THE ANTI-VIRAL ROLE OF IL-17 DURING HSV-2 INFECTION

INVESTIGATING THE ROLE OF IL-17 IN MEDIATING ANTI-VIRAL T CELL IMMUNITY IN THE FEMALE REPRODUCTIVE TRACT DURING HSV-2 INFECTION

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TITLE: Investigating the role of IL-17 in mediating anti-viral T cell immunity in the female reproductive tract during HSV-2 infection

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ABSTRACT

Herpes simplex virus type 2 (HSV-2) is one of the most predominant sexually transmitted infections (STIs) in the world, with over 417 million individuals infected globally. Despite its widespread impact, there is still no cure or vaccine available for HSV-2. Rates of infection are higher in women compared to men, and the female reproductive tract (FRT) is the primary site of infection. Immune responses in the FRT are highly regulated by the presence of female sex hormones, and studies have shown that hormones can differentially affect susceptibility to infection. Our lab has been studying the effects of the hormonal microenvironment on genital HSV-2 infection for over a decade, and we and others have shown that the hormone estradiol (E2) is protective against HSV-2. Recent studies from our lab have reported that E2 appears to modulate $CD4^+$ T cell immunity in the FRT, which is known to be critical for protection against HSV-2. In particular, E2 treatment resulted in robust IL-17^+ $CD4^+$ T cell responses, which coincided with enhanced T_h1 immunity. While the protective anti-HSV-2 role of T_h1 cells is well described in literature, little has been published on the importance of anti-viral T_h17 immunity. The central objective of this dissertation was to further investigate the role of IL-17 in mediating anti-viral T cell immunity in the FRT during HSV-2 infection. We hypothesized that IL-17 enhances anti-viral protection against HSV-2 infection by mediating more efficient $CD4^+$ T cell immunity in the FRT.

First, we examined the importance of IL-17 during HSV-2 infection (Chapter 2). We showed that while IL-17 did not appear to play an important role during primary HSV-2 infection, it was critical for mediating protective immunity during the secondary recall

response. We found that in the absence of IL-17, there was a compromised *in vivo* anti-viral T_h1 recall response following intravaginal HSV-2 re-exposure and challenge, resulting in poor disease outcomes. These findings highlight a novel anti-viral role for IL-17 in the FRT, in which IL-17 mediates efficient T_h1 immunity.

Second, we examined the influence of IL-17 produced by innate sources, which represents the majority of IL-17 produced under homeostatic conditions, on the induction of T cell immunity in the FRT (Chapter 3). We found that in the absence of innate IL-17, vaginal dendritic cells were severely impaired at priming T_h17 responses *in vitro*. Furthermore, this defect in T_h17 induction was associated with reduced IL-1 production. We also characterized the primary source of innate IL-17 in the FRT and showed that $\gamma\delta^+$ T cells were the predominant source of innate IL-17, and that these cells were modulated by both E2 and microbiota. These findings suggest that overall IL-17 production in the FRT is modulated by a variety of factors found in the local microenvironment, including innate sources of IL-17, E2 and the vaginal microbiota.

Finally, we examined the effect of IL-17 and E2 on the establishment of memory T cells following HSV-2 immunization (Chapter 4). We found that following intranasal vaccination, E2 enhanced memory T_h1 and T_h17 populations in both the upper respiratory tract, as well as the FRT. In particular, E2 treatment resulted in greater establishment of vaginal $CD4^+$ tissue-resident memory T (T_{RM}) cells, a subset of memory cells known to be critical for protection against infection. Furthermore, we found that E2 enhanced vaginal T_h1 T_{RM} cells through an IL-17-dependent mechanism. These findings highlight a novel

mechanism for E2-mediated protection against HSV-2 infection, in which E2 enhances the establishment of anti-viral $CD4^+$ memory T cell populations during vaccination.

This dissertation summarizes the original contributions and work completed during the last five years to understand numerous ways IL-17 influences anti-viral protection in the FRT. Taken together, these findings provide critical insight on prospective strategies to improve mucosal vaccines and generate more efficient protection against viral infections.

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throughout my time in the lab. I will never forget the countless hours we spent doing hundreds of ovariectomies; I think I might finally be as quick as you were! Thanks for being such an awesome lab mate, and even better friend. Sara, thank you for helping me get through graduate school. We battled through many "firsts" together, be it graduate courses or committee meetings, and I could not have survived without you! Thanks for always being there for me, no matter what, and helping me stay sane. Varun, thank you for everything you have done for me. I started off with very little technical skills and you taught me (almost) everything in your arsenal. My research ideas stemmed directly from your findings, and so I honestly would not have been able to do this work without you. I am still in awe at all of the complicated work you did, and it is an honour to have worked alongside you in order to help bring that work to fruition. I will never forget the 80 mouse sac we worked on for over 24 hours straight, which "kind of" worked, nor the countless times we would come in at 2:00 am to start our sorting experiments that did not end until 10:00 pm. Thanks for always letting me bounce ideas off of you and giving me critical, but insightful, feedback. You have helped mould me into the researcher that I am today. I would also like to thank Danielle, Ramtin and Emma, who were instrumental in helping me complete this work, and Jeff, Haley and Andrew, for providing endless laughs and being great listeners. I am extremely grateful for the friendships that I gained within the lab. Next, to Jann and JP, thank you for providing countless hours of laughter and fun. Jann, thank you for always cooking me the most delicious food and helping me de-stress, especially by playing entertaining games. I will always be thankful that the Miller lab was serendipitously placed next to the Kaushic lab. JP, thank you for being an incredible mentor and friend. Meeting

you changed the way I approached graduate school, and I will never forget how you helped reinvigorate my passion for student and community engagement. I continue to look to you for inspiration and I know you are destined for great things. I know that no matter where life takes us all, we C. Auctioneers will stick together! Finally, to my "second lab", the Stämpflis. Danya, Josh and Steve, thank you for not only helping me explore completely new areas of research, but for always lending an ear when needed. You were all always there to listen and provide moral support whenever I needed it most. From sporting events, to gallivanting through the UK, and everything in between, I am beyond grateful for the friendships I have with each of you. My time during graduate school would have not been the same without each of the individuals mentioned above, and countless others I have had the pleasure to work and interact with.

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support. Zahra, thanks for being such a supportive and encouraging friend. You have always believed in me, which means a lot. I am lucky to have such a strong group of women in my life, and I take none of these friendships for granted.

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television shows and movies with you. I mainly looked forward to coming home every weekend because of the time we got to spend together. To my entire family, I could not have asked for a better support system, not just through this process, but throughout life. I cannot emphasize enough how grateful I am for all of you.

I would like to finish this section by sharing some final thoughts. I fell in love with learning at a very young age, and always said that if I could be a student forever, I would. Completing this thesis has been the greatest learning experience of my life. Not only have I acquired a great deal of knowledge, but more importantly, I have experienced tremendous personal growth along the way. I will forever be an advocate for the importance of education, without which nothing would be possible. I cannot wait to one day share this work with my beautiful nieces, Rhea and Ariya, and goddaughter Isabella, and tell them that they too, can accomplish anything they set their minds to. I am so proud of this thesis. It sometimes felt like there were more lows than highs, and I doubted myself more often than I would like to admit, but as they say, nothing worth having comes easy. I can only hope that my research will have some type of impact on humankind. But if nothing else, this work represents a journey of true dedication and perseverance.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

APPENDIX II

APPENDIX III

APPENDIX IV

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ACK – Ammonium-Chloride-Potassium

AMP – anti-microbial peptide

ANOVA – analysis of variance

APC – antigen-presenting cell

CAF01 – Cationic Adjuvant Formulation no. 1

C. albicans – Candida albicans

CASP1 – caspase 1

CCR – C-C chemokine receptor

CD – cluster of differentiation

CFSE – carboxyfluorescein succinimidyl ester

cLN – cervical lymph node

C. muridarum – Chlamydia muridarum

CSC – cell stimulation cocktail

C. trachomatis – Chlamydia trachomatis

CVL – cervicovaginal lavage

CXCR – C-X-C chemokine receptor

DAPI – 4′,6-diamidino-2-phenylindole

DC – dendritic cell

Depo – Depo-Provera

DMPA – depot medroxyprogesterone acetate

DNA – deoxyribonucleic acid

E2 – estradiol

EC – epithelial cell

ER – estrogen receptor

ERE – estrogen response element

ERKO – estradiol receptor knockout

FBS – fetal bovine serum

FcRn – neonatal Fc receptor

FDR – false discovery rate

FGT – female genital tract

FMO – fluorescence minus one

FOXP3 – forkhead box P3

FRT – female reproductive tract

FSH – follicle-stimulating hormone

F. tularensis – Francisella tularensis

FTY720 – Fingolimod

gB – glycoprotein B

G-CSF – granulocyte-colony stimulating factor

gD – glycoprotein D

GF – germ-free

HC – hormonal contraceptive

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV-1 – human immunodeficiency virus 1

HPV – human papillomavirus

HSV-1 – herpes simplex virus type 1

HSV-2 – herpes simplex virus type 2

ICAM-1 – intercellular adhesion molecule 1

IFN – interferon

IFNα/β$R^{-/-}$ – interferon alpha/beta receptor knockout

IFN-γ – interferon gamma

Ig – immunoglobulin

IgA – immunoglobulin A

IgG – immunoglobulin G

IL – interleukin

IL-17$A^{-/-}$ – interleukin-17A deficient

IL-1β – interleukin-1 beta

IL-17R – interleukin-17 receptor

ILC – innate lymphoid cell

iLN – iliac lymph node

IN – intranasal

IVAG – intravaginal

KEGG – Kyoto Encyclopedia of Genes and Genomes

KO – knockout

LFA-1 – lymphocyte function-associated antigen 1

LH – luteinizing hormone

LN – lymph node

MCP-1 – monocyte chemoattractant protein 1

M-CSF – macrophage colony-stimulating factor

MFI – median fluorescence intensity

MHC – major histocompatibility complex

MIP-1β – macrophage inflammatory protein-1beta

mRNA – messenger ribonucleic acid

M. tuberculosis – Mycobacterium tuberculosis

NALT – nasal-associated lymphoid tissue

NF-κB – nuclear factor kappa B

N. gonorrhoeae – Neisseria gonorrhoeae

NK – natural killer

NKT – natural killer T

NLR – nucleotide-binding oligomerization domain-like receptor

NLRP3 – nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3

NOD – nucleotide-binding oligomerization domain

PAMP – pathogen-associated molecular pattern

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

pDC – plasmacytoid dendritic cell

PFU – plaque forming unit

pIgR – polymeric immunoglobulin receptor

PMA – phorbol 12-myristate 13-acetate

PPI – protein-protein interaction

PRR – pattern recognition receptor

OT-II Tg – ovalbumin receptor transgenic

OVA – ovalbumin

OVX – ovariectomized

P4 – progesterone

RANTES – regulated on activation, normal T-cell expressed and secreted

RIG-I – retinoic acid-inducible gene-I

rIL-1β – recombinant interleukin-1 beta

rIL-17 – recombinant interleukin-17

RLR – retinoic acid-inducible gene-I-like receptor

RNA – ribonucleic acid

ROR-γ – retinoic-acid-receptor-related orphan nuclear receptor gamma

RPMI – Roswell Park Memorial Institute

S1PR1 – sphingosine-1-phosphate receptor 1

SEM – standard error of the mean

sIgA – secretory IgA

SPF – specific pathogen-free

STAT – signal transducer and activator of transcription

STI – sexually transmitted infection

TC – tissue cells

T_{CM} – central memory T

TCR – T cell receptor

T_{EM} – effector memory T

Tg – transgenic

TGF-β – transforming growth factor beta

T_h1 – T helper 1

T_h2 – T helper 2

T_h17 – T helper 17

TK – thymidine kinase

TK^- – thymidine kinase-deficient

TLR – Toll-like receptor

TNF-α – tumor necrosis factor alpha

T_{reg} – regulatory T cell

T_{RM} – tissue-resident memory T

uNK – uterine natural killer

URT – upper respiratory tract

VCAM-1 – vascular cell adhesion molecule 1

VLA-4 – integrin alpha4 beta1

WT – wildtype

WHO – World Health Organization

α – alpha

α-MEM – alpha-minimal essential medium

β – beta

δ – delta

γ – gamma

γδ – gamma delta

DECLARATION OF ACADEMIC ACHIEVMENT

This thesis is presented in the "sandwich doctoral thesis" format, based on guidelines provided by the School of Graduate Studies at McMaster University. Chapter 1 serves as a central overview of the themes which will be discussed throughout this document. This is followed by the presentation of three independent, but thematically-related, research studies (Chapters 2-4), two of which are published and one which is in the process of being submitted for publication at this time. Finally, Chapter 5 serves as the discussion and conclusion section, where the collective body of work is summarized, and the overall impact of the research is considered. Although I was the primary contributor for most of all the work presented in this thesis, the research was done in collaboration with several colleagues. In the following section, I have outlined and detailed my contributions to the work presented in this thesis, along with the roles and contributions of other individuals who were also critically involved.

CHAPTER 2

TITLE: Novel role for interleukin-17 in enhancing type 1 helper T cell immunity in the female genital tract following mucosal herpes simplex virus 2 vaccination

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AUTHOR CONTRIBUTION: P.B. and C.K. conceived and designed the experiments. P.B., V.C.A., P.V.N. and D.V. performed the experiments. P.B., V.C.A. and C.K. analyzed the data. M.R.S. provided materials and assisted with data analysis. P.B. and C.K. wrote the manuscript. We thank Kristen Mueller for providing technical assistance.

SCIENTIFIC CONTRIBUTION: IL-17 plays a critical role in mediating efficient anti-viral T_h1 immunity in the female reproductive tract and is essential for inducing optimal protection against HSV-2 infection. IL-17 also influences vaginal T_h1 tissue-resident memory cell populations.

CHAPTER 3

TITLE: IL-17 production by $\gamma\delta^+$ T cells is critical for inducing T_h17 responses in the female genital tract and regulated by estradiol and microbiota

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AUTHOR CONTRIBUTION: V.C.A. and P.B. contributed equally to this work. V.C.A., P.B. and C.K. conceived and designed the experiments. V.C.A., P.B., S.E.D. and R.S. performed the experiments. V.C.A., P.B., D.P.S. and C.K. analyzed the data. M.J. and M.R.S. provided materials and assisted with data analysis. V.C.A., P.B., D.P.S. and C.K. wrote the manuscript. We thank Kristen Mueller for providing technical assistance.

SCIENTIFIC CONTRIBUTION: Innate IL-17 is primarily secreted by $\gamma\delta^+$ T cells in the female reproductive tract and is modulated by both E2 and microbiota. Innate IL-17 also conditions vaginal dendritic cells to prime potent T_h17 responses, likely via an IL-1β-dependent pathway.

CHAPTER 4

TITLE: Estradiol enhances anti-viral $CD4^+$ tissue-resident memory T cell responses following mucosal vaccination through an IL-17-mediated pathway

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AUTHOR CONTRIBUTION: P.B. and C.K. conceived and designed the experiments. P.B., R.G., J.J.C. and D.T. performed the experiments. P.B. and C.K. analyzed the data. A.G.B and M.R.S. provided materials and assisted with data analysis. P.B. and C.K. wrote the manuscript. We thank Emma Yu and Dr. Varun Anipindi for providing technical assistance.

SCIENTIFIC CONTRIBUTION: Estradiol enhances $CD4^+$ anti-viral memory T cell populations in the upper respiratory tract and female reproductive tract following intranasal immunization, likely via an IL-17-dependent pathway.

CHAPTER 1: Background and Objectives

1.1 Introduction

Sexually transmitted infections (STIs) are among the most common communicable diseases in the world, and are associated with high levels of morbidity and mortality (1). STIs can be caused by different types of pathogens including bacterial, viral and fungal species. The most prevalent STIs include chlamydia, gonorrhoea, syphilis, trichomoniasis, hepatitis B, herpes simplex virus (HSV), human immunodeficiency virus-1 (HIV-1) and human papillomavirus (HPV) (1). While effective treatments are available for several STIs, there is still no cure or viable vaccine available for many of these pathogens including HSV and HIV. Additionally, STIs can lead to further complications, as infected individuals are more likely to acquire and transmit HIV (2). Although most STIs are transmitted through sexual contact, including oral, vaginal and anal sex, some can be spread by non-sexual contact such as through blood or blood products (1). Furthermore, STIs can also be transmitted from mother-to-child during pregnancy and childbirth, leading to greater risk of stillbirth, low birth weight, neonatal conjunctivitis and congenital deformities (1).

According to the World Health Organization (WHO), more than 1 million STIs are acquired daily, affecting the quality of life of men, women and children worldwide, and placing a substantial financial strain on health care systems (1, 3). STIs can have a significant impact on sexual and reproductive health, and are associated with physical, psychological and social consequences. Interestingly, rates of STIs are often higher in women compared to men, and further complications beyond initial primary infection disproportionately affect women in many ways as well (4). Firstly, many STIs are

transmitted more easily from males to females. For instance, the risk of HSV-2 transmission during unprotected sexual acts from females to males was shown to be 1.7 transmissions/1000 unprotected acts, while the rate from males to females was 28.5 transmissions (5). Likewise, the risk of HIV seroconversion per heterosexual act is approximately 2-fold higher for females compared to males (6). Increased risk of transmission in women can be attributed to several factors, including social and behavioural practices. For example, in areas where STIs are extremely prevalent, there is often a power imbalance between sexes, and the use of protective barriers such as condoms is dependent on the male partner (7). Societal practices related to vaginal hygiene, such as vaginal wiping, cleansing or douching, have also been associated with increased risk of STIs (8).

There are also many biological factors which are related to increased susceptibility in women. These include the anatomical composition of the female reproductive tract (FRT), where during the act of sexual intercourse, there is a greater surface area of mucosa exposed to infected bodily fluids and greater physical trauma to the mucosal tissue (9). Additionally, females are more likely to be asymptomatic when infected, and so they remain undiagnosed for longer periods of time, which can delay the initiation of treatment considerably (4). For instance, as many as 70-90% of women with chlamydial infection are asymptomatic, compared to 50% of men (10). There are also sex differences in immune responses, and these are largely mediated by the presence of female sex hormones and the vaginal microbiome. Hormones can directly influence immune responses in the FRT, as many immune cells express receptors for the endogenous female sex hormones estrogen (E2) and progesterone (P4) (11-13). The effects of these hormones on immunity in the FRT

will be discussed in great detail throughout this dissertation. Along with endogenous hormones, the use of hormonal contraceptives (HCs) can influence susceptibility as well. Specifically, the use of depot medroxyprogesterone acetate (DMPA), an injectable HC, is associated with enhanced risk of HIV-1 infection (14-16). Hormones and the microbiota are known to regulate inflammation in the FRT, further influencing susceptibility (17-19).

Beyond the scope of initial infection, untreated STIs can cause serious sequelae in women, including pelvic inflammatory disease, infertility, chronic pelvic pain and ectopic pregnancy. For example, chlamydial or gonorrheal infection which spreads to the upper genital tract can lead to pelvic inflammatory disease, infertility and ectopic pregnancy. In contrast, infertility due to STIs is much more rare in males (4). Similarly, HPV infection in women can also cause cellular changes that lead to cervical cancer (1). Furthermore, STIs are also more dangerous in women due to several risks associated with pregnancy. As mentioned previously, vertical transmission of an STI from mother-to-child during childbirth can lead to severe complications including miscarriage, premature labour, stillbirth, congenital deformities and infant death (4). Current strategies to help combat STIs centre around comprehensive sexual education, counselling, and safe-sex measures such as condom use. However, there are still many barriers against protection which include societal practices, lack of education and public awareness, limitation of resources and stigmatization (1). Due to the tremendous burden of disease caused by sexually transmitted pathogens, especially in women, it is critical to better understand how to protect against STIs. As such, the focus of this dissertation will be centered around understanding the mechanisms influencing susceptibility of infection in the FRT.

