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MATERNAL IMMUNITY: PREIMPLANTATION PREPARATION AND SPATIAL DISTRIBUTION OF CCL19

Ashleigh Elbert
Pittsburg State University, aelbert@gus.pittstate.edu

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Ashleigh R. Elbert

Pittsburg State University

Pittsburg, Kansas

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Ashleigh R. Elbert

APPROVED:

Thesis Advisor _____
Dr. Virginia Rider, Biology Department Chair

Committee Member _____
Dr. Phillip Harries, Department of Biology

Committee Member _____
Dr.Tuhina Banerjee Department of Chemistry.

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MATERNAL IMMUNITY: PREIMPLANTATION PREPARATION AND THE ROLE OF CCL19

An Abstract of the thesis by
Ashleigh R. Elbert

The maternal uterus in mammals undergoes extensive remodeling in preparation for implantation of the semi-allogenic embryo. Activation of the T cell homing receptor, CCR7, regulates multiple aspects of adaptive immunity. Genetic deletion of CCR7 reduces T regulatory (T Reg) cell migration into mouse uteri and decreases embryo implantation. CCL19 and CCL21 are the sole ligands for CCR7. It is hypothesized that CCL19 expression could attract T reg cells into the pre-implantation uterus and provide local immune suppression prior to implantation. Sprague Dawley rat uteri were removed from pregnant rats at days 3-6 of pregnancy. To determine the spatial distribution of this ligand, rat uteri were fixed, embedded in paraffin and uterine sections were analyzed by immunochemistry. The influence of sex steroids on the spatial distribution of CCL19 expression was evaluated in uterine sections from rats at each day (3-6) of pregnancy. Upon spatial analysis, at day 3 of pregnancy CCL19 expression was limited to the luminal and glandular epithelium and was otherwise absent from the stroma. Expression appeared in the anti-mesometrial uterine stroma at day 4, 5 and 6 of pregnancy and appeared most robust at day 6. These results suggest that unlike the constitutive expression of this ligand in primary and secondary lymphoid tissue, the spatial distribution and expression of CCL19 is likely regulated by female sex steroids that fluctuate in the uterus during preparation for implantation.

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CHAPTER I

INTRODUCTION

The mammalian uterus undergoes extensive remodeling in preparation for embryo implantation (1,2). It is established that the female sex steroids progesterone and estradiol are required for implantation in mammals (1, 2, 3). It has been suggested that the maternal immune system must be suppressed in target areas of the uterus in order to modulate the immune response to the semi-allogeneic embryo. If this immune modulation does not occur, implantation is negatively impacted, leading to failed pregnancy.

Evidence suggests that the proper regulation of this immune suppression is critical to a successful implantation and that T regulatory (Treg) cells play a crucial role in the modulation of this response (1-21). However, more needs to be known about what regulates these Treg cells in the uterine environment.

The Rider laboratory is currently working on understanding how the modulation of the immune system may be regulated by female sex steroids in the uterus prior to implantation in pregnancy. The laboratory is currently looking specifically at the regulation and spatial distribution of the chemokines CCL19 and CCL21, the sole ligands for C-C chemokine receptor 7 (CCR7), in rat uterine tissue. This thesis specifically

examines the expression and distribution of CCL19 and its role in the recruitment of CCR7. It is expected that this information will lead to a better understanding of CCR7 signaling prior to implantation. This knowledge could eventually aid in the assistance of those with fertility issues.

CCR7 is a T cell homing receptor that regulates multiple aspects of adaptive immunity. Deletion of CCR7 reduces T regulatory (Treg) cell migration into the mouse uterus and decreases embryo implantation. Thus, the focus of the current study is to evaluate the expression of chemokine CCL19 which could attract CCR7 positive Treg cells to the uterus prior to embryo implantation. A second goal of the study, was to correlate CCL19 expression to the changing hormonal environment of the rat uterus as it prepares for implantation. In particular, the role, progesterone and estradiol may have, if any on the expression and spatial distribution of CCL19.

For this thesis project, rats were utilized for their many benefits (size, ease of breeding, similarities amongst animal models). Rats, mice, and pigs are all have discoidal, decidual placenta types, and their hormonal signaling is similar. The rat is a common model despite some developmental and anatomical differences (22,23). In rats, the implantation window is a period of 24 hours between day 5 and 6 of pregnancy thus in looking at preimplantation preparation, we harvested the uterine tissue at Days 3,4,5 and 6 of pregnancy, respectively. During this period the uterus undergoes changes induced by the hormones estrogen and progesterone (1-6). By harvesting uterine tissue on each of these days the goal was to capture a clear image of how the hormonal changes on each of these days impacts the expression of CCL19.

There are several important structures in the rodent uterus that are of note. A cross-section through a rat's uterine horn can be divided into a mesometrial half and an anti-mesometrial half. Implantation occurs on the anti-mesometrial portion of the tissue, while blood and nutrients enter through the mesometrium. A cross-section clearly shows a lumen and multiple glands that carry secretions to the uterus as shown in Figure 1 (A) and (B).

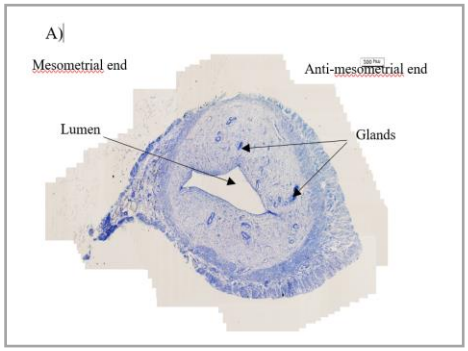

Figure 1: A) Sprauge Dawley rat uterine cross section (6µm thickness) stained with methyl green stain. Gland and luminal structures labeled and mesometrial and antimesometrial orientation shown.

In this study, we used immunohistochemistry to examine the spatial distribution of CCL19 in pregnant Sprague-Dawley rats uterine cross sections. This spatial data will be used assist in the determination of the expression of CCL19 across days 3-6 of pregnancy, and compared with non-pregnant, hormone treated uteri to better understand the hormonal control of CCL19. This thesis explores the idea that in Sprague-Dawley rat uterine tissue, the chemokine CCL19 is modulated via the hormones estrogen and progesterone that fluctuate in preparation for embryo implantation. The uterus undergoes differentiation, in which there are cellular changes in the endometrium in preparation for

pregnancy and implantation. Decidualization transforms the stromal cells and thus changes the signaling that occurs in order to increase endometrial vascular permeability (12). The hypothesis is that expression of chemokine CCL19 will differ in response to the hormonal changes of the uterine environment between days 3-6 of pregnancy in preparation for implantation.

