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Immune Cell Profile in the Epididymis of Leptin and Leptin Receptor Knockout Mice

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Immune Cell Profile in the Epididymis of Leptin and Leptin-Receptor Knockout Mice

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The signatories of the committee members below indicate that they have read and approved the thesis of Hilary Skalski in partial fulfillment of the requirements for the degree of Master of Health Science in Biomedical Sciences.

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Dedication

I would like to dedicate this research to my parents, Jean and Felix Skalski, and my sister Lizzy. I could not have gotten through these last three years without your love and support. Thank you, mom and dad, for being examples of where an education and hard work can take you. Lizzy, thank you for helping me laugh through even the hardest of days. I thank all three of you for being my rock and always believing in me. I love you to the moon and back!

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Abstract

Genetically-modified mice lacking the leptin hormone (Ob) or leptin receptor (Db) have reduced testosterone and decreased sperm production leading to infertility. Leptin receptors are also expressed by immune cells, suggesting leptin can regulate immune responses. Recent studies have focused on identifying the immune cells present within the epididymis and found cells expressing markers typically associated with dendritic and macrophage cells. These cells likely function against infection and to induce tolerance to sperm. The effects of leptin on the immune system and the presence of immune cells within the epididymis raises the possibility that alterations in the immune cell profile and/or function may contribute to the infertility observed in leptin-altered mice. The purpose of this study was to investigate potential differences in the epididymal immune cell profile in normal, Ob, and Db mice. The epididymis was collected from C57, Ob and Db mice at 16 weeks of age (n=6 per group) and divided into three regions (IS/caput, corpus, and cauda). Each region was processed for incubation with antibodies to innate immune cell markers (CD11b, CD11c, F4/80) or adaptive immune cell markers (CD4, CD8, CD19) and counted by flow cytometry. The percentage of CD8+ cells was significantly lower in the cauda of Ob and Db mice compared to the C57 mice. In all groups of mice, the percentage of CD11b+ cells was highest in the IS/caput and significantly lower in the cauda. Within each region, the percentage of CD11b+ cells was similar between all mouse models. The largest population of CD11b+ cells was also CD11c+ and F4/80+, though no difference was seen between epididymal regions or genetic backgrounds. The percentage of CD11b+ cells that were CD11c+ and F4/80- was significantly lower in the cauda of Ob and Db

mice compared to C57. The percentage of CD11b+ cells that were CD11c- and F4/80+ was significantly lower in the corpus of Ob and Db mice compared to C57. These results suggest that leptin may alter subpopulations of innate immune cells within specific epididymal regions, but the overall profile of innate immune cells, and therefore the response of the innate immune system, is likely unaffected.

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Abbreviations

ARC = arcuate nucleus

C57 = wild-type mouse model

CD4 = unique immune cell marker found on Helper T cells

CD8 = unique immune cell marker found on Cytotoxic T cells

CD11b = immune cell marker found on cells on myeloid-lineage

CD11c = unique immune cell marker found on dendritic cells

CD19 = unique immune cell marker found on B lymphocytes

CD45 = immune cell marker found on all immune cells

CD64 = unique immune cell marker found on macrophages

CD206 = unique immune cell marker found on macrophage and dendritic cells

DC = dendritic cells

Db = leptin-receptor deficient mouse model

FSH = follicle stimulating hormone

F4/80 = unique immune cell marker found on macrophage cells

GnRH = gonadotropin-releasing hormone

HPG = Hypothalamic-Pituitary-Gonadal axis

LEPR = leptin-receptor

LH = luteinizing hormone

MP = mononuclear phagocytes

Mφ = macrophage cells

NK = natural killer cells

NPY = neuropeptide Y

Ob = leptin-deficient mouse model

Tc = Cytotoxic T cells

Th = Helper T cells

Treg = Regulatory T cells

Chapter 1 Introduction

Introduction

Males contribute up to 60% of infertility seen in couples (Katib, 2015). Male obesity might contribute to this infertility, as an increase in BMI over 25 kg/m^2 is correlated with up to a 17% increase in infertility when compared to a BMI <25 kg/m^2 (Sallmén *et al.*, 2006). The hormone leptin is produced mostly in adipose tissue and its function is satiety. The mutation of leptin receptor's long form (Ob-Rb) is known for its connection with severe obesity in both human and mouse models (reviewed in Wasim *et al.*, 2016). This is because circulating leptin reaches the hypothalamus and binds with the Ob-Rb receptor to reduce appetite and help reduce food intake as well as increase energy consumption and metabolism (Friedman, 2002; reviewed in Williams *et al.*, 2009).

Leptin not only affects metabolism, but also the reproductive tract by interacting with the hypothalamic-pituitary-gonadal (HPG) axis. In leptin-altered obese mouse models, Ob mice lack leptin and Db mice lack the long form of the leptin-receptor (Fantuzzi and Faggioni, 2000). Leptin-altered mouse models show a decrease in reproductive hormones and exhibit subfertility/infertility (reviewed in Teerds *et al.*, 2011; Martins *et al.*, 2017).

The testis is the major focus of research on the effects of leptin for infertility, while the post-testis system, including the epididymis, is underexplored. Research on the epididymis is important though, as work in the Pearl lab found a decrease in the number of epididymal sperm in Ob and Db mice when compared to the control mice, indicating a loss of sperm (Pearl and Doherty, 2018). In addition to decreased sperm

counts, the Pearl lab found histological evidence of inflammation in the initial segment of the epididymis.

Not only is there a relationship between leptin and the reproductive tract, but also the immune system. Several immune cells have leptin-receptors including monocytes, neutrophils, NK cells, DC cells and T and B lymphocytes (reviewed in Carlton *et al.*, 2012). Altering leptin signaling can therefore impact the function of various immune cells (reviewed in Scotece *et al.*, 2014). While, the impact of altered leptin signaling has been studied, the immune cell population in the epididymis of Ob and Db mice have not been investigated.

Purpose

The purpose of this study was to determine the effects of altered leptin signaling on the epididymis, specifically its immune cell profile.

Scope

Altered leptin signaling causes changes to the male reproductive tract. We will reconfirm that there are relevant reproductive parameter changes to the male Ob and Db mice compared to the C57 wild type, as previously observed. This includes measurements of body, epididymal fat pad, testis and epididymal weights as well as epididymal sperm counts and testis to epididymal distance. To prove that leptin can have direct effects on the epididymis, immunohistochemistry (IHC) of 16-week C57 control mice will then be used to determine if cells in caput, corpus and cauda of the epididymis express the leptin receptor. In regards to the immune cell profile, there is

some evidence of inflammation in previous histological sections containing the initial segment of the epididymis in the leptin-altered mouse models. Therefore, we plan to investigate the types and distribution of immune cells in the epididymis between the C57, Ob, and Db mouse models.

Assumptions

1. Leptin signaling acts directly within the reproductive tract to affect infertility.
2. Altered leptin signaling can lead to changes in immune cell populations within a tissue.

Hypothesis

Leptin and leptin-receptor deficient mice have altered epididymal immune cell profiles.

Significance

Males contribute to a significant amount of infertility seen in couple. In males, obesity incrementally increases the likelihood of infertility. Therefore, it is important to investigate the relationship between obesity and decreased fertility. Ob and Db mice which lack functional leptin signaling, show an increase in body weight as well as decreased reproductive parameters. In order to better understand the relationship between leptin-induced obesity and infertility, these two mouse models have been used. Immune cells also have receptors for leptin and as a result, concentrations are altered in the male reproductive tract of Ob and Db mice. This paper, to our knowledge, is the

first to investigate the effects of altered leptin-signaling on the immune cell profile in the murine epididymis.

Chapter 2 Review of Literature

Obesity in Regards to Reproductive Health

According to the CDC, from 2015 to 2016 the US national obesity rate for adult males was 38% (Hales, 2017). This is a significant increase from the estimated 11.7% in 1991 or even the 17.9% in 1998 (Katib, 2015). Due to this rapid increase, several authors suggest that obesity is a growing epidemic (Katib, 2015; El Salam, 2018). In 2015 to 2016 the males of usual reproductive age, 20 to 39, specifically had an obesity rate of 34.8% (Hales, 2017). This number is concerning as in one study, percentage of infertility increased 5% once an overweight BMI was obtained (BMI of >25 kg/m^2) as compared to just under the overweight category. This percentage of infertility increased each BMI interval and was the highest at a 17% increase in infertility in the highest BMI category of 35+ kg/m^2 (Sallmén *et al.*, 2006).

Infertility is defined as the absence of conception by a couple after one year of regular unprotected intercourse (Zegers-Hochschild *et al.*, 2009). A common misconception is to link infertility only with females. On the contrary, up to 30% of infertility cases are due solely to the male partner. An additional 30% of infertility cases have both the male partner and the female partner contributing to the infertility (Katib, 2015). This leads to the question of how much the male infertility rate of 9% is, and will be, affected by the growing rate of obesity (Chandra and Stephen, 2013).

Anatomy and Physiology of the Male Reproductive System

There are several structures that make up the male reproductive system. First, there are the two testes in the external scrotal sac of most mammals. The testes are

composed of up to 900 seminiferous tubules, each averaging more than a half-meter in length. These seminiferous tubules are grouped together into about 250 lobules. The avascular seminiferous tubules contain Sertoli cells whose basal surface is attached to the basement membrane and developing germ cells. The Sertoli cell cytoplasm envelopes and supports germ cells throughout spermatogenesis. Spermatogenesis is the process where the circular spermatogonia transform into primary and secondary spermatocytes and then to spermatozoa over approximately 35 days in mice and 64 days in humans (Kerr *et al.*, 2006). As sperm undergo this process, they migrate from the basement membrane towards the lumen of the tubule (Smith and Walker, 2015). Each testis can form up to 60 million sperm each day (Hall, 2016). Sperm are composed of several parts, including the head, the acrosome cap, the midpiece with mitochondria and a centriole, and the flagellum tail (McKinley *et al.*, 2019).

Tight junctions between adjacent Sertoli cells form a blood-testis barrier. In a healthy testis, this blood-testis barrier protects the sperm in the tubules from the adjacent interstitial space's immune cells, which might consider the sperm a foreign substance due to their unique protein composition (McKinley *et al.*, 2019). The interstitial space surrounding the seminiferous tubules contain blood vessels, lymphatic vessels, nerves and Leydig cells. These interstitial Leydig cells produce and secrete testosterone, which is a primary steroid hormone of the testis and is fundamental for maintaining spermatogenesis (Smith and Walker, 2015).

Once released, the sperm travel down their seminiferous tubule with fluid from Sertoli cells, collect at the rete testis and then to the efferent ducts (Cooper and Jackson, 1972; Hall, 2016; McKinley *et al.*, 2019). The efferent ducts originate from the

mesonephric tubules, as compared to the mesonephric duct, where most of the male reproductive system develops (Linder, 1971). This series of efferent ducts, covered by connective tissue and fat, provides a connection from the testis to the epididymis. The wall of the ducts is composed of a thin layer of smooth muscle and connective tissue supporting a columnar epithelium. This, along with an increase in peritubular capillaries, helps mark the transition from the rete testis, as that was composed of cuboidal cells (Robaire and Hermo, 1988). There are three sections of the efferent ducts including the proximal zone, the conus region and the terminus. The proximal region connects the ducts to the rete testis. Next, is the conus region, which is larger in diameter followed by the narrow terminus region connecting the efferent ducts to the initial segment of the epididymis (Ilio and Hess, 1994).

The number of efferent ducts at the testis' end range from 2 to 33 depending on species. In humans, they vary from 6 to 15 (Jonté and Holstein, 1987), whereas mice range from 3 to 5 (Barack, 1968). The number of ducts that transition to the epididymis also varies with species. For example, the ducts in mice usually join to form one common duct that morphs into the epididymis' initial segment (Cooper and Jackson, 1972). On the other hand, humans have several ducts that enter the epididymis separately (Macmillan, 1953).

Along with the varied converging patterns of epididymal ducts, there is a possibility of mistaking blind-ending tubules for stretched and broken efferent ducts. Blind-ending tubules have been seen in human males as well as other mammals. One study on rats, showed that 60% of the efferent ducts dissected contained blind-ending tubules (Guttroff *et al.*, 1992). These tubules are connected to the lumen of either the

epididymis or extratesticular rete testis, but not connected to the lumen of the reproductive tract on the other end. The end that is not connected to the lumen is sealed off at a blunt end (Hemeida *et al.*, 1978). The appearance of blind-ending tubules vary from normal efferent ducts in several ways. Differences include that the tubules contain small diameters, are surrounded by thick dense connective tissue, contain fewer lysosomes in the cytoplasm of non-ciliated cells and their epithelium stain more intensely. Moreover, sperm are generally not present in the lumen of blind-ending tubules (Guttroff *et al.*, 1992).

The efferent ducts are a major site for fluid reabsorption, with up to 94.8% of luminal fluids being removed as sperm passes through (Man *et al.*, 1997).
Unlike most of the male reproductive tract which depend on androgen receptors (AR) for regulation, the efferent ducts rely on estrogen receptors (ER). In particular, ERα serves a major role in the efferent ducts' regulation. This receptor is important in regulation as ERα knockout mice (ERαKO) show dilated efferent ducts up to 297%, suggesting the lack of reabsorption of luminal fluid (Hess *et al.*, 1997; Hess *et al.*, 2000). ERαKO mice also show reduced efferent duct epithelial height by 48% and show a 60% increase in prevalence of blind-ending tubules as compared to wild-type mice. Additionally, microvilli on non-ciliated cells show a reduction in both length (by 64%) and overall presence on some cells. In regard to ciliated cells, the cilia number was also reduced and appeared to beat off sync from one another. This could affect the even reabsorption of fluid as the cilia are used to mix luminal fluids (Ilio and Hess, 1994; Hess *et al.*, 2000).

After the efferent ducts, the sperm travel through the single convoluted tube called the epididymis. The epididymis can reach up to one meter in length in a mouse, while a human's can reach up to six meters. The epididymis, like the efferent ducts, is made up of several regions along its length. Most species include three major regions called the caput, the corpus and the cauda. These regions can be further broken up into more morphologically and molecularly distinct regions that are unique for different species (Cornwall, 2009). For example, there is evidence that the epididymis of a mouse can now be separated into as many as 10 distinct regions (Johnston *et al.*, 2005). In most species, from the caput down to the cauda, the luminal diameter increases and the epithelial cells height decreases (Robaire *et al.*, 2010a).

There are several different cell types making up the epithelium of the epididymis including principal cells, basal cells, clear cells, halo cells, narrow cells and apical cells (Robaire and Hermo, 1988; Robaire and Hinton, 2015). The most common type of cell is the columnar-shaped principal cell, which has apical cilia and is seen from the initial segment to the cauda. Its functions are fluid and nutrient exchange. Basal cells are also spread throughout the epididymis and vary in appearance by region of the epididymis. They can be dome shaped by the basement membrane but have cytoplasmic projections that go in between epithelial cells towards the lumen. These projections are thought to sample the lumen and then signal to the neighboring principal and clear cells (Robaire and Hermo, 1988; Robaire and Viger, 1995; Shum *et al.*, 2008; Robaire and Hinton, 2015). Halo cells are also seen throughout the epididymis. These cells are now known to be T lymphocytes as well as monocytes with past research describing them as immunocompetent cells that help prevent sperm autoimmunity (Robaire and Hermo,

1988; Robaire and Hinton, 2015). Narrow cells on the other hand are only in the initial segment and clear cells are in the caput, corpus, and cauda. These two cell types maintain luminal pH, which is important for sperm maturation. Also, like in the testis, there is a blood-organ barrier, which in the epididymis is called with blood-epididymal barrier. This barrier is made by tight junctions between the epithelial cells of the epididymis. In contrast to the blood-testis barrier though, the blood-epididymal barrier's apical end is not as elaborate and therefore allows for potential leukocyte invasion (Nashan *et al.*, 1989; Nashan *et al.*, 1990; Da Silva *et al.*, 2011; Hedger, 2011; Michel *et al.*, 2015).

