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. A histogram was then created showing CD3 positive cells.

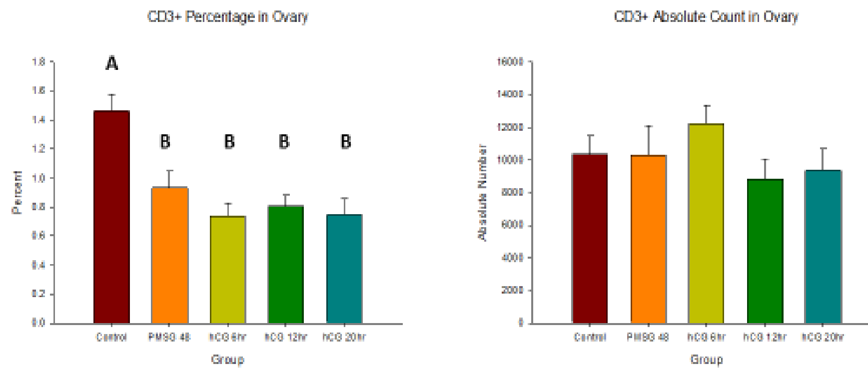

Figure 20. Percentage (left) and absolute number (right) of CD3+ T cells in the ovary during ovulation.

Results do not indicate any real trend. There was a significant difference between the control group compared to all four other groups in percentage graph. Different letters show significant difference. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

F4/80+ Macrophages

No significant difference was found for F4/80 positive cells, although an increasing trend was noticed in macrophage numbers. F4/80 positive cells are likely appearing in the ovary during ovulation.

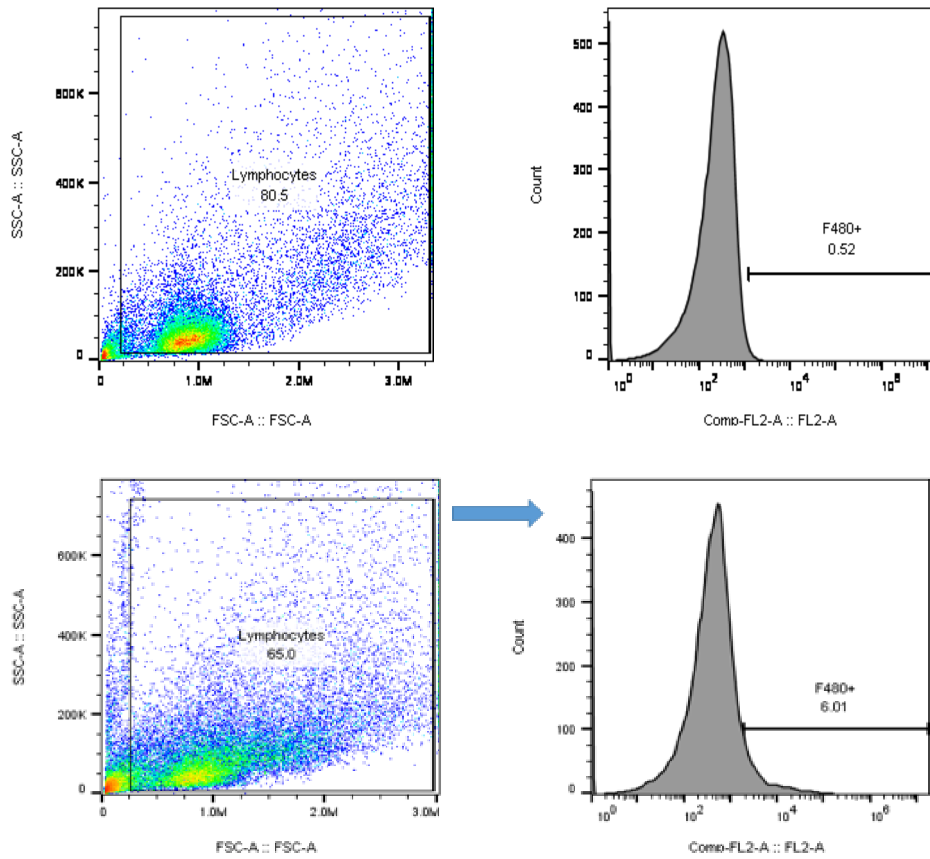

Figure 21. Gating strategy for F480+ cells.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. A histogram was then created displaying all F480 positive cells.

