COMPARING THE EFFECTS OF NET AND DMPA ON SUSCEPTIBILITY TO HSV-2 INFECTION AND EFFECTS ON IMMUNE CELLS

COMPARING THE EFFECTS OF NET AND DMPA ON SUSCEPTIBILITY TO HSV-2 INFECTION AND EFFECTS ON IMMUNE CELLS

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ABSTRACT

Background

HSV-2 was estimated to infect 491 million people worldwide, with women disproportionately affected by HSV-2. Understanding factors that influence susceptibility to HSV-2 in women is important in preventing infections. Through various studies, the progestin-based contraceptive DMPA exhibited immunosuppressive effects, and has shown increased susceptibility to HIV and HSV-2. Studies comparing DMPA to other contraceptives like NET suggest that NET may be safer. In vivo NET effects have not been characterized thoroughly to better understand the effect of NET on susceptibility to HSV-2. Therefore, this study aimed to compare the effects of NET and DMPA in mouse models that affect susceptibility to HSV-2. We hypothesized that NET treated mice will have decreased susceptibility to HSV-2 compared to DMPA but elevated compared to normal mice.

Method of study

Ovariectomized mice were treated with DMPA (2mg) and NET (2 mg injections, 2.5 mg pellets or 5 mg pellets) for 10 days and intravaginally immunized with HSV-2 TK^-, then intravaginally challenged with WT HSV-2 ~4-7 weeks later. Primary intravaginal WT HSV-2 challenges were conducted in ovariectomized and normal mice after 10 days of DMPA and NET treatment. Viral titers, pathology and survival were examined. Mucus production in the vagina was investigated through immunohistology. Effects of hormones on immune cells were explored in the lymph nodes, spleens, and vaginal tracts through flow cytometry.

Results

Increased mucus was consistently observed in the vaginal tracts of mice after treatment with NET 2.5 mg and 5 mg treated mice, but not with DMPA Therefore, NET treated mice displayed reduced viral shedding and delayed pathology compared to DMPA treated mice. No significant changes occurred in immune cells analyzed post DMPA and NET treatment, although there were trends of increased T cells in progestin treated mice. However, more experiments need to be conducted to confirm observed trends.

Conclusion

NET treatment in mice results in mucus production in the vaginal tract, a potential mechanism impeding intravaginal HSV-2 infection and could be applied to other STIs. This provides insight into protective effects of NET compared to DMPA allowing women to make informed decisions regarding hormonal contraceptives.

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All experiments were conceived and designed by Sidney Pa, Dr. Charu Kaushic and Dr. Amy Gillgrass. All experiments were conducted by Sidney Pa with assistance from Dr. Christina Hayes, Dr. Charu Kaushic and Dr. Amy Gillgrass. Sidney Pa wrote this thesis dissertation with contributions from Dr. Charu Kaushic.

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AMPs – antimicrobial peptides

APCs – antigen presenting cells

AR – androgen receptor

CD – cluster differentiation

cGAS – cGMP-cAMP synthase

DC – dendritic cell

DMPA – depot medroxyprogesterone acetate

ETG – etonorgestrel

FRT – female reproductive tract

G-CSF – granulocyte colony stimulating factor

gB – envelope glycoprotein B

gC – envelope glycoprotein C

gD – envelope glycoprotein D

gH – envelope glycoprotein H

gK – envelope glycoprotein K

gL – envelope glycoprotein L

gM – envelope glycoprotein M

GM-SCF – granulocyte-macrophage colony-stimulating factor

GR – glucocorticoid receptor

HIV – human immunodeficiency virus

HPV – human papillomavirus

HSV-1 – Herpes simplex virus type 1

HSV-2 – Herpes simplex virus type 2

IFN – interferon

IgA – immunoglobulin A

IgG – immunoglobulin G

IL – interleukin

IRF3 – interferon regulatory factor 3

IUD – intrauterine device

JAK/STAT – Janus kinase/signal transducers and activators of transcription

LAT – latent associated transcript

LNG – levonorgestrel

MCP-5 – murine monocyte chemoattractant protein

MIP-1a – macrophage inflammatory protein-1a

MPA – medroxyprogesterone acetate

MR – mineralocorticoid receptor

MUC-1 – mucin-1

MyD88 – myeloid differentiation primary-response protein 88

NET – norethisterone

NET-A – norethisterone acetate

NET-EN – norethisterone enanthate

NF-κB – nuclear factor kappa beta

NK – natural killer

NKT – natural killer T cells

OVX – ovareictomized

PAMPs – pattern associated molecular patterns

PAS – Periodic acid-Schiff

PBMCs – peripheral blood mononuclear cells

pDC – plasmacytoid dendritic cells

PFU – plaque forming units

PMNs – polymorphonuclear leukocytes

PR – progesterone receptor

PRRs – pattern recognition receptors

RIG-I – retinoic acid inducible gene I

S1PR1 – sphingosine-1-phosphate receptor 1

STING – stimulator of interferon genes

STIs – sexually transmitted infections

TK^- – thymidine kinase deficient

TLRs – toll like receptors

TNF – tumor necrosis factor

TRM – tissue resident memory T cells

U_L – unique long sequence

U_S – unique short sequence

WT – wildtype

WT – wildtype

CHAPTER 1: INTRODUCTION

1.1 Epidemiology of HSV-2

HSV-2 (Herpes simplex virus type 2) is a sexually transmitted lifelong viral infection which causes genital herpes [1]. In 2016, it was estimated that 491.5 million people aged 15-49 were infected with HSV-2 globally [2]. The prevalence of HSV-2 infections is much higher in females compared to males; females accounted for an estimated 313.5 million (63.8%) of these infections and males accounted for an estimated 178 million globally in 2016 (36.2%) [2]. The highest prevalence occurred in Africa, with 102.9 million infections in women, accounting for 43.99% of infections in women worldwide[2]. Within Africa, the prevalence of infection in males was 59.3 million, accounting for 25.4% of infections in males globally[2]. HSV-2 remains one of the most common sexually transmitted infections, with an estimated 23.9 million new infections in 2016[2]. Of these new infections, women made up 14.7 million (61.5%), while males made up 9.2 million (38.5%)[2]. HSV-2 disproportionately affects women and is a global burden with no cure for the disease[2]. This emphasizes the need for a better understanding of the factors that affect susceptibility to HSV-2 infections.

1.2 Structure, pathogenesis and transmission of HSV-2

HSV-2 is part of the Herpesviridae family and consists of linear double stranded DNA that is 150-200 nm long[3 4]. The HSV-2 DNA contains approximately 150 000 base pairs, encoding at least 84 proteins[3 4]. HSV-2 contains viral DNA in its core, with an icosapentahedral capsid, a tegument, a layer of proteins surrounding the capsid and an envelope[3 4]. HSV-2 contains 2 regions, with a unique long sequence (U_L) and a unique short sequence (U_S)[3 4]. The envelope

contains viral proteins called glycoproteins which include gB, gC, gD, gH, gK, gL, and gM[3 4]. These glycoproteins facilitate viral entry, however not all are required[3 4].

HSV-2 is transmitted sexually through contact with the skin, sores or bodily fluids in the genital regions of a seropositive individual to a seronegative individual[3]. HSV-2 infects mucosal membranes and the skin[3]. This infection typically presents with sores in the genital regions, however it is possible for HSV-2 to present oral lesions and sores that are commonly caused by HSV-1[5]. Individuals can also be asymptomatic, still shedding virus, thus transmitting it to others[5].

HSV-2 infects primarily epithelial cells in the mucosal lining, through gB and gC binding to heparan sulfate proteoglycans on the surface of the target cell[4]. Other surface cell receptors include herpes viral entry mediator A and nectin-1α, which can be bound by gD[4]. The glycoproteins that are required for the viral envelope to fuse with the host's cell membrane are gB, gD and the glycoprotein complex gH/gL[4]. The viral capsid can enter the nucleus of the host where viral replication occurs and infected cells can then release HSV particles after the viral DNA is incorporated into the nucleus[4]. The tegument proteins on the surface of the virus can interfere with the cell's ability to detect virus allowing the infection to progress[4]. Viral genes are transcribed in order of immediate (alpha) genes, then early (beta) and late (gamma) genes, where replication occurs in three rounds[5]. This starts with immediate proteins regulating replication, early proteins synthesizing and packaging the DNA, then late proteins which are the virion proteins[3 5]. The total time for the replication cycle to complete is 12-16 hours[6]. HSV-2 can lyse or burst the infected cell causing inflammation and ulcers to form and the virions are then released in envelopes to infect other cells and nerve endings[3 4].

In addition to the active infection of epithelial layer, HSV-2 also causes latent infection where it migrates to the dorsal root ganglia[5]. Expression of the LAT (latent associated transcript) preserves the virus reservoir and allows the virus to stay dormant as well as promoting survival of the host cell[3 5]. During reactivation lytic genes are produced, and through anterograde transport, the virus can travel back to the original site of infection[3 5]. Reactivation can occur due to many different stressors and stimuli where individuals have episodes of symptoms present, including ulcers and viral shedding[1 5]. Viral shedding can occur frequently and can occur when an individual is asymptomatic as well[1].

1.3 Distribution of immune cells in the human FRT

The human female reproductive tract (FRT) is comprised of the upper and lower tract which have different distributions of immune cells to aid in fighting off pathogens (Figure 1)[7 8]. The upper tract is made up of the fallopian tubes, endocervix and uterus, which is also lined by a single layer of columnar epithelial cells[7 9]. The lower tract is comprised of the ectocervix and vagina, lined with layers of stratified squamous epithelial cells[7 9]. CD3+ T cells are distributed throughout the FRT, however there are more CD8+ T cells present and less CD4+ T cells present overall [7 10 11].

1.3.1 Immune cell distribution in the uterus

The uterus contains lymphoid aggregates, consisting of a B cell core surrounded by T cells[12]. T cells and macrophages are also present in the uterus and continue to be present in similar densities throughout the menstrual cycle[12]. Neutrophils have also been found in high cell numbers in the uterus[13]. NK (natural killer) cells increase greatly from the proliferative to

secretory phase of the menstrual cycle[9 12]. NK cells in the uterus play a role in tissue remodelling during pregnancy, in which uterine NK cells show a phenotypic and functional difference from circulating NK cells[12]. Uterine NK cells express high CD56 and low CD16, lacking expression of FcγRIII, differing from NK cells found in the blood[9 12 14].

1.3.2 Immune cell distribution in the cervix

In the human cervix lymphoid aggregates are commonly found, which consists of a B cell core, surrounded by T cells (Figure 1)[7 15 16]. Additionally, antigen presenting cells (APCs) represent the major immune cells in the cervix, accounting for approximately 37-55% of CD45+ cells[7 8 17]. The endocervix consists of mucus-producing glands which help fight off microbial pathogens[9]. Of the CD45+ population, almost 50% was found to be CD3+ cells[8 10 18]. The total CD3+ cell population in the ectocervix has been found to be almost double that in the endocervix[7 17]. The ectocervix contains approximately 40% CD4+ T cells and 60% CD8+ T cells[7 17]. Furthermore, it was found that approximately 70% of the CD4+ T cells here are effector memory T cells or effector T cells[7 17]. Most of the CD8+ T cells in the ectocervix were found to be effector T cells[7 17 19]. A similar pattern was seen in the population of B cells in the ectocervix, which is typically less than 1% in the ectocervix and endocervix[7 17]. The endocervix was found to contain the most IgG (immunoglobulin G) and IgA (immunoglobulin A) antibody-producing cells[9 20]. The NK cell population in the ectocervix was found to be an average of 2.7% of the immune cells, in which the endocervix and ectocervix showed similar ranges for NK cells[7 17].

1.3.3 Immune cell distribution in the endometrium

The human endometrium contains lymphoid aggregates with a B cell core, with T cells surrounding this and macrophages surrounding the T cells[7 15 18]. Lymphoid aggregates are usually larger in the secretory phase, containing around 3000-4000 cells, and are smaller in the proliferative phase containing around 300-400 cells[7 9 21]. The endometrium has a small population of CD3+ T cells present, with 66% of them being CD8+ T cells and 33% being CD4+ T cells (Figure 1)[7 22 23]. NK cells are present in different proportions in the endometrium depending on the stage of the menstrual cycle[7 17]. After ovulation, NK cells increase in numbers during the secretory phase up until menstruation, but have a small population during the proliferative phase[7 21-23]. Neutrophils are present in very low numbers in the endometrium but increase prior to and during menstruation, accounting for up to 6-15% of the total immune cells[22 23]. Macrophages are present in all phases of the menstrual cycle, but increase during secretory phase and menstruation[7 23].

1.3.4 Immune cell distribution in the vagina

There is a large CD45+ population in the lower FRT, with approximately 50% of these cells being CD3+ throughout the menstrual cycle[11]. The lower vagina consists of a large portion of CD8+ T cells in the lamina propria, with few CD4+ T cells (Figure 1)[7 8 19]. A small population of macrophages and DCs are found in the lower vagina[7]. In the upper vagina, CD8+ T cells are abundant, with DCs being the next largest population[7 9 19]. CD8+ T cell aggregates are commonly found in the vaginal epithelium[9 19 21]. Studies have also shown macrophages present in the vagina, but these are usually found in low cell numbers[7 19]. It was reported that B cells in the vagina represent approximately 10% of leukocytes, present in low numbers

throughout the menstrual cycle[7 8]. From cervicovaginal secretions, IgG was found to be more abundant than IgA[24]. Studies have also shown that the populations of immune cells in the FRT fluctuate during the menstrual cycle, however, there does not seem to be a large difference between the populations of T cells and APCs in the vagina throughout the menstrual cycle[7 19].

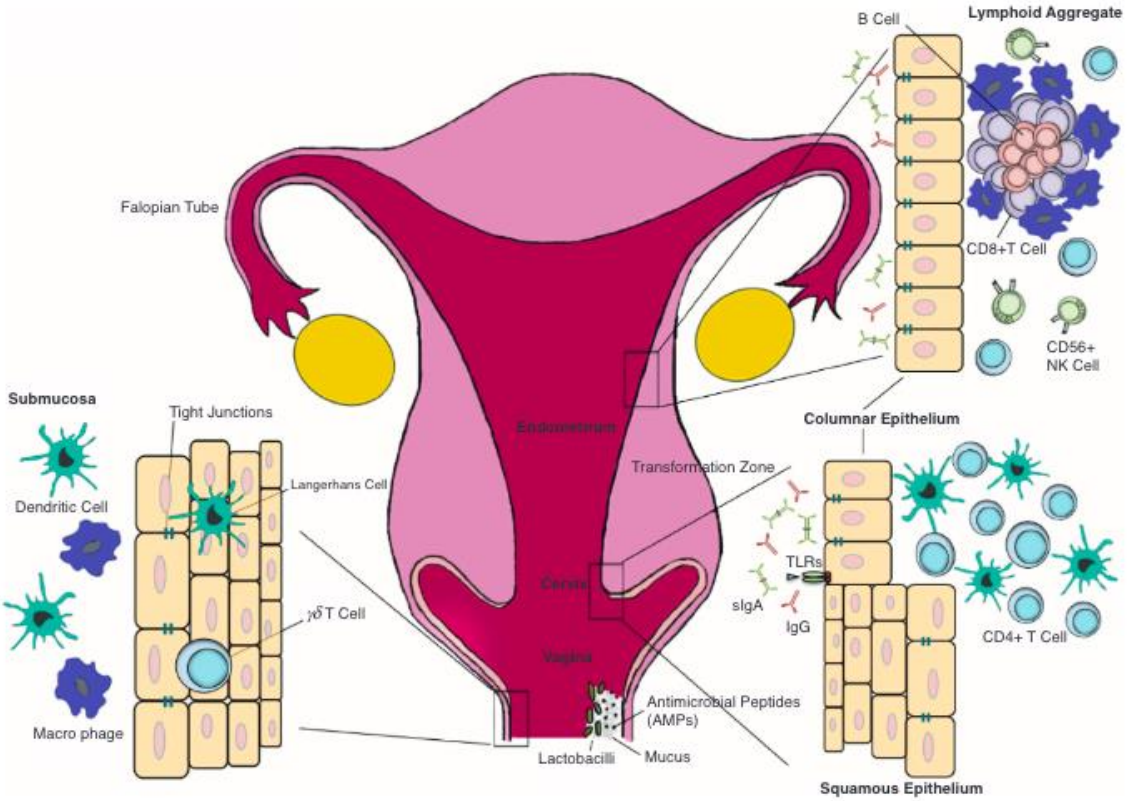


Figure 1. Diagram of distribution of various immune cells in different parts of the female reproductive tract. Tight junctions, AMPs, TLRs, as well as immune cells including NK cells, CD4+ T cells, CD8+ T cells, macrophages, dendritic cells, B cells and antibodies are outlined in the different areas of the FRT which provide protection against external pathogens. Figure retrieved from (Nguyen et al. 2014)[9].

1.4 Immune cells in the mouse reproductive tract

While humans and mice have similar immune cells, the distribution and density of immune cells in the reproductive tract differ. By examining dendritic cell distribution in the vagina of mice, it was found that dendritic cells were found in the lamina propria[25 26]. Additionally, during the metestrus stage of their cycle, neutrophils are present in the lumen[25 26]. During metestrus and diestrus, there are large amounts of Langerhans cells[25 26]. Another study also showed that NK cells are present in the endometrium as well, showing similar patterns of NK cells during pregnancy to humans[27]. Studies examining immune cells in the mouse genital tract have shown that following intravaginal infection with HSV-2, the mouse genital tract produces HSV-specific CD4+ and CD8+ T cells, which produced interferon gamma (IFN-γ)[28]. Studies have also showed that tissue resident memory T cells (TRMs) are present in the mouse reproductive tract following intranasal and intravaginal immunization with an attenuated strain of HSV-2, with mainly CD4+ TRMs present, which are able to provide protection against subsequent challenge[29-31]. Another study also showed that intravaginal immunization against HPV (human papillomavirus) also induced CD8+ TRMs in the mouse vagina[31 32]. Macrophages are also present in the endometrium, increasing in density during the secretory phase similar to humans[26 33 34]. In the mouse uterus macrophages and dendritic cells are present[26 34 35]. The mouse uterus was found to contain IgA and IgG producing plasma cells as well as in the endometrium[24 36].

1.5 Innate Immune Response to HSV-2

1.5.1 Epithelial cell related innate immunity

Epithelial cells line the reproductive tract and are held together by a variety of cell-cell junctions to maintain a physical barrier against external pathogens[37]. Epithelial cells and neutrophils secrete antimicrobial peptides (AMPs), which can be antibacterial, antifungal and antiviral[9 38]. These AMPs include defensins, lysozymes, protease inhibitors, lactoferrin and calthelicidin which have been found in the FRT, but lactoferrin and lysozyme have been shown to impede the spread of HSV to other cells[9 38 39]. HSV-2 is first recognized by the immune system through the PAMP (pattern associated molecular pattern) -PRR (pattern recognition receptors)[5] recognition system. PRRs, like toll like receptors (TLRs), are present on epithelial cells[5]. The immune system recognizes the virus glycoproteins and nucleic acids as PAMPs by signalling through TLRs, which then triggers an innate immune response[5]. There are several TLRs that recognize HSV, such as TLRs 1, 2, 3 5, 6, and 9, which have been detected on the surface of epithelial cells in the vagina and cervix[5 40 41]. Recognition of HSV-2 through TLRs stimulates the secretion of type I interferons through the transcription factor MyD88 (myeloid differentiation primary-response protein 88). Studies have shown HSV-2 is recognized through TLR9, which is required for IFN-α secretion, where the absence of TLR9 interferes with this[42]. Type I interferons are important for viral clearance in the innate immune response which include IFN-α and IFN-β. Studies have also indicated that TLR2 can recognize HSV-2, activating the transcription factor NF-κB (nuclear factor-κB)[43 44]. A study obtained bone marrow derived DCs from TLR knockout mice, which included knockouts of TLR2, TLR9, and MyD88, and examined their immune responses towards HSV-2 infection [40 42]. It was found that bone marrow derived cells from these mice had reduced interleukin-6 (IL-6) and interleukin-

12 (IL-12) responses when stimulated *in vitro* with different HSV strains[42]. TLR and MyD88 signalling is required for the induction of Th1 responses, which link innate and adaptive immune responses[45]. Type I IFNs, can induce expression of TLR3, which recognizes double-stranded RNA and has been shown to induce activation of DCs[46]. In a study that immunized mice with HSV-2 gD with poly I:C as an adjuvant (ligand for TLR3), an antiviral immune response was elicited and provided protection against a lethal dose of HSV-2[46]. Another study explored the TLR7 and 8 ligand, R-848, showing that this treatment in mice provided little protection against intravaginal HSV-2 infection compared to mice treated with CpG (the TLR9 agonist) and untreated mice[47]. The R-848 treated mice showed increased pathology and lower survival compared to the other groups 2 weeks after infection, showing that TLR7 and 8 may not be one of the main TLRs involved in the antiviral HSV-2 immune response[47].

HSV has also been shown to bind to cytosolic receptors, which recognize viral infections independent of TLRs[1 5]. One of said cytosolic receptors is IFI16 (interferon-γ-inducible protein 16), which senses nucleic acids and another one is RIG-I (retinoic acid inducible gene I), both responsible for the type 1 interferon response[5 41]. IFI16 also signals through the transcription factors IRF3 (interferon regulatory factor 3) and NF-κB to induce type 1 IFNs and IL-6 production[5 41]. Additionally, cGMP-cAMP synthase (cGAS) is another cytosolic receptor which can activate the transmembrane adaptor STING (stimulator of interferon genes) to cause an IRF3 dependent production of type I IFNs[5 41]. These pathways promote transcription of proinflammatory cytokines such as type I IFNs, IL-12, IL-18, inducing IFN-γ production from T cells, contributing to the antiviral immune response against HSV-2[5 41].

1.5.2 Other innate immunity

Type I interferons signal through the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway, part of the first line of defense against a viral infection[48 49]. This pathway regulates transcription of cytokines to control survival, activation and proliferation of cells[48]. Studies have assessed the role of type I interferons using IFN-α and IFN-β knockout mice infected with HSV-2[48]. These mice showed high viral titers, severe disease progression and lower survival compared to mice with functioning type I IFN production[48]. IFN-α and IFN-β were found to be required for induction of Th1 responses and viral clearance in a primary infection, as well as early anti-viral immunity[48 49]. However, immunization with an attenuated thymidine kinase (TK^-) deficient HSV-2 strain, Lyons, showed that IFN-α and IFN-β were not required for induction of protective Th1 responses[48]. Type I IFNs can activate other innate immune cells such as NK cells, macrophages, and dendritic cells (DCs), to be recruited to the site of infection, being critical in this innate immune response and contributing towards triggering of adaptive immune response against viruses[5 41 48 49].

DCs are an important component of the innate immune response as they are able to sense foreign- and self-antigens, present them to T cells, resulting in T cell activation or inactivation[41]. Infection with HSV-2 can decrease expression of co-stimulatory molecules such as CD80 and CD86, present on the cell surface of DCs, which can affect the ability for DCs to activate T cells[41]. Antigen presentation to CD8+ T cells can also be affected, as inhibition of autophagy in DCs can be caused by HSV-2[41]. HSV-2 can also reduce the capacity for DCs to migrate to the lymph nodes from infected tissues, also impeding antigen presentation and antiviral immune responses[41]. Plasmacytoid dendritic cells (pDCs) are a rare subset of DCs that circulate throughout the body, and upon viral infection, will be recruited to the site of

infection as part of the innate immune response. Theses pDCs have been found in the lungs, Peyer's patches and liver[50-52]. Through activation of TLR9, plasmacytoid dendritic cells (pDCs) can produce type I interferons[41 53 54]. A study that investigated the role of pDCs in intravaginal HSV infections found that pDC depleted mice and TLR9 deficient mice showed similar outcomes, such as lower survival, higher viral titers and reached higher pathology scores earlier than the wildtype (WT) mice[54]. This demonstrated that pDCs and TLR9 were both necessary to control viral replication in the vagina[54]. However, Th1 differentiation was not affected when pDCs were depleted, showing similar levels of IFN-γ secreted by the pDC depleted mice and WT mice[54]. Another study found that depletion of these cells in mice did not affect viral load or survival[53]. However, pDCs were required for type I interferon production, activation of NK cells and CD8+ T cell responses after systemic HSV-2 infection[53]. These studies suggest that pDCs may not be required, but are beneficial in HSV-2 infections, aiding in the immune response.

In the vaginal epithelium, neutrophils are the dominant leukocyte population present[55]. Through depletion of polymorphonuclear leukocytes (PMNs) in mice, the role of neutrophils in HSV-2 infection were assessed[55]. Mice were immunized with HSV-2 TK^- and 24 hours afterwards, neutrophils were found to be the main leukocytes present in the vaginal mucosa[55]. There was an influx of neutrophils early on, but on day 3 post infection, the population started to decline[55]. In the absence of neutrophils, the HSV-2 infection was not effectively cleared, which was evident through the high viral titers in these mice[55]. This shows that neutrophils are an important component of the innate immune response towards HSV-2.

Studies have shown conflicting results in elucidating the role of NK cells in HSV-2 infections[56]. It has been found that type I interferons can induce IL-15 production from cells to

promote survival and proliferation of NK cells[49 56 57]. To deplete NK and natural killer T cells (NKT cells), IL-15 knockout mice were infected with HSV-2[56]. Compared to control mice, the IL-15 knockout mice showed lower survival[56]. Additionally, they showed increased susceptibility to low doses of HSV-2 compared to immunodeficient mouse groups, which included RAG-2 deficient mice that lacked all lymphoid cells, RAG-2 deficient mice that lacked T and B cells, and CD-1 deficient mice that lacked NKT cells or did not express IFN-γ[49 56]. The IL-15 knockout mice showed viral titers comparable to the RAG-2 deficient mice which were more susceptible to HSV-2 by 100-fold[49 56]. IL-15 knockout mice also showed a reduction in IFN-γ production during the initial days post infection, demonstrating that the NK and NKT cells are the main source of IFN-γ here[57]. However, depletion of NK cells in mice was shown to reduce IFN-γ production early on, which is crucial for the innate immune response[57 58]. Another study also showed that NK cell depletion in mice was associated with high HSV-2 levels in tissues including vaginal tissue, spinal cord and brain stem[59].

The role of macrophages was assessed through a study examining macrophages from mice after infection with HSV-2[58]. HSV induced IFN-α and β, RANTES and TNF-α production in macrophages[58]. Post HSV-2 infection, murine peritoneal macrophages showed increased levels of expression of various chemokines which included RANTES, CXCL2, CXCL13, MIP1-α and MCP-5[58 60]. These chemokines can attract various immune cells such as T cells, monocytes, NK cells, basophils and mast cells to the site of infection to coordinate an immune response[58].

1.6 Adaptive Immune Response to HSV-2

1.6.1 T cell mediated immunity to primary HSV-2 infection

In the adaptive immune response CD4+ and CD8+ T cells play an important role in clearing viral infections such as HSV-2. Studies have explored how critical these T cells are in HSV-2 infections through depleting mice of these T cells and examining how well they are able to clear the virus[61]. Studies have highlighted that CD4+ T cells provide better protection against HSV-2 and that CD8+ T cells alone are not effective in providing protection[49 57 61 62]. A study with CD8+ T cell deficient mice showed higher viral titers after HSV-2 infection compared to wildtype (WT) mice[61]. The CD4+ T cell deficient mice showed higher viral titers compared to the CD8+ T cell deficient mice[61]. This demonstrates that CD8+ T cells do play a role in viral clearance, however, CD4+ T cells play a more critical role[61]. An additional study showed that CD4+ T cell knockout mice showed greater disease severity post HSV-2 infection compared to mice with functioning CD4+ T cells[63]. The production of the cytokines IFN-γ, IL-2 and IL-4 were also reduced from the mice depleted of CD4+ T cells and mice depleted of CD8+ T cells, however the reduction was greater in the CD4+ T cell deficient mice[61]. This further demonstrates the importance of CD4+ T cells in aiding in the clearance of HSV-2 infections, as the production of these various cytokines can also affect the progression of infection[61]. Additional studies have shown that CD4+ T cells are required to prime CD8+ T cells and allow for cytotoxic CD8+ T lymphocyte migration to infection areas in various tissues, occurring through the secretion of IFN-γ as well as eliciting the production of chemokines such as CXCL9 and CXCL10[64 65]. Other studies have also shown that in HSV-2+ women, a higher frequency of CD4+ T cells have been detected compared to CD8+ T cells[66 67].

1.6.2 The role of T cells in an immunization and challenge model of HSV-2

The previously mentioned studies focused on primary HSV-2 infections, so studies have assessed if the importance of these cells changes in an immunization and challenge model. It was found that CD4+ T cell knockout mice could not mount a protective response after immunization with HSV-2 TK^- and subsequent challenge with WT HSV-2[68]. These mice showed high disease scores and eventually died, similar to the control unvaccinated group[68]. They additionally showed low IgG antibody titers[68]. When CD8+ T cell knockout mice were immunized with HSV-2 TK^- and challenged with WT HSV-2, they survived and did not show any signs of severe disease progression[68]. This indicates that CD4+ T cells are required to mount an effective immune response against HSV-2, but CD8+ T cells do not show the same critical role as CD4+ T cells[68 69]. When these T cell knockout mice were administered IFN-γ, it was found that this cytokine was able to clear the infection, also playing a critical role in this immune response to HSV-2[68]. Further studies which have neutralized IFN-γ as well as using mice deficient in IFN-γ have shown that the absence of IFN-γ does not allow for viral clearance in which mice still result in high HSV-2 titers, as well as decreased survival compared to mice with functioning IFN-γ production[70 71]. This also showed that intravaginal immunization was not effective in IFN-γ knockout mice highlighting the importance of IFN-γ production from HSV-specific CD4+ T cells in clearing HSV-2 infection[69 71].

1.6.3 Memory T cells and tissue resident memory T cells

Memory T cells also play a role in protecting against infection, and recurrent infections. TRMs are particularly important in providing protection in a local area against recurrent HSV-2 infections[29 72 73]. Studies have also shown TRMs are important for chronic HSV-2

infections, in which they are more effective than effector memory T cells and are able to respond to the infection quickly due to their location[29 72 73]. One study explored whether TRMs or circulating memory T cells are responsible for providing protection against HSV-2 by using parabiotic combinations of groups of nonimmunized and intravaginally immunized mice with HSV-2 TK^- [30]. Groups of mice were treated with sphingosine-1-phosphate receptor 1 (S1PR1) to prevent lymphocytes from migrating out of the lymph nodes, depleting the circulating memory lymphocyte population to represent the mice with only TRMs present[30]. Mice with TRMs were able to control the infection better than mice with circulating memory T cells, shown through their lower viral titers, lower disease scores and higher survival[30]. When immunized CD4+ T cell knockout and CD8+ T cell knockout mice were paired with a WT mouse, the CD8+ T cell knockout pair showed better protection against infection compared to the CD4+ T cell knockout pair[30]. This further emphasizes the importance of CD4+ T cells in HSV-2 infections[30]. Additionally, it was found that IFN-γ secretion from macrophages induced the production of the chemokines CCL5 and CXCL9, which are required for TRM retention in the vagina[30]. A study by our lab showed that intranasal immunization with an attenuated strain of HSV-2 under estrogen treatment results in a higher CD4+ T cell population than CD8+ T cells in the FRT[29]. Furthermore, another study showed that HSV-specific TRMs provide better protection against HSV-2, in which high numbers of these TRMs, particularly CD8+ TRMs can provide better immunity due to the production of IFN-γ[72 74]. This outlines the importance of memory T cells in HSV-2 infection, particularly TRMs.

1.6.4 B cells

B cells and the antibodies they produce also aid in the immune response against HSV-2. When B cell knockout mice were intravaginally infected with HSV-2 TK^-, inflammation and high viral titers were seen as compared to WT mice[68]. Another study also demonstrated that B cell deficient mice were able to clear intravaginal HSV-2 infection, showing lower viral titers, where mice still showed effective T cell responses comparable to control mice[75]. Additionally, one study demonstrated that B cell deficient immunized mice were able to survive high and low dose intravaginal HSV-2 challenges, but that controlling viral load was mediated by T cells[69]. Furthermore, in secondary HSV-2 challenge in mice, it has been shown that B cells play a role in stimulating the production of IFN-γ from memory CD4+ T cells to control HSV-2 infection by presenting viral antigens to these cells[76]. Together, these studies suggest that B cells are not required in the immune response against HSV-2 to clear the infection, however, still contribute towards controlling the infection.

1.6.5 Antibodies

The main antibody present in the lower human female reproductive is IgG[77 78]. One study explored how effective these antibodies are in providing protection against HSV-2 in mice, where vaginal secretions from immunized mice with HSV-2 TK^- and nonimmunized mice were passively transferred into the vaginas of nonimmunized mice[79]. It was determined that purified IgG in vaginal secretions from immunized mice were able to effectively neutralize the virus, showing low viral shedding and low disease scores[79]. When examining the ability of IgA to neutralize virus, viral shedding was higher compared to IgG, showing that IgG is the main antibody responsible for neutralizing the virus[79 80]. Through an IgA knockout mouse model,

studies have demonstrated that these mice are effectively immunized with HSV-2 TK^- and show protection comparable to normal mice when challenged with HSV-2[81]. These studies demonstrate the importance of IgG in HSV-2 infections, particularly in immunization and challenge models.

1.7 Hormones

1.7.1 The menstrual cycle in women

During the menstrual cycle, women undergo fluctuations in the sex hormones, estrogen and progesterone[24 82]. The menstrual cycle has four phases: the menstrual phase, proliferative or follicular phase, mid-cycle and secretory or luteal phase[24]. During the proliferative phase estrogen levels rise and peak just before ovulation[24]. Ovulation then occurs at the mid-cycle, where estrogen levels decrease[24]. Progesterone levels increase moving into the secretory phase[24]. The entire menstrual cycle lasts on average 21-35 days but differs between women[24].

1.7.2 Mouse estrous cycle

Similar to women, female mice also undergo a cycle in which fluctuations in sex hormones occur[83]. This is referred to as the estrous cycle and is comprised of four stages, including metestrus, proestrus, estrous and diestrus[83]. The estrus stage is similar to the proliferative phase that women undergo in that a peak in estrogen levels also occurs in mice[83]. Diestrus is comparable to the secretory phase in women, with high progesterone levels[83]. The mouse estrous cycle lasts for about 4 to 5 days before repeating again[83]. Each stage lasts for about 1 day, with diestrus lasting about 2-3 days[83].

Studies have shown that mice are not susceptible to HSV-2 infections during the estrus stage of their cycle[84 85]. In one study that staged mice on the various stages of the estrous cycle, the number of mice in each cycle that were susceptible to intravaginal HSV-2 was recorded[84]. Mice were more susceptible to HSV-2 during the diestrus phase of their cycle, with all mice in diestrus becoming infected and continuing to show increasing viral titers post infection[84]. Additionally, diestrus treated mice showed lower survival after infection compared to mice infected under other stages, in which mice in estrus showed little to no viral shedding post infection[84]. This has also been demonstrated in a study in our lab, where mice in diestrus and estrus were infected with varying doses of HSV-2[85]. Mice in diestrus were easily infected, whereas mice in estrus were only susceptible to HSV-2 at higher doses[85].

1.7.3 Effect of sex hormones on FRT immune cells and responses

Various studies have shown that sex hormones can affect immune cells present in the FRT and the immune responses that occur here[9 24]. As previously stated in Section 1.3, there are different distributions of immune cells throughout the human FRT[9 24]. However, the distribution of immune cells can change with fluctuations of hormones throughout the menstrual cycle[24]. Additionally, fluctuations in sex hormones can affect epithelial cells[24]. As estrogen levels rise, proliferation of epithelial cells increases in the uterus and vagina, shown in mice in a previous study from our lab[24 86-88]. Progesterone treatment in mice has shown to thin the vaginal epithelium, however the same has not been demonstrated in human studies[24 88]. Furthermore during the estrogen high phase of the cycle, mucus in the human FRT is thin and has a consistency similar to water, compared to the thick and viscous consistency present during the secretory phase, in which mucus can provide protection to epithelial cells from pathogens[9

24 89]. It is also thought that during the secretory phase of the menstrual cycle, there is a window of vulnerability towards STIs, as the FRT is preparing for implantation and preventing fetal rejection, suggesting that immune responses allow for increased susceptibility to STIs[24 82 90].

1.7.4. Mouse model of HSV-2 infection

Mice are a useful animal model to study HSV-2, as they exhibit similar symptoms to humans with HSV-2 which include lesions, redness, swelling and viral shedding[91]. The virus is able to infect the genital region in mice as well as spreading to the nervous system[91 92]. However, primary HSV-2 infections can be lethal to mice, in which severe symptoms include hind limb paralysis and immobility[92]. Depending on the dose of HSV-2 mice are infected with, death can occur between 6-8 days post infection[92]. As mentioned previously, the stage of the estrus cycle influences whether mice are susceptible to HSV-2, with mice being more susceptible to infection during diestrus[84]. Therefore, many studies have developed a routine of giving mice progesterone or a progestin-based hormone treatment prior to intravaginal HSV-2 infection[80 91]. This treatment synchronizes all mice in the diestrus stage of their estrous cycle and ensures all mice can undergo HSV-2 infection at the same time[80 91].

In addition to primary HSV-2 infections, studies have also assessed different mouse models of HSV-2 immunizations. McDermott et al. developed a mouse model to intravaginally immunize mice against HSV-2, which will confer protection after subsequent challenge intravaginally[93]. To immunize mice, a strain of HSV-2 was made by partially deleting the TK gene[93]. This TK gene is required for viral DNA synthesis in quiescent cells such as the sensory neurons[93]. This resulted in mice becoming infected with nonlethal HSV-2, but the virus cannot spread to the neurons[93]. This therefore prevented severe disease outcomes which occur with the WT strain

of HSV-2 which includes the TK gene[93]. Mice infected with the HSV-2 TK^- strain still showed viral shedding, however, were able to survive the infection and showed protection when intravaginally challenged with WT HSV-2[93]. After intravaginal immunization, HSV-2 specific antibodies have been found in the vagina and in vaginal secretions[79 80].

Other studies, as well as many studies from our lab, have frequently utilized an ovariectomized mouse model to study HSV-2 infections[88 94-98]. In this model, the ovaries are surgically removed from female mice and afterwards mice are given 2 weeks to recover and to allow the endogenous hormones to dissipate[88 96 97]. Studies from our lab have also shown that this ovariectomized mouse model is highly susceptible to HSV-2 infections without the need for hormone treatments, as shown through their viral shedding and increasing pathology scores each day post HSV-2 infection[88]. This is beneficial for studying effects of hormone treatments in HSV-2 infections in a mouse model, as specific effects of exogenous hormone treatment can be studied without the influence of endogenous hormones.

1.7.5 Effects of estrogen in HSV-2 infections in mice

Several studies have demonstrated the beneficial effects of estrogen treatment in primary HSV-2 infection[29 88 96]. Mouse studies have shown that treatment with estrogen results in lower HSV-2 pathology scoring, lower viral titers, and greater survival as compared to other treatments including progesterone[88]. Estrogen treated mice also showed thickening of vaginal epithelium, making it impermeable to the virus, providing protection against HSV-2[88]. In a primary HSV-2 infection, estrogen treatment is effective in providing protection against infection in mice[88]. More recent studies in our lab have shown a mechanism of how estrogen enhances antiviral immune responses to HSV-2 infection in mice[96]. These mice treated with estrogen showed

better Th1 and Th17 responses in the vaginal tract through specific programming of DCs, providing greater protection against HSV-2 compared to untreated mice[96]. This was demonstrated through the analysis of vaginal tissue from mice, in which mice were immunized intranasally with HSV-2 TK-, then challenged intravaginally with WT HSV-2[96]. The estrogen treated mice showed higher IFN-γ and IL-17 expression early on within days 1 and 3 post HSV-2 infection compared to control mice with no estrogen treatment[96]. Additionally, CD11c+ dendritic cells were found to be the main cell population responsible for priming Th17 responses in the genital tract, which occurred through an IL-1ß dependent pathway, contributing to protection against HSV-2[96]. Another study from our lab showed that estrogen treatment in mice intranasally immunized against HSV-2 resulted in enhanced Th17 and Th1 responses in the FRT as well as greater CD4+ memory T cell populations in the FRT to provide protection against subsequent HSV-2 infection[29].

A study from our lab showed that intravaginal immunization with HSV-2 TK^- is not effective after mice received estrogen treatment[97]. This was demonstrated by the absence of viral shedding after intravaginal immunization in estrogen treated mice[97]. Therefore, when these mice were challenged with WT HSV-2, they were not protected and succumbed to infection, eventually dying[97]. These mice also showed very high viral shedding post challenge, thinning of the vaginal epithelium from infection, as well as showing little to no HSV-2 specific antibody titers[97]. However, it has been shown that intravaginal immunization on day 5-7 after estrogen treatment, resulted in some protection against subsequent WT HSV-2 challenge[95]. Thus, these studies demonstrate that treatment with hormones can have differing effects depending on the infection and immunization.

1.7.6 Effects of progesterone in HSV-2 infections in mice

Studies have also shown that progesterone treatment can affect susceptibility to HSV-2 infections[85 88 90 99 100]. Studies from our lab showed that progesterone treatment in mice showed a 10-fold increased susceptibility to HSV-2 compared to normal untreated mice in diestrus[85]. Progesterone treatment in mice has also been show to result in high pathology scores earlier on and high viral titers post intravaginal HSV-2 infection compared to mice given estrogen treatment or no treatment[88]. We also showed lower survival compared to other groups[88]. Additionally, histological analysis of the vaginal tract of mice, showed that progesterone treatment results in thinning of the vaginal epithelium, allowing virus to easily enter as barrier permeability was increased[88]. There was also extensive HSV-2 specific staining observed in the vaginal tracts of the progesterone treated mice 24 hours after infection[88]. Therefore, progesterone treatment does not provide protection against a primary HSV-2 infection in mice. Additional studies on progesterone treatment in human or murine T cells have shown that it can promote Th1 responses towards Th2 responses, as well as showing decreased IFN-γ production which could negatively affect immune responses to HSV-2 as this cytokine is important for viral clearance[99-101].

In an immunization and challenge model, progesterone treatment in mice showed different outcomes compared to a primary infection[97]. Ovariectomized mice under different hormone treatments including progesterone, estrogen, estrogen + progesterone and saline were immunized intravaginally with HSV-2 TK^-[97]. Progesterone treated mice showed viral shedding post immunization, but estrogen treated mice showed no viral shedding post immunization[97]. Three weeks later when mice were challenged intravaginally with WT HSV-2, no viral shedding was detected in progesterone treated mice[97]. When vaginal tracts from mice were analysed through

histology, the progesterone treated mice also showed no positive HSV-2 specific staining[97]. Vaginal washes from the mice were also collected and mice treated with progesterone resulted in high levels of HSV-2 specific antibodies as well[97]. Progesterone treatment results in an effective intravaginal immunization with HSV-2 TK^- and therefore mice show protection when subsequently challenged with WT HSV-2[97]. In an immunization and challenge model, progesterone treatment prior to intravaginal immunization with HSV-2 can be beneficial in providing protection against subsequent challenge in mice.