CHAPTER II

REVIEW OF THE LITERATURE

Autoimmune disorders and immune cells

Autoimmune disorders including lupus, cancer, type 1 diabetes, Chrone's disease, HIV and those that impact embryo implantation and placentation among others, can be life altering. These autoimmune disorders share the inability of the immune cells of the body to recognize self from non-self. In these cases, an immune response occurs against the body's own cells leading immune cells to attack the body's own tissues and cause an immune response. These autoimmune attacks are not caused by outside invaders such as a virus or bacteria, but rather the very family of immune cells called T cells and natural killer cells (NK cells) (14) that are meant to defend the body from these infections. Through their capability to recognize and kill virus-infected or transformed target cells and remove them, in autoimmunity, NK cells attack the very body they are trying to defend (14). This becomes a problem when these cells turn this defense system against normally functioning cells within the body and attack the healthy cells instead. In contrast, this same response can be required within the body as is the case with preparation for implantation in early pregnancy. Some inflammatory response is required in order to create a habitable environment for the embryo to implant. However, the response must be modulated in order to prevent rejection of the semi-allogenic embryo.

Current research exploring Treg cells and recruitment mechanisms, is ongoing. One current area of research into their function is the investigation of the specific role of immune molecules known as chemokines and their receptors.

What are Chemokines

Chemokines are a family of signaling molecules that have roles in immune responses, inflammatory processes, and homeostasis (7),and thus, can act as disease modulators (7). Chemokines, also known as cytokine proteins, are a group of small structurally and functionally related proteins. These protein molecules are typically 8–14 kDa in size (24). Due to their small size, Zlotnik and Yoshie (2000) indicated that, they were among the first molecular families to be analyzed fully using genomics and bioinformatics techniques. Chemokine proteins can be found in all vertebrate species, and as of 2011, approximately fifty chemokines have been identified in the human body and the number continues to increase (24).

These molecules play a fundamental role in both homeostasis through chemotaxis, and the function of the immune system in combating disease and inflammatory processes (24). In short, these chemokines have a wide range of effects on a wide array of different cell types. They can vary from those commonly expressed to those that act with great specificity (24). One example of specificity is the family of chemokines that are exclusively expressed in the decidua of the uterus of a pregnant female (25). In the case of chemokines, their function as immune modulators and inflammatory mediators is far more complicated than initially assumed.

One of the most essential functions of chemokines is their homeostatic function via chemotaxis. This is an important role in which they control the movement or migration of cells both during the process of development and throughout normal cellular function. They aid the process of migration by acting as a chemoattractant and leading molecule to their proper target receptors. These functions include aiding in the process of angiogenesis (the formation and maintenance of blood vessels) formation and maturation of lymphoid organs and tissues as well as leukocyte maturation (7).

Chemokine molecules also have roles in a wide variety of inflammatory and immune responses through chemoattraction of innate and adaptive immune cells (7). Here they can assist in getting immune cells to their proper target receptors. Chemokines with a role in inflammatory processes can be induced during an immune response to infection to stop viruses and bacteria at the site of infection. They exert their biological effects by interacting with G protein-linked transmembrane receptors called chemokine receptors, which are found on the surfaces of their target cells. They play a critical part in the defense system of the body through their action with neutrophils, monocytes, lymphocytes, and eosinophils (24). Many autoimmune diseases can be traced back to a deficiency or malfunction of a chemokine or its receptor (26).

How Chemokines are Classified

Despite their extensive and complex impact on many tissues, chemokines can be grouped into one of four families based on their chemical structure. CXC, CC, CX³C, and

XC (7). Placement into these families is based on the arrangement of the N-terminal cystine residues of their chemical structure.

Chemokines can also be grouped by function, based on whether they play a role in homeostasis (homeostatic chemokines) or in the inflammatory process (inflammatory chemokines)(7). Homeostatic chemokines are expressed constitutively in a tissue and are usually involved in cell migration. Inflammatory chemokines are strongly upregulated as an immune or inflammatory response to stimuli (24). However, according to Widdison and Coffey (7) many chemokines can play dual roles and function in both homeostasis and inflammatory processes. The current nomenclature now has the delineation CC or CXC and then L for ligand or R for receptor followed by an assigned number. Interestingly, many chemokine proteins can act on the same receptor or multiple receptors, evident in *Systematic classification of vertebrate chemokines based on conserved synteny and evolutionary history* by Nomiyama et al. (10). Chemokine CCL19 is classified by chemical structure and function into the CC family. They are amongst a unique subset of this family however that are non clustered and can act both in a homostatic and an inflammatory manner (7).

Chemokine Phylogeny

Nomiyama et al. (2013) discussed how genes involved in host defense undergo rapid evolution (10). Their rapid evolution presumably is in response to their need to constantly adapt to keep up with the ever changing needs of the immune system. Their rapid mutation rates, however makes it difficult to trace them back to a common gene

ancestor. More specifically, they undergo duplication which makes phylogenetic coalescence and classification complicated. In some cases, whole genome duplication occurs further complicating the process (10). Yet another complication comes from some of the various organisms that chemokines help defend against, viruses. Data shows that some viruses encode mimics that imitate the function of chemokines and other host immune cells in order to evade detection(6). However, as the field of genomics has grown and more genomes are sequenced, another piece is added to the puzzle. It has become more attainable to get past these complications and sort out the complicated web of proteins that are chemokines. Nomiyama et al.(2013) explored chemokines, looking for orthologous genes(1). Orthologous genes are those descended from a single gene in the last common ancestor between the species (10). They surveyed 18 representative vertebrate species for chemokine genes and identified 553 genes.

Nomiyama et al.(2013) used phylogenetic analysis and conserved synteny analysis to search for for orthologous genes(10). Phylogenetic analysis is widely used to determine orthologs in multiple genomes. (10). It was determined that the first split in vertebrate chemokines was between jawed gnathostomes and jawless agnathans.

However, this method of analysis was insufficient for determining the orthologous relationships of many of the chemokine genes (10). According to Nomiyama et al. (2103): “phylogenetic analysis on its own was insufficient because, phylogenetic trees of vertebrate chemokines exhibit many collapsed or poorly supported nodes(10). This is due in part to the short alignment of chemokine domains used for tree construction, and also to gene duplications followed by gene losses or rapid lineage-specific gene expansions. In addition, most genomes are still incomplete, and the ortholog assignments can be wrong.”

To combat these issues and supplement the data from the phylogenetic analysis they also used conserved synteny analyses. Conserved synteny describes preserved co-localization of genes on chromosomes of different species. During evolution, rearrangements to the genome by such processes as chromosome translocations may separate two loci, resulting in the loss of synteny between them.

Conserved synteny analysis, discovered that the 553 chemokine genes could be traced back to two ancestral species (10). This analysis seems to support the same conclusion as the phylogenetic analysis and supports the hypothesis that all chemokines are derived from the gnathostomes and agnathans (10).