1.2 Female reproductive tract

1.2.1 Anatomy

The FRT is essential for supporting reproduction and is comprised of multiple components which allow for this primary function to occur. The FRT is divided into two main compartments: 1) the upper FRT, comprised of the endocervix, uterus, fallopian tubes and ovaries, and 2) the lower FRT, comprised of the vagina and ectocervix (Fig. 1.1). Both compartments have phenotypically distinct epithelial cell (EC) layers, which act as the first line of defense against microbes. The epithelial layer in the FRT acts as both a physical deterrent against pathogens, as well as an immunological barrier. The upper FRT is lined with a monolayer of tightly joined columnar ECs, with tight junction proteins such as claudin and occludin present to help provide an impermeable barrier which prevents the entry of foreign molecules (20, 21). Conversely, the lower FRT is lined primarily with a multi-layer, non-keratinized, stratified squamous epithelium. Unlike the upper FRT, the lower FRT epithelium does not contain classical tight junction proteins and instead has mainly adherens junctions and desmosomes, which allows for the transportation of small pathogens and molecules across the barrier (21, 22). In humans, the transition between the two types of epithelium occurs at the transformation zone, and this region is the most immunologically active site of the FRT; thus, making it greatly susceptible to pathogen entry (23). A layer of stromal fibroblasts lies beneath the epithelium, providing structural and functional support. Leukocytes are dispersed throughout the stroma and account for 6-20% of the total cells in the FRT, with larger cell numbers present in the upper FRT compared to the lower FRT (24).

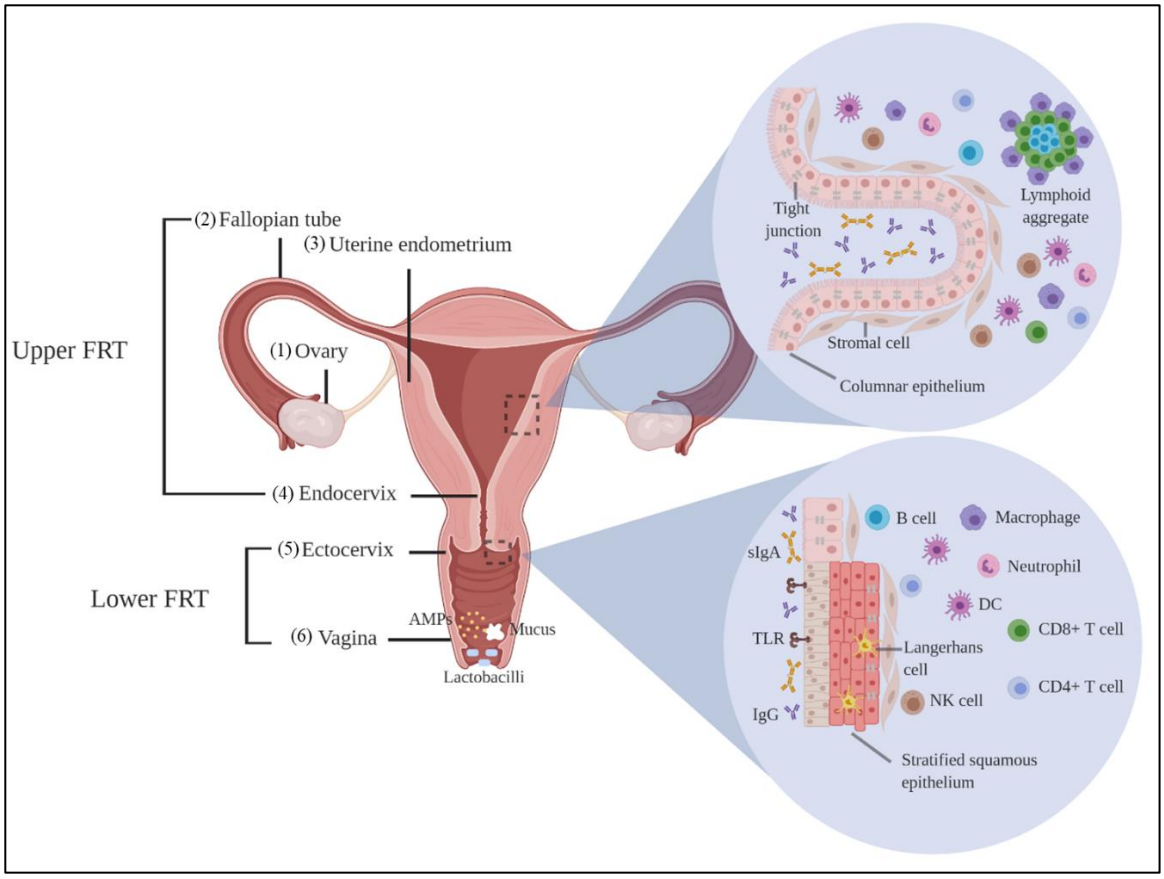

Figure 1.1: Structure and cellular composition of the female reproductive tract. The female reproductive tract (FRT) can be divided into two compartments: the upper FRT, comprised of the (1) ovaries, (2) fallopian tubes, (3) uterus and (4) endocervix, and the lower FRT, comprised of the (5) ectocervix and (6) vagina. The lower FRT is lined with a multi-layered, stratified squamous epithelium and has many components related to innate immunity including tight junction proteins, mucus, anti-microbial peptides (AMPs), and the presence of Toll-like receptors (TLRs). There are also several innate immune cells such as natural killer (NK) cells, neutrophils, Langerhans cells, dendritic cells (DCs), and macrophages, as well as adaptive immunes cells (T cells and B cells) and immunoglobulins (Igs), which survey the local environment for potentially dangerous organisms. The keratinized squamous epithelium transitions into a singular, columnar epithelial cell layer located between the ectocervix and endocervix, known as the transformation zone. The upper FRT includes lymphoid aggregates composed of a core of B cells, surrounded primarily by $CD8^+$ T cells and an outer layer of macrophages, along with a variety of other immune subsets. sIgA, secretory IgA. Created with BioRender.

1.2.2 Mucosal immunity

The mucosal immune system is comprised of immune processes which are distinct from those that occur systemically, and provides protection at sites with mucosal membranes such as gastrointestinal, respiratory and urogenital tissues, which are exposed to external environmental factors. The mucosal immune system in the FRT is especially unique, as the environment must be adapted to facilitate critical physiological events such as fertilization, implantation and pregnancy, while remaining vigilant towards providing protection against pathogens (20). Furthermore, the FRT is a complex mucosal site where various immune components including structural tissue cells, innate and adaptive immune cells, and soluble factors work in conjunction to allow for these processes to occur, while being constantly regulated by factors including female sex hormones and the presence of commensal microbiota (25, 26). Most innate immune cells have unique phenotypic and functional adaptations that are specific to the FRT microenvironment (25, 26). Likewise, there is a distinct compartmentalization of adaptive immune cells as well (25, 26). Innate immunity in the FRT acts as the first line of defence against incoming pathogens. This includes the physical barrier formed by ECs which line the FRT, secretions produced by innate cells such as mucus, anti-microbial peptides (AMPs), cytokines and chemokines, and immune cell subsets including neutrophils, natural killer cells (NK cells), macrophages and dendritic cells (DCs), all which work together to initiate antigen-specific adaptive immunity (27-29). Adaptive immune responses include $CD4^+$ and $CD8^+$ T cells, as well as immunoglobulin G (IgG) and IgA production by B cells (25, 26).

1.2.3 Innate immunity

Innate immunity in the FRT is largely mediated by several factors such as mucus, AMPs and epithelial barriers, which are involved in preventing pathogen entry, as well as other cells and factors including EC responses and immune cells (macrophages, DCs, neutrophils and NK cells), which recognize pathogens and act locally to decrease pathogen load and initiate adaptive immunity (27-29).

1.2.3.1 Epithelial cells

The epithelium acts as a barrier to protect against extraneous factors, some of which may be detrimental. The ECs which line the FRT mucosa are the first cells to initiate an innate immune response following recognition of microorganisms by germ-line-encoded receptors called pattern recognition receptors (PRRs) (30). PRRs such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are found on host innate immune cells and recognize conserved pathogen-associated molecular patterns (PAMPs) expressed by microorganisms (31, 32). Interactions between TLRs and PAMPs initiate recruitment of immune cells, as well as the production of AMPs, which are involved in protection against invading microbes (33-36). Expression of other receptors including NOD1, NOD2 and RIG-1 has also been detected in the FRT (37). NLRs such as NOD1 and NOD2 play a role in initiating anti-bacterial responses, and RIG-1 is involved in inducing anti-viral responses against intracellular viruses (37).

Upon activation, ECs secrete mucus, AMPs, cytokines and chemokines, all of which can help prevent the entry of pathogens and/or regulate immunity in the FRT. Mucus

is composed of mucins, which are glycosylated proteins that can physically trap pathogens in a thick gel phase; thus, acting as a physical barrier (38-41). Additionally, the pH of the mucus can also affect the transmission of pathogens. Typically, the mucus in the FRT is acidified to a pH of 4-5, and it has been reported that this acidic environment can slow the rate of viral infection (42, 43). Cervical mucus also contains anti-microbial factors which assist in protecting against pathogens (44, 45). Likewise, AMPs such as defensins, protease inhibitors and enzymes are produced by ECs and other immune cells in the FRT (46). These small proteins and peptides are secreted both constitutively, as well as following stimulation with microorganisms, and provide protection by helping to directly and/or indirectly kill or inhibit the growth of microorganisms such as bacteria (*Neisseria gonorrhoeae (N. gonorrhoeae)*, *Chlamydia trachomatis (C. trachomatis)*), fungi (*Candida albicans (C. albicans)*) and viruses (HIV-1, HSV-2) (47-54). Finally, ECs release a variety of cytokines and chemokines which act as chemical messengers between cells to regulate critical aspects of immunity (38, 47). This includes pro-inflammatory chemokines and cytokines, such as interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein 1 (MCP-1), granulocyte-colony stimulating factor (G-CSF), tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein-1beta (MIP-1β), which are critical for recruiting and activating immune cells in response to pathogens (55).

1.2.3.2 Antigen-presenting cells

Antigen-presenting cells (APCs) are critical cells involved in innate immunity and include DCs and macrophages, which often derive from common myeloid precursors. As such, both APC subsets share several common surface markers including CD11c, CD11b,

CD14 and major histocompatibility complex (MHC) class II (56). Based on the cytokine microenvironment present, myeloid cells may differentiate into either DCs or macrophages. Macrophages account for approximately 10% of leukocytes present in the FRT (24, 29, 57, 58). Unlike gut macrophages, endometrial macrophages express CD14, CD68, and co-stimulatory molecules CD40, CD80 and CD86 (59). Along with antigen-presentation, macrophages are also involved in the phagocytosis of foreign molecules and pathogens.

DCs residing in the FRT are responsible for surveying the surrounding environment and processing antigens in order to activate adaptive immunity; thus, bridging generally non-specific innate immune responses and inducing a more direct, antigen-specific adaptive immune response. During periods of homeostasis, DCs in the FRT are highly phagocytic and express several types of PRRs which allows them to recognize a large repertoire of pathogens (60). Once the DCs have taken up and processed the antigen, they undergo a maturation process which involves increased MHC class II expression, leading to migration of DCs to the local draining lymph nodes (LNs) where they prime antigen-specific T cells (61, 62). Upon pathogen recognition, DCs also release cytokines and chemokines which regulate activation and recruitment of other immune cells to the site of infection. DCs are found throughout the FRT, and depending on their anatomical location, differ in regard to their phenotype and function. DCs are present in highest numbers in the upper FRT (24), and subsets include $CD1a^+$ and $CD11c^+$ DCs, and $CD123^+$ plasmacytoid DCs (pDCs) (63). In the lower FRT, DCs are localized within the epithelium, and subsets include Langerhans cells present in the vaginal epithelium (64) and $CD11c^+$ DCs found in

the submucosal stroma (65). Little is known regarding the distinct functions of the different vaginal DC subsets, and how they differ from other mucosal DCs.

1.2.3.3 Neutrophils and NK cells

Neutrophils are innate cells present throughout the FRT, with highest levels found in the fallopian tubes and numbers waning from the upper FRT into the vaginal mucosa (24). Studies done using tissue biopsies have shown that the upper FRT expresses high levels of neutrophil-attracting ligands (24, 66-68), thus explaining the higher numbers found in the upper compartment. The roles of neutrophils in the FRT include responding to pathogens through phagocytosis, secretion of cytokines and release of AMPs.

NK cells are a critical component of the innate immune response generated in the FRT. NK cells are able to kill certain tumor and virally-infected cells, and also help generate early immune protection against bacterial, viral and fungal pathogens by secreting immunoregulatory cytokines (69). Additionally, they play a fundamental role in remodeling the uterine tissue during pregnancy (70, 71). NK cells are found throughout the FRT, and account for 10% to 30% of leukocytes present in the FRT of non-pregnant women (72). NK cells in the uterus are phenotypically distinct from NK cells in circulation; unlike NK cells in the blood which are $CD16^+$ $CD56^-$, uterine NK (uNK) cells in the FRT express CD56 and very little CD16 ($CD56^+$ $CD16^-$) (73). uNK cells also express CD69 and CD9, but not CD57 and CD8 (74-76). Furthermore, uNK cells have been shown to have a distinct gene profile that is very different from blood NK cells (76).

1.2.3.4 Gamma delta T cells

Apart from traditional innate immune subsets, other innate or innate-like cells are also present in the FRT including gamma delta ($\gamma\delta^+$) T cells. $\gamma\delta^+$ T cells are predominantly localized in mucosal tissues, and rapidly activate upon exposure to pathogens and pro-inflammatory cytokines released by APCs (77). Similar to traditional $CD4^+$ and $CD8^+$ T cells, $\gamma\delta^+$ T cells originate from the thymus. During T cell development, $CD4^-$ $CD8^-$ thymocytes may rearrange their T cell receptor (TCR) γ and δ genes to become $\gamma\delta^+$ T cells (78). $\gamma\delta^+$ T cells develop early in the fetus and provide immunity prior to the generation of adaptive immune responses (79). Unlike traditional T lymphocytes, $\gamma\delta^+$ T cells use their TCRs directly as PRRs to respond to pathogens (77, 80). As such, it has been suggested that $\gamma\delta^+$ T cells may play an important role in the early response to bacterial, fungal, parasitic and viral pathogens (77). Their ability to resolve infections is related to the production of inflammatory molecules such as interferon-gamma (IFN-γ) and IL-17, as well as recruitment of pathogen-clearing immune cells such as neutrophils, macrophages and NK cells (81-83).

1.2.4 Adaptive immunity

The adaptive immune system in the FRT is initiated following activation of innate immunity in order to generate antigen-specific responses to help eliminate pathogens, and is comprised of two types of responses: 1) humoral immunity mediated by B cells and antibodies, and 2) cellular immunity facilitated by T cells. Adaptive immunity also consists of memory responses, which generate efficient protection against re-exposure to specific antigens.

1.2.4.1 B cells and antibodies

Humoral immunity consists of the production of antibodies which can bind to antigens and effectively inhibit cell entry or neutralize the biological activity of pathogens. Following antibody binding, pathogens are eliminated through phagocytosis mediated by macrophages or by the complement system. Unlike in the gut mucosa, IgG is the predominant antibody response in the FRT compared to IgA (84). Studies looking at human cervicovaginal lavages (CVLs) demonstrate that levels of IgG are 2- to 6-fold higher than that of IgA (85). Although B cells are a minor cell population in the FRT, both IgG- and IgA-producing plasma cells are present primarily in the upper FRT. Majority of antibodies in the lower FRT are IgG antibodies, while the upper FRT consists predominantly of IgA antibodies, and this is largely due to the location of their respective receptors (26). ECs in the lower FRT express the neonatal IgG receptor (FcRn), which mediates the transport of IgG into FRT secretions (86). Meanwhile, IgA transport is dependent on the polymeric Ig receptor (pIgR) and unlike FcRN expression, pIgR is expressed primarily by ECs in the upper FRT (26). IgA-secreting plasma cells produce dimeric IgA, which binds to pIgR to form secretory IgA (sIgA). It remains unclear what the sources of IgG and IgA are in the FRT, and it is believed that both locally- and plasma-derived antibodies contribute to the total Ig populations present in FRT secretions.

1.2.4.2 T cells

Antigen-presentation and T cell activation generally occurs in the iliac LNs (iLNs) which drain the FRT, followed by migration of effector $CD8^+$ and $CD4^+$ T cells to the FRT. T cells are the most abundant leukocytes present in the FRT, constituting approximately

40–50% of the leukocyte population, and are distributed amongst all compartments of the FRT (24, 84, 87-91). In the lower FRT, T cells are dispersed throughout the stroma, while within the uterus, T cells are primarily found in lymphoid aggregates (26). Additionally, $CD4^+$ and $CD8^+$ T cells are equally abundant in the lower FRT, whereas there are more $CD8^+$ than $CD4^+$ T cells in the upper FRT (26). Overall, $CD8^+$ T cells account for the majority of T cells present in the FRT (35–50%), compared to $CD4^+$ T cells which make up 25% of the T cell population (24, 87). T cell recruitment to the FRT is dependent on expression of C-X-C chemokine receptor 3 (CXCR3) and C-C chemokine receptor type 5 (CCR5) ligands, CXCL9 and CXCL10, which are expressed in the upper FRT and by CVLs (92).

1.2.4.3 $CD8^+$ T cells

$CD8^+$ T cells mediate protection primarily through cytotoxic activity. $CD8^+$ T cells recognize infected cells through interactions between the TCR and peptide-bound MHC class I molecules expressed on the cell surface. Upon recognition of infected cells, $CD8^+$ T cells initiate apoptosis through perforin- and granzyme-mediated cytolysis, and can also produce cytokines such as IFN-γ (93). $CD4^+$ T cell help may also be required for the generation of long-lasting $CD8^+$ memory T cell responses (94). The primary mechanism of protection exhibited by $CD4^+$ T cells is IFN-γ secretion by T helper 1 (T_h1) cells, which can help mediate $CD8^+$ T cells and block viral replication (92, 95). Organized lymphoid aggregates are present in the uterine endometrium and are composed of a B cell core that is surrounded by $CD8^+$ T cells, and encapsulated by macrophages (58). Lymphoid aggregates develop during the proliferative phase of the cycle as a result of immune cells

which traffic into the endometrium during this time, and their cellular composition and size is highly dependent on hormone levels (58, 96, 97).

1.2.4.4 $CD4^+$ T cells

Naïve $CD4^+$ T cells are activated upon interaction with DCs, and the nature of the antigen, co-stimulatory signals and cytokine signals direct these cells to differentiate into effector T helper subsets including T_h1, T_h2, T_h17 and regulatory T cells (T_{regs}) (98). Two primary subsets of $CD4^+$ T cells in the FRT include T_h1 and T_h17 cells. A cytokine milieu consisting of DC-derived IL-12 leads to the differentiation of T_h1 cells, which produce IFN-γ and regulate cellular immunity against intracellular bacterial and viral infections, including HSV-2 (99-101). T_h1 cells are regulated by the master transcription factor T-box transcription factor (T-bet) (102). Exposure of naïve $CD4^+$ T cells to APC-derived polarizing cytokines such as transforming growth factor beta (TGF-β), IL-6, IL-21, IL-23 and IL-1β leads to the differentiation of T_h17 cells (76). Interestingly, different combinations of these cytokines can all direct T_h17 differentiation, demonstrating the plasticity of T_h17 cells (103-106). For instance, some studies have shown IL-6 and IL-1-independent pathways of T_h17 differentiation (106, 107), while others have shown that IL-1 signalling is critical (108, 109). The primary effector response of T_h17 cells involves the induction of cytokines that are pro-inflammatory in nature including IL-17A, IL-17F and IL-22, as well as the recruitment of neutrophils. Phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3) leads to the transcription of retinoic-acid-receptor-related orphan nuclear receptor gamma (ROR-γ), the master transcription factor which regulates T_h17 cells (110). T_h17 cells play a fundamental role in the resolution

of extracellular fungal and bacterial infections at mucosal surfaces (111, 112), however, aberrant T_h17 responses can lead to autoimmune or chronic inflammatory diseases (113-117). Within the FRT, T_h17 cell immunity and IL-17 production have been shown to play a role in genital tract infections with *N. gonorrhoeae*, *C. trachomatis* and *C. albicans* (118-128). However, the role of T_h17 immunity during viral infections, such as HSV-2, remains largely unknown and this is a topic which will be further discussed in this dissertation.