1.9 Hormonal contraceptives and HSV-2 susceptibility

1.9.1 Hormonal contraceptives and global use

Based on differences in the effects of sex hormones in HSV-2 infections, studies have started to examine the effects of hormonal contraceptives, which include different combinations of these sex hormones. Of the 1.9 billion women in the world that are of reproductive age (15-49 years), there are 842 million women who use modern methods of contraceptives, which include barrier methods and hormonal contraceptives, reported from United Nations[102]. There are an estimated 80 million women who use traditional methods which include periodic abstinence or withdrawal, as estimated in 2019 from the United Nations[102]. There are many different types and formulations of hormonal contraceptives worldwide[103]. Injectable hormonal contraceptives are long lasting hormonal contraceptives that provide a peak level of hormones that will gradually decrease in the body over a long period of time[103]. Data from the United Nations in 2019 stated that there has been an increase in the use of injectable hormone contraceptives, which was found to be 17 million in 1974, but increased to 74 million in 2019[102]. Injectable contraceptives are most used in Sub-Saharan Africa, with 9.6% of women

of reproductive age using this method[102]. With such a large population of the world using hormonal contraceptives, it is important to understand what additional effects these hormonal contraceptives may have in users.

1.9.2 DMPA

DMPA (depot medroxyprogesterone acetate) is the most popular injectable hormonal contraceptive, particularly popular in Sub-Saharan Africa [103]. DMPA is an injectable hormonal contraceptive given every 3 months, at a dose of 150 mg [103]. It is a synthetic progestin, which is made structurally similar to progesterone and mimics the actions of progesterone[103 104]. The active component of DMPA is MPA (medroxyprogesterone acetate)[105]. By mimicking the actions of progesterone, MPA shows high affinity for the progesterone receptor (PR) but has also been shown to have affinity for other steroid receptors such as androgen receptor (AR) and very high affinity for the glucocorticoid receptor[103 106-109]. Through binding to steroid receptors, DMPA can alter the gene expression of various genes regulating immune function and metabolism[103 105 109 110]. It can alter expression by causing transactivation, to increase the rate of transcription of genes or cause transrepression, which decreases the rate of transcription of genes[103 105 107 110].

1.9.3 NET

Norethisterone is also an injectable hormonal contraceptive, which has been gaining interest in research. It is not as popular as DMPA and is mainly used in South Africa[103]. This contraceptive is available in two different formulations, an oral pill and an injectable[103]. The oral contraceptive is norethisterone acetate (NET-A) and the injectable contraceptive is

norethisterone enanthate (NET-EN), given at a dose of 200 mg every 2 months[103 111 112]. These various formulations are all metabolized to the compound norethisterone (NET) once given[105]. Similar to DMPA, NET is also a synthetic progestin, showing high affinity for the progesterone receptor[103 105 110]. In addition to the progesterone receptor, NET also shows affinity for the androgen and mineralocorticoid receptor[103 104 109]. NET can also have effects on the rate of transcription of genes, however it does not show the same high affinity for the GR that DMPA does[103 105 108 109]. Therefore, NET does not seem to show the same effects that are associated with DMPA use[103 108].

1.9.4 Steroid receptor-mediated effects of DMPA and NET

DMPA has been shown to have very high affinity for the glucocorticoid receptor (GR)[105]. Typical ligands for the glucocorticoid receptor are glucocorticoids which are anti-inflammatory and immunosuppressive[105]. Through this mechanism DMPA is thought to suppress the immune system by causing transrepression of genes regulating cytokine expression, therefore affecting immune responses, and affecting susceptibility to infections such as HSV-2 and human immunodeficiency virus (HIV)[103 105].

One study assessed the binding affinities of MPA, NET-A (norethisterone acetate) and progesterone to the GR[105 108]. MPA was found to have the highest binding affinity to the GR compared to other hormonal contraceptives and showed higher transactivation for the GR compared to NET-A and progesterone when examined in human lung carcinoma A549 cells and human embryonic kidney HEK293 cells [108]. Additionally, an IL-8 promoter construct was examined where MPA acted as an agonist for transrepression of the GR compared to NET-A and progesterone, demonstrating the immunosuppressive effects of MPA[108]. Another study used

endocervical epithelial cells (End1/E6E7) and cervical epithelial cells (HeLa) to examine hormone treatment and their effects on the expression of various genes regulated through the GR[105]. MPA was found to act as an agonist to the GR, decreasing mRNA expression of pro-inflammatory cytokines, such as IL-6 and IL-8[105]. MPA also upregulated mRNA expression of anti-inflammatory cytokines[105]. However, with NET treatment, little changes were seen in mRNA expression, but these changes were not significant[105]. Similar results were seen when examining the effects of MPA in human ectocervical Ect1/E6E7 cells, in which MPA downregulated the pro-inflammatory gene RANTES, but upregulated RANTES in human vaginal epithelial cells Vk2/E6E7[110]. However, NET did not show any effects on RANTES in these cell lines[110]. Through a study using human peripheral blood lymphocytes, MPA was shown to cause transrepression of IL-2 through the GR, PR and AR[107]. Through these various studies mentioned, all have demonstrated the effects of MPA through the GR, and other steroid receptors and some of their immunosuppressive effects on genes.

1.9.5 Observational studies on hormonal contraceptives and risk for STI acquisition

Numerous studies have analyzed the associated risk for STI acquisition with hormonal contraceptive use in women, focusing on HIV acquisition. A meta-analysis of various observational studies was performed to assess DMPA use and its associated risk for HIV in women in Sub Saharan Africa[113]. Hazard ratios were defined for all these studies, resulting in most showing an increased risk for HIV acquisition with the use of DMPA, whereas only a few studies showed no increased risk[113]. Another meta-analysis assessed 18 different studies of individual patient data which examined hormonal contraceptive use and HIV infection in Sub-Saharan Africa[114]. The risk for HIV infection was compared between women using combined

oral contraceptives, DMPA, NET-EN and women not using hormonal contraceptives[110]. There was an increased hazard ratio associated with DMPA use, which was higher than hazard ratios found for other contraceptives[110]. These studies provide evidence that there is an increased risk for HIV-1 acquisition associated with progestins, particularly DMPA. However, there are conflicting studies which have not found an associated increase for STI acquisition with the use of DMPA. One study recruited women from Zimbabwe, Thailand and Uganda to assess their risk for HIV acquisition based on hormonal contraceptive use, for which there was no associated risk found with contraceptive use, including DMPA[115]. Another study examined incidences of STIs including C. trachomatis, N. gonorrhoeae, T. vaginalis and its association with hormonal contraceptive use in women from Uganda, Zimbabwe, Malawi and South Africa[116]. Some of the contraceptives that women used included DMPA, implants, NET-EN and copper IUD (intrauterine device)[116]. Through this study there were no associations found between the use of progestins and incidences of STIs, however there were higher incidences of T. vaginalis in copper IUD users[116]. Additionally, one study compared MPA and NET treatment on cervical and ectocervical explants, showing that MPA treatment at a dose of 10 nM (physiologically relevant to peak serum levels seen in women using DMPA) resulted in increased R5-tropic viral replication of HIV[117]. With these contradicting studies, there is no consensus as to whether DMPA increases the risk for STI acquisition or not.

1.9.6 Effects of DMPA and NET on the immune system in human studies

Many studies have focused on characterizing the effects of DMPA on the immune system. A study assessed whether DMPA affects susceptibility to HIV by collected vaginal, endocervical and rectal swabs from women in New Jersey aged 18-35 years old that were using DMPA[118].

They found that in 3 separate visits that occurred over 3 months, there was a decrease in CD4+ T cells in the cervical lumen as well as a decrease in several cytokines, including G-CSF (granulocyte colony stimulating factor), GM-SCF (granulocyte-macrophage colony-stimulating factor), MIP-1α, IL-1β, IL-10, TNF-α, IL-17 and IFN- γ[118]. Michel et al. examined the effect of several hormonal contraceptives such as DMPA, the NuvaRing and combined oral contraceptives in women 19-40 years old[119]. The use of DMPA was associated with lower IFN-α production from human pDCs as well as decreased levels of TNF-α, MIP-1α, MIP-1β, IL-6 and IL-8[119]. These various cytokines and chemokines listed play an important role in recruiting neutrophils, macrophages and lymphocytes to the site of infection[119]. From another study, ectocervical biopsy tissues were taken from women using DMPA for a month and from women who were not using hormonal contraceptives[98]. Results from this study demonstrated that women on DMPA had decreased desmoglein-1, which is a component of desmosomes creating the cell-cell junctions between epithelial cells, indicating that the vaginal permeability is increased[98]. This same group isolated DCs from human PBMCs (peripheral blood mononuclear cells) to explore the effects of MPA on DCs[120]. MPA treatment decreased CD40 and CD80 expression on DCs, which impaired their ability to promote T cell differentiation and as a result, CD4+ and CD8+ T cell proliferation was reduced[120]. PBMCs were taken from premenopausal healthy women, which were incubated with different progestins, including progesterone, NET, MPA, LNG (levonorgestrel) and ETG (etonogestrel) for 24 hours[121]. MPA inhibited IFN-γ production significantly from pDCs and T cells. NET showed little inhibition, with the highest level of IFN-γ production[121]. Furthermore, a study examined cervical T cells from adolescent women after treatment with different hormonal contraceptives, which found that long term NET-EN treatment for 16 weeks did not result in any changes in

frequency of Th17 cells as well as Th17 related cytokines[122]. These changes were compared to baseline levels in women prior to NET-EN treatment, as well as women using combined oral contraceptives[122]. These T cell subsets were analyzed as they have been identified as a subtype of CD4+ cells that are susceptible to HIV[122]. Another study also obtained PBMCs from healthy women 30, 90 and 180 days after receiving hormones, including DMPA, NET-EN or copper IUD[123]. It was found that MPA treatment resulted in a decrease in IFN-γ and TNF-α producing CD4+ and CD8+ T cells after 180 days compared to baseline[123]. Additionally, NET-EN treatment for 90 and 180 days was associated with a decrease in IL-4 producing CD4+ and CD8+ T cells[123]. This shows that DMPA and NET treatment long term can have different effects on immune cells and cytokine production. Overall, these studies indicate that DMPA typically has more effects on the immune system compared to other hormonal contraceptives such as NET which shows little to no significant changes. These changes can negatively impact the immune response to infections, suggesting that DMPA could be providing negative effects on the immune system.

1.9.7 Effects of DMPA and NET on susceptibility to HSV infections in mouse models

Studies have used mouse models to investigate effects of contraceptive use on the immune system and how this affects susceptibility to infections such as HSV-1 and HSV-2. One study investigated the effects of MPA in HSV-1 infections by treating ovariectomized mice with MPA that were latently infected with HSV-1[98]. In the trigeminal ganglia, the MPA treated mice showed decreased TNF and IFN-γ production compared to no treatment groups[98]. This showed that MPA inhibited the production of these cytokines, specifically in CD8+ T cells, therefore impairing their function[98]. It was also found that MPA treated mice infected with HSV-1 had

reduced T cell expansion of various T cell populations in the trigeminal ganglia[124]. This pattern was seen in CD8+ T cells, CD4+ T cells, memory CD8+ T cell precursors, HSV-1 specific CD8+ T cells and HSV-1 specific CD4+ T cells[124]. The T cell expansion reduction was seen 8 days post infection, and this continued up to 35 days post infection[124]. Additionally, NK cells, NKT cells, and macrophage populations were also reduced[124]. Decreased expression of CD70 and CD80 was also seen in DCs from MPA treated mice[124]. Due to the reduced CD70 and CD80 expression, proliferation of HSV-specific CD8+ T cells was reduced[124]. The reduction in T cells and reduced expression of co-stimulatory molecules that occurred with MPA treatment can greatly affect immune responses to infection.

Studies from our lab have also characterized some effects of DMPA in mice with HSV-2, exploring the effects of short term and long-term treatment with DMPA[94]. Mice with long term DMPA treatment resulted in higher HSV-2 viral titres and higher pathology scores earlier on compared to the mice that were on short term DMPA treatment[94]. Furthermore, long term DMPA treatment resulted in impaired Th1 responses and decreased HSV IgA and IgG levels in mice[94]. Additional studies from our lab showed that with DMPA treatment, mice immunized with recombinant gB of HSV-2 and boosted with the adjuvant CpG ODN resulted in lower IgA and IgG antibody titers compared to the previous level of antibodies present 2 weeks prior to DMPA treatment[85]. MPA was also found to inhibit the production and transcription of pro-inflammatory cytokines IL-6 and IL-8[125]. This shows that the treatment length of DMPA can result in different outcomes post infection with HSV-2. One study from our lab also showed that DMPA treatment in mice results in decreased desmoglein-1 expression, weakening the vaginal epithelial barrier[126]. Altogether the results from these studies indicate that DMPA treatment

negatively alters the immune system and the physiology of the vagina in mice which does not result in protection against HSV-2.

A recent study compared the effects of NET and DMPA in mice, showing that both treatments resulted in thinning of the vaginal epithelium, but that DMPA treated mice had more severe thinning, increasing permeability to external pathogens[127]. During HSV-2 infection, NET treated mice showed greater survival and reached high pathology scores later than DMPA treated mice[127]. When DMPA and LNG treatment was compared in mice, both contraceptives increased susceptibility to HSV-2 as indicated by the increased pathology and decreased survival compared to the control group[128]. However, the DMPA treated mice showed lower survival and increased pathology scores earlier on post infection compared to LNG treated mice[128]. The DMPA and LNG treated mice also showed decreased descmocollin-1 and desmoglein-1α protein expression in vaginal tissue, indicating increased barrier permeability[128]. Similar to the in vitro and human studies that were previously discussed, animal studies also show that DMPA showed more severe outcomes after infection with HSV-1 and HSV-2 compared to other contraceptives[94 127 128]. Thus, these collective studies suggest that there may be an alternative contraceptive that should be used over DMPA.

CHAPTER 2: RATIONALE AND HYPOTHESIS

HSV-2 is one of the most common sexually transmitted infections, with women disproportionately affected by this infection globally[2]. However, it is unclear what factors are specifically causing the differential prevalence of infection in women compared to men. One of the factors thought to affect the prevalence is sex hormones. Many women around the world use hormonal contraceptives, which can include synthetic progestins such as DMPA and NET, and it is important to understand how these contraceptives affect women and susceptibility to STIs[102]. While studies have explored physiological effects with different hormonal contraceptives, whether these contraceptives influence susceptibility to STIs in women is still unclear.

DMPA is a widely used injectable contraceptive, especially popular in Sub-Saharan Africa, which has been demonstrated to increase susceptibility to HSV-2 and HIV-1 infections [94 102 113 115 126-130]. This is concerning, and more research is needed to determine the underlying mechanism of how DMPA increases susceptibility to these STIs. Several studies have explored the effects of DMPA on immune cells, cytokines, chemokines, as well as susceptibility to HIV-1 and HSV-2 infections in vivo and in vitro[94 98 105 113 114 118-120 124 126-129]. In murine models, pre-treatment with DMPA for 5 days results in an effective immunization with inactivated HSV-2, where mice show protection when subsequently challenged[94]. However longer treatments with DMPA increase susceptibility and decrease immune responses[94]. Furthermore, when comparing DMPA to other injectable hormonal contraceptives as well as progestins in general, other formulations do not show the same susceptibility to HSV-2 and HIV-1, or immunosuppressive effects seen with DMPA[117 119 123 127 128]. Further research of other injectable hormonal contraceptives can provide information on the different options available that are potentially safer than DMPA.

An injectable progestin-based contraceptive that could be an alternative to DMPA that has not been well researched is NET. Through the studies comparing NET to DMPA, it has been shown that NET has little effect on the immune system, such as insignificant changes on cytokine secretion compared to DMPA[105 117-119 123 128]. Additionally, there is evidence that NET treatment in mice does not result in the same increased susceptibility to HSV-2 and HIV-1 infections as DMPA[127]. However, this is the only study to our knowledge that has tested this in animal models, which examined pathology, survival and vaginal barrier permeability in mice after HSV-2 and HIV-1 infections separately[127]. This study also utilized intact mice with endogenous sex hormones, and as such, effects seen may not be solely due to NET treatment as the influence of endogenous sex hormones may also have an influence on outcomes[127]. Furthermore, it is unknown whether NET treatment alters the immune response in the context of HSV-2 infections to increase or decrease infection severity. Since many studies have only tested NET effects *in vitro* and have found that NET does not show the same immunosuppressive effects seen with DMPA, it is important to explore this *in vivo*[105 110 121 131]. Further research is required to assess the risks of these alternative hormonal contraceptives to determine whether they show the same increased susceptibility to STIs that is seen with DMPA and whether other options should be considered over DMPA. Therefore, the aim of this project is to determine the effects of NET treatment on HSV-2 susceptibility and immune cell populations in mice compared to DMPA treated mice. *It is hypothesized that NET treated mice will have decreased susceptibility to HSV-2 infection compared to DMPA but elevated compared to mice with no hormone treatment.* It is predicted that post intravaginal immunization against HSV-2, NET treated mice will be successfully immunized and be protected post challenge, similar to DMPA treated mice. It is also expected that NET will show better outcomes post primary HSV-2 infection compared to DMPA

treatment and it will not have an immunosuppressive effect on the immune system in the FRT. This hypothesis was addressed by the following aims:

Aim 1: Examine the effect of NET on susceptibility to HSV-2 compared to DMPA

Aim 2: Investigate the effect of NET on the physiology of the vaginal tract

Aim 3: Examine the effects of NET on immune cell phenotypes in mice

CHAPTER 3: MATERIALS AND METHODS

3.1 Mice

Six-to-eight-week-old female C57BL/6 mice were ordered from Charles River laboratories (Saint-Constant, Quebec, Canada). Mice were housed in the Central Animal Facility at McMaster University. Mice were maintained in ventilated cages under specific pathogen-free conditions that was standard temperature controlled as well as under a 12-hour light/dark cycle. Mice were left untouched one week after arrival to allow them to acclimate to their environment. All animal studies were approved by and in compliance with the Animal Research Ethics Board (AREB) McMaster University.

3.2 Ovariectomies

Similar to previous protocols that have been conducted in other studies from our lab, mice were administered the analgesic carprofen (5 mg/kg) or buprenorphine (0.05 mg/kg) subcutaneously 30 minutes prior to the start of surgery[95-97 132-134]. Mice were then given ketamine/xylazine intraperitoneally (150 mg of ketamine/10 mg of xylazine/ml). Once the mice reached surgical plane, the surgery site on both sides of the mice (under the ribcage), were shaved and sanitized with iodine surgical scrub and isopropyl alcohol. A local anesthetic, bupivacaine (4 mg/kg), was also injected into the surgical areas intradermally as an added measure of anesthesia. Incisions were made through the skin and peritoneal wall. Forceps were used to locate the ovarian fat pad, which was then pulled out of the mice. The region below the ovary and ovarian fat pad was clamped down and the ovary was removed with a surgical blade. The peritoneal and muscle layers were then sutured shut, and the skin was stapled to close the opening. Mice were given ~1 ml of saline (0.9% NaCl) subcutaneously and were laid on a heat pad until they recovered from

the anesthetic. Mice were monitored for a minimum of 5 days following surgery to ensure mice recovered properly. For the first two days after surgery mice were given daily doses of carprofen (5 mg/kg) for pain management and received the dietary supplement Nutrigel to aid in recovery. Once the incisions were properly healed on day 7-10 post-surgery, staples were removed from each side of the mice. Mice were given 2 weeks to recover and to allow the endogenous hormones to dissipate.

3.3 Collection of vaginal washes

To collect vaginal washes from mice, 30 µl of 1X PBS (phosphate buffered saline) was pipetted into the vaginal tract of mice two times consecutively. Approximately 60 µl was collected in total and was stored at -80°C until needed for later use.

3.4 Staging for diestrus

Vaginal washes were collected to determine which stage of the estrous cycle mice were in. Approximately 10 µl of the vaginal wash was pipetted onto a glass slide and viewed under a microscope. The cells and the amount of each cell types were observed and compared to images of vaginal washes from mice under different stages of the estrus cycle which include proestrus, estrus, metestrus and diestrus[135]. Vaginal washes from mice under the estrus stage of their cycle shows a majority of nucleated epithelial cells with few nucleated epithelial cells[135]. The metestrus stage is characterized by mainly nucleated epithelial cells with a small portion of leukocytes and fewer nucleated epithelial cells present[135]. Vaginal washes from mice in the proestrus stage consists mainly of nucleated epithelial cells, a smaller portion of cornified epithelial cells with few leukocytes[135]. The vaginal washes from mice in the diestrus stage is

composed of primarily leukocytes[135]. There can be a portion of nucleated epithelial cells and few cornified, but majority are leukocytes[135].

3.5 Hormone Treatments

DMPA (depot medroxyprogesterone acetate) or Depo-Provera was administered as a subcutaneous injection in the scruff of the neck at a dose of 2 mg diluted in saline (0.9% NaCl) as this dose resulted in serum levels in mice that were similar to serum levels seen in women using DMPA[136-142]. The injectable NET formulation or 19-Norethidrone (Sigma-Aldrich Cat. 68-22-4) was suspended in 100% ethanol, further diluted with PBS to 70 μg, 250 μg and 1 mg, administered subcutaneously to mice. Norethisterone 2.5 mg and 5 mg 21-day release pellets (Innovative Research of America, Cat. P-171-2.5MG-25 and P-171-5MG-25, respectively) and progesterone 10 mg 21-day release pellets (Innovative Research of America, Cat. P-131-10MG-25) were surgically inserted into the scruff of mice. These progesterone pellets were previously used in another study from our lab, which corresponds to high progesterone levels during women's menstrual cycle[96 143].

3.6 Hormone pellet insertion

Briefly, carprofen (5 mg/kg) was subcutaneously injected into the mice, and 30 minutes later, mice were anesthetized with isoflurane. The back of the neck was shaved and sterilized with surgical iodine scrub and isopropyl alcohol. A small incision was made into the skin and forceps were used to form a pocket underneath the skin where the hormone pellet was then placed. The incision was stapled shut, mice were given ~1 ml of saline subcutaneously, and recovered on a

heat pad. Mice were monitored for 5 days post-surgery to ensure proper recovery. Staples were removed 7-10 days after surgery once the incision healed.

3.7 Intravaginal HSV-2 TK$^-$ Immunization

Ten days after hormone treatment, mice were anesthetized with an injectable anesthetic (150 mg of ketamine/kg with 10 mg of xylazine/kg). Mice were intravaginally infected with 10^4 PFU of HSV-2 TK$^-$ (thymidine kinase deficient) and left on their backs with the lower body elevated on a heat pad until recovery from anesthesia (approximately 45 minutes) to allow for viral infection.

3.8 Intravaginal WT HSV-2-333 Infection

Ten days after immunization, mice were anesthetized with an injectable anesthetic (150 mg of ketamine/kg with 10 mg of xylazine/kg). Mice were intravaginally infected with $5x10^3$ PFU of WT (wildtype) HSV-2 (strain 333) and left on their backs on a heat pad for approximately 45 minutes to allow the virus to infect.

3.9 Genital Pathology Scoring

Mice were monitored daily post infection following a 5-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 around genital area; 4, genital ulceration with severe redness; and 5, severe genital ulceration extending to surrounding area and/or hindlimb paralysis. Mice were euthanized by cervical dislocation once they reached pathology scores of 4 or 5, indicating endpoint. Cumulative pathology scores were calculated by multiplying the highest pathology score of each mouse in each group by the number of mice that

displayed this score and the number of days the highest pathology score was observed. Average pathology scores were calculated based on the sum of the cumulative pathology scores for each group and divided by the number of mice in each group.

3.10 Vero cell culture

African green monkey kidney epithelial cells (Vero; ATCC CCL81) were grown in T-150 flasks with alpha minimum essential medium (α-MEM) (GIBCO Laboratories, Burlington, Canada), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Invitrogen, Burlington, Canada), 1% L-glutamate (BioShop Canada Inc., Burlington, ON, Canada) and 1% HEPES (Invitrogen, Burlington, Canada). Once cells reached 100% confluency, cells were washed with 1X PBS, then incubated with 1X trypsin-EDTA for 5 minutes in a 37°C incubator. Cells were resuspended in supplemented α-MEM and seeded into multiple flasks or 12 well plates, as needed.

3.11 Vero plaque assay

Viral titers were quantified through a plaque assay on Vero cells. A confluent monolayer of Vero cells were seeded into a 12 well plate and left to grow to approximately 60-70% confluency in supplemented a-MEM. Vaginal washes were thawed on ice and serially diluted to 10^{-2} to 10^{-7} in supplemented a-MEM lacking FBS, which were then added to the 12 well plate of Vero cells. Plates were incubated with the various diluted vaginal washes for 2 hours and were rocked every 15 minutes within the 2 hours to allow the virus to distribute evenly when infecting cells. After these 2 hours, supplemented α-MEM was added on top of the monolayers to stop new viral adsorption. After 48 hours of incubation, media was aspirated out of each well, plates were fixed

and stained with crystal violet for approximately 15-20 minutes, then rinsed in water. The plates were left to dry overnight. An average number of plaques for each sample were counted under a light microscope and PFU calculated based on the dilution factor.

3.12 Serum collection from mice

Blood was collected from mice at various timepoints including 1 week 3 weeks and 6 weeks post hormone treatment. Blood was collected through endpoint cardiac puncture to obtain approximately 1 ml of blood. Blood collection was performed by staff at the Central Animal Facility at McMaster University. Blood samples were centrifuged at 10 000 rpms for 10 minutes, to separate serum from blood. Serum was collected and stored at -80°C for future use.

3.13 Hormone level measurement in mouse serum

Serum from mice under various hormones including DMPA, NET 2.5 mg pellets and NET 5 mg pellets. Serum samples were sent to the Small Molecule Biomarker Core at the University of Pittsburgh School of Pharmacy (Pittsburgh, Pennsylvania) to measure serum through ultra-performance liquid chromatography paired with mass spectrometry (UPLC-MS) [144]. Briefly, 10 µl of the internal standard (20 ng/ml testosterone-d3) was added to each 500 µl sample[144]. To perform liquid-liquid extraction, 3 ml of N-butyl chloride was added to the samples[144]. Samples were then centrifuged at 4000 rpm for 5 minutes[144]. The supernatant was drawn and dried off with nitrogen at 40 °C, then samples were reconstituted in 50 µl of 50:50 (acetonitrile:water)[144]. For UPLC-MS, 7.5 µl of the sample was used[144]. An Acquity UPLC system (Waters) was used with an Acquity BEH C18 column (Waters)[144]. The mobile phase A contained 2 mM ammonium acetate with 0.1% formic acid and mobile phase B contained 2

mM ammonium acetate methanol[144]. The gradient started with 50% 2 mM ammonium acetate with 0.1% formic acid held for 0-0.5 minutes, then decreased to 15% for 3.5-4.5 minutes, then back to the initial 50% concentration for 4.5-6.5 minutes[144]. A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific) with a heated electrospray ionization (HESI) source was used to perform Tandem mass spectrometric (MS/MS)[144]. Spray voltage was set to 3000 V in positive polarity with collision gas pressure, auxiliary gas and sheath gas set to 1.5 mTorr, 50 and 10 (arbitrary units), respectively[144]. Ions were detected based on m/z (mass-charge ratio) of 299.2 $\rightarrow$ 231.2 for norethindrone (collision energy = 21V), m/z 387.2 $\rightarrow$ 327.2 for medroxyprogesterone acetate (collision energy = 25V), and m/z 292.1 $\rightarrow$ 97.1 for d3-testosterone (collision energy = 20V)[144]. Data acquisition and processing was performed with Xcalibur software v4.0 (Thermo Scientific, San Jose, CA, USA)[144].

3.14 Tissue Collection and Processing

3.14.1 Vaginal tissue processing

Mice were euthanized by cervical dislocation post challenge and tissues were collected for flow cytometry. The pelvic bone was cut to expose the vaginal tract was removed from mice and placed in cold Roswell Park Memorial Institute (RPMI) media (Invitrogen, Burlington, Canada). Vaginal tissue was processed by washing the vaginal tracts with fresh media. The vaginal tracts were then cut open with a surgical blade to expose the interior and then cut into smaller pieces. Vaginal tissue was pooled from 1-2 mice. Collagenase A (Cedarlane, Cat. 11088793001) was added to 15 mL of RPMI at a mass of 0.00157 g/mL to digest the tissue. The vaginal tissue suspension was stirred at 37°C with magnetic stirring rods for an hour, which was repeated for two rounds of digestion. The suspension was then filtered through a 40 µm filter, where

remaining tissue was also pushed through the filter. This mixture was then filtered again through a 40 µm filter. Afterwards, all samples were centrifuged at 1200 rpm at 4°C for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 300 µl of RPMI supplemented media (10% fetal bovine serum (FBS), 100 IU/ml of penicillin, 100 ug/ml of streptomycin, 1% L-glutamine, 0.1% 2-mercaptoethanol, 1x nonessential amino acids, and 1x sodium pyruvate).

3.14.2 Spleen processing

The spleens were aseptically removed and placed in cold PBS. The lymph nodes and spleen processing followed similar techniques to each other. A needle was used to scratch the wells of a 6-well plate to easily break apart the tissue. To create single cell suspensions from spleen, the tissues were homogenized with the end of a syringe on scratched 6 well plates. PBS was used to rinse off the syringe and to create liquid mixture. The spleen samples were treated with ACK lysis buffer 10 minutes at room temperature to lyse red blood cells. All samples were washed with PBS and placed into the centrifuge for 5 minutes at 1500 RPMs at 4°C to obtain a pellet of cells. The pellets were resuspended in 300 µl of RPMI supplemented media. Cells from all samples were then counted in a hemocytometer.

3.14.3 Iliac lymph node processing

The iliac lymph nodes were aseptically removed and placed in cold PBS. The iliac lymph nodes were processed following similar techniques to the spleen processing. To create single cell suspensions from the lymph nodes, the tissues were homogenized with the end of a syringe on 12 well plates. All samples were washed with PBS and centrifuged for 5 minutes at 1500 RPMs at

4°C to obtain a pellet of cells. Cell pellets were resuspended in in 300 µl of RPMI supplemented media. Cells were then counted in a hemocytometer.

3.15 Flow Cytometric Analysis

Staining was done on $1x10^6$ cells. A cell stimulation cocktail of ionomycin, PMA, brefeldin A, and monensin (eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) (500X), Thermo Fisher Scientific Cat. 00-4975-03) were used to stimulate maximum intracellular cytokine secretion from cells. Cells were stimulated overnight for 16 hours as recommended from the manufacturer's protocol. Samples were then stained with allophycocyanin (APC)-ef780 viability dye (eBioscience Cat. 65-0865-14) for 30 minutes on ice. Samples were then incubated with FC block (rat anti-mouse CD16/32, BD Biosciences Cat. 553142) for 15 minutes to decrease nonspecific FC receptor binding. An antibody panel was used that was similar to a previous study that was done in our lab[29 132]. Vaginal tract samples were stained using antibodies for cell surface markers which included phycoerythrin (PE-cf594) labelled hamster anti-mouse CD3 (BD Biosciences Cat. 562286) and brilliant violet (BV421) labelled anti-CD4 (VWR Cat. CA10756-660) at a dilution of 1:50. The lymph node and spleen samples were stained with these antibodies as well but at a dilution of 1:100. All samples were stained with brilliant violet (BV786) rat anti-mouse labelled CD8 (BD Biosciences Cat. 563332), PE hamster anti-mouse labelled CD69 (BD Biosciences Cat. 553237) and BV510 rat anti-mouse labelled CD103 (BD Biosciences Cat. 563087) at a 1:100 dilution for lymph node and spleen samples. All samples were also stained with alexa fluor (AF700) rat anti-CD44 (VWR Cat. 103026) at a dilution of 1:200. The BD Transcription Factor Buffer Set (BD Biosciences Cat. 562574) was used for intracellular staining and instructions from this kit were followed for

washing, fixing, and staining cells. Cells were stained for the intracellular markers fluorescein isothiocyanate (FITC) labelled IFN-γ (Thermo Fisher Cat. 11-7311-81) and APC labelled IL-17 (Thermo Fisher Cat. 17-7177-81). Samples were washed and suspended in FACs buffer and sorted through the Cytoflex Flow Cytometer (McMaster Core Flow Facility). The data was analyzed using FlowJo (version 10.8.0).

Table 1. Outline of surface and intracellular fluorophore antibodies used for flow cytometric analysis of immune cells from lymph nodes, spleen and vaginal tissue including dilution factors used.

Marker	Fluorophore	Supplier	Catalogue number	Dilution for spleen and LN	Dilution for vaginal tissue
LIVE/DEAD	APC-ef780 viability dye	eBioscience	65-0865-14	1:100	1:100
CD3	PE-cf594	BD Biosciences	562286	1:100	1:50
CD4	BV421	VWR	CA10756-660	1:100	1:50
CD8	BV786	BD Biosciences	563332	1:100	1:100
CD69	PE	BD Biosciences	553237	1:100	1:100
CD103	BV510	BD Biosciences	563087	1:100	1:100
CD44	AF700	VWR	103026	1:200	1:200
IFN-γ	FITC	Thermo Fisher	11-7311-81	1:100	1:100
IL-17	APC	Thermo Fisher	17-7177-81	1:100	1:100

3.16 Immunohistochemistry of Vaginal Tissue

Vaginal tissue was collected from mice and fixed in 10% formalin or methacarn (60% methanol, 30 % chloroform and 10% glacial acetic acid) for at least 48-72 hours. Vaginal tracts were then cut into two pieces, placed in cassettes and transferred to 70% ethanol. Samples were taken to McMaster Immunology Research Center (MIRC) Histology Core Facility for processing, in

which H&E staining, PAS (Periodic Acid Schiff) staining, anti-MUC-1 (mucin-1) (antibody info), staining as well as HSV-2 specific staining (antibody info) was performed. Images were obtained at 10X magnification using a Zeiss Axio Imager M2 microscope with Hamamatsu Flash4.0 camera using Zeiss Zen Pro software.

3.17 Enzyme linked immunosorbent assay (ELISA) for Quantification of MUC1

Mucin-1 protein concentration was quantified in the vaginal washes from mice using a mouse MUC1 ELISA kit (Novus Biologicals Cat. NBP2-76697). The manufacturer's instructions were followed using vaginal washes that were pooled from mice from day 1-3, 4-6 and 7-10 post hormone treatment. Vaginal washes from control mice were diluted 1:2 in PBS and vaginal washes from NET treated mice were diluted 1:10 in PBS. Capture antibody was added at a volume of 100 µl to the 96 well plate provided with the kit, which was sealed and incubated for 2 hours. The plate was then washed with 1X wash buffer two times. The reagent diluent was added to the plate and was sealed and incubated for 60 minutes. The plate was washed with 1X wash buffer two more times. The diluted samples and standards were added to wells at a volume of 100 µl, sealed and incubated at room temperature for 2 hours. The washing step was repeated. The detection antibody was added at a volume of 100 µl to each well, which was sealed, covered and incubated for 2 hours. The substrate solution was added at 100 µl per well, which was covered and incubated for 20-30 minutes. After 50 µl of stop solution was added to stop the enzymatic reaction. The absorbance of each well was measured in optical density (OD) at 450 nm using a microplate reader. The lower limit of detection was 0.156 ng/ml and the upper limit of detection was 10 ng/ml. Results were reported as OD units.

3.18 Statistical Analysis

GraphPad Prism version 8.1.1 (GraphPad Software, San Diego, California) was used for statistical analysis. The Mantel-Cox log-rank test was used to calculate statistically significant differences in survival. Significant differences in vaginal epithelial thickness as well as immune cell populations and counts were determined using one-way ANOVA with Tukey's multiple comparisons tests or a two tailed unpaired nonparametric t test.

CHAPTER 4: RESULTS

4.1 Aim 1: Examine the effect of NET on susceptibility to HSV-2 compared to DMPA

4.1.1 Determining an optimal in vivo dose of NET

Few studies have tested the contraceptive NET in animal models, and an optimal dose of this hormone that is physiologically similar to the dose humans use has not been documented. To determine the appropriate dose of NET to give mice, calculations were done based on previous studies using NET in mice as well as animal toxicity studies of NET. Qusipe Calla et al. used a dose of 5 mg of NET in mice, which was administered as a 1 mg injection subcutaneously for 5 days consecutively[127]. A previous study in our lab quantified the serum levels of MPA in mice and found that a 2 mg injection of DMPA results in serum levels similar to the serum levels of women using DMPA as a contraceptive[136]. Compared to the 2 mg dose of DMPA that is administered to mice, 5 mg of NET seemed high. To determine the doses of NET to test in mice, the clinical dose of 200 mg of NET was converted to an animal equivalent dose, which was converted to a mg/kg dose and calculated based on the average 20-25-gram weight of a mouse. This resulted in a 70 μg dose of NET. Toxicity studies from the European Chemicals Agency gave various doses of NET injections ranging from 1-50 mg/kg to mice weekly over an extended period of time, up to 72-73 weeks, in which the LOAEL (lowest observed adverse effect level) was observed to be 10 mg/kg, in which adverse effects reported included tumour formation [145]. Based on the above studies and calculations two doses of NET (250 μg and 1 mg) were tested in mice (Table 2) to determine whether a single administration of NET at these doses would result in the same adverse effects stated in these toxicity studies[145]. The 250 μg dose was used since a one-time administration of the 250 μg dose would be expected to be safer and less likely to cause adverse effects compared to continuous weekly administrations over a year as done in the toxicity

studies[145]. The 1 mg NET dose was considered a higher dose and was used to compare to the dose used in the study by Quispe Calla et al[127]. These various doses were tested in normal intact mice to determine how long each dose would last in their system, which was demonstrated by examining how long they remained in diestrus stage, which is a key physiological effect of progestins (Table 1). This would reveal which dose of NET would keep mice in diestrus long enough to allow them to be infected with HSV-2, as well as allowing the hormones a sufficient period of time to impact the physiology and immunity in the vaginal tract.

To test these different doses of NET in mice, 3 groups of 3 mice were subcutaneously administered 70 μg, 250 μg and 1 mg of NET. Vaginal washes were taken from mice daily post hormone treatment to examine the stage of the estrus cycle mice were in and how long they remained in this stage. The diestrus stage lasts for 2-4 days, but longer diestrus stages that last 7-15 days are associated with thinning of the vaginal epithelium which is ideal for enhancing HSV-2 infection[94]. As detailed in Table 2, NET 70 μg and 250 μg were not sufficient doses to keep mice in diestrus for a long period of time; the 70 μg dose kept 2/3 mice in diestrus for 4-6 days, while the other mouse in this group only stayed in diestrus for 2 days. The 250 μg dose resulted in 2/3 mice staying in diestrus for approximately 3-7 days, with one mouse only staying in diestrus for 1 day. There was variability between how long each mouse remained in diestrus within each group. The 1 mg dose kept 2/3 mice in diestrus for approximately 13-14 days, which was the longest compared to the other doses tested. The other mouse in the 1 mg group stayed in diestrus for only 4 days. Additionally, a DMPA treated mouse was staged for diestrus as a positive control, since DMPA is known to keep mice in diestrus for a long period of time and was used as a comparison when examining vaginal washes and determining the stage of the estrus cycle mice were in. The DMPA treated mouse remained in diestrus for the entire 20 days that all mice were

being staged. The 1 mg dose of NET seemed to be the most effective at keeping mice in diestrus for the longest period of time, similar to how long mice stayed in diestrus with DMPA treatment.

Table 2. Estrous cycle stages over 22 days post hormone treatment with NET injection doses of 70 µg, 250 µg and 1 mg, as well as DMPA 2 mg injection, in female C57BL/6 mice**.**

Day (D) post hormone treatment	NET 70 µg injection			NET 250 µg injection			NET 1 mg injection			DMPA 2 mg injection
	M1	M2	M3	M1	M2	M3	M1	M2	M3	M1
D1	E	M	P	P	P	E	M	M	M	P or M
D2	D	M or P	D	M or P	D	D	D	P	M	M
D3	D	D	D	D	D	D	D	D	D	D
D4	D	D	D	P	D	D	D	D	D	D
D5	D	P	D	E	D	P	D	D	D	D
D6	E	E	D	P	D	E	D	D	D	D
D7	E	E	D	D	D	E	D	M	D	D
D8	E	D	M or P	D	D	D	D	D	D	D
D9	M	D	P	D	M or P	D	D	D	D	D
D10	D	P	E	P	M	D	D	E	D	D
D11	D	E	D	M	M	E	D	E	D	D
D12	D	P	D	D	M or P	M or P	D	D	D	D
D13	D	D	D	D	D	D	D	D	D	D
D14	E	P or M	P or M	P	D	D	D	P	D	D
D15	P	P or M	P or M	P	D	D	D	P or M	D	D
D18							D	E	D	D
D20							P	D	P	D

E: estrus, D: diestrus, P: proestrus, M: metestrus
N= 3 for each NET dose tested, N=1 for the DMPA group.

4.1.2 Intravaginal immunization and challenge with HSV-2 in mice treated with NET 2 mg injection

Based on these results, we determined that a 1 mg NET injection would be optimal to use for the next experiment. However, with variability occurring between mice within each group, including the 1 mg group, a higher dose would more likely ensure mice would stay in diestrus more consistently for a longer period of time. Therefore, a dose of 1 mg for 2 days consecutively was used for a total of 2 mg of NET, as this was the dosing schedule used by Quipse Calla et al. [127]. To answer the question of how NET treatment affects immunization and challenge with HSV-2 compared to DMPA and progesterone, this dose of NET was tested in mice in the experiment outlined in Figure 2. Mice were first ovariectomized to eliminate endogenous hormones. After 2 weeks when the endogenous hormones have dissipated, hormone treatments as outlined in Figure 2 were administered to mice, and they were then immunized intravaginally with 10^4 PFU (plaque forming units) of HSV-2 TK^-. The NET 2 mg dose was compared to DMPA treatment to determine whether NET treatment shows similar outcomes to DMPA with HSV-2 susceptibility. Progesterone was used for comparison as DMPA, and NET are synthetic progestins which are made structurally similar to progesterone and are made to mimic the actions of progesterone. Four weeks after hormone treatment, mice were intravaginally challenged with $5x10^3$ PFU of WT HSV-2.

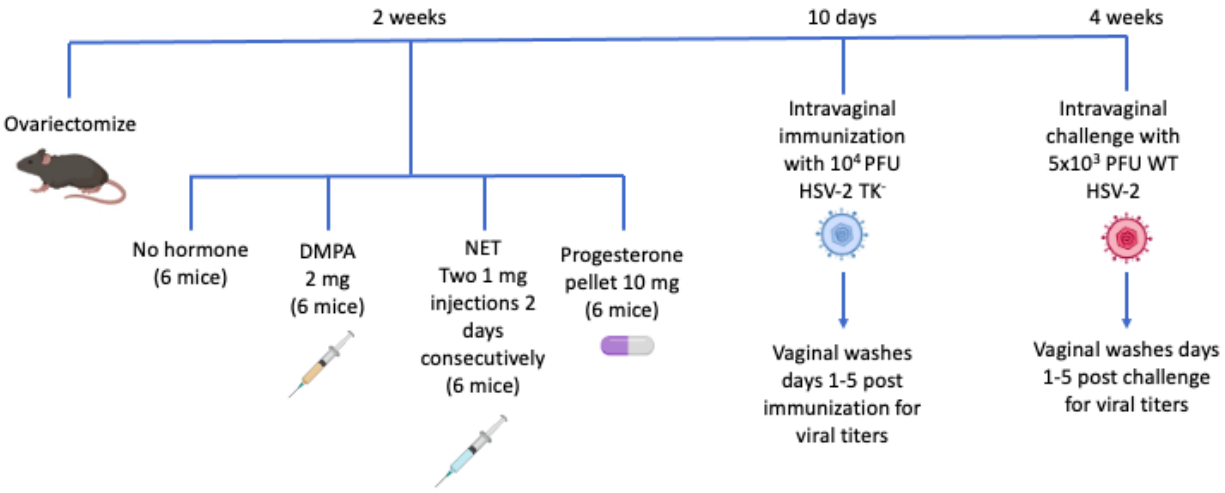


Figure 2. Outline of immunization and challenge in mice treated with NET 2 mg injection compared to mice treated with DMPA 2 mg injection, progesterone 10 mg pellet and a no hormone control group. Six- to eight-week-old female C57BL/6 mice were ovariectomized and 2 weeks later were given different hormone treatments. Ten days after hormone treatment, mice were immunized intravaginally with HSV-2 TK- 10^4 PFU and vaginal washes were collected for 5 days post immunization. Four weeks after immunization when their exogenous hormones have dissipated, mice were challenged intravaginally with WT HSV-2 $5x10^3$ PFU and vaginal washes were collected for 5 days post challenge. N= 6 mice per group.