A prominent feature of the chemokine system is its high degree of promiscuity that allows a single receptor to bind several chemokines and a single chemokine to bind several receptors (10). Receptor promiscuity is an evolutionary clue as to why the chemokine system is so robust (10). Among the chemokines; the inflammatory and related plasma/platelet chemokines seem to be the most promiscuous (10). Their genes reside within the two major clusters in the genomes of mammals. In contrast, the chemokine CCL19 and its cohort CCL21 have a low degree of promiscuity and only bind to one receptor CCR7. Conversely, CCR7 only binds CCL19 and CCL21 no other chemokines bind to it.

Receptor CCR7 and its functions.

Receptor CCR7 is the sole receptor for CCL19 and CCL21 and is a requirement for their recruitment (28). This receptor has 8 main functions: Chemotaxis, adhesion

and/or integrin avidity, proliferation, changes in cytoarchitecture, endocytosis, migratory speed modulation, differentiation, survival, and invasive functions (8). Of major interest is its role in directing the migration of dendritic cells in order to facilitate an immune response (29). Myeloid Dendritic cells (DCs) are found in their immature state in the epithelia and interstitial space of organs (29). These DCs are stimulated when they come into contact with chemokines such as CCL19 and use receptors such as CCR7 to travel to target tissues (29). CCR7 in particular has only two ligands that bind to it CCL19 and CCL21. According to Sanchez and Blanco(29) in "The multiple personalities of the chemokine receptor CCR7 in dendritic cells" that stimulation of CCR7 with ligand CCL19 and or its cohort CCL21 positively regulates the rate of endocytosis of mature DCs and increases migratory speed of these DCs (29). Sanchez and Blanco also assert that stimulation of CCR7 can through this process lead to the secretion of inflammatory cytokines (29). In many respects, CCL19 and CCL21 are the same in their actions, however, it has been discovered that CCL19 alone can impact the cytoarchitecture. In T-cells it can desensitize CCR7 to respond to a second stimulus of the chemokine while CCL21 can not(29). Despite their similarities, CCL19 and CCL21 only share 32% amino acid identities and CCL21 contains 30 additional amino acids with two extra cytosines in the carboxyl terminus there by suggesting different signaling capabilities (29). In contrast to the constitutive nature of this receptor/chemokine relationship in lymphoid tissues throughout the body (28), this thesis explores the idea that in uterine tissue in particular, the chemokine is modulated via the hormones estrogen and progesterone that fluctuate in preparation for embryo implantation. It has been demonstrated that an absence of CCR7 resulted in implantation failure due to the lack of proper changes in the

endometrium and improper inflammatory response to prevent rejection of the embryo (30).

Rat Development

For this thesis project rats were chosen for their many benefits. Size, ease of breeding, similarities amongst animal models. Rats, mice, and pigs are all have discodal, decidual placenta types, and their hormonal signaling is similar . The rat is a common model despite some developmental and anatomical differences such as the absence of a gallbladder and liver structure differences (23).

In adult female mammals, the uterus undergoes remodeling in response to estradiol and progesterone. In the rodent, estradiol stimulates the proliferation of luminal and glandular epithelial cells at days 2 and 3 post coitum. At day 4 there is a proliferative switch in the cell division to the stroma in response to increasing progesterone in circulation (19). In rats, the implantation window is a period of 24 hours between Day 5 and 6 of pregnancy thus in looking at preimplantation preparation, we harvested the uterine tissue at Days 3,4,5 and 6 of pregnancy, respectively, in order to observe in the pregnant rat the differences in expression of CCL19 prior to and 1 day after implantation. During this period the uterus undergoes decidualization and remodeling induced by the hormones estrogen and progesterone released.

Implantation and the role of T Regs

As stated above, implantation in Sprague-Dawley rats happens early in pregnancy and the window of implantation is a twentyfour hour period between day 5 and 6 of pregnancy. Embryo implantation is considered the point at which attachment of the semi-allogenic embryo to the uterine wall occurs (31). Proper implantation requires a precise sequence of cell proliferation which in rodent uteri is stimulated by progesterone and estradiol (19). The Rider laboratory has previous investigated the progesterone-dependent proliferative switch (19). During these early preimplantation days there are changes in the stroma localized to the implantation chamber in which its diameter enlarges in comparison to the rest of the lumen and there is a stratification of the luminal epithelium. In order for this process to happen successfully, a delicate balance of hormone and immune responses must occur (19, 30, 31, 32). There must be an inflammatory response that is strong enough to cause the morphological changes required of the uterine wall in order to be a habitable environment for the impending embryo. At the same time the inflammatory response and the Treg cells responsible must be modulated in order to accept the semi-allogenic embryo which due to foreign DNA from the male sperm causes a risk of rejection. Rejection of the embryo during the period of implantation is amongst the leading causes of spontaneous miscarriage in pregnancy. The balance between the pro and anti-inflammatory actions of the uterus as it prepares for the impending embryo is a key component (30). According to Sposito and Santos (31), during this window, the endometrium undergoes morphological changes in response to estrogen and progesterone that help facilitate apposition attachment and embryo invasion and includes changes in the luminal epithelium (31). At the same time, the recruitment of dendritic cells and

Tregs among other molecules in response to these hormones is crucial and these changes are mediated by a host of molecules including CCR7 and its ligands CCL19 and CCL21 (8, 11, 26, 33). This thesis explores one molecule in particular, CCL19. It is speculated that expression of CCL19 by the uterus is modulated by the fluctuation of the estrogen and progesterone released during this process in order to recruit CCR7 and by extension Tregs that aid in the modulation of the inflammatory processes mentioned above.

It is speculated that CD4+ T regs such as those recruited by CCR7 play a large role in the tolerance of the embryo both during and after implantation (30). They do this by regulating the immune response of the maternal uterus and preventing what is often referred to as a hostile womb (30). It is also speculated that Tregs help to counteract or modulate the pro-inflammatory actions that occur in preparation for impending implantation. It has been observed that in response to hormones such as estrogen and progesterone, mediators such as CCL19 was strongly upregulated and that homing of Tregs to the uterus is dependent on CCR7 and its ligands CCL19 and CCL21 that are present in the stromal cell line and recruit and direct the migration of dendritic cells and Tregs (30).

The Role of Hyaluronan

Hyaluronan, also known as hyaluronic acid (HA) is an extracellular matrix(ECM) glycosaminoglycan (GAG) present in the stroma of pregnant and pseudo-pregnant mouse uteri prior to decidualization(21, 22). Decidualization is accompanied by a clearance of HA and then a five to six-fold increase occurs at the time of implantation (21, 22). It is thought that as part of its hydrophilic nature, HA can contribute to an expansion of the

ECM and allow penetration of migrating cells (21,22). Due to this expansion of the ECM, HA also has a functional role in modulation of trophoblast migration and invasion by providing a HA rich ECM which causes the expansion and allows space for the invasion and migration to occur (21,22).

