EXAMINING THE EFFECT OF ESTRADIOL ON B CELL RESPONSES AGAINST HERPES SIMPLEX VIRUS TYPE-2

EXAMINING THE EFFECT OF ESTRADIOL ON B CELL RESPONSES AGAINST HERPES SIMPLEX VIRUS TYPE-2

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ABSTRACT

Problem: Herpes simplex virus type-2 (HSV-2) is one of the most prevalent sexually transmitted infections in the world, and rates of infection are higher in women compared to men. Furthermore, vaccines developed against HSV-2 have failed at various stages of clinical trials, due to their inability to induce protective mucosal immunity. In animal models, intranasal (IN) immunization with attenuated HSV-2 (TK^-) virus has been shown to confer protection against wildtype HSV-2 challenge. Since IN immunization serves as a more practical and less intrusive vaccination strategy, further studies are warranted to characterize optimal immune responses following IN immunization. We have previously demonstrated that estradiol (E_2) treatment promotes enhanced protection against HSV-2 through enhanced anti-viral T cells responses. However, the effect of E_2 on B cell responses, which were recently shown to be critical in protecting the host following IN immunization, remain poorly understood. Therefore, in this study we aimed to examine if following IN immunization, E_2 enhances the memory B cell (MBC) and antibody-secreting plasma cell populations within the secondary lymphoid tissues and nasal effector sites, and whether this enhancement leads to an overall better protection against intravaginal IVAG WT-HSV-2 challenge.

Methodology: Ovariectomized (OVX) mouse model of HSV-2 were pre-treated with E_2 or placebo pellets. Subsequently, both groups were immunized intranasally with TK^- HSV-2. Four weeks later nasal associated lymphoid tissues, nasal mucosa, cervical and iliac lymph nodes, spleen and vaginal tract were collected and processed and MBC and antibody-secreting plasma cells were characterized by flow cytometric analysis. HSV-2 specific IgM and IgG antibody responses in serum and vaginal secretions were measured by ELISA. In parallel experiments, animals were IVAG challenged with WT-HSV-2 and the B cell subsets were characterized as above.

Results: The formation of MBC subsets, as seen by the presence of $CD19^+$ IgD^- cells and the heterogenous expression of CD73, CD80, and PD-L2, were observed four-weeks post immunization within the cervical and iliac lymph nodes and spleen, which

were further enhanced in the presence of E_2. Additionally, E_2-treated mice had increased number of $B220^-$ $CD138^+$ $IgG2c^+$ plasma cells within the nasal mucosa following immunization. These enhancements translated into increased levels of HSV-2 specific IgG2b and IgG2c antibodies within the serum and vaginal secretions of E_2-treated mice at four-weeks post IN immunization. Upon IVAG challenge, E_2-treated mice, but not control mice, were protected. Since the antibody isotypes that were enhanced in E2 treated mice are correlated with Th17 responses, E2 mediated antibody enhancement was tested in IL-17 knockout mice. E_2 treatment in IL-17-knockout mice failed to induce similar responses observed in WT mice, indicating that the enhancement of B cells and antibodies seen following E2 treatment was mediated in an IL-17 dependent manner.

Conclusion: This study highlights the importance of sex-dependent differences in vaccine-induced immunity. Specifically, the findings from this study will provide valuable information for the design of a potentially efficacious mucosal vaccine strategy, whereby immunization in the context of E2 could significantly enhance antigen-specific antibody responses in the genital tract.

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work to my loved ones who I've lost during my time as a graduate student. To my grandparents, your memories are something I will always hold onto. To Kiana, I will never understand why you had to leave so young. You were supposed to be here making a better life for yourself and fulfilling your dreams. We were supposed to create so many more memories together, but instead I will cherish the ones we already have and hold on to them dearly. You are truly missed.

DECLARATION OF ACADEMIC ACHIEVEMENT

All the experiments were conceived and designed by Ramtin Ghasemi and Dr. Charu Kaushic. Ramtin Ghasemi performed all experiments. Ramtin Ghasemi wrote this dissertation with contributions from Dr. Charu Kaushic.

Table of Contents

DESCRIPTIVE NOTES I
ABSTRACT II
ACKNOWLEDGEMENTS IV
DECLARTION OF ACADEMIC ACHIEVEMENTS vi
TABLES OF CONTENTS vii
LIST OF FIGURES AND TABLES ix
LIST OF ABBREVIATIONS xi

CHAPTER 1: INTRODUCTION 1

1.1 EPIDEMIOLOGY AND FEMALE SUSCEPTIBILITY TO HSV-2 1
1.2 BIOLOGICAL PROPERTIES AND REPLICATION OF HSV-2 2
1.3 PATHOGENESIS OF HSV-2 3
1.4 ANTIVIRAL TREATMENTS FOR HSV-2 4
1.5 HSV-2 VACCINE DEVELOPMENT AND CLINICAL ADVANCEMENTS TO DATE 6
1.6 ENDOCRINE CONTROL OF THE IMMUNE SYSTEM IN THE HUMAN FEMALE GENITAL TRACT 10
1.7 MURINE MODEL OF HSV-2 11
1.8 INNATE IMMUNE RESPONSES TO HSV-2 12
1.9 ADAPTIVE IMMUNE RESPONSES TO HSV-2 14
1.9.1 CELL-MEDIATED IMMUNITY 14
1.9.2 HUMORAL IMMUNITY 16
1.10 UPPER RESPIRATORY TRACT AS ANTI-VIRAL INDUCTIVE SITE 19
1.11 THE ROLE OF FEMALE SEX HORMONES ON SUSCEPTIBILITY TO HSV-2 INFECTION IN MURINE ANIMAL MODEL 21
1.12 COGNATE T-B CELL INTERACTION DRIVES B CELL ISOTYPE CLASS SWITCH RECOMBINATION 23

CHAPTER 2: RATIONALE AND HYPOTHESIS 24

CHAPTER 3: MATERIAL AND METHODS 28

3.1 ANIMAL MODEL PROTOCOLS 28
3.1.1 MICE 28
3.1.2 OVARIECTOMIES 28
3.1.3 HORMONAL PELLET IMPLANTATION 28
3.1.4 TK$^-$HSV-2 IN IMMUNIZATION 29
3.1.5 WT HSV-2 INTRAVAGINAL CHALLENGE 29
3.1.6 COLLECTION OF VAGINAL SECRETIONS 29
3.1.7 SERUM COLLECTION 30
3.1.8 GENITAL PATHOLOGY 30
3.1.9 PLAQUE ASSAYS 30
3.2 TISSUE ISOLATION AND PROCESSING 31
3.2.1 VAGINAL TRACT 31
3.2.2 NASOPHARYNX-ASSOCIATED LYMPHOID TISSUE 32
3.2.3 NASAL MUCOSA 32
3.2.4 LYMPH NODES AND SPLEEN 33

3.3 FLOW CYTOMETRIC ANALYSIS **33**
3.4 ANTI-HSV-2 IgM AND IgG ELISA **34**

CHAPTER 4: RESULTS **36**

4.1 CHARACTERIZE B CELL AND ANTIBODY RESPONSES POST-IMMUNIZATION AND POST-CHALLENGE AND DETERMINE THE EFFECT OF ESTRADIOL **36**
4.1.1 ASSESSMENT OF B CELL RESPONSES FOLLOWING INTRANASAL IMMUNIZATION AND INTRAVAGINAL CHALLENGE AND DETERMINING THE EFFECT OF ESTRADIOL 37
4.1.2 CHARACTERIZING B CELLS AND ANTIBODY RESPONSES FOLLOWING INTRANASAL IMMUNIZATION AND DETERMINING THE EFFECT OF ESTRADIOL 47
4.1.3 DETERMINING WHETHER ESTRADIOL INCREASES THE REPERTOIRE OF MEMORY B CELLS AND ANTIBODY-SECRETING PLASMA CELLS IN THE ABSENCE OF ANTIGENIC STIMULATION 60
4.2 DETERMINE WHETHER IL-17 MEDIATES ESTRADIOL-ENHANCED B CELL RESPONSES THROUGH AUGMENTED TH1 AND TH17 CELLS **65**
4.2.1 ELUCIDATING WHETHER THE ESTRADIOL EFFECT OBSERVED POST IMMUNIZATION IS MEDIATED BY IL-17 65
4.2.2 DETERMINING WHETHER THE E_2 EFFECT OBSERVED ON B CELL RESPONSES POST-INTRAVAGINAL CHALLENGE IS MEDIATED BY IL-17 75
4.3 FIGURE SUMMARY **78**

CHAPTER 5: DISCUSSION **80**

5.1 SUMMARY OF FINDINGS AND IMPLICATIONS **80**
5.2 LIMITATIONS **96**
5.1 FUTURE DIRECTIONS **99**
5.2 CONCLUSION **100**

CHAPTER 6: REFERENCES **101**

LIST OF FIGURES AND TABLES

Figure 1. Flow cytometric gating strategy for characterizing memory B cell and plasma cell subsets.

Figure 2. Vaginal viral shedding within the first five days post IVAG challenge.

Figure 3. E_2-treated mice have increased proportion of plasma cells at day 5 post IVAG challenge.

Figure 4. E_2-treated mice have increased levels of HSV-2 specific IgG2b and IgG2c in serum and vaginal secretion at day 5 post IVAG challenge.

Figure 5. Intranasal immunization experimental design.

Figure 6. $B220^+$ B cells are not present in the FGT in the absence of active local infection.

Figure 7. E_2-treated mice have increased serum but not vaginal levels of HSV-2 specific IgG2b and IgG2c at four weeks post IN immunization.

Figure 8. E_2-treated mice have increased memory B cell formation within peripheral secondary lymphoid tissues following intranasal immunization.

Figure 9. The proportion of IgG2c-secreting plasma cells is enhanced in nasal mucosa of E_2-treated mice four weeks post IN immunization.

Figure 10. Experimental design of E_2 treatment of immunologically naïve mice.

Figure 11. E_2 does not enhance the formation of MBCs within the secondary lymphoid tissues of immunologically naïve mice.

Figure 12. The proportion of IgG2c-secreting plasma cells is not altered in nasal mucosa of naïve E_2-treated mice.

Figure 13. In the absence of IL-17, the effect of E_2 on MBC formation was ablated following intranasal immunization.

Figure 14. E_2 -mediated enhancement of IgG2c-secreting plasma cells within the nasal mucosa is abrogated in IL-$17^{-/-}$ mice at four weeks post intranasal immunization.

Figure 15. In the absence of IL-17, E_2-treated mice have similar serum levels of IgG2b and IgG2c at four weeks post IN immunization.

Figure 16. In the absence of IL-17, E_2-treated mice have similar B cell profile in FGT to their placebo counterpart follow IVAG challenge with WT-HSV-2.

Figure 17. Study Summary.

Supplementary Figure. HSV-2 specific IgG ELISA optimization.

Table 1. B cell phenotype based on surface marker expression.

LIST OF ABBREVIATIONS

APC: Antigen presenting cells

CTL: Cytotoxic T lymphocyte

DC: Dendritic cells

DNA: Deoxyribonucleic acid

DMPA: Depo-medroxyprogesterone acetate

ELISA: Enzyme-linked immunosorbent assay

ERKO: Estrogen receptor knockout

FGT: Female genital tract

GC: Germinal center

HIV-1: Human immunodeficiency virus

HSP: Heat shock protein

HSV-2: Herpes simplex virus type-2

IgA: Immunoglobin A

IgD: Immunoglobulin D

IgG: Immunoglobin G

IgM: Immunoglobulin M

IFN: Interferon

IRF: Interferon regulatory factors

IL-4: Interleukin 4

IL-8: Interleukin 8

IL-17: Interleukin 17

IN: Intranasal

IVAG: Intravaginal

LC: Langerhans cells

LAT: Latency-associated transcript

M: Microfold cells

MBC: Memory B cells

MCP-1: Monocyte Chemoattractant Protein

NALT: Nasopharynx-associated lymphoid tissue

NK: Natural killer cells

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

OVX: Ovariectomized

PRRs: Pattern recognition receptors

pDC: Plasmoacytoid dendritic cells

SIgA: Secretory immunoglobulin A

STI: Sexually transmitted infection

TCR: T cell receptor

Tfh: T follicular helper

TK: Thymidine kinase

T_{RM}: Tissue resident memory

TNF-α: Tumour Necrosis Factor

URT: Upper respiratory tract

Vhs: virion host shutoff

WT: Wildtype

CHAPTER 1: INTRODUCTION

1.1 Epidemiology and female susceptibility to HSV-2

Herpes Simplex virus-2 (HSV-2), the primary cause of genital herpes, is one of the most prevalent sexually transmitted infections (STI) in the world. HSV-2 primary infects the genital mucosal epithelial layer and establishes latency in sensory ganglia.[1] Globally, an estimated 417 million people between the age 15-49 years were living with HSV-2 infection in 2012 and an estimated 19.2 million are newly infected annually.[1] Moreover, the patterns of infection is drastically higher in developing countries with seroprevalence reaching a maximum of 70% in sub-Saharan Africa.[2] Furthermore, the prevalence and incidence of HSV-2 differ between sexes; women account for more than 60% of the infections. A large proportion of the individuals infected with HSV-2 do not experience clinical signs and symptoms constantly, however, periodically may experience symptomatic reactivation of the disease.[3] During these symptomatic episodes, infected individuals may have painful genital lesion. Importantly, immunosuppressed individuals may experience these episodes more frequently.[3,4] There are also comorbidities that may arise such as pregnancy complications, proctitis, and meningitis.[5,6] Of note, individuals seropositive for HSV-2 are at an increased risk of acquisition of other sexually transmitted infections.[7] In particular, the risk of human immunodeficiency virus (HIV-1) acquisition is three fold higher in HSV-2-infected individuals and antiviral therapy with acyclovir fails to reduce this risk.[7,8]

1.2 Biological properties and replication of HSV-2

HSV-2, a member of Herpesvirus family, consists of a core containing a relatively large double stranded, linear deoxyribonucleic acid (DNA) genome enclosed by icosahedral capsid.[9] The capsid is tightly adhered to tegument; an amophrous layer containing viral proteins critical for infection initiation. Loosely surrounding the tegument is a lipid bilayer which consists of polyamines, lipids, and glycoproteins. The glycoproteins mediate virus entry and fusion between the viral envelope and cell membrane, which is subsequently followed by viral replication within the host cell.

Transmission of genital herpes most frequently occurs through close contact of abraded skin or mucosa with body fluids of an infected person, typically during sexual intercourse.[3] Upon transmission, HSV-2 primarily infects the epithelial cells in the genital mucosa.[3] The attachment and entry of HSV-2 into the epithelial cells involves a cascade of interactions mediated by the viral glycoproteins (gB, gC, gD, gH, and gL).[10] The first step involves the binding of the highly conserved gB or gC to heparin sulfate chains expressed on cell surface proteoglycans.[11] Next the glycoprotein D (gD), a key determinant in viral tropism, interacts with its complementary cell-surface receptors nectin 1, HVEM, or modified heparin sulfate, and through unknown mechanisms activates the fusogenic activity of gB, gH, and gL.[12] Subsequently, the gH and gL form a heterodimer while gB forms a homotrimer and stimulate viral fusion. Upon entry, the de-enveloped nucleocapsid is transported to the host cell nucleus through the nuclear pores where the viral DNA is introduced into

the cellular nucleoplasm.[13] Since HSV-2 undergoes a lytic replication cycle within the vaginal epithelial cells, the macromolecular synthesis pathways are suppressed in this cell type through the action of virion host shutoff (vhs). Subsequently, the VP16 protein expression activates the immediate-early (α) regulatory genes which are required for the successive transcriptional activation and expression of the delayed-early (β) genes.[14] These genes encode for a variety of proteins and ultimately induce transcription and expression of late (γ) genes which are translated into structural components of the virion.[10] Lastly, the viral capsid and tegument proteins are formed and the viral replication process is complete.[10]

1.3 Pathogenesis of HSV-2

Following the lytic replication, infectious virions go on to infect the surrounding epithelial cells or ultimately invade the nerve endings present throughout the vaginal tissue and establish latency.[15] Within the axon termini, the nucleocapsid is transported to the neuronal cell body of the neuron in a ganglion.[16] The retrograde transport of the virion is mediated by the movement of dynein along the microtubules that line the length of the axon.[15] Once the virion reaches the soma of the neuron, it undergoes a similar mechanism to that used during lytic replication to exude its DNA into the nucleus. Within the nucleus, the HSV DNA circularizes and forms episomes.[16] The exact mechanism by which latency is initiated is still unclear; however the latency-associated transcript (LAT), highly expressed in neuronal nucleus, appears to suppress lytic gene expression and induce latency.[16] Reactivation of HSV-2 from latency leads to expression of the lytic genes. Subsequently, the virus

is transported from the cell body back into axonal termini of the initial site of infection by anterograde transport and released onto the epithelial cells. The reinfection of the epithelium by the virus allows for lytic replication and subsequent host cell lysis; in turn leading to the symptoms commonly associated with genital herpes.[17]

The most severe symptoms of HSV-2 are often associated with primary exposure, however many patients are often unaware of infection.[18] In clinically symptomatic episodes of the infection, release of virus from nerve endings into the epithelium create genital ulcers, which expand further by cell-associated HSV-2.[19] Additional lesions may appear in close proximity; however these develop from cell-free virus released from the initial site of infection.[19] Mathematical studies suggest that virus reactivation and virus shedding may occur even in the absence of clinical symptoms. In a study by Schiffer et al[20], mathematical modeling revealed a constant virus reactivation in a small number of neurons at a given time, which may translate into a continuous low-grade release of HSV-2 into the genital epithelium that may be undetectable. Consequently, the risk of transmission of HSV-2 from an infected individual to a susceptible partner is present even in the absence of clinical symptoms.

1.4 Antiviral treatments for HSV-2

The current treatments available for HSV-2 fail to eliminate the disease and prevent viral transmission to other hosts. Instead, these treatments aim to alleviate the clinical symptoms, reduce transmission and recurrence.[21] Currently, the standard treatment for clinical use are acyclic nucleoside analogues which act by targeting the viral DNA synthesis.[22] These include Acyclovir, Valacyclovir, and Famciclovir.

Typically, Acyclovir is the first choice as a therapeutic against HSV-2. By competing with deoxyguanosine triphosphate for phosphorylation by HSV-2 thymidine kinase, it terminates the elongation of viral DNA. Specifically, the triphosphate form of Acyclovir inhibits the viral DNA polymerase by being incorporated into the growing viral DNA chain. This leads to chain termination since the 3′ hydroxyl groups which are required for further linkage are missing.[23] Valacyclovir is a prodrug of Acyclovir and has which is converted into Acyclovir by the hepatic enzyme valacyclovir hydrolase after ingestion.[18] Famciclovir is a prodrug of Penciclovir. It has similar mechanism of action to Acyclovir and Valacyclovir and acts by utilizing thymidine kinase for phosphorylation to Penciclovir-triphosphate.[24] However, unlike Acyclovir and Valacycloir, Penciclovir-triphosphate is not an obligate chain terminator.[21]

In addition to the commonly used drugs mentioned, other alternative treatments options are available in case of acyclovir/valacyclovir resistance which may develop in immunosuppressed or HIV-1 positive indiviudals.[21] One such drug is Foscarnet which is a pyrophosphate analogue. It acts by binding near the pyrophosphate-binding site of viral DNA polymerase. This blocks the cleavage of pyrophosphate moiety from deoxynucleotide triphosphates, effectively terminating DNA chain elongation. [25] Given that this drug does not act by utilizing thymidine kinase, it is also an effective treatment option against TK^- strains of HSV-2. In addition to Fosarnet, a new class of drug called Pritelivir is currently being tested in Phase II clinical trials.[21,26] Unlike other treatments against HSV-2, Pritelivir is a helicase blockers and acts by binding to the helicase-primase complex. This effectively inhibits

DNA synthesis and in turn viral replication. In fact, both *in vivo* and *in vitro* studies have shown that Pritelivir has superior efficacy to acyclovir and valacyclovir in treating HSV-2 as indicated by greater reduction of clinical symptoms and viral shedding.[26,27]

Although these antiviral treatments are important for limiting disease severity, they cannot eliminate the disease. Furthermore, HSV resistant strains to the currently available antiviral treatments are emerging.[21] More importantly, several studies have indicated that individuals infected with HSV-2 are at an increased risk of acquiring HIV-1.[3,7,28] Therefore, a prophylactic or therapeutic vaccine against HSV-2 which can eliminate the disease is still favoured.

1.5 HSV-2 Vaccine development and clinical advancements to date

An effective vaccine against HSV-2 has been highly sought after given the shortcomings of the treatments currently available. A number of different formulations have been tested but a successful HSV-2 vaccine remains elusive.

Early attempts include subunit vaccines enriched in viral glycoproteins which were tested in randomized, blinded, and placebo controlled clinical trials.[29–35] Although these vaccines elicited virus-specific antibodies and stimulated T cell proliferation to some extent, no difference was observed in protection against HSV-2 between the vaccinated and placebo groups. Subsequently, another promising subunit vaccine, called gD2-alum/MPL, was clinically tested.[36] This vaccine consisted of recombinant gD2 glycoproteins and a mixture aluminium hydroxide, and a 3-deacylated monophosphoryl lipid A adjuvants and was able to induce neutralizing

antibody and $CD4^+$ T cell responses. Further testing revealed that the vaccine was not efficacious in men or HSV-1 seropositive women, however it was successful in reducing HSV-2 disease by 70% and HSV-2 infection by 40% in the subgroup of women who were HSV-1 and HSV-2 seronegative. Vaccinated individuals showed reduced symptomatic reoccurrence of HSV-2 disease by 73% and new acquisition of HSV-2 infection by 40% in this subgroup.[37] Further testing with the same vaccine did not show any efficacy in any cohort.[38] Just recently, a therapeutic subunit vaccine called GEN-003 completed a successful phase II trial.[39] This subunit vaccine consisted of a transmembrane deleted mutant of glycoprotein D, and an internal fragment of immediate early protein ICP4 combined with Matrix M2. The results indicate the vaccine was efficacious since it reduced viral shedding and recurrence rates and shortened duration of recurrence of vaccinated individuals.[39] Unfortunately, the developers of GEN-003 are not conducting further evaluations.

Peptide-based vaccines against HSV-2 have also been explored. These vaccines are specifically designed to contain T cell epitopes that are known to elicit cell-mediated responses and protect against HSV-2.[40] Typically, the peptides are non-covalently complexed to heat shock proteins (HSP) which act as delivery vehicle for the peptides. To date, only the HerpV vaccine has shown promising efficacy in clinical trials.[41] This vaccine consisted of 32 synthetic HSV-2 peptides predicated to contain HSV-specific T cell epitopes and Phase I testing found the vaccine to be both safe and highly immunogenic by eliciting broad $CD4^+$ and $CD8^+$ T cell responses in HSV-2 positive participants.[41] Furthermore, results from phase II trials found a 15%

reduction in viral shedding and 34% reduction in viral load of the vaccinated participants.[42] Despite promising results, further testing of this vaccine has halted.