1.2.5 IL-17 in the FRT

IL-17 was originally cloned in the early 1990s and initially known as cytotoxic T lymphocyte-associated antigen 8 (129). Although first recognized as a new cytokine in 1995, the importance of IL-17 remained obscure until almost a decade later, when a novel population of $CD4^+$ T helper cells, T_h17 cells, were discovered and characterized by their secretion of IL-17 (130, 131). Following this discovery, many studies have examined the role of IL-17 in several disease models.

The IL-17 family of cytokines includes six similarly structured ligands (IL-17A to IL-17F), of which, IL-17A (IL-17) and IL-17F are most closely related. IL-17 cytokines signal through five receptor subunits: IL-17RA through IL-17RE (132, 133). IL-17 and IL-17F exist either as homodimers or as a heterodimer, and signal through an obligate dimeric IL-17RA and IL-17RC complex (134). There are two primary signalling pathways initiated upon IL-17 binding to its receptor complex (reviewed in (133, 135)). The first is a canonical pathway, which leads to the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase pathways, and results in transcriptional activation of downstream, pro-inflammatory target genes. The second, noncanonical pathway, leads to the

stabilization of messenger ribonucleic acid (RNA) transcripts which encode for intrinsically unstable targets including cytokines and chemokines. Overall, at the transcriptional and post-transcriptional level, IL-17 enhances the production of immune mediators including chemokines, cytokines, AMPs and other inflammatory effectors.

IL-17 has several immunoregulatory functions (reviewed in (136)). During infection, IL-17 is actively involved in neutrophil and monocyte recruitment through enhanced induction of various chemoattractants including CXCL1, CXCL2 and CXCL5 (137, 138). IL-17 is also known to modulate neutrophil production by inducing G-CSF, which is involved in promoting the expansion and survival of neutrophils (139). Additionally, IL-17 plays an important role in maintaining epithelial barrier integrity by regulating the induction of AMPs during homeostasis conditions when the barrier is intact, and then by inducing immune mediators upon the loss of barrier integrity (136). As such, IL-17 is considered a key cytokine involved in the clearance of extracellular bacteria and fungi. However, aberrant IL-17 production in some cases has been linked to autoimmune or chronic inflammatory diseases (132).

The production of IL-17 is linked to several immune subsets. As mentioned previously, IL-17 is traditionally considered to be a T_h17 cytokine. T_h17 cells are a subset of activated $CD4^+$ T cells which secrete signature cytokines IL-17A and IL-17F, as well as IL-21 and IL-22 (138). In addition to T_h17 cells, other cell types are also capable of producing IL-17. This includes innate and innate-like lymphocytes such as $\gamma\delta^+$ T cells (140), NK cells, natural killer T (NKT) cells (141) and group 3 innate lymphoid cells (ILCs) (142, 143). These additional sources of IL-17 have been found to play an important role in

early immune responses against pathogens and tend to accumulate at mucosal surfaces, emphasizing the importance of IL-17 in not just adaptive, but innate immunity as well. Different pro-inflammatory cytokines including IL-1β and IL-23 are known to induce IL-17 production by these cells. Interestingly, $\gamma\delta^+$ T cells in particular have been shown to be the primary source of IL-17 production in various settings of tissue homeostasis and infection (reviewed in (144)). Unlike traditional T lymphocytes, $\gamma\delta^+$ T cells can acquire their effector function during thymic development (79); thus, allowing them to respond earlier and more rapidly to pathogens in comparison to T_h17 cells.

The role of IL-17 has been demonstrated to be critical in helping clear infections in several mucosal tissues. Since the FRT is such a critical site for enabling reproductive success, immune responses in this tissue should be well understood to help facilitate positive reproductive health. However, little is known regarding the role of IL-17 during infections in the FRT. As such, we and others have focused on the role of this important immunoregulatory factor in the context of genital tract infections. For instance, vaginal infection with *N. gonorrhoeae* is known to induce IL-17, which plays an important role in both the recruitment of neutrophils and prompt clearance of the infection (123). IL-17 also plays an anti-fungal role in the FRT. Studies have shown that vulvo-vaginal candidiasis infection results in a strong IL-17 response, which corresponds with the infiltration of neutrophils into the FRT, while inhibition of IL-17 leads to exacerbation of the disease (122). Unlike the role of IL-17 during bacterial and fungal infections at mucosal sites, very limited studies have examined the importance of IL-17 in viral infections, especially in the context of the FRT. A study by Kim et al. (145) examined the role of IL-17 during primary

infection with HSV-2, and found that in the absence of IL-17, mice demonstrated slower disease progression and delayed death. This resulted in the conclusion that IL-17 plays a pathogenic role during HSV-2 infection. However, it is difficult to say that this conclusion is completely accurate since the scope of this work is limited to a single study. As such, this has been an area of interest in our lab and the following dissertation focuses on innate and adaptive IL-17 production in the FRT, and the role it plays during HSV-2 infection.

1.3 Herpes-simplex virus type 2

1.3.1 Epidemiology and pathogenesis

HSV-2, the virus causing genital herpes, is one of the most common STIs worldwide, with over 400 million individuals infected globally and approximately 17 million new infections occurring each year (146). Rates of infection are especially alarming in sub-Saharan Africa, where prevalence is as high as 80% amongst women between the ages of 15-49 (146). HSV-2 first infects genital ECs, and then travels via retrograde transport along nerve axons to the dorsal root ganglia, where it establishes life-long latency (147). Here, the neuronal cells act as a reservoir for the latent virus, which can be activated due to factors including stress and hormonal changes. Reactivation leads to anterograde transport of the virus, resulting in productive replication in the FRT which may be associated with painful genital ulcers or, more frequently, is asymptomatic (148). Along with sexual transmission, HSV-2 can also be transmitted during labor to the neonate through direct mucosal or skin contact. Consequently, HSV-2 infection has long-term health implications, such as mother-to-child transmission during pregnancy, central and peripheral nervous system infections, and increased risk of co-infections with other STIs (149-152). In particular, HSV-2 infection is associated with a 2- to 3-fold increased risk of HIV-1 acquisition (153) and up to a 5-fold increase in transmission of HIV-1 (154). Studies indicate that in areas of high HSV-2 prevalence, between 25-35% of all HIV incidence is directly attributable to HSV-2 infection (154, 155). It remains unclear exactly why or how HSV-2 infection results in increased susceptibility to HIV, although, some potential mechanisms include enhanced facilitation of HIV entry due to breaches in the epithelium

caused by genital lesions and increased presence of activated $CD4^+$ T cells that persist in the lesions to control HSV-2 reactivation, which can act as target cells for HIV (156).

1.3.2 Viral structure, replication and transmission

HSV-2 belongs to the *Herpesviridae* family, a large family of double-stranded DNA enveloped viruses (157). Herpesviruses are further classified into subfamilies (α-, β- or γ-subfamilies) based on their biological functions and sequence similarities (158). HSV-1 and HSV-2 are two serotypes of the HSV virus and are closely related members of the α-herpesvirus subfamily, with a homology of 83% (159). Both HSV-1 and HSV-2 primarily infect ECs, however HSV-1 infection commonly results in orofacial infections, whereas HSV-2 infection primarily results in genital and/or neonatal infections (158).

The herpes virion is composed of a double-stranded DNA genome of 150 kbp enclosed in an icosahedral capsid surrounded by tegument, an amorphous structure which includes several viral proteins involved in the initial phase of viral infection and replication, and encased in an outer envelope embedded by several glycoproteins (160). Entry of herpesviruses into cells occurs through similar mechanisms amongst the different subfamilies, and the common goal is to cause lytic infection and establish latency. In the case of HSV, the target of latency is neural cells (160). Adsorption of HSV to host cells is mediated by viral glycoproteins, gB and gC, which attach to heparan sulphate, a glycosaminoglycan sugar moiety found on host cell surface proteoglycans (161). gD binds to one of the HSV receptors, herpes virus entry mediator, nectin 1 or nectin 2, and recruits accessory glycoproteins gB, gH, and gL, which are all involved in facilitating membrane fusion (157). HSV entry occurs when the virion fuses with the plasma membrane of the

host, which results in the release of tegumented nucleocapsids into the cytoplasm (157). The HSV nucleocapsid is transported via the microtubule network to the nuclear membrane pore where the virion utilizes host machinery to replicate and transcribe the viral genome (162). Replication of the virus is initiated by the expression of immediate-early genes, which are responsible for initial viral transcription and involved in evasion of the host innate immune response (158). This is followed by the expression of the early proteins responsible for replication of the viral DNA genome, and eventually the expression of the late genes, which promote expression of structural proteins including capsid, tegument and envelope proteins (158). Both the late protein products and the replicated DNA are further assembled into progeny virions, which are released from the infected cell by exocytosis or lysis of the cell (158). The released viral progenies are then able to infect other cells in the surrounding environment.

Most primary HSV-2 infections are asymptomatic, thus infected individuals are usually unaware that they are infected and can go undiagnosed (163). As a result, subclinical viral shedding accounts for much of the transmission of HSV (164), as shedding occurs even in the absence of clinical reactivation of the virus. Transmission of HSV-2 occurs relatively efficiently with a median number of 40 sexual acts before HSV-2 acquisition (165). Although transmission transpires predominantly through sexual contact with an infected individual who is shedding virus both symptomatically or asymptomatically, direct contact other than sexual intercourse can result in viral spread if an area of viral shedding comes into contact with skin or mucous membranes (164). Similarly, abrasions on mucosal surfaces can also act as an entry point for the virus (40).

In more severe cases, clinical manifestations usually begin with macules and papules, which progress to painful lesions along with swollen regional LNs. The disease can spread systemically, causing HSV encephalitis, which can prove to be fatal.

1.3.3 Treatment and vaccine development

There are no known curative treatments or therapeutic vaccines available for HSV-2 infection. Current therapeutic strategies consist of treatment with anti-viral drugs administered either at the start of early signs of recurrence or taken daily to reduce frequency of recurrences, recurrent virus shedding and the risk of transmission (166, 167). These anti-viral drugs include nucleoside analogues such as acyclovir. Acyclovir is a guanosine analogue available in both oral and intravenous formulations that inhibits HSV DNA polymerase, which prevents viral replication (166, 167). The process through which this occurs involves phosphorylation mediated by viral thymidine kinase (TK), and so the drug only becomes activated in virally-infected cells, making it a very safe therapeutic option (168). The use of acyclovir reduces symptomatic viral shedding and also shortens the duration of the reactivation episode (169-171). Since the development of acyclovir, other therapeutic drugs including valacyclovir, penciclovir, trifluridine and famciclovir have also been approved as anti-viral options for HSV-2 treatment. These additional drugs offer higher bioavailability and most require less frequent dosing (168). Although these anti-viral therapeutics are effective for treating HSV-2, they can be costly, require daily adherence, cannot completely supress outbreaks and do not eliminate the disease. Therefore, there is a clear need for the development of other treatment options such as prophylactic or therapeutic vaccines.

Ideally, a preventative vaccine would address the major concerns regarding HSV-2 infection, and preferably elicit both neutralizing antibody responses and cell-mediated immunity. To accomplish this, several factors must be taken into consideration, including the type of vaccine, the route of immunization, the delivery system and the use of specific adjuvants; all of which can influence vaccine efficacy. The primary types of preventative vaccines use prophylactic strategies to prevent viral acquisition amongst uninfected individuals. The most commonly used product for HSV vaccines in clinical trials has been glycoprotein subunit vaccines (172). HSV subunit vaccines use gD, with or without gB, to generate protective immune responses. gD is expressed on the surface of HSV and is responsible for most neutralizing antibody activity, thus making it a good target (172). Although pre-clinical studies conducted in animal models used for testing these early subunit vaccines demonstrated promising outcomes, including the induction of neutralizing antibodies, these vaccines failed to show efficacy for HSV-2 prevention in phase III clinical trials (173-175). For instance, a vaccine formulation with recombinant gD and gB in a MF59 adjuvant resulted in the production of neutralizing antibodies and $CD4^+$ T cell responses, and although the duration and severity of the first outbreak was reduced, there was no significant decrease in disease occurrence (174). In fact, the overall efficacy of this vaccine was only 9% in preventing HSV-2 infection in seronegative partners amongst serodiscordant heterosexual couples. Most recently, Herpevac, an adjuvanted gD2 vaccine, failed to prevent HSV-2 disease or acquisition (176). It did show moderate efficacy in prevention of HSV-1 infection and disease, with a vaccine efficacy of 58%, and this was attributed to increased titers of antibody to gD2 (176, 177). However, due to overall lack

of efficacy, further investment into these subunit prophylactic vaccines has declined considerably. There are currently other types of prophylactic vaccine candidates in early phases of testing, including a live, replication-deficient DNA vaccine, HSV-529. This is the first time a replication-defective HSV-2 vaccine has been tested in a phase I clinical trial. Early results are promising, as it was reported that the vaccine was safe and well tolerated, and elicited better neutralizing antibodies and $CD4^+$ T cell responses in HSV-naïve participants, compared to those previously infected with HSV (178).

There is also great interest in developing therapeutic HSV-2 vaccines, especially following disappointing results seen from early prophylactic vaccine trials. Therapeutic vaccines are designed for people who are already infected, and the goal would be to either replace anti-viral drugs or be used in conjunction with them to help reduce HSV-2 recurrences and shedding (179). There are two candidates which have met their endpoints in recent phase II clinical trials. The most advanced, GEN-003, is a subunit vaccine comprised of a deletion mutant of gD2 and a portion of infected cell protein 4, along with a novel Matrix-M2 adjuvant to stimulate T cell immunity (172). In dose-ranging trials, it was reported that immunization with GEN-003 reduced viral shedding by up to 52%, along with a 65% decrease in percentage of days with genital lesions (179-182). Furthermore, both antibody and T cell responses increased with vaccination, some persisting for up to 12 months. However, there was no correlative effect between these immune parameters and the reduction in viral shedding, and further work is being done to develop this promising vaccine. The second candidate, HerpV, is a polypeptide vaccine comprised of 32 HSV antigens, human heat shock protein 70 and adjuvanted with QS-21. Peptide-based vaccines,

such as HerpV, consist of specific peptides that contain T cell epitopes which have been shown to be protective against HSV, and are usually complexed with heat shock proteins that act as vehicles for the peptides (172). In a small trial, HerpV was shown to reduce shedding by just 15% in HSV-2 positive individuals, but it did demonstrate broad $CD4^+$ and $CD8^+$ T cell responses (183). There are currently many ongoing vaccine candidates that are entering phase I/II trials, as well as novel vaccine strategies undergoing pre-clinical testing in animal models.

Along with the different types of vaccines mentioned (subunit, peptide-based and DNA-based), others have also examined the efficacy of live-attenuated formulations. In pre-clinical animal studies, live-attenuated vaccines have shown to induce broad and long-lasting immunity; leading to lower frequency of HSV-2 recurrences (184). However, safety concerns such as the risk of reversion back to a wildtype (WT) phenotype and retained pathogenic potential have inhibited the development of these formulations. Another vaccine strategy that has gained traction over the years in pre-clinical models is the prime and pull strategy. This strategy relies on vaccination to elicit systemic T cell responses (prime), followed by the recruitment of activated T cells to the target effector site via local administration of a T cell attractant (pull) (185). The purpose is to bring in effector T cells which can then establish long-term protective immunity. This strategy has been successfully shown to recruit $CD8^+$ T cells specifically to the FRT following prophylactic HSV immunization, and following the pull, mice were completely protected against primary challenge with HSV-2 (186). Similarly, in the guinea pig model of recurrent HSV-2 using a therapeutic subunit vaccine, recurrent HSV disease was reduced following prime

and pull (185). Finally, it has also been shown in several animal studies that immunizing via mucosal routes (intranasally or intravaginally) leads to improved protection in the FRT compared to systemic immunization (187-191). The HSV-2 candidate vaccines described thus far have been delivered by injection, and associated problems include safety, compliance and most importantly, lack of antigen-specific protection at mucosal sites (192, 193). A possible way to overcome these limitations is through the use of mucosal immunization. Mucosal vaccination is considered more cost effective, less invasive and poses no risk of occupational needlestick injury (193). Additionally, mucosal vaccination is effective at priming both mucosal and systemic immune responses (192, 194). There are currently very limited mucosal vaccines licensed for human use, mainly due to issues concerning lack of effective delivery systems which are able to preserve vaccine antigen integrity and adjuvanticity (192). However, this field is expanding and more work is being done to counteract these shortcomings (195). Due to the lack of efficacy with many of the HSV-2 vaccine formulations tested in clinical trials thus far, perhaps more unconventional strategies such as live-attenuated vaccines, prime and pull methods or mucosal routes of immunization should be further explored in future studies.

1.3.4 Mouse model of infection

The availability of animal models has significantly advanced our understanding of host-pathogen interactions for HSV-2. There are multiple animal models used to study HSV-2 infection which reflect numerous aspects of human disease. In particular, the mouse model of HSV-2 has been extensively used and has provided extremely useful insights into viral mechanisms involved during infection, as well as protective immune responses

generated during infection. Although mice shed virus in vaginal secretions following infection, and also develop genital ulcers similar to those observed in humans, there are some limitations to this model. Unlike human infection, HSV-2 infection in mice can often result in a lethal acute disease. Moreover, the mouse model lacks a critical aspect of human disease, as HSV-2 does not spontaneously reactivate in mice (196). Conversely, the guinea pig model can be used to recapitulate HSV-2 recurrence, and thereby acts as a useful model to study HSV-2 latency and reactivation (196). However, the reagents available to study HSV-2 in guinea pigs are quite limited. Recently, there has been some success in the development of a large animal model using rhesus macaques which can mimic acute infection and subsequent sub-clinical reactivation of HSV-2 (197). In this model, macaques inoculated intravaginally with a WT strain of HSV-2 demonstrated clear signs of acute infection including viral shedding in genital washes, increased expression of proteins associated with tissue damage and inflammation, and tissue-based evidence of HSV-2 replication in the FRT. Infected animals also developed T cells and antibody responses against HSV-2. Furthermore, there was evidence of latent infection and reactivation, as seen by spontaneous HSV-2 shedding post-infection in majority of the animals. However, viral shedding was undetectable for more than 40 days following initial viral inoculation, demonstrating a clear limitation to this model. Altogether, the mouse model remains the most practical and cost-efficient model to study HSV-2 host-pathogen interactions.

Several modifications have been made to mitigate the limitations of the mouse model, making it a valid model to study mechanisms associated with HSV-2 infection. Early studies showed that only pre-pubescent mice are susceptible to HSV-2, as the murine

vaginal epithelium becomes too thick after puberty for the virus to be able to penetrate this barrier (198). It has also been shown that HSV-2 infection is dependent on the stage of the estrous cycle that the mice are experiencing (199). Normal mice are generally unsusceptible to HSV-2 during estrus due to high levels of E2 which results in keratinization of the epithelium (200). However, mice inoculated during the diestrus stage, when P4 levels are high, demonstrate significant viral shedding and external pathology (200). To mimic conditions of diestrus in a controlled and consistent manner and to ensure mice can be infected by HSV-2 intravaginally, most studies pre-treat mice with Depo-Provera (Depo), an injectable formulation of DMPA, prior to infection (200, 201). This is an effective strategy to make the mice susceptible to infection, although the use of hormones is less than ideal considering hormones are known to modulate immune responses (25, 26), especially in the FRT. Instead, our lab performs ovariectomies (OVX) to remove the source of endogenous hormones in female mice and this method also makes the mice susceptible to HSV-2 infection. Another aspect that needed to be addressed was determining viral strains that do not lead to lethal outcomes. Several strategies have been developed to study the virus at less lethal formulations in the form of attenuated strains, replication-impaired strains, inactivated viruses, viral protein subunits and DNA vaccines (202). Early murine vaccine studies by McDermott et al. (1984) found that a live attenuated, thymidine kinase-deficient (TK^-) strain of HSV-2 caused only mild infection in the FRT, without the neurological illness associated with WT virus (198). Deletion of the TK gene results in a viral strain which replicates in vaginal keratinocytes and causes latent infection in the dorsal root ganglia, but does not reactivate (203, 204). Moreover, using this strain primes the

immune system for subsequent challenge with WT virus, in which the mice become immune to HSV-2 exposure (198). Consequently, we extensively use TK^- HSV-2 to understand anti-HSV-2 immunity.