No hormone, progesterone and DMPA treated mice all showed viral shedding post immunization, indicating that viral entry occurred easily (Figure 3a, b and c). When challenged with WT HSV-2, the no hormone, DMPA and progesterone treated mice all displayed little to no viral shedding post challenge (Figure 3a, b and c). This indicates that mice under these hormone treatments were effectively immunized and were not infected once challenged with WT HSV-2.

However, this was not seen in the mice treated with NET 2 mg injection (Figure 3d). Little viral shedding occurred in 2/6 mice post immunization and 4/6 mice did not shed any virus indicating that the immunization was not effective. Post challenge, all NET treated mice were shedding virus

and 2/6 successfully immunized NET treated mice showed viral shedding for the first 3 days post challenge but decreased viral shedding was seen on day 4 and 5. The NET treated mice that were not effectively immunized (4/6) showed high viral shedding each day post challenge up to day 5. As outlined in Table 1, there was variability and inconsistencies seen in the number of days mice stayed in diestrus after hormone treatment. This occurred with each dose of NET that was tested. Since the NET hormone used in this experiment was a powder made up into a suspension, there may have been inconsistencies in the dose of hormone administered during injections. Therefore, one possibility was that the immunization may not have been effective in all NET treated mice since mice may have received unequal doses of hormones.

Even if inconsistencies occurred with the doses of NET that mice received, there were still clear differences in susceptibility to HSV-2 between NET and DMPA treated mice. Specifically, DMPA treated mice were susceptible to infection, were immunized effectively, and were not infected post challenge, whereas NET treated mice that did show viral shedding post immunization were still shedding virus post challenge (Figure 3c and d). Thus, NET treated mice are susceptible to infection, but this was inconsistent, and they are not susceptible to the same extent as progesterone and DMPA.

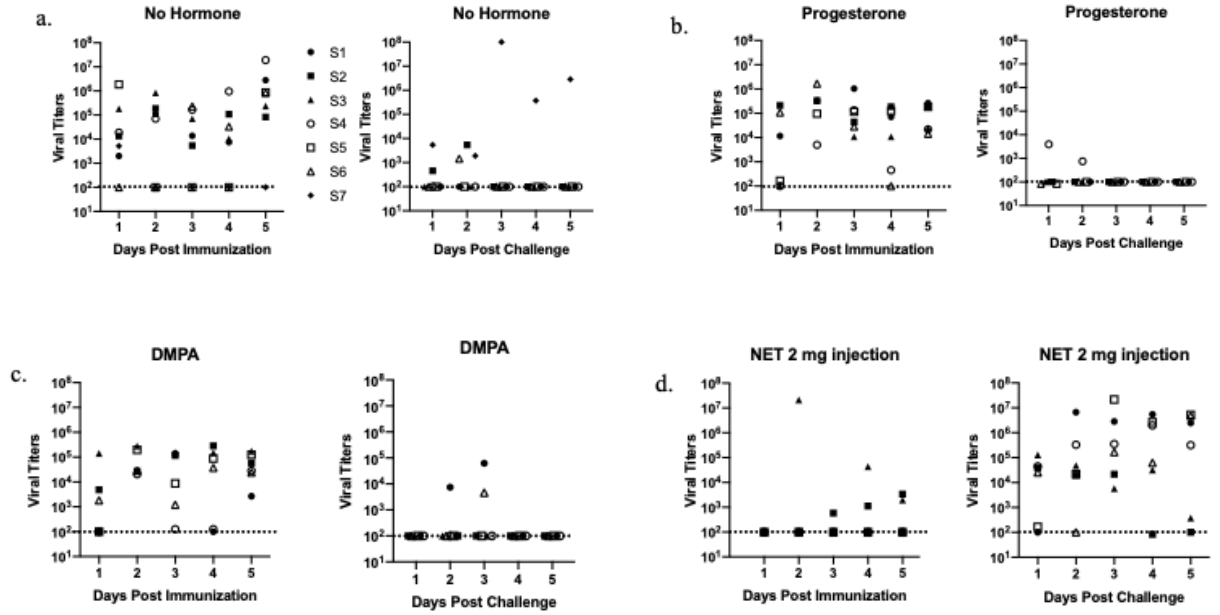


Figure 3. Viral titers of vaginal washes from OVX mice with no hormones or treated with progesterone, DMPA, or NET 2 mg injection post intravaginal immunization with 10^4 PFU of HSV-2 TK^-, as well as post intravaginal challenge with $5x10^3$ PFU of WT HSV-2. There was a no hormone control group (a) (n=7) as well as mice treated with progesterone pellet 10 mg (b) (n=6), DMPA 2 mg (c) (n=6), or NET 2 mg injection (d) (n=6). Vaginal washes were collected for 5 days post immunization and 5 days post challenge. Each symbol shape represents a different mouse, and their viral titers for each day is shown. Dashed line indicates the lower limit of detection**.**

4.1.3 Staging for diestrus in NET and progesterone treated mice

Considering the estrous cycle and HSV-2 susceptibility inconsistencies seen following NET injection, hormone pellets were next tested in mice as an alternate method to provide a more consistent dose of hormones. Hormone pellets were inserted under the skin on the back of the neck of mice and as per manufacturer description, sustained amount of the hormone is released from the pellet each day for a set time period (see Materials and Methods section 3.6 for more details).

Previous studies in our lab have used these hormone pellets which are known to last in the mouse's system for a long period of time (21-28 days)[96 133]. In the study by Quispe Calla et al. mice received 1 mg of NET each day for 5 consecutive days, resulting in a total dose of 5 mg [127]. However, based on calculations and toxicity studies, this dose of NET may be too high to be administered to mice daily[145]. A NET 5 mg pellet would consistently release ~0.238 mg/day of NET over 21 days, a safer dose to administer to mice based on the collective studies[127 145]. Additionally, with a 250 μg injection 2/3 mice stayed in diestrus for up to 7 days at most (Table 1). We estimated that the 0.238 mg/day dose would be similar to the 250 μg injection but would effectively keep mice in diestrus longer as it would be released consistently over 21 days. However, the hormones may take time to dissipate, and they may last in the mouse's system longer than the intended 21 days, as seen in our studies before[146]. Therefore, the NET 5 mg pellets were tested in intact mice to determine the duration of NET in serum and how long it effectively keeps mice in diestrus. Table 3 displays the staging for mice treated with NET 5 mg pellets compared to mice treated with progesterone 10 mg pellets, which has been shown to provide a dose of progesterone that is physiologically similar to progesterone levels in women during the luteal or secretory phase of their cycle (progesterone high phase of the cycle)[143]. NET 5 mg pellet treated mice remained in diestrus for 38-43 days, which was comparable to the 37-43 days observed in 10 mg progesterone pellet treated mice. Therefore, NET hormone pellets were more effective and consistent in keeping mice in diestrus compared to the injections, likely due to the dose of NET being more consistent between mice.

Table 3. Staging for diestrus in female C57BL/6 mice over 46 days post NET 5 mg pellet or progesterone 10 mg pellet.

Days (D) post hormone treatment	NET 5 mg pellet			Progesterone 10 mg pellet		
	M1	**M2**	**M3**	**M1**	**M2**	**M3**
D1	D	D	D	D	D	D
D2	D	D	D	D	D	D
D3	D	D	D	D	D	D
D4	D	D	D	D	D	D
D5	D	D	D	D	D	D
D6	D	D	D	D	D	D
D7	D	D	D	D	D	D
D8	D	D	D	D	D	D
D9	D	D	D	D	D	D
D10	D	D	D	D	D	D
D11	D	D	D	D	D	D
D14	D	D	D	D	D	D
D16	D	D	D	D	D	D
D18	D	D	D	D	D	D
D21	D	D	D	D	D	D
D22	D	D	D	D	D	D
D23	D	D	D	D	D	D
D25	D	D	D	D	D	D
D28	D	D	D	D	D	D
D30	D	D	D	D	D	D
D32	D	D	D	D	D	D
D35	D	D	D	D	D	D
D37	D	D	D	D	P or M	D
D38	D	M or D	D	D	M	M
D40	D	D	Euthanized*	D	M	M
D43	D	M or D		M	M	M
D44	D	M				

D45	D	M				
D46	M	M				

E: estrus, D: diestrus, P: proestrus, M: metestrus

One mouse in the NET 5 mg pellet group was euthanized due to bladder swelling and urinary retention. N = 3 for each group.

4.1.4 Intravaginal immunization and challenge with HSV-2 in mice treated with NET 5 mg

The NET 5 mg pellet was then tested in an HSV-2 immunization and challenge experiment as outlined in Figure 4 to assess the effect of NET treatment on HSV-2 susceptibility. Mice were first ovariectomized to eliminate their endogenous hormones, then administered DMPA 2 mg injection, NET 5 mg pellet or progesterone 10 mg pellet hormone treatment. A no hormone control group was also included. Ten days after hormone treatment mice were intravaginally immunized with 10^4 PFU of HSV-2 TK^-, and approximately 5-7 weeks later, mice were intravaginally challenged with $5x10^3$ PFU of WT HSV-2.

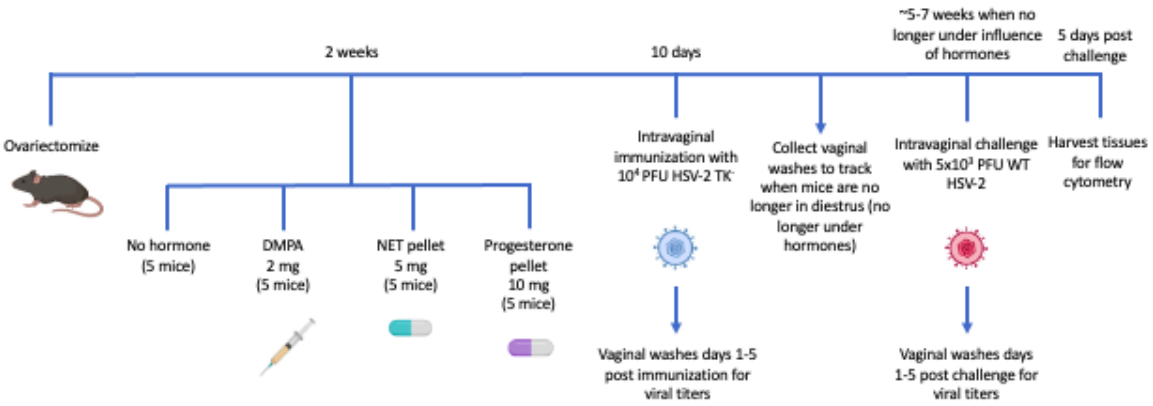


Figure 4. Outline for immunization and challenge in mice treated with NET 5 mg pellet compared to mice treated with DMPA 2 mg injection, progesterone 10 mg pellet and a no hormone control group. Six- to eight-week-old C57BL/6 mice were ovariectomized and 2 weeks later, were given their hormone treatments. After 10 days of hormone treatment, mice were intravaginally immunized with 10^4 PFU of HSV-2 TK^- and vaginal washes were collected for 5 days post immunization. When hormones effects had worn off, after about 5-6 weeks, mice were challenged intravaginally with $5x10^3$ PFU of WT HSV-2 and vaginal washes were collected for 5 days post challenge. On day 5 post challenge, vaginal tracts, spleens and iliac lymph nodes were collected for immune cell analysis through flow cytometry. N= 5 per group.

All mice in the no hormone, progesterone and DMPA treatment groups were shedding virus post immunization, an indicator of successful immunization (Figure 5a, b and c). Therefore, when these mice were subsequently challenged, they showed little to no viral shedding, indicating that the immunization was effective.

Mice treated with the NET 5 mg pellet showed no viral shedding on day 1 post immunization. Additionally, 3/5 mice NET treated mice were shedding virus post immunization indicating effective immunization on day 2-5, whereas the other 2/5 NET treated mice did not shed virus at all indicating ineffective immunization (Figure 5d). Indeed, the effectively immunized mice did

not shed virus post challenge, but no viral shedding was detected in mice that were not effectively immunized either. Treatment with the NET 5 mg pellet also resulted in adverse effects, where 2/5 mice had bladder swelling and urinary retention, one of which had to be euthanized before the HSV-2 challenge. These adverse effects indicated that the NET 5 mg pellet may be too high of a dose to be administered to mice.

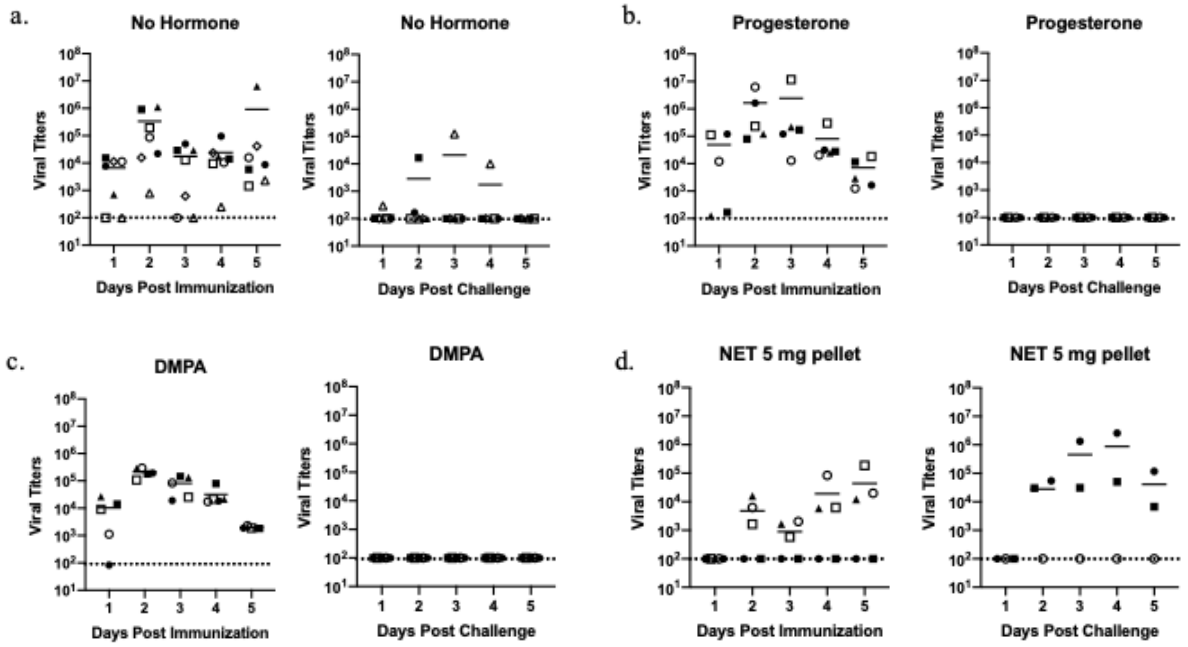


Figure 5. Viral titers of OVX mice with no hormones or treated with progesterone, DMPA, or NET 5 mg pellet post intravaginal immunization with 10^4 PFU of HSV-2 TK^- and after intravaginal challenge with $5x10^3$ PFU of WT HSV-2. There was a no hormone control group (a) (n=7) and mice treated with progesterone pellet 10 mg (b) (n=5), DMPA 2 mg (c) (n=5), or NET 5 mg pellet (d) (n=5). Vaginal washes were collected for 5 days post immunization and post challenge. Each symbol shape represents a different mouse, their viral titers for are shown for each day. Dashed line indicates the lower limit of detection.

4.1.5 Measurement of NET and MPA levels in mouse serum

Based on the adverse events associated with the NET 5 mg pellet, a lower dose of NET was tested next. This lower dose was 2.5 mg and the pellet would release NET at a rate of ~0.119 mg/day. To ascertain which NET dose was physiologically relevant, an experiment was conducted to examine serum levels of NET in mice given 2.5 mg and 5 mg pellets to compare to levels in women using NET as a contraceptive in order to determine which dose of NET would be optimal to give to mice. This was additionally done with mice treated with DMPA as well, to confirm our dose of DMPA is accurate through this method of hormone measurement in serum. Ovariectomized mice were used as well as normal mice to determine whether the presence of endogenous hormones affect serum levels of NET and DMPA. Mice were administered hormone treatments which included NET 2.5 mg pellet, NET 5 mg pellet and DMPA 2 mg injection and at 1 week post hormone treatment, serum was collected from mice. Comparing the serum levels from ovariectomized mice given NET 2.5 mg at 1 week, the average serum level of NET was 15.56 ng/ml ± 13.75 which was within the ~10-20 ng/ml range of women using NET as a contraceptive (Figure 6a) [147-153]. The average serum level of NET in normal NET 2.5 mg treated mice was 12.80 ng/ml ± 9.30, still falling within the observed range in women, but is lower than the average serum levels in ovariectomized mice treated with NET 2.5 mg. At 1 week post treatment, the ovariectomized NET 5 mg treated mice had an average serum level of 20.31 ng/ml ± 14.02, slightly higher than the average serum level from ovariectomized and normal NET 2.5 mg treated mice, but still fell within the 10-20 ng/ml range. For the normal NET 5 mg treated mice the average serum level of NET was 10.21 ng/ml ± 1.05, still falling within the observed range. However, the serum level in the normal NET 5 mg treated group was also lower than the ovariectomized NET 5 mg treated group. The first week post treatment is also close to the time mice are immunized with HSV-2 TK^-, and

as such, mouse NET serum levels should be comparable to human levels during immunization. Serum levels in normal and ovariectomized mice treated with NET 2.5 mg and NET 5 mg all resulted in serum levels that are within the range seen in women 1 week after using NET as a contraceptive. This suggests that both the NET 2.5 mg and NET 5 mg dose would be optimal to administer to mice since they both result in serum levels similar to the levels seen in women. When serum levels of MPA from ovariectomized mice treated with DMPA after 1 week were compared to serum levels observed in women using DMPA as a contraceptive after 1 week, serum levels in mice were comparable (Figure 6b). Mouse serum levels were on average 3.15 ng/ml ± 1.18 ng/ml which fell within the range seen in women which ranged from 1-8 ng/ml[136-142 154]. However, when comparing serum levels of MPA in normal mice treated with DMPA, serum levels differed from levels observed in women. The average serum level in normal mice treated with DMPA was 0.61 ng/ml ± 0.31, falling well below the 1-8 ng/ml range. Thus, this confirmed that the dose of DMPA used in ovariectomized mice results in similar levels observed in women, but in normal mice with endogenous hormones, serum levels are not similar. This suggests that the presence of these hormones should be considered when determining optimal doses of hormones to administer to mice.

To further compare the doses of NET and DMPA, serum from mice was collected at 3 weeks and 6 weeks post hormone treatment to measure serum levels of NET and MPA to compare to serum levels observed in women using these contraceptives. This would further validate whether the doses used in these experiments replicate serum levels seen in women at these timepoints after taking these hormonal contraceptives. However, this analysis was only done in ovariectomized mice as there was not a large difference in serum levels between normal and ovariectomized mice after 1 week of hormone treatment with NET. At 3 weeks post treatment, the serum levels of NET

2.5 mg treated mice were low compared to women, with an average of 0.43 ng/ml ± 0.66 in mice and ~3-6 ng/ml in women (Figure 6c) [147-153]. At 3 weeks post hormones, average serum levels from NET 5 mg pellet treated mice were still high compared to serum levels seen in women. The average serum level from mice treated with NET 5 mg pellets was 26.04 ng/ml ± 13.52 at 3 weeks, which was still higher than serum levels seen in women after 1 week and much higher than levels seen in women at 3 weeks. At 3 weeks post DMPA treatment in ovariectomized mice, the average serum level of MPA in mice was 0.04 ng/ml ± 0.01 (Figure 6d). The observed serum levels of MPA in women using DMPA after 3 weeks was found to be an average of ~1 ng/ml or less than 1 ng/ml. The serum levels from mice were lower than the average 1 ng/ml, but the levels after 1 week were still comparable to those seen in women, suggesting that the dose of DMPA is still optimal to be administering to mice.

At 6 weeks post treatment, the serum levels in ovariectomized mice treated with NET 2.5 mg pellets were below the lower limit of quantitation which indicates that the hormones are no longer present in the serum, or they are too low to be detected (Figure 6e). At 6 weeks the mice treated with NET 5 mg pellets still resulted in high serum levels with an average of 7.07 ng/ml ± 2.34, whereas in women the serum levels are around 1 or <1 ng/ml[147-153]. This showed that the NET 5 mg dose was very high and is not a good representation of a physiologically relevant dose of NET, as the serum levels exceed the observed serum levels seen in women using NET. While after 1 week the serum levels observed in mice treated with NET 2.5 mg or NET 5 mg were similar to those observed in women, these serum levels at 3-week and 6-week timepoints show that NET 5 mg may be too high. Previous experiments showed that mice treated with NET 5 mg pellets resulted in 1/3 mice euthanized due to bladder swelling and urinary retention (Table 3) and 2/5 NET 5 mg treated mice euthanized in the next experiment due to the same adverse effects (Figure

4 and 5). The mice treated with NET 2.5 mg pellets did not result in these adverse effects and still resulted in similar serum levels of NET seen in women and thus was used in subsequent experiments.

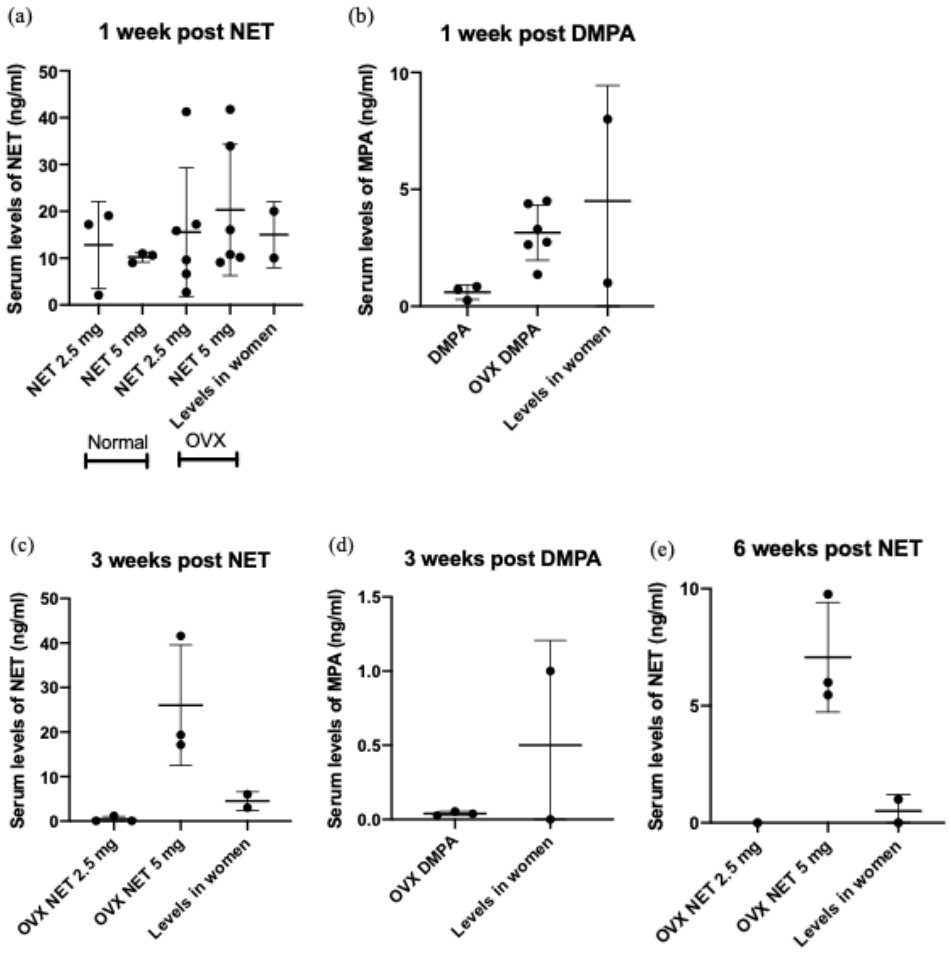


Figure 6. Serum levels of NET and MPA in mice treated with these hormones compared to serum levels that are seen in women using NET and DMPA as a contraceptive. Six- to eight-week-old C57BL/6 mice were ovariectomized and 2 weeks later, were administered NET 2.5 mg pellets, NET 5 mg pellets, or DMPA 2 mg injection and serum was collected after hormone treatment. Serum was analyzed through high performance liquid chromatography paired with mass spectrometry. Serum levels for women were extracted from women that were using NET or DMPA

as a contraceptive from various studies to compare to the various timepoints serum was collected from mice[103 136-142 147-154]. Serum levels of NET were measured 1 week after treatment with NET 2.5 mg and NET 5 mg in normal and ovariectomized mice (a), 3 weeks after treatment with NET 2.5 mg in ovariectomized mice (c) and 6 weeks after treatment with NET 2.5 mg and NET 5 mg in ovariectomized mice (e). Serum levels of MPA were measured 1 week after DMPA treatment (b) in ovariectomized and normal mice and 3 weeks after DMPA treatment in ovariectomized mice (d). N= 3 mice for each group.

4.1.6 Intravaginal immunization and challenge with HSV-2 in mice treated with NET 2.5 mg

The next experiment was done to test the NET 2.5 mg pellets in mice in an immunization and challenge model to assess how this concentration of hormone affected susceptibility to HSV-2, as the previous results from Figure 6 indicate that NET 2.5 mg is a more optimal dose (Figure 7). Mice were first ovariectomized and then given their hormone treatments two weeks later including DMPA, progesterone, NET 2.5 mg pellet and a no hormone control group. Ten days after hormone treatment, mice were intravaginally immunized with 10^4 PFU of HSV-2 TK^- and 5-7 weeks later, mice were intravaginally challenged with $5x10^3$ PFU of WT HSV-2.

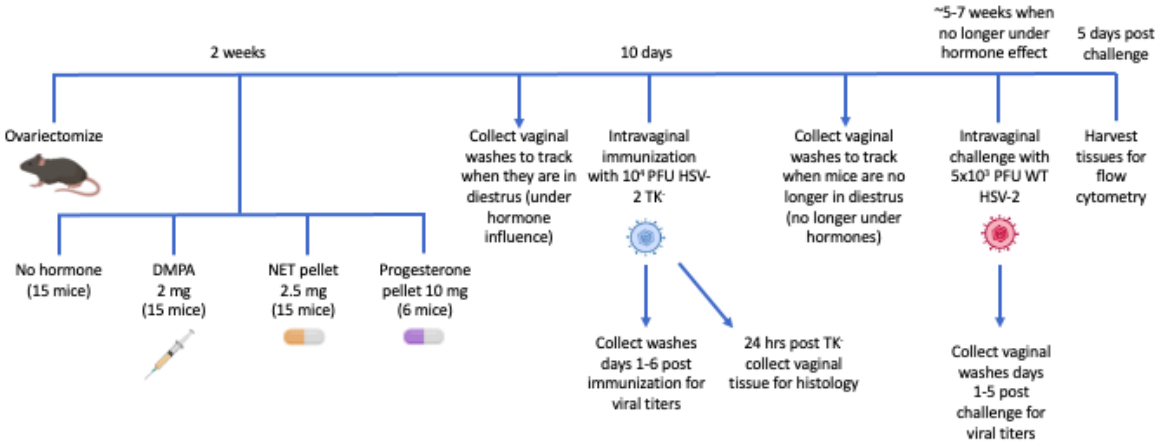


Figure 7. Outline of immunization and challenge in mice treated with NET 2.5 mg pellet compared to mice treated with DMPA 2 mg injection, progesterone 10 mg pellet and a no hormone control group. Mice were ovariectomized and were treated with different hormone treatments. Ten days after hormone treatment, mice were immunized intravaginally with 10^4 PFU of HSV-2 TK$^-$ and ~5-6 weeks later when their hormones have dissipated, mice were challenged intravaginally with $5x10^3$ PFU of WT HSV-2. Vaginal washes were collected for 6 days post immunization and 5 days post challenge. On day 5 post challenge, tissues were collected for T cell analysis through flow cytometry.

The no hormone group showed viral shedding post immunization, and no viral shedding post challenge, indicating successful immunization to HSV-2, with the exception of one mouse (Figure 8a). Said mouse that did not shed virus post immunization did shed virus post challenge, demonstrating that the immunization was not effective. In the progesterone treated group, all mice except for one showed viral shedding post immunization (Figure 8b). Progesterone treated mice were shedding virus on day 1-3 post challenge which ceased by day 4, indicating that the immunization was still effective. All of the DMPA treated mice showed viral shedding post immunization (Figure 8c). Similar to the progesterone treated mice, DMPA treated mice were

shedding virus on day 1-3 post challenge. However, by day 4, the DMPA treated mice stopped shedding virus, demonstrating that the immunization was effective. NET 2.5 mg pellet treatment resulted in viral shedding post immunization in 9 out of 15 mice (Figure 8d). Comparing the NET 2.5 mg post immunization titers to no hormone, progesterone, and DMPA treated mice, NET 2.5 mg treated mice showed a delay in viral shedding on day 1 post immunization and viral shedding was much lower compared to other experimental groups. Post challenge, all NET treated mice were shedding virus, indicating that the immunization was not as effective as compared to the other groups. Therefore, NET 2.5 mg pellet treated mice did not show similar susceptibility as DMPA treated mice, as NET treated mice are not able to be effectively immunized the same way DMPA treated mice are. These results were similar to the previous experiment where NET 5 mg had been used. This showed that there may be an underlying mechanism associated with NET treatment that is different than progesterone and DMPA, not allowing mice to become immunized effectively. These results were different from what has been previously reported.

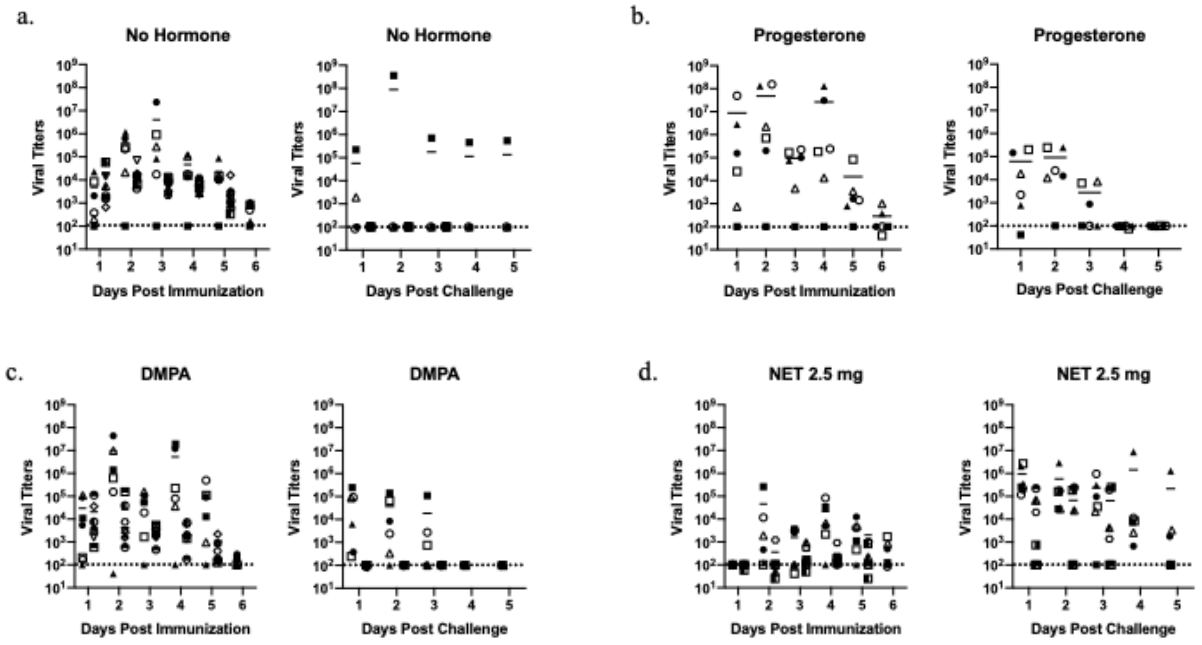


Figure 8. Viral titers of OVX mice with no hormones or treated with progesterone, DMPA, or NET 2.5 mg pellet post intravaginal immunization with 10^4 PFU of HSV-2 TK^- and after intravaginal challenge with $5x10^3$ PFU of WT HSV-2. There was a no hormone control group (a) (n=15) or mice were treated with progesterone 10 mg pellet (b) (n=6), DMPA 2 mg (c) (n=15), or NET 2.5 mg pellet (d) (n=15). Vaginal washes were collected for 5 days post immunization and challenge. Each symbol shape represents a different mouse and their viral titers each day after infection. Data is representative of two separate experiments. Dashed line indicates the lower limit of detection. Data was analyzed through a one-way ANOVA; however, no significance was found between average viral shedding of each mouse compared to each treatment group.

4.1.7 Primary intravaginal HSV-2 challenge in NET 2.5 mg, DMPA and progesterone treated mice

In order to understand if there are specific mechanisms that result in decreased viral susceptibility in NET treatment, we switched to the primary infection model, instead of the

immunization/challenge model. Differences in pathology and survival post primary HSV-2 infection in ovariectomized and normal mice were then investigated following treatment with NET, DMPA, and progesterone. Mice were first ovariectomized and two weeks later were administered hormone treatments including NET 2.5 mg pellets, as well as DMPA, progesterone and a no hormone group for comparison (Figure 9). Additionally, one group of mice were not ovariectomized and given NET 2.5 mg pellets to determine whether there are differences in pathology and survival in mice with and without the influence of their endogenous hormones. Ten days after hormone treatment, mice were intravaginally challenged with WT HSV-2 and followed for survival, pathology and viral shedding to determine differences in susceptibility.

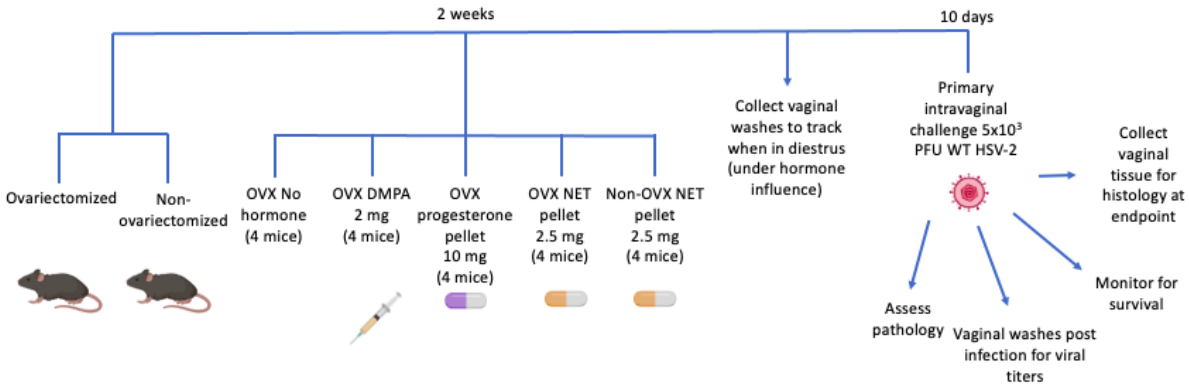


Figure 9. Outline of primary intravaginal challenge with WT HSV-2 in mice under different hormone treatments. Six- to eight-week-old C57BL/6 mice were ovariectomized and given 2 weeks to allow hormones to dissipate, while four mice were not ovariectomized. After 2 weeks, hormone treatments are given to mice, which include DMPA 2 mg injection and progesterone 10 mg pellet in OVX mice, NET 2.5 mg pellet in OVX mice and non-OVX mice, and a no hormone OVX control group. At 10 days of hormone treatment, mice were challenged intravaginally with $5x10^3$ PFU of WT HSV-2. Each day post infection vaginal washes were collected to measure viral

titers. Pathology was also assessed, and mice were monitored for survival. Once mice reached endpoint (pathology score of 4 or 5), mice were euthanized, and vaginal tracts were collected for histology.

Survival was monitored in mice post HSV-2 primary infection (Figure 10). The OVX no hormone group displayed the lowest survival rate, with all mice reaching endpoint by day 6. The OVX no hormone group also showed a statistically significant difference in survival rate compared to all other groups. The OVX DMPA group follow with the next lowest survival rate, with all mice reaching endpoint on day 8. The OVX progesterone treated group also resulted in a low survival rate but reached endpoint a day later than the OVX DMPA treated mice, which reached endpoint at day 8 post infection and the OVX progesterone treated group reached endpoint on day 9 post infection.

Both OVX and normal NET 2.5 mg treated groups showed the highest survival rates compared to all other groups (Figure 10). The OVX NET 2.5 mg treated mice all reached endpoint by day 9, the same day as the OVX progesterone group. However, OVX NET 2.5 mg treated mice started to reach endpoint on day 8, whereas OVX progesterone treated mice had started to reach endpoint on day 6. This indicated that the OVX progesterone treated group was more susceptible to infection compared to the NET treated groups. The OVX NET 2.5 mg treated group presented a lower survival rate than the normal NET 2.5 mg treated group, with one mouse that survived the infection. These results show that although mice did not survive in most groups, NET 2.5 mg may have decreased susceptibility to HSV-2 infection compared to DMPA and progesterone. There was a statistically significant difference in survival rates comparing OVX DMPA and OVX NET 2.5 mg as well as OVX DMPA compared to normal NET 2.5 mg. There may be differences in

susceptibility to HSV-2 in OVX and normal mice treated with NET 2.5 mg, where the influence of endogenous hormones can affect survival rates after HSV-2 infection. This was indicated by the lower survival rate with OVX NET 2.5 mg treated mice, and the one mouse in the normal NET 2.5 mg treated group that was not infected and survived. Altogether, this data suggests that there are differences in susceptibility to HSV-2 infection in DMPA and NET treated mice, where NET treatment resulted in delayed HSV-2 infection and end point compared to DMPA.

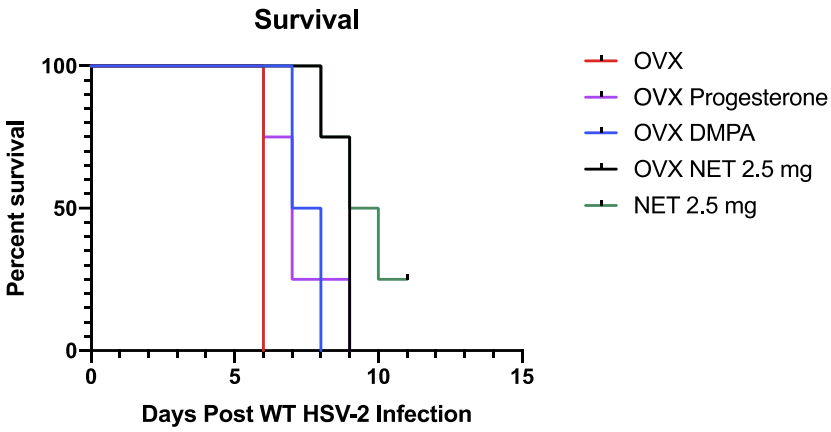


Figure 10. Survival curves of ovariectomized mice with treated with progesterone 10 mg pellet, DMPA 2 mg injection, NET 2.5 mg pellet, no hormone, as well as a non-OVX NET 2.5 mg pellet treatment group after intravaginal infection with $5x10^3$ PFU WT HSV-2. Mice were euthanized once they reached a pathology score of 4 or 5, which indicated endpoint. Data was analyzed through log-rank (Mantel-Cox) test. A statistically significant difference was found between the survival of the OVX group and other groups, showing a p-value of 0.0082** for OVX vs OVX DMPA, OVX vs OVX NET 2.5 mg, and OVX vs NET 2.5 mg. The OVX group also showed a statistically significant difference compared to OVX progesterone with a p-value of 0.0404*. OVX DMPA showed a statistically significant difference compared to OVX NET 2.5 mg with a p-value of 0.0280* and compared to NET 2.5 mg with a p-value of 0.0280*.

The OVX no hormone group (Figure 11a) showed high viral shedding in the first two days post infection, which is consistent with previous data from our lab indicating that this group is very susceptible to HSV-2[88]. In the OVX progesterone (Figure 11b) and OVX DMPA (Figure 11c) treated groups, showed similar patterns of viral titers, where the viral titers increased in the first 2-3 days post infection and peaked on day 3-4 post infection. In the OVX and normal NET 2.5 mg group (Figure 11d and e), there was a delay in viral shedding that occurred on day 1 post infection, in which no shedding was detected. Similar to the progesterone and DMPA treated groups, both groups of NET 2.5 mg treated mice show increased viral titers over the first 3-4 days post infection, but the peak viral titers occurred on day 5 post infection. Comparing the viral titers from OVX and normal mice treated with NET 2.5 mg pellets, the viral titers are similar. However, in the normal NET 2.5 mg treated group, there was one mouse that did not show any viral shedding, indicating that the mouse was not infected, suggesting that there may some mechanism of NET preventing viral entry from occurring.

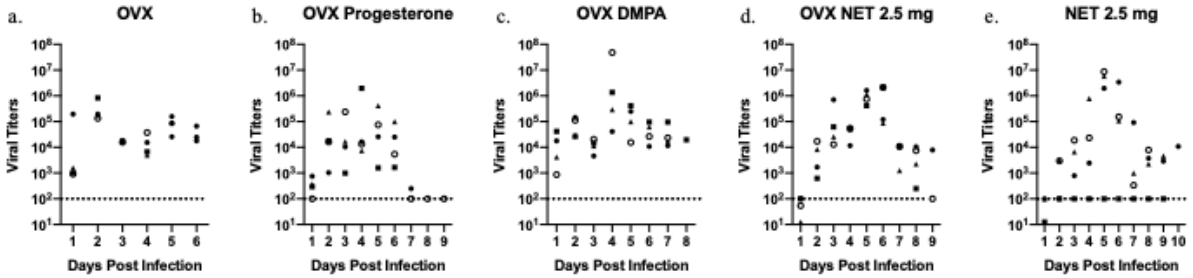


Figure 11. Viral titers post primary infection with $5x10^3$ PFU of WT HSV-2 IVAG in mice while under different hormone treatments. Groups include an OVX no hormone control (a) (n=4), OVX progesterone treated (b) (n=4), OVX DMPA treated (c) (n=4), OVX NET 2.5 mg (d) (n=4) and non-OVX NET 2.5 mg (e) (n=4). Vaginal washes were collected daily until each mouse reached endpoint. Each symbol represents a different mouse and their viral titers each day after infection. Dashed line indicates the lower limit of detection.