Typically, given their superior safety profile, subunit vaccines and peptide-based vaccines are preferred over live attenuated virus-based vaccines.[43] Live attenuated vaccines retain their pathogenic potential and bear the risk of reverting to wildtype virus. However, these vaccines are capable of eliciting more robust, longer-lasting and broader immunity.[44] As such, a number of live-attenuated vaccines candidates against HSV-2 has been developed and clinically tested.[3,40] One of the first live-attenuated vaccines clinically tested was the HSV-1 mutant deficient in glycoprotein gH, known as DISC which had reduced replication capabilities and protected against intravaginal (IVAG) HSV-2 in guinea pigs.[45] In phase I clinical trial, this vaccine was found to be well tolerated and immunogenic. However it failed to meet clinical endpoints and its development was halted.[46] Recently, phase I testing was completed for a live attenuated, replication-deficient HSV-2 vaccine (HSV529).[47] This vaccine lacks the U_L5 and U_L29 genes which are critical for viral DNA synthesis and replication.[48] The results from this trial indicate that the vaccine is both safe and elicits neutralizing antibodies and a modest $CD4^+$ T cell responses.[47] However, further testing is required to prove it's efficacy against HSV-2.

A radically new approach to vaccination is DNA-based vaccines which are becoming increasingly popular. While they are able to elicit comparable B cells and T cell responses, they outcompete other formulations by having superior stability and safety and lower manufacturing costs.[3] To date, three DNA-based vaccine candidates

have reached Phase II of clinical trials.[49,50] The first candidate, named VCL-HB01/HM01, was constructed by cloning coding DNA sequences of gD2, UL46 and UL47 genes into plasmid DNA containing the hCMV immediate early promoter.[49] Although VCL-HB01/HM01 was found to be immunogenic, it failed to meet primary endpoints during phase II testing.[51] The second DNA-based vaccine, called COR-1, recently completed a successful phase IIa testing..[50,52] This vaccine is mixture of two plasmids in equal proportion. The first plasmid contains full length gD DNA sequence while the second consists of ubiquitin-fused truncated gD2 to enhance cytotoxic T cell responses. Even though vaccine-induced antibody responses were not observable, cell-mediated responses were observed in 95% of the vaccinated participants. Currently, phase IIb testing is underway to test and improve its efficacy.[52]

All in all, a multitude of vaccine candidates with different formulations have failed to show efficacy in clinical trials. This indicates that in addition to vaccine formulation and adjuvant used, other factors that modulate vaccine induced immunity must be taken into account. For example, the finding that gD2-alum/MPL vaccine was only protective in women highlights the importance gender-specific immune responses which should be considered during vaccine development. Another important factor is the route of immunization. Genital HSV-2 is a mucosal infection and a number of animal studies have shown that mucosal immunization elicits enhanced local immune responses and leads to superior protection.[37,53,54] This is mainly attributed to the existence of a common mucosal immune system that consists of the respiratory, gastrointestinal, and genital mucosa.[55–57] However, the exact

dynamics are not fully understood and further research is required to better understand how a mucosal HSV-2 vaccine could elicit enhanced anti-viral immunity in the FGT.

1.6 Endocrine control of the immune system in the human female genital tract

The sex hormones also influence distinct immune compartments of the human FGT throughout the menstrual cycle to maintain equilibrium between effectively fighting infectious pathogens and immune regulation and tissue modelling that is required for successful implantation and pregnancy.[58] While immune cell numbers in the lower FGT are not altered and remain constant throughout the menstrual cycle, immune cell numbers in the endometrium are known to increase during the late secretory phase and menstruation.[59] Furthermore, the dynamic leukocyte population account for 6-20% of all cells in the FGT. The T cells constitute 40-50% of leukocyte population in the FGT and they are differentially distributed between the upper and lower FGT. $CD8^+$ T cells predominate in the uterus and endometrium, whereas in the lower FGT, both $CD4^+$ and $CD8^+$ T cells are equally abundant.[60] During the proliferative phase of the menstrual cycle, $CD8^+$ T cells in the uterus are found as scattered T cells and small aggregate in the stroma or as intraepithelial lymphocytes. While their numbers stay relatively constant, uterine $CD8^+$ T cells undergo a uterine site-specific condensation in the lamina basalis, resulting in lymphoid aggregate formation. Lymphoid aggregates, which mainly consist of a B cell core, surrounded by memory CD8+ T cells and macrophages, peak in size at mid-cycle and persist during the secretory phase.[61] Furthermore, during the secretory phase $CD8^+$ cytotoxic T

lymphocyte (CTL) activity is suppressed in the endometrium, presumably to minimize rejection of the allogeneic sperm and the semi-allogeneic fetus.[61]

In contrast to T cells, B cells constitute only a small population throughout the FGT. Plasma cells producing IgG or IgA are predominately found in the cervix and, to a lesser extent, in the vagina.[62] In contrast to other mucosal surfaces, cervico-vaginal secretions are characterized by greater amount IgG than IgA.[62–64] Both uterine and cervico-vaginal levels of IgA and IgG are regulated by the sex hormones. In the endometrium, IgA and IgG levels increase during ovulation, whereas in cervical secretion, both IgG and IgA levels are lowest at the mid-secretory phase.[62,65] The decrease of IgA and IgG levels in the lower FGT is thought to reduce sperm-specific antibodies.

Macrophages constitute 10-20% of the FGT leukocytes and their population remains constant throughout the menstrual cycle. On the other hand, NK cells constitute 30-40% of total immune cells in FGT and have distinct site-specific phenotypes. Ectoervical and vaginal NK cells are phenotypically similar to the cytotoxic NK cells in the blood.[66] Conversely, endometrial NK cells display low cytotoxic activity and their IFN-γ expression is suppressed by epithelial cell production of TGF-β. Furthermore, the cytotoxicity of NK cells and their perforin production is inhibited by P4.[67,68]

1.7 Murine model of HSV-2

The immunological features and the pathogenesis of HSV-2 infection have been extensively investigated in mouse models of IVAG HSV-2 infection.[69–71] Normal

cycling female mice are refractory to HSV-2 IVAG infection during estradiol-dominated estrus phase and only become susceptible during progesterone-dominated diestrus phase of their estrous cycle.[72] To overcome the variation in susceptibility to HSV-2 infection, many studies utilize Depo-provera (Depo-medroxyprogesterone acetate, (DMPA)) injection prior to infection.[70] However, administration of DMPA has been found to increase susceptibility to various STIs including HSV-2. Specifically, we have shown that DMPA-treated mice are 100-fold more susceptible to HSV-2 infection compared to untreated mice.[73]

Murine infections with wild-type HSV-2 (WT-HSV-2) are often fatal at high inoculum and low inoculum fail to establish infection. The development of less virulent, live attenuated strain of HSV-2 (TK^- HSV-2) containing a partial deletion of the thymidine kinase gene has been instrumental to the understanding of protective immunity against genital herpes.[74] Immunization with TK^- HSV-2 in DMPA treated mice elicited immunity against subsequent challenge with WT-HSV-2 and this strain of the virus has been used extensively to investigate immune responses against HSV-2 in a vaccination model.[74,75]

1.8 Innate immune responses to HSV-2

The infection of the genital mucosa by HSV-2 elicits immediate innate and subsequent adaptive responses in the female genital tract (FGT). Initially, the virus engages with pattern recognition receptors (PRRs) expressed on target keratinocytes and tissue-resident hematopoietic cells.[76]. Viral recognition by the PRRs initiates intracellular signaling pathways which activate the nuclear factor kappa-light-chain-

enhancer of activated B cells (NF-κB) transcription factor and interferon regulatory factors (IRF); ultimately leading to proinflammatory cytokine and chemokine (TNF-α, IL-8, MCP-1, and CXCL9) secretions and type I interferon (IFN) production.[77] A number of studies have shown type I IFN to be an essential component of the innate immunity against HSV-2 infection. It influences a number of immune populations which include plasmoacytoid dendritic cells (pDC) and natural killer (NK) cell activation, induction of optimal T helper 1 (Th1) development and cytokine production, activated T cell survival, and virally induced inducible NO synthetase activation.[78–81]

A number of different innate immune cells have been shown to be critical for induction of adaptive immune responses in addition to early control of viral replication. Initially, Langerhans cells (LC) in the epidermis become infected with HSV-2.[82,83] The infected LCs undergo apoptosis and are taken up by bystander migratory DCs which migrate to lymph nodes draining the vaginal mucosa and together with lymph node-resident DCs prime antigen-specific T cells.[84] In addition to the conventional DC, pDC, which are characterized by plasma cell morphology and absence of antigen presentation, are stimulated and secrete large quantities of type 1 IFN which limit viral replication during early stages of infection.[85] Meanwhile, activated NK cells eliminate virally infected cells by inducing apoptosis through the release of perforin and granzyme B.[86] In addition, they secrete IFN-γ which is essential in early control of viral replication by inducing nitric oxide production in epithelial cells, macrophages, and DCs.[87]

1.9 Adaptive immune responses to HSV-2

The role of the adaptive immune system in antiviral defense against genital HSV-2 infection has been well characterized. While earlier studies focused on antibody-mediated immunity, later studies determined a crucial role of cellular immunity, specifically $CD4^+$ T cells, against HSV-2.[3]

1.9.1 Cell-mediated immunity

Cytotoxic $CD8^+$ T cells are typically regarded as the hallmark of protection against vast majority of viral infections, however their protective role against HSV-2 infection in the mouse model has been shown to be insignificant.[88,89] Early studies indicated that virus-specific $CD8^+$ T cells have a protective role against HSV-2 since depletion of $CD8^+$ T cells prior to HSV-2 challenge led to increased viral titres and slightly delayed virus clearance.[90] However, later studies demonstrated that $CD8^+$ knockout mice were still protected against subsequent challenge with WT-HSV-2.[91]

In contrast to $CD8^+$ T cells, $CD4^+$ T cells have been shown to be central for protection against HSV-2 challenge in the mouse model. Both depletion studies and knockout models of $CD4^+$ T cells have shown that immunity following WT-HSV-2 challenge is principally dependent on $CD4^+$ T cells since $CD4^+$ depleted or knockout mice ultimately succumbed to infection.[90,92] Precisely, it is the secretion of IFN-γ by Th1 cells that have been shown to be protective as IFN-γ–deficient mice were not protected against HSV-2 challenge even though the $CD4^+$ T cell population was present.[91,92] Furthermore, in addition to Th1 cells, we have learned that Th17 cells contribute to HSV-2 antiviral immunity.[93,94] In our study, we used IL-17 knockout

(IL17$^{-/-}$) mice to determine its role during primary infection, re-exposure, and vaccination of HSV-2.[93] Although the survival and viral shedding did not differ between WT and IL-17$^{-/-}$ mice during primary genital infection; IL-17$^{-/-}$ mice had significantly higher rate of mortality, cumulative genital pathology, and viral shedding along with decreased IFN-γ levels in comparison to WT mice upon re-exposure to HSV-2.[93] Furthermore, the proportion of IFN-γ^{+} CD4^{+} tissue resident (T_{RM}) cells was significantly reduced in IL-17$^{-/-}$ post immunization and this coincided with attenuated Th1 responses upon challenge.[93]

In addition to CD4^{+} and CD8^{+} T cells, γδ^{+} T cells are present in abundance in the FGT.[95] Within the FGT, these subsets bear the highly invariant V1δ T cell receptor (TCR) and primarily modulate the homeostasis of the epithelium they reside in. A number of studies have examined the role of γδ^{+} T cells in context of HSV-2 immunity. While Milligan et al.[96] illustrated that γδ^{+} T cells are not critical for HSV-2 clearance, other studies have shown that V1δ TCR deficient-mice have impaired systemic Th1 responses and were more susceptible to IVAG HSV-2 infection.[97] Furthermore, we recently identified that, in addition to Th1 responses, IL-17 producing γδ^{+} T cells are critical for Th17 priming in the FGT.[98] Specifically, we found that vaginal antigen presenting cells (APCs) isolated from IL-17$^{-/-}$ mice were unable to prime Th17 responses in APC–T cell cocultures and further identified γδ^{+} T cells as the primary source IL-17 in the FGT.[98]

1.9.2 Humoral immunity

The role of B cells and different immunoglobulins in the context of HSV-2 infection has been studied extensively since the 1980s.[99–101]

The early studies demonstrated the presence of HSV-2 specific immunoglobulin G (IgG) and not secretory immunoglobulin A (SIgA) within the vaginal secretions and suggested differential responses with respect to the route of exposure to HSV-2.[71,102] As first demonstrated by McDermott et al[102], IVAG exposure to TK^- HSV-2 elicited a robust HSV-2 specific IgG within the vaginal tract and sera of mice while intraperitoneal exposure with the same virus only elicited HSV-2 IgG in sera; as such, the antiviral antibody response originated locally and was independent of serum-borne IgG. Further studies revealed enrichment of IgG and IgA plasma cells within the vagina following IVAG immunization which was further enhanced after challenge; however systemic immunization routes failed to established such population within the FGT.[103] The protective role of HSV-2 specific vaginal IgG and SIgA was studied by neutralization experiments. Affinity-purified IgG isolated from the vaginal secretions effectively neutralized HSV-2 while S-IgA isolated from the same secretions failed to do so.[104] In addition, immune protection against HSV-2 challenge of IgA knockout mice was found to be similar to the wild-type stain indicating that mucosal immunity against HSV-2 was independent of IgA.[105] In contrast, IgG was found to be the main immunoglobulin involved in protection, since adoptive transfer of serum IgG from immunized mice into the FGT of naïve mice

reduced viral load after HSV-2 infection.[106] Furthermore, a recent study found IgG2b and IgG2c to be the primary IgG isotypes secreted in response to HSV-2.[107]

A number of studies have demonstrated that resistance to lethal WT-HSV-2 challenge in immunized mice is independent of B cells and antibody responses, however their exact role against HSV-2 remains controversial.[90,91,108] In one study, Dudley et al[109] demonstrated that immunized B cell-deficient (μMT) mice had significantly higher viral titers of HSV-2 at 24-hours post challenge and experienced a 2-day delay in virus clearance compared to immunized WT mice. This indicates that deficiency of B cells in immunized animals does not appear to affect the outcome of disease after WT-HSV-2 challenge, however these mice do not fare as well as their WT counterparts, suggesting that B cells may play a role during early control of infection. This was further elucidated in a study by Iijima et al,[110] in which depletion of DCs or B cells prior to WT-HSV-2 challenge did not influence the Th1-mediated immunity and complete protection was persistent. However collective depletion of both DCs and B cells abrogated Th1-mediated protection, suggesting that B cells primarily served as APCs. The exact role of B cells in augmenting the Th1 response observed is not clearly understood and further studies are required to elucidate the phenotypic and functional properties of the B cells involved in restimulating the Th1 cells within the FGT.

An important mediator of induced humoral immune responses can be the route of primary exposure or immunization. To date, all the studies demonstrating immunity against HSV-2 is independent of B cell responses employ IVAG route of

immunization.[90,91,108,109] As later demonstrated by Iijima and Iwasaki[111], IVAG immunization establishes significantly greater proportion of virus-specific $CD4^+$ T_{RM} cells within FGT which coincides with the dominant Th1 immunity observed and ultimately protection against HSV-2 is conferred locally. Recently, in a study by the same group showed that B cells are absent in the FGT following IVAG immunization and only infiltrate into the tissue upon subsequent IVAG challenge.[112] Specifically, upon challenge, memory B cells (MBCs) are recruited into vaginal mucosa in a CXCR3-dependent manner and differentiate into plasma cells. Consequently, the protection is primarily T cell mediated and B cells are not required.

In contrast to IVAG immunization, intranasal (IN) immunization heavily relies on B cells; as B cell-deficient (J_HD) and (μMT) mice were not protected against subsequent WT-HSV-2 challenge.[96,107] Additionally, passive transfer of HSV-2-specific serum to immunized μMT mice restored protection. This revealed that protection following IN immunization is mediated by the secreted antibodies present in the circulation rather than the B cells themselves.[96,107] More importantly, as demonstrated by Iijima and Iwasaki[107], IN immunization fails to control viral replication locally and the protection conferred by this route of immunization primarily occurs at the neuronal tissues where circulating antibodies diffuse into and prevent the virus from infecting neuronal cells. It is important to note that in this study, researchers treated the mice with DMPA to render them susceptible to IVAG HSV-2 challenge. As previously mentioned, DMPA treatment significantly increases susceptibility to STIs by suppressing local immune responses.[73]As such, it is

important to recognize that in a natural hormonal environment, IN immunization may be able to establish enhanced local immunity against HSV-2.

Although IVAG immunization provides a more robust immunity to HSV-2; a viable human vaccine must be clinically practical. Currently, the social acceptability of IVAG immunization is unknown, whereas IN immunization is already used with certain vaccines.[113] Therefore, further studies are required to characterize and maximize the B cell responses generated following IN immunization.

1.10 Upper respiratory tract as anti-viral inductive site

A myriad of studies utilize the nasal route as an immunization site to increase vaccine-induced immunity to HSV-2 in the FGT. Surprisingly, none of these studies have examined the immune response elicited at site of induction, the upper respiratory tract (URT). Studies involving other pathogens and antigen models have elucidated the immunological events that occur following antigen entry in the URT.

Immune responses within the human URT are generated in the mucosal lymphoid tissues of the Waldeyer's ring and the cervical lymph nodes they drain.[114]. While rodents lack Waldeyer's ring, they possess nasopharynx-associated lymphoid tissue (NALT), which consists of two paired lymphoepithelial structures in nasal passages.[115] Upon IN vaccination, antigens are deposited into the nasal mucosa. Subsequently, specialized epithelial cells, known as microfold (M) cells, overlaying NALT transport antigens across and deliver it to the underlying DCs and lymphocytes.[116] Alternatively, antigens may be taken up by the local APCs that exists below the epithelium of the nasal mucosa and transported to the draining cervical

lymph nodes.[117] In both lymphoid tissues, adaptive immune responses are induced. Subsequently, NALT-activated lymphocytes drain into the cervical lymph nodes from which effector subsets migrate out to regional secretory tissues related to the airways where they carry out their function.[118] More importantly, during activation, NALT-derived lymphocytes develop a selective homing potential which allows their migration to peripheral lymph nodes and other mucosal sites.[119,120] In fact, studies conducted in humans and mice have both have shown the presence of NALT-derived plasmablasts in the FGT.[121,122] As such, immune responses induced in URT following IN immunization can ultimately confer protection in the FGT.

In addition to imprinting lymphocytes to preferentially migrate to mucosal sites, NALT is highly plastic with regards to the type of immune response induced.[123] While other canonical mucosal lymphoid organs such as Peyer's Patches solely stimulate IgA-secreting B cell differentiation and promote IL-10-producing T cells; NALT is capable of priming both IgG or IgA-secreting B cells and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells following IN infection or vaccination. For example, IN immunization using protein antigens and the adjuvant chlorea toxin induces a Th2-biased $CD4^+$ T cell and IgA-secreting B cell responses.[124] On the other hand, IN immunization with protein-containing microparticles elicits an IgG response.[120] In fact, Heritage et al.[120] illustrated that these IgG responses were elicited locally in NALT, as they were initially only detectable in NALT and appeared in cervical lymph nodes and spleen at a later timepoint. The plastic nature of NALT has the potential to design vaccines that

could induce tailored immunity which makes IN immunization more desirable in comparison to traditional approaches.

1.11 The role of female sex hormones on susceptibility to HSV-2 infection in murine animal model

The pathogenesis of infectious diseases as well as vaccination outcomes vary between sexes.[125] In females, the ovarian sex hormones, progesterone and estrogen, have been shown to have profound effects on susceptibility to STIs and FGT immune responses.[126] Our lab, specifically, has studied the role of female sex hormones on mouse models of HSV-2 infection and vaccination. Initially, we demonstrated an increased susceptibility to genital HSV-2 infection in mice after treatment with DMPA, a long-acting progestational formulation, or a saline suspension of progesterone (P-sal).[73] Further studies were conducted in ovariectomized (OVX) mice in which exogenous administration of estradiol or progesterone allowed us to assess the direct effect of female sex hormones on immune responses against genital HSV-2. In one study, we demonstrated that mice immunized under the influence of E_2 had better survival rates, reduced cumulative genital pathology, and significantly lower viral titers compared to those immunized under the influence of P_4.[53] Furthermore, IN immunization under the influence of E_2 enhanced the serum and vaginal virus-specific IgG levels and lead to better overall protection.[53] Parallel to these studies, we showed that E_2 treatment lead to earlier recruitment and higher proportion of Th1 and Th17 cells to the FGT post-challenge.[94] Specifically, we found that E_2 enhances the proportion of IL-17-secreting $\gamma\delta^+$ T cells in FGT. Subsequently, these subsets condition

the local vaginal dendritic cells to become potent inducer of Th17 responses. Specifically, the local vaginal APCs isolated from estrogen receptor knockout (ERKO) mice induced 12-fold lower IL-17 levels compared to WT control when co-cultured with CD4+ T cells.[94,98] In a follow up study, we examined the role of IL-17 in host defense against HSV-2 and found that Th17 augments Th1 and T_{RM} responses and lead to enhanced antiviral immunity against HSV-2.[93]

Until now, we have studied the effect of female sex hormones on distinct components of immune system involved in protection against HSV-2. However, the role of female sex hormones on the B cell response against HSV-2 remains to be studied. In contrast to IVAG immunization with TK^- HSV-2 where humoral immunity plays an insignificant role, the humoral immune response is critical in establishing immunity against WT-HSV-2 during IN immunization.[107] Therefore, it is of great importance to understand the immunomodulatory effect of E_2 and P_4 on such immune population in order to develop means of maximizing B cell responses and enhance anti-viral immunity in the FGT.

1.12 Cognate T-B cell interaction drives B cell isotype class switch recombination

A class of $CD4^+$ T cells, namely T follicular helper (Tfh) cells, specialize in orchestrating B cell maturation and differentiation and ultimately shape the long-lived humoral immunity against a wide range of pathogens.[127] The interaction between Tfh and B cells begins outside of the follicles within secondary lymphoid organs (SLO). Subsequently, these interactions continue in germinal centers (GC), a

microanatomical structure where antigen-specific B cells undergo isotype class switch recombination, immunoglobulin affinity maturation, and differentiate into MBCs and plasma cells.[127]

The class of humoral response induced following pathogen encounter depends on the nature of the pathogen. As such, the GC Tfh cells involved in priming B cells are functionally heterogenous. Depending on the infectious agent, GC Tfh cells produce the signature cytokines of their corresponding Th effector subsets. For instance, IL-4 has been shown to direct isotype switching to IgG1 in type 2 responses contrasting IFN-γ driven switch to IgG2 in type 1 responses.[128] Additionally, IL-17, secreted by Th17 cells, has been shown to induce IgG3 class switching.[129] As previously mentioned, E_2 treatment enhances both IFN–γ and IL-17-secreting $CD4^+$ T cells. As such, these subsets may augment B cell responses and enhance humoral immunity in the context of HSV-2.

CHAPTER 2: RATIONALE & OBJECTIVES

An ideal vaccine against HSV-2 would be expected to induce sterile immunity in the FGT; thus, preventing the virus from establishing latency. Sterile immunity is established when the local virus-specific antibodies induced by immunization are sufficient to efficiently neutralize the invading pathogen and prevent the establishment of infection.[130] The goal of developing an antibody-mediated vaccine against HSV-2 has been pursued since the 1980s. Although each candidate vaccine induced systemic immune responses, they were unsuccessful in providing protection in the genital tract.[3] In order to generate local anti-viral immunity in the genital mucosa, a number of studies have highlighted the importance of mucosal immunization.[103,106,131–134] Earlier studies demonstrated the critical role of IFN-γ secreting $CD4^+$ T cells, but not antibodies, in protecting against WT-HSV-2 following IVAG immunization.[91,92,110] Later studies exhibited that mucosal protection is comparable following intravaginal or IN immunization, but IN is a more practical and less intrusive vaccination strategy.[50,51] Interestingly, protective immunity against HSV-2 following IN immunization is B cell dependent.[96,107] Further studies are required to identify strategies to maximize B cell responses in the FGT, in order to develop vaccination strategies that induce enhanced protection following IN immunization.