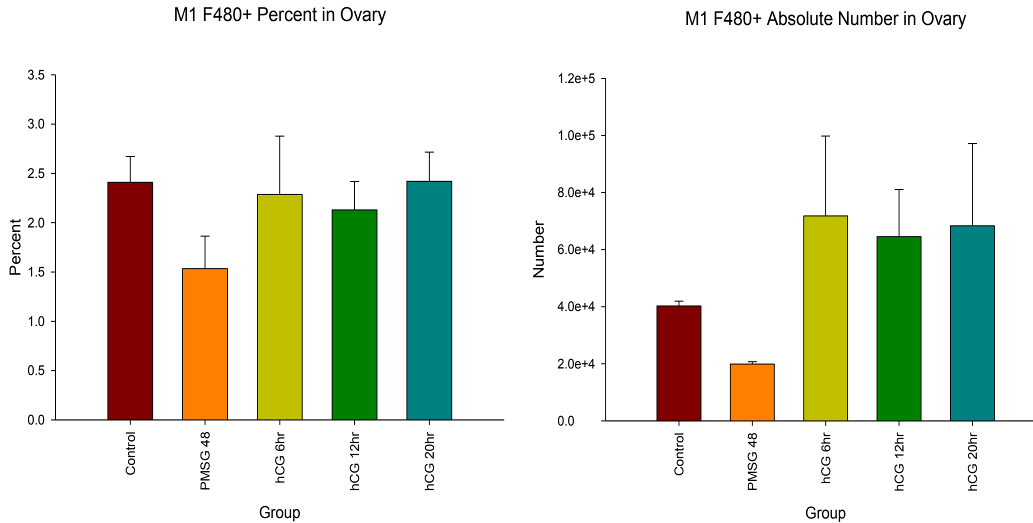

Figure 22. Percentage (left) and absolute number (right) of F480+ T cells in the ovary during ovulation.

Results suggest an increase in absolute numbers of macrophages during ovulation. No significant difference was found between groups. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

Recruitment of Leukocytes from Spleen

In percentage, a significant decrease, particularly for the $ly6c^{high}$ monocyte, in the spleen was demonstrated for groups hCG 6hr, hCG 12hr, and hCG 24hr compared to the control. There was also a significant decrease in groups hCG 6hr compared to PMSG 48hr. For absolute number, a significant decrease in the spleen was demonstrated in groups hCG 6hr and hCG 24hr compared to the control group. The spleen appears to be losing $ly6c^{high}$ monocytes during times that coincide with ovulation.

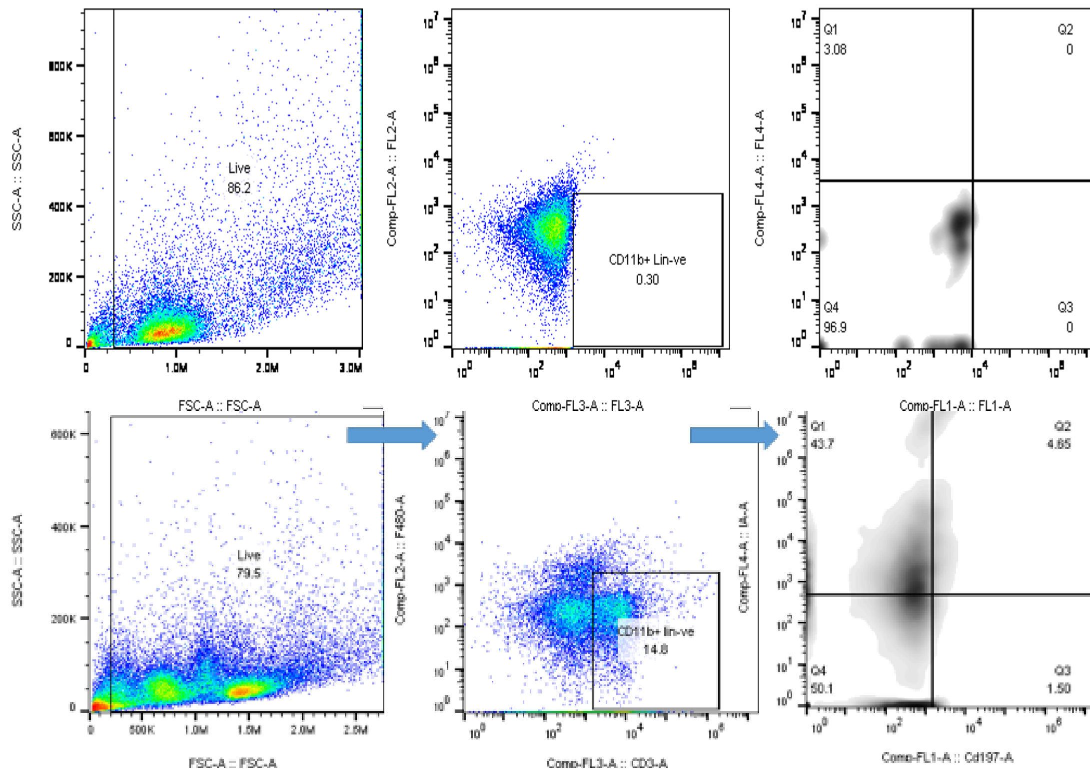

Figure 23. Gating strategy for splenic leukocytes.