CHAPTER III

MATERIALS AND METHODS

Tissue acquisition and fixation

Pregnant and hormone treated Sprague-Dawley rats from Charles River Laboratory were used to provide uterine tissue for examination. For the hormone treatment, the rats were treated via the following protocol: Sexually mature (150-170 g body weight) Sprague-Dawley rats were bilaterally ovariectomized and rested for 10 days. The rats were housed on a 14 h light:10 h darkness cycle at the Pittsburg State University and provided rodent chow and water *ad libitum*. Animals were treated in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals. The Pittsburg State University Animal Care Committee approved protocols for the care and the use of rats. 3 OVX rats were injected subcutaneously (s.c.) only with sesame oil to serve as a control group (OVX), 3 rats were injected with progesterone (2 mg; Sigma-Aldrich) dissolved in sesame oil for three consecutive days (OHE). On the fourth day, 3 more rats were treated with progesterone a in the same manner as the OHE test group then given a single injection of estradiol 17-β (0.2 µg; Sigma-Aldrich) s.c. This hormone regimen increases the number of synchronously proliferating stromal cells and is meant to replicate the hormonal

environment of pregnant animals (1). Uterine horns were removed at 6 h post estradiol injections when a significant number of stromal cells are in S phase of the cell cycle (3).

For the pregnant tissue trials, 12 rats were obtained from Charles Rivers Laboratory having been shipped overnight after sperm was confirmed in a vaginal smear which for purposes of the experiment is considered day 1 of pregnancy and arrival on site at day 2 of pregnancy. The rats were also housed on a 14h light:10 h darkness cycle at Pittsburg State University upon arrival and were provided rodent chow and water *ad libitum*. Animals were treated in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals. The Pittsburg State University Animal Care Committee approved protocols for the care and the use of rats. Beginning on Day 3 of suspected pregnancy, three rats per day through day 6 had both Uterine horns surgically removed. For each rat, one uterine horn was frozen in liquid nitrogen and stored at -80ºC for use preservation and later use, while the other was fixed in 4% paraformaldehyde and paraffin embedded.

The fixation process started with obtaining 1000 ml beaker of fresh paraffin and heating it to 60ºC in a warming oven. Once tissue was harvested, it was placed in 4% paraformaldehyde on a nutator at 4ºC for one hour. Next the tissue was washed three times for five minutes each in 1X PBS at 4ºC. Next the tissue was washed two times for thirty minutes each in 70% ethanol at 4ºC. Next the tissue was washed two times for forty minutes each in 95% ethanol at 4ºC. Next the tissue was washed two times for forty minutes each in 100% ethanol at 4ºC. Next the tissue was washed two times for 30 minutes each in xylene at room temperature. The tissue was then placed in the 60ºC pre-warmed paraffin

for 30 minutes. The tissue was then transferred to fresh paraffin pre-warmed to 60ºC and left in warmer overnight. The tissue was then transferred to a fresh dish of paraffin and cooled to room temperature. The tissue was then labeled and stored at 4ºC for use in sectioning blocks.

Sectioning and Immunohistochemistry

Per protocol in the Rider laboratory, paraffin embedded uterine tissue was sectioned at 6 μm, placed into a water bath at 52°C and four sections were attached to each ++ charged super frost excel microscope slide (Fisher brand™). The slides were then placed onto a warming tray for twelve to twenty four hours then removed and either placed into a -20°C freezer or processed using the immunohistochemistry protocol within twenty four hours if kept at room temperature.

Once tissue slides were prepped as described above, deparaffinization and rehydration of the mounted tissue slides was performed by placing the slides in 3 washes of xylenes for five minutes each followed by a five-minute wash in a 50/50 solution of xylene and ethanol. This is followed by a gradient of ethanol washes: two five minute washes in 100% ethanol, followed by a five minute wash in 95%, 70%, and 50% ethanol each respectively. The slides were then washed twice in distilled water for five minutes each. The tissue sections then underwent antigen unmasking by being placed for ten minutes in sodium citrate prewarmed to 96°C in a water bath. Slides were then allowed to cool at room temperature on the bench for thirty minutes. Once cooled, endogenous

peroxidase activity was blocked by incubation in 3% hydrogen peroxide in distilled water for twenty minutes. The slides were then washed three times for five minutes each in a 1X phosphate buffered saline solution (1X PBS). The tissue sections were then rinsed with distilled water and circled with a PAP pen (wax) and rinsed in 1X PBS for five minutes after which they were then placed into a humid chamber and the tissue was blocked using 100 µl -400 µl of a 5% goat serum (Vector) solution for twenty minutes. The goat serum was then removed, and sections were blocked with 100 µl-400 µl of a 5% CCL19 primary antibody solution. The sections on the control slides were blocked with the stock solution of 5 mls of 1X PBS with 75 µl of goat serum and incubated for twelve to twenty four hours at 4°C.

Following incubation for a minimum of twelve hours, the primary antibody and/or control solution was removed and the sections were then washed three times for five minutes each in 1X PBS before being incubated in 100 µl-400 µl of a goat, anti-rabbit secondary antibody solution that was diluted to 25% in goat serum before being diluted to 1% in 1X PBS. The slides were then incubated for one hour at room temperature. Thirty minutes into the incubation process, Avidin-Biotin Complex (Vector, VectaStain ABC Kit) (ABC) reagent was mixed in 1X PBS according to the manufacturer's instructions and was placed on a nutator for thirty minutes at room temperature. After the one hour incubation period, the secondary antibody was removed and the slides were rinsed twice for five minutes each in 1X PBS and the slides were then incubated in 100 µl-400 µl of ABC reagent at room temperature for one hour. The ABC reagent was then removed, and the sections were washed twice for five minutes each in 1X PBS. During the second 1X PBS wash Vector ImmPACT DAB-Peroxidase stain was prepared (100 µl – 400 µl). The stain

was applied to the sections for two minutes and then the slides were immediately placed into distilled water. Sections were then counterstained with 0.5% histological grade methyl green that had been warmed in a water bath to 45°C for five minutes. The sections were then washed twice for 5 minutes each in distilled water. The sections were then dehydrated through a gradient of ethanol and xylene dips then allowed to airdry. Fisher Permount was applied to mount a coverslip.