In order to induce enhanced immune responses against HSV-2 in FGT, its microenvironment must be taken into account. An important factor that has been shown to significantly modulate the immune responses generated within the FGT are

the female sex hormones. Specifically, the female sex hormones, estrogen and progesterone, have been shown to influence immune responses elicited within this site in response to various STIs. The influence of these sex hormones in the context of FGT immunity against HSV-2 has been extensively studied in our lab and further validated by others. Initial studies conducted involved IVAG or IN immunization of female mice with TK^- HSV-2 and subsequent IVAG challenge with WT-HSV-2.[53,135] The overall conclusion from both studies indicated that E_2 treatment provided protection against HSV-2 with better outcomes as indicated by lower vaginal pathology and viral shedding.[53,135] Further mechanistic studies revealed that E_2 provided protection following IN TK^- HSV-2 immunization by enhancing different arms of the immune system. Specifically, E_2 was found to induce an IL-$1\beta^{high}$ $CD11c^+$ DC population which primed enhanced Th17 responses in the murine FGT.[94] Furthermore, the Th17 cells were found to augment Th1 and T_{RM} responses.[78] E_2 treatment also led to an earlier, and greater accumulation, of IFN-γ $CD4^+$ cells in the vagina post-immunization and challenge.[93,94] Furthermore, recent findings from our lab indicate that both Th1 cells and Th17 cells are primed within the local site of immunization, the URT, post IN immunization (Bagri et al, unpublished). Specifically, we find that both IFN-γ-secreting $CD4^+$ cells as well as IL-17-secreting $CD4^+$ cells are induced one week post-IN immunization within the cervical lymph nodes and NALT. Furthermore, these responses are enhanced in the presence of E_2, indicating that the effect of E_2 is not limited to FGT. In a subsequent study, our lab demonstrated that E_2 treatment enhanced HSV-2 specific serum and vaginal IgG levels.[53] Of note, this finding was

further validated by Pennock et al.[136] where they showed increased titers of neutralizing HSV-2 specific IgG in the presence of E_2. Furthermore, data from our lab and others suggests that unlike T cells, B cells are normally absent within the FGT and only infiltrate into the tissue in response to HSV-2 infection or challenge, post-immunization.[112] Although a number of studies have shown the importance of IgG in protecting against HSV-2, little is known about the cellular and histologic source of HSV-2 specific antibodies that are detected within the genital lumen.[49] The effector HSV-2 specific B cells subsets that are involved in antiviral immunity may be induced within the URT. Furthermore, the exact influence of E_2 on B cell differentiation and the underlying mechanism for the enhanced IgG levels observed under the influence of E_2 remains to be elucidated.[53] One plausible mechanism is that the presence of Th17 cells observed only in E_2-treated mice may accelerate B cell differentiation and ultimately induce greater proportion of IgG-secreting cells. The work undertaken for this thesis addresses this possibility.

I hypothesized that E_2 treatment will enhance the formation of MBCs within secondary lymphoid tissues and antibody-secreting cells within nasal effector sites following IN immunization. We also hypothesize that upon IVAG challenge, these enhancements will lead to greater migration of MBCs and antibody-secreting cells to the FGT leading to enhanced HSV-2 antiviral immunity.

I addressed this hypothesis through the following aims:

1. Characterize HSV-2 specific B cell and antibody responses post-immunization and post-challenge and determine the effect of E_2.
2. Determine whether IL-17 mediates E_2-enhanced B cell responses through augmented Th1 and Th17 cells.

CHAPTER 3: MATERIAL AND METHODS

3.1 – Animal Model Protocols

3.1.1 - Mice

6-8 weeks old female C57BL/6 were purchased from Charles River Laboratories (Saint-Constant-Quebec). IL-17$^{-/-}$ mice were bred and maintained internally at the Central Animal Facility (McMaster University, Hamilton, ON). All animals were and housed and maintained under standard controlled conditions in the Central Animal Facility. All experiments and protocols used in this study were in compliance with AREB (Animal research ethics board) at McMaster University.

3.1.2 – Ovariectomies

The endogenous source of sex hormones was removed by ovariectomies (OVX) as previously described.[137] Briefly, mice were given analgesic (Temgesic 0.03mg/mL) through subcutaneous injection 30 minutes prior to the surgery. Next, they were anaesthetized intraperitoneally with injectable anesthetics (150 mg of ketamine/kg–10 mg of xylazine/mL). Ovaries were located proximally near the hind limbs of the murine abdomen and excised through incisions. The incisions were sutured and surgically clipped. The animals were monitored two weeks post-surgery and allowed to recover before any experiments were conducted.

3.1.3 – Hormonal Pellet Implantation

21-day formulation of 17β estradiol (cat# E-121 0.010mg/pellet) were purchased from Innovative research of America (Sarasota, FL, USA). The implanted pellet is formulated to release 476 ng/mouse/day of E_2 for a guaranteed period of 21 days.

Implantation was done as previously described.[53] Briefly, OVX mice were anaesthetized intraperitoneally with 120uL of injectable anaesthetic (150 mg of ketamine/kg–10 mg of xylazine/mL). The surgical site was located between the ears of the mouse. A small incision was made in the outer skin, and the pellet was placed inside the cavity. The skin was seal with surgical clips and the mice were monitored for 14 days.

3.1.4 – TK$^-$ HSV-2 IN immunization

At two weeks following pellet implantation, OVX'd mice were anaesthetized using gaseous anesthetic (Isofluorane-Bimeda-MTC, Cambridge, ON). The mice were then immunized with 5 μL of thymidine kinase-deficient (TK$^-$) HSV-2 strain 333 at 10^4 plaque forming unit (PFU)/mouse into each nare with micropipette.

3.1.5 – WT HSV-2 Intravaginal Challenge

Six to eight weeks following IN immunization, mice were anaesthetized using injectable anesthetics (150 mg of ketamine/kg–10 mg of xylazine/mL) given interperitoneally, laid on their back, and inoculated intravaginally with 10 μl of wildtype HSV-2 strain 333 at 10^4 PFU/mouse.

3.1.6 – Collection of Vaginal Secretions

Vaginal secretions were collected daily for 5 consecutive days from day 21–27 following IN immunization and for 5 consecutive days following IVAG WT HSV-2 challenge by pipetting 30 μL of PBS twice consecutively into and out of the vagina 5-6 times to give a total volume of approximately 60 μL and stored at - 80 ^{0} C until used.

3.1.7 – Serum Collection

Serum samples were collected on day 28 post IN immunization and day 5 post IVAG challenge. Briefly, animals were anesthetized using gaseous anesthetic Isofluorane-Bimeda-MTC, Cambridge, ON). For post IN immunization, blood samples were collected by retro-orbital bleeding using the micro-capillary tubes. For post IVAG challenge, blood samples were collected by cardiac puncture. In either case, the samples were allowed to clot at room temperature for 30 minutes. The clotted blood was then centrifuged at 8000 x g force for 10 minutes and the serum was collected and stored at -80^0 C to be used later.

3.1.8 – Genital pathology

Genital pathology following challenge with WT HSV-2 was monitored daily until the end of the experiment and was scored on a five-point scale: 0, no infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina; 3, severe swelling and redness of both vagina and surrounding tissue and hair loss in genital area; 4, genital ulceration with severe redness, swelling, and hair loss of genital and surrounding tissue; 5, severe genital ulceration extending to surrounding tissue. Animals were euthanized when they reached stage 5 as per approved procedures of Animals Utilization Protocol.

3.1.9 – Plaque assays

Plaque assays were used to quantify the viral replication in the vaginal epithelium. The vagina washes collected and stored at -80^0 C are first thawed on ice. They were

then diluted from 10^{-2} to 10^{-7} in serum free alpha-MEM media. Each dilution is added onto a monolayer of plated vero cell cultures. Plates were incubated with 10 uL for 2hours to facilitate viral entry and later overlaid with human immune serum to prevent new attachment of remaining virus in the samples. After 48 hours, each plaque formed among the verso signified a viral PFU. Plates were then stained with crystal violet and visualized on a light microscope to count the total plaques. To obtain the total viral count in a lavage sample, the counted plaques were multiplied by the dilution factor.

3.2 – Tissue Isolation and Processing

3.2.1 – Vaginal Tract

The vaginal tract was isolated and process according to a standardized protocol previously described.[54] Briefly, mice were sacrificed by cervical dislocation and a large incision was made at their abdomen through the outer skin and the peritoneal wall exposing the inner organs. The vaginal tract was located by following the uterine tubes towards the posterior end of the animal. The vaginal canal was exposed by splitting the pelvic bone with a scissors. The tissue was lifted with forceps, the entire canal was opened all the way to the exterior of the vaginal and excised just before the open end. The excised vaginal tracts were placed on ice-cold RPMI 1640 media. Tissue processing commenced with an initial thorough wash of the isolated tracts with fresh media. Then tissue was placed on polystyrene 10cm plates with 2mL of fresh RPMI-1640 media. The vaginal tract was opened to expose the inner epithelial layer. Scalpel blades ere used to cut the tissue into small pieces. Vaginal tissue pieces were digested

in 15 mL of RPMI 1640 containing 0.00157 g/mL collagenase A (Roche Life Science, USA) at 37 ° C on a stir plate, stirring with a magnetic bar for 1 hour, followed by a second round of digestion. The suspension was processed by filtration through 40 μm filter. The resultant homogenous cellular suspension was spun down and the pellet was resuspended in 1mL of FACS buffer.

3.2.2 – Nasopharynx-Associated Lymphoid Tissue

NALT tissues were isolated, processed and provided by personnel from Dr. Martin Stampflï's laboratory: Joshua McGrath (PhD) and Danya Thayaparan (PhD). The tissue was isolated accordingly to previously published protocol.[138] Briefly, animals were sacrificed by cervical dislocation. Subsequently, the lower jaw, tongue and connective tissues were removed exposing the soft palate of the upper jaw. The palate was peeled back, revealing the pair NALT structures that were positioned at the posterior of the hard palate. The palate was isolated using forceps and washed with 250 μL of RPMI-1640 media and cultured in 1mL of RPMI-1640 media in a 1.5 mL Eppendorf. Subsequently, the collected tissue was processed by placing tissue pieces between two frosted glass slides and grounding it to homogeneity. Finally, the resultant homogenous cellular suspension was spun down and the pellet was resuspended in 250 μL of FACS buffer.

3.2.3 – Nasal Mucosa

Nasal mucosa was isolated, processed and provided by personnel from Dr. Martin Stampflï's laboratory: Joshua McGrath (PhD) and Danya Thayaparan (PhD). Briefly, animals were sacrificed by cervical dislocation. After excising the NALT, the skull was

dissected and nasal mucosa was obtained by resecting 1/3 of mucosa from nasal septum exposed by extending incision of nasal skin and bone below nares to inner cavity. Nasal mucosa was removed from the nasal septum mucosa and washed extensively with RPMI-1640 media. Single cell suspension was obtained by filtering the tissue pieces through a 40 μm. The resultant homogenous cellular suspension was spun down and the pellet was resuspended in 500 μL of FACS buffer.

3.2.4 – Lymph Nodes and Spleen

Cervical lymph nodes draining the URT and iliac lymph nodes draining the small intestines and vagina was collected. 6-well plates were scarred with a needle and lymph nodes or spleen were mechanically disrupted by placing the tissue in 1 mL of PBS inside each well and grounding the tissue to homogeneity using a 3mL syringe. The resultant suspension was spun down. For lymph nodes, the pellet was resuspended in 1 mL of FACS buffer. For spleen, 2mL/spleen ACK lysis buffer (Sigma Aldrich, Canada) for 2 minutes at room temperature to lyse the red blood cells. The resultant homogenous cellular suspension was spun down and pellet was resuspended in 5 mL of FACS buffer.

3.3 – Flow Cytometric Analysis

The cells isolated from processing were differentially counted on hemocytometer to exclude fibroblasts or epithelial cells. Staining was performed at 3 x 10^6 cells/sample. Samples were divided and incubated with their respective combination of conjugated antibodies for 30 minutes on ice. The samples were then washed with FACS buffer to wash away unbound antibody. Cells were then fixed with BD

fixation/permeabilization (BD Biosciences, Canada) solution before staining with intracellular antibodies. Finally, cells were washed with 1xBD Perm/wash buffer and suspended in 250 μL for analysis on the BD Fortessa.

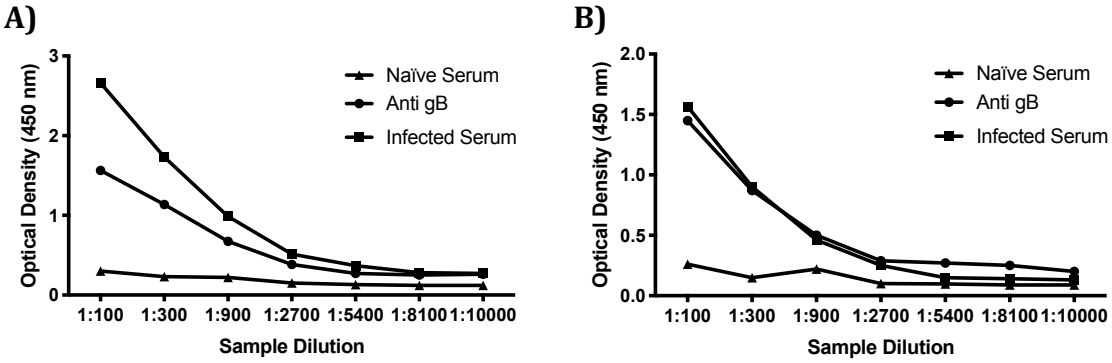
3.4 – Anti-HSV-2 IgM and IgG ELISA

3.4.1 – ELISA for experimental samples

Nighty-six well plate were coated with UV-inactivated WT-HSV-2 (104 pfu equivalent per 100 μL) for virus specific Ig measurements and incubated overnight at 4° C. Next day, the plates were washed with PBS-Tween 20 and blocked with 5% casein for 6 hrs. Four-fold dilution of serum or two-fold dilution of vaginal secretion samples were plated in wells (100 μL per well) and incubated overnight. After washing with PBS-Tween 20, BIOTIN-conjugated anti-mouse IgM (Southern Biotech, Cat#:1020-08), IgG (Southern Biotech, Cat#:1030-08), IgG1 (Southern Biotech, Cat#:1070-08), IgG2b (Southern Biotech, Cat#:1090-08), IgG2c (Southern Biotech, Cat#:1078-08), or IgG3 (Southern Biotech, Cat#:1100-08), was added to each well at 1:500 dilution, followed by washing. Subsequently, 100 μL extravidin–peroxidase (1:2000 diluted in PBS) was added to each well. Next, the plate was developed by the addition of 100□□L TMB solution. Reactions were stopped with 1N H2SO4 and absorbance was measured 450 nm. Endpoint titers was determined by calculating two times the mean background optical density value of non-immunized serum or vaginal secretion. The last dilution of experimental sample above this cut-off was considered to be the endpoint titer.

3.4.2 – ELISA optimization

HSV-2 specific ELISA was optimized and developed by UV inactivating HSV-2. Briefly, 10^6 PFU/ml of WT-HSV-2 suspended in 60 ml of PBS was inactivated using UV Stratalinker 2400 (Stratagene) set at 3000 energy dose (μJoules x 100). Subsequently, plaque assays were performed to ensure that the virus was not infectious. Next, to ensure that viable HSV-2 antigens were intact after UV-inactivation, nighty-six well plate were coated with 100 μL of UV-inactivated WT-HSV-2 (Diluted to 10^4 PFU/ml) per well or 2.5 g/ml of recombinant gB protein (ProSpec, cat#:HSV-226) and subsequently incubated with serum from HSV-2 infected mice. Anti-gB specific antibody (Sigma Aldrich, cat#:SAB4700766) was used as a positive control, while serum from unimmunized mice was used as a negative control (Supplementary Figure).



Supplementary Figure. HSV-2 specific IgG ELISA optimization. A) Wells coated with 100 μL of UV inactivated HSV-2 at 10^4 PFU. **B)** Wells coated with 2.5 μg of recombinant gB protein.

CHAPTER 4: RESULTS

4.1 – Aim 1: Characterize B cell and antibody responses post-immunization and post-challenge and determine the effect of estradiol.

As shown by Oh et al.,[112] B cells are typically absent in the FGT and only enter the FGT following IVAG HSV-2 exposure. Furthermore, a number of studies have demonstrated an important role for early B cell response and IgG antibodies in the context of HSV-2 immunization and challenge.[86] However, the exact functional phenotype and localization of these B cells remains to be elucidated.[139] In the context of vaccination, both MBCs and antibody-secreting plasma cells would be expected to play a critical role during subsequent anti-viral responses.[140] After vaccination, the pre-existing anti-viral antibodies serve as a first line of defense against subsequent exposure to the virus. Meanwhile, MBCs serve as a back-up system and rapidly reactivate to produce antibodies upon secondary exposure with the same virus. More importantly, virus-specific MBCs play a critical role when exposed to a similar but different strain of virus or when viral escape mutants are encountered.[140] Therefore, virus-specific B cell responses are paramount to vaccine induced immunity.[140] As such, we sought out to characterize the effector B cell and antibody responses upon IN immunization (priming) and following IVAG wildtype viral challenge.

Conventionally, MBCs were identified solely by the expression of CD27 in humans.[139] However, it is now recognized that a heterogenous population is induced upon antigenic stimulation with differing effector functions. In mice, multiple strategies have been employed to identify surface markers associated with MBCs and

findings from these studies suggest that MBC subsets can be divided into subsets based on the expression of CD73, CD80, and PD-L2 (Table 1).[141,142] As such, these three markers were used in our experiments to identify MBC subset following HSV-2 exposure (Table 1). Furthermore, plasma cells were identified by the lack of expression of $B220^-$ and presence of $CD138^+$ (Table 1). Additionally, intracellular staining was used to identify the specific immunoglobulin expressed by both plasma and MBCs and to determine the source of HSV-2-specific IgG reported in previous studies. Lastly, specific IgG subclasses (IgG1, IgG2b, IgG2c and IgG3) and IgM levels in serum and vaginal secretions were measured by enzyme-linked immunosorbent assay (ELISA). Since recombinant isotype class switching to IgG occurs in a T cell dependent manner, identification of the specific subclass of IgG will help us determine the specific $CD4^+$ T cell subset is involved in enhancing B cell differentiation and class switch recombination in E_2 conditions (*Aim* 2, Section 4.2). As previously mentioned, a number of studies have shown that IFN-γ^+ $CD4^+$ T cells induce recombinant isotype class switching to IgG2 while others have demonstrated that IL-17^+ $CD4^+$ T cells induce recombinant isotype class switching IgG3.[128,129,143] Therefore, the identification of the specific subclass that is enhanced under E_2 will allude to the main T cell subset that mediates E_2-enhanced B cell responses (*Aim* 2, Section *4.*2)

4.1.1 – Aim 1.1 - Assessment of B cell responses following intranasal immunization and intravaginal challenge and determining the effect of estradiol

Our lab has consistently observed that IN immunization in the presence of E_2 leads to decreased susceptibility to WT-HSV-2 challenge.[53,94,137] Specifically upon challenge, E_2-treated mice had significantly lower pathology score and viral shedding

which translated into increased survival rates. As discussed earlier, our lab has previously shown that this enhanced protection was, at least in part, due to augmented $CD4^+$ T cells responses within the FGT.[94] Furthermore, our lab has previously documented that E_2-treated mice have increased levels of HSV-2 specific IgG which may also contribute to E_2-mediated enhanced immunity.[53] While it is well-established that B cells are critical for conferring protection following IN immunization, it remains unclear whether B cells contribute to this enhanced E_2-mediated protection.

Currently, it is not clear whether the humoral protection conferred following IN immunization stems from the antibody-secreting long-lived plasma cells primed during immunization or MBCs that infiltrate the vaginal tract post-immunization and differentiate into plasma cells in the context of a challenge. To determine the major B cell population involved in protection against WT-HSV-2 IVAG challenge, we sought to determine the phenotype of the B cells infiltrating the vaginal tract post IN immunization-IVAG challenge as they transit from secondary lymphoid organs. Furthermore, we expected E_2 to enhance this infiltration since previous studies from our lab indicate an increased establishment of DCs and $CD4^+$ memory T cells under E_2 treatment (Bagri et al. unpublished).[94] Specifically, these memory T cells which are IFN-γ^+CD4^+ T_{RM}s residing within the FGT are stimulated upon IVAG challenge to secrete IFN-γ, which leads to the subsequent induction of CXCR3 ligands.[112] As B cell migration into the FRT occurs in a CXCR3-dependent manner, this increase in CXCR3 ligands within the vaginal mucosa thus leads to increased infiltration of B cells.

Finally, vaginal secretions and blood were collected after challenge to determine the antibody profile elicited by the IVAG WT-HSV-2 challenge.

As shown in the experimental outline shown in Fig. 1A, WT C57BL/6 mice were OVX as previously described. Two weeks later, one group of OVX mice were implanted with E_2 pellets. Two weeks later, both groups were immunized intranasally with TK$^-$ HSV-2 at 10^4 PFU/mouse. Six weeks later, vaginal smears of E_2-treated mice were observed to ensure that the effect of E_2 had waned. Subsequently, all animals were challenged with WT-HSV-2 at 10^4 PFU/mouse. Vaginal washes were collected, and endpoint monitoring were conducted daily. Animals were sacrificed 5 days after challenge. Blood was collected via cardiac puncture. The immunological tissues associated with B cell systemic and local immune responses which include iliac lymph nodes, spleen, and vaginal mucosa were collected, processed and stained with a cocktail of antibodies. Effector B cell subsets were characterized by polychromatic flow cytometric analysis (Fig. 1B). HSV-2 specific antibody ELISA was optimized using UV inactivated HSV-2 followed by gB specific antibody (positive control), serum from naïve mice (negative control) or unknown sample serum dilutions, followed by IgM or IgG subclass specific secondary antibody (Section 3.4 of Material & Methods). End point titres for each subclass was determined and compared between treatment groups.

As shown in Figure 2, after IVAG WT-HSV-2 challenge, viral titres were detectable in vaginal secretions of almost all placebo mice while only 50% of E_2-treated mice had no detectable viral titres. This is consistent with our previous studies

where we have shown that IN immunization in the presence of E_2 leads to superior protection against IVAG challenge.[53,94]Furthermore, flow cytometric analysis of genital tract cell isolates revealed that after challenge with WT-HSV-2, $B220^+$ B cells were a substantial fraction of the genital tract cells of placebo animals while this population was a much lower fraction in genital tract of E_2-treated mice (Fig 3), indicating that the not only the protection levels but also the mechanisms of protection differed between the groups (Fig 3A). Specifically, vaginal tissue isolated from E_2-treated mice contained significantly lower proportion of $B220^+$ B cells (0.22% of live cells) compared to placebo control (2.86% of live cells) (Fig. 3B). However, no difference was observed between E_2-treated and placebo mice with regards to the total number of $B220^+$ B cells (Fig. 3B). Interestingly, as shown by Fig. 3C, vaginal tissue isolated from E_2-treated mice had significantly greater proportion of $CD138^+$ $B220^-$ plasma cells (11.3% of live cells) in comparison to placebo mice (3.89% of live cells). A similar trend was observed with respect to total number of $CD138^+$ $B220^-$ plasma cells although these differences were not significant. Overall, the increased proportion of $CD138^+$ $B220^-$ plasma cells observed in the E_2-treated mice indicates that local plasma cells are most likely responsible for enhanced antibody response to the virus in response to challenge.[53]

Table 1. B cell phenotype in mice based on surface marker expression.