1.3.5 Intranasal route of immunization

The mouse model also allows for the exploration of mucosal vaccination strategies. As mentioned previously, it is well established that mucosal vaccination leads to better immunity in the FRT compared to systemic routes of immunization. Although local immunization induces the strongest immune responses, intranasal (IN) immunization can also generate protection at distal sites such as the FRT. Studies have shown that IN immunization provides better protection in the FRT compared to systemic immunization, and similar protection to intravaginal (IVAG) immunization (191, 205, 206). Multiple studies have shown that both IVAG and IN immunization with TK^- HSV-2 leads to comparable protection against subsequent genital HSV-2 challenge, and this is mediated primarily by $CD4^+$ T cell production of IFN-γ (191, 207, 208). A primary benefit of IN immunization is that it generates a balanced, protective mucosal and systemic immune response which is comprised of both cellular and humoral immunity. Additionally, IN immunization is considered a more feasible, less invasive and clinically relevant method of vaccination. Although IN immunization is mainly being used for respiratory pathogens, there are some IN HIV-1 vaccine candidates in the pipeline (195, 209), demonstrating the potential use of IN vaccines for urogenital pathogens in the near future.

Interestingly, the mechanism by which IN immunization results in protection in the FRT is not yet fully understood. Studies examining the kinetics of T cell priming and

dissemination following IN immunization have shown that $CD4^+$ T cells are first activated in the nasal mucosa around day 3, followed by those in the cervical LNs (cLNs) at day 5 (210-215). The resulting antigen-specific $CD4^+$ T cells can then be found in peripheral LNs, including the iLNs, by day 7, and subsequently in the FRT. Briefly, following IN immunization, DCs in the nasal mucosa obtain antigens and can activate T cells directly in the nasal-associated lymphoid tissue (NALT) or can migrate to the cLNs and present antigen here (210-215). During this process, T cells can be primed by DCs to express markers which allow them to traffic to specific tissues. Induction of tissue-specific homing markers by DCs has been well described in both the gut and skin. For instance, gut DCs induce expression of integrin α4β7 and chemokine receptor CCR9 on T cells through the production of retinoic acid, a metabolite of vitamin A (216, 217), while skin-derived DCs imprint skin-specific markers such as E- and P-selectin ligands and chemokine receptors CCR4 and CCR10 (218). The phenotype associated with vaginal T cell homing, as well as the mechanism involved, however, is relatively unknown. Both CCR5 and CXCR3 expression by T cells has been implicated in homing to the FRT, but only following IVAG immunization (92, 215, 219). It remains largely unknown which chemokine receptors and corresponding ligands direct T cells to the FRT following IN immunization. A recent study by Joo et al. (2019) examined mechanisms by which nasally primed antigen-specific effector cells migrate from the nasal mucosa to the genital mucosa following IN TK^- HSV-2 immunization, and they found that the CCR5-CCL5 chemokine pathway is required for the migration and retention of nasally primed effector cells to the FRT (220). Based on the limited work done in this area, more research is required to understand this mechanism.

1.3.6 Anti-viral immunity

The host and viral determinants of HSV-2 infection in humans is poorly understood; thus, a lot of knowledge and insight comes from animal studies (221). Both innate and adaptive immunity play an important role in mediating protection against HSV-2 infection. The initial response to HSV-2 involves the innate immune system, which attempts to control viral replication immediately following infection, and also initiates adaptive immune responses which are critical for viral clearance (222) (Fig. 1.2).

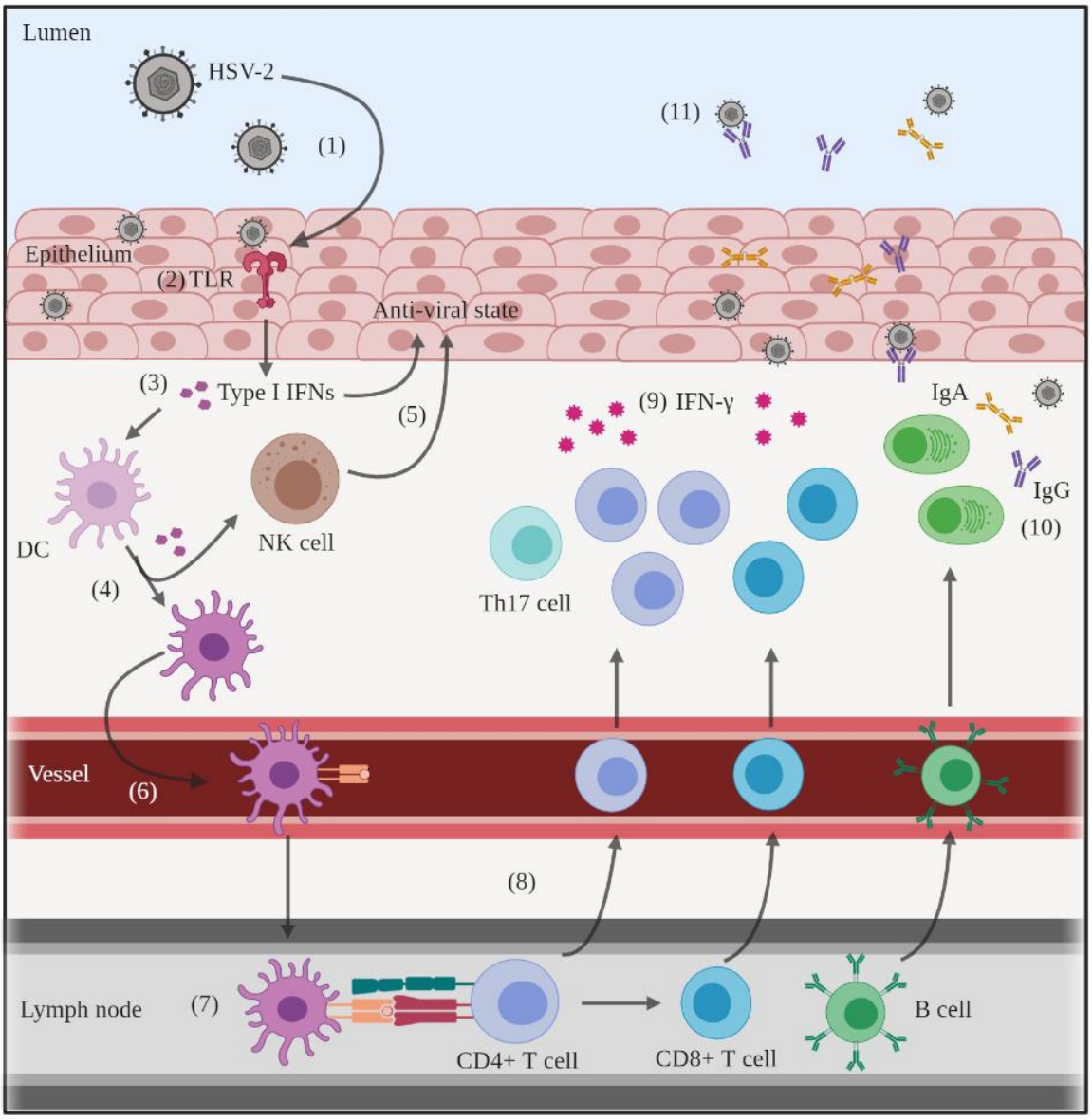

Figure 1.2: Innate and adaptive immunity following genital HSV-2 infection. (1) HSV-2 infects the vaginal epithelium and is recognized by Toll-like receptors (TLRs) present on epithelial cells and other immune cells (2). This leads to the initiation of innate immune responses including the production of pro-inflammatory cytokines and chemokines, as well as type I interferons (IFNs) (3), which are involved in conferring an anti-viral state in the surrounding epithelium by inhibiting the translation of viral mRNA. Type I IFNs are also involved in the maturation of dendritic cells (DCs) (4) and promoting IL-15 production, which leads to the activation of natural killer (NK) cells. (5) NK cells also contribute to establishing an anti-viral state by releasing IFNs and inducing apoptosis of virally-infected cells. (6) Mature DCs carry viral antigens to the draining lymph node, where they initiate adaptive immunity by stimulating HSV-2-specific $CD4^+$ T cells (7), which then help induce

HSV-2-specific $CD8^+$ T cells and B cells that differentiate into effector cells and enter circulation (8). HSV-2-specific effector $CD4^+$ T cells (T_h1 and T_h17 cells), cytotoxic $CD8^+$ T cells, and activated B cells travel into the female reproductive tract, where they are all involved in viral clearance. (9) $CD4^+$ T cells release IFN-γ, which is critical for viral clearance. $CD8^+$ T cells also release IFN-γ and help kill infected cells. (10) Plasma cells release antibodies including HSV-2-specific immunoglobulin G (IgG) and IgA, which can cross into the vaginal lumen and help neutralize HSV-2 and prevent re-infection. Created with BioRender.

1.3.6.1 Innate immunity

Genital ECs are the first to respond to HSV-2, and rapidly secrete pro-inflammatory cytokines and chemokines involved in mediating anti-viral immunity, as well as attracting other immune cells to the FRT. Recognition of the virus is facilitated by TLRs, specifically TLRs 2, 5 and 9, which recognize viral PAMPs such as viral glycoproteins, and HSV-2-associated RNA and DNA (223-225). The importance of TLR-mediated protection against HSV-2 was demonstrated in studies which reported that single nucleotide polymorphisms in human TLR 2 (226) and TLR 3 (227) are associated with increased susceptibility to HSV-2. Additionally, an *in vitro* study showed that primary genital ECs were able to inhibit HSV-2 replication following treatment with TLR 3, TLR 5 and TLR 9 ligands (224). Similar results were seen in mouse studies where mucosal immunization with TLR 3 and 9 ligands showcased protection against subsequent WT HSV-2 challenge (228-231).

TLR activation leads to the production of type I IFNs (IFN-α and IFN-β), which initiate an anti-viral state in the surrounding cells by blocking or reducing viral infection (36, 232, 233). Many studies support the importance of type I IFN, where infection with HSV-2 in IFN α/β receptor knockout (KO) (IFNα/βR$^{-/-}$) mice resulted in increased viral replication and pathogenesis, leading to decreased survival compared to WT mice (233). Moreover, HSV-2 immunization failed to protect IFNα/βR$^{-/-}$ mice against subsequent challenge with WT virus. Furthermore, humans with severe HSV-2 infection have also shown defective type I IFN signalling (227). IFNs also activate multiple effector immune cells including NK cells and DCs (233, 234). NK cells play an important anti-viral role by activating perforin- and granzyme B-mediated apoptosis of infected cells, as well as by

releasing IFN-γ (234). IFN-γ is a critical component of the immune response against HSV-2, and during the early anti-viral response following HSV-2 infection, IFN-γ activates the inducible nitric oxide synthase gene which is responsible for the production of nitric oxide in ECs, macrophages and DCs (235). Nitric oxide interferes with viral replication, and helps to protect against HSV-2 infection (222). However, some animal studies have reported conflicting findings regarding the role of NK cells, with one study suggesting increased susceptibility of infection in the absence of NK cells (236), while others showing little importance for NK cells in HSV-2 clearance (207, 237). In human studies, patients with defects in NK cells demonstrated increased rate of HSV infection (238). The primary role of DCs during infection is to transport HSV-2 peptides to the iLNs, where they initiate adaptive immunity by stimulating HSV-2-specific T and B cells (239). In addition to conventional DCs, a specialized subset of DCs called pDCs, have also been shown to play a role in protecting against HSV-2. pDCs, which express TLRs, are able to recognize HSV-2 DNA and secrete IFN-α following HSV-2 infection (240).

Ultimately, the innate immune system plays an essential role during initial HSV-2 infection. Although mechanisms such as TLR signalling and subsequent type I IFN production, induction of pro-inflammatory cytokines, and effector functions of immune cells such as NK and DCs, all attempt to control initial viral infection, the innate response is not sufficient for viral clearance and full protection requires a subsequent adaptive immune response.

1.3.6.2 Adaptive immunity

The importance of adaptive immunity in mediating protection against HSV-2 has been established extensively, especially in animal models. Both B and T cells have been shown to be involved in the clearance of HSV-2, as well as for generating immunological memory against the virus (241-243). HSV-2 infection induces antigen-specific IgG and IgA responses in the genital tract of humans (244) and mice (245, 246), with IgG acting as the primary neutralizing antibody (247). These antibodies can help neutralize the virus before it infects the epithelium (245, 248). For instance, early studies in mice demonstrated that IgG antibodies from HSV-2 vaccinated mice could neutralize HSV-2 *in vitro*, and adoptive transfer of serum IgG from immunized mice into non-immunized mice could reduce the viral load following HSV-2 infection (245). Similarly in humans, the presence of maternal antibodies specific for HSV-2 were shown to reduce neonatal transmission of the virus (249). Although these results indicate that HSV-2-specific antibodies contribute to HSV-2 clearance, they are not required for viral clearance and as mentioned previously, human vaccine strategies relying on antibodies for protection have failed to demonstrate efficacy against HSV-2 (173). This has also been seen in several animal studies, where following HSV-2 immunization, protection against subsequent HSV-2 challenge was T cell-mediated and antibody-independent (208, 250-252). Interestingly, recent studies have indicated that although B cells are not required for the IVAG model of immunization, they are required for protection seen in the IN model (253).

Along with humoral immunity, cellular immunity plays a critical role in the adaptive immune response to HSV-2. Although B cell deficient mice have been shown to

be capable of clearing HSV-2, protection is severely compromised following T cell depletion (254). Furthermore, the transfer of T cells from an immunized donor mouse to a non-immunized recipient was able to confer resistance against HSV-2 challenge in the recipient, but B cell transfer did not produce the same result (250). Cytotoxic $CD8^+$ T cells play an important role in anti-viral immunity and although HSV-2-specific $CD8^+$ T cells are induced following infection (251), it is the production of IFN-γ by these cells that plays an important role in viral clearance rather than their cytotoxic activity (241, 245). In addition, studies using $CD8^+$ T cell KO mice, as well $CD8^+$ T cell depletion techniques, have shown that immunized mice were still protected against IVAG HSV-2 challenge (207, 208), providing further evidence that $CD8^+$ T cells are dispensable for protection. Interestingly, the importance of $CD8^+$ T cells has been showcased in humans, where the infiltration of $CD8^+$ T cells within recurring genital lesions has been shown to correlate with viral clearance (255), and $CD8^+$ T cells have also been associated with modulating HSV-2 reactivation (256). It was shown that 8 weeks following the clearance of HSV-2, $CD8^+$ T cells remained present around the peripheral nerve endings, which act as the site of HSV-2 reactivation (256). More recent studies have demonstrated that these $CD8^+$ T cells continue to express granzymes and anti-viral cytokines even during clinical quiescence, suggesting that $CD8^+$ T cells play an active immunosurveillance role after lesion clearance (257). In contrast, others have found that although patients with frequent recurrences of HSV-2 outbreaks had persistent antigen-specific $CD8^+$ T cells present, they failed to prevent virus reactivation or initiate clearance of infection (258). Moreover, as

many studies have shown, $CD8^+$ T cells alone are not sufficient for viral clearance, and $CD4^+$ T cell help is critical for mediating protection (245, 251, 259).

As shown by several studies using T cell-depleted mouse models, $CD4^+$ T cells are predominantly responsible for protection against HSV-2, rather than $CD8^+$ T cells. For instance, $CD4^+$, but not $CD8^+$, T cell-depleted mice demonstrated delayed viral clearance and reduced protection (245, 251). In addition, T cell lines expanded from cervical cytobrush samples collected from HSV-2-infected women and examined for reactivity to HSV-2, demonstrated that of the HSV-2-specific $CD3^+$ T cells detected, 91.3% were $CD4^+$ whereas only a median of 3.9% were $CD8^+$ (260). HSV-2-specific $CD4^+$ T cells expanded from the cervix also exhibited greater breadth in terms of antigenic reactivity, as compared to $CD8^+$ T cells. The primary function of $CD4^+$ T cells that is associated with viral clearance is the production of IFN-γ. This was shown in a study where mice depleted of $CD4^+$ T cells, which also had decreased levels of IFN-γ in vaginal secretions, had protective immunity restored following administration of exogenous IFN-γ (208). Furthermore, IFN-γ deficient mice showed lack of protection against HSV-2, even in the presence of $CD4^+$ T cells (208). Studies examining HSV-2 lesions have also reported that $CD4^+$ T cell infiltration occurs earlier than $CD8^+$ T cell infiltration (255), and these $CD4^+$ T cells continue to express IFN-γ after lesion healing (156). The secretion of IFN-γ by $CD4^+$ T cells also leads to the production of CXCL9 and CXCL10, which recruit $CD8^+$ T cells to the site of infection (92, 95). Once effector T cells are established in the FRT following HSV-2 infection, memory populations persist for a long period of time and are able to respond rapidly to secondary infection. One such memory population, tissue-resident memory (T_{RM}) cells, have been

shown to be critical for generating protection against HSV-2 challenge (261), and this concept will be discussed in detail within this dissertation.

Other T cells present in the FRT that have also been shown to play a role during HSV-2 infection include T_{regs} and IL-17-producing cells. In animal studies, Lund et al. (2008) showed that in the absence of T_{regs}, mice had poor disease outcomes and increased viral titers, which was linked to decreased type I IFN levels in the FRT (262). However, in a separate study, T_{regs} were shown to suppress $CD4^+$ and $CD8^+$ T cell effector functions, resulting in increased viral titers (263). Likewise, human T_{regs} isolated from the blood of HSV-2-infected patients suppressed $CD4^+$ memory T cells *in vitro* (264). Similarly, the role of IL-17-producing T cells during HSV-2 infection is also uncertain. Kim et al. (2012) showed that following HSV-2 infection, the deaths of IL-17A deficient (*IL-17A*$^{-/-}$) mice were delayed compared to WT mice (145), suggesting that IL-17-producing T cells may play a pathogenic role in HSV-2 infection. However, this study only examined IL-17 in the context of a primary infection, and with a very high inoculation dose of HSV-2. Furthermore, this study used Depo to make mice susceptible to HSV-2, and several studies have shown that Depo can significantly downregulate endogenous hormone levels and decrease mucosal anti-viral responses to HSV-2 (201). Therefore, the use of Depo may have prevented accurate assessment of the role of IL-17. Overall, further research is needed to better understand the role of IL-17-producing cells during HSV-2 infection, and this is a primary focus of the research presented here.

1.3.6.3 Tissue-resident memory T cells

Effective protection against HSV-2 entails the establishment of long-lasting memory responses. Following T cell activation, $CD4^+$ and $CD8^+$ T cells differentiate into different populations of effector cells with multiple functions. Effector T cells are responsible for helping to clear the pathogen, as they are able to migrate to the site of infection in order to help control the spread of the invading pathogen, ultimately leading to its clearance (265). Once the infection is resolved, a majority of these effector cells die, leaving behind a heterogeneous pool of memory cells (266). Originally, two types of memory T cells were identified: central memory T cells (T_{CM}), found in secondary lymphoid organs, and effector memory T cells (T_{EM}), found primarily in non-lymphoid tissues (267-269). T_{CM} cells are a subset of memory T cells that express secondary lymphoid homing molecules vascular addressin L-selectin (an adhesion molecule also known as CD62L), and CCR7, which allows entry into LNs from the blood. T_{CM} cells recirculate through secondary lymphoid organs and can proliferate extensively in response to re-stimulation (267-269). Conversely, T_{EM} cells are a subset of memory T cells which lack the expression of L-selectin and CCR7 (267-269). T_{EM} cells can be found in non-lymphoid tissues and can exert immediate effector functions, including the production of cytokines such as IFN-γ (267-269). In addition, T_{EM} cells have receptors which allow them to access peripheral tissues, thus playing an important role in peripheral immune surveillance (270, 271).