The transit time for sperm through the epididymis takes 2 to 6 days in a human and 10 to 13 days in rodents (Cornwall, 2009). The sperm can then be stored in the cauda of the epididymis for long periods of time, even extending past 30 days (Orgebin-Crist *et al.*, 1975). Movement through the epididymis is propelled by the smooth muscle layer surrounding the tubule. This muscle layer increases in thickness from the proximal to distal segments (Baumgarten *et al.*, 1971). Even though the sperm are much more concentrated, due to the fluid being absorbed in the efferent ducts, sperm still need to undergo maturation as they traverse through the epididymis. Therefore, in general, the four main functions of the epididymis are 1) absorptive and secretory activities creating a luminal environment conducive for sperm maturation, 2) development of sperm motility, 3) development of sperm fertilizing ability and 4) the transport and storage of sperm (Robaire and Hinton, 2015).

After the cauda of the epididymis, the sperm travel through the vas deferens. At the base of the bladder, the vas deferens joins with the proximal end of the seminal

vesicle duct to then form the ejaculatory duct of the prostate. After the ejaculatory duct, the sperm continue through the prostatic urethra, which urine also travels through. Once the mixture exits the prostate, it enters the membranous urethra just inferior to the prostate and then finally the penile urethra, where it will then exit the male's body (McKinley *et al.*, 2019).

Normal Endocrine Regulation with Emphasis on the HPG Axis

The hormonal regulation of the male reproductive system is through the Hypothalamic-Pituitary-Gonadal (HPG) axis (Figure 1). In this sequence, there is a pulsatile release of gonadotropin-releasing hormone (GnRH) from the arcuate nucleus (ARC) of the hypothalamus, which travels through the hypothalamic-hypophyseal portal blood system, to the anterior pituitary gland. Once at the anterior pituitary, GnRH binds to its gonadotropin-releasing hormone receptor (GnRHR) on the cells' surface. This stimulates the production and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the gonadotrope cells of the anterior pituitary. LH then travels in the blood and attaches to its G protein-coupled receptors on the surface of testis' Leydig cells to stimulate the production of testosterone from cholesterol (Robaire *et al.*, 2010b). Testosterone can bind to its receptors in Sertoli cell nuclei, which allows testosterone to have an indirect effect on spermatogenesis. Alternatively, testosterone can travel in the bloodstream, where it can go to peripheral tissues. The testes, as well as the peripheral adipose tissue, may convert the testosterone to estradiol (reviewed in Teerds *et al.*, 2011). FSH can also stimulate the Sertoli cells to promote spermatogenesis (Figure 1) (Robaire *et al.*, 2010b).

This HPG axis is a negative feedback system with differential regulation of LH and FSH. The increase in FSH causes inhibin to be produced in the Sertoli cells. Inhibin suppresses the release of FSH from the anterior pituitary. Concurrently, testosterone or estradiol at the hypothalamus bind to their receptors on KISS1 neurons in the hypothalamus and subsequently inhibit the release of kisspeptins (Teerds *et al.*, 2011). GnRH neurons in the hypothalamus require these kisspeptins to bind to their G-protein coupled receptor, GPR54, to stimulate the release of GnRH. Therefore, the absence of kisspeptin signaling prevents the release of GnRH (Messager *et al.*, 2005). Additionally, KISS1 represses the action of neuropeptide Y (NPY) in the hypothalamus, whose function is to inhibit GnRH release. Therefore, an increase in kisspeptin will prevent NPY from impeding GnRH release, therefore promoting the release of GnRH (Teerds *et al.*, 2011).

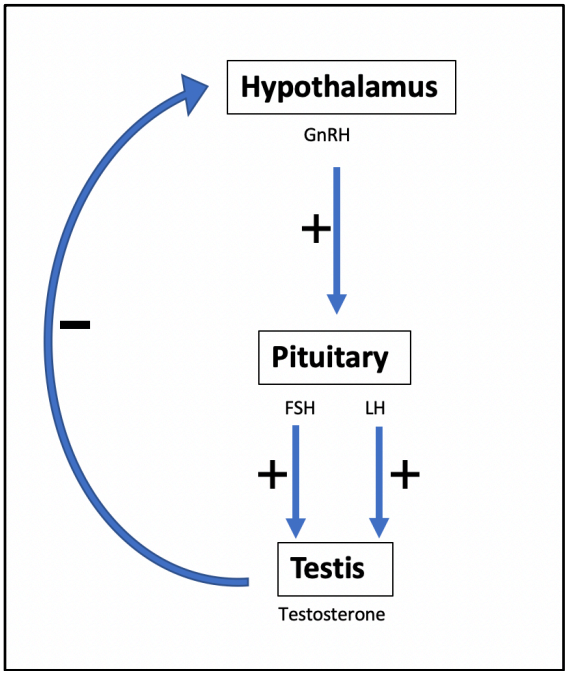


Figure 1: Diagram of the Hypothalamic-Pituitary-Gonadal (HPG) Axis
GnRH=gonadotropin-releasing hormone, FSH, follicle stimulating hormone, LH= luteinizing hormone.

Leptin and the Male Reproductive Tract

In addition to the classic components of the HPG axis, another factor interacting with this axis is a 146 amino acid protein named leptin. In 1950, Jackson Laboratory discovered a spontaneous strain of mice that were morbidly obese due to overeating and lacked energy expenditure. Scientists believed these mice lacked satiety signals and labelled this mysterious missing satiety gene the *ob* gene, after the obesity its functional absence caused. Therefore, mice that lack a functional gene were called Ob mice. Another similar spontaneous mouse strain was discovered in 1965. These mice were said to lack a functioning *db* gene, which was named *db* after the diabetes that these mice acquired along with the obesity (Kobayashi *et al.*, 2000; Wauman *et al.*, 2017). These Db mice produced the satiety factor missing in Ob mice but could not respond to it. This led scientists to believe that the *db* gene encoded for the *ob* receptor (Fantuzzi and Faggioni, 2000). Then in 1994, the *ob* gene was isolated on chromosome 6 in mice and its protein product was named leptin, derived from the Greek word “leptos” meaning “thin” (Zhang *et al.*, 1994).

The majority of leptin is produced by adipose tissue, but is also produced in skeletal muscle, joint tissues, stomach and the placenta (Señarís *et al.*, 1997; Wang *et al.*, 1998; Bado *et al.*, 1998; Scotece *et al.*, 2014). Expression of leptin was also discovered in the male reproductive tract, specifically in spermatocytes of humans and mice as well as spermatozoa in humans (Aquila *et al.*, 2005; Ishikawa *et al.*, 2007; Herrid *et al.*, 2008).

Leptin receptors are highly expressed in the hypothalamus as well as the lung and kidney. Lower expression can also be found in the skeletal muscle, heart, liver, small intestines, pancreas, spleen and immune cells (Fei *et al.*, 1997; Matteis *et al.*, 1998; Chen *et al.*, 1999; Margetic *et al.*, 2002; Francisco *et al.*, 2018). Leptin receptor expression can also be found in male reproductive structures including spermatogonia and Leydig cells in mice, as well as spermatozoa and adult Leydig cells in humans (El-Hefnawy *et al.*, 2000; Jope *et al.*, 2003; Ishikawa *et al.*, 2007).

There are 6 isoforms of the leptin receptor, which are named Ob-Ra through Ob-Rf (Wasim *et al.*, 2016). Forms Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf are classified as short forms due to their shorter intracellular domains, while Ob-Rb is the long form capable of STAT signal transduction and Ob-Re is secreted (Landry *et al.*, 2013). Leptin receptors in the hypothalamus with signaling capabilities are in the long Ob-Rb category, while the short isoforms like Ob-Ra appear to predominate most peripheral tissues (Tartaglia, 1997; Tsiotra *et al.*, 2000). The mutation of this long Ob-Rb form is known for its connection with severe obesity in both human and mouse models (Wasim *et al.*, 2016). This is because circulating leptin reaches the hypothalamus and binds with its Ob-Rb form's receptors to reduce appetite and help reduce food intake as well as increase energy consumption and metabolism (Friedman, 2002; Williams *et al.*, 2009). Therefore, an increase in adipose tissue should produce increased amounts of leptin, which with the hypothalamus would inhibit appetite until the fat stores are depleted. The depletion of fat would then decrease the amount of leptin released and allow an increase in appetite once more (Friedman and Halaas, 1998).

Leptin is not only involved in satiety and obesity, but also in bone metabolism, immune responses, and reproductive function through the HPG axis. There are no leptin receptors on the GnRH neurons, though leptin still plays an important indirect role in GnRH release (Quennell *et al.*, 2009). In normal weight subjects, leptin released from adipose tissue travels to the hypothalamus and binds to receptors on KISS1 neurons, which promotes the release of GnRH (Irwig *et al.*, 2004). Moreover, the KISS1 neurons can also stimulate GnRH release by inhibiting NPY neurons, which inhibit GnRH release (Fu and van den Pol, 2010). Therefore, by inhibiting the NPY neurons, GnRH can be released again (Schwartz *et al.*, 1996).

Leptin dysfunction can be caused by either a genetic predisposition or a prolonged elevation of leptin levels. Congenital leptin dysfunction is a rare cause of severe early-onset obesity, with only 12 subjects identified. Leptin receptor mutations are even rarer, with only 8 subjects identified (Wabitsch *et al.*, 2015; Wasim *et al.*, 2016). The phenotypic features of the subjects with the receptor mutation are less severe than ones with the leptin deficiency (Farooqi *et al.*, 2007). This suggests that leptin can function without the use of the traditional leptin receptor by means of another pathway. Both congenital mutations, leptin deficiency and leptin receptor deficiency, can be positively impacted by leptin replacement therapy (Wasim *et al.*, 2016).

A second mechanism of leptin dysfunction is from leptin resistance caused by chronically increased leptin from obesity. Since adipose tissue is the major producer of leptin, circulating levels of leptin correlate with the amount of adipose tissue in the subject (Maffei *et al.*, 1995). Therefore, high levels of circulating leptin are seen in most obese individuals (Maffei *et al.*, 1995; Considine *et al.*, 1996). Leptin circulates in the

blood in both a bound and free form. Around 50% of leptin is bound in lean individuals, while in obese individuals, most of the leptin is free (Sinha *et al.*, 1996). Some explanations for this acquired resistance are leptin transport saturation across the blood-brain barrier, inability to activate or bind to the leptin receptor or its downstream signals, and the degradation or downregulation of leptin receptors (El-Haschimi *et al.*, 2000; Zabeau *et al.*, 2003).

In regards to the HPG axis, leptin resistance causes the Ob-Rb receptor on the KISS1 neurons to no longer function properly (Teerds *et al.*, 2011). Due to this malfunction, the KISS1 neural expression in the arcuate nucleus is decreased, so inhibition of NPY is reduced in both Ob and Db mice and consequently GnRH release is doubly diminished (Stephens *et al.*, 1995; de Luca *et al.*, 2005; Smith *et al.*, 2006). As a result, LH, FSH, and testosterone levels will also be reduced (Cunningham *et al.*, 1999; Tsatsanis *et al.*, 2015). Even though its been shown that FSH and LH receptor gene expression in Ob mice were significantly lower versus the control mice, this cannot necessarily be implied that FSH and LH levels are reduced in obese human males (Martins *et al.*, 2017). In a study of human males, researchers noticed that the obese males had similar LH and FSH values as the non-obese controls (Isidori *et al.*, 1999). While there are discrepancies between species relating to gonadotropins, both the mouse and human models show that increased leptin levels correlate with decreased testosterone levels, with the Ob mice having a 51% decrease in testosterone levels, as compared to the wild-type mice. This reduction of testosterone can result in impaired spermatogenesis through fewer spermatocytes, elongated spermatids and mature spermatozoa (Martins *et al.*, 2017). NADPH oxidase 5 was also shown to be reduced,

which is vital for sperm motility (Chen *et al.*, 2015). Testicular atrophy in the form of decreased testicular mass and increased germ cell apoptosis was also noted in the Ob mice when compared to the wild-type mice (Bhat *et al.*, 2006; Martins *et al.*, 2017).

Ob and Db mice are considered sterile, though one study found that Ob male mice could occasionally reproduce (Mounzih *et al.*, 1997; Ewart-Toland *et al.*, 1999). The importance of leptin for fertility was additionally shown when Ob mice were given leptin treatments (Mounzih *et al.*, 1997). After the final treatment, the mouse's body weight and epididymal fat pad decreased, whereas testicular weight, mature sperm count in the seminiferous tubules and normal Leydig cell morphology increased (Mounzih *et al.*, 1997; Cleary *et al.*, 2001; Hoffmann *et al.*, 2016). These leptin-treated Ob mice all successfully impregnated lean females, showing successful restoration of fertility. Furthermore, they showed that weight loss alone is not enough to make Ob mice fertile, as leptin-deficient mice who lost weight through a diet, were still unable to impregnate lean females (Mounzih *et al.*, 1997).

There is a category of obese men with fertility problems that are resistant or insensitive to leptin. To further study the leptin-obesity-infertility relationship, several studies have used the previously mentioned genetically modified Ob and Db mouse models. Mice that lack the leptin gene are called Ob mice. Mice that lack only the long (Ob-Rb) form of the receptor are called Db mice. The other forms of the Ob-R receptor are believed to be unaffected in these Db mice (Friedman and Halaas, 1998). Overall, both mutant forms of mice have impaired leptin receptor communication and consequently both the Ob and Db modified strains of mice present with obesity, even early on at 3 to 5 weeks of age (Harlan, 2007). Additionally, at 3 months of age, Ob

mice in particular were 83% larger in body mass compared to wild-type mice (Martins *et al.*, 2017). Due to these results, leptin-deficient obese mouse models are used as a model to study the effects of obesity on male reproduction.

Previous Laboratory Findings

The Pearl lab has previously investigated key reproductive fertility parameters in C57, Ob and Db mice at 8 and 16 weeks of age. They found a significant increase in both the Ob and Db body weight compared to the C57. Conversely both the epididymides and testicles weighed less in both of the leptin-altered models as compared to the C57. Additionally, there appeared to be an increased distance between the initial segment of the epididymis and the superior aspect of the testis in the two leptin-altered models, when compared to the C57 control mice but this was not quantified. If real, this increase in distance might indicate a stretching of the efferent ducts due to the increase in adipose tissue.

When analyzing the sperm count in the testis at 16 weeks, production in Ob mice was decreased compared to the C57 wild-type, though Db mice were similar to C57. On the other hand, both the Ob and Db mice showed significantly decreased sperm counts at 16 weeks in the epididymis as compared to the C57 mice (Figure 2). This indicates, that the Db specifically had decreased sperm counts going from the testis to the epididymis. Additionally, all mouse models had the same sperm count in the epididymis at 8 weeks of age. Therefore, this decrease in epididymal sperm count at 16 weeks on both Ob and Db could explain why some breeding studies suggest a limited ability of Ob and Db to produce offspring.

