THESIS

MODELING HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1) INFECTION IN THE MALE REPRODUCTIVE TRACT (MRT) USING HUMANIZED MICE

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ABSTRACT

MODELING HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1) INFECTION IN THE MALE REPRODUCTIVE TRACT (MRT) USING HUMANIZED MICE

Thirty-five million people are currently living with human immunodeficiency virus (HIV) globally. While 9.7 million infected people are receiving antiretroviral therapy, 2.3 million people are newly infected every year. Transmission via semen is one of the most prevalent methods of HIV-1 transmission, accounting for up to 80% of new infections every year. However, the source of infected leukocytes and the method of virus replication in semen and/or in the male reproductive tract (MRT) is not well described. It has been shown that infected germinal cells such as spermatogonia and spermatocytes as well as infected leukocytes are present in the MRT of HIV infected human patients and simian immunodeficiency virus (SIV) infected macaque models. Studies have also shown that the seminal viral strain differs from the serum viral strains in HIV infected human patients. Furthermore, HIV-1 continues to persist in the semen and in the MRT of infected male patients on highly active antiretroviral therapy (HAART) despite undetectable serum viral load. To investigate the spread and replication of HIV-1 in the MRT in human patients, development of an animal model is essential.

Use of humanized mice allows researchers to study the transmission, pathogenesis and drug interactions of HIV-1 in the human immune system *in vivo*. The human-hematopoietic stem cell (hu-HSC) mouse model created by transplantation of HSC into $Rag2^{-/-}\gamma c^{-/-}$ mice, has shown excellent susceptibility to HIV-1 and ability to sustain high viremia for months. However, so far no studies have been conducted to demonstrate whether or not HIV-1 infiltrates and replicates in

the semen or MRT of infected humanized mice. The presence of HIV-1 in the semen or MRT of infected humanized mice could play a vital role in using humanized mice for describing the pathogenesis mechanism of the virus in semen and drug development in reducing seminal HIV-1 viral load in human patients.

The aim of this study is to model HIV-1 infection in the MRT using humanized mice to 1) evaluate the severity of disease using histologic scoring of MRT and morphometric analysis of the testis and epididymis in viremic and negative control mice, 2) characterize leukocytes in the testis and epididymis of viremic and negative control mice via immunohistochemistry, 3) assess the presence of HIV-1 viral RNA in the MRT of viremic mice via *in situ* hybridization (HIV-1 mRNA), and 4) assess seminal viral loads from vaginal plugs of viremic mice via qRT-PCR.

We showed that both viremic (16/25; 64%) and negative control (9/28; 32%) mice developed histopathologic lesions in their testis and epididymis parallel to the lesions found in the testes and epididymis of chronically infected HIV-1 and AIDS patients. When broken down by two age groups, 5-7 month old and 8-12 month old, the viremic mice from both age groups showed significantly higher frequency of testicular oligospermia/azoospermia ($p<0.05$) compared to the age-matched negative control groups. Five to seven month old viremic mouse group showed significantly higher frequency of testicular germinal cell degeneration ($p<0.05$) compared to the age-matched negative control group. The histopathological lesion scores were significantly higher in both 5-7 month old and 8-12 month old viremic mouse group compared to that of age-matched negative control mouse groups ($p<0.05$), suggesting the lesions in viremic mice are related to HIV-1 infection. Morphometric analysis of the testicular and epididymal epithelium however, did not show any statistical differences on the levels of germinal cell degeneration between the viremic (n=8) and negative control (n=4) group.

We were able to identify the presence of CD4 positive cells in viremic (4/22; 22%) and negative control (7/17; 41%) mice in the testicular and epididymal interstitium. The majority of the CD4 cells were associated with histopathologic lesions within primary reproductive organs. RNA *in situ* hybridization showed successful infiltration of HIV-1 infected cells in the MRT of viremic mice (9/11; 82%), most often found in penis or prepuce (6/11; 55%), and less often in seminal vesicles (4/11; 9%), testes (3/11; 27%), epididymides (1/11; 9%) and prostate glands (1/11; 9%), suggesting that HIV-1 indeed can infect and infiltrate the MRT via CD4 positive cells. Vaginal plugs failed to show detectable HIV-1 viral loads using qRT-PCR. Our study offers first glance at HIV-1 infection in the MRT of viremic humanized mice and provides further evidence that leukocytes play an important role in HIV-1 infection in the MRT.

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Chapter 1

Introduction: Review of Literature

1.1 HIV and Epidemiology

Human Immunodeficiency virus (HIV) is an enveloped, positive-sense, single-stranded RNA virus of the lentivirus family. The lentivirus family is recognized as five different serogroups based on the host mammal species. This includes the bovine lentivirus group, equine lentivirus group, feline lentivirus group, ovine/caprine lentivirus group and the primate lentivirus group in which HIV and Simian immunodeficiency virus (SIV) are included. Lentiviruses have a unique ability to target myeloid lineage cells such as macrophages and dendritic cells as well as CD4 lymphocytes (Sellon et al., 1992; Levy, 1993; Magnani et al., 1995). HIV selectively infects and destroys CD4+ T lymphocytes, causing severe immunodeficiency in infected patients (Klatzmann et al., 1984; Masur et al., 1989). The advanced stage of HIV disease is defined as Acquired Immunodeficiency Syndrome (AIDS), clinically characterized by CD4 counts less than 350 per mm^3 of blood and/or overt clinical signs of opportunistic infections such as pneumocystis pneumonia or esophageal candidiasis (WHO, 2007).

HIV is found throughout the world and is prevalent in sub-Saharan Africa, accounting for 70% of new infections yearly (UNAIDS, 2013) (Figure 1). Worldwide, an estimated 36.9 million people are living with HIV and about 2 million people became newly infected in 2014 (UNAIDS, 2015). The earliest report of HIV dates back to 1981 with five cases of *Pneumocystis carinii* pneumonia in healthy young homosexual men in Los Angeles, CA. At the time, it was described as “cellular-immune dysfunction” related to “sexual contact” (CDC, 2001). Since then, tremendous efforts have been made worldwide for the diagnosis, control and prevention of HIV. While 15.8 million people living with HIV had access to antiretroviral therapy globally, 1.2

million people died from AIDS-related causes in 2014. The continued development of a clinically safe and efficacious vaccine along with improved therapeutic regime against this disease are crucial.

1.2 HIV Transmission

HIV can be transmitted via bodily fluids such as blood, semen, pre-seminal fluid, rectal fluids, vaginal fluids and breast milk. Sexual transmission remains the main route of HIV transmission between people, accounting for 80% of new infections worldwide (Royce et al., 1997; Geneva: World Health Organization, 2005). New HIV infections amongst adults and adolescents in the United States and 6 dependent areas (American Samoa, Guam, Northern Mariana Islands, Puerto Rico, Republic of Palau, and US Virgin Islands), are caused by male to male sexual contact (67%), followed by heterosexual contact (24.2%), injection drug use (6.1%), male to male sexual contact and injection drug use (2.7%), and others such as hemophilia, blood transfusion, perinatal exposure (0.2%), thus placing men who have sex with men (MSM) the highest risk group for HIV infection (CDC, 2014). It has been shown in heterosexual transmission studies that male-to-female transmission is 2.3 times higher than female-to-male transmission (Nicolosi et al., 1994), placing HIV in semen the most prevalent vector in transmission of this disease.

1.3 HIV in Lymph Nodes

Lymph nodes are lymphatic organs located throughout the body linked by lymphatic vessels and responsible for both cellular and humoral immune responses. They consist of germinal follicles where B lymphocytes proliferate, medulla where macrophages are located and communicate to the efferent vessels, and surrounded by the paracortex where T lymphocytes proliferate. They provide structural background to support maturation, proliferation and

migration of T and B lymphocytes, macrophage, and interactions between these immune cells with antigen presenting cells against pathogens.

In early HIV-1 infection, the patients suffer from enlarged lymph nodes, further described by lymphadenopathy due to follicular hyperplasia (excessive B cell proliferation) (Metroka et al., 1983) and with antiretroviral therapy, the enlarged lymph nodes diminish dramatically (Bucy et al., 1999). As the disease progresses with severe depletion of circulating CD4+ T cells, the lymph nodes become smaller and fibrotic, histologically described by follicular involution (Paiva et al., 1996). Due to the high efficiency and capacity of HIV to infect CD4+ T cells, HIV antigen is accumulated in high numbers in lymphoid organs. Follicular dendritic cells (FDCs), found only in lymphoid tissues, persistently harbor viral particles on their surface and serve as reservoir (Tenner-Racz et al., 1988; B. A. Smith et al., 2001). In addition, FDCs contribute in migrating T lymphocytes to the germinal center microenvironment, further increasing HIV antigen concentration in the lymph nodes and destruction of lymphoid cells (Burton et al., 2002).

1.4 HIV in Brain

1.4.1 HIV-Associated Neurocognitive Disorder (HAND)

While HIV can effectively infect CD4+ T cells and cause acquired immunodeficiency syndrome (AIDS), it can also affect the brain and cause neurological disorders known as HIV-associated neurocognitive disorders (HAND). There are multiple ways that HIV can affect the central nervous system (CNS); AIDS opportunistic infections such as crytpococcosis or toxoplasmosis, tumor development such as primary lymphoma, co-infections including tuberculosis or hepatitis C virus, or HIV infection itself leading to destruction of neuronal tissues and subsequent inflammation (McArthur, 1987; Almeida, 2015). There are also different types of HAND which include asymptomatic neurocognitive impairment (ANI), mild neurocognitive

disorder (MND), and HIV-associated dementia (HAD), described by the severity of cognitive, motor and behavioral abnormalities (Grant et al., 2014; Kaul et al., 2005). Twenty to 30 % of chronically infected HIV patients showed symptoms of HAD prior to availabilities of therapies, which is the most debilitating and severe form of HAND (Gonzalez-Scarano & Martin-Garcia, 2005; Kaul et al., 2005). With the introduction and widespread use of highly active antiretroviral therapy (HAART), the cases of HAD, opportunistic CNS infections, and primary lymphoma has decreased (Sacktor, 2002). However, about 40% of HIV patients on HAART continues to show mild symptoms of HAND that continues to diminish the quality of the patients' lives (Sacktor, 2002; McArthur, 2004).

1.4.2 Blood-Brain Barrier

The brain is protected by a highly selective permeable barrier called blood-brain barrier (BBB) that keeps circulating blood, antigens and inflammatory cells from entering the central nervous system. The BBB consists of brain endothelial cells with specialized tight junctions (TJs), and capillary basement membrane in which astrocyte end-feet and pericytes are embedded. Substances with high lipid solubility such as O_2 and CO_2 diffusely travel across the BBB, but the entrance of substances with high water solubility and leukocytes are limited by the endothelial TJ (Grieb et al., 1985). Nutrients such as glucose and amino acids utilize carrier-mediated transporter in the endothelium (Cornford & Cornford, 1986; Fuglsang et al., 1986). These carrier-mediated BBB transporters, especially the P-glycoprotein is known to also protect the CNS by actively pumping neurotoxins and drugs back to the vasculature (Laterra & Betz, 1999). In addition, brain capillary endothelial cells contain drug and neurotoxic metabolizing enzymes that minimizes the brain exposure to drugs and toxins (Minn, et al., 1991; Faissner et al., 2014).

1.4.3 HIV in Brain

It is known that HIV can be detected in the brain during the early infection, within 1-2 weeks of detectable plasma viral level (Davis et al., 1992; Gray et al., 1993). The source of HIV in the brain is thought to be infected monocytes crossing the BBB that later differentiate into macrophages (Albright et al., 2003). There are two proposed mechanisms in which HIV causes neuronal cell damage and symptoms of HAND. The first mechanism is where the direct contact of viral particles released from the infected monocytes causes the neuronal cell death (Gonzalez-Scarano & Martin-Garcia, 2005; Kaul et al., 2005). The other proposed mechanism is indirect. Soluble factors are released by the macrophages and microglia, triggering inflammatory cascades in the bystander neurons leading to neuronal cell destruction (Gonzalez-Scarano & Martin-Garcia, 2005; Kaul et al., 2005).

The exact mechanism in which these HIV-infected monocytes cross the BBB into the brain is yet to be determined. Astrocyte end-feet are located between the brain capillary endothelium and neurons, and play an important role in sustaining the BBB by releasing growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) to form the TJs (Wong et al., 2013; Cabezas et al., 2014). Astrocytes are the most abundant glial cells in the brain and they maintain the homeostasis of the brain by modulating and controlling synaptic activity via releasing neurotransmitters (Kimelberg & Nedergaard, 2010). HIV-1 utilizes a CD4 independent viral entry mechanism to infect astrocytes (Harouse et al., 1989; Brack-Werner et al., 1992; Liu et al., 2004; Lopez-Herrera et al., 2005), raising the possibility that astrocytes at the BBB may serve as the initial passage of HIV-1 from the periphery to the CNS.

1.5 HIV in Semen and the Male Reproductive Tract

1.5.1 Semen Production and Spermatogenesis

Semen is produced by the male reproductive organs and consists of spermatozoa, seminal fluids, prostatic fluids and proteins (WHO, 1999). Spermatozoa are produced in seminal tubules of testes, mature and are stored in the epididymis. With stimulation, the mature spermatozoa travel through vas deferens and admix with seminal fluids from seminal vesicles, and prostatic fluids from prostate glands before being deposited via the urethra of penis (Peate & Nair, 2015) (Figure 2 and 3). The bulbourethral gland, also known as a Cowper's gland, also contributes to the total semen volume by producing mucus (Eroschenko, 2008).

Sperm development (spermatogenesis) occurs in the seminal tubules of the testes. The immature germinal cells, known as the spermatogonia are derived from the basement membrane of the seminal tubules and undergo mitosis to develop into primary spermatocytes (de Kretser et al., 1998). Through divisions, these cells mature into secondary spermatocytes and spermatids, crossing a barrier formed by Sertoli cells (the blood-testis barrier; BTB) and moving closer to the lumen of the seminiferous tubules, and finally become tailed spermatozoa and deposited into the lumen (de Kretser et al., 1998) (Figure 4). Spermatogenesis is orchestrated by endocrine and paracrine hormones such as follicle stimulating hormone (FSH) produced by the anterior pituitary glands, and testosterone, produced by the Leydig cells located in the interstitium of testes (Sofikitis et al., 2008). Sertoli cells, located within the germinal cell layer of the seminal tubules, also contribute to spermatogenesis by secreting glycoproteins such as transferrin, androgen-binding protein (ABP), and stem cell factors to drive the maturation and migration of the germinal sperm cells (Dimitriadis et al., 2015).

1.5.2 Testicular Defense Mechanisms

Like the brain, testes are considered immune privileged organs, protected by blood-testis barrier (BTB) (Figure 5). Because of its unique anatomical location between the spermatogonial germinal cells and post-meiotic germinal cells (spermatocytes and spermatids), the BTB serves as a platform to provide the spermatogonia with nutrients and hormones needed to further differentiate while protecting the post-meiotic germinal cells from the blood vessels in the interstitium (Cheng & Mruk, 2012). Post-meiotic germinal cells transiently express antigens during spermatogenesis and can elicit autoimmune responses if exposed outside of the BTB (Meinhardt & Hedger, 2011). The BTB prevents the production of antisperm antibodies and male infertility by isolating these germinal cells. Sertoli cells have been shown to secrete immunosuppressive molecules such as cytokines and prostaglandins to further block immune responses against these transiently expressed antigens (Meinhardt & Hedger, 2011).

Unlike the BBB, BTB consists of coexisting TJs, gap junctions, adhesions junctions and desmosomes formed exclusively by the Sertoli cells (Siu & Cheng, 2004). These junctions, consist of actin-filament bundles and ectoplasmic specialization, form a dynamic ultrastructure and reconfigures to aid the movement of spermatogonia germinal cells into the lumen (Mital et al., 2011). Each junction produces numerous transmembrane proteins such as claudin, N-cadherin, actin, and vimentin to maintain and strengthen the integrity of BTB (Cheng & Mruk, 2012). Unlike the BBB, blood vessels, lymphatics, or nerves do not penetrate through the seminiferous tubules but they are isolated in the interstitium between the tubules (Cheng & Mruk, 2010; Cheng & Mruk, 2012), allowing BTB to regulate the entry of substances into the lumen.

Spermatogenesis is a dynamic process that is carefully synchronized by proliferation and apoptosis of germinal cells in order to balance an essential proportion of germinal cells at different stages and Sertoli cells (Rodriguez et al., 1997). In the adult mammalian testis, as much as 75% of germinal cells are discarded by the process of apoptosis (Blanco-Rodriguez, 1998; Print & Loveland, 2000). Sertoli cells play an important role in phagocytizing these apoptotic and damaged germ cells before they induce inflammatory responses (Nakanishi & Shiratsuchi, 2004).

While there is a negative correlation between the number of immune cells and spermatogenesis, testicular resident macrophages have been shown to produce anti-inflammatory cytokines such as IL-3, IL-4 and IL-10 in the presence of antigen (Bhushan et al., 2011; Winnall et al., 2011). Leydig cells, found in the interstitium of testes, synthesize androgens needed for spermatogenesis. Androgens such as testosterone and luteinizing hormone are known to suppress the autoimmune process and maintain BTB (Meng et al., 2005; Page et al., 2006). Other factors such as programmed death receptor-1/programmed death ligant-1 (PD-1/PD-L1) and growth arrest specific gene 6 (Gas6)/Protein S (ProS)-Tyr3, Axl and Mer (TAM) system additionally are expressed in the testis and further contribute to the negative immune-regulatory environment of these organs (Wang et al., 2005; Cheng et al., 2009).