Based on the average pathology scores on each day post infection, the OVX no hormone group is the most susceptible compared to all other groups (Figure 12). This group reached high pathology scores earlier than other groups, which occurred on day 6 post infection. OVX no hormone mice also displayed a high average pathology score of 5 between all mice within the group (Table 4). Both the OVX progesterone and OVX DMPA groups reached high pathology scores later than the OVX no hormone group, reaching their highest scores on day 7 (Figure 12). From Table 3, the OVX DMPA group showed a higher pathology score overall of 4.5 compared to the 3.75 of the OVX progesterone group, indicating higher susceptibility to HSV-2 associated with DMPA treatment. The OVX NET 2.5 mg group did not start showing pathology until day 5, much later than the other groups, which started showing pathology on day 2 and 3 post infection. The OVX NET 2.5 mg treated group had an average overall pathology score of 5, whereas the normal NET 2.5 mg treated group had an average pathology score of 3. The normal NET 2.5 mg treated group

had one mouse in the group that did not present any pathology. Pathology scores demonstrate that the OVX no hormone mice are the most susceptible to HSV-2 primary infection, with OVX DMPA and OVX progesterone as the next most susceptible groups to HSV-2. Both normal and OVX NET 2.5 mg treated groups took longer to reach endpoint indicating a delayed infection and possible lower susceptibility to HSV-2 compared to OVX no hormone, OVX DMPA and OVX progesterone treated mice, although that was not reflected in the average pathology scores because of the scoring system.

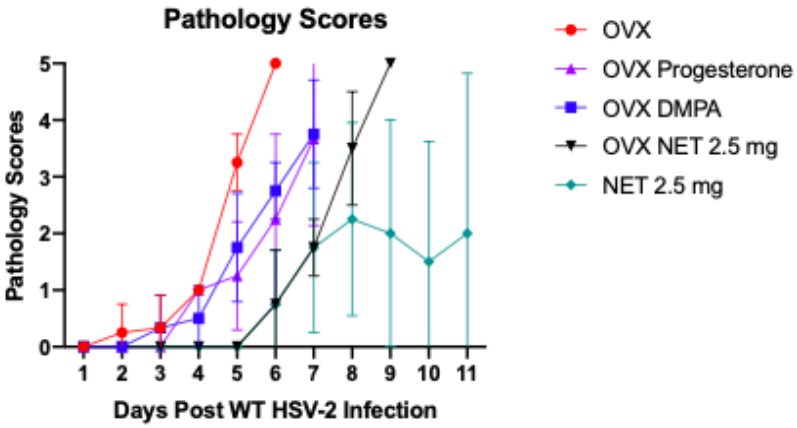


Figure 12. Pathology scores of ovariectomized mice with no hormone (n=4), DMPA (n=4), NET 2.5 mg pellet (n=4) or progesterone pellet treatment (n=4), as well as a group of normal mice treated with NET 2.5 mg (n=4) over 11 days post intravaginal infection with $5x10^3$ PFU of WT HSV- 2. Each point represents the average pathology score at each day post infection ± standard deviation. Each number of the pathology score represents a different level of disease severity, 0: no pathology; 1: slight redness & inflammation; 2: genital swelling & redness; 3: genital & surrounding area swelling, redness & hair loss; 4: genital ulceration; 5: ulceration & hair loss in surrounding areas, hind limb paralysis.

Table 4. Cumulative pathology scores for OVX mice (n=4), OVX progesterone treated mice (n=4), OVX DMPA treated mice (n=4), OVX NET 2.5 mg treated mice (n=4) and NET 2.5 mg (n=4) treated mice.

Group	Highest Pathology score	No. of mice	No. of days	No. of days until endpoint was reached	Cumulative pathology	Average pathology score/Mouse ± SEM
OVX	5	4	1	6	20	5 ± 0
OVX Progesterone	2	1	1	7	2	3.75 ± 1.5
	4	2	1	6 and 7	8	
	5	1	1	7	5	
OVX DMPA	3	1	1	7	3	4.5 ± 0.645
	3	1	2	7	6	
	4	1	1	7	4	
	5	1	1	7	5	
OVX NET 2.5 mg	5	4	1	8 and 9	20	5 ± 0
NET 2.5 mg	0	1	11	Endpoint not reached	0	3 ± 0
	4	3	1	11	12	

Cumulative pathology scores were calculated by tabulating the highest pathology score of each mouse in each treatment group for the number of days the highest pathology score was observed. The total pathology for the group is the sum of the pathology scores for all mice in each group,

where average pathology was calculated by dividing the cumulative pathology score by the number of mice in each group.

4.1.8 Primary intravaginal HSV-2 infection in OVX and normal mice treated with NET 2.5 mg, NET 5 mg and DMPA

Since the previous experiment indicated that there could be differences in susceptibility in the effect of NET in OVX versus non-OVX or normal mice, this next experiment was conducted to explore the susceptibility to HSV-2 in NET 2.5 mg and NET 5 mg treated mice in ovariectomized and normal mice which was compared to DMPA treated normal and ovariectomized mice (Figure 13). The normal control mice were staged and infected while in the diestrus stage of their cycle. This was done to determine if there was a difference in susceptibility with or without the influence of endogenous hormones. Since most of the mice reached end point in all groups in previous experiment, a lower dose of virus was used for primary challenge to see if clear differences could be seen in survival. Ten days after hormone treatment, mice were intravaginally infected with $5x10^3$ PFU of WT HSV-2 and pathology and survival were monitored each day post infection.

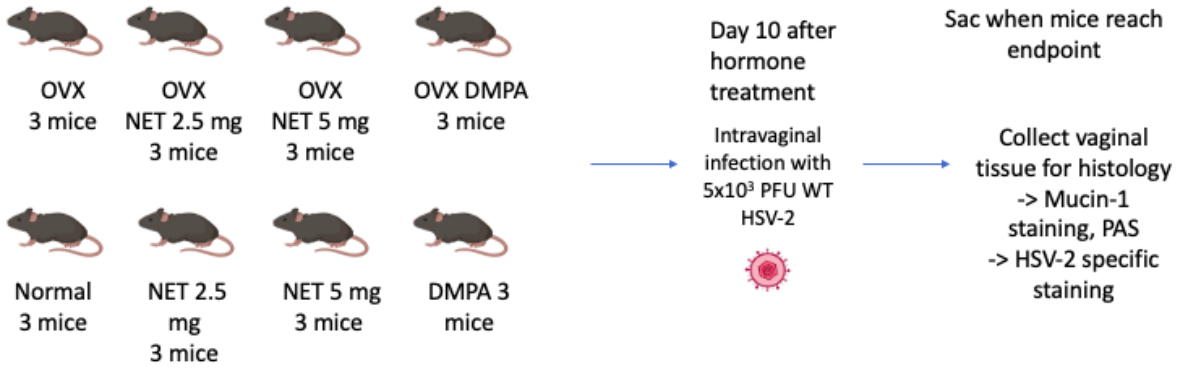
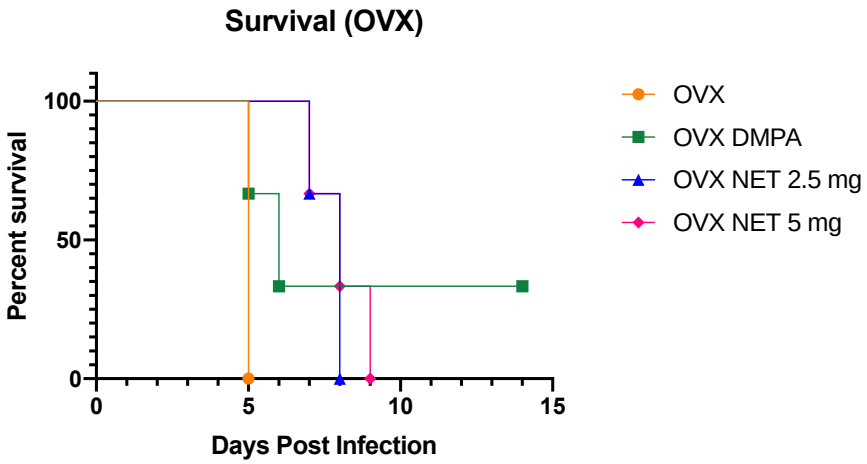


Figure 13. Outline of primary intravaginal challenge with WT HSV-2 in ovariectomized and normal mice under different hormone treatments. Six- to eight-week-old C57BL/6 mice were ovariectomized and given 2 weeks to allow hormones to dissipate, while 4 groups of mice were not ovariectomized. After 2 weeks, hormone treatments are given to mice, which include DMPA 2 mg injection, progesterone 10 mg pellet, NET 2.5 mg pellet in OVX mice and non-OVX mice, and an OVX no hormone control group. On day 10 post hormone treatment mice were intravaginally infected with $5x10^3$ PFU of WT HSV-2. Pathology scores were recorded for mice each day post infection and vaginal tracts were collected for histology once mice reached endpoint. Vaginal tissue was stained with Periodic acid–Schiff (PAS) to stain neutral and acidic mucins as well as mucin-1.

Mice were monitored for survival each day post HSV-2 infection. Similar to the results in Figure 10, OVX no hormone mice showed the lowest rate of survival (Figure 14), all reaching endpoint by day 5, indicating high susceptibility to HSV-2. The OVX DMPA group resulted in 2/3 mice reaching endpoint by day 6, except for the one uninfected mouse which survived. The OVX NET 2.5 mg and OVX NET 5 mg showed the next lowest survival rates, reaching endpoint at day 8 and 9, respectively. The normal NET 2.5 mg group had 1/3 mice reaching endpoint by day 9 post

infection, with 2 mice surviving (Table 5). The normal NET 5 mg group had 2/3 mice that reached endpoint by day 11, which occurred later than the NET 2.5 mg group. The NET 5 mg group also had 1 mouse survive the infection. All mice in the normal DMPA and normal groups survived the infection. Comparing the ovariectomized and normal DMPA treated mice to NET treated mice, DMPA treated mice showed improved survival compared to NET treated mice, as all ovariectomized NET treated mice reached endpoint but one DMPA treated mouse did not. This shows that OVX NET treated mice may be more susceptible to HSV-2 infection than normal NET treated mice, as they all reached endpoint and the normal NET treated groups had mice surviving the infection. This indicates that the influence of endogenous hormones can affect HSV-2 infection outcomes.

(a)



(b)

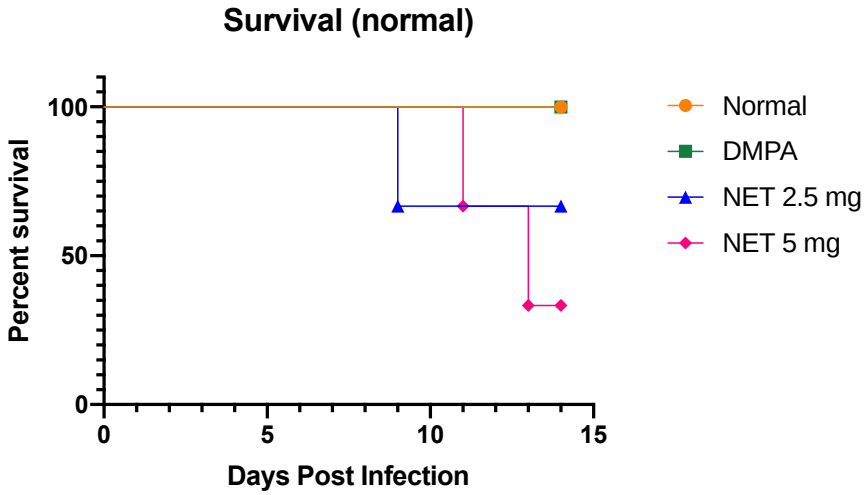


Figure 14. Survival of ovariectomized and normal mice under different hormone treatments after intravaginal WT HSV-2 infection. Ovariectomized (a) and normal mice (in diestrus) (b) were administered DMPA, NET 2.5 mg pellets or NET 5 mg pellets. These groups also included OVX and normal mice with no treatment as controls. Mice were monitored each day and euthanized once they reached a pathology score of 4 or 5, which indicated endpoint. N=3 for each group.

Average pathology scores for each day in each group is shown in Figure 15. Similar to the previous survival experiment conducted in Figure 9, the OVX no hormone group reached high pathology scores earlier on than other groups. As seen in Table 5, the OVX no hormone group had a high cumulative pathology score of 4. They reached their highest pathology scores on day 5.

The next group that showed high pathology scores early on was the OVX DMPA group, which is also consistent with the results in Figure 12. The OVX DMPA group reached endpoint on day 5 and 6, as indicated in Table 5. However, there was one mouse in this group that did not present any pathology post infection and was most likely not infected. The normal DMPA treated group also did not show any pathology post infection and was also likely not infected, which could have occurred due to this being a low dose infection of HSV-2.

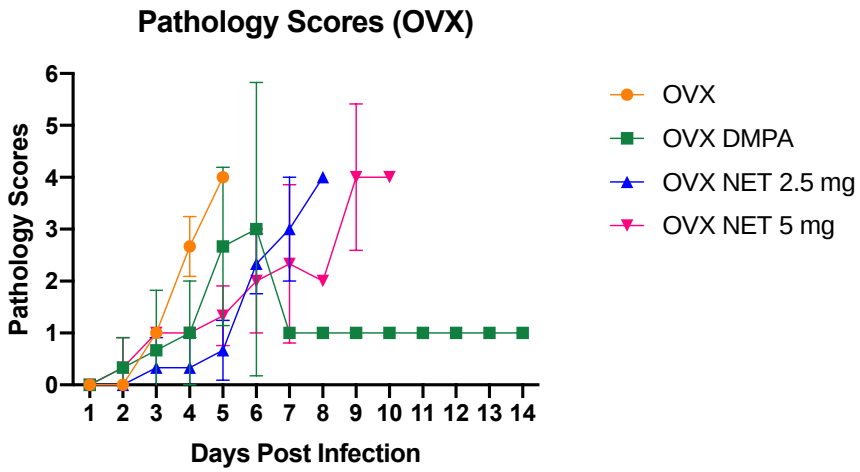
The next most susceptible group was the OVX NET 2.5 mg treated group, reaching high pathology scores by day 8. The next most susceptible group was the OVX NET 5 mg treated group, which reached their highest pathology scores on day 10 and 11. Both groups showed similar average pathology scores calculated in Table 5. The OVX NET 2.5 mg group had an average pathology of 4 ± 0 and the OVX NET 5 mg group showed an average pathology score of 4.33 ± 1.22. There were 2/3 mice in the normal NET 2.5 mg group that did not result in any pathology post infection, which could be due to the mechanism that may be preventing viral entry. Normal NET treated mice showed little to no viral shedding post infection as shown in Figure 3, 5 and 8. In the normal NET 5 mg group, 1/3 of the mice did not develop pathology, which could also be due to the same mechanism.

Compared to the OVX groups, the normal NET treated groups showed lower average pathology scores of 1.66 ± 0 for NET 2.5 mg and an average score of 3 ± 0.408 for NET 5 mg as indicated in Table 5, which is lower than the 4 and 4.33 associated with the OVX NET groups at similar

doses, respectively. This further confirms that there is a difference in susceptibility to HSV-2 in NET treated mice with and without the influence of endogenous hormones.

The normal no hormone control mice did not develop any pathology post HSV-2 infection. From a previous study in our lab, mice in diestrus showed viral shedding after infection with 10^5 PFU of WT HSV-2, while in this experiment a low dose of $5x10^3$ PFU of WT HSV-2 was used[85]. This lower dose may not have been sufficient to cause lethal infection the normal mice in diestrus. A lower dose was used, as ovariectomized mice and the use of progestins make mice very susceptible to infection with HSV-2[85 88]. A high dose would likely result in mice reaching endpoint very early on post infection, where differences in pathology and survival would not be captured.

(a)



(b)

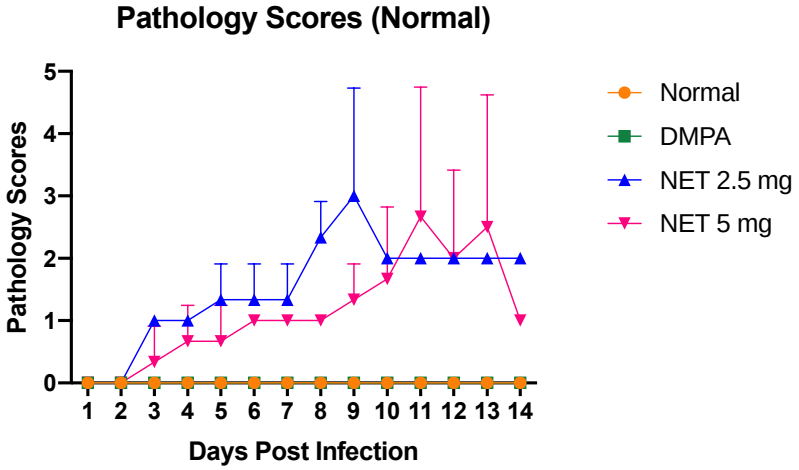


Figure 15. Average pathology score of each treatment group on each day post infection intravaginally with $5x10^3$ PFU WT HSV-2. Treatment groups in the ovariectomized groups include OVX no hormone, OVX DMPA, OVX NET 2.5 mg and OVX NET 5 mg (a). Treatment groups in the normal mice include normal no hormone (in diestrus), DMPA, NET 2.5 mg and NET 5 mg (b). N=3 for each group.

Table 5. Table of cumulative pathology scores for OVX mice, OVX DMPA treated mice, OVX NET 2.5 mg treated mice and OVX NET 5 mg treated mice, as well as normal no hormone and normal mice treated with DMPA, NET 2.5 mg and NET 5 mg.

Group	Pathology score	No. of mice	No. of days	No. of days until endpoint was reached	Cumulative pathology	Average pathology/ mouse ± SEM
OVX	4	3	1	5	12	4 ± 0
OVX DMPA	0	1	14	endpoint was not reached	9	3 ± 0.408
	4	1	1	4		
	5	1	1	5		
OVX NET 2.5 mg	4	3	1	7 and 8	12	4 ± 0
OVX NET 5 mg	4	2	1	5 and 6	8	4.33 ± 1.22
	5	1	1	7 and 9	5	
Normal	0	3	14	endpoint was not reached	0	0
DMPA	0	3	14	endpoint was not reached	0	0
NET 2.5 mg	0	2	14	endpoint was not reached	0	1.66 ± 0
	5	1	1	9	5	
NET 5 mg	0	1	1	endpoint was not reached	0	3 ± 0.408
	4	1	1	11	4	
	5	1	1	12	5	

Pathology was monitored for 14 days and mice were euthanized once they reached a pathology score of 4 or 5 indicating endpoint. Cumulative pathology scores were calculated by tabulating the highest pathology score of each mouse in each treatment group for the number of days the highest pathology score was observed. The total pathology for the group is the sum of the pathology scores for all mice in each group, where average pathology was calculated by dividing the cumulative pathology score by the number of mice in each group. N=3 for each group.

Overall, we found that injections of NET suspensions were not effective in providing a consistent dose of NET to mice. This led towards testing hormone pellets of NET, which were much more effective at providing a consistent dose of hormones in mice. Through testing two different doses of NET, which included NET 2.5 mg and NET 5 mg, NET 2.5 mg was found to be a more optimal dose reflecting serum levels of women using NET as a contraceptive and did not result in adverse effects like the NET 5 mg dose. When these two doses of NET were tested in mice in immunization and challenge models, they did not show similar results to DMPA treated mice. NET treated mice consistently showed a delay and lower titers in viral shedding. Additionally, ineffective intravaginal immunizations occurred in NET treated groups that resulted in high viral shedding post challenge. Furthermore, in primary infections, OVX NET treated mice showed a delay in HSV-2 infection than DMPA treated mice in terms of pathology scores and survival rates. There was also a difference in the pathology and survival post HSV-2 infection seen in OVX and normal NET treated mice, indicating a higher susceptibility associated with OVX NET treated groups compared to normal NET treated groups.

4.2 Aim 2: Investigate the effect of NET on the physiology of the vaginal tract

The previous results indicated that NET treatment in mice resulted in lower susceptibility to HSV-2 infections compared to DMPA and progesterone treatment. It is known that DMPA treatment causes thinning of the vaginal epithelium, which is a key mechanism since it compromises the integrity of the vaginal epithelial barrier, allowing viral entry to occur easily[94]. However, the effect of NET hormone pellets at the dose used for these experiments has not been explored on vaginal thickness and physiology.

4.2.1 Examining mucin in the vaginal tract of mice under different stages of the estrus cycle and OVX and normal mice treated with NET 2.5 mg, NET 5 mg and DMPA

To explore the underlying mechanism responsible for the lower susceptibility to HSV-2 infection observed with NET, histology of the vaginal tract was examined. Ovariectomized and normal mice were given NET 2.5 mg and NET 5 mg pellets, and ten days later the vaginal tracts were collected to examine the effects of the hormone treatments on the vaginal physiology (Figure 16). Periodic Acid Schiff (PAS) staining was performed on vaginal tissue, which is known to stain polysaccharides including neutral and acidic mucins. In the literature, expression of specific mucins in the vaginal tracts of mice has not been extensively researched. However, one study showed that mucin-1 expression is present in the vaginal tracts of mice, therefore, muc-1 staining was also performed in these samples[155].

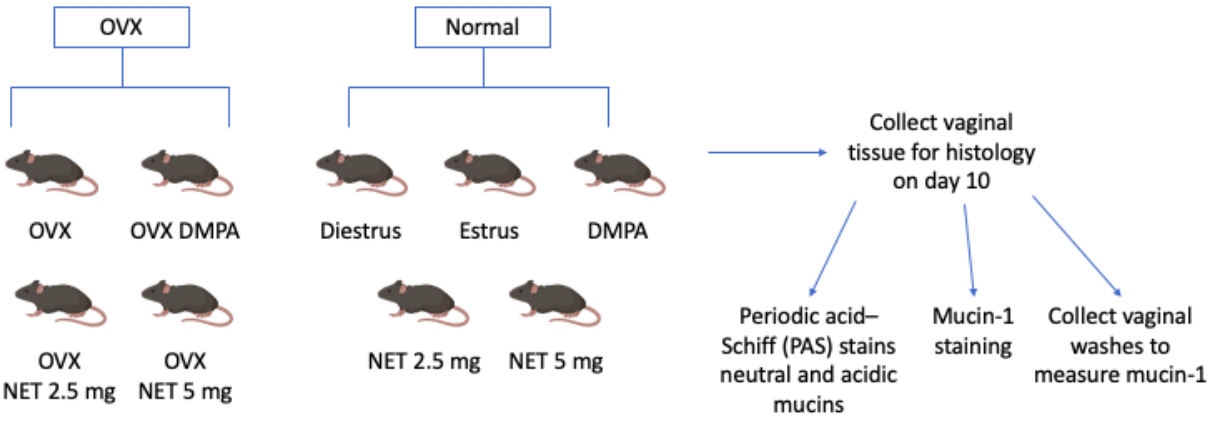


Figure 16. Experimental outline for visualization of mucus in vaginal tracts of mice under different hormone treatments. Four groups of six- to eight-week-old C57BL/6 mice were ovariectomized and given two weeks to recover, and mice with intact ovaries were included to evaluate effects with endogenous hormones. Mice were administered NET 2.5 mg, NET 5 mg, DMPA 2 mg injection and control mice were not given hormone treatments. There were three mice in each treatment group. Ten days after hormone treatment, vaginal tracts were collected for PAS and mucin-1 staining to examine mucin production. N= 3 for each group.

In the OVX no hormone, estrus, and diestrus groups, all mice showed very little to no positive PAS staining, which was consistent in all 3 mice in each group (Figure 17). Similar to the PAS staining, the OVX no hormone, estrus and diestrus mice displayed little positive mucin-1 staining, also seen in all 3 mice in each group (Figure 17). All mice in the OVX DMPA group did not show any positive PAS staining but showed a thin lining of positive mucin-1 staining similar to the OVX, estrus and diestrus mice. All mice in the DMPA group showed little positive PAS and mucin-1 staining, which was also similar to the OVX, diestrus and estrus mice. More positive PAS and mucin-1 staining was observed in the OVX and normal NET treated mice compared to the OVX and normal controls. The OVX NET 5 mg and OVX NET 2.5 groups consistently showed

similar positive mucin-1 and PAS staining in all 3 mice in each group. There were 2/3 mice in the NET 2.5 mg group that consistently showed similar positive mucin-1 and PAS staining. There were 2/2 mice in the NET 5 mg group also showed positive mucin-1 and PAS staining, which visually looked similar to the NET 2.5 mg group. Together, these show that there is more mucus lining the vaginal tracts of NET treated mice compared to the ovariectomized and normal control groups. This could be creating a barrier to stop or delay viral entry, a potential mechanism of NET that results in lower susceptibility to HSV-2 infection.

These images also outline thinning of the vaginal epithelium in OVX no hormone and OVX DMPA treated mice. This was also evident when the vaginal thickness was quantified (Figure 18). The estrus group showed a thicker vaginal epithelium compared to the OVX, OVX NET 2.5 mg and NET 2.5 mg group. Additionally, the OVX no hormone group showed thinner vaginal epitheliums compared to the diestrus group and the diestrus group showed a thinner vaginal epithelium compared to the estrus group. The NET treated groups did not show a difference in vaginal epithelium thickness, demonstrating that both doses result in similar thickness. While there were no significant differences in vaginal thickness between the OVX and OVX or normal NET and DMPA treated groups, the OVX no hormone and OVX DMPA groups showed similar thickness. The OVX and OVX DMPA groups also had the lowest reported thickness in the vaginal epithelium. This would indicate the OVX no hormone and OVX DMPA group are much more susceptible to HSV-2 viral entry compared to other groups. With the NET treated groups not showing the same thickness as the OVX no hormone and OVX DMPA treated groups, this could indicate that they are not as susceptible to HSV-2 compared to these groups.

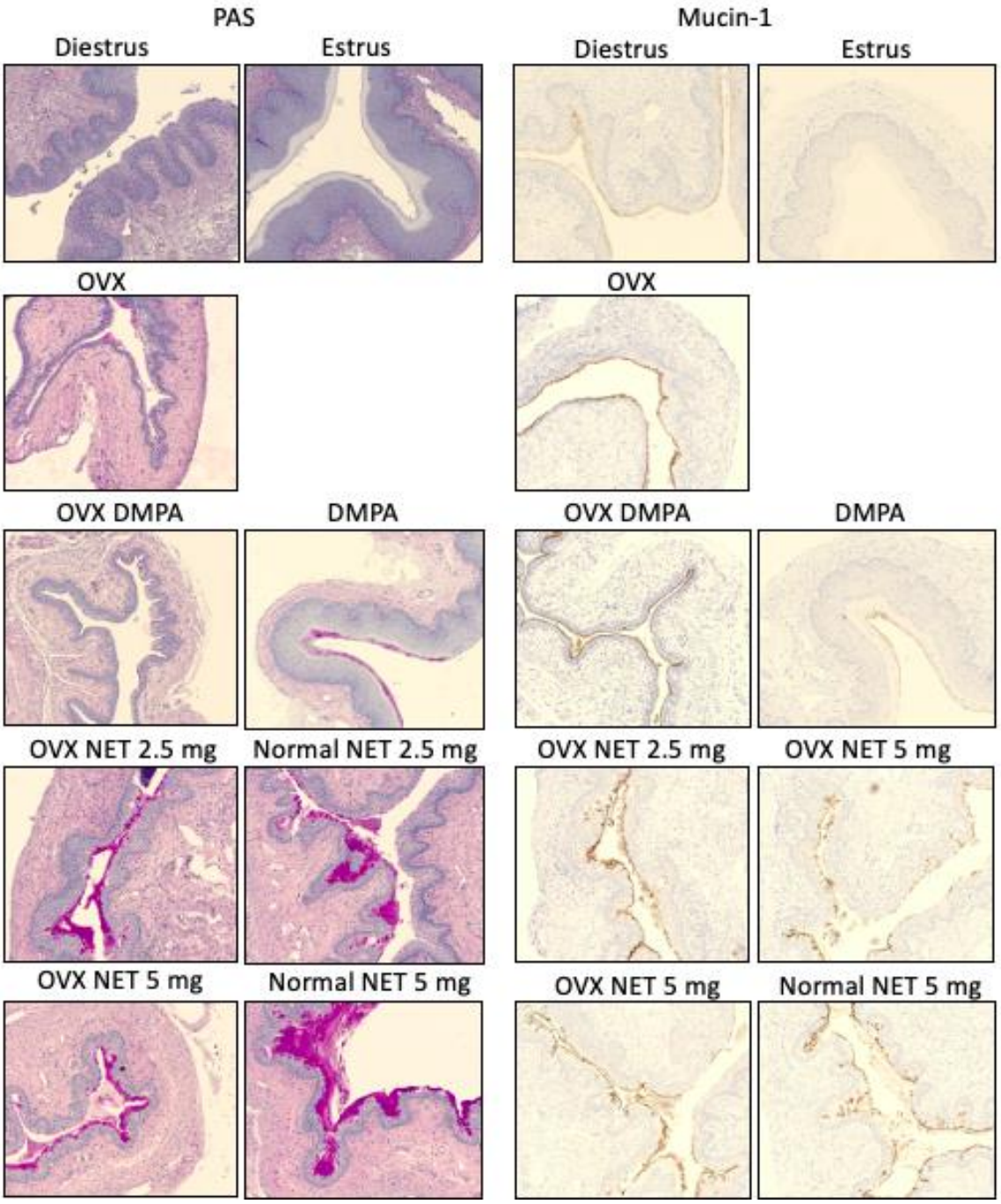


Figure 17. PAS and mucin-1 staining of the vaginal tracts of mice under different hormone treatments. Images of the vaginal tracts from OVX and normal mice under different hormone treatments, including NET 2.5 mg, NET 5 mg and DMPA 2 mg, after 10 days of hormone treatment, as well as mice in estrus and diestrus to compare different stages of the estrous cycle.

N= 3 for OVX, n=3 for OVX NET 2.5 mg, n=2 for OVX NET 5 mg, n=2 for OVX DMPA, n= 3 for NET 2.5 mg, n=2 for NET 5 mg, n=2 for DMPA, n=3 for estrus, n=3 for diestrus. Vaginal tissue was fixed in methanol-Carnoy solution. Slides were stained with Periodic acid–Schiff (PAS) to stain neutral and acidic mucins (left) and mucin-1 (right). Images were taken at 10X using a Zeiss microscope. Each image is a representative image of the 2-3 mice in each treatment group.

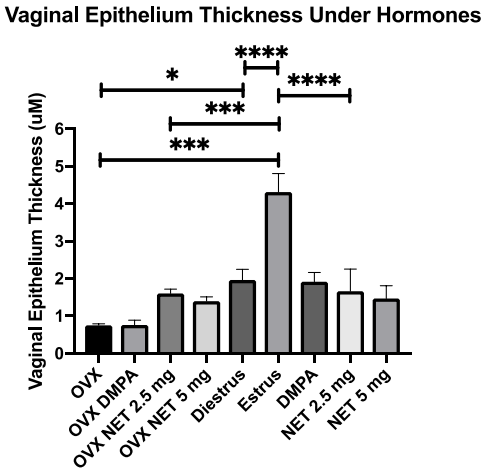
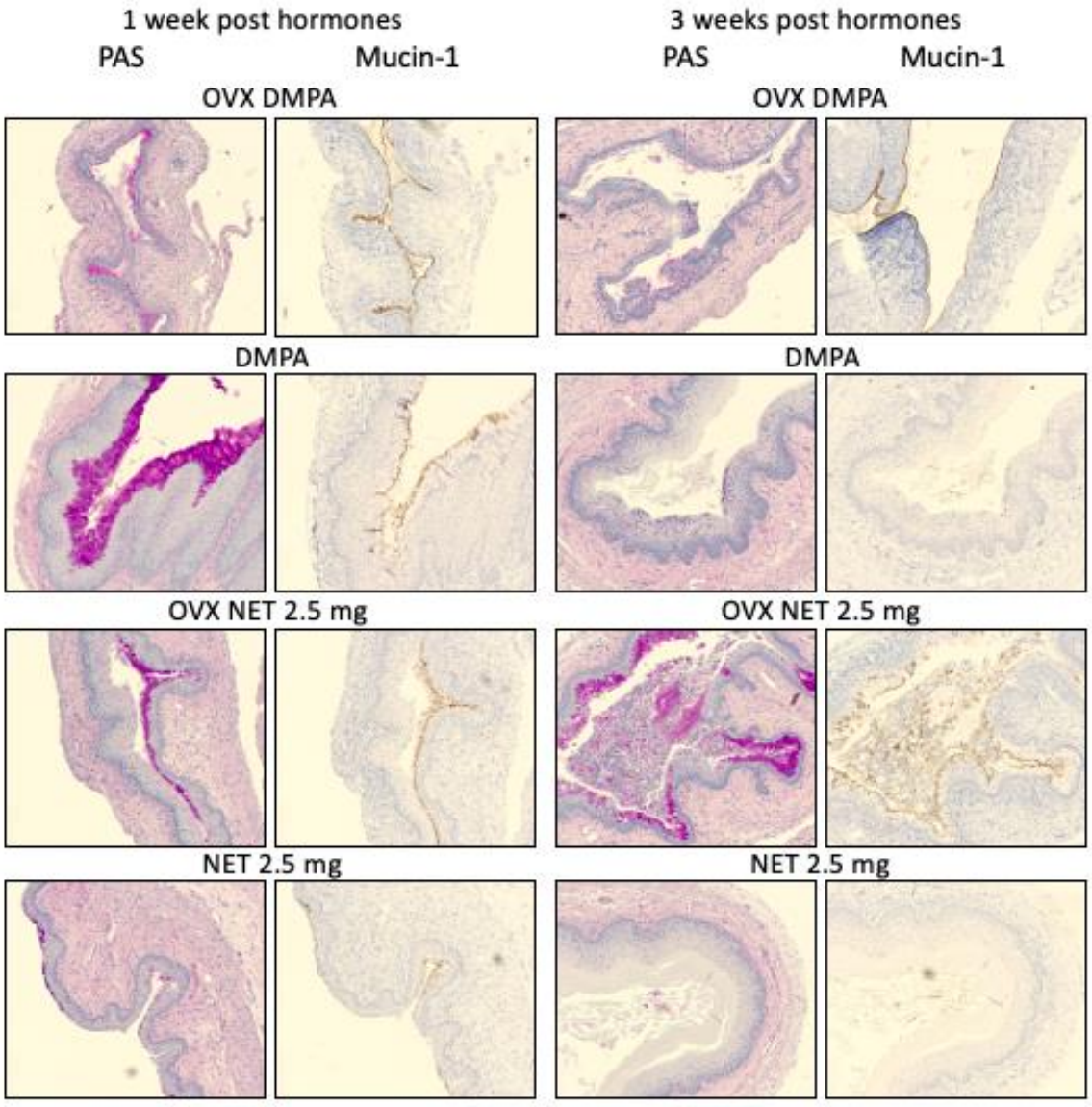


Figure 18. Vaginal epithelium thickness measurements in mice under different hormone conditions. On day 10 post hormone treatment, vaginal tracts were collected from ovariectomized and normal mice for histology. Hormone treatments include DMPA, NET 2.5 mg and NET 5 mg pellets. Control groups include an ovariectomized no hormone control as well as normal mice in the diestrus and estrus stage of the estrous cycle. N= 3 for OVX, n=3 for OVX NET 2.5 mg, n=2 for OVX NET 5 mg, n=2 for OVX DMPA, n= 3 for NET 2.5 mg, n=2 for NET 5 mg, n=2 for DMPA, n=3 for estrus, n=3 for diestrus. ImageJ software was used to measure the thickness of the vaginal epithelium for each mouse in each group. Vaginal epithelium thickness is reported as an average measurement of thickness for each mouse with the standard deviation. There was a statistically significant difference comparing the thickness of the vaginal epithelium from the

estrus group compared to the OVX no hormone, OVX NET 2.5 mg, diestrus and NET 2.5 mg. Data were analyzed with a one-way ANOVA and Tukey's multiple comparisons test. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$.

4.2.2 Examining mucin in the vaginal tract of NET 2.5 mg and DMPA treated mice over 6 weeks of hormone treatment



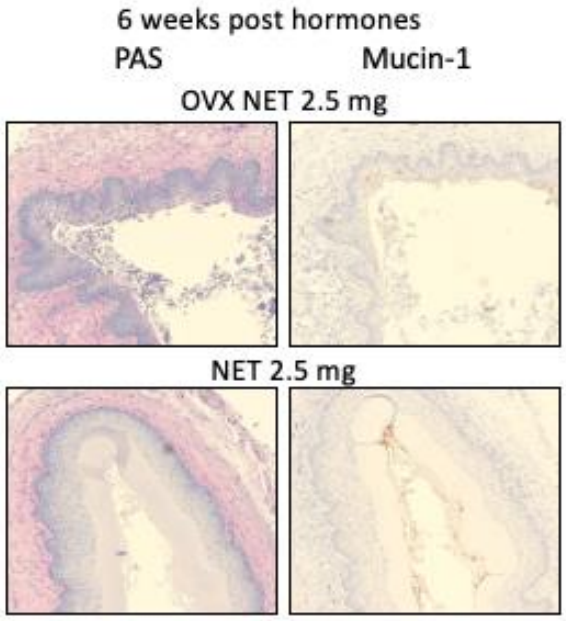


Figure 19. Images of mucin-1 and PAS staining on vaginal tracts of mice under different hormones over 6 weeks. Ovariectomized and normal mice were treated with DMPA and NET 2.5 mg pellets. Vaginal tracts were collected from mice at 1 week, 3 weeks and 6 weeks post hormone treatment for histology. Tissues were stained with PAS and mucin-1 to visualize mucus in the vaginal tract. Images were taken at 10x magnification on the Zeiss microscope. N= 1 per group,

As mucus was visible in Figure 17 in the vaginal tracts of NET treated mice after 10 days of hormone treatment, vaginal tracts from NET treated mice were examined at 1 week, 3 weeks and 6 weeks post hormone treatment. This was done to determine whether the presence of mucus changes over time after treatment and whether this effect of NET is still ongoing after the 21 days in which hormones are no longer being released from the pellet. For comparison, vaginal tracts were also collected from DMPA treated mice 1 week and 3 weeks post hormone treatment as well. After 1 week of hormone treatment, all hormone groups show positive PAS staining and mucin-1 staining (Figure 19). This indicates that at 1 week after hormone treatment, there are still high

enough levels of the endogenous hormones in the mouse's system to cause mucus production in the vaginal tract. After 3 weeks, the OVX DMPA and normal DMPA group show slightly diminished PAS and mucin-1 staining. The OVX NET 2.5 mg group still showed positive PAS and mucin-1 staining, however, the normal NET 2.5 mg group showed little positive PAS and mucin-1 staining after 3 weeks. This indicates that at 3 weeks for both DMPA and NET, the hormones may be starting to dissipate in the mouse's system and may not be in high enough levels to lead to high mucus production in the vaginal tracts, as seen after 1 week. Although the one mouse in the OVX NET 2.5 mg group, did show lots of positive PAS and mucin-1 staining present in the vaginal tract after 3 weeks. Furthermore, after 6 weeks of hormone treatment, both the OVX NET 2.5 mg and normal NET 2.5 mg groups showed very little positive PAS and mucin-1 staining. At this time point there is less mucus present in the vaginal tracts of NET treated mice in both ovariectomized and normal mice, which is when the hormones will be dissipated from the mice.

From histology images in Figure 17, it is clear that the ovariectomized and normal NET treated mice show more positive PAS and mucin-1 staining compared to the other groups. This showed the amount of mucus that is present on the cell surface. However, to be able to quantify the amount of secreted mucus in the vaginal tract, vaginal washes were collected from normal mice for days 1-10 post hormone treatment. The normal mice were used in this experiment as in the histology images, lots of mucus was visibly present in the vaginal tracts and was more visible than the ovariectomized groups (Figure 17). An ELISA would quantify the amount of secreted mucus over the first 10 days after hormone treatment and would also be indicative of how much mucus would be present at the time of HSV-2 immunization or challenge which was the timeline used for previous experiments (Figures 2, 4, 7, 9 and 13).

The OD values for the NET 2.5 mg treated groups, as well as the NET 5 mg treated groups showed a trend of increased secreted mucin-1 protein in the vaginal washes over the 10 days post hormone treatment (Figure 20). The standard deviation between samples was too large to obtain any statistical significance. This provides preliminary evidence that the NET treated groups show increased mucus present in the vaginal tract 4-6 days after hormone treatment.

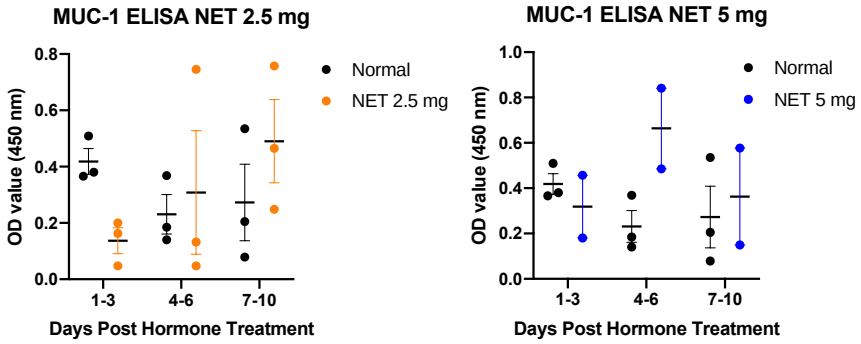


Figure 20. OD values for mucin-1 protein expression in vaginal washes from mice. Normal mice were given hormone treatments which incldued NET 2.5 mg and NET 5 mg as well as a no hormone control group. Each day after hormone treatment, vaginal washes were collected from mice for up to 10 days. Vaginal washes from mice were pooled from days 1-3, 4-6 and 7-10 from each mouse in each group. N= 3 for normal mice, n=3 for NET 2.5 mg and n=2 for NET 5 mg. The muc-1 concentration was quantified through a MUC-1 ELISA. Data are presented as optical density units (OD) with standard error of the mean (SEM).

4.2.3 Examining mucin in the vaginal tract of NET 2.5 mg and DMPA treated mice after intravaginal immunization with attenuated HSV-2

To determine the effect of the hormones on the vaginal epithelium after HSV-2 immunization, vaginal tracts were collected from mice under different hormone treatments after immunization

for histological analysis. Mice were ovariectomized and two weeks later treated with DMPA, NET 2.5 mg pellets or NET 5 mg pellets. Ten days after hormone treatment, mice were intravaginally immunized with 10^4 PFU of HSV-2 TK$^-$ (Figure 21). Vaginal tissue was collected and H&E staining as well as PAS staining were performed to visualize the vaginal epithelium thickness and mucus. Ovariectomized mice were used in this experiment to determine whether mucus is still visible in the vaginal tract after immunization and if this is influencing the infection.

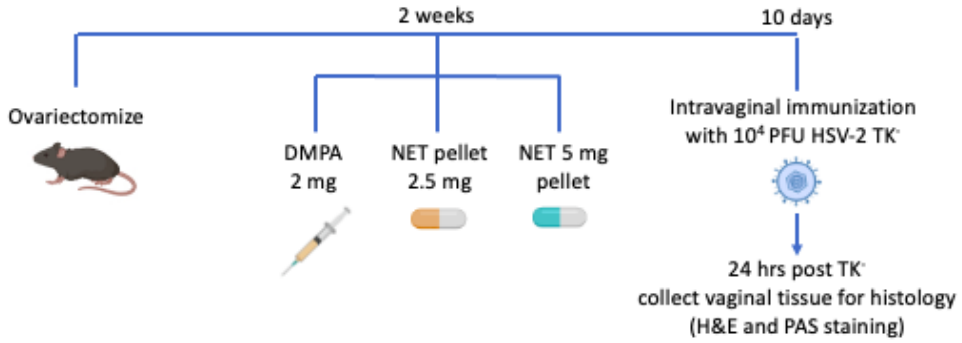


Figure 21. Experiment outline of collection of vaginal tracts for histology from mice under different hormone treatments after intravaginal immunization. Six- to eight-week-old C57BL/6 mice were ovariectomized and treated with different hormones, including DMPA, NET 2.5 mg and NET 5 mg. Ten days after hormone treatment, mice were immunized intravaginally with 10^4 PFU of HSV-2 TK$^-$. Vaginal tissue from mice were collected for histology 24 hours after intravaginal immunization, fixed in methanol-Carnoy solution, and Hematoxylin and eosin (H&E) and Periodic acid–Schiff (PAS) staining was performed. N=2 for each group.