	B220	CD19	CD73	CD80	PD-L2	CD138
Naïve B cells	✓	✓				
MBC	✓	✓	✓			
MBC	✓	✓		✓		
MBC	✓	✓			✓	
MBC	✓	✓	✓	✓		
MBC	✓	✓		✓	✓	
MBC	✓	✓	✓		✓	
MBC	✓	✓	✓	✓	✓	
Plasma cells	✓					✓

Plasma cells were identified by gating $CD138^{+}$ $B220^{-}$ cells off of live cells. Each MBC subset was identified by individually gating CD73, CD80, or PD-L2 off of IgD^{-} $CD19^{+}$ live B cells.

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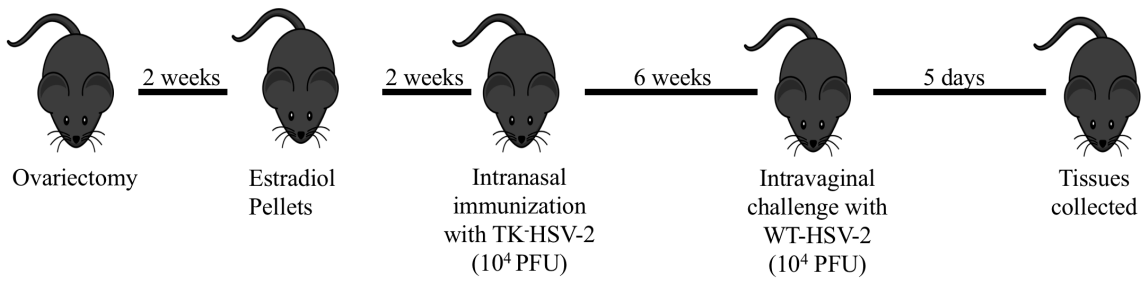

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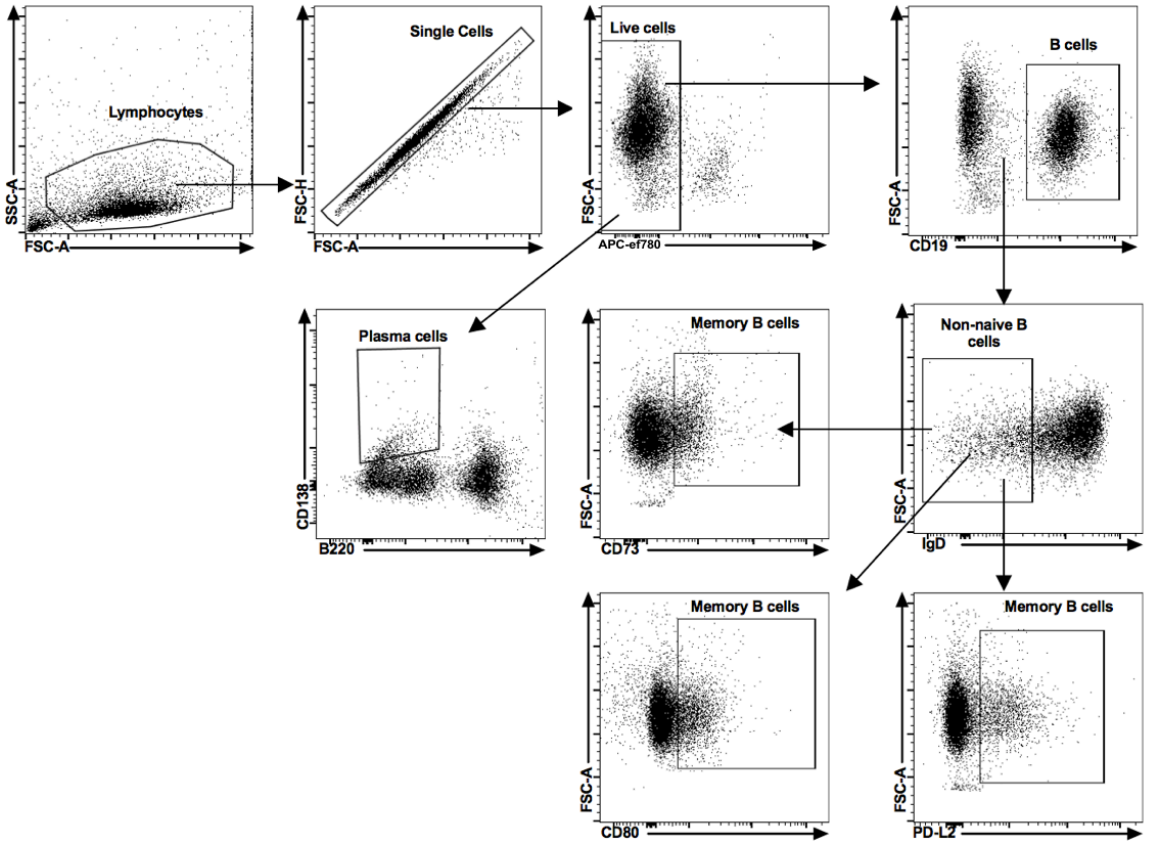

Figure 1. Flow cytometric gating strategy for characterizing memory B cell and plasma cell subsets. **A)** WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK$^-$ HSV-2 at 10^4 pfu/mouse. Six weeks later, both groups were challenged with WT-HSV-2 at10^4 pfu/mouse. **B)** CD19$^+$ B cells were gated of total live cells. Subsequently, each MBC subset was gated off of IgD$^-$ CD19$^+$ B cells. Also, off of live cells, we also gated CD138$^+$B220$^-$ plasma cells.

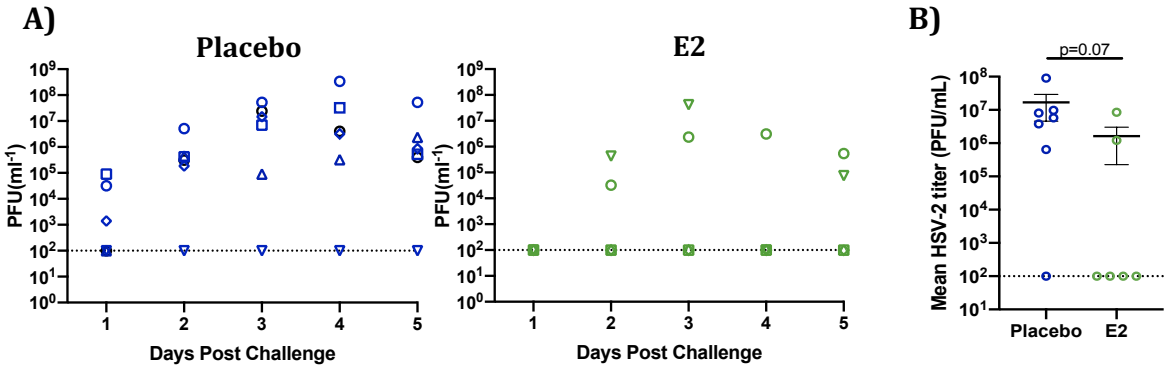

Figure 2. Vaginal viral shedding within the first five days post IVAG challenge. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Six weeks later, both groups were challenged with WT-HSV-2 at10^4 pfu/mouse. **A)** Vaginal washes were taken daily to determine viral shedding following challenge until day 5 when animals were sacrificed. Each dot represents an animal at the given timepoint. **B)** HSV-2 shedding was calculated using a Vero cell-based plaque assay, and data represents the viral loads (means ± SEMs) over 5 days. Data shown is a representative of three independent experiments with similar findings (placebo n=6-7/group, E_2=5-6/group).

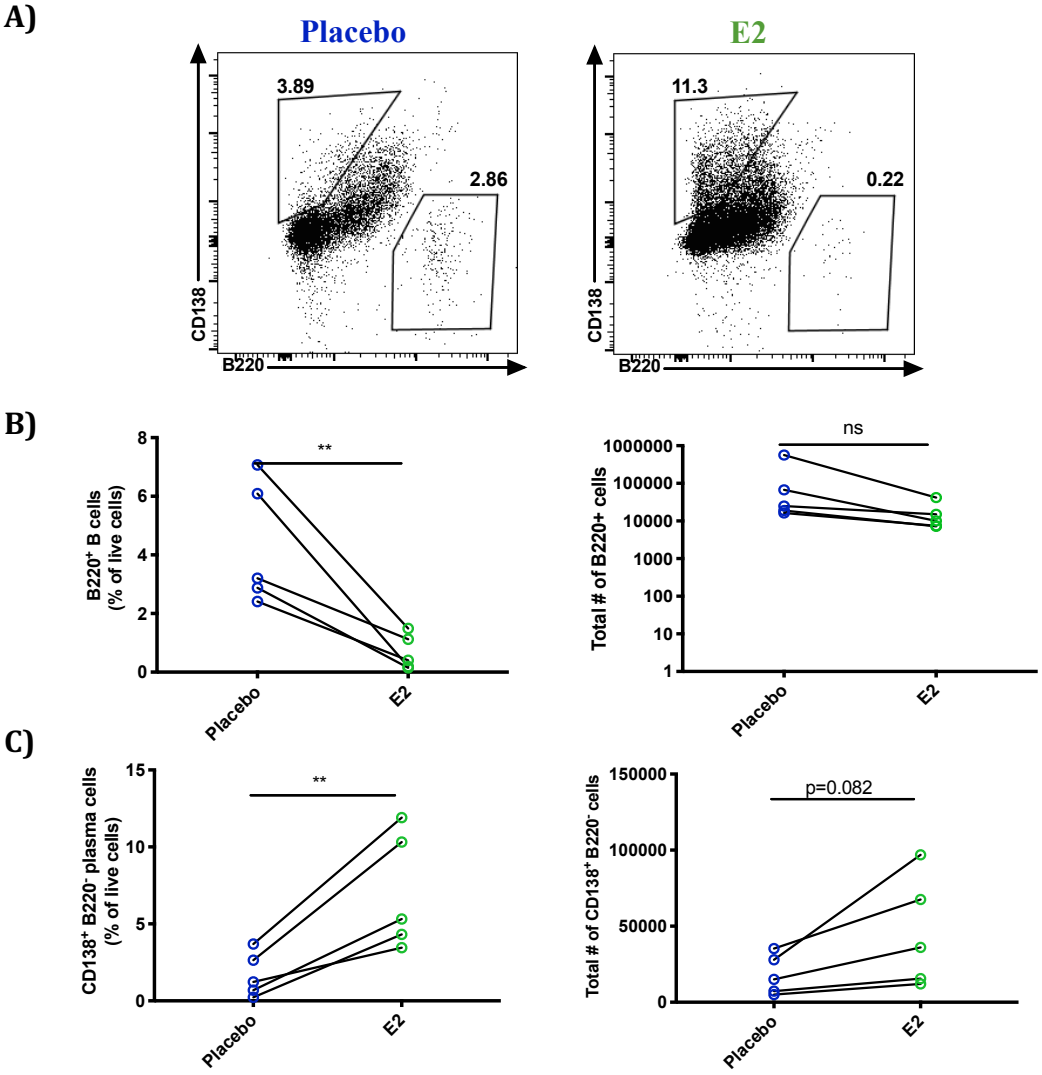

Figure 3. E_2-treated mice have increased proportion of plasma cells at day 5 post IVAG challenge. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Six weeks later, both groups were challenged with WT-HSV-2 at10^4 pfu/mouse. Vaginal tissues were collected at day 5 post IVAG challenge, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. **A)** $B220^+$ and $B220^-CD138^+$ cells were gated of total live cells and their proportion was compared between E_2-treated and placebo mice. **B)** The differences in percentages and total cell numbers of $B220^+$ cells and **C)** the differences in percentages and total cell numbers of $B220^-$ $CD138^+$ cells after HSV-2 challenge in the vaginal tract were compared between placebo and E_2-treated mice. For **(A)** data shown is a representative of five independent experiments with similar findings (n=3 to 5 vaginal tracts pooled/group). For **(B)** and **(C)** data shown is pooled from five independent experiments.

IgM, IgG1, IgG2b, IgG2c, and IgG3 antibody responses to WT-HSV-2 challenge were measured using an ELISA. Vaginal secretions and serum collected from E_2-treated mice had comparable HSV-2 specific IgM endpoint titers to placebo mice. Furthermore, both groups had similar serum levels of HSV-2 specific IgG1 (Fig. 4A). Consistent with our hypothesis, E_2-treated mice had significantly greater HSV-2 specific IgG2b and IgG2c titers compared to placebo controls in both serum and vaginal secretions (Fig. 4A & 4B). Finally, no significant levels of HSV-2 specific IgG3 was detected in serum or vaginal secretions of either treatment group (Fig. 4A & 4B). Overall, these observations support previous findings from our lab and further revealed the specific subclass of IgG that was found to increase in the presence of E_2.[53]

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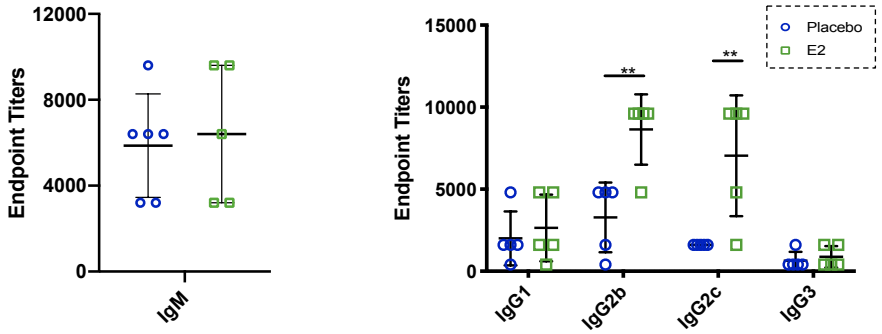

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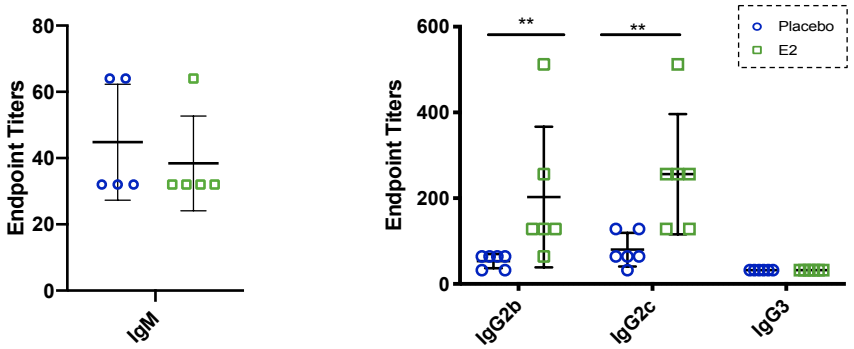

Figure 4. E_2-treated mice have increased levels of HSV-2 specific IgG2b and IgG2c in serum and vaginal secretion at day 5 post IVAG challenge. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Six weeks later, both groups were challenged with WT-HSV-2 at10^4 pfu/mouse. Vaginal washes were taken daily following challenge until day 5 when animals were sacrificed. Serum samples were collected on day 5 post challenge. HSV-2 specific IgG1, IgG2b, IgG2c, IgG3 were measured by ELISA and endpoint tiers were determined. Each dot represents an animal. HSV-2 specific IgG antibody titers at day 5 post challenge in **A)** Serum samples and **B)** vaginal secretions. Data shown are representative of two separate experiments showing similar results. Each data point represents the serum or vaginal secretion of a single animal.

4.1.2 – Aim 1.2 - Characterizing B cells and antibody responses following intranasal immunization and determining the effect of estradiol

The establishment of MBCs and long-lived plasma cells is critical in generating immune responses against WT-HSV-2 during challenge. As demonstrated by Iijima and Iwasaki[90], IN immunization with TK^- HSV-2 induces a potent B cell response that is fundamental to mice survival following IVAG challenge with WT-HSV-2. However, in this study the researchers did not identify the cellular and histologic source of HSV-2 specific B cells that were primed during immunization. One of our goals in this study was to examine the major immunological sites following IN immunization and determine the cellular and histologic source of HSV-2 specific humoral responses. Specifically, we examined the local inductive sites in the URT, peripheral secondary lymphoid tissues and effector sites to obtain a comprehensive understanding of B cell responses following IN immunization. The inductive sites that were examined were the NALT and the draining cervical lymph nodes. Iliac lymph nodes and spleen were also examined since early studies indicate that these sites are involved in cellular trafficking from URT to the FGT.[120,144] Finally, the effector sites that we examined were nasal mucosa and the FGT. Previous studies indicate that antibody-secreting plasma cells reside in nasal mucosa. As such, nasal mucosa may be the primary source of circulating HSV-2 antibodies following IN immunization.

In addition to identifying the cellular and histologic source of HSV-2 specific B cells, we sought to determine the effect of E_2 on these subsets following IN immunization. Pervious findings from our lab indicate the E_2-mediated enhancements observed with respect to $CD4^+$ T cell responses occurs prior following IN

immunization when E_2 levels are at its peak. As such, functional B cell subsets were compared between E_2-treated and placebo mice at four weeks following IN immunization. We predicted that E_2 treatment would enhance both MBC and plasma cell populations within the secondary lymphoid tissues and effector tissues, respectively. Furthermore, given our previous findings that E_2 treatment leads to enhanced Th1 and Th17 responses (Bagri et al. unpublished), we speculate that such enhancements would ultimately lead to greater levels of IgG2c and IgG3 in the serum and vaginal washes of E_2-treated mice following IN immunization and enhance protection against subsequent exposure to HSV-2.

Briefly, one group of WT OVX C57BL/6 mice were implanted with 21-day release E_2 pellets while the placebo group received placebo pellets (Fig. 5). Subsequently, both groups were immunized via the intranasal route with TK^- HSV-2 at 10^4 PFU/mouse. Animals were sacrificed at four weeks following immunization. NALT, cervical lymph nodes, nasal mucosa, spleen, iliac lymph nodes, and genital tract were collected, processed, and stained for polychromatic flow cytometric analysis. Vaginal wash and blood were collected before animals were sacrificed to determine HSV-2 specific IgG subclasses (IgG1, IgG2b, IgG2c and IgG3) and IgM levels by ELISA.

As expected, very few B cells were present in the FGT of either treatment groups following IN immunization (Fig. 6A). Specifically, as illustrated in Fig. 6B., vaginal tissues isolated from both E_2-treated mice and placebo mice contained similar proportion (0.42% vs 0.54% of live cells, respectively) and total number of $B220^+$ B cells (680 vs 1072, respectively). A small percentage of plasma cells, characterized as

$CD138^+$ $B220^-$, were present in the FGT at four weeks post IN immunization (Fig.6A). Furthermore, both E_2-treated mice and control mice had comparable proportion (1.43% vs 1.48% of live cells, respectively) and total number (1701 vs 1552, respectively) of $CD138^+$ $B220^-$ plasma cells. Overall, these findings indicate that the FGT is not an immunologically active site with respect to B cell responses following IN immunization.

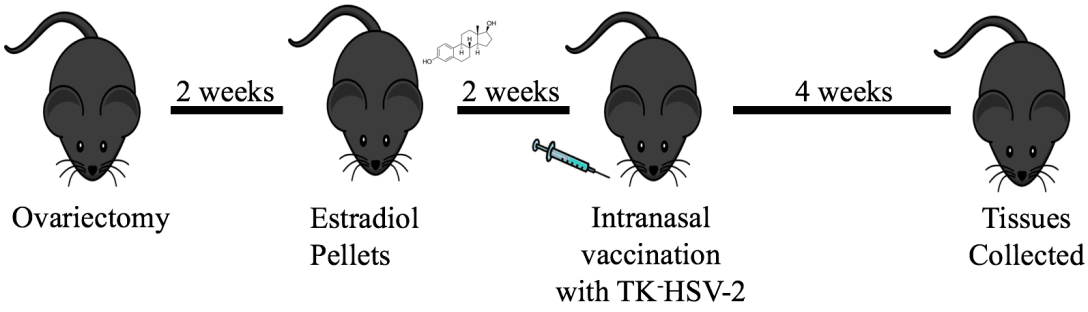

Figure 5. Intranasal immunization experimental design. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Four weeks later animals were sacrificed and NALT, cervical and iliac lymph nodes, spleen and vaginal tissues were collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry.

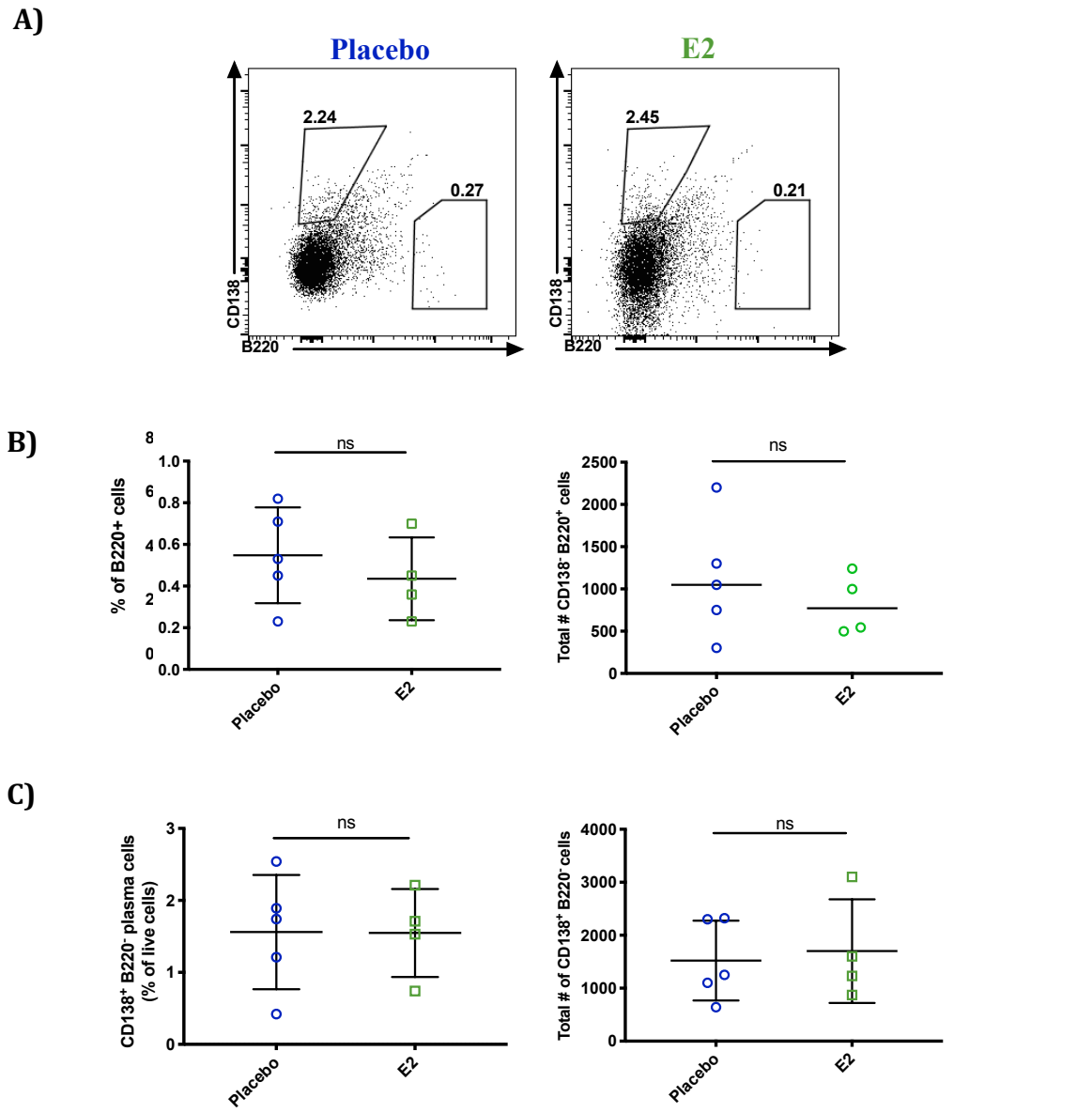

Figure 6. B220⁺ B cells are not present in the FGT in the absence of active local infection. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK⁻ HSV-2 at 10^4 pfu/mouse. Four weeks later animals were sacrificed, and vaginal tissues were collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. **A)** CD138⁺ and B220⁺ cells were gated of total live cells and their proportion was compared between E_2-treated and placebo mice. The differences in percentages and total cell numbers of B220⁺ cells **(B)** and CD138⁺ B220⁻ cells **(C)** in the vaginal tract were compared between placebo and E_2-treated mice. For **(A)** data shown is a representative of four independent experiments with similar findings (n=3 to 5 vaginal tracts pooled/group). For **(B)** and **(C)** data shown is pooled from four independent experiments.