1.5.3 HIV in Semen and the MRT

HIV-1 infection significantly impairs a male fertility, characterized by low number and concentration of sperm, low volume of semen, and abnormal sperm morphology and motility (Dondero et al., 1996; Umapathy et al., 2001; Nicopoullos et al., 2004). Low CD4+ cell count has been positively correlated with low sperm counts and negatively correlated with normal sperm morphology (Nicopoullos et al., 2011). Histologically there are degeneration and loss of

testicular germinal cells, varying severity of oligospermia and azoospermia (decrease or absence of sperm production), peritubular fibrosis, and leukocyte infiltration in testicular and epididymal interstitium (Chabon et al., 1987; Dalton & Harcourtwebster, 1991; Pudney & Anderson, 1991; Shevchuk et al., 1999) (Figure 6 and 7). HIV-1 infection is also associated with erectile dysfunction and hypogonadism due to hypothalamic-pituitary dysfunction (Tindall et al., 1994; Crum-Cianflone et al., 2007; Zona et al., 2012; Perez et al., 2013).

While HIV in semen plays an important role in the transmission of HIV, the source of HIV in semen or in the MRT remains unclear. HIV-1 is detected in the semen as free viral particles and as cell-associated virus in lymphocytes and macrophages (Ho et al., 1984; Zagury et al., 1984), and can be detected just a few weeks after primary infection (Tindall et al., 1992). It was originally thought that the only source of HIV-1 in the semen was the infected leukocytes from the blood. However, phylogenetic analysis has shown a diverged subpopulation of HIV-1 in semen (seminal cells and seminal fluids) and in infected seminal leukocytes (Zhu et al., 1996; Paranjpe et al., 2002; Ghosn et al., 2004). There is also much evidence demonstrating a distinct evolution of virus in semen (Byrn & Kiessling, 1998; Coombs et al., 1998; Kiessling et al., 1998), and development of drug resistant mutation (Kroodsma et al., 1994; Hecht et al., 1998). HIV shedding in semen can be intermittent throughout the disease progression and even on HAART with no detectable plasma viral load (Zhang et al., 1998; Bujan et al., 2004). This suggests compartmentalization of HIV-1 and existence of a potential reservoir population or local production within the MRT that are separate from the blood, driven by the unique immune privileged environment of MRT.

Although, the source of HIV-1 in semen and in the MRT is undefined, many studies have demonstrated either testicular germinal/spermatozoa and/or leukocytes as the origin of seminal

viral load and seminal transmission. A study done by Muciaccia et al., demonstrated the presence of HIV-1 proviral DNA in asymptomatic seropositive and male AIDS patients in all stages of differentiating testicular germinal cells, including spermatogonium to round spermatids, as well as infected leukocytes in testes (Muciaccia, Filippini, et al., 1998; Muciaccia, Uccini, et al., 1998) (Fugre 7). Other studies showed the presence of HIV-1 nucleic acids in spermatogonia, spermatocytes, spermatids, and infected macrophages in HIV-infected males (Nuovo et al., 1994; Shevchuk et al., 1998), suggesting clonal infection as well as leukocyte infection leading to the shedding of HIV in the semen.

Both spermatozoa and testicular germinal cells lack CD4 and CCR5/CXCR4 (Habasque et al., 2002), receptors required for HIV-1 to infect cells, but alternative receptors such as GalAAG have been described for HIV-1 to enter cells, independent of CD4 presence (Brogi et al., 1998; Gadella et al., 1998). Other alternative receptors used by HIV to infect spermatozoa in *in vitro* studies include, 160 kDa sperm protein (Bandivdekar et al., 2003) and mannose receptor (Cardona-Maya et al., 2006; Cardona-Maya et al., 2011). But whether or not the entire virion can be internalized using these alternative receptor is unknown, and it has been demonstrated the binding of HIV-1 to these alternative receptors is inhibited by the seminal plasma (Gadella et al., 1998). This suggests that if spermatozoa infection were to occur, it can only be in seminal-fluid-free organs such as testes and epididymides and not in seminal vesicles or prostate glands. While the mechanisms used by HIV-1 to cross the mucosal epithelium is yet to be defined, an *in vitro* study, demonstrated that an alternative receptor found on spermatozoa, heparine sulfate, allowed HIV-1 binding to spermatozoa and enhanced the subsequent viral transmission to dendritic cells (Ceballos et al., 2009).

Others have demonstrated HIV detection only in leukocytes in testis, epididymis, and prostate of AIDS patients but not in germinal cells (Pudney & Anderson, 1991) (Figure 6). Unlike the testicular germinal cells and spermatozoa, seminal and testicular lymphocytes and monocytes express CD4 on their surfaces (Gobert et al., 1990; Gil et al., 1995). Studies have shown cells expressing CXCR4, CCR5, CD4 and DC-SIGN in the testicular interstitium (Habasque et al., 2002; Roulet et al., 2006), suggesting that the testes can sustain productive infection by replicating in testicular macrophages. Furthermore, HIV-1 has been located in macrophages of human seminal vesicles *in vitro* and *in vivo* (Deleage et al., 2011), providing additional evidence of HIV-1 infection in leukocytes. Sperm washing technique is often done for HIV seropositive males to separate seminal fluids from the mature sperm cells in order to help HIV positive couples conceive. Many studies have demonstrated successful separation of HIV containing (cell-free form and cell-associated form in leukocytes) seminal fluids and were able to detect no HIV in the spermatozoa leading to HIV free conceptions (Hanabusa et al., 2000; Kato et al., 2006), further supporting the leukocyte theory.

1.5.4. HIV Sexual Transmission by Cell-Associated Virus and Cell-Free Virus

In a host, HIV-1 disseminates either by diffusing viral particles (cell-free virus) or by direct cell-to-cell transmission (cell-associated virus) (Zhong et al., 2013). Clinically, the concentration of cell-free virus in the blood plasma (viremia) is the gold standard predictor of the disease process. Therefore, subsequent development of anti-retroviral therapy drugs (ARTs) and broadly-neutralizing antibodies (bNabs) has been focused on blocking cell-free virus transmission (Cihlar & Fordyce, 2016; Smith & Derdeyn, 2016).

Both cell-associated proviral DNA and cell-free RNA virion of HIV are present in the semen of infected individuals as infected leukocytes in semen and as free floating virion in the

seminal plasma accordingly (Anderson et al., 2010). However, whether cell associated virus, cell-free virus, or both are the source of sexually transmitted HIV-1 is yet to be defined. (Zhu et al., 1996; Butler et al., 2010; Gianella et al., 2012). Recent studies have demonstrated that cell-associated virus play an important role in sexual transmission of HIV (Salle et al., 2010; Kolodkin-Gal et al., 2013), and it has been established in *in vitro* studies that cell-to-cell transmission is 2-3 times more efficient than cell-free virus dissemination (Carr et al., 1999; Chen et al., 2007). Several studies have been conducted to block the sexual transmission of cell-associated HIV transmission in *in vivo* and in *in vitro* models using topical microbicides and passive immunization (Matoba et al., 2004), antibodies that block the attachment of leukocytes to epithelial cells (Romer et al., 2009), and chemoprophylactic antiretroviral drugs (Cranage et al., 2008). These discoveries are limited to *in vitro* or in pre-clinical stages. Therefore, studies investigating HIV-1 infection in the MRT to further define the potential mechanisms of HIV pathogenesis and transmission in these tissues is critical to aid in the development of novel therapeutics, targeting both cell-associated and cell-free virus.

1.6 Animal Models

1.6.1 HIV-1 Infection in Chimpanzees

The origin of HIV-1 is known to be SIV from chimpanzees (*Pan troglodytes troglodytes*) and gorilla (*Gorilla gorilla gorilla*) located in west central Africa (Keele et al., 2006). Chimpanzees are human's closest living animal with 98% genetic similarity. While chimpanzees are susceptible to chronic HIV-1 infection and can maintain high plasma viral load (Saksela et al., 1993), they rarely develop disease progression (Watanabe et al., 1991). HIV-1 infected human patients experience depletion in CD4+ T cells as well as dysfunction of remaining CD4+ T cells (Iyasere et al., 2003). Most HIV-1 infected chimpanzees do not experience CD4+ T cell

depletion or decrease in CD4+ T cell's ability to proliferate and function (Rutjens et al., 2008). However, because of this lack of disease progression, HIV-1 studies in these animals has provided us with invaluable evolutionary perception and key features of immune system in HIV-1 infection and replication (Gao et al., 1999; Decker et al., 2009; de Groot et al., 2010).

1.6.2 SIV/SHIV Infection in Nonhuman Primates

Because HIV-1 only replicates in humans and chimpanzees (Watanabe et al., 1991), alternative lentiviral infections in animal models have been developed to study the complex pathogenesis, replication and transmission of HIV-1. This includes feline immunodeficiency virus (FIV) in feline models and simian immunodeficiency virus (SIV) or SIV/HIV chimeric virus (SHIV) in nonhuman primates which causes severe immunodeficiency in the host animals post-infection (Haigwood, 2004; Yamamoto et al., 2010). These studies have greatly advanced our knowledge on how retroviruses cause immunodeficiency in these animals, especially the SIV/SHIV infection of nonhuman primates (Fauci & Desrosiers, 1997).

The natural hosts of SIV are African nonhuman primates. Sooty mangabeys (*Cercocebus atys*) in particular are the main model for the natural infection of SIV (SIVsmm). It has been established that SIVsmm gave rise to HIV-2 in humans and SIVmac in macaques (genus *Mucaca*) (Hirsch et al., 1989; Marx et al., 1991). While SIVsmm results in high viral replication and elicits innate and adaptive immunity in the host species at initial infection, it does not cause the depletion of central memory CD4+ T cells or develop AIDS which is comparable to SIVmac and HIV-1 (Chahroudi et al., 2012).

Unlike SIV infection in African monkeys, SIV infection in Asian monkeys demonstrates a similar pathogenesis as seen in HIV infection in humans. SIVmac infection in rhesus macaques (*Macaca mulatta*) cause severe depletion of CD4+ T cells particular in gut-associated lymphoid

tissues (GALT) and leads to simian AIDS with chronic infection (Veazey et al., 1998; Mattapallil et al., 1999). SIVmac251 and SIVmac239, the two most common SIV strains used for nonhuman primate research, were derived from rhesus macaques of Indian descend and are infectious amongst other Asian macaques used for research such as pig-tailed macaques (*Macaca nemestrina*), cynomoglus macaques (*Macaca fascicularis)* and rhesus macaques of Chinese origin (Hatziioannou & Evans, 2012).

In addition to similar systemic pathogenesis of SIV infection, macaques have also been used to study the pathogenesis of SIV in the MRT and semen due to the physiologic and immunologic similarities. High viral load has been shown in testis and epididymis of SIV and SHIV infected male macaques which were associated with increased numbers of infected leukocytes found in the interstitium of both organs at acute infection (Shehu-Xhilaga et al., 2007). They were also able to demonstrate infected germinal cells [only in spermatogonia population and no other advanced germinal cells such as spermatocytes or spermatids] in juvenile macaques using immunofluorescence (Shehu-Xhilaga et al., 2007) which has been reported only once before in HIV-1 infected patients using a less sensitive technique (Muciaccia, Uccini, et al., 1998).

The presence of productively infected CD4+ T cells and macrophages in the semen of SIV infected macaques at all stages of SIV infection has been shown (Bernard-Stoecklin et al., 2013). A more recent study demonstrated localization of SIV in the MRT, specifically in the urethra, epididymis and prostate of chronically infected macaques (Matusali et al., 2015). While the animals on HAART showed decreased number of leukocytes in all three organs compared to the untreated group, the urethra contained the highest proportion of SIV in both groups (Matusali et al., 2015). Furthermore, infected macrophages were found only in the urethra of treated groups

regardless of detectable viremia level in the plasma (Matusali et al., 2015), suggesting chronically infected cells reside in the urethra, contributing to the persistent shedding of the virus during treatment, independent of viremia.

SIV or SHIV infection in macaque model has contributed greatly to our understanding of HIV-1 transmission, pathogenesis, innate and adaptive immunity and vaccine development (Apetrei et al., 2012; Hatziioannou & Evans, 2012; Evans & Silvestri, 2013). However, because of the molecular and genetic differences between HIV and SIV/SHIV and the immunogenic differences between humans and nonhuman primates, many pre-clinical trials of vaccines and therapeutics that have been developed in nonhuman primate models have not translated into success in clinical trials in humans (Warren & Levinson, 1997; Warren, 2002; Apetrei et al., 2012).

1.6.3 HIV-1 Infection in Humanized Mice

Although studies with SIV and SHIV have greatly contributed to our current knowledge on HIV infection, obtaining nonhuman primates for such research is difficult due to the availability, cost and ethical concerns. In addition, the biological and genetic differences between HIV and SIV/SHIV (Shibata et al., 1995; Pollom et al., 2013) and nonhuman primate model's inefficiency in translating into human trials (Watkins et al., 2008) requires alternative *in vivo* models using HIV-1 itself. Humanized mice are immunodeficient mice that do not reject human cells or tissue engraftment and therefore are able to produce circulating human immune cells. They are smaller in size, easier to use and more cost-effective in comparison to nonhuman primate models and have great advantages in studying human-specific diseases.

The early discoveries of athymic nude mice lacking in T cells and severe combined immunodeficiency mice (SCID) lacking in T and B cells in the 1960s to 1980s provided the first

models for immune deficient mice and successful xenotransplantation (Fogh & Giovanella, 1978; Bosma et al., 1983). The introduction of *Prdkc*^{scid} gene in non-obese diabetic (NOD)/SCID mice was a key advancement of generation of humanized mice. The NOD/SCID mice are homozygous for autosomal recessive spontaneous mutation (*Prdkc*^{scid} or commonly known as *scid*). The *Prkdc* gene encodes for a protein kinase DNA-activated catalytic polypeptide (Bosma & Carroll, 1991). This is a DNA repair function mutation, and a failure of rearrangement mutation for genes that code for antigen-specific receptors on lymphocytes (Bosma & Carroll, 1991). The mice with *scid* mutation lack functional T and B cells, which enable engraftment of human cells (Bosma & Carroll, 1991). The development of NOD/SCID and NOD/Rag1^{null} mice also allowed for subsequent generation of NOD/SCID/B2m^{null} and NOD/ Rag1^{null}Pfp^{null} mice that are devoid of mature B and T cells, natural killer (NK) cell cytotoxic activity, and display high levels of human peripheral blood mononuclear cells (PBMCs) when engrafted with human cells (Christianson et al., 1997; Shultz et al., 2003).

Most recently, the introduction of knockout Interluekin-2 receptor subunit gamma (*IL-2rγ*) into the NOD/SCID and Rag1/2^{null} mice resulting in NOD/SCID/γc^{null} mice and Rag1/2^{null}γc^{null} mice created a severe immunodeficiency in multiple immune cells (Ito et al., 2002; Brehm et al., 2010). *IL-2rγ* encodes for the common and signaling components for six distinct interleukins (IL-2, IL-4, IL7, IL-9, IL-15 and IL-21) which is required for differentiation and function of multiple hematopoietic cells (Cao et al., 1995; Ito et al., 2008). Unlike the parent immunodeficient mice, Rag1−/−γc−/− or Rag2−/−γc−/− have a multidysfunctional naïve immune system. When engrafted with human CD34+ stem cells intrahepatically at an early age, these mice (known as Rag-hu or hu-HSC mice) are capable of *de novo* multilineage human hematopoiesis and able to generate human T and B cells, macrophages, dendritic cells and

natural killer cells (Akkina, 2013a, Akkina, 2014) (Figure 8). Most importantly, hu-HSC can sustain long-term chronic HIV-1 infection for more than one year and display depletion of CD4+ T cells during the chronic infection as well as susceptible to mucosal exposure of HIV-1 (Berges et al., 2006; Berges et al., 2010).

1.6.3.1. Vaginal Plugs

Vaginal plugs, also known as copulatory plugs or mating plugs, are coagulated semen from a male animal that physically adhere to, and occlude the female reproductive tract after mating. It can be found in many species of animals including mice, rats, guinea pigs, hamsters, certain nonhuman primate species and even snakes, nematodes and mosquitos (Martan & Shepherd, 1976; Devine, 1977; Huck et al., 1987; Dixson & Anderson, 2002; Lejnieks, 2007; Palopoli et al., 2008; Rogers et al., 2009; Dean, 2013). Studies of vaginal plugs have been extensively done in laboratory mice and rats due to their research demands and availabilities. Vaginal plugs of rodents are made of secretions from seminal vesicles, prostate glands, coagulating glands, bulbourethral glands and spermatozoa (Matthews & Adler, 1977; Matthews & Adler, 1978; Carballada & Esponda, 1992;). The coagulatory factor of the vaginal plug is due to the enzymatic interaction between the transglutaminase from coagulating glands and proteins in seminal vesicle secretion (SVS) at ejaculation (Williams-Ashman, 1984; Tseng et al., 2012). Proteins of *Transglutaminase IV (Tmn4)* has been identified in human ejaculates (Pilch & Mann, 2006) and the ortholog proteins of *Tmn4* has also been identified in mouse ejaculates (Dean et al., 2011).

In mice, plugs remain intact in the female cervix and vagina for up to 48 hours (Voss, 1979) and are visible to the naked eye at the vaginal opening of mated females, therefore the presence or absence of "plugs" are commonly used to determine the mating frequency in a

laboratory breeding colony. Vaginal plugs are known to promote male's competitive fertilization by decreasing the chance for that particular female to re-mate, and promote efficient sperm transport into female reproductive tract (Martan & Shepherd, 1976; Sutter et al., 2015; Sutter & Lindholm, 2016).

1.7 Hypothesis and Specific Aims

HIV-1 in seminal viral load plays a key role in transmitting this disease in humans but little is known about the source and pathogenic mechanism of the virus in semen or the MRT. No studies have been done so far demonstrating HIV-1 infection in the MRT or semen using humanized mice.