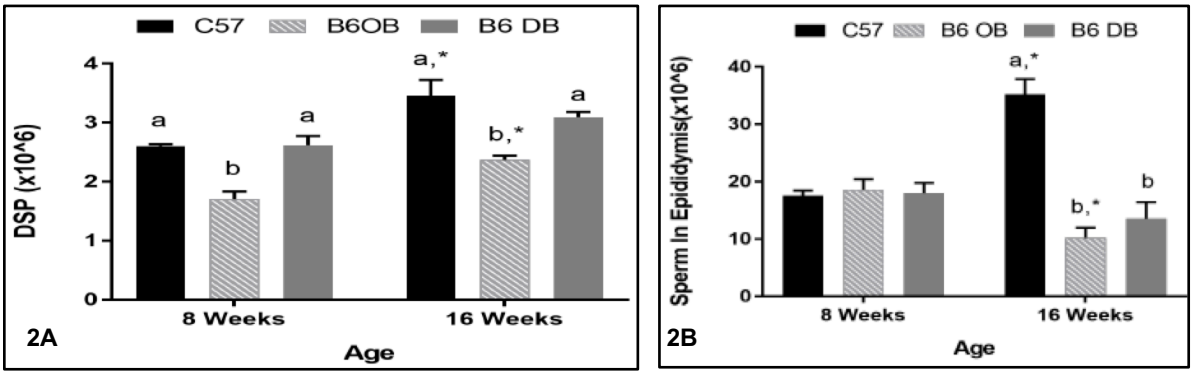


Figure 2: Total Testis and Epididymal Sperm Counts

2A and 2B) The Ob mouse model has decreased testis sperm count compared to the C57 and Db mouse models at 16 weeks. In the epididymis, both the Ob and Db models have decreased sperm counts at 16 weeks of age as compared to the C57 mouse model. This finding is also significant from the 8-week mouse models' epididymal sperm count as there was no significant difference then in any model. "*a*" vs. "*b*" indicates significant differences between genetic backgrounds at 16 weeks of age. " * " indicates significant difference between ages within a genetic background (Pearl and Doherty, 2018).

Histologically, in the Ob and Db mouse models there were decreased epididymal cauda tubule and lumen diameters, as well as increased epithelial cell height as compared to the C57. Limited investigation also showed unidentified cellular debris in the epididymal lumens of the Ob and Db mice, but was absent in the C57 mice (Figure 3). These altered epididymal appearances might indicate a blockage or obstruction of luminal contents along the male reproductive tract. Additionally, there is some indication of inflammation in the initial segment of the epididymis (Figure 3).

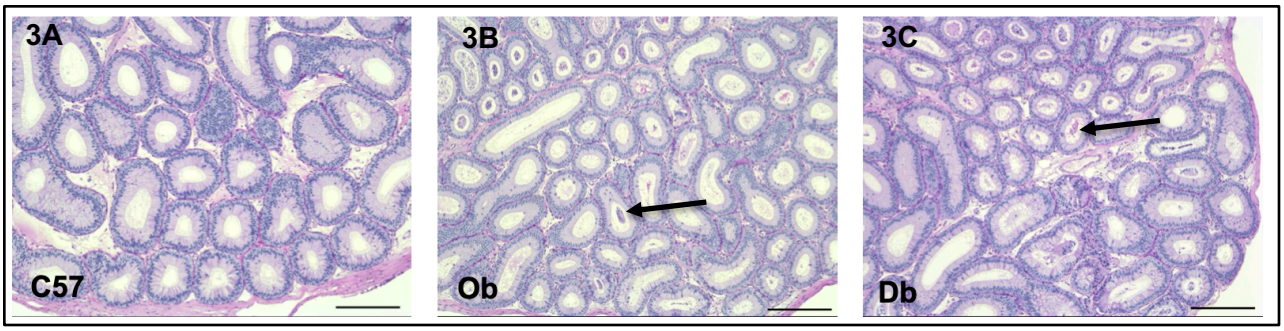


Figure 3: Histological Differences in the Initial Segment of the Epididymis in C57 Versus Ob and Db Obese Mice
3A) Cross-section of the C57 wild-type epididymis with nothing in the lumen. 3B) There is unidentified debris in the Ob initial segment epididymal lumen (black arrow) 3C) There is the same debris in the Db's epididymal lumen (black arrow) (Pearl and Doherty, 2018).

Immune System Components and their Basic Functions

The innate and adaptive immune systems provide protection against invading organisms. The innate immune system involves components like complement, neutrophils, macrophages (Mφ), dendritic cells (DC) and natural killer (NK) cells. These cells provide non-specific defense against a wide range of pathogens (Parham, 2005). The adaptive immune system, on the other hand, is acquired and is slower to respond. It reacts to specific antigens and can develop memory for improved responses to future infections. The adaptive immune system includes T and B lymphocyte cells and functions with the help from antigen-presenting cells like dendritic cells and macrophage cells (Parham, 2005).

The innate immune system is initiated immediately and can recognize various common antigens on pathogens. The complement system is made up of plasma proteins made by the liver and cover the surface of bacteria and virus particles to help other immune cells phagocytose them (Parham, 2005). Macrophages phagocytose and

kill invading microorganisms, dead cells and cellular debris. Macrophages are usually the first cell to sense a pathogen. They secrete cytokines to recruit neutrophils and other leukocytes to the macrophage's infected area (Parham, 2005). Neutrophils can also phagocytose and kill microorganisms. They are shorter-lived than macrophages and form pus when they die at the site of infection (Parham, 2005). NK cells can also enter infected tissue and kill cells that contain viruses (Parham, 2005). Dendritic cells on the other hand leave their infected tissue and activate T and B cells of the adaptive immune system (Parham, 2005).

The innate immune system also activates the adaptive immune system. B cells have specific cell-surface pathogen receptors called immunoglobulins. B cells become activated and differentiate into effector B cells, or plasma cells, which secrete specific immunoglobulins that are now called antibodies. Antibodies can neutralize a pathogen by binding to a part of it, thus preventing the pathogen from binding with other cells, growing, or replicating. Antibodies can also help with opsonization by binding with a matching antigen on a pathogen and covering the pathogen's surface with the antibodies. Macrophages and neutrophils can then bind with the antibody and phagocytose the pathogen (Parham, 2005). T cells on the other hand have very specific T-cell receptors that are never secreted and these cells can differentiate into either Helper T (Th) cells or Cytotoxic T (Tc) cells. Tc cells kill infected cells, like the NK cells of the innate immune response. Th cells on the other hand, secrete cytokines that help other immune cells become full activated effector cells. Th1 cells for example, help macrophages phagocytose better, while Regulatory T cells (Tregs) regulate T cell

activity to prevent unnecessary tissue damage and stop the immune response once the infection is over (Parham, 2005).

The Immune System and Leptin

Leptin plays a role in inflammation, infections and immune responses. Leptin receptors have been found on leukocytes including monocytes/macrophages, neutrophils, NK cells, DC cells and T and B lymphocytes (Zhao *et al.*, 2003; Mattioli *et al.*, 2005; Bruno *et al.*, 2005; Papathanassoglou *et al.*, 2006; Carlton *et al.*, 2012). Leptin is described as a cytokine hormone with a structure similar to Interleukin 2 and 6 (Otero *et al.*, 2006). Leptin increases the activation and the ability of neutrophils to undergo chemotaxis, while LEPR mutated animals had decreased neutrophil chemotaxis (Montecucco *et al.*, 2006; Naylor *et al.*, 2014). Additionally, leptin promotes macrophage chemotaxis as well as increasing their cytokine release and phagocytosis of dead cells (Gruen *et al.*, 2007; Amarilyo *et al.*, 2014; Dayakar *et al.*, 2016). Conversely, leptin-receptor knockout mice have macrophages with decreased phagocytosis and diminished killing activity (Mancuso *et al.*, 2012). Also due to leptin, NK cells increase their cytotoxicity and longevity. Leptin-receptor deficient (Db) mice on the other hand, showed decreased NK cell function with an increased apoptotic rate (Tian *et al.*, 2002; Zhao *et al.*, 2003; Lo *et al.*, 2009). DC cells also undergo less frequent apoptosis with leptin as well as an increase in migration (Lam *et al.*, 2006). Alternatively, Db mice had a reduced number of DC cells due to increased apoptosis as well as a poor capacity to stimulate T cell proliferation (Lam *et al.*, 2006). Leptin deficient (Ob) mouse models' DC

cells had reduced maturation and inflammatory cytokines but they were more efficient in inducing Tregs than wild-type mice (Moraes-Vieira *et al.*, 2014; Francisco *et al.*, 2018).

In regard to adaptive immunity, leptin secreted by human adipocytes promote T effector cell proliferation and limit Treg cell population expansion (Wagner *et al.*, 2013). Tregs (CD4+CD25+) are suppressors of autoimmunity and limit effector T cells as the end of infection nears. With decreased levels of leptin, effector T cells are reduced and Tregs are increased (Rosa *et al.*, 2007). Additionally, this trend in increased Tregs and resistance to autoimmunity is seen in Ob and Db mice; administration of leptin decreased the Treg population back down to the amount seen in the wild-type (Matarese *et al.*, 2010). With leptin, B cells had increased proliferation and cytokine production along with a decrease in apoptosis (Lam *et al.*, 2010). Then, with a lack of leptin in Ob and Db mice, there was a decrease in the number of B lymphocytes, which recovered with leptin treatment (Bennett *et al.*, 1996; Claycombe *et al.*, 2008; Francisco *et al.*, 2018).

As for the type of leptin receptor expressed in these immune cells, neutrophils only express the short, Ob-Ra, form (Francisco *et al.*, 2018). From this finding, neutrophils should not be affected by the Ob-Rb knockout seen in Db mice. Macrophages and DC cells on the other hand do express the long form (Sánchez-Margalet *et al.*, 2003; Mattioli *et al.*, 2005). Tsiotra et al. (2000) additionally found that mononuclear cells (T cells, B cells, NK cells and monocytes) in humans expressed both the long and short form in all samples tested. Specifically, they found that the short form was expressed an average of 8 times more than the long form in the samples. This relative difference in expression was reduced though in subjects with an overweight BMI

(BMI>26), which was also associated with significantly increased leptin levels. Additionally, the expression of both isoforms was reduced in overweight subjects compared to in lean individuals (BMI<25). This supports that a reduction in receptors is why leptin resistance occurs in throughout the body in obese individuals (Tsiotra *et al.*, 2000).

Immune System of the Male Reproductive Tract

Spermatogenesis begins at puberty, while the immune cells have matured and had immune tolerance set around the time of birth. Therefore, these now matured sperm have molecules on their surface that may be seen as foreign to the immune system's tolerance mechanisms (Guerau-de-Arellano *et al.*, 2009). Immunological infertility is suggested to be the primary cause of unexplained infertility in males. In sub-Saharan African, the rate of infertile males with anti-sperm antibodies is 26.7% (Ekwere, 1995; Emeghe and Ekeke, 2017). Even in developed countries, where treatment to sexually transmitted infections is readily available, sperm autoantibodies are present in up to 10% of infertile males. This prevalence of autoimmune infertility supports that human sperm cells are commonly being seen as foreign by the immune system (Baker *et al.*, 1983; Lenzi *et al.*, 1997). While most autoimmunity throughout the human body is due to reactions against antigens that are normally ignored and is caused by a disruption of normal regulatory controls, autoimmunity in the reproductive tract involves antigens that would not normally be ignored (Aitken *et al.*, 1987; Shetty *et al.*, 1999; Bohring *et al.*, 2001).

Protection for the sperm then relies on several mechanisms and structures. First, there is the blood-testis barrier, which separates the interstitial space from the seminiferous tubules that contain the sperm. These tubules have occluding junctions between adjacent Sertoli cells, which separate the epithelial cells into two regions, the basal region and the more apical adluminal region. The adluminal portion contains the maturing germ cells and with these tight junctions, are protected from many different types of immune cells. Therefore, in a normal testis, lymphocytes are rarely, if ever, observed in the seminiferous tubules. Still, even though they are rarely seen in the basal layer, the immune cells could invade that outer layer of the tubule (Hedger, 2015).

Outside of the tubules, the interstitial compartment contains the Leydig cells, nerves, blood vessels and lymphatics of the testis. Specifically for immune cells in a healthy host, this compartment normally contains mostly macrophages, but also NK cells, T cells and possibly mast cells or eosinophils (el-Demiry *et al.*, 1985; Pöllänen and Niemi, 1987; Hutson, 1994; Tompkins *et al.*, 1998; Anton *et al.*, 1998; Hedger, 2015). The lymphatics in the interstitial space then exit to the local lumbar lymph nodes (Möller, 1980; Itoh *et al.*, 1998).

The majority of research on the immune system in the testis is in rat and mouse models. For a healthy mouse model, ratio of Leydig cells to macrophage cells in the interstitial space is 4-5:1 (Hume *et al.*, 1984; Hedger, 2015). These two cells types are thought to communicate as they have cytoplasmic interdigitations interlinking them together. Leydig cells and macrophages also undergo similar morphological and cytoplasmic size changes when there is testicular pathology (Geierhaas *et al.*, 1991; Hutson, 1992). Macrophages play a role in Leydig cell development and maintenance of

steroidal production in adulthood (Gaytan *et al.*, 1994b; Gaytan *et al.*, 1994a; Gaytan *et al.*, 1994c). Studies have also shown that an increase in number and maintenance of macrophages actually depends on Leydig cells, though it did not appear that androgens were directly involved (Wang *et al.*, 1994; Meinhardt *et al.*, 1998). DC cells in the interstitial space are also under evaluated. Rival *et al.* (2007) though found that DC cells play a vital role in the initiation and maintenance of autoimmunity in the testis, with an increase in DC numbers with the progression of disease (Rival *et al.*, 2007). In humans, there is also a population of mast cells in the interstitial tissue. Their role is unknown, but it has been suggested that they might play a role in the local innate immune system and possibly testicular blood flow (Hedger, 2015). The majority of T cells in the interstitium of the testis are consistent with the phenotype of activated or memory T cells, which could be a sign of autoimmunity or just previous infections. These T cells are specifically skewed towards CD8+ cells. There are a high amount of anergic and apoptotic T cells in this tissue though as compared to T cells in other tissues. Recently, Regulatory T cells (Tregs) and NK cells have also been seen (Tompkins *et al.*, 1998; Hedger, 2015). Neutrophils differ from the other innate cells mentioned, as after testicular damage is the only time that they are anywhere in the testis (el-Demiry *et al.*, 1985; Flickinger *et al.*, 1997). The number of lymphocytes and macrophages also greatly increases in the interstitial space with testicular infection. This increase in immune cell count has a potential for the cells to break through the blood-testis barrier, which is associated with a decrease in spermatogenesis (Kohno *et al.*, 1983; Doncel *et al.*, 1989; Jahnukainen *et al.*, 1995). While there are sparsely located lymphocytes in the interstitial tissue and none in the actual seminiferous tubules, immune cells are

present in the epithelium of the rete testis. This is because the rete testis does not have a secure blood-testis barrier like the seminiferous tubules (Dym and Romrell, 1975; el-Demiry *et al.*, 1985).

One big difference between the epididymis and the testis is the normal presence of immune cells in the epididymal epithelium. Within the epididymal epithelium are macrophages, dendritic cells and T cells. These immune cells have previously been referred to in literature as halo cells, which were thought to be some type of immunocompetent cells (Robaire and Hermo, 1988; Serre and Robaire, 1999). The epididymis has a tendency for larger amounts of epithelial and peritubular leukocytes to be at the initial segment and caput as compared to the cauda. Likewise, the caput also has more immunoregulatory molecule expression, like Activin A and indoleamine 2,3-dioxygenase (IDO) (Flickinger *et al.*, 1997; Seiler *et al.*, 1999; Hedger, 2015).

Epididymal mononuclear phagocytes (MP's) consist of macrophages and dendritic cells. These cells have their nucleus at the basal region of the epithelium and project multiple intraepithelial dendrites that go towards the lumen of the tubule. These cells can be morphologically and antigenically confused with basal cells of the epididymis, though morphologically, basal cells only have one projection coming off each cell. Both cell types' projections decrease in length going down the epididymal segments (Mullen *et al.*, 2003; Da Silva *et al.*, 2011; Shum *et al.*, 2014). It has been proposed that the macrophages phagocytose the old and excessive sperm, while dendritic cells sample antigens in the lumen to show to the interstitial CD4+ T cells and local lymph nodes (Cooper *et al.*, 2002; Da Silva *et al.*, 2011). When it comes to T cells seen in the epithelium, there tend to be more CD8+ Cytotoxic T cells rather than CD4+

T Helper cells and Tregs (Ritchie *et al.*, 1984). B cells are not usually seen in the murine or human epididymis (el-Demiry *et al.*, 1985; Flickinger *et al.*, 1997).

