PROTECTIVE T CELL RESPONSES AGAINST GENITAL HSV-2 INFECTIONS

INVESTIGATING THE ROLES OF LOCAL MUCOSAL IMMUNITY AND ESTRADIOL IN THE GENERATION OF PROTECTIVE T CELL RESPONSES AGAINST SECONDARY HSV-2 INFECTIONS IN THE FEMALE GENITAL TRACT

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TITLE: Investigating the roles of local mucosal immunity and estradiol in the generation of protective T cell responses against secondary HSV-2 infections in the female genital tract

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ABSTRACT

Genital herpes is one of the most prevalent sexually transmitted infections in the world and recent estimates indicate that over 500 million people are infected by herpes simplex virus type 2 **(HSV-2**). Although women are more susceptible to HSV-2 infections than men and the female genital tract is the primary site of HSV-2 infection, little is known about the generation of protective immune responses in this distinct microenvironment and how these immune responses protect against genital HSV-2 infections in women. Previous studies in our lab using an HSV-2 mouse model have found that protection against genital HSV-2 challenge in immunized mice correlates with the induction of inducible vaginal associated lymphoid tissues (**iVALTs**). The appearance of these structures coincides with the clearance of the virus, which suggests that protective immune responses against HSV-2 could be generated in the genital mucosa itself, without the help of draining lymph nodes. In addition, these iVALTs primarily consist of CD4+ T cells, underlying the importance of identifying and characterizing the type and function of T cells that are present in the genital mucosa and how these T cells provide specific protection against HSV-2 challenge, which has not been examined. In addition, previous studies have shown that estradiol (**E2**) has a protective effect against sexually transmitted viral infections, but the mechanisms by which E2 protects against these viral sexually transmitted infections (**STIs**) has not been examined. Therefore, the work undertaken in this thesis determines the conditions under which the genital mucosa can generate protective immune responses against HSV-2 infection and also characterizes the T cell responses that are generated in the genital

mucosa of immunized mice following HSV-2 challenge. We also determined the conditions under which E2 enhances protection against genital HSV-2 infections and examined the mechanism by which E2 regulates protective T cell responses in the genital mucosa following HSV-2 infection.

We first examined whether local immunization could establish effective antiviral memory responses directly in the female genital tract (**FGT**), without the help of draining lymph nodes, following HSV-2 infection. We found that even in the absence of secondary lymphoid organs (**SLOs**), mice immunized intravaginally (**IVAG**) with attenuated thymidine kinase-negative (**TK**$^-$) HSV-2 were completely protected against genital HSV-2 challenge, but they showed delayed viral clearance and prolonged genital pathology post-immunization compared to WT mice. Although local viral-specific antibody responses were compromised and T cell-mediated anti-HSV-2 responses were delayed in the absence of SLOs, the immune responses generated in the genital mucosa were effective in protecting against HSV-2 challenge.

Next, we determined whether immunization, either locally in the genital mucosa or distally via the nasal mucosa, with a non-replicating virus vaccine could establish protection against HSV-2 challenge, in the presence or absence of SLOs, to further understand the requirements for generating local effector responses in the genital tract. To do this, we immunized WT and LTα-/- mice IVAG or intranasally (**IN**) with a subunit vaccine (HSV-2 gD plus CpG), a heat-inactivated virus vaccine (heat-inactivated HSV-2 plus CpG), or a live attenuated virus vaccine (TK$^-$ HSV-2). All groups of mice were then

challenged IVAG with WT HSV-2. Mice immunized by either route, with non-replicating vaccine, were not protected against genital HSV-2 challenge and succumbed to infection. However, treating WT and LTα-/- mice with E2 prior to immunization resulted in enhanced protection against HSV-2 challenge.

Lastly, we examined the mechanism by which E2 treatment leads to enhanced protection of immunized mice following HSV-2 infection. We found that E2 treatment increased the recruitment of mucosal effector CD4+CD103+ T cells into the vaginal tract of immunized mice, following HSV-2 challenge. In addition, E2 treatment upregulated Th17 cells in the vaginal tract of mice following HSV-2 challenge, which was accompanied by an early IFNγ+ Th1 response and a decreased TNFα+ T cell response compared to mock controls.

These results suggest that the use of highly immunogenic live-attenuated virus based vaccines delivered via mucosal routes can provide protection in the genital tract against subsequent genital HSV-2 infections. However, if a less immunogenic vaccine formulation, such as a subunit or inactivated virus based vaccine, or a lower dose of the attenuated virus based vaccine is delivered, either locally or distally, additional mucosal adjuvants or hormones may be required to elicit protection in the genital tract. Furthermore, the hormonal microenvironment during immunization should be a critical consideration in the development of mucosal HSV-2 vaccines as hormones present at the time of immunization may significantly alter the induction of protective immune responses in the female genital tract.

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you. I hope that this will encourage you to always believe in yourself and follow your dreams.

PREFACE

This thesis is prepared in standard format as outlined in the "Guide for the preparation of Master's and Doctoral Theses" available through the School of Graduate Studies at McMaster University. Chapter 1 of this thesis serves as a general introduction. The body of this thesis consists of 3 chapters (Chapter 2-4), each one an independent study, one of which is published. The published study included in this thesis was written by the author of this thesis, who is also the first author of the study. The preamble section preceding each chapter describes the contributions of other authors to the multi-authored works. Finally, the discussion section (Chapter 5) summarizes the conclusions of this thesis and draws out the overall implications.

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LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
Depo	Depo-Provera
DMPA	Depot medroxyprogesterone acetate
E2	Estradiol
ERs	Estradiol receptors
ERKO	Estradiol receptor alpha knockout
FcRn	Neonatal Fc receptor
FGT	Female genital tract
GPR-30	G protein receptor 30
GR	Glucocorticoid receptor
HIV	Human immunodeficiency virus
HSV-2	Herpes simplex virus type 2
ICP47	Infected cell protein 47
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
IN	Intranasal
IVAG	Intravaginal
iVALTs	Inducible vaginal associated lymphoid tissues
LA	Lymphoid aggregate
LCMV	Lymphocytic choriomeningitis virus
LN	Lymph node
LTα	Lymphotoxin alpha
MHCII	Major histocompatibility class II
NALT	Nasal associated lymphoid tissue
NI	Nonimmunized
NK	Natural killer
OVX	Ovariectomy
P4	Progesterone
pDC	Plasmacytoid dendritic cells
pIgR	Polymeric immunoglobulin receptor
PMA	Phorbol 12-myristate 13-acetate
poly(I:C)	Polyinosinic-polycytidylic acid
PR	Progesterone receptor
PRR	Pattern recognition receptors
RANTES	Regulated on activation, normal T cell expressed and secreted
S	Saline
S-IgA	Secretory IgA

SIV	Simian immunodeficiency virus
SLOs	Secondary lymphoid organs
SPL	Splenectomized
STD	Sexually transmitted disease
STIs	Sexually transmitted infections
TGF-β	Transforming growth factor β
TK	Thymidine kinase
TK^- HSV-2	Thymidine kinase-negative herpes simplex virus type 2
TNF	Tumor necrosis factor
TLR	Toll-like receptors
WT	Wild type

CHAPTER 1

GENERAL INTRODUCTIONS AND OBJECTIVES

1.1 – Herpes Simplex Virus Type 2 (HSV-2)

1.1.1 – Epidemiology

HSV-2 is a sexually transmitted virus that is the major causative agent of genital herpes, a highly prevalent infection among sexually active individuals. The prevalence of HSV-2 increases in adolescents with initiation of sexual activity, and steadily increases throughout adulthood (Fleming *et al.*, 1997). As of 2008, it was estimated that 536 million people aged 15-49 are infected with HSV-2 globally, and approximately 23.6 million people in this age group become newly infected with HSV-2 every year (Looker *et al.*, 2008). In the United States, it was estimated that 17% of the population between the ages of 14-49 is seropositive for HSV-2 and 58% is seropositive for HSV-1, which is emerging as a major cause of genital herpes infections (Xu *et al.*, 2006). Similarly, in Canada, the seroprevalence of HSV-2 among people aged 14 to 59 was 13.6%, or an estimated 2.9 million people, as of 2011 (Rotermann *et al.*, 2013). Globally, the seroprevalence rate for HSV-2 is much higher, with the highest rates reaching 70% among women in Sub-Saharan Africa and 95% in some populations of HIV-infected persons and female sex workers (Gupta *et al.*, 2007; Looker *et al.*, 2008).

1.1.2 – Women and HSV-2

Women are more susceptible to HSV-2 than men, with the estimated risk of a female contracting HSV-2 from infected males being 80% following a single contact. The seroprevalence in women is up to twice as high as men, and increases with age (Xu *et al.*, 2006). This may be due to the more efficient transmission of HSV-2 from men to

women since one study found that 4 out of 13 women in steady relationships with HSV-2 positive men seroconverted over 3 years, compared with no seroconversions among 16 seronegative men with HSV-2 positive female partners (Bryson *et al.*, 1993). This may partly be due to the higher rate of disease recurrences in men which may make them more infectious (Benedetti *et al.*, 1994).

1.1.3 – HSV-2 Structure and Replication

HSV-2 is a member of the alphaherpesvirus subfamily of the *Herpesviridae* family. HSV-2 is transmitted as a large, double-stranded DNA virus. This double-stranded DNA genome consists of about 150 Kbp and is packaged into an icosahedral capsid which contains 162 capsomers, and in turn, is enclosed by the tegument, a layer of proteins. The tegument is then covered by a bilayer lipid membrane which consists of at least 10 glycosylated and several nonglycosylated viral proteins, lipids and polyamines (reviewed in (Spear, 2004)). This enveloped virion is designed to protect the viral genome from adverse conditions in the extracellular environment and to permit cell invasion so that the viral genome can be released into the cell nucleus for expression of its genes.

HSV-2 can infect a number of different cell types, including epithelial cells of the skin and mucosa, neurons, cells of the immune system and many others (Spear *et al.*, 2006). To initiate infection, HSV-2 must first attach to cell surface receptors. Viral surface glycoproteins mediate attachment and penetration of the virus into cells. Five viral glycoproteins have been implicated in the viral entry process: gB, gC, gD, gH and

gL, all of which are essential for viral entry except gC (Spear, 2004; Campadelli-Fiume *et al.*, 2007). The initial interaction between virus and host cell is mediated via the binding of HSV-2 gB and/or gC to heparin sulfate proteoglycans on the cell surface. After the initial attachment to cells, HSV-2 penetrates the cell by either fusing the viral envelope with the plasma membrane or with the membrane of an intracellular vesicle (Clement *et al.*, 2006; Campadelli-Fiume *et al.*, 2007). This membrane fusion event requires essential participation from viral glycoproteins gB, gD, gH and gL, as well as a number of cellular receptors, such as nectin-1 and -2, herpesvirus entry mediator, and 3-O sulfated heparin sulfate (Shukla *et al.*, 1999; Spear, 2004; Heldwein *et al.*, 2006; Campadelli-Fiume *et al.*, 2007). Fusion of the viral envelope with the host cell membrane results in the release of the viral capsid and tegument proteins into the cellular cytoplasm. Once the viral capsid is released into the cytoplasm, it is transported to the nucleus, where it attaches to a nuclear pore. The viral DNA is then released into the nucleus where it undergoes replication. Finally, viral replication occurs, which consists of the following key events: transcription, DNA synthesis, capsid assembly, DNA packaging, and envelopment (Whitley *et al.*, 1998).

The synthesis of viral gene products, both RNA and proteins, takes place in three sequential steps. The first viral gene products produced are called the α or immediate early proteins. There are six immediate early proteins, five of which regulate the reproductive cycle of the virus and one, called infected cell protein 47 (**ICP47**), blocks the presentation of antigenic peptides on the infected cell surface, thereby helping the

virus evade detection by the immune system. These proteins are also essential for the synthesis of the second set of viral proteins, known as β or early proteins. The β-gene products are viral enzymes, such as the thymidine kinase (**TK**), an enzyme that plays an important role in the replication of the viral genome (Roizman, 2001). Finally, the third set of viral proteins are the structural components of the virus and assemble to form the capsid and tegument, as well as incorporate into the nuclear membrane for the eventual envelopment of the virions. Once these proteins are synthesized, the process of viral assembly begins, starting with the formation of the pro-capsid from scaffolding and capsid proteins. Newly synthesized DNA is then packaged into the capsid, which then attaches to the inner nuclear membrane and is rapidly enveloped and released into the space between the inner and outer nuclear membranes. Here, the virions become encased in transport vesicles and are transported to the extracellular space. This entire viral replication process takes approximately 18 hours (reviewed in (Whitley *et al.*, 1998)).

1.1.4 – HSV-2 Pathogenesis

HSV-2 enters the body through direct infection of the mucosal surfaces or cracks in the skin and initiates cytolytic replication in epithelial cells at the site of entry (Whitley *et al.*, 1998). Although HSV-2 can infect the skin and other mucosal surfaces, such as the oral mucosa, it is primarily associated with genital tract infections (Lafferty *et al.*, 1987; Corey, 1994). Disease is caused by a direct cytopathic effect of the replicating virus, resulting in cell damage in the genital mucosa which causes epithelial cells to detach and form fluid-filled blisters containing cell debris, inflammatory cells and progeny virions

(Nathwani & Wood, 1993). During a primary infection, the virus penetrates the genital mucosa and enters the peripheral sensory nerves closest to the infected cells or spreads via the lymphatic system to local and regional lymph nodes. Upon entering the sensory nerves, the DNA-containing nucleocapsid is taken up by the sensory nerve axons and travels to sensory or autonomic nerve root ganglia via retrograde transport (Mertz *et al.*, 1985). Neuronal HSV-2 infections do not lead to cell death, instead HSV-2 enters a latent state in the sensory ganglion where it persists for the life of the individual. The HSV-2 genome is maintained in an unintegrated latent state, with expression of a few proteins, but without active replication or cytotoxicity (Roizman & Taddeo, 2007). Recurrent episodes take place when HSV-2 is reactivated within the neurons and is transported in the peripheral nerves back to the mucosal or skin surface. Reactivation of HSV-2 can result in clinically evident disruption of the mucosa, or viral shedding might occur in the absence of clinically recognized symptoms (Kimberlin & Rouse, 2004). Reactivation and replication of latent HSV-2 can be triggered by a number of factors, including local trauma (eg. surgery or UV light), systemic stimuli (eg. immunosuppression or fever), stress or hormonal changes.

Although asymptomatic and atypical presentation is most frequent, primary genital HSV-2 infections classically present with localized symptoms, including pain, itching and burning at the site of the infection and regional swollen lymph nodes, usually within 4-7 days after sexual exposure. Primary infections usually begin with macules and papules and progress to mucocutaneous lesions (Kimberlin & Rouse, 2004). Within 2-3

weeks, 75% of patients have new lesions and existing lesions progress to vesicles, pustules and can coalesce into ulcers (Corey *et al.*, 1983a). Skin ulcers crust and then heal, whereas lesions in the mucous membrane heal without crusting (Corey *et al.*, 1983a).

About 70-90% of people with symptomatic HSV-2 will have a recurrence within the first year (Lafferty *et al.*, 1987; Benedetti *et al.*, 1994). For some patients, prodrome is the earliest sign of recurrence and precedes the development of lesions by hours to days. Prodrome symptoms include tingling, itching, paraesthesias and pain. Lesions in recurrences are more likely to be fewer in number and pain tends to be mild (Corey *et al.*, 1983a). In most patients, the rate of symptomatic reactivation naturally and gradually decreases with time (Benedetti *et al.*, 1999). Whereas primary infections can last up to two to six weeks, lesions in recurrent disease only last for approximately one week (Habif, 1996).

1.1.5 – HSV-2 Transmission

HSV-2 infection is acquired through close contact with an infected individual who has symptomatic lesions or asymptomatic viral shedding from their skin or genital secretions (Corey, 1994). Even though transmission of HSV-2 is more efficient from symptomatic lesions due to higher viral titers in the lesions, asymptomatic viral shedding is thought to account for the majority of genital HSV-2 transmissions (Kinghorn, 1994; Wald *et al.*, 2000). This is because asymptomatic shedding occurs much more frequently than symptomatic reactivation of the disease. Most HSV-2 transmissions occur during

periods of subclinical shedding and transmission occurs relatively efficiently with the median number of 40 sexual acts prior to HSV-2 acquisition among serodiscordant couples (Mertz *et al.*, 1992; Wald *et al.*, 2006). In 50-90% of transmissions, the source contact is unaware of being infected.

1.1.6 – The Immune Response to HSV-2

HSV-2 infection in humans triggers both innate and adaptive immune responses, which have been suggested to aid in the control and clearance of the virus, respectively (Roth *et al.*, 2013a). The importance of the host immune response against HSV-2 infection is demonstrated by the severe, prolonged ulcerations that occur in patients with AIDS or after solid organ or stem cell transplantation (Naraqi *et al.*, 1977; Meyers *et al.*, 1980; Siegal *et al.*, 1981). The mucosal immune system of the genital tract is the first line of defense against genital HSV-2. Since women are more susceptible to HSV-2 than men and the female genital tract is the primary site of infection, this thesis will focus on the immune responses generated in the female genital tract in response to genital HSV-2 infections.

Stratified squamous epithelial cells along the lower female genital tract produce antimicrobial factors, such as β-defensins, secretory leukocyte protease inhibitors, and lysozymes, which contribute to an antimicrobial milieu (Wira & Fahey, 2004). HSV-2 is recognized by both innate immune cells and non-immune cells via pattern recognition receptors (**PRR**), such as toll-like receptors (**TLR**), which bind to highly conserved pathogen-associated molecular patterns, such as viral DNA (Paludan *et al.*, 2011).

Interestingly, host mutations associated with increased susceptibility to HSV-2 infections and childhood mortality occur at loci for TLR2 and TLR3, respectively, indicating the importance of these innate recognition receptors in protection against HSV infections in humans (Bochud *et al.*, 2007; Zhang *et al.*, 2007). Following the recognition of HSV-2 by these TLR, plasmacytoid dendritic cells (**pDC**) and other cells, such as conventional dendritic cells (**DCs**), macrophages and fibroblasts, produce type I interferon (**IFN**), which includes IFNα and IFNβ (Rasmussen *et al.*, 2007). Type I IFN initiates an antiviral response in surrounding cells, which produce antiviral molecules, such as protein kinase R, to block or reduce viral infection (Akira & Takeda, 2004). Type I IFNs are also involved in a number of other antiviral mechanisms. For example, type I IFN expression induces the maturation of DCs and the activation of natural kill (**NK**) cells and antigen-specific CD8+ T cells at the site of infection, as well as the recruitment of DC and monocytes to the site of infection (Uematsu & Akira, 2006; Martinez *et al.*, 2008; Gill *et al.*, 2011; Iijima *et al.*, 2011). A number of studies show that treatment of susceptible human cells with type I IFN *in vitro* results in inhibition of HSV-2 replication (Fish *et al.*, 1983; Leventon-Kriss *et al.*, 1987). Our lab has previously shown that primary human genital epithelial cells, which are one of the first cells to encounter HSV-2 following acquisition or during reactivation, can be significantly protected against HSV-2 infection following treatment with ligands for TLR3, 5 and 9, due to production of type I IFN (Nazli *et al.*, 2009). In animal models, inhibition of IFNβ signaling pathways is associated with increased HSV-2 viral burden and disease (Conrady *et al.*, 2011; Gill *et al.*, 2011). However, human biopsy studies reveal that extremely low levels

of IFNα and IFNβ are found in the genital tract during the course of HSV-2 infection, despite the presence of a large number of cells capable of synthesizing these molecules, raising questions about the importance of type I IFNs during the course of human HSV-2 infection (Peng *et al.*, 2009).

Another important aspect of the innate immune response that is induced following HSV-2 infection is the activation of NK cells. When activated, NK cells function mainly by either killing virus-infected cells or producing large amounts of cytokines, such as IFNγ (Wira *et al.*, 2005). IFNγ production can indirectly clear virus by inducing nitric oxide production, a potent obstructer of viral replication, from epithelial cells, DCs and macrophages (Bogdan, 2001). NK cells are important in controlling HSV-2 infections as individuals who lack NK cells or have defective NK cell function have increased susceptibility to herpesvirus infections (Orange, 2006). In addition, studies using NK and NKT cell deficient mice showed a lower rate of survival and higher viral titers in the vaginal tract among HSV-2 infected mice (Ashkar & Rosenthal, 2003). The role of NK cells during the course of human HSV-2 infection, on the other hand, remains unclear. Earlier studies found low numbers of NK-like cells at the site of lesions (Cunningham *et al.*, 1985), whereas later studies suggested a local enrichment of NK cells at the site of HSV lesions (Koelle *et al.*, 1998). However, no apparent correlation between NK cell activity and viral clearance was found.

The innate immune system also provides stimuli to initiate the adaptive immune response. The adaptive immune response against HSV-2 is primarily responsible for

viral clearance and the generation of long-term memory. The role of the adaptive immune response in clearing the virus has been clearly established by a study that observed the temporal correlation between clearing of the virus and the number of infiltrating antigen-specific CD4+ and CD8+ T cells at the site of a lesion (Koelle *et al.*, 1998). Evidence exists in both mouse models and humans substantiating the importance of effector CD8+ T cells in clearing HSV-2 infection within the genital mucosa (Koelle *et al.*, 1998; Parr & Parr, 1998; Dobbs *et al.*, 2005; Schiffer *et al.*, 2010). The effector function of CD8+ T cells primarily involves inducing apoptosis in pathogen infected cells and depends on the production of IFNγ (Dobbs *et al.*, 2005). In humans, the infiltration of a large cytotoxic CD8+ T lymphocyte (**CTL**) population within the genital lesions correlates with viral clearance (Koelle *et al.*, 1998). CD8+ T cells have the ability to shape the severity and frequency of HSV-2 reactivation. In humans, CD8+ T cells act like sentinels around the peripheral nerve endings (the sites of reactivation) up to 8 weeks after HSV-2 resolution (Zhu *et al.*, 2007). A similar finding was observed in mice infected with an attenuated strain of HSV-2, whereby memory effector T cells persisted in the genital tract for 6 weeks (Tang & Rosenthal, 2010). However, CD8+ T cells alone are not sufficient for viral clearance, CD4+ T cell help is required (Gill & Ashkar, 2009; Nakanishi *et al.*, 2009).

CD4+ Th1 cells are an important source of IFNγ, have cytotoxic effector activity for HSV-infected cells, and localize to human HSV-2 genital lesions (Koelle *et al.*, 1998). Early studies established that CD4+ T cell depletion in mice led to an increase in

HSV-2 viral burden and a decrease in cell-mediated lysis and IFNγ production compared to CD8+ T cell depletion (Milligan & Bernstein, 1997; Milligan *et al.*, 1998). The protection conferred by CD4+ T cells is linked to their ability to secrete IFNγ because the administration of exogenous IFNγ to immunized, CD4+ T cell-deficient mice restored protective immunity against genital HSV-2 (Harandi *et al.*, 2001a). Futhermore, IFNγ-deficient mice showed a lack of protection against HSV-2 even in the presence of CD4+ T cells, confirming the critical role of Th1 CD4+ T cells secreting IFNγ (Harandi *et al.*, 2001a). IFNγ secretion by CD4+ T cells was also shown to be important for the production of CXCL9 and CXCL10 chemokines, which recruited CD8+ T cells into the vaginal epithelium (Nakanishi *et al.*, 2009). Although CD4+ T cell responses are critical against HSV-2, depletion of both T cell populations resulted in a greater viral burden within the vaginal mucosa compared with depletion of either subset alone (Milligan *et al.*, 1998). Evidence thus far indicates that in response to infection, B cells and DC stimulate CD4+ T cells to secrete IFNγ, resulting in the secretion of CXCL9 and CXCL10, leading to CD8+ T cell activation and migration to the site of infection (Iijima *et al.*, 2008; Nakanishi *et al.*, 2009).

B cells also contribute to the adaptive immune response against HSV-2 by producing neutralizing antibodies against viral envelope glycoproteins. HSV-2-specific immunoglobulin G (**IgG**) and immunoglobulin A (**IgA**) have been found in the genital tracts of both humans (Ashley *et al.*, 1992) and mice (McDermott *et al.*, 1990) following HSV-2 infection. These antibodies are naturally produced during the course of HSV-2

infections and can be detected in >70% of asymptomatic, HSV-1 and HSV-2 infected women (Mbopi-Keou *et al.*, 2003). However, in mice, IgG has been shown to be the primary HSV-2-specific antibody in vaginal secretions over IgA (McDermott *et al.*, 1990).

1.1.7 – Treatment and Vaccine Trials

We have recently reviewed the current state of HSV-2 vaccines and provided insight into the development of future vaccines (see Appendix A). Currently there is no treatment available to eliminate HSV-2 infection. Patient management includes the provision of information, counselling and antiviral therapy (Mindel, 1993). Acyclovir was introduced in the early 1980s and when administered either intravenously or orally, it has been shown to produce a clinical benefit in primary genital herpes infections (Nilsen *et al.*, 1982; Bryson *et al.*, 1983; Corey *et al.*, 1983b; Mertz *et al.*, 1984; Mindel, 1993). Acyclovir acts as a specific inhibitor of herpesvirus DNA polymerase, thereby inhibiting viral replication (Gnann *et al.*, 1983). Acyclovir reduces the clinical severity of the disease episode, shortens its duration, prevents complications and reduces symptomatic viral shedding (Mindel *et al.*, 1982; Kinghorn, 1993; Wald *et al.*, 1996). Other therapeutic antiviral agents include famciclovir, valaciclovir, penciclovir, topical trifluridine and intravenous floscarnet (Patel & Barton, 1995).

While antivirals are effective for treating HSV-2 infections, they are expensive and do not prevent or eliminate the disease. Therefore, the development of a prophylactic HSV-2 vaccine would be the best and cheapest option to address the problems associated

with HSV-2 infections globally. Subunit vaccines were one of the earliest and most utilized vaccine strategies due to their safety, simplicity and cost effectiveness. In the 1970s and 1980s, a vaccine enriched in HSV-2 envelope glycoproteins was tested clinically and was found to be immunogenic and appeared to decrease the severity of recurrent HSV-2 disease, but it did not undergo further development (Cappel *et al.*, 1985). From the mid-1980s to early 1990s, randomized, blinded, placebo-controlled trials of vaccines consisting of a mixture of envelope glycoproteins were conducted. While these vaccines induced neutralizing antibodies and lymphoproliferative responses to HSV-2, they did not provide protection against primary HSV infection (Ashley *et al.*, 1985; Mertz *et al.*, 1990; Mester *et al.*, 1990). In the late 1990s, Chiron developed a prophylactic vaccine formulation consisting of truncated recombinant gB2 and gD2 with the adjuvant MF59, which was tested in phase I trials and found to induce HSV-2-specific neutralizing antibodies and CD4+ T cell lymphoproliferative responses. However, upon further testing during phase III trials, the overall efficacy of this vaccine was only 9% in preventing HSV-2 infection in HSV-2 seronegative partners among serodiscordant heterosexual couples or participants enrolled in sexually transmitted disease (**STD**) clinics. It also showed no efficacy on the duration of the first clinical episode of genital HSV-2 or the frequency of subsequent recurrences (Straus *et al.*, 1994; Corey *et al.*, 1999; Langenberg *et al.*, 1999). More recently, Glaxo-Smith-Kline developed a vaccine consisting of a truncated recombinant gD2 formulated with an alum-monophosphoryl lipid adjuvant. During phase I trials, this vaccine induced both neutralizing antibodies and CD4+ T cell immune responses, but later clinical trials

revealed a lack of efficacy in men or HSV-1 seropositive women. Upon further analysis, it was found that this vaccine reduced HSV-2 disease by 70% and HSV-2 infection by 40% in a subgroup of HSV-1 and HSV-2 seronegative women (Stanberry *et al.*, 2002). However, a follow-up study consisting of more than 8000 HSV-1 and HSV-2 seronegative women revealed that this vaccine was not efficacious in decreasing HSV-2 disease or preventing infection (Cohen, 2010).

In addition to subunit vaccines, peptide-based vaccines have also been explored. These vaccines consist of specific peptides that contain T cell epitopes which have been shown to be protective against HSV disease and are typically complexed with heat shock proteins, which are used as vaccine vehicles for the peptides. Phase I clinical trials were conducted to evaluate the safety and immunogenicity of these T cell epitope-based vaccines (Koelle *et al.*, 2008; Wald *et al.*, 2011). Only the HerpV vaccine study has shown any efficacy as a candidate vaccine by inducing broad CD4+ and CD8+ T cell responses in HSV-2-positive participants (Wald *et al.*, 2011).

Compared to subunit and peptide vaccines, live attenuated virus based vaccines have the advantage of imparting long-lasting and broad immunity. Their disadvantages are safety concerns, including reversion back to a wildtype phenotype. In the late 1980s, a live vaccine (R7020) containing a portion of the unique short region of HSV-2 encoding gD, gG, gI and part of gE was inserted into a deletion mutant of HSV-1 strain F. This vaccine induced antibodies in HSV-seronegative participants but overall was found to be poorly immunogenic (Meignier *et al.*, 1988). Another vaccine consisting of

an HSV-1 mutant deficient in glycoprotein gH, known as DISC (disabled infectious single cycle), was found to reduce HSV-2 replication and provided protection against HSV-2-induced disease (McLean *et al.*, 1994). When administered systemically, but not via the mucosal route, in the guinea pig, DISC also caused a 36% reduction in recurrent lesions (McLean *et al.*, 1994; Boursnell *et al.*, 1997). In phase I clinical trials, a DISC HSV-2 mutant with a deletion of the gH gene was tested in individuals with symptomatic HSV-2. The vaccine was well tolerated and induced neutralizing antibody and lymphoproliferative T cell responses and 83% of vaccinees also developed HSV-2-specific CTLs. However, no differences were observed between treatment and placebo groups in terms of the number of recurrences, healing of lesions and asymptomatic viral shedding, thereby halting further development (de Bruyn *et al.*, 2006). In the early 2000s, clinical trials were conducted on a live attenuated virus vaccine composed of an HSV-2 mutant lacking the UL39 gene, which codes for one of the subunits of the viral ribonucleotide reductase, and found a decreased number of self-reported genital herpes recurrences among individuals who received the vaccine compared with the placebo control group (Casanova *et al.*, 2002). Despite the promising results of this and other live attenuated vaccines, further development was stalled due to safety concerns and acceptability.

Therefore, despite decades of effort, the development of an efficacious HSV-2 vaccine has remained elusive. An ideal HSV-2 vaccine would be expected to induce sterile immunity in the genital tract, in order to ensure that the virus does not become

latent. Thus, an ideal vaccine should induce a robust early innate response as well as a combination of HSV-2-specific antibodies and T cells, directly in the genital tract that neutralizes the virus either prior to entry and/or during replication. Although current and past vaccine candidates have been shown to induce systemic immune responses, they have been unsuccessful in providing protection in the genital tract. Serious consideration needs to be given to vaccine formulations and delivery strategies that induce more robust immunity in the genital mucosa, where HSV-2 is likely to be acquired or shed following reactivation (Roth *et al.*, 2013a). This information was recently reviewed by our lab (see Appendix A).

1.2 – The Female Genital Tract

1.2.1 – Anatomy of the Human Female Genital Tract

The human female genital tract (**FGT**) can be divided into two different compartments: the lower genital tract and the upper genital tract. The lower genital tract consists of the vagina and ectocervix and the upper genital tract consists of the endocervix, uterus, Fallopian tubes and ovaries. The vagina meets the external organs at the vulva, which includes the labia, clitoris and urethra. The cervix attaches the vagina to the uterus, while the Fallopian tubes, which are attached to the uterus and juxtaposed to the ovaries, allow the passage of the egg from the ovaries to the uterus (Marieb, 2012).

1.2.2 – Mucosal Immunity in the Female Genital Tract

The immune system of the FGT is part of the integrated mucosal immune system. Although the FGT has a number of characteristics that are similar to the mucosal immune

system and distinct from systemic immunity, it is also a unique immunological site that is required to protect the mucosa from a variety of pathogens without compromising the development of an allogenically distinct fetus. This requires special adaptations in the immune system of the FGT so that different components of the immune response, such as humoral and cellular immunity, are differentially modulated to extend protection against sexually transmitted pathogens during all stages of the menstrual cycle while facilitating fertilization and fetus implantation, if necessary (Nguyen *et al.*, 2014). Hormonal changes during the menstrual cycle regulate the immune system throughout the FGT in a way that optimizes conditions for successful sperm migration, fertilization, implantation and pregnancy (Mor & Cardenas, 2010; Wira *et al.*, 2010). In addition to sex hormones, the presence of a microbiome, primarily dominated by the bacterial species *Lactobacillus*, is critical for the development and shaping of innate and adaptive immune responses in the FGT (Mirmonsef *et al.*, 2011; Brotman *et al.*, 2014).

1.2.3 – Barrier Mechanisms in the Female Genital Tract

The mucosal lining of the FGT, composed of epithelial cells and mucus, provides the primary physical and immunological barrier between the FGT and the external environment. The upper FGT, consisting of the endocervix, endometrium and Fallopian tubes, is lined with a single layer of columnar epithelial cells which are joined by tight junctions (Wira *et al.*, 2005; Ferreira *et al.*, 2014). In contrast, the lower FGT, consisting of the vagina and ectocervix, is lined with multiple layers of non-keratinized stratified squamous epithelium attached to a basement membrane. The outer squamous layer

effectively protects the underlying tissue from abrasions during intercourse. A lack of tight junctions in the squamous epithelial layers permits the movement of small molecules, including various pathogens, within epithelial spaces between cells (Blaskewicz *et al.*, 2011). In addition to forming a physical barrier, epithelial cells of the FGT express PRR including TLRs that recognize pathogen-associated molecular patterns on microorganisms *in vitro* (reviewed in (Kaushic, 2011)). Upon stimulation, PRRs mediate the secretion of cytokines, chemokines and antimicrobial peptides (Nazli *et al.*, 2009; Wira *et al.*, 2011).

Protecting the genital epithelial cells from direct contact with pathogens is a layer of mucus, composed of a family of glycosylated proteins, known as mucins, which physically trap pathogens in a thick gel phase. Interspersed within mucin complexes is an aqueous phase containing immunoglobulins and antimicrobial peptides, the primary function of which is to prevent viable pathogens from infecting epithelial mucosa (Ming *et al.*, 2007).

1.2.4 – Innate Immune Cells in the Female Genital Tract

Many innate immune cells, both resident and recruited, are present in the FGT and provide defense against invading pathogens. In the steady state, macrophages, Langerhans cells and DCs are sentinels of the mucosal immune system that constantly survey and process antigens from the external environment, which provides important information and signals to the host immune system (Banchereau & Steinman, 1998). DCs in particular serve the important function of bridging the innate responses with the

initiation of adaptive immunity. Following infection, several cell types are mobilized to the vaginal tissue, including neutrophils, monocytes, pDCs and NK cells. At later timepoints, antigen-specific T cells and B cells enter the tissue to provide pathogen-specific immune defense.

Mucosal DCs are recognized by their unique ability to recognize and respond to antigens by inducing host immune responses that can range from tolerogenic to the induction of antigen-specific adaptive immunity (Iwasaki, 2007). Typically, tissue-resident DCs, including those in the FGT, have an immature phenotype. In the steady state, Langerhans cells in the epithelium and DCs in the submucosa are highly phagocytic and express several PRRs that can recognize a wide array of microorganisms (Iwasaki, 2007). After pathogen recognition by PRR, Langerhans cells and DCs undergo maturation and migrate to the draining lymph nodes to prime naïve T cells and B cells (Cella *et al.*, 1997; Janeway & Medzhitov, 2002).

Neutrophils are found throughout the FGT, particularly in the Fallopian tubes, but they are also present in lower numbers in the upper and lower genital tracts (Givan *et al.*, 1997). During HSV-2 infections, a large number of neutrophils are recruited to the infected vaginal mucosa and are required for protection during primary and secondary challenges (Krzyzowska *et al.*, 2011). Neutrophils express TLR 1-9 and respond to pathogens through phagocytosis, production of oxidative compounds, and release of antimicrobial peptides (Selsted & Ouellette, 1995).

NK cells are also present throughout the FGT in significant numbers, and they can make up anywhere from 10-30% of leukocytes in the FGT of non-pregnant women (Givan *et al.*, 1997). In the uterus, NK cell populations increase from the proliferative to the secretory phase. In the later stages of the secretory phase, NK cells can make up to as much as 70% of the leukocytes in the endometrium (Croy *et al.*, 2006). Although it is unclear whether this increase in numbers is a consequence of either local proliferation or recruitment from peripheral blood, the significant accumulation of NK cells late in the menstrual cycle is likely associated with preparation for the extensive uterine remodeling that occurs at implantation and during the first trimester of pregnancy.

1.2.5 – T cells in the Female Genital Tract

Adaptive immunity is a pathogen-specific driven response following antigen presentation and stimulation of T cells by antigen-presenting cells (**APC**s). A number of cells in the FGT can present antigens to T cells, including macrophages, DCs, Langerhans cells and under certain conditions, epithelial cells of the cervix and endometrium (Hickey *et al.*, 2011). CD4+ and CD8+ T cells are found throughout the human FGT, but in the upper FGT, CD8+ T cells are more abundant compared to CD4+ T cells. Organized lymphoid aggregates (**LA**), composed of a B cell core surrounded by CD8+ T cells with an outer halo of macrophages, have been found in the stratum basalis of the uterine mucosa (Yeaman *et al.*, 1997). Most T cells in the human lower genital tract are localized at the stromal/epithelial interface. However, significant numbers of intraepithelial CD8+ T cells were found dispersed within the vaginal and ectocervical

squamous epithelium, whereas only the ectocervix contained significant numbers of intraepithelial CD4+ T cells (Pudney *et al.*, 2005). Interestingly, inflamed vaginal and cervical samples contained higher proportions of intraepithelial lymphocyte populations compared to non-inflamed tissue (Pudney *et al.*, 2005).

Recent studies have found that Th17 cells are also present in the FGT, typically following exposure to inflammatory conditions (McKinnon *et al.*, 2011; Robertson *et al.*, 2013). Naïve CD4+ T cells differentiate into Th17 cells following antigen presentation by DCs in the presence of a combination of cytokine signals, such as interleukin (**IL**)-6, IL-1β, transforming growth factor β (**TGF-β**) and IL-23 or IL-21 and TGF-β, or IL-1β, IL-6 and IL-23 (Korn *et al.*, 2009). The primary effector response of Th17 cells involves the induction of inflammatory cytokines, such as IL-17A, IL-17F and IL-22, and recruitment of neutrophils. Th17 cells are typically involved in the resolution of extracellular fungal and bacterial infections (Aujla *et al.*, 2008; Ishigame *et al.*, 2009), but can also lead to autoimmunity or chronic inflammatory diseases (Guglani & Khader, 2010). In the human FGT, IL-17 and Th17 cells are associated with a normal lactobacilli (Kirjavainen *et al.*, 2009). In addition, a number of genital tract infections in mice has been shown to induce Th17 cell responses. For example, infection with *Chlamydia* results in accumulation of Th17 cells in the upper FGT, which have been shown to play an accessory role in the recruitment of neutrophils and produce IL-17, which contributes to the development of genital pathology (Scurlock *et al.*, 2011). Similarly, *N. gonorrhoeae* elicits Th17 responses that trigger infiltration of neutrophils and production

of antimicrobial proteins (Feinen *et al.*, 2010; Feinen & Russell, 2012). IL-17 also plays a protective role in *C. albicans* infections, with candidiasis leading to a strong IL-17 response and infiltration of neutrophils into the genital tract (Pietrella *et al.*, 2011; Hernandez-Santos & Gaffen, 2012).

Even though Th17 cell responses are typically associated with fungal or bacterial infections, several studies have shown that Th17 cells are also involved in viral infections, including respiratory syncytial virus, rotavirus, human immunodeficiency virus (**HIV**) and HSV (Hashimoto *et al.*, 2004; Hashimoto *et al.*, 2005; Maek *et al.*, 2007; Smiley *et al.*, 2007; Kim *et al.*, 2008; Kim *et al.*, 2012). In HIV infection, production of IL-17 by peripheral blood T cells is enhanced compared with control subjects (Maek *et al.*, 2007). Exogenous IL-17 exaggerates the inflammatory response following rhinovirus infection in the lungs or intraperitoneal vaccinia virus infection, leading to accelerated mortality in the latter (Patera *et al.*, 2002; Wiehler & Proud, 2007). The role of Th17 cells has also been examined in HSV-1 infections as well. One study found that IL-17 is expressed in the corneas of individuals infected with herpetic stromal keratitis (HSK) (Maertzdorf *et al.*, 2002). Similarly, another found that IL-17 is rapidly expressed in the cornea after HSV-1 infection (Molesworth-Kenyon *et al.*, 2008). Another study showed that HSV infection results in the upregulation of IL-23 expression, a cytokine that is important in the induction of IL-17 cells, but there were only minimal detectible CD4+ IL-17 producing responses in the eye (Kim *et al.*, 2008). They also observed that mice lacking IL-23 had significantly more severe ocular lesions compared to WT mice,

suggesting that the IL-23/IL-17 axis is not likely to be the main mediator of SK. Thus, although IL-17 has been found to be expressed in herpetic lesions, the importance of Th17 cells in HSV-1 infections is still relatively unexplored. In addition to HSV-1, studies have also started to examine the role of Th17 cells in HSV-2 infections. One study found that, in the absence of IFNγ, immunization with TK^- HSV-2 induced the secretion of IL-17 from HSV-2-specific CD4+ T cells, but the adoptive transfer of these cells did not induce viral clearance following infection with WT HSV-2, suggesting that Th17 cells do not protect against genital HSV-2 infections (Johnson *et al.*, 2010). Another study also found that IVAG immunization with TK^- HSV-2 did not induce production of IL-17 from CD4+ T cells and that infecting IL-17A-/- with WT HSV-2 resulted in delayed death compared to WT mice (Kim *et al.*, 2012). These results suggest that IL-17 does not have any direct role in the protection against genital HSV-2 infections and perhaps even has negative impact on protective immunity. However, further studies are required to determine the precise role of Th17 cells in HSV-2 infections.

1.2.6 – Humoral Immunity in the Female Genital Tract

Humoral immunity is characterized by the production of antibodies that bind to free as well as cell associated antigens, thereby inhibiting cell entry and/or neutralizing the biological activity of a pathogen. Antibody binding also facilitates pathogen elimination by the subsequent phagocytosis by macrophages or fixing by the complement system. CD4+ T cells play a role in humoral immunity by driving B cell maturation to antibody-secreting plasma cells (Kutteh & Mestecky, 1994).