Given that following IVAG challenge with WT-HSV-2 in IN immunized mice, E_2-treated mice had increased levels of HSV-2-specific IgG2b and IgG2c in both serum and vaginal secretions (Section 4.1.1*)*, we next sought out to determine whether such differences were established following IN immunization. As such, HSV-2 specific IgM, IgG1, IgG2b, IgG2c, and IgG3 levels in serum and vaginal secretions at four weeks post IN immunization were measured by ELISA as previously discussed. The serum antibody levels mirrored those observed post IVAG challenge (Fig. 7A). E_2-treated mice had significantly greater serum levels of HSV-2 specific IgG2c compared to control mice. Similarly, while not statistically significant, HSV-2 specific IgG2b was found to be greater in serum of E_2-treated mice. Both E_2-treated and control mice had comparable serum levels of HSV-2 specific IgM and IgG1 while HSV-2 specific IgG3 was undetectable in both groups. Surprisingly, very little to no HSV-2 specific antibodies were detectable in vaginal secretions of E_2-treated and placebo mice (Fig. 7B). It is important to note that this experiment was conducted only once and further validation is required. These findings affirm the notion that E_2 enhances B cell responses upon immunological priming, but indicate that likely this effect is first observed systemically and not in distant tissues such as the FGT.

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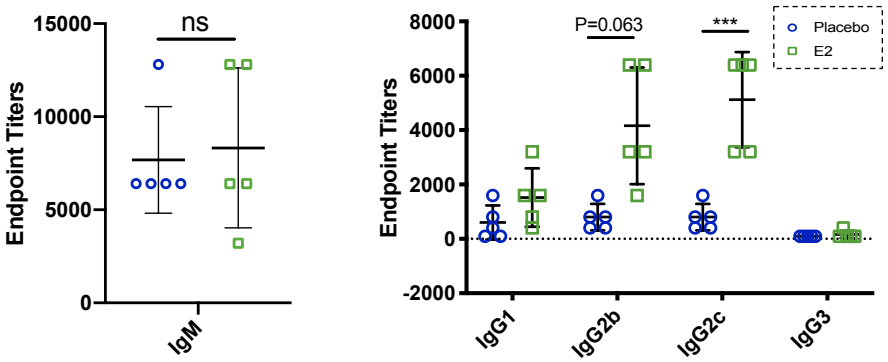

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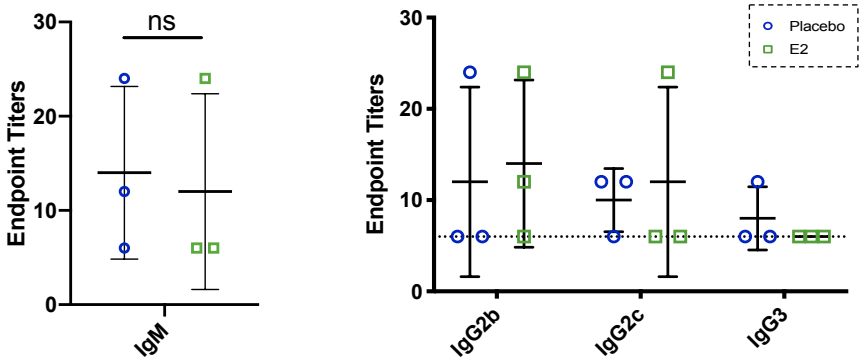

Figure 7. E_2-treated mice have increased serum but not vaginal levels of HSV-2 specific IgG2b and IgG2c at four weeks post IN immunization. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Six weeks later, both groups were challenged with WT-HSV-2 at10^4 pfu/mouse. Vaginal washes were collected from day 21-25 post-immunization and pooled for each animal. Serum samples were collected at day 28 post-immunization via orbital bleeding. HSV-2 specific IgG1, IgG2b, IgG2c, IgG3 were measured by ELISA and endpoint tiers were determined. HSV-2 specific IgG antibody titers were determined in **A)** Serum samples and **B)** vaginal secretions. For serum, data shown are representative of two separate experiments showing similar results (n=5-6 animals/group). Vaginal secretion ELISA results are from one experiment (secretions collected from day 21-27 post IN immunization were pooled for each animal) (n=3 animals/group). Each data point represents a single animal.

The results so far indicated that the cellular source of HSV-2 specific antibodies detected post-immunization lies outside of the FGT. As such we examined other immunological sites to identify and characterize MBCs and antibody-secreting plasma cells induced following IN immunization. Since the MBCs are defined by the heterogenous expression of CD73, CD80, and PD-L2, we measured the frequency and total number of individual subsets (individual frequency and total number, respectively) as well as the combination of all individual frequencies (combined frequency). We further determined whether these populations are influenced by E_2 treatment and are ultimately responsible for the superior responses observed.

At four weeks post-IN immunization, MBCs were found within the NALT, cervical and iliac lymph nodes and spleen of E_2-treated and placebo mice. The NALT and cervical lymph nodes isolated from E_2-treated mice had comparable individual frequency, combined frequency and total number of MBCs to those isolated from control mice (Fig. 8A & 8B). However, the combined frequency of MBCs was significantly greater in the iliac lymph nodes (32% vs 66% of live cells) and spleen isolated from E_2-treated mice (21% vs 40% of live cells) (Fig 8C.ii and Fig 8D.ii). When analyzed as individual subsets, similar significant increases in frequency were likewise observed for most MBC populations (Fig 8C.i and Fig 8D.i). The only subsets not in line with this observation were PD-L2$^+$ and CD80$^+$ MBCs isolated from iliac lymph nodes and PD-L2$^+$ MBC isolated from spleen. It is important to note that the significant differences observed in iliac lymph nodes and spleen were mainly associated the frequency and not total number of cells seen in MBC subsets (Fig 8C.iii

and Fig 8D.iii). This observation indicates that E_2 treatment leads to the enrichment of MBCs within the $CD19^+$ pool.

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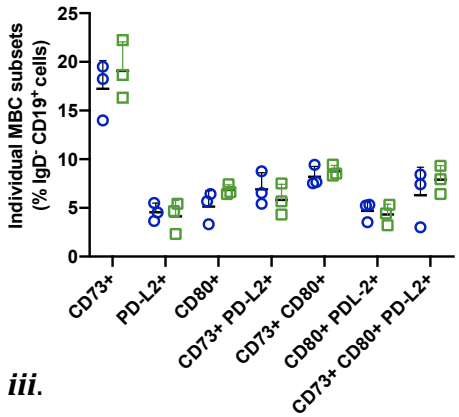

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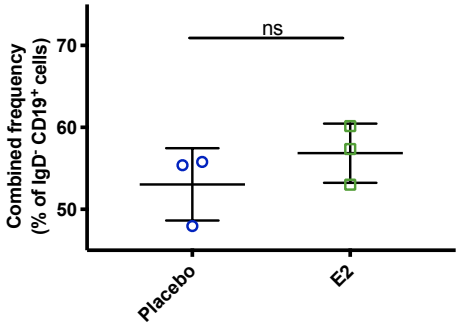

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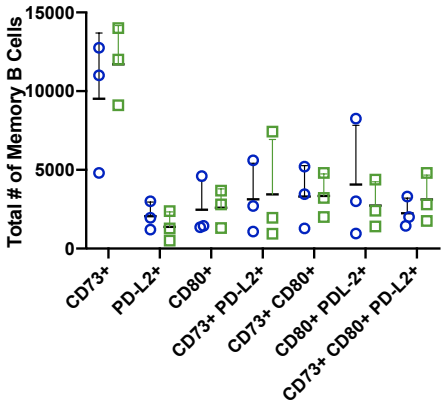

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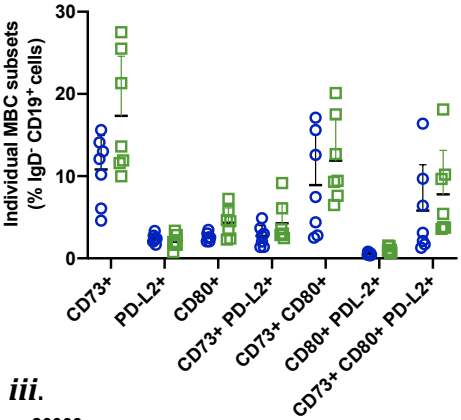

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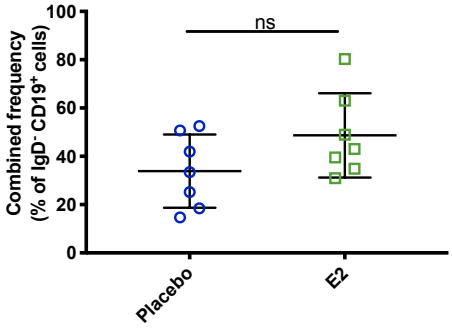

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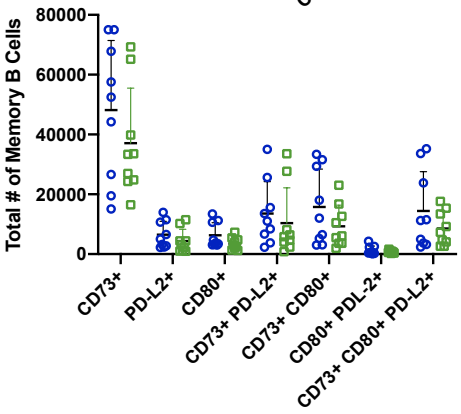

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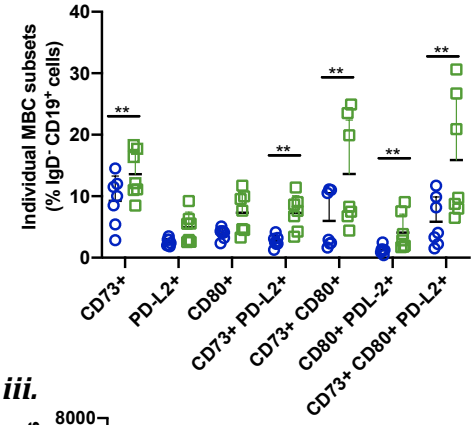

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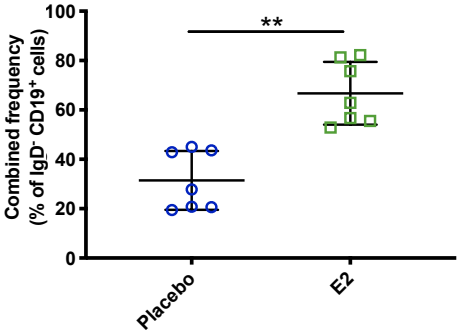

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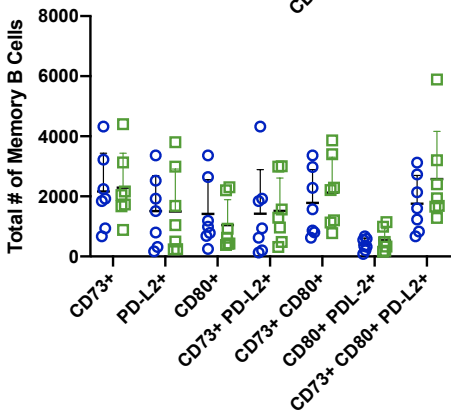

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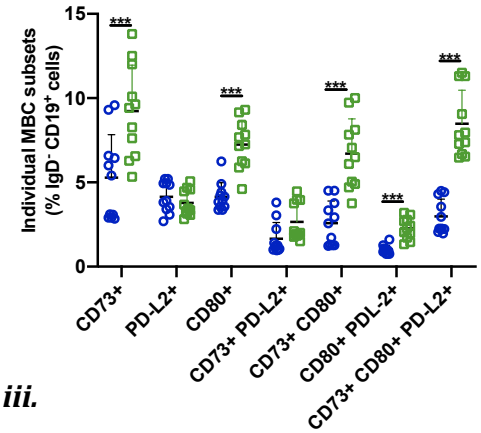

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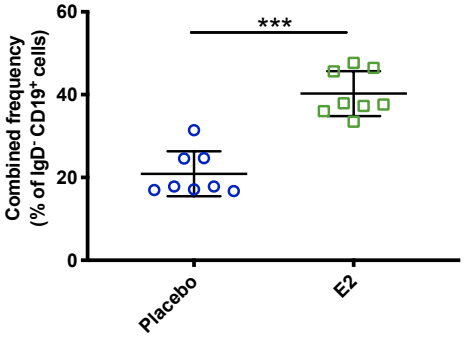

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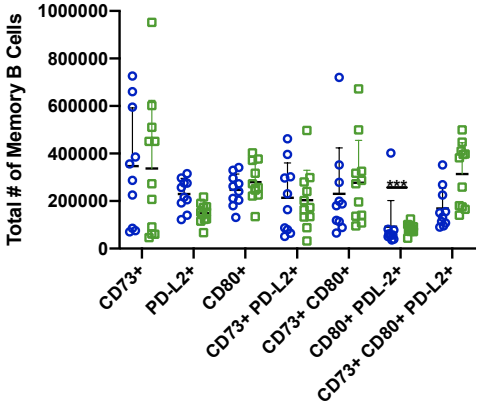


Figure 8. E_2-treated mice have increased memory B cell formation within peripheral secondary lymphoid tissues following intranasal immunization. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Four weeks later, cervical lymph nodes, iliac lymph nodes and spleen were collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. Memory B cells were gated of IgD^- $CD19^+$ cells and identified by the heterogenous expression of CD73, CD80, or PD-L2. The differences in percentages of individual subsets (***i***), the combined frequency of all subsets (***ii***), and total cell number of memory B cells (***iii***) following intranasal immunization was examined in **A)** NALT, **B)** cervical lymph node, **C)** iliac lymph nodes, and **D)** spleen. (○) Represents placebo mice while(□) represents E_2. Data shown is pooled from three independent experiments exhibiting similar findings (NALT of each group was pooled and process as one sample) (n=3 to 5 animals/group).

Finally, antibody-secreting plasma cells were identified within the nasal mucosa of both placebo and E_2-treated mice four weeks post-IN immunization (Fig. 9A). Intracellular staining revealed that E_2-treated mice had enhanced frequency of $IgG2c^+$ $CD138^+$ $B220^-$ cells (3% vs 8% of live cells) (Fig. 9B). This observation points to the possibility that HSV-2 specific antibodies detected in serum likely stem from plasma cells residing in the nasal mucosa.

In summary, we have characterized the effector B cell subsets that are generated following IN immunization with TK^- HSV-2 within different immunological sites. While the MBC compartment is primarily localized within the secondary lymphoid tissues, the plasma cells predominantly localize within the nasal effector sites. Furthermore, E_2 treatment was found to enhance both subsets. Of note, there is no sign of any MBC or plasma cells in FGT at 4 weeks post-immunization. The exact mechanism by which E_2 enhances B cell responses remains to be elucidated, however based on our previous findings, these could be a result of the augmented T cell responses. The involvement of Th1 and/or Th17 cells is also indicated by the enhanced IgG2b, and IgG2c antibody titres which are products of class switch recombination, a process heavily reliant on T cell interaction.[127]

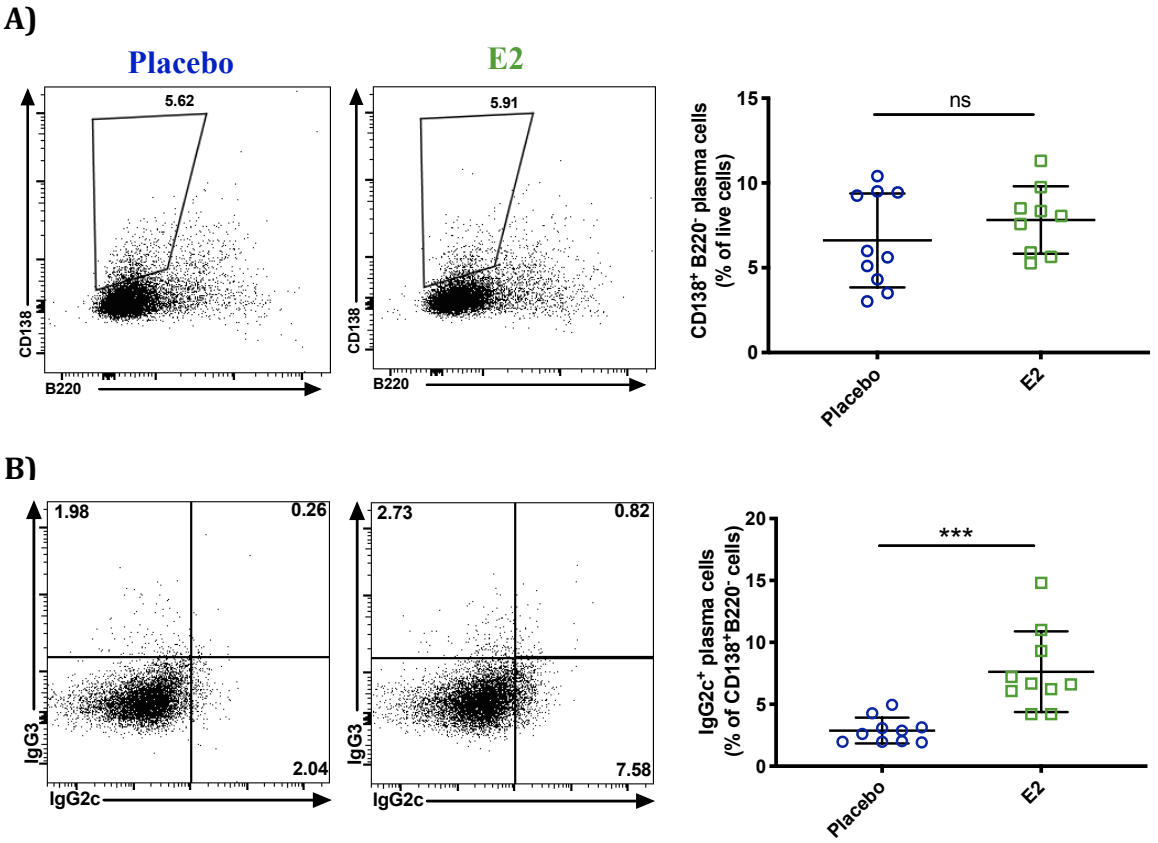


Figure 9. The proportion of IgG2c-secreting plasma cells is enhanced in nasal mucosa of E_2-treated mice four weeks post IN immunization. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK$^-$ HSV-2 at 10^4 pfu/mouse. Four weeks later, nasal mucosa was collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. **A)** B220$^-$ CD138$^+$ were gated of total live cells and their proportion was compared between E_2-treated and placebo mice. **B)** Cells were stained for intracellular IgG2c and IgG3 and the frequency of these population were compared between placebo and E_2-treated mice. Data shown is pooled from three independent experiments. Each data point represents a single animal. (n=3-4/group).

4.1.3 – Aim 1.3 – Determining whether estradiol increases the repertoire of memory B cells and antibody-secreting plasma cells in the absence of antigenic stimulation

As observed in Section 4.1.2, animals pre-treated with E_2 had enhanced B cell responses at four weeks post IN immunization. This raises the question of whether these enhancements are inherent effects of E_2 or only occurs following pathogenic encounter. As such, we sought to characterize the functional B cell subsets in immunologically naïve mice and determine the effect of E_2. Specifically, we examined the URT and the peripheral secondary lymphoid tissues where the E_2 effect post IN immunization was observed in the earlier experiments. Furthermore, our experimental design mimicked the IN immunization model to ensure that the same amount of time had elapsed after E_2 treatment.

Specifically, WT OVX C57BL/6 mice were implanted with either 21-day release E_2 or placebo pellets (Fig. 10). Six weeks later, animals were sacrificed. NALT, cervical lymph nodes, nasal mucosa, spleen, iliac lymph nodes, and genital tract were collected, processed, and stained for polychromatic flow cytometric analysis.

As depicted in Fig. 11, E_2 treatment does not enhance the formation MBC subsets within the secondary lymphoid tissues in the absence of pathogenic stimulation. Put differently, no difference was observed between E_2-treated and control mice with respect to the individual and combined frequencies of MBCs across all tissues examined. Furthermore, both groups had comparable proportion of $CD138^{+}B220^{-}$ cells residing within the nasal mucosa. More importantly, intracellular

staining revealed that the frequency of $IgG2c^+$ $CD138^+B220^-$ cells remained unaltered despite E_2-treatment.

Overall these findings indicate the E_2-mediated enhanced B cell responses do not occur in immunologically naïve animals. This allows us to conclude that the enhanced responses observed following IN immunization occur in antigen-specific manner.

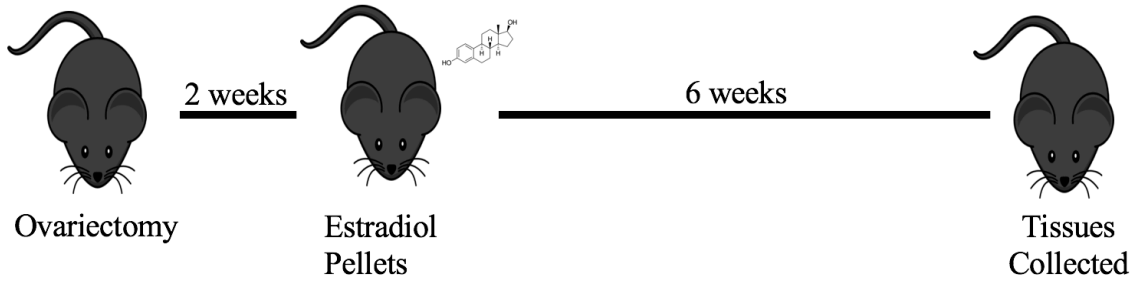
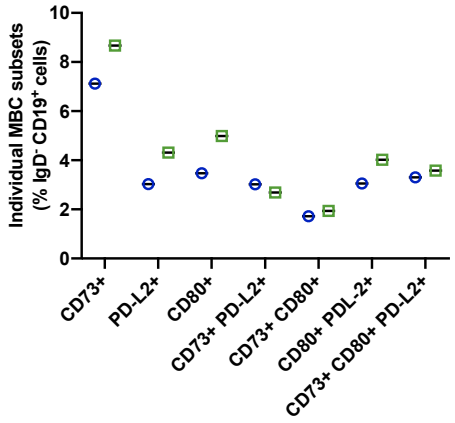


Figure 10. Experimental design of E_2 treatment of immunologically naïve mice. WT OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Four weeks later animals were sacrificed and NALT, cervical and iliac lymph nodes, spleen and vaginal tissues were collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry.