Like in the testis, the interstitial space surrounding the epididymis normally contains macrophages, DC cells and T cells (Ritchie *et al.*, 1984; Nashan *et al.*, 1989; Yeung *et al.*, 1994; Da Silva *et al.*, 2011; Shum *et al.*, 2014). The majority of lymphocytes surrounding the epididymis in the interstitium are F4/80+ macrophages, while less than 2% are T cells (Nashan *et al.*, 1989). The F4/80 expression is lower in the interstitium though when compared to the epithelium. Also differing from the epithelium is that the T cell population in the interstitium has more CD4+ than CD8+ T cells (Ritchie *et al.*, 1984).

With damage to the epididymis, there is a potential for development of sperm autoimmunity, though the severity is decreased in epididymal regions closer to the testis (de Kretser *et al.*, 1998; Hedger, 2015). The lower amount of autoimmunity seen in the more proximal epididymal segments indicates a higher amount of local immunoregulatory mechanisms. Due to the epididymis being less restricted to immune cells as compared to the testis, the epididymis relies much more on tolerance than the testis does to protect the sperm. Immunosuppressive factors might diffuse from the testis to the epididymal fluid in the caput, explaining why there are more immunoregulatory molecules expressed in that area of the epididymis than the rest of the segments (de Kretser *et al.*, 1998). Another suggestion is that epididymal secretions cause modifications to the sperms' surface, which obscures sperm antigens (Eddy *et al.*, 1985).

Lymphatic drainage of the epididymis is favored around the cauda as compared to the other sections of the epididymis (Hirai *et al.*, 2010). While the lumbar lymph nodes drain the testis, the lymph nodes that drain the scrotum are the inguinal lymph nodes (Itoh et al., 1998; Jha, 2019).

Physical barriers do not provide all of the protection seen in the male reproductive tract, as there are also local immunoregulatory and immunosuppressive mechanisms to provide protection for the sperms' antigens (Meinhardt and Hedger, 2011). It has been explained that the immune cells that enter the male reproductive tract are modified to limit their proinflammatory activity and antigen-specific immune responses are very controlled. Hedger (2015) states that the immune privilege seen in the testicle is caused by several mechanisms including (1) maintenance of tolerance, (2) the blood-testis barrier, (3) testicular cells' reduced immunogenicity, (4) macrophages in the tissue being anti-inflammatory (5) Sertoli and Leydig cells having immunosuppressive properties, and (6) production of immunoregulatory/immunosuppressive cytokines locally (Hedger, 2015). Therefore, the male reproductive tract has a unique and varied immune system that still requires more research to understand its complexity.

Chapter 3 Methodology

Animal Model

The C57BL/6J mice were chosen for this study as it is an established model for the study of the male reproductive system, especially the Ob and Db strains in regards to obesity caused by leptin malfunction. Eighteen mice (6 of each genetic background) ranging from 6 to 10 weeks were acquired from Jackson Laboratory. Mice were housed 3 per cage with free access to standard mouse chow and water on a 12 hour on/off light cycle. Housing occurred for 6-10 weeks depending on initial age when received. All mice were sacrificed at 16 weeks of age. All procedures were approved by Grand Valley State University's Institutional Animal Care and Use Committee (IACUC).

When the mice reached an age of 16 weeks, they were euthanized by CO_2 and body weight was collected. We then opened up the ventral abdomen and cut the diaphragm. On side 1 for each mouse, the epididymal fat pad was removed with the testis and epididymis attached. The distance between the superior edge of the testis to the beginning of the epididymis was measured with a digital caliper (Figure 4). The testis, epididymis, and fat pad were then separated and individually weighed. The testis was frozen on dry ice and stored at -80°C. The epididymis was processed for tissue dissociation, while the fat pad was discarded. On side 2, the testis, epididymis, and associated fat pad structures were removed and the distance between the testis and epididymis was once more measured. The testis and proximal epididymis (initial segment, caput, corpus) remained intact and was fixed in Bouin's fixative. The cauda was separated, weighed and frozen on dry ice and stored at -80°C. After the

reproductive structures were gathered and measured, the bilateral inguinal lymph nodes were dissected from each mouse and processed for tissue dissociation (Figure 5).

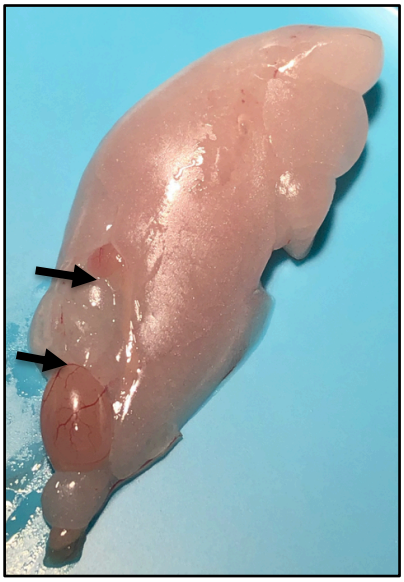


Figure 4: 16-Week Db Mouse Epididymal Fat Pad with Testis and Epididymis Attached
Black arrow heads indicate where the points of the caliper were positioned for measuring the distance between the superior edge of the testis to the head of the epididymis.

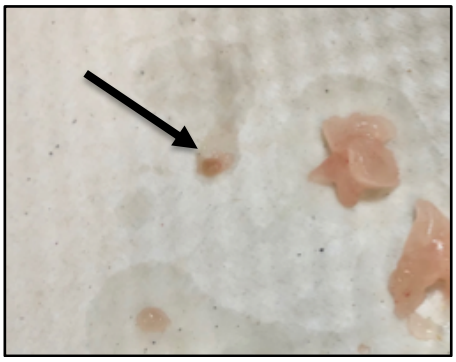


Figure 5: 16-Week Db Mouse Inguinal Lymph Node Dissected from Surrounding Abdominal Cavity Fat
Black arrow indicates the slightly darker inguinal lymph node. Some lighter-colored abdominal fat is seen on the right.

Sperm Counts

The frozen cauda was thawed and processed for determination of sperm number. Each cauda and 2mls of filtered solution containing 0.9% NaCl with 0.05% Triton-X was placed into a test tube. The samples were then homogenized in the solution using an OmniTip tissue homogenizer for 30 seconds and transferred into 50ml conical tubes with solution added to it until it reached a volume of 10ml. The samples were then placed in the refrigerator for 24 hours.

After 24 hours, a portion of the mixture containing the sperm heads was loaded into a Bright-Line® hemacytometer and placed under a microscope using phase contrast at 40x. The sample was then counted by two different individuals who counted each sample two times (Figure 6). Counts were either obtained from five different 4x4 squares or, if smaller sperm counts, all 25 of the 4x4 squares were counted (Figure 7A and 7B). If one of the highlighted 4x4 squares was obscured, due to a bubble or clumping, then an adjacent 4x4 square was selected. After cleaning off the hemacytometer with DI water, this process was repeated for each sample.

Once counting was completed, the four sperm counts from each animal were then averaged and multiplied by 5 to get the total number of sperm per 25 square grid. That count was then multiplied by 10,000 to find how many sperm per ml of sample and then multiplied by 10 to find number of sperm per full 10ml sample. The total number of sperm in the 10ml is equal to the total number of sperm per cauda. Sperm per mg of cauda was also obtained by dividing sperm per cauda by the weight of the cauda in mg.

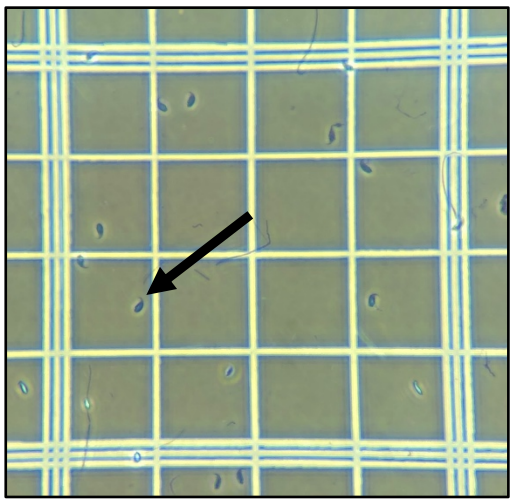


Figure 6: Example of Sperm on a 4x4 Counting Square within a Hemacytometer
Sperm heads were counted if they were on or inside the middle of the three border lines surrounding the 4x4 counting square. Example of a counted sperm head (black arrow). Image shown used a 40x objective.

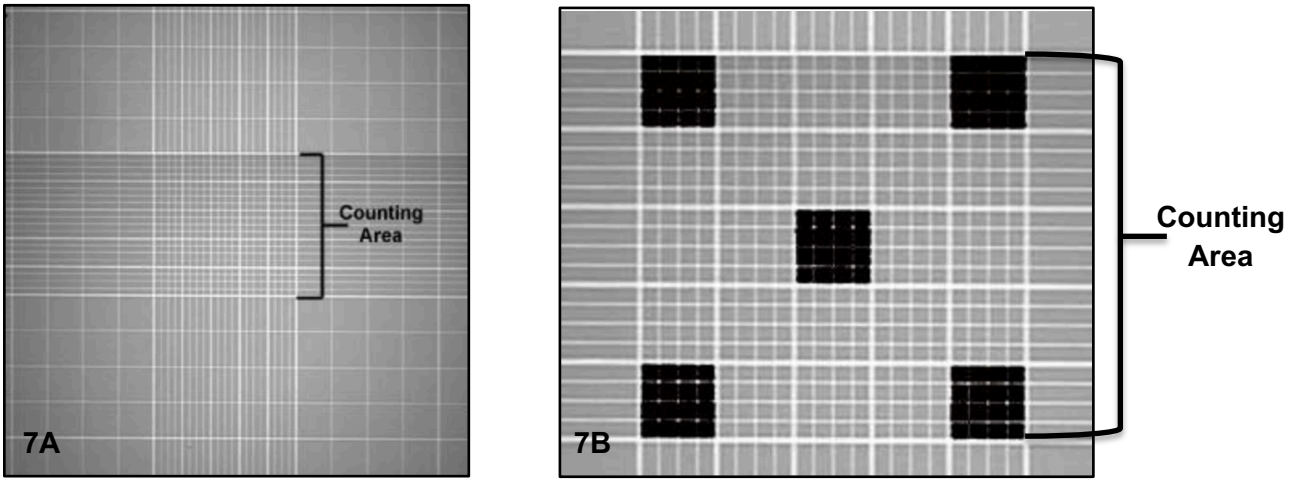


Figure 7: The Counting Area for a Hemocytometer
7A) “Counting Area” indicates section of slide that contained the sperm sample. This whole 5x5 area was used for counting when lower sperm counts were seen. 7B) Zoomed in picture of picture A. Each of the five blackened areas represents a 4x4 square within the larger 5x5 “Counting Area”. Each of the five 4x4 blackened areas were counted when sperm counts were larger.

Immunohistochemistry (IHC)

Tissues from an additional 18 mice (6 per genetic background) previously fixed in the Pearl lab were used for IHC of leptin receptor. Paraffin blocks containing the epididymis were cut with a microtome at 5μm and sections adhered onto slides. Slides were later dewaxed in Fisher's CitriSolv® and rehydrated sequentially in 100%, 95% and then 70% alcohol solutions. Samples were then rinsed in tap water for 5 minutes and incubated in 0.3% H_2O_2 and Fisher's HistoPrep™ Methanol for 30 minutes. After a wash in PBST, antigen retrieval with heat and Vector's Antigen Unmasking Solution was performed, followed by another wash in PBST. Slides were then placed in humidifying chambers with Vectastain® Elite ABC Rabbit IgG kit blocking serum added to each slide to incubate for 20 minutes. After dumping off the blocking serum, Invitrogen's primary leptin receptor (LepR) polyclonal rabbit IgG antibody at a concentration of 1:1,000 (or PBST or NRS for negative controls at 1:10,000) was added to the slides to incubate for 24 hours at 4°C.

After 24 hours, the primary antibody was removed, slides washed with PBST, and biotinylated secondary antibody was added for 30 minutes. Slides were then washed once more in PBST and an ABC enzyme solution was added to incubate for 30 minutes. Vector® NovaRED™ peroxidase substrate was then added and subsequently rinsed off in tap water. Slides were then rinsed with DI water and counterstained with ImmunoMaster Hematoxylin. Finally, slides were dehydrated in increasing alcohol concentrations and CitriSolv® before being cover slipped with CoverSafe™ Histology mounting medium. Immunostaining was visualized with a Nikon Eclipse Ni-U Compound microscope and images captured using NiS elements software.

Flow Cytometry

Tissue Digestion, Antibody Incubation, and Flow Cytometry

Flow cytometry was performed on cells isolated from the epididymal regions and the inguinal lymph nodes. Upon dissection, tissues were individually placed in FACS buffer (PBS with 1% FBS) on ice. The epididymis samples were separated into initial segment/caput, corpus and cauda and then minced further. Each of the three minced sections were then mixed with prewarmed FACS buffer and left for 10 minutes in an attempt to allow the sperm to separate from the rest of the tissue sample. After settling, the supernatant was removed and the remaining tissue sample was resuspended in 10ml of FACS with Sigma's 1.0mg/ml collagenase type I and 1.0mg/ml collagenase type II for 45 minutes in a 37°C shaking water bath.

The enzyme-digested epididymis regions and inguinal lymph nodes were poured and crushed through a 70-μm nylon mesh cell strainer into a conical tube using a syringe plunger. The samples were then centrifuged at 4°C for 7 minutes, supernatant was again removed, and the pellets/cells resuspended in 2ml of FACS buffer. The cell suspension was transferred into 1ml flow cytometry tubes (1ml for the innate and 1ml for the adaptive panel) and centrifuged for 5 minutes at 1500rpm. After once more discarding the supernatant, the FACS-diluted Tonbo's Anti-Human/Mouse or Thermo Fisher's (for Live/Dead) innate and adaptive antibody panels were added and mixed into each tube. Specifically, for the innate panel CD11b at 1:400 (all innate immune cells), CD11c at 1:400 (dendritic cells), F4/80at 1:400 (macrophage cells) and Live/Dead at 1 μL per mL (dead cells) were used. For the adaptive panel CD4 at 1:400 (Helper T cells),

CD8 at 1:400 (Cytotoxic T cells), CD19 at 1:500 (B cells), and Live/Dead at 1 μL per mL (dead cells) were used. Samples were then covered from light and incubated for 30 minutes at 4°C. After incubation, 750μl of FACS buffer was added and tubes were once again centrifuged for 5 minutes and then supernatant discarded. The final pellets were resuspended in 200μl of FACS buffer and stored and protected from light at 4°C until each sample was run through a Beckman Coulter CytoFLEX flow cytometer. Inguinal lymph node samples were run through the machine for roughly 3 minutes at fast speed (60μl/ml). The caput and corpus samples were run for approximately 3.5 minutes at medium speed (30μl/min). The epididymal cauda segment samples were run at slow speed for roughly 4.5-5 minutes at slow speed (10μl/min).

Gating Strategy

After samples were sorted by flow cytometry, data was saved onto a flash drive for later analysis using the software program FlowJo™ Version 10.7. For all lymph node samples, we first gated for singlet cells with SSC-H on the x-axis and SSC-A on the y-axis (Figure 8A). Next, we gated to isolate the live cells with FSC-A on the x-axis and Live/Dead-A on the y-axis (Figure 8B).

For inguinal lymph nodes incubated with antibodies to innate immune cell markers, we then gated for cells that highly expressed CD11b, which is a marker found on all innate immune cells. The x-axis was set to CD11b and the y-axis was FSC-A (Figure 8C). Then from the CD11b+ cells, we gated areas with high expression of specific innate immune cell markers CD11c (dendritic cells) and F4/80 (macrophage cells). This graph had F4/80-A on the x-axis and CD11c-A on the y-axis. The three

areas on this graph included CD11c+ and F4/80+ cells, CD11c+ and F4/80- cells, and CD11c- and F4/80+ cells (Figure 8D).