An unstained sample of ovarian cells was used to create gates yielding positive populations that were 1% or less which were then applied to all samples. Cell other than leukocytes were gated out first based off of small size. CD11b+ and lineage negative cells were then selected and narrowed into either ly6c high or low monocytes. These cells were further defined by lack of expression of IA, F480, and CD11c. Ly$6c^{high}$ monocytes fell into the lower right quadrant while

Ly6c^{low} monocytes fell into the lower left quadrant.

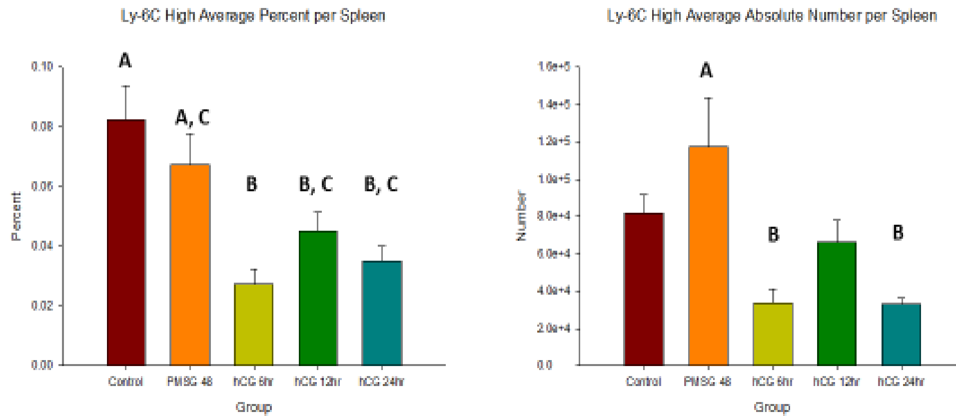

Figure 24. Percentage (left) and absolute number (right) of ly6c^{high} monocytes in the spleen at different time points during ovulation.

Demonstrates a trend that leukocyte numbers, particularly for ly6c^{high} monocytes, fall during ovulation. Groups hCG 6hr, hCG 12hr, and hCG 24hr are significantly decreased compared to the control group for percentage. Group hCG 6hr is also significantly lower compared to group PMSG 48hr for percentage. For absolute numbers, groups hCG 6hr and hCG 24hr are significantly lower than group PMSG 48hr. Different letters show significant difference. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).

Cumulous Oocyte Complex Counts

Oviducts were first isolated from the ovaries in mice taken at hCG hour 20. The ampulla of the oviduct was then cut, expelling most COCs from the oviduct in a clump. Fine tweezers were then used to tease remaining COCs from the oviduct. The oocytes in these complexes were then counted under microscope and then averaged to determine ovulation rate. Ovulation rates ranged from 14 to 29. Reduced rates in trials 2, 4, and 5 had one mouse that did not have distinct ampullas full of COCs, which lowered the overall ovulation rate.