Processing and Analysis

Once slides were processed via the immunohistochemistry protocol and mounted and dried completely, they were imaged and analyzed in order to evaluate presence and spatial distribution of the CCL19 antibody via presence of positive DAB staining which appears brown against the blue appearance of the background/secondary stain. For evaluation of each treatment group (Day 3-6 of pregnancy), representative sections were selected from 5 separate replicate slides (with 4 sections per slide to choose from) for each day of pregnancy and examined and compared. Representative sections were chosen based on quality of the section. Images of representative sections were taken utilizing a BX41 olympus microscope at 10 X magnification and processed using cellSans imaging software which is linked to the microscope and takes stacked images of what is tracked in the field of view and allowing it to be saved as a jpg image.

CHAPTER IV

RESULTS

Chemokine CCL19 has previously been shown to be present in the uterine stromal cell line, and CCL19 expression was detected in the uteri of Sprauge-Dawley rats at all days of pregnancy examined (Day 3-6) when compared to control sections as seen in figure 2 and expression seems to intensify and spread into the stroma and cells secrete the antibody (see figure 3). At Day 3 of pregnancy, chemokine CCL19 is present but positive cells appear to be restricted to the glandular and luminal epithelium. Close examination shows brown stained DAB positive cells in the glands and epithelium of sections from Rat 1, Rat 2, and Rat 3 as seen in figures 4A, 5A, and 6A. However, no positive cells appear in the stroma. This can be compared to the negative control slide which were not exposed to the CCL19 antibody shown in figure 2 in which no brown DAB positive cells can be seen, instead the only stain present is the counter stain methyl green which manifests as a blue stain on the cells. This result suggests that at day 3 of pregnancy prior to the proliferative switch and increased levels of progesterone and estradiol, the expression of CCL19 is limited to the epithelial tissue and expression of the ligand is present but low as inflammatory processes are not yet occurring. At Day 4, the expression of CCL19 appears to increase and extend a small amount into the stroma near the site of implantation around the lumen in the anti-mesometrial region of the section.

DAB positive cells can be noted both in the luminal and glandular epithelium as well as in the stroma of the anti-mesometrial region. Cells in the mesometrial region of the section remain negative as seen in figures 4B, 5B, and 6B. At Day 5 of pregnancy, as the uterus undergoes decidualization the trend of expansion into the stroma continues and the expression of positive cells in the stroma appears more intense and expands further into the stroma as seen in figures 4C, 5C, and 6C. It was also noted that at Day 5 we were able to capture a close-up image of the ligand being excreted from the luminal epithelium as seen in figure 3. It can be speculated that this increased expression is in response to the increased progesterone and estradiol present as the increased cell proliferation and endometrial changes occur in preparation for implantation at the end of Day 5. Examination of sections at Day 6 of pregnancy, we see the expansion and intensification of stromal expression increase even more into the anti-mesometrial region as seen in figures 5D, and 6D. Close examination shows that in Days 5 and 6 in particular, it appears that not only are the nuclei of the cells appearing to demonstrate a positive stain, but it appears that the cells are secreting into the extra cellular matrix (ECM) as you can see positive DAB reaction around the cells in the matrix despite quite extensive blocking. As a confirmation, a round of sections in tissue from hormone treated rats in which non-pregnant rats were ovariectomized and rested for ten days as described above. Three were given a s.c. injection sesame oil for three days and thus received no hormone treatment(OVX) then uterine horns were removes. Three more OVX rats were treated with 2 mg of progesterone for three days (0 hE) and the uterine horns were removed, and three more rats were treated with 2 mg of progesterone for three days as well as given a single s.c injection of 0.2 ug of estradiol (6 hE) six hours before the uterine horns were

removed. One round of these sections was analyzed to demonstrate that the same pattern occurs in response to a similar hormonal environment in non-pregnant rats exposed to increase levels of estradiol and progesterone as described above. These comparisons also showed a pattern of increased expression in the rat uteri. Those treated without hormones are shown to have positive cells in the glandular and luminal epithelium as seen in figure 7A although there is some stromal expression which does differ from the results at Day 3 in a pregnant rat. Despite this difference the increased expression levels in those treated with progesterone and those treated with progesterone and estradiol tends to fall in line with what we see in day 4 and day 6 of pregnancy respectively. as seen in figure 7B and 7C.

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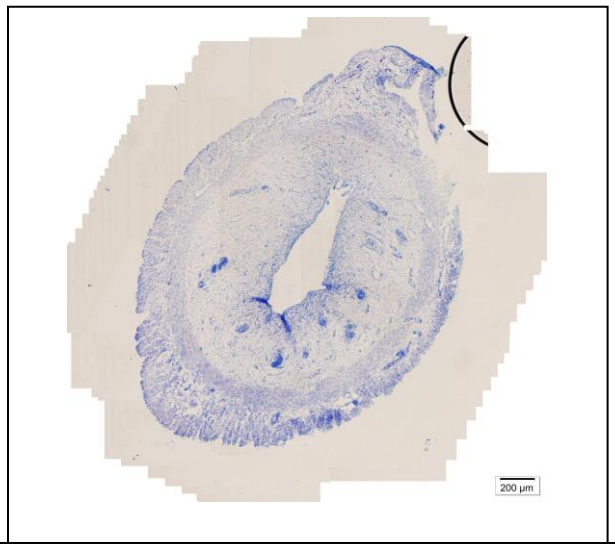

***Figure 2**: Representative Control Slide. This slide represents sections that have no positive DAB reaction. Each round of immunohistochemistry performed had control slides that were not exposed to the primary antibody thus should demonstrate no positive brown staining.*