For several years it was unknown whether these T_{EM} cells were continuously recirculating, or whether they could permanently reside within non-lymphoid tissues. New

technological approaches were utilized to determine if memory T cells can be retained in peripheral tissues. Parabiosis experiments, which surgically conjoin mouse pairs to create shared circulation, provided evidence for $CD4^+$ memory T cell retention in lung tissues (272). In addition, parabiosis studies looking at HSV-2 infection in the dorsal root ganglia and skin (273, 274), as well as lymphocytic choriomeningitis virus infection in the small intestine (275), showed that memory $CD8^+$ T cells can remain in tissues following infection, without recirculating through the blood. The discovery that a portion of T_{EM} cells did not re-enter circulation and instead, remained resident in non-lymphoid tissues, resulted in the characterization of a novel subset of non-circulating memory T cells, known as tissue-resident memory T cells (T_{RM} cells) (273, 275). T_{RM} cells are essentially clonally expanded memory T cells which enter peripheral tissues, where they are retained long-term and are able to survive without depending on replenishment from circulating T cells. They have distinct functional, phenotypic, and transcriptomic profiles, all of which are dependent on their site of residency (276, 277).

Motility of T_{RM} cells is limited to within the tissues in where they are resident, and the inability of T_{RM} cells to recirculate via the bloodstream is an important defining feature of these cells (265). Furthermore, T_{RM} cells are identified based on the expression of specific markers, which vary based on the tissue and subset of cells being examined. The most common markers expressed by most T_{RM} cells are CD69 and CD103 (265). CD69 is a marker associated with T cell activation, while CD103 is the α chain of the αEβ7 integrin, a molecule that binds to E-cadherin (278). Many T_{RM} cells also express high levels of CD44 (a cell-surface glycoprotein involved in cell adhesion), CD11a (a cell adhesion integrin)

and low levels of CD62L (261). It is important to note that $CD8^+$ and $CD4^+$ T_{RM} cells in some mucosal sites in both humans and mice do not express CD103 (279, 280). Likewise, there have been populations of T_{RM} cells found within the FRT of mice which are negative for both CD69 and CD103 (281). In fact, $CD4^+$ T_{RM} cells in the vaginal tract generally do not express CD103, and CD103 expression is detected in only 10% of $CD4^+$ T_{RM} cells in the vagina following immunization with TK^- HSV-2 (261). This represents one of the biggest difficulties in studying T_{RM} populations, as there may be substantial phenotypic variability within this memory T cell subset, as well as overlap with other memory and effector populations. As a result, it is best to use a large panel of markers in order to identify T_{RM} cells in a more confident manner. In addition, T_{RM} cells are better identified based on their lack of ability to recirculate. There are also other T cell subsets which permanently reside within tissues, including T_{regs}, NKT cells, NK cells, $\gamma\delta^+$ T cells and ILCs, and so these subsets may also assist T_{RM} cells in providing protection at peripheral sites.

Due to their close proximity to the site of pathogen exposure, T_{RM} cells can rapidly respond to infection and thus, represent a critical population which can be targeted to improve HSV-2 vaccination. In general, upon re-exposure to a pathogen, circulating memory T cells are re-stimulated in draining secondary lymphoid organs, where they proliferate and generate large numbers of secondary effector T cells, which can then migrate to infected tissues (282). However, reliance on the recall of circulating memory T cell populations, which can take from hours to days to appear at the site of infection, may result in a delay during which the pathogen can spread and initiate disease (265). Also, by the time circulating T_{EM} cells arrive at the site of infection, they may have difficulty

accessing the infected tissue region, or be largely outnumbered by the replicating pathogen. Furthermore, studies have shown that following primary infection, protection of peripheral tissues can decrease over time. This can be due to changes in homing molecule expression, as effector T cells differentiate into memory cells (275, 281), along with a decline in the overall number of T_{EM} cells, as cells transition into T_{CM} cells (283). Therefore, circulating memory T cells may not provide sufficient protection against re-infection in peripheral tissues and T_{RM} cell-mediated surveillance of tissues results in a more rapid response to infection. It is likely that the main role of T_{RM} cells in peripheral tissues is to delay the spread of the pathogen and to recruit recirculating T cells from circulation to the site of infection. For instance, a study by Schenkel et al. (284) showed that upon antigen re-exposure within the mouse vaginal mucosa, $CD8^+$ T_{RM} cells immediately triggered an anti-viral response by secreting cytokines that initiated immune responses including humoral immunity, maturation of DCs and activation of NK cells.

Although $CD8^+$ T_{RM} cells have been extensively studied, less is known about $CD4^+$ T_{RM} cells. Similarly, even less has been reported in literature about the generation of tissue-resident IL-17^+ $CD4^+$ T cells (285-288). This is partly due to the fact that unlike $CD8^+$ T_{RM} cells, which can be consistently distinguished based on the high expression of CD103, there is no definitive residency-only marker identified for $CD4^+$ T_{RM} cells (265). Also, while $CD4^+$ T_{RM} cells have primarily been identified and studied in the lungs and skin, little is known about these cells in the FRT. Iijima and Iwasaki recently described a protective role for $CD4^+$ T_{RM} cells in the FRT against lethal HSV-2 infection (261). They used parabiotic mice to show that following IVAG HSV-2 immunization, a pool of $CD4^+$ T cells remained

resident within the FRT. Upon lethal challenge with HSV-2, mice relying only on circulating memory cells were unable to suppress viral replication and many succumbed to infection, while those with T_{RM} cells were completely protected. The T_{RM} cell population ($CD4^+$ $CD62L^-$ $CD44^+$ $CD11a^+$ $CD69^+$ $CCR7^{lo}$) was readily available to respond to subsequent HSV-2 challenge and generated immediate protection via IFN-γ production, while mice relaying on circulating memory T cells did not induce IFN-γ production until much later. They also found that while $CD8^+$ T_{RM} cells localized in the epithelium, $CD4^+$ T_{RM} cells assembled within aggregates of macrophages. These clusters of leukocytes, which also include NKT cells, NK cells, $\gamma\delta^+$ T cells and ILCS, are called memory lymphoid clusters. Although the local migration of these T_{RM} cells was not directly examined, they are believed to have restricted movement in the proximity of the memory lymphoid clusters and may require local chemokine expression for maintenance, as abrogation of chemokine signals (CCL5 and CXCL9) disrupted the clusters. Furthermore, the non-circulating $CD4^+$ T_{RM} cells produced IFN-γ, which stimulated the macrophages to produce chemokines such as CCL5. Similar clusters of memory T cells were also found in the brain after vesicular stomatitis virus infection (289) and in the intestinal lamina propria after *Yersinia pseudotuberculosis* infection (290). In the guinea pig model of genital HSV-2 infection, IFN-γ-secreting T cell responses were developed in the genital tract, lumbosacral ganglia, spinal cord and spleen following infection, and populations of HSV-specific $CD4^+$ and $CD8^+$ T_{RM} cells were maintained in the genital tract and sensory ganglia for up to 150 days post-infection (291).

Interestingly, in the study by Iijima and Iwasaki, these $CD4^+$ T_{RM} cells were absent following IN immunization (261). This was attributed to the fact that the FRT is considered a restrictive tissue, and so it is believed that local infection or inflammation is required to recruit T_{RM} precursors and maintain T_{RM} cells (266), and that $CD4^+$ T_{RM} cells in the genital mucosa may require antigen to persist (261). However, in a separate study, an IFN-γ^+ $CD4^+$ T cell population was observed in the FRT up to 3 weeks post-IN immunization (191), albeit these cells were not characterized as T_{RM} cells. As such, the establishment of $CD4^+$ T_{RM} cells following IN HSV-2 immunization has not been reported. Likewise, very little has been reported regarding the effect of hormones on T_{RM} cells. A recent study characterized resident T cell populations, defined as $CCR7^{hi}$ $CD4^+$, in the lower FRT of women during the P4-high phase of the menstrual cycle, and suggested that the frequency of this population positively correlated with P4 levels in the blood (292). However, the study was not able to clearly distinguish between T_{RM} cells and circulating memory T cells.

Overall, it appears that T_{RM} cells are a critical subset of memory T cells which provide optimal protection against pathogens, including HSV-2; thus, making these cells an important factor to focus on during vaccine development. Although few studies have looked at T_{RM} cells in human tissues, primarily due to the issue of obtaining samples and isolating sufficient numbers of cells, T_{RM} cells have been identified in various tissues (265, 277, 293-295). For instance, using human skin biopsies, Zhu et al. (256) showed that HSV-2-specific $CD8^+$ T cells persisted in the skin adjacent to nerve endings for more than two months following HSV-2 clearance. Additionally, patients with persistent HSV-2 infection of the genital skin were shown to have $CD8^+$ T cells at the dermal-epidermal junction

months after lesion healing, while $CD4^+$ T cells were found mainly in the dermis. This is similar to responses in the skin of mice following HSV-1 infection (281). Furthermore, recent evidence suggests that mouse and human memory T cell populations are similar, as both species express common memory markers (296-298). Parallels shown between mice and human memory cells indicate that there is potential for findings in mice to be translated into humans.

1.4 Sex hormones and the female reproductive tract

1.4.1 Menstrual cycle in women

The menstrual cycle consists of a series of changes that occur in women of reproductive age, with the intent to prepare the uterus for pregnancy. The average length of the menstrual cycle is 28 days, although this varies greatly among women (21-35 days) (26). The changes which occur throughout the cycle are primarily mediated by the cyclic secretion of sex hormones, E2 and P4. The menstrual cycle is divided into four stages: the proliferative/follicular phase, mid-cycle (ovulation), the secretory/luteal phase and the menstrual phase (26). During the proliferative phase, the endometrium develops, and follicles grow under the influence of rising levels of E2, along with the presence of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). This results in the thickening of the uterus. Rising levels of E2, as well as a surge in LH, then triggers the process of ovulation mid-cycle, which is marked by the release of an ovum (egg) by a follicle. The onset of the secretory phase occurs following ovulation and is characterized by high levels of P4 released from the corpus luteum, which is the remains of the dominant follicle. This helps to maintain the endometrium in order to prepare for potential implantation of an embryo. In the absence of implantation, the cycle ends with menstruation. During this time, the corpus luteum regresses and levels of E2 and P4 drop, resulting in shedding of the uterine lining and the onset of menses (26).

1.4.2 Estrous cycle in mice

The reproductive cycle in rodents, including mice, is called the estrous cycle. Unlike the menstrual cycle, the length of the estrous cycle is just 4-5 days (299-301). The estrous

cycle is divided into four phases: proestrus, estrus, metestrus and diestrus. Throughout these phases, the vagina and uterus go through morphological changes which can be tracked based on the proportions of three cell types present in vaginal secretions: ECs, cornified cells and leukocytes. Proestrus lasts for no more than a day, and consists of increasing levels of E2, as well as the development of the uterine lining. During proestrus, there is a predominance of large nucleated ECs, with a few cornified cells present. This is considered the pre-ovulatory day, and overnight, both LH and FSH levels start to rise. This signals ovulation, which occurs during the estrus phase. During estrus, there is a loss of mitotic figures and progressive shedding of the cornified epithelial layers. Estrus is characterized by the presence of irregularly-shaped, cornified squamous ECs that appear in clusters and have no visible nucleus. E2 levels remain elevated throughout the morning and fall back to basal levels by the afternoon. This is followed by the metestrus phase, where the corpora lutea grow and produce P4. In the metestrus stage, there is a mix of cell types, with primarily leukocytes and a few nucleated ECs and/or cornified squamous ECs. In the absence of pregnancy, the cycle terminates during the next phase, diestrus, with regression of the corpus lutea. Diestrus is a slightly longer phase and predominantly consists of leukocytes. The lining of the uterus is not shed, but is reorganized for the next cycle (300, 301). Similar to what occurs during the human menstrual cycle, the cyclic changes observed throughout the estrous cycle are largely modulated by E2 and P4. The levels of E2 peak during the estrus phase, while P4 levels peak during diestrus (302).

1.4.3 Estradiol

Sex hormones are fat-soluble steroid compounds under control of the hypothalamus–pituitary–adrenal gland axis, and play a major role in the development of the reproductive system by exerting profound effects on cell growth, development, differentiation and homeostasis (303). While the natural presence of hormones, E2 and P4, as well exogenous administration of HCs, are all known to modulate immunity in the FRT, the focus here will be examining the role of E2.

It is well documented that estrogens are critical regulators of growth, differentiation and function in a wide array of tissues including the FRT, mammary gland, and skeletal and cardiovascular systems (303, 304). Estrogens can have either pro- or anti-inflammatory roles, depending on the context of factors involved. 17-β E2 is the predominant, bioactive estrogen found in reproductive-age women. E2 is primarily produced by the ovaries, which account for ~95% of E2 found in circulation, while the adrenal cortex is a secondary source for E2 (305). Physiological serum levels of E2 fluctuate between 10^{-9} M to 10^{-12} M throughout the normal menstrual cycle, and can reach levels as high as 10^{-8} M during pregnancy (306). E2 regulates human physiology via diffusion through the plasma membrane of target cells and signalling through intracellular estrogen receptors (ERs). ERs are members of a large family of nuclear receptors that function as ligand-activated transcription factors (307). There are two forms of ERs which have been identified, ERα and ERβ, both of which are widely expressed by various cells and tissues (308). Although both ERα and ERβ have similar homology at their DNA- and ligand-binding domains and share overlapping roles during E2 signalling, the expression and tissue distribution of these

receptors varies depending on the tissue/cell type (304). For instance, ERα expression has been reported in the endometrium, ovaries, and hypothalamus, while ERβ is more widely expressed by the kidneys, brain, lung, heart, intestinal mucosa, prostate and endothelial cells (309). These receptors are also expressed by many immune subsets including lymphocytes, macrophages, NK cells and DCs (310, 311). While ERα appears to be ubiquitously expressed by most immune cells, ERβ is less universally expressed (312). In the traditional genomic pathway of signalling, E2 ligands diffuse across the plasma and nuclear membranes to bind to appropriate receptors, which induces an activating conformational change within the ER by mediating dimerization of receptor proteins (307). The activated, homodimerized ER then binds to specific DNA estrogen response elements (EREs) located in the regulatory regions of E2-responsive genes, and this results in either transcriptional trans-activation or -repression of these target genes. Along with hormone-mediated activation, ERs can be modulated indirectly by extracellular signals in the absence of E2, and can also participate in signalling mechanisms which do not involve direct DNA binding (304). Furthermore, E2 can also elicit rapid, non-genomic signal events by binding to a membrane-bound receptor called the G-protein receptor 30, which is found in numerous tissues and cells (313, 314). For instance, binding of E2 to G-protein receptor 30 results in modulation of intracellular calcium and potassium currents, and stimulation of the PI3K/AKT/mTOR pathway, which can lead to the phosphorylation of transcription factors that indirectly regulate gene expression (313).

1.4.4 Influence of E2 on immune responses in the FRT

It is well established that E2 influences immunity in women. The role of E2 in disease, however, is complex. Although women are less susceptible to infectious diseases compared to men, primarily due to enhanced inflammatory responses, a number of studies have shown that women are also more likely to suffer from autoimmune conditions such as systemic lupus erythematosus, Sjogren's syndrome, multiple sclerosis and rheumatoid arthritis (12, 315). Therefore, the heightened inflammatory response observed in women is advantageous in certain situations, but can also result in increased immune responses against self-antigens. This paradoxical role of E2 is further complicated because the effect of E2 is often concentration-dependent, and the mechanisms by which low and high physiological doses of E2 differentially influence immune responses are unclear.

E2 is known to regulate immune cell frequencies and function though direct and indirect mechanisms in the FRT. In most mammalian species, E2 induces proliferation of the epithelial layer of the uterus, producing a thickened epithelium (316). E2 also modulates several immune mechanisms associated with ECs, such as the expression of tight junction proteins (317, 318) and TLRs (319-322), and the secretion of AMPs (48), cytokines and chemokines (323-326). As mentioned above, whether E2 acts in an inflammatory or anti-inflammatory manner is largely concentration-dependent (315). For example, low E2 levels induce the expression of inflammatory mediators including TNF-α, IL-1β and IL-6, and increase migration of leukocytes to the site of inflammation (315). Conversely, high E2 levels, similar to those observed during pregnancy, act in an anti-inflammatory manner by inhibiting inflammatory mediators (327). Prior to ovulation, when E2 levels are high, E2

represses inflammation at the epithelial surface, which suggests that E2 helps mediate an environment which is conducive for sperm entry (328). During this period, E2 inhibits secretion of IL-6, IL-8, TNF-α and NF-κB (48, 329). One of the primary ways E2 helps restrict inflammation is by regulating the function of NF-κB, a key transcription factor involved in mediating inflammation. E2 is involved in preventing the degradation of NF-κB inhibitors, as well as restricting its cytoplasmic-to-nuclear translocation (330, 331).

Changes in hormone levels during the menstrual cycle can also influence antigen presentation in the FRT and modulate the number of DCs and macrophages present. For instance, frequencies of macrophages and DCs are highest in the menstrual phase of the cycle, compared to other stages (332, 333). Animal studies have also shown that uterine ECs produce higher amounts of G-CSF during the E2-high phase of the estrous cycle in rodents, and consequently, frequencies of macrophages and DCs were highest during this phase (334, 335). Additionally, E2 can also affect DC differentiation and function by acting on DC precursors, as well as on mature DC activation; although examining these effects has largely been limited to *in vitro* studies (12). These studies demonstrated that high doses of E2 can enhance differentiation of human and murine bone marrow-derived DCs, increase expression of costimulatory molecules CD80, CD86 and CD40 and MHC II (336-339), and enhance production of cytokines (340). In our own studies, we reported novel effects of E2 on DC priming in the FRT *in vivo*. Specifically, we showed that in the murine vaginal tract, $CD11c^+$ DCs prime greater T_h17 responses following E2 treatment (341). The effect of E2 on the function of DCs in the vaginal tract is an ongoing area of research that will be addressed throughout this dissertation.

Adaptive immunity in the FRT is also influenced by hormones. Studies show that total IgG and IgA titers in CVLs are highest during the proliferative phase of the cycle when E2 levels are high, while the lowest levels of IgG occur during ovulation (342-344). Hormones also modulate the expression of pIgR, thereby affecting IgA levels. Animal studies using a rat model have shown that IgA, IgG and pIgR levels are increased in the uterus during E2-high phases of the cycle, as well as following exogenous E2 treatment of OVX rats (345, 346). Additionally, both IgG and IgA levels were significantly reduced in CVLs collected from post-menopausal women post-hysterectomy (347). E2 levels are also known to influence T cell responses. In lymphoid aggregates found in the uterine endometrium, the activity of cytotoxic $CD8^+$ T cells is severely reduced during the secretory stage (58, 348), when E2 levels are low, while the greatest cytotoxic activity is observed during the proliferative phase. Furthermore, lymphoid aggregates are largely absent in post-menopausal women (96), providing further evidence that sex hormones regulate aggregate formation and size.

E2 also differentially affects different T helper cell subsets. Low concentrations of E2 have shown to promote T_h1 responses and cell-mediated immunity, whereas high E2 concentrations have been shown to promote T_h2 responses and humoral immunity (13, 315). For instance, studies have shown that the binding of E2 to its receptors increases IFN-γ transcription via EREs in the promoter region of the IFN-γ gene (12, 13). This was seen using human T cells, where E2 treatment greatly increased the activity of the IFN-γ promoter (349). Additionally, studies have shown that low concentrations of E2 also upregulated mitogen activated protein kinase, Tbet, and certain miRNAs to increase

production of IFN-γ by T cells (350, 351). Likewise, we have shown in a rodent model that E2 treatment in OVX mice enhanced IFN-γ production by $CD4^+$ T cells in the FRT (341). Studies also show that during the proliferative phase of the cycle, when E2 levels are high, there is a significant expansion of forkhead box P3 $(FOXP3)^+$ T_{regs} in the uterus (352), which may play a critical role in inducing immune tolerance for successful implantation and for tissue breakdown and repair (352, 353). When examining the effect of E2 on T_h17 cells, a study by Rodriguez-Garcia et al. (2014) found decreased numbers of $T_{h1}7$ cells in the endometrium compared to the cervix in pre-menopausal women (91). However, as demonstrated by animal studies, the effect of E2 on T_h17 cells appears to be dose-dependent. For example, studies showed that E2 deficiency in OVX mice induced T_h17 cell differentiation (354), while treatment with high doses of E2 decreased IL-17 production by T_h17 cells (355). Furthermore, we have shown that E2 enhances T_h17 priming by vaginal DCs *in vivo* (341). Based on the conflicting results regarding the effect of E2 on T_h17 immunity in the FRT, we examined this relationship further, and the findings are presented in this dissertation.