The Central Hypothesis is that viremic male humanized mice (hu-HSC) will harbor HIV-1 in their reproductive tract, and could therefore be used as an *in vivo* model for investigating the pathogenesis and replication mechanism of HIV-1 in the MRT. This will be accomplished by evaluating 4 specific aims:

1) Evaluate the severity of disease using histologic scoring of the MRT and morphometric analysis of testis and epididymis in viremic and negative control mice
2) Characterize leukocytes in testis and epididymis of viremic and negative control mice via immunohistochemistry
3) Assess the presence of HIV-1 viral RNA in MRT of viremic mice via *in situ* hybridization (HIV-1 mRNA)
4) Assess seminal viral loads from vaginal plugs of viremic mice via qRT-PCR.

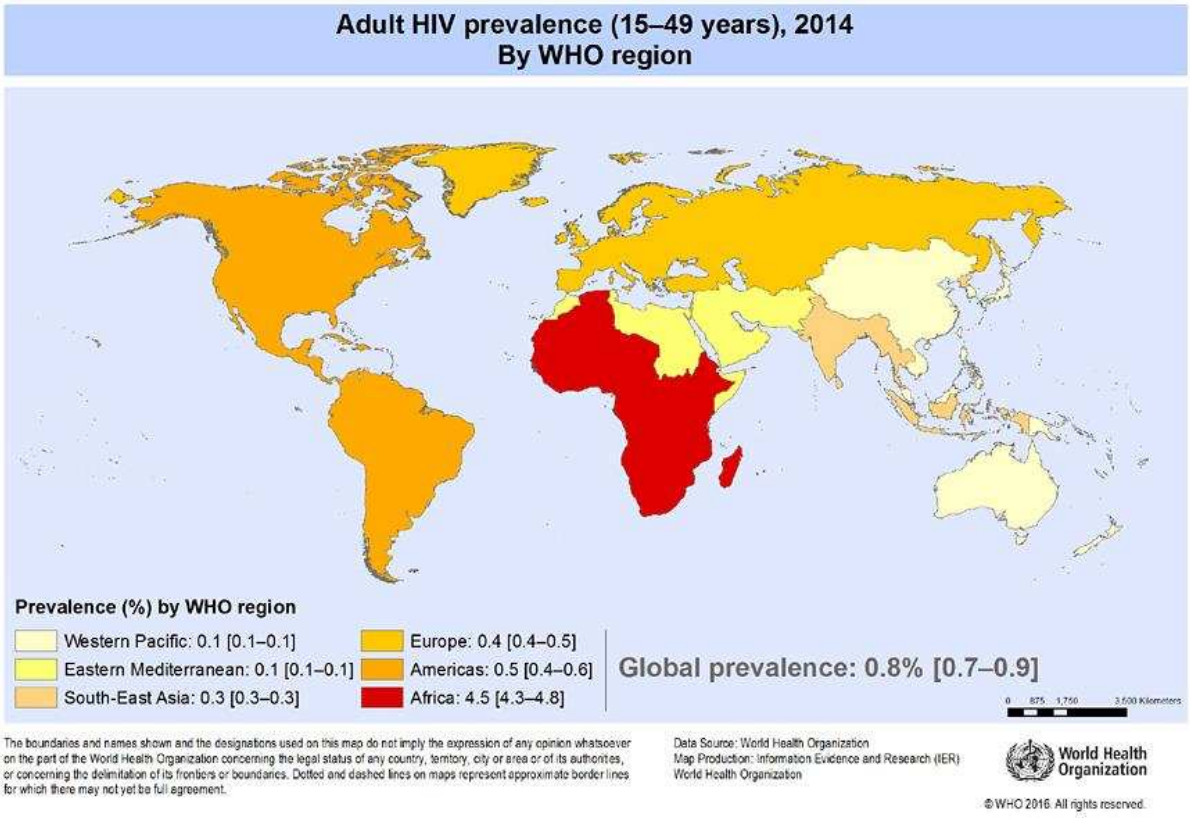

Figure 1: Adult HIV Prevalence, 2014. Globally, 36.9 million people were living with HIV at the end of 2014 and an estimated 0.8% of adults aged 15-49 years worldwide are living with HIV. Sub-Saharan Africa remains most severely affected area, with nearly 1 in every 20 adults living with HIV and accounting for about 70% of the people living with HIV worldwide. (WHO, 2016)

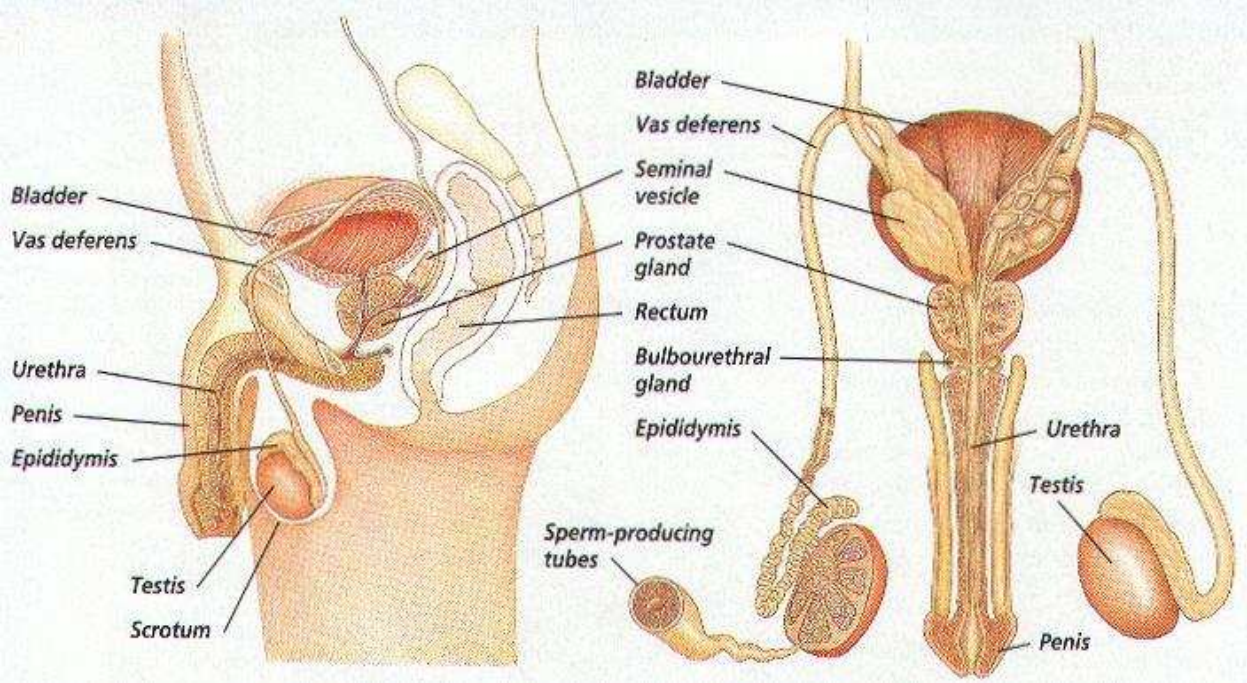

Figure 2: Human Male Reproductive System – the External Organs. Human male reproductive system consists of testes, epididymis, vas deferens, penis, and accessory male reproductive organs such as seminal vesicles, prostate gland and bulbourethral glands. (Advisor, 2016)

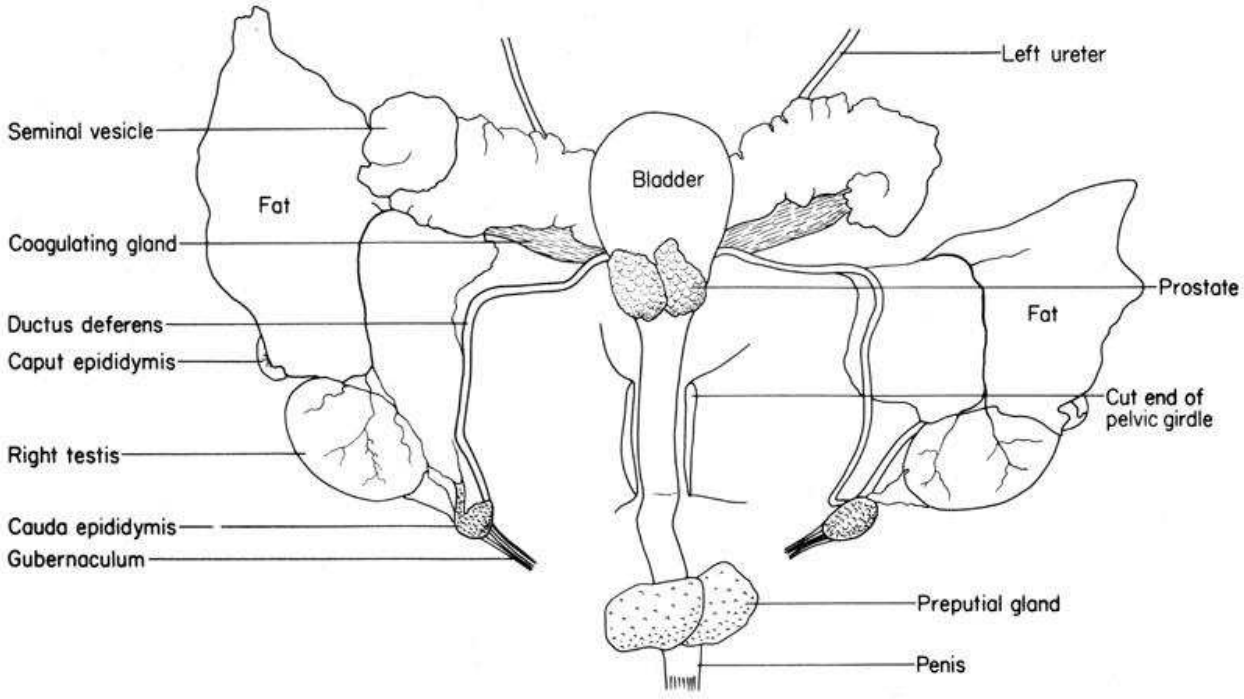

Figure 3: Mouse Male Reproductive System – the External Organs. Mouse male reproductive system consists of testes, epididymis, vas deferens, penis, and accessory male reproductive organs such as seminal vesicles, coagulating gland, and prostate gland. (Cook, 1965)

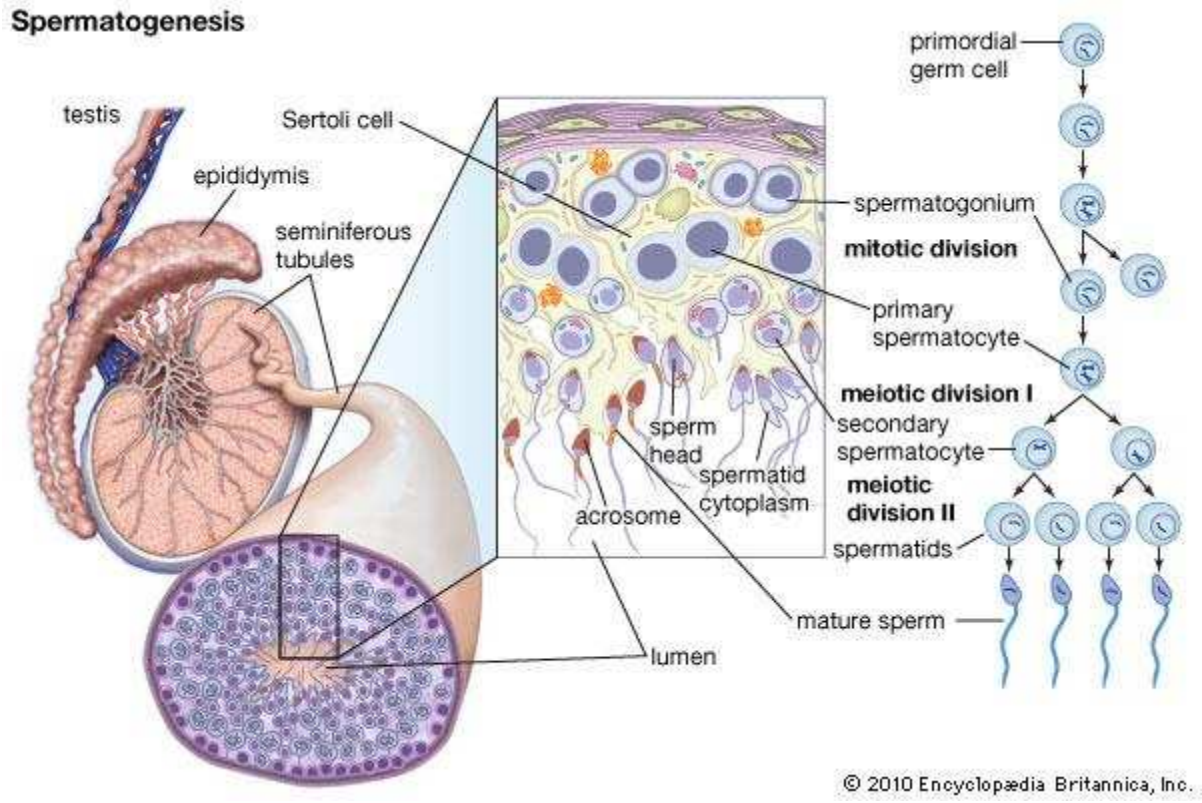

Figure 4: Spermatogenesis. Sperm cells are made from seminiferous tubules of testes. The spermatogonia are derived from primordial germ cells and undergo mitotic division to differentiate into more spermatogonia and primary spermatocytes. Through meiotic divisions, the primary spermatocytes further differentiate into secondary spermatocytes, spermatids and mature sperms. The mature sperms are stored in epididymis until ready to leave the male body. (Britannica, 2016)

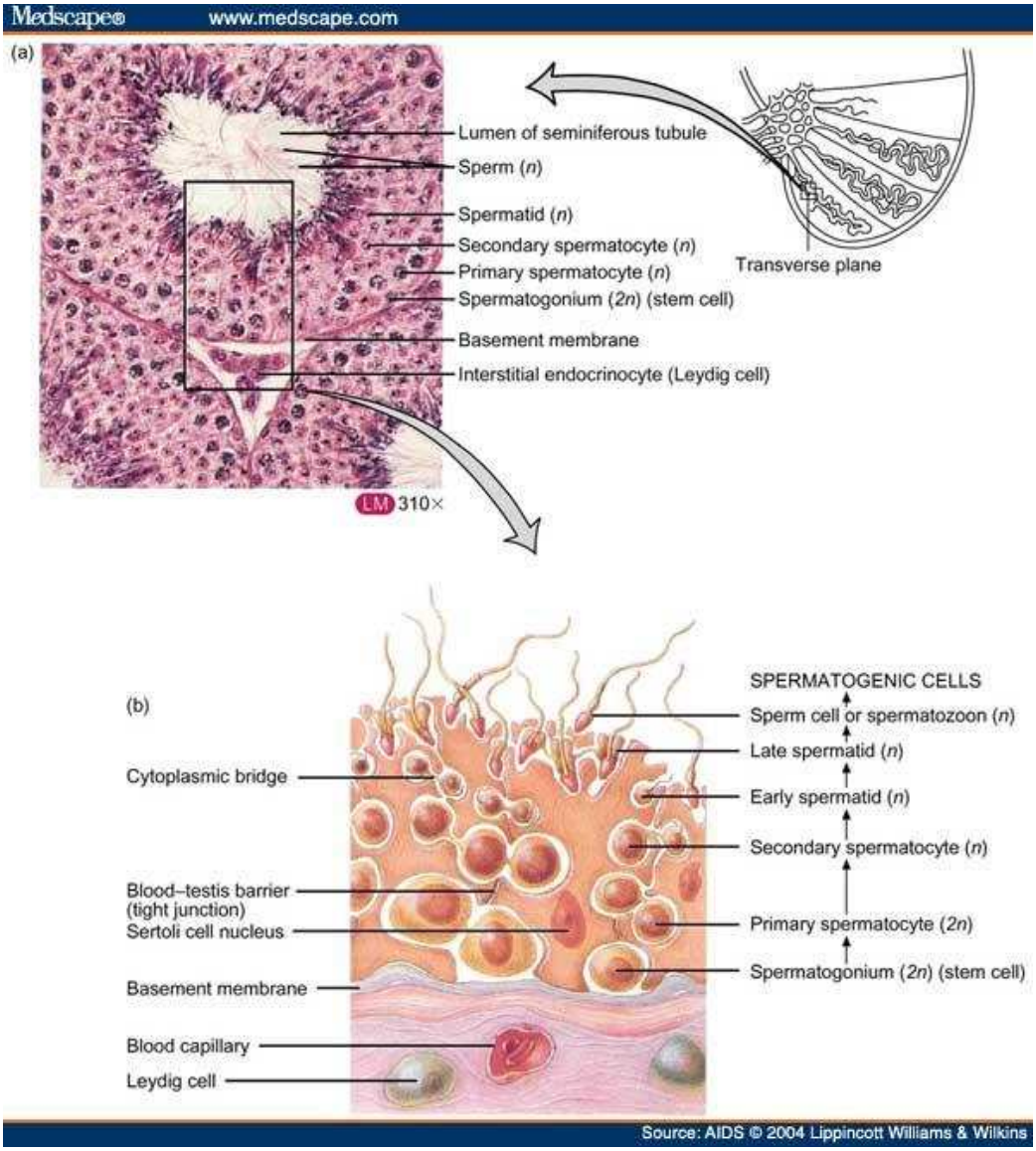

Figure 5: Blood-Testis Barrier. (a) Transverse section of seminiferous tubules depicting blood capillaries within interstitium; (b) Higher magnification image of area between seminiferous tubules and interstitium. The blood-testis barrier is located in seminiferous tubule between the Sertoli cells, physically separating the blood capillaries of interstitium from the germinal cells. (Lowe et al., 2004)