Immunohistochemical studies that have examined the FGT for the presence of antibody-secreting cells found that in humans the endocervix contains the most IgG and IgA antibody-producing and antibody-containing cells compared to the ectocervix, vagina and Fallopian tubes (Russell & Mestecky, 2002). Unlike the other mucosal surfaces such as the gastrointestinal and respiratory surfaces where IgA is the dominant isotype, the FGT is unique in that both IgG and secretory IgA (**S-IgA**) are present in genital secretions. Studies of IgG and IgA ratios in human lower FGT secretions indicate that levels of IgG are twofold to sixfold higher than that of IgA in cervical mucus and cervico-vaginal lavage fluid (Hickey *et al.*, 2011). In contrast to other studies of cervical mucus, endocervical secretions have been found to have higher levels of IgA compared to IgG, which most likely corresponds to the increased presence of IgA secreting plasma cells in the endocervix (Quesnel *et al.*, 1997). The source of IgG in the FGT is likely from both IgG in circulation and local production, as secreted by plasma cells present in the FGT. The transport of IgA and IgG into the lumen of the FGT depends on the expression of their respective transporter proteins, polymeric immunoglobulin receptor (**pIgR**) and neonatal Fc receptor (**FcRn**), on the genital epithelium. The pIgR has been studied in detail for its ability to transport IgA (Kutteh *et al.*, 1998) and recent studies have identified FcRn expression on genital epithelial cells as the primary receptor that transports IgG across the epithelium into the lumen (Li *et al.*, 2011).

1.3 – Mouse Model of HSV-2

1.3.1 – Animal Models of HSV-2

This information has recently been reviewed by our lab (see Appendix A). A number of animal models have been developed to study the immune response to HSV-2 as well as to test the efficacy of potential HSV-2 vaccines (Scriba, 1976; McDermott *et al.*, 1984; Parr *et al.*, 1994; Yim *et al.*, 2005). The guinea pig is the most clinically relevant animal model for testing therapeutic vaccine candidates against genital herpes caused by HSV-2 infection (Armerding *et al.*, 1981; Scriba, 1981; Scriba & Tatzber, 1981). Similar to humans, intravaginal inoculation of HSV-2 into guinea pigs leads to acute replication and disease at the site of infection, establishment of a latent viral reservoir within the innervating sensory neurons, and periodic reactivation leading to viral shedding and recurrent genital lesions (Bourne *et al.*, 1994; Krause *et al.*, 1995; Dudek & Knipe, 2006; Dudek *et al.*, 2011). Since guinea pigs develop both acute and recurrent illness, this model has allowed for the evaluation of both prophylactic and therapeutic vaccine strategies (Bernstein, 2001). A number of different vaccine formulations have been tested using this model, including live-attenuated virus, live-virus vector, DNA and glycoprotein-based vaccines, and have been shown to be successful in preventing clinically apparent infection and reducing disease severity (Boursnell *et al.*, 1997; Da Costa *et al.*, 1997; Bourne *et al.*, 2005; Hoshino *et al.*, 2005; Natuk *et al.*, 2006; Hoshino *et al.*, 2008; Hoshino *et al.*, 2009; Brans & Yao, 2010).

While the guinea pig model closely mimics human HSV-2 infection and disease, one disadvantage of this model is that fewer reagents are available to study the immune response generated by immunizations and HSV-2 infections. One alternative to this model is the model developed using the cotton rat, which has the advantage of greater availability of immunological reagents to study the immune response to HSV-2. Cotton rats have been shown to be readily infected by HSV-2 and primary infection resembles that seen in humans, with the development of genital lesions followed by complete recovery (Yim *et al.*, 2005). Following primary infection, rats develop spontaneous clinical recurrences that mimic human disease and infection. This model was developed in 2005 by Yim, *et al.*, but no further studies have been conducted using the cotton rat to study HSV-2 infection or vaccine development (Yim *et al.*, 2005).

1.3.2 – Mouse Models of HSV-2 Infection and Vaccination

Arguably, the mouse model of HSV-2 infection has provided the most insight into the protective immune responses generated following HSV-2 infection and immunization with various HSV-2 vaccine formulations. While mice are not naturally susceptible to HSV-2, they can be infected intravaginally with HSV-2 following treatment with progesterone. An early mouse model of HSV-2 found that infecting progestin-treated mice intravaginally with wild type (**WT**) HSV-2 results in infection of the vaginal epithelium followed by a lethal neurological illness (Parr *et al.*, 1994). However, when progestin-treated mice are inoculated intravaginally with a thymidine kinase-negative, attenuated strain of HSV-2 (**TK$^-$ HSV-2**), they only develop primary vaginal infections

without the neurological illness that is associated with the WT virus. Further, mice inoculated with TK^- HSV-2 are protected against subsequent challenge with an otherwise lethal dose of WT HSV-2 (McDermott *et al.*, 1984; Parr *et al.*, 1994). Immunization with other HSV-2 mutants besides TK^- HSV-2 also provides protection against subsequent WT HSV-2 challenge. For example, immunization with a recombinant HSV-2 lacking virion host shutoff protein or with a highly attenuated replication-competent HSV-1 mutant, HF10, protected mice from genital disease caused by HSV-2 (Smith *et al.*, 2002; Luo *et al.*, 2012).

In addition to live attenuated virus vaccines, HSV-2 mouse models have also been used to study other vaccine formulations, such as replication-defective mutants or recombinant glycoproteins and DNA vaccines (Lee *et al.*, 2002; Hoshino *et al.*, 2005). Previous studies have found that intravaginal immunization with an HSV-2 gD based vaccine combined with a mucosal adjuvant, such as CpG, can induce protective immune responses against subsequent intravaginal HSV-2 challenge (Harandi *et al.*, 2003; Tengvall *et al.*, 2005). One study compared the efficacy of recombinant gD2, a plasmid expressing gD2 and HSV-2 with the essential genes *UL5* and *UL29* deleted (*dl5-29)* vaccines against genital HSV-2 infections and found that both *dl5-29* and recombinant gD2 were highly effective in decreasing acute and recurrent disease and reducing latent viral load, and both were superior to the plasmid vaccine alone or the plasmid vaccine followed by a single dose of *dl5-29* (Hoshino *et al.*, 2005). Furthermore, a DNA vaccine combining gD2 and gB2 proved to provide more effective humoral and cellular immune

responses and better protection against genital HSV-2 challenge compared to individual gene vaccines (Lee *et al.*, 2002).

Other studies have successfully shown protection against vaginal challenge, following systemic subcutaneous immunization using gD-based liposomal formulations (Olson *et al.*, 2009) or following gD based vaccine (gD-AS04) given in estradiol-treated mice (Pennock *et al.*, 2009), to enhance immune responses in the genital tract. In addition, immunization with subunit vaccines at a distal site, and not just the vaginal tract, can elicit protection against subsequent genital viral challenge. Gallichan, *et al.* have shown that intranasal immunization of mice with HSV-1 gB plus CpG mediates protective immunity in the female genital tract against subsequent HSV-2 challenge (Gallichan *et al.*, 2001b). Another study found that intranasal immunization with HSV-2 glycoprotein gD in combination with IC31 adjuvant induced protective immunity against genital herpes infection in mice (Wizel *et al.*, 2012).

1.3.3 – Innate Immune Responses in HSV-2 Mouse Model

The HSV-2 mouse model has provided unparalleled insight into the induction of immune responses following genital HSV-2 infections (Parr & Parr, 1997b; Parr & Parr, 2003). The mucosal immune system of the mouse vaginal tract is the primary site for induction of innate immune responses against HSV-2. Similar to humans, TLRs, such as TLR3 and TLR9 which recognize ssRNA and CpG DNA, respectively, recognize HSV-2 during infection in mice and have been implicated in generating an immune response against HSV-2. In mice, the mucosal administration of TLR3 and 9 ligands,

polyinosinic-polycytidylic acid (**poly(I:C)**) and CpG, respectively, provides protection against subsequent WT HSV-2 challenge (Gill *et al.*, 2006). However, mucosal administration of other TLR ligands, such as ligands to TLR2, TLR4 and TLR5, provides little to no protection against HSV-2 challenge. Although the precise mechanism of this TLR-mediated protection has not been determined, studies have found a correlation between TLR-mediated innate protection against HSV-2 and the production of IFNβ. In addition, the local delivery of IFNβ alone protected mice against genital HSV-2 challenge (Gill *et al.*, 2006). Many studies support the importance of type I IFN in generating innate immunity against HSV-2 (Conrady *et al.*, 2011; Gill *et al.*, 2011). The production of IFNβ induces an antiviral state in surrounding cells and also activates multiple effector cells during the innate immune response to HSV-2, including NK cells and pDCs. Although the exact role of the innate immune response against HSV-2 has not been completely elucidated, studies thus far indicate that induction of early innate response aids in controlling viral replication early in infection.

1.3.4 – Adaptive Immune Responses in HSV-2 Mouse Model

Studies using mouse models have found that both antibody- and cell-mediated adaptive immune responses are generated following genital HSV-2 infections. Protection against infections at mucosal sites often correlates with levels of S-IgA and studies have shown that S-IgA is the predominant antibody by weight in vaginal mucus of progestin-treated mice and that plasma cells of the IgA isotype are present in the vaginal mucosa of these mice (Parr & Parr, 1998). However, nearly all of the HSV-2-specific antibody

present in TK-HSV-2-immunized mice, following WT challenge, is of the IgG isotype (Parr & Parr, 2003). Studies using IgA-deficient mice have also shown that IgA is not critical for protection against HSV-2 (Parr *et al.*, 1998). In addition, following immunization with TK-HSV-2, IgG is the main protective antibody found in vaginal secretions and it is able to neutralize WT HSV-2 *in vitro* (Parr & Parr, 1997a). Adoptive transfer of serum IgG from immunized mice into the vaginal lumen of naïve mice also reduced the viral load and pathological signs of disease (Parr & Parr, 1997a). These results demonstrate that mice inoculated with an attenuated strain of HSV-2 develop sufficient antibody response, capable of protecting them from a subsequent challenge with an otherwise lethal dose of HSV-2. Nonetheless, the effectiveness of antibody-mediated protection remains a controversial topic. Subsequent studies found that the passive transfer of immune serum or anti-HSV-2 monoclonal antibodies failed to protect against vaginal infection in murine models (McDermott *et al.*, 1990; Morrison *et al.*, 2001).

Studies using B cell-deficient mice have also shown a conflicting role for humoral immunity in protection against HSV-2. One study found that B cell-deficient mice had higher levels of virus protein titers in the vaginal epithelium and secretions following HSV-2 infection compared to WT controls (Parr & Parr, 2000). In addition, another study showed that while immunized B cell-deficient mice were able to control viral replication in the genital mucosa, they were not completely protected (Morrison *et al.*, 2001). However, other studies have shown that B cell-deficient mice immunized with

TK^- HSV-2 are completely protected against WT HSV-2 challenge, and antibody-mediated protection was only compromised following T cell depletion (Dudley *et al.*, 2000; Parr & Parr, 2000; Harandi *et al.*, 2001a). This is consistent with the finding that T cell immunity was required for protection of the vaginal mucosa, even in the presence of high HSV-2-specific antibody titers (Milligan *et al.*, 1998). These results suggest that while humoral immunity may play an early and beneficial role in primary genital HSV-2 infection, ultimately cellular-mediated immunity is required for HSV-2 clearance and protection.

Generally, viral clearance in mouse studies has been associated with the development of cytotoxic CD8+ T cells. Accordingly, studies have found that the induction of an HSV-2-specific CD8+ T cell response protected mice against HSV-2 infection and depletion of CD8+ T cells *in vivo* resulted in reduced vaginal immunity against HSV-2 infection (Blaney *et al.*, 1998; Parr & Parr, 1998). However, other mouse models have found that CD8-/- mice and mice depleted of CD8+ T cells were still protected against HSV-2 after immunization, similar to WT mice (Milligan & Bernstein, 1997; Blaney *et al.*, 1998). This suggests that while CD8+ T cells are important for viral clearance, they are not critical in providing protection against HSV-2 infection.

Several studies have demonstrated that Th1 CD4+ T cells are required to provide protective immunity against HSV-2. Depletion of CD4+ T cells from immune mice reduced their ability to clear HSV-2 infections (Kuklin *et al.*, 1998; Milligan *et al.*, 1998), whereas the transfer of T cells from mice immunized with TK^- HSV-2 to naive mice

provided protection against subsequent HSV-2 challenge (McDermott *et al.*, 1989). The ability of CD4+ T cells to secrete IFNγ is an important aspect in the protection against HSV-2 infections as depletion of $CD4^+$ T cell leads to decreased levels of vaginal IFNγ secretion (Milligan & Bernstein, 1997). However, if exogenous IFNγ is injected into CD4-deficient, TK^- HSV-2 immunized mice, protection is restored following HSV-2 challenge (Harandi *et al.*, 2001a). Since IFNγ stimulates the secretion of CXCL9 and CXCL10, which recruit CD8+ T cells to the site of infection, the decreased levels of IFNγ found in CD4+ T cell-deficient or depleted mice resulted in reduced mobilization of CD8+ T cells to the vaginal epithelium of these mice (Nakanishi *et al.*, 2009). These findings were supported by an earlier study which found that depleting CD4+ T cells resulted in a greater reduction of IFNγ production and subsequent cytotoxic T cell activity compared to depleting CD8+ T cells (Milligan & Bernstein, 1995). It was also observed that CD4+ T cell depletion resulted in significantly elevated levels of viral titers in the vaginal tissue as well as delayed viral clearance compared to CD8+ T cell depletion. However, the greatest viral burden was observed when both T cell subsets were depleted (Milligan *et al.*, 1998). These results suggest that while IFNγ-producing CD4+ T cells are an essential part of anti-HSV-2 immunity, effective protection against HSV-2 infection requires a balance between both CD4+ and CD8+ T cells. However, studies identifying and characterizing the specific T cell responses that are generated in the genital mucosa of immunized mice following IVAG HSV-2 challenge are lacking.

1.3.5 – Lymphotoxin Alpha Knockout Mouse Model

Lymphotoxin alpha (**LTα**) is a member of the tumor necrosis factor (**TNF**) family and plays an important role in the development of secondary lymphoid organs (**SLOs**) (Gommerman & Browning, 2003; Tumanov *et al.*, 2003a; Tumanov *et al.*, 2003b). TNF family members, including LTα, have been shown to be essential for the proper development of lymphoid tissues, including lymph nodes (**LN**) and Peyer's patches, and is also important in the maintenance of the correct architecture of SLOs, including the spleen (De Togni *et al.*, 1994; Banks *et al.*, 1995; Alimzhanov *et al.*, 1997). Knockout mice for both LTα and LTβ have been used in studies to demonstrate the importance of LT in lymphoid organogenesis. Whereas LTβ-/- mice maintain both the cervical and mesenteric LNs, LTα-/- mice lack all LNs, including mucosal ones (Alimzhanov *et al.*, 1997; Koni *et al.*, 1997). LTα-/- mice have also been shown to have significantly disorganized lymphoid structures within the spleen (Alimzhanov *et al.*, 1997). Since SLOs provide an optimal microenvironment where APCs and antigen-specific T and B cells can interact together to initiate an efficient immune response, $LT\alpha^{-/-}$ mice, which lack SLOs, provide an effective tool for examining whether the genital tract mucosa can initiate and sustain specific T cell responses to HSV-2 infections.

Although no studies have been done to examine HSV-2-specific T cell responses in the genital tract of LTα-/- mice, antiviral immune responses have been examined in both LTα-/- and LTβ-/- mice. In LTβ-/- mice, there are severely diminished cytotoxic CD8+ T cell responses to lymphocytic choriomeningitis virus (**LCMV**), leading to viral

persistence, but the major functions of T cells and DC were still found to be intact (Berger *et al.*, 1999). Another study using LTβ-/- mice found that protective T cell responses could be generated in the sacral LN, leading to protection against genital HSV-2 infection (Soderberg *et al.*, 2004). LTα-/- mice have been shown to generate delayed but effective primary and memory T- and B-cell responses to influenza (Moyron-Quiroz *et al.*, 2004; Moyron-Quiroz *et al.*, 2006). In another study, LTα-/- mice failed to develop effective CD8+ T cell responses and clear the virus, but they were still able to mount protective immune responses following a systemic infection with HSV (Lee *et al.*, 2000; Lund *et al.*, 2002). In addition, studies have shown that the disruption of the splenic microarchitecture seen in LTα-/- mice results in impaired IgG responses (Fu *et al.*, 1997). These studies suggest that protective antiviral T cell responses can be generated in the absence of SLOs. However, it has not been determined whether protective T cell responses can be generated in the genital tract of LTα-/- mice following HSV-2 infection.

1.4 – Female Sex Hormones and Hormonal Contraceptives

1.4.1 – Estrogen

It is well established that estrogens are essential regulators of female reproductive activity, with the ovary, uterus, mammary gland and central nervous system as target tissues. More recent evidence also supports a role for estrogen in the function of non-reproductive tissues such as the skeletal and the cardiovascular systems (Nilsson & Gustafsson, 2011). (17)β-estradiol (**E2**) is the dominant estrogen found in women, with

physiological serum levels fluctuating between 10^{-9} M to 10^{-12} M throughout the normal menstrual cycle and reaching as high as 10^{-8} M during pregnancy (Stricker *et al.*, 2006; Abbassi-Ghanavati *et al.*, 2009). The ovaries represent the most important source of circulating E2 (95%), with the adrenal cortex also contributing (5%) (Vrtacnik *et al.*, 2014).

E2 exerts its effects by binding to estrogen receptors (**ERs**). Two intracellular estrogen receptors are best known and studied, ERα and ERβ, and the tissue distribution and level of expression within tissues of both receptors varies. ERα is found in endometrium, breast cancer cells, ovarian stromal cells, liver, kidney, adipose tissue and hypothalamus. In contrast, ERβ expression has been documented on ovarian granulosa cells, kidney, brain, lung, bone, heart, intestinal mucosa, prostate and endothelial cells (Bouman *et al.*, 2005; Fish, 2008). These receptors are also expressed by a number of immune cells, such as T cells, B cells, DCs, macrophages, neutrophils and NK cells, as well as non-immune cells, such as genital epithelial cells (Fish, 2008). The binding of E2 to ERα or ERβ in the cytoplasm of target cells causes conformational changes that enable receptor dimerization, translocation to the nucleus and binding to the estrogen response elements located in or near the promoters of target genes, thereby promoting gene expression (Vrtacnik *et al.*, 2014).

More recently, a membrane-bound receptor for E2 has also been described (Filardo *et al.*, 2002; Thomas *et al.*, 2005). G-protein receptor 30 (**GPR-30**) is a member of the 7 transmembrane G-protein coupled receptor family and is found in the uterus,

ovaries, mammary glands, gastrointestinal system, central and peripheral nervous system, pancreas, liver, kidney, adrenal and pituitary glands, bone tissue, cardiovascular system and a number of immune cells. In contrast to the ERs, when E2 binds to the membrane-bound GPR-30, it results in the rapid non-genomic signaling events, such as mobilization of intracellular calcium and activation of various protein kinase cascades that can eventually lead to indirect changes in gene expression due to phosphorylation of various transcription factors (Vrtacnik *et al.*, 2014).

1.4.2 – Progesterone

In addition to E2, progesterone (**P4**) is another sex hormone that is produced during the menstrual cycle. The major sources of P4 during the menstrual cycle are the ovaries and corpus luteum, with the adrenal glands and central nervous system also contributing (Graham & Clarke, 1997; Schumacher *et al.*, 2007). The physiological serum levels of P4 normally fluctuate between 10^{-7} M and 10^{-10} M during a standard menstrual cycle and peak at 10^{-6} M during the third trimester of pregnancy (Kratz *et al.*, 2004; Stricker *et al.*, 2006; Abbassi-Ghanavati *et al.*, 2009). P4 plays a role in a number of functions within the ovary and uterus, including release of mature oocytes from the ovary, differentiation of the endometrium, facilitation of embryo implantation, and maintenance of pregnancy. In addition, P4 also functions in the mammary gland by helping prepare for milk secretion and suppressing milk protein synthesis during parturition, and in the brain by mediating signals required for sexually responsive behavior (Graham & Clarke, 1997).

P4 exerts its effects by binding to the progesterone receptor (**PR**), which is a member of the nuclear receptor superfamily of transcription factors capable of activating and repressing transcription of their target genes. Upon binding P4 in the cytoplasm, the PR dimerizes, enters the nucleus and binds DNA to regulate the expression of target genes. PR has two isoforms, PRA and PRB, which can homodimerize or heterodimerize to generate increased specificity and regulation upon binding to progesterone response elements of target genes (Wetendorf & DeMayo, 2012). Both isoforms of PR are expressed in the ovary, uterus, mammary glands, brain, pancreas, thymus, bone, blood vessels and central nervous system (Rajaram & Brisken, 2012). In addition, PRs are also present on a number of immune cells, including NK cells, T cells, B cells, monocytes, macrophages and dendritic cells (Xu *et al.*, 2011).

1.4.3 – Depo-Provera

Depo-Provera (**Depo**) is the commercial name for the injectable synthetic progestin, depot medroxyprogesterone acetate (**DMPA**). It is most commonly used in women as an oral or subcutaneous injectable hormonal contraceptive. As such, Depo works by inhibiting gonadotropin secretion from the pituitary, thereby blocking follicular development, which in turn reduces the ovarian production of E2 and prevents ovulation. It also causes thickening of the cervical mucus and thinning of the endometrium (Guilbert *et al.*, 2009). Depo has been shown to have a binding affinity for PR that is 100 times greater than P4 (Philibert *et al.*, 1999), and it also binds and signals through ER, glucocorticoid receptor (**GR**), androgen receptor and mineralocorticoid receptor

(Kemppainen *et al.*, 1999; Philibert *et al.*, 1999; Koubovec *et al.*, 2005; Africander *et al.*, 2011). Serum levels of Depo range from 10^{-9} M immediately following administration to 10^{-12} M, which is usually measured approximately 3 months following use (Goldman, 2000).

1.4.4 – The Menstrual Cycle

The menstrual cycle is the cycle of natural changes that occur in the uterus and ovary to prepare the uterus for pregnancy. In humans, the length of a menstrual cycle varies greatly, with 28 days being the average length. Each cycle can be divided into three phases based on events in the ovary (ovarian cycle) or in the uterus (uterine cycle). The ovarian cycle consists of the follicular phase, ovulation and luteal phase whereas the uterine cycle consists of menstruation, proliferative phase and secretory phase (Marieb, 2012).

The first day of menstrual bleeding marks the beginning of the menstrual cycle (see Appendix B). Increasing amounts of E2 during the follicular phase cause the discharge of blood to slow then stop and the uterine lining to thicken. In the ovary, follicles begin to develop under the influence of a complex interplay of hormones. Approximately mid-cycle, a surge in the production of luteinizing hormone causes a follicle to release an ovum, or egg, in an event called ovulation. After ovulation, the remains of the follicle in the ovary becomes the corpus luteum, which produces large amounts of P4. This increase in P4 production causes the endometrium (uterine lining) to prepare for potential implantation of an embryo to establish pregnancy. If implantation

does not occur within approximately two weeks, the corpus luteum will involute, causing sharp drops in the levels of P4 and E2, leading to shedding of the uterine lining and menstruation (Marieb, 2012).

1.4.5 – The Mouse Estrus Cycle

The reproductive cycle in mice is called the estrus cycle, and lasts approximately 4-5 days (see Appendix C). This cycle can be divided into four stages: proestrus, estrus, metestrus and diestrus. Proestrus lasts one day and consists of increasing E2 levels, leading to one or several follicles of the ovary starting to grow. Under the influence of E2, the uterine lining starts to develop. During estrus, luteinizing hormone and follicle stimulating hormone levels start to rise and ovulation occurs. This stage lasts 12-18 hours, with E2 levels remaining elevated at the beginning and then returning to basal levels. During the following day, metestrus, the corpora lutea grow and produce P4. In the absence of pregnancy, the diestrus stage terminates with the regression of the corpus lutea. The lining of the uterus is not shed, but is reorganized for the next cycle (Caligioni, 2009).

In mice, the identification of the stage of estrous cycle is based on the proportion of cell types observed in the vaginal secretion (see Appendix C). During proestrus, there is a predominance of nucleated epithelial cells, which may appear in clusters or individually. Occasionally, some cornified cells may appear. Estrus is characterized by cornified squamous epithelial cells, which occur in clusters. There is no visible nucleus, the cytoplasm is granular, and the shape is irregular. In the metestrus stage, there is a mix

of cell types with a predominance of leukocytes and a few nucleated epithelial and/or cornified squamous epithelial cells. Diestrus stage primarily consists of leukocytes (Caligioni, 2009).

1.4.6 – Regulation of Host Immune Responses by the Menstrual Cycle

The female menstrual cycle can have dramatic effects on immune responses generated in the FGT. While immune cells are present in the FGT over the course of the menstrual cycle, their numbers and distribution varies with the phase of the menstrual cycle and the site examined (Wira *et al.*, 2010). In the uterus, both the size and cellular composition of LAs varies with the stage of the menstrual cycle. The B cell core was most often seen in large aggregates present in the late proliferative and secretory stages of the menstrual cycle. In addition, LAs were significantly larger during the secretory (3000-4000 cells) than the proliferative stage (300-400 cells) (Wira *et al.*, 2010). Coincident with LA formation in the uterus, CD8+ cytotoxic T cell activity is highest during the proliferative phase but significantly suppressed in the uterus and Fallopian tubes during the secretory phase of the cycle. In contrast, in the ectocervix and vagina, CD8+ cytotoxic T cell activity was measurable in tissues from women at the proliferative or secretory stages of the menstrual cycle (White *et al.*, 1997).

Humoral immunity in the FGT is also regulated by hormones and varies with site examined and stage of the menstrual cycle. The frequency of B cells in tissues and the rate of antibody production and transepithelial transport can result in fluctuation of antibody levels in genital secretions during the menstrual cycle (Kutteh *et al.*, 1996; Lu *et*

al., 1999). One study found that high levels of P4 produced during the luteal phase of the menstrual cycle results in decreased levels of both IgA and IgG in cervical mucus (Kutteh *et al.*, 1998). Another study found that IgA and IgG levels in cervical mucus were decreased by 10- to 100-fold at mid-cycle relative to that seen early in the proliferative phase, only to rise toward the end of the menstrual cycle (Wira *et al.*, 2010).

1.4.7 – Impact of Female Sex Hormones and Hormonal Contraceptives on Susceptibility to Sexually Transmitted Infections

Both the stage of menstrual cycle and the use of hormonal contraceptives have been shown to influence susceptibility to initial infection with a number of sexually transmitted infections (**STIs**), including HSV-2, Candidiasis, gonorrhoea, HIV and Chlamydia in women (Brabin, 2002). Several studies have found that the use of Depo can increase susceptibility to HIV infection and that women who use progesterone-based contraceptives have accelerated HIV disease progression and mortality compared to women who did not (Lavreys *et al.*, 2004; Stringer *et al.*, 2007; Leclerc *et al.*, 2008). Similarly, a more recent study that followed 8663 study participants for 52 weeks and found that Depo use significantly increased the risk of HIV acquisition compared to participants using oral contraceptives or no hormonal contraceptives (Crook *et al.*, 2014). In addition, studies have also found that Depo use inhibits T cell and pDC activation, suppresses cytokine production by activated mononuclear cells and pDCs, and increases the levels of the cytokine regulated on activation, normal T cell expressed and secreted (**RANTES**), which could be factors underlying a possible association between Depo use

and increased risk of HIV acquisition (Huijbregts *et al.*, 2014; Morrison *et al.*, 2014). However, other studies have found no association between hormonal contraceptive use and HIV acquisition and no association between Depo use and increased detection of either plasma or cervical HIV-1 RNA in women (Morrison *et al.*, 2007; Day *et al.*, 2014). These results suggest that, in humans, the use of hormonal contraceptives may increase risk of acquiring HIV, but further studies are required to understand the relationship between hormonal contraceptive use and acquisition of STIs.

To further elucidate the role that female sex hormones and hormonal contraceptives play in acquisition of STIs, several studies have been conducted using animal models. In rhesus macaques, it was found that susceptibility to IVAG simian immunodeficiency virus (**SIV**) was highest during the progesterone-high, luteal phase compared to the estrogen-high, follicular phase (Sodora *et al.*, 1998). In addition, treating rhesus macaques with progesterone increased SIV acquisition by more than 7-fold (Marx *et al.*, 1996), whereas the systemic administration of estrogen protected OVX female rhesus macaques against IVAG SIV infection (Smith *et al.*, 2000). Mouse studies have also been done to study the effects of Depo treatment on HSV-2 infection, these are discussed in more detail in the following sections.

1.4.8 – Regulation of Host Immune Responses by Progesterone and Progesterone-Based Hormonal Contraceptives

In addition to regulating susceptibility to STIs, female sex hormones and hormonal contraceptives also influence the underlying immune responses. P4

significantly affects the infiltration of lymphocytes, macrophages and NK cells into the FGT and has been shown to exert a number of effects on T cell-mediated immunity, including inhibiting CTL activity and blocking perforin expression in T cells (Inoue *et al.*, 1996; Arici *et al.*, 1999; Borel *et al.*, 1999; Szekeres-Bartho *et al.*, 2001; Yeaman *et al.*, 2001; Cherpes *et al.*, 2008). In addition, P4 also impairs the function of a number of other types of immune cells. For example, P4 decreases NK cell function and FcγR expression on monocytes, thereby reducing the two arms of antibody-dependent cell cytotoxicity (Scanlan *et al.*, 1995; Yovel *et al.*, 2001; Gomez *et al.*, 2002). P4 also inhibits TLR9-induced IFNα production by both human and mouse pDCs (Hughes *et al.*, 2008). In addition to inhibiting IFNα production, P4 also influences production of other cytokines, generally by increasing Th2-type cytokine responses, but also by decreasing Th1-type cytokine production by CD4+ and CD8+ T cells (Piccinni *et al.*, 1995; Enomoto *et al.*, 2007). In both human and mouse T cells, P4 inhibits the differentiation of Th17 and decreases production of IL-17A. Recently, it was shown that P4 inhibits Th17 response in murine vaginal gonococcal infection (Xu *et al.*, 2013). Taken together, the impaired cellular functions and cytokine production observed following P4 treatment may contribute to the increased susceptibility to HIV observed in both women and animal models.

1.4.9 – Regulation of Host Immune Responses by Estrogen

Extensive research has been done to study the effects of estrogen on immune responses in women. Estrogen can either have pro-inflammatory or anti-inflammatory

effects depending on the concentration. At low concentrations, one study found that estrogen increases the production of pro-inflammatory cytokines, including TNFα, IL-6 and IL-1β, and also inhibits Th2 cytokine production and increases migration of leukocytes to the site of inflammation (Straub, 2007). In contrast, another study found that estrogen inhibits production of TNFα, IL-6 and IL-1β by T cells, macrophages and DCs, and induces Th2 cytokine production, thereby having an anti-inflammatory effect (Zang *et al.*, 2002). Estrogen can also have anti-inflammatory effects at higher concentrations, by inhibiting cell-mediated immunity and decreasing the expression of a number of activation markers (Attanasio *et al.*, 2002; Enomoto *et al.*, 2007).

E2 also has varying effects on Th17 cells as well. It has been shown that E2 suppresses experimental autoimmune encephalitis, a Th17 cell mediated disease, and E2 inhibits IL-17 production by murine lymphocytes (Jansson *et al.*, 1994; Bebo *et al.*, 2001). In addition, OVX mice have increased Th17 cells in bone marrow, which was reversed by E2 supplementation (Tyagi *et al.*, 2012). However, another study found that E2 promotes IL-17 production by stimulated splenocytes in mice (Khan *et al.*, 2010). Therefore, while the impact of E2 on Th17 cells is still controversial, it appears to depend on the tissue and disease context.

E2 also influences immune responses in the FGT. In OVX rats, E2 treatment increases the levels of IgA and IgG in the uterus, but decreases the levels of these antibodies in cervicovaginal secretions (Wira & Sandoe, 1977; Wira & Sullivan, 1985). E2 also affects the activity of a number of immune cells as well. For example, E2

treatment decreases antigen presentation in the vagina (Wira *et al.*, 2002), down-regulates CTL activity (White *et al.*, 1997; Zang *et al.*, 2002), and decreases migration of inflammatory T cells and macrophages into the genital tract (Straub, 2007), all of which could contribute to the protective effects of estrogen treatment against sexually transmitted viral infections.

1.4.10 – Hormonal Regulation of Susceptibility and Immune Responses to HSV-2

Results from recent vaccine trials where protection was found primarily among HSV-1 and HSV-2 seronegative women underlines the importance of considering the role of female sex hormones on immunization and the generation of immune responses against genital HSV-2 infection. While the effect of female sex hormones or hormonal contraceptive use on susceptibility and immune responses to HSV-2 has not been studied in women, a number of studies have been conducted using HSV-2 mouse models. These mouse models rely on altering the hormonal environment of the FGT to induce infection. Without altering the hormonal environment of the FGT, susceptibility to vaginal HSV-2 infection is dependent on the stage of the estrous cycle in mice. Female mice are susceptible to genital HSV-2 infection at diestrus, the stage of the reproductive cycle when P4 levels are highest, but not at estrus, when E2 levels are highest (Parr & Parr, 2003). Therefore, to make all mice susceptible to genital HSV-2 infection, a model was developed that pretreated mice with Depo prior to infection (McDermott *et al.*, 1984). Our lab has shown that Depo-treated mice had prolonged diestrus that lasted more than 4 weeks and coincided with a 100-fold increase in susceptibility to genital HSV-2

compared to untreated mice in diestrus. In contrast, mice pretreated with P4 were in diestrus stage for 4 to 6 days and showed a 10-fold increase in susceptibility compared to untreated mice. In addition to altering susceptibility to genital HSV-2, Depo treatment had inhibitory effects on immune responses to HSV-2. Depo-treated, immunized mice had a significantly decreased antibody response and were not protected following intravaginal (**IVAG**) HSV-2 infection, whereas P4-treated mice were completely protected (Kaushic *et al.*, 2003). In addition, the length of time mice are exposed to Depo prior to immunization also alters immune responses. Mice immunized IVAG with TK^- HSV-2 following longer (15 days) exposure to Depo failed to show protection when challenged with WT HSV-2, whereas mice that were immunized shortly after Depo treatment (5 days) were fully protected. The longer exposure to Depo also resulted in delayed viral clearance and significantly decreased IFNγ and HSV-2-specific antibody responses, suggesting that longer exposure to Depo results in decreased immune responses to HSV-2 that fail to protect mice from subsequent genital challenge (Gillgrass *et al.*, 2003).

In order to avoid these impaired immune responses associated with Depo pretreatment, our lab has established an alternative mouse model to further study the effects of hormones on susceptibility and immune responses to HSV-2. In this model, mice are ovariectomized (**OVX**) to remove any endogenous hormones, thereby making them susceptible to genital HSV-2. Following OVX, mice are administered either E2, P4 or saline (**S**) as a control. Mice are then immunized IVAG with TK^- HSV-2 and

challenged 4 weeks later with WT HSV-2. Due to the 4 week interval between immunization and challenge and the short half-life of hormones in blood, the mice are no longer under the influence of hormones at the time of challenge. Thus, the hormones present at the time of immunization influence the outcome of subsequent HSV-2 challenge. In parallel studies, mice were also immunized intranasally (**IN**) with TK^- HSV-2 following hormone treatments and challenged IVAG with WT HSV-2. The overall conclusion from these studies was that E2 treatment provided protection from HSV-2 infection with better outcomes on vaginal pathology and viral shedding, whereas P4- or S-treated mice were highly susceptible to infection and show chronic pathology and inflammation, as well as high viral titers in vaginal secretions (Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008). Protection in these mice correlated with the induction of vagina-associated lymphoid tissues (**iVALTs**), a transient lymphoid structure found in the vaginal lamina propria. Immunohistochemical analyses indicate that these iVALTS are composed of an outer ring of $CD11c^+$ DCs, whereas the majority of cells present are $CD3^+$ and $CD4^+$ (Gillgrass *et al.*, 2005b). The appearance of these structures coincided with the clearance of virus, suggesting that iVALTS play a critical role in protection against HSV-2. Since these iVALTs primarily consist of CD4+ T cells, it is important to identify and characterize the specific T cell responses that are present in the genital mucosa and how these T cells provide protection against HSV-2 challenge, which has not been examined. In addition, the hormonal regulation of these HSV-2-specific T cell responses in the genital tract of mice has also not been examined.

1.5 – Rationale, Hypothesis and Objectives

Results from recent vaccine trials where protection was found primarily among HSV-1 and HSV-2 seronegative women underlines the importance of considering gender-specific differences on immunization strategies and the generation of immune responses. Although women are more susceptible to HSV-2 infections than men and the female genital tract is the primary site of HSV-2 infection, little is known about the generation of protective immune responses in this distinct microenvironment and how these immune responses protect against genital HSV-2 infections in women. Understanding the underlying immune responses against HSV-2 infections in the FGT is essential to developing successful vaccine strategies that will provide protection at this primary site of transmission and acquisition. Most vaccine strategies have focused on stimulating IgG and IgA antibodies in the genital tract, but it has become apparent that strong local T cell responses are required for protection against HSV-2. In fact, studies in our lab using an HSV-2 mouse model have found that protection against genital HSV-2 challenge in immunized mice correlates with the induction of iVALTs. Since these iVALTs primarily consist of CD4+ T cells, it is important to identify and characterize the specific T cell responses that are present in the genital mucosa and how these T cells provide protection against HSV-2 challenge, which has not been examined.

Furthermore, other studies suggest that the local microenvironment in the genital tract plays a role in generating effective antiviral immune responses following immunization. Therefore, we wanted to determine whether protective immune responses

could be induced locally in the genital mucosa, against HSV-2 infection, in the absence of SLOs. We also wanted to determine what type of vaccine formulation and routes of immunization are required to induce these protective immune responses in the genital mucosa, which has not been determined.

Given that, in the absence of SLOs, mice can generate effective antiviral immune responses following immunization in other mucosal tissues, we *hypothesized that the protective antiviral immune responses following both local and distal immunization with a robust immunogenic vaccine could be generated in the genital mucosa itself, without the help of LN, and that factors present in the local microenvironment, such as the presence of the female sex hormone estradiol, can enhance protection against HSV-2 infection by enhancing antiviral T cell responses in the genital tract.* Specifically, we hypothesized that IVAG or IN immunization of LTα-/- mice with the live attenuated TK^- HSV-2 vaccine could induce protective antiviral T cell responses in the genital tract of mice, which could provide protection against a subsequent HSV-2 challenge. However, since LTα-/- mice have previously been shown to have delayed immune responses to viral infections, we hypothesized that using a less robust, non-replicating vaccine, administered either locally or at a distal mucosal site, will not induce a robust enough immune response, and thereby not protect against subsequent HSV-2 challenge. To test this, we immunized WT and LTα-/- OVX mice, either IVAG or IN, with a live attenuated virus vaccine (TK^- HSV-2), a subunit vaccine (HSV-2 gD plus mucosal adjuvant CpG) or an inactivated whole virus vaccine (heat-inactivated HSV-2 plus CpG) and then

challenged IVAG with WT HSV-2. Survival and gross genital pathology were monitored for 3 weeks, and viral shedding was measured for 5 days post-challenge. T cell responses were also examined post-challenge by flow cytometry.

In addition, since we and others have previously found that E2 can enhance protection against HSV-2 and other STIs, we hypothesized that treating mice with E2 prior to immunization, even with a non-replicating vaccine at a distal mucosal site, should result in enhanced protection against HSV-2 challenge. To test this hypothesis, we treated WT and LTα-/- OVX mice with E2 pellets one week prior to IN immunization and then mice were challenged IVAG with WT HSV-2. Survival and pathology were monitored post-challenge, and viral titers were measured for 5 days post-challenge. Lastly, since CD4+ T cell responses are required for protection against genital HSV-2, we hypothesized that the enhanced protection observed in E2-treated mice will be due to enhanced functions of specific CD4+ T cell responses in the genital tract of immunized mice following HSV-2 challenge. To test this, cells from the vaginal tracts, draining lymph nodes and spleens of E2-treated (or mock controls), IN immunized mice were collected on different days post-HSV-2 challenge and analyzed by flow cytometry to characterize the specific T cell responses.

Therefore, the overall objective of this work was to identify and characterize the protective T cell responses that are generated locally in the genital mucosa of immunized mice following genital HSV-2 challenge and to determine what vaccine formulation(s) and route(s) of immunization are required to effectively induce these protective T cell

responses in the genital mucosa. These objectives were addressed through the following aims:

1) To examine local protective anti-HSV-2 T cell responses in the genital tract of mice immunized IVAG with a live attenuated virus followed by genital HSV-2 challenge.
2) To determine whether local protective immune responses could be generated in the genital tract following immunization, either locally or distally, with non-replicating vaccines rather than the live attenuated virus vaccine.
3) To determine how E2 pretreatment influences protective T cell responses in the genital tract of immunized mice following HSV-2 challenge.

Chapter 2

Delayed but effective induction of mucosal memory immune responses against genital HSV-2 in the absence of secondary lymphoid organs

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This study examined whether local immunization in the absence of SLOs could establish effective antiviral memory responses in the female genital tract following HSV-2 infection. We found that even in the absence of SLOs, mice immunized IVAG with TK^- HSV-2 were completely protected against genital HSV-2 challenge, but they showed delayed viral clearance and prolonged genital pathology post-immunization compared to WT mice. Although local viral-specific antibody responses were compromised and T cell-mediated anti-HSV-2 responses were delayed in the absence of SLOs, the immune responses generated were effective in protecting against mucosal HSV-2 challenge in the genital mucosa.

Dr. Charu Kaushic, Sudha Bhavanam, Hong Jiang and I were responsible for the design and interpretation of the experiments. Sudha Bhavanam, Hong Jiang and I were responsible for the generation and analysis of the data. Amy Gillgrass, Karen Ho and Victor H. Ferreira provided technical assistance. Dr. Charu Kaushic and I wrote and edited the manuscript.

Delayed but effective induction of mucosal memory immune responses against genital HSV-2 in the absence of secondary lymphoid organs

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ABSTRACT

To examine whether local immunization in the absence of secondary lymphoid organs (**SLOs**) could establish effective anti-viral memory responses in the female genital tract, we examined immunity in the vaginal tracts of LTα-/- mice, LTα-/- SPL (splenectomized) and control C57BL/6 (**WT**) mice. All three groups of mice were immunized intravaginally (**IVAG**) with attenuated (**TK**$^{-}$) herpes simplex virus type 2 (**HSV-2**) and challenged 4-6 weeks later with wild-type (**WT**) HSV-2. Both groups of LTα-/- mice exhibited delayed viral clearance and prolonged genital pathology after immunization. Following IVAG WT HSV-2 challenge, LTα-/- and LTα-/- SPL mice had significantly lower levels of HSV-2-specific IgG and IgA in the vaginal secretions. Although the frequency of B and T cells in the vaginal mucosa was comparable or higher in both groups of LTα-/- mice, lower frequency of HSV-2-specific interferon-γ (**IFNγ**)-producing CD3+ T cells was seen after immunization and after challenge, compared with WT group. Despite this, immunized mice in all three groups showed complete sterile protection against IVAG WT HSV-2 challenge. These results show that even in the absence of SLOs, IVAG immunization generates effector memory immune responses at genital mucosa that can provide antiviral protection against subsequent viral exposures. This will inform new strategies to design mucosal vaccines against sexually transmitted infections.