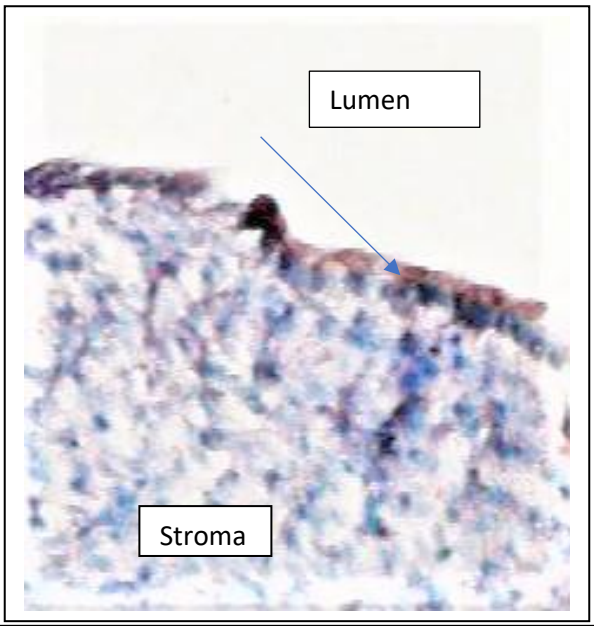

***Figure 3**: Up-close image of the luminal epithelium excreteing CCL19 into the Extra Cellular Matrix and Lumen.*

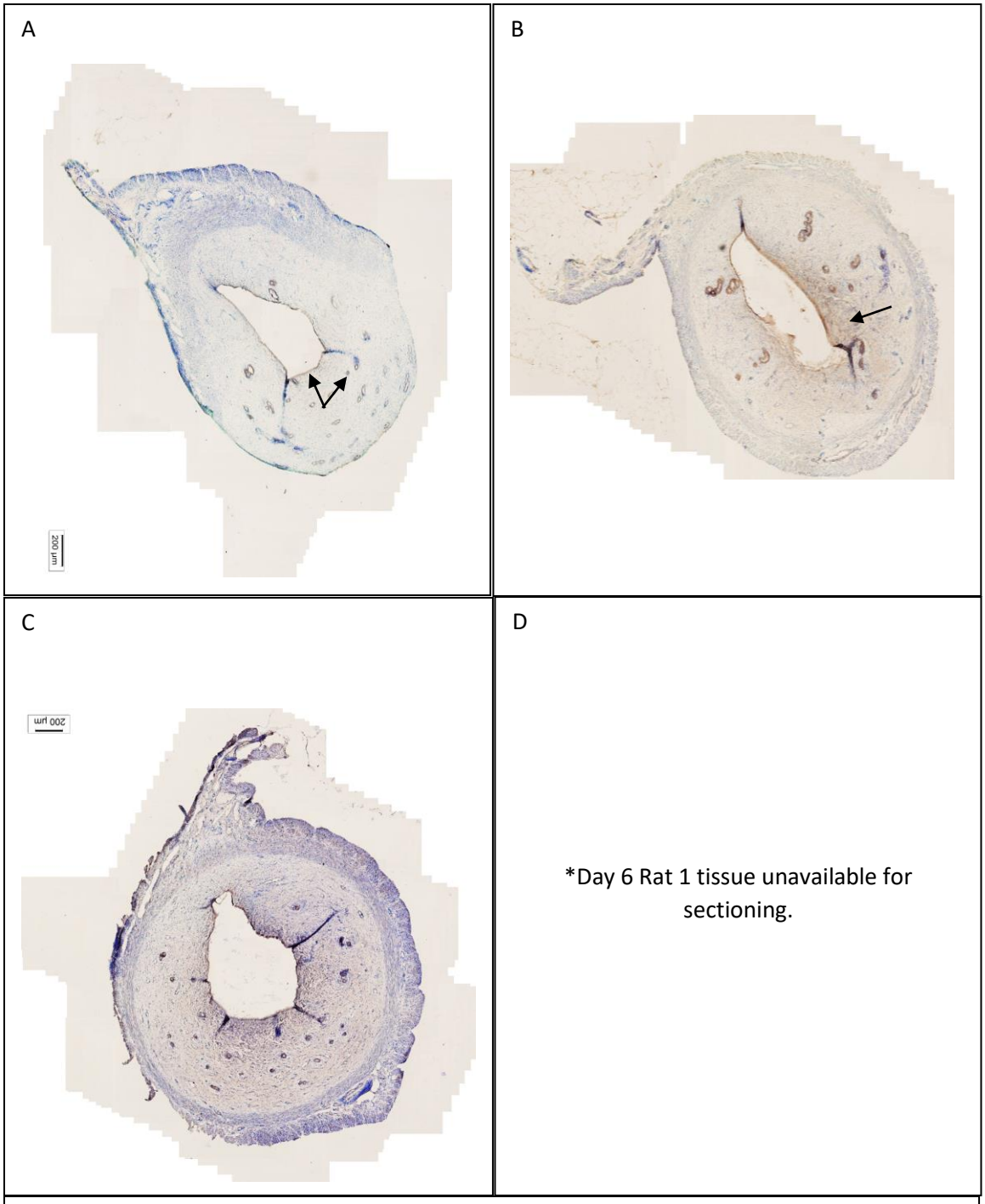

Figure 4: *Animal 1 A) Day 3of pregnancy B) Day 4 of pregnancy C) Day 5 of pregnancy D) Day 6 of pregnancy*