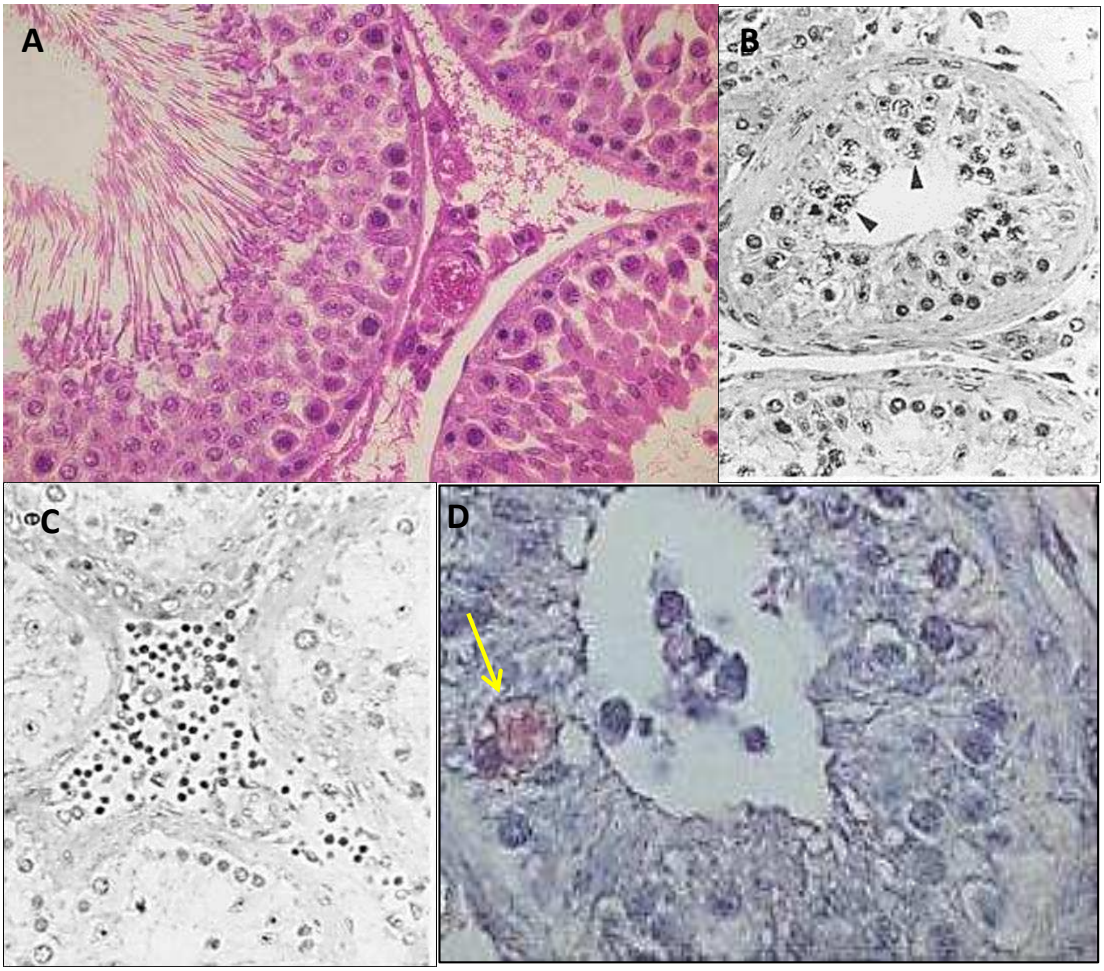

Figure 6: Histopathologic Lesions in Testes of Chronically Infected HIV and AIDS Human Patients – Leukocyte Involvement. (A) Negative control seminal tubules: heterogeneous population of germinal cells with tailed spermatids in the lumen of seminal tubules (normal spermatogenesis). Interstitium contains a blood vessel and leydig cells. (B) AIDS patient seminal tubules: azoospermia with only spermatocytes present; (C) AIDS patient seminal tubules: increased numbers of mononuclear cells in interstitium; (D) Detection of HIV-1 in seminal tubule of AIDS patients by immunocytochemistry: cell staining positive (yellow arrow) with the cocktail of anti-HIV-1 antibodies present in the seminiferous epithelium. (Pudney & Anderson, 1991)

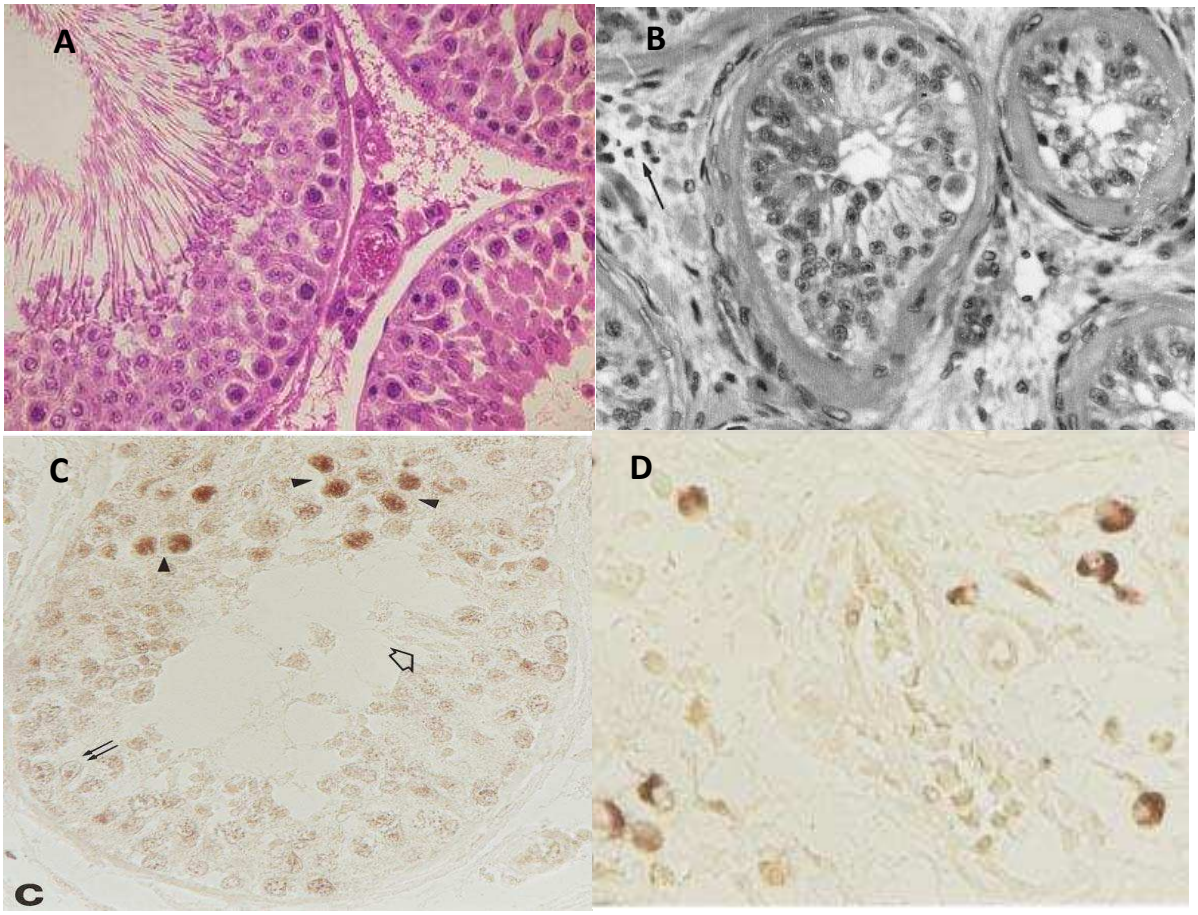

Figure 7: Histopathologic Lesions in Testes of Chronically Infected HIV and AIDS Human Patients – Germinal Cell and Leukocyte Involvement. (A) Negative control seminal tubules: heterogeneous population of germinal cells with tailed spermatids in the lumen of seminal tubules (normal spermatogenesis). Interstitium contains a blood vessel and leydig cells; (B) AIDS patient seminal tubules: Spermatogenesis is impaired and only spermatogonia and few spermatocytes are present. Peritubular fibrosis and interstitial infiltration of monocytes (arrow) are evident; (C-D) *In situ* PCR hybridization for HIV-1 proviral DNA of AIDS patient: positive cells are spermatocytes (arrowheads). Sertoli cells (double arrows) are not stained. Mononuclear positively staining cells are observed only in the interstitium. (Muciaccia, Uccini, et al., 1998)

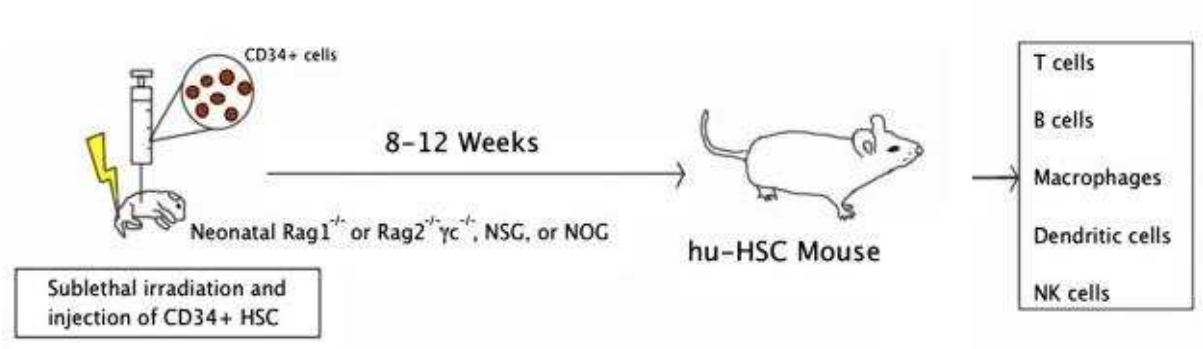

Figure 8: Schematic for Generation of hu-HSC (Rag-hu) Mice. (R. Akkina, 2013b)

Chapter 2

Materials and Methods

2.1 Generation of Humanized Mice

Humanized Rag1−/−γc−/− or Rag2−/−γc−/− (hu-HSC) were generated with human fetal liver derived CD34+ stem cells. CD34+ hematopoietic stem cells were isolated and cultured as previously described (Akkina et al., 1994; Bai et al., 2000; Veselinovic et al., 2016). CD34+ purity was determined by flow cytometry. Neonatal BALB/c-Rag2−/−γc−/− or BALB/c-Rag1−/−γc−/− were preconditioned by irradiation at 350 rads and injected intrahepatically with 0.2-1x10^6 CD34+ cells per mouse (Berges et al., 2008; Veselinovic et al., 2016). Eight to 12 weeks post engraftment, mice were checked for the human common leukocyte antigen CD45 by flow cytometry as previously described (Berges et al., 2006).

2.2 HIV-1 Infection of Humanized Mice and Viremia/Engraftment Level Monitoring

Adult hu-HSC mice were inoculated with 100-150uL of either CCR5 tropic HIV-1 lab strain BaL-1 (0.9×10^5 i.u.) or CXCR4 tropic HIV-1 lab strain NL4-3 (1.2×10^5 i.u.) intraperitoneally. Mouse viremia was monitored by HIV-1 RNA copies in plasma as well as by monitoring for CD4 depletion. Briefly, plasma viral loads of HIV-1 were detected by qRT-PCR on the RNA extracted from 25uL-50uL of EDTA treated plasma with E.Z.N.A Viral RNA Kit following manufactures protocol (Omega bio-tek). QRT-PCR reaction mixes were prepared following the iTaq Universal Probes One-Step Kit (Bio-rad) and ran with HIV-1 LTR RNA standards using previously published assay (F. Rouet et al., 2005). The samples were processed and analyzed using a Bio-Rad C1000 Thermal Cycler with a CFX96 Real-Time System (Bio-rad) (Rouet et al., 2005; Veselinovic et al., 2016).

Engraftment and CD4 T cell levels were monitored by flow cytometry of mouse blood. Fifty to 75µl of heparinized blood was collected and stained for CD45/CD3/CD4 as previously described (Berges et al., 2006). Briefly, whole blood was collected and blocked with Fc-block (Jackson Immuno Research Laboratories) for 5 minutes. Samples were then stained with hCD-45-FITC (eBioscience), hCD-3-PE (eBioscience) and hCD-4-PE-CY5 (BD Pharmingen) antibodies. Cells were incubated at room temperature in the dark for 30 min then subjected to RBC lysis using an RBC lysing kit (BD Biosciences). After RBC lysis, stained cells were washed twice with wash buffer (BD Biosciences) and analyzed using BD Accuri C6 FACS analyzer.

2.3 Specimen Collection

Tissues (lymph nodes, spleen, testes, epididymides, vas deferens, seminal vesicles, prostate glands and penis/urethra) were collected immediately after carbon dioxide euthanasia. The tissues were either stored at -80°C in OCT or fixed in 4% paraformaldehyde (or 10% formaldehyde) for further processing.

2.4 Immunohistochemistry

Anti-CD4 at a concentration of 1:250 was used (ab70951; Abcam). Rabbit IgG Vectastain Elite ABC Kit (PK-6101; Vector Laboratories) was used as an avidin/biotin-based system and secondary antibody. Tissue sections (5 um) were deparaffinized, rehydrated through Histoclear II (HS-202; National Diagnostics) and graded alcohol series, and incubated in an antigen retrieval solution (10mL/L Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0) for 45 minutes in a water bath at 97°C. After 30 minutes of cooling to room temperature, the slides were incubated with 0.03% H_2O_2 to inactivate endogenous peroxidases and incubated with 0.3M glycine for 15 minutes, then with a blocking serum solution (10% FBS in TTBS with 1%

BSA) for 30 minutes. The slides were incubated for 2-hour at room temperature with the serum solution as control or serum diluted antibodies. Specific binding of the primary antibody was revealed using biotinylated secondary antibody, peroxidase-conjugated streptavidin and 3,3' diaminobenzidine substrates (SK-4100; Vector Laboratories). The nuclei were counter-stained with hematoxylin and TTBS. No staining was observed with control serum. The sections were photographed by a microscope coupled to a digital macrocamera.

2.5 Vaginal Plug Collection

Once infected male mice showed viremia via qRT-PCR, a trio housing was set up for mating. Each cage housed one viremic male, one engrafted female (hu-HSC or hu-PBL) and one unengrafted/non-irradiated female for one to two months as long as the plasma viral load of viremic male mouse was sustained. Each morning, the females were checked for the presence of a vaginal plug and if present, they were placed in a separate cage until the next morning when the vaginal plug is easily removed with forceps. The plug was then immediately placed on dry ice and stored at -80°C. Negative control vaginal plugs were also collected from unengrafted/non-irradiated/uninfected pairs from in-house breeding colony.

2.6 Vaginal Plug RNA Extraction

The vaginal plugs were thawed to room temperature and weighed, and 180uL Buffer ATL (939011; Qiagen) per 25mg of a sample and 40uL of Protein Kinase (151028410; Qiagen) were added to each sample and incubated overnight at 57°C. Once completely dissolved, RNA was extracted using RNA Clean and Concentrator^{TM}-5 (R1013; Zymo Research). TRIzol (15596-026; Ambion) was added equivalent to 15 times the total volume and incubated for 5 minutes at room temperature. Chloroform (C7559; Sigma), 0.2mL per 1mL of TRIzol was added and the tube was vigorously shaken manually for 15 seconds. After 3 minute of room temperature

incubation, the sample was centrifuged at 4°C for 15 minutes, separating the organic phase from RNA containing aqueous phase. The aqueous phase was transferred to Zymo Spin IC Column and centrifuged. The column was then washed with 400uL RNA Wash Buffer and in-column DNAse I treatment was performed using DNA Digestion Buffer and DNAse I. The column was further washed with 400uL RNA Prep Buffer, 700uL RNA Wash Buffer and 400uL RNA Wash Buffer and centrifuged each time and flow through were discarded. The column was then transferred to warm RNAse free tube and RNA was eluted with 25uL of RNase and DNase free water and centrifuged. The final eluted RNA sample was stored at -80°C.

2.7 VP - Quantification of HIV-1 Viral Load by qRT-PCR

Vaginal plug viral loads of HIV-1 were detected by qRT-PCR on the RNA extracted with RNA Clean and Concentrator^{TM}-5 (R1013; Zymo Research) following manufactures protocol. QRT-PCR reaction mixes were prepared following the iTaq Universal Probes One-Step Kit (Bio-rad) and ran with HIV-1 LTR RNA standards using previously published assay (F. Rouet et al., 2005). The samples (8uL each) were processed and analyzed using a Bio-Rad C1000 Thermal Cycler with a CFX96 Real-Time System (Bio-rad) (Rouet et al., 2005; Veselinovic et al., 2016).

2.8 Morphometric Analysis

One testis and one epididymis from viremic mice (n=8) and negative control mice (n=4) sacrificed at peak viremia and engraftment level, was collected in 4% paraformaldehyde with PBS buffer for less than 24 hours and transferred to 80% ethanol for less than 24 hours (or 10% formaldehyde with PBS buffer for less than 24 hours and transferred to 70% ethanol for less than 24 hours). The testis and epididymis were sectioned, embedded in paraffin and processed and stained for hematoxylin-eosin for optical microscopy analysis.

The tissue was examined under an optical microscope connected to a computerized digital imaging system and data were analyzed using the software NIS Elements – Nikon. The user was blinded to experimental group to which the testes and epididymis belonged.

Five sections (top, bottom, left, right and center) were selected per testis and seminal tubules within each section were counted for tubular density. From the 5 sections, 10 round to nearly round seminal tubules were chosen at random for the morphometric evaluation per mouse. Each structure was manually traced around outside of seminal tubules and tubule lumen to obtain the area within, using the computer mouse (Trindade et al., 2013) at magnification at 200x for morphometric evaluation. The epithelium area was obtained by the difference between total seminal tubule area and lumen area. The proportion of lumen area over total tubular area was calculated by dividing the lumen area by the total tubule area and multiplying it by 100. The percent epithelium area was obtained by subtracting the percent lumen from 100. The calculations were made in 10 seminal tubules per mose. The thickness of epithelium was measured from its basement to free surface on the same 10 tubules.