Keywords: secondary lymphoid structures, immunization, HSV-2, protection, mucosal immunity, genital tract immunity

INTRODUCTION

The presence of a common immune system at mucosal sites, including intestinal, lung and genitourinary tract, is well recognized. Some characteristics such as homing markers are shared among these sites; however, there are distinct differences between individual mucosal tissues (Youngman, 2005; Brandtzaeg, 2010). Studies have also shown that effective memory T cell responses are generated in the lung mucosa during influenza infection in the absence of secondary lymphoid structures (**SLOs**) (Moyron-Quiroz *et al.*, 2006). The site for the maintenance and generation of these memory responses was shown to be induced bronchial associated lymphoid tissues (Moyron-Quiroz *et al.*, 2004). In a mouse model of genital herpes infection, we have also documented the development of induced vaginal-associated lymphoid tissue (**iVALT**) aggregates in the genital mucosa of immunized mice, which correlated with protection against herpes simplex virus type 2 (**HSV-2**) infection following challenge. These iVALTs were found to contain primarily CD4+ T cells, B cells and CD11c+ antigen-presenting cells (Gillgrass *et al.*, 2005b). Furthermore, our studies suggested that the local microenvironment in genital tract plays a role in generating effective antiviral immune responses following immunization (Gillgrass *et al.*, 2005a; Gillgrass *et al.*, 2005b). We therefore decided to examine whether protective effector memory responses could be induced in the genital mucosa, against a viral infection, in the absence of SLOs. To do this, we utilized a lymphotoxin alpha (**LTα**) knockout mouse model.

LTα alpha is a member of the tumor necrosis factor family. The tumor necrosis factor family members, including LTα, have been shown to be essential for the proper development of lymphoid tissues, including lymph nodes (**LNs**) and Peyer's patches, and for maintenance of the correct architecture of SLOs, including the spleen (De Togni *et al.*, 1994; Banks *et al.*, 1995; Alimzhanov *et al.*, 1997; Gommerman & Browning, 2003; Tumanov *et al.*, 2003a; Tumanov *et al.*, 2003b). Knockout mice for both LTα and LTβ and have been used in studies to demonstrate the importance of LT in lymphoid organogenesis. Whereas LTβ-/- mice maintain both the cervical and mesenteric LNs (Alimzhanov *et al.*, 1997; Koni *et al.*, 1997), LTα-/- mice lack all LNs, including mucosal ones (Alimzhanov *et al.*, 1997). LTα-/- mice have also been shown to have significantly disorganized lymphoid structures within the spleen. Thus, LTα-/- mice, which lack SLOs, provide an effective tool for examining the role of SLOs in the generation and maintenance of adaptive immune responses to infectious agents. Antiviral immune responses in the absence of SLOs have been examined in both LTα-/- and LTβ-/- mice (Berger *et al.*, 1999; Lee *et al.*, 2000; Kumaraguru *et al.*, 2001; Lund *et al.*, 2002; Soderberg *et al.*, 2004). LTα-/- mice have been shown to generate delayed but effective responses to influenza and MHV-68 (Lee *et al.*, 2000; Lund *et al.*, 2002). However, during systemic infection with HSV, LTα-/- mice failed to develop effective $CD8^+$ T cell responses and clear the virus (Kumaraguru *et al.*, 2001). Whether adequate antiviral memory immune responses can be mounted in the genital mucosa in response to mucosal immunization in the absence of SLOs is not known.

Genital tract mucosa is an important target for inducing immune responses against sexually transmitted pathogens. Genital herpes is one of the most common sexually transmitted infections (Corey & Wald, 1999). As of 2008, it was estimated that 536 million people aged 15-49 are infected globally, and approximately 23.6 million people become newly infected every year (Looker *et al.*, 2008). Despite the significant global health burden from HSV-2, attempts to develop herpes vaccines have failed since the 1930s (Cunningham & Mikloska, 2001; Koelle & Corey, 2003). The most recent trial of a glycoprotein D (**gD**)-based HSV-2 vaccine showed a lack of efficacy, despite previous trials where partial protection was observed in women who were seronegative for both HSV-1 and HSV-2 (Stanberry *et al.*, 2002; Belshe *et al.*, 2012). The failure of conventional HSV-2 vaccines reiterate the importance of examining the local microenvironment in the female genital tract (Kaushic, 2009; Kaushic *et al.*, 2011).

In the present study we examined whether intravaginal (**IVAG**) immunization in the absence of SLOs could establish effective anti-HSV-2 memory responses in the female genital tract and hypothesized that the genital mucosa itself, without the help of any draining LNs, is capable of generating a protective antiviral immune response. LTα-/- mice were ovariectomized (**OVX**) to make mice susceptible to HSV-2 infection, and splenectomized (**SPL**) to remove all SLOs. Mice were then immunized IVAG with an attenuated strain of HSV-2, thymidine kinase-negative (**TK**$^{-}$) HSV-2, and 4-6 weeks later challenged IVAG with wild-type HSV-2. LTα-/- mice, as well as wild-type (**WT**) controls, showed complete protection against genital HSV-2 challenge, although both

groups of LTα-/- mice exhibited delayed viral clearance and prolonged genital pathology after immunization. Although local viral-specific antibody responses were compromised and T cell-mediated anti-HSV-2 responses were delayed in LTα-/- mice, the immune responses generated were effective in protection against mucosal HSV-2 challenge in the genital mucosa.

RESULTS

Protection against HSV-2 challenge in LTα-/- mice following IVAG immunization with TK-HSV-2. Previously, we have shown that following IVAG immunization with TK^- HSV-2, WT OVX female mice are completely protected against IVAG challenge (Gillgrass *et al.*, 2005b). Removal of ovaries (OVX) makes mice susceptible to genital HSV-2 infection without hormonal manipulation (Gillgrass *et al.*, 2005a; Gillgrass *et al.*, 2005b). As LTα-/- mice completely lack draining LNs as well as Peyer's patches, we first examined whether IVAG immunization with TK^- HSV-2 would result in protection. To ensure absence of all SLO, we removed the spleens in LTα-/- mice. Both LTα-/- OVX and LTα-/- OVX+SPL, along with WT OVX controls, were then immunized IVAG with 10^5 PFU of TK^- HSV-2, as previously described (Gillgrass *et al.*, 2005b). At 4-6 weeks after immunization, mice were challenged IVAG with 10^5 PFU of WT HSV-2. All three groups of mice showed 100% survival against HSV-2 infection after challenge (Figure 1). To show that all mice did in fact become infected, nonimmunized control groups were included. Following OVX and/or SPL, all three groups of mice were infected IVAG with 10^5 PFU of WT HSV-2. Mice in all three groups succumbed to infection within a week, as expected (Figure 1). Thus, IVAG immunization induced HSV-2-specific immune responses in all three groups of immunized mice, resulting in complete protection against high-dose genital HSV-2 challenge.

Genital pathology in LTα-/- mice after immunization and after challenge. To determine if the protection in all three groups of mice was comparable, gross pathology

of the genital area was examined daily following TK^- HSV-2 immunization and WT HSV-2 challenge. In all, 7 of 9 WT, 8 of 10 LTα-/-, and 10 of 10 LTα-/- SPL mice showed external pathology starting 6 to 7 days after immunization (Figure 2A). Although a majority of mice in the WT group showed mild pathology symptoms, higher pathology was noted in more mice in both groups of LT mice (Figure 2 A and Table 1). Assessment of cumulative pathology scores for each group and average pathology per mouse, as described before (Bhavanam *et al.*, 2008), showed that mice in the WT group had the lowest average pathology score (29) compared to both LTα-/- (40) and LTα-/- SPL (62) mice after immunization (Table 1). Following WT HSV-2 challenge 6 weeks after immunization, none of the mice in any of the three groups showed any pathology up to 21 days after challenge (Figure 2B). In non-immunized control groups, LTα-/- and LTα-/- SPL mice developed pathology more rapidly than WT mice (Figure 2C).

Viral titers in LTα-/- and LTα-/- SPL mice after immunization and after challenge. Viral titers were measured in vaginal washes after immunization and after challenge to measure the control of viral replication and shedding in the genital tract by local immune responses. Previous studies have shown that viral shedding in vaginal washes occurs for 3-5 days after immunization in WT mice before virus is cleared (Gillgrass *et al.*, 2005a; Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008). In the present study, similar results were seen in WT mice (Figure 3A). However, LTα-/- and LTα-/- SPL mice had prolonged viral shedding (Figure 3A). Overall the viral titers were not significantly higher ($p>0.05$) in the LTα-/- and LTα-/- SPL mice after immunization compared with WT mice. Mice in

all three groups did not show any viral shedding after HSV-2 challenge (Figure 3B). In non-immunized controls, there was significant viral shedding in all three groups of mice on day 1 after infection that persisted over next 3-5 days, after which the vaginal pathology was too high to collect samples from the mice (Figure 3C).

To confirm that there was no viral shedding after challenge, a more sensitive real-time PCR assay was performed to measure HSV-2 DNA (Supplementary Figure 1). Viral shedding measured by real-time PCR confirmed the results seen by plaque-forming assay (Figure 3), with no viral shedding seen 7-12 days after immunization and no shedding after challenge in all three groups of immunized mice. These results indicate that IVAG immunization with TK^- HSV-2 in WT and both groups of LTα-/- mice provided sterile immunity against subsequent HSV-2 challenge.

Immune cell populations in the vaginal mucosa of WT and LTα-/- mice after immunization and after challenge. The complete protection against genital HSV-2 challenge in the absence of LNs in LTα-/- mice and LNs and spleen in LTα-/-SPL groups led us to examine the immune cell populations in the vaginal mucosa of these mice before and after immunization, as well as after challenge by flow cytometric analysis. The vaginal mucosa of control, unimmunized LTα-/- mice contained a higher frequency of CD3+CD4+ T cells (2.79%) compared to WT mice (0.63%; Figure 4A). Although the increase in CD4+ T cells was >20-fold in WT mice compared with naïve WT mice on day 14 after immunization, in the LTα-/- mice this increase was less remarkable (2.7-fold), suggesting delayed induction of T cell responses (Figure 4A and Table 2). On day

22 after immunization and day 3 after challenge, the frequency of $CD4^+$ T cells in the vaginal mucosa of all three groups of mice was comparable.

Similar to CD3+CD4+ T cells, higher frequency of CD3+CD8+ T cells was observed in the vaginal mucosa of naïve LTα-/- mice (1.10% compared to 0.33%, Table 2). On day 22, the highest percentage of CD3+CD8+ T cells was noted in LTα-/- SPL mice (6.27%) (Table 2). After challenge, a 1.5-2-fold increase in frequency of $CD3^+CD8^+$ T cells was observed, particularly in LTα-/- SPL mice (9.51±0.03%) compared with WT (6.14±0.41%) and LTα-/- (6.45±1.69%) mice.

The B cell population in the vagina was identified by co-expression of CD19 and major histocompatibility complex class II (MHCII). In naïve LTα-/- mice, B cells were present at ~10 times higher frequency (4.34%) compared with WT mice (0.063%; Figure 4B and Table 2). On day 22 after immunization, the B cell population in the vaginal tract was seen to increase 10-fold and 2-fold in WT and LTα-/- mice, respectively (Figure 4B). After challenge, WT mice demonstrated a 3.5-fold increase in B cell percentages compared with day 22 after immunization, whereas a small (LTα-/-, 2-fold) or no (LTα-/- SPL) increase in B cell frequency was seen in LTα-/- mice.

In addition to T and B cells, the infiltration of professional antigen-presenting cells was also examined in all three groups of mice after immunization and challenge (Table 2). A small number of MHCII+CD11b+ cells and MHCII+CD11c+ cells were identified in the vaginal tracts of control mice and after immunization (Table 2).

However, by day 22 after immunization and day 3 after challenge, highest frequency of antigen-presenting cells was seen in WT mice (Table 2).

Previous studies in our lab have found that protection against secondary HSV-2 challenge in WT mice correlated with the development of iVALTs, consisting of CD3+ CD4+ T cells surrounded by MHC Class II+ CD11c+ antigen-presenting cells (Gillgrass *et al.*, 2005b). We therefore examined whether protection against secondary HSV-2 challenge in LTα-/- mice correlated with iVALT formation (Supplementary Figure 2). Although LTα-/- mice had a distinct increase in the number of CD4+ T cells present in the vaginal tract after challenge, they were nonpreferentially distributed throughout the tissue and did not form iVALT structures. WT mice had more MHCII+ and CD11c+ cells present, which were localized along with CD4+T cells, in lymphoid aggregates. Neither WT nor LTα-/- mice showed many CD8+ cells in the vaginal tracts after challenge.

Overall, these results indicate that in the naïve LTα-/- mice there was an increased frequency of B and T cells compared with WT. Following IVAG immunization, although CD4+ T cell numbers increased rapidly in the vaginal mucosa of WT mice, the increase in CD4+ T cells was gradual in LTα-/- mice, but by day 22 after immunization, all three groups had comparable number of CD4+ T cells and this was maintained after challenge. Unlike CD4+ T cells, CD8+T cells and B cells were detected at higher frequency in vaginal mucosa of LTα-/- mice, compared to WT mice, both after immunization and after challenge. Furthermore, although iVALT formation was observed

after challenge in WT mice as reported previously, no lymphoid aggregates were observed in LTα-/- after challenge.

IgG and IgA levels in vaginal secretions and serum of immunized mice before and after HSV challenge. The increase in frequency of T and B cell populations after immunization and after challenge suggested that effector immune responses were present in the vaginal mucosa of LTα-/- and LTα-/- SPL mice, despite the absence of SLOs. Therefore, we first examined systemic and local antibody responses following TK^- HSV-2 immunization and HSV-2 challenge to determine B cell responses. HSV-2-specific serum immunoglobulin G (**IgG**) levels were increased significantly by almost 100-fold following HSV-2 challenge in WT mice compared with the levels observed after immunization (Figure 5A). However, the IgG response was not significantly increased in the serum samples of LTα-/- and LTα-/- SPL mice after challenge. In the vaginal washes, only the WT mice showed significant booster responses after challenge compared with post-immunization levels of HSV-2-specific IgG (Figure 5B). Anti-HSV-2 immunoglobulin A (**IgA**) levels in the vaginal washes of WT mice were quite low but did show some booster response after challenge (Figure 5C). The HSV-2-specific IgA levels in LTα-/- and LTα-/- SPL mice were negligible and no booster response was observed. These results indicate that although LTα-/- mice did generate some IgG antibody responses, the overall HSV-2-specific IgG levels were significantly compromised compared with WT mice. Interestingly, the mucosal IgA responses were virtually absent in LTα-/- mice.

To examine if the decreased levels of IgA in LTα-/- mice were in part due to defects in antibody production in these mice that has been previously reported, we examined the total IgG and IgA levels in serum and vaginal washes of nonimmunized control WT, LTα-/- and LTα-/- SPL mice (Supplementary Figure 4). With the exception of vaginal IgG levels, significantly decreased levels of IgG and IgA were present in the serum and vaginal washes of both groups of LTα-/- mice.

Comparison of total and HSV-2-specific antibody responses in the vaginal secretions of LTα-/- mice after challenge. As flow cytometric analysis results indicated increased proportions of B cells in the LTα-/- groups, but the HSV-2-specific antibody titers were lower in vaginal tract of LTα-/- groups compared to WT mice, we examined whether the impairment in antibody secretion was HSV-2 specific. Total and specific antibody responses in LTα-/- mice were compared with WT mice. WT and LTα-/- mice had similar levels of total IgG, whereas LTα-/- SPL mice had marginally reduced levels (not significant, $p>0.05$) compared with WT mice (Figure 6A). However, both groups of LTα-/- mice had significantly reduced HSV-2-specific IgG responses in the vaginal tract after HSV-2 challenge compared with WT mice (Figure 6B). This suggests that in the absence of SLOs, the ability to mount specific antibody responses in the local mucosa following immunization was compromised.

T cell functional responses in the vaginal mucosa: HSV-2-specific IFNγ-producing T cells, after immunization and after challenge. IVAG TK^- HSV-2 immunization has been reported to induce protective immune responses that are primarily mediated by

interferon-γ (**IFNγ**)-secreting $CD4^+$ T cells (Milligan *et al.*, 1998; Harandi *et al.*, 2001b; Harandi *et al.*, 2001a) . Therefore, we examined the presence of IFNγ-producing CD3+ T cells in the vaginal mucosa of WT and LTα-/- mice. CD3+CD4+ and CD3+CD8+ T cells were analyzed for the expression of intracellular IFNγ directly following fresh isolation of these cells from the vaginal tract and following *in vitro* restimulation. CD3+CD4+ and CD3+CD8+ T cells isolated 22 days after immunization from the vaginal mucosa of WT mice showed higher percentages of IFNγ production compared to both groups of LTα-/- mice (Figure 7A,B, left). However, these differences in cell percentages did not translate into differences in cell numbers, as all three groups of mice had similar numbers of IFNγ+ T cells (both CD4+ and CD8+; Figure 7B, right). Following *in vitro* restimulation, WT mice had higher percentages of IFNγ+ CD4+ and CD8+ T cells compared with LTα-/- mice (Figure 7C). Surprisingly, there was no increase in IFNγ+ T cells following *in vitro* restimulation compared with cells that were analyzed fresh out of the vaginal tract. In contrast to UV-HSV-2, stimulation with CD3/CD28 dynabeads significantly increased the frequency of IFNγ+ T cells (Figure 7D). Thus, the decreased numbers of IFNγ+ T cells in LTα-/- mice were not due to poor T cell viability.

At three days after challenge, flow analysis of freshly isolated vaginal tract immune cells showed that WT mice had higher percentages and total cell counts for both CD4+IFNγ+ and CD8+IFNγ+ cells compared to LTα-/- mice (Figure 8A,B). Following *in vitro* restimulation with UV-HSV-2, the percentage of cells (both CD4+ and CD8+)

that were positive for intracellular IFNγ increased by approximately twofold in all three groups of mice (Figure 8C,D, left). This percentage increased further when T cells were nonspecifically stimulated in the presence of CD3/CD28 dynabeads (Figure 8D left). As expected, *in vitro* restimulation of vaginal cells with UV-HSV-2 resulted in higher percentages (1.7- to 2.5-fold) and total cell numbers (2- to 4-fold) of IFNγ+ T cells in both WT and LTα-/- mice compared with LTα-/- SPL mice (Figure 8D). These results suggest that although LTα-/- mice have similar percentages and total cell numbers of CD3+CD4+ and CD3+CD8+ cells (Figure 4 and Table 2), the number of HSV-2-specific IFNγ-producing T cells is decreased in LTα-/- compared with WT mice.

Further analysis showed that both after immunization (70-90%) and after challenge (44-85%), a majority of IFNγ+ CD4+ T cells were CD44+CD62L- in concurrence with the previously described effector memory phenotype of T cells in this model (Supplementary Figure 3) (Tang & Rosenthal, 2010). Similar results were seen for IFNγ+ CD8+ T cells (not shown).

DISCUSSION

Previously, we have shown that WT OVX mice are completely protected against IVAG HSV-2 challenge following IVAG immunization with an attenuated strain of HSV-2 (Gillgrass *et al.*, 2005b) (Gillgrass *et al.*, 2010). In the present study, we determined whether memory effector responses against genital herpes infection were induced in the absence of SLOs and could effectively protect against a viral challenge in the genital mucosa. We used LTα-/- mice, which lack all LNs, and surgically removed the spleens to ensure the absence of SLOs. We found that although the absence of SLOs delayed the induction and decreased the magnitude of immune responses following mucosal IVAG immunization, effector memory T cell responses and IgG antibody responses were present in the vaginal mucosa of LTα-/- mice that resulted in sterile protection following HSV-2 challenge in the genital tract. Although in our studies we used same parent strain of virus for immunization and challenge, others have shown that similar protection is seen against other strains of HSV-2 (Johnson *et al.*, 2010) (Harandi *et al.*, 2001a).

To examine the quality of immune protection generated in LTα-/- mice following mucosal IVAG immunization, gross genital pathology and viral shedding were examined. In the absence of SLOs, viral clearance from genital tract was delayed, resulting in longer period of viral shedding and higher genital pathology, indicating that immune responses were generated more slowly in LTα-/- mice following immunization with TK^- HSV-2. However, as all three groups of mice exhibited no vaginal pathology or viral shedding

following HSV-2 challenge, this indicated that the immune responses generated were effective and sufficient to provide complete protection against a high-dose viral challenge. To characterize the immune responses that were responsible for providing this sterile protection observed after challenge, HSV-2-specific IgG and IgA antibody levels were measured. In WT mice, serum and vaginal IgG levels were significantly increased after challenge, compared with after immunization. However, in both groups of LTα-/- mice, the booster response in serum and vaginal IgG was not significant and the overall levels of HSV-2-specific IgG after challenge were significantly lower as compared with WT mice. IgA responses were almost completely absent in LTα-/- mice. This supports previous studies that concluded that HSV-specific antibody responses are not the most critical component of the protective immune responses in this model and when present, levels of HSV-2-specific IgG rather than IgA levels, correlate with viral shedding and sterile immunity (Parr & Parr, 1997a; Gillgrass *et al.*, 2010).

The findings of lower IgG antibody responses in LTα-/- mice compared with WT mice and the absence of IgA responses could be explained by the observations that isotype switching, and humoral immune responses in general, are impaired in LTα-/- mice (Banks *et al.*, 1995; Fu *et al.*, 1997). Studies have shown that the disruption of the splenic microarchitecture seen in LTα-/- mice results in impaired IgG responses, rather than an intrinsic defect in LTα-/- B cells and T cells (Fu *et al.*, 1997). This was further confirmed in our current studies where total IgA levels in serum and vaginal secretions of unimmunized LTα-/- mice were significantly decreased (Supplementary Figure 4).

It has been well established that IVAG immunization with TK^- HSV-2 induces protective immunity that is primarily mediated by IFNγ-secreting CD4+ T cells (Milligan *et al.*, 1998; Harandi *et al.*, 2001b) (Harandi *et al.*, 2001a; Parr & Parr, 2003). The remarkable increase in frequency of CD4+ T cells following HSV-2 challenge in LTα-/- mice, which was comparable to the responses seen in WT mice, suggests that even in the absence of SLOs, mice were still able to mount a protective, effector memory T cell response following IVAG immunization. To determine whether this T cell response was specific for HSV-2, the number of HSV-2-specific, IFNγ-producing CD3+ T cells were measured in the vaginal mucosa after immunization and after challenge. The results showed that LTα-/- mice had a lower frequency of HSV-2-specific, IFNγ-producing CD4+ T cells and CD8+ T cells present in the vaginal mucosa compared with WT mice. Thus, the combined lack of TNFR/LTβR signaling and SLOs appear to decrease the magnitude of HSV-2-specific IFNγ+ T cell responses compared with that seen in WT mice. Overall, the delayed generation and decreased magnitude of immune responses following immunization did not affect the quality of protection in LTα-/- mice, as there was no viral shedding or pathology detected following challenge. One possible explanation for complete protection despite decrease in overall immune responses could be that the extent of cellular and antibody responses generated in WT mice are quite robust and possibly exceed the magnitude of responses absolutely required for protection. Our results are in agreement with previous studies that have shown that although LTα-/- mice have impaired cytotoxic and cytokine-mediated effector functions, they can still

mount protective immune responses to a systemic infection of HSV (Lee *et al.*, 2000; Lund *et al.*, 2002).

Previous studies have shown that the lung mucosa of LTα-/- mice makes effective primary and memory T and B cell responses to influenza (Moyron-Quiroz *et al.*, 2004; Moyron-Quiroz *et al.*, 2006). In addition, whereas Soderberg, *et al.* found that protective T cell responses to HSV-2 infection can be generated in the sacral LNs in LTβ-deficient mice, the present study indicates that the genital mucosa itself, like the lungs, can mount protective antiviral memory responses in complete absence of SLOs. Interestingly, unlike the LTβ-deficient mice where IgG and IgA antibody levels were maintained, in the present study, the absence of sacral LN in LTα-/- mice appeared to severely compromise HSV-2 specific antibody responses (Soderberg *et al.*, 2004). Collectively, these studies point to the importance and autonomy of local mucosal responses in protecting against mucosal infections.

Although this study provides promising evidence regarding the independence of mucosal immune responses in the genital mucosa, the results raise important considerations that need to be addressed. Further studies are needed to determine whether, as has been previously shown in WT mice, in the absence of SLOs (i) immunization with subunit vaccines, with or without mucosal adjuvants, rather than with live attenuated virus, can replicate the protection seen in this study and (ii) immunization via nasal mucosa can provide protection in the genital mucosa (Tengvall *et al.*, 2005) (Gallichan *et al.*, 2001a; Olson *et al.*, 2009) (Bhavanam *et al.*, 2008). This information

will allow a more realistic assessment of the implication of this study for mucosal vaccine strategies.

Overall, the results from this study provide further support for mucosal vaccines for sexually transmitted viral infections. Conventional vaccine strategies against HSV-2 and HIV-1 have been unsuccessful for over two decades, including the latest efficacy trial of HSV-2 gD-2 vaccine (Belshe *et al.*, 2012). As an important goal of vaccination against sexually transmitted infections is to induce and maintain immunity in the female genital tract, the finding that protective immune responses can be generated in the absence of SLOs indicates that immunization that generates effector memory responses at genital mucosa can provide adequate protection against subsequent viral exposures. The results also imply that once memory effector responses are generated, they can protect against genital tract infections independently and without involvement of other secondary lymphoid structures.

METHODS

Animals and surgeries. Female C57BL/6 and LTα-/- mice, 6-8 weeks old, were purchased from Jackson Laboratory (Maine, USA). All mice were housed and maintained under standard temperature controlled conditions in special pathogen free rooms that followed a 12-h day and 12-h night schedule. All animal protocols were approved by McMaster Animal Research Ethics Board. The ovaries were removed by making two bilateral incisions through the peritoneal wall, exteriorizing the fat, ligating the blood vessels and removing the ovaries. The spleen was exteriorized through the incision on left side and removed by ligating the splenic vasculature. The incisions were closed by surgical clips and animals allowed to recover for 10 days.

HSV-2 inoculation and challenge. Groups of mice were immunized 10-14 days following OVX or OVX+SPL. Mice were anesthetized using injectable anesthetic (150 mg of ketamine/kg-10 mg of xylazine/ml) given intraperitoneally, placed on their backs, and immunized intravaginally with 10^5 PFU (10 µl) of TK^- HSV-2, strain 333. At 4-6 weeks following immunization, mice were challenged intravaginally with 10^5 PFU (10 µl) of wild-type HSV-2, strain 333.

Collection of vaginal washes and serum. Serum was collected on day 24 following IVAG immunization and day 3 following IVAG HSV-2 infection. Blood samples were obtained by retro-orbital bleeding, clotted at room temperature, and the serum was collected, re-centrifuged, and stored at -70 °C. Vaginal washes were collected for 5 consecutive days following both IVAG immunization and challenge, by pipetting twice

with 30 µl of phosphate-buffered saline in and out of the vagina 6-8 times to give a total of 60 µl per mouse and stored at -70 °C.

Genital pathology and viral titration. Genital pathology following TK^- HSV-2 immunization and HSV-2 strain 333 challenge was monitored daily and was scored on a five-point scale, as described before (Gillgrass *et al.*, 2005a). Viral titers in vaginal washes were determined by plaque assay, as described before (Gillgrass *et al.*, 2005a). Briefly, Vero cells were grown to confluence in 12-well plates: vaginal washes were diluted (10^{-1} to 10^{-7}) and added to Vero cell monolayers. Infected monolayers were incubated at 37 °C for 2 h and then overlaid with α-modified Eagle's medium supplemented with 0.05% human immune serum globulin (Canadian Blood Services). At 48 h after infection, monolayers fixed and stained with crystal violet, and viral plaques were counted. The number of PFU ml^{-1} was calculated by taking into account the dilution factors.

Real-time PCR for HSV-2 DNA. Viral DNA was isolated from vaginal wash samples using QIAamp MinElute Virus Spin Kit (QIAGEN Sciences, MD) according to the manufacturer's guidelines. Each viral DNA sample (5 µl) was added to 12.5 µl RT^2 Real-Time SYBR Green/ROX PCR Master Mix (SA Biosciences), 0.6 µM each forward and reverse primers (HSV DNA-pol; F: 5'- ATG GTG AAC ATC GAC ATG TAC GG – 3', R: 5' - CCT CCT TGT CGA GGC CCC GAA AC – 3'; Integrated DNA Technologies, IA) and 7.2 µl of water. Samples underwent 40 cycles of PCR, and the fluorescent dyes in each reaction were read automatically during PCR cycling in a

7900HT Fast Real-Time PCR System (Applied Biosystems). To quantify the amount of viral DNA in each sample, a standard curve was generated using known quantities of HSV-2 Quantitated Viral DNA (Advanced Biotechnologies, MD).

Enzyme-linked immunosorbent assay for anti-HSV-2 gB IgG and IgA. HSV-2 gB-specific antibody titers were determined by an enzyme-linked immunosorbent assay, as described before (Bhavanam *et al.*, 2008). Briefly, 96-well flat-bottom Maxisorp plates (Invitrogen, Burlington, ON, Canada) were coated with 2.5 μg ml^{-1} of recombinant gB protein (Chiron, Emeryville, CA) or purified antibodies (goat anti-mouse IgG or goat anti-mouse IgA, both purchased from Southern Biotech, Birmingham, AL) overnight at 4 °C. Twofold serial diluted samples were added and incubated overnight at 4 °C. Biotin-labeled goat anti-mouse IgG or goat anti-mouse IgA (Southern Biotech) was used at a concentration of 1 μg ml^{-1} and plates were developed with extravidin-peroxidase and tetramethyl benzidine (Sigma, St. Louis, MO) and read at an optical density of 450 nm. End point titers were determined and expressed as mean titers. The mean background optical density value of nonimmune mice was taken two times as the cutoff for determining positive values.

Vaginal cell preparation and culture. The vaginal tissue was minced and digested 2X in 15 ml of RPMI containing 150 mg ml^{-1} collagenase A (Roche, Manheim, Germany) for 1 h each at 37 ^{0}C and supernatants were collected. The remaining tissue and supernatants was passed through a 40 μm filter (Small Parts, Miami Lakes, FL) to collect single cells. Cells were centrifuged for 10 min at 1,200 r.p.m. and resuspended in 0.2%

bovine serum albumin for flow cytometric analysis or 10% fetal calf serum/RPMI for cell culture. Cells were tested for HSV-2-specific IFNγ production by *in vitro* stimulation with 10^4 PFU ultraviolet (UV)-inactivated HSV-2. As negative and positive controls, cells were either left unstimulated or stimulated with $2x10^5$ CD3/CD28 dynabeads (Invitrogen) + 50 U ml^{-1} interleukin-2 (Peprotech, Rocky Hill, NJ), respectively. Cultures were incubated in the presence of GolgiPlug (BD Biosciences, Mississauga, Canada) for the last 4 h of 24 h culture.

Flow cytometry. Cell preparations were resuspended to a density of 10^6 cells per ml and stained for cell surface markers using the following antibodies for 30 min at 4^0C: FITC anti-mouse MHCII, eFluor-450 anti-mouse CD11b, PE anti-mouse CD11c, Pacific blue anti-mouse CD3, PE anti-mouse CD4, PE-Cy7 anti-mouse CD8 (eBioscience, San Diego, CA), and PE-Cy7 anti-mouse CD19 (BD Biosciences). For intracellular cytokine staining, cells were resuspended in Fixation/Permeabilization solution (BD Biosciences) and washed and resuspended in 1x BD Perm/Wash buffer and stained with either FITC anti-mouse IFNγ (Biolegend) or FITC IgG1 isotype control (eBioscience). Stained cells were analyzed by flow cytometric analysis using a BD LSRII Flow Cytometer and analyzed using FlowJo Software (TreeStar Inc, OR).

Statistical Analysis. Statistical analysis was performed using Graph Pad Prism 3.02 (GraphPad Software, San Diego, CA). Viral shedding and antibody titers were analyzed by one-way analysis of variance followed by Tukey's post-test. Statistical significance

was defined as $p<0.05$. For antibody titers, Grubbs' test was performed to remove any outliers.

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DISCLOSURE

The authors have declared no conflict of interest.

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FIGURES

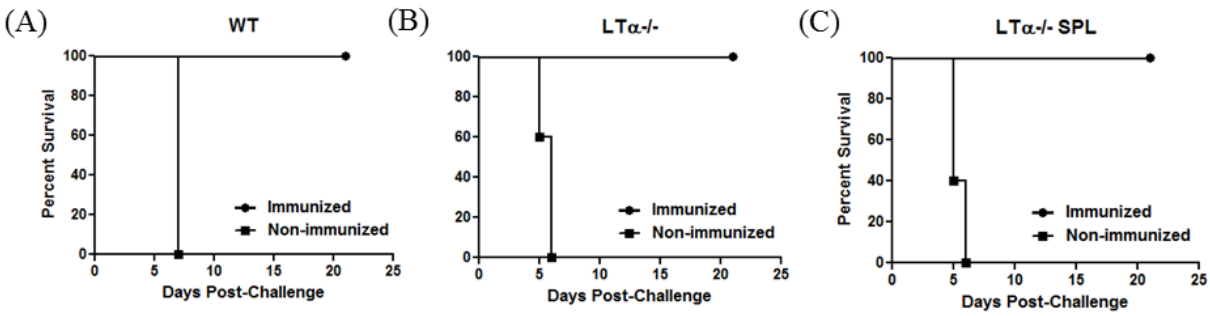


Figure 1: LTα-/- mice immunized IVAG with TK$^-$ HSV-2 are completely protected against genital HSV-2 challenge. (A) WT OVX, (B) LTα-/- OVX and (C) LTα-/- OVX+SPL mice were immunized IVAG with 10^5 PFU of TK$^-$ HSV-2, or left unimmunized as a control. At 4-6 weeks following immunization mice were challenged IVAG with HSV-2, strain 333 and monitored daily for survival. Mice in all three immunized groups showed 100% survival. The results shown are pooled from three separate experiments. n=21 for WT OVX and LTα-/- OVX; n=18 for LTα-/- OVX+SPL. For nonimmunized (NI) controls, n=5 for all three groups of mice. HSV-2, Herpes simplex virus type 2; IVAG, intravaginal; LTα, lymphotoxin-α; OVX, ovariectomized; SPL, splenectomized; TK$^-$, thymidine kinase-negative; WT, wild type.

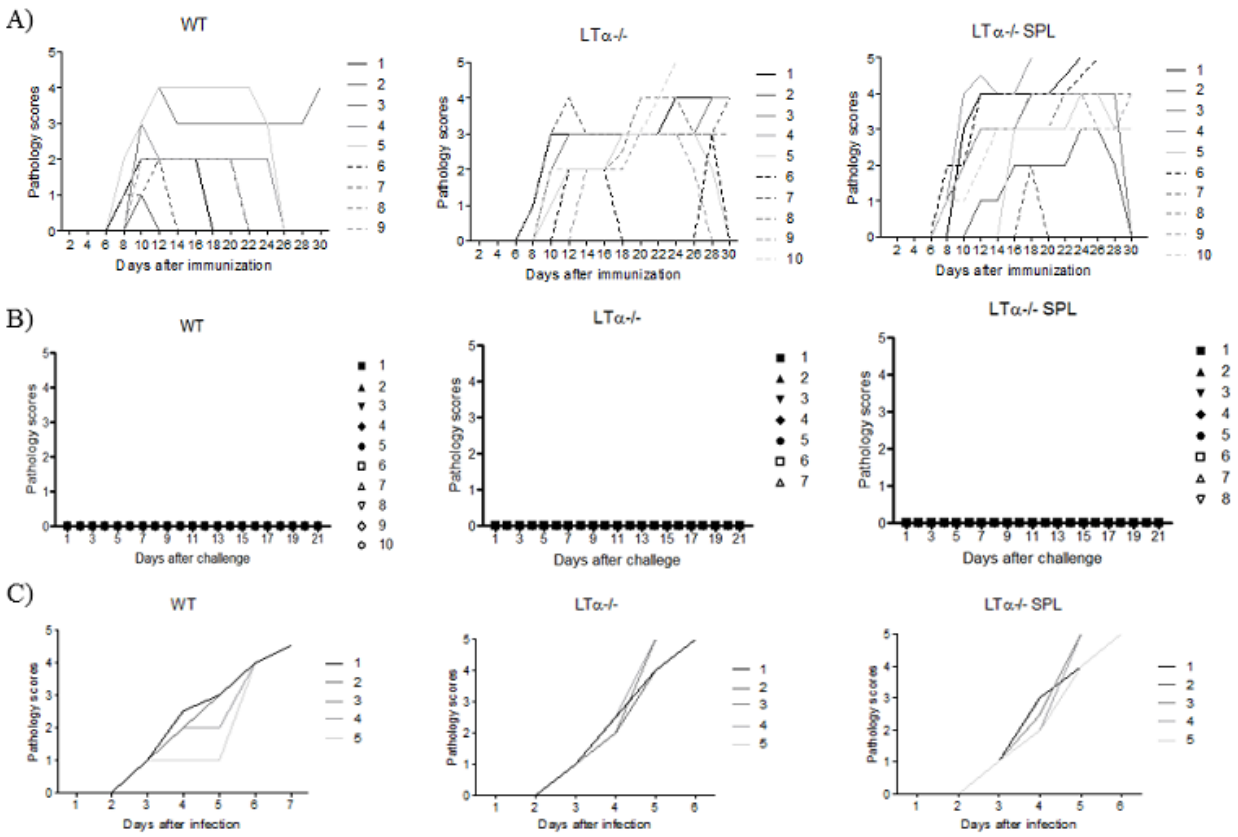


Figure 2: LTα-/- mice have higher pathology after immunization with TK$^-$ HSV-2 compared with WT mice, but no differences in pathology were seen after challenge. WT OVX, LTα-/- OVX and LTα-/- OVX+SPL mice were immunized IVAG with 10^5 PFU of TK$^-$ HSV-2, or left unimmunized as a control. At 4-6 weeks following immunization, mice were challenged IVAG with HSV-2, strain 333. Vaginal pathology was monitored daily (A) after immunization and (B) after challenge and (C) after infection in nonimmunized mice. The results shown are representative of four separate experiments with similar results. HSV-2, Herpes simplex virus type 2; IVAG, intravaginal; LTα,

lymphotoxin-α; OVX, ovariectomized; SPL, splenectomized; TK^-, thymidine kinase-negative; WT, wild type.

Table 1: Pathology scores of WT OVX, LTα-/- OVX, and LTα-/- OVX+SPL mice following IVAG TK$^-$ HSV-2 immunization.

Treatment group (total number of mice)	Pathology score	Number of mice	Average number of days	Cumulative pathology	Average pathology per mouse
WT OVX (9)	4	2	8	64	29
	3	3	9	81	
	2	6	9	108	
	1	2	3	6	
	0	9	18	0	
LT OVX (10)	4	5	5	100	40
	3	7	10	210	
	2	6	7	84	
	1	4	1	4	
	0	10	15	0	
LT OVX + SPL (10)	4	7	14	392	
	3	8	7	168	62
	2	7	3	42	
	1	8	2	16	
	0	10	10	0	

Abbreviations: HSV-2, Herpes simplex virus type 2; IVAG, intravaginal; LTα, lymphotoxin-α; OVX, ovariectomized; SPL, splenectomized; TK$^-$, thymidine kinase-negative; WT, wild type.
Cumulative pathology scores were determined by tabulating the number of mice with a given pathology score and the average number of days that score was observed for each group, taking into consideration that each mouse had varying levels of pathology throughout the experiment. The average pathology score per mouse was then calculated for each group.

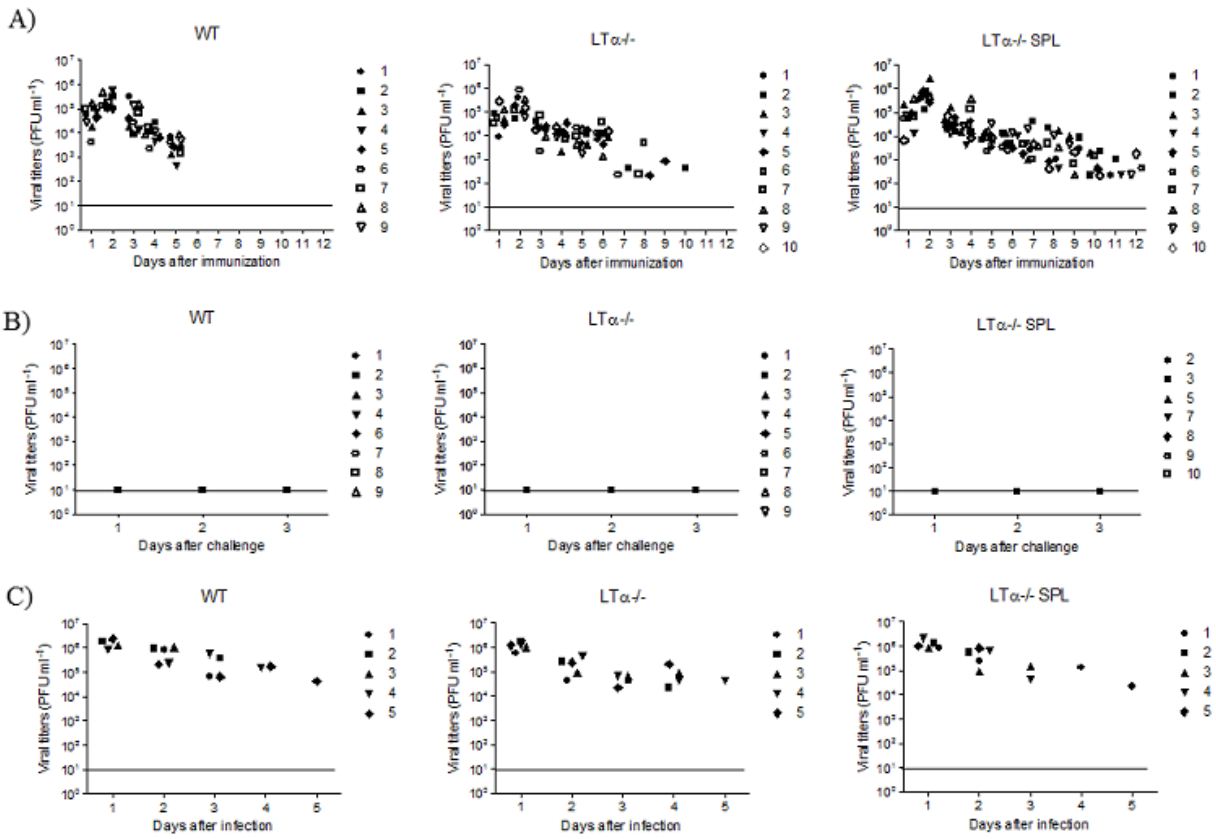


Figure 3: Viral titers of WT and LTα-/- mice following IVAG TK$^-$ HSV-2 immunization and HSV-2, strain 333 challenge. Vaginal washes were collected daily (A) after immunization and (B) after challenge, and (C) in nonimmunized control mice after infection, from all three groups and viral plaque assays were done. Plaques were counted and viral titers were expressed as PFU ml^{-1}. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay. Results are representative of four separate experiments with comparable results. HSV-2, Herpes simplex virus type 2; IVAG, intravaginal; LTα, lymphotoxin-α; SPL, splenectomized; TK$^-$, thymidine kinase-negative; WT, wild type.