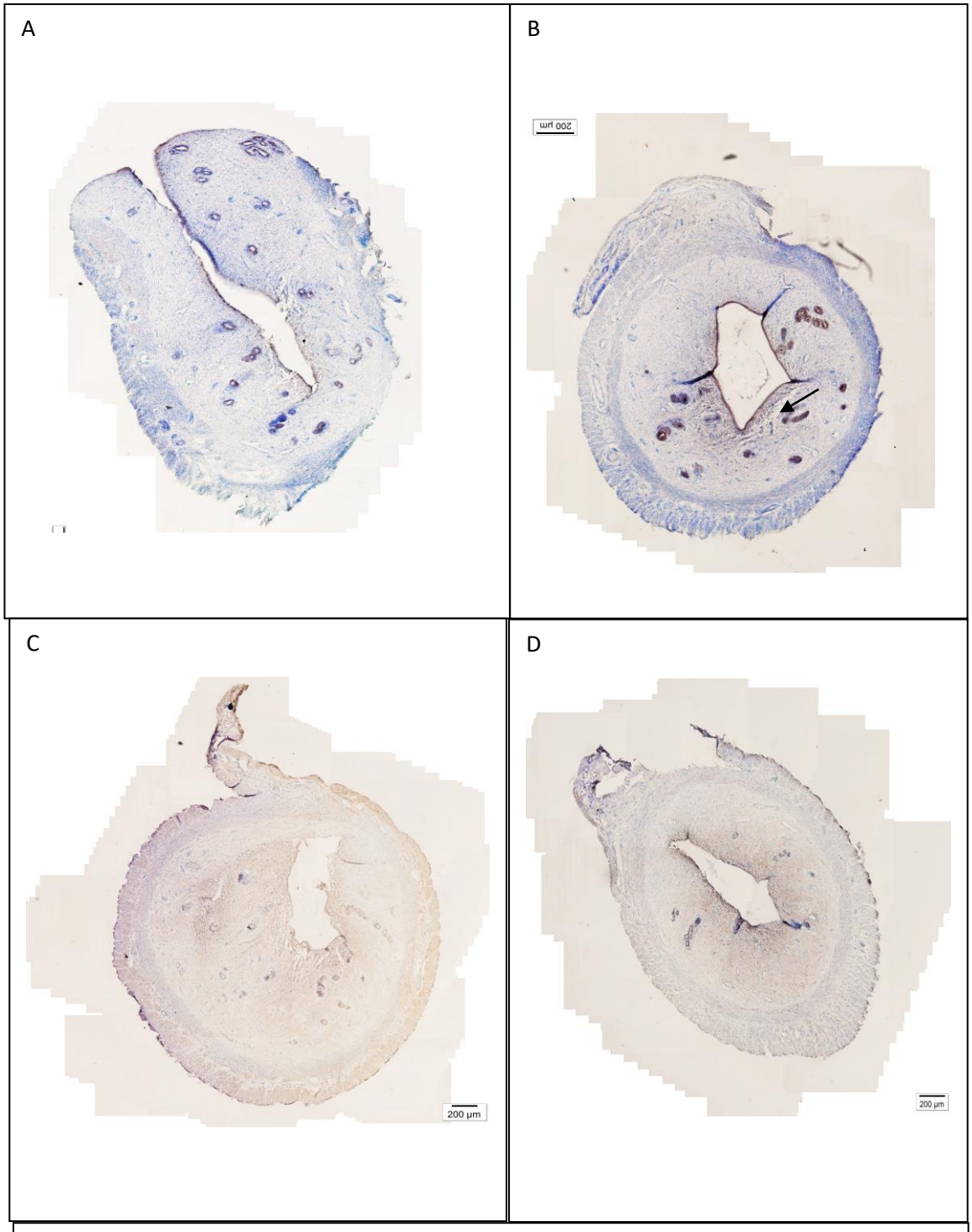

Figure 5: *Animal 2 representative slides A) Day 3 of pregnancy B) Day 4 of pregnancy C) Day 5 of pregnancy D) Day 6 of pregnancy*

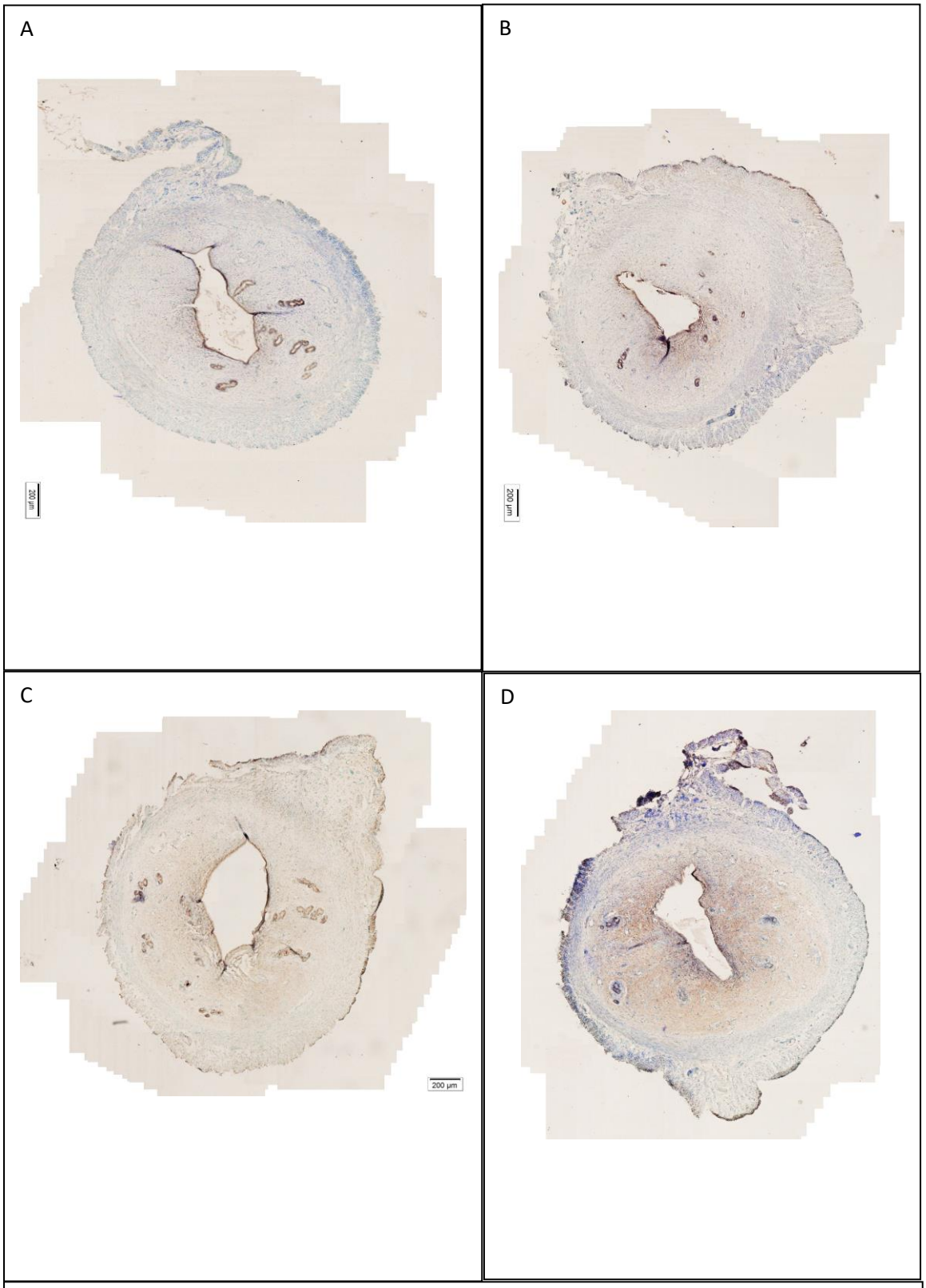

Figure 6 *: Animal 3 representative slides A) Day 3 of pregnancy B) Day 4 of pregnancy C) Day 5 of pregnancy D) Day 6 of pregnancy*