Three sections (head, tail, and middle) were selected per epididymis and seminal tubules within each section were counted for tubular density. From the 3 sections, 10 round to nearly round seminal tubules were chosen at random for morphometric evaluation per animal. The same method was applied to obtain the values from epididymis.

2.9 Statistical Analysis

The morphometric parameters and the average histopathologic scores were analyzed by unpaired two sample student's t test. A P value less than 0.05 was considered significant.

2.10 *In Situ* Hybridization- RNAscope

To detect HIV viral RNA in the different tissues, a novel next generation RNA in situ hybridization technology was used as previously described (Smedley et al., 2014). Each series of specific HIV target probes were designed to hybridize to viral RNA in gag, pol, vif, vpx, vpr, tat, rev, env, and nef genes which target ~4kb of the viral genome. The target probe design strategy was described previously (Wang et al., 2012). After in situ hybridization all slides were counterstained with hematoxylin, mounted in Permount, and scanned at high magnification (×400) using the ScanScope AT2 System (Aperio Technologies), yielding high-resolution data from the entire tissue section. Representative regions of interest were identified and high-resolution images extracted from these whole-tissue scans.

Chapter 3

Results

3.1 Viremic hu-HSC Mice Showed Histopathologic Lesions in Their Testis and Epididymis That are Potentially Related to HIV-1 Infection

Testis, epididymis, seminal vesicles, prostate glands, penis/urethra, and vas deferens were collected from 25 viremic hu-HSC mice and 28 negative control hu-HSC mice. At the time of collection, the average viremia level was 4.83 E+04copies/mL (1.3 E+03 – 5.90 E+06 copies/mL), the average age was 8-month-old (5 to 12-month old), and the average duration of infection was 12 weeks (4 to 22 weeks post infection). The tissue samples were processed in paraffin blocks and stained with hematoxylin and eosin for histopathologic evaluation by a board certified veterinary pathologist.

Both viremic and negative control groups showed histopathologic lesions in the testis and/or epididymis but lesions found in viremic samples were more severe and more frequent compared to the negative control samples. Out of 25 viremic mice, 16 mice (64%) showed histopathologic lesions in the testis and/or epididymis compared to 9 out of 28 negative control mice (32%). The lesions found in the testes of viremic mice include various levels of germinal cell degeneration and loss, multinucleated giant sperm cells, testicular interstitial leukocyte infiltration, apoptosis and vacuolization of germinal cells, interstitial fibrosis, testicular granuloma, oligospermia and azoospermia (Figure 9). The lesions found in the epididymis of viremic mice include oligospermia, azoospermia, epididymal granuloma, tubular ectasia and leukocyte infiltration (Figure 10). Out of 8 NL4-3 virus infected mice, 4 mice (50%) had histopathologic lesions only in the testis. Out of 17 Bal virus infected mice, 9 mice (9/17; 53%)

had lesions only in the testis, 1 mouse (1/17; 6%) had lesions only in the epididymis, and 4 mice (4/17; 24%) had lesions in both.

The lesions found in the testis of negative control mice include various levels of germinal cell degeneration and loss, multinucleated giant sperm cells, testicular interstitial leukocyte infiltration, mineralization, fibrosis, oligospermia and azoospermia. The lesions found in the epididymis of negative control mice include oligospermia and epididymal granuloma. Out of 28 negative control mice, 6 mice (6/28; 21%) had lesions only in testis, 2 mice (2/28; 7%) had lesions only in epididymis, and 1 mouse (1/28; 3%) had lesions both.

The lesions were scored based on the presence of testicular germinal cell degeneration (Deg), multinucleated giant spermatids (MGS), testicular oligospermia or azoospermia (T O/A), other testicular lesions (T Other), epididymal oligospermia or azoospermia (E O/A) and other epididymal lesions (E Other) (Table 1 and 2). If a category of lesion was present in a mouse, a score of one was given regardless of the severity, and the total scores were added. Mice were grouped based on age, 5-7 month old and 8-12 month old, and the average of each category of lesion and total scores were compared between viremic and negative control mice per age group. (Figure 11). The lesions were observed in 11/13 (85%) of 8-12 month old viremic mice compared to 9/17 (53%) of 8-12 month old negative control mice (Table 1). These lesions were also observed 5/12 (42%) of 5-7 month old viremic mice compared to no lesions observed in 5-7 month old negative control mice (Table 2). Both the 5-7 month old viremic mouse group and 8-12 month old viremic mouse group showed significantly higher frequency of testicular oligospermia/azoospermia ($p<0.05$) compared to the age-matched negative control group. 5-7 month old viremic mouse group showed significantly higher frequency of testicular germinal cell degeneration ($p<0.05$) compared to the age-matched negative control group. In addition, the

average of total histopathological lesion scores was significantly higher both in 5-7 month old viremic mouse group and 8-12 month old viremic mouse group compared to that of age-matched negative control mouse groups ($p<0.05$) (Figure 11).

Out of thirteen 8-12 month old viremic mice, 8 mice (8/13; 62%) had testicular germinal cell degeneration, 4 mice (4/13; 31%) had multinucleated giant spermatids, 6 mice (6/13; 46%) had testicular oligospermia or azoospermia, 4 mice (4/13; 31%) had epididymal oligospermia or azoospermia, 4 mice (4/13; 31%) had other testicular lesions, and 2 mice (2/13; 15%) had other epididymal lesions which are listed in Table 1 and 2. Out of seventeen 8-12 month old negative control mice, 5 mice (5/17; 29%) had testicular germinal cell degeneration, 2 mice (2/17; 12%) had multinucleated giant spermatids, 1 mouse (1/17; 6%) had testicular oligospermia or azoospermia, 1 mouse (1/17, 6%) had epididymal oligospermia or azoospermia, 5 mice (5/17; 29%) had other testicular lesions and 2 mice (2/17; 11.8%) had other epididymal lesions which are listed in Table 1 and 2.

Out of twelve 5-7 month old viremic mice, 4 mice (4/12; 36%) had testicular germinal cell degeneration, 2 mice (2/12; 17%) had multinucleated giant spermatids, 5 mice (5/12; 42%) had testicular oligospermia or azoospermia, and 3 mice (3/12; 25%) had other testicular lesions. No lesions were observed in eleven 5-7 month old negative control mice.

Overall, out of 25 viremic mice, 12 mice (12/25; 48%) had testicular germinal cell degeneration, 6 mice (6/25; 24%) had multinucleated giant spermatids, 11 mice (11/25; 44%) had testicular oligospermia or azoospermia, 4 mice (4/25; 16%) had epididymal oligospermia or azoospermia, 7 mice (7/25; 28%) had other testicular lesions and 1 mouse (2/25; 8%) had other epididymal lesions. Out of 28 negative control mice, 5 mice (5/28; 18%) had testicular germinal cell degeneration, 2 mice (2/28; 7%) had multinucleated giant spermatids, 1 mouse (1/28; 4%)

had testicular oligospermia or azoospermia, 1 mouse (1/28, 4%) had epididymal oligospermia or azoospermia, 5 mice (5/28; 18%) had other testicular lesions and 2 mice (2/28; 7%) had other epididymal lesions.

There were no histopathologic lesions noted in any other organs (spleen, lymph nodes, penis/urethra, vas deferens, seminal vesicles and prostate glands).

We compared the tubular number density, epithelium thickness and percent lumen/epithelium of testes and epididymis in viremic mice (n=8) to those of negative mice (n=4) using morphometric analysis (Figure 12) and found no significant difference between the two groups (Figure 13 and 14). Out of the 8 viremic mice used for this analysis, 4 of them had histopathologic lesions in the testis/epididymis. Three out of those 4 mice had testicular germinal cell degeneration and 1 out of those 4 mice had testicular oligospermia/azoospermia. Out of the 4 negative control mice used for this analysis, 2 mice had histopathologic lesions in the testis/epididymis and 1 of those 2 mice had testicular germinal cell degeneration. The numerical values are depicted in Table 3.

3.2. CD4 Positive Cells are Present in the Testicular and Epididymal Interstitium of hu-HSC Mice

Staining of testicular and epididymal tissues with CD4 antibody established the presence of CD4 positive cells in the testicular or epididymal interstitium, indicative of the presence of T helper cells, monocytes, macrophages or dendritic cells in these tissues of hu-HSC mice (Figure 15). Out of 22 viremic mice, 4 (22%) showed CD4 positive cells in the testis or epididymis. Out of the 4 with positive staining, one had CD4 positive cells only in the epididymis, and the other three had CD4 positive cells only in the testis. The CD4 cells found in the testis or epididymis of all four mice were associated with histopathologic changes in the tissues.

Out of 17 negative control mice, 7 (41%) showed CD4 positive cells in the testis or epididymis. Out of the 7 with lesions, 4 had CD4 positive cells in the epididymis, 1 had CD4 positive cells in the testis, and two had in both. Out of the 7, CD4 cells in 5 of them were associated with histopathologic lesions in the testis or epididymis and 2 were free of any changes to the tissues.

In general, CD4 positive cells were rare to few in numbers. This lower numbers of CD4 cells in viremic mice is likely due to virus mediated cell depletion. The CD4 positive cells were found in the testicular interstitium and epididymal interstitium only and never in the lumen of either testiss or epididymis. Two negative control mice had epididymal granuloma in which the CD4 positive cells surrounded the lesion. One Bal virus infected viremic mouse had testicular granuloma in which the CD4 positive cells surrounded the lesions.

3.3. Viral Dissemination is Seen in MRT of hu-HSC Mice Subsequent to HIV-1 Infection

To verify dissemination of the virus, the MRT sections obtained from viremic mice at various durations of infection were subjected to RNA in situ hybridization to detect HIV-1 sequences. HIV-1 positive cells were detected in the spleens of infected mice while as expected, the uninfected spleens were negative.

A total of 11 mice were collected at 13-22 weeks-post-infection (average of 16 weeks-post-infection), and HIV-1 positive cells were detected in at least one MRT section of 9 mice (9/11; 82%). The MRT most often harbored by HIV-1 positive cells were penis and prepuce (6/11; 55%), followed by seminal vesicles (5/11; 45%), testes (3/11; 27%), epididymis (1/11; 9%) and prostate glands (1/11; 9%) (Figure 16). While spleens of viremic hu-HSC showed high numbers of infected cells, the numbers of infected cells found in MRT were relatively low.

Out of 4 Bal virus infected mice collected at 13 weeks-post-infection, HIV-1 positive cells were detected in at least one MRT section of all 4 mice (100%). The MRT most often harbored by HIV-1 positive cells were penis and prepuce (4 out of 4), followed by seminal vesicles (1 out of 4) and testes (1 out of 4).

Out of 7 NL4-3 virus infected mice collected at 14-22 week-post-infection, HIV-1 positive cells were detected in at least one MRT section of 5 mice (71%). The MRT most often harbored by HIV-1 positive cells were seminal vesicles (4 out of 7), followed by penis and prepuce and testes (2 out of 7), epididymis (1 out of 7), and prostate (1 out of 7). One animal (J2111) in particular showed a large aggregation of infected cells in the spleen and infected cells in multiple organs including testis, epididymis and multiple sections of seminal vesicles. This animal had higher level of viremia among the group (1.89E + 05 copies/mL), one of the most severe testicular germinal cell degeneration and loss with epithelial apoptosis and vacuolation and lesion-associated CD4 positive cells present in the testes.

3.4 Vaginal Plugs (VP) Failed to Show Presence of HIV-1

A total of 19 experimental and 8 negative control VPs were collected and processed. The 19 experimental VPs came from a total of 9 males that had high viremia throughout their trio-housing with the females, averaging about 2.28E+05 copies/mL. Only 3 out of 19 cases of VP collection were retrieved from humanized female mice and the rest were retrieved from unengrafted/non-irradiated $Rag2^{-/-}\gamma c^{-/-}$ female mice. All VPs had undetectable level of HIV-1 RNA in qRT-PCR as shown in Table 4.

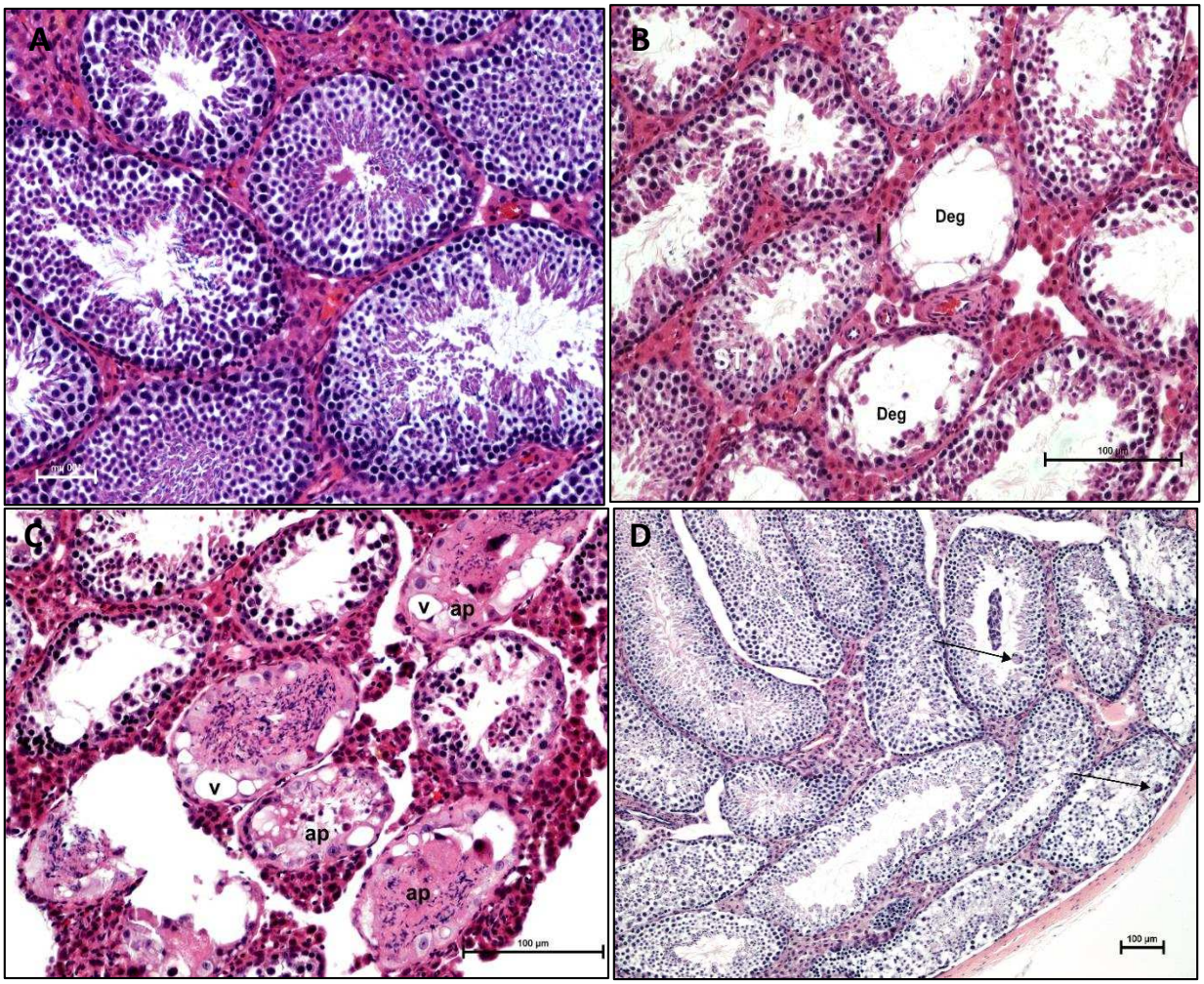

Figure 9: Histopathologic Lesions in Testis of Viremic hu-HSC Mice. (A) Negative control 10-month-old hu-HSC mouse testes: heterogeneous population of germinal cells with tailed spermatids in the lumen of seminal tubules (normal spermatogenesis). Interstitium contains blood vessels and leydig cells (200x); (B) Viremic 12-month-old hu-HSC mouse testes: severe degeneration of seminiferous epithelium (Deg) and oliospermia present (200x); (C) Viremic 12-month-old hu-HSC mouse testes: severe degeneration, vaculation (v) and apoptotic (ap) seminiferous epithelium present. Azoospermia evident (200x); (D) Viremic 8-month-old hu-HSC mouse testes: moderate degeneration of seminiferous epithelium with multinucleated spermatids (arrows) (100x).