1.4.5 Influence of E2 on susceptibility to HSV-2

The WHO estimates that more than one million individuals acquire an STI daily (3), many of which do not have a viable cure or vaccine currently available. Rates of several STIs, including HSV-2, are higher in women compared to men, and so understanding how female sex hormones influence susceptibility is critical for the development of protective measures. This is especially important in areas with high incidences of STIs, as the use of injectable progestin-based contraceptives such as DMPA, has been linked to increased

susceptibility (356). In particular, studies have shown that compared to other HCs, DMPA use increases HIV-1 acquisition by 1.4-fold in sub-Saharan Africa (356), a region where DMPA is the most common form of HC used and coincidently, rates of HIV-1 infection are the highest compared to any other region in the world (357, 358). Both the stage of the menstrual cycle and the use of HCs have been shown to influence susceptibility to a number of other STIs as well, including candidiasis, gonorrhoea and chlamydia (359). However, the effect of hormones on HSV-2 infection and shedding in humans is conflicting, as some human studies have linked DMPA use with increased susceptibility to HSV-2, while others report no effect of HC use or menstrual cycle timing on HSV-2 shedding or lesion frequency in infected women (360-364). For example, the most recent study suggests that the follicular phase of the cycle may be associated with a higher frequency of HSV-2 shedding compared to the luteal phase in women who are not taking any HC, while there were no differences observed in lesion frequency or any noted impact of HC use (364). Therefore, further studies are required. Attempts to understand the underlying mechanisms by which hormones differentially affect susceptibility have largely only been studied in animal models. In general, studies have demonstrated that P4 and progestin-based formulations increase susceptibility to STIs, while E2 has a protective effect (200, 365-372).

Our lab has been extensively studying the effect of female sex hormones on the outcome of HSV-2 infection in both primary infection and vaccinated mice for over a decade (25). We, and others, have shown that E2 has a greater protective effect against sexually transmitted pathogens with no immunopathology, whereas P4 leads to protection,

but with increased inflammation and immunopathology. In the context of HSV-2, mice can only become infected during the P4-dominant phase of the estrous cycle and are unable to be infected during the E2-dominant phase (245). We have shown that mice immunized IN or IVAG under the influence of E2 are completely protected from genital HSV-2 challenge, with limited viral shedding and genital pathology (370, 371, 373). Conversely, early studies in our lab found that DMPA or P4 treatment in mice increased susceptibility to genital HSV-2 infection by 100- or 10-fold (200, 368), respectively, and this was largely due to compromised mucosal immune responses. These observations were confirmed by Pennock et al. (2009), where E2 treatment improved vaccine outcomes in a study examining an HSV-2 gD peptide-based vaccine formulation (374). Additionally, others have shown that while progestin-based HCs diminish the genital mucosal barrier in mice, leading to increased mucosal epithelial permeability and subsequently, increased susceptibility to HSV-2 infection, concurrent treatment of mice with DMPA and E2 restored mucosal barrier function and prevented HSV-2 infection (375, 376). Along with our findings in animal models, we have also shown using *in vitro* studies with a vaginal cell line that P4 treatment confers greater susceptibility to HSV-2 infection compared to E2 treatment (377). Non-human primate studies done by others using rhesus macaque monkeys support this data, as subcutaneous implants of P4 made the monkeys more susceptible to simian immunodeficiency virus infection, while E2 was able to protect against infection (366, 367). However, the underlying mechanism by which E2 mediates protection remains relatively unclear, and this has been a major area of focus in our research, including the work presented in this dissertation.

In other studies where enhanced protection was observed in the presence of E2, several methods of protection were speculated. It was theorized that: 1) the thickening of the vaginal epithelium by E2 could act as a physical barrier of protection against viral infection (378), 2) the decrease in the pH of the FRT mediated by E2 could make the microenvironment less hospitable to pathogens (379), and 3) the primarily lactobacilli dominate microbiome observed in the presence of E2 could help prevent infection (380, 381). It is possible that all of these factors may be involved, but there is no conclusive evidence to support these claims. Furthermore, there remains a lack of cellular-mediated evidence to explain the differential immunity observed in the presence of certain hormones. In our lab, we recently showed that E2 treatment directly induced murine vaginal DCs to prime robust IL-17^+ T_h17 responses *in vivo*, which coincided with enhanced anti-viral IFN-γ^+ T_h1 responses and protection against HSV-2 challenge (341). Although it is well known that immune cells including DCs and T cells express sex hormone receptors and can be regulated by hormones, this was the first indication that E2-mediated protection against HSV-2 involves differential priming of T cell responses by vaginal DCs. Considering T cells play a critical role in protection against HSV-2, these findings prompted us to further understand the axis between E2, IL-17 and immune protection against HSV-2, and this work will be discussed thoroughly in this dissertation.

1.5 Summary

HSV-2 continues to be one of the most predominant STIs globally, especially in women (146). Increased susceptibility in women has been linked to sex hormones, which are present in the FRT and can modulate immunity and susceptibility to infection (26). Previous attempts at HSV-2 vaccine development have focused on eliciting antibody responses to mediate protection, however, limited success has been observed (168, 172). Furthermore, studies have demonstrated a critical role for T cells in generating protective immunity against HSV-2 (156, 241, 245, 251, 252, 255, 261). Our lab has been studying the effect of the hormonal microenvironment on genital HSV-2 infection for over a decade. In early studies, we showed that E2 treatment was protective against HSV-2 infection (369-371) and recently, we found that E2 treatment induced potent T_h17 responses in the FRT, which coincided with an earlier, and greater, accumulation of mucosal IL-17^+ and IFN-γ^+ $CD4^+$ effector T cells post-challenge (341). While the protective anti-viral role of T_h1 cells during HSV-2 infection is well described in literature, little has been published on the importance of anti-viral T_h17 immunity. Thus, the primary objective of this dissertation was to further investigate the role of IL-17 in mediating anti-viral T cell immunity in the FRT during HSV-2 infection. Further knowledge about factors which enhance anti-viral immunity in the FRT can provide insight on effective strategies for generating optimal immunity during vaccine development for STIs.

1.6 Central Hypothesis and Specific Aims

Central Hypothesis: IL-17 enhances anti-viral protection against HSV-2 infection by mediating more efficient $CD4^+$ T cell immunity in the FRT.

Specific Aim 1: Determine if IL-17 is critical for enhancing anti-viral T_h1 responses in the FRT following HSV-2 infection.

Objective 1: Examine the differences in protection between *IL-17A*$^{-/-}$ and WT mice following primary HSV-2 infection and challenge.

Objective 2: Examine *in vivo* T_h1 responses in *IL-17A*$^{-/-}$ and WT mice following HSV-2 challenge.

Specific Aim 2: Determine if innate IL-17 is critical for the induction of adaptive T_h17 responses in the FRT.

Objective 1: Characterize the primary source of innate IL-17 production in the FRT.

Objective 2: Examine the effect of innate IL-17 on the induction of T_h17 responses in the FRT.

Objective 3: Examine the effect of factors such as E2 and microbiota on innate IL-17 production in the FRT.

Specific Aim 3: Determine if IL-17 is critical for E2-mediated enhancement of vaginal $CD4^+$ memory T cells following HSV-2 immunization.

Objective 1: Characterize the establishment of $CD4^+$ memory T cells in E2- and placebo-treated mice following IN HSV-2 immunization.

Objective 2: Test the anti-viral efficacy of vaginal $CD4^+$ T_{RM} cells generated following IN HSV-2 immunization, in E2- and placebo-treated mice.

Objective 3: Examine the role of IL-17 in the establishment of vaginal $CD4^+$ T_{RM} cells following IN HSV-2 immunization.

CHAPTER 2: Novel role for interleukin-17 in enhancing type 1 helper T cell immunity in the female genital tract following mucosal herpes simplex virus 2 vaccination

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In this study, we demonstrated a novel role for IL-17 in regulating anti-viral IFN-γ^+ T_h1 cell immunity in the FRT, and showed that IL-17 is essential for inducing effective protection against HSV-2 infection. We found that in the absence of IL-17, immunized IL-17-deficient mice were unable to overcome IVAG HSV-2 challenge and demonstrated poor disease outcomes, including higher mortality, genital pathology, viral shedding and levels of pro-inflammatory cytokines and chemokines in the FRT, compared to WT mice. This was shown to coincide with impaired T_h1 cell responses following HSV-2 challenge.

Dr. Charu Kaushic and I conceived and designed the experiments. Dr. Varun Anipindi, Philip Nguyen, Danielle Vitali and I performed the experiments. Drs. Charu Kaushic, Varun Anipindi and I analyzed and interpreted the data. Dr. Martin Stämpfli provided materials and assisted with data analysis. Dr. Charu Kaushic and I wrote and edited the manuscript.

ABSTRACT

It is well established that interferon-gamma (IFN-γ) production by $CD4^+$ T cells is critical for anti-viral immunity against herpes simplex virus type 2 (HSV-2) genital infection. However, the role of interleukin-17A (IL-17A) production by $CD4^+$ T cells in HSV-2 anti-viral immunity is yet to be elucidated. Here, we demonstrate that IL-17A plays an important role in enhancing anti-viral T helper type 1 (T_h1) responses in the female genital tract (FGT), and is essential for effective protection conferred by HSV-2 vaccination. While IL-17A did not play a critical role during primary genital HSV-2 infection, seen by lack of differences in susceptibility between IL-17A deficient mice (*IL-17A*$^{-/-}$) and wild-type (WT) C57BL/6 mice, it was critical for mediating anti-viral responses post-challenge/re-exposure. Compared to WT mice, *IL-17A*$^{-/-}$ mice (1) infected intravaginally and re-exposed or (2) vaccinated intranasally and challenged intravaginally, demonstrated poor outcomes. Following intravaginal HSV-2 re-exposure or challenge in vaccinated mice, *IL-17A*$^{-/-}$ mice had significantly higher mortality, greater disease severity, higher viral shedding and higher levels of pro-inflammatory cytokines and chemokines in vaginal secretions. Furthermore, *IL-17A*$^{-/-}$ mice had impaired T_h1 cell responses post-challenge/re-exposure, with significantly lower proportions of vaginal IFN-γ^+ $CD4^+$ T cells. The impaired T_h1 cell responses in *IL-17A*$^{-/-}$ mice coincided with smaller populations of IFN-γ^+ $CD4^+$ tissue-resident memory T cells (T_{RM}) in the genital tract post-immunization. Taken together, these findings describe a novel role for IL-17A in regulating anti-viral IFN-γ^+ T_h1 cell immunity in the vaginal tract. This strategy could be exploited to enhance anti-viral immunity following HSV-2 vaccination.

INTRODUCTION

Genital herpes, caused primarily by herpes simplex virus type 2 (HSV-2), is one of the most predominant viral sexually transmitted infections (STIs) in the world (1). Recent estimates show that 417 million people between the ages of 15-49 are infected with HSV-2 globally, and approximately 19.2 million in this age group become newly infected each year (1). Similar to many other STIs, rates of HSV-2 infection are disproportionally higher in women, with approximately 14.8% of women infected compared to 8.0% of men, globally (1). Efforts to develop effective vaccines against STIs such as HSV-2 have been unsuccessful, as there is little understanding of factors in the local microenvironment of the female genital tract (FGT) that determine the outcome of exposure to HSV-2, making it difficult to develop preventative interventions (2).

To study the immune mechanisms involved in generating protection against HSV-2 infection, a well-established mouse model of genital HSV-2 infection is commonly used as it closely recapitulates many aspects of human infection. Mouse studies have demonstrated that both humoral and cell mediated responses are involved in generating protection against genital HSV-2 infection. Vaccine strategies that can induce robust local antibody responses or passively transfer antibodies from immunized mice, can protect mice against subsequent intravaginal HSV-2 challenge (3). However, it is well known that $CD4^+$ T helper 1 (T_h1) cell immune responses and production of interferon gamma (IFN-γ) are critical for protection both following natural resolution of infection, as well as following immunization (4-5). Therefore, preventative strategies that aim to induce strong T_h1 cell immunity in the genital tract should be able to effectively control HSV-2 infection.

Along with T_h1 cell immunity, genital HSV-2 infection also induces a T_h17 immune response. T_h17 cells are a subset of activated $CD4^+$ T cells, characterized primarily by the secretion of cytokine interleukin-17 (IL-17), as well as IL-21 and IL-22 (6). The IL-17 family of cytokines includes six ligands (IL-17A to IL-17F), and T_h17 cells generally produce IL-17A and IL-17F (6). T_h17 cell immunity and IL-17A production in particular, has been shown to play a fundamental role in resolution of fungal and bacterial infections in the genital tract including *Candida albicans* (*C. albicans*), *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Chlamydia trachomatis* (*C. trachomatis*) (7-12); however, aberrant T_h17 cell responses can lead to autoimmune conditions or chronic inflammatory diseases in other tissues (6, 13, 14). Although IL-17 has been studied in the context of bacterial and fungal infections in the genital tract, the role of IL-17 in genital anti-viral responses, particularly during HSV-2 infection, is less understood. Kim et al. (2012) reported there was a significant delay in the deaths of IL-17A deficient (*IL-17A*$^{-/-}$) mice compared to C57BL/6 control mice following primary genital HSV-2 infection, implying that IL-17A may have a pathogenic effect (15). Conversely, we have recently shown that T_h17 cell immunity and IL-17A production may be involved in mediating better protection following intravaginal HSV-2 challenge (16). Evidently, the role of IL-17A in host defence against genital HSV-2 infection remains unresolved, and thereby, is the focus of the current study.

In addition to anti-bacterial and fungal immunity, IL-17 has also been shown to contribute to the generation of efficient T_h1 cell immunity against intracellular pathogens (17-19). In the pulmonary *Mycobacterium tuberculosis* (*M. tuberculosis*) vaccination model, the absence of IL-17 resulted in reduced and delayed IFN-γ responses and

consequently, delayed bacterial clearance (17). Similarly, in IL-17 receptor A deficient mice (IL-17RA), genital tract infection with *Chlamydia muridarum* (*C. muridarum*) also resulted in reduced IFN-γ production (18). Bai et al. (2009) further showed that reduced chlamydia-specific T_h1 cell response was related to impaired dendritic cell (DC) induction of IFN-γ responses upon IL-17A neutralization (19). Parallel to these studies, we recently reported that enhanced T_h1 cell immunity against HSV-2 in the genital tract involves T_h17 cell responses (16). We showed that estradiol treatment in mice, which is known to protect against HSV-2 (20-23), enhanced anti-viral immunity by priming vaginal DCs to induce T_h17 cell responses following HSV-2 immunization. This resulted in increased production of IL-$17A^+$ $CD4^+$ (T_h17) cells in estradiol-treated mice, and coincided with earlier recruitment and increased proportions of IFN-γ^+ $CD4^+$ (T_h1) cells in the genital tract post-challenge (16). This was the first study to suggest that T_h17 responses in the vaginal tract may be augmenting IFN-γ-T_h1 cell immunity. However, it was not determined whether this enhanced anti-viral immunity was due to IL-17A alone, or the result of other cytokines produced by T_h17 cells; thus, emphasizing the importance of examining directly and in more detail, the role of IL-17A in the genital tract model of HSV-2 infection.

In light of these findings, the aim of the present study was to investigate the role and mechanism of IL-17A in regulating IFN-γ-T_h1 cell immunity to genital HSV-2 infection. Using IL-17A deficient mice (*IL-17A*$^{-/-}$), we found no difference in disease severity compared to wild-type (WT) C57BL/6 mice, following primary genital HSV-2 infection. However, following HSV-2 re-exposure, *IL-17A*$^{-/-}$ mice had significantly poorer disease outcome compared to WT mice. To further examine the role of IL-17A in post-

challenge anti-viral responses, *IL-17A*$^{-/-}$ mice were vaccinated intranasally with an attenuated strain of HSV-2 [thymidine kinase deficient (TK^{-})] and demonstrated poor disease outcomes following intravaginal challenge with WT HSV-2. In addition, *IL-17A*$^{-/-}$ mice had significantly higher levels of pro-inflammatory cytokines and chemokines in vaginal secretions. Most importantly, *IL-17A*$^{-/-}$ mice showed impaired T_h1 responses, following both re-exposure and challenge. Upon examination, the attenuated recall responses in *IL-17A*$^{-/-}$ mice were found to correlate with a smaller population of vaginal CD4^{+} tissue-resident memory T cells (T_{RM}) post-vaccination. Our data therefore suggest that IL-17A is critical for the induction of optimal T_h1 cell responses and overall protection against genital HSV-2 infection.

RESULTS

IL-17A is not critical during primary genital HSV-2 infection

We recently showed that enhanced protection against HSV-2 involved greater vaginal T_h17 cell responses, which coincided with increased T_h1 cell responses (16); however, it was not established if the protective effect of T_h17 cell immunity was mediated directly by IL-17A. Therefore, we decided to further investigate, in greater detail, the direct role of IL-17A in mediating HSV-2 anti-viral immunity in the genital tract.

In order to assess if *IL-17A*$^{-/-}$ mice were more susceptible than WT mice to primary intravaginal infection with HSV-2, ovariectomized (OVX) *IL-17A*$^{-/-}$ and WT mice were inoculated with sub-lethal doses of WT HSV-2 virus (10^1, 10^2, 10^3 PFU/mouse). Survival, genital pathology, and viral shedding were monitored daily to determine disease severity and compare susceptibility to infection. We found that following primary genital infection, there were no significant differences in survival between *IL-17A*$^{-/-}$ and WT mice (Fig 1A). Comparable rates of survival corresponded with similar severity of genital pathology between *IL-17A*$^{-/-}$ and WT mice (Fig 1B), and similar numbers of *IL-17A*$^{-/-}$ and WT mice shedding virus post-infection (Fig 1C). Overall, regardless of the infectious dose used, there were no differences in mortality, disease pathology, and viral shedding between *IL-17A*$^{-/-}$ and WT mice, demonstrating that IL-17A does not appear to play a critical role during primary genital HSV-2 infection.

IL-17A contributes to anti-viral responses following genital HSV-2 re-exposure

We then examined whether *IL-17A*$^{-/-}$ mice would show compromised anti-viral responses following HSV-2 re-exposure, as this would determine the role of IL-17A during memory recall responses in the FGT. Previous studies with the intracellular pathogen *M. tuberculosis* have shown that although IL-17 is not critical during primary infection, it is important for augmenting memory responses (17).

Intravaginally pre-exposed *IL-17A*$^{-/-}$ and WT mice (10^2 PFU/mouse) were re-exposed to a higher dose of HSV-2 ($5x10^3$ PFU/mouse), and survival, genital pathology and viral shedding were monitored daily. We found that following intravaginal HSV-2 re-exposure, *IL-17A*$^{-/-}$ mice had poor disease outcomes and demonstrated higher rate of mortality (Fig 2). Post-re-exposure, 78% of WT mice survived compared to only 22% of *IL-17A*$^{-/-}$ mice (Fig 2A), and *IL-17A*$^{-/-}$ mice developed more than twice as high average cumulative genital pathology (*IL-17A*$^{-/-}$: 13.3; WT: 5.8) (Table 1, Fig 2B). We also collected vaginal washes and found that a greater percentage of *IL-17A*$^{-/-}$ mice were shedding virus compared to WT mice (*IL-17A*$^{-/-}$: 89%; WT: 44%) (Fig 2C), and had higher viral shedding in the vaginal tract (p = 0.003) (Fig 2D). We repeated these re-exposure experiments with other viral doses (primary exposure: 10^1 and 10^3 PFU/mouse; re-exposure: $5x10^2$ and $5x10^4$ PFU/mouse) and consistently found decreased protection in *IL-17A*$^{-/-}$ mice (Table 1). In addition to quantifying viral shedding, cytokine and chemokine concentrations were also measured in vaginal secretions. 48 hours following re-exposure, *IL-17A*$^{-/-}$ mice had significantly lower levels of IFN-γ (*IL-17A*$^{-/-}$: 86.45 ± 15.34 pg/mL; WT: 252.11 ± 31.97 pg/mL; p = 0.016) (Fig 2E). Interestingly, levels of pro-inflammatory cytokines and chemokines including IL-6 (p = 0.006), tumor necrosis factor alpha (TNF-

α) (p = 0.038), regulated on activation, normal T-cell expressed and secreted (RANTES) (p = 0.032), monocyte chemoattractant protein 1 (MCP-1) (p = 0.009), macrophage colony-stimulating factor (M-CSF) (p = 0.038), macrophage inflammatory protein 1 alpha (MIP-1α) (p = 0.017) and beta (MIP-1β) (p = 0.017), were significantly higher in *IL-17A*$^{-/-}$ mice compared to WT mice (Fig 2E). These findings showcase significant differences between WT and *IL-17A*$^{-/-}$ mice upon intravaginal HSV-2 re-exposure. In the absence of IL-17A, mice demonstrated higher rates of mortality, more severe genital pathology and greater viral shedding, along with higher concentrations of pro-inflammatory factors and lower levels of IFN-γ in vaginal secretions. Together, these results indicate that IL-17A plays an important role in the anti-viral responses within the genital tract following HSV-2 re-exposure.