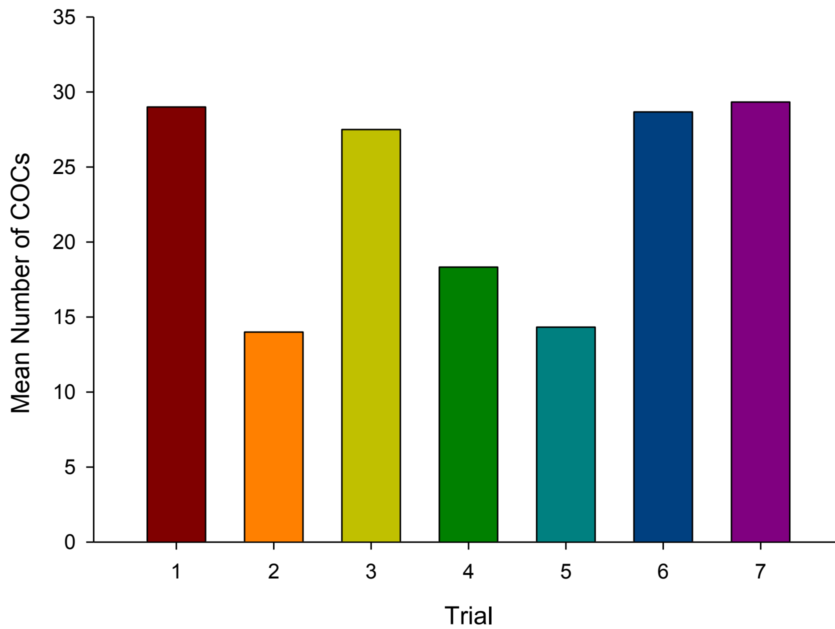

Figure 25. Ovulation rate determined by counting cumulous oocyte complexes in hCG 20hr mice.

Oviducts were isolated in hCG 20hr mice and the ampulla was cut to release cumulous oocyte complexes which were then counted. Numbers for both ovaries in mice were averaged to determine ovulation rate. Average ovulation rate ranges between approximately 14 and 29. Results represent mean of n=3 mice for seven experiments.

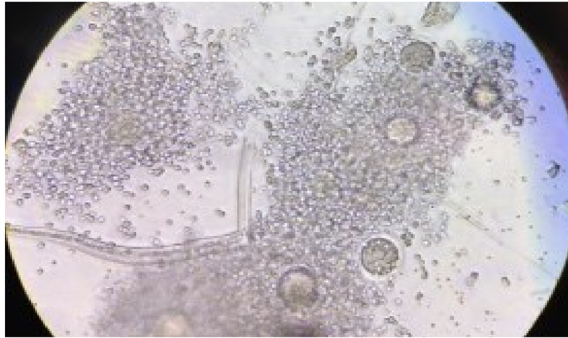

Figure 26. Cumulous oocyte complexes under the microscope.

Five distinct oocytes along with numerous small cumulous cells can be seen. Each visible oocyte was counted and averaged per group to determine ovulation rates.

Immunohistochemistry

Ovary, oviduct, and spleen tissue were all stained with hematoxylin and eosin for basic cell structures. A rat anti-mouse B220 primary antibody was utilized to detect B cells in all three tissues. Cells stained positive for B220 in the spleen, but not in the ovary or oviduct, suggesting B cells are not present or necessary in ovulation.

Chapter 4

Discussion

Leukocyte infiltration into the ovaries is a key factor in the physiological process of ovulation, that is now well recognized as an inflammatory response. We identified several leukocyte populations infiltrating the ovarian tissue, including monocytes, macrophages, and granulocytes including neutrophils and eosinophils. Previous studies have identified a variety of leukocyte products found in the ovaries, including proteases, cytokines, and chemokines, that promote leukocyte recruitment and inflammation. The presence of leukocytes and their products indicates they are necessary for facilitating ovarian processes, such as steroidogenesis, breakdown of the basement membrane of the follicular wall for ovulation, angiogenesis and vascularization of the corpus luteum, and degradation of a collapsing corpus luteum.

Referring to Figure 7, which shows the total number of cells in the ovary, it seems clear that prior to ovulation, the cell totals begin to increase, and the ovary seems to double in size. This was evident by observing ovaries taken from mice at each time point during ovulation, as control mice had very small, pale ovaries, and mice at any stage during ovulation had much bigger, pink ovaries. Upon close examination, many of these ovaries also had multiple follicles visible. This indicates that leukocytes must serve some function in the process of ovulation, although we believe they likely serve multiple purposes. Many cells that come into the ovary prior to ovulation likely serve in an inflammatory role, while the cells coming in after ovulation probably serve reparative functions.

The ly6c^{high} monocyte is one of the first cells we have identified that are recruited the ovary prior to ovulation. These monocytes seem to peak in number at hCG hour 6, which is before ovulation occurs in mice. This means they are most likely involved in inflammatory processes, as they can differentiate into M1-like macrophages and help to break down the wall of follicles in the ovary so that the oocyte can be released. The numbers of these monocytes quickly decrease, as their role finishes when ovulation occurs. This may indicate that they are either leaving the ovary or differentiating into other cell types, including ly6c^{low} monocytes, which can then differentiate into M2-like macrophages for tissue repair. The large number of ly6c^{high} monocytes may simply arrive in the ovary before ovulation occurs simply to serve as a source of both types of macrophages, since they are versatile and can become either one based on cellular cues in the tissue environment they infiltrate. The population of ly6c^{high} cells is transient, as these are short lived cells that down-regulate ly6c expression quickly and differentiate into macrophages[27,31].