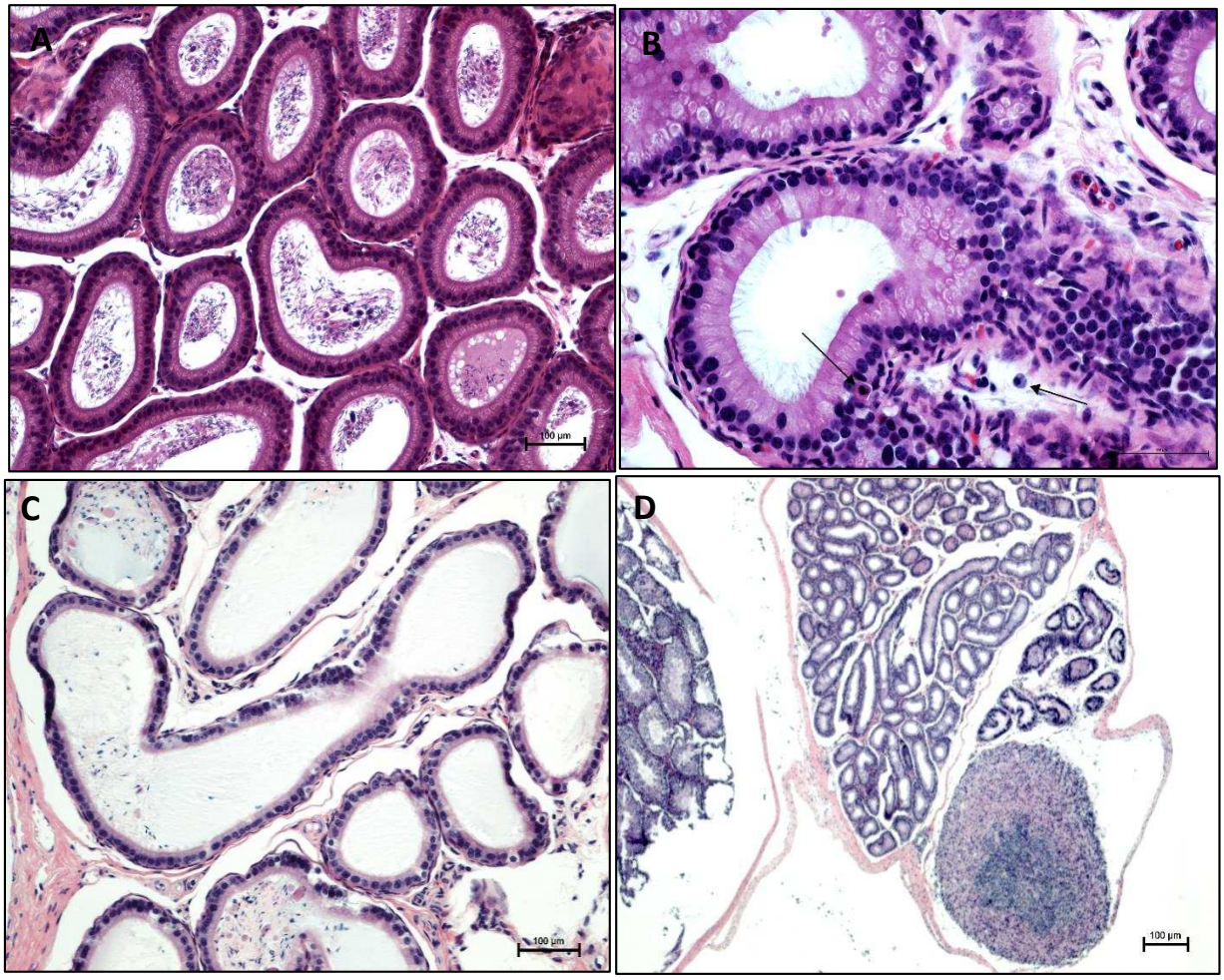

Figure 10: Histopathologic Lesions in Epididymis of Viremic hu-HSC Mice. (A) Negative control 10-month-old hu-HSC mouse epididymis: presence of maturing sperm in lumen (100x); (B) Viremic 12-month-old hu-HSC mouse epididymis: absence of maturing sperm in lumen – oligospermia and presence of mononuclear cells in interstitium (200x); (C) Viremic 8-month-old hu-HSC mouse epididymis: severe oligospermia (200x); (D) Viremic 10-month-old hu-HSC mouse: presence of epididymal spermatic granuloma (40x).

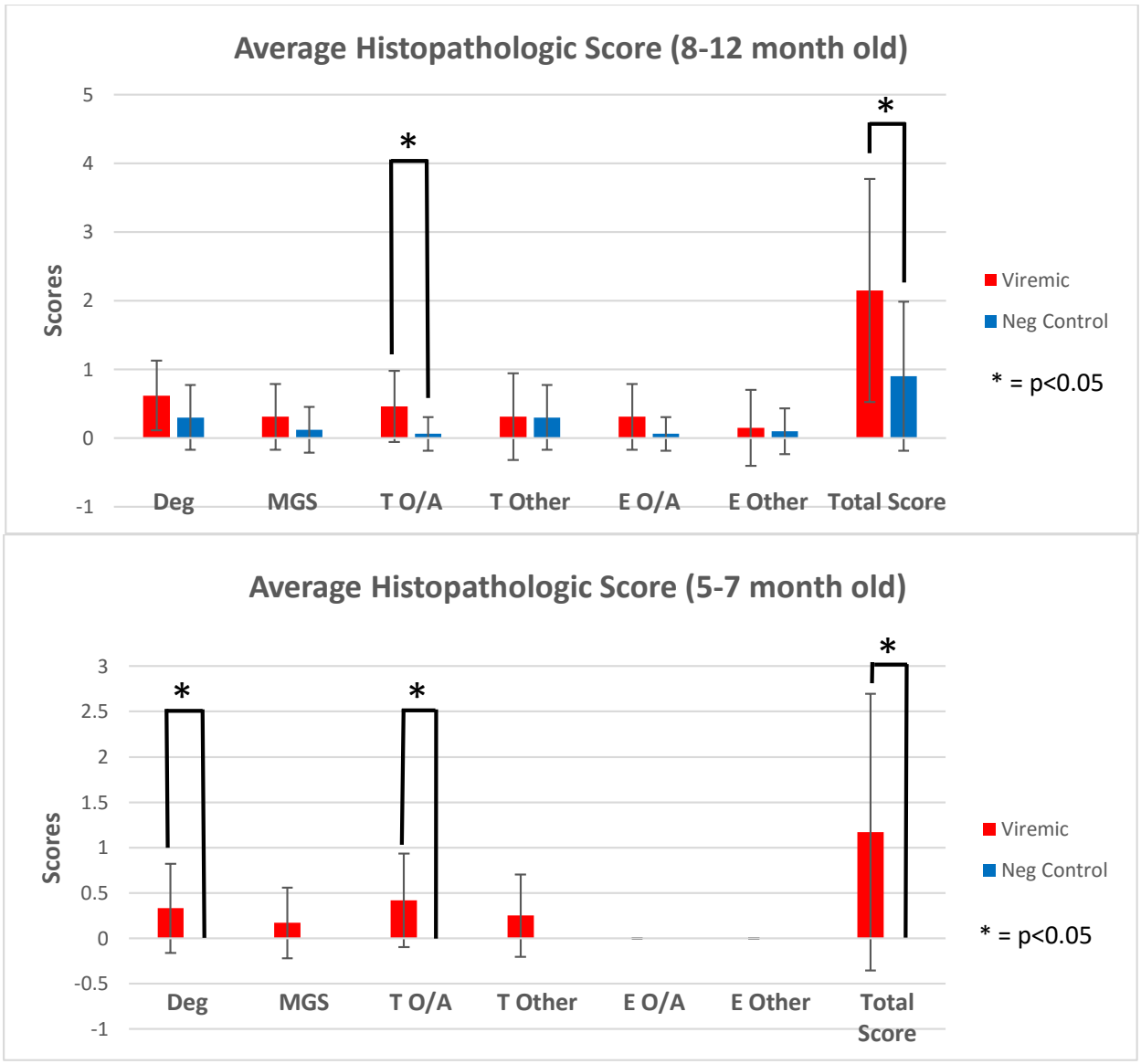

Figure 11. Average Histopathologic Scores Based on Six Categories and Total Average Scores in Viremic and Negative Control Mice – Two Age Groups. Lesions were scored by the presence of: testicular germinal cell degeneration (Deg), multinucleated giant spermatids (MSG), testicular oligospermia/azoospermia (T O/A), other testicular lesions (T Other), epididymal oligospermia/azoospermia (E O/A), and other epididymal lesions (E Other). The mice were further broken down by two age groups (5-7 month old and 8-12 month old). Both the 5-7 month old viremic mouse group and 8-12 month old viremic mouse group, showed significantly higher frequency of testicular oligospermia/azoospermia ($p<0.05$) compared to age-matched negative control groups. The 5-7 month old viremic mouse group showed significantly higher frequency of testicular germinal cell degeneration ($p<0.05$) compared to age-matched negative control group. The average of total histopathological lesion scores was significantly higher in both 5-7 month old viremic mouse group and 8-12 month old viremic mouse group compared to that of age-matched negative control mouse groups ($p<0.05$; asterisk).

Table 1. Histopathological Lesion Scoring in Testis and Epididymis of 8-12 Month Old Viremic (top) and Negative Control (bottom) Mice. Superscripts under "T Other" and "E Other" Columns: a- epididymal granuloma and epididymal interstitial inflammatory infiltrate; b- testicular interstitial inflammatory infiltrate; b- testicular interstitial fibrosis; c- testicular cyst; d- apoptotic testicular germinal cells and testicular vacuolation; e- testicular fibrosis; f- focal testicular vacuolation; g- testicular interstitial inflammatory infiltrate; h- epididymal granuloma; i- epididymal granuloma; j- focal testicular mineralization; k- focal testicular vacuolation.

8-12 month old viremic mice								
Animal ID	**Age (month)**	**Deg**	**MGS**	**T O/A**	**T Other**	**E O/A**	**E Other**	**Total Score**
2292	8	0	0	0	0	0	0	0
2294	8	1	1	1	0	0	2[a]	5
1728	8	1	0	1	1[b]	1	0	4
1711	8	1	1	1	0	1	0	4
J2040	8	0	0	0	1[c]	0	0	1
J2247	8	0	0	0	0	0	0	0
J1981	9	1	0	1	0	0	0	2
J2181	9	1	0	0	0	0	0	1
J2072	10.5	0	0	0	0	1	0	1
J2073	10.5	1	0	0	0	0	0	1
J2074	10.5	1	1	1	0	0	0	3
J2075	10.5	0	1	1	0	1	0	3
J2111	12	1	0	0	2[d]	0	0	3
Total		**8**	**4**	**6**	**4**	**4**	**2**	**28**

8-12 month old negative control mice								
Animal ID	**Age (month)**	**Deg**	**MGS**	**T O/A**	**T Other**	**E O/A**	**E Other**	**Total Score**
J2228	9.5	0	0	0	0	0	0	0
2239	9	1	0	0	0	0	0	1
2234	10	0	0	0	0	0	0	0
2335	10	1	0	0	1[e]	0	0	2
2233	10	0	0	0	0	0	0	0
2224	10	0	0	0	0	0	0	0
2232	10	1	1	1	0	0	0	3
J2084	10.5	1	0	0	1[f]	0	1[g]	3
J2085	10.5	0	0	0	1[h]	0	0	1
J2086	10.5	1	1	0	0	0	0	2
2211	10.5	0	0	0	0	0	1[i]	1
1825	10.5	0	0	0	1[j]	0	0	1
2193	11	0	0	0	0	0	0	0
1793	12	0	0	0	0	0	0	0
1797	12	0	0	0	0	0	0	0
1799	12	0	0	0	0	0	0	0
2146	12	0	0	0	1[k]	1	0	2
Total		**5**	**2**	**1**	**5**	**1**	**2**	**16**

Table 2. Histopathological Lesion Scoring in Testis and Epididymis of 5-7 Month Old Viremic (top) and Negative Control (bottom) Mice. Superscripts under "T Other" Columns: a- testicular interstitial inflammatory infiltrate; b- testicular interstitial fibrosis; c- testicular interstitial inflammatory infiltrate.

5-7 month old viremic mice								
Animal ID	**Age (month)**	**Deg**	**MGS**	**T O/A**	**T Other**	**E O/A**	**E Other**	**Total Score**
1919	6	1	1	1	1[a]	0	0	4
1875	7	0	0	1	0	0	0	1
1874	7	0	0	0	0	0	0	0
2337	7	0	0	0	0	0	0	0
2338	7	1	0	1	0	0	0	2
J2270	7	0	0	0	0	0	0	0
J2259	7	0	0	0	0	0	0	0
J2271	7	0	0	0	0	0	0	0
J2278	7	0	0	0	1[b]	0	0	1
J2283	7	1	0	1	0	0	0	2
2392	7	1	1	1	1[c]	0	0	4
2394	7	0	0	0	0	0	0	0
total		**4**	**2**	**5**	**3**	**0**	**0**	**14**

5-7 month old negative control mice								
Animal ID	**Age (month)**	**Deg**	**MGS**	**T O/A**	**T Other**	**E O/A**	**E Other**	**Total Score**
J2401	**5**	0	0	0	0	0	0	0
J2402	**5**	0	0	0	0	0	0	0
J2403	**5**	0	0	0	0	0	0	0
J2405	**5**	0	0	0	0	0	0	0
J2379	**6**	0	0	0	0	0	0	0
J2380	**6**	0	0	0	0	0	0	0
J2381	**6**	0	0	0	0	0	0	0
1900	**7**	0	0	0	0	0	0	0
1901	**7**	0	0	0	0	0	0	0
1902	**7**	0	0	0	0	0	0	0
1903	**7**	0	0	0	0	0	0	0
Total		**0**	**0**	**0**	**0**	**0**	**0**	**0**

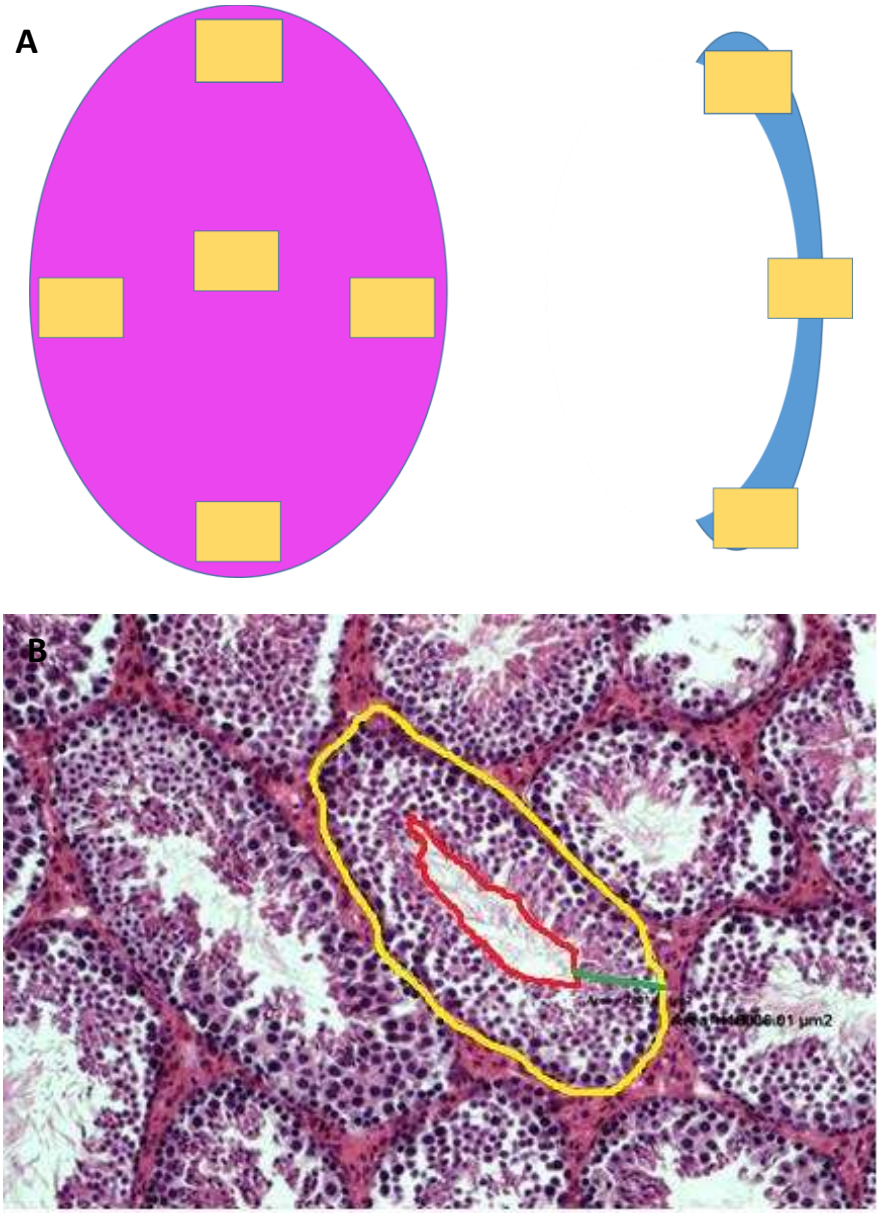

Figure 12: Morphometric Analysis Method. (A) Schematic for measurement of Tubular number density (# of tubules/mm^2); (B) Image for measurement of Total area in yellow (μm^2), lumen area in red (μm^2), epithelium area (μm^2), and epithelium thickness in green (μm) (200x).