IL-17A mediates efficient anti-viral T_h1 responses following genital HSV-2 re-exposure

Next, we sought to better understand why *IL-17A*$^{-/-}$ mice were more susceptible to HSV-2 re-exposure and demonstrated increased mortality (Fig 2). Since IFN-γ is known to play a key role in clearance of HSV-2 and we saw lower levels of IFN-γ in the vaginal secretions of *IL-17A*$^{-/-}$ mice (Fig 2E), we decided to examine the *in vivo* T cell responses in the vaginal tissue in order to quantify IFN-γ production by $CD4^+$ T cells.

Following intravaginal primary infection and re-exposure described above, vaginal tissues and iliac lymph nodes (that drain the genital tract) were collected 3 days later to phenotype and functionally characterize the *in vivo* T cell responses. $CD4^+$ T cells were gated based on total, live $CD3^+$ cells in the vagina (Fig 3A), and isotype controls for IFN-

γ and IL-17A were included (Fig 3B). To examine IL-17A production by $CD4^+$ T cells, vaginal cells were stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and higher proportions of total T_h1 and T_h17 cells were seen in WT mice compared to *IL-17A*$^{-/-}$ mice (Fig 3C), with insignificant IL-17A detected in *IL-17A*$^{-/-}$ mice. Following *in vivo* challenge alone, and in the absence of *in vitro* stimulation, vaginal tissue from *IL-17A*$^{-/-}$ mice contained lower proportions of IFN-γ^+ T_h1 cells (10.6% of $CD4^+$ T cells) compared to WT mice (26.1% of $CD4^+$ T cells) (Fig 3D). Data compiled from multiple independent experiments consistently showed that following re-exposure, *IL-17A*$^{-/-}$ mice had significantly lower IFN-γ production by vaginal $CD4^+$ T cells, compared to WT mice (*IL-17A*$^{-/-}$: 8.42 ± 1.18% of $CD4^+$ T cells; WT: 20.86 ± 3.89% of $CD4^+$ T cells; p = 0.016) (Fig 3E), and significantly lower total number of vaginal IFN-γ^+ $CD4^+$ T cells (*IL-17A*$^{-/-}$: 6304 ± 883; WT: 15624 ± 2912; p = 0.008) (Fig 3E). Notably, this difference was not present in the lymph nodes, where *IL-17A*$^{-/-}$ and WT mice had very similar proportions of IFN-γ^+ $CD4^+$ cells (*IL-17A*$^{-/-}$: 2.32 ± 0.27% of $CD4^+$ T cells; WT: 1.94 ± 0.27% of $CD4^+$ T cells; p = 0.374) and similar total number of IFN-γ^+ $CD4^+$ cells (*IL-17A*$^{-/-}$: 14562 ± 1673; WT: 12194 ± 1678; p = 0.400) (Fig 3F and 3G); thereby suggesting that memory $CD4^+$ T cells, which are readily available to induce IFN-γ production, are compartmentalized in the vaginal tract specifically. Together, these data suggest that the inability of *IL-17A*$^{-/-}$ mice to effectively resolve HSV-2 re-exposure is likely due to impaired local IFN-γ^+ T_h1 cell responses, indicating that IL-17A plays a critical role in mediating efficient anti-viral T_h1 cell immunity in the genital tract.

IL-17A is essential for establishing efficient T_h1 cell responses in the female genital tract following HSV-2 vaccination

Based on the above results which suggested that IL-17A was critical for augmenting T_h1 cell responses (Fig 3), we wanted to examine whether IL-17A would also play a role in enhancing immune responses following HSV-2 vaccination. To test anti-viral immune responses to HSV-2 vaccination, mice were immunized intranasally with an attenuated strain of HSV-2 (TK^- HSV-2), followed by intravaginal challenge with WT HSV-2. Intranasal immunization generates immunity against intravaginal HSV-2 challenge, comparable to local immunization, and has been frequently used by us and others to generate potent immune responses in the genital tract (20, 24-26).

When WT and *IL-17A*$^{-/-}$ mice were immunized intranasally, we found once again, that *IL-17A*$^{-/-}$ mice were more susceptible to intravaginal HSV-2 challenge (Fig 4). There was considerably lower survival in *IL-17A*$^{-/-}$ mice (*IL-17A*$^{-/-}$: 20%; WT: 70%) (Fig 4A), and this coincided with more than twice as high average cumulative genital pathology (*IL-17A*$^{-/-}$: 14.4; WT: 5.2) (Table 2, Fig 4B). There were also more *IL-17A*$^{-/-}$ mice shedding virus (*IL-17A*$^{-/-}$: 80%; WT: 30%) (Fig 4C), with higher viral shedding in the vaginal tract (p = 0.003) (Fig 4D). In vaginal secretions collected post-challenge, *IL-17A*$^{-/-}$ mice had significantly lower levels of IFN-γ (*IL-17A*$^{-/-}$: 129.80 ± 38.94 pg/mL; WT: 438.80 ± 93.77 pg/mL; p = 0.017) (Fig 4E). Levels of other pro-inflammatory cytokines and chemokines (IL-6: p = 0.0006; TNF-α: p = 0.0003; RANTES: p = 0.0002; MCP-1: p = 0.029; M-CSF: p = 0.029; MIP-1α: p = 0.006; MIP-1β: p = 0.0006) were significantly higher in *IL-17A*$^{-/-}$ mice compared to WT mice (Fig 4E). These results confirmed that following intranasal

vaccination, IL-17A plays an important role in the anti-viral immune response post-HSV-2 challenge.

When we examined T cell responses in the vaginal tract post-challenge, there was a clear difference in the T_h1 cell response generated in *IL-17A*$^{-/-}$ mice compared to WT mice. Consistently, there was significantly lower IFN-γ production by $CD4^+$ T cells in the vaginal tissue of immunized *IL-17A*$^{-/-}$ mice post-challenge (*IL-17A*$^{-/-}$: 10.39 ± 1.16% of $CD4^+$ T cells; WT: 26.60± 2.01% of $CD4^+$ T cells; $p = 0.002$) (Fig 5A), and significantly lower total number of vaginal IFN-γ^+ $CD4^+$ T cells in *IL-17A*$^{-/-}$ mice (*IL-17A*$^{-/-}$: 7782 ± 872; WT: 19923 ± 1504; p = 0.002) (Fig 5B). However, there was no difference in the lymph nodes, where the percentage of IFN-γ^+ $CD4^+$ T cells (*IL-17A*$^{-/-}$: 1.53 ± 0.33% of $CD4^+$ T cells; WT: 1.55 ± 0.23% of $CD4^+$ T cells; $p = 0.963$) and the total number of IFN-γ^+ $CD4^+$ T cells (*IL-17A*$^{-/-}$: 9596 ± 2067; WT: 9722 ± 1438; $p = 0.963$) was similar between *IL-17A*$^{-/-}$ and WT mice (Fig 5C and 5D). Taken together, these results suggest that IL-17A is important for mounting a proficient T_h1 recall response in the FGT that can protect against HSV-2 challenge, following vaccination.

The absence of IL-17A is associated with decreased vaginal T_{RM} cells following HSV-2 vaccination

Our results demonstrating more robust $CD4^+$ T cell responses in the vaginal tract compared to lymph nodes (Fig 3 and 5), indicated that the anti-viral immune response observed upon HSV-2 re-exposure/challenge was compartmentalized in the vaginal tract. These findings suggest there is a population of $CD4^+$ T cells generated post-immunization,

which are localized in the vaginal tract and readily available to respond to future HSV-2 exposure. As such, we next focused on examining the tissue-resident memory T cell population (T_{RM}) established in the vaginal tract post-immunization. We wanted to see if the IFN-γ-producing $CD4^+$ cells we had observed were consistent with the phenotype of $CD4^+$ T_{RM} cells, and if absence of IL-17A would affect the induction of this population, thereby explaining the subsequent inefficient T_h1 response generated post-challenge.

Following vaccination with TK^- HSV-2, vaginal cells were isolated, pooled and stimulated to assess IFN-γ production. $CD4^+$ T cells were identified in the vaginal tract and further classified based on previously reported cell-surface markers associated with vaginal T_{RM} populations (27). Following gating strategies previously used in literature, $CD4^+$ T cells were gated based on live, $CD3^+$ cells, followed by gating based on the positive expression of adhesion molecule CD44 and negative expression of the retention marker CD103 ($CD44^+$ $CD103^-$), and finally, gated based on the positive expression of T cell activation and retention molecule CD69 and negative expression of the adhesion molecule CD62L ($CD69^+$ $CD62L^-$). We found that post-immunization, *IL-17A*$^{-/-}$ mice had a smaller population of $CD4^+$ T_{RM} cells ($CD4^+$ $CD44^+$ $CD103^-$ $CD69^+$ $CD62L^-$) compared to WT mice (*IL-17A*$^{-/-}$: 45.0%; WT: 68.7%) (Fig 6A). Data compiled from multiple independent experiments consistently showed that post-immunization, *IL-17A*$^{-/-}$ mice had significantly smaller populations of $CD4^+$ T_{RM} cells compared to WT mice (*IL-17A*$^{-/-}$: 56.53 ± 5.82%; WT: 75.77 ± 3.70%; $p = 0.05$) (Fig 6B), and significantly lower total number of vaginal $CD4^+$ T_{RM} cells (*IL-17A*$^{-/-}$: 21521 ± 2212; WT: 28791 ± 1406; $p = 0.05$) (Fig 6B). Furthermore, when focusing specifically on the population of $CD4^+$ T cells producing IFN-

γ, using the same gating strategy, we found that there was a smaller population of IFN-γ^+ $CD4^+$ T_{RM} cells ($CD4^+$ IFN-γ^+ $CD44^+$ $CD103^-$ $CD69^+$ $CD62L^-$) in the vaginal tract of *IL-17A*$^{-/-}$ mice (*IL-17A*$^{-/-}$: 51.2%; WT: 70.9%) (Fig 6C). This was true across multiple independent experiments, where *IL-17A*$^{-/-}$ mice consistently had significantly lower proportions (*IL-17A*$^{-/-}$: 54.90 ± 2.11%; WT: 75.83 ± 2.52%; p = 0.003) and total cell numbers (*IL-17A*$^{-/-}$: 8862 ± 801; WT: 13149 ± 497; p = 0.01) of IFN-γ^+ $CD4^+$ T_{RM} cells (Fig 6D). Overall, these results suggest that IL-17A helps establish a proficient IFN-γ^+ $CD4^+$ T_{RM} cell population post-immunization, and consequently, mediates efficient anti-viral T_h1 responses post-challenge.

DISCUSSION

The generation of robust IFN-γ-T_h1 cell responses is critical for protective immunity against HSV-2 infection and recent studies suggest that IL-17 potentiates T_h1 immunity, thereby playing an important role in controlling infections with intracellular pathogens (17-19, 28, 29). In the current study, we investigated the role of IL-17A in host defense against genital HSV-2 infection. We found that in the absence of IL-17A, protection against both intravaginal HSV-2 re-exposure following primary genital infection, as well as intravaginal HSV-2 challenge following intranasal immunization, was significantly decreased. This was evident as *IL-17A*$^{-/-}$ mice had higher rates of mortality and viral shedding, and more severe disease pathology, as compared to WT mice. In addition, there were higher levels of inflammatory cytokines and chemokines in the vaginal secretions of *IL-17A*$^{-/-}$ mice. Furthermore, poor disease outcomes in the absence of IL-17A also coincided with lower T_h1 responses compared to WT mice, with significantly less IFN-γ production by vaginal $CD4^+$ T cells following re-exposure, as well as post-challenge in immunized mice. Finally, IL-17A was shown to be important for establishing proficient IFN-γ^+ $CD4^+$ T_{RM} cell populations in the FGT post-immunization. To the best of our knowledge, this is the first study demonstrating that IL-17A plays a critical role in mediating efficient anti-viral T_h1 responses in the female genital tract, thereby improving HSV-2 vaccination efficacy.

The function of IL-17A, a cytokine primarily produced by T_h17 cells, in reproductive tract infections initiated by exposure to fungal and bacterial pathogens has been reported by several studies. IL-17A can help control overgrowth of the fungus *C.*

albicans by upregulating pro-inflammatory cytokines, anti-microbial peptides, and recruiting neutrophils through the secretion of neutrophil-recruiting chemokines (10, 30). In addition, T_h17 deficiency is associated with chronic candidiasis (31). Similarly, IL-17A also plays a protective role in defence against bacterial STIs. During murine genital tract infection with *N. gonorrhoeae*, IL-17A is important for the recruitment of neutrophils and resulting clearance of infection (11), while the absence of IL-17A leads to prolonged infection (7). However, the role of IL-17A during viral infections in the genital tract has not been well described. A study by Kim et al. (2012) reported that the death of *IL-17A*$^{-/-}$ mice was significantly delayed following intravaginal infection with HSV-2 compared to WT mice, suggesting that IL-17A-producing TCR $\gamma\delta^+$ $CD4^-$ $CD8^-$ T cells play a pathogenic role during HSV-2 infection (15). However, this study only examined the role of IL-17A in the context of a primary infection with a lethal dose of virus, focusing on IL-17A$^+$ TCR$\gamma\delta^+$ T cells; an approach starkly different than the one used in the current study. Here, we examined the role of IL-17A in closer detail following HSV-2 challenge as opposed to only after primary infection, as *IL-17A*$^{-/-}$ mice showed no differences in disease outcome compared to WT mice following primary infection with non-lethal doses of virus (Fig 1). However, following intravaginal challenge, *IL-17A*$^{-/-}$ mice consistently demonstrated greater mortality and genital pathology, along with higher viral shedding compared to WT mice (Fig 2 and 4; Table 1 and 2). Studies examining the role of IL-17 during infection with other intracellular pathogens such as *M. tuberculosis* show similar results, where although IL-17 is dispensable during the primary infection, it appears to play a critical role in inducing immune responses following vaccination (17, 28). Similar to the role of IL-17

in the *M. tuberculosis* model, our results also show that IL-17 is critical for inducing efficient immune response to HSV-2 post-immunization.

In addition, the study by Kim et al. used a lethal dose of HSV-2 (15) and it is possible that this may mask any effect that IL-17A has in terms of the efficiency in the immune response generated. When administered such a high dose of virus, mice may be able to compensate for the lack IL-17A by producing greater amounts of other protective cytokines and immune factors, making it difficult to assess the efficiency of the immune response generated. Interestingly, we observed significantly increased pro-inflammatory cytokine and chemokine production in vaginal washes collected from *IL-17A*$^{-/-}$ mice, even while using lower viral doses (Fig 2D and 4D). It is possible the increased production of pro-inflammatory cytokines in the absence of IL-17A is a compensatory mechanism which is implemented to help protect the mice against HSV-2 infection, and using even higher viral doses further amplifies this compensatory effect. Similarly, a study examining the role of IL-17 during genital *C. muridarum* infection found that IL-17RA KO mice exhibited increased TNF-α production, which acted as a compensatory mechanism to effectively control chlamydial genital infection (18). Furthermore, other studies including that by Kim et al. utilize medroxyprogesterone acetate (MPA) to make mice susceptible to HSV-2. MPA is an injectable progestin-based formulation that thins the vaginal epithelium and makes mice susceptible to infection. Several studies including our own have shown that MPA can significantly dampen mucosal anti-viral responses to HSV-2 (32, 33). Specifically, prolonged exposure to MPA has been shown to decrease levels of HSV-2-specific mucosal immune responses following intravaginal immunization. Therefore, the

use of MPA may have impacted the results of the study by Kim et al., resulting in an inaccurate assessment of the role of IL-17A during HSV-2 infection. To avoid compromised immune responses as a result of MPA treatment and the presence of endogenous hormones, we use an alternative model in which mice undergo an ovariectomy surgery in order to remove the source of endogenous hormones altogether. This procedure naturally thins the vaginal epithelium, making the mice susceptible to intravaginal HSV-2 infection. This allows us to study anti-viral immune responses in the female genital tract under no hormone or clearly defined hormonal conditions, and is the technique that was used in the present study.

Several studies show that IL-17 potentiates T_h1 cell immune responses and plays a critical role in controlling infections with intracellular pathogens (17-19, 28, 29). The contribution of IL-17 to the development of T_h1 cell immunity against intracellular pathogens has been shown during infection with pulmonary *M. tuberculosis*, *Francisella tularensis* (*F. tularensis*) and *C. muridarum*, as well as genital infection with *C. muridarum*. Khader et al. (2007) reported using the pulmonary *M. tuberculosis* vaccination model, where like anti-HSV-2 immunity, IFN-γ^+ $CD4^+$ T cell responses play a key role in generating effective immunity, that T_h17 responses induced in the lung following immunization play a key role in accelerating T_h1 cell responses (17). This resulted in the early resolution of bacteria post-challenge, and in the absence of IL-17, IFN-γ responses and clearance of the bacteria were significantly delayed. Similarly, a study examining the role of T_h17 cell immunity in host resistance to the intracellular bacterium *F. tularensis* showed that T_h1 cell immunity was compromised in the absence of IL-17A (29). In

addition, studies looking at chlamydial infection found that IL-17A is important for the development of T_h1 responses in both the lung and genital tract. Within the vaginal tract, Scurlock et al. (2011) found that IL-17RA mice demonstrated reduced IFN-γ production following genital *C. muridarum* infection (18). Furthermore, Bai et al. (2009) reported that mice treated with anti-IL-17A antibodies showed significantly delayed clearance of *C. muridarum* and increased disease severity in the lung. This corresponded with significantly reduced chlamydia-specific T_h1 responses and was related to impaired DC induction of IFN-γ responses in the absence of IL-17A (19). Parallel to these studies, we recently showed that IL-17A in the vaginal tract may be augmenting anti-viral IFN-γ-T_h1 cell responses in the vaginal tract (16). We found that HSV-2 vaccination under the influence of female sex hormone estradiol resulted in increased production of IL-17A by $CD4^+$ cells, which coincided with earlier recruitment and increased proportions of vaginal IFN-γ^+ $CD4^+$ cells post-challenge (16); however, it was not established if IL-17A was directly responsible for the increase in IFN-γ. Interestingly, similar to what was shown by Bai et al. (2009) in the *C. muridarum* model, we also found in *in vitro* co-cultures conducted with vaginal cells from *IL-17A*$^{-/-}$ mice and ovalbumin (OVA)-specific OT-II transgenic $CD4^+$ T cells, vaginal antigen presenting cells (APCs) from *IL-17A*$^{-/-}$ mice were significantly impaired at inducing IFN-γ production compared to vaginal APCs from WT mice; suggesting there is an intrinsic impairment in the priming of T_h1 cell responses by vaginal DCs in *IL-17A*$^{-/-}$ mice (16).