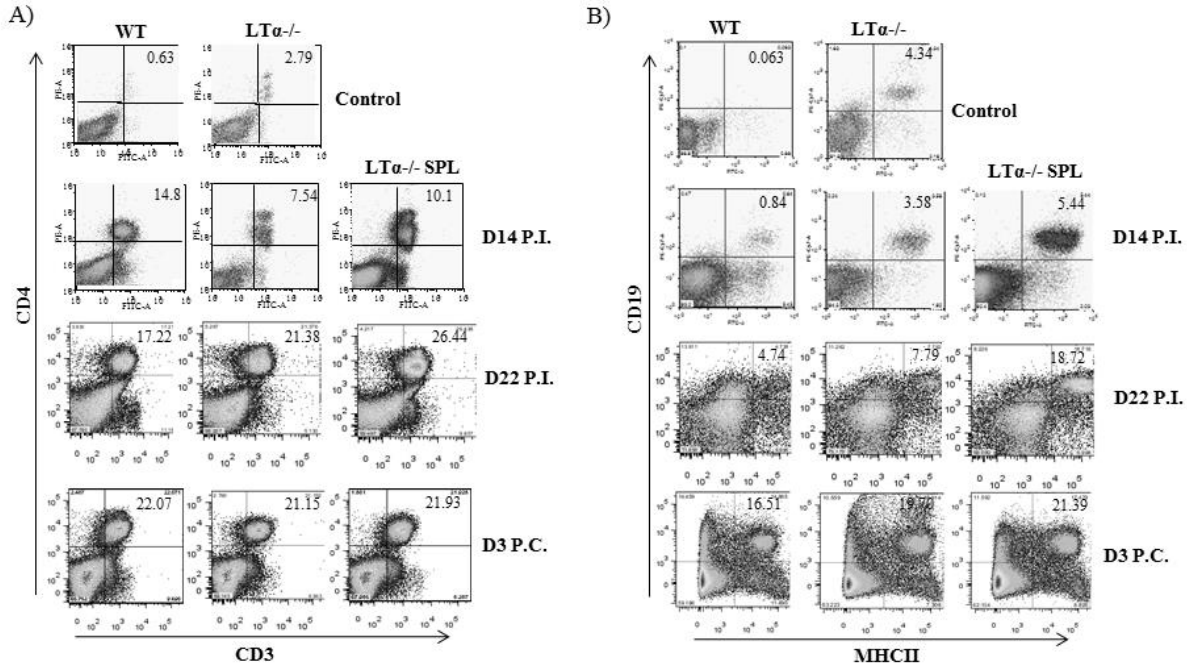


Figure 4: Flow cytometric analysis of immune cell populations in the vaginal mucosa of LTα-/- mice after immunization and after challenge. WT OVX, LTα-/- OVX and LTα-/- OVX+SPL mice were immunized IVAG with 10^5 PFU of TK^- HSV-2. At 6 weeks after immunization, mice were challenged IVAG with HSV-2, strain 333. Vaginal tracts were excised on day 14 and 22 after immunization and day 3 after challenge and single cell preparations were made as described in Methods. Vaginal cell preparations were stained with a panel of antibodies and analyzed for surface expression of markers for B cells, T cells and APCs by flow cytometry. Viable cells were gated on lymphocytes. B cells were defined as percentage of cells expressing MHCII and CD19. T cell subsets were determined by expression of CD3, CD4 and CD8. Dendritic cells were defined as percentage of cells expressing MHCII and CD11c and macrophages as percentage of

cells expressing MHCII and CD11b. Dot blots are shown for T cells (A) and B cells (B). Dot blots are shown for (A) T cells and (B) B cells. The WT control and day 3 postchallenge data are representative of two separate experiments, whereas n=1 for day 14 and 22 postimmunization data, as a result statistical analysis was not performed. The percentage of various cell populations, as well as the averages and s.d. for experiments with n=3, in control (unimmunized mice), days 14 and 22 (D14 and D22) after immunization, and day 3 (D3) after challenge is shown in Table 2. APC, antigen-presenting cell; HSV-2, Herpes simplex virus type 2; IVAG, intravaginal; LTα, lymphotoxin-α; MHCII, major histocompatibility complex class II; OVX, ovariectomized; PC, postchallenge; PI, postimmunization; SPL, splenectomized; TK^-, thymidine kinase-negative; WT, wild type.

Table 2: Immune cell populations in the vaginal tract of WT OVX, LTα-/- OVX, and LTα-/- OVX+SPL mice.

Cell type	Markers	Treatment group	Cell percentages		
			WT OVX	LTα−/− OVX	LTα−/− OVX+SPL
T cells	CD3+CD4+	Control	0.50±0.19	2.79	NA
		After immunization (D14)	14.80	7.54	10.70
		After immunization (D22)	17.22	21.38	26.44
		After challenge (D3)	19.07±2.69	21.43±4.44	23.29±1.51
	CD3+CD8+	Control	0.33±0.01	1.10	NA
		After immunization (D14)	2.42	1.30	2.31
		After immunization (D22)	3.35	4.17	6.27
		After challenge (D3)	6.14±0.41	6.45±1.69	9.51±0.03
B cells	MHCII+CD19+	Control	0.45±0.54	4.34	NA
		After immunization (D14)	0.84	3.58	5.44
		After immunization (D22)	4.74	7.79	18.72
		After challenge (D3)	16.51±1.80	19.70±2.29	21.39±5.52
Dendritic cells	MHCII+CD11c+	Control	1.17	1.02	NA
		After immunization (D14)	2.23	0.65	0.83
		After immunization (D22)	7.32	4.10	4.85
		After challenge (D3)	21.14±3.08	15.40±1.12	14.68±6.22
Macrophages	MHCII+CD11b+	Control	1.37	1.46	NA
		After immunization (D14)	2.61	1.27	2.74
		After immunization (D22)	6.28	4.12	4.90
		After challenge (D3)	12.56±1.03	8.50±0.36	8.05±1.20

Abbreviations: LTα, lymphotoxin-α; MHCII, major histocompatibility complex class II; NA, not applicable; OVX, ovariectomized; SPL, splenectomized; WT, wild type. N=3 for WT control and day 3 (D3) postchallenge experiments—cell percentages are expressed as average±s.d. As the results for the three independent experiments were very similar, we assessed different time points after immunization as single experiments.

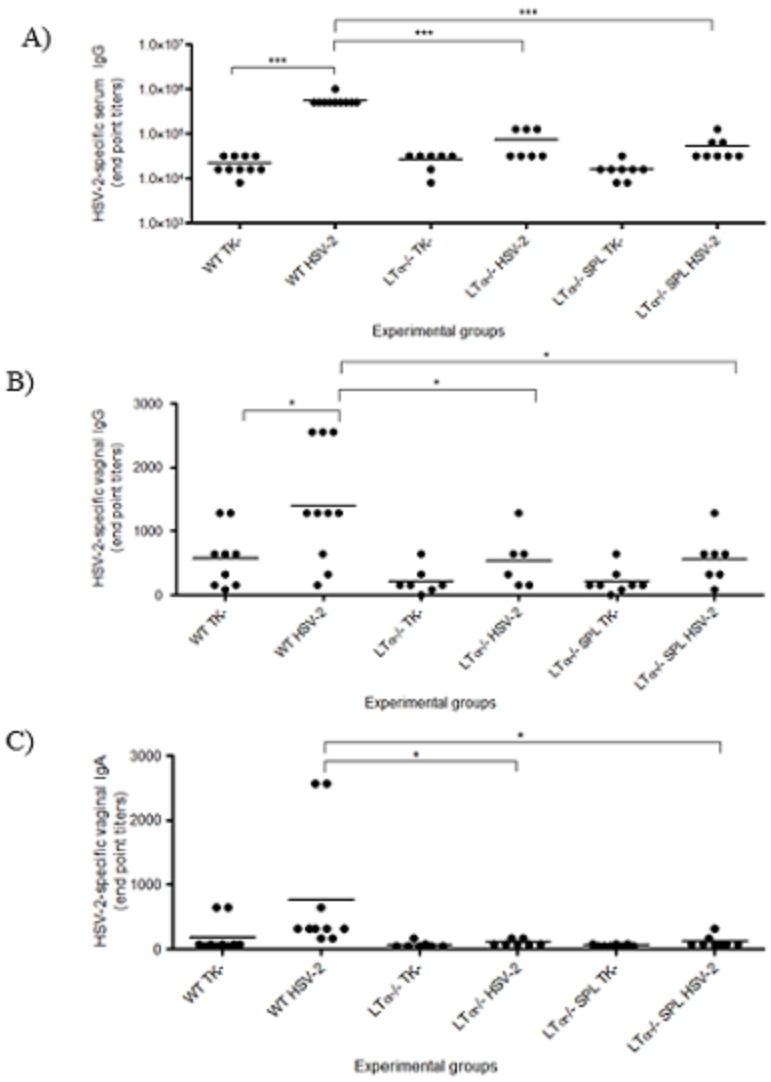


Figure 5: HSV-2-specific antibody titers in vaginal washes and serum samples of WT OVX, LTα -/- OVX, and LTα -/- OVX+SPL mice both after immunization and after challenge. Vaginal washes were collected for 5 consecutive days after TK^- HSV-2 immunization and pooled for each animal; serum samples were collected on day 24 after immunization. Vaginal washes were collected days 1-5 after challenge and pooled for each animal; serum samples were collected on day 3 after challenge. HSV-2-specific IgG

antibodies were measured in (A) serum and (B) vaginal washes and (C) IgA antibodies were measured in vaginal washes by enzyme-linked immunosorbent assay (ELISA), and endpoint titers were determined. Each dot indicates an individual mouse. Bars show mean values for the group. Data is representative of two experiments with comparable results. TK- : after immunization; HSV-2: after challenge. Statistical differences were determined by one-way analysis of variance followed by Tukey's post-test. *, $p<0.05$; ***, $p<0.001$ compared with after immunization. HSV-2, Herpes simplex virus type 2; IVAG, intravaginal; LTα, lymphotoxin-α; OVX, ovariectomized; SPL, splenectomized; TK^-, thymidine kinase-negative; WT, wild type.

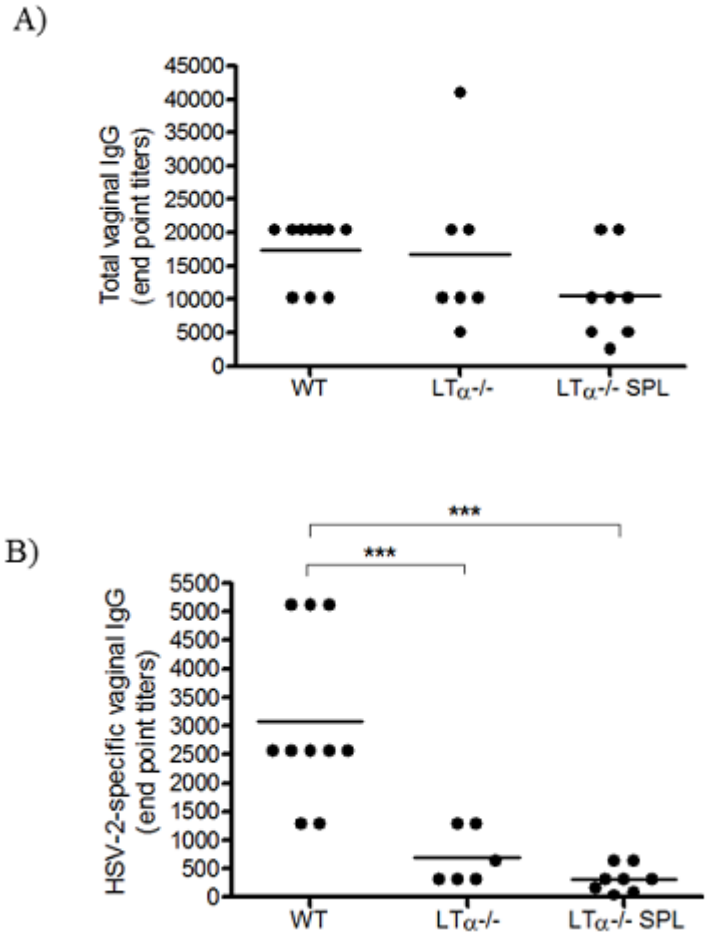


Figure 6: Total and HSV-2-specific IgG in vaginal washes at day 5 after challenge. WT OVX, LTα-/- OVX and LTα-/- OVX+SPL mice were immunized IVAG with 10^5 PFU of TK^- HSV-2. At 4 weeks after immunization, mice were challenged IVAG with HSV-2, strain 333. Vaginal washes were collected on days 1-5 after challenge in all three groups of mice. (A) Total IgG and (B) HSV-2-specific IgG antibodies in vaginal washes were measured by enzyme-linked immunosorbent assay (ELISA) and end point titers were determined. Each dot represents an individual animal. Bars show mean values for the group. Data are representative of two experiments with similar results. Statistical

differences were determined by one-way analysis of variance followed by Tukey's post-test. * $p<0.05$, ***$p<0.001$ compared with WT. HSV-2, Herpes simplex virus type 2; IVAG, intravaginal; LTα, lymphotoxin-α; OVX, ovariectomized; SPL, splenectomized; TK^-, thymidine kinase-negative; WT, wild type.

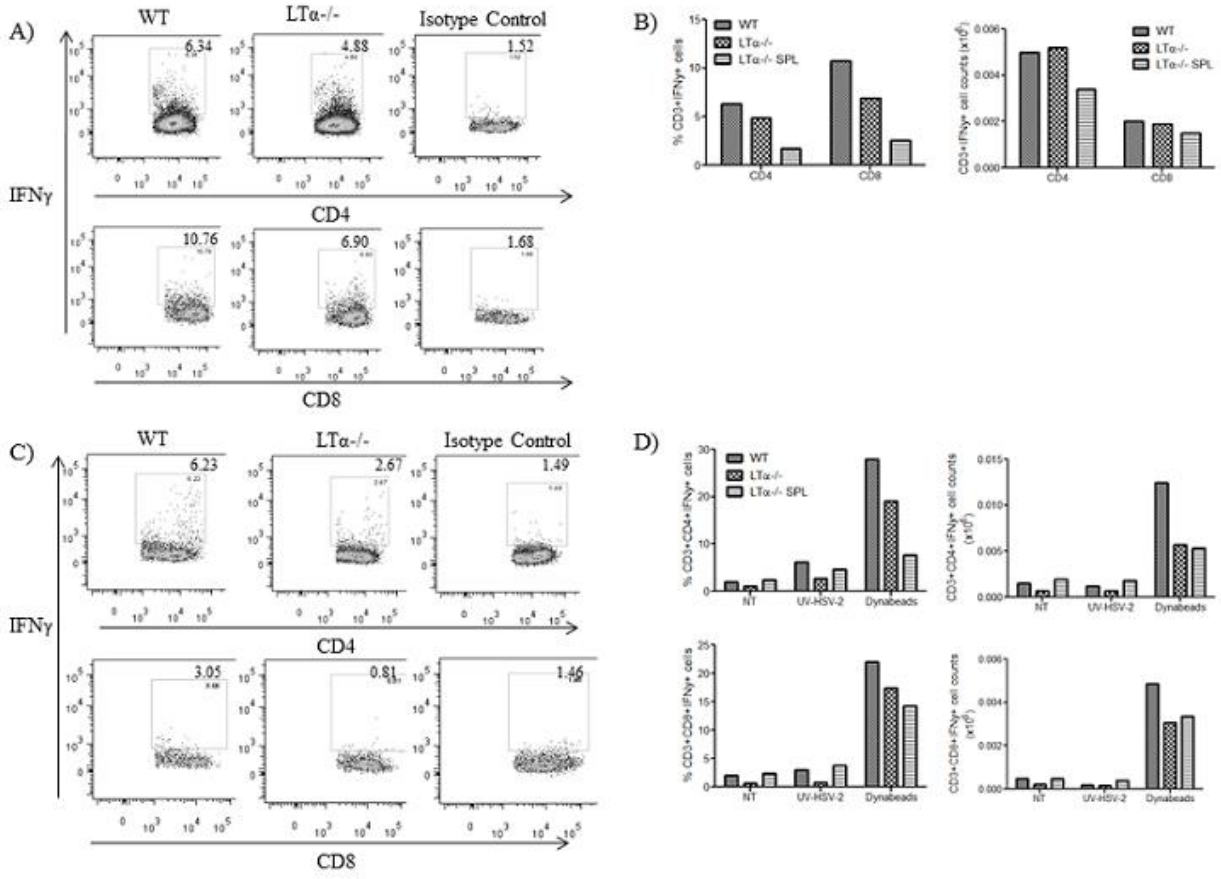


Figure 7: Flow cytometric analysis of IFNγ+ cells in vaginal tract day 22 after immunization. WT OVX, LTα-/- OVX and LTα-/- OVX+SPL mice were immunized IVAG with 10^5 PFU of TK^- HSV-2. Vaginal tracts were excised on day 22 after immunization and lymphocytes were isolated by enzymatic digestion. (A, B) Cells were stained for flow cytometric analysis the same day after isolation using the T cell markers, CD3, CD4, CD8 and intracellular IFNγ. (C, D) Lymphocytes ($5x10^5$) were plated in 96-well plates and left NT or stimulated with UV-inactivated HSV-2 (UV-HSV-2; 10^4 PFU) or 5 μl of CD3/CD28 dynabeads + IL-2 (50 U ml^{-1}) as a positive control. Cells were incubated at 37 ^{0}C overnight and analyzed by flow cytometry the next day for the

presence of IFNγ+ T cells. Data are representative of 2 separate experiments with similar results. HSV-2, Herpes simplex virus type 2; IFNγ, interferon-γ; IL-2, interleukin-2 IVAG, intravaginal; LTα, lymphotoxin-α; NT, unstimulated; OVX, ovariectomized; SPL, splenectomized; TK^-, thymidine kinase-negative; UV, ultraviolet; WT, wild type.

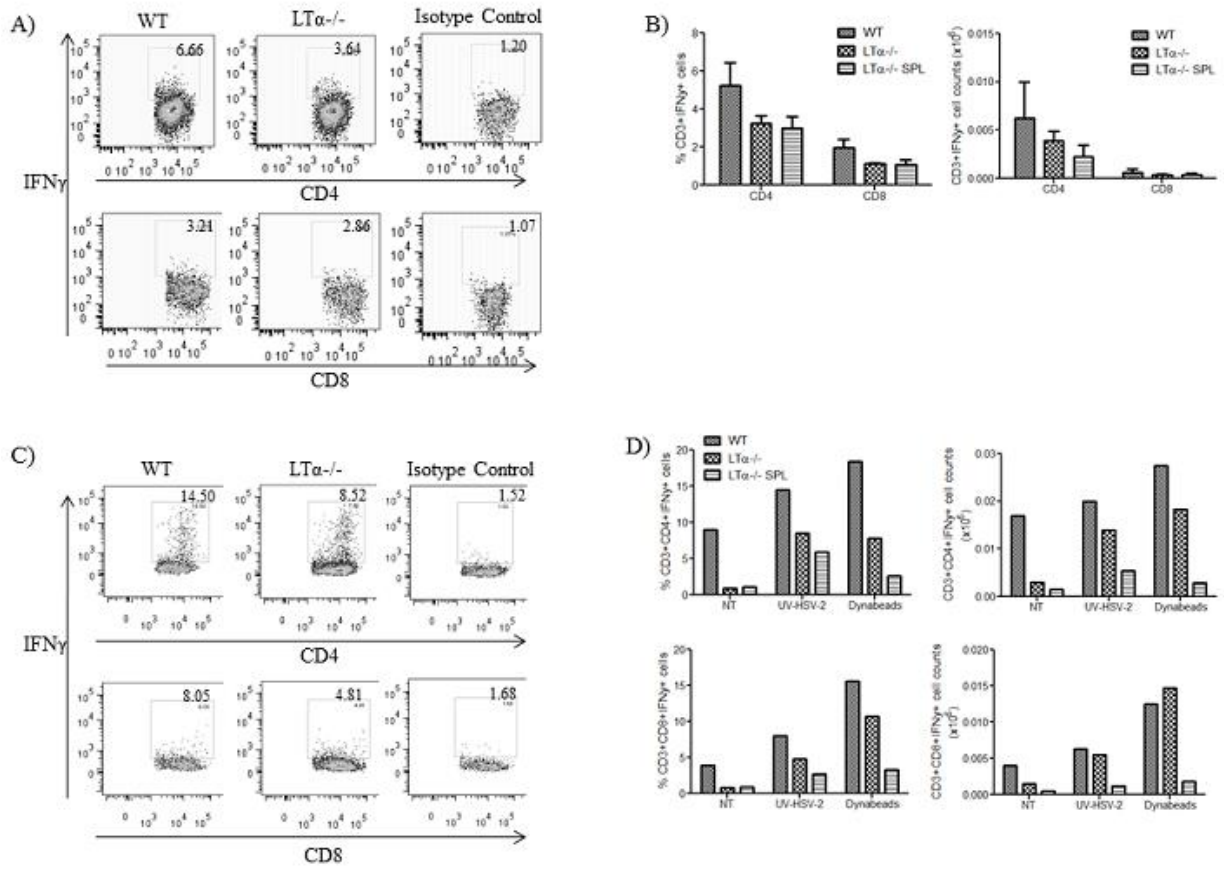
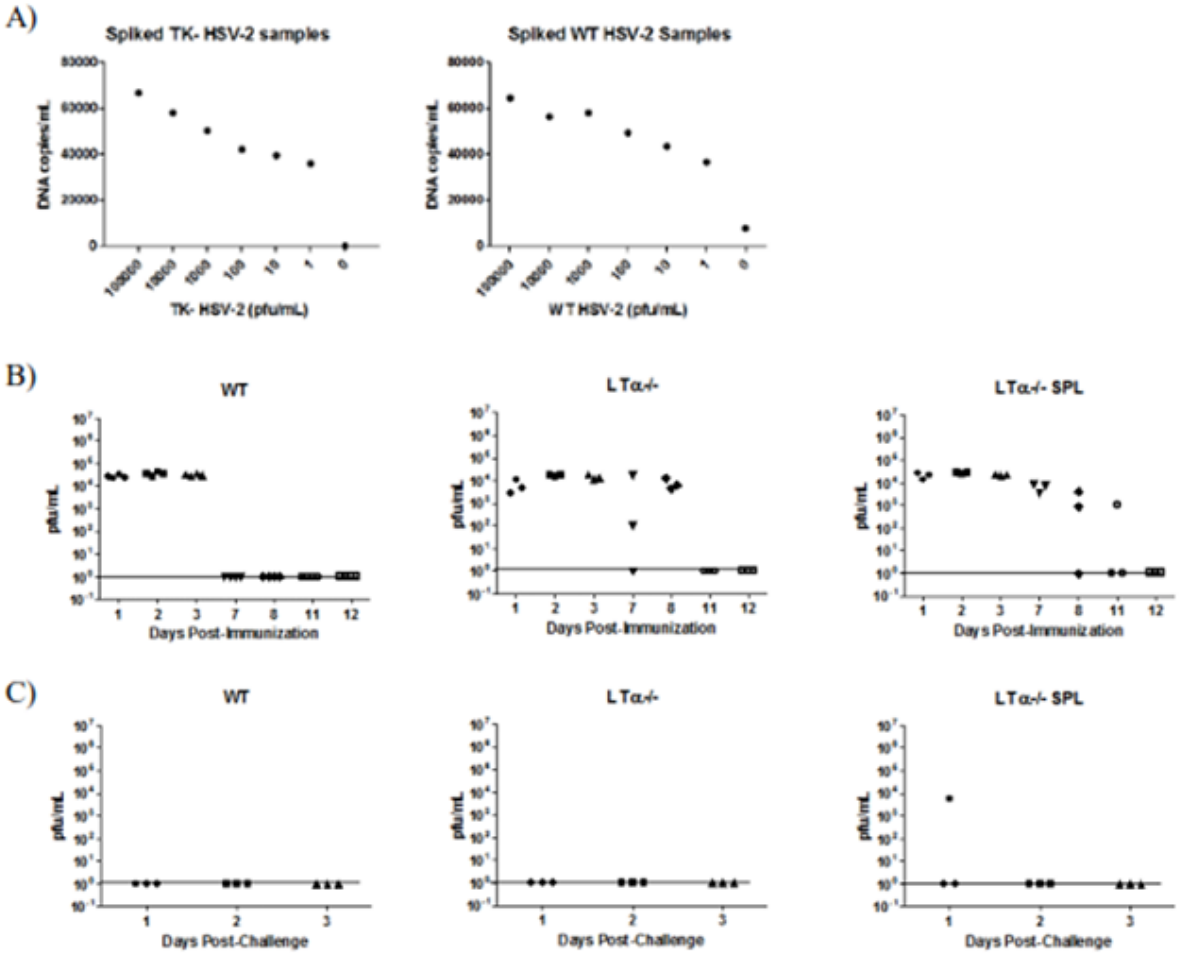
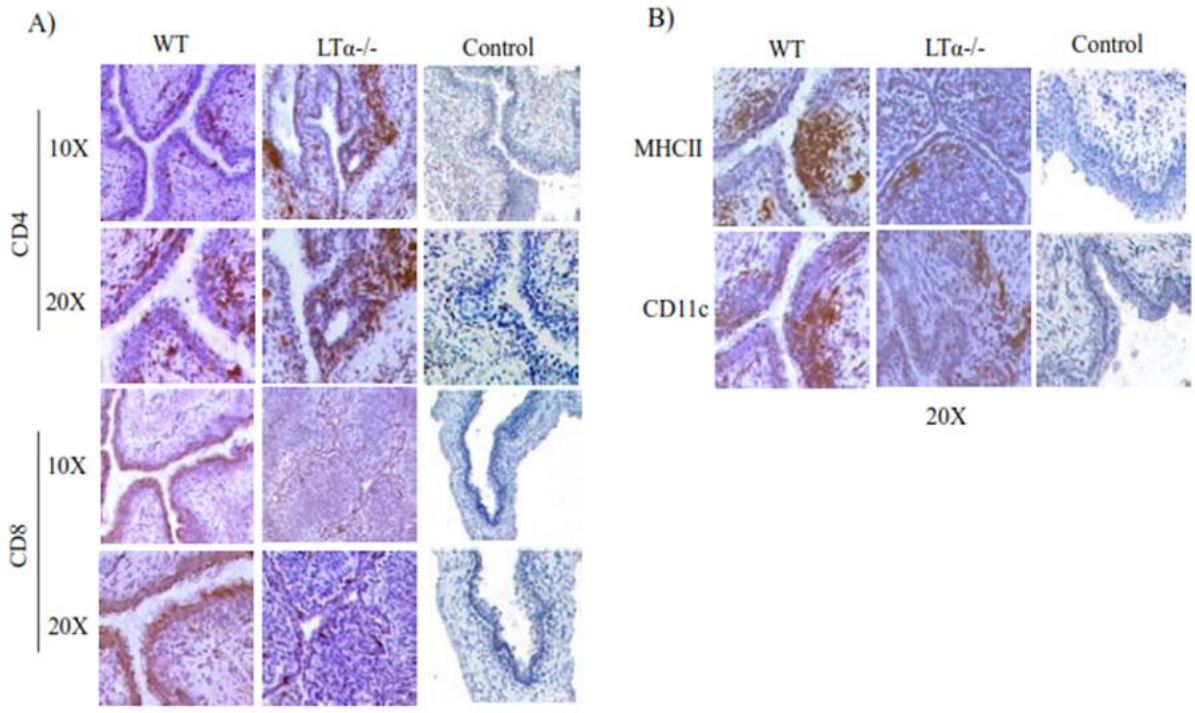


Figure 8: Flow cytometric analysis of IFNγ+ cells in vaginal tract day 3 after challenge. WT OVX, LTα-/- OVX and LTα-/- OVX+SPL mice were immunized IVAG with 10^5 PFU of TK^- HSV-2. At 6 weeks after immunization, mice were challenged IVAG with 10^5 PFU of WT HSV-2, strain 333. Vaginal tracts were excised on day 3 after challenge and lymphocytes were isolated by enzymatic digestion. (A, B) Lymphocytes were stained for flow cytometric analysis the same day after isolation using the T cell markers: CD3, CD4, CD8 and intracellular IFNγ. (C, D) Lymphocytes were plated in 96-well plates and left NT or stimulated with UV-inactivated HSV-2 (10^4 PFU) or 5 µl of CD3/CD28 dynabeads + IL-2 (50 U ml^{-1}) as a positive control. Cells were incubated at 37^0C

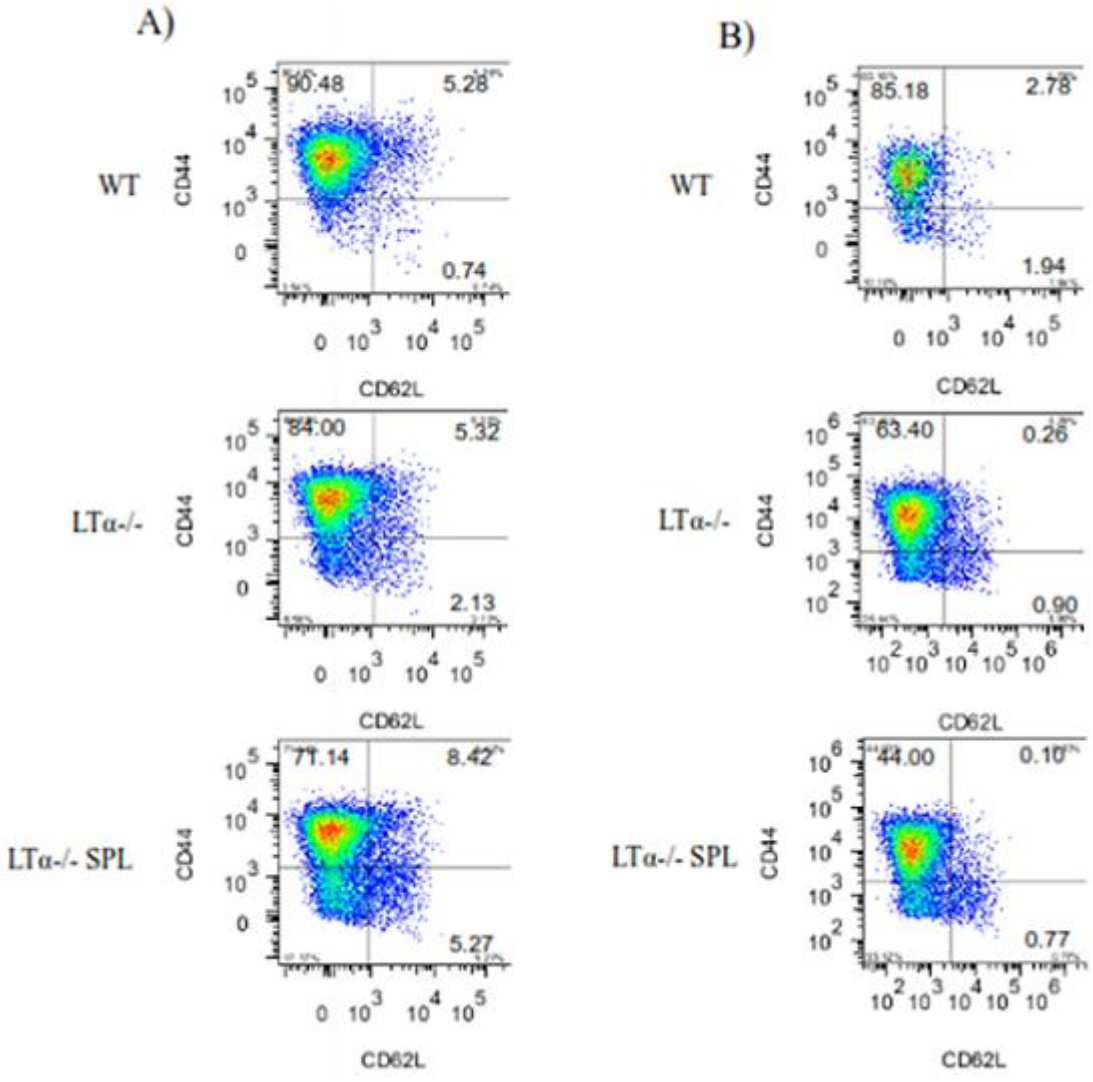
overnight and analyzed by flow cytometry the next day for the presence of IFNγ+ T cells. Data are representative of 2 separate experiments with similar results. HSV-2, Herpes simplex virus type 2; IFNγ, interferon-γ; IL-2, interleukin-2 IVAG, intravaginal; LTα, lymphotoxin-α; NT, unstimulated; OVX, ovariectomized; SPL, splenectomized; TK^-, thymidine kinase-negative; UV, ultraviolet; WT, wild type.



Supplementary Figure 1: Real-time PCR analysis of vaginal washes. Vaginal washes from uninfected mice were spiked with known concentrations of TK^- HSV-2 and WT HSV-2 (A), or collected at different days post-immunization (B) and post-challenge (C). Viral DNA was isolated using a QIAamp MinElute Virus Spin Kit and real-time PCR was performed to determine the expression of HSV-2 DNA polymerase gene.

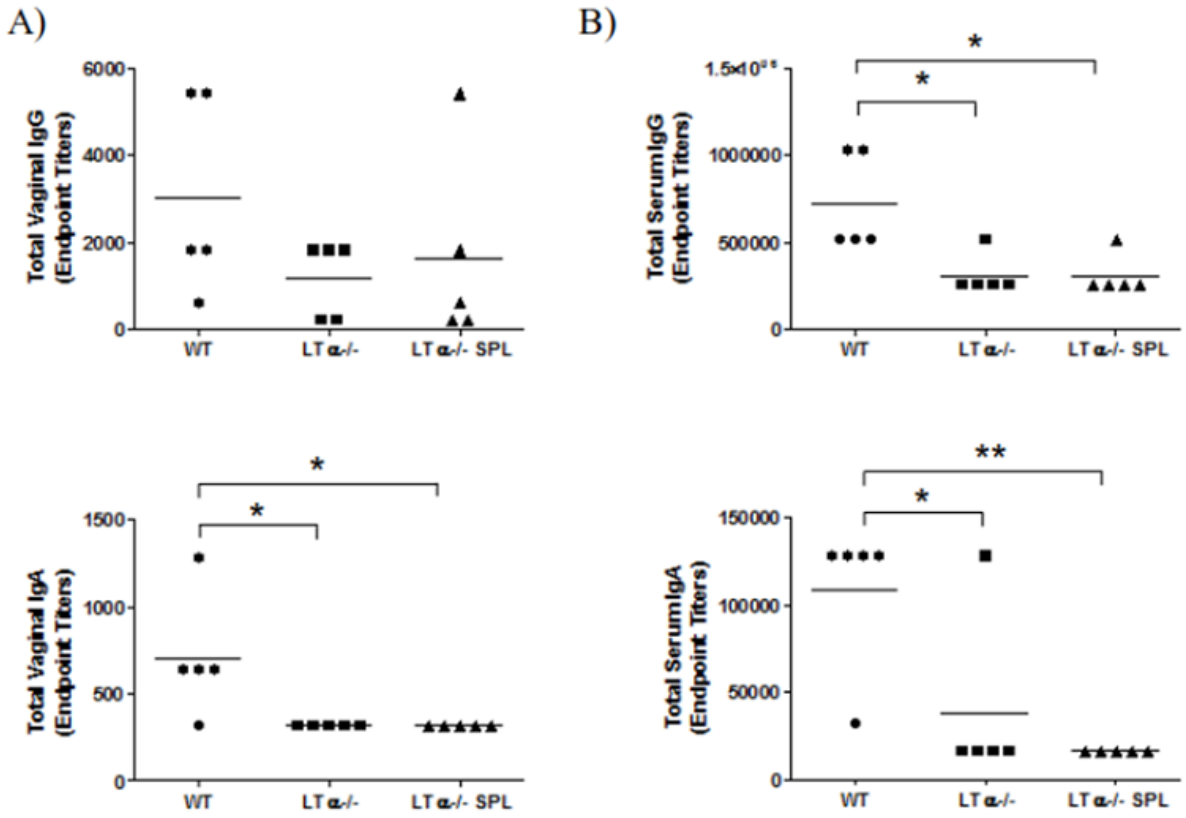


Supplementary Figure 2: iVALT formation in vaginal mucosae of immunized mice challenged with wild-type HSV-2. WT and LTα-/- mice were ovariectomized and immunized IVAG with attenuated HSV-2. Four weeks after immunization, mice were challenged IVAG with wild-type HSV-2 (10^5 PFU). Post-challenge, mice were sacrificed and immunohistochemical staining was done to localize immune cells. Representative tissue sections are shown. Original magnification, x100 or x200.



Supplementary Figure 3: Flow cytometric analysis of CD4+T cell populations in the vaginal tract post-immunization and post-challenge. WT OVX, LTα-/- OVX and LTα-/- OVX+SPL mice were immunized IVAG with 10^5 PFU of TK^- HSV-2. Four weeks post-immunization, all 3 groups of mice were challenged IVAG with 10^5 PFU of WT HSV-2. Vaginal tracts were excised on day 22 post-immunization (A) or day 3 post-challenge (B) and lymphocytes were isolated by enzymatic digestion. Lymphocytes were stained for

flow cytometric analysis the same day after isolation using the T cell markers: CD3 and CD4; and activation/memory markers: CD44 and CD62L.



Supplementary Figure 4: Total IgG and IgA antibody titers in vaginal washes and serum samples of WT OVX, LTα -/- OVX, and LTα -/- OVX+SPL non-immunized mice. Vaginal washes (A) and serum samples (B) were collected from WT OVX, LTα -/- OVX, and LTα -/- OVX+SPL mice. Total IgG and IgA antibodies were measured by ELISA, and endpoint titers were determined. Each dot indicates an individual mouse. Bars show mean values for the group. Statistical differences were determined by one-way ANOVA. *, $p<0.05$; **, $p<0.01$.

Chapter 3

Understanding the role of vaccine formulation, route of mucosal immunization and estradiol in the induction of protective local immune responses against HSV-2 challenge in the genital tract

Kristy L. Roth and Charu Kaushic.

This study examined whether local or distal mucosal immunization with a non-replicating vaccine could establish protection in the female genital tract, in the presence or absence of SLOs, following HSV-2 infection. We found that while both WT and LTα-/- mice were not protected against genital HSV-2 infections following intravaginal or intranasal immunization with a subunit vaccine, a heat-inactivated virus vaccine or a live-attenuated virus vaccine, pretreating mice with estradiol prior to immunization enhanced protection in the genital tract of these mice, irrespective of the vaccine formulation administered.

Dr. Charu Kaushic and I were responsible for the design and interpretation of the experiments. I was responsible for the generation and analysis of the data. I wrote this chapter with feedback and suggestions from Dr. Kaushic.

ABSTRACT

Previously, we found that local immunization with a live attenuated virus vaccine can generate effector memory immune responses in the genital mucosa, in the absence of secondary lymphoid organs (**SLOs**), and protect against subsequent viral exposure. In the current study, we determined what vaccine formulations and route(s) of immunization could establish protection against HSV-2 infection in the female genital tract, in the presence or absence of SLOs to further understand the requirements for developing a vaccine formulation that could generate local effector responses in the genital tract. To do this, we immunized WT and LTα-/- mice intravaginally (**IVAG**) or intranasally (**IN**) with a subunit vaccine (HSV-2 gD plus CpG), a heat-inactivated virus vaccine (heat-inactivated HSV-2 plus CpG), or a live attenuated virus vaccine (**TK$^-$ HSV-2**). All groups of mice were then challenged IVAG with WT HSV-2. Mice immunized by either route, with non-replicating vaccine, were not protected against genital HSV-2 challenge and succumbed to infection. However, treating WT and LTα-/- mice with estradiol (**E2**) prior to immunization resulted in enhanced protection against HSV-2 challenge. These results suggest that future immunization strategies against genital tract infections should consider the influence of female sex hormones, those produced endogenously during the menstrual cycle as well as those administered exogenously, such as hormonal contraceptives, in the induction of genital tract immunity following vaccination.

INTRODUCTION

Herpes simplex virus type 2 (**HSV-2**) is the major causative agent of genital herpes infections. Present statistics show that approximately one in four sexually active individuals are infected with HSV-2, making it one of the most common sexually transmitted infections. Despite decades of effort, an efficacious HSV-2 vaccine has not been developed. In the last large scale vaccine clinical trial, it was found that systemic administration of a recombinant subunit vaccine consisting of the HSV-2 glycoprotein gD2 formulated with an alum-monophosphoryl lipid adjuvant reduced HSV-2 disease by 70% and HSV-2 infection by 40% in a subgroup of women who were seronegative for both HSV-1 and HSV-2, but it did not protect against HSV-2 in men or HSV-1 seropositive women (Stanberry *et al.*, 2002; Bernstein *et al.*, 2005). However, a recent follow-up study consisting of more than 8000 HSV-1 and HSV-2 seronegative women found that the vaccine did not decrease HSV-2 disease or prevent infection (Cohen, 2010). Despite the failure of this vaccine, the results of these studies suggest that gender-specific differences should be considered when developing future vaccine strategies. Since the female genital tract is the primary site of infection, it is important to understand how the immune responses in this distinct microenvironment are generated and can protect against HSV-2 infections.

Previous studies in our lab using an HSV-2 mouse model have found that local immunization can generate local effector memory immune responses in the genital mucosa and protect against subsequent viral exposure (Roth *et al.*, 2013b). In this study,

lymphotoxin-alpha knockout (**LTα-/-**) mice, which lack all lymph nodes, were ovariectomized (**OVX**) to induce susceptibility to HSV-2 infections, and then immunized intravaginally (**IVAG**) with an attenuated HSV-2 virus, TK^- HSV-2. Four weeks after immunization, the mice were challenged IVAG with WT HSV-2. LTα-/- mice were completely protected against genital HSV-2 challenge, but they showed delayed viral clearance and prolonged genital pathology post-immunization compared to WT mice. Although local viral-specific antibody responses were compromised and T cell-mediated anti-HSV-2 responses were delayed in LTα-/- mice, the immune responses generated were effective in protecting against mucosal HSV-2 challenge in the genital mucosa (Roth *et al.*, 2013b). These results show that even in the absence of secondary lymphoid organs (**SLOs**), IVAG immunization generates antiviral immune responses in the genital mucosa that can protect against subsequent HSV-2 challenge.

While this study provided promising evidence regarding the independence of mucosal immune responses in the genital mucosa, the results raised important considerations for the design of future mucosal vaccines. While live attenuated virus vaccines induce a robust immune response against genital HSV-2 infection they are not representative of the mainstream efforts in the development of an HSV-2 vaccine. Further studies are required to examine whether more traditional vaccine strategies, such as immunizing with an inactivated virus or subunit vaccine could also induce protective immune responses in the genital tract of immunized mice following HSV-2 infection. In addition, it is also important to determine whether protective immune responses can be generated in the genital mucosa following immunization via a distal mucosa, such as

nasal mucosa, compared to the first study where the mucosal challenge was delivered via the same route as immunization. This information would allow a more realistic assessment of the requirements for generating local immune responses in the genital tract as part of designing a mucosal vaccine strategy.