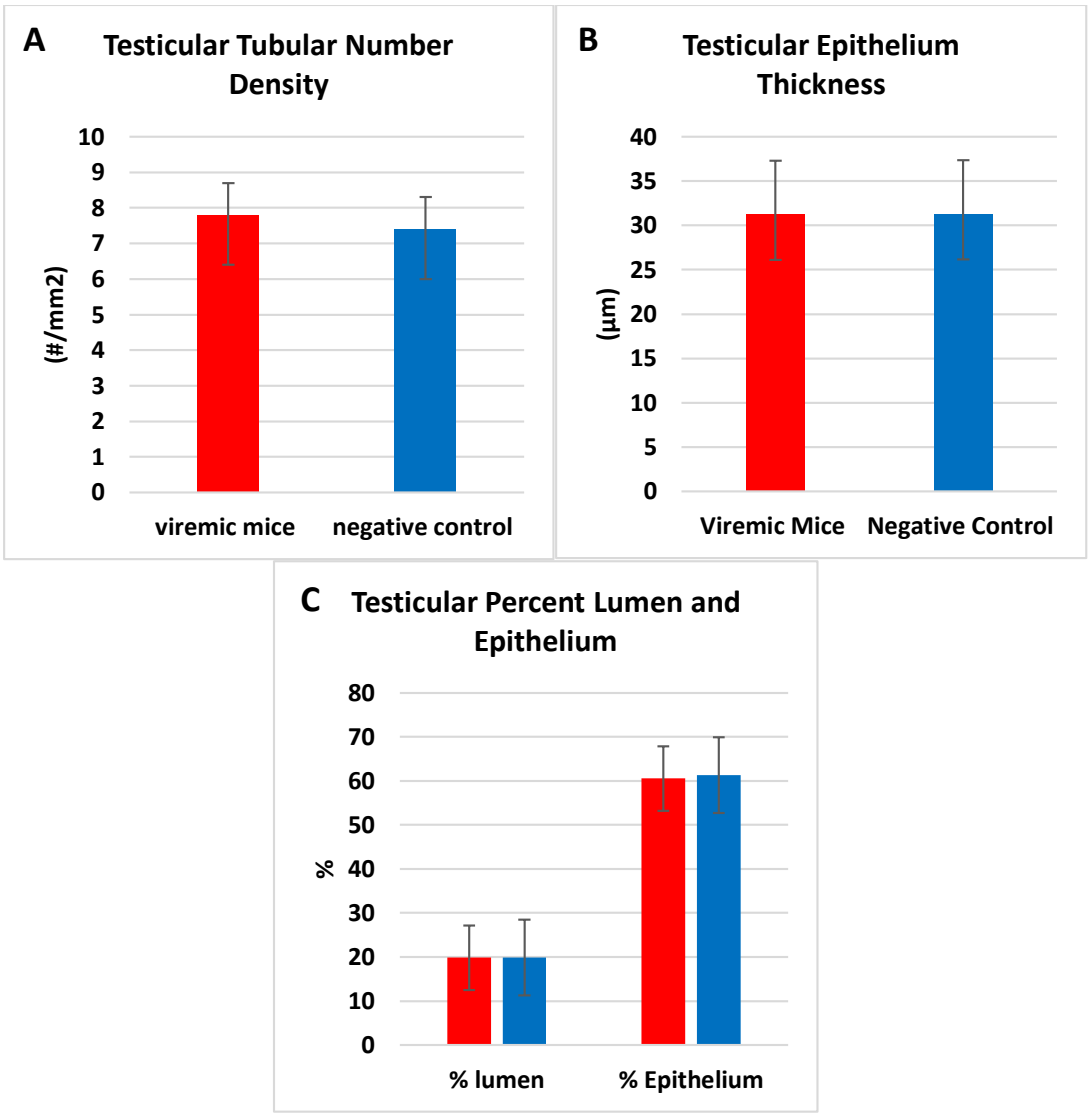

Figure 13: Morphometric Analysis of Testicular Epithelium – Measure of Degenerative Seminiferous Epithelium. (A) Testicular tubular number density in testis of viremic (n=8; red) vs. negative control (n=4; blue) hu-HSC; p=0.5527; (B) Testicular epithelium thickness in testis of viremic vs. negative control hu-HSC; p=0.2451; (C) Testicular percent lumen and epithelium in testis of viremic vs. negative control hu-HSC; percent lumen: p=0.9951 and percent epithelium: p=0.9951. No significant differences were found between the groups.

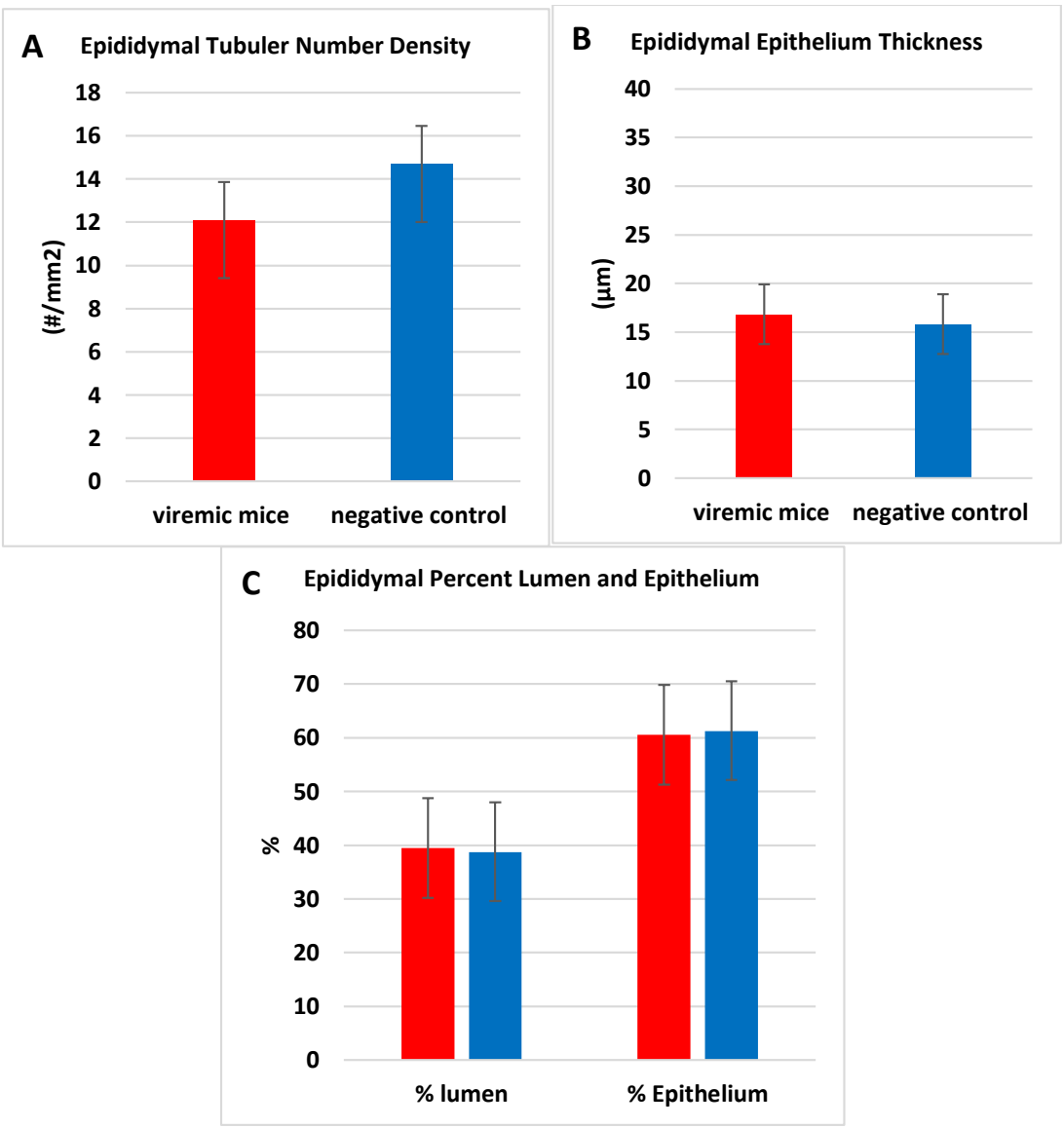

Figure 14: Morphometric Analysis of Epididymis Epithelium – Measure of Degenerative Epididymal Epithelium. (A) Epididymal tubular number density in testis of viremic (n=8; red) vs. negative control (n=4; blue) hu-HSC; p=0.0702; (B) Epididymal epithelium thickness in testis of viremic vs. negative control hu-HSC; p=0.7349; (C) Epididymal percent lumen and epithelium in testis of viremic vs. negative control hu-HSC; percent lumen: p=0.8998 and percent epithelium: p=0.8998. No significant differences were found between the groups.

Table 3. Values of Morphological Parameters Obtained from Testicular Seminiferous Tubules and Epididymal Epithelium of Viremic and Control Mice.

TESTIS	viremic mice (N=8)	negative control (N=4)	P value
Tubular number density (#/mm^2)	7.8 ± 0.9	7.4 ± 1.4	0.5527
Total Area (µm^2)	16010.5 ± 4465.01	18105.99 ± 4654.15	0.4667
Lumen Area (µm^2)	3030.78 ± 1515.31	3613.88 ± 1667.20	0.5558
Epithelium Area (µm^2)	11621.6 ± 3713.28	14492.11 ± 3981.75	0.2451
Epithelium Thickness (µm)	31.27 ± 6.05	31.305 ± 5.15	0.9923
% lumen	19.88 ± 7.33	19.85 ± 8.61	0.9951
% Epithelium	80.12 ± 7.33	80.15 ± 8.61	0.9951
EPIDIDYMIS	**viremic mice** (N=8)	**negative control** (N=4)	**P value**
Tubular number density (#/mm^2)	12.08 ± 1.78	14.67 ± 2.67	0.0702
Total Area (µm^2)	11065.71 ± 4059.27	9518 ± 3780.23	0.5394
Lumen Area (µm^2)	4740.19 ± 2268.45	3695.13 ± 1676.63	0.4371
Epithelium Area (µm^2)	6325.39 ± 2322.93	5823.09 ± 2431.59	0.7349
Epithelium Thickness (µm)	16.78 ± 3.11	15.81 ± 3.03	0.6189
% lumen	39.46 ± 9.26	38.73 ± 9.16	0.8998
% Epithelium	60.54 ± 9.26	61.27 ± 9.16	0.8998

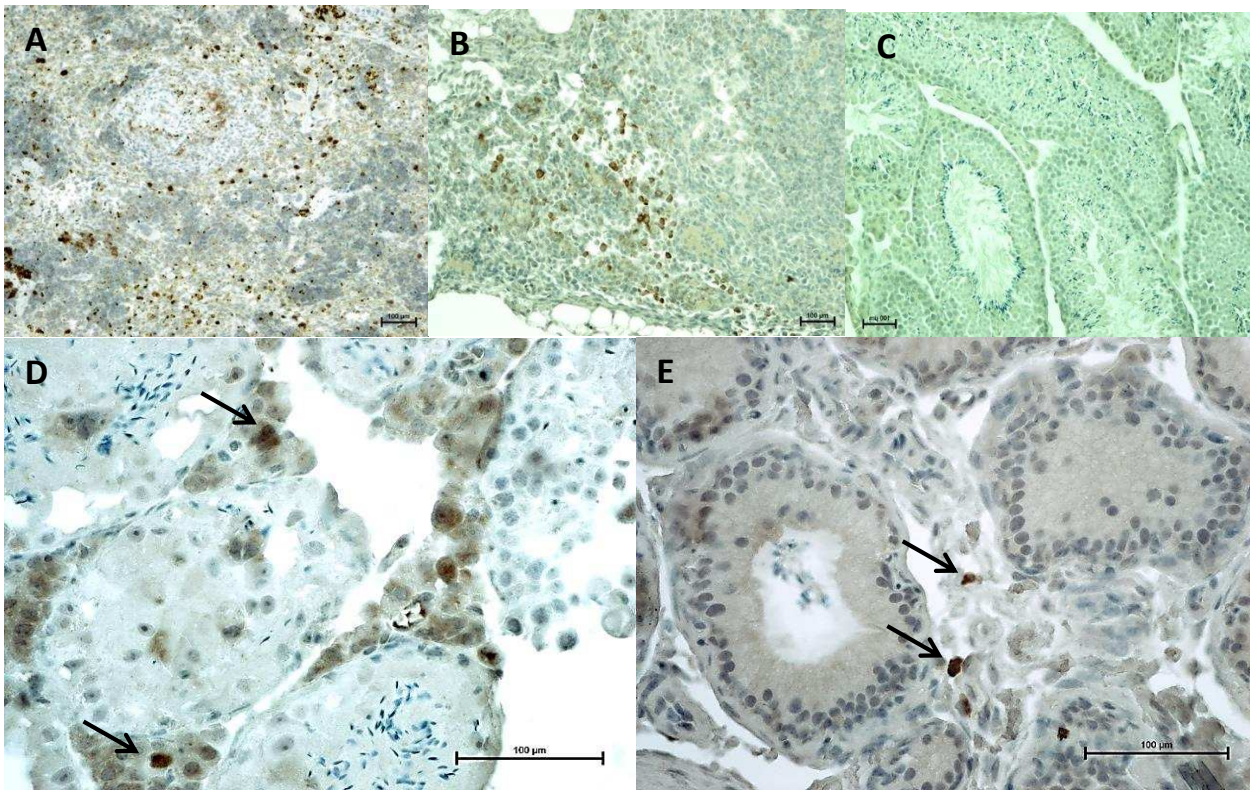

Figure 15: Immunohistochemistry of CD4 Positive Cells in Testes and Epididymis of hu-HSC Mice. (A) Positive control, CD4 staining in hu-HSC mouse spleen (100x); (B) Positive control, CD4 staining in hu-HSC mouse lymph node (100x); (C) Negative CD4 staining in viremic hu-HSC mouse testes (100x); (D) Positive CD4 staining in viremic hu-HSC mouse testicular interstitium (arrows) (200x); (E) Positive CD4 staining in viremic hu-HSC epididymal interstitium (arrows)(200x).

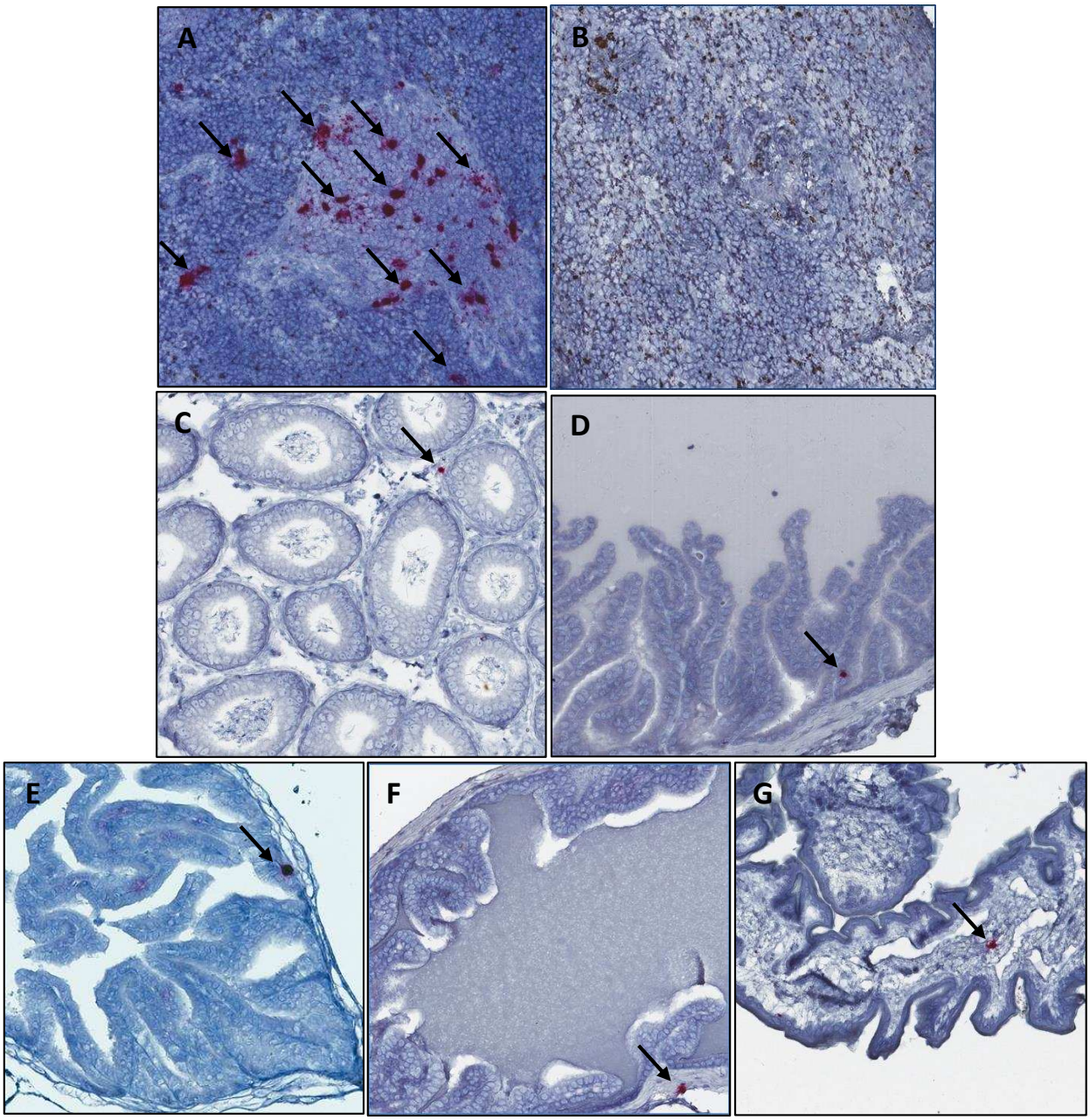

Figure 16: Identification of HIV-1 Infected Cells in the MRT of Viremic Mice by *in situ* Hybridization (100x). (A) Positive control, viremic hu-HSC mouse spleen with HIV-1 infected cells (arrows); (B) Negative control, negative control hu-HSC mouse spleen showing no signal; (C-G) Viremic hu-HSC mouse MRT displaying infected cells (arrows); (C) Testes; (D) epididymis; (E) Seminal Vesicles; (F) Prostate; (G) Prepuce.

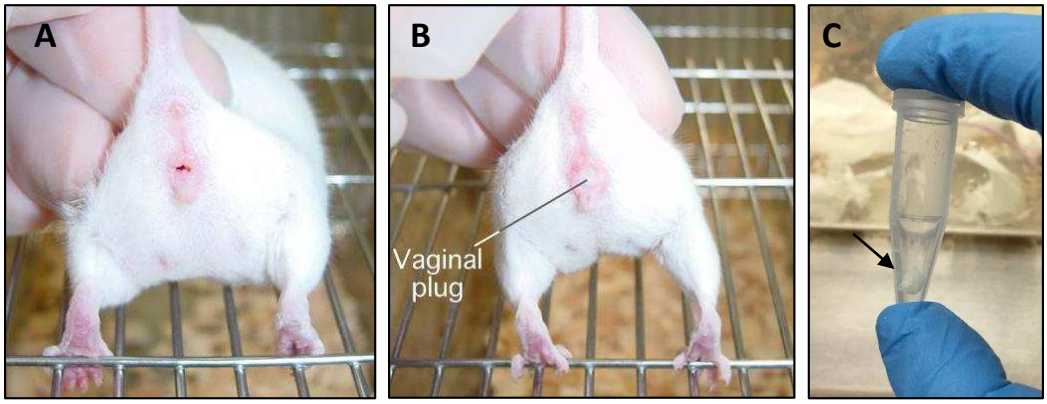

Figure 17. Vaginal Plugs (VP). A) No VP present after being housed with a male for 24 hours, this pair has not mated; B) VP present after being housed with a male for 24 hours, this pair has mated over night; C) VP collected after retrieved from vaginal opening (arrow).