A)

i.



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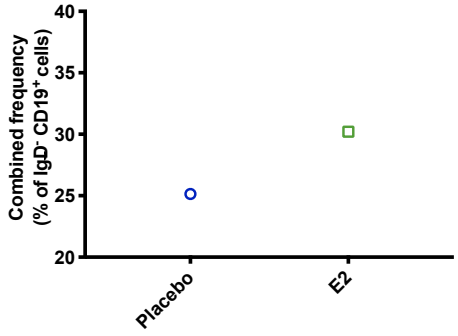
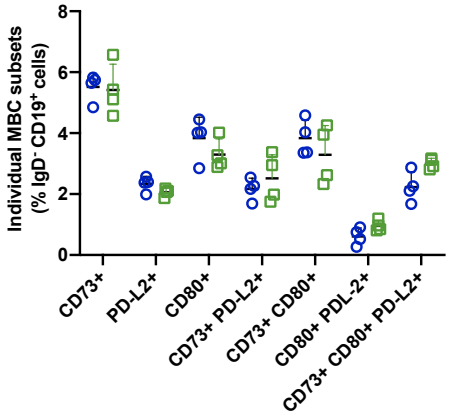


B)

i.



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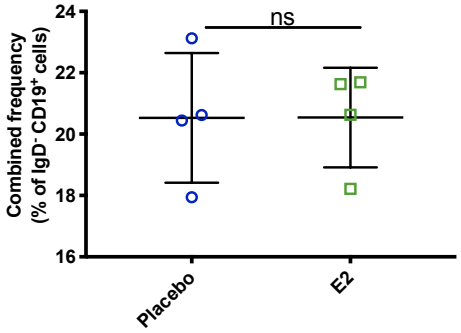
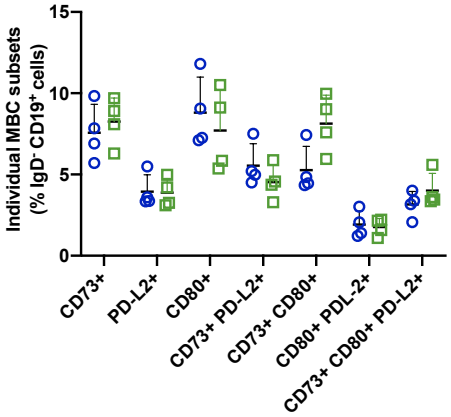


C)

i.



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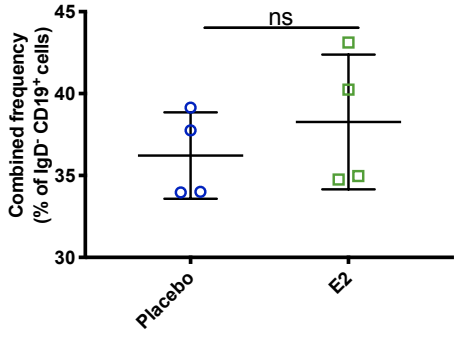


D)

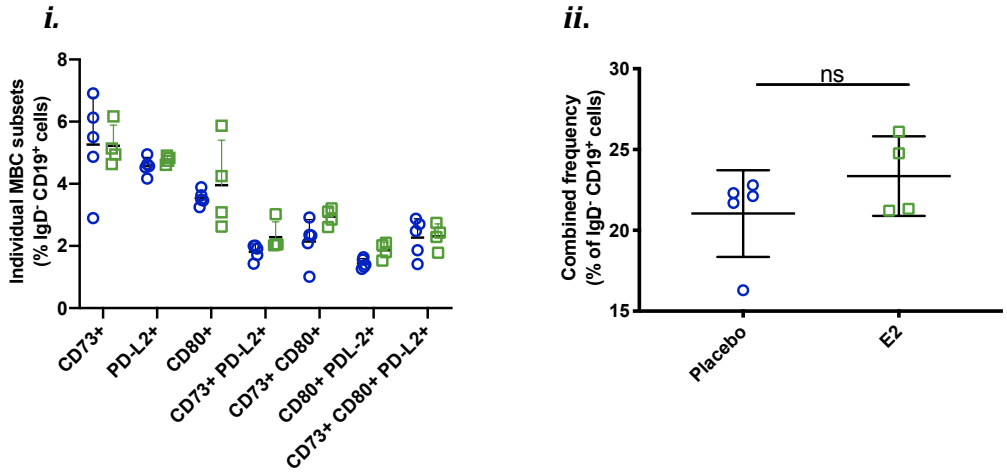


Figure 11. E_2 does not enhance the formation of MBCs within the secondary lymphoid tissues of immunologically naïve mice. WT OVX mice were implanted with E_2 or placebo pellets. Six weeks later the NALT, cervical lymph nodes, iliac lymph nodes and spleen were collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. Memory B cells were gated of IgD^- $CD19^+$ cells and identified by the heterogenous expression of CD73, CD80, or PD-L2. The differences in percentages of individual subsets (**i**) and the combined percentages of all subsets (**ii**) following intranasal immunization was examined in **A)** NALT, **B)** cervical lymph nodes, **C)** iliac lymph node and **D)** spleen. (○) Represents placebo mice while (□) represents E_2. Data shown is from one single experiment (NALT of each group was pooled and process as one sample), (placebo n=6-7/group, E_2=5-6/group).

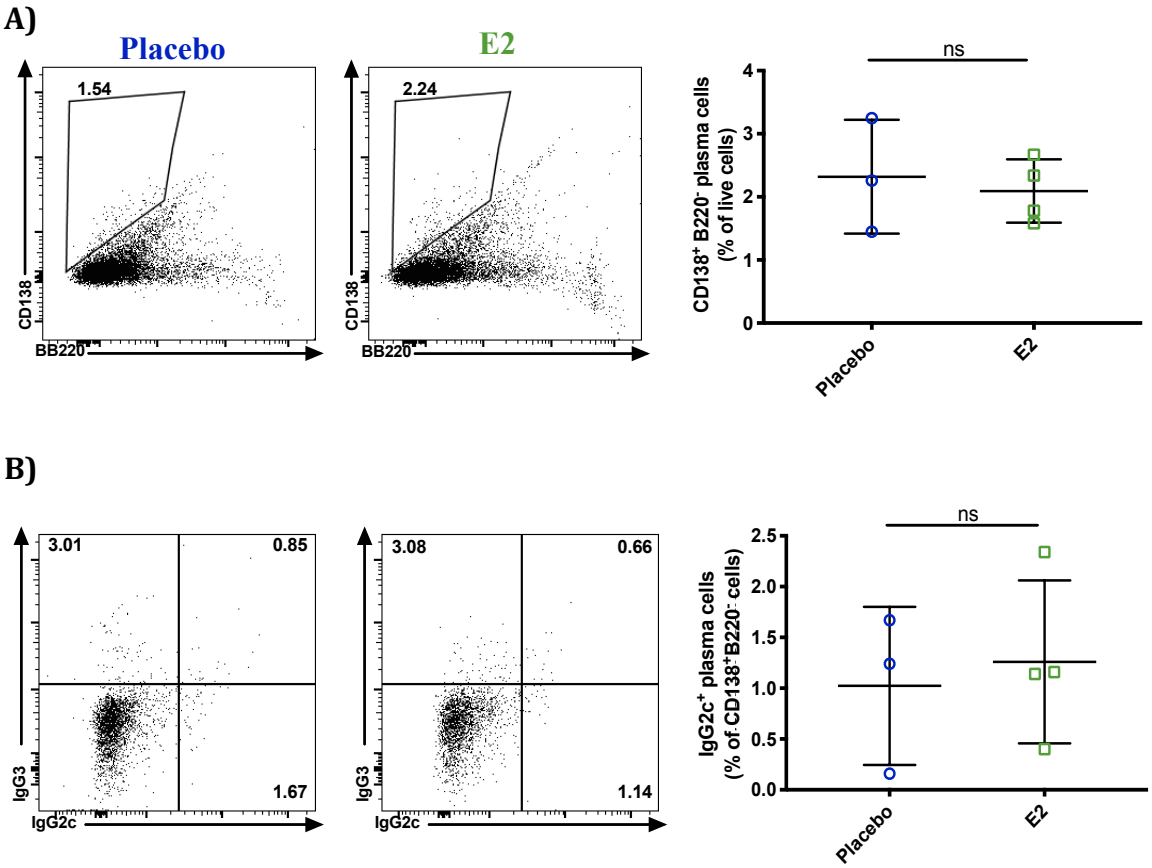


Figure 12. The proportion of IgG2c-secreting plasma cells is not altered in nasal mucosa of naïve E_2-treated mice. WT OVX mice were implanted with E_2 or placebo pellets. Six weeks later, nasal mucosa was collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. **A)** $B220^-$ $CD138^+$ were gated of live cells and their proportion was compared between E_2-treated and mock mice. **B)** Cells were stained for intracellular IgG2c and IgG3 and the frequency of these population were compared between mock and E_2-treated mice. Data shown is from one single experiment. Each data point represents a single animal (n=3-4/group).

4.2 – Aim 2: Determine whether IL-17 mediates estradiol-enhanced B cell responses through augmented Th1 and Th17 cells.

$CD4^+$ T cells play a crucial role in orchestrating and refining the humoral arm of adaptive immune responses. Their dynamic interactions with B cells drives B cell maturation and differentiation into MBCs and plasma cells and further dictates isotype class switch recombination and immunoglobulin affinity maturation.[140] In line with this notion, we postulated that the E_2-mediated enhancement of B cell responses observed following IN immunization and IVAG challenge (*Aim* 1, Section 4.1) occur in a T cell-dependent manner and therefore, is a direct consequence of the augmented Th1 and Th17 responses previously observed.[94] Since these $CD4^+$ T cells enhancements are abrogated in IL-$17^{-/-}$ mice (Bagri et al. unpublished), we postulated that IL-17 is also required for E_2-mediated enhancement of B cell responses observed in *Aim* 1 (Section 4.1). Therefore, to determine whether the enhanced B cell responses observed in the presence of E_2 is T cell dependent and mediated by IL-17, we characterized effector B cell and antibody responses upon IN immunization and IVAG challenge using IL-$17^{-/-}$ mice.

4.2.1 – Aim 2.1 - Elucidating whether the estradiol effect observed post immunization is mediated by IL-17

Recent findings from our lab indicate that the E_2-mediated augmentation of $CD4^+$ T cell responses following IN immunization is established not only in the FGT but also outside of the FGT. In fact, we found that these subsets are primarily enriched within the site of immunization where immune responses to IN TK^- HSV-2 is initially elicited. Put differently, E_2 augmentation of IFN-γ-secreting $CD4^+$ cells and IL-17-

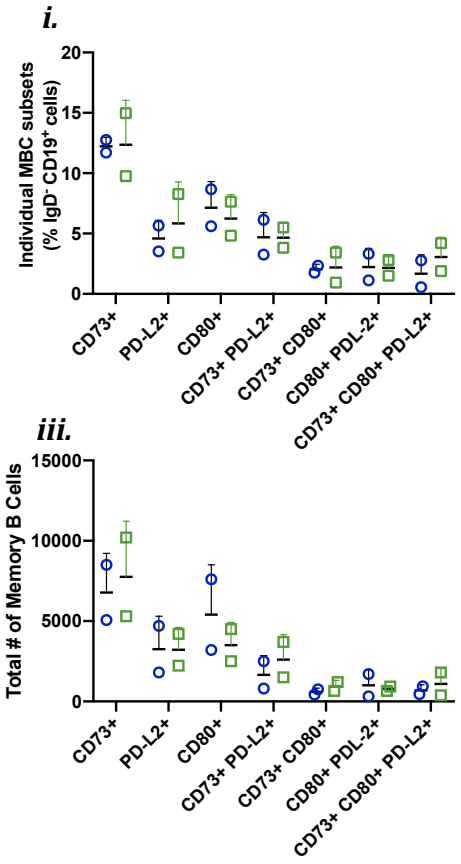
secreting $CD4^+$ cells can occur as early as one-week post IN immunization within the URT while these enriched subsets only appear in the FGT at four weeks post IN immunization. Furthermore, these effects were ablated when our lab conducted parallel experiments using IL-$17^{-/-}$ mice (Bagri et al. unpublished). These findings complement the observations made in this study, where E_2 mediated enhancements of B cell responses are initiated following IN immunization. As such, if these enhanced humoral responses are induced through a $CD4^+$ T cell dependent manner, then they would be absent or significantly reduced in E_2-treated IL-$17^{-/-}$ mice. This was addressed by examining the local inductive sites in the URT, peripheral secondary lymphoid tissues and effector sites following IN immunization of IL-$17^{-/-}$ mice.

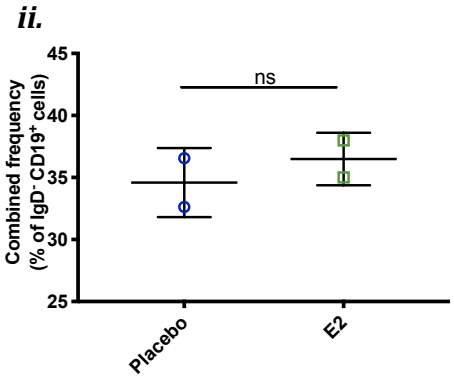
Using the same experimental design as outlined by Fig. 5, IL-$17^{-/-}$ C57BL/6 mice were ovariectomized to remove the endogenous source of sex hormones. Next, one group of IL-$17^{-/-}$ OVX mice were implanted with 21-day release E_2 or placebo pellets Two weeks later, both groups were immunized via the intranasal route with TK^- HSV-2 at 10^4 PFU/mouse. Animals were sacrificed at four weeks following immunization. NALT, cervical lymph nodes, nasal mucosa, spleen, iliac lymph nodes, and genital tract were collected, processed, and stained for polychromatic flow cytometric analysis. Vaginal wash and blood were collected before animals were sacrificed to determine HSV-2 specific IgM, IgG1, IgG2b, IgG2c and IgG3) by ELISA.

In these experiments, E_2-treated IL-$17^{-/-}$ mice had similar MBC profile to their placebo counterpart (Fig. 13). Specifically, the NALT, cervical and iliac lymph nodes and spleen isolated from E_2-treated IL-$17^{-/-}$ mice had comparable individual

frequency, combined frequency and total number of MBCs. Furthermore, regardless of treatment group or the tissue examined, IL-17$^{-/-}$ mice had very little to no double or triple positive MBC subsets (CD73^{+}CD80^{+}, CD73^{+}PD-L2^{+}, CD80^{+}PD-L2^{+}, or CD73^{+}CD80^{+}PD-L2^{+}). These results indicate that the differentiation of B cells into MBC subsets is attenuated in the absence of IL-17. More importantly, the E_2 enrichment of MBCs does not occur in the absence of IL-17.

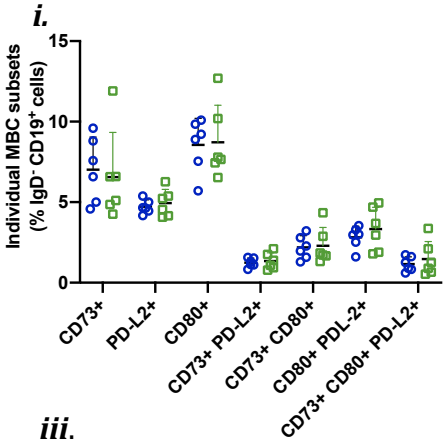
A)

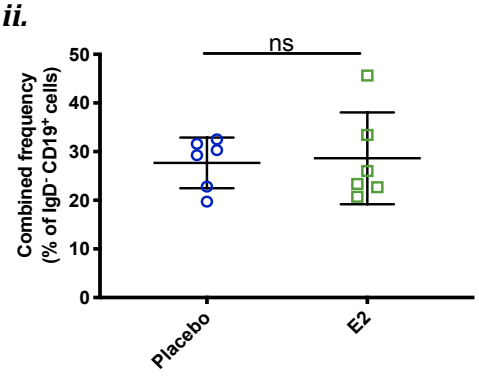

i.
Individual MBC subsets (% IgD- CD19+ cells)
20
15
10
5
0
CD73+
PD-L2+
CD80+
CD73+ PD-L2+
CD73+ CD80+
CD80+ PDL-2+
CD73+ CD80+ PD-L2+

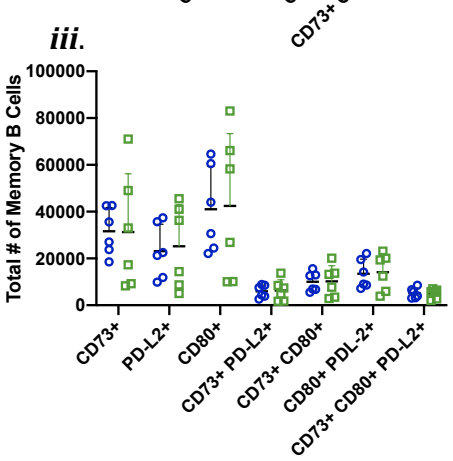

ii.
Combined frequency (% of IgD- CD19+ cells)
45
40
35
30
25
ns
Placebo
E2

iii.
Total # of Memory B Cells
15000
10000
5000
0
CD73+
PD-L2+
CD80+
CD73+ PD-L2+
CD73+ CD80+
CD80+ PDL-2+
CD73+ CD80+ PD-L2+

B)

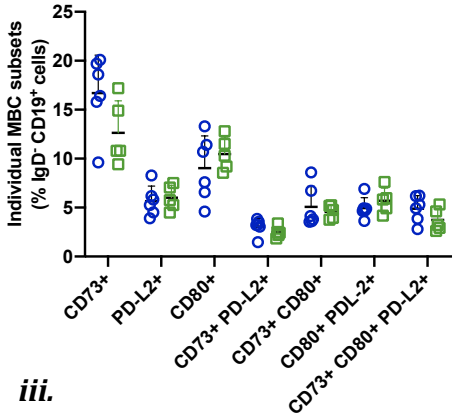

i.
Individual MBC subsets (% IgD- CD19+ cells)
15
10
5
0
CD73+
PD-L2+
CD80+
CD73+ PD-L2+
CD73+ CD80+
CD80+ PDL-2+
CD73+ CD80+ PD-L2+


ii.
Combined frequency (% of IgD- CD19+ cells)
50
40
30
20
10
0
ns
Placebo
E2


iii.
Total # of Memory B Cells
100000
80000
60000
40000
20000
0
CD73+
PD-L2+
CD80+
CD73+ PD-L2+
CD73+ CD80+
CD80+ PDL-2+
CD73+ CD80+ PD-L2+

C)

i.



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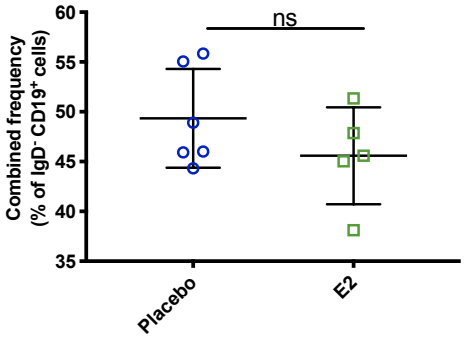

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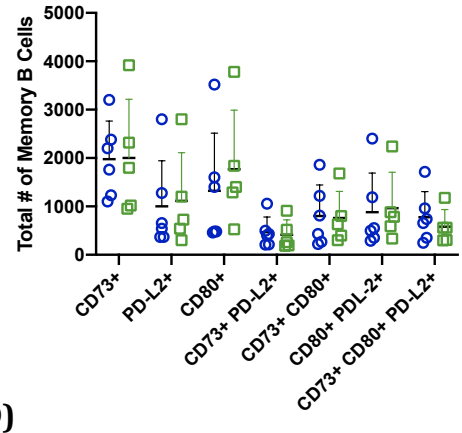
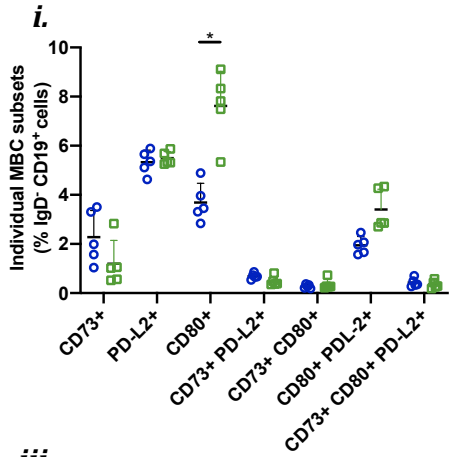
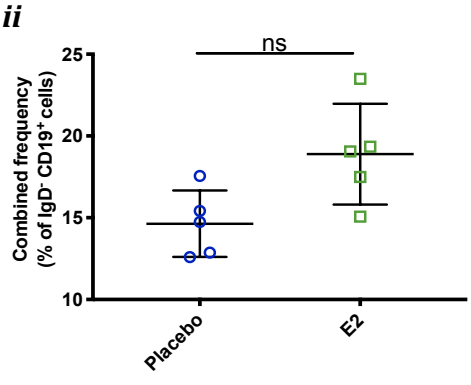


D)

i.



ii



iii.

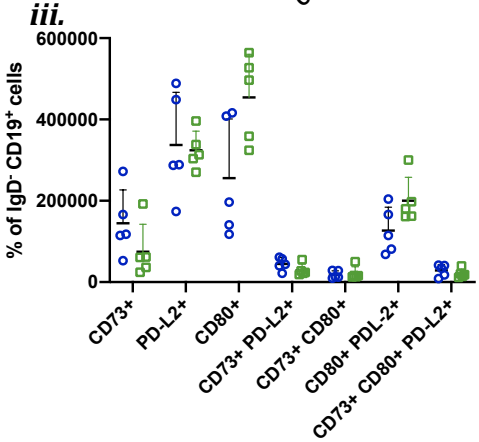


Figure 13. In the absence of IL-17, the effect of E_2 on MBC formation was ablated following intranasal immunization. IL-$17^{-/-}$ OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Four weeks later, cervical lymph nodes, iliac lymph nodes and spleen were collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. Memory B cells were gated of IgD^- $CD19^+$ cells and identified by the heterogenous expression of CD73, CD80, or PD-L2. The differences in percentages of individual subsets **(i)**, the cumulative percentages of all subsets **(ii)**, and total cell number of memory B cells **(iii)** following intranasal immunization was examined in **A)** NALT, **B)** cervical lymph node, **C)** iliac lymph nodes, and **D)** spleen.(○)Represents placebo mice while (□) represents E_2. Data shown is pooled from two independent experiments showing similar findings (NALT of each group was pooled and process as one sample) (n=3-5 mice/group).

Secondly, we examined the antibody-secreting plasma cells residing within the nasal mucosa of E_2-treated and placebo IL-$17^{-/-}$ mice at four-week post IN immunization (Fig. 14A). In congruence with the MBC data, both groups had comparable proportion of $CD138^+B220^-$ cells. More interestingly, intracellular staining revealed the proportion of $IgG2c^+CD138^+B220^-$ cells was similar between the two groups (Fig. 14B) which further substantiates the role of IL-17 and augmented $CD4^+$ T cells in E_2-enhanced B cell immunity.