Using the genital herpes mouse model, previous studies have found that IVAG immunization with an HSV-2 gD based vaccine combined with a mucosal adjuvant, such as CpG, can induce protective immune responses against subsequent IVAG HSV-2 challenge (Kwant & Rosenthal, 2004; Tengvall *et al.*, 2006). Other studies have successfully shown protection against vaginal challenge, following systemic subcutaneous immunization using gD-based liposomal formulations (Olson *et al.*, 2009) or following gD based vaccine (gD-AS04) given in estradiol (**E2**)-treated mice (Pennock *et al.*, 2009), to enhance immune responses in the genital tract. In addition, immunization with subunit vaccines at a distal site, and not just the vaginal tract, can elicit protection against subsequent genital viral challenge. Gallichan, *et al.* have shown that intranasal (**IN**) immunization of mice with HSV-1 gB plus CpG mediates protective immunity in the female genital tract against subsequent genital HSV-2 challenge (Gallichan *et al.*, 2001b). Another study found that IN immunization with HSV-2 glycoprotein gD in combination with IC31 adjuvant induced protective immunity against genital herpes infection in mice (Wizel *et al.*, 2012). However, none of these studies examined whether protective anti-HSV-2 immune responses could be generated against HSV-2 infections in the genital tract following local or distal immunization with a subunit vaccine without the involvement of secondary lymphoid structures such as draining LN to facilitate

generation of effector memory responses. Therefore, we wanted to determine whether immunization, either locally in the genital tract, or distally via the nasal mucosa, with a subunit vaccine, an inactivated virus vaccine or a live attenuated virus vaccine could provide protection in the genital mucosa without the involvement of systemic immunity. To address these questions, we immunized WT and LTα-/- mice with recombinant HSV-2 glycoprotein gD and mucosal adjuvant CpG, or with inactivated virus vaccines, both IVAG and IN, to determine the immunization conditions required to elicit protection in the genital tract of mice in the presence or absence of SLOs following genital HSV-2 challenge.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice, 6-8 weeks old, and female LTα$^{-/-}$ were purchased from Jackson laboratory (Maine, USA). LTα-/- mice on a 129 background were backcrossed 8-10 generations on to B6 background. ERα knockout (ERKO) mice were bred internally (McMaster University, Hamilton, ON, Canada). All mice were housed and maintained under standard temperature controlled conditions in special pathogen-free rooms that followed a 12-h day and 12-h night schedule. The Animal Research Ethics Board at McMaster University approved all of the animal protocols in this study.

Ovariectomy and splenectomy. The ovaries were removed from all mice and for some experiments, spleens were removed from the LTα-/- mice by making two bilateral incisions, followed by small incisions through the peritoneal wall. The ovaries were excised through the incisions by grasping the fat, ligating the blood vessels and removing the ovaries. The spleens were exteriorized through the same incisions and removed by ligating the splenic vasculature. The incisions were closed by surgical clips. The mice were allowed to recover for at least 10 days before the start of experiments.

Hormone treatments. For Depo-treated mice, five days before the first immunization and again before challenge, mice were injected subcutaneously with 2mg of Depo-Provera (Depo) (Upjohn, Don Mills, Ont., Canada). 17β-estradiol pellets were purchased from Innovative Research of America (Sarasota, Florida, USA). Mice were anaesthetized using injectable anaesthetic (150 mg of ketamine/kg-10 mg of xylazine/ml) given intraperitoneally, hormone pellets were implanted subcutaneously on the lateral side of

the neck. The concentration of hormones in the 17β-estradiol pellets was 476 ng/mouse/day, designed to be released equally for 21 days. For mock control groups, mice were anaesthetized as before and incisions were made in the lateral side of the neck, but pellets were not inserted.

Immunization. Mice were immunized either intravaginally or intranasally with 5 μg HSV-2 recombinant glycoprotein gD (Meridian Life Science, Inc., Memphis, TN, USA) plus 30 μg CpG ODN (5'-TCCATGACGTTCCTGACGTT-3') (Life Technologies Inc., Burlington, ON), or 10^4 PFU heat-inactivated HSV-2 plus CpG, or thymidine kinase-negative (TK^-) HSV-2 strain 333, at a dose of $1x10^3$ PFU or $1x10^5$ PFU. For intravaginal immunization, mice were anaesthetized using injectable anaesthetic (150 mg of ketamine/kg-10 mg of xylazine/ml) given intraperitoneally, placed on their backs, and immunized intravaginally with 10 μl of the inoculum. For intranasal immunization, mice were anaesthetized using the gaseous anaesthetic, isoflorane, and immunized with 5 μl of the inoculum into each nare with a micropipette. All groups of mice were immunized twice, two weeks apart. Heat-inactivation of HSV-2 was performed by heating the virus at 56^0C for 30 min, as previously described (Croughan & Behbehani, 1988). To ensure the virus was completely inactivated, viral titers were measured by VERO plaque assay.

WT HSV-2 challenge. Three to six weeks following the second immunization, mice were anaesthetized using injectable anaesthetic (150 mg of ketamine/kg-10 mg of xylazine/ml) given intraperitoneally, placed on their backs, and challenged intravaginally with 10 μl of wild-type HSV-2 strain 333 at a dose of $5x10^3$ PFU or $1x10^5$ PFU. Mice

were kept on their backs, under the influence of anaesthesia, for 45 min to 1 h to allow the inoculum to infect.

Collection of vaginal washes. Vaginal washes were collected for 5 consecutive days post-HSV-2 challenge. Vaginal washes were collected by pipetting 30 µl of PBS twice consecutively in and out of the vagina 6-8 times to give a total volume of 60 µl and stored at -70^{0}C until use.

Viral replication. Viral titers in vaginal washes were determined by plaque assay. Vero cells were grown in α-modified Eagle's medium supplemented with 5% fetal calf serum, 1% penicillin-streptomycin, and L-glutamine (all purchased from GIBCO Laboratories, Burlington, Canada). Vero cells were grown to confluence in 12-well plates. Vaginal washes were diluted (10^{-1} to 10^{-4}) and added to Vero cell monolayers. Infected monolayers were incubated at 37^{0}C for 2 h and were rocked every 15 min for viral absorption. Infected monolayers were overlaid with α-MEM supplemented with 0.05% human immune serum globulin (Canadian Blood Services). Infection was allowed to occur for 48 h at 37^{0}C. Monolayers were then fixed and stained with crystal violet, and viral plaques were counted under a light microscope. The number of PFU/ml was calculated by taking a plaque count for every sample and taking into account the dilution factors.

Genital pathology. Following infection with HSV-2, genital pathology was monitored daily and was scored on a five-point scale: 0, no infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina; 3, severe swelling and redness of both

vagina and surrounding tissue and hair loss in genital area; 4, genital ulceration with severe redness, swelling, and hair loss of genital and surrounding tissue; 5, severe genital ulceration extending to surrounding tissue. Animals were euthanized when they reached stage 5 as per approved procedures of Animals Utilization Protocol.

RESULTS

Intravaginal immunization of Depo-treated WT and LTα-/- mice with HSV-2 gD plus CpG protects against low dose HSV-2 challenge. Previous studies by Tengvall, *et al.,* immunized WT mice with gD plus CpG IVAG and found that following challenge with a lethal dose of HSV-2 ($9x10^4$ PFU), 80% of mice survived (Tengvall *et al.*, 2006). In these experiments, mice were treated with Depo-Provera (**Depo**) prior to immunization and challenge to thin the vaginal epithelium and allow the vaccine and challenge virus to enter the vaginal tract of these mice. We first repeated these studies in order to optimize the non-replicating vaccine dose and protocol in our hands and ensure reproducibility in our lab, since we had not previously used the gD plus CpG vaccine formulation in our model. We treated both WT and LTα-/- mice with Depo, and 5 days later immunized IVAG with 5 µg of gD plus 30 µg of CpG, similar to the previous study (Tengvall *et al.*, 2006). Two weeks later, the immunization was repeated. Three to five weeks following the second immunization, mice were treated with Depo and then challenged 5 days later with a lethal dose of HSV-2, at either a high ($1x10^5$ PFU) or a low ($5x10^3$ PFU) dose. A pilot study in our lab was done to determine the minimum lethal dose for WT and LTα-/- mice and it was found that all non-immunized mice challenged IVAG with $5x10^3$ PFU WT HSV-2 succumbed to infection by day 7 post-challenge, whereas only half of the mice challenged with $1x10^3$ PFU WT HSV-2 succumbed to infection (data not shown). Therefore, we decided to use $5x10^3$ PFU WT HSV-2 for the low dose challenge. It was found that only 20% of WT mice survived challenge with high dose HSV-2, whereas

80% of both WT and LTα-/- mice survived challenge with low dose HSV-2 (Figure 1A). All three groups of mice developed significant genital pathology, but WT and LTα-/- mice challenged with low dose HSV-2 were able to recover from this increased pathology whereas WT mice challenged with high dose HSV-2 were not (Figure 1B). All three groups of mice also showed significant viral shedding in the vaginal tract from days 1-5 post-challenge (Figure 1C). LTα-/- mice did not receive the high dose challenge based on previous pilot studies showing 100% mortality in these mice when challenged with a high dose of WT HSV-2. These results indicated that as shown in previous studies, Depo-treated mice immunized IVAG with a non-replicating vaccine were protected, however high protection (80%) was seen only against low dose WT HSV-2 challenge.

Intravaginal immunization of WT and LTα-/- OVX mice with HSV-2 gD plus CpG does not protect against low dose HSV-2 challenge. The primary model used in our lab to make mice susceptible to HSV-2 infection is to ovariectomize (OVX) mice. Previous studies have shown that Depo treatment modifies susceptibility to HSV-2 infection by thinning the vaginal epithelium and alters immune responses in the genital tract based on the length of Depo treatment (Kaushic *et al.*, 2003). In addition, Depo-treatment also superimposes a synthetic progestin on endogenous hormones already present in the genital tract, making it difficult to determine the individual effects of hormones. However, the OVX model makes mice susceptible to HSV-2 infection by removing the ovaries and thereby eliminating the production of endogenous hormones in

the genital tract, without compromising the immune responses to infection (Gillgrass *et al.*, 2005a). Therefore, using the OVX model instead of Depo-treatment allows one to better define the conditions required to induce protective immune responses in the genital tract of immunized mice following HSV-2 challenge. Using this OVX model, next we determined whether WT and LTα-/- OVX mice are protected against low dose HSV-2 challenge following IVAG immunization with gD plus CpG, similar to Depo-treated mice. WT and LTα-/- mice were OVX, and we also removed the spleen from one group of LTα-/- mice (LTα-/- SPL) to remove all secondary lymphoid organs. All groups of mice were then immunized twice IVAG with gD plus CpG. Five weeks following the second immunization, mice were challenged IVAG with low dose ($5x10^3$ PFU) HSV-2. Both groups of LTα-/- mice succumbed to infection before day 10 post-challenge, and all WT mice succumbed to infection by day 20 post-challenge (Figure 2A). All three groups of mice developed significant pathology (Figure 2B) and shed virus (Figure 2C) post-challenge. This indicated that while Depo-treated, immunized mice were protected against low dose HSV-2 challenge, OVX mice were more susceptible to infection and were not protected against HSV-2 challenge when immunized IVAG with a non-replicating subunit vaccine.

Intravaginal immunization of WT and LTα-/- OVX mice with heat-inactivated HSV-2 plus CpG does not protect against low dose HSV-2 challenge. Since OVX mice were not protected against HSV-2 infection when immunized with a single recombinant glycoprotein, we wanted to determine whether using a more polyclonal non-

replicating vaccine formulation (ie. one that contains multiple antigens that could lead to broader stimulation of the immune system, thereby leading to a polyclonal T cell and antibody response) could elicit better protection in these mice. We IVAG immunized OVX mice twice, two weeks apart, with heat-inactivated HSV-2 plus the mucosal adjuvant CpG. Five weeks following the second immunization, mice were challenged with low dose WT HSV-2. WT mice only showed 20% protection following challenge, whereas all LTα-/- mice succumbed to infection (Figure 3A). A closer examination revealed that while all LTα-/- mice succumbed more quickly to infection (by day 8 post-challenge), 6 of 10 WT mice succumbed to infection by day 9 post-challenge. Of the remaining 4 WT mice, 2 succumbed to infection by day 19 post-challenge and 2 survived the genital HSV-2 challenge (Figure 3A). In addition, both groups of mice developed significant pathology and showed viral shedding post-challenge (Figure 3B, C). Therefore, even with an inactivated whole virus vaccine delivered IVAG, OVX mice are not protected against HSV-2 challenge.

Intranasal immunization of WT and LTα-/- OVX mice with HSV-2 gD plus CpG does not protect against low dose HSV-2 challenge. Previous studies in our lab found that IN immunization of mice with TK^- HSV-2 leads to protection against genital HSV-2 challenge (Bhavanam *et al.*, 2008). Similarly, other studies have also shown that IN immunization with a subunit vaccine induces protective immunity against genital HSV-2 infection in mice (Gallichan *et al.*, 2001b; Wizel *et al.*, 2012). We wanted to determine whether IN immunization with a subunit vaccine could protect OVX mice against

subsequent genital HSV-2 challenge, and whether mice that lack lymph nodes (LTα-/- mice) could also be protected. Therefore, WT and LTα-/- mice were immunized IN twice, two weeks apart, with HSV-2 gD plus CpG. Five weeks following the second immunization, mice were challenged IVAG with low dose ($5x10^3$ PFU) HSV-2. All WT mice succumbed to infection by day 15 post-challenge, whereas both groups of LTα-/- mice all succumbed to infection by day 9 post-challenge (Figure 4A). All 3 groups of mice developed genital pathology and showed viral shedding post-challenge (Figure 4B, C). Therefore, in our model, even though WT showed longer survival compared to LTα-/- mice, ultimately neither group was protected against genital HSV-2 challenge following IN immunization with a subunit vaccine.

Intranasal immunization of WT and LTα-/- OVX mice with heat-inactivated HSV-2 plus CpG does not protect against low dose HSV-2 challenge. Since IN immunization with a single subunit vaccine did not elicit protection in the genital tract following low dose HSV-2 challenge, we wanted to test whether IN immunization with an inactivated whole virus vaccine could provide protection against genital HSV-2. Previous studies have shown that heat inactivation of HSV-2 was effective as both a prophylactic and therapeutic vaccine in small clinical trials (Weitgasser, 1977; Nasemann & Wassilew, 1979). WT and LTα-/- OVX mice were immunized twice IN with heat-inactivated HSV-2 plus CpG and then challenged with low dose HSV-2 administered IVAG. Both groups of mice developed genital pathology and shed virus post-challenge, and all mice succumbed to infection by day 9 post-challenge (Figure 5). This suggests that even with

a more polyclonal vaccine formulation, IN immunization of WT or LTα-/- OVX mice does not protect against genital HSV-2 challenge.

Intranasal immunization of WT OVX mice with high dose TK$^-$ HSV-2 protects against low dose HSV-2 challenge. In our previous experiments, we showed that WT mice are not protected against genital HSV-2 challenge following IN immunization with either a single subunit vaccine (gD plus CpG) or with an inactivated whole virus vaccine (heat-inactivated HSV-2 plus CpG) (Figures 4 and 5). However, IN immunization with gD plus CpG prolonged the survival of WT mice following genital HSV-2 infections to 15 days post-challenge, compared to 9 days post-challenge in LTα-/- mice following IN immunization with heat-inactivated HSV-2 plus CpG. This suggests that IN immunization with a subunit vaccine results in better protection against genital HSV-2 challenge compared to an inactivated virus vaccine. Since it is possible that the heat-inactivation of the virus resulted in denaturation of the surface proteins, thereby altering their antigenic structure, we next determined whether IN immunization with a more antigenic live, attenuated virus vaccine (TK$^-$ HSV-2) could provide better protection against subsequent genital HSV-2 infection. Previous studies in our lab have shown that IN immunization of WT mice with $1x10^5$ PFU TK$^-$ HSV-2 elicits protection in the genital tract against subsequent genital WT HSV-2 challenge (Bhavanam *et al.*, 2008). However, when LTα-/- mice were immunized IN with $1x10^5$ PFU TK$^-$ HSV-2, all mice succumbed to infection by day 8 post-immunization (data not shown). Therefore, we lowered the immunization dose to $1x10^3$ PFU TK$^-$ HSV-2. WT and LTα-/- mice were

immunized IN with $1x10^3$ PFU TK^- HSV-2 twice, two weeks apart, and then challenged IVAG with low dose ($5x10^3$ PFU) WT HSV-2. As a control, WT mice were also immunized IN with $1x10^5$ PFU TK^- HSV-2, and showed complete protection against subsequent low dose ($5x10^3$ PFU) HSV-2 infection, with no accompanying genital pathology and no viral shedding after day 2 post-challenge (Figure 6) in agreement with our previous studies (Bhavanam *et al.*, 2008). However, WT mice immunized IN with $1x10^3$ PFU TK^- HSV-2 only showed about 40% protection against low dose challenge, whereas LTα-/- mice were not protected against genital HSV-2 challenge (Figure 6A). Whereas only 40% of WT mice had succumbed to infection by day 9 post-challenge, over 80% of LTα-/- mice had succumbed to infection by day 9 post-challenge, suggesting that LTα-/- may have a slower response to genital HSV-2 infections, and therefore succumb to infection more quickly, compared to WT mice. Both WT and LTα-/- mice immunized with $1x10^3$ PFU TK^- HSV-2 developed significant genital pathology and showed viral shedding until day 5 post-challenge (Figure 6B, C). Since IN immunization with $1x10^5$ PFU TK^- HSV-2 provided protection against subsequent genital challenge in WT mice, but IN immunization with $1x10^3$ PFU TK^- HSV-2 did not provide adequate protection, this suggests that there may be a threshold of live attenuated virus that is required for mounting a fully protective immune response against genital HSV-2 infections.

Chemokine pull does not enhance protection in intranasally immunized mice following genital HSV-2 infection. Since none of the vaccine strategies that we had

tried so far provide protection against genital HSV-2 challenge, we wanted to find a way to enhance the protective immune response in the genital tract. A recent study by Shin and Iwasaki found that they could establish a protective memory T cell pool in the vaginal tract of mice following parenteral vaccination using a 'prime and pull' vaccine strategy (Shin & Iwasaki, 2012). Briefly, mice were immunized subcutaneously with TK^- HSV-2 to elicit systemic T cell responses (prime), followed by recruitment of activated T cells into the genital tract by IVAG administration of the chemokines, CXCL9 and CXCL10 (pull), where these T cells mediated protective immunity against subsequent HSV-2 infections. To determine whether this prime-pull strategy could enhance protection in our IN model, we immunized mice with heat-inactivated HSV-2 plus CpG, as described above, and then administered CXCL9 and CXCL10 IVAG three weeks following the second immunization. Two days later, mice were challenged with low dose HSV-2. As seen in Figure 7, all mice developed significant pathology and succumbed to infection by day 8 post-challenge. Given the lack of indication that this strategy could work in our model, we did not further pursue this line of experiments.

Estradiol pre-treatment enhances protection against genital HSV-2 infections in intranasally immunized WT and LTα-/- OVX mice. Previous studies in our lab demonstrated that pre-treatment of mice with estradiol (**E2**) prior to either IVAG or IN immunization leads to protection following genital HSV-2 infections with no accompanying pathology (Bhavanam *et al.*, 2008; Gillgrass *et al.*, 2010). In addition, in a follow up to our study, Pennock, *et al.*, found that E2 enhances genital herpes vaccine

efficacy in mice (Pennock *et al.*, 2009). Therefore we decided to determine in this model of OVX WT and LTα-/- mice, whether pre-treating mice with E2 prior to IN immunization with various vaccine formulations could enhance protection following genital HSV-2 challenge. WT and LTα-/- mice were treated with E2 pellets and then immunized IN one week later with gD plus CpG, heat-inactivated HSV-2 plus CpG, or TK^- HSV-2. Immunization was repeated two weeks later and then mice were challenged IVAG with low dose WT HSV-2 five weeks later. Whereas WT and LTα-/- mice that were not treated with E2 were not protected following HSV-2 challenge, all 3 groups of E2-treated WT mice showed over 75% protection following HSV-2 challenge (Figure 8A). In E2-treated LTα-/- mice, 100% survived challenge following IN immunization with TK^- HSV-2, but only 40% survived infection following IN immunization with heat-inactivated HSV-2 plus CpG (Figure 9A). In both WT and LTα-/- immunized group, less than half the mice developed genital pathology, although viral shedding was seen in most mice post-challenge showing that protection was not sterile in E2-treated mice (Figure 8B, C and Figure 9B, C), in agreement with our previous studies (Gillgrass *et al.*, 2010). These results show that treating mice with E2 prior to IN immunization can improve vaccine efficacy and help protect mice against subsequent genital HSV-2 infection, regardless of the presence of secondary lymphoid tissues.

Intranasal immunization, with or without estradiol pre-treatment, does not protect against genital HSV-2 challenge in estradiol receptor knockout mice. To further examine the role of E2 in the enhanced protection observed in the previous experiments,

we used estradiol receptor alpha knockout (ERKO) mice as a control. OVX ERKO mice were treated with E2 pellets, or left untreated, and then immunized twice IN with TK^- HSV-2. Three weeks after the second immunization, mice were challenged IVAG with low dose WT HSV-2. Both groups of mice succumbed to infection, and showed significant pathology and viral shedding post-challenge (Figure 10). These results indicate that E2 signaling is essential for the enhanced protection observed in IN immunized mice, post-HSV-2 challenge.

DISCUSSION

Previously, we reported that WT mice, as well as LTα-/- mice, are completely protected against WT HSV-2 challenge following IVAG immunization with TK^- HSV-2 (Roth *et al.*, 2013b). However, these experiments administered a robust replicating live attenuated vaccine locally in the genital tract of mice. Whether these mice are also protected following immunization with non-replicating vaccine formulations, such as subunit or inactivated virus vaccines, or when immunized at a distal site instead of in the vaginal tract, had not been determined. Here we show that while Depo-treated WT or LTα-/- mice are protected against a low dose viral challenge following IVAG immunization with HSV-2 glycoprotein gD plus mucosal adjuvant CpG, OVX mice immunized IVAG with gD plus CpG, or heat-inactivated HSV-2 plus CpG, are not protected following HSV-2 challenge. These results suggest that OVX mice are more susceptible to HSV-2 infection than Depo-treated mice and that OVX mice require a more robust IVAG vaccine formulation, such as an attenuated virus vaccine, to be protected against a subsequent viral challenge. One possible explanation for the increased susceptibility in OVX mice is because while OVX removes the endogenous production of female sex hormones in the genital tract, Depo treatment superimposes a progestin-based contraceptive on endogenous hormones already present in the FGT. Since Depo-treated mice still have E2 present, this could be providing some protection against genital HSV-2 infection. Therefore, the absence of E2 in OVX mice may increase susceptibility to HSV-2 infections compared to Depo-treated mice.

We also examined whether IN immunization with different vaccine formulations could protect WT and LTα-/- OVX mice against a subsequent genital HSV-2 challenge. First, we found that mice immunized IN with a non-replicating subunit vaccine (HSV-2 gD plus CpG) were not protected against a low dose genital HSV-2 challenge, in contrast to previous studies which have shown that IN immunization with an HSV-2 glycoprotein plus adjuvant provides protection against subsequent genital HSV-2 challenge (Gallichan *et al.*, 2001b; Wizel *et al.*, 2012). However, there are a number of differences between those studies and ours. For example, while Wizel¸*et al.* used 5 µg of HSV-2 gD (similar to our experiment), they used the adjuvant IC31®, which combines a synthetic antimicrobial peptide with a non-CpG motif-bearing oligonucleotide (Wizel *et al.*, 2012). IC31® has been shown to form a vaccine depot at injection sites, enhance the association of antigen to APCs, and elicit a potent antigen-specific Th1 response and a strong antigen-specific CTL response (Yamada & Natori, 1994; Schellack *et al.*, 2006; Lingnau *et al.*, 2007; Riedl *et al.*, 2008), which could account for the better protection observed in this study compared to our results. In addition, Wizel, *et al.* immunized mice three times, compared to twice in our model, and they treated mice with Depo prior to genital HSV-2 challenge to make mice susceptible. As previously mentioned, OVX are more susceptible to genital HSV-2 challenge compared to Depo-treated mice, which could explain the difference in protection observed in the two studies. Similarly, Gallichan, *et al.* also used Depo treatment to make mice susceptible to genital HSV-2 challenge and they also used HSV-2 glycoprotein gB (Gallichan *et al.*, 2001b), which may be more

immunogenic and thereby lead to better protection following HSV-2 challenge compared to gD, which was used in our experiments.

Since IN immunization with a non-replicating subunit vaccine did not provide protection against genital HSV-2 infection, we next wanted to determine whether IN immunization with a vaccine formulation that contains multiple antigens, instead of a single antigen, and stimulates a polyclonal T cell and antibody response could provide better protection against genital HSV-2 challenge. However, IN immunization with heat-inactivated HSV-2 plus CpG adjuvant did not provide protection against subsequent genital HSV-2 challenge. In fact, IN immunization with gD plus CpG prolonged survival in WT mice following genital HSV-2 challenge compared to IN immunization with heat-inactivated HSV-2 plus CpG (15 days vs. 9 days, respectively). Therefore, one possible explanation for why IN immunization with heat-inactivated HSV-2 had no protective effect against subsequent genital HSV-2 challenge is that heat inactivation of microbes can result in the denaturation of surface proteins, which thereby alters their antigenic structure. The immune response that is generated using this heat-inactivated vaccine may not be effective against a subsequent challenge with the natural pathogen. While previous studies have shown that heat inactivation of HSV-2 can be result in an efficacious vaccine (Weitgasser, 1977; Nasemann & Wassilew, 1979), it is possible that our heat-inactivated HSV-2 vaccine was not as effective at inducing protective immune responses against genital HSV-2 infections.

Since IVAG or IN immunization with a subunit or heat-inactivated virus vaccine did not induce protection against a subsequent genital HSV-2 challenge, we next determined whether IN immunization with a more polyclonal and antigenic live, attenuated virus vaccine (TK^- HSV-2) could result in better protection. Compared to subunit and inactivated virus vaccines, live attenuated virus vaccines stimulate a more robust innate immune response and a stronger antibody and CD8+ T cell response and therefore should provide better protection against a genital HSV-2 challenge. Previous studies in our lab have shown that IN immunization of WT mice with $1x10^5$ PFU TK^- HSV-2 can protect against a high dose ($1x10^5$ PFU) HSV-2 challenge (Bhavanam *et al.*, 2008). However, when LTα-/- mice were administered $1x10^5$ PFU TK^- HSV-2 during a pilot study in our lab, all mice succumbed to infection by day 8 post-immunization. This suggests that the nasal mucosa, without the help of draining lymph nodes, is not capable of mounting a protective immune response against a high dose immunization with a live, attenuated virus. Therefore, the IN immunization dose of TK^- HSV-2 was lowered to $1x10^3$ PFU. While IN immunization of WT mice with high dose TK^- HSV-2 resulted in protection against subsequent genital HSV-2 challenge, IN immunization with low dose TK^- HSV-2 did not result in protection in either WT or LTα-/- mice. This suggests that a threshold level of live, attenuated virus is required in order for IN immunization to mount a fully protective immune response against a subsequent genital HSV-2 challenge.

With none of the vaccine strategies that we had tried so far providing protection against genital HSV-2 challenge, we wanted to enhance T cell responses in the genital

tract of mice following immunization to elicit better protection against genital HSV-2 infections. A recent study described a promising new vaccine strategy that establishes a memory T cell pool within peripheral tissues to protect against subsequent viral exposure (Shin & Iwasaki, 2012). Following a genital HSV-2 infection, IFNγ-secreting CD4+ T cells induce the expression of chemokines CXCL9 and CXCL10, which then mediate the recruitment of effector CD8+ T cells to the infected tissue (Nakanishi *et al.*, 2009). Therefore, Shin and Iwasaki used the topical application of both CXCL9 and CXCL10 to recruit effector T cells into the vaginal tract of mice in the absence of infection. In this study, mice were immunized subcutaneously to elicit systemic T cell responses (prime), followed by recruitment of activated T cells into the genital tract by intravaginal administration of the chemokines, CXCL9 and CXCL10 (pull), where such T cells mediate protective immunity against subsequent HSV-2 infections (Shin & Iwasaki, 2012). To determine whether this vaccine strategy could enhance protection in our intranasal model, we immunized mice with heat-inactivated HSV-2 plus CpG, and then administered CXCL9 and CXCL10 intravaginally three weeks following the second immunization. Two days later, mice were challenged with low dose WT HSV-2. In our model, the prime-pull vaccine strategy did not enhance protection against genital HSV-2 challenge, and all mice succumbed to infection by day 8 post-challenge. However, there were a number of differences in the design of our experiment compared to the previous study. For example, Shin and Iwasaki observed that the prime-pull vaccine strategy was most effective at recruiting T cells when mice were immunized subcutaneously and the chemokines were administered 5 days later. When the chemokines were administered at

later time points following immunization (either day 15 or day 28 post-immunization), the recruitment of T cells into the vaginal tract of mice was not as effective (Shin & Iwasaki, 2012). In our study, mice were IN immunized twice, two weeks apart, and the chemokines were not administered until 3 weeks after the second immunization. Therefore, it is possible that we administered the chemokines too late following immunization to effectively promote the recruitment of T cells into the vaginal tract. However, we did not examine T cell populations following chemokine administration to determine whether there was an increase in T cell recruitment into the vaginal tracts of mice. Therefore, future studies would be required determine whether this vaccine strategy could be used to enhance T cell recruitment and protect against genital HSV-2 challenge.

Although IN immunization with a subunit vaccine, a heat-inactivated whole virus vaccine and a low dose of a live, attenuated virus vaccine did not result in protection against subsequent genital HSV-2 challenge, when mice were treated with E2 prior to immunization, irrespective of the vaccine formulation that was administered, they were protected against HSV-2 challenge, suggesting that E2 enhances protection in these mice. It has been well established that E2 treatment in mice leads to increased keratinization of vaginal tract epithelial cells, which prohibits HSV-2 from crossing the epithelial cell barrier and infecting mice. To ensure that better protection was not due to a physical barrier in the genital tract, mice were treated with E2 prior to IN immunization and were not challenged with WT HSV-2 until 8 weeks following E2 administration. Vaginal

washes were collected prior to WT HSV-2 challenge to ensure that the mice were no longer under the effects of E2 treatment (data not shown and V. Anipindi, unpublished observations). In addition, HSV-2 viral shedding was measured for 5 days post-challenge (Figure 8C and Figure 9C) and all groups of mice developed genital pathology post-challenge, suggesting that the WT HSV-2 virus did in fact infect the mice (Figure 8B and Figure 9B). Similarly, previous studies in our lab also found that E2 treatment prior to IN or IVAG immunization with TK^- HSV-2 resulted in non-sterile protection against subsequent genital HSV-2 challenge (Bhavanam *et al.*, 2008; Gillgrass *et al.*, 2010). To further verify the role of E2 in enhancing protection following HSV-2 challenge in IN immunized mice, ERKO mice were treated with E2 or left untreated, and immunized IN with TK^- HSV-2. Following genital HSV-2 challenge, both groups of ERKO mice succumbed to infection, suggesting that E2 signaling is required for the enhanced protection in these mice.

These results are in accordance with a number of other animal studies that have found that E2 treatment can protect against sexually transmitted infections. Previous studies in our lab have found that treating mice with E2 prior to immunization protects against genital HSV-2 challenge with no accompanying pathology (Bhavanam *et al.*, 2008; Gillgrass *et al.*, 2010). Another HSV-2 mouse model found that immunizing mice with a subunit HSV-2 gD/ASO4 vaccine in the presence of E2 improved vaccine efficacy and disease prevention (Pennock *et al.*, 2009). Similar to the findings on HSV-2, studies have also shown that female rhesus macaques are less susceptible to SIV infection during

the follicular ovarian phase, when estrogen levels are highest compared to the luteal phase, when progesterone levels are highest (Sodora *et al.*, 1998). The systemic administration of estrogen also protects against SIV infection in OVX female rhesus macaques (Smith *et al.*, 2000). These studies from both mice and non-human primates suggest that the presence of estrogen can prevent or decrease the risk of viral infections in the female genital tract. Therefore, the timing of vaccination during the menstrual cycle or under the influence of exogenous estrogen should be considered for the development of future vaccines to elicit optimal protection in women.

However, even though this study, as well as previous studies, has shown that E2 can enhance protection against genital HSV-2 infections, there are a number of limitations associated with using exogenous estrogen as a vaccine adjuvant or administering vaccines during estrogen-high phases of the menstrual cycle. For instance, the human menstrual cycle is highly variable among different women and vaccinating women when estrogen levels are highest would be challenging. In addition, the administration of exogenous estrogen has been shown to have a number of adverse effects, including increased risk of coronary heart disease, stroke or endometrial cancer (Rossouw *et al.*, 2002; Lepine *et al.*, 2010). Therefore, future studies are required to examine the safety and efficacy of administering estrogen as a vaccine adjuvant or to determine whether immunization during estrogen-high phase of the menstrual cycle is practical.

Previously we had found that while LTα-/- mice had higher levels of genital pathology and viral shedding post-challenge compared to WT mice, they were still protected against genital HSV-2 challenge when immunized IVAG with a high dose ($1x10^5$ pfu) of TK^- HSV-2 (Roth *et al.*, 2013b). These results suggest that local immunization with a high dose of an attenuated virus vaccine can generate effective immune responses in the genital mucosa, without the help of draining lymph nodes. These immune responses can then provide antiviral protection against subsequent genital HSV-2 infections. In the present study, although neither WT nor LTα-/- OVX mice were protected against a genital HSV-2 infection following IN immunization with a subunit vaccine, a heat-inactivated virus vaccine or a low dose of an attenuated virus vaccine, we did observe that WT mice showed prolonged survival following HSV-2 challenge in both IN and IVAG immunized mice, compared to LTα-/- mice. This suggests that, similar to our previous study (Roth *et al.*, 2013b), the immune responses generated in LTα-/- mice may be delayed and decreased in magnitude compared to WT mice, thereby resulting in less protection against genital HSV-2 infection following immunization with a less potent vaccine formulation or immunization at a distal mucosal surface. However, when WT mice were treated with E2 prior to IN immunization with any of the 3 vaccine formulations, they were protected (80% survival in all 3 groups) against subsequent genital HSV-2 challenge. In contrast, when LTα-/- mice were treated with E2 prior to IN immunization, they showed 80% survival when immunized with low dose TK^- HSV-2 but only 40% survival when immunized with heat-inactivated HSV-2 plus CpG. Therefore, while in our previous study immunization at the same mucosa with high dose

of a live attenuated virus does not require assistance from secondary lymphoid organs such as LN (Roth *et al.*, 2013b), this study shows that a less robust immunization, either because of a weaker vaccine formulation or because of immunization via a distal mucosa, requires the help of SLOs to mount an efficient protective response against subsequent genital HSV-2 challenge.

Overall, the results from this study have a number of implications for the design of future prophylactic HSV-2 vaccines. First, the use of highly immunogenic live-attenuated virus based vaccines delivered via mucosal routes, either IVAG or IN, can provide protection in the genital tract against subsequent HSV-2 infections. However, if a less immunogenic vaccine formulation, such as a subunit or inactivated virus based vaccine, is delivered either locally or distally, or a lower dose of the attenuated virus based vaccine is delivered via a distal mucosal surface, additional mucosal adjuvants or hormones may be required to elicit protection in the genital tract.

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FIGURES

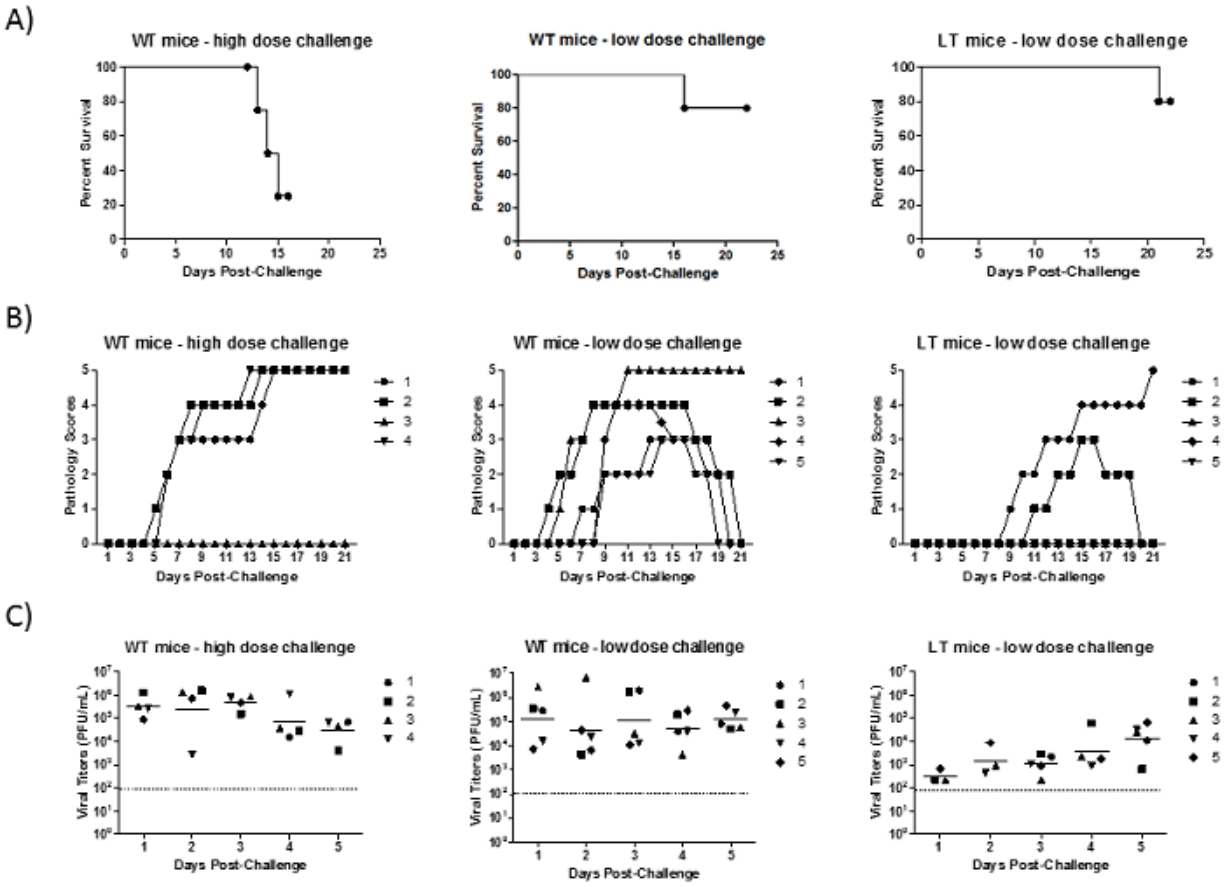

Figure 1: IVAG immunization of Depo-treated WT and LTα-/- mice with HSV-2 gD plus CpG protects against low dose HSV-2 challenge. WT and LTα-/- mice were injected subcutaneously with 2mg of Depo-Provera (Depo) and then immunized intravaginally 5 days later with 5 µg HSV-2 gD plus 30 µg CpG. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $1x10^5$ PFU WT HSV-2, strain 333 (high dose challenge) or $5x10^3$ PFU WT HSV-2, strain 333 (low dose challenge). (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done.