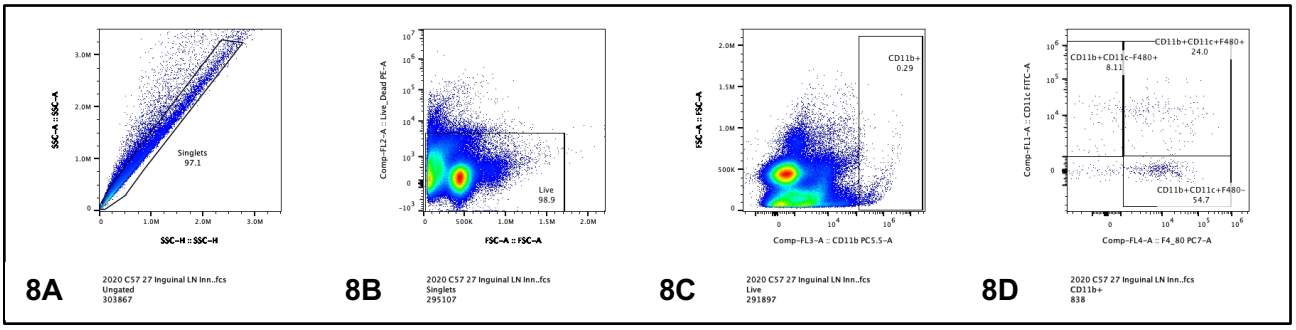


Figure 8: Lymph Node Gating for Dendritic (DC) and Macrophage (Mφ) Cells
8A) Gating for singlet cells. 8B) Gating for live cells. 8C) Gating for CD11b+ (innate immune) cells. 8D) Gating for macrophage and dendritic cells, which commonly express either/both CD11c and F480.

For inguinal lymph nodes incubated with antibodies to adaptive immune cell markers, we again kept the same gates as the innate inguinal lymph nodes for first singlet cells and then live cells (Figure 9A and 9B and 10A and 10B). Then, we put a gate to isolate the area where the immune cells should be with the x-axis being FSC-A and the y-axis being SSC-A (Figures 9C and 10C). After that gate, we then gated for T Helper cells (Th) and B cells with CD19-A on the x-axis and CD4-A on the y-axis (Figure 9D). We then changed the y-axis to CD8-A to gate for the Cytotoxic T cells (Tc) (Figure 10D).

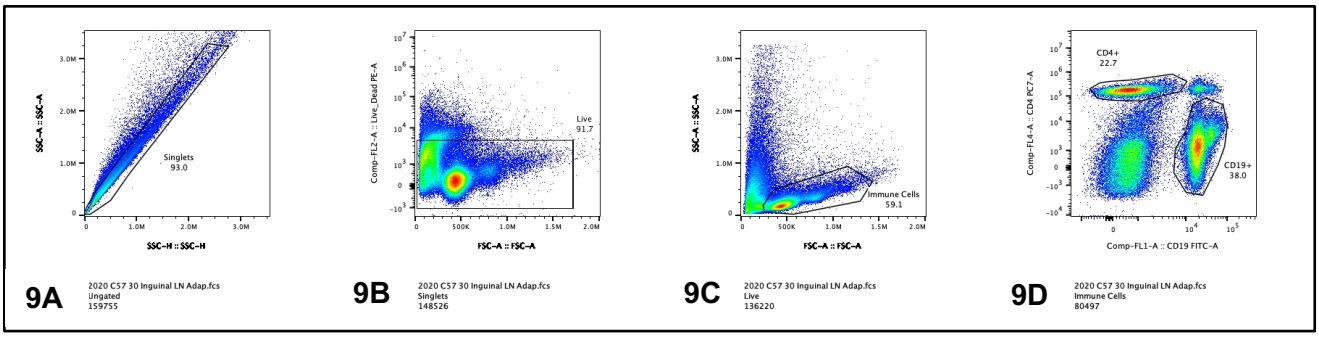


Figure 9: Lymph Node Gating for Helper T (Th) and B Cells
9A) Gating for singlet cells. 9B) Gating for live cells. 9C) Gating for immune cells. 9D) Gating for Th and B cells.

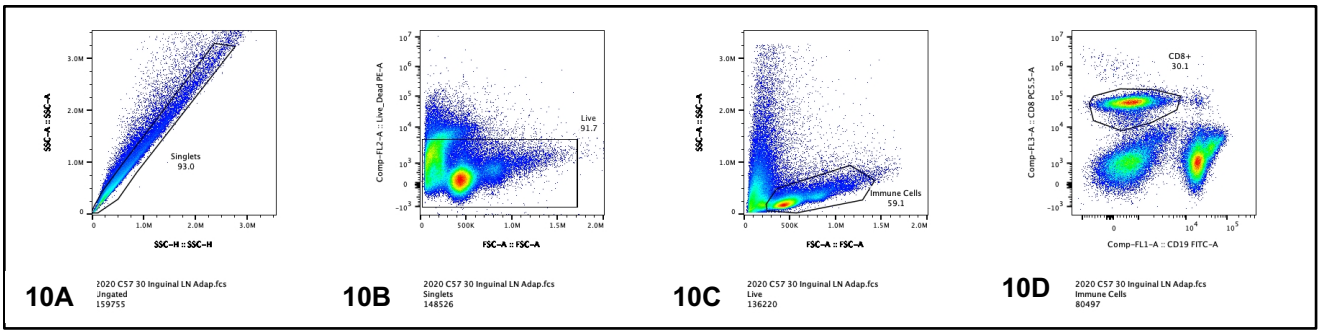


Figure 10: Lymph Node Gating for Cytotoxic T (Tc) Cells
10A) Gating for singlet cells. 10B) Gating for live cells. 10C) Gating for immune cells. 10D) Gating for Tc cells.

For the epididymal regions incubated with antibodies to innate immune cell markers, we used similar gating as we did for the inguinal lymph nodes. Therefore, we first kept the same gates for singlet cells, then live cells, and then CD11b+ cells. Then out of the CD11b+ cells, we gated for the CD11c+ and F4/80+ cells, the CD11c+ and F4/80- cells, and the CD11c- and F4/80+ cells (Figures 11A - 11D).

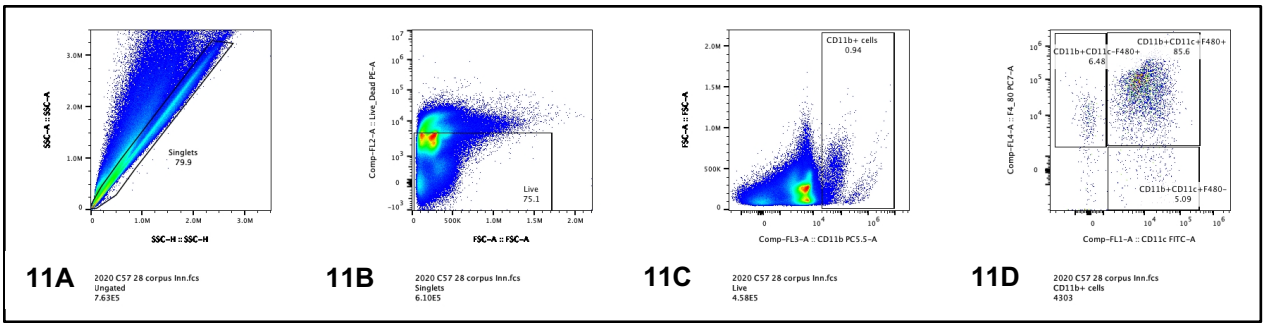


Figure 11: Epididymis Gating for Potential DC and Mφ Cells
11A) Gating for singlet cells. 11B) Gating for live cells. 11C) Gating for CD11b+ (innate immune cells). 11D) Gating for potential macrophage and dendritic cells by gating for their common markers (CD11c and F4/80).

When gating epididymal segments incubated with antibodies to adaptive immune cell markers, we first gated for singlet cells and then live cells using the same gates as for the inguinal lymph nodes (Figure 12A, 13A, 14A and 12B, 13B, 14B). Then we gated for specific adaptive immune cell markers CD4 (T helper cells), CD8 (Cytotoxic T cells) and CD19 (B cells) with those markers on the x-axis and FSC-A on the y-axis (Figures 12C, 13C and 14C).

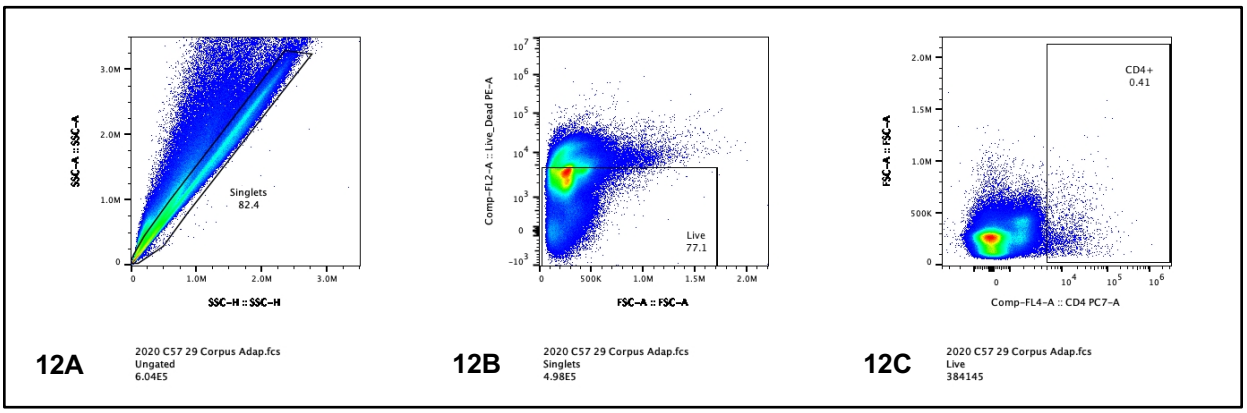


Figure 12: Epididymis Gating for Th (CD4+) Cells
12A) Gating for singlet cells. 12B) Gating for live cells. 12C) Gating for CD4+ cells.

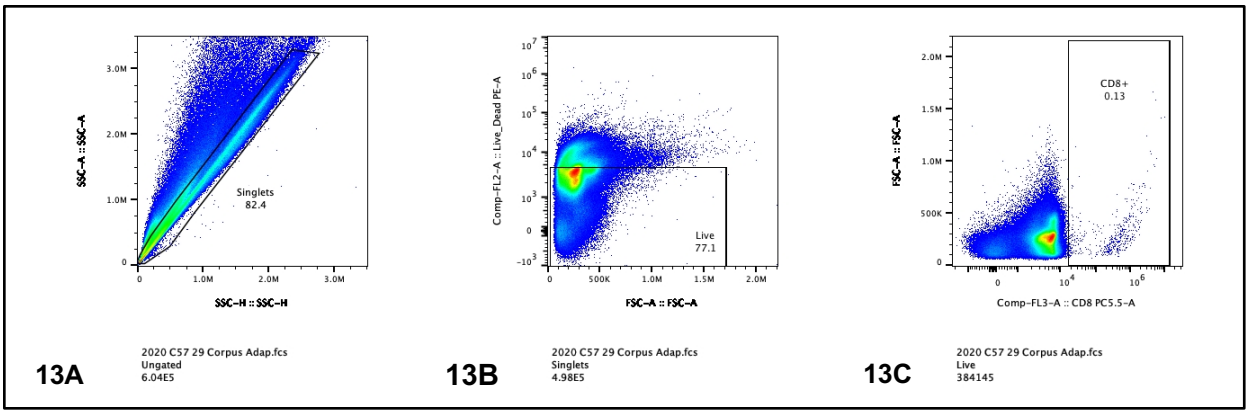


Figure 13: Epididymis Gating for Tc (CD8+) Cells
13A) Gating for singlet cells. 13B) Gating for live cells. 13C) Gating for CD8+ cells.

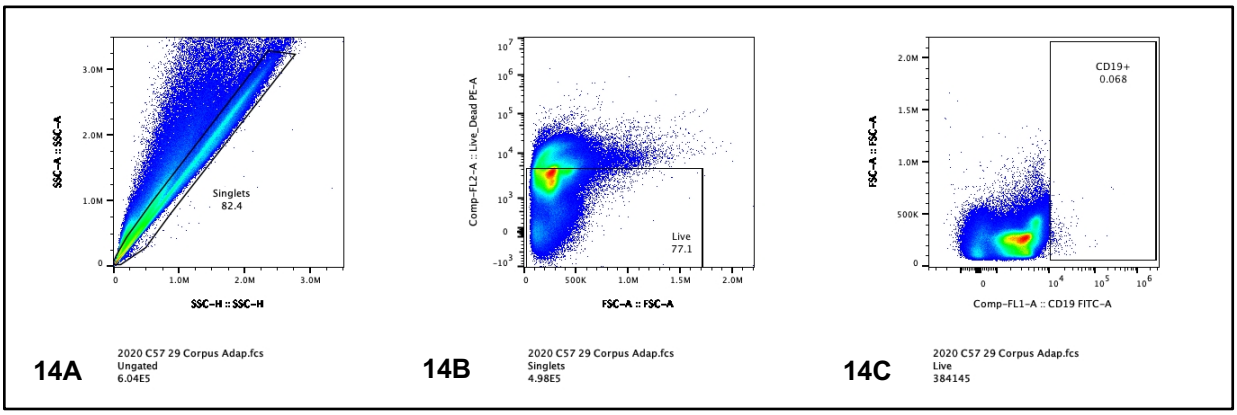


Figure 14: Epididymis Gating for B (CD19+) Cells
14A) Gating for singlet cells. 14B) Gating for live cells. 14C) Gating for CD19+ cells.

Data/Statistical Analysis

All statistical analyses were performed using GraphPad Prism (Version 8). Body, organ weights, testis to epididymis distance and sperm counts were analyzed by ANOVA. If the overall ANOVA was significant ($p<0.05$), differences between groups were determined using Tukey's multiple comparison test. Values reported are means ± SEM. For immunohistochemistry, a positive staining for leptin-receptor in the

epididymis was indicated by a deep red color when compared to the much lighter negative control NRS. Data are reported as positive or negative for each region.

Lymph node cell populations identified by flow cytometry were analyzed by ANVOA. If the ANOVA was significant, differences between groups were analyzed by Tukey's multiple comparison test. Epididymis cell populations identified by flow cytometry were analyzed by a two-factor ANOVA with region and genetic background as the factors. If the ANOVA was significant, differences between groups within a region and differences between regions within a group were analyzed by Tukey's multiple comparison test.

Innate immune cell data is presented as 1) the percentage of live cells that are CD11b+; 2) the percent of CD11b+ cells that are CD11c+ and/or F4/80+; 3) the percentage of CD11b+ cells that are CD11c+ and F4/80-; and 4) the percentage of CD11b+ cells that are CD11c- and F4/80+.

Adaptive immune cell data for lymph nodes is presented as the percentage of immune cells positive for CD4, CD8 or CD19. Adaptive immune cell data for the epididymis is presented as the percentage of live cells within a region positive for CD4, CD8, or CD19.

Chapter 4 Results

Reproductive Parameters

The body weights of the leptin-disrupted mouse models (Ob and Db) were both significantly larger weighing 56.15 ± 2.45g and 56.55 ± 3.00g respectively compared to the control (C57) mice at 27.94 ± 0.517g ($p<0.0001$) (see Figure 15A). Likewise, the Ob and Db epididymal fat pads weighed significantly more at 1.69 ± 0.09g and 1.91 ± 0.15g versus the control at 0.218 ± 0.02g ($p<0.0001$) (see Figure 15B). Conversely, the average testis weight of the Ob and Db mouse models were significantly less at 0.0771 ± 0.006g ($p=0.0026$) and 0.0843 ± 0.06g ($p=0.0185$) as compared to the control mice at 0.107 ± 0.003g (see Figure 15C). Similarly, the average epididymis weight of the Ob and Db mice was significantly less at 0.0313 ± 0.003g ($p=0.0005$) and 0.0345g ± 0.001 ($p=0.0044$) when compared to the control mice at 0.0454 ± 0.002g (see Figure 15D).