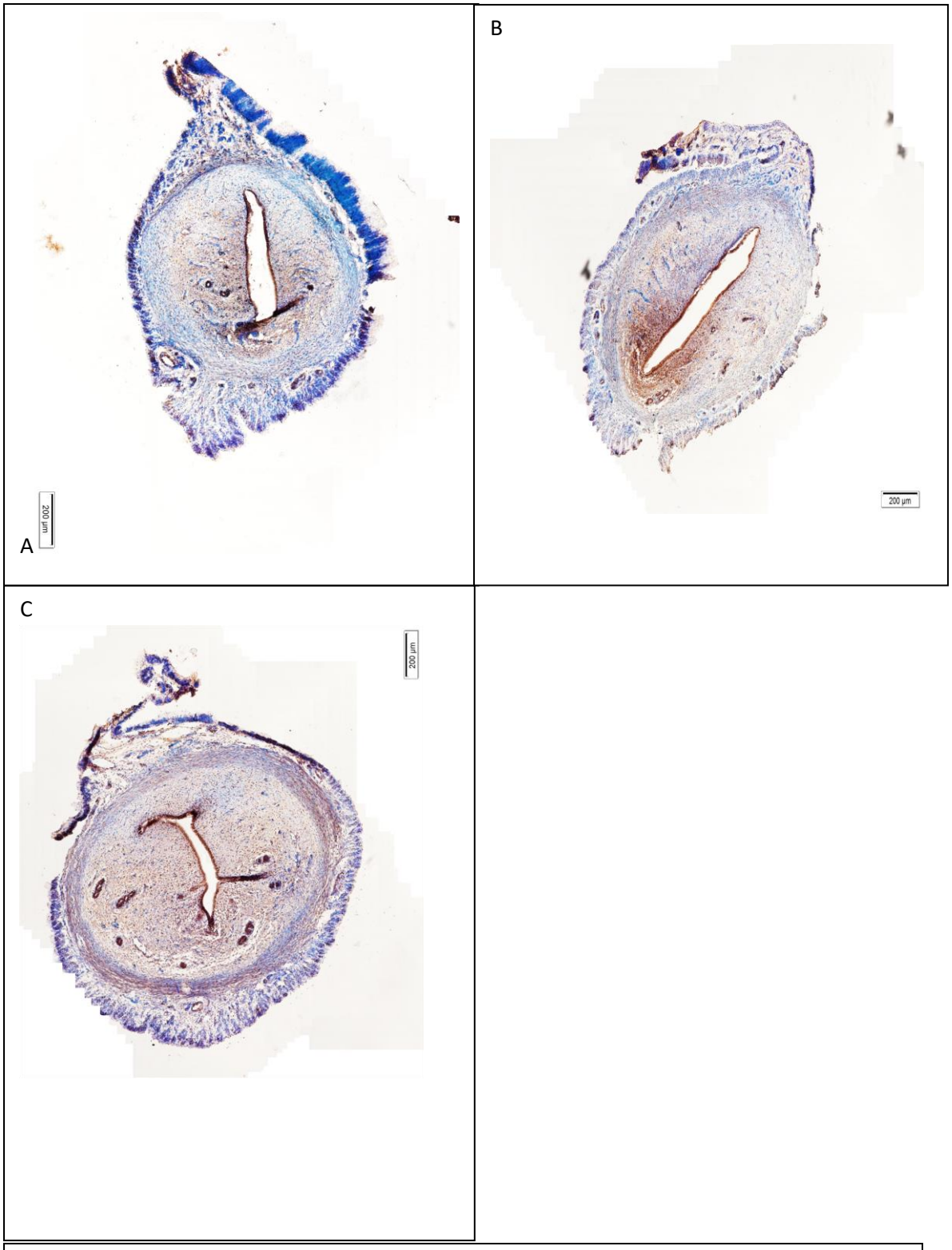

Figure 7: *A) OVX overectomized rats (no hormone treatment. B) OHE progesterone treated rats per protocol C) 6HE progesterone+ estradiol treated rats per protocol*

CHAPTER V

SUMMARY AND CONCLUSION

The chemokine CCL19 is crucial in recruiting CCR7 and by extension Treg cells. This is essential in the modulation of the inflammatory processes that are critical within the body. Modulation of the maternal immune system in particular is very crucial in ensuring a positive pregnancy outcome (36). According to Sisti et al.(36) "acceptance of the developing fetus by the maternal immune system is defined as tolerance" and that "both male and female immune-mediated events are necessary for a successful pregnancy outcome"(36). In the process of exploring expression of CCL19, this thesis is demonstrating one way in which the maternal uterus achieves this tolerance and there by a successful pregnancy.

Throughout most of the body, CCL19 is constitutively released and is a major player in the immune response of the body to invaders such as cancer and HIV (12, 35) through recruitment of CCR7 which attracts Treg cells and dendritic cells. This constituative release appears not to be the case in the maternal uterus. It was discussed by Nancy et al. (37) that "Treg cells are unable accumulate in the decidua"(37) thus they must somehow be recruited. Nancy et al. further demonstrated that chemokine gene silencing impacted feto-maternal tolerance at both ends of the spectrum, too much expression of the Treg cells caused rejection, too little expression made the decidua

susceptible to infections and immune attack. The findings in this thesis demonstrate that CCL19 appears to be hormonally regulated within uterine tissue and their expression is modulated in order to aid in the maintenance of this delicate balance of Tregs through specific localized expression which in turn will selectively recruit CCR7 in the anti-mesometrial region of the uterus. Thus, proper expression of CCL19 and its receptor CCR7 is critical for implantation success and by extension successful pregnancy and according to Hauser et al. " asymmetric architectural organization and polarized distribution of signaling molecules is necessary for directional cell migration" and one of the most important chemokine receptors in this process is CCR7 and its chemokines CCL19 and CCL21(35).

It has been demonstrated in previous research that the lack of CCR7 leads to implantation failure which is a leading cause of spontaneous miscarriage (16, 31, 32,33). Through the experiments reported in this thesis, it can be asserted that unlike the constitutive nature of CCL19 expression throughout the body and its role in inflammatory processes of the immune system at large, the chemokine in question CCL19 plays a different role in uterine tissue. It has been demonstrated through spatial analysis of antibody staining that CCL19 expression is modulated by the hormone environment of the maternal uterus as it prepares for impending implantation. Expression increases in localized manner in response to the increased levels of estradiol and progesterone that occur during pre-implantation preparation as the proliferative switch initiates decidualization (1-6).

This modulated expression may help to enhance and at the same time restrict the inflammatory processes required to prevent a hostile uterine environment and prevent

fetal-maternal rejection. It has also been demonstrated through the examination of non-pregnant hormone treatment of ovariectomized sprauge-dawley rats that this response is hormone regulated and not influenced by the presence of or in response to the embryo itself. Thus we can conclude that increased levels of estradiol and progesterone lead to increased expression of chemokine CCL19 and it can be speculated that based on previous research this would lead to increased recruitment of CCR7 in areas of expression and lead to a targeted migration of Tregs and dendritic cells where expression of CCL19 is increased and an absence when expression is absent. Dendritic cells have two duties, to be aware of pathogens and aid Tregs in stoping them, also have to prevent attack on innocuous antigens and harm of healthy tissue(38). Localized expression of CCL19 is speculated to be crucial in this process.

These findings are a crucial part of the puzzle going forward in gaining knowledge of implantation preparation of the maternal uterus as the semi-allogenic embryo attaches. As stated above, research such as that by Heitmen et al. (16) has explored CCR7 and found T reg cell depletion impairs implantation. This research is a step further in understanding the mechanisms behind that implantation failure.

Further research into CCL21 and its role is on-going. This along with further exploration of both chemokines in hormone treated tissue will further support the theory that both chemokine CCL19 and by extension CCR7 in the maternal uterus are hormonally regulated.

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