Plaques were counted and viral titers were expressed as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

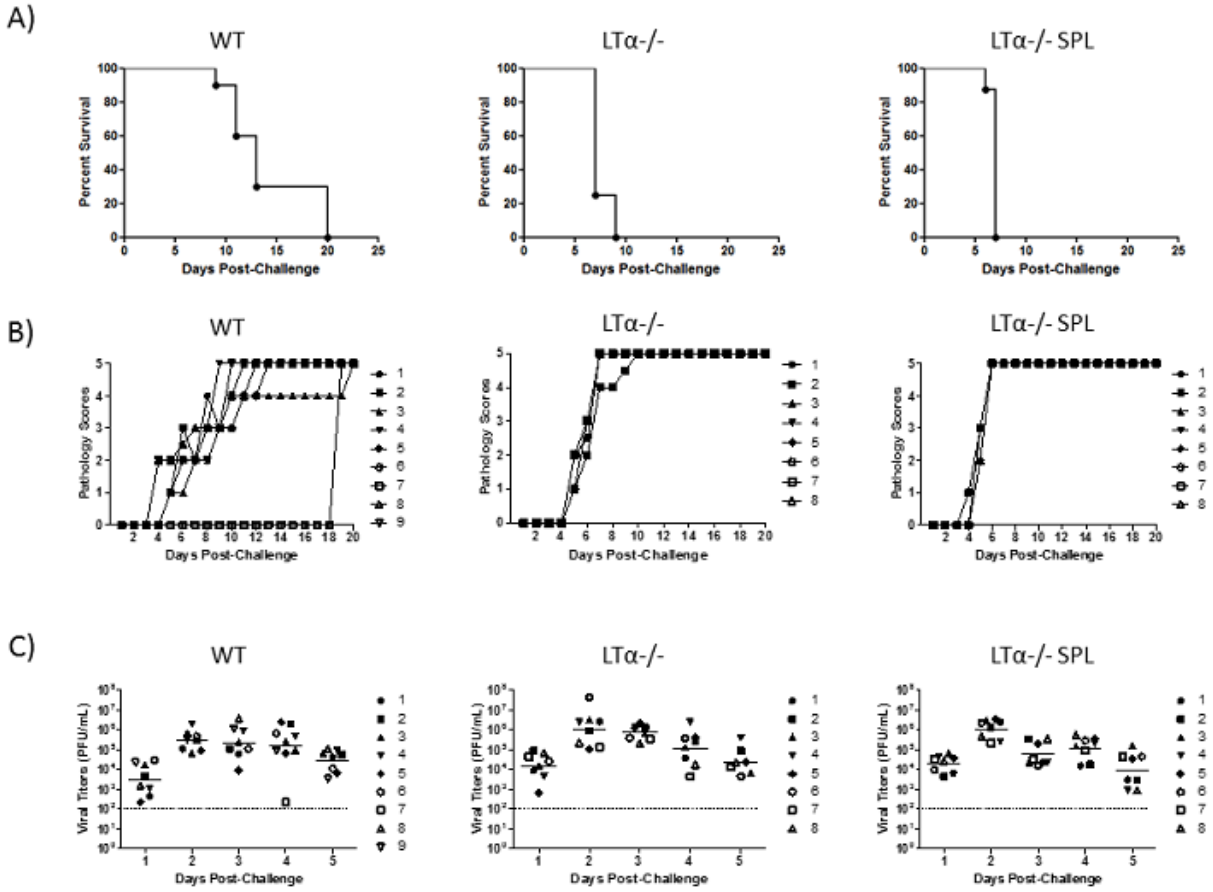

Figure 2: IVAG immunization of WT and LTα-/- OVX mice with HSV-2 gD plus CpG does not protect against low dose HSV-2 challenge. WT and LTα-/- OVX mice were immunized intravaginally with 5 µg HSV-2 gD plus 30 µg CpG. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done. Plaques were counted and viral titers were expressed as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

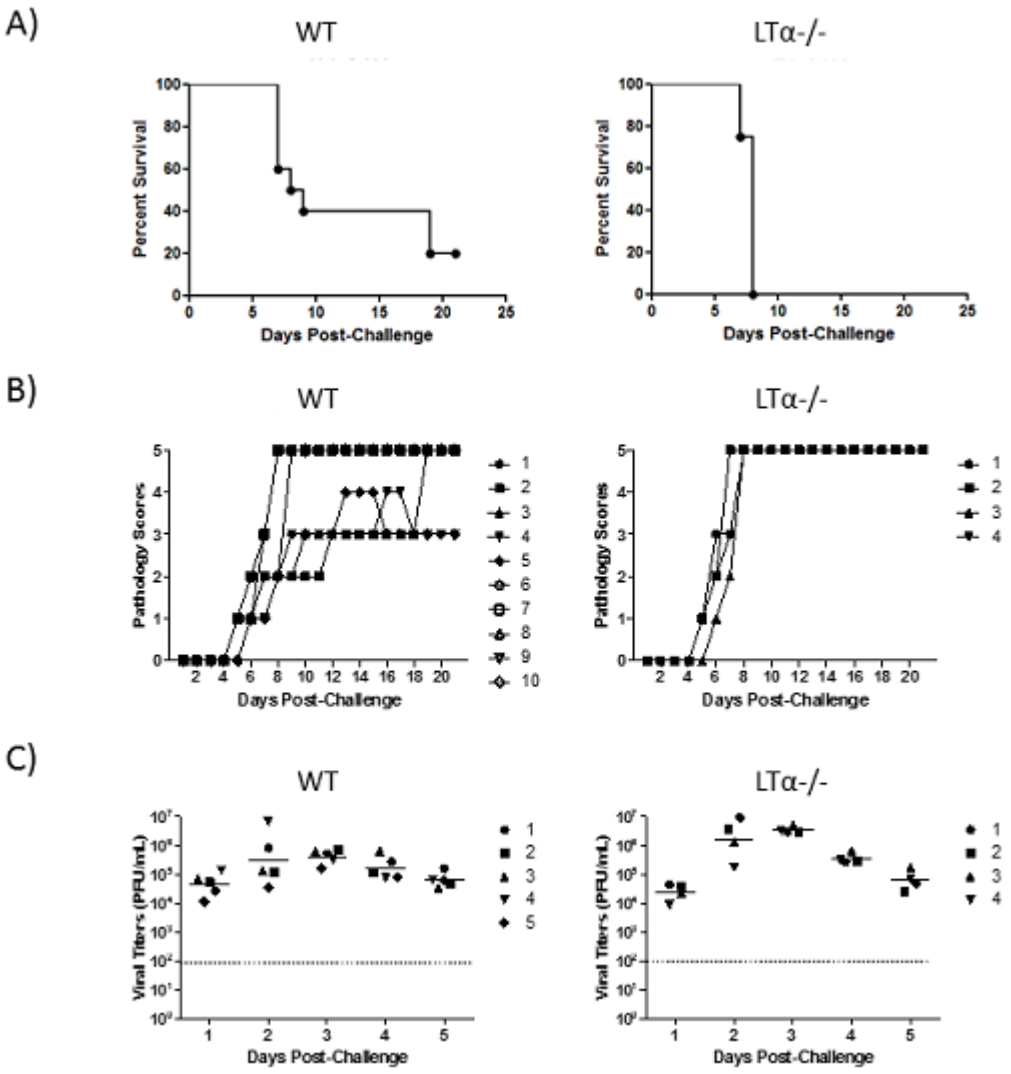

Figure 3: IVAG immunization of WT and LTα-/- OVX mice with heat-inactivated HSV-2 plus CpG does not protect against low dose HSV-2 challenge. WT and LTα-/- OVX mice were immunized intravaginally with $1x10^4$ PFU heat-inactivated HSV-2 plus 30 µg CpG. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done. Plaques were counted and viral titers were expressed

as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

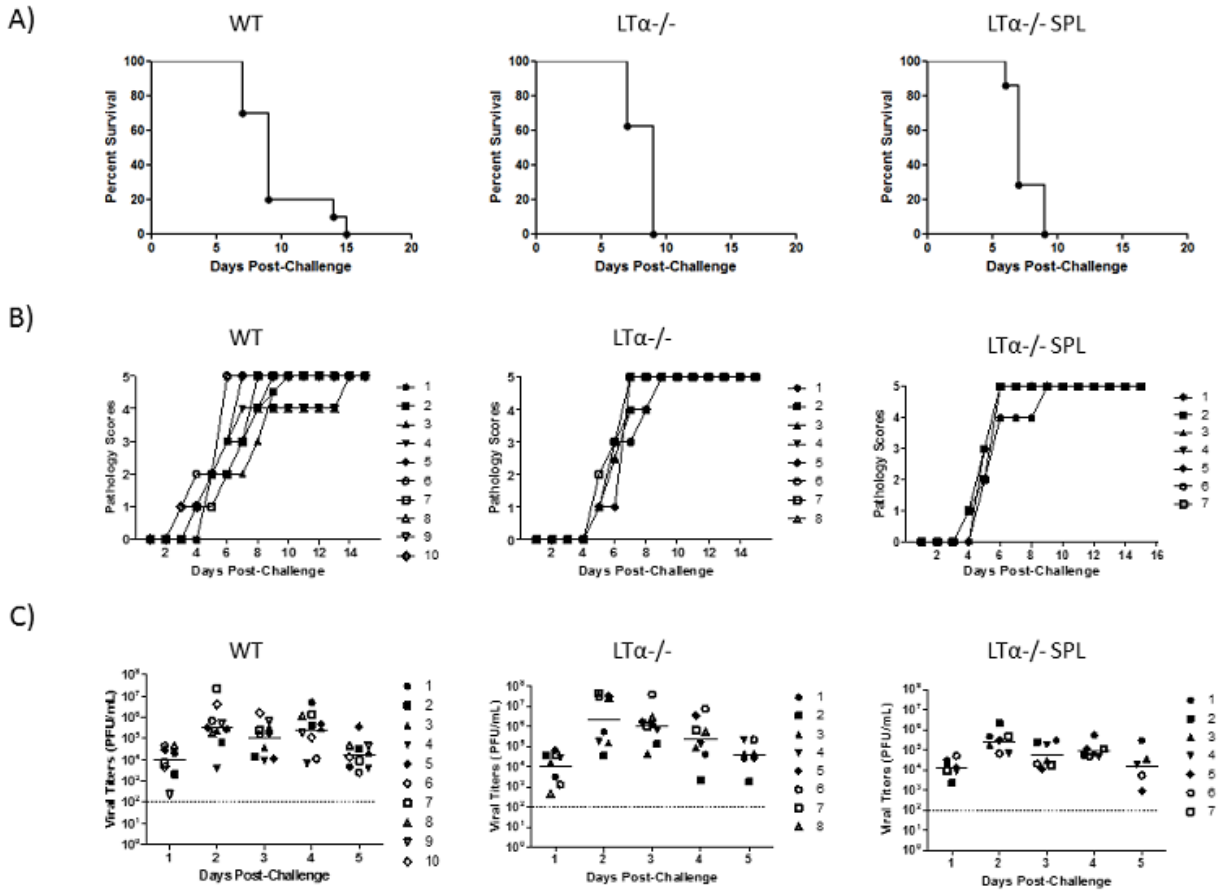

Figure 4: IN immunization of WT and LTα-/- OVX mice with HSV-2 gD plus CpG does not protect against low dose HSV-2 challenge. WT and LTα-/- OVX mice were immunized intranasally with 5 µg HSV-2 gD plus 30 µg CpG. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done. Plaques were counted and viral titers were expressed as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

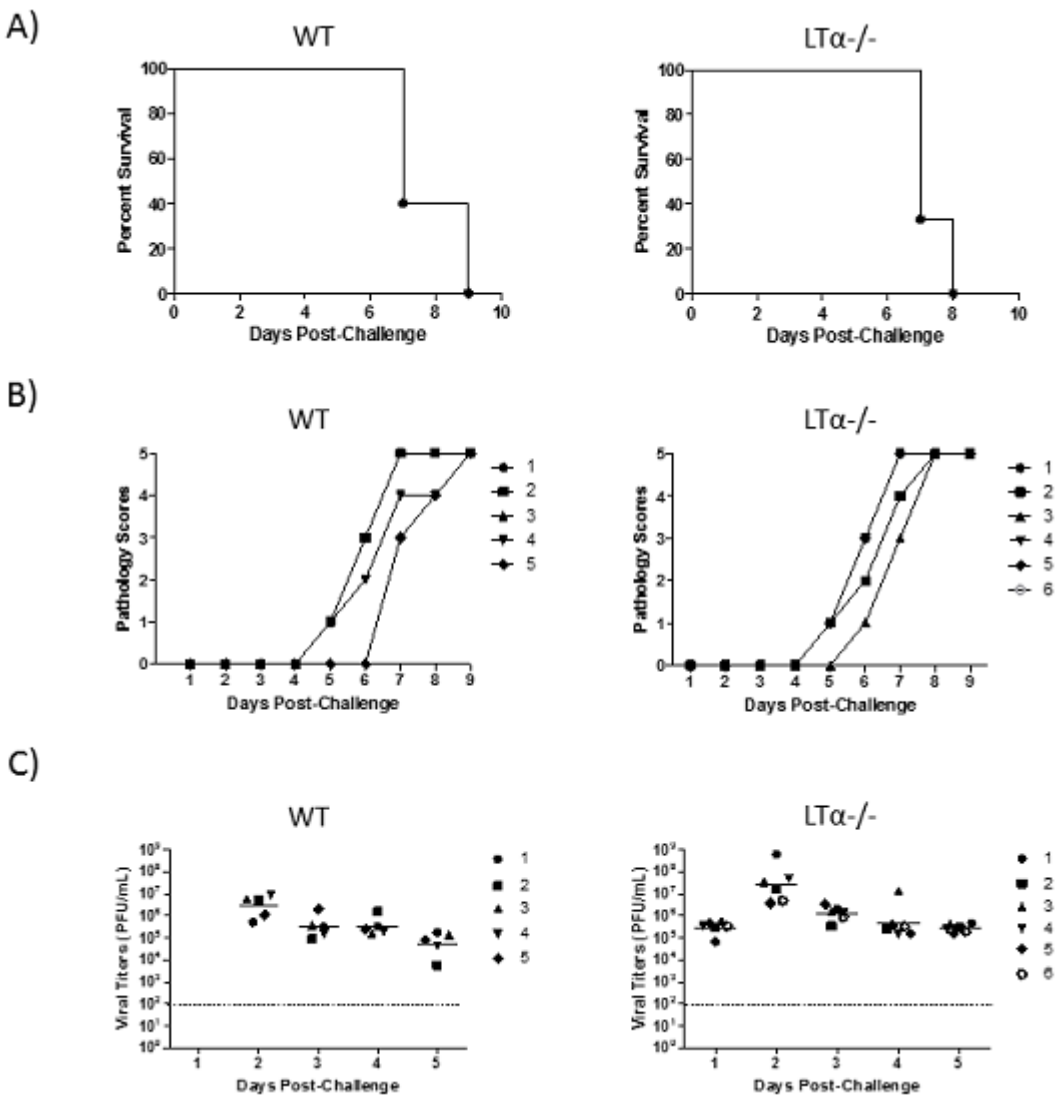

Figure 5: IN immunization of WT and LTα-/- OVX mice with heat-inactivated HSV-2 plus CpG does not protect against low dose HSV-2 challenge. WT and LTα-/- OVX mice were immunized intranasally with $1x10^4$ PFU heat-inactivated HSV-2 plus 30 µg CpG. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done. Plaques were counted and viral titers were expressed -

as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

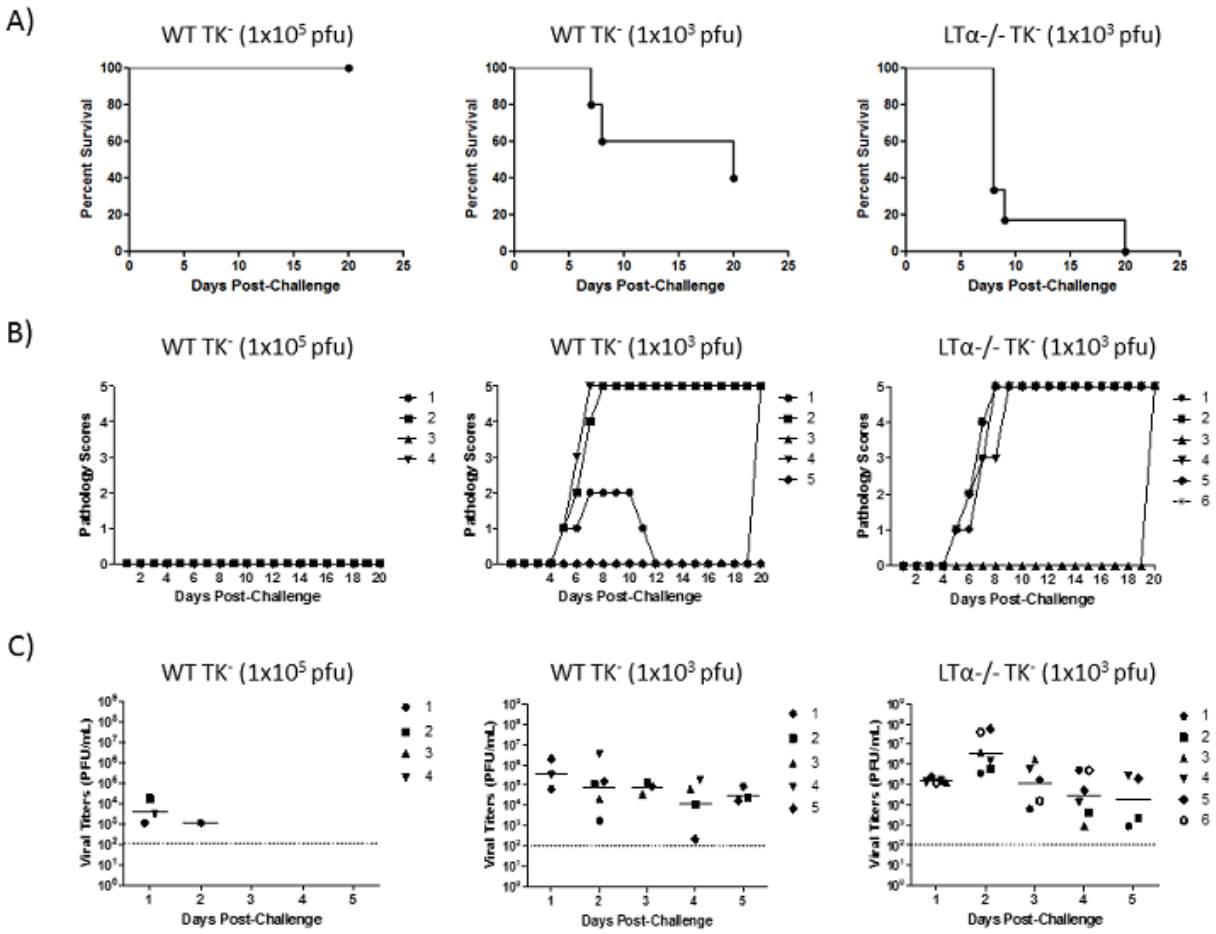

Figure 6: IN immunization of WT OVX mice with high dose TK$^-$ HSV-2 protects against low dose HSV-2 challenge. WT and LTα-/- OVX mice were immunized intranasally with 1x10^5 PFU TK$^-$ HSV-2 or 1x10^3 PFU TK$^-$ HSV-2. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with 5x10^3 PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done. Plaques were counted and viral titers were expressed as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

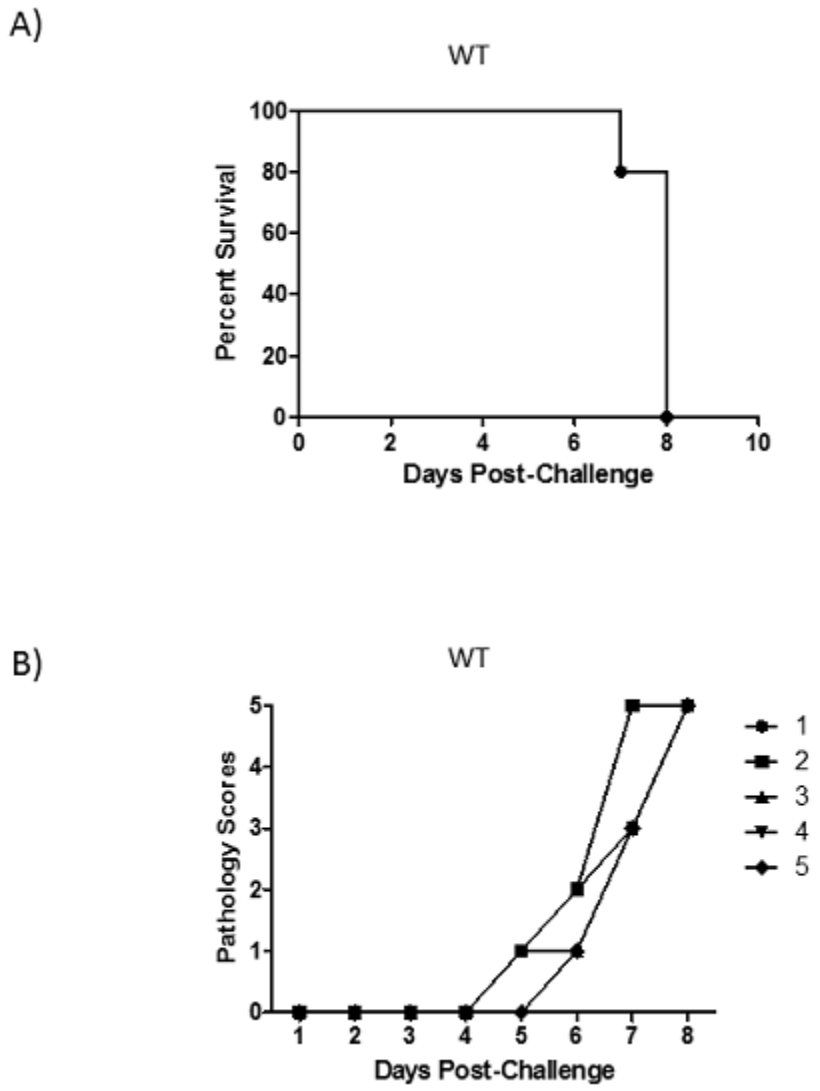

Figure 7: Chemokine pull does not enhance protection in IN immunized mice following genital HSV-2 infection. WT OVX mice were immunized intranasally with $1x10^4$ PFU heat-inactivated HSV-2 plus 30 µg CpG. Two weeks later, the immunization was repeated. Three weeks following the second immunization, mice were treated intravaginally with 3 µg CXCL9 and 3 µg CXCL10. Two days following chemokine treatment, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival and (B) pathology were monitored daily post-challenge.

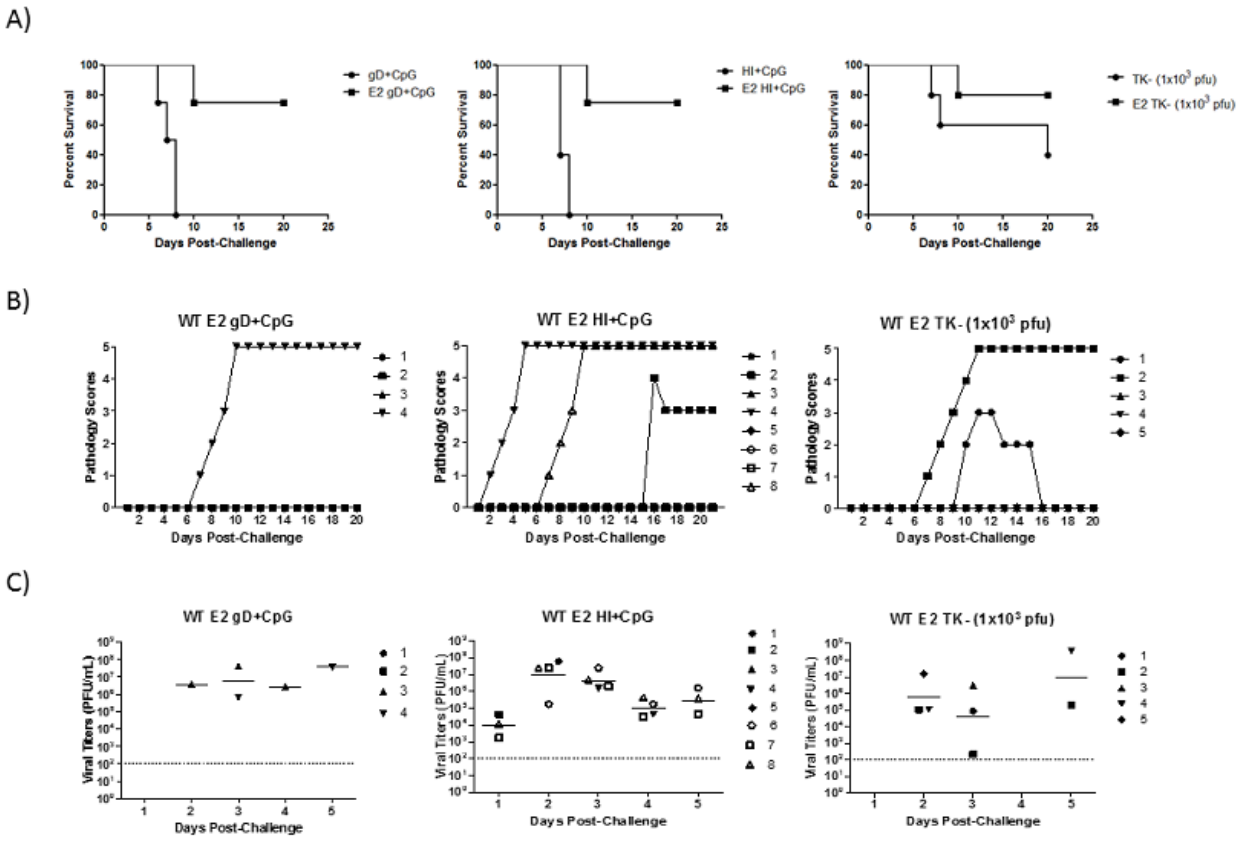

Figure 8: Estradiol pre-treatment enhances protection against genital HSV-2 infections in IN immunized WT OVX mice. WT OVX mice were treated with estradiol pellets and one week later were immunized intranasally with 5 µg HSV-2 gD plus 30 µg CpG, $1x10^4$ PFU heat-inactivated HSV-2 plus 30 µg CpG, or $1x10^3$ PFU TK^- HSV-2. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done. Plaques were counted and viral titers were expressed as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

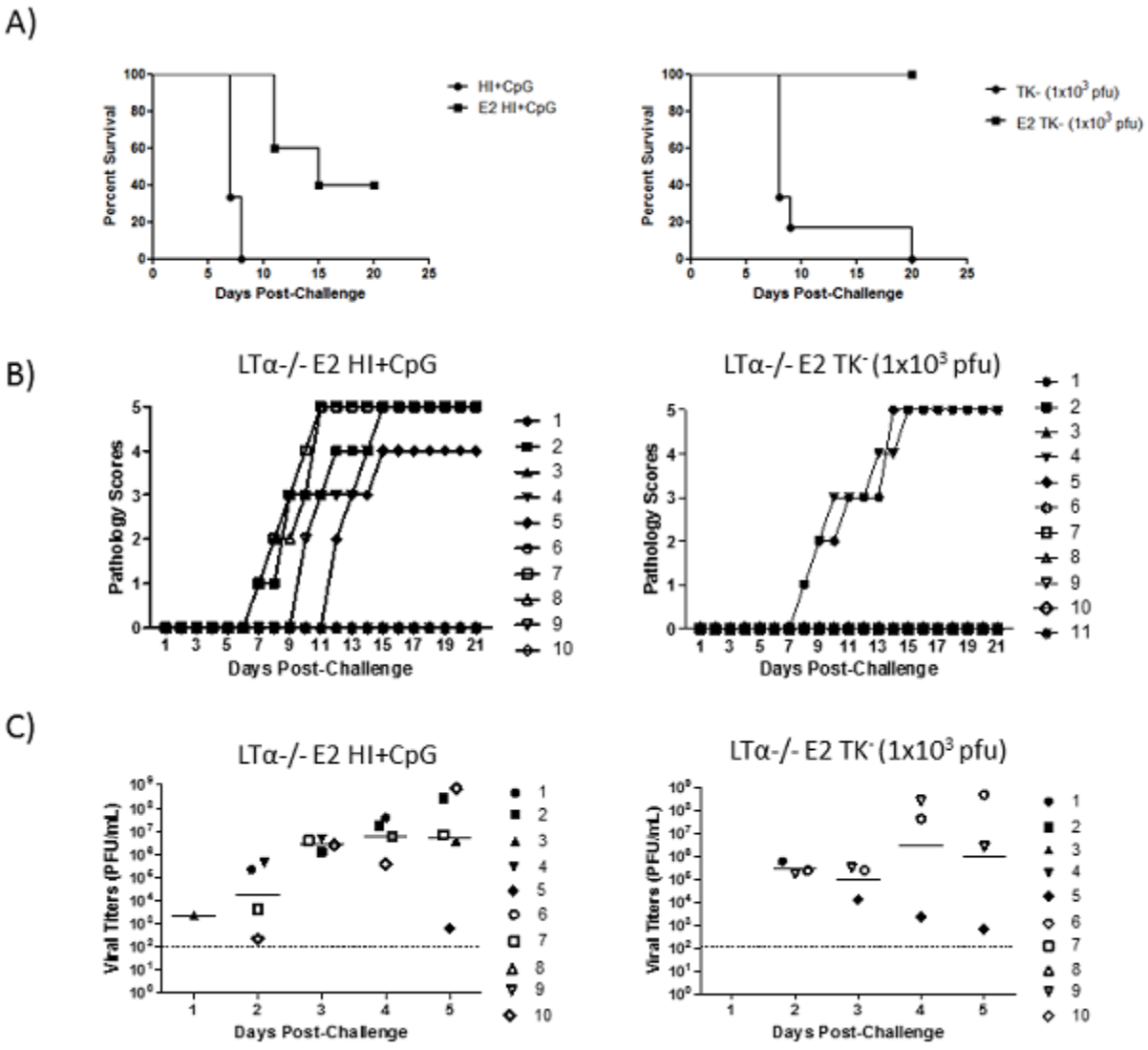

Figure 9: Estradiol pre-treatment enhances protection against genital HSV-2 infections in IN immunized LTα-/- OVX mice. LTα-/- OVX mice were treated with estradiol pellets and one week later were immunized intranasally with $1x10^4$ PFU heat-inactivated HSV-2 plus 30 µg CpG or $1x10^3$ PFU TK^- HSV-2. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were

done. Plaques were counted and viral titers were expressed as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

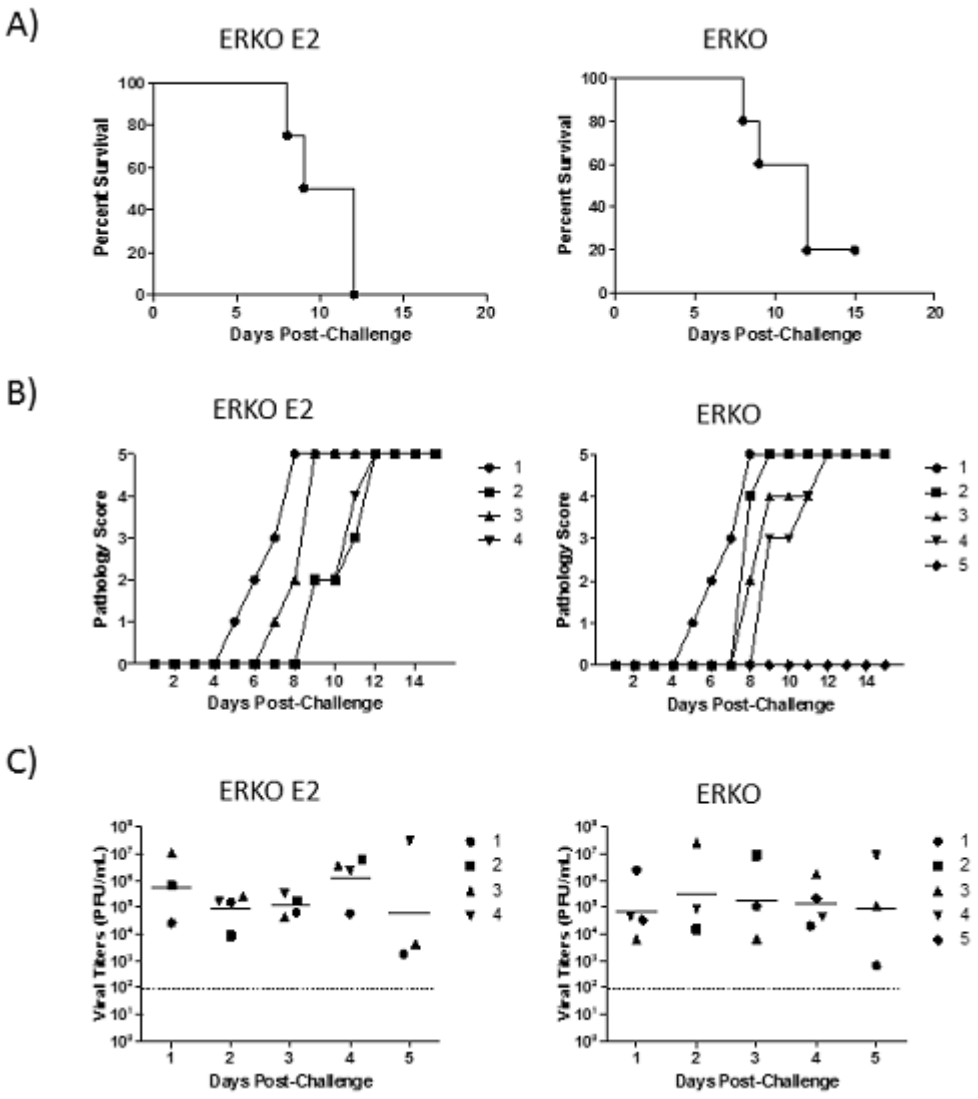

Figure 10: IN immunization, with or without estradiol pre-treatment, does not protect against genital HSV-2 challenge in estradiol receptor knockout mice. ERKO OVX mice were treated with estradiol pellets, or left untreated, and one week later were immunized intranasally with $1x10^3$ PFU TK^- HSV-2. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. (A) Survival was monitored daily post-challenge. (B) Pathology was monitored daily post-challenge. (C) Vaginal washes were collected daily post-challenge from all three groups and viral plaque assays were done. Plaques were

counted and viral titers were expressed as PFU/mL. Each symbol represents a single animal. The dashed line shows the lower detection limit of the assay.

Chapter 4

Estradiol increases IL-17-producing Th17 and IFNγ-producing Th1 responses in the genital tract of immunized mice following HSV-2 challenge

Kristy L. Roth, Varun Anipindi and Charu Kaushic

This study examined the mechanism by which estradiol treatment leads to enhanced protection of immunized mice following HSV-2 infection. We found that estradiol treatment increased the recruitment of mucosal effector CD4+CD103+ T cells into the vaginal tract following HSV-2 challenge. In addition, estradiol treatment upregulated Th17 cells in the vaginal tract of mice following HSV-2 challenge. This increase in Th17 cells was accompanied by an early IFNγ response and a decreased TNFα response compared to mock controls.

Dr. Charu Kaushic, Varun Anipindi and I were responsible for design and interpretation of the experiments. I performed all experiments, with some help from Varun Anipindi. Varun Anipindi and I were responsible for the generation and analysis of the data. I wrote this chapter with feedback and suggestions from Dr. Kaushic.

ABSTRACT

Previously we have shown that estradiol (**E2**) treatment prior to immunization can enhance vaccine efficacy in an HSV-2 mouse model. To determine the mechanism by which E2 improves vaccine efficacy, we examined the presence of CD4+ T cells locally in the vaginal tract and draining lymph nodes, as well as systemically in the spleen, of E2-treated or mock-treated control immunized mice, following HSV-2 challenge. E2 treatment enhanced the recruitment of mucosal effector CD4+CD103+ T cells into the vaginal tract of immunized mice following genital HSV-2 challenge. These vaginal tract CD4+ T cells had a Th17 phenotype, which was unique to the vaginal tract of E2-treated mice as cells from the draining lymph nodes and spleens and vaginal tract from mice not treated with E2 did not produce significant quantities of IL-17, suggesting that effector Th17 cell responses localize to the vaginal tract following genital HSV-2 challenge in mice immunized following E2 treatment. Notably, in E2-treated, immunized mice, the increase in Th17 cells correlated with an early increase in IFNγ+ Th1 cells and decreased proportions of TNFα+ CD4+ T cells in the vaginal tract following HSV-2 infection compared to mock-treated mice. Overall, these results suggest that the vaginal tract is a unique microenvironment regulated by E2, which preferentially induces mucosal Th17 cell responses, that is accompanied by an accelerated IFNγ response and decreased TNFα responses, thereby providing a possible explanation for how E2 treatment results in better protection against subsequent genital HSV-2 challenge with no accompanying pathology.

INTRODUCTION

Genital herpes is one of the most prevalent sexually transmitted infections in the world and recent estimates indicate that over 500 million people are infected by HSV-2 (Looker *et al.*, 2008). Women are more susceptible to HSV-2 infection than men, and the seroprevalence in women is twice as high as men (Xu *et al.*, 2006). These gender-specific differences in susceptibility to sexually transmitted infections can be attributed, at least in part, to the presence of the female sex hormones, estradiol (**E2**) and progesterone (**P4**), in the female genital tract. A number of clinical and epidemiological studies illustrate that sex hormones influence genital tract infections in women (Sonnex, 1998). Both the stage of menstrual cycle and/or use of hormonal contraceptives are known to affect infection with candidiasis, gonorrhea, HSV-2, HIV and *Chlamydia* in women (Crowley *et al.*, 1997; Martin *et al.*, 1998; Sonnex, 1998). In rhesus macaque models, subcutaneous implants of P4 increased susceptibility to vaginal SIV infection, whereas estrogen was able to protect against SIV (Marx *et al.*, 1996; Smith *et al.*, 2000). Studies in mouse models also show similar effects of hormones on sexually transmitted infections (Gillgrass *et al.*, 2005a; Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008).

Previous studies in our lab have also shown that the presence of E2 and P4 in the FGT can influence the generation of antiviral immune responses following HSV-2 challenge in immunized mice. For example, intravaginal (**IVAG**) or intranasal (**IN**) immunization with live attenuated HSV-2 (**TK$^-$ HSV-2**) under the influence of E2 leads to greater protection with no pathology, whereas immunization under the influence of P4

leads to protection, but with greater inflammation and immunopathology (Bhavanam *et al.*, 2008; Gillgrass *et al.*, 2010). More recently, we have also found that following IN immunization with a subunit vaccine (HSV-2 gD plus mucosal adjuvant CpG), an inactivated virus vaccine (heat-inactivated HSV-2 plus CpG) or a live attenuated virus vaccine (TK^- HSV-2), mice were not protected against a subsequent genital HSV-2 challenge. However, when mice were treated with E2 prior to IN immunization, they showed increased protection following HSV-2 challenge. These observations suggest that E2 treatment leads to improved protection against genital HSV-2 infection, but the precise mechanism by which this occurs has not been examined.

Both antigen presenting cells (**APCs**), such as dendritic cells (**DCs**), and T cells are critical for the induction of antiviral immune responses against HSV-2. Submucosal DCs in the vagina sample HSV-2 antigens and present them to antigen-specific CD4+ T cells in the local draining lymph nodes 48-72h after infection, thereby inducing the differentiation of these CD4+ T cells into IFNγ+ Th1 cells, which are critical for the clearance of infection (Zhao *et al.*, 2003; Iijima *et al.*, 2007). The local microenvironment at the time of antigen presentation can greatly influence the differentiation of T cell responses. For example, the presence of cytokines IL-12, IFNα/β and TNFα can induce CD4+ T cells to differentiate into Th1 cells, whereas the presence of IL-6, TGFβ and IL-23 can induce the differentiation of Th17 cells (Kapsenberg, 2003; Reis e Sousa, 2006; Weaver *et al.*, 2006; Manel *et al.*, 2008). In our previous studies where E2 treatment led to protection with no accompanying pathology whereas P4

treatment resulted in protection with increased genital inflammation and immunopathology following HSV-2 challenge, the only difference in these mice was the hormone environment in the genital tract at the time of immunization. This suggests that the outcome of viral challenge in immunized mice was dependent on the hormones present at the time of immunization. Since the microenvironment greatly influences the priming of DCs, and subsequent T cell activation and differentiation, this suggests that the hormone treatments prior to immunization must prime vaginal tract DCs to induce differential T cell responses, which are then responsible for the outcome of genital HSV-2 infection.

The mechanism by which female sex hormones influence antiviral T cell responses in the genital tract has not been determined. Therefore, in this study, we wanted to determine how E2 treatment leads to enhanced protection in immunized mice following HSV-2 challenge. To do this, we examined the different types of T cell responses present locally in the vaginal tract and draining lymph nodes, as well as systemically in the spleen, of mice treated with E2, or left untreated (mock), and then immunized with TK^- HSV-2 and challenged IVAG with WT HSV-2. We show that E2 treatment prior to immunization induces potent Th1 and Th17 responses, but decreased TNFα+ T cell responses, in the genital tract of mice following HSV-2 challenge. Understanding the ability of female sex hormones within the genital tract microenvironment to influence $CD4^+$ T cell responses is essential for developing vaccine

formulations that can induce optimal immune responses against sexually transmitted infections (**STIs**) in the genital mucosa.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice, 6-8 weeks old, were purchased from Jackson laboratory (Maine, USA). All mice were housed and maintained under standard temperature controlled conditions in special pathogen-free rooms that followed a 12-h day and 12-h night schedule. The Animal Research Ethics Board at McMaster University approved all of the animal protocols in this study.

Ovariectomy. The ovaries were removed by making two bilateral incisions, followed by small incisions through the peritoneal wall. The ovaries were excised through the incisions by grasping the fat, ligating the blood vessels and removing the ovaries. The incisions were closed by surgical clips and the mice were allowed to recover for at least 10 days before the start of experiments.

Hormone treatments. Mice were treated with 17β-estradiol pellets and progesterone pellets, which were purchased from Innovative Research of America (Sarasota, Florida, USA). Mice were anaesthetized using injectable anaesthetic (150 mg of ketamine/kg-10 mg of xylazine/ml) given intraperitoneally, hormone pellets were implanted subcutaneously on the lateral side of the neck. The quantity of hormones in the 17β-estradiol and progesterone pellets was as follows: 476 ng/mouse/day and 476 μg/mouse/day, respectively, designed to be released equally for 21 days. For mock control groups, mice were anaesthetized as before and incisions were made in the lateral side of the neck, but pellets were not inserted.

Immunization. Mice were immunized intranasally with thymidine kinase-negative (TK^-) HSV-2, strain 333, at a dose of $1x10^3$ PFU. Mice were anaesthetized using the gaseous anaesthetic, isoflorane, and immunized with 5 μl of the inoculum into each nare with a micropipette. All mice were immunized twice, two weeks apart.

WT HSV-2 challenge. Four weeks following the second IN immunization, mice were anaesthetized using injectable anaesthetic (150 mg of ketamine/kg-10 mg of xylazine/ml) given intraperitoneally, placed on their backs, and challenged intravaginally with 10 μl of wild-type HSV-2, strain 333 at a dose of $5x10^3$ PFU. Mice were kept on their backs under the influence of anaesthesia for 45 min to 1 h to allow the inoculum to infect.

Lymph node and spleen cell preparation. Iliac lymph nodes that drain the genital tract and spleens were dissected, and a single cell suspension was prepared by teasing the LN or spleen. Debris was allowed to settle for 2 min, and supernatant containing single cells was recovered and spun centrifuged at 5000 x g for 10 min. LN cells were washed with RPMI 1640 medium containing 5% FBS and plated at a density of $2x10^6$ cells/well in 24 well plates for flow cytometric analyses. Splenic red blood cells were then lysed by resuspending the cell pellets in 2 ml of ACK lysis buffer. Samples were incubated at room temperature for 5 min and then centrifuged at 5000 x g for 10 min. Spleen cells were washed with RPMI 1640 medium containing 5% FBS and plated at a density of $2x10^6$ cells/well in 24 well plates for flow cytometric analyses.

Vaginal cell preparation. Vaginal tracts were removed, cut lengthwise and minced with scalpel blades. The tissues were digested in 15 ml of RPMI containing 0.00157 g/ml

collagenase A (Roche, Manheim, Germany) for 1 hr at 37^0C. The supernatants were collected and the tissues were digested in another 15 ml of collagenase A in RPMI for 1 hr at 37^0C. At the end of digestion, supernatants were removed and added to that already collected. The remaining tissue was passed through a 40 μm filter (Small Parts, Miama Lakes, USA). All supernatants were then passed through a 40 μm filter into a 50 ml Falcon tube. Tubes were centrifuged for 10 min at 1200 rpm at 4^0C. Cells were resuspended with RPMI 1640 medium containing 5% FBS and plated at a density of $1x10^6$ cells/well in 24 well plates for flow cytometric analyses and $5x10^5$ cells/well in 96 well plates for cell proliferation analyses.

Flow cytometry. Vaginal tract, LN and spleen cell preparations in 24 well plates were treated with 2 μl of Protein Transport Inhibitor Cocktail (eBioscience) with or without Cell Stimulation Cocktail (eBioscience) and incubated at 37^0C for 18 hrs. The Cell Stimulation Cocktail is a cocktail of phorbol 12-myristate 13-acetate (**PMA**) and ionomycin, which non-specifically activates T cells. All cells were then aliquoted into tubes, centrifuged at 5000 rpm for 5 min, and then resuspended in 100 μl of 0.2% BSA-PBS. Cells were incubated with Fc block (anti-mouse CD16/32; eBioscience) for 10 min to reduce nonspecific immunofluorescent staining and then stained for cell surface markers using the following antibodies: Brilliant violet 785 anti-mouse CD3, Alexa Fluor 700 anti-mouse CD44, Brilliant violet 605 anti-mouse CD62L, Brilliant violet 510 anti-mouse CD103 and PE/Cy7 anti-mouse CD8a (all purchased from Biolegend), and Brilliant violet 421 anti-mouse CD4 (purchased from BD Horizon). Cells were incubated

with these antibodies on ice for 30 min in the dark. Cells were then washed with 1 ml of 0.2% BSA-PBS and stained for intracellular cytokines using Transcription Factor Buffer Set (BD Pharmingen) following manufacturer's protocol. Briefly, cells were resuspended in 1 ml of Fix/Perm buffer and incubated on ice for 40-50 min in the dark. Cells were washed with 1 ml of Perm/Wash buffer and resuspended in 100 μl of Perm/Wash buffer and stained for intracellular markers using the following antibodies: FITC anti-mouse IFNγ or FITC rat IgG1 isotype control, (purchased from BD Pharmingen), APC rat anti-mouse IL-17A or APC rat IgG1 isotype control, Brilliant violet 650 anti-mouse TNFalpha or Brilliant violet 650 rat IgG1 isotype control (all purchased from Biolegend). Cells were incubated on ice for 40-50 min in the dark and then washed with 1 ml Perm/Wash buffer. Cells were resuspended in 400 μl of 0.2% BSA-PBS and put into a 5 ml polystyrene round-bottom tube with cell-strainer cap (BD Falcon, Franklin Lakes, NJ, USA). Stained cells were analyzed by flow cytometric analysis using a BD LSRII Flow Cytometer System. Results were analyzed using FlowJo Software (BD Bioscience Pharmingen).