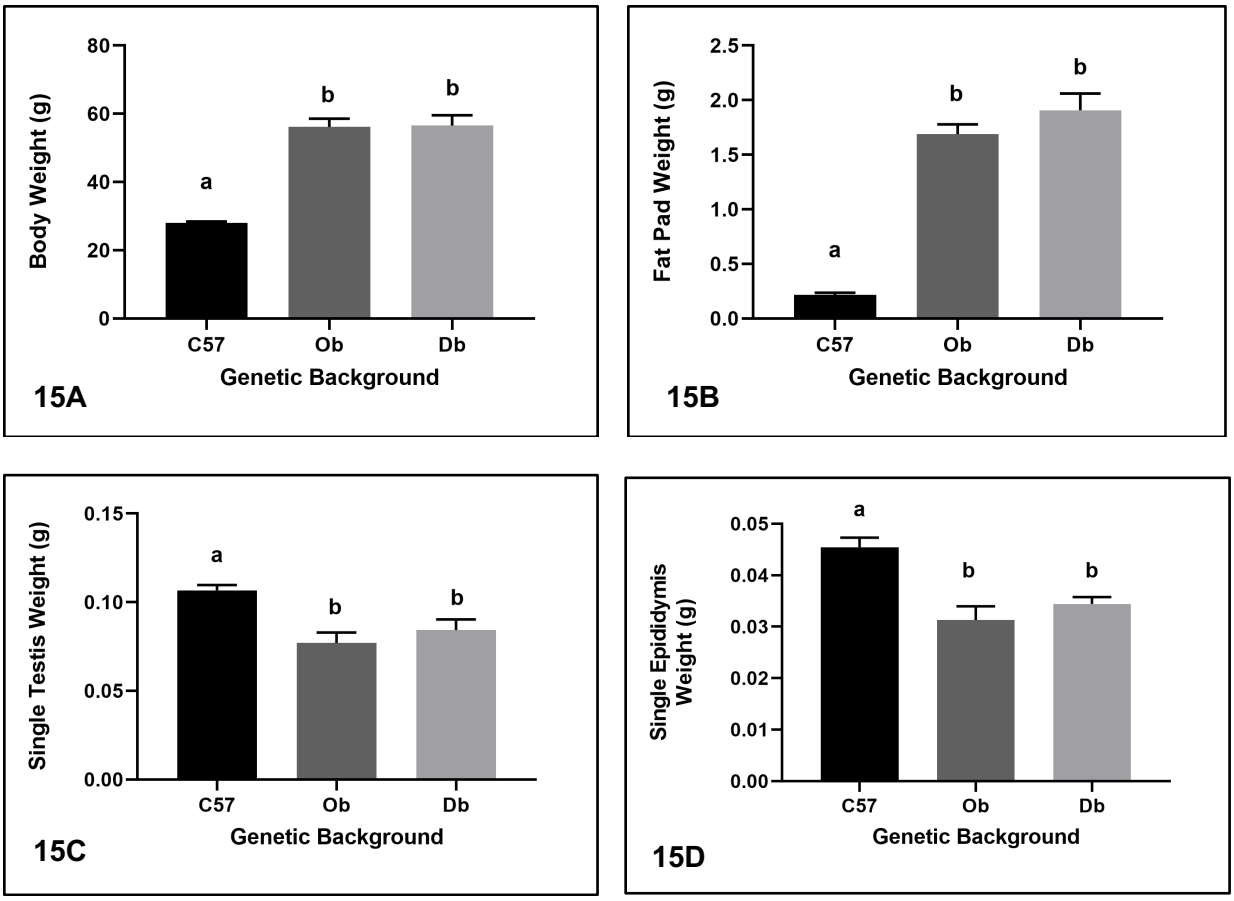


Figure 15: Body and Tissue Weights of C57, Ob and Db Mice
15A) Significantly larger body weight of Ob and Db compared to C57. 15B) Significantly larger Ob and Db fat pad weight versus C57. 15C) Significantly smaller testis weight in Ob and Db compared to C57. 15D) Significantly smaller epididymis weight in Ob and Db versus C57. *("a" vs "b" indicates significant difference between genetic models)*

Unmeasured, the distance from the superior edge of the testis to the initial segment of the epididymis previously appeared farther upon visual inspection. Quantitatively it was shown to be significantly longer in the Ob and Db models at 6.02 ± 0.41mm and 6.40 ± 0.36mm as compared to 2.91 ± 0.26mm in the control model ($p<0.0001$) (Figure 16).

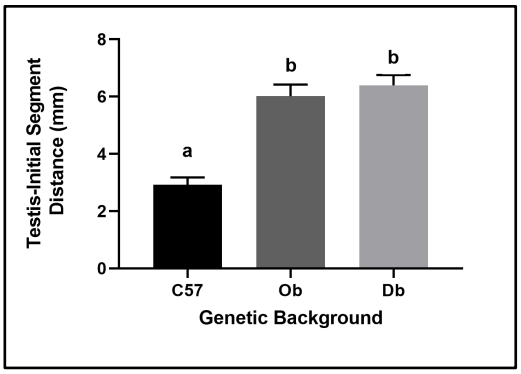


Figure 16: Distance between Testis and Initial Segment
There is an increased testis to initial segment of the epididymis in the Ob and Db mouse models as compared to the C57. *("a" vs "b" indicates significant difference between genetic models)*

The average sperm per epididymal cauda was significantly lower in the Ob and Db models at $8.35x10^6 \pm 2.06$ and $5.67x10^6 \pm 1.49$ respectively ($p<0.0001$) as compared to the control mice at $24.7x10^6 \pm 0.811$ (Figure 17A). When calculating the average as sperm per mg of cauda, the Db model still had significantly less sperm at $1.07x10^6 \pm 0.164$ when compared to C57 at $1.81x10^6 \pm 0.102$ ($p=0.0300$), though neither were significantly different from the Ob at $1.27x10^6 \pm 0.250$ ($p=0.7409$;$p=0.1197$) (Figure 17B).

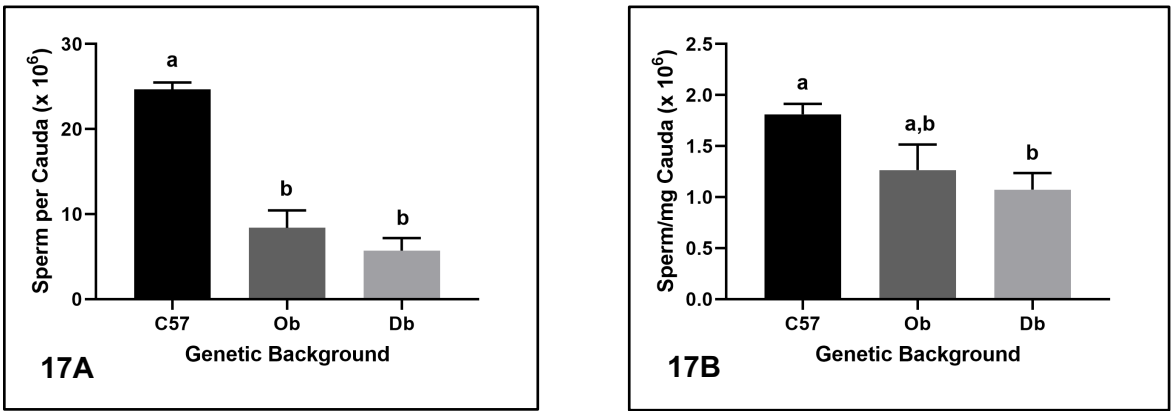


Figure 17: Epididymal Sperm Counts
17A) The sperm per cauda was significantly lower in the Ob and Db as compared to the C57. 17B) The sperm per mg of cauda was significantly lower in the Db as compared to the C57, but the Ob was not significantly different from the C57 or Db. *("a" vs "b" indicates significant difference between genetic models)*

Immunohistochemistry (IHC)

In regards to the basic anatomy of the samples, the tubule segments contained pseudostratified epithelium which lined the basement membrane. Within the epithelial cell layer, there were principal cells with stereocilia projecting into the lumen. The lumen contained sperm and increased in size going down the segments, which led to a decrease in epithelial cell height. Outside of the basement membrane, the tubule was surrounded by smooth muscle and then dense connective tissue was in between all the tubule segments (Figures 18 A-F).

The initial segment (IS)/caput and corpus segments incubated with NRS were negative (Figures 18A and 18C). The cauda was mostly negative except for some non-specific immunostaining of the dense connective tissue surrounding the tubule (Figure 18E). The caput was negative for leptin receptor immunostaining (Figure 18B). The corpus and cauda conversely, had positive immunostaining, specifically in the principal cell cytoplasm (Figures 18D and 18F).

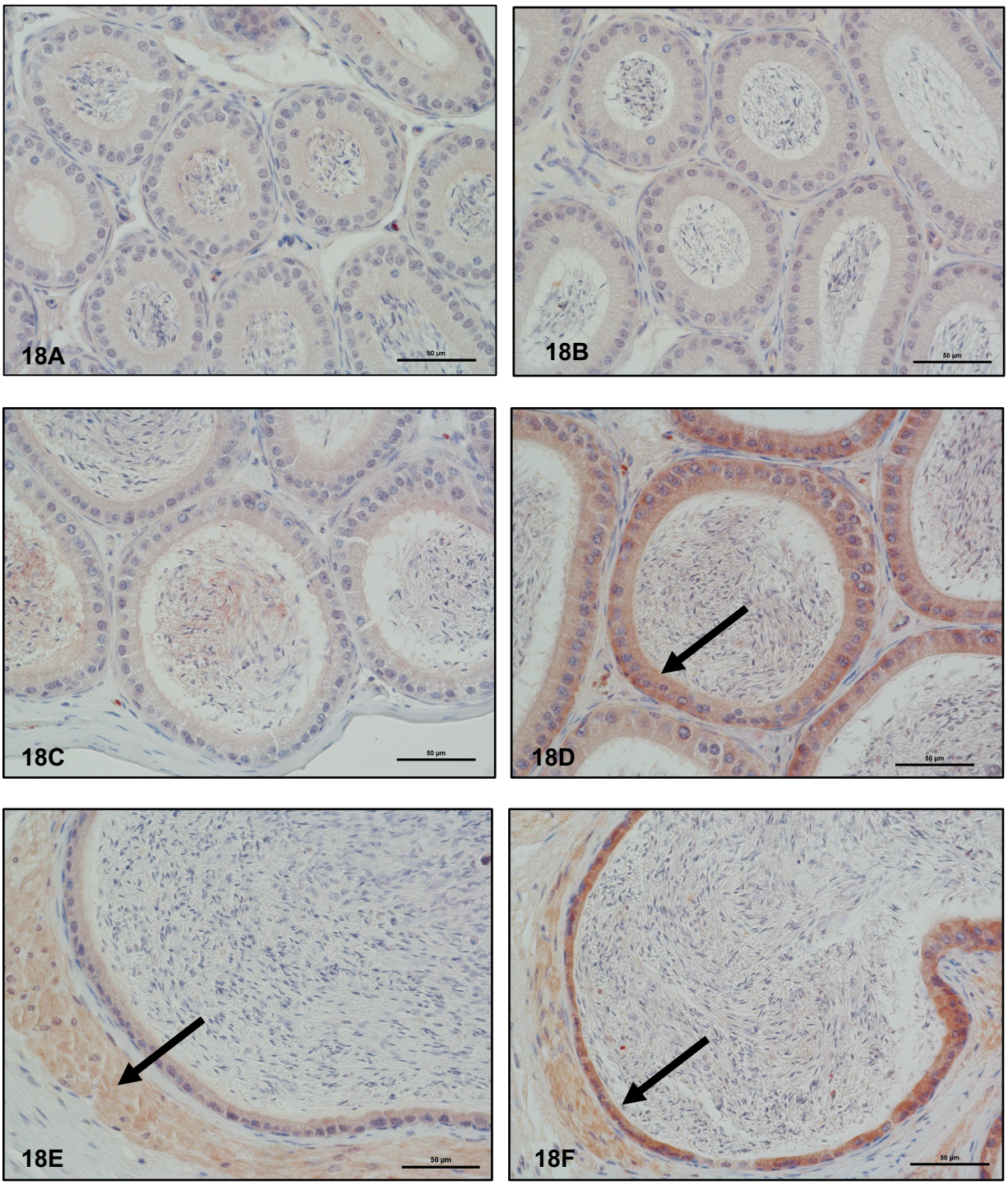


Figure 18: IHC Images of the C57 Caput, Corpus and Cauda (50 μm Scale)
18A) IS/Caput segment with NRS. 18B) IS/Caput segment when stained for leptin receptor. 18C) Corpus segment with NRS. 18D) Positive principal cell cytoplasm in corpus segment when stained for leptin receptor (arrow). 18E) Cauda segment with NRS; arrow indicates non-specific interstitium staining. 18F) Positive principal cell cytoplasm in cauda segment when stained for leptin receptor (arrow).

Flow Cytometry

Inguinal lymph nodes

The percentage of CD11b+ cells from the gated live cells in the inguinal lymph nodes was not significantly different between genetic backgrounds. The percentage in C57 was 0.332 ± 0.041%, Ob was 0.282 ± 0.025%, and Db was 0.335 ± 0.070% (p=0.74) (Figure 19). The percentage of CD11b+ cells that were then also CD11c+ and F4/80+ was significantly larger in the Ob and Db models at 53.5 ± 9.17% and 58.7 ± 5.7% when compared to the C57 model at 23.4 ± 2.84% (p=0.01; p=0.002) (Figure 20A). The percentage of CD11b+ cells that were CD11c+ but F4/80- was significantly lower in Ob and Db mice at 30.4 ±7.36% and 25.9 ± 3.94% as compared to C57 mice at 56.3 ± 2.41% (p=0.005; p=0.0009) (Figure 20B). The percentage of CD11b+ cells that were F4/80+ but CD11c- was not significantly different among mouse models with C57 at 3.71 ± 1.44%, Ob at 3.95 ± 1.18%, and Db at 5.26 ± 1.05% (p=0.64) (Figure 20C).

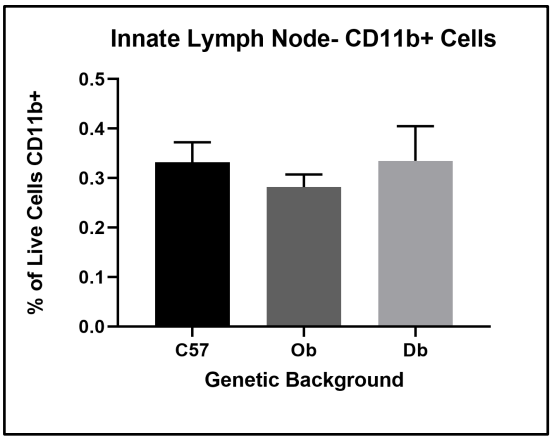


Figure 19: Percentage of Cells Expressing CD11b in Inguinal Lymph Nodes
Percentages of live cells in the inguinal lymph nodes that express high amounts of CD11b (innate immune cell marker) are not statistically different between the genetic backgrounds.