In the OVX NET 2.5 mg and OVX NET 5 mg treated mice post-immunization (Figure 22), the vaginal tracts showed similar thickness in the vaginal epithelium as seen in Figure 17 and 18. Similar trends occurred, showing that all mice in the OVX DMPA group showed thinner vaginal epithelium compared to the 2 mice in each of the OVX NET treated groups. The vaginal epithelium

thinning is not seen in the NET treated groups and could be a potential mechanism that does not allow them to be easily infected in the same way this occurs in DMPA treated mice.

In the vaginal tracts of all the OVX NET 2.5 mg treated mice, there is a thin layer of positive PAS staining lining the epithelium after immunization (Figure 22). However, in the two OVX NET 5 mg treated mice, there was much more positive PAS staining compared to the OVX NET 2.5 mg and OVX DMPA groups. The OVX DMPA group, showed little to no positive PAS staining.

Overall, these images demonstrated that after HSV-2 immunization, the effects of NET treatment on the vaginal tract are still present. Therefore, this influences outcomes after infection, in which higher positive PAS staining is present in the NET treated groups, and potentially providing a barrier to virus.

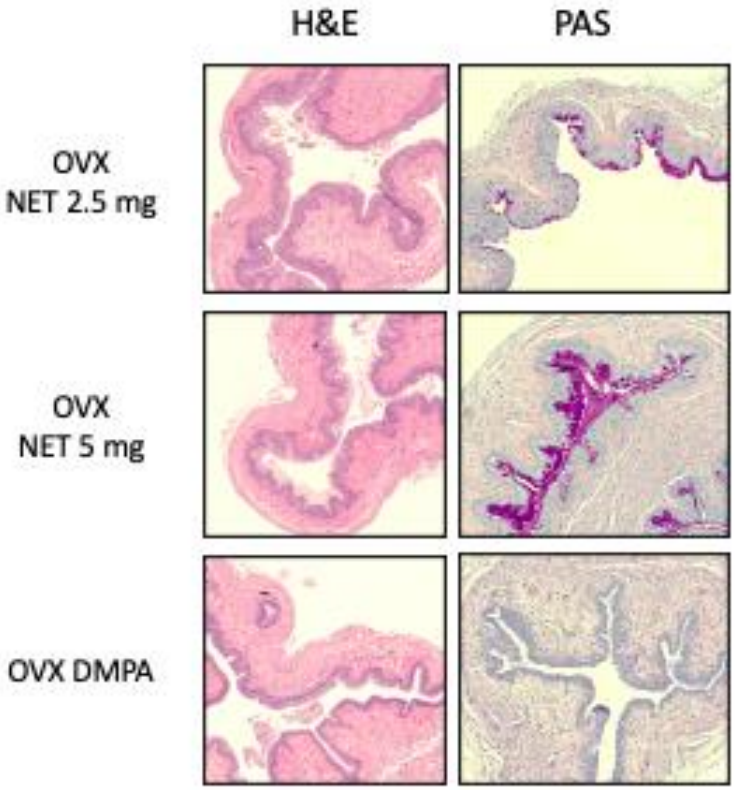


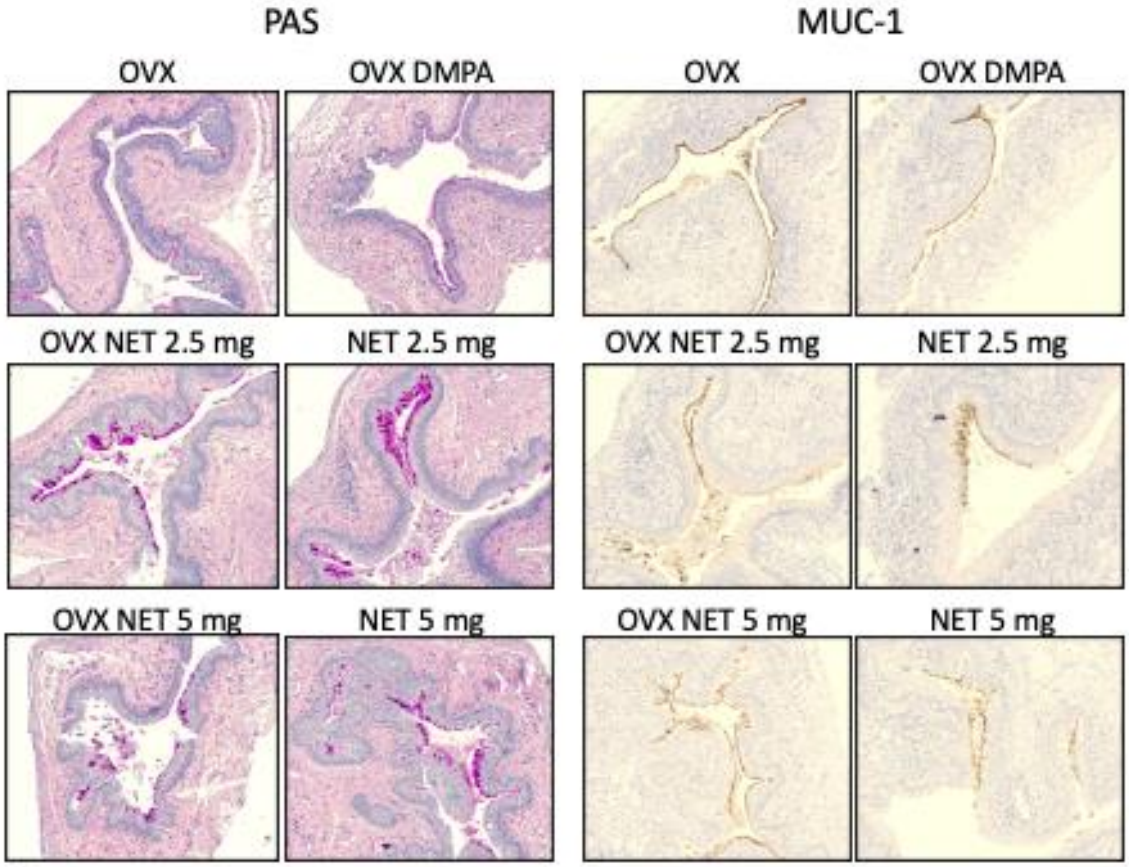
Figure 22. Images of the vaginal tracts from ovariectomized mice under different hormone treatments, including NET 2.5 mg, NET 5 mg and DMPA, post-HSV-2 immunization. Slides were stained with Hematoxylin and eosin (H&E) and Periodic acid–Schiff (PAS) to stain neutral and acidic mucins. N=2 for each group. Images were taken at 10x using the Zeiss microscope.

4.2.4 Examining mucin and HSV-2 infection in the vaginal tract of OVX and normal mice treated with NET 2.5 mg, NET 5 mg and DMPA

In these previous experiments done (Figure 2, 4, 7, and 9), vaginal washes were collected from mice to measure viral shedding post infection. However, it is possible that performing vaginal washes could be disrupting the mucus formation in the vaginal tract that would be occurring in NET treated mice and could be affecting results. Therefore, the experiment outlined in Figure 13 was done to determine whether there is a difference in susceptibility to HSV-2 infection in

ovariectomized and normal mice with NET treatment compared to DMPA treated mice. Vaginal washes were not performed in this experiment to prevent disruption of any mucus formation in the vaginal that could affect outcomes after HSV-2 infection.

As previously shown in Figure 14, 15 and Table 5, the normal and normal DMPA treated groups of mice did not reach endpoint post intravaginal WT HSV-2 infection and therefore vaginal tracts were not collected from these mice as they survived the infection. Similar to Figure 17, the OVX mice and OVX DMPA groups showed little positive PAS staining as well as positive mucin-1 staining lining the vaginal epithelium (Figure 23). This trend was seen in all three of the mice from the OVX group and the 2 mice in the OVX DMPA group. The OVX NET 2.5 mg, normal NET 2.5 mg, OVX NET 5 mg, and normal NET 5 mg groups showed more positive PAS and mucin-1 staining than the OVX and OVX DMPA treated groups. The PAS and mucin-1 staining in the 3 mice in the OVX NET treated groups and 1 mouse in the NET 2.5 mg, and 2 mice in the NET 5 mg groups displayed positive PAS and mucin-1 staining lining the vaginal epithelium. Interestingly, only the OVX and OVX DMPA treated groups had positive HSV-2 specific staining in the vaginal tracts, but none of the 3 mice in OVX NET 2.5 mg and OVX NET 5 mg groups presented any positive HSV-2 specific staining. There was also no positive HSV-2 specific staining in the 2 mice that reached endpoint in the normal NET 2.5 mg and the 1 mouse that reached endpoint in the normal NET 5 mg treated mice. This could be due to the effect of NET, where mucus formation as well as a thicker vaginal epithelium may have prevented viral entry into the vaginal tract where virus may have leaked out. All images of NET treated vaginal tracts showed an intact vaginal epithelium, indicating that the virus did not get into the vaginal tract. This provides further evidence to show that NET treatment in mice does not increase susceptibility to intravaginal HSV-2 infection as seen with DMPA treatment.


PAS
MUC-1
OVX
OVX DMPA
OVX
OVX DMPA
OVX NET 2.5 mg
NET 2.5 mg
OVX NET 2.5 mg
NET 2.5 mg
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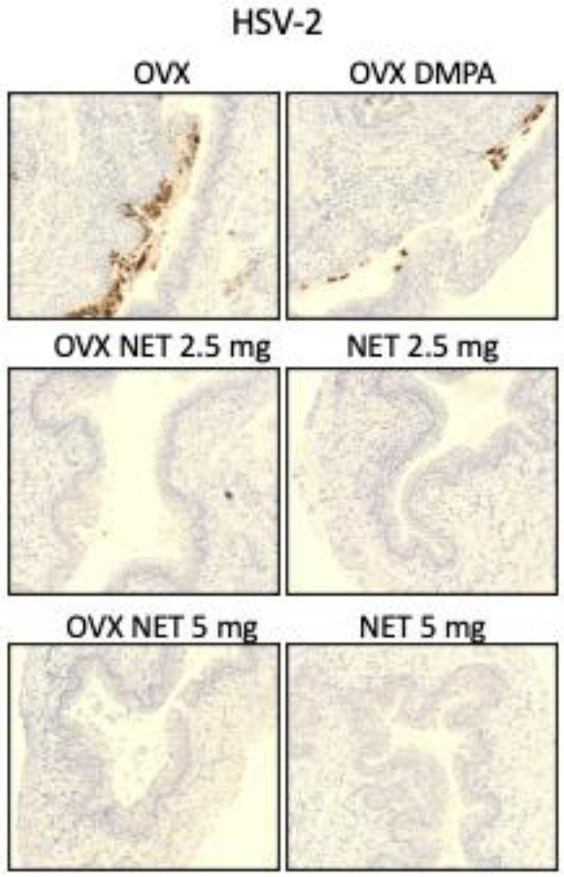

Figure 23. Images of the vaginal tracts from ovariectomized and normal mice after intravaginal WT HSV-2 infection under the influence of different hormones. Ovariectomized and normal mice were treated with DMPA, NET 2.5 mg pellets and NET 5 mg pellets. Vaginal tracts were collected from mice for histology once they reached endpoint after intravaginal $5x10^3$ PFU WT HSV-2 infection. Periodic acid–Schiff (PAS) staining, mucin-1 staining, and HSV-2 specific staining was done on vaginal tract samples. N=3 for OVX, OVX NET 2.5 mg, OVX NET 5 mg, n=2 for OVX DMPA, normal NET 5 mg and n=1 normal NET 2.5 mg. Images were taken at 10x using the Zeiss microscope.

Altogether through these histological images of the vaginal tracts, NET treated ovariectomized and normal mice show more positive staining for mucins compared to no hormone ovariectomized mice, normal mice and mice treated with DMPA. This was demonstrated through the PAS and MUC-1 staining that was performed. Additionally, these experiments also showed that NET treatment in mice does not result in thinning of the vaginal epithelium which is seen with DMPA treatment. Both results provide evidence to demonstrate that these two mechanisms can contribute towards preventing viral entry. Therefore, NET treated mice do not show the same increased susceptibility to HSV-2 infections seen with DMPA treatment in mice and therefore results in better outcomes post HSV-2 infection.

4.3 Aim 3: Examine the effects of NET on immune cell phenotypes in mice

Previous studies on DMPA have shown that treatment with this hormone can have effects on the immune system, such as altering the proportion of cells and expression of cytokines and chemokines. This was demonstrated in a study by Cherpes et al., where MPA treatment in mice resulted in a decrease in T cell subsets in the trigeminal ganglia as well as decreased T cell expansion post HSV-1 infection[98]. Additional studies on cytobrush samples, as well as vaginal swabs from women using DMPA, have shown that over time, a decrease in levels of various chemokines and cytokines was observed[118]. These cytokines and chemokines include IL-6, GM-CSF, GSCF, and MIP-1[118]. However, few studies have examined the effects of NET *in vivo*, and it is unknown whether this progestin shows similar or different effects on the immune system compared to DMPA. As mentioned previously, DMPA treatment can exert immunosuppressive effects, such as suppressing cytokine production due to its high affinity for the GR, but NET does not show this high affinity for the GR[105 110 121]. Therefore, NET is not

expected to show the same outcomes as DMPA treatment and may not show the same effects on immune cells.

4.3.1 NET- OVX and Normal – 1 week treatment

This first experiment was done to examine the effect of NET treatment alone in mice (Figure 24). Ovariectomized and normal mice were administered NET 2.5 mg pellets; immune cells from their spleen, lymph node and vaginal tissues were analyzed to determine whether NET treatment has any effect on the proportion or phenotypes of immune cells. This was also done to determine if there are different effects in ovariectomized mice with no hormones given that normal mice possess endogenous hormones that could influence these outcomes.

The immune cells that were analyzed were CD4+ and CD8+ cells as well as CD4+IFN-γ+ and CD8+IFN-γ+ cells, which were gated from CD3+ cells (for more details see Materials and Methods section 3.15). As mentioned previously, in another study MPA was shown to suppress IFN-γ production *in vitro* to a much higher extent compared to other progestins such as NET[121]. Memory T cell subsets were also analyzed with the cell surface markers CD69, CD103 and CD44. CD69 is a surface marker for T cell activation and retention in memory T cells, which has been previously used in our studies as a marker for tissue resident memory T cells[30 132 156]. CD44 is an adhesion molecule that is expressed and maintained in memory T cells[30 132 157]. Positive expression of CD44 and CD69 from CD4+ cells was used to distinguish CD4+ memory T cells. CD103 is also known as integrin alpha E and is a mucosal homing marker that is typically expressed in CD4 and CD8+ memory T cell subsets[30 158]. The positive expression of CD44 and CD103 were also used to distinguish CD8+ memory T cells.

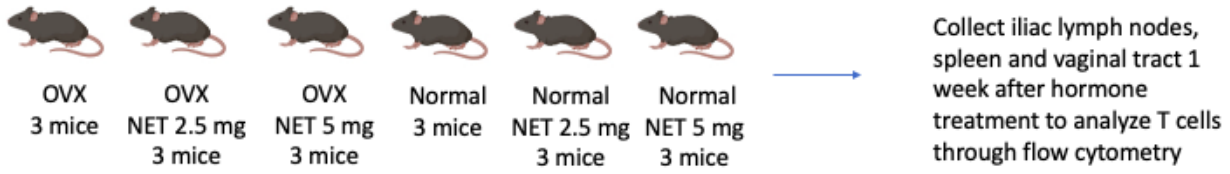

Figure 24. Outline of tissues that will be analyzed in ovariectomized and normal mice after receiving hormone treatments for 1 week. Three groups of 6–8-week-old C57BL/6 mice were ovariectomized and given 2 weeks to allow hormones to dissipate, while 3 groups of mice were not ovariectomized. Ovariectomized and normal mice with intact ovaries were then administered hormone treatments which included NET 2.5 mg and NET 5 mg pellets as well as no hormone control groups. One week after hormone treatment, iliac lymph nodes, spleens and vaginal tracts were collected to analyze immune cells through flow cytometry to determine their effect on immune cells. N=3 for each group.

4.3.1.1 Lymph node- 1 week post NET treatment in OVX and Normal mice

In the normal and ovariectomized NET 2.5 mg and NET 5 mg groups, CD4+ and CD8+ cells were analyzed in the lymph nodes, however due to low cell counts, comparisons could not be made. However, after an overnight stimulation of 16 hours in lymph node cultures, sufficient cells were present to do IFN-γ analysis. In Figure 25 and 26, the ovariectomized and normal NET 2.5 mg treated groups showed trends of higher percentages and cell counts of IFN-γ+ cells compared to the no hormone and NET 5 mg groups.

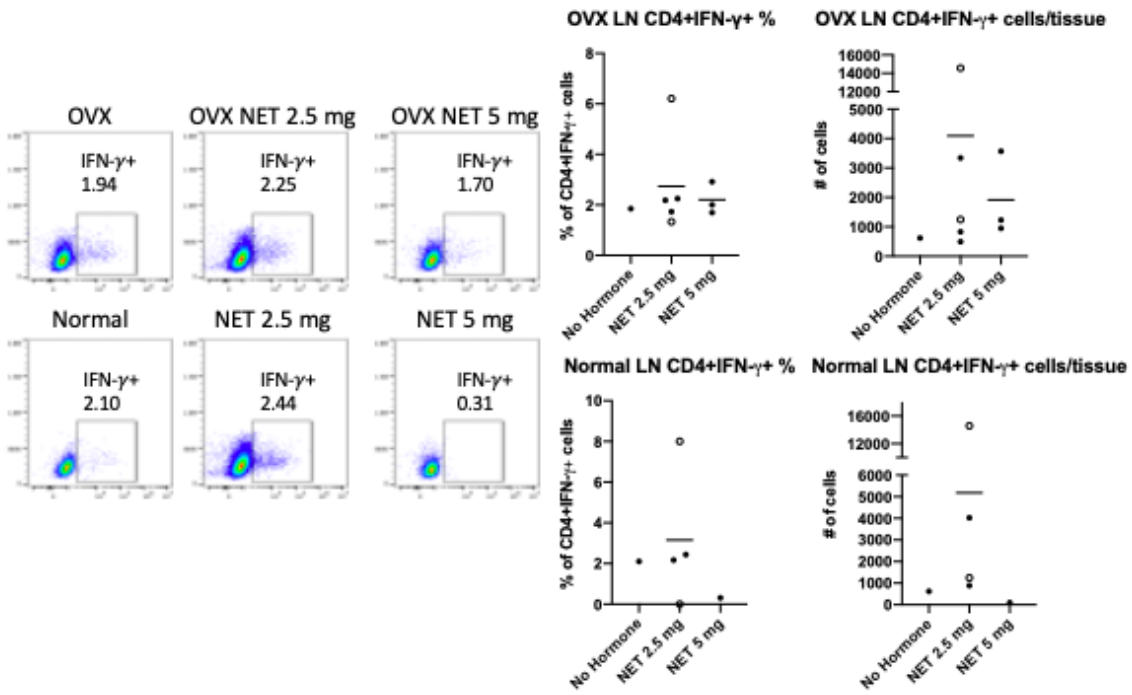

Figure 25. Graphs of the percentage of CD4+IFN-γ+ cells and cell counts per tissue from iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with NET 2.5 mg and NET 5 mg pellet. A no hormone control group was also included. Flow plots show a representative image for each group. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. This experiment was repeated twice with similar results. N=1 for OVX no hormone, n=5 for OVX NET 2.5 mg, n=3 for OVX NET 5 mg, n=1 for normal no hormone, n=4 for normal NET 2.5 mg and n=1 for normal NET 5 mg. Different symbols are representative of 2 different experiments. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

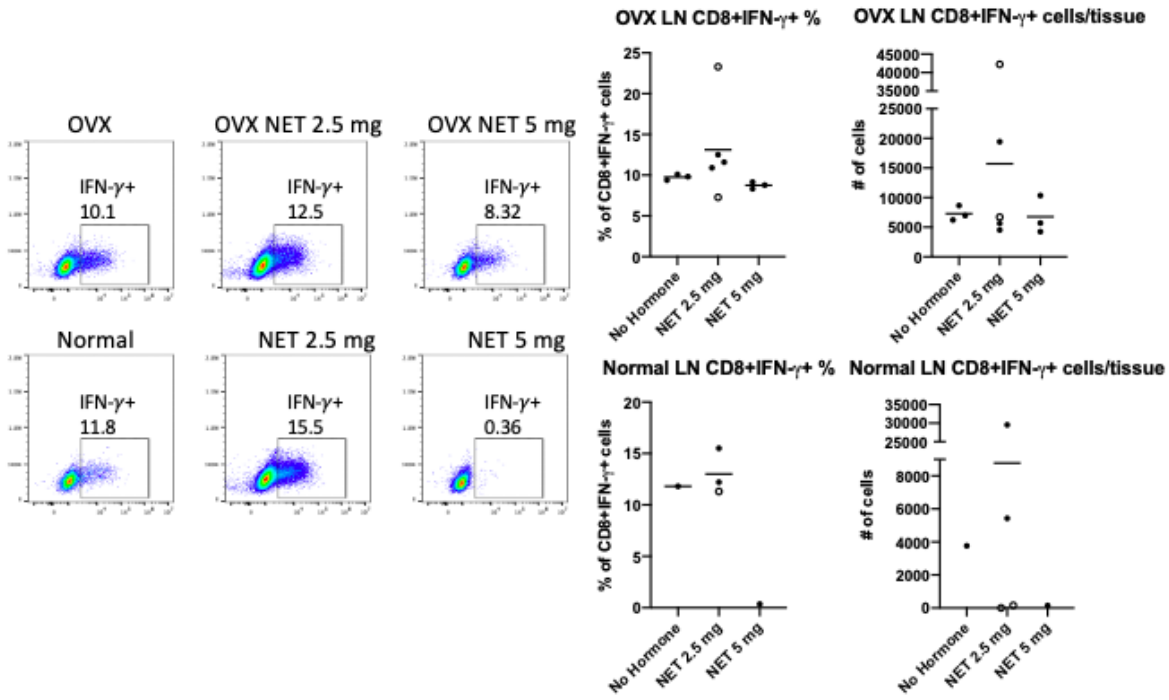

Figure 26. Graphs of the percentage of CD8+IFN-γ+ cells and cell counts per tissue from iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with NET 2.5 mg and NET 5 mg pellet. A no hormone control group was also included. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. N=3 for OVX no hormone, n=5 for OVX NET 2.5 mg, n=3 for OVX NET 5 mg, n=1 for normal no hormone, n=4 for normal NET 2.5 mg and n=1 for normal NET 5 mg. Flow plots show a representative image for each group. Different symbols are representative of 2 different experiments. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

4.3.1.2 Spleen- 1 week post NET treatment in OVX and Normal mice

In the spleens from normal and ovariectomized mice treated with NET 2.5 mg and NET 5 mg, CD4+ and CD8+ cells were analyzed, but no significant differences were found between any of the NET treated groups or control groups (Figure 27). There were trends of increased cell counts in the CD4+ cells in the OVX groups, as well as CD8+ cells in the OVX and normal groups. By examining the IFN-γ+ cells from CD4+ and CD8+ cells, the ovariectomized and normal groups treated with NET 5 mg showed trends of increased CD4+IFN-γ+ and CD8+IFN-γ+ cell counts compared to the no hormone and NET 5 mg groups (Figure 28 and 29).

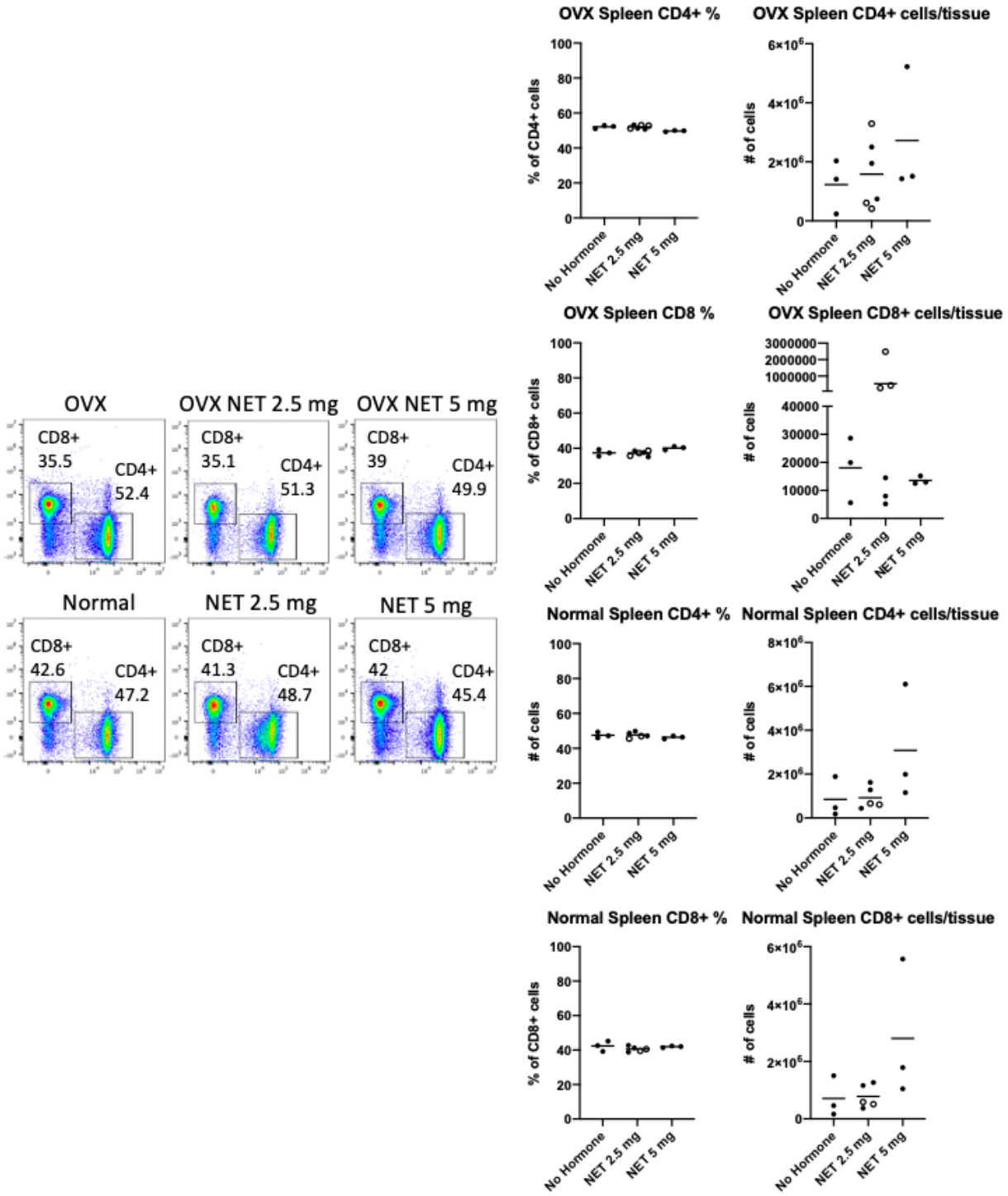

Figure 27. Graphs of the percentage and cell counts of CD4+ and CD8+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with NET 2.5 mg and NET 5 mg pellet. A no hormone control group was also included. Cells were left unstimulated. N=3 for OVX no

hormone, n=6 for OVX NET 2.5 mg, n=3 for OVX NET 5 mg, n=3 for normal no hormone, n=5 for normal NET 2.5 mg and n=3 for normal NET 5 mg. Flow plots show a representative image for each group. Different symbols are representative of 2 different experiments. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

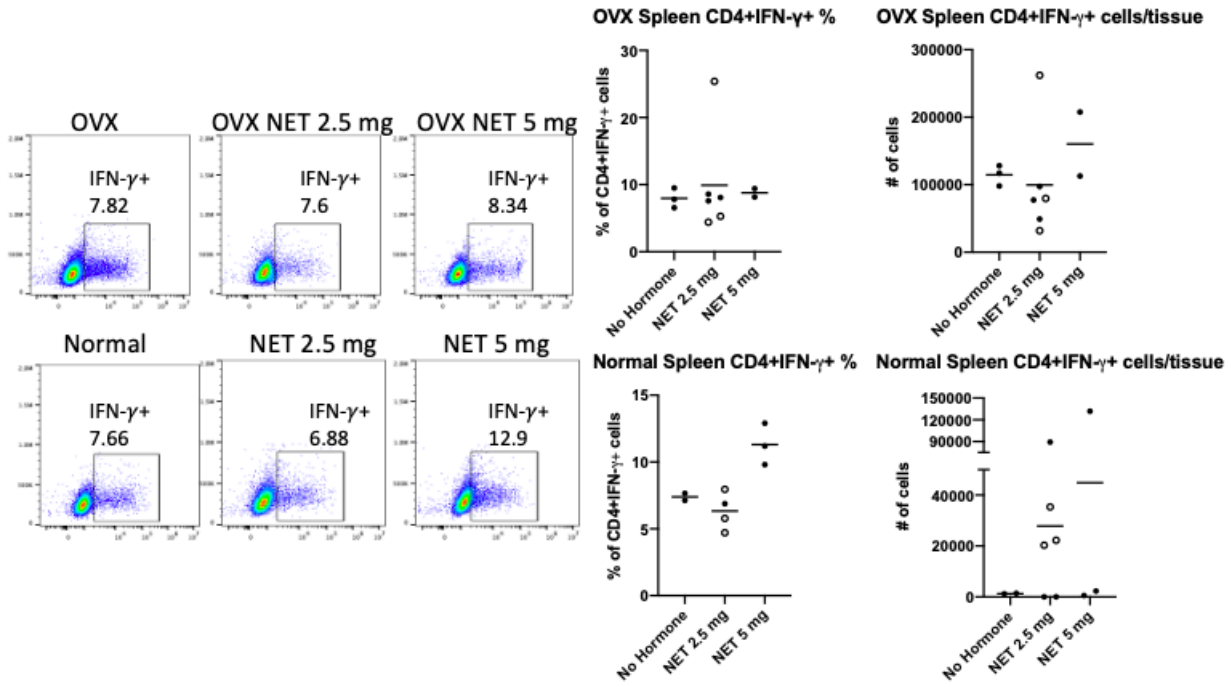

Figure 28. Graphs of the percentage and cell counts of CD4+IFN-γ+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with NET 2.5 mg and NET 5 mg pellet. A no hormone control group was also included. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. Flow plots show a representative image for each group. N=3 for OVX no hormone, n=6 for OVX NET 2.5 mg, n=2 for OVX NET 5 mg, n=2 for normal no hormone, n=6 for normal NET 2.5 mg and n=3 for normal NET 5 mg. Different symbols are representative of 2 different experiments. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

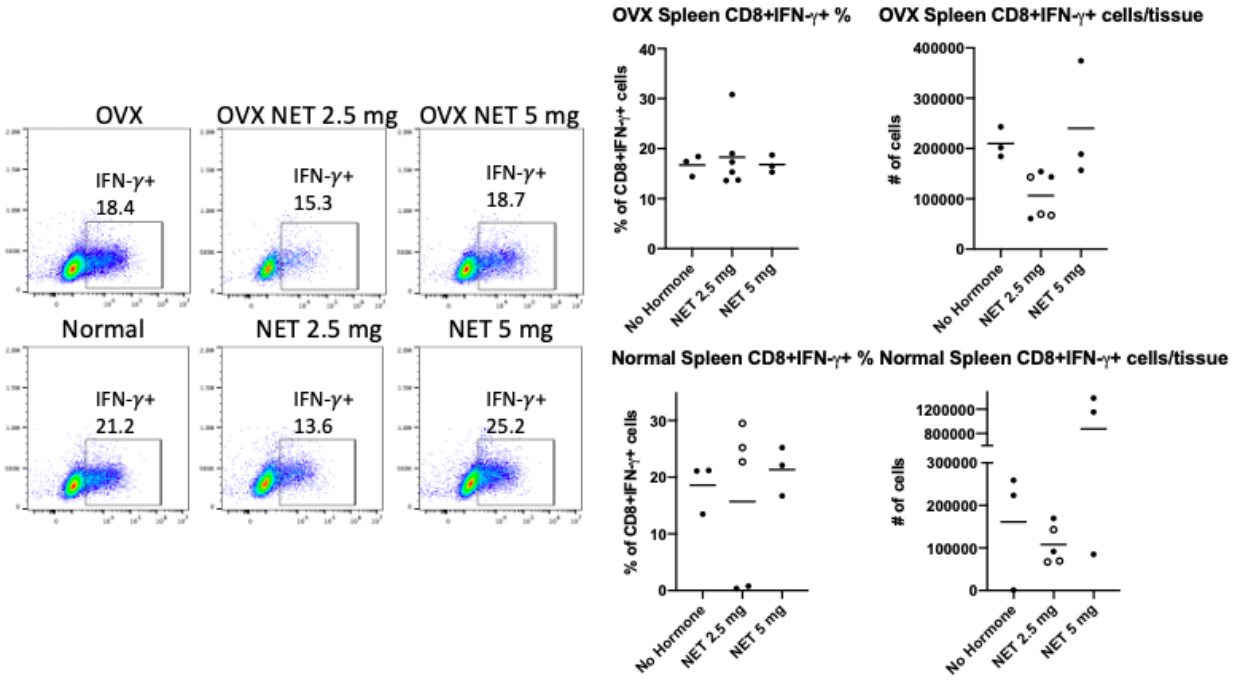

Figure 29. Graphs of the percentage of CD8+IFN-γ+ cells and cell counts per tissue from the spleens of ovariectomized and normal mice 1 week after treatment with NET 2.5 mg and NET 5 mg pellet. A no hormone control group was also included. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. Flow plots show a representative image for each group. N=3 for OVX no hormone, n=6 for OVX NET 2.5 mg, n=3 for OVX NET 5 mg, n=3 for normal no hormone, n=5 for normal NET 2.5 mg and n=3 for normal NET 5 mg. Different symbols are representative of 2 different experiments. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

4.3.1.3 Vaginal Tract- 1 week post NET treatment in OVX and Normal mice

In the vaginal tracts of these mice, CD4+ and CD8+ cells were analyzed as well. There were trends of increased CD4+ and CD8+ cells in the ovariectomized and normal NET 2.5 mg groups compared to the ovariectomized and normal control groups, as well as the normal and ovariectomized NET 5 mg groups (Figure 30).

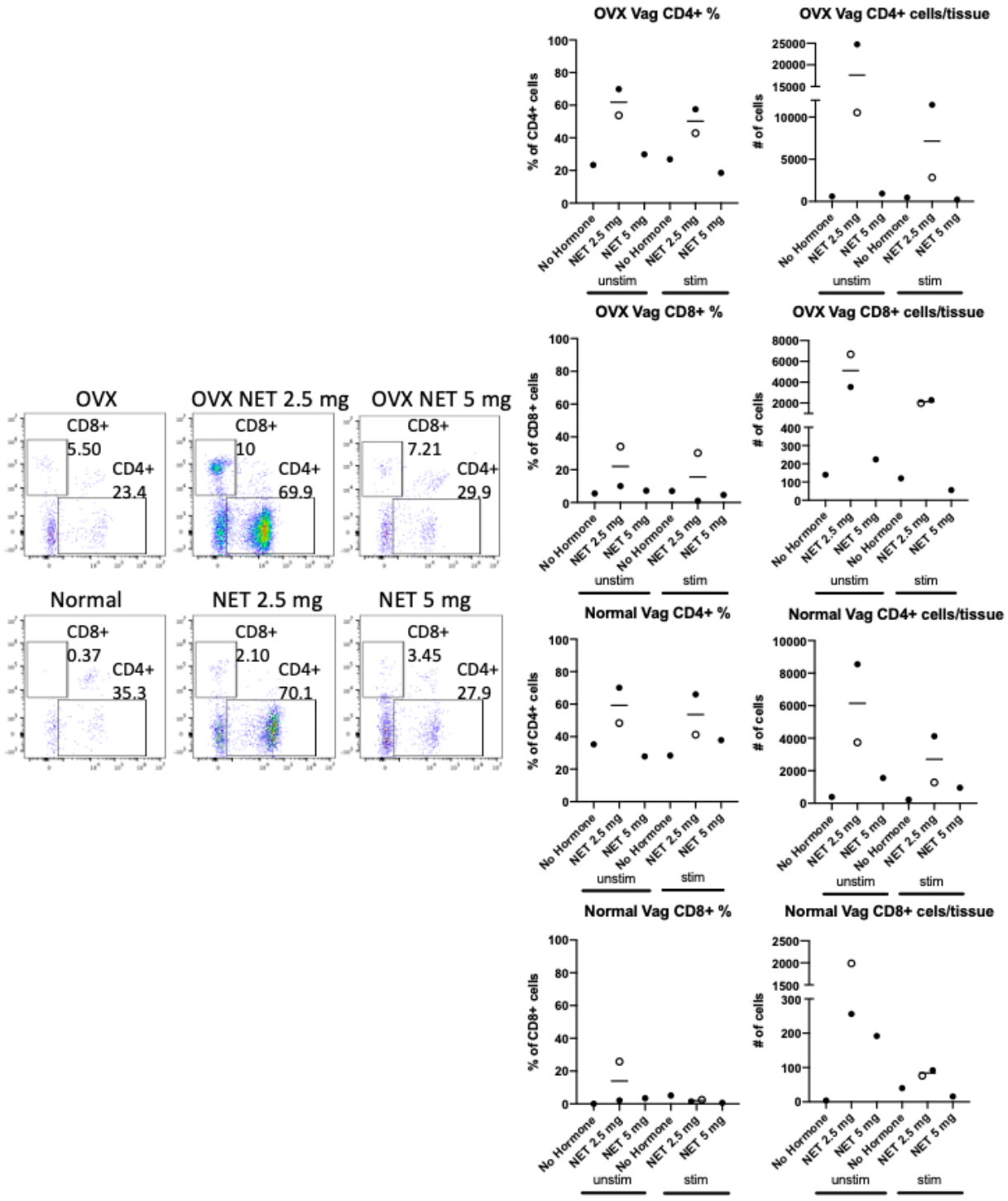

Figure 30. Graphs of the percentage and cell counts of CD4+ and CD8+ cells from the vaginal tracts of ovariectomized and normal mice 1 week after treatment with NET 2.5 mg and NET 5 mg pellet. A no hormone control group was also included. Cells were left unstimulated. N=1 for OVX,

OVX NET 5 mg, normal no hormone mice, and normal NET 5 mg. N=2 for OVX NET 2.5 mg and normal NET 2.5 mg. Flow plots show a representative image for each group. Different symbols are representative of 2 different experiments. No statistical analysis was done because of low n numbers.

Table 6. Summary of trends observed in percentages and cell counts in different immune cell phenotypes in various tissues after 1 week of hormone treatment including NET 2.5 mg and NET 5 mg in normal and ovariectomized mice.

Treatment group	Tissue	Cell types	Trend in Percentage of cells	Trend in Cell counts
OVX NET 2.5 mg	Lymph nodes	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	↑	↑
		CD8+IFN-γ+	↑	↑
	Spleen	CD4+	-	-
		CD8+	-	↑
		CD4+IFN-γ+	-	↑
		CD8+IFN-γ+	-	↓
	Vagina	CD4+	↑	↑
		CD8+	-	↑
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
OVX NET 5 mg	Lymph nodes	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	↑	↑
		CD8+IFN-γ+	↑	↑
	Spleen	CD4+	-	↑
		CD8+	-	↑
		CD4+IFN-γ+	-	↑
		CD8+IFN-γ+	-	-
	Vagina	CD4+	-	-
		CD8+	-	-
		CD4+IFN-γ+	-	-
		CD8+IFN-γ+	-	-
Normal NET 2.5 mg	Lymph nodes	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	-	↑
		CD8+IFN-γ+	-	↑
	Spleen	CD4+	-	-

		CD8+	-	-
		CD4+IFN-γ+	-	↑
		CD8+IFN-γ+	-	↓
	Vagina	CD4+	↑	↑
		CD8+	-	↑
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
Normal NET 5 mg	Lymph nodes	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	-	-
		CD8+IFN-γ+	-	-
	Spleen	CD4+	-	↑
		CD8+	-	↑
		CD4+IFN-γ+	↑	↑
		CD8+IFN-γ+	-	↑
	Vagina	CD4+	-	-
		CD8+	-	-
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND

ND (not done) indicates that there were not enough cells to run the samples and/or to perform an analysis. Trends are shown in comparison to the ovariectomized and normal control mice.

4.3.2 DMPA- OVX and Normal – 1 week treatment

A similar experiment to Figure 18 was done but with DMPA treated mice to examine the effects of DMPA treatment on immune cells after 1 week in normal and ovariectomized mice (Figure 31). This was done due to a previous study in our lab showed that long term DMPA treatment in mice resulted in different effects compared to short term treatment as well as other studies showing effects of DMPA on immune cells[94 98 105 110 118 121].

Figure 31. Two groups of 6–8-week-old C57BL/6 mice were ovariectomized and given 2 weeks to allow hormones to dissipate, while two groups of mice were not ovariectomized. Ovariectomized and normal mice with intact ovaries were then administered DMPA. There was also a no hormone control group and an ovariectomized control group. One week after hormone treatment, iliac lymph nodes, spleens and vaginal tracts were collected to analyze immune cells through flow cytometry to determine their effect on immune cells. N=3 mice for each group.

4.3.2.1 Lymph node- 1 week post DMPA treatment in OVX and Normal mice

In the iliac lymph nodes, there were trends of increased CD4+ and CD8+ cell counts in the OVX DMPA group compared to all other groups (Figure 32). This trend was also seen in the cell counts from CD4+ IFN-γ+ and CD8+ IFN-γ+ cells, as well as memory T cell subsets including CD4+CD44+, CD4+CD69+, CD8+CD44+ and CD8+CD103+ cells (Figure 33-38).