Two cell types that do not seem to be playing a role in ovulation are the dendritic cells and T cells. Cells that stained positive for CD11c are generally accepted to be dendritic cells, and cells that stain positive for CD3 are T cells. From our results, absolute numbers of dendritic cells and T cells in the ovaries do not seem to be increasing or decreasing. This would suggest that they likely are not playing a major role in ovulation. It is logical that T cells would not play a role because they are involved in acquired immunity, and ovulation is a naturally occurring process with no foreign particles involved. However, other studies have indicated the presence of T cells in the

ovaries during corpus luteum development, so more studies should be done to determine their role.

The M2-like macrophages were designated as CD206+ cells. These cells did not really exhibit any significant pattern or trend in our results. If anything, it looks as if there may have been an increase in M2-like macrophages after the resolution of ovulation at hCG 20hr. This would make sense because these macrophages tend to help repair tissue and clean up cellular debris left behind from other cells. These assays should be repeated with a more specific antibody, however, as the antibody used for CD206 did not demonstrate sufficient effectiveness. This may have been due to low CD206 expression or some other reason, but it made it difficult to differentiate M2-like macrophages from other cell types. Therefore it is advisable to repeat the flow cytometry assays to better identify this population of cells.

M1-like macrophages were designated as double positive for CD197 and IA. The results show an increase in these cells during ovulation. As these are inflammatory in nature, this seems probable. However, as seen with the CD206 antibody, the CD197 antibody did not produce good results, making it hard to differentiate M1-like macrophages from other populations. This may also have been because of low CD197 expression. This population should also be better differentiated, perhaps by using a different antibody or by searching for a different unique M1 macrophage antigen.

Any cells that stained positive for ly6g were designated as neutrophils. According to our results, it appears that neutrophils doubled in number throughout ovulation. Neutrophils are part of the innate immune system and are highly inflammatory, so it would make sense that they would be involved in ovulation. They

could potentially be helping follicles rupture by releasing inflammatory cytokines to degrade the follicular wall until the oocyte is released. Others suggest that neutrophils may be recruited to the ovary by other leukocytes, possibly by monocytes.

We also used a general marker for macrophages, called F4/80, to identify them in the ovary. Our results, although not significant, show that after administering hCG, F4/80 positive cells double in absolute number in the ovary. These would include both M1-like macrophages, for promoting inflammation and follicular rupture, and M2-like macrophages, for repairing tissue and bringing ovarian tissue back to the start of the ovulatory process. The numbers for generic macrophages remain high throughout ovulation because both M1 and M2 populations play important roles and are active at different points throughout ovulation.

The spleen is a known lymphoid organ, and in our case, may act as a source of cell donation for the ovaries. Our results show immature control mice having many more leukocytes in their spleen than mice taken during ovulation. The spleen, just as it donates white blood cells to a site of injury, may also be donating some leukocytes to the ovaries and other reproductive tissues to help the process of ovulation occur. This is probably due to the fact that ovulation requires inflammatory factors to occur and reparative factors to help restore tissue after ovulation occurs.

Counting cumulous oocyte complexes (COCs) proved to be a challenging task, as each pair of ampullas had to be separated from the ovaries in a delicate manner so as not to tear the ampullas and lose any COCs. This process was made significantly easier by using fine scissors and tweezers, which could make precise cuts without tearing the ovaries or ampullas. The complexes are also very mucous-like and tend to stick to each

other, making it difficult to count. However, there was a distinct bulge in every ampulla that actually held all the COCs, making them easier to locate. If the distinct bulge was not present on the ampulla, it usually meant no or very few COCs would be found. Overall, the results for ovulation rates ranged between 14 and 29 COCs. Lower ovulation rates usually had one mouse that did not have distinctive ampulla bulges, causing the average to be reduced. These ovulation rates are a good basis for future experiments investigating the effect of specific compounds on ovulation. For instance, we would expect lowered ovulation rates from mice that were given a drug that would stop the activity of luteinizing hormone.

When I began this project, I used one panel of antibodies for flow cytometry, which was designed for isolating ly6c^{high} and ly6c^{low} monocytes, and CD11c+/IA+/F480+ cells, which would have included macrophages and dendritic cells. After learning the basics of flow cytometry, I designed new panels that were designed to isolate macrophages, and later narrowed it down to a panel for M1-like macrophages and a panel for M2-like macrophages. However, there was one antibody from each of these panels that was very inefficient, and therefore we could never obtain conclusive results on these macrophages. The M1 panel antibody that did not work well was CD197, and the M2 panel antibody was CD206. It is likely that CD197 is not significantly expressed, which made it difficult to isolate from other cell types. The antibody for CD206 is for a receptor that is found inside the cell, so we utilized intracellular staining. Again, we were unable to effectively identify M2-like cells from other cell types. Other flow markers for M2-like macrophages that may potentially work better for future panels include CD64 and MerTK[34].