RESULTS

Estradiol-treated mice have increased IL-17 production by vaginal tract cells. Ongoing studies in our lab have found that vaginal tract DCs, under the influence of different hormones, can differentially regulate T cell responses *in vitro.* Specifically, DCs isolated from the vaginal tract of mice during the estrogen-high estrus stage, or from mice treated with exogenous E2, prime T cells *in vitro* to produce significant quantities of IL-17 compared to DCs isolated from the vaginal tracts of mice with no hormones present (OVX mice), during the progesterone-high diestrus stage or from non-hormone-treated or P4-treated mice (Anipindi, Roth, *et al,* in review). However, whether this occurs *in vivo* as well has not been determined. Therefore, first we wanted to determine whether vaginal tract cells from E2-treated mice produced higher levels of IL-17, compared to P4-treated mice. To do this, we treated OVX mice with E2 or P4 pellets for 14 days. The vaginal tracts of these mice were collected and cells were analyzed by flow cytometry for IL-17 production. As expected, the vaginal tracts from E2-treated mice had higher proportions of IL-17+ cells compared to vaginal tracts from P4-treated mice (Figure 1).

Estradiol-treated mice have increased proportion of vaginal tract CD4+CD103+ mucosal T cells following HSV-2 challenge. To determine why E2-treated mice are better protected following HSV-2 infections, we examined the presence of T cells in the vaginal tract, draining lymph nodes and spleen of immunized mice on different days post-challenge. WT OVX mice were treated with E2 pellets, or left untreated (mock), and

then IN immunized twice, two weeks apart, with $1x10^3$ PFU TK^- HSV-2. Four weeks following the second immunization, mice were challenged IVAG with $5x10^3$ PFU WT HSV-2. On days 1, 3 and 5 post-challenge, the vaginal tracts, draining lymph nodes and spleens of these mice were collected, and single cell suspensions were cultured overnight with or without *in vitro* stimulation and then analyzed by flow cytometry for the presence of different types of T cells by staining for cell surface markers and intracellular cytokines.

First, we examined the presence of CD3+ cells and found that E2-treated mice had higher frequency of vaginal tract CD3+ cells on all 3 days post-challenge compared to mock-treated mice (Figure 2A and Table 1). Similar increased proportions of CD3+ cells were also observed in the draining lymph nodes, but the proportions of CD3+ cells in the spleens were similar between E2-treated and mock-treated mice (Table 1). Despite higher proportion of CD3+ cells, the proportion of CD4+ and CD8+ T cells in the vaginal tracts of E2-treated mice was lower compared to mock-treated mice on all 3 days post-challenge, with no observable differences in the lymph nodes or spleens (Figure 2B and Table 1). E2-treated mice had a higher percentage of vaginal tract CD3+CD4+ cells expressing the mucosal marker CD103 (36.5%) compared to mock-treated mice (12.0%), whereas very few cells (<15%) expressed this mucosal marker in the lymph nodes or spleen (Figure 2C and Table 1) and there was no difference between E2-treated and mock-treated mice. These results suggest that E2 treatment increases the

recruitment of mucosal CD4+CD103+ effector T cells in the vaginal tract of immunized mice following HSV-2 challenge.

Estradiol treatment upregulates percentage of Th17 cells and IFNγ+ Th1 cells in the vaginal tract of immunized mice following HSV-2 challenge. To examine the functionality of these CD3+CD4+ T cells, we measured the production of intracellular cytokines IFNγ and IL-17. There was a higher proportion of T cells expressing IFNγ and IL-17 in the vaginal tract compared to T cells from the draining lymph nodes or spleen (Figure 3A, B and Table 2), showing that the highest proportions of effector T cells were localized to the vaginal tract following HSV-2 challenge. E2-treated mice showed a higher proportion of IFNγ+ Th1 and IL-17+ Th17 CD4+ T cells at earlier time points, days 1 and 3 post-challenge compared to mock controls (Figure 3A & Table 1). By day 5 post-challenge, a higher proportion of Th17 cells remain in the vaginal tract of E2-treated mice compared to mock, but the frequency of Th1 cells was lower in E2-treated mice compared to mock. In addition, an increased proportion of multi-functional IFN-γ^+ IL-17^+ $CD4^+$ T cells was observed in E2-treated mice (d1: 1.81%, d3: 4.5%, d5: 1.71%) which were largely absent (<1%) in mock controls (Figure 3 and Table 2). To determine if the increase in Th17 and IFNγ+ Th1 cells seen following *in vivo* challenge was representative of the total T cell population in the vaginal mucosa, Th1 and Th17 cells were compared between E2-treated and mock controls following *in vitro* total T cell stimulation, at all three days post-challenge (Figure 3B, Table 2). *In vitro* stimulation upregulated the proportion of T cells expressing IL-17 and IFNγ in both E2-treated and

mock control mice, but E2-treated mice still had increased proportions of both Th17 and Th1 cells on all 3 days post-challenge (Figure 3B and Table 2). Overall, since mock control mice do not have a significant Th17 response and have a delayed IFNγ+ Th1 response compared to E2-treated mice, this suggests that the increased Th17 response observed in E2-treated mice may lead to an early enhancement of IFNγ+ T cell responses in the vaginal tracts of E2-treated mice post-HSV-2 challenge. This Th17-induced early enhancement of IFNγ+ T cell responses in E2-treated mice could thereby result in better protection against subsequent HSV-2 challenge.

Estradiol treatment upregulates Th17 responses but decreases TNFα+ T cell responses in the vaginal tract of immunized mice following genital HSV-2 challenge. To further characterize the functionality of the CD3+CD4+ T cell populations, the production of intracellular cytokines IL-17 and TNFα was measured on all 3 days post-challenge. In the vaginal tract, in contrast to the increased proportions of IL-17+ and IFNγ+ T cells, E2-treated mice had lower proportions of TNFα+ T cells on days 3 and 5 post-challenge compared to mock control mice (Figure 4A and Table 3). However, following *in vitro* stimulation, the total T cell population in the vaginal mucosa of E2-treated mice had similar levels of TNFα+ T cells compared to mock control mice (Figure 4B and Table 3), suggesting that while T cells in the vaginal tract of mice are capable of producing TNFα, E2 treatment preferentially increases IL-17 and IFNγ production, but decreases TNFα production following HSV-2 challenge. In addition, both the draining lymph nodes and spleen had much higher proportions of TNFα+ cells compared to the

vaginal tract, but E2 treatment did not affect the percentages of these cells in the lymph nodes or spleens (Table 3). Overall, these results suggest that while E2 enhances the frequency of IL-17+ T cells, it decreases the frequency of TNFα+ T cells in the vaginal tract of mice following HSV-2 challenge.

DISCUSSION

Previously we have shown that the presence of E2 during immunization improves antiviral protection against genital HSV-2 infections by enhancing survival and decreasing external genital pathology (Gillgrass *et al.*, 2005a; Bhavanam *et al.*, 2008; Gillgrass *et al.*, 2010). However, the precise mechanisms by which E2 enhances protection in the genital tract has not been previously examined. Here, we show that E2-mediated enhanced protection correlated with increased mucosal CD4+ Th17 cell responses and accelerated IFNγ+ Th1 cell responses in the genital tract following HSV-2 challenge. In addition, we also show that E2-treated mice had decreased TNFα+ T cell responses compared to mock controls. While the contribution of IFNγ+ Th1 cells to antiviral responses and viral clearance during HSV-2 infection is well understood, this is the first report indicating that Th17 cells may be involved in enhancing antiviral immune responses against HSV-2 infection in the genital tract.

At present, it is unclear how this E2-induced Th17 bias affects antiviral responses in the FGT. Although IL-17-producing Th17 cell responses have been found to play a role in clearance and protection against vaginal *Candida* and *N. gonorrhoeae* infections (Feinen *et al.*, 2010; Pietrella *et al.*, 2011; Hernandez-Santos & Gaffen, 2012), their role in genital HSV-2 infections has not been examined. However, a recent study conducted on the intracellular bacterium, *Mycobacterium tuberculosis* (**Mtb**), showed that immunization caused an accelerated IFNγ response by CD4+ T cells in the lung during subsequent Mtb infection. Th17 responses induced in the lung following immunization

played a critical role in the rapid induction of Th1 IFNγ responses and depletion of IL-17 during challenge reduced the accumulation of IFNγ-producing CD4+ T cells in the lungs, thereby leading to delayed bacterial clearance (Khader *et al.*, 2007). Similarly, in the present study, we found that E2-treated mice have increased IL-17+ Th17 cell responses and accelerated IFNγ+ Th1 cell responses in the genital tract following HSV-2 challenge. Therefore, it is possible that E2 treatment results in increased IL-17-producing Th17 cell responses following immunization, which leads to enhanced protection following a subsequent genital HSV-2 infection by rapidly inducing IFNγ+ Th1 cell responses. However, it does not appear that this IL-17 production is HSV-2-specific, since *in vitro* restimulation with UV-inactivated HSV-2 increased IFNγ production, but did not increase IL-17 production (data not shown). This suggests that while Th17 cells are present in the vaginal tracts of E2-treated mice, they may not be specific for HSV-2. However, further studies are required to determine the proportion and type of T cells that are HSV-2-specific.

In addition to the increased IL-17 production, it was also observed that E2-treated, immunized mice have lower proportions of TNFα+ CD4+ T cells following HSV-2 challenge compared to mock-treated mice. Since the presence of TNFα has been shown to be associated with increased immunopathology (Iwamoto *et al.*, 2007), lower levels of TNFα production by vaginal tract CD4+ T cells could also contribute to the enhanced protection and absence of genital inflammation and immunopathology observed in E2-treated, immunized mice following genital HSV-2 challenge. In other studies, we have

observed P4 treatment leads to increased proportions of TNFα+ cells in the vaginal tracts of mice following IVAG immunization with TK^- HSV-2 (V. Anipindi, unpublished data). Since P4-treated, immunized mice are protected following genital HSV-2 challenge, but show significant genital inflammation and immunopathology, this suggests that increased proportions of TNFα+ cells may contribute to the increased immunopathology observed in these mice. In addition, other studies in our lab have found that P4-treated, immunized TNFKO mice show protection following genital HSV-2 challenge with no accompanying genital pathology (data not shown), which supports the results shown here, suggesting that the decreased proportions of TNFα+ T cells in the vaginal tracts of E2-treated, immunized mice could contribute to the lack of pathology observed in these mice following genital HSV-2 challenge. However, the precise role of TNFα in HSV-2 infections has not been determined and further studies are required to examine the role of this cytokine in HSV-2-induced immunopathology.

It was also observed that E2-treated, immunized mice had a higher proportion of CD3+CD4+ T cells expressing the mucosal homing marker CD103 compared to mock controls. CD103, also known as integrin αE, binds integrin β7 to form the heterodimeric integrin molecule αEβ7 (Hadley *et al.*, 1997). It has been shown to be selectively expressed on >90% of intestinal intraepithelial lymphocytes and on 45-50% of lamina propria T cells in both mice and humans (Cerf-Bensussan *et al.*, 1987; Kilshaw & Baker, 1988; Russell *et al.*, 1994). In addition, it has also been found on T cells in the genitourinary tract (Pudney & Anderson, 1995). In contrast, αEβ7 is only expressed on

less than 5% of peripheral blood lymphocytes in humans and on only 15% of splenic T cells in mice (Cerf-Bensussan *et al.*, 1987; Lefrancois *et al.*, 1994). Similarly, we also observed a higher proportion of T cells expressing CD103 in the vaginal tracts of mice post-challenge compared to T cells from the draining lymph nodes or spleen. In addition, several studies have implicated CD103 in homing to various mucosal epithelial sites including the FGT (Cerf-Bensussan *et al.*, 1987; Schon *et al.*, 1999; Shacklett *et al.*, 2000), and one study found that the expression of CD103 by CD8+ T cells mediated the accumulation of these cells in the cervix (Kiravu *et al.*, 2011). Therefore, since it was observed that E2-treated mice had higher proportions of vaginal tract T cells expressing CD103 compared to mock controls, this suggests that E2 treatment may result in increased homing of T cells into the vaginal tracts of mice following HSV-2 challenge. Previous studies have shown that increased homing of T cells to the genital mucosa can provide better protection against subsequent genital HSV-2 challenge (Gebhardt *et al.*, 2009; Shin & Iwasaki, 2012), suggesting that the increased proportions of CD103+ T cells in E2-treated, immunized mice may be responsible for the enhanced protection against genital HSV-2 challenge observed in these mice compared to mock controls.

In addition to having increased proportions of CD103+ T cells in the vaginal tracts of mice compared to the draining lymph nodes and spleen, the vaginal tract also had higher proportions of IL-17+ and IFNγ+ T cells whereas the lymph nodes and spleen had higher proportions of TNFα+ T cells. These results suggest that there is a distinct compartmentalization of T cells between the vaginal mucosa and the lymph nodes and

spleen. In support of these results, previous studies have also shown that vaginal tract T cells are distinct from those found in other tissues (Ibraghimov & Lynch, 1994; Ibraghimov *et al.*, 1995; Perry *et al.*, 1998). In addition, ongoing studies in our lab have found that cells from the vaginal mucosa are phenotypically and functionally distinct from those found in secondary lymphoid organs or other mucosal tissues. More specifically, it was observed that DCs from the vaginal mucosa of E2-treated mice were programmed to induce Th17 cells, a phenomenon that was not observed in the lymph nodes and spleen (V. Anipindi, unpublished data). Therefore, the E2-mediated effect on DCs to prime Th17 responses may be a vaginal tissue specific phenomenon. Furthermore, the ability of E2 to specifically prime vaginal tract DCs to induce a Th17 phenotype could explain the increased IL-17+ Th17 response observed following genital HSV-2 challenge in E2-treated, immunized mice, thereby providing a potential mechanism by which E2 treatment leads to enhanced protection following genital HSV-2 challenge.

Overall, the results from this study show a potential mechanism by which E2 treatment leads to enhanced protection against HSV-2 infections following immunization. In the absence of E2, IN immunization leads to the production of both IFNγ and TNFα by vaginal tract T cells, which leads to protection against subsequent genital HSV-2 challenge with increased immunopathology. However, when E2 is present during immunization, vaginal tract T cells become IL-17-producing Th17 cells, which leads to the accelerated recruitment of IFNγ-producing Th1 cells. In addition, E2 treatment prior

to immunization results in decreased TNFα+ T cells, in the vaginal tract of mice following HSV-2 challenge. This early IFNγ response in the presence of E2 could lead to enhanced viral clearance and survival following HSV-2 challenge. In addition, the decreased TNFα response following HSV-2 challenge could result in the decreased pathology observed in E2-treated mice. Therefore, the hormonal microenvironment during immunization should be a critical consideration in the development of mucosal HSV-2 vaccines as hormones present at the time of immunization may significantly alter the induction of protective immune responses in the female genital tract.

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FIGURES

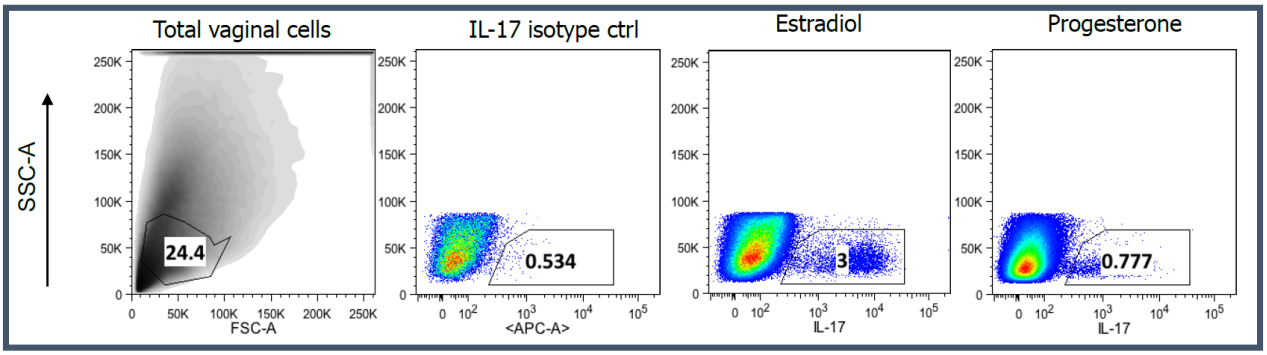

Figure 1: Estradiol-treated mice have increased IL-17 production by vaginal tract cells. WT OVX mice were treated with E2 or P4 hormone pellets for 14 days, vaginal tract cells were isolated and stained for IL-17 production by flow cytometry. Viable cells were gated on lymphocytes and then for their expression of intracellular IL-17.

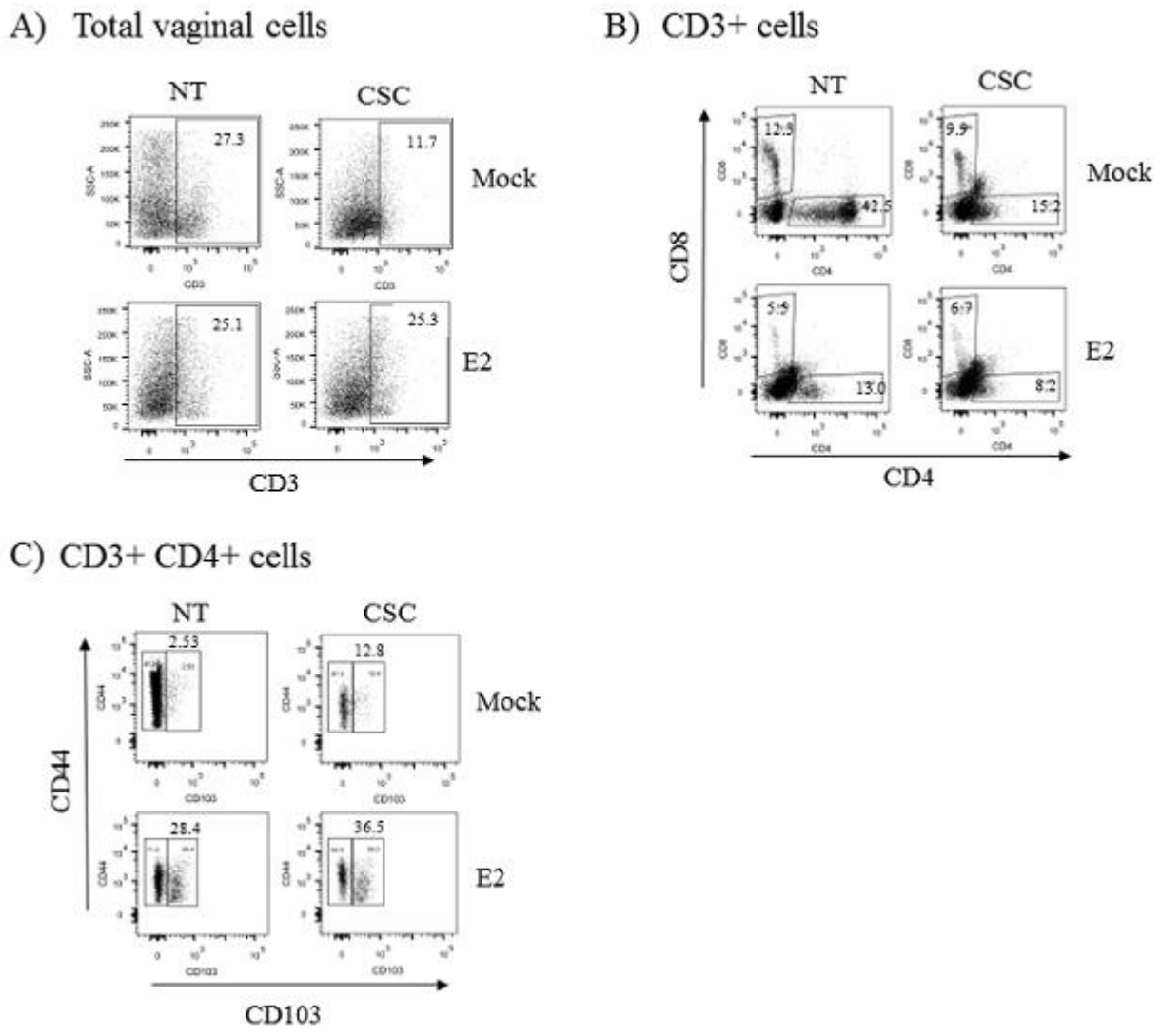

Figure 2: Estradiol-treated mice have increased proportion of vaginal tract CD4+CD103+ mucosal T cells following HSV-2 challenge. WT OVX mice were treated with estradiol pellets, or left untreated (mock), and one week later were immunized intranasally with $1x10^3$ PFU TK^-HSV-2. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. On day 5 post-challenge, the vaginal tracts of

these mice were collected and cells were cultured *in vitro* overnight with cell stimulation cocktail (CSC), or were left untreated (NT). Cells were then stained for (A) CD3; (B) CD4 and CD8; (C) CD44 and CD103 and analyzed by flow cytometry. Viable cells were gated on lymphocytes (A) and then CD3+ cells (B) and CD3+CD4+ cells (C).

		NT						CSC					
		d1 P.C.		d3 P.C.		d5 P.C.		d1 P.C.		d3 P.C.		d5 P.C.	
		Mock	E2	Mock	E2	Mock	E2	Mock	E2	Mock	E2	Mock	E2
Vaginal Tract													
	CD3+	16.5	21.9	12.7	30.0	27.3	25.1	22.0	23.9	11.6	25.0	11.7	23.3
	CD3+CD4+	10.5	6.51	15.8	5.75	42.5	10.8	7.20	5.17	11.4	4.22	13.2	8.25
	CD3+CD4+ CD103+	12.8	33.8	19.2	50.3	2.53	28.4	16.4	49.1	22.9	49.6	12.0	36.5
Lymph Node													
	CD3+	57.9	64.7	35.2	44.3	30.7	50.5	56.0	67.5	25.6	38.7	27.8	49.2
	CD3+CD4+	42.4	44.7	32.6	37.9	51.2	44.5	45.2	43.8	43.1	40.6	53.3	45.0
	CD3+CD4+ CD103+	4.74	6.73	6.34	7.59	5.34	10.4	4.15	5.10	10.1	6.86	5.50	9.16
Spleen													
	CD3+	30.0	30.8	24.9	23.9	24.9	23.0	31.9	25.5	27.0	27.4	27.0	19.0
	CD3+CD4+	44.6	33.9	45.0	47.5	46.3	31.7	44.7	40.6	43.1	43.7	43.2	39.6
	CD3+CD4+ CD103+	8.12	10.3	8.61	6.89	10.1	11.4	10.2	14.4	8.21	10.1	12.3	15.9

Abbreviations: CSC, cell stimulation cocktail; d1, day 1; E2, estradiol; NT, no treatment; P.C., post-challenge.

Numbers shown are cell percentages.

Table 1: T cell populations in the vaginal tract, draining lymph nodes and spleen of estradiol-treated, or mock-treated, intranasally immunized mice on different days following intravaginal HSV-2 challenge.

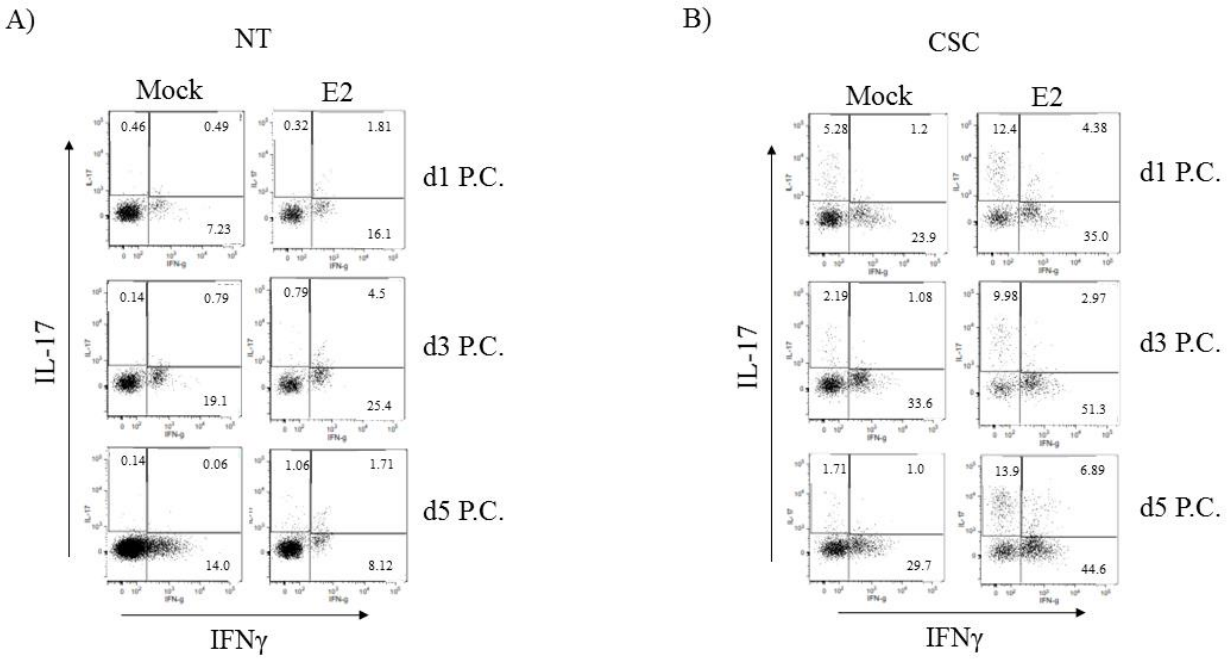

Figure 3: Estradiol treatment upregulates percentage of Th17 cells and IFNγ+ Th1 cells in the vaginal tract of immunized mice following HSV-2 challenge. WT OVX mice were treated with estradiol (E2) pellets, or left untreated (mock), and one week later were immunized intranasally with $1x10^3$ PFU TK^-HSV-2. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. On days 1, 3 and 5 post-challenge, the vaginal tracts of these mice were collected and cells were cultured *in vitro* overnight with (A) no treatment (NT), or with (B) cell stimulation cocktail (CSC). The following day, cells were stained with a panel of antibodies and analyzed by flow cytometry for the following T cell markers: CD3, CD4, IFNγ and IL-17. Viable cells were gated on lymphocytes and then their surface expression of CD3 and CD4.

		NT						CSC					
		d1 P.C.		d3 P.C.		d5 P.C.		d1 P.C.		d3 P.C.		d5 P.C.	
		Mock	E2	Mock	E2	Mock	E2	Mock	E2	Mock	E2	Mock	E2
Vaginal Tract													
	IL-17$^+$	0.9	2.1	0.9	5.2	0.2	2.8	6.5	17.0	3.3	13.0	2.7	20.9
	IFNγ$^+$	8.2	18.5	20.7	31.3	18.3	10.5	27.0	40.6	36.3	56.9	35.7	54.3
	IL-17$^+$ IFNγ$^+$	0.5	1.8	0.8	4.5	0.1	1.7	1.2	4.4	1.1	3.0	1.0	6.9
Lymph Node													
	IL-17$^+$	1.1	1.9	1.5	1.6	0.8	2.6	3.7	3.0	5.4	5.0	3.3	4.0
	IFNγ$^+$	0.5	1.0	0.7	0.7	0.7	1.7	2.0	1.8	5.6	2.9	11.6	3.5
	IL-17$^+$ IFNγ$^+$	0.2	0.4	0.3	0.3	0.2	0.7	0.4	0.4	1.0	0.5	1.4	1.0
Spleen													
	IL-17$^+$	1.7	1.8	2.0	0.8	3.1	2.5	4.2	5.8	2.9	4.2	6.3	5.5
	IFNγ$^+$	1.0	1.3	1.1	0.8	1.3	2.0	8.4	9.7	5.4	7.6	8.6	9.5
	IL-17$^+$ IFNγ$^+$	0.4	0.4	0.5	0.2	0.6	0.7	1.2	1.5	0.6	1.0	1.7	1.3

Abbreviations: CSC, cell stimulation cocktail; d1, day 1; E2, estradiol; NT, no treatment; P.C., post-challenge.

Numbers shown are cell percentages.

Table 2: IL-17+ and IFNγ+ functional phenotype of CD3+CD4+ T cells in the vaginal tract, draining lymph nodes and spleen of estradiol-treated, or mock-treated, intranasally immunized mice on different days following intravaginal HSV-2 challenge.

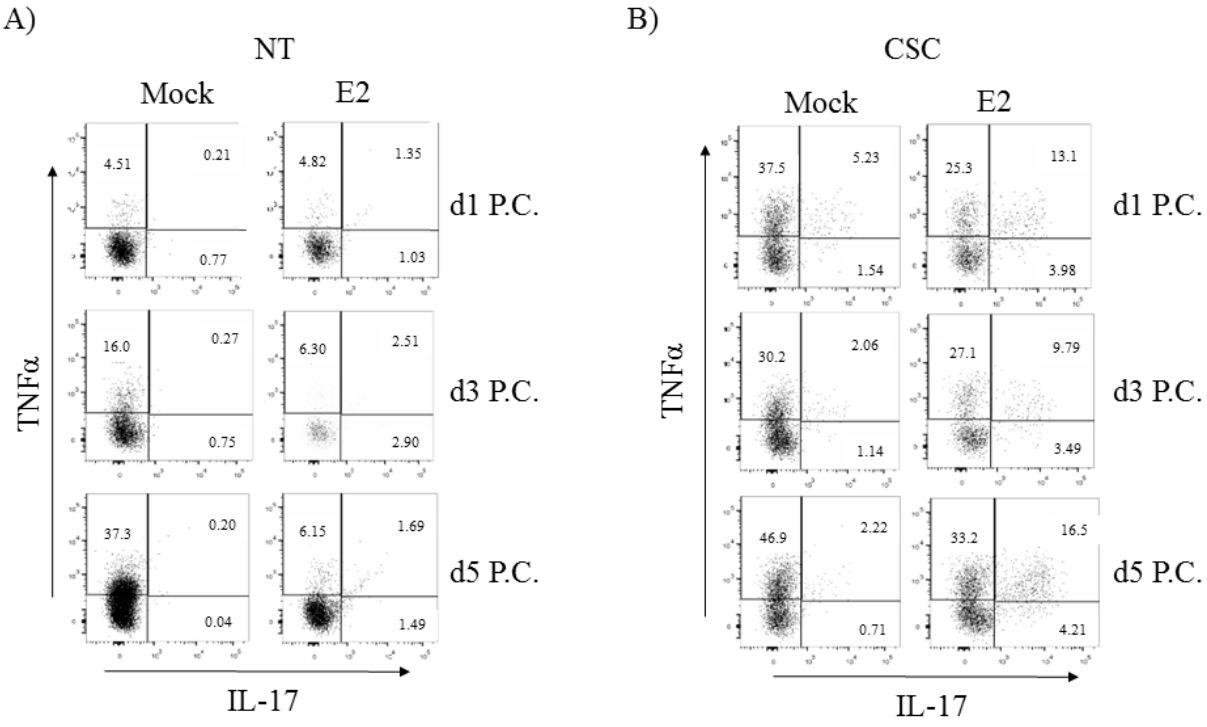

Figure 4: Estradiol treatment upregulates Th17 responses but decreases TNFα+ T cell responses in the vaginal tract of immunized mice following genital HSV-2 challenge. WT OVX mice were treated with estradiol (E2) pellets, or left untreated (mock), and one week later were immunized intranasally with $1x10^3$ PFU TK^-HSV-2. Two weeks later, the immunization was repeated. Five weeks following the second immunization, mice were challenged with $5x10^3$ PFU WT HSV-2, strain 333. On days 1, 3 and 5 post-challenge, the vaginal tracts of these mice were collected and cells were cultured *in vitro* overnight with (A) no treatment (NT), or with (B) cell stimulation - cocktail (CSC). The following day, cells were stained with a panel of antibodies and analyzed by flow cytometry for the following T cell markers: CD3, CD4, IL-17 and

TNFα. Viable cells were gated on lymphocytes and then their surface expression of CD3 and CD4.

		NT						CSC					
		d1 P.C.		d3 P.C.		d5 P.C.		d1 P.C.		d3 P.C.		d5 P.C.	
		Mock	E2	Mock	E2	Mock	E2	Mock	E2	Mock	E2	Mock	E2
Vaginal Tract													
	IL-17$^+$	1.0	2.4	1.0	5.41	0.2	3.2	6.8	17.1	3.2	13.3	2.9	20.7
	TNFα$^+$	4.7	6.0	16.3	8.8	37.5	7.8	42.7	38.4	32.3	36.9	49.1	49.7
	TNFα$^+$ IL-17$^+$	0.2	1.4	0.3	2.5	0.2	1.7	5.2	13.1	2.1	9.8	2.2	16.5
Lymph Node													
	IL-17$^+$	1.4	2.7	2.1	2.2	1.2	3.6	4.8	4.3	7.1	6.6	4.4	5.3
	TNFα$^+$	46.6	48.7	52.1	52.2	43.4	49.3	87.1	83.8	83.4	84.3	85.4	80.7
	TNFα$^+$ IL-17$^+$	1.0	1.8	1.4	1.4	0.9	2.6	4.6	4.0	6.7	6.3	4.3	4.9
Spleen													
	IL-17$^+$	2.3	2.5	2.8	1.1	4.1	3.4	5.6	7.4	4.0	5.5	7.9	7.0
	TNFα$^+$	42.5	44.6	53.3	42.7	51.0	55.9	87.4	86.3	83.0	87.2	85.9	84.7
	TNFα$^+$ IL-17$^+$	1.6	1.7	2.1	0.8	3.0	2.6	5.2	7.1	3.7	5.2	7.6	6.6

Abbreviations: CSC, cell stimulation cocktail; d1, day 1; E2, estradiol; NT, no treatment; P.C., post-challenge.

Numbers shown are cell percentages.

Table 3: IL-17+ and TNFα+ functional phenotype of CD3+CD4+ T cells in the vaginal tract, draining lymph nodes and spleen of estradiol-treated, or mock-treated, intranasally immunized mice on different days following intravaginal HSV-2 challenge.

CHAPTER 5

DISCUSSION

5.1 – Summary and Context of the Research

Genital herpes is one of the most prevalent sexually transmitted infections in the world and recent estimates indicate that over 500 million people are infected by HSV-2 (Looker *et al.*, 2008). Although women are more susceptible to HSV-2 infections than men and the female genital tract is the primary site of HSV-2 infection, little is known about the generation of protective immune responses in this distinct microenvironment and how these immune responses protect against genital HSV-2 infections in women. Previous studies in our lab using an HSV-2 mouse model have found that protection against genital HSV-2 challenge in immunized mice correlates with the induction of iVALTs, which are composed of primarily CD3+ and CD4+ T cells, $CD11c^{+}$ APCs, and B cells (Gillgrass *et al.*, 2005b). The appearance of these structures coincides with the clearance of the virus, which suggests that protective immune responses against HSV-2 could be generated in the genital mucosa itself, without the help of draining lymph nodes. Therefore the microenvironment in the genital mucosa could play an important role in determining the type of immune response. However, this has not been previously examined. Since these iVALTs primarily consist of CD4+ T cells, it is important to identify and characterize the type and function of T cells that are present in the genital mucosa and how these T cells provide specific protection against HSV-2 challenge, which has not been examined. Therefore, the work undertaken in this thesis examined the conditions under which the genital mucosa can generate protective immune responses

against HSV-2 infection and also characterized the T cell responses that are generated in the genital mucosa of immunized mice following HSV-2 challenge.

Although studies in both mice and non-human primates have suggested that E2 has a protective effect against sexually transmitted viral infections (Sodora *et al.*, 1998; Smith *et al.*, 2000; Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008; Pennock *et al.*, 2009), the underlying mechanisms by which E2 protects against these viral STIs has not been examined. Our own previous studies showed IN or IVAG immunization under the influence of E2 leads to greater protection following genital HSV-2 challenge with no accompanying pathology, whereas immunization under the influence of P4 leads to protection, but with greater inflammation and immunopathology (Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008). However, the specific immune responses that are responsible for this difference in pathology between E2- and P4-treated mice were not apparent in these studies. Therefore, we particularly wanted to determine the conditions under which E2 enhances protection against genital HSV-2 infections and examine the mechanism by which E2 regulates protective T cell responses in the genital mucosa following HSV-2 infection.

First, we wanted to examine whether local immunization could establish effective antiviral memory responses directly in the FGT, without the help of draining lymph nodes, following HSV-2 infection. We found that even in the absence of secondary lymphoid organs, mice immunized IVAG with TK^- HSV-2 were completely protected against high dose ($1x10^5$ PFU) genital HSV-2 challenge, but they showed delayed viral

clearance and prolonged genital pathology post-immunization compared to WT mice. Although local viral-specific antibody responses were compromised and T cell-mediated anti-HSV-2 responses were delayed in the absence of SLOs, the immune responses generated were effective in protecting against mucosal HSV-2 challenge in the genital mucosa. These results showed that even in the absence of SLOs, IVAG immunization with a live attenuated virus generated effector memory immune responses at genital mucosa that can provide antiviral protection against subsequent viral exposures.

While live attenuated viruses induce a robust immune response against genital HSV-2 infection as well as significant protection against WT HSV-2 in the genital herpes mouse model, live attenuated virus vaccines are not representative of the mainstream efforts to develop an HSV-2 vaccine. Therefore, we wanted to examine whether more traditional vaccine strategies, such as immunizing with an inactivated virus or subunit vaccine, either locally in the genital tract or distally via the nasal mucosa, could also induce protective immune responses in the genital tract of immunized mice following HSV-2 infection and whether these protective responses could occur in the absence of SLOs. We found that both WT and LTα-/- mice were not protected against genital HSV-2 infections following IVAG or IN immunization with a subunit vaccine (HSV-2 gD + CpG), a heat-inactivated virus vaccine (heat-inactivated HSV-2 + CpG) or a low dose of a live-attenuated virus vaccine (TK^- HSV-2). Nevertheless, WT mice showed longer survival compared to LTα-/- mice following HSV-2 challenge. This suggests that, similar to our previous study (Roth *et al.*, 2013b), the immune responses generated in

LTα-/- mice may be delayed and decreased in magnitude compared to WT mice, thereby resulting in decreased and delayed protection against genital HSV-2 infection following immunization with a non-replicating vaccine formulation or immunization at a distal mucosal surface. More interestingly, we found that pretreating mice with E2 prior to immunization enhanced protection in the genital tract of both WT and LTα-/- mice, irrespective of the vaccine formulation administered, and this protection required signaling through the ER as IN immunized ERKO mice were not protected against subsequent genital HSV-2 challenge. Therefore, while immunization at the same mucosa with high dose of a live attenuated virus does not require assistance from secondary lymphoid organs such as LN (Roth *et al.*, 2013b), these results suggest that a less robust immunization, either because of a weaker vaccine formulation or because of immunization via a distal mucosa, requires the help of SLOs to mount an efficient protective response against subsequent genital HSV-2 challenge. However, immunization under the influence of E2 can overcome this requirement for SLOs to mount an effective immune response and can enhance protection against genital HSV-2 challenge, even in the absence of SLOs.

Lastly, we examined the mechanism by which E2 treatment leads to enhanced protection of immunized mice following HSV-2 infection. Even in the absence of infection, E2 treatment was found to increase the production of IL-17 by vaginal tract cells compared to P4 treatment. We found that E2 treatment increased the recruitment of mucosal effector CD4+CD103+ T cells into the vaginal tract in immunized mice,

following HSV-2 challenge. In addition, E2 treatment upregulated Th17 cells in the vaginal tract of mice following HSV-2 challenge. This Th17 cell response was found to localize to the vaginal tract as T cells from the draining lymph nodes and spleens of HSV-2 challenged mice did not produce significant quantities of IL-17, suggesting that effector Th17 cells localize to the vaginal tract following HSV-2 challenge. This increase in Th17 cells was also accompanied by an early IFNγ+ Th1 response and a decreased TNFα+ T cell response compared to mock controls. These results suggest that the vaginal tract is a unique microenvironment regulated by E2, which preferentially induces mucosal Th17 cell responses that are accompanied by early enhancement of IFNγ responses and decreased TNFα responses. This could explain why E2 treatment results in efficient protection against subsequent genital HSV-2 challenge with no accompanying pathology.

The results summarized in this thesis have significantly improved our understanding of how the microenvironment of the genital tract can influence the generation of protective antiviral immune responses during the immunization of mice. A fundamental theory of immunology is that primary adaptive immune responses are initiated in SLOs, such as LNs, Peyer's patches or spleen (Barker & Billingham, 1968; Goodnow, 1997; Zinkernagel *et al.*, 1997). These SLOs are organized to recruit naïve lymphocytes from the blood and to promote their interaction with activated APCs from surrounding tissues (Goodnow, 1997). Once lymphocytes have been activated and clonally expanded in these SLOs, the resulting effector cells localize to the infected or

inflamed tissues and perform their effector functions (Weninger *et al.*, 2001). While this has been widely accepted as the central dogma for the activation of primary adaptive immune responses, recent studies have shown that effective memory T cell responses can be generated in the lung mucosa during influenza or Mtb infections in the absence of SLOs (Moyron-Quiroz *et al.*, 2006; Day *et al.*, 2010). With respect to genital HSV-2 infections, Soderberg *et al.* used LTβ-/- mice, which have mucosal LNs, to show that the sacral LN provides a site for the generation of protective immune responses against genital HSV-2 infection (Soderberg *et al.*, 2004). A recent study expanded on these findings and found that the vaginal mucosa itself, despite the lack of structured lymphoid tissues, can act as an inductive site during primary CD8+ T cell immune responses (Wang *et al.*, 2015). This study shows that the vaginal mucosa can support both the local immune priming of naïve CD8+ T cells and the local expansion of antigen-specific CD8+ T cells. Similarly, our study found that the genital mucosa itself, without the help of SLOs, can generate protective antiviral immune responses following genital HSV-2 challenge in mice immunized IVAG with a high dose of a live, attenuated virus vaccine.