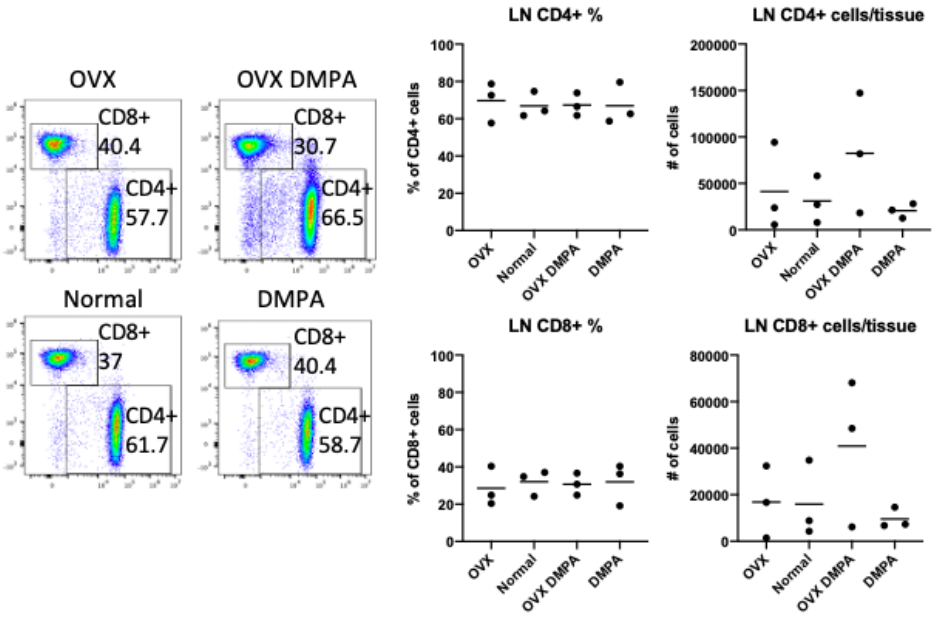

Figure 32. Graphs of the percentage and cell counts of CD4+ and CD8+ cells from the iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

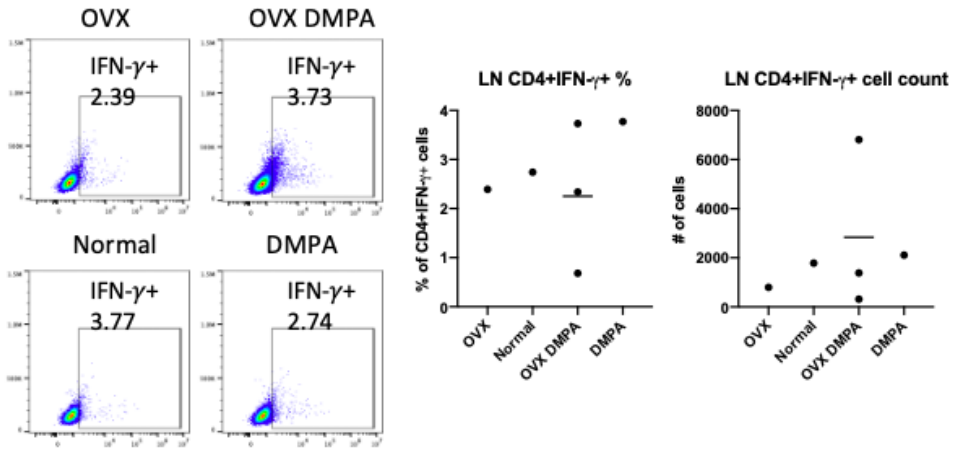

Figure 33. Graphs of the percentage and cell counts of CD4+ IFN-γ+ cells from the iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. Not all samples were used due to low cell numbers, in which no analysis was done. N=1 for OVX no hormone, n=3 for OVX DMPA, n=1 for normal no hormone and n=1 for normal DMPA. Flow plots show a representative image for each group.

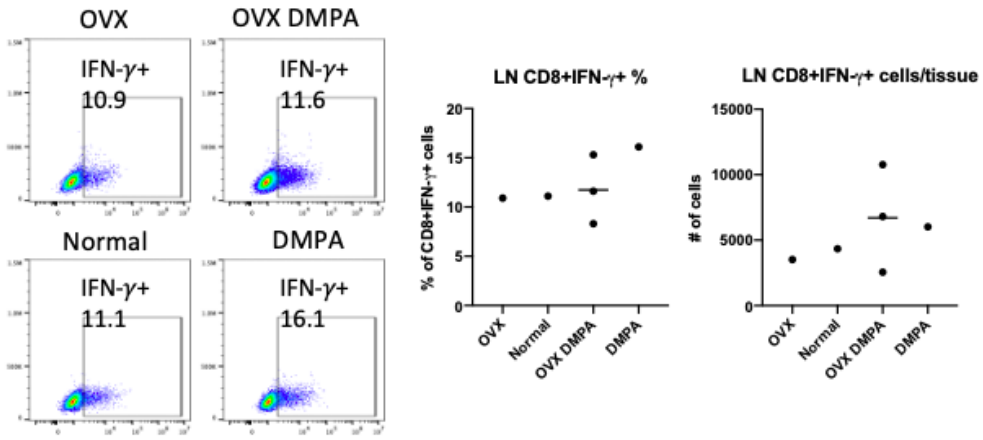

Figure 34. Graphs of the percentage and cell counts of CD8+ IFN-γ+ cells from the iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. Not all samples were used due to low cell numbers, in which no analysis was done. N=1 for OVX no hormone, n=3 for OVX DMPA, n=1 for normal no hormone and n=1 for normal DMPA. Flow plots show a representative image for each group.

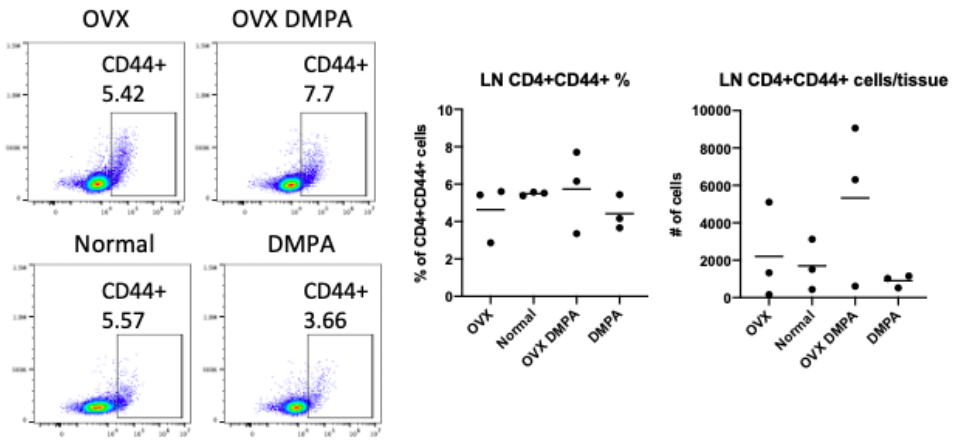

Figure 35. Graphs of the percentage and cell counts of CD4+CD44+ cells from the iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

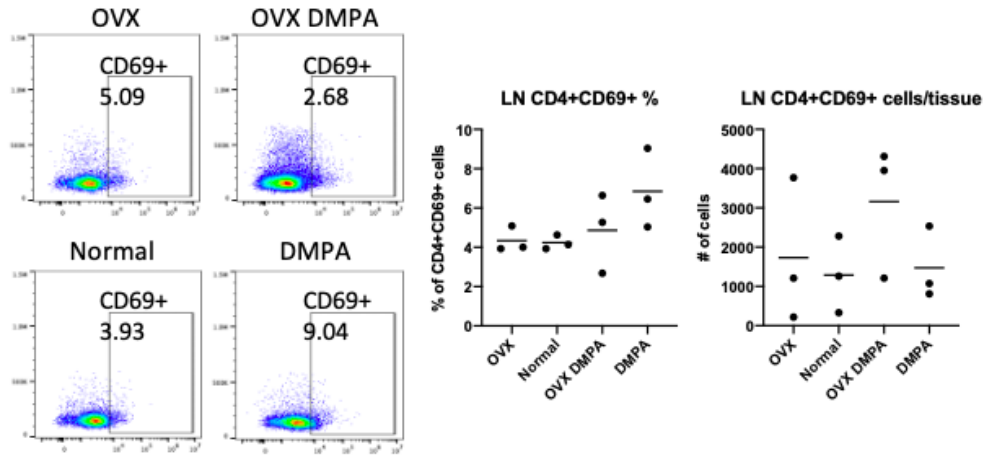

Figure 36. Graphs of the percentage and cell counts of CD4+CD69+ cells from the iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a

representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

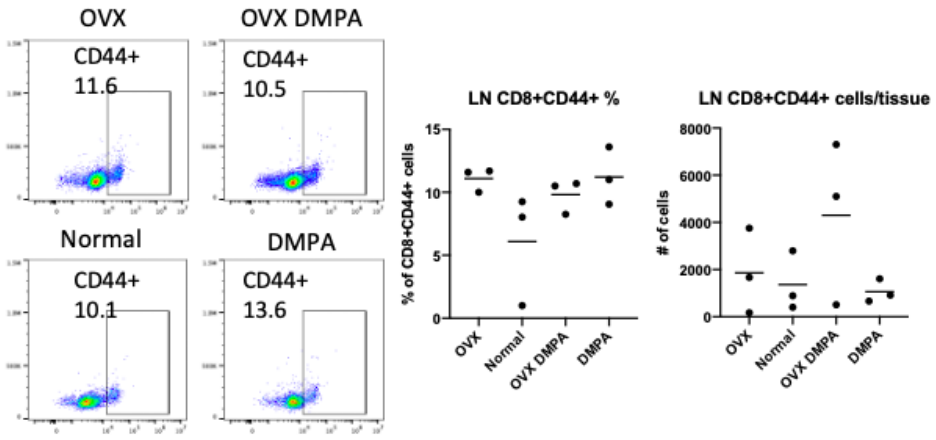

Figure 37. Graphs of the percentage and cell counts of CD8+CD44+ cells from the iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

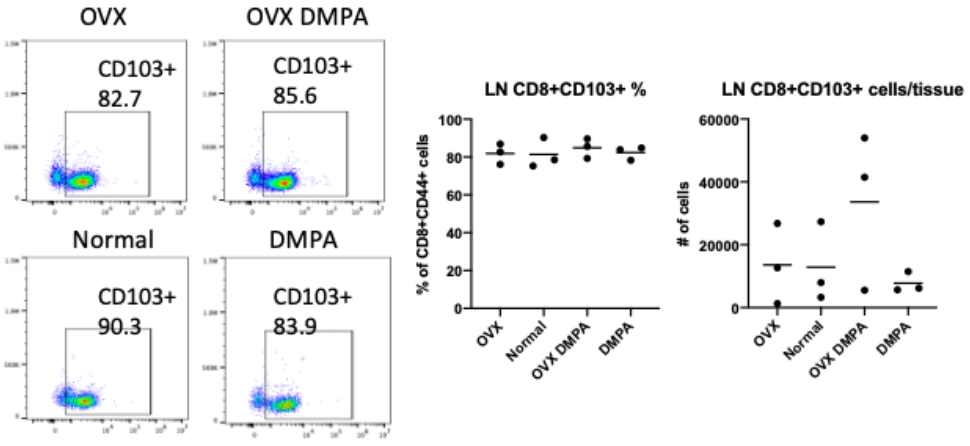

Figure 38. Graphs of the percentage and cell counts of CD8+CD103+ cells from the iliac lymph nodes of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

4.3.2.2 Spleen- 1 week post DMPA treatment in OVX and Normal mice

The same immune cell phenotypes that were analyzed in the lymph nodes were also analyzed in the spleen. No significant differences or trends were seen comparing the proportion and cell counts of CD4+ and CD8+ cells from the control groups to the DMPA treated mice (Figure 39). When CD4+IFN-γ+ cells were analyzed in the spleen, the ovariectomized control group showed higher percentages of CD4+IFN-γ+ cells compared to the normal, OVX DMPA, and normal DMPA treated groups (Figure 40). This was found to be statistically significant. This trend was also seen in the cell counts of CD4+IFN-γ+ cells but was not statistically significant. No significant differences or trends were observed in the CD8+IFN-γ+ cells (Figure 41).

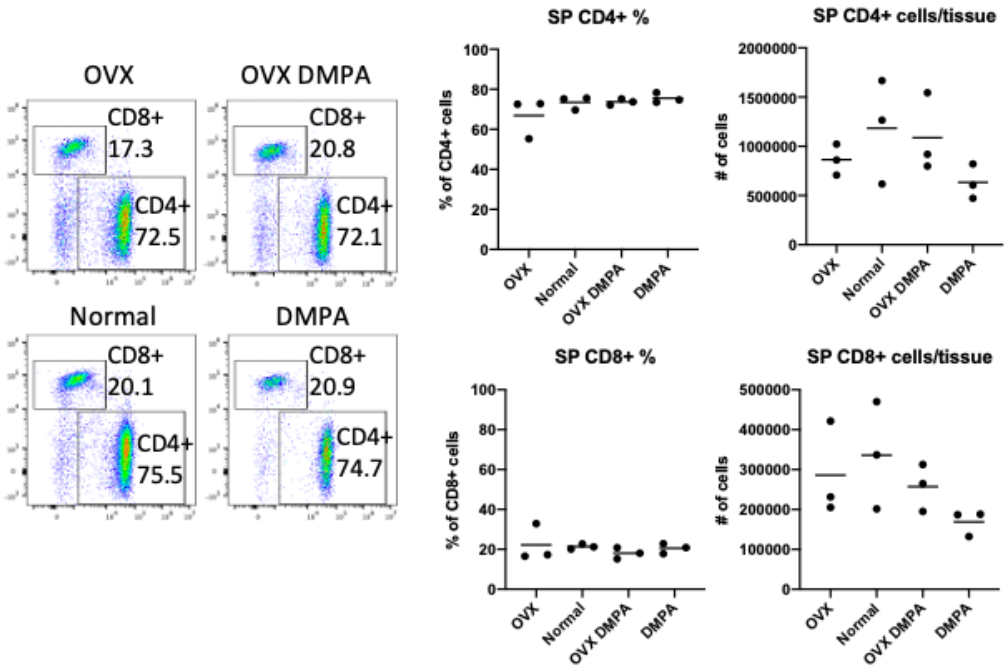

Figure 39. Graphs of the percentage and cell counts of CD4+ and CD8+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

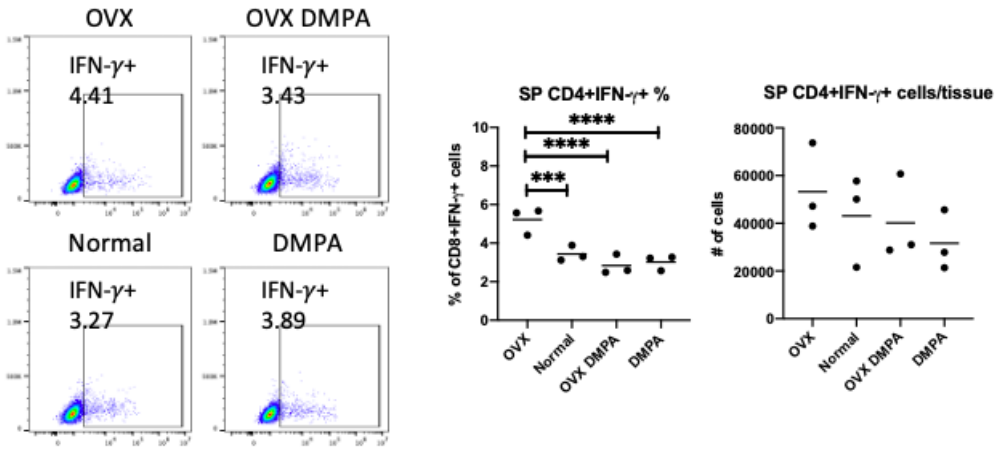

Figure 40. Graphs of the percentage and cell counts of CD4+IFN-γ+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. ***p<0.001, ****p<0.0001.

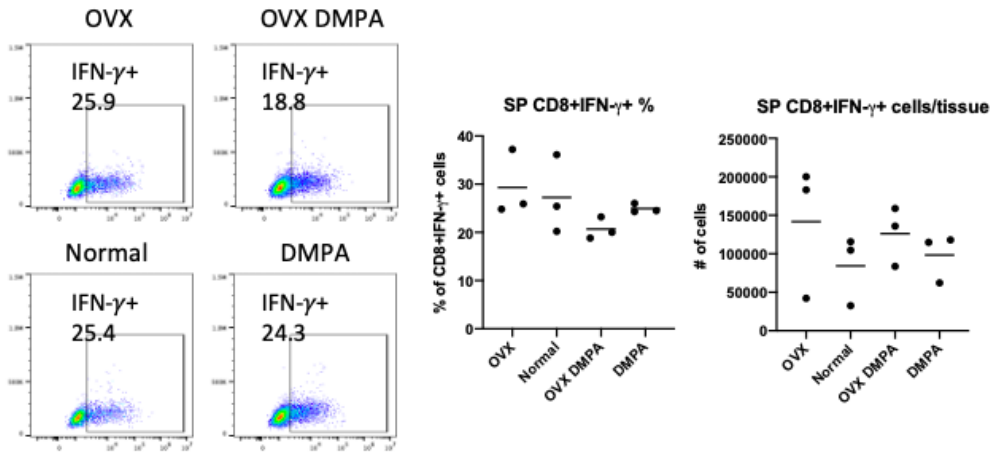

Figure 41. Graphs of the percentage and cell counts of CD8+IFN-γ+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

There were no significant differences seen in the CD4+CD44+ cells in the spleen (Figure 42). Furthermore, there was a significant decrease in the percentage of CD4+CD69+ cells comparing the OVX control to the normal control group (Figure 43). There was also a significant increase in the percentage of CD4+CD69+ cells in the spleen comparing the normal DMPA treated group to the OVX control group. There was also a significant difference between the OVX DMPA and normal DMPA group in the percentage of CD4+CD69+ cells, with the normal DMPA group showing higher percentages of cells. There were no trends or significant differences in the percentages and cell counts of CD8+CD44+ and CD8+CD103+ cells (Figure 44 and 45).

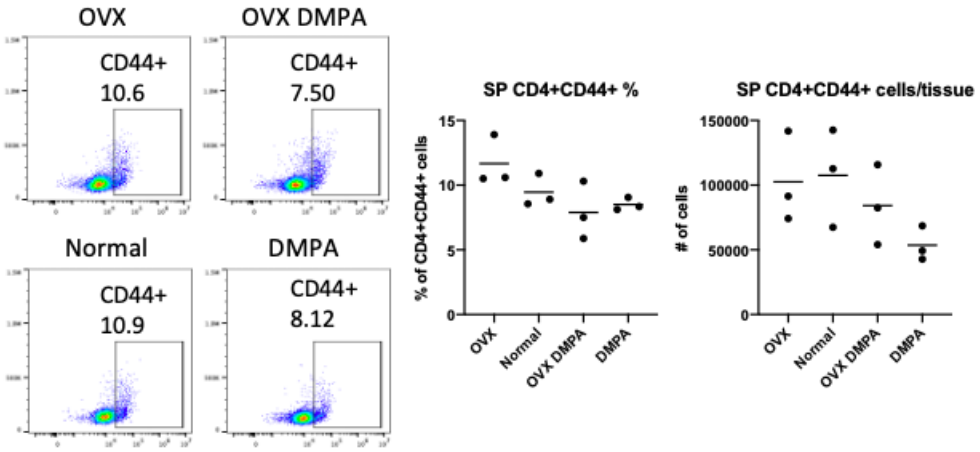

Figure 42. Graphs of the percentage and cell counts of CD4+CD44+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

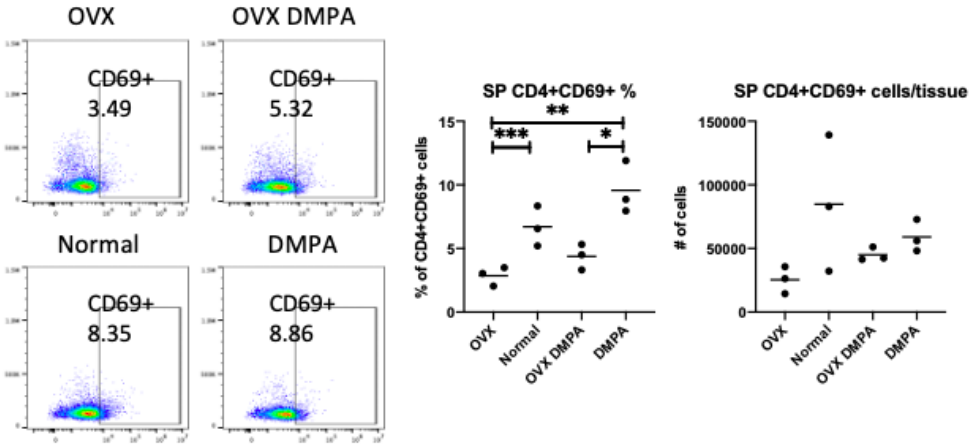

Figure 43. Graphs of the percentage and cell counts of CD4+CD69+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. N=3 for each group. Flow plots show a representative image for each group.

Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. **p<0.01, ***p<0.001, ****p<0.0001.

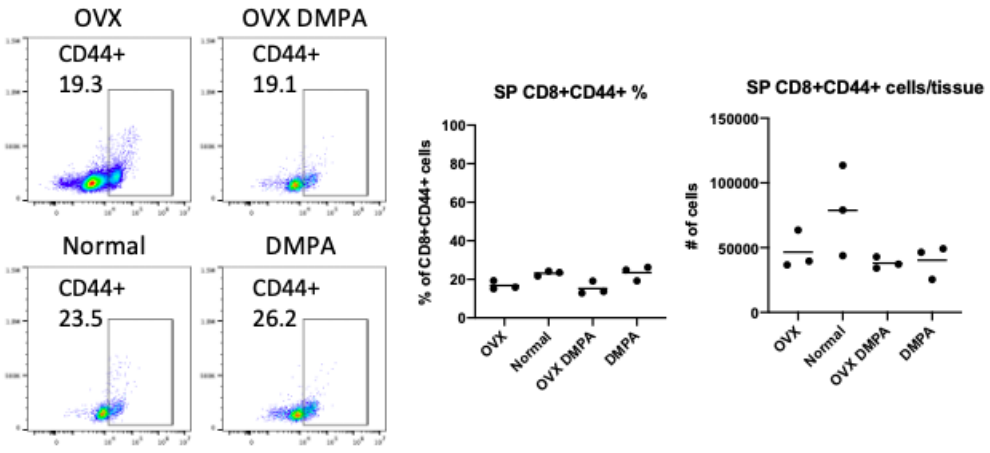

Figure 44. Graphs of the percentage and cell counts of CD8+CD44+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

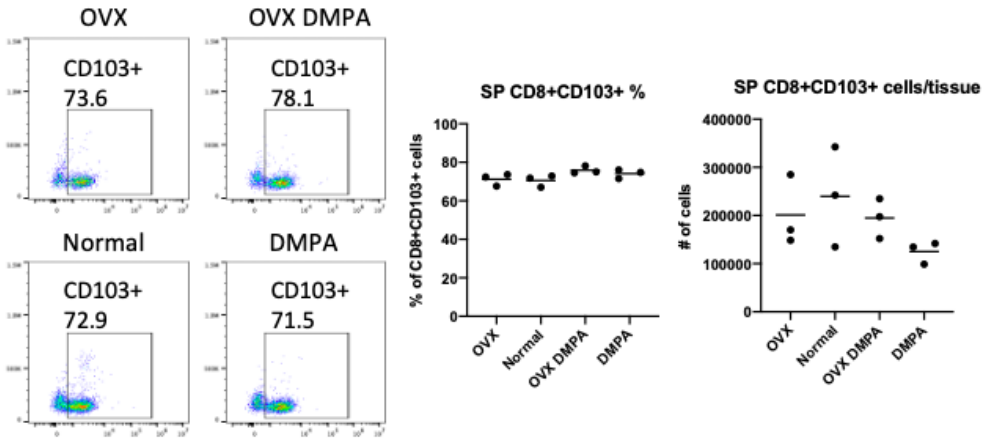

Figure 45. Graphs of the percentage and cell counts of CD8+CD103+ cells from the spleens of ovariectomized and normal mice 1 week after treatment with DMPA. No hormone control groups were also included. Cells were left unstimulated. N=3 for each group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

4.3.2.3 Vaginal tract- 1 week post DMPA treatment in OVX and normal mice

The same phenotypes of immune cells from the vaginal tracts of these mice were also analyzed, however, due to low cell counts no comparisons could be made.

Table 7. Summary of trends observed in percentages and cell counts in different immune cell phenotypes in various tissues after 1 week of hormone treatment with DMPA in normal and ovariectomized mice.

Treatment group	Tissue	Cell types	Trend in Percentage of cells	Trend in Cell counts
OVX DMPA	Lymph nodes	CD4+	-	↑
		CD8+	-	↑
		CD4+IFN-γ+	-	-
		CD8+IFN-γ+	-	-
		CD4+CD44+	-	↑
		CD4+CD69+	-	↑
		CD8+CD44+	-	↑
		CD8+CD103+	-	↑
	Spleen	CD4+	-	-
		CD8+	-	-
		CD4+IFN-γ+	↓****	↓
		CD8+IFN-γ+	-	-
		CD4+CD44+	-	-
		CD4+CD69+	-	-
		CD8+CD44+	-	-
		CD8+CD103+	-	-
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND
DMPA	Lymph nodes	CD4+	-	-
		CD8+	-	-
		CD4+IFN-γ+	-	-
		CD8+IFN-γ+	-	-
		CD4+CD44+	-	-
		CD4+CD69+	-	-

		CD8+CD44+	-	-
		CD8+CD103+	-	-
	Spleen	CD4+	-	-
		CD8+	-	-
		CD4+IFN-γ+	-	-
		CD8+IFN-γ+	-	↓
		CD4+CD44+	-	↓
		CD4+CD69+	↑	-
		CD8+CD44+	-	-
		CD8+CD103+	-	-
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND

ND (not done) indicates that there were not enough cells to run the samples and perform an analysis. Trends are shown in comparison to the ovariectomized and normal control mice. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. ****$p<0.0001$ indicating statistical significance in comparison to control.

4.3.3 NET versus DMPA- OVX and Normal – 3 week treatment

Similar to the experiment done in Figure 24, tissues were collected from normal and ovariectomized mice to determine the effect of hormones after 3 weeks of treatment (Figure 46). This was done to determine if there are differing effects due to longer hormone treatment with NET and DMPA treatment.

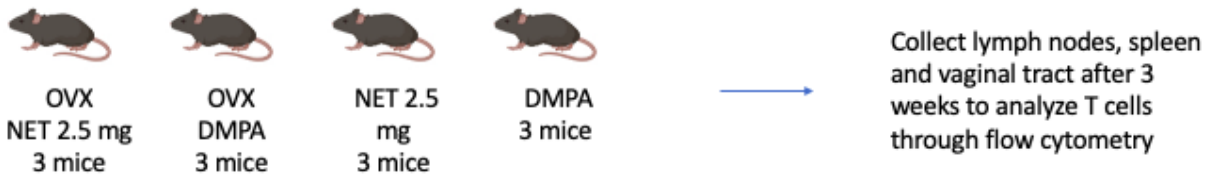

Figure 46. Outline of tissues collected after 3 weeks of hormone treatment with NET 2.5 mg and DMPA 2 mg in ovariectomized and normal mice. Three groups of 6–8-week-old C57BL/6 mice were ovariectomized and given 2 weeks to allow hormones to dissipate, while 3 groups of mice were not ovariectomized. Ovariectomized and normal mice with intact ovaries were then administered hormone treatments which included NET 2.5 mg and DMPA. Three weeks after hormone treatment, iliac lymph nodes, spleens and vaginal tracts were collected to analyze immune cells through flow cytometry to determine their effect on immune cells. N=3 for each group.

4.3.3.1 Lymph node- 3-week post DMPA or NET treatment in OVX and Normal mice

In the iliac lymph nodes, the OVX DMPA group showed a significantly higher percentage of CD4+ cells compared to OVX NET 2.5 mg (Figure 47). The trend was also reflected in the cell counts of CD4+ cells but was not found to be statistically significant. There was a significant decrease in the percentage of CD8+ cells in OVX DMPA group compared to the OVX NET 2.5 mg group. There was also a trend of lower cell counts of CD8+ cells in the OVX NET 2.5 mg compared to the OVX DMPA group. The normal NET 2.5 mg and DMPA groups showed similar percentages of CD4+ and CD8+ cells. The trend of the OVX DMPA group showing higher cell counts than the OVX NET 2.5 group in CD4+ and CD8+ cell counts was also reflected in the

normal NET 2.5 mg and normal DMPA group. In the CD4+ and CD8+ cells, IFN-γ+ cells were examined but no comparisons were made due to low cell counts.

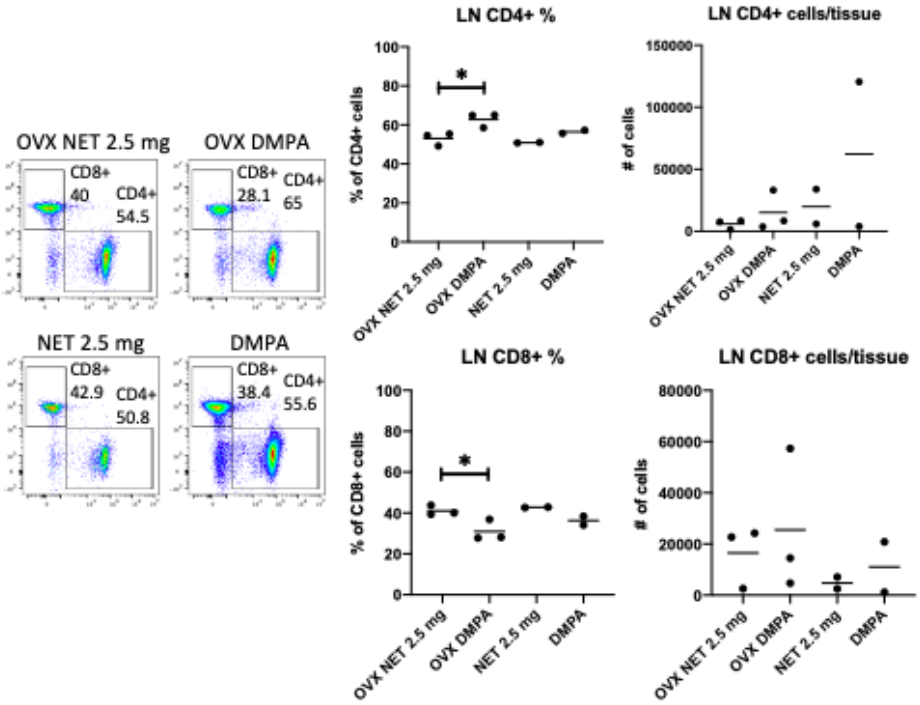

Figure 47. Graphs of the percentage and cell counts of CD4+ and CD8+ cells in the iliac lymph nodes of ovariectomized and normal mice after 3 weeks of treatment with NET 2.5 mg and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=2 for NET 2.5 mg and n=2 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t test. *$p<0.05$.

In the lymph nodes, CD4+ and CD8+ memory T cell subsets were also examined and there were trends of increased CD4+ and CD8+ memory T cell subsets in the OVX DMPA and normal DMPA groups compared to the OVX and normal NET 2.5 mg treated groups (Figures 48-51).

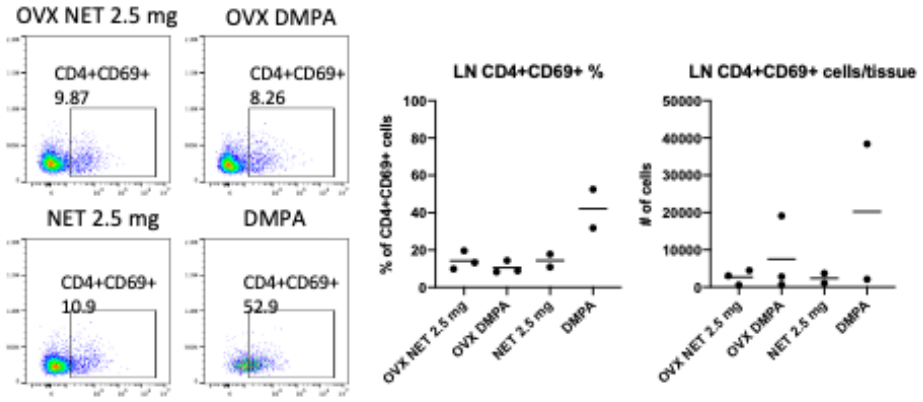

Figure 48. Graphs of the percentage and cell counts of memory T cell subsets which include CD4+CD69+ cells from the iliac lymph nodes of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=2 for NET 2.5 mg and n=2 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

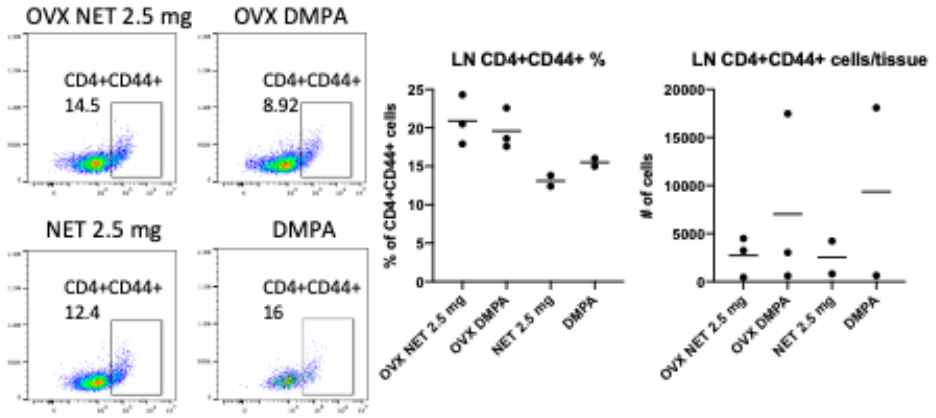

Figure 49. Graphs of the percentage and cell counts of memory T cell subsets which include CD8+CD44+ cells from the iliac lymph nodes of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=2 for NET 2.5 mg and n=2 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

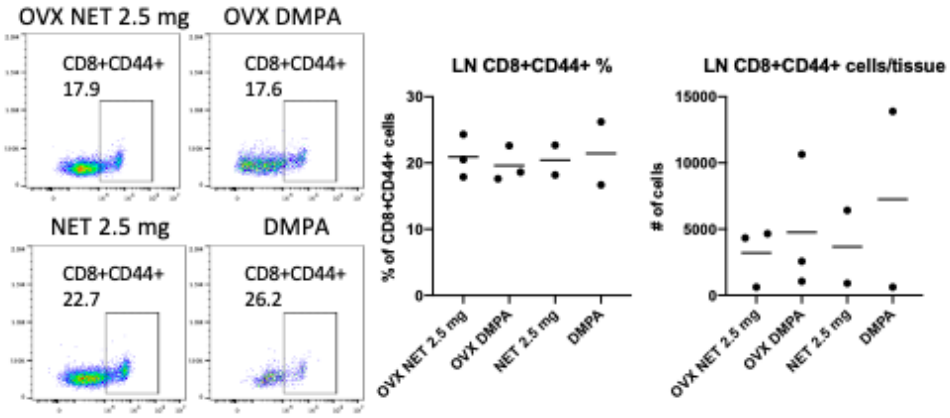

Figure 50. Graphs of the percentage and cell counts of memory T cell subsets which include CD8+CD44+ cells from the iliac lymph nodes of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET

2.5 mg, n=3 for OVX DMPA, n=2 for NET 2.5 mg and n=2 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA with Tukey's multiple comparisons test.

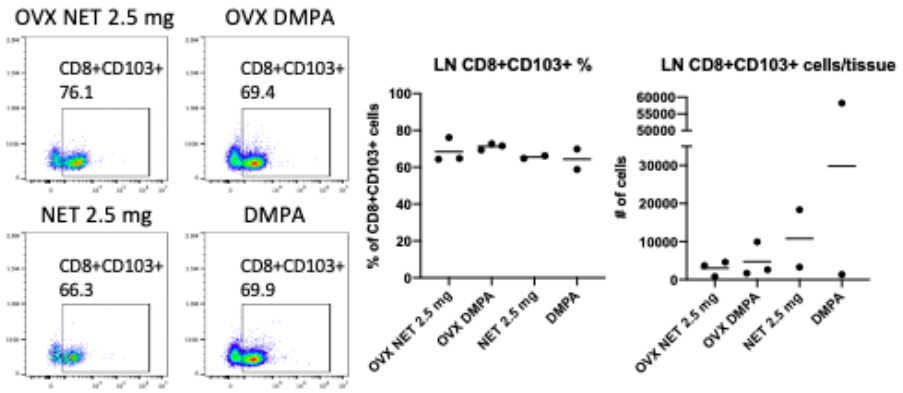

Figure 51. Graphs of the percentage and cell counts of memory T cell subsets which include CD8+CD103+ cells from the iliac lymph nodes of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=2 for NET 2.5 mg and n=2 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

4.3.3.2 Spleen- 3 week post DMPA or NET treatment in OVX and Normal mice

The same immune cell phenotypes were examined in the spleen. There was a significantly higher percentage of CD4+ cells in the OVX DMPA group compared to OVX NET 2.5 mg. In the OVX NET 2.5 mg group, mice showed significantly lower CD8+ cell counts compared to the OVX DMPA group (Figure 52). There was also a trend of lower CD4+ cell counts in the OVX NET 2.5 mg group compared to OVX DMPA. In the normal NET 2.5 mg and normal DMPA group, mice

showed similar trends in the cell counts of CD4+ and CD8+ cells but were not significantly different.

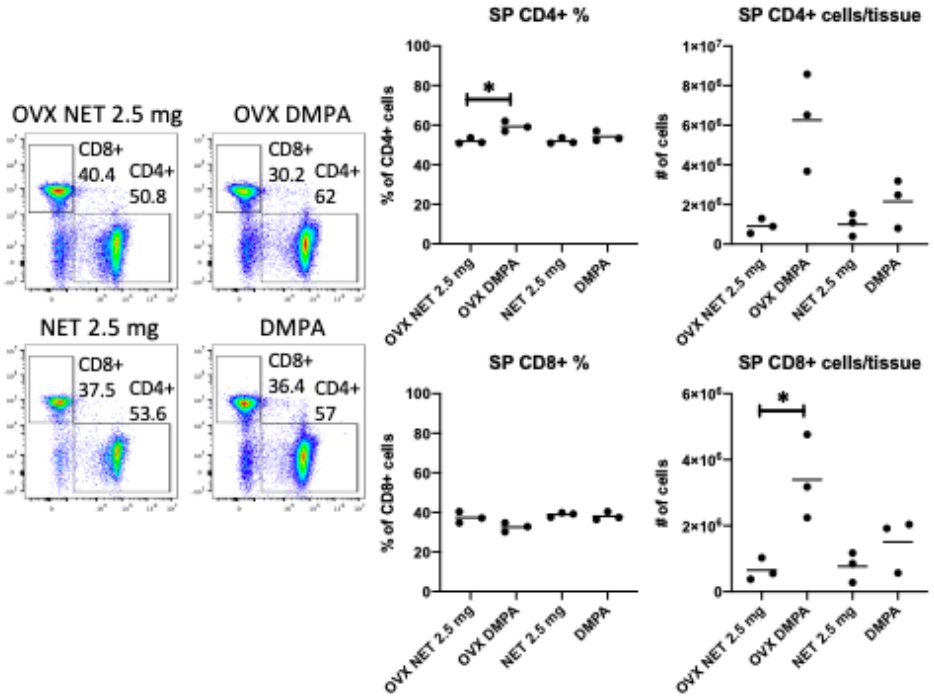

Figure 52. Graphs of percentage and cell counts of CD4+ and CD8+ cells from the spleens of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=3 for NET 2.5 mg and n=3 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *p<0.05.

Comparing the CD4+IFN-γ+ cells in the spleens from the OVX NET 2.5 mg group, there is was significantly lower cell counts compared to the OVX DMPA group (Figure 53). In the CD8+IFN-γ+ cells, a similar trend was observed but was not statistically significant (Figure 54). When analyzing the normal NET 2.5 mg group and normal DMPA group, there are trends of higher

CD4+IFN-γ+ and CD8+IFN-γ+ cells in the normal NET 2.5 mg group compared to the normal DMPA group.

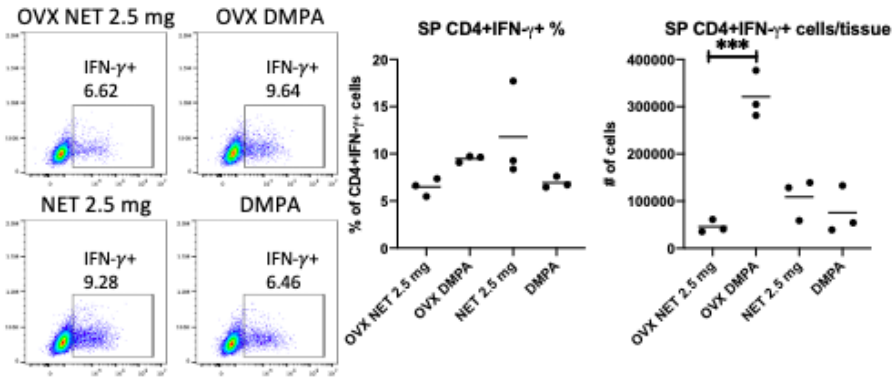

Figure 53. Graphs of percentage and cell counts of CD4+IFN-γ+ cells from the spleens of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=3 for NET 2.5 mg and n=3 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. ***p<0.001.

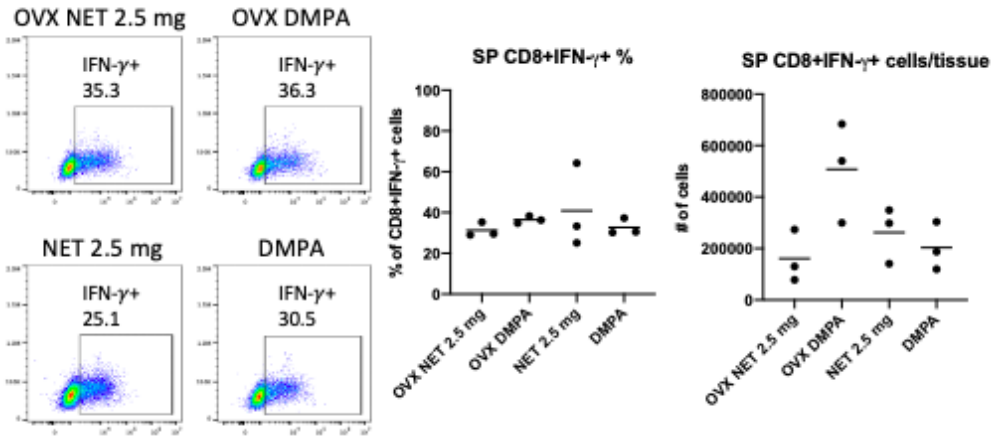

Figure 54. Graphs of percentage and cell counts of CD8+IFN-γ+ cells from the spleens of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=3 for NET 2.5 mg and n=3 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

Memory T cell subsets were also examined in the spleen, in which there were lower cell counts for CD4+CD44+ cells in the OVX NET 2.5 mg group compared to the OVX DMPA group, which was found to be statistically significant (Figure 55). This trend was also seen in the CD4+CD69+ cell counts from the OVX NET 2.5 mg group compared to the OVX DMPA group (Figure 56). However, when comparing the normal NET 2.5 mg and normal DMPA groups, there were no significant differences seen in the percentages and cell counts of CD4+CD44+ and CD4+CD69+ cells.

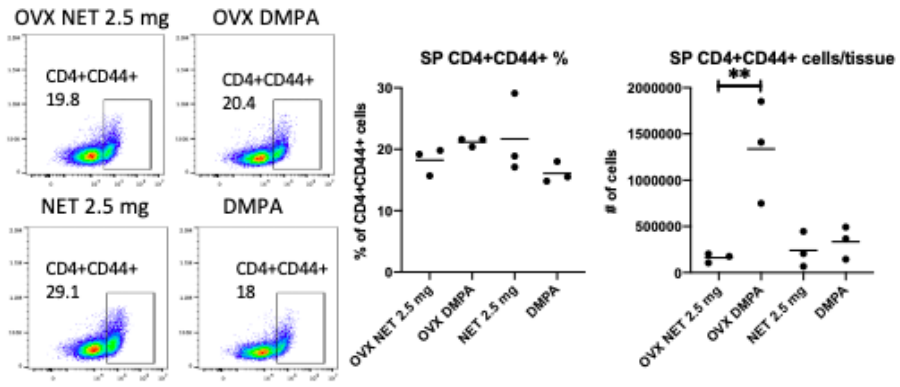

Figure 55. Graphs of percentages and cell counts of the memory T cell subset CD4+CD44+ cells from the spleens of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=3 for NET 2.5 mg and n=3 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. **p<0.01.