Our immunohistochemistry experiments were more recent attempts to identify the cells, and were not fully completed. We were only able to successfully stain a few leukocyte markers. We used formalin fixed tissues, which creates problems by masking our antigens, making them not accessible to antibodies. Another possible problem may have been due to our use of a microwave to heat the tissue before we stained, which may have denatured our antigens beyond antibody recognition.

Future Aims

One of the first priorties is to complete the immunohistochemistry assays. We must first identify useful antibodies to stain specific white blood cells using the spleen tissue as a control, and then progress to ovarian tissue to identify where in the ovary these cells were going. This will help to elucidate the location of the cells infiltrating the ovary, as flow cytometry only indicates how many were entering. Without knowing where these cell types are located in the ovary, it is harder to say with certainty what their function is. After we are able to identify where these cells are going, it would also be of interest to stain for specific cytokines and chemokines, particularly those that are inflammatory in ovarian tissue, taken before and during ovulation, or cytokines that are able to mend tissue in ovary sections taken after ovulation is over. This would further clarify the function of many of these cells.

It may also be informative to use cell cultures of ovarian tissue to test for cytokine and chemokine production. After getting an idea of baseline production, the addition of specific leukocyte populations should cause increased production of particular cytokines and chemokines. It may be interesting to see how $ly6c^{high}$ monocytes affect cytokine production. As an alternative, cell growth could be altered in response to particular

cytokines and chemokines, supporting the idea that leukocytes are promoting tissue remodeling after ovulation. Cell culture could provide new insight into chemokine activities during ovulation.

Another project would be to search for a better marker to use for the M1 and M2-like panels. RT-PCR could also be used to identify M1- and M2-like macrophages in the ovaries, since there are not very efficient antibody markers available. RT-PCR would be able to quantify levels of mRNA using macrophage markers that are not as easily detected via flow cytometry.

Together with narrowing down macrophage involvement, we could perform more experiments looking for neutrophils and determining their role in ovulation. Designing a flow panel to identify them is easily manageable and since they are an innate white blood cell, they appear to be a good candidate for playing a role in ovulation as well. Ovulation is naturally occurring so it would make sense that these innate cells, which are inflammatory cells, might be involved at some level. Furthermore, many studies have also been able to determine that neutrophils do appear in the ovary, particularly around the basement membrane of mature follicles. As monocytes have been shown to be able to regulate neutrophil activity in response to commensals and their products, it would be of interest to discern whether they also regulate neutrophils in ovarian tissue[37].

Further studies should be conducted on the effect of estrogen on ovulation. Estrogen is generally regarded to be an anti-inflammatory hormone and is highly involved in the process of ovulation. Estrogen levels remain elevated prior to ovulation, and once they drop, ovulation is able to occur. An influx of leukocytes is also seen after estrogen levels drop, coinciding with ovulation. This suggests that estrogen acts as one

of the key signals for leukocyte recruitment. Preliminary experiments administering estradiol to mice were performed following super ovulation, but more experiments need to be done to determine the precise effect it had on leukocyte infiltration. Along with this aspect, it would be ideal to see the effect of estradiol inhibitors on ovulation. For instance, estrogen inhibitors should block the anti-inflammatory effect of estrogen, potentially allowing leukocytes to enter prematurely in the ovary. This would no doubt have an effect on ovulation success, possibly causing premature follicle rupture.

It would also be a good idea to see the effect that administering leukocyte inhibitors has on ovulation. If leukocytes are truly are involved in helping ovulation occur, then blocking their migration to the ovary should effectively reduce the ability to ovulate. This should be easy to discern by looking to see whether ovarian cell counts increase after ovulation, and by checking ovulation rate by counting COCs. If cell counts remain low and similar to control mice, and ovulation rates are low, it would effectively support the role leukocytes have in ovulation. Inhibitors for monocytes and neutrophils would be a great starting point for this idea, specifically looking at inhibitors for CCR2, which is a major chemokine found on monocytes. INCB3344 is a drug that has been shown to be an effective and efficient antagonist for blocking monocyte recruitment. This drug can be administered orally to mice and binds to CCR2 on monocytes, effectively inhibiting monocyte extravasation[46,47]. A drug candidate for blocking fractalkine and ly6c^{low} monocytes is AZ12201182[39,43]. This particular drug is specific to the receptor CX_3CR1 and can inhibit the pro-survival effects of fractalkine and also block mRNA production of epiregulin[43]. An option for inhibiting neutrophil infiltration is the drug DF 2156A, which is a potent, non-competitive, allosteric inhibitor for both the

CXCR1 and CXCR2 receptors[45]. It is important to note that by blocking one type of leukocyte, the lack of that particular cell's influence and products could indirectly cause decreased recruitment of other white blood cells.