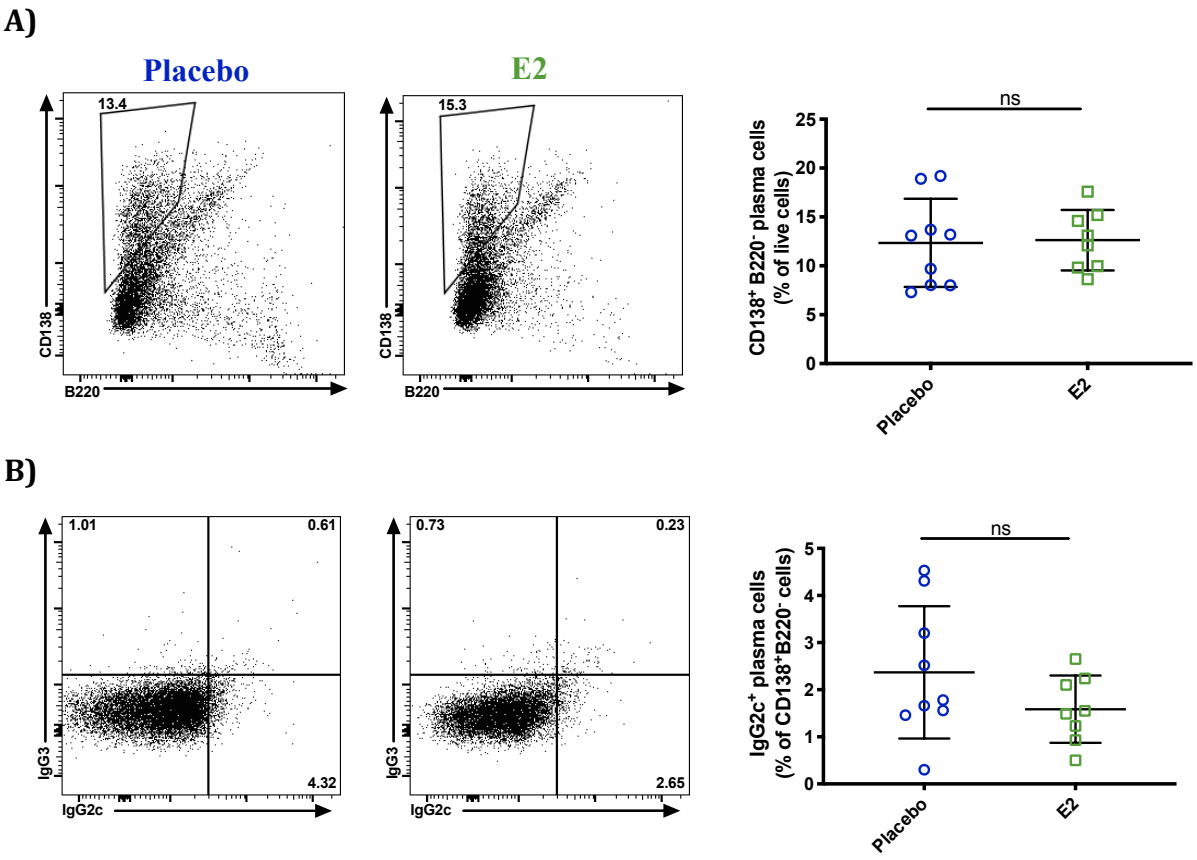


Figure 14. E_2 -mediated enhancement of IgG2c-secreting plasma cells within the nasal mucosa is abrogated in IL-17$^{-/-}$ mice at four weeks post intranasal immunization. IL-17$^{-/-}$OVX mice were implanted with E_2 or placebo pellets. Six weeks later, nasal mucosa was collected, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. A) B220$^-$ CD138$^+$ were gated of total live cells and their proportion was compared between E_2-treated and placebo mice. B) Cells were stained for intracellular IgG2c and IgG3 and the frequency of these population were compared between placebo and E_2-treated mice. Data shown is pooled from two independent experiments (n=5-6 animals/group).

Next, serum levels of HSV-2 specific IgM, IgG1, IgG2b, IgG2c, and IgG3 at four weeks post IN immunization were measured and compared between E_2-treated and placebo IL-$17^{-/-}$ mice (Fig. 15). Similar to observations made in Section 4.1.2, both groups had comparable levels of HSV-2 specific IgM and IgG1 while IgG3 remained undetectable. However, unlike findings of Section 4.12, no difference was observed between E_2-treated and placebo IL-$17^{-/-}$ mice with respect to serum HSV-2 specific IgG2b and IgG2c levels. This finding is consistent with the observations above where the cellular source of IgG2c antibodies were found to be the same between the two groups.

Finally, similar to WT mice, B cells were absent in the FGT of both E_2-treated and placebo IL-$17^{-/-}$ mice (data not shown).

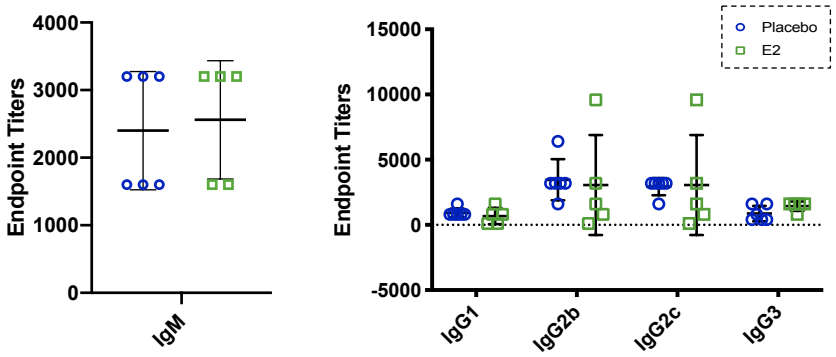


Figure 15. In the absence of IL-17, E_2-treated mice have similar serum levels of IgG2b and IgG2c at four weeks post IN immunization. IL-17$^{-/-}$OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK$^-$ HSV-2 at 10^4 pfu/mouse. Eight weeks later, both groups were challenged with WT-HSV-2 at10^4 pfu/mouse. Vaginal washes were collected from day 21-25 post-immunization and pooled for each animal. Serum samples were collected at day 28 post-immunization via orbital bleeding. HSV-2 specific IgG1, IgG2b, IgG2c, IgG3 were measured by ELISA and endpoint tiers were determined. HSV-2 specific IgG antibody titers were determined in serum samples. The figure is representative of three independent experiments. Each data point represents the serum of a single animal.

4.2.2 – Aim 2.2 - Determining whether the estradiol effect observed on B cell responses post-intravaginal challenge is mediated by IL-17

While $B220^+$ B cells only infiltrated into the FGT of placebo WT mice following IVAG WT HSV-2 challenge, E_2-treated WT mice had greater proportion of $CD138^+B220^-$ plasma cells (Section 4.1.1). The enhanced proportion of plasma cells within the FGT led to increased levels of local HSV-2 specific IgG2b and IgG2c which translated to superior protection against HSV-2. Further analysis revealed that these enhancements were established following IN immunization (Section 4.1.2) and dependent and required the presence of IL-17 (Section 4.2.1). As such, we postulated the in the absence of IL-17, E_2-treated mice would have similar disease outcome and B cell responses to their control counterpart following IVAG WT HSV-2 challenge.

Using the same experimental design as outlined by Fig. 1, IL-$17^{-/-}$ OVX C57BL/6 mice were implanted with either E_2 placebo pellets. Two weeks later, both groups were immunized intranasally with TK^- HSV-2 at 10^4 PFU/mouse. Six weeks later, vaginal smears of E_2-treated mice were observed to ensure that the effect of E_2 had waned. Subsequently, all animals were challenged with WT-HSV-2 at 10^4 PFU/mouse. Vaginal washes were collected, and endpoint monitoring were conducted daily. Animals were sacrificed 5 days after challenge. Blood was collected via cardiac puncture. Iliac lymph nodes, spleen, and vaginal tract were collected, processed and stained with a cocktail of antibodies. Effector B cell subsets were characterized by polychromatic flow cytometric analysis.

Unlike observations made in Section 4.1.1 where protection of WT E_2-treated mice following IVAG challenge was independent of $B220^+$ B cells infiltration into the

FGT, in the absence of IL-17$^{-/-}$, protection of both groups relied on the recruitment of B220^{+} B cells into the FGT following IVAG challenge (Fig. 16). In fact, the proportion of B220^{+} B cells was found to be comparable between the two groups (5.38% vs 5.18% of live cells). Furthermore, both groups have similar frequency of CD138^{+}B220^{-} cells indicating the homing of antibody-secreting cells is disrupted in E_2-treated IL-17$^{-/-}$ mice (Fig. 16). These findings affirm the notion that in the absence of IL-17, E_2-mediated enhancement of B cell responses is ablated. It is important to note these experiments have been conducted only once and further validation is required to draw conclusions.

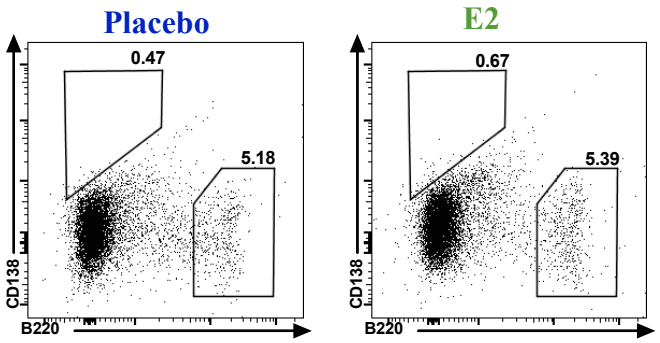
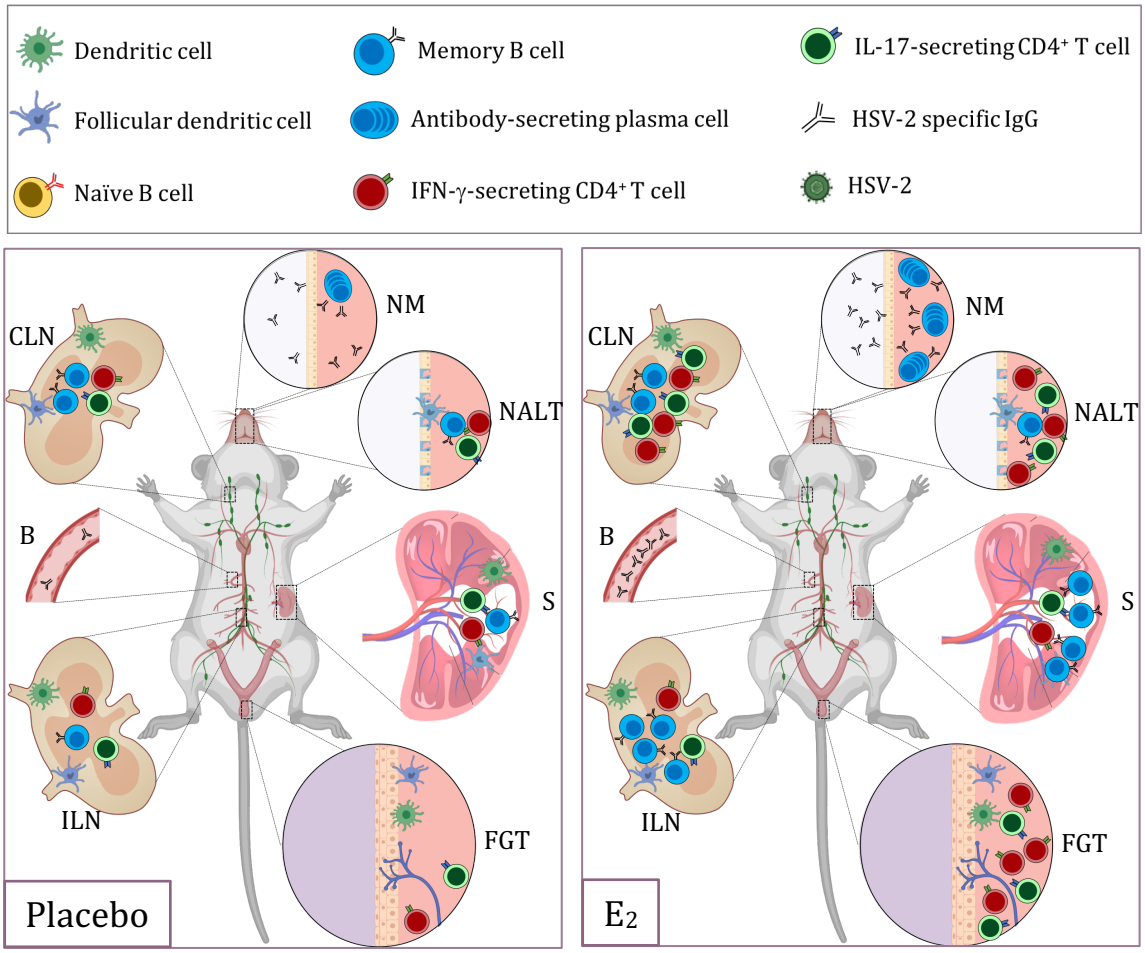


Figure 16. In the absence of IL-17, E_2-treated mice have similar B cell profile in FGT to their placebo counterpart follow IVAG challenge with WT-HSV-2. IL-$17^{-/-}$ OVX mice were implanted with E_2 or placebo pellets. Both groups were IN immunized with TK^- HSV-2 at 10^4 pfu/mouse. Six weeks later, both groups were challenged with WT-HSV-2 at10^4 pfu/mouse. Vaginal tissues were collected at day 5 post IVAG challenge, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry**.** $B220^+$ and $B220^-CD138^+$ cells were gated of total live cells and their proportion was compared between E_2-treated and placebo mice (n=3 to 5 vaginal tracts pooled/group). This experiment was conducted once.

4.3 – Study Summary

A)



B)

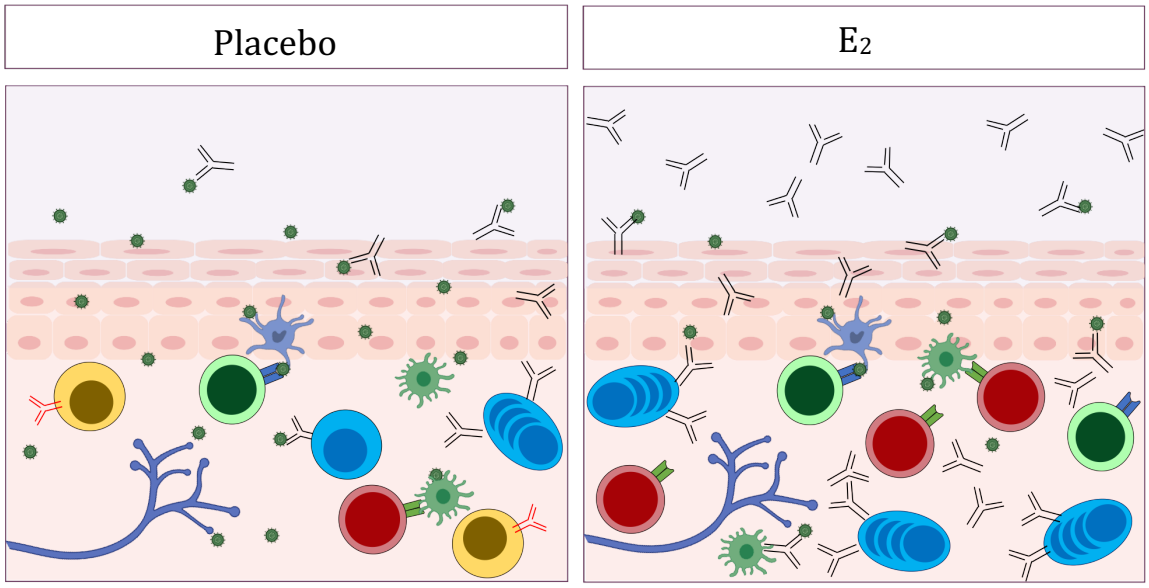


Figure 17. Study Summary. A) At four weeks post IN immunization, MBCs and antibody-secreting plasma cells are compartmentalized into different tissues. Specifically, various MBC subsets are present within the secondary lymphoid tissues and these subsets are enriched in the iliac lymph node and spleen when IN immunization occurs in the presence of E_2. Antibody-secreting plasma cells were localized within the nasal mucosa and were also enhanced in the presence of E_2. **B)** Immune responses within the FGT at 5 days post IVAG challenge. E_2-treated mice have significantly greater proportion of antibody-secreting plasma cells which translate to greater levels of HSV-2-speicific IgG within the vaginal lumen. Conversely, in placebo-treated mice, infiltration of naïve B cells and MBCs is observed. This difference in immune phenotype explains the underlying mechanism by which superior protection is observed under E_2. B: blood, CLN: cervical lymph node, ILN, iliac lymph node, NM: nasal mucosa, and S: spleen.

CHAPTER 5: DISCUSSION

5.1 – Summary of Findings and Implications

The challenges of developing an efficacious vaccine against HSV-2 requires further research into better understanding the host antiviral responses and identifying strategies to maximize these responses. While ideally an optimal vaccine would establish sterile immunity, whereby the virus is neutralized and therefore unable to establish any productive infection, this feat is yet to be achieved. While this remains the ultimate goal of HSV-2 vaccine development, other modes of protection can still provide considerable clinical improvement. For example, more clinically feasible prophylactic measures that would control the virus locally and prevent dissemination of the virus into the neurons thereby mitigating the development of latency and a chronic infection would also be extremely significant. Similarly, a therapeutic vaccine that can prevent the reactivation of HSV-2 and thereby preventing viral shedding and reduce the rates transmission would be highly valued. In fact, a clinical proof of this concept is the Varicella zoster virus (VSV) vaccine which prevents herpes zoster, caused by reactivation of latent VZV.[145] This is the only vaccine used in a clinical setting that confers protection in a T cell-mediated manner in which vaccine-induced IFN-γ^+ $CD4^+$ T cells line around the neuronal tissue and prevent viral reactivation.[145] While a HSV-2 vaccine providing either of the aforementioned modes of protection has not been achieved, a better understanding of the immune responses against HSV-2 can be provide insights into the most feasible vaccine strategy.

To date, extensive research utilizing animal models of HSV-2 have underscored the fundamental role of cell mediated-immunity during the natural course of infection.[90,92] While cell mediated immunity is also vital for generating immunological memory against the virus following vaccination, optimal vaccine-induced protection still requires antibody-mediated immunity that can prevent productive viral infection and its subsequent establishment of latency. Put differently, mathematical modeling reveals that an effective T cell-mediated HSV-2 vaccine requires high density of T_{RM}'s established throughout the vaginal epithelium to prevent viral spread, which may not be feasible in a clinical setting.[146] As such, a clinically efficacious HSV-2 vaccine must induce high levels of HSV-2 specific antibodies in addition to establishing T_{RM}s in the vaginal mucosa to prevent latent infection of neurons. One strategy to achieve these responses is immunization via mucosal routes.[131–134] Mechanistic studies reveal that the mode of protection differs depending on the mucosal route that the vaccine is administered. IVAG immunization provides complete protection by establishing high concentration of T_{RM} within the FGT.[111] This leads to viral clearance within the FGT and stops its spread to other tissues.[107] Conversely, protection conferred by IN immunization appears to occur viaHSV-2 specific antibodies which neutralize the virus within the dorsal root ganglion and the spinal cord and prevent its incorporation into the host genome within the neurons.[107] Even though IVAG immunization is superior in generating local immune responses within the FGT, it's clinical practicality remains unknown and untested.[113] On the other hand, vaccination via the IN route has proven to be an effective strategy in a clinical setting.[113] However, although it is well

known that protection conferred by IN immunization is antibody-mediated, it still remains unclear how and where these responses are elicited.[96,107] As a result, in an attempt to fill the gap in the current literature, one of the goals of this project was to gain a comprehensive understanding of the B cell responses elicited following IN immunization and determine their role in protection following IVAG challenge. Furthermore, given the pressing need to identify strategies to improve vaccine-induced immunity against HSV-2, we tested the efficacy of exogenous E_2 as one strategy to enhance antiviral immunity.

The overall hypothesis of the current study was that E_2 treatment will enhance the formation of MBCs within secondary lymphoid tissues and antibody-secreting cells within nasal effector sites following IN immunization. Upon IVAG challenge, these enhancements will lead to greater migration of MBCs and antibody-secreting cells to the FGT leading to enhanced HSV-2 antiviral immunity. To address this hypothesis, we formulated two goals for this study: 1) Characterize B cell and antibody responses post-immunization and challenge and determine the effect of E_2, 2) Determine whether IL-17 mediate E_2-enhanced B cell responses through augmented Th1 and Th17 cells. These objectives were addressed by using a mouse model of HSV-2. Flow cytometric analysis was used to characterize MBCs and antibody-secreting plasma cells following both IN immunization and IVAG challenge while HSV-2 specific antibody levels were measured by ELISA.

The first goal of our study was to investigate the B cell responses elicited

following IN immunization. Early studies in the HSV-2 mouse model primarily focused on T cell responses and the humoral immunity generated by B cell responses.[71,102,104,133] Then the finding that B cells were not required for protection following IVAG immunization shifted the focus of the field onto better understanding the T cell responses and the mechanisms by which they conferred protection.[91,96,108,109,111,147] Consequently, there exists a large gap between our current understanding of antiviral B cell responses compared to antiviral T cell responses in this model. In an effort to close this gap, we sought out to investigate the B cell responses generated following IN immunization in the absence of sex hormones by utilizing an ovariectomized mouse model of HSV-2. We examined various sites pertaining to the URT and the FGT, characterized the different effector B cell subsets and tried to identify the cellular and tissue source of HSV-2 specific antibodies previously reported. Our findings indicate that IN immunization elicited B cell differentiation into MBCs and antibody-secreting plasma cells. More interestingly, these subsets were found to be compartmentalized into different tissues. The MBCs mainly localized within the local and peripheral secondary lymphoid tissues while the antibody-secreting plasma cells primarily resided within the nasal mucosa. More importantly, our result indicated that these plasma cells were likely the cellular source of HSV-2 specific antibodies detected in the serum at four weeks post IN immunization. This is likely given as we did not identify any significant plasma cell population within other sites. In line with findings from previous studies, these antibodies were predominately of IgG2b and IgG2c subclasses.[107,143] Finally, since IN

immunization with TK$^-$ HSV-2 leads to migration and retention of effector T cells within the FGT, we sought to determine whether B cell recruitment into FGT occurred in a similar manner.[148] Surprisingly, at four weeks post IN immunization, B cells did not infiltrate into FGT. The absence of B cells within the FGT translated to very minimal levels of HSV-2-specific antibodies in the vaginal lumen. These findings indicate that the B cell responses elicited following IN immunization are primarily established outside of the FGT which mirror the observations made by Oh et al.,[112] where IVAG immunization failed to recruit and retain any effector B cell within the FGT.

Although HSV-2 specific antibodies were scarce in the FGT following IN immunization, other studies have reported their presence following secondary IVAG exposure/challenge to the virus.[53,103,107,134] As such, our next goal was to investigate the B cell responses elicited following IN immunization-IVAG challenge and try to determine the source of the vaginal HSV-2 specific antibodies. Our findings indicate that IVAG challenge elicited the recruitment of B cells into the FGT. Specifically, at 5 days post IVAG challenge, both CD138$^+$B220$^-$ plasma cells and CD138$^-$B220$^+$ B cells were present within the FGT. More importantly, the establishment of CD138$^+$B220$^-$ plasma cells led to detectable levels of HSV-2 specific IgM, IgG2b and IgG2c within the vaginal lumen. These findings indicate that the HSV-2-specific antibodies detected within the vaginal lumen upon challenge to the virus are secreted by local antibody-secreting plasma cells. Unfortunately, given the limited availability of vaginal samples, we opted to measure HSV-2 specific IgG3 rather than IgG1 for reasons explained later.