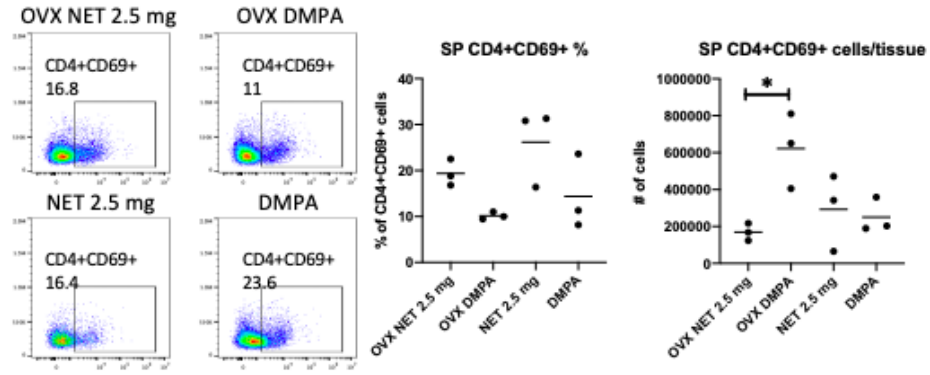

Figure 56. Graphs of percentages and cell counts of the memory T cell subset CD4+CD69+ cells from the spleens of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=3 for NET 2.5 mg and n=3 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *p<0.05.

When the CD8+ memory T cell subsets were analyzed, the OVX NET 2.5 mg group shows significantly lower cell counts of CD8+CD44+ and CD8+CD103+ cells compared the OVX DMPA group (Figure 57). This trend was also seen in the cell counts of CD8+CD44+ and CD8+CD103+ cells in the normal NET 2.5 mg and normal DMPA groups but was not statistically significant.

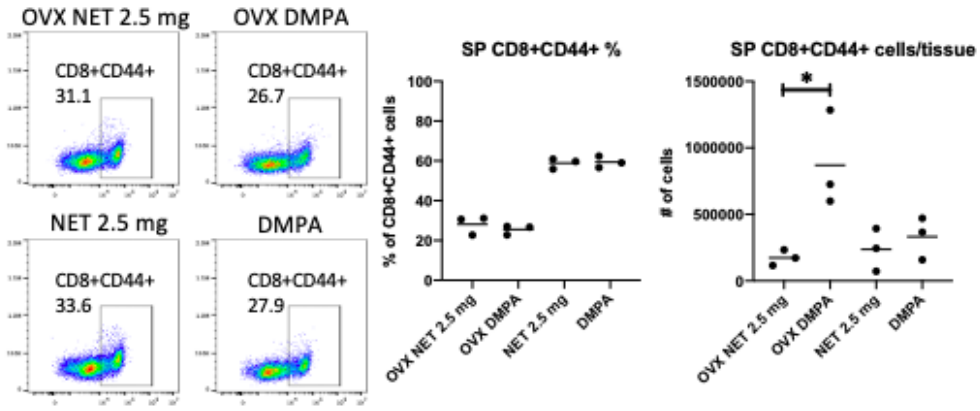

Figure 57. Graphs of percentages and cell counts of the memory T cell subset CD8+CD44+ cells from the spleens of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. Cells were left unstimulated. N=3 for OVX NET 2.5 mg, n=3 for OVX DMPA, n=3 for NET 2.5 mg and n=3 for DMPA. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *$p<0.05$.

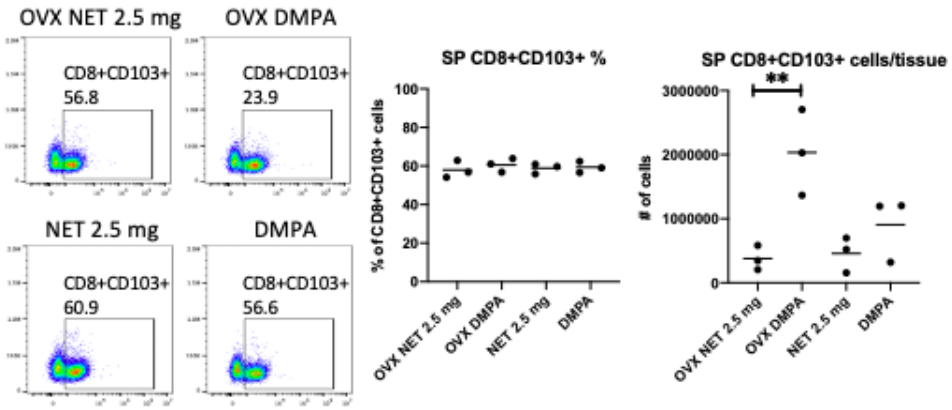

Figure 58. Graphs of percentages and cell counts of the memory T cell subset CD8+CD103+ cells from the spleens of ovariectomized and normal mice 3 weeks after treatment with NET 2.5 mg pellet and DMPA. N=3 per group. Cells were left unstimulated. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

4.3.3.3 Vaginal tract- 3 week post DMPA or NET treatment in OVX and Normal mice

T cell subsets were also examined in the vaginal tracts of ovariectomized, and normal mice treated with NET 2.5 mg and DMPA, but due to low cell counts and sample size, no comparisons could be made.

Table 8. Summary of trends observed in immune cells from mice after 3 weeks of treatment with NET 2.5 mg or DMPA in ovariectomized and normal mice.

Treatment group	Tissue	Cell types	Trend in Percentage of cells	Trend in Cell counts
OVX NET 2.5 mg	Lymph nodes	CD4+	↓*	↓
		CD8+	↑*	↓
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	-	↓
		CD4+CD69+	-	↓
		CD8+CD44+	-	-
		CD8+CD103+	-	-
	Spleen	CD4+	↓*	↓
		CD8+	-	↓*
		CD4+IFN-γ+	-	↓***
		CD8+IFN-γ+	-	↓
		CD4+CD44+	-	↓ **
		CD4+CD69+	-	↓ *
		CD8+CD44+	-	↓ *
		CD8+CD103+	-	↓ **
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND
Normal NET 2.5 mg	Lymph nodes	CD4+	-	↓
		CD8+	-	↓
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	-	↓
		CD4+CD69+	↓	↓
		CD8+CD44+	-	↓

		CD8+CD103+	-	↓
	Spleen	CD4+	-	↓
		CD8+	-	↓
		CD4+IFN-γ+	-	↑
		CD8+IFN-γ+	-	↑
		CD4+CD44+	-	-
		CD4+CD69+	-	-
		CD8+CD44+	-	-
		CD8+CD103+	-	-
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND
OVX DMPA	Lymph nodes	CD4+	↑*	↑
		CD8+	↓ *	↑
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	-	↑
		CD4+CD69+	-	↑
		CD8+CD44+	-	↑
		CD8+CD103+	-	-
	Spleen	CD4+	↑*	↑
		CD8+	-	↑*
		CD4+IFN-γ+	-	↑ ***
		CD8+IFN-γ+	-	↑
		CD4+CD44+	-	↑ **
		CD4+CD69+	↓	↑
		CD8+CD44+	-	↑ *
		CD8+CD103+	-	↑ **
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND

		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND
DMPA	Lymph nodes	CD4+	-	↑
		CD8+	-	↑
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	-	↑
		CD4+CD69+	-	↑
		CD8+CD44+	-	↑
		CD8+CD103+	-	↑
	Spleen	CD4+	-	↑
		CD8+	-	↑
		CD4+IFN-γ+	↓	↓
		CD8+IFN-γ+	-	↓
		CD4+CD44+	-	-
		CD4+CD69+	-	-
		CD8+CD44+	-	↑
		CD8+CD103+	-	↑
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND

ND (not done) indicates that there were not enough cells to run the samples and perform an analysis. Trends are shown to compare OVX NET 2.5 mg to OVX DMPA and normal NET 2.5 mg to normal DMPA. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.3.4 NET - OVX and Normal – 6 week treatment

This next experiment analyzing immune cells after 6 weeks of hormone treatment, was done to investigate whether there are any effects of NET treatment in ovariectomized and normal mice with the influence of hormones over different periods of time (Figure 59). As detailed in Table 3, NET pellets keep mice in diestrus for ~45 days. At approximately 6 weeks, the hormone pellets would have started to dissipate and may be present in the mouse's system at very low levels, so this would determine whether there are any long-lasting effects of the hormone treatment in mice and if this would affect immune cell phenotypes and proportions. This was only examined in mice treated with NET hormone pellets, as DMPA treatment only lasts in mice for approximately 3-4 weeks. A previous study in our lab has also shown that DMPA serum levels in mice decrease to very low levels that are less than 2 ng/ml at 3 weeks[136]. By 6 weeks it is expected that DMPA would no longer be in the mouse's system and would not show any effects.

Figure 59. Outline of tissues collected from ovariectomized and normal mice for flow cytometric analysis after 6 weeks of hormone treatment with NET 2.5 mg. One group of 6–8-week-old C57BL/6 mice were ovariectomized and given 2 weeks to allow hormones to dissipate, while the other group of mice were not ovariectomized. OVX and normal mice with intact ovaries were then administered hormone treatments which included NET 2.5 mg pellets. N=3 per group. Six weeks after hormone treatment, iliac lymph nodes, spleens and vaginal tracts were collected to analyze immune cells through flow cytometry to determine their effect on immune cells.

4.4.1 Lymph node- 6 week post NET treatment in OVX and Normal mice

Iliac lymph nodes were collected from ovariectomized, and normal mice treated with NET 2.5 mg, however, there were low cell counts and no comparisons could be made.

4.4.2 Spleen- 6 week post NET treatment in OVX and Normal mice

When the spleens were examined, there were no significant differences found between the OVX NET 2.5 mg and normal NET 2.5 mg group in terms of the percentages and cell counts of CD4+ and CD8+ cells (Figure 60). In the CD4+IFN-γ+ and CD8+IFN-γ+ cells, there was a trend of decreased cells in the OVX NET 2.5 mg compared to the normal NET 2.5 mg group (Figures 61 and 62). When CD4+ and CD8+ memory T cell subsets were analyzed in the spleen, no trends or significant differences were observed (Figure 63-66). Memory T cell subsets included CD4+CD44+, CD4+CD69+, CD8+CD44+ and CD8+CD103+ cells.

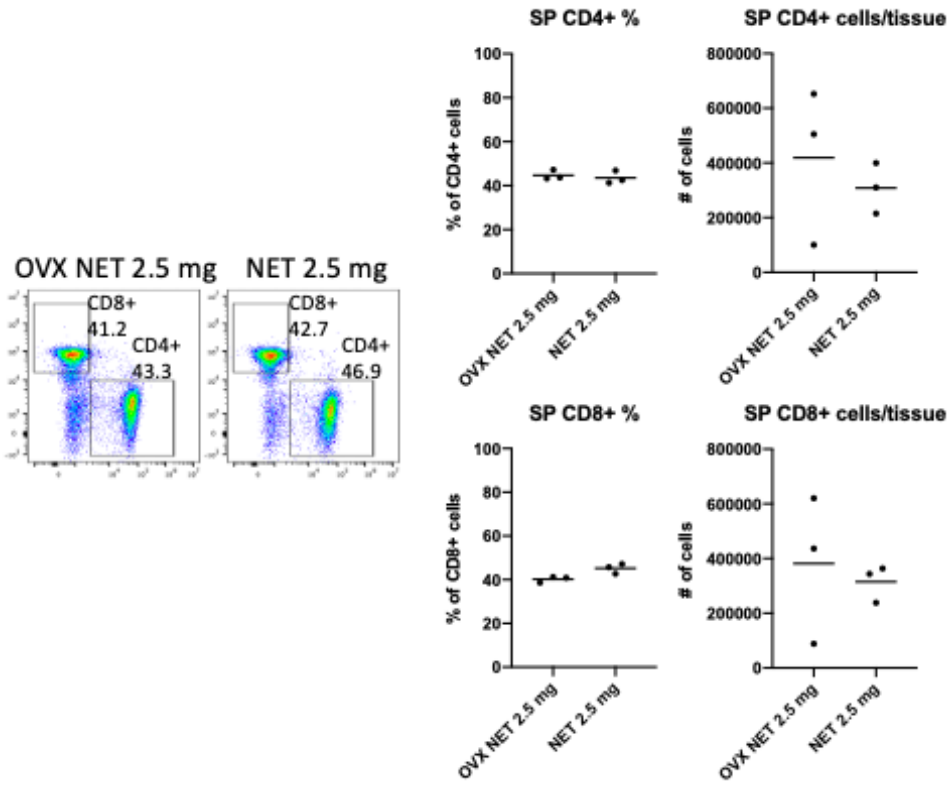

Figure 60. Graphs of percentages and cell counts of CD4+ and CD8+ cells from the spleens of ovariectomized and normal mice 6 weeks after treatment with NET 2.5 mg pellet. N=3 per group. Cells were left unstimulated. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t test.

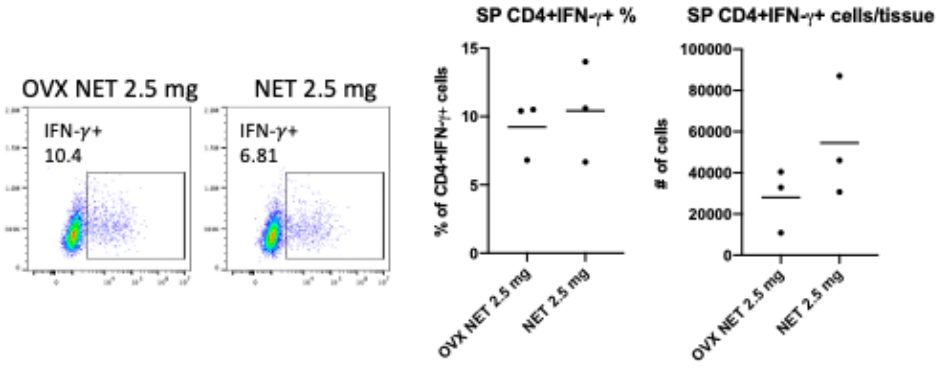

Figure 61. Graphs of percentages and cell counts of CD4+ IFN-γ+ cells from the spleens of ovariectomized and normal mice 6 weeks after treatment with NET 2.5 mg pellet. N=3 per group. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t comparisons test.

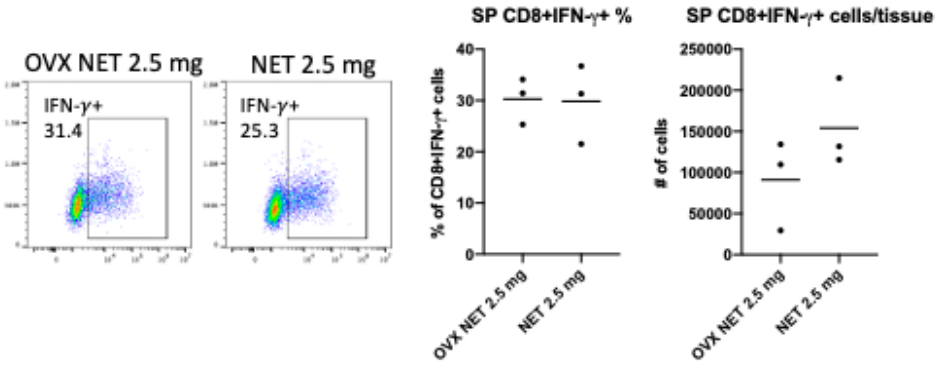

Figure 62. Graphs of percentages and cell counts of CD8+IFN-γ+ cells from the spleens of ovariectomized and normal mice 6 weeks after treatment with NET 2.5 mg pellet. N=3 per group. Cells were stimulated in vitro with a cell stimulation cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 16. hours. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t test.

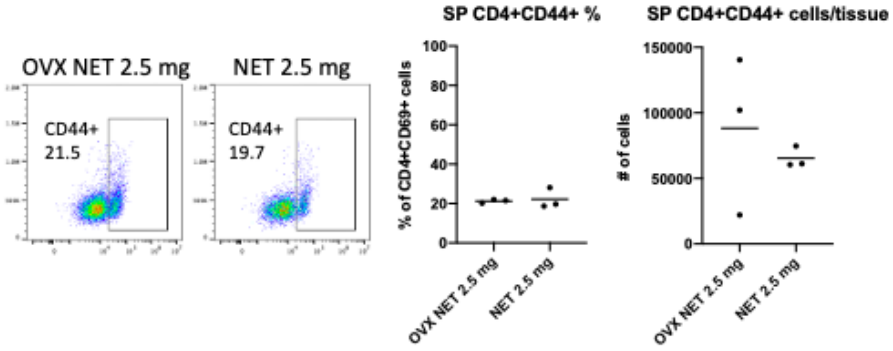

Figure 63. Graphs of percentages and cell counts of CD4+CD44+ cells from the spleens of ovariectomized and normal mice 6 weeks after treatment with NET 2.5 mg pellet. N=3 per group. Cells were left unstimulated. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t test.

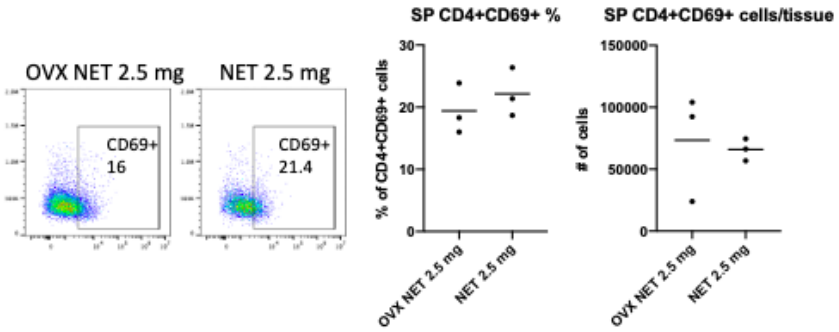

Figure 64. Graphs of percentages and cell counts of CD4+CD69+ cells from the spleens of ovariectomized and normal mice 6 weeks after treatment with NET 2.5 mg pellet. N=3 per group. Cells were left unstimulated. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t test.

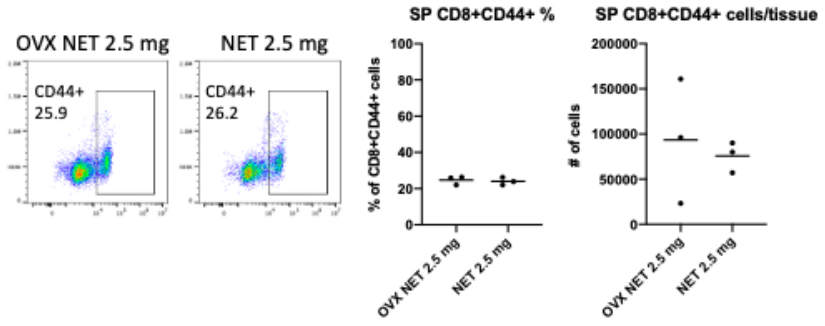

Figure 65. Graphs of percentages and cell counts of CD8+CD44+ cells from the spleens of ovariectomized and normal mice 6 weeks after treatment with NET 2.5 mg pellet. N=3 per group. Cells were left unstimulated. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t test.

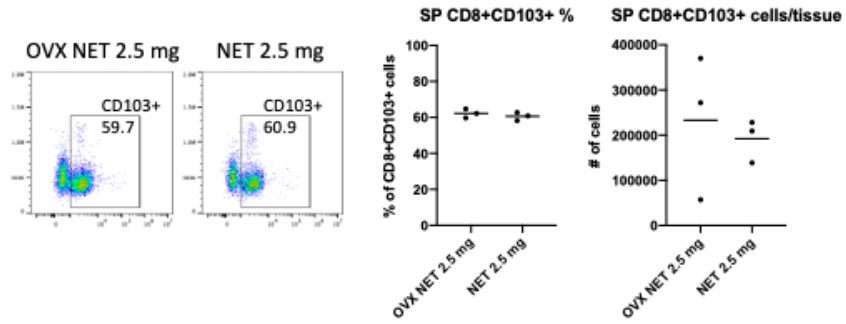

Figure 66. Graphs of percentages and cell counts of CD8+CD103+ cells from the spleens of ovariectomized and normal mice 6 weeks after treatment with NET 2.5 mg pellet. N=3 per group. Cells were left unstimulated. Flow plots show a representative image for each group. Data were analyzed using a two-tailed unpaired t test.

4.4.3 Vaginal tract- 6 week post NET treatment in OVX and Normal mice

Vaginal tracts were also examined for these same T cell subsets but due to low cell counts and sample size, no comparisons were made.

Table 9. Summary of trends observed in immune cells from ovariectomized and normal mice after 6 weeks of treatment with NET 2.5 mg.

Treatment group	Tissue	Cell types	Trend in Percentage of cells	Trend in Cell counts
OVX NET 2.5 mg	Lymph nodes	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND
	Spleen	CD4+	-	↓
		CD8+	-	↓
		CD4+IFN-γ+	-	↓
		CD8+IFN-γ+	-	↓
		CD4+CD44+	-	-
		CD4+CD69+	-	-
		CD8+CD44+	-	-
		CD8+CD103+	-	-
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND
Normal NET 2.5 mg	Lymph nodes	CD4+	ND	ND

		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND
	Spleen	CD4+	-	↓
		CD8+	-	↓
		CD4+IFN-γ+	-	↑
		CD8+IFN-γ+	-	↑
		CD4+CD44+	-	-
		CD4+CD69+	-	-
		CD8+CD44+	-	-
		CD8+CD103+	-	-
	Vagina	CD4+	ND	ND
		CD8+	ND	ND
		CD4+IFN-γ+	ND	ND
		CD8+IFN-γ+	ND	ND
		CD4+CD44+	ND	ND
		CD4+CD69+	ND	ND
		CD8+CD44+	ND	ND
		CD8+CD103+	ND	ND

ND (not done) indicates that there were not enough cells to run the samples and perform an analysis. Trends are shown to compare OVX NET 2.5 mg and normal NET 2.5 mg. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

Overall, there were some trends and differences seen in the proportions of immune cells and cell counts seen in different hormone treatment groups. After 1 week of DMPA treatment in ovariectomized mice, there was a trend of increased cell counts for CD4+, CD8+, CD4+IFN-γ+, CD8+IFN-γ+, as well as the CD4+ and CD8+ memory T cells in the iliac lymph nodes compared to the no hormone controls (Figures 32-38) (Table 7). The trend of increased CD4+ and CD8+ memory T cells was also a trend observed in the iliac lymph nodes after 3 weeks of DMPA

treatment compared to NET 2.5 mg treated mice (Figures 47-51). In the spleens of normal and ovariectomized DMPA treated mice, after 3 weeks there were also trends of increased CD4+ and CD8+ memory T cells compared to NET 2.5 mg treated mice (Figures 55-58).

In the ovariectomized and normal NET 2.5 mg treated groups, there were no consistent trends observed between 1 week, 3 weeks and 6 weeks post treatment in the iliac lymph nodes. After 1 week, there were trends of increased CD4+IFN-γ+ and CD8+IFN-γ+ percentages and cell counts in the lymph nodes in the OVX NET 2.5 mg, OVX NET 5 mg and normal NET 2.5 mg groups compared to the OVX no hormone and normal no hormone controls (Table 6). In the spleens, after 1 week of NET 2.5 mg treatment in ovariectomized and normal mice there were trends of increased CD8+ cells as well as CD4+ cells in ovariectomized NET 2.5 mg treated mice compared to the no hormone controls (Figure 25) (Table 6). In the vaginal tracts of ovariectomized and normal mice treated with NET 2.5 mg and NET 5 mg, there were trends of increased CD4+ and CD8+ cells (Figure 30) (Table 6). After 3 weeks of treatment with NET 2.5 mg, there were trends of decreased CD4+ and CD8+ cell counts in the spleens of ovariectomized mice compared to the OVX DMPA treated mice (Figure 39 and Table 8). However, after 6 weeks of treatment, there seemed to be little effects from the hormones as little to no trends were observed (Table 9). There seem to be small trends and changes that occur with hormone treatment, but more experiments would need to be conducted to validate these trends and changes.

CHAPTER 5: DISCUSSION

5.1 Summary of results

Millions of women worldwide use hormonal contraceptives, including NET and DMPA which are the most popular injectable hormonal contraceptives[102]. Many studies have shown that there is an associated risk for HIV acquisition with the use of DMPA, demonstrated in clinical studies, *in vivo* and *in vitro*[114 118 122 126 127 130 136 159 160]. A meta-analysis examining DMPA, NET-EN and combined oral contraceptive use over various studies found that there was a 50% increased risk for HIV acquisition with the use of DMPA[114]. However, there was a no increased risk for HIV acquisition when the use of combined oral contraceptives and NET-EN were examined[114]. Additional studies in animal models have also shown that DMPA/MPA treatment increased susceptibility to HIV-1 resulting in increased HIV-1 copy numbers in plasma[126 129 136]. However, a large clinical trial conducted recently, evaluating DMPA use in women in Zambia, South Africa, Kenya and Eswatini named the ECHO Trial, found no association between DMPA use and risk for HIV acquisition, compared to other common contraceptive methods[115 161]. However, the conclusions of this trial has been questioned as the study had limitations and led to misinterpretations regarding their conclusions[161 162]. For example, there were issues with the low power of the study to detect differences below 50% in comparing different contraceptive use and the risk for HIV acquisition. In addition, there was no control group, without any contraceptive use, included in the trial for comparison and the overall rate of HIV-1 infection was very high in all 3 arms of the trial[161 162].

Nevertheless, the debate whether DMPA use poses a risk to women in terms of increasing susceptibility to STIs such as HSV-2 and HIV is still not settled. Various studies have examined the effects of DMPA and have found associated negative effects including risk for STI acquisition,

specifically HIV [113-115]. Therefore, it is important to explore the effects of DMPA and whether they affect outcomes to other infections, such as HSV-2 infections. It is also important to explore other synthetic progestins that are used as hormonal contraceptives to determine whether they pose the same risk. Another hormonal contraceptive that has been compared to DMPA is NET, in which *in vitro* studies suggest that NET does not show the same immunosuppressive effects as DMPA and might be a promising alternative to DMPA[122 130 131]. Studies have shown that MPA treatment inhibits cytokine production of T cells, including IFN-γ and TNF-α, but this was not seen with NET-EN treatment as mentioned in Section 1.9.6[121 123]. However, few studies have characterized the effects of NET in vivo. The studies described in this thesis are among the few to show the specific effects of varying doses of NET in mice.

From the literature, *in vivo* studies that examined the effects of NET in mice have shown that NET increases genital permeability, increasing susceptibility to HSV-2 as shown through pathology and survival as well as HIV-1 demonstrated through HIV-1 copy numbers in plasma [127]. When this was compared to DMPA, effects of NET were not as severe as DMPA[127]. In our study, mice treated with all doses of NET tested (2 mg injection, 2.5 mg and 5 mg pellets) resulted in low viral shedding after intravaginal immunization with attenuated HSV-2 TK^- as well as mice not shedding virus at all (Figure 3, 5, 8 and 11). These mice showed viral shedding post intravaginal challenge with WT HSV-2, indicating that indeed, the immunization was not effective. However, in DMPA treated mice, the immunization was consistently effective, in which mice showed little to no viral shedding when challenged with WT HSV-2 (Figure 3, 5, 8 and 11). HSV-2 TK^- has a lower rate of replication compared to WT HSV-2 and it could be possible that there is not enough viral replication occurring to allow for successful immunization in NET treated mice. In immunization

and challenge models, unlike DMPA, NET treatment does not allow for effective intravaginal immunization to provide protection in a challenge with HSV-2.

Our experiments that examined susceptibility to HSV-2 in mice under different hormone conditions demonstrate that NET treated mice show delayed primary HSV-2 infection compared to DMPA treated mice. This was displayed through delayed viral shedding and pathology scores (Figure 11, 12, 14 and 15). Furthermore, 3/6 mice in the normal NET treated groups did not show any pathology and survived the infection, which also occurred in all normal DMPA treated mice as well as 1/3 mice in the OVX DMPA group. However, overall survival in NET treated mice was not improved compared to DMPA treated mice, as their survival was similar. Overall susceptibility was not decreased in primary infection for NET treated mice and is similar to DMPA treated mice. In a primary challenge, the more virulent WT strain of HSV-2 is used which is able to replicate much faster than the attenuated HSV-2 TK^- strain. The WT strain has a higher capability of viral replication and once virus reaches the vaginal epithelial cells, it can replicate quickly to other epithelial cells and spread to the neurons infecting the central nervous system. Thus, hind limb paralysis can occur in mice indicating they have reached endpoint. However, in immunization with HSV-2 TK^- the virus does not have the same replication capacity as the WT strain. Thymidine kinase is required to replicate in the neurons to cause a lethal infection, which the TK^- strain is incapable of. This explains the differences in susceptibility seen in immunization and challenge models, in which NET treated mice were not consistently shedding virus and did not reach endpoint once immunized. However, in primary infection NET treated mice can develop severe pathology reaching endpoint once the virus has reached the epithelium and spreads quickly. In an immunization and challenge model NET treated mice show a lower susceptibility to intravaginal immunization with HSV-2 TK^- compared to DMPA treated mice, which results in an effective

intravaginal immunization. In a primary challenge NET treated mice show a delayed HSV-2 infection, but overall susceptibility to HSV-2 does not differ from DMPA treated mice.

To gain insight into potential mechanisms through which NET provides protection towards HSV-2 infection, the physiology of the vaginal tract was examined in NET- and DMPA-treated mice. Mucus staining depicted more mucus lining the vaginal tract of NET treated mice compared to DMPA treated mice, supported by a trend of higher mucus quantities in vaginal washes of NET treated mice (Figure 17, 19, 20, 22 and 23). This increase in mucus production when mice are under the NET treatment could be preventing viral entry. Furthermore, immunohistochemistry demonstrates that at endpoint, unlike DMPA treatment, HSV-2 was not present in the vaginal tract of NET treated mice, further providing evidence that NET treatment inhibits intravaginal HSV-2 infection (Figure 23). The vaginal epithelium thickness was also quantified, and NET treated mice do not show the same thinning of the vaginal epithelium that occurs with DMPA treatment (Figure 18). This indicates that viral entry would not occur as easily in NET treated mice as it does in DMPA treated mice. Altogether these findings suggest that NET treatment results in ineffective HSV-2 immunization in mice compared to DMPA but does not show reduced susceptibility to primary HSV-2 infections compared to DMPA.

The FRT contains cervicovaginal mucus, which has been shown to have the ability to block viral entry inhibiting the spread of the virus. It has been found that cervicovaginal mucus also contains antimicrobial properties playing a protective role[163-165]. As demonstrated in other studies, human cervicovaginal mucus from HIV negative women was found to hinder the movement of HIV-1, providing a barrier to viral entry[166]. Another study showed through confocal microscopy that HIV-GFP (green fluorescent protein) labelled virions were trapped and suspended in cervical mucus in primary cervical samples[167]. An additional study has also showed that cervicovaginal

mucus was able to reduce the rate of HSV-2 transmission in the mouse vagina[168]. Furthermore, when the cervicovaginal mucus was removed from the mouse, susceptibility to HSV-2 increased by approximately 30%[168]. One study also showed that most mammalian viruses are larger than average pore sizes found in the cervicovaginal mucus, and showed that through adhesive interactions, HSV becomes trapped in the cervicovaginal mucus, reducing the diffusion of HSV[169]. All doses of NET delayed HSV-2 infection compared to DMPA in mice. Unlike DMPA treated mice, NET treated mice fail to become immunized with attenuated HSV-2 TK^- and had delayed infection following WT HSV-2 primary infection. These findings suggest that enhanced mucus production may be a mechanism through which NET inhibits or even prevents intravaginal infection with HSV-2 in mice.

Lastly, to determine whether hormone treatments had any effects on immune cell populations present in various tissues, immune cells were analyzed in mice under NET and DMPA treatments. Overall, there were little changes in immune cell subsets. After 1 week of DMPA treatment in ovariectomized mice, there were trends of increased T cells in iliac lymph nodes (Figure 32-38 and Table 7). This trend was also observed in the lymph nodes of mice after 3 weeks of DMPA treatment compared to NET 2.5 mg treatment in ovariectomized and normal mice (Figure 47-51 and Table 8). After 1 week of NET 2.5 mg treatment, there were also trends of increased T cells and IFN-γ+ cells from CD4+ and CD8+ cells in lymph nodes, spleens, as well as the vaginal tissue from ovariectomized and normal mice (Figure 25-30 and Table 6). These experiments would need to be repeated to confirm these trends, but this suggests that progestin-based hormone treatments could be increasing T cell subsets which could affect immune responses to infection.

5.2 Comparison of *in vivo* doses of NET

As there has only been one other study that has used NET in animal models done by Quispe Calla et al., it was important to determine an appropriate dose to administer to mice[127]. To optimize NET dose and administration in our mouse model, injection versus insertion of hormone pellets of various doses were evaluated. Injections of NET gave inconsistent results, as they failed to keep mice in diestrus consistently for longer than 1-2 weeks. This was necessary as diestrus is the stage of estrus cycle that mice need to remain in for HSV-2 infection to occur [84 91]. Our results showed that NET 2.5 mg pellet treatment led to serum levels in mice that was found to be an average of 15.56 ng/ml that were comparable to serum levels observed in women using NET as a contraceptive after 1 week which ranged from approximately 10-20 ng/ml (Figure 6a)[147-153]. Similar to the NET 2.5 mg pellet, serum levels from mice treated with NET 5 mg pellet also led to an average serum level of 20.31 ng/ml. This also falls within the range seen in women, however, is towards the higher end of the range. Our serum analysis of different doses of NET in ovariectomized and normal mice showed similar serum levels at 1 week post hormone treatment, which also still fell within the observed range seen in women. The average serum level for normal NET 2.5 mg pellet treated mice was 12.80 ng/ml and the average serum level for normal NET 5 mg pellet treated mice was 10.21 ng/ml. Although the study by Quispe Calla et al. used a NET injection dose equivalent to 5 mg pellets, in our model this dose led to adverse effects, including bladder distention in some mice. Furthermore, 5mg NET serum levels at 3 weeks (26.04 ng/ml) and 6 weeks (7.07 ng/ml) post hormone treatment that exceeded those reported in humans, which was ~3-6 ng/ml at 3 weeks and ~1 or <1 ng/ml at 6 weeks post hormone treatment(Figure 6c and e)[127 147-153]. It is unclear why study by Quispe Calla et al. needed to use a higher dose of NET as our results indicate that the NET 2.5 mg pellet was sufficient to keep mice in diestrus for a long

period of time and to allow mice to become susceptible to HSV-2 infection[127]. Our study is the only one to our knowledge that has used these NET hormone pellets in mice and has tested various doses and routes of administration in OVX and intact mice.

5.3 Vaginal epithelium thickness in NET vs DMPA treated mice

Due to the decreased success of HSV-2 TK^- immunization in NET treated mice, the effects of hormone treatment on the vaginal epithelium were investigated using histology. The OVX no hormone control group and OVX DMPA treated group showed the thinnest vaginal epithelium (Figure 18). This has also been shown in studies from our lab, in which DMPA treated mice showed the lowest level of Dsg1 protein expression in the vaginal tract and correlated with increased permeability of the vaginal epithelial barrier allowing infection to occur easily[126]. Quispe Calla et al demonstrated that DMPA and NET treated mice showed lower Dsg1 protein expression in the vaginal tracts compared to mice in estrus[127]. However, NET treated mice in our study did not show the same thinning of the vaginal epithelium as DMPA treated mice. Therefore, this is another mechanism of NET could result in lower susceptibility to HSV-2 infection compared to DMPA.

5.4 Mucus production as a barrier to HSV-2 infection in NET treated mice

Mucus provides a physical barrier in the vaginal tract, which is one mechanism of how progestin-based hormonal contraceptives can prevent pregnancy but could also be a method of protection against pathogens[24 170]. Mucus is a viscous gel that lines the epithelium which is secreted from the endocervix[171]. There are various mucins that make up the mucus in the vaginal tract, including cell surface mucins (MUC1, MUC4 and MUC16) in humans[166]. Secreted mucins or

gel-forming mucins include MUC5AC, MUC5B and MUC6[166]. Studies have shown that the amount of mucus present in the vaginal tract fluctuates with hormones; specifically, mucus is thinner during the proliferative phase when estrogen levels are high, which allows sperm to move through the FRT, but during the secretory phase when progesterone levels are high, mucus is thicker to block entry into the FRT[24]. The same principal may be relevant to viral entry. Indeed, compared to normal mice, a trend of higher mucin-1 quantification was observed in NET treated mice (Figure 20). Moreover, NET treated mice had much more vaginal tract mucus visualized by PAS and mucin-1 staining compared to the controls (Figure 17, 19, 22 and 23). Cervicovaginal mucus has been shown to hinder the movement of HIV-1 virus particles ex vivo, providing a barrier[166]. There also seems to be variability in the mucus present in the vaginal tract of each mouse in the NET treated groups, which could also explain the variability seen within groups regarding successful infection with HSV-2. NET treated mice with more vaginal mucus present would therefore have the capacity to inhibit virus from reaching the epithelium and would result in better outcomes post HSV-2 infection compared to NET treated mice that show less mucus present in the vaginal tract.

5.5 Comparison of survival and pathology of NET and DMPA treated mice after primary HSV-2 infection

We then evaluated the effects of our optimized dose of NET to DMPA in our murine model of primary HSV-2 infection. We found that ovariectomized NET treated mice show a 1 day delay in viral shedding (Figure 11), develop pathology later than DMPA treated mice, reaching higher pathology scores between day 8 and 10, while ovariectomized DMPA treated mice reached high pathology scores between day 5 and 7 (Figure 12 and 15). Additionally, normal DMPA treated

mice did not develop pathology throughout the course of infection (Figure 12 and 15). Furthermore, it was found that all ovariectomized NET treated mice succumbed to infection, while 2/3 normal NET 2.5 mg and 2/3 normal NET 5 mg treated mice did not develop any pathology and were able to survive the infection (Figure 10, 12, 14 and 15). DMPA treated mice showed improved survival overall compared to NET. This was demonstrated through the 1/3 mice in the ovariectomized DMPA group that survived and all 3 of the normal DMPA treated mice that survived (Figure 14). Quispe Calla et al. showed that compared to NET, DMPA treated mice showed higher susceptibility, demonstrated through the pathology scores and survival post infection[127]. In this study, NET treated mice reached high pathology scores indicating endpoint by day 9 and 10. However, they used NET injections which we demonstrated to be inconsistent, while we used NET pellets that were more consistent (Table 2 and 3). Furthermore, the immunofluorescence images of the vaginal epithelium of NET treated mice from their study did not show mucus production[127]. Due to the different doses and formulations, their mice may not have had the mucus production seen in our results. Overall, these discrepancies in HSV-2 pathogenesis may be due to the influence of endogenous hormones in combination with differing serum hormone levels between our studies.

This provides further evidence to suggest that in NET treated mice the mucus in the vaginal tract is acting as a barrier to trap and slow down the viral infection, giving less accessibility of the virus to the vaginal epithelium. HSV-2 TK^- is an attenuated virus and is limited in its ability to replicate, which could also explain the lower viral shedding[93]. With less virus reaching the vaginal epithelium, low numbers of virus would be present to start the infection and would therefore result in the delayed viral shedding and lower viral titers as well as delayed pathology compared to DMPA treated mice when infected with HSV-2 TK^-. In primary infections with WT HSV-2, the

mucus in the vaginal tract from NET treated mice could still be blocking and trapping virus. Our results still showed a delay in viral shedding in NET treated mice after infection with WT HSV-2, but once the virus reaches the vaginal epithelium it is able to replicate and cause a lethal infection even with low amounts of virus present[93]. This would also explain the delayed pathology in which NET treated mice reached endpoint later than DMPA treated mice.

5.6 Effect of NET and DMPA on immune cell phenotypes and frequencies

Other studies have showed that DMPA can exert immunosuppressive effects and alter cytokines, chemokines and immune cells *in vitro* and *in vivo*[105 107 110 118-121 124 125]. Therefore, immune cell phenotypes and frequencies were examined in NET treated mice and compared to DMPA treated mice to determine whether there are any effects on immune cells that may impact that immune response to HSV-2. Flow cytometry experiments showed little changes in immune cells between DMPA and NET treatment in mice. One week of DMPA treatment results in an increase in CD4+ and CD8+ T cells as well as CD4+ and CD8+ memory T cell subsets in the lymph nodes. Cherpes et al. showed that MPA inhibits T cell proliferation in ex vivo cultures from the trigeminal ganglia of HSV-1 infected mice[98]. However, it is important to note that in their study, high doses of MPA were used including 10 mg and 50 mg which is much greater than our validated 2 mg injection dose used in mice, based on steady state serum levels seen in women [98]. Since studies have shown that immunosuppression occurs with DMPA treatment through the GR, it is possible that the high doses of MPA used in other studies could cause immunosuppressive effects on cells such as decreasing proliferation, which may not be seen with the physiologically relevant doses used in our study that mimic human serum levels [105 107-110 118]. Another study has also shown that DMPA treatment increases cervical CCR5+CD4+ T cells, which would align

with our results of increased T cells with DMPA treatment but was seen in the lymph nodes[103 118]. However, studies that have examined effects of NET on T cells and cytokine production have shown little to no changes[121 122 131]. Studies have shown that MPA treatment inhibits cytokine production from T cells in vitro such as IFN-γ, but that NET treatment showed the highest level of IFN-γ production[98 105 107 110 121 131]. However, in our studies, when CD4+IFN-γ+ and CD8+IFN-γ+ cells were analyzed, the DMPA treated groups did not show any trends or differences compared to NET 2.5 mg treated mice. In our results, there was a trend of increased CD4+IFN-γ+ and CD8+IFN-γ+ cells in the lymph nodes of mice after 1 week of NET 2.5 mg treatment compared to no hormone control mice, which is similar to the in vitro studies previously mentioned here[121 122 131]. This suggests that NET treatment does not inhibit cytokine production the same way that DMPA does. However, to further confirm the trends and differences seen, our experiments would need to be repeated.

5.7 Strengths and Limitations

A strength of this study is that using an ovariectomized mouse model allowed us to study the effects of single hormones without the influence of endogenous hormones. It is important to determine what effects these hormones can have separately from endogenous hormones that could change these effects. In addition to studying the effects of these hormones individually in mice, there were also normal mice used in several experiments with intact ovaries and endogenous hormones that were used for many of the experiments discussed as well. This addressed the question of whether there are different effects seen with these hormone treatments alone and with the influence of endogenous hormones, which replicates the effects expected to be seen in humans that are using these hormonal contraceptives. Furthermore, the dose of NET used in this study was

validated through a more advanced and newer method of quantifying hormone levels in serum. Previous studies have used an old technique of radioimmunoassay, however, this study used ultra-performance liquid chromatography-mass spectrometry that quantified the levels of hormones in mouse serum, which is also used to quantify hormones levels in serum samples from women on these hormonal contraceptives[112 144 147-151 153]. Additionally, we tested various doses of NET in ovariectomized and intact mice, which has not been done before. This allowed us to validate the dose of NET in mice in conditions that replicate conditions in women that are using NET as a contraceptive.