Table 4. Vaginal Plug Samples. Uneng = unengrafted; LOD = level of detection.

Sample ID	Neg vs. exp	Male	Female	viremia (copies/mL)	Virus strain	qRT-PCR Result
1	neg	uneng	uneng	-	-	Below LOD
2	neg	uneng	uneng	-	-	Below LOD
3	neg	uneng	uneng	-	-	Below LOD
4	neg	uneng	uneng	-	-	Below LOD
5	neg	uneng	uneng	-	-	Below LOD
6	neg	uneng	uneng	-	-	Below LOD
7	neg	uneng	uneng	-	-	Below LOD
8	neg	uneng	uneng	-	-	Below LOD
9	exp	J2072	humanized	7.46E+05	NL4-3	Below LOD
10	exp	J2073	uneng	2.05E+04	NL4-3	Below LOD
11	exp	J2104	humanized	4.20E+05	NL4-3	Below LOD
12	exp	J2270	uneng	4.20E+05	Bal	Below LOD
13	exp	J2247	uneng	8.29E+04	Bal	Below LOD
14	exp	J2270	uneng	4.20E+05	Bal	Below LOD
15	exp	J2181	uneng	1.56E+05	Bal	Below LOD
16	exp	J2283	uneng	2.72E+04	Bal	Below LOD
17	exp	J2270	humanized	4.20E+05	Bal	Below LOD
18	exp	J2247	uneng	8.29E+04	Bal	Below LOD
19	exp	J2181	uneng	1.56E+05	Bal	Below LOD
20	exp	J2259	uneng	4.34E+04	Bal	Below LOD
21	exp	J2270	uneng	4.20E+05	Bal	Below LOD
22	exp	J2283	uneng	2.72E+04	Bal	Below LOD
23	exp	J2259	uneng	4.34E+04	Bal	Below LOD
24	exp	J2181	uneng	1.56E+05	Bal	Below LOD
25	exp	J2181	uneng	1.56E+05	Bal	Below LOD
26	exp	J2278	uneng	7.76E+05	Bal	Below LOD
27	exp	J2181	uneng	1.56E+05	Bal	Below LOD
28	water	-	-	-	-	Below LOD
29	water	-	-	-	-	Below LOD
30	water	-	-	-	-	Below LOD

Chapter 4

Discussion and Future Directions

Acquired immune deficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV) is one of the most destructive pandemic disease in history. Currently, about 36.9 million people are living with HIV globally (UNAIDS, 2015). Access to antiretroviral therapy has increased in many parts of the world, but the number of new infections continue to rise in the millions, globally. Sexual transmission is the most common route of HIV transmission between people, accounting for 80% of new infections worldwide (Royce et al., 1997; Geneva: World Health Organization, 2005). While seminal HIV is the most prevalent vector in transmission of this disease, little is known about the mechanisms and pathogenesis of HIV in semen or the male reproductive tract (MRT).

Chronic HIV-1 infection is known to cause infertility and histopathologic changes to MRT of human patients (Dondero et al., 1996; Pudney & Anderson, 1991; Umapathy et al., 2001; Nicopoullos et al., 2004). Infected cells have been identified in the MRT, and HIV-1 can be detected in semen as early as few weeks after primary infection in human patients (Brett Tindall et al., 1992). Phylogenetic analysis of nucleotide sequences of the virus also demonstrated that there's genetic variance between the virus found in semen and plasma within an infected individual (Byrn & Kiessling, 1998; Coombs et al., 1998; Kiessling et al., 1998), suggesting compartmentalization and local replication of the virus in the MRT. Continued shedding of HIV-1 in the semen despite antiretroviral therapy with no detectable plasma viral load and identification of drug resistant mutation of the seminal virus further supports this theory of viral compartmentalization in (Bujan et al., 2004; Zhang et al., 1998). However, the mechanism by which HIV-1 enters the MRT and sheds to semen is still not well defined. In

addition, testes are considered immune privileged organs with physical, immunological and hormonal defense systems to sustain normal spermatogenesis (Siu & Cheng, 2004; Meng et al., 2005; Page et al., 2006; Bhushan et al., 2011; Winnall et al., 2011). Because of this protected environment and resistance to entrance of particles such as drugs or circulating immune cells, investigating the source of HIV-1 infection in these organs can be challenging.

Few earlier studies have demonstrated possibilities of both testicular germinal cells and leukocyte involvement in the pathogenesis and shedding of HIV in semen or the MRT using autoptic samples from deceased chronically infected HIV and AIDS patients (Muciaccia, Uccini, et al., 1998. Pudney & Anderson, 1991). Fortunately, with the help of antiretroviral therapy, the life expectancy of HIV patients has increased dramatically and the development of AIDS in these patients have decreased worldwide. But because of this, the feasibility and practicality of studies involving patient tissues have decreased over the decades, and development of animal models to study this disease in these particular organs became a focus.

Both humans and chimpanzees are susceptible to HIV-1 infection (Saksela et al., 1993), but chimpanzees do not experience the depletion or dysfunction of CD4+ T cells seen in humans (Rutjens et al., 2008), and they rarely develop AIDS (Watanabe et al., 1991). Other lentivirus models that causes immunodeficiency in host animals have been investigated and especially studies of simian immunodeficiency virus (SIV) or simian human immunodeficiency virus (SHIV) in nonhuman primates have tremendously advanced our knowledge on the disease process (Fauci & Desrosiers, 1997). Rhesus macaques have been used most commonly for studies of SIVmac infection which have shown parallel disease progression as humans such as CD4+ T cell depletion and dysfunction, and development of simian AIDS with chronic infection (Veazey et al., 1998; Mattapallil et al., 1999). Furthermore, SIV infection causes similar clinical

and histopathologic changes in spermatogenesis and the MRT of infected rhesus macaques (Baskerville, 1992). It has been shown that seminal CD4+ T cells and macrophages, as well as testis, epididymis, prostate, and urethra are productively infected with SIV in this animal model (Bernard-Stoecklin et al., 2013; Matusali et al., 2015).

Because of the genetic differences between HIV and SIV and the immunogenic difference between humans and nonhuman primates (Shibata et al., 1995; Pollom et al., 2013), humanized mice have been used widely as an alternative animal model to study HIV-1. There are many types of humanized mice, including the human cell engraftment in severe combined immunodeficient mice (SCID) to the most recent $Rag2^{-/-}\gamma c^{-/-}$ models. Hu-HSC (Rag-hu) mice, created by intrahepatic human CD34+ stem cell engraftment in irradiated $Rag2^{-/-}\gamma c^{-/-}$ mice, have been an excellent animal model for HIV-1, with the ability to sustain long-term *de novo* multilineage human hematopoiesis, chronic HIV-1 infection and subsequent depletion of CD4+ T cells (Berges et al., 2006; Berges et al., 2010; Akkina, 2013a, Akkina, 2014). Vaginal, rectal and intestinal mucosal susceptibility to HIV-1 infection in this model has also been established (Berges et al., 2008) but no studies so far have been done to demonstrate HIV-1 infection in the MRT or semen in humanized mice.

In this project, we used HIV-1 infected hu-HSC mice to determine the characteristics of HIV-1 infection in the MRT. The MRT of viremic hu-HSC male mice were collected for histopathologic evaluation, immunohistochemistry, and *in situ* hybridization. In addition, vaginal plugs, collected from female mice that had mated with a viremic male mouse, were processed for presence of HIV-1 RNA using qRT-PCR. Our data demonstrate 1) the presence of potential HIV-1 related histopathologic lesions in testes and epididymis of viremic mice, 2) the presence of CD4+ cells in the testicular and epididymal interstitium of humanized mice, 3) the presence of

productive, HIV-1 infected leukocytes in the MRT (penis and prepuce, seminal vesicles, testes, epididymis, prostate glands) of viremic mice, 4) absence of viral loads in vaginal plugs from viremic mice.

The histopathologic lesions found in the mice were similar to that of chronically infected HIV and AIDS patients which included testicular germinal cell degeneration and loss, testicular and epididymal oligospermia and azoospermia, testicular and epididymal interstitial infiltration of leukocytes and testicular interstitial fibrosis (Figures 9 and 10). In order to analyze the mice and to isolate lesions that may be a result of HIV infection, the experimental mice were broken down into two age groups, 5-7 month of age and 8-12 month of age. These age groups were then examined to determine the role that HIV infection plays in the MRT, which allowed us to differentiate the effects that infection has, from that of age-related lesions. The degenerative lesions in the MRT were observed in both viremic and negative control mice in 8-12 month old group, however, viremic mice group had statistically significant increase in the frequency of these lesions (Figure 11). When the younger group of mice (5-7 month old) were examined, only the viremic mice showed the same degenerative lesions as observed in the older (8-12 month old) viremic mice. From this, we can conclude that while age plays a role in degenerative changes observed in the older group of mice (8-12 month old), HIV infection does result in an increased frequency of pathological lesions in the MRT of viremic mice from both age groups. This impact that HIV has on the MRT of viremic mice mimics that observed in the MRT of human patients with HIV-1 infection. To further substantiate the effects of HIV infection in the MRT, devoid of the impacts of age (Creasy et al., 2012), an investigation of histopathologic evaluation of MRT in additional younger viremic mice is underway.

Chronically infected HIV human patients suffer from decreased testosterone level and subsequent secondary pituitary androgen deficiency (Klein, Lo, Santoro, & Dobs, 2005). This condition contributes to clinical signs such as weight loss, fatigue, decreased in libido and sperm count. The cause for this decreased testosterone in HIV-infected men are multifactorial including poor nutritional and clinical support, drug use, opportunistic infections and age (Kietsiriroje, 2015). Gonatotropin hormones (GnRH), produced by the hypothalamus, are responsible for releasing follicular stimulating hormones (FSH) and lutinizing hormone (LH) from the anterior pituitary, the two hormones required for normal production of testosterone and spermatogenesis (Scanlon & Sanders, 2014). Secondary androgen deficiency seen in HIV-1 infected human patients are due to hypothalamic dysfunction where low level of testosterone does not trigger increased number of LH (Ashby et al., 2014). While not measured in our study, decreased testosterone and hormonal dysfunctions caused by the HIV-1 infection may have resulted in the degenerative changes in the MRT of viremic mice.

The morphometric evaluation and analysis of testicular germinal epithelium and epididymal epithelium did not show any significant differences between the viremic and negative control group (Table 3; Figures 13 and 14). This may be due to a small sample size and also may be due to smaller numbers of randomly selected tubular profile. In previous studies that used morphometric analysis in testes in rodent models, they have randomly chosen 100 to 200 seminal tubules per testes for evaluation (Absalan et al., 2008; Trindade et al., 2013), while in our study, we have randomly chose 10 seminal tubules per testis and epididymis. The lack of significant difference in our results can additionally be from the possibility that HIV-1 infection may not cause severe diffuse histopathologic lesions in testes or epididymides of humanized mice. Another explanation for this could be the fact that both viremic and negative control mice were

once irradiated at a dose of 350 rads (3.5 Gys) at 1-4 days old as a part of humanization protocol. Radiation exposure of 8 Gy is known to cause histopathologic alteration to the MRT in rodents (Samarth & Samarth, 2009).

We were also able to demonstrate the presence of human CD4+ cells in the testes and epididymides of humanized mice by immunohistochemistry. Confirmation of CD4+ cells were done with two control tissues, lymph nodes and spleen (Figure 15). Except for the few cases of testicular and epididymal granuloma where there were abundant CD4+ cells surrounding the lesions, CD4+ cells in general were scarce in numbers (one to five) in these tissues. The lower numbers of CD4 cells in viremic mice is likely due to virus mediated cell depletion. The fact that the CD4+ cells were limited to testicular and epididymal interstitum and never in the lumen or in the germinal cells is significant. An *in vitro* and *in situ* study done by Roulet et al., has demonstrated that CD4+ cells represented about 8% of total interstitial cells in normal human testes and further identified these cells as testicular resident macrophages (Roulet et al., 2006). They also identified CCR5 and CXCR4 receptors amongst 3-4% of all testicular interstitial cells and were able to demonstrate low numbers of productively infected cells in testicular interstitium in *in vitro* HIV-1infection using *in situ* hybridization for HIV RNA (Roulet et al., 2006). Additionally, a study showed expression of CD4, CCR5 and CXCR4 in human testicular interstitial macrophages (Habasque et al., 2002), further supporting testicular interstitial leukocytes as a potential reservoir for HIV-1.

The presence of productively infected cells in the MRT of viremic humanized mice has never before been demonstrated until this study. HIV-1 infected cells were found in the MRT of 9 out of 11 (82%) viremic mice, most often in penis and prepuce. HIV-1 and SIV infected cells have been demonstrated in testes, epididymides, prostate glands, seminal vesicles, and penis of

men or macaques respectively. Despite the fact that humanized mice lack human germinal or epithelial cells, we were able to show that HIV-1 infected leukocytes are indeed present in the MRT, most likely T-lymphocytes and macrophages, supporting the leukocyte involvement of the MRT infection. In addition, HIV-1 infected cells were found in the penis and prepuce in 6 out of 11 (55%) and in the seminal vesicles in 5 out of 11 (45%) of viremic mice, compared to other tissues of the MRT. Previous studies have shown that vasectomy had an insignificant effect on seminal viral load (D. J. Anderson et al., 1991; Krieger et al., 1998), suggesting most HIV-1 in semen may arise distal to testes, epididymides and vas deferens. The high prevalence of HIV-1 infected cells detected in prepuce is most likely due to its high degree of vascularization and subsequent presence of virus in the vessels. Seminal vesicle secretion accounts for large percentage of seminal fluid volume and therefore are likely to represent one of the main source of virus in semen.

Our study failed to detect HIV-1 viral load from the vaginal plugs (VP). We have chosen this indirect and non-invasive method of collecting semen from mice after careful consideration. The method of collecting semen from mice have been well established for artificial insemination and *in vitro* fertilization purposes. This usually requires either surgical or post mortem dissection of the caudal epididymis, and the mature spermatozoa are carefully teased out in a solution (Del Val & Robledano, 2013). There are many vasculatures in the epididymis including the branches of *epididymalis caudalis* in which divided into 2-3 smaller vessels that even further branches into numerous fine capillaries along the caudal parts of epididymis (Chubb & Desjardins, 1982). In consultation with experts in this field, in order to avoid the possible plasma viral loads that may exist in the blood contamination that is inevitable during this procedure, we opted not to go forward with this particular method. A whole tissue PCR of the MRT after complete removal of

blood with exsanguination and buffer saline perfusion, may increase the sensitivity of detection of the low numbers of infected cells in these tissues and seminal fluids while eliminating the contamination from the circulating virus in the vessels.

Another method considered to obtain semen from mice was electroejaculation under sedation. A study by Anderson et al., has shown that average of 1.5uL ejaculated volume can be collected a mouse via electroejaculation (Anderson et al., 1983). After consulting with the researchers that have used electroejaculation to collect mouse or rat semen, due to the high morbidities and mortalities (urethral blockage, azotemia, and retrograde ejaculation in the bladder) associated with the procedure and inconsistent results, we decided not to use this method either. The last method considered to obtain semen from mice was to euthanize the female after mating and to collect semen from their reproductive tract but this method would require culling of the female mice. VP in mice are coagulated form of semen, and are visible with naked eyes. After 24 hours of deposition, it is easily removed with gentle manipulation from the female reproductive tract. Therefore, this method of semen collection was chosen as a non-invasive method to decrease the chance of morbidity and mortality and to decrease the number of animals that would need to be euthanized. Often, when the female we collected a VP from returned to the trio-housing, we were able to collect another VP from the same female the next day.

Our study failed to show HIV-1 RNA in the vaginal plugs using qRT-PCR (Table 4). One of the possible causes for this would be that humanized mice may not harbor detectable level of HIV-1 in their semen. When negative samples were spiked with the virus, the assay showed appropriate detection of HIV-1 RNA levels. The detection level of qRT-PCR is 10^1- 10^6 copies/uL, and since most of the experimental samples showed no detectable levels of HIV-1

RNA with assay, we can conclude that our samples either did not contain any HIV-1 RNA or that our samples contained HIV-1 RNA below 10 copies/uL. Seminal viral load in infected humans and macaques (SIV) without treatment are usually in low number. An average seminal viral load of 330 copies/mL in one study (Hanabusa et al., 2000), 515copies/mL in another (Liuzzi et al., 1996), and 575 copies/mL in macaques (Matusali et al., 2015) have been reported. Other methods such as ddPCR which can detect 1 copy/uL could provide more sensitive data. Another reason for the undetectable level of HIV-1 RNA in the vaginal plugs maybe due to the fact that it was collected 24 hour post-deposition. Vaginal plugs that were checked daily during the trio-housing, were tightly adhered to the vaginal canal at presentation, and only after 24 hours, they were easily manipulated and removed. HIV is sensitive to change in pH level (Ongradi et al., 1990) and the lower pH of vaginal environment could have inactivated the virions and degraded the viral RNA over the 24 hours.

Overall, our study demonstrated for first time, the evidence of HIV-1 infection of the MRT in humanized mice via leukocyte involvement. Further characteristics of the CD4 staining cells in testes, epididymides and other tissues of the MRT as well as simultaneous *in situ* hybridization and immunohistochemistry may provide more concrete identity of the involved leukocytes.

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