As such, HSV-2 specific IgG1 antibodies could also be present within the vaginal lumen after secondary IVAG challenge. Although we did not identify the mechanism by which B cells were recruited into the FGT upon IVAG challenge, we postulate that the mechanism is similar to that described by Oh et al.,[112] where the $CD4^+$ T_{RM}'s established within the FGT post-IVAG immunization secrete IFN-γ upon IVAG challenge and induce the expression of CXCR3 ligands (CXCR9 and CXCR10) within the vaginal mucosa; thereby recruiting B cells into the FGT in a CXCR3-dependent manner.

These findings have a number of clinical implications. First, our observations complements the findings of Oh et al.,[112] and reveal that regardless of the route of mucosal immunization, B cells are only recruited into FGT upon secondary exposure/challenge. Put differently, both IVAG and IN immunization induce similar B cell responses but require a secondary exposure/local challenge within the FGT microenvironment to facilitate entry of antibodies into the FGT tissue. However, neither vaccination route seems capable of establishing effector B cell subsets within the FGT, following immunization. Accordingly, high levels of HSV-2 specific antibodies within the circulation following immunization does not translate to their presence within the vaginal mucosa. These observations help explain, at least in part, the underlying reason why antibody-mediated HSV-2 vaccines developed to date have all failed to confer protection in humans.[149] Specifically, as indicated by murine studies, the concentration of HSV-2 specific IgG required to confer protection against HSV-2 within the vaginal lumen is high. Therefore, the absence of these antibodies in the

local tissue upon viral entry into the FGT renders the vaccines ineffective.[150,151] Moreover, findings from our study illustrates that IN immunization is as equally effective as IVAG immunization with respect to generating antibody-mediated responses. Therefore, given its equal immunogenicity and superior practicality in a clinical setting, future studies should utilize the IN route of immunization and primarily focus on identifying strategies to recruit and retain effector B cell subsets within the FGT. One such strategy that may be successful in accomplishing this goal is "the prime and pull" strategy described by Shin and Iwasaki[152]. Specifically, CXCR3 ligands may be applied to the vaginal cavity, a few weeks after IN immunization, to pull the effector B subsets into the FGT and generate luminal HSV-2 specific antibodies. It is important to emphasis that solely establishing effector B cells within the FGT will not guarantee complete protection upon vaginal exposure since the extremely high levels of HSV-2-specific IgG required may not be achieved.[102] As such, moving forward, other factors that influence vaccine-induced immunity must be taken into account to overcome the challenges that we currently face with respect to developing a viable vaccine against the HSV-2.

One important factor that is commonly overlooked during the rational design of an efficacious vaccine against HSV-2 is how sex hormones modulate immune responses. By taking their effects into consideration, one can utilize the endogenous sex hormones, specifically E_2, as a means of eliciting superior vaccine-induced immune responses against not only HSV-2 but other infectious agents as well.[3] Findings from our lab showcase the success of such approach in the mouse model of

HSV-2.[53,94,135,137,145,146] Our lab has demonstrated that IN immunization in the presence of E_2 leads to reduced viral replication and confers superior protection upon IVAG challenge with WT-HSV-2.[135,153,154] Further mechanistic studies revealed the presence of E_2 enhances the establishment of effector $CD4^+$ T cells within the FGT and leads to increased luminal levels of HSV-2 specific IgG upon IVAG challenge.[53,94] Given the importance of luminal antibodies in protection against HSV-2, we next sought out to examine the influence of E_2 on vaginal B cell responses following IN immunization-IVAG challenge to determine how E_2 treatment led to increased luminal levels of HSV-2 specific IgG. This was done by comparing the B cell and antibody responses elicited within the FGT of placebo and E_2-treated mice at day 5 post IVAG challenge. We initially hypothesized that E_2 treatment, through the enhanced $CD4^+$ T cells, will augment the B cell responses observed under placebo conditions and lead to greater recruitment of both MBCs and antibody-secreting plasma cells post IVAG challenge. However, our findings revealed that the B cell profile within the FGT of E_2-treated mice was considerably different to their placebo counterpart after IVAG challenge. Specifically, at day 5 post IVAG challenge, $B220^+$ B cells were not recruited into the FGT of E_2-treated mice. Instead, E_2 treatment induced greater establishment of vaginal antibody-secreting plasma cells which led to increased luminal HSV-2 specific IgG2b and IgG2c detect at day 5 post challenge. These findings provide further insight into how IN immunization in the presence of E_2 confers superior protection upon IVAG challenge. Particularly, E_2 treatment imprints a microenvironment within FGT which allows the preferential recruitment of the antibody-secreting plasma cells rather than

MBCs or naïve B cells in face of IVAG challenge. Although we did not examine the specific factors that mediate the differential B cell recruitment remains unclear, we speculate that it is likely primarily mediated by the enhanced $CD4^+$ T cell responses that are established within the FGT following IN immunization. Specifically, as demonstrated by Bagri et al. (unpublished), IN immunization in the presence of E_2 leads to significantly greater proportion of IFN-γ and IL-17-secreting $CD4^+$ T_{RM}'s within the FGT. Based on these findings in addition to observations made by Oh et al.,[112] upon IVAG challenge in our study, these enhanced $CD4^+$ T_{RM}'s would induce greater levels of CXCR3 ligands within the vaginal mucosa. Consequently, this would lead to the recruitment of more differentiated effector B cells which highly express CXCR3 and are preferentially recruited into FGT. In fact, these more differentiated effector B cells could be antibody-secreting plasma cells that are recruited into the FGT or MBCs that exhibit more memory-like phenotype (co-expressing CD73, CD80, and PD-L2) and can readily differentiate into plasma cells upon infiltrating the FGT.[139] Regardless, the findings from the current study illustrate that by day 5 post IVAG challenge, plasma cells constitute the only B cell population within the FGT of E_2-treated mice. This finding is highly significant since plasma cells readily secrete luminal HSV-2 specific antibodies and contribute to the early control and protection of the virus. Furthermore, the antibodies secreted by these cells are thought to be of higher affinity for the virus.[142] This is thought to occur due to the antigen affinity-dependent manner by which MBC and plasma cell differentiation occurs. As described in detail by Zuccarino-Catania et al.[142], of those B cells that enter the GC center, B cells

with low affinity B cell receptors (BCR) receive little to no cognate T cell help and are driven towards a MBC phenotype with less-memory like phenotype (do not co-express CD73, CD80, and PD-L2). Conversely, those with high affinity BCR strongly interact with cognate T cells and differentiate into more memory like MBCs and plasma cells.[142,155]As such, our identification of enhanced antibody-secreting plasma cells within the FGT suggest that the secreted antibodies by these cells are of high affinity for the virus. Overall, these findings provide further insight into the immunological mechanisms by which E_2 treatment enhances protection against HSV-2. Specifically, in this study, we have discovered how the augmentation of vaccine-induced immunity in the presence of E_2 translates to the establishment of antibody-secreting plasma cells within the FGT following challenge, which leads to enhanced levels of luminal HSV-2 specific IgG2b and IgG2c. This ultimately confers superior protection against HSV-2.

Although the exogenous E2 pellets that are widely used, including in our studies, state that they are 21-day release pellets, our previous studies indicate that they typically provide sustained release between 25 to 47 days post pellet implantation. However we showed that in all cases the E_2 levels become undetectable by day 47 (6-7 weeks).[156] Since in this study, the animals were intravaginally challenged at day 56 post pellet implantation, the enhanced B cell responses observed in the E_2-treated group post-challenge could expect to have been induced during immune priming, since there is no detectable E2 levels at the time of challenge. In fact, the IN TK^- HSV-2 was administered at day 14 post pellet implantation when the levels

of E_2 were at its peak. Accordingly, to determine whether E_2-mediated enhancement occurs following IN immunization, we characterized the B cell and HSV-2 specific antibody responses at four weeks post IN immunization and compared these responses between E_2-treated mice and their placebo counterparts. The first indication that E_2-mediated enhancement does indeed occur following immune induction was the observation that the serum of E_2-treated mice contained significantly greater levels of HSV-2-specific IgG2c. Our subsequent investigation into secondary lymphoid tissue and effector sites revealed that E_2-mediated enhancement of B cell responses was observed outside of the FGT since both B cells and luminal HSV-2 specific antibodies levels were virtually absent in the FGT of both groups. These findings are supported by the notion that B cell recruitment into the FGT is dependent on preceding establishment of IFN-γ^+ $CD4^+$ T_{RM} within the tissue and their subsequent stimulation.[112] Interestingly, outside of the FGT, we found E_2 treatment to enhance both MBC and plasma cell subsets within distinct sites. Specifically, at four weeks post IN immunization, iliac lymph nodes and spleen isolated from E_2-treated mice had significantly greater proportion of individual MBC subsets. Furthermore, combination of these individual subsets revealed that E_2-mediated enhancement occured by enriching the MBCs within the B cell pool. Interestingly, while the same MBC subsets were present within the local inductive sites (NALT and cervical lymph nodes), the E_2-mediated enhancement was not observed, as both E2-treated and placebo mice had comparable proportion of MBC subsets. While further investigation is required to better understand these mechanisms by which this selective enhancement is

occurring, we speculate that our current understanding with regards to the URT and the $CD4^+$ T cell responses may help explain these observations. Specifically, as mentioned earlier, our lab recently discovered that IN immunization in the presence of E_2 leads to greater establishment of IFN-γ^+ and IL-17^+ $CD4^+$ T_{RM}'s only within the NALT and cervical lymph nodes and not the iliac lymph nodes or spleen (Bagri et al, unpublished). These E_2-mediated enhancements were observed as early as one week post IN immunization and persisted until at least 4 weeks later (Bagri et al, unpublished). We believe that a small subset of these $CD4^+$ T_{RM}'s, which reside within these sites permanently, serve as cognate Tfh cells and help B cells differentiate to functional effector subsets. As such, upon differentiating into MBCs or plasma cells, these effector B cells may transit out of the NALT and cervical lymph nodes and migrate to peripheral secondary lymphoid tissues and effector mucosal sites.[119,120] In support of this, Heritage et al.[120] demonstrated that upon IN immunization, IgG responses were initially detectable in the NALT and cervical lymph nodes and only appeared in the spleen at a later timepoint. This could likewise be the case in our study, where IN immunization in the presence of E_2 leads to increased proportion of $CD4^+$ T_{RM}'s within local inductive sites which may result in enhanced rate of B cell differentiation and increased proportion of effector B subsets which then migrate to other sites where we observe the E_2 mediated enhancement. In support of this mechanism, we also found E_2 treatment to enhance the establishment of $IgG2c^+CD138^+B220^-$ plasma cells within the nasal mucosa (mucosal effector site). Additionally, this finding complemented our earlier observations and revealed that

the enhanced HSV-2-specific IgG2c detected in the serum of E_2-treated mice stems from the augmented IgG2c-secreting plasma cells localized within the effector sites of the URT. Finally, to ensure that the E_2-mediated enhancements were indeed specific to the IN immunization and did not occur in a non-specific manner, we conducted a parallel experiment in which neither groups received IN immunization. As expected, none of the E_2-mediated enhancements that were seen post IN immunization were observed in immunologically naïve mice. Altogether, these results indicate that functional B responses are augmented with E_2 treatment only in the context of IN immunization.

More importantly, the findings from this study further substantiate the applicability of sex hormones as a means of eliciting superior vaccine-induced immunity against HSV-2. A thorough understanding of such factors may allow us to overcome the obstacles faced when developing an efficacious vaccine against the virus. As previously mentioned, one of the reasons why most of the vaccines tested to date have failed to confer protection against HSV-2 has been their inability to induce adequately high levels of HSV-2-specific IgG within vaginal lumen.[150,151] In this study, we demonstrated how such potent levels may be achieved in the vaginal lumen by administering the vaccine in the presence of high *in vivo* levels of E_2. Such strategy could be employed in premenopausal women during the E_2-high phase of their cycle, but also used in conjunction with exogenous E_2, such as in the form of hormonal contraceptives or in hormonal therapy in post-menopausal women. Furthermore, this strategy is not only limited to viruses that infect the FGT, but also those that target

other sites.[157–161] In fact, this phenomena has been extensively studied in the context of Influenza A virus.[157,158,161] While the majority of these were conducted in animal models of the disease, importantly, a few studies have showcased it in a clinical setting. Most notably, Engelmann et al.[162] demonstrated, that the efficacy of the seasonal trivalent inactivated influenza vaccine was significantly reduced in postmenopausal women in comparison to premenopausal women. Interestingly, postmenopausal women that received E_2 therapy had significantly increased plasma levels of virus-specific IgG.[162] These findings, in addition to our own, thus could have important implications for the design and execution of future vaccines. Moreover, these findings underscore the importance of taking the effect of sex hormones into account when studying immune responses to various pathogens.

Our previous studies revealed that the superior protection observed with E_2 treatment was related to increased Th1 and Th17 immunity in the FGT.[94] Specifically, we found this to be dependent on the secretion of IL-1β by vaginal $CD11c^+$ DCs which augmented Th17 responses within the FGT.[94] Subsequently, these augmented responses resulted in a microenvironment rich in IL-17, a cytokine critical for inducing IFN-γ^+ $CD4^+$ T cell immunity in the context of HSV-2.[93,98] In the current study, E_2 treatment led to the enhancement of HSV-2 specific IgG2b and IgG2c levels. In order for a B cell to undergo recombinant isotype switching to IgG, the help of its cognate T cell is required.[127] Due to this reliance on T cells, we sought to understand if the absence of IL-17 would likewise result in abrogation of E_2-mediated enhancements of the B cell responses observed after both IN immunization and IVAG challenge. As

such, we conducted experiments parallel to those in section 4.1.1 and 4.1.2 using IL-17$^{-/-}$ mice. Interestingly we found that the E_2-mediated enhancements of B cell responses that were observed following IN immune priming were no longer present in the absence of IL-17. Specifically, E_2-treated and placebo IL-17$^{-/-}$ mice had comparable proportion of MBCs across all of their secondary lymphoid tissues and IgG2c^{+}-secreting plasma cells within the nasal mucosa. More importantly, upon IVAG challenge, similar B cell responses were observed in FGT of both E_2-treated and placebo IL-17$^{-/-}$ mice. Furthermore, at day 5 post-challenge, comparable proportion of CD138^{+}B220^{-} plasma cells and B220^{+} B cells were present in the FGT of both groups. This contrasted the observation made in section 4.1.1 where protection upon IVAG challenge in E_2-treated WT mice was independent of B220^{+} B cells infiltration into the FGT. Instead, these mice had significantly greater establishment of CD138^{+}B220^{-} plasma cells within the FGT in comparison to their placebo counterpart. Therefore, these findings indicate that E_2-mediated enhancement of B cell responses were impaired in the absence of IL-17. Although the specific role of IL-17 in enhancing these B cell responses remains to be elucidated, we speculate that this is an indirect effect where the augmented IFN-γ^{+} CD4^{+} T cells mediated through IL-17 under E_2 high conditions, induces greater B cell differentiation. This is further supported by the observation that only HSV-2 specific IgG2 subclasses were enhanced upon E_2 treatment. As previously mentioned, IFN-γ^{+} CD4^{+} T cells drives B cell recombinant isotype class switching to IgG2.[128] Importantly this phenomena has been previously documented in the context of HSV-2. Specifically, Johnson et al.[163] demonstrated that

post IVAG immunization, the serum levels of HSV-2 specific IgG2 in IFN-$\gamma^{-/-}$ mice, in contrast to WT mice, was not significantly different to the serum of immunologically naïve mice. The finding that IFN-γ is critical in eliciting IgG2 responses against HSV-2 leads us to believe that a similar mechanism is responsible for our observations. Specifically, we believe that IN immunization in the presence of E_2 leads to increased levels of IL-17 which in turn enhances Th1 and Th17 responses within the inductive sites of URT and the FGT. Consequently, a subset of these enhanced Th1 subsets serve as cognate Tfh cells to induce B cell maturation and differentiation. Ultimately, this translates into increased IgG2 responses. Overall, these findings reveal multiple immunological mechanisms that are modulated by E_2. An understanding of these mechanisms can be implemented in future vaccine regiments. As previously stated, this could as simple as administering the vaccine at the E_2 high phase of the menstrual cycle in premenopausal women or in conjugation with exogenous E_2. However, our more nuanced understanding of the mechanisms responsible for E_2-mediated enhancements have broader implications that may impact a wider population. For instance, future vaccine regimens may use IL-17 or IL-1β as an adjuvant to induce similar enhancements observed in our study. This approach would not preclude males, prepubescent girls, postmenopausal women or populations where hormonal therapy is not suitable or preferred. While IL 1β as an mucosal adjuvant has been examined in the context of Influenza A in mice, further investigation into the safety and efficacy into these potential cytokine based adjuvants is still required.[164,165]

5.2 – Limitations

While the findings from this study provide further insight into the mechanisms by which E_2 modulates B cell responses elicited in the context of immunization and challenge, there are a number of limitations in these studies that are important to consider. First and foremost, the findings from our study mainly stemmed from experiments conducted in the mouse model of HSV-2. While mice serve to be important model to study the immunobiology of various human pathogens, they do not completely mimic that of the human body. For example, in this study, we did not examine HSV-2-specific IgA responses. This decision was primarily based on previous murine studies demonstrating that mucosal immunity against HSV-2 was independent of IgA.[53,105] However, this may not necessarily be the case in humans since some IgA effector functions in humans is mediated through Fcα receptor (CD89) which is absent in mice.[166] As such, while the lack of critical role for IgA as a neutralizing antibody seen in murine studies may be clinically translatable, its other functions that require the presence Fcα receptor would not be captured during in our model. Therefore, the relative contribution of IgA in antiviral HSV-2 immunity remains to be further studied. Another limitation to our study was that mice were ovariectomized and given 21-day release E_2 pellets which led to high physiological levels of E_2 that remained elevated for several weeks. While this allowed us to study its effect on B cell responses in isolation, it lacks the physiological resemblance of women where E_2 levels fluctuate through the menstrual cycle and there is also the presence of progesterone throught the menstrual cycle.[62] Consequently, the enhanced

B cell responses observed in our model may not occur to the same extent or be more variable under physiological conditions. An additional limitation in this study was that we did not determine whether the enhanced MBC subsets observed E_2 treatment group post IN immunization were all antigen specific. The results from our ELISA support the hypothesis that E_2 enhance HSV-2-specific plasma cell differentiation and leads to increased HSV-2-specific IgG antibody titres. However, it remains unclear whether the enhanced MBCs subsets observed within the peripheral secondary lymphoid tissues post-IN immunization are antigen specific and contribute to viral control upon challenge or they are enhanced due to non-specific bystander effect.[167] As previously described MBC and plasma cell differentiation occurs in an antigen affinity dependent manner.[168] As such, the enhanced MBC subsets observed in our E_2 treatment group may be a direct result of E_2's promotion of B cell survival and proliferation rather than antigen specific stimulation.[169] Lastly, in this study, luminal levels of HSV-2-specific IgG subclasses were not readily detectable at four weeks post IN immunization. This observation was not consistent with other studies which have previously reported the presence of HSV-2 specific IgG within the FGT after IN or IVAG immunization.[53,71,106,143,170] It is important to note in these studies, the level of luminal HSV-2 specific IgG was significantly lower than those measured in serum. In fact, in a study by Ye et al.[143], the endpoint titer of luminal HSV-2-specific IgG following two rounds of IN immunization was measured to be around 1:50. In this study, the researchers measured the total level of HSV-2-speific IgG which included all of the IgG subclasses. In our study, our objective was to determine the levels of IgG2b, IgG2c,

and IgG3 at four weeks post IN immunization and at 5 days post IVAG challenge. Given the finding that the levels of total HSV-2-specific IgG within the vaginal mucosa following immunization is extremely low, our ELISA assay may not have the sensitivity that was required to detect specific subclasses. To resolve this discrepancy between the literature and our study, future studies should first measure HSV-2-specific IgG levels within the vaginal secretion at four weeks post IN immunization before measuring specific subclasses.

5.3– Future Directions

The findings from this study provide a comprehensive understanding of the antiviral B cell responses elicited in the context of HSV-2 and showcase how E_2 can augment these responses. As previously mentioned, the mode of protection differs depending on the route of mucosal immunization.[107,111] Protection conferred by IVAG immunization occurs locally and is primarily mediated by highly concentrated $CD4^+$ T_{RM}s within the FGT. On the other hand, IN immunization fails to control the virus locally but confers neuroprotection by an antibody-mediated mechanism.[107] In this study, we demonstrated how IN immunization in the presence of E_2 enhances functional B cell responses. Additionally, findings by Bagri et al. (unpublished) reveal that establishment of $CD4^+$ T_{RM}s within the FGT is also enhanced. Despite these findings, it remains unclear whether the augmented local T cell responses are able to control the virus locally or protection actually occurs by HSV-2 specific antibodies blocking viral entry into neurons in the dorsal ganglia. This can be addressed by conducting further studies in which ovariectomized WT and HEL-BCR Tg mice are

intranasally immunized in the presence of E_2, later challenged intravaginally and survival and disease outcomes are assessed and compared between the two groups. The B cells of HEL-BCR Tg mice will be able to serve as APCs and support T cell responses, however they bear an irrelevant BCR (against hen egg lysozyme, HEL) and cannot mount an antiviral antibody response.[171] As such, this approach will highlight the relative contribution of HSV-2-specific antibodies in E_2-mediated protection induced following IN immunization.

Previously, we have demonstrated that both Th1 and Th17 responses are augmented with E_2 treatment and the absence of IL-17 impaired these enhancements.[94] Likewise, in this study, we found that IL-17 is required for the enhancement of B cell responses observed in the presence E_2. While our findings allude to the involvement of $CD4^+$ T cells in enhancing E_2-mediated B cell responses, further studies are required to identify the specific $CD4^+$ T cell subset that is involved. The finding that only IgG2 responses were enhanced suggests that augmented IFN-γ $CD4^+$ T cells are responsible for the E_2-mediated enhancement of B cell responses. This can be addressed by generating mixed bone-marrow chimeric mice which lack IFN-γ producing $CD4^+$ T cells and subsequently assessing the B cell responses elicited following IN immunization in the presence of E_2. Theoretically, E_2-mediated enhancements of Th17 responses will still occurring. As such, the findings from such study will reveal the role of Th1 cells in modulating the elicited B cell responses.

Conclusion

In conclusion, findings from this study demonstrate the importance of sex-dependent differences in vaccine-induced immunity and particularly highlight the role of E_2 as a modulator of humoral immunity. Specifically, we showcased how IN immunization in the presence of E_2 leads to the enrichment of various MBC subsets within peripheral secondary lymphoid tissues and antibody-secreting plasma cells within the nasal effector sites. Subsequently, upon IVAG exposure to the WT virus, these enhancements allow for a more rapid and robust response within the FGT by establishing increased levels of HSV-2-specific IgG within the vaginal lumen and ultimately conferring superior protection. With the current lack of an efficacious vaccine for HSV-2, this has important implications for the rational and practical design of a viable vaccine. Furthermore, this strategy is not limited to HSV-2, but can be broadly applied to vaccination strategies against a number of virally infectious agents. Additionally, through the elucidation of the exact mechanisms by which this hormone-mediated enhancement occurs, more nuanced approaches can be taken to achieve the same enhancement of vaccine-induced immunity as was observed under the influence of E2. Overall, this study highlights the importance and lays the foundation of considering sex-hormones in the design of more optimal vaccination strategies that can have significant implications for the protection against a number of pathogens.

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