While there are many similarities between mice and humans, this model does not replicate all aspects of HSV-2 infections that occur in humans. For example, HSV-2 infections in humans can reactivate and have recurrent episodes of viral shedding and symptoms. However, mice do not experience recurrent infections, and productive HSV-2 infection in this model is lethal[83 172]. Additionally, mice are only susceptible to HSV-2 infections during the diestrus stage of their estrus cycle, which is why progestin-based hormones allow mice to become susceptible to infection[83]. This is not seen in humans, as women can become infected with HSV-2 during any stage of the menstrual cycle. Taking these limitations into consideration, mouse models still provide valuable insights into HSV-2 infections that are applicable to humans; mice still become virally infected in the vagina, showing pathology and immune responses similar to humans, making it a useful model to study this disease[5 173]. Furthermore, limitations of our data include that suspected cause of delayed infection or no infection occurring in NET treated mice was due to mucus. However, to validate this, more direct experiments would need to be conducted to confirm this mechanism. More evidence would be needed to provide direct proof that NET is causing mucus production and that this is directly inhibiting viral infection. In addition to this, the flow cytometric analysis of

immune cells in mice treated with DMPA and NET were preliminary and would need to be repeated due to low n numbers to strengthen the trends and patterns reported in this study.

5.8 Future Directions

The first experiments that would need to be done are the experiments examining immune cells after hormone treatment with NET in comparison to DMPA. Our results show preliminary findings but would need to be repeated to confirm the trends observed. Various studies have examined the effects of DMPA and NET in cervicovaginal lavage samples and vaginal swabs from humans which have provided information of levels and expression of various chemokines and cytokines and how these hormones affect these levels compared to baseline measurements[118 121 122 131]. It would be interesting to explore more of the effects DMPA and NET have on immune cells as well as cytokines and chemokines in mice to determine if DMPA and NET show differences and if DMPA exerts immunosuppressive effects. The next experiment would be to determine if mucin in the vaginal tract is the cause of the decreased susceptibility to HSV-2 observed in NET treated mice. Experiments to correlate the mucin production in individual mice under NET treatment and the effects of this on HSV-2 susceptibility would address whether mucus in the vaginal tract is inhibiting viral infection. Experiments depleting mucin in NET treated mice and examining how this affects outcomes such as pathology, viral titers and survival would also address whether mucin is responsible for the decreased susceptibility to HSV-2. Since these novel findings have elucidated effects NET can have on murine vaginal barrier function, it would be interesting to obtain vaginal biopsies and cervicovaginal lavage samples from women using NET as a contraceptive to examine vaginal epithelial thickness and secreted mucus to compare to our results seen in mice. As the

results from our study indicate that NET treatment in mice results in thickening of the vaginal epithelium and increased mucus production in the vaginal tract, a study comparing NET treatment in women would determine whether the same results occur in humans. Evaluating HSV-2 infection rates of women using NET and DMPA would provide further evidence to support mucus production as a protective mechanism of NET treatment, which results in lower susceptibility to HSV-2 compared to DMPA treatment.

5.9 Significance

Overall, this study shows that NET treatment results in lower susceptibility to HSV-2 infections compared to DMPA treatment in mice and that the same may be true for humans. This is the first study to our knowledge that has shown an association between NET treatment and improved vaginal barrier in mice which is associated with protection against HSV-2 infection compared to DMPA. These findings suggest that in addition to its contraceptive effect, increased mucus production by NET may also decrease susceptibility to STIs through a mechanism by hindering the infection. If this is shown to be true in women, then NET may be a better option as an injectable hormonal contraceptive compared to DMPA, as many of the negative effects that are seen with the use of DMPA are not observed with the use of NET, which has been demonstrated in studies in vitro and in vivo. This is important as many women use these hormonal contraceptives and women need to know the effects of these contraceptives to make informed decisions on what the safest options are for them.

CHAPTER 6: CONCLUSION

It is known that women are disproportionately affect by HSV-2 and it is important to understand and examine factors that are influencing their increased risk for infection. Hormones are also known to influence susceptibility to HSV-2 infections, which is why additional effects of hormonal contraceptives should be studied. This study investigated the effects of DMPA and NET 2.5 mg and 5 mg pellets in mouse models to determine the effect of these hormones in mice alone as well as their effects in intravaginal immunization and challenge models with HSV-2 and primary intravaginal HSV-2 infections. This was done in the ovariectomized mouse model as well as normal mice to explore the effects of these hormones alone and with the influence of endogenous sex hormones.

The study aimed to examine susceptibility to HSV-2 in mice treated with NET 2.5 mg and 5 mg pellets as well as DMPA 2 mg injection. The main findings of this study have shown that NET and DMPA treated mice show differences in susceptibility to intravaginal immunization with attenuated HSV-2 and that NET treatment does not allow for effective immunization, but DMPA does. Additionally, NET and DMPA do not show a difference in susceptibility to primary HSV-2 infection as demonstrated by their similar survival. However, NET treated mice show a delayed primary HSV-2 infection as indicated by their delayed pathology and viral shedding. Furthermore, histological analysis of the vaginal tract displayed mucus present in NET treated mice, which could contribute to a delayed infection with HSV-2, a potential protective mechanism that is not observed in DMPA treated mice. Lastly, immune cells were analyzed in mice treated with NET and DMPA and trends of increased T cells were associated with progestin treated mice. These trends in immune cells are preliminary findings and more experiments would need to be done to confirm these trends.

This study provides more information on the effects of NET alone and in the context of HSV-2 infections in mouse models. This is the first study to our knowledge that has shown this mechanism of mucus production in NET treated mice in hindering HSV-2 infection. This exhibits a protective mechanism associated with NET treatment that could decrease susceptibility to other STIs.

CHAPTER 7: APPENDIX

These next experiments were done to determine whether there are differences in the effects of these hormones on immune cells after immunization and challenge with HSV-2. We were interested in the effects of these hormones after primary challenge, however, in a primary challenge, the immune system is coping with the infection. The immune system will be overwhelmed as mice will eventually reach endpoint after a primary HSV-2 infection. The effects of the hormones may be unclear in a primary challenge, while with immunization and challenge, it will show the effects of these hormone treatments on the development of immune responses after immunization and the response to a challenge. This would also determine whether there are different effects with hormone treatment alone and whether this changes with infection.

7.1 Examine the effect of NET 5 mg in OVX mice after HSV-2 immunization and challenge

Mice were first ovariectomized and two weeks later were given DMPA 2 mg injection, NET 5 mg pellet and progesterone 10 mg pellet (Figure 4). This also included a no hormone control group. Ovariectomized mice were used as the experiments described previously that examined immune cell phenotypes after hormone treatment did not show significant differences between normal and ovariectomized mice. Mice were then intravaginally immunized with 10^4 PFU of HSV-2 TK^- ten days after hormone treatment to determine the effect of hormones on immunization. Mice were then intravaginally challenged with $5x10^3$ PFU of WT HSV-2 approximately 4-6 weeks later when mice were longer under the influence of hormones. On day 5 post challenge, tissues were collected, and flow cytometry was performed to examine immune cell phenotypes to investigate whether these hormones had any changes on immune cells and how immunization was affected by this. At the time of challenge, the hormones would not have effects any longer.

7.1.1 Lymph node – immunization and challenge under progesterone, DMPA and NET 5 mg

In the iliac lymph nodes, no significant differences were observed in the percentages of CD4+, CD8+ cells and memory T cell subsets (Figures 67-69).

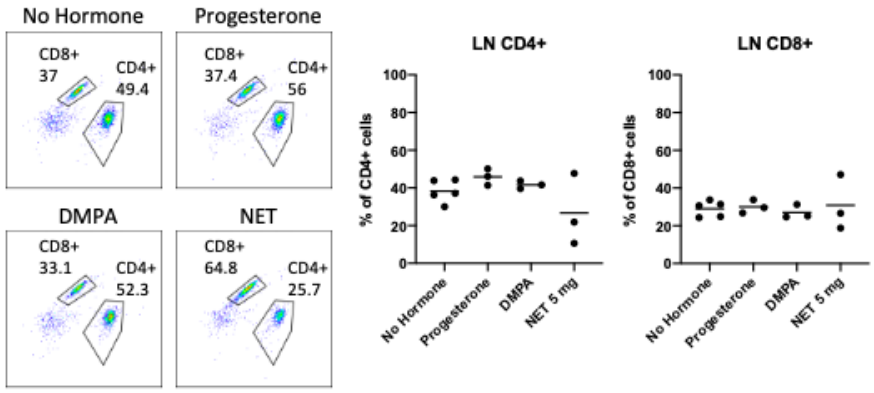

Figure 67. Graphs of percentages of CD4+ and CD8+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

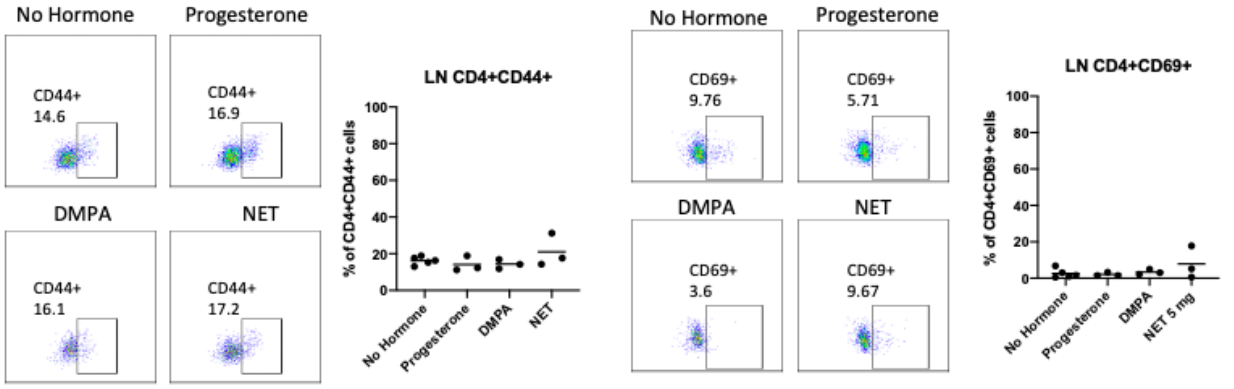

Figure 68. Graphs of percentages of the memory T cell subsets, CD4+CD44+ and CD4+CD69+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

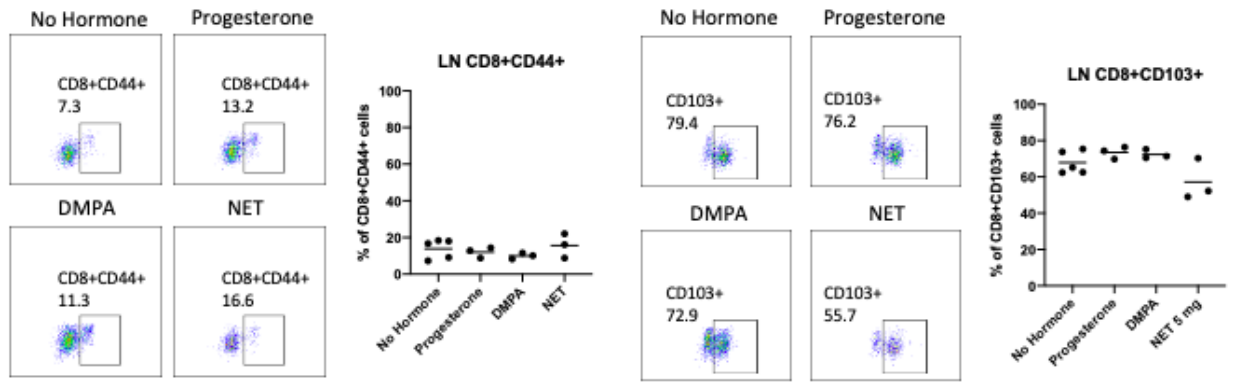

Figure 69. Graphs of percentages of the memory T cell subsets, CD8+CD44+ and CD8+CD103+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

7.1.2 Spleen – immunization and challenge under progesterone, DMPA and NET 5 mg

Cells in the spleen were analyzed, in which the NET 5 mg group showed significantly lower percentages of CD4+ cells compared to the progesterone group (Figure 70). The DMPA group also showed significantly lower percentages of CD4+ cells compared to the progesterone group. The NET 5 mg group showed higher percentages of CD8+ cells compare to the DMPA, progesterone and no hormone control group, which was found to be statistically significant.

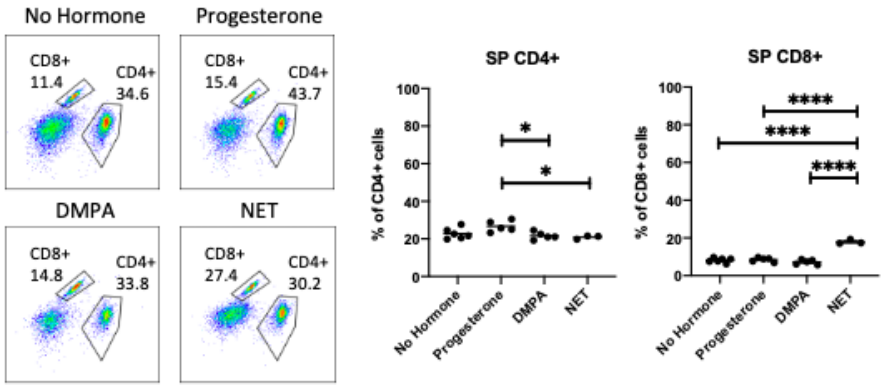

Figure 70. Graphs of percentages of CD4+ and CD8+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *p<0.05, ****p<0.0001.

CD4+ and CD8+ memory T cell subsets were also examined in the spleen (Figures 71 and 72). The percentage of CD8+CD103+ cells in the NET 5 mg group was significantly lower than the

DMPA and progesterone group (Figure 72). In all other memory T cell subsets, no trends or statistically significant differences were found.

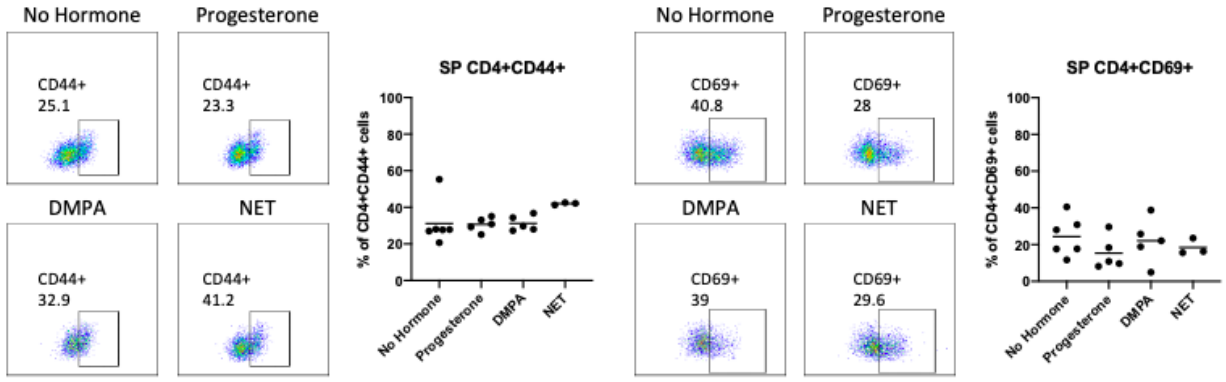

Figure 71. Graphs of percentages of the memory T cell subsets, CD4+CD44+ and CD4+CD69+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

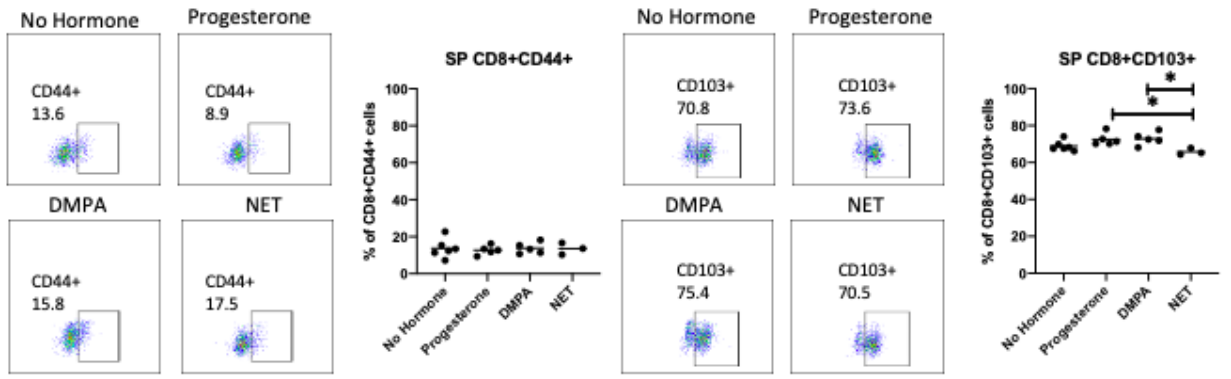

Figure 72. Graphs of percentages of the memory T cell subsets, CD8+CD44+ and CD8+CD103+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *$p<0.05$.

7.1.3 Vaginal tissue – immunization and challenge under progesterone, DMPA and NET 5 mg

Additionally, the vaginal tracts were analyzed and due to low cell numbers and sample size, no trends or comparisons could be made.

7.2 Examine the effect of NET 2.5 mg in OVX mice after HSV-2 immunization and challenge

Similar to the previous experiment, an immunization and challenge experiment was done as outlined in Figure 7 but using the NET 2.5 mg pellet rather than the NET 5 mg pellet. This was done to compare the results seen in the immunization and challenge with NET 5 mg and NET 2.5 mg to determine if there are different effects on immune cells with different doses of NET. This

was also done to compare NET treatment to DMPA as well as progesterone, as NET and DMPA are synthetic progestins.

7.2.1 Lymph node – immunization and challenge under progesterone, DMPA and NET 2.5 mg

In the iliac lymph nodes, the NET 2.5 mg group showed higher CD4+ cell counts compared to the progesterone and no hormone group (Figure 73). Similar trends were seen in the cell counts for CD8+, CD4+CD44+, CD4+CD69+, CD4+IFN-γ+, CD8+IFN-γ+, CD8+CD44+ and CD8+CD103+ cells (Figures 73-79).

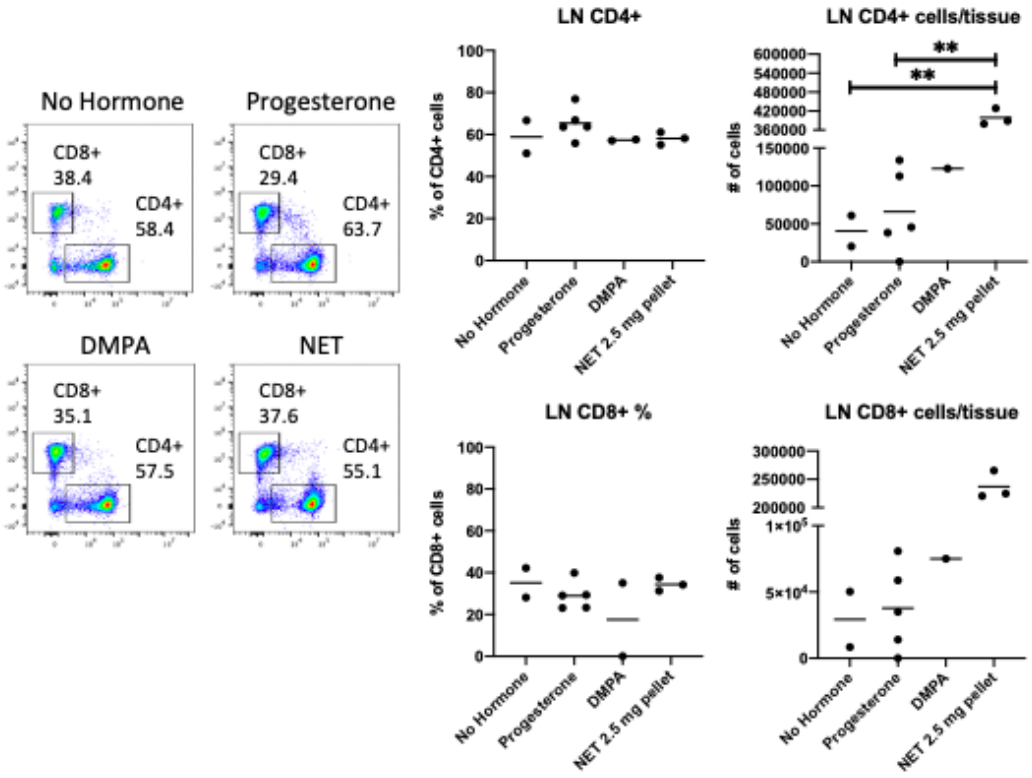

Figure 73. Graphs of percentages of CD4+ and CD8+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group.

Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. **p<0.01.

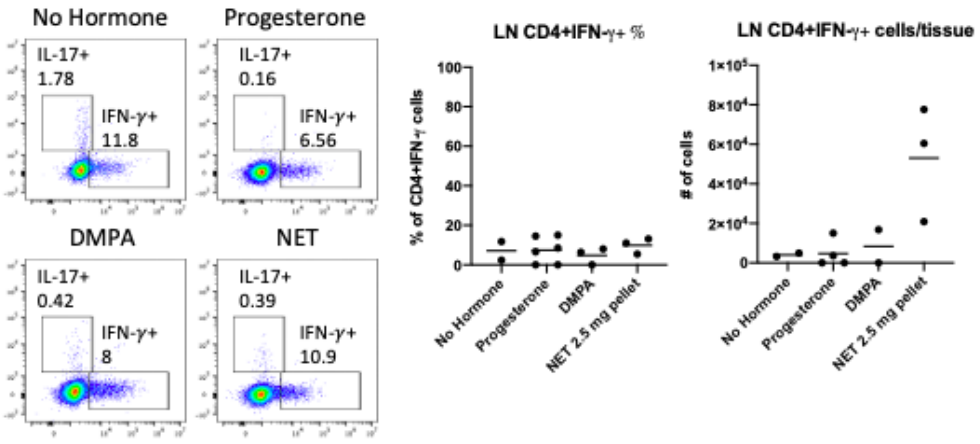

Figure 74. Graphs of percentages of CD4+IFN-γ+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

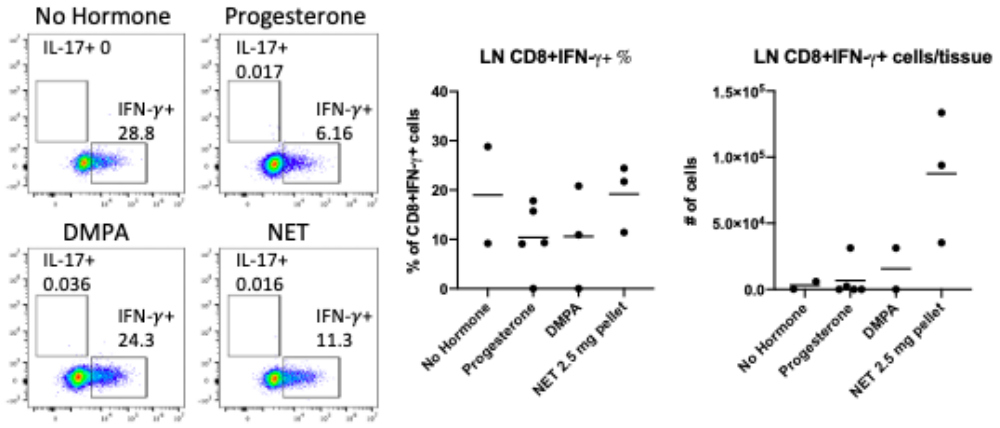

Figure 75. Graphs of percentages of CD8+IFN-γ+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

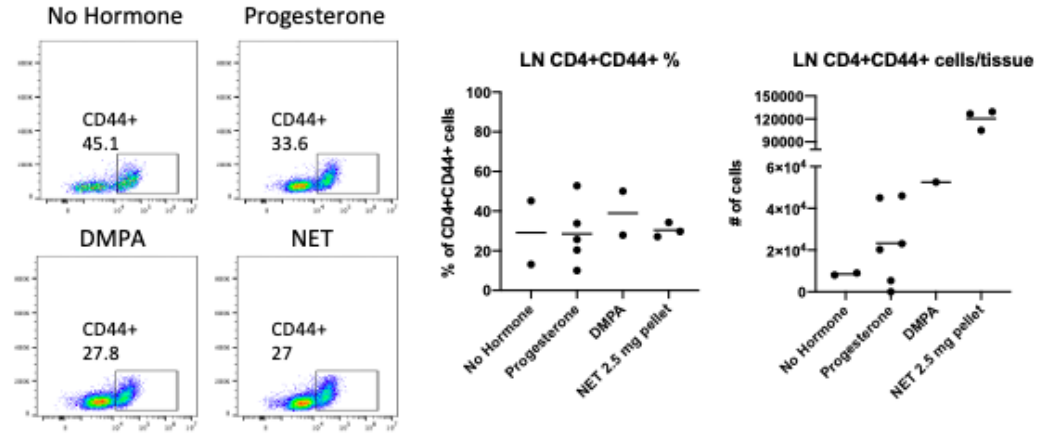

Figure 76. Graphs of percentages of CD4+CD44+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group.

Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

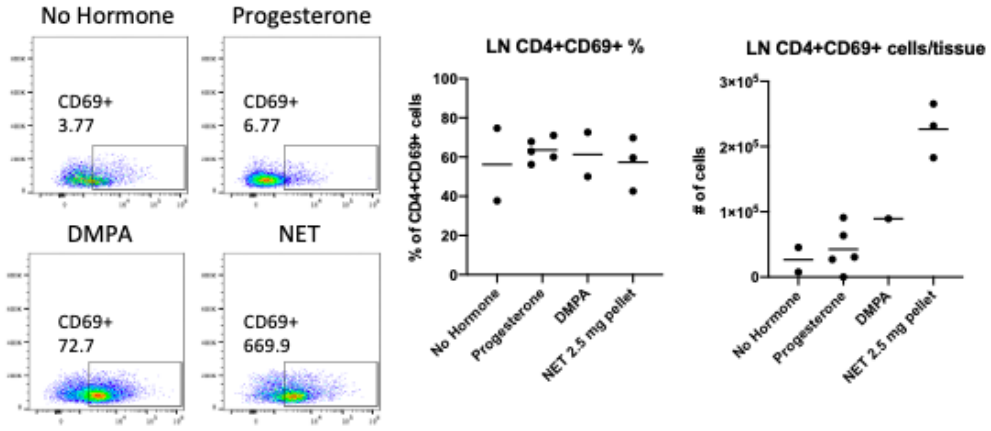

Figure 77. Graphs of percentages of CD4+CD69+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

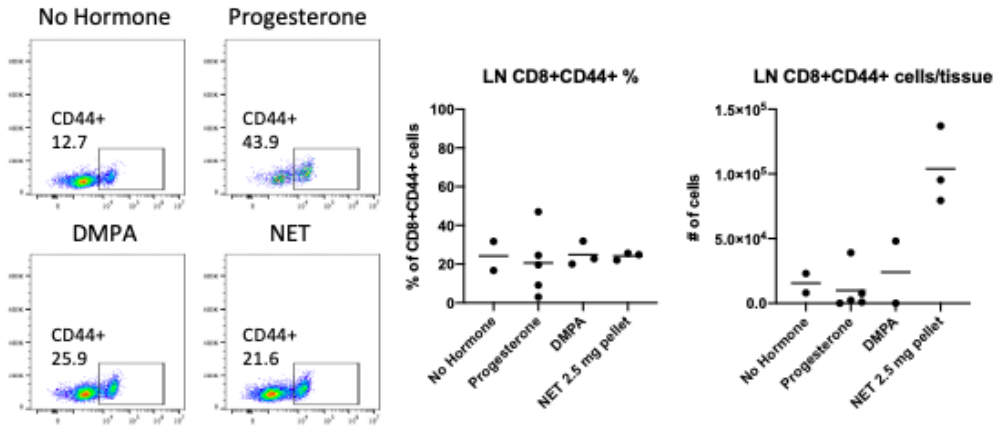

Figure 78. Graphs of percentages of CD8+CD44+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

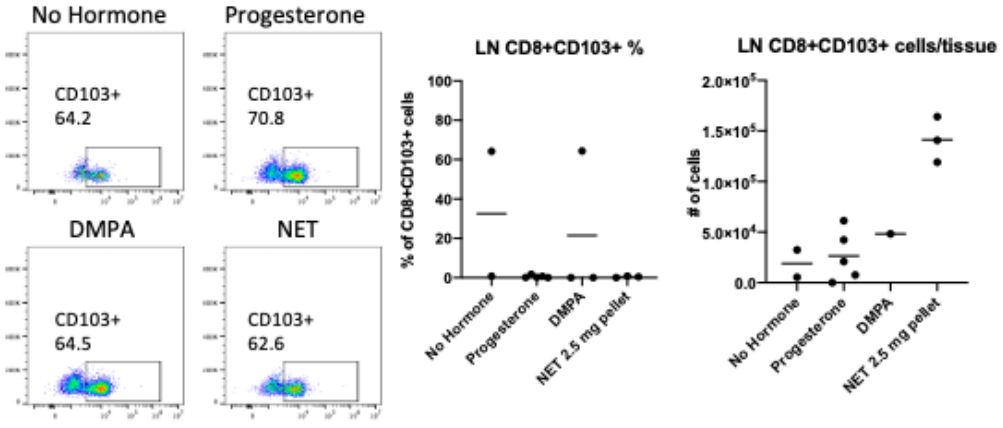

Figure 79. Graphs of percentages of CD8+CD103+ cells from the iliac lymph nodes of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group.

Flow plots show a representative image for each group. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

7.2.2 Spleen – immunization and challenge under progesterone, DMPA and NET 2.5 mg

In the spleen samples, the no hormone control group displayed lower percentages of CD4+ cells compared to the progesterone and DMPA groups which was statistically significant (Figure 80). Additionally, the no hormone group displayed significantly higher percentages of CD8+ cells compared to the progesterone and DMPA groups. The no hormone control group also showed significantly lower percentages of CD4+IFN-γ+ cells compared to the DMPA, which showed the highest percentage of CD4+IFN-γ+ cells (Figure 81). This was also reflected in the cell counts of CD4+IFN-γ+ cells. In the spleen, CD8+IFN-γ+ cells were also examined; however, no significant differences were found between groups (Figure 82).

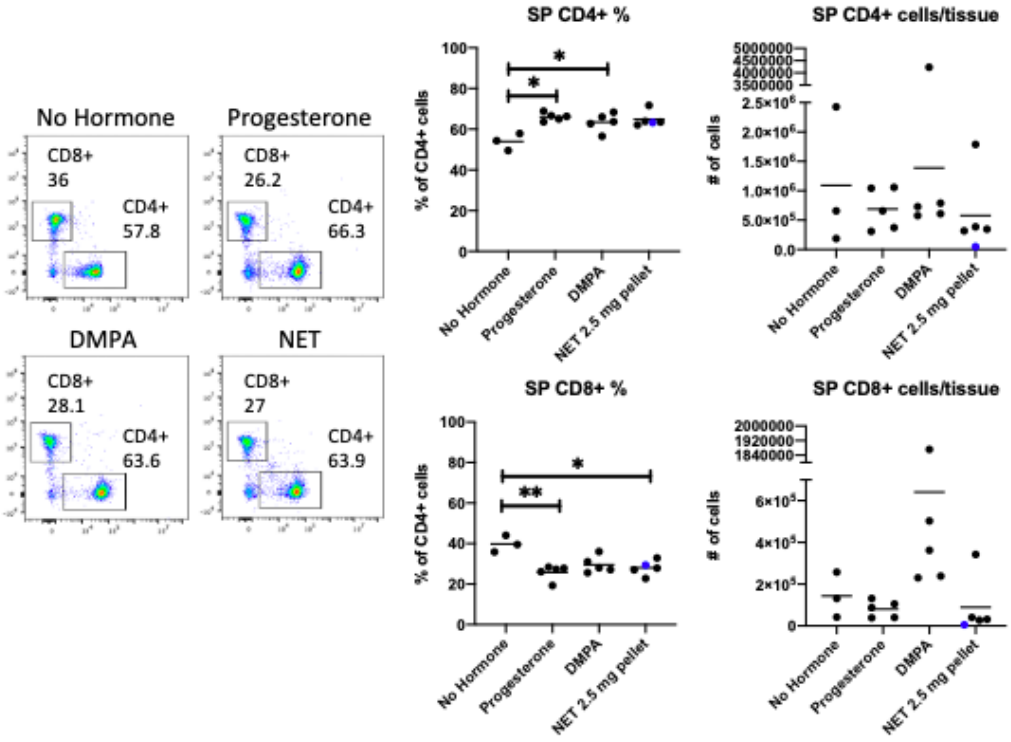

Figure 80. Graphs of percentages of CD4+and CD8+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. The blue points indicate one mouse that was not successfully immunized. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *$p<0.05$, **$p<0.01$.

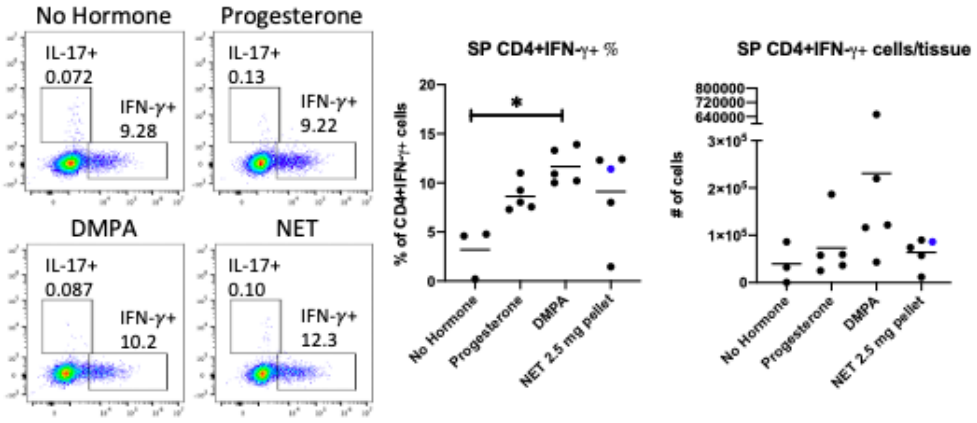

Figure 81. Graphs of percentages of CD4+IFN-γ+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. The blue points indicate one mouse that was not successfully immunized. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *p<0.05.

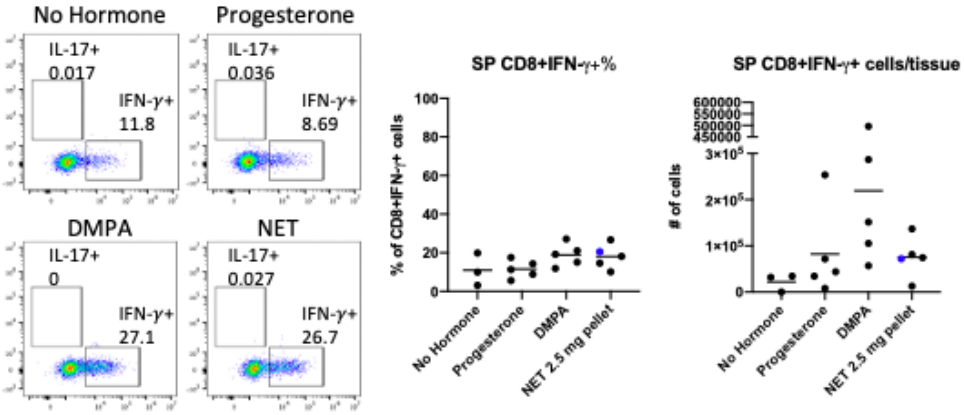

Figure 82. Graphs of percentages of CD8+IFN-γ+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a

representative image for each group. The blue points indicate one mouse that was not successfully immunized. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

There was also one mouse in the NET 2.5 mg group that was not successfully immunized. This is represented in Figures 80-86 by the blue points. This one mouse that was not immunized showed low cell counts for CD4+ and CD8+ cells, compared to other mice in the NET group that were immunized (Figure 80). Overall, the one mouse that was not immunized shows similar percentages and cell counts for the various immune cell phenotypes analyzed that look similar to the rest of the immunized mice in the NET group. This showed that a lack of immunization did not create large differences in immune cell phenotypes and cell counts between the immunized mice.

Memory T cell subsets were also analyzed in the spleen. The no hormone group also displayed a lower percentage of CD4+CD44+ cells compared to the DMPA group, which was statistically significant (Figure 83). When other memory T cell subsets were analyzed, no significant differences were observed (Figures 84-86).

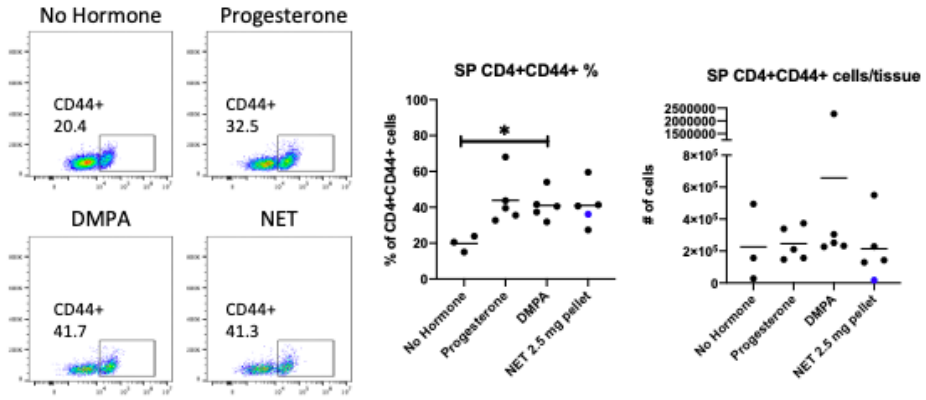

Figure 83. Graphs of percentages of CD4+CD44+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments

include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. The blue points indicate one mouse that was not successfully immunized. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. *p<0.05.

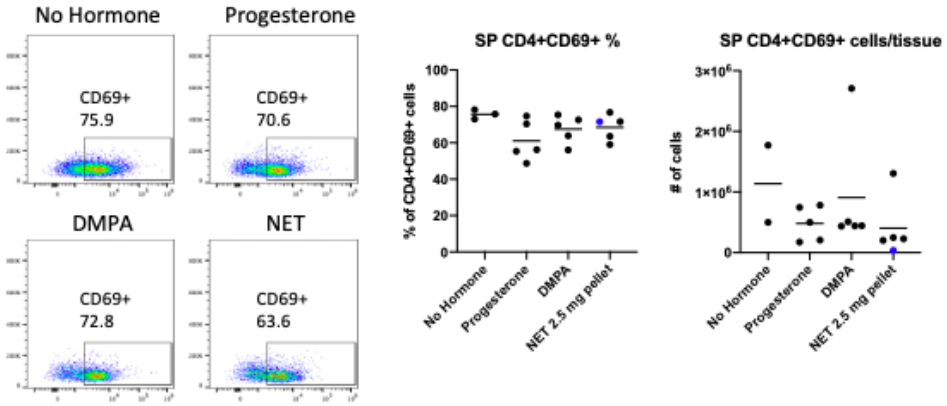

Figure 84. Graphs of percentages of CD4+CD69+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. The blue points indicate one mouse that was not successfully immunized. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

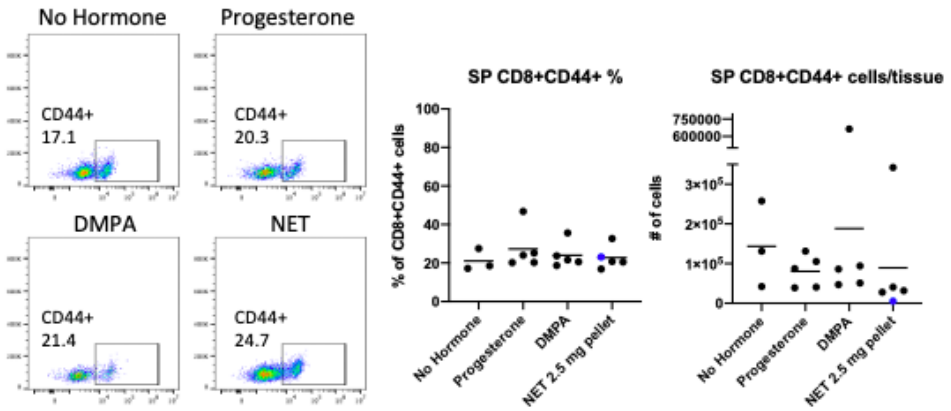

Figure 85. Graphs of percentages of CD8+CD44+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. The blue points indicate one mouse that was not successfully immunized. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

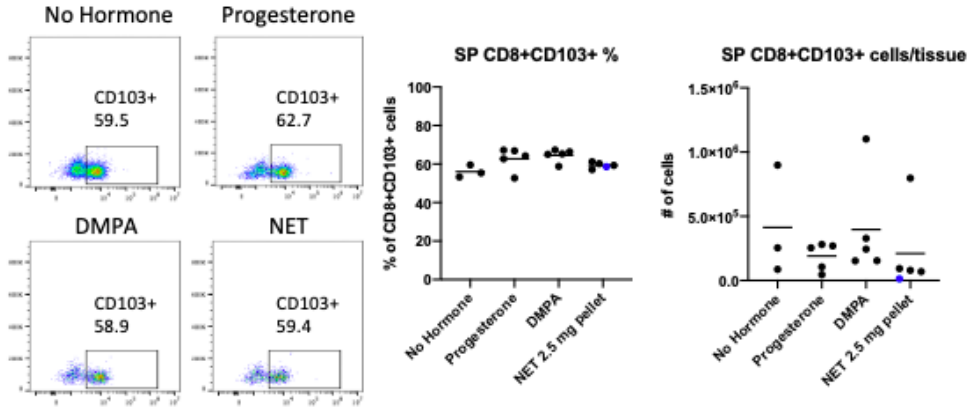

Figure 86. Graphs of percentages of CD8+CD103+ cells from the spleens of ovariectomized mice after immunization and challenge under different hormone treatments. The hormone treatments include progesterone, DMPA, NET 2.5 mg and a no hormone control group. Flow plots show a representative image for each group. The blue points indicate one mouse that was not successfully immunized. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test.

7.2.3 Vaginal tract – immunization and challenge under progesterone, DMPA and NET 2.5 mg

Cells in the vaginal tract were also analyzed, however, no significant differences were observed between these samples.

Overall, the NET 5 mg treated group showed some trends of increased CD8+ cells in the spleen compared to the DMPA group (Figure 70). Compared to the progesterone group, the NET 5 mg treated group showed displayed higher percentages of CD4+ cells in the spleen, while the DMPA group displayed lower percentages of CD4+ cells compared to progesterone (Figure 70). However, with the NET 2.5 mg treatment, in the iliac lymph nodes there were trends of increased CD4+, CD8+, CD4+CD44+, CD4+CD69+, CD4+IFN-γ+, CD8+IFN-γ+, CD8+CD44+ and CD8+CD103+ cells (Figures 73-79). In the spleens, there were trends of decreased cells in the ovariectomized no hormone control group in CD4+, CD4+IFN-γ+, CD8+IFN-γ+ and CD4+CD44+ cells compared to the hormone treated groups (Figures 80-83). These results show some differences in immune cell proportions and numbers between different hormone treatments as well as no hormone control mice. However, more experiments would need to be conducted to further validate the trends observed and whether hormone treatment does affect immune cells and have any influence on immune responses.

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