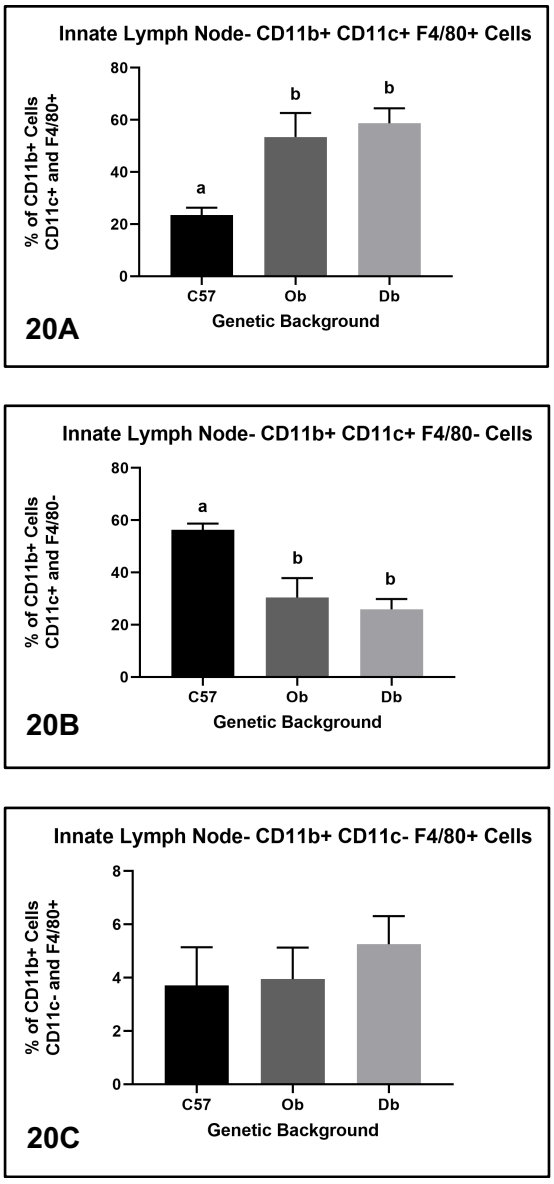


Figure 20: Expression of CD11c and F4/80 among CD11b+ cells from Inguinal Lymph Nodes

20A) Percentage of the CD11b+ cells that also express high amounts of CD11c (DC cell marker) and F4/80 (Mφ marker) are significantly higher in leptin-modified models when compared to C57. 20B) Percentage of the CD11b+ cells that express high amounts of CD11c but not F4/80 was smaller in the Ob and Db models when compared to the C57 model. 20C) Percentage of the CD11b+ cells that express high amounts of F4/80 but not CD11c was not significantly different among the genetic backgrounds.

When looking at the panel for adaptive immune cell markers in the inguinal lymph nodes, none of the genetic backgrounds were significantly different for any of the markers. For the frequency of T-helper (Th) cells, which highly express CD4 (Th marker) and have a low expression of CD19 (B cell marker), percentages were not significantly different with C57 at 21.3 ± 0.975%, Ob at 20.9 ± 3.83%, and Db at 18.6 ± 2.06% (p=0.685) (Figure 21A). The frequency of cytotoxic T cells (Tc), which have high expression of CD8 (Tc marker) and low expression of CD19 (B cell marker) were also not significantly different at 29.3 ± 1.27% for C57, 24.7 ± 3.54% for Ob, and 22.4 ± 1.32% (p=0.0833) (Figure 21B). Lastly, B cell frequency which highly express CD19 (B cell marker) and have low expression of CD4, was also not significantly different at 35.5 ± 2.38% for C57, 38.3 ± 8.36% for Ob, and 40.9 ± 2.79% for Db (p=0.725) (Figure 21C).

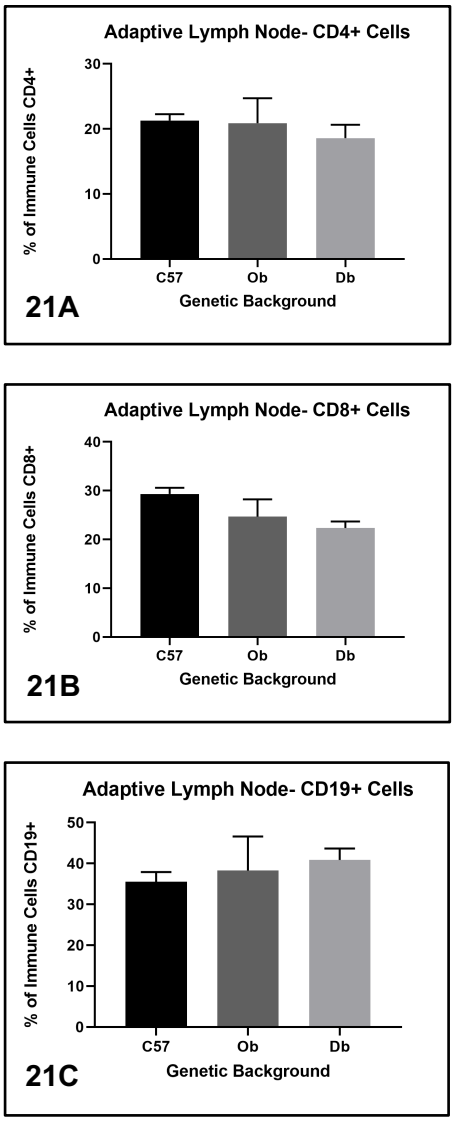


Figure 21: Adaptive Immune Cell Marker Expression in Cells from Inguinal Lymph Nodes

21A) No significant difference between genetic models in frequency of cells that have high expression of CD4 and low expression of CD19 (gated as Th cells). 21B) No significant difference between genetic models in frequency of cells that have high expression of CD8 and low expression of CD19 (gated as Tc cells). 21C) No significant difference between genetic models in frequency of cells that have high expression of CD19 and low expression of CD4 (gated as B cells).

Epididymal segments

After looking at the inguinal lymph nodes, we analyzed and compared the innate and adaptive immune cell markers in the IS/caput, corpus and cauda epididymal segments for the three mouse models. The percentage of CD11b+ cells from the gated live cells was significantly different between epididymal segments of the same genetic background. Specifically, the percentages in the IS/caput segments of all three models were significantly higher than the cauda with C57 at 2.43 ± 0.37% and 0.86 ± 0.57% (p=0.033), Ob at 3.21 ± 0.23% and 0.72 ± 0.16% (p=0.0004), and Db at 2.97 ± 0.32% and 1.10 ± 0.41% (p=0.0088). In addition to being larger than the cauda, the Ob mouse model's IS/caput percentage was also significantly larger than the corpus' which was 1.1 ± 0.11% (p=0.003). There were no significant differences between genetic backgrounds of the same epididymal segment (p=0.78) (Figure 22). When looking at CD11b+ cells that were then also CD11c+ and F4/80+ there was no significant difference between genetic backgrounds or epididymis segments (p=0.21; p >0.05) (Figure 23A). In percentage of cells that were CD11b+ and then were CD11c+ but F4/80-, the only significant difference between epididymal segments of the same genetic background was in the C57 model. In this model, the cauda at 8.91± 4.66% was significantly higher than the IS/caput and corpus at 1.28 ± 0.20% and 3.35 ± 0.64 (p=0.0041; p= 0.045) (Figure 23B). In regards to the difference in percentages in that same cell type between genetic models in the same epididymal segment, the Ob and Db models had significantly lower percentages in the cauda at 3.0 ± 0.30% and 2.44 ± 0.30% as compared to the C57 at 8.91 ± 4.66% (p=0.031; p=0.017) (Figure 23B). Lastly, when comparing the percentage of CD11b+ cells that were then F4/80+ but

CD11c-, the only significant difference between epididymal segments of the same genetic background was that the corpus of C57 at 16.8 ± 6.9% was significantly higher than C57's cauda at 5.68 ± 0.65% (p=0.013) (Figure 23C). In regard to differences in percentages of the same cell type among different genetic background with the same epididymal segment, the Db corpus at 6.74 ± 1.1% was significantly lower than C57 at 16.8 ± 6.9% (p=0.027) but neither was significantly different from the Ob at 8.47 ± 1.5% (p=0.89; p=0.079) (Figure 23C).

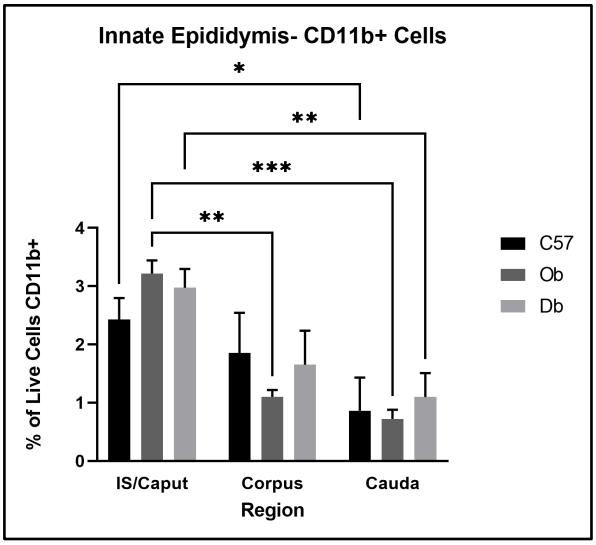
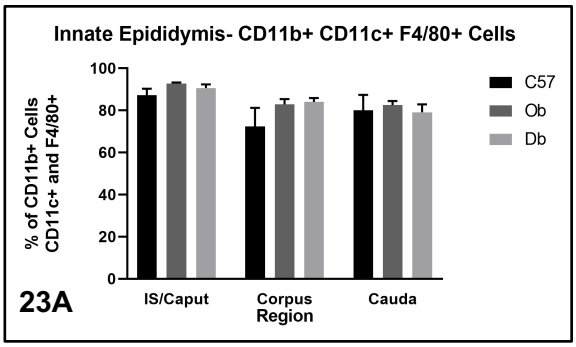
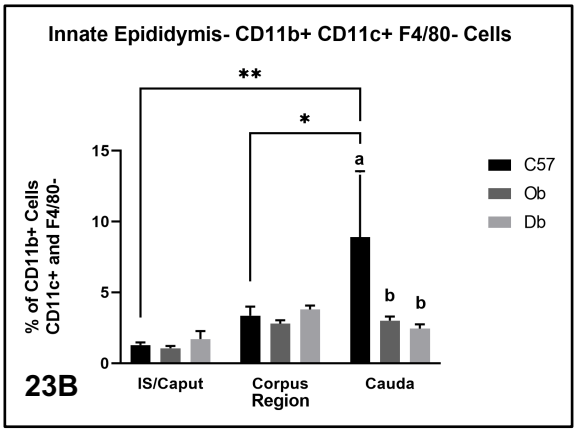


Figure 22: Percentage of Cells Expressing CD11b in Epididymal Segments (IS/Caput, Corpus, and Cauda)
The percentage of CD11b+ cells in the gated live cells differed among epididymal segments of the same genetic background. All three genetic backgrounds had a higher percentage in the IS/caput than the cauda. Additionally, the Ob mice had a significantly larger percent in the IS/caput as compared to the corpus. *("*" indicates significant difference between epididymal segments of the same genetic background; "a" vs "b" indicates significant difference between genetic backgrounds of the same epididymal segment)*





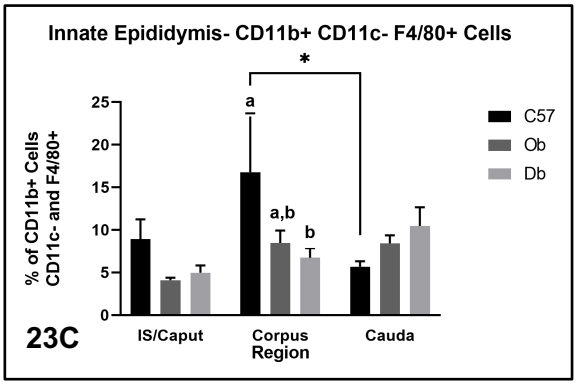
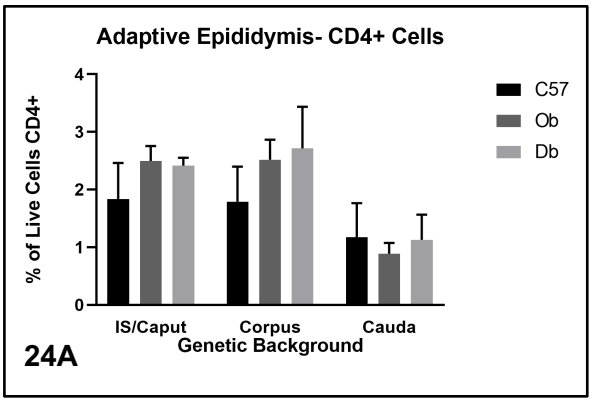
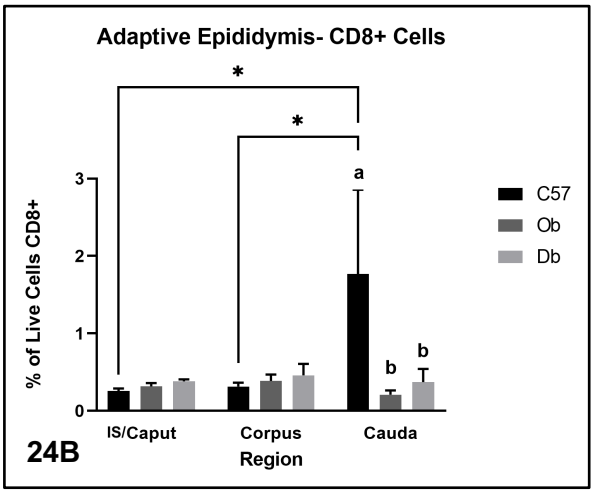


Figure 23: Expression of CD11c and F4/80 Among CD11b+ Cells from Epididymal Segments (IS/Caput, Corpus, and Cauda)

23A) There was no significant differences in percentage of CD11b+ cells that were also CD11c+ and F4/80+ between either different genetic backgrounds or epididymal segments in the mouse models. 23B) The C57 mouse model's cauda percentage of CD11b+ cells that were also CD11c+ but F4/80- was significantly higher than the IS/caput and corpus. Additionally, the Ob and Db caudas' percentages were significantly lower than the C57 cauda. 23C) The C57 corpus percentage was significantly higher in CD11b+ cells that were then F4/80+ and CD11c- when compared to C57's cauda. Additionally, the Db's corpus percentage was significantly lower than the C57 corpus. *("*" indicates significant difference between epididymal segments of the same genetic background; "a" vs "b" indicates significant difference between genetic backgrounds of the same epididymal segment)*

The adaptive immune system markers of CD4, CD8, and CD19 were then analyzed for the three epididymal segments among the different genetic backgrounds. There was no significant difference in the percentages of CD4+ cells between genetic models or epididymal segments ($p=0.44$; $p>0.05$) (Figure 24A). There were significant differences though in percentages of CD8+ cells. Between genetic backgrounds, the percentage of CD8+ cells in the cauda was significantly lower in the Ob at $0.21 \pm 0.054\%$ and the Db at $0.37 \pm 0.17\%$ as compared to the C57 at $1.77 \pm 1.1\%$ ($p=0.013$; $p=0.029$) (Figure 24B). In regards to CD8+ cells between epididymal segments of the same genetic background, there was also a significant increase in CD8+ cells in the cauda of C57 mice as compared to their IS/caput and corpus which are $0.26 \pm 0.031\%$ and $0.31 \pm 0.05\%$ ($p=0.017$; $p=0.022$) (Figure 24B). There was no significant difference in the percentage of CD19+ cells between genetic models or epididymal segments ($p=0.12$; $p=0.16$) (Figure 24C).