Due to chemokine redundancy, leukocytes may still be recruited to tissue using other routes, so an alternative to merely blocking chemokines and their receptors would be to use knockout mice that do not express the chemokine receptors at all. To test how important monocytes in particular are to ovulation, running the same experiments on CCR2 knockout mice would be advantageous. These mice should have normal numbers of white blood cells, but monocytes and macrophages would not express CCR2, making them much less likely to be recruited to tissue[36]. Monocyte infiltration into the ovary should be reduced, and in theory would result in reduced ovulation rate.

In summary, we were able to positively identify the presence of numerous leukocytes in the ovaries, including monocytes, macrophages, and neutrophils. We were able to narrow down specifically $ly6c^{high}$ monocyte infiltration as occurring before ovulation, supporting the notion that these inflammatory leukocytes promote degradation of the basement membrane of the follicle. We were also able to correlate influx of leukocytes in the ovary to a decreased number in the spleen, suggesting the spleen as a source for additional leukocytes. The presence of these white blood cells in the ovaries supports the need for leukocyte involvement in successful ovulation. Reproductive immunology, although a fairly recent field of study, proves to be a promising approach to determining means to help increase fertility in women who have a variety of either reproductive or immune diseases. The results substantiate this idea and suggest that novel therapies could be developed based on the immune response.

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Appendix A:

Immunohistochemistry Results

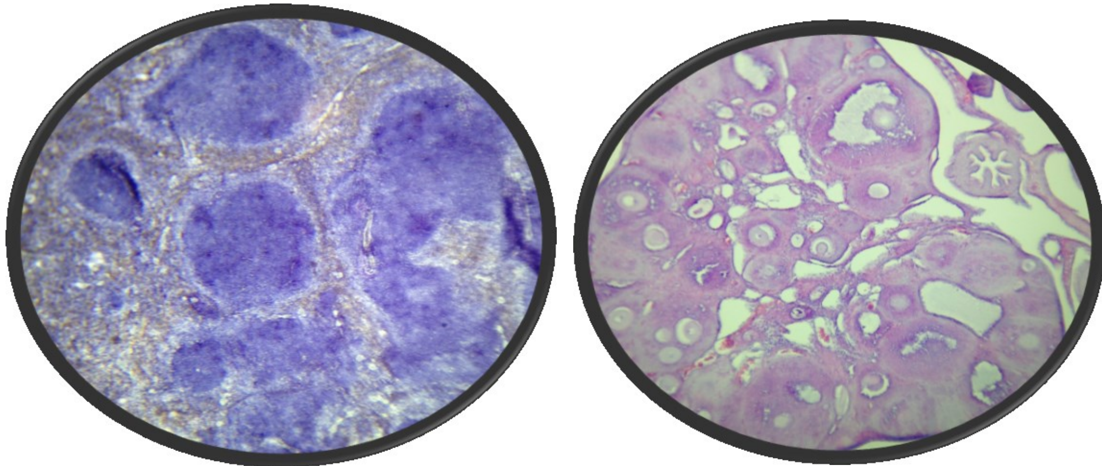

Figure 27. Tissue from spleen and ovary stained with B220.

Splenic tissue (left) stained positive for the antibody B220, indicating the presence of B cells. Ovarian tissue (right) did not stain positive for the antibody B220, indicating no B cells were present.