Previous studies in LTα-/- mice have shown that protective antiviral T cell responses are generated in the induced bronchus associated lymphoid tissue in the lung mucosa following influenza infection (Moyron-Quiroz *et al.*, 2004). Similar lymphoid structures, called iVALTs, have been found in the genital mucosa following intravaginal HSV-2 infections (Gillgrass *et al.*, 2005b). However, in contrast to the lung, here we have shown, for the first time, that iVALTs do not form in immunized LTα-/- mice

following genital HSV-2 challenge. We have also shown, for the first time, that although the absence of SLOs delayed the induction and decreased the magnitude of immune responses following mucosal IVAG immunization with TK^- HSV-2, effector memory T cell responses and IgG antibody responses were present in the vaginal mucosa of LTα-/- mice that resulted in sterile protection following HSV-2 challenge in the genital tract. While other studies have shown that LTα-/- mice have delayed but effective immune responses against influenza and MHV-68 infections (Lee *et al.*, 2000; Lund *et al.*, 2002), we are the first to show that this also occurs in response to HSV-2 infections in the genital mucosa.

Previous studies have shown IVAG or IN immunization with HSV-2 glycoproteins plus adjuvant can protect against subsequent genital HSV-2 challenge in Depo-treated mice (Harandi *et al.*, 2003; Tengvall *et al.*, 2005; Wizel *et al.*, 2012). We are the first to show that, in OVX mice, IVAG or IN immunization with HSV-2 glycoprotein gD plus CpG adjuvant does not protect against genital HSV-2 infections in either WT or LTα-/- mice. In addition, we have also shown, for the first time, that both WT and LTα-/- OVX mice are not protected against genital HSV-2 challenge following IVAG or IN immunization with heat-inactivated HSV-2 plus CpG or a low dose of TK^- HSV-2. These results suggest that OVX, which removes endogenous hormones, seems to induce higher susceptibility to HSV-2 infections compared to Depo treatment, which superimposes a synthetic progestin upon the endogenous hormones, estradiol and progesterone. While previous studies in our lab and others have shown that E2 can

enhance protection in WT OVX mice immunized with live attenuated virus vaccine or glycoprotein based vaccines following genital HSV-2 challenge (Gillgrass *et al.*, 2005b; Pennock *et al.*, 2009), here we have expanded these studies and shown that E2 can also enhance protection against genital HSV-2 infections in LTα-/- mice immunized IN with either heat-inactivated HSV-2 plus CpG or low dose TK^- HSV-2.

In addition, these previous studies have stopped short of determining how E2 helps enhance protection against genital HSV-2 infections. While some studies suggest that E2 enhances protection by thickening the vaginal epithelium and thereby preventing the virus from penetrating the mucosal lining, in this study we waited for the physical effects of E2 on the vaginal epithelium to wane before challenging the mice IVAG with HSV-2. Therefore, the protective effects of E2 in this model are not because of a thick and impenetrable vaginal epithelium. Rather, the physiological effects of E2 occur during the immunization event, not during genital HSV-2 challenge.

Here we show, for the first time, that E2 increases IL-17 production by vaginal tract CD4+ T cells both prior to, and following genital HSV-2 infection. We also show that E2 treatment results in early enhancement of IFNγ+ Th1 responses and decreased TNFα+ T cell responses in the vaginal tract of immunized mice following HSV-2 challenge. Ongoing studies in our lab have shown that DCs from the vaginal mucosa of E2-treated mice were programmed to induce Th17 cells, a phenomenon that was not observed with cells from other tissues (Anipindi, Roth, *et al*, in review). This suggests that E2 treatment specifically primes vaginal tract DCs to induce a Th17 phenotype,

which could explain the increased Th17 response observed following genital HSV-2 challenge in E2-treated, immunized mice and thereby provide a potential mechanism by which E2 treatment leads to enhanced protection following genital HSV-2 challenge. In addition, preliminary results from our lab have shown that P4 treatment leads to increased proportions of TNFα+ cells in the vaginal tracts of mice following IVAG immunization with TK^- HSV-2 (V. Anipindi, unpublished data). Since P4-treated, immunized mice are protected following genital HSV-2 challenge, but show significant genital inflammation and immunopathology, this suggests that increased proportions of TNFα+ cells may contribute to the increased immunopathology observed in these mice. Other studies in our lab have also found that P4-treated, immunized TNFKO mice show protection following genital HSV-2 challenge with no accompanying genital pathology (data not shown), which supports the results shown here, suggesting that the decreased proportions of TNFα+ T cells in the vaginal tracts of E2-treated, immunized mice could contribute to the lack of pathology observed in these mice following genital HSV-2 challenge.

5.2 – Implications of this Study

Our results from the experiments using LTα-/- mice provide insight into the independence of the genital mucosa in generating protective antiviral immune responses against sexually transmitted infections. While it has long been accepted that adaptive immune responses are initiated in SLOs, recent studies have found that mucosal tissues can function independently of SLOs and generate and maintain effective memory immune responses to various pathogens. Previous studies using LTα-/- mice, which lack

all lymph nodes, have found that the lung mucosa could generate effective antiviral immune responses against both influenza and MHV-68 infections (Lee *et al.*, 2000; Lund *et al.*, 2002). Even though no one has previously examined whether the genital mucosa could generate effective immune responses against viral infections, a recent study found that a preexisting pool of resident memory CD4+ T cells present in the vaginal mucosa is required for providing antiviral defense against genital HSV-2 infections and that mice relying only on circulating memory CD4+ T cells were impaired in their ability to suppress viral replication and protect against genital HSV-2 infection (Iijima & Iwasaki, 2014). This study suggests that the genital mucosa is capable of mounting a protective memory T cell response against viral infections. In addition, another recent study has shown that the vaginal mucosa itself, without the help of any secondary lymphoid tissues, can act as an inductive site during primary CD8+ T cell immune responses (Wang *et al.*, 2015). This study showed that the vaginal mucosa supports the local immune priming of naïve CD8+ T cells and the local expansion of antigen-specific CD8+ T cells. Similarly, here we have shown that local immunization with a live attenuated virus vaccine can establish effective antiviral immune responses in the female genital tract, independent of SLOs. While subsequent studies using less robust vaccine formulations, or a lower dose of TK^- HSV-2, given either locally or distally via the nasal mucosa, did not provide protection against subsequent genital HSV-2 challenge, WT mice did show prolonged survival compared to LTα-/- mice. In addition, treating mice with E2 prior to IN immunization enhanced protection in both WT and LTα-/- mice, irrespective of the vaccine formulation administered. However, E2-treated, WT mice showed better

protection compared to E2-treated, LTα-/- mice when a weaker vaccine formulation was administered. Therefore, these results suggest that the use of highly immunogenic live-attenuated virus based vaccines administered locally can provide protection in the genital tract against subsequent HSV-2 infections, without the help of SLOs. However, a less robust immunization, either because of a weaker vaccine formulation or because of immunization via a distal mucosa, requires the help of SLOs to mount an efficient protective response against subsequent genital HSV-2 challenge. Nevertheless, these results provide further support for the independence of the genital mucosa from SLOs in generating protective antiviral immune responses and suggest that future vaccines should focus on stimulating protective immune responses in the genital mucosa. Multiple unsuccessful vaccine strategies have demonstrated that the generation of systemic immune responses does not provide protection in the genital mucosa (Cappel *et al.*, 1985; Mertz *et al.*, 1990; Straus *et al.*, 1994). Since HSV-2 and other viral STIs are mucosal infections that enter the host and initiate infection in the genital tract, vaccines that elicit specific antiviral immune responses within the mucosal tissue are critical for providing protection against genital infection.

Another important aspect for the development of future HSV-2 vaccines to protect the lower genital tract against infection is to determine which route of immunization provides the best protection against reinfection. The data provided in this thesis demonstrates that both IVAG and IN immunization can induce protective immune responses in the female genital tract, in both the presence and absence of SLOs. While it

is not surprising that IVAG and IN immunization with different vaccine formulations can protect against genital HSV-2 infections, as many studies have previously shown similar results (reviewed in (Roth *et al.*, 2013a)), it is somewhat surprising that IN immunization, under the influence of E2, can protect against genital HSV-2 infections in the absence of SLOs. Since LTα, in addition to being required for the development and organization of SLOs, is also important for the development and function of lymphatic vasculature (Mounzer *et al.*, 2010), one would expect that the trafficking of lymphocytes from a distal site of immunization to the genital mucosa might be impaired in mice lacking LTα. In fact, this appears to be the case since IN immunization does not protect LTα-/- mice against genital HSV-2 challenge unless E2 is present at the time of immunization. This suggests that E2 treatment increases the homing of mucosal T cells into the vaginal tract of immunized mice to protect against subsequent HSV-2 challenge. In support of this, we found that in WT mice, E2 treatment lead to increased proportions of CD103+ T cells in the vaginal tract of mice following HSV-2 challenge compared to mock control mice. Therefore, there is increased mucosal homing of T cells in E2-treated WT mice, which could result in the enhanced protection observed in these mice. Since LTα-/- mice are only protected against genital HSV-2 challenge when IN immunized under the influence of E2, this suggests that a similar phenomenon occurs in LTα-/- mice as well. However, the immune responses generated following IN immunization in the absence of SLOs still need to be characterized and future studies should determine how these protective immune responses traffic to the genital tract following HSV-2 challenge. Nevertheless, these results suggest that immunization via either local or distal mucosal surfaces, under

the influence of E2, can optimize the induction of immune responses in the genital tract that can protect against subsequent viral exposures.

Our finding that E2 enhances protection against genital HSV-2 infection is not surprising. In fact, several animal studies have found that E2 can enhance protection against not only HSV-2, but other STIs as well (Smith *et al.*, 2000; Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008; Pennock *et al.*, 2009). Previous studies in our lab have found that E2 treatment prior to immunization results in protection against subsequent genital HSV-2 challenge with better outcomes on vaginal pathology and viral shedding compared to non-treated or P4-treated mice (Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008). A subsequent study also found that E2 treatment improves efficacy of an HSV-2 vaccine in mice, by improving prophylaxis and disease prevention (Pennock *et al.*, 2009). Similarly, E2 treatment has also been found to protect OVX rhesus macaques against IVAG challenge with SIV (Smith *et al.*, 2000). The results presented in this thesis, along with these previous findings, illustrate the importance of the hormonal environment in the FGT and demonstrates how it plays an important role in the outcome of infection as well as the induction of mucosal immune responses. However, only a limited number of studies have examined the effects of female sex hormones on vaccination outcomes in women. For example, one study found that women who were given IVAG immunizations with a cholera vaccine containing killed vibrios and cholera toxin B subunit during either the E2-dominant follicular phase or the P4-dominant luteal phase of the ovarian cycle only induced cervical IgA2-restricted antibodies against the bacterial

LPS vaccine component when immunized during the follicular phase of the cycle (Kozlowski *et al.*, 2002). Another study found that women who were given IVAG immunizations with a recombinant Cholera toxin B subunit and immunogen showed no differences in their resulting immune responses to the antigen whether they were using P4-containing intra-uterine devices, on the oral contraceptive pill, or were using no contraception at all (Wassen & Jertborn, 2006). These results indicate that the magnitude of the immune responses elicited in the FGT could be influenced by the phase of the menstrual cycle for vaccines administered IVAG in women. Therefore, the timing of vaccination during the menstrual cycle and the influence of exogenous hormones such as hormonal contraceptives need to be considered for the induction of optimal immune responses in the genital tract. Since E2 treatment has been shown to enhance protection against viral STIs in animals, future mucosal vaccines against STIs could exploit this strategy to elicit optimal protection in women.

The finding that E2 treatment prior to immunization results in increased proportions of vaginal tract IL-17+ T cells following HSV-2 infection suggests that IL-17-producing Th17 cells may play a role in the enhanced protection against HSV-2 in E2-treated mice. We also found that E2 treatment increases IL-17 production by vaginal tract cells in the absence of infection, which suggests that IL-17 may play a role in steady state functions of the FGT or may be involved in protection against other genital tract infections. Previous studies have shown that Th17 cells are typically involved in the resolution of extracellular fungal and bacterial infections (Aujla *et al.*, 2008; Ishigame *et*

al., 2009). Therefore, the presence of increased levels of IL-17 production in the vaginal tract following E2 treatment suggests that this Th17 cell response may be important for protection against bacterial or fungal infections, which are dominant in the FGT. In fact, previous studies have found that IL-17-producing Th17 cell responses play a role in clearance and protection against vaginal *Candida* and *N. gonorrhoeae* infections (Feinen *et al.*, 2010; Pietrella *et al.*, 2011; Hernandez-Santos & Gaffen, 2012). Therefore, a pre-programmed Th17 response in the vaginal tract may represent a useful adaptation for the efficient generation of immune responses to protect the genital mucosa against infection.

However, the precise role of Th17 cell responses induced by E2 following viral infection is less clear. One study found that, in the absence of IFNγ, immunization with TK^- HSV-2 induced the secretion of IL-17 from HSV-2-specific CD4+ T cells, but the adoptive transfer of these cells did not induce viral clearance following infection with WT HSV-2, suggesting that Th17 cells do not protect against genital HSV-2 infections (Johnson *et al.*, 2010). Another study also found that IVAG immunization with TK^- HSV-2 did not induce production of IL-17 from CD4+ T cells and that infecting IL-17A-/- with WT HSV-2 resulted in delayed death compared to WT mice (Kim *et al.*, 2012). These results suggest that IL-17 may have negative effects in the protection against genital HSV-2 infections. However, in both of these studies, mice were treated with Depo prior to immunization and challenge, which could decrease or inhibit the production of IL-17 by CD4+ T cells, since in our results E2-treated mice had higher levels of IL-17 production from vaginal tract cells compared to P4-treated mice. In

addition, Depo treatment may also affect the immune responses generated following HSV-2 challenge. Therefore, further studies are required to determine the precise role of Th17 cells in genital HSV-2 infections.

5.3 – Strengths of this Study

5.3.1 – HSV-2 Mouse Model

Utilizing an HSV-2 mouse model is one of the primary strengths of this thesis. While other animal models, such as the guinea pig and cotton rat models, have been used to study genital HSV-2 infection and disease, the HSV-2 mouse model has provided the most insight into the generation of protective immune responses following HSV-2 immunization and infection. Both the guinea pig and cotton rat models closely mimic human HSV-2 infection and disease, and both models develop spontaneous clinical recurrences similar to humans (Scriba, 1976; Yim *et al.*, 2005). However, these models have fewer reagents available to study the immune responses generated following immunization or HSV-2 infection, and the immune responses generated in both species are not as well characterized compared to mice. Mice also have the advantage of being cheaper, and there is also a large variety of knockout and transgenic mouse strains that allow one to more easily study the contribution of various aspects of the immune system to HSV-2 infection and disease.

5.3.2 – OVX Model vs. Depo-Provera Model

Since mice are not naturally susceptible to HSV-2 infections, it is imperative to manipulate the hormone environment in the genital tract in order to induce susceptibility. Many other studies utilizing the HSV-2 mouse model treat mice with Depo prior to IVAG infection to make mice susceptible to HSV-2. However, it has been shown that Depo treatment can effect both susceptibility and immune responses to HSV-2. For example, studies in our lab have found that Depo pretreatment can increase susceptibility to genital HSV-2 by 100-fold compared to untreated mice. Depo pretreatment also significantly decreased HSV-2-specific antibody levels in vaginal washes following IVAG immunization with HSV-2 glycoprotein gB and also decreased protection of mice immunized IVAG with TK^- HSV-2 following genital HSV-2 challenge (Kaushic *et al.*, 2003). Previous studies in our lab have also shown that Depo treatment can alter immune responses based on the length of time the mice are exposed to Depo prior to immunization (Gillgrass *et al.*, 2003). Longer exposure to Depo (15 days) prior to IVAG immunization with TK^- HSV-2 led to poor innate and adaptive immune responses to HSV-2 that failed to protect mice from subsequent genital HSV-2 challenge. In contrast, mice that were immunized shortly after Depo treatment (5 days) were fully protected and showed no genital pathology, no viral shedding and higher IFNγ+ Th1 and HSV-2-specific antibody responses following genital HSV-2 challenge. This immunosuppressive effect is likely mediated by Depo binding not only to the PR but also to the GR (Govender *et al.*, 2014), which has been shown to have potent anti-

inflammatory and immunosuppressive activity (Kadmiel & Cidlowski, 2013). In addition to Depo treatment affecting both susceptibility and immune responses to HSV-2 infections, it also superimposes a progestin-based contraceptive on endogenous hormones already present in the FGT, making it difficult to determine the effects of individual hormones. In contrast, the OVX model of genital HSV-2 infection, which removes the ovaries of the mice and thereby eliminates the production of sex hormones in the genital tract, makes mice susceptible to HSV-2 without compromising the generation of antiviral immune responses. The OVX mouse model also has the advantage of having very defined hormonal conditions. Therefore, outcomes of infection can be clearly correlated to the absence of hormones, instead of the mix of hormones present in the Depo model. The OVX model also allows one to study the effects of individual sex hormones on genital HSV-2 infections without interference from endogenous hormones.

5.4 – Limitations of this Study

5.4.1 – Murine Model of a Human Infection

While the HSV-2 mouse model is preferable to the other animal models available for studying HSV-2 infections, it is far from ideal. There are a number of challenges associated with utilizing an HSV-2 mouse model to study the immune response to a human disease. While there are many advantages to using a mouse model to study immune responses to genital HSV-2 infection, it is important to bear in mind the limitations of employing a mouse model to the study of human HSV-2 infection. First and foremost, mice are not naturally susceptible to genital HSV-2 infections. Mice are

only susceptible to genital HSV-2 infections during the progesterone-high, diestrus stage of the estrus cycle, whereas humans are can contract HSV-2 anytime during their menstrual cycles. Therefore, mice require either Depo pretreatment, which thins the vaginal epithelium to allow virus entry into the FGT, or OVX, which removes endogenous hormones, in order to become susceptible to genital HSV-2. Second, mouse HSV-2 infections do not completely mimic the human disease. For example, mice develop a severe neurological illness following HSV-2 infection as well as hindlimb paralysis, which is absent in humans, and mice also do not develop spontaneous reactivation of HSV-2 following primary infection. Finally, it cannot be assumed that what happens in one species will occur to the same degree in another, thus direct comparison is not possible. While these limitations will likely restrict the degree to which we can determine how the human immune system will respond to immunization and HSV-2 infections, the model nonetheless provides significant insight into the development of future HSV-2 vaccines.

5.4.2 – Hormones

While we have decided to use hormone levels associated with physiological concentrations found in the blood of mice, tissue levels of these hormones may actually exceed that which we used in these studies. We still have an incomplete understanding of hormone concentrations present in the actual genital tract of both mice and women during the estrus or menstrual cycle, respectively, and so the estradiol and progesterone concentrations used in this study are based on hormone levels found in the blood. Studies

in female pigs have shown that blood collected from arteries proximal to genital tract contained sex hormone levels up to 69% greater than those measured in distal blood (Stefanczyk-Krzymowska *et al.*, 1998), suggesting that genital tract hormone levels may be higher than serum blood levels since that is where the majority of female sex hormones are produced. Also, while our OVX model, which removes endogenous hormones via the removal of the ovaries, allows us to study the role of each sex hormone individually, it does not take into consideration that *in vivo*, both E2 and P4 are usually present throughout the cycle and at variable levels and relative concentrations (Marieb, 2012). Thus, our results should be viewed cautiously since they do not take the complexity of hormone cycling into consideration. Since we have suggested that E2 pretreatment could potentially enhance protection against genital HSV-2 infections following immunization, and therefore be considered for the development of future HSV-2 vaccines, future studies are required to determine whether E2 has similar protective effects when other hormones are present as well, as would be the case in most women receiving an HSV-2 vaccine. However, using a mouse model to study the effects of both E2 and P4 on HSV-2 infection in hormone-intact mice would be challenging since the presence of E2 in the genital tract physically prevents HSV-2 infections in mice. Nevertheless, studies could be conducted in women to determine the effectiveness of HSV-2 vaccines during different phases of the menstrual cycle to determine whether immunization during the E2-high, follicular phase could improve vaccine efficacy.

In addition, we found that the 21 day release E2 pellets actually lasted longer than 21 days. Since E2 thickens the vaginal epithelium in mice and prevents virus entry, it is

important to wait until the physiological effects of E2 have waned prior to genital HSV-2 challenge. Therefore, prior to genital HSV-2 challenge, we collected vaginal washes from each mouse and examined them under the microscope to determine which cells were present, which allowed us to determine whether the mouse was still under the physiological effects of E2 or if it had begun the estrus cycle again. It was found that the E2 pellets lasted at least 4-5 weeks after implantation, and therefore we had to wait at least 3 weeks following the second immunization to challenge the mice. Since the HSV-2 gD and heat-inactivated HSV-2 vaccine formulations are not as robust as the live attenuated TK^- HSV-2 vaccine, it is possible that the immune responses generated following immunization did not last long enough to protect against subsequent genital HSV-2 challenge. In future experiments, the HSV-2-specific T cell responses induced post-immunization should be examined to determine how long they persist following immunization with each vaccine formulation. This will allow us to determine the best time to challenge mice with WT HSV-2 following immunization.

5.4.3 – Induction of Immune Responses

Results presented in this thesis show that, in the absence of SLOs, mice can generate effective antiviral immune responses following local immunization with TK^- HSV-2 that can protect against a subsequent viral exposure in the genital tract (Roth *et al.*, 2013b). However, it is unknown whether these protective immune responses are induced directly in the genital mucosa. Even though we have shown that the immune responses are not induced in the draining lymph nodes or spleen, we did not rule out the possibility that the initial induction of protective memory immune responses could have

occurred in a location other than the genital mucosa. As an alternative, previous studies have shown that the bone marrow can function as a site of lymphocyte priming to systemic antigens when lymph nodes are unavailable (Tripp *et al.*, 1997; Feuerer *et al.*, 2003). However, a recent study has shown that while circulating memory CD4+ T cells can provide some protection against genital HSV-2 infection and disease, a preexisting pool of local CD4+ T cells in the genital mucosa is required for full protection against genital HSV-2 infection (Iijima & Iwasaki, 2014), suggesting that a local CD4+ T cell response can be mounted from the genital mucosa. Therefore, it is possible that, in the absence of SLOs, memory lymphocytes are primed and proliferate locally, in the genital mucosa, or at systemic sites, such as the bone marrow, and subsequently migrated to the genital tract to exert their effector functions. Further studies are required to determine where these protective immune responses are induced following immunization.

5.4.4 – Vaccine Formulations

While experiments conducted for this thesis use a number of different vaccine formulations to test the role of SLOs in generating protective immune responses against genital HSV-2 infections in immunized mice, there are a number of limitations that should be considered before using these formulations for the development of future vaccines. First, the use of the TK^- HSV-2 virus vaccine has a number of safety concerns. This live attenuated virus lacks thymidine kinase, and is therefore replication defective and has been shown to cause primary vaginal infections without the neurological illness that is associated with the WT virus (McDermott *et al.*, 1984). Since primary infection is still established following IVAG immunization with TK^- HSV-2, this would not be an

ideal vaccine candidate for humans. Live attenuated virus vaccines have the advantage of providing a prolonged exposure of viral antigen to the immune system as well as an innate immune response that aids in the generation of protective adaptive immune responses, thereby resulting in a large population of T and B cells, as well as memory cells to protect against subsequent viral exposures. Therefore live attenuated virus vaccines usually only require one dose in a lifetime and do not require repeated boosters. However, there are a number of disadvantages to using live attenuated virus vaccines. First, is the possibility of the virus reverting back to its virulent form. Second, live attenuated vaccines cannot be administered to immunocompromised individuals because even though the virus vaccine is attenuated, or weakened, compared to WT virus, it could still cause disease in an immunocompromised individual.

The use of a heat-inactivated HSV-2 vaccine also has a number of disadvantages. For example, inactivated virus vaccines do not induce a robust innate immune response or a strong antibody or CD8+ T cell response, which could explain the lack of protection observed in our model. Since inactivated virus vaccines do not replicate there also may not be enough antigen present to elicit a strong memory T cell response. Even with the repeated immunization used in our model, it is possible that using a heat-inactivated virus vaccine does not provide enough antigen to stimulate sufficient T cell expansion to provide an effective memory T cell response that protects against genital HSV-2 challenge 3 weeks following the second immunization. Therefore, increasing the dose of heat-inactivated HSV-2 used for immunization may help enhance protection against subsequent HSV-2 challenge. Future studies should also be conducted to examine

whether immunization with heat-inactivated HSV-2 can induce HSV-2-specific memory CD4+ T cells in the genital tract prior to HSV-2 challenge. Another disadvantage of using a heat-inactivated virus vaccine is that heat inactivation of microbes results in the denaturation of surface proteins, which thereby alters their antigenic structure. Therefore, the immune response that is generated using this heat-inactivated vaccine may not be effective against the natural pathogen. Since the heat-inactivated HSV-2 vaccine used in this thesis did not provide protection against WT HSV-2 challenge, it is possible that this may be due to the denaturation of the surface proteins of the virus. Future experiments could alter the inactivation process of the virus, by using either UV-inactivation or chemical inactivation, which may provide better protection against genital HSV-2 infections. However, as with heat-inactivation, it is essential to use the lowest dose possible of UV or chemical inactivation to inactivate the virus, without completely destroying the antigenic structure, which can be measured by Vero plaque assay.

Lastly, we also used an HSV-2 glycoprotein gD vaccine to determine whether a subunit vaccine could provide protection against genital HSV-2 challenge when administered either IVAG or IN in mice lacking SLOs. Subunit vaccines are the most utilized vaccine strategies due to their simplicity, safety and cost effectiveness. However, a number of clinic trials have already been conducted using HSV-2 glycoprotein vaccines and found a lack of efficacy in protecting against genital HSV-2 infections (Ashley *et al.*, 1985; Cappel *et al.*, 1985; Mertz *et al.*, 1990; Mester *et al.*, 1990; Straus *et al.*, 1994; Corey *et al.*, 1999; Langenberg *et al.*, 1999; Stanberry *et al.*, 2002; Bernstein *et al.*, 2005; Cohen, 2010). Therefore, while a simple HSV-2 glycoprotein gD vaccine may not

provide protection against genital HSV-2 infection in OVX mice or in humans based on results of previous clinical trials, an improved subunit vaccine could potentially be used for future HSV-2 vaccines. For example, a subunit vaccine currently in phase I/IIa clinical trials, which consists of T cell and antibody directed epitopes meant to induce a balanced T cell and antibody response, could potentially provide adequate protection against HSV-2 (Genocea-Biosciences, 2012).

5.5 – Future Directions

5.5.1 – Induction of Immune Responses in the Absence of Secondary Lymphoid Organs

The results presented in this thesis provide a number of promising directions for future research. Firstly, while we have found that, in the absence of SLOs, mice can generate effective antiviral immune responses following local immunization with TK^- HSV-2 that can protect against a subsequent viral exposure in the genital tract, it is unknown whether these protective immune responses are induced directly in the genital mucosa. As an alternative, previous studies have shown that the bone marrow can function as a site of lymphocyte priming to systemic antigens when lymph nodes are unavailable (Tripp *et al.*, 1997; Feuerer *et al.*, 2003). Therefore, it is possible that, in the absence of SLOs, memory lymphocytes are primed and proliferate locally, in the genital mucosa, or at systemic sites, such as the bone marrow, and subsequently migrated to the genital tract to exert their effector functions. To distinguish these possibilities, mice could be treated with the immunomodulatory agent, FTY720, which has been shown to sequester lymphocytes and dendritic cells in tissues and prevent trafficking between

organs (Cyster, 2005; Lan *et al.*, 2005). A recent study used FTY720 to show that naïve CD8+ T cells could be primed locally in the vaginal mucosa independent of LNs (Wang *et al.*, 2015). Therefore, a similar experiment could be used to show whether naïve CD4+ T cells could also be primed locally in the genital mucosa without the help of draining LNs or other systemic sites, such as the bone marrow. Treating mice with FTY720 following immunization or challenge would cause cells to remain within the vaginal tissue, thereby allowing us to determine whether HSV-2-specific protective T cell responses could be generated in the genital mucosa. If, following FTY720 treatment, immunized LTα-/- mice have similar responses to genital HSV-2 challenge as non-FTY720-treated LTα-/- mice, then we could conclude that the genital mucosa itself is capable of generating effective antiviral immune responses which protect against genital HSV-2 infections.

Secondly, since we found that LTα-/- mice are protected against genital HSV-2 infection following IN immunization, in the presence of E2, it would be interesting to determine where these protective immune responses are generated as well. Previous studies using LTα-/- mice have shown that while LTα is essential for the development of lymph nodes and Peyer's patches, it is not required for the development of nasal associated lymphoid tissue (**NALT**). However, it was also found that NALT of LTα-/- mice was disorganized and lymphopenic, suggesting that the organization and recruitment of lymphocytes within NALT remained dependent on LTα (Harmsen *et al.*, 2002). Therefore, it is possible that immune responses are initiated in NALT of LTα-/-

mice following IN immunization. To determine if this occurs in our model, we could isolate NALT following IN immunization (Asanuma *et al.*, 1997) and analyze the cells by flow cytometry to determine whether HSV-2-specific T cells are present. We could also collect nasal washes to measure viral shedding (TK^- HSV-2) and HSV-2-specific antibody responses following immunization. In addition, to determine whether these immune responses are induced locally, mice could once again be treated with FTY720 following immunization, and then challenged IVAG with WT HSV-2. If FTY720-treated LTα-/- mice show similar protection against genital HSV-2 challenge and can generate protective antiviral immune responses to a similar extent as non-treated LTα-/- mice, then we can conclude that antiviral immune responses are generated locally within the NALT.

5.5.2 – Lack of Protection in WT OVX Mice Immunized with Non-Replicating Vaccines

Results presented in this thesis show that while WT OVX mice immunized IVAG or IN with high dose of a live attenuated virus vaccine are protected against genital HSV-2 challenge, WT OVX mice immunized IVAG or IN with a subunit or heat-inactivated virus vaccine are not protected following genital HSV-2 challenge. However, the immune responses that are responsible for the difference in protection between the different vaccine formulations has not been determined. Therefore, future studies should be conducted to determine the differences in immune responses between the protective live attenuated virus vaccine and the non-protective subunit or inactivated virus vaccines. To do this, we could measure the different CD4+ T cells present in the vaginal tract and draining lymph nodes of mice following both immunization and challenge. Total and

HSV-2-specific cells could be analyzed by flow cytometry for the production of IFNγ, IL-17 and TNFα. In addition to CD4+ T cells, cytotoxic CD8+ T cells and neutralizing antibodies are also important for protection against genital HSV-2 infections (Koelle *et al.*, 1998; Parr & Parr, 1998; Mbopi-Keou *et al.*, 2003; Dobbs *et al.*, 2005; Schiffer *et al.*, 2010). Therefore, we could also measure cytotoxic activity of T cells isolated from the vaginal tracts and draining lymph nodes of mice following both immunization and challenge, as well as the presence of neutralizing HSV-2-specific IgG and IgA antibodies in vaginal washes and blood collected following immunization and challenge in all groups of mice. The characterization of both T cells and antibodies present in mice immunized with a subunit, inactivated or live attenuated virus vaccine both following immunization as well as following challenge will allow us to determine why the live attenuated virus provides protection against genital HSV-2 infections but the non-replicating virus vaccines do not provide protection.

5.5.3 – Role of E2-Induced Th17 Response in HSV-2 Infection

Results presented in this thesis show that E2 treatment prior to IN immunization results in increased protection following HSV-2 challenge. To determine the mechanism by which E2 treatment enhances protection against genital HSV-2 challenge, we examined the presence of different types of T cells in the vaginal tract of mice post-challenge. It was found that E2-treated mice had a large population of vaginal tract Th1 and Th17 cells following HSV-2 infection. To further characterize these T cell responses, we could measure the levels of cytokines present in nasal washes post-

immunization and vaginal washes post-challenge. In addition, NALT cells and vaginal tract cells could be collected post-immunization and post-challenge, respectively, and cultured *in vitro.* Supernatants collected from these *in vitro* cultures as well as nasal and vaginal washes could then be analyzed by ELISA for the presence of IFNγ, IL-17 and TNFα. In addition, it is also important to determine whether these cells are HSV-2-specific. To do this, cells isolated from the NALT or vaginal tract of mice following immunization or challenge, respectively, could be stimulated with UV-inactivated HSV-2 *in vitro* and IFNγ, IL-17 and TNFα production measured by intracellular cytokine staining. If UV-HSV-2 stimulation increases the production of IFNγ, IL-17 and/or TNFα, then we can conclude that these T cell responses are specific for HSV-2.

Furthermore, it would be valuable to determine whether this E2-induced Th17 cell response is what causes the enhanced protection in these mice. Ongoing studies in our lab are using IL-17KO mice to determine if IL-17 responses are important for protection against HSV-2 challenge in E2-treated mice. WT and IL-17KO mice will be OVX, treated with E2 pellets and then immunized IN one week later with TK^- HSV-2. Following immunization, mice will be challenged IVAG with WT HSV-2. Since previous studies in our lab have found that E2 treatment results in decreased genital pathology in immunized mice following genital HSV-2 challenge, we will first examine the external genital pathology of mice post-challenge. If IL-17-producing T cells are involved in E2-induced enhanced protection against HSV-2 challenge, we would expect to see increased genital pathology in IL-17KO mice compared to WT mice. In addition,

genital tract HSV-2-specific T cell responses should also be examined post-challenge in IL-17KO mice. Previous studies conducted on the intracellular bacteria, Mtb, have shown that Th17 responses induced in the lung following immunization played a critical role in the rapid induction of IFNγ+ Th1 responses. In addition, the absence of these IL-17 responses resulted in delayed IFNγ responses in the lung (Khader *et al.*, 2007). Since Mtb and HSV-2 are both intracellular pathogens and protective immune responses against both pathogens are dependent on IFNγ+ Th1 responses, it is possible that the Th17 responses induced by vaginal DCs in E2-treated mice following immunization lead to enhanced protection by inducing IFNγ+ Th1 responses, which protect against subsequent HSV-2 infections. To test this, IFNγ T cell responses in the genital tract of IL-17KO mice should be examined. If E2-induced IL-17 responses enhance protection by inducing IFNγ+ Th1 responses, we would expect to see decreased IFNγ responses in the genital tract of IL-17KO mice following HSV-2 challenge, compared to WT mice.

5.5.4 – Mechanism by which P4 Increases Inflammation in HSV-2 Mouse Model

In contrast to E2 enhancing protection in our HSV-2 mouse model, previous studies in our lab have also found that P4-treated mice immunized either IVAG or IN with TK^- HSV-2 are protected following IVAG HSV-2 challenge but develop significantly increased inflammation and immunopathology compared to E2-treated mice (Gillgrass *et al.*, 2005b; Bhavanam *et al.*, 2008). Since the only difference among different experimental groups was the hormonal environment at the time of immunization, we examined functions of APCs 24 hours after immunization with TK^-

HSV-2. It was found that DCs and macrophages in the vaginal tract of P4-treated mice had the highest proportion of TNFα+ cells compared to E2- or saline-treated mice (V. Anipindi, unpublished data). In accordance with these findings, results from this thesis show that E2-treated, immunized mice have decreased levels of TNFα+ T cell responses in the vaginal tract of mice post-HSV-2 challenge. This suggests that the increased inflammation and immunopathology observed in P4-treated mice could be due to increased levels of TNFα in the genital tract of immunized mice. TNFα has been shown to cause pathology, when in excess amounts, in response to Mtb infections (Jacobs *et al.*, 2007). However, whether TNFα is associated with increased pathology in the genital tract following HSV-2 challenge in immunized mice has not been previously examined. While it seems unlikely that TNFα produced post-immunization by DCs and macrophages is responsible for the increased pathology observed 3-4 weeks later, following HSV-2 challenge, it is possible that TNFα is also being produced post-challenge, thereby leading to increased immunopathology. To examine the role of TNFα in mediating pathology post-challenge, it should first be determined whether vaginal tract cells from P4-treated, immunized mice have increased production of TNFα following HSV-2 challenge, which can be done by measuring TNFα production from different cell types via intracellular cytokine staining. Ongoing studies in our lab are also examining the role of TNFα in mediating immunopathology following HSV-2 infections by using TNFKO mice. If P4-treated, immunized TNFKO mice show decreased levels of immunopathology following genital HSV-2 challenge, compared to P4-treated WT mice,

this would suggest that P4-induced TNFα production plays a role in HSV-2 infections and potentially leads to increased immunopathology observed in these mice.

5.6 – Concluding Remarks

Overall, the results from this thesis have a number of implications for the design of future HSV-2 vaccines. Since an important goal of vaccination against STIs is to induce and maintain immunity in the FGT, the finding that protective immune responses can be generated in the absence of SLOs indicates that immunization generates effector memory responses at the genital mucosa can provide adequate protection against subsequent viral exposures. The results also imply that once memory effector responses are generated, they can protect against genital tract infections independently and without involvement of other secondary lymphoid structures, which is supported by a recent study showing that memory CD4+ T cells residing in the vaginal mucosa can provide better antiviral defense and increased protection against genital HSV-2 infections compared to mice relying only on circulating memory CD4+ T cells, which were impaired in their ability to suppress viral replication and protect against genital HSV-2 infection (Iijima & Iwasaki, 2014).

The use of highly immunogenic live-attenuated virus based vaccines delivered via mucosal routes, either IVAG or IN, can provide protection in the genital tract against subsequent HSV-2 infections. However, if a less immunogenic vaccine formulation, such as a subunit or inactivated virus based vaccine, or a lower dose of the attenuated virus based vaccine is delivered, either locally or distally, additional mucosal adjuvants or

hormones may be required to elicit protection in the genital tract. The administration of exogenous estrogen in women combined with an HSV-2 vaccine may be required to optimize the induction of protective immune responses in the female genital tract.

The potential mechanism by which E2 treatment leads to enhanced protection against HSV-2 infections following immunization is possibly by increasing Th17 responses in the genital tract. In addition, E2 treatment prior to immunization results in an earlier and increased presence of Th1 cells, but decreased the presence of TNFα+ T cells, in the vaginal tract of mice following HSV-2 challenge, which could lead to the enhanced protection and decreased pathology observed in these mice. Therefore, the hormonal microenvironment during immunization should be a critical consideration in the development of mucosal HSV-2 vaccines.

As a whole, this work not only provides important considerations for future HSV-2 vaccine strategies, but it also suggests potential mechanisms and future directions for the work that may have significant clinical implications.

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APPENDIX

Appendix A – Review on HSV-2 Vaccines

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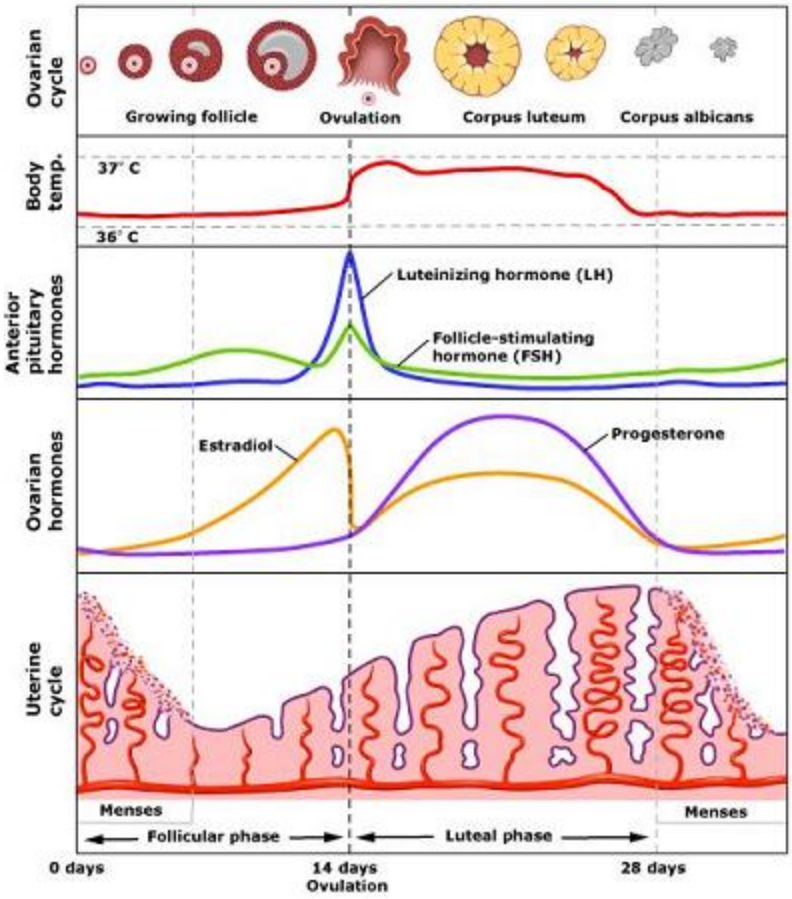
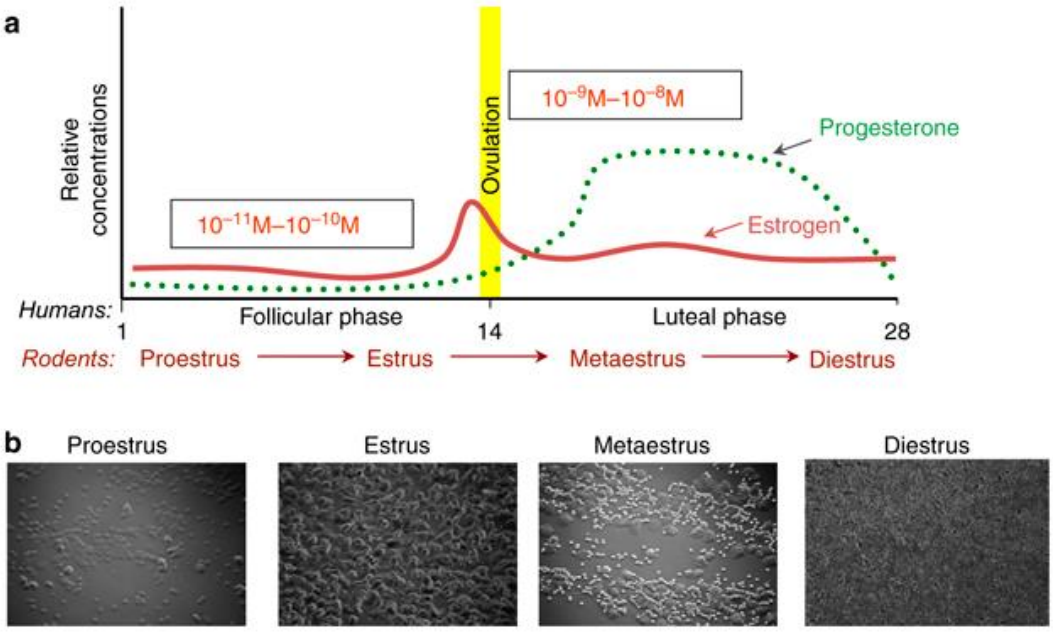
http://www.sciencedirect.com.libaccess.lib.mcmaster.ca/science/article/pii/S0882401012001933

Appendix B – The Human Menstrual Cycle



http://www.sportsmd.com/SportsMD_Articles/id/309/n/birth_control_and_the_female_athlete.aspx#sthash.SCwebzUD.dpbs

Appendix C – The Rodent Estrus Cycle



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Title: Delayed but effective induction of mucosal memory immune responses against genital HSV-2 in the absence of secondary lymphoid organs

Author: K L Roth, S Bhavanam, H Jiang, A Gillgrass, K Ho et al.

Publication: Mucosal Immunology

Publisher: Nature Publishing Group

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