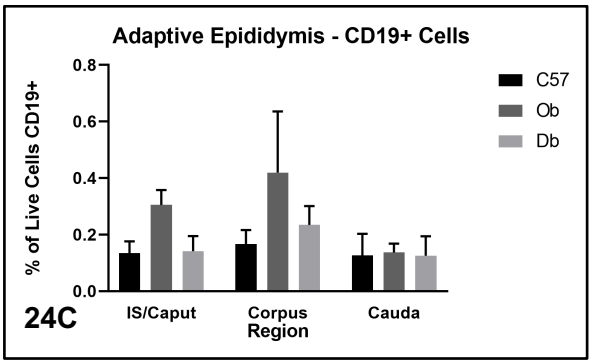


Figure 24: Adaptive Immune Cell Marker Expression in Cells from Epididymal Segments (IS/Caput, Corpus, and Cauda)

24A) There is no significant difference in percentages of CD4+ cells between the genetic backgrounds or the epididymal segments. 24B) The percentage of CD8+ cells was higher in the C57 cauda compared to the C57 IS/caput and corpus. Also, the Ob and Db's cauda percentages are lower than the C57 cauda's percentage. 24C) There is no significant difference in percentages of CD19+ cells between the genetic backgrounds or the epididymal segments. *("*" indicates significant difference between epididymal segments of the same genetic background; "a" vs "b" indicates significant difference between genetic backgrounds of the same epididymal segment)*

Chapter 5 Discussion and Conclusions

This study includes the first immunohistochemical description of leptin receptor expression in the mouse epididymis and the first description of immune cell composition in the Ob and Db mouse epididymis.

Previous research show that Ob and Db mice have increased body weight and fat over the C57 (Kobayashi *et al.*, 2000; Wasim *et al.*, 2016; Wauman *et al.*, 2017). Our Ob and Db mice have double the body weight and eight times the epididymal fat pad weight versus the C57 mice. Previous research also shows that Ob and Db mice have reduced reproductive parameters including a decrease in testosterone, fewer spermatocytes and mature spermatozoa, and decreased testicular mass (Mounzih *et al.*, 1997; Cleary *et al.*, 2001; Bhat *et al.*, 2006; Hoffmann *et al.*, 2016; Martins *et al.*, 2017). In accordance with this previous research, the testis and epididymal weights were significantly reduced in the Ob and Db mice as compared to the C57. Additionally, the average sperm per cauda was reduced in Ob and Db mice versus the control. Not reported before, but quantified here, is that the distance from the superior edge of the testis to the initial segment of the epididymis is double the length in the Ob and Db mouse models as compared to the C57 mice. This increase in distance might indicate stretching of the efferent ducts and be a potential cause for the decrease in epididymal sperm count, and thus decreased fertility, seen in these leptin-altered mouse models.

Future work on the topic of the effects of altered leptin signaling in the post-testis environment could develop from the now quantified increased distance between the superior edge of the testis and the initial segment of the epididymis. As mentioned in our results, the obese Ob and Db mouse models had double the length in between

these structures as compared to the C57 wild-type. This increase in distance could indicate a stretching of efferent ducts in between the two structures and present a possibility that the efferent ducts are altered due to this change in arrangement. If the efferent ducts are altered due to stretching, this increased distance could account for the decrease in sperm count previously seen going from the testis to the epididymis in Db mice (Pearl and Doherty, 2018). Alteration of the efferent ducts' configuration could be investigated by dissecting the efferent ducts out from the epididymal fat pad and comparing their appearances between the C57 and leptin-altered mouse models. One idea is that the decreased sperm counts could be due to breakages in the efferent ducts due to stretching. The difficulty with this idea is that efferent ducts are very small and might be hard to dissect out without breaking connections between ducts. Additionally, blind-ending tubules are commonly seen among the efferent ducts in mammals and can be mistaken for broken efferent ducts (Guttroff *et al.*, 1992). There are histological differences though to these blind-ending tubules like containing small diameters, are surrounded by thick dense connective tissue, and their epithelium stain more intensely (Guttroff *et al.*, 1992). Therefore, investigating the stretching and potential breakage of the efferent ducts might be difficult.

Immunohistochemistry on C57 epididymal segments revealed that leptin-receptor is expressed in the mouse epididymis. Expression was observed in the principal cell cytoplasm of the corpus and cauda segments, but not in the initial segment or caput. Therefore, not only does leptin have an indirect effect on male reproductive structures through the HPG axis (Cunningham *et al.*, 1999; Irwig *et al.*, 2004; Quennell *et al.*,

2009; Teerds *et al.*, 2011; Tsatsanis *et al.*, 2015), but leptin could also have a direct effect on the corpus and cauda of the epididymis.

Leptin has an indirect effect on the epididymis due to its interaction with the HPG axis. Leptin, which is mostly produced in adipose tissue, binds with its receptors on KISS1 neurons to stimulate the release of GnRH in the hypothalamus (Irwig *et al.*, 2004; Scotece *et al.*, 2014). GnRH then travels to the anterior pituitary to allow production and release of LH and FSH, which travels to the testis. LH attaches to the Leydig cells in the interstitial space to promote the synthesis of testosterone. Both testosterone and FSH can stimulate spermatogenesis in Sertoli cells (Robaire *et al.*, 2010b). Mice with leptin signaling interrupted have testicular atrophy and impaired spermatogenesis (Martins *et al.*, 2017).

Our results indicate that the corpus and cauda of the epididymis can also be directly impacted by leptin. The corpus is where sperm mature, start developing motility, and acquire fertilizing ability. The cauda is where the sperm are stored until ejaculation and anywhere from 50-80% of sperm that are in the epididymis are in the cauda at any given time (Robaire and Hinton, 2015). Cauda epithelial cells also secrete factors to help maintain the luminal environment and a pH that keeps sperm in a “dormant” state. Another suggestion is that epididymal secretions cause modifications to the sperms’ surface, which obscures sperm antigens (Eddy *et al.*, 1985). Additionally, while the majority of fluid absorption to increase sperm concentration occurs in the initial segment and caput, additional absorption occurs in the corpus and cauda (Turner, 1984; Robaire and Hinton, 2015). Our finding of leptin-receptor in the principal cells of the corpus and cauda is relevant as principal cells are vital for fluid and nutrient exchange in the

epididymis (Robaire and Hinton, 2015). Therefore, since leptin receptor is expressed in the corpus and cauda, it's a possibility that these epididymal functions are partially regulated by leptin and may be impaired when leptin signaling is altered.

We hypothesized that there would be a difference in immune cell percentages for Ob and Db mice as compared to the C57 mice. We reasoned this since leptin receptors have been found on leukocytes including monocytes/macrophages, neutrophils, NK cells, DC cells and T and B lymphocytes (Zhao *et al.*, 2003; Mattioli *et al.*, 2005; Bruno *et al.*, 2005; Papathanassoglou *et al.*, 2006; Carlton *et al.*, 2012), then altering leptin signaling would cause changes to the immune cell concentrations. The inguinal lymph nodes in our study did not show any significant difference in adaptive immune cell population percentages, though there were some differences in the innate immune cell population percentages. The percentage of CD11b+ cells that were CD11c+ and F4/80+ were increased in Ob and Db mice as compared to C57 mice. Conversely, the percentage of CD11b+ cells that were CD11c+ and F4/80- were decreased in Ob and Db mice as compared to the C57. While there were few significant differences in immune cell profiles in the inguinal lymph nodes, there were more changes in the immune cell percentages in the epididymis.

The cell populations that showed significant difference between epididymal segments of the same genetic background were 1) CD11b+ cells, 2) CD11b+/CD11c+/F4/80- cells, 3) CD11b+/CD11c-/F4/80+ cells, and 4) CD8+ cells. The CD11b+ cells were significantly higher in the C57's and Db's IS/caput as compared to the cauda. The Ob also had a significantly higher CD11b+ cell percentage in the IS/caput, which was higher than both the corpus and the cauda. In regards to the

CD11b+/CD11c+/F4/80- cells, only the C57 showed any differences, with the cauda having a significantly higher percentage as compared to both the IS/caput and corpus. Likewise, the CD8+ cells showed an increased percentage in the C57 cauda as compared to the C57 IS/caput and corpus. Similarly, with the CD11b+/CD11c-/F4/80+ cells, only the C57 showed any difference, with now the corpus being significantly higher in percentage as compared to the cauda. Overall, in the C57 model the CD11b+ cells and the CD11b+/CD11c-/F4/80+ cells tended to decrease going down the epididymis, while the CD11b+/CD11c+/F4/80- cells and CD8+ cells increased going down the epididymis. The only cell population that changed with epididymal segment in the Ob and Db models was CD11b+, which like C57, decreased from IS/caput to cauda.

The trend of decreasing immune cell populations down the epididymal segments is consistent with previous research in the murine epididymis. Previous research found that macrophage cells (F4/80+), DC cells (CD11c+) and CD8+ (Tc) cells were more prevalent in the caput epididymis as compared to the cauda (Flickinger *et al.*, 1997; Hedger, 2015). We found that CD11b+ cells (all innate immune cells) and CD11b+/CD11c-/F4/80+ cells (potential Mφ cells) in C57 agreed with previous research, while the CD11b+/CD11c+/F4/80- cells (potential DC cells) and CD8+ cells (Tc cells) in C57 showed the opposite trend. Still, one study did agree with our trend of increased potential dendritic cells in the cauda as compared to the more proximal segments (Mendelsohn *et al.*, 2020).

It's been argued that this higher amount of dendritic cells seen in the cauda is because it's the location for sperm storage. These DC cells might protect the sperm from the immune system while they are awaiting ejaculation. Additionally, since bacteria

external to the body can enter distally in the reproductive tract, these DC cells could be used to help regulate an immune response against invading pathogens as DC cells activate T cells (Mendelsohn *et al.*, 2020). Alternatively, the macrophage cell population is reported to be higher in the caput as compared to the cauda (Mendelsohn *et al.*, 2020). It's been suggested that this cell population may be used to establish an immune privileged environment for sperm, against both pathogens and autoimmune responses. Macrophages in the epididymis accomplish this by their dendrites that extend out into the epididymal lumen to sample for potential antigens. Macrophages are known for their role in clearing debris and initiating an innate immune response (Hume, 2008; Mendelsohn *et al.*, 2020).

The cell populations that showed any significant differences between genetic backgrounds of the same epididymal segment were 1) CD11b+/CD11c+/F4/80- cells, 2) CD11b+/CD11c-/F4/80+ cells, and 3) CD8+ cells. For CD11b+/CD11c+/F4/80- cells, the Ob's and Db's cauda had significantly smaller percentages when compared to the C57 cauda. Likewise, when looking at the CD8+ cells, the Ob and Db caudas had significantly smaller percentages than the C57 cauda's. Along the same lines, when looking at the CD11b+/CD11c-/F4/80+ cells, the Db corpus had a significantly smaller percentage than the C57 corpus' but neither was different from the Ob's. Overall, the differences that were seen in immune cell profiles between genetic backgrounds tended to show a decrease in percentage of immune cells with altered leptin function.

The decrease in percentage of immune cells in epididymis Ob and Db mice is consistent with previous literature of non-epididymal immune cell populations in Ob and Db mice. We found that CD11b+/CD11c+/F4/80- cells (probable DC cells),

CD11b+/CD11c-/F4/80+ cells (probable Mφ cells), and CD8+ cells (Tc cells) were all reduced in various portions of the Ob and Db mouse models as compared to C57. Previous literature states that leptin increases migration of DC cells as well as increases their longevity, while leptin-altered mice have increased apoptosis, reduced maturation, and poor capacity to stimulate T cell proliferation (Lam *et al.*, 2006; Moraes-Vieira *et al.*, 2014; Francisco *et al.*, 2018). Additionally, leptin promotes the macrophage function, while leptin-altered models have decreased functioning (Gruen *et al.*, 2007; Mancuso *et al.*, 2012; Amarilyo *et al.*, 2014; Dayakar *et al.*, 2016). In regards to Tc cells, leptin causes an increase in effector T cells and reduces the amount of Tregs (Wagner *et al.*, 2013). Conversely, decreased amount of leptin leads to a decrease in the amount of Tc cells and Tregs are increased (Rosa *et al.*, 2007).

A decrease in epididymal innate immune cells could be problematic for the sperm of leptin-signaling altered mice. Mφ and DC cells are thought to protect the sperm from both pathogens and an immune response to self. Sperm antibodies are thought to be the most common cause of unexplained infertility, with their presence in up to 10% of male infertility cases in developed countries (Baker *et al.*, 1983; Lenzi *et al.*, 1997). Moreover, the decrease in tolerance mechanisms for the sperm could be a bigger problem since the blood-epididymal barrier is not as elaborate as the blood-testis barrier and could therefore let outside immune cells interact with the sperm more easily (Hedger, 2015).

The immune cell populations that did not show any significant differences in the epididymis were 1) CD11b+/CD11c+/F4/80+ cells 2) CD4+ cells and, 3) CD19+ cells. The opposing findings to previous research and limited number of significant differences

seen in the immune cell profiles may be due to our chosen immune cell markers and gating strategies.

One difficulty when deciding how to identify epididymal immune cells is in regards to the innate immune cells, specifically the mononuclear phagocytes (MP's) macrophage and dendritic cells. Previous research describes epididymal immune cells as having an overlap of innate immune cell markers on their surface, so previous studies have called these groups of cells collectively as heterogenous MP's. Murine mononuclear phagocytes (MP's) typically express markers such as CD45 (all immune cells), CD11b (myeloid-lineage immune cells), CD206 (in macrophages and dendritic cells), CD11c (in dendritic cells), and F4/80 and CD64 (in macrophage cells) (Mullen *et al.*, 2003; Da Silva *et al.*, 2011; Azad *et al.*, 2014; Mendelsohn *et al.*, 2020). Previous research has found that the typical CD11c surface marker of dendritic cells can also be expressed on macrophages, which usually express F4/80 (Hume, 2008). This is relevant to our study as our largest population of CD11b+ cells in the epididymis were both CD11c+ and F4/80+.

All of these innate immune cell markers are commonly used in research regarding the immune cell profiles in the murine epididymis. Shum *et al.* (2014) found epididymal epithelial MP cells that express either CD11c or F4/80 are seen with their nucleus in the basal region of the epididymal epithelium and are more common in the initial segment of the epididymis. Both these MP cell types project multiple intraepithelial dendrites that reach towards the lumen, though decrease in length as get closer to the cauda. Mullen *et al.* (2003) describes a similar type of F4/80+ cells with identical sounding shape and dispersion but was CD11b-, which is not common for an innate immune cell (Mullen *et*

al., 2003). The MP cells seen in Shum *et al.* (2014) appear morphologically similar to basal cells (BC) of the epididymis, which can also sample the luminal environment, though BC only have one projection coming off of each cell in the initial segment and the projection disappears once in the caput. Additionally, these BC cells express KRT5 (BC marker keratin 5) and not F4/80, while MP's express F4/80 and not KRT5 (Shum *et al.*, 2014). Da Silva et al (2011) additionally describes several cell populations with the stellate morphology in between epithelial cells, which they call DC cells. One of the three populations is CD11c+, CD11b+, 103+ and F4/80+. The second is also CD11c+, CD11b+, but 103- and F4/80-. The third population is CD11c- and F4/80+ (Da Silva *et al.*, 2011). Recently, Mendelsohn *et al.* (2020) described DC cells in the epididymal epithelium as being CD11c+/CD64-/ MHCII+ and Mφ cells as being CD11c+/CD64+/F4/80+. They additionally found that the Mφ -like cells were more common in the initial segment, whereas the dendritic-like cells were more common in the cauda (Mendelsohn *et al.*, 2020).

In regards to the epididymal interstitium, Mullen *et al.* (2003) also found F4/80+ interstitial cells, though they were also CD45+ and CD11b-, which CD11b was not tested in Shum *et al.* (2014) (Mullen *et al.*, 2003). Additionally, CD11c+ cells are expressed in the interstitium, though less frequent than F4/80+ cells (Shum *et al.*, 2014). On the other hand, Da Silva et al. (2011) found an interstitial population of CD11c- cells that were CD206+ (Da Silva *et al.*, 2011). Therefore, due to the overlap of immune cell surface markers, more research needs to be done on further differentiating the molecular profile of epididymal innate immune cells. This unique epididymal immune

cell profile though might contribute to why the epididymis has such a low rate of cancer, with only 0.03% of all male cancers are in the epididymis (Yeung *et al.*, 2012).

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