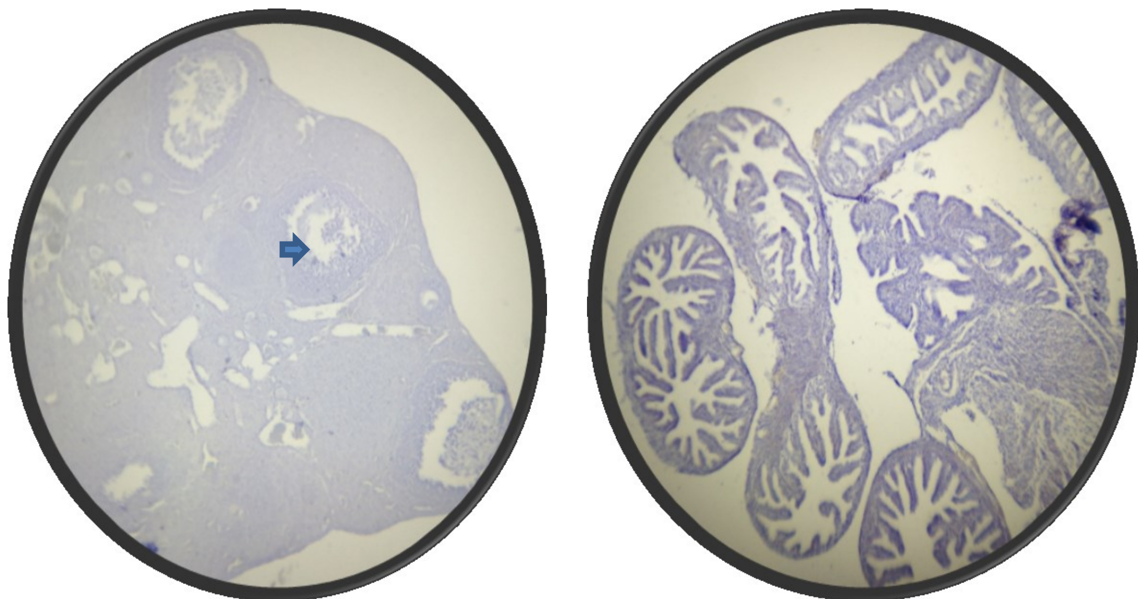

Figure 28. Additional H&E stained tissue.

Ovarian tissue (left) and oviduct tissue (right) stained with hematoxylin and eosin. The arrow points out a follicle in the ovarian tissue.

Appendix B:

Preliminary Estrogen Results

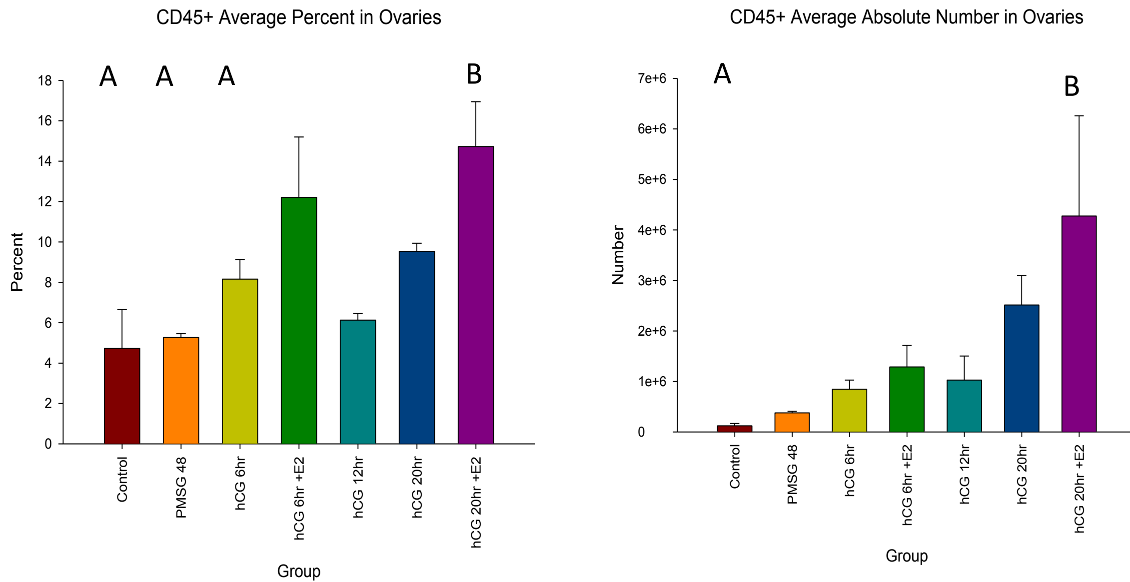

Figure 29. Leukocyte infiltration into ovaries.

Estrogen is causing an increase in leukocyte infiltration compared to the same groups that did not receive estrogen. For percentage, group hCG 20 plus estrogen was significantly different from groups control, PMSG 48hr, and hCG 12hr. The group hCG 20hr plus estrogen was also significantly different from the control group for absolute numbers. Different letters show significant difference. Results represent mean ± SEM of n=3 mice and are representative of 3 experiments ($p<0.05$, ANOVA + Tukey).