The present study advances our understanding regarding the *in vivo* role of IL-17A, by focusing on the ability of *IL-17A*$^{-/-}$ mice to initiate an IFN-γ^+ $CD4^+$ T cell response *in*

vivo in the context of HSV-2 infection. To assess the differentiation of $CD4^+$ T cells into IFN-γ^+ T_h1 cells, vaginal cells were isolated following *in vivo* HSV-2 challenge, and left unstimulated to measure spontaneous cytokine production. We have previously shown that cytokine production, including IFN-γ, by vaginal $CD4^+$ T cells is comparable between cells which have been stimulated with UV-inactivated HSV-2 *in vitro* post-challenge, and cells grown in culture following *in vivo* HSV-2 challenge, without further *in vitro* antigen challenge (34). This is likely because at three days post *in vivo* challenge, T cells in the vaginal tract are already activated. Therefore, we consider T cell responses following *in vivo* challenge to be good surrogate measures of antigen-specific T cell responses. Similar to the reported *in vitro* results regarding impaired T cell priming in the absence of IL-17A (16), we show that there is a significant decrease in IFN-γ production by vaginal $CD4^+$ T cells *in vivo* following HSV-2 challenge (Fig 3 and 5). Evidently, lower IFN-γ production in the absence of IL-17A is enough to critically impact survival and disease outcomes, as having an approximately two-fold lower IFN-γ^+ $CD4^+$ T cell responses in the vaginal tract post-re-exposure (Fig 3) was sufficient to lower survival from 78% seen in WT mice, to 22% seen in *IL-17A*$^{-/-}$ mice (Fig 2). Comparable trends were also seen in vaccinated *IL-17A*$^{-/-}$ mice, as a loss in IFN-γ^+ $CD4^+$ T cell efficiency post-challenge (Fig 5), resulted in survival decreasing from 70% in WT mice to only 20% in *IL-17A*$^{-/-}$ mice (Fig 4). Since IFN-γ plays a critical role in host protection against HSV-2 in the mouse model of infection, decreased IFN-γ production in the vaginal tract can help explain the inability of *IL-17A*$^{-/-}$ mice to overcome HSV-2 challenge.

Our findings suggest that IL-17A plays an in important role in mediating efficient anti-viral immune responses in the vaginal tract following HSV-2 vaccination, and based on previous work, this appears to be linked to a defect in the priming of T_h1 cell responses in the absence of IL-17A (16). Interestingly, IL-17A also been shown to play a role in augmenting long-lasting memory responses in the lung (17). In the current study, we found that the protective IFN-γ^+ $CD4^+$ T cell response was compartmentalized in the vaginal tract, and that in the absence of IL-17A, there was an impaired IFN-γ response in vaccinated mice post-challenge (Fig 3 and 5). To better understand how IL-17A may be involved in generating efficient recall responses, we examined the repertoire of T cells in the vaginal tracts of *IL-17A*$^{-/-}$ and WT mice following HSV-2 vaccination, in order to compare the phenotype and functional characteristics of the T cells which localize to the vaginal tract post-immunization. Specifically, we looked at $CD4^+$ T_{RM} cells, a newly described subset of memory T cells, to examine if IL-17A is involved in establishing long-lasting, efficient tissue-resident populations. T_{RM} cells are clonally expanded memory T cells which enter peripheral tissues, where they are retained long-term and are able to survive without depending on replenishment from circulating T cells (35). $CD8^+$ and $CD4^+$ T_{RM} cells established in the vaginal tract post-immunization have been shown to be involved in mediating better protection against HSV-2 challenge compared to circulating memory T cells on their own (27, 36), however, this is a new area of research in the field, and very little is known about these populations in the genital mucosa. In particular, it has been shown that vaginal $CD4^+$ T_{RM} cells are established in extremely low numbers, making them especially difficult to detect and characterize. For this reason, post-immunization, we

stimulated our cells *in vitro* with a cell stimulation cocktail including PMA and ionomycin in order to assess maximum IFN-γ production by $CD4^+$ T_{RM} cells. Although this stimulation process may have resulted in T cell responses which are not entirely HSV-2-specific, it allowed us to measure the maximum ability of the vaginal cells to induce cytokine responses, which closely mimic those produced in response to HSV-2. Based on the limited published data, we utilized markers (CD44, CD103, CD69, CD62L) which have been shown to identify tissue-resident memory populations specifically in the vaginal tract (27, 36). CD44 is a cell adhesion molecule and cell activation marker that is highly expressed by T_{RM} cells in most mucosal tissues. In addition, CD69 is another activation marker found to be expressed by T_{RM} cells and is thought to play a role in T_{RM} cell retention within periphery tissues, while CD62L is a lymph node homing receptor that is expressed by central memory T cells and not T_{RM} cells. Unlike other mucosal tissues, vaginal $CD4^+$ T_{RM} cells do not highly express the conventional memory and mucosal homing marker, CD103, as CD103 expression was shown to be detected in only 10% of $CD4^+$ T_{RM} cells in the vagina following infection with TK^- HSV-2 (27). We thereby characterized $CD4^+$ T_{RM} cells as follows: $CD4^+$ $CD44^+$ $CD103^-$ $CD69^+$ $CD62L^-$. Interestingly, we found that following HSV-2 vaccination, there was a smaller population of $CD4^+$ T_{RM} cells in the vaginal tract of *IL-17A*$^{-/-}$ mice, as compared to WT mice (Fig 6A and 6B). Furthermore, the population of vaginal IFN-γ^+ $CD4^+$ T_{RM} cells was also smaller in *IL-17A*$^{-/-}$ mice (Fig 6C and 6C). This suggests that in the absence of IL-17A, mice are also impaired in their ability to generate a proficient pool of local, vaginal $CD4^+$ T_{RM} cells following HSV-2 vaccination. Since it has been shown that T_{RM} cells are the primary source of IFN-γ early on during HSV-2 challenge

(27), this can help explain why *IL-17A*$^{-/-}$ mice have a less efficient T-cell response upon subsequent HSV-2 exposure. However, with the limited knowledge available regarding the phenotype and the functional characteristics of vaginal CD4$^+$ T_{RM} cells, further work is ongoing to better understand the importance of this T cell population and how IL-17A may be involved in the establishment of these tissue memory cells. In ongoing studies, we are optimizing a model using transgenic mice expressing CD4$^+$ T cell receptors specific for the HSV-2 glycoprotein-D-derived epitope, which will allow us to better study HSV-2-specific CD4$^+$ T cell responses and bypass the use of stimulatory markers, which may be influencing the expression of our T_{RM} cell markers.

In summary, our study provides insight regarding a novel anti-viral role for IL-17A in the genital tract, in which IL-17A improves mucosal vaccination efficacy by mediating efficient T_h1 cell immunity. We show for the first time, that IL-17A contributes significantly to the generation of protective anti-viral T_h1 cell immunity in the genital tract. In the absence of IL-17A, there is a compromised anti-viral T_h1 cell response following intravaginal HSV-2 re-exposure and challenge, resulting in poor disease outcomes. This has important implications in terms of HSV-2 vaccine development, as a hallmark of an effective vaccination strategy is the ability to generate a rapid, robust local effector response, and we propose that HSV-2 vaccine strategies which induce IL-17A production in the genital tract may promote more effective T_h1 cell immunity.

MATERIALS AND METHODS

Animals

6-8 week-old female C57BL/6 mice were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada). *IL-17A*$^{-/-}$ mice (C57BL/6 background) were generated by Dr. Yoichiro Iwakura (University of Tokyo, Tokyo, Japan) (37) and bred internally in the Central Animal Facility at McMaster University. All mice were maintained under specific pathogen-free (SPF) and standard temperature controlled conditions that followed a 12-hour light/dark cycle, and given low-fat mouse chow. Routine quality assurance was done by serology and PCR to ensure mice remained SPF, and included testing dirty bedding sentinels, direct resident animals and exhaust air duct samples of racks. All animal studies performed were approved by, and in compliance with, the Animal Research Ethics Board (AREB) at McMaster University.

Ovariectomy surgeries

Endogenous hormones in mice were depleted by ovariectomies (OVX). Prior to OVX, mice were administrated analgesic (Temgesic) subcutaneously, and after thirty minutes, received an injectable anesthetic preparation of Ketamine and Xylazine intraperitoneally. Ovaries were removed by making two bilateral incisions, followed by small incisions through the peritoneal wall, and excised through the incisions. Incisions were closed using surgical clips and mice recovered for 10-14 days before the start of experiments.

Viral infection

For primary HSV-2 infection, OVX mice were anaesthetized intraperitoneally and intravaginally infected with 10 µl of WT HSV-2 strain 333 (10^1, 10^2 or 10^3 PFU/mouse). After inoculation, mice were placed on their backs for approximately 30-45 minutes to allow for the inoculum to infect the vaginal tract. For re-exposure experiments, mice infected with 10^2 PFU of WT HSV-2, were re-exposed intravaginally 6 weeks later with a higher dose of WT HSV-2 ($5x10^3$ PFU/mouse). For intranasal immunization experiments, OVX mice were anaesthetized using isoflurane. The mice were then immunized intranasally with 5 µl of thymidine kinase-deficient (TK^-) HSV-2 strain 333 (10^2 PFU/mouse) into each nare with a micropipette, for a total of 10 ul. Six weeks following intranasal immunization, mice were challenged intravaginally with WT HSV-2 ($5x10^3$ PFU/mouse). To examine T_{RM} cell populations following vaccination, mice were immunized intravaginally as described above, with TK^- HSV-2 strain 333 (10^4 PFU/mouse).

Collection of vaginal washes

Vaginal washes were collected daily for up to 6 consecutive days post-HSV-2 re-exposure or challenge by pipetting 30 µl of phosphate-buffered saline (PBS) into the vagina 5-6 times. This was repeated to give a total volume of approximately 60 µl and stored at -70ºC until use.

Genital pathology

Following infection with HSV-2, genital pathology was monitored daily and scored on a 5-point scale: 0, no infection; 1, slight redness of external vagina; 2, swelling and

redness of external vagina; 3, severe swelling and redness of both vagina and surrounding tissue and hair loss in genital area; 4, genital ulceration with severe redness; 5, severe genital ulceration extending to surrounding tissue. Animals were euthanized by cervical dislocation when they reached stage 4/5. To compare groups, cumulative pathology scores were determined by tabulating the number of mice with the highest score of pathology they achieved and the number of days that score was observed. Mice that did not survive the challenge were given the highest pathology score for the duration of the experiment to accurately reflect overall pathology for each group. The sum of these scores for all the mice was the total level of pathology for each group and the average pathology score per mouse for each group was then calculated.

Viral titration

Viral titers in vaginal washes were determined by viral plaque assay on Vero cell (ATCC, Manassas, USA) monolayers. Vero cells were grown in supplemented α-minimal essential medium (α-MEM) (GIBCO Laboratories, Burlington, Canada) supplemented with 5% fetal bovine serum (FBS) (GIBCO, Burlington, Canada), 1% penicillin-streptomycin (Invitrogen, Burlington, Canada), L-glutamate (BioShop Canada Inc., Burlington, Canada), and 1% HEPES (Invitrogen, Burlington, Canada). For plaque assays, Vero cells were grown to confluence in 12-well plates. Vaginal lavages were removed from -70°C and thawed on ice. Samples were diluted (10^{-2} to 10^{-7}) in α-MEM and added to monolayers. Infected monolayers were incubated at 37°C for 2 hours and were rocked every 15 minutes to facilitate viral absorption. Infected monolayers were then overlaid with α-MEM to stop viral adsorption. Infection was allowed to occur for 48 hours at 37°C.

Monolayers were then fixed and stained with crystal violet, and viral plaques were counted under a light microscope. The number of PFU/mL were calculated by taking a plaque count for every sample and accounting for the dilution factors.

Multiplex cytokine and chemokine assay

Vaginal washes collected from mice post-re-exposure and post-challenge were analyzed for cytokines and chemokines, using the 31-Plex Mouse Cytokine/Chemokine Discovery Luminex Assay from Eve Technologies (Calgary, Canada), as per the manufacturer's protocol.

Single-cell preparation and cultures

Three days post-challenge, iliac lymph nodes (LN), which drain the genital tract, were removed and a single cell suspension was prepared by mechanically disrupting the tissues. Debris was allowed to settle, and the supernatant containing single cells was collected and centrifuged for 5 minutes (1500 rpm) at 4°C. Cells then were re-suspended in 1 mL of RPMI 1640 media supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% L-glutamine, 0.1% 2-mercaptoethanol, 1x non-essential amino acids and 1x sodium pyruvate (Gibco Life Technologies, Burlington, ON, Canada). Finally, mononuclear cells were counted and cell preparations were seeded in a 24-well plate at $3x10^6$ cells/mL, in previously described supplemented RPMI 1640 media.

Vaginal tracts were removed, pooled, cut lengthwise, washed to remove mucous, and minced into 2-4 mm pieces. The 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. Following the first digestion, supernatants were collected, and the tissues were digested for a second time in another 15 mL of collagenase A in RPMI for 1 hour. At the end of the second digestion, supernatants were collected. The remaining tissue was passed through a 40 μm filter (Small Parts, Miami Lakes, USA), and then all collected supernatants were passed through a 40 μm filter into a 50 mL Falcon tube. Vaginal cell samples were then centrifuged for 10 minutes (1200 rpm) at 4°C. Cells were re-suspended in 1 mL of previously described supplemented RPMI 1640 medium, mononuclear cells were counted, and cell preparations were seeded in a 24-well plate at a density of $5x10^5$-$1x10^6$ cells/well in previously described supplemented RPMI 1640 media. Cells were either left unstimulated (brefeldin A and monensin) or underwent *in vitro* stimulation with 2μl/mL cell stimulation cocktail (CSC) plus protein transport inhibitors (500X) [cocktail of PMA, ionomycin, brefeldin A and monensin (eBioscience, San Diego, CA, USA)] for 16 hours at 37°C.

Flow cytometry

Following 16 hours of incubation at 37°C, with or without CSC, cells were collected and stained with allophycocyanin (APC)-ef780 viability dye (eBioscience) for 30 minutes. Cells were washed and incubated for 5-10 minutes with 2 ul of Fc block (anti-mouse CD16/32; eBioscience) to reduce nonspecific Fc receptor staining. Cells were then stained for cell surface markers using the following antibodies at concentrations based on manufacturer specification sheets: peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD4; phycoerythrin (PE)-CF594-labeled hamster anti-mouse CD3; Brilliant Violet 510 (BV510)-labeled rat anti-mouse CD103; Brilliant Blue 515 (BB515)-labeled rat anti-

mouse CD62L; PE-labeled hamster anti-mouse CD69 (BD Biosciences, Mississauga, ON, Canada); BV421-labeled anti-mouse CD4; Alexa Fluor 700 (AF700)-labeled anti-mouse CD44 (BioLegend, San Diego, CA, USA). Cells were incubated with these antibodies for 30 minutes, and then permeabilized and fixed using the Transcription Factor Buffer Set (BD Biosciences) following manufacturer's protocol. Cells were then stained for intracellular markers using the following antibodies: BV421- and fluorescein isothiocyanate (FITC)-labeled rat anti-mouse IFN-γ or BV421- and FITC-labeled rat IgG1 isotype control (BD Biosciences), and APC-labeled anti-mouse IL-17A or APC-labeled rat IgG2 isotype control (eBioscience). The validity of intracellular staining was verified by fluorescence minus one (FMO) controls, and/or appropriate isotype controls. Data was collected by flow cytometric analysis using a BD LSRII Flow Cytometer System (BD Bioscience Pharmigen) and results were analyzed using FlowJo software.

Statistical analysis

Statistical analysis and graphical representation was performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). The Mantel-Cox log-rank test was used to calculate significant differences in survival. Differences between the groups were identified using the unpaired, non-parametric, two-tailed t-test and multiple comparison Mann-Whitney test with 95% confidence interval, with the ROUT method used to identify outliers. The Bonferroni correction was used to correct for multiple measures in the cytokine analysis. Data are expressed as mean ± standard error of mean. Statistical significance was defined as *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

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FIGURES AND TABLES

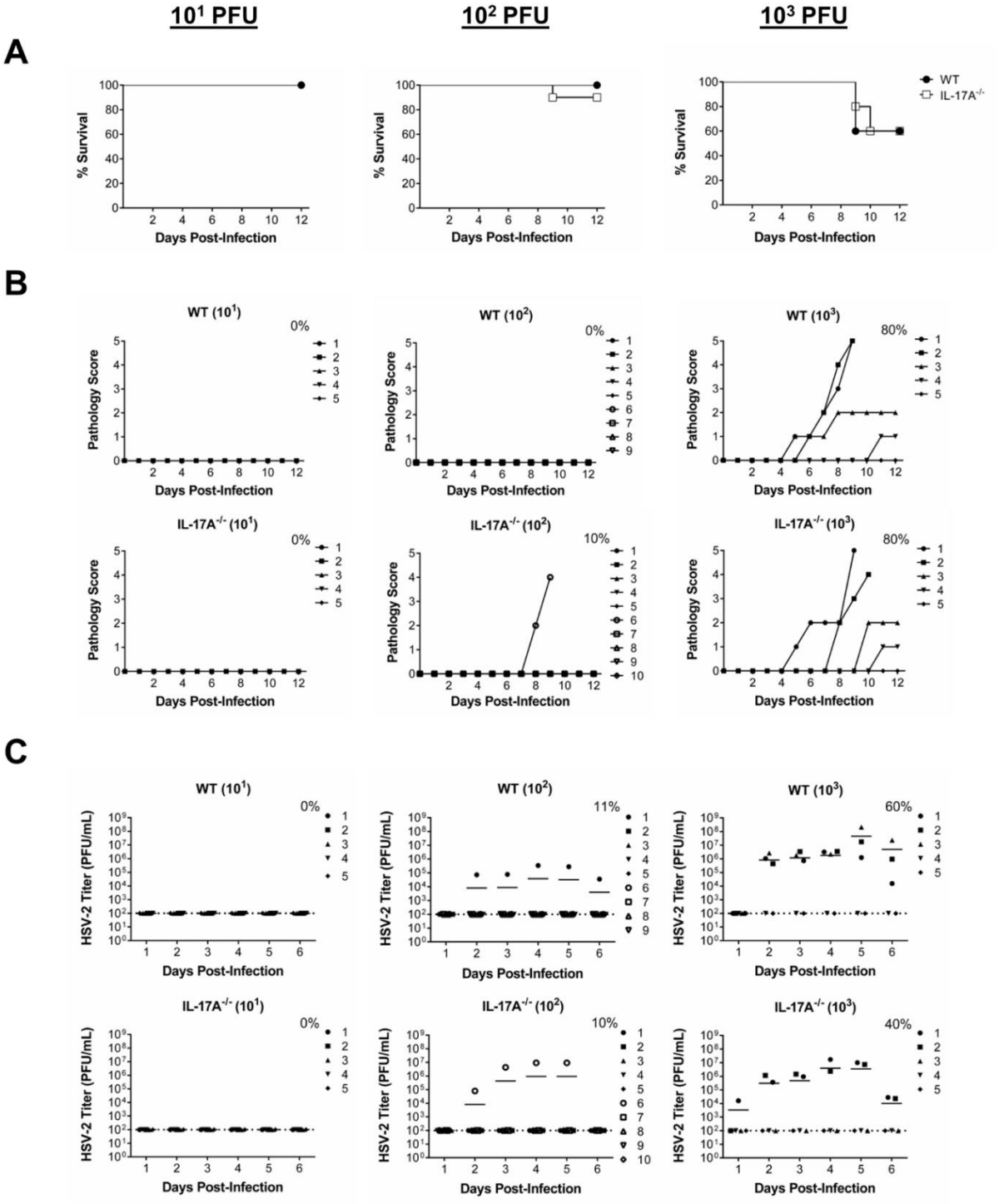

Figure 1: *IL-17A*$^{-/-}$ mice demonstrate no significant difference in susceptibility to primary intravaginal HSV-2 infection. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=5-10/group) were infected intravaginally with sub-lethal doses of WT HSV-2 (10^1, 10^2 or 10^3 PFU/mouse). Survival was monitored (A) and pathology scores were recorded on a scale of 0 to 5 (B) for 12 days post-infection. Data points superimposed on the x axes of panel B indicate mice without genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. (C) Vaginal washes were collected daily for 6 days post-infection and HSV-2 shedding was assessed using a Vero cell-based assay. The bars in panel C indicate mean PFU per milliliter of shed virus. The dotted lines in panel C indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. The percentages in panel C represent maximum numbers of mice that shed virus on any given day. Each symbol represents a single animal. The results are representative of those from two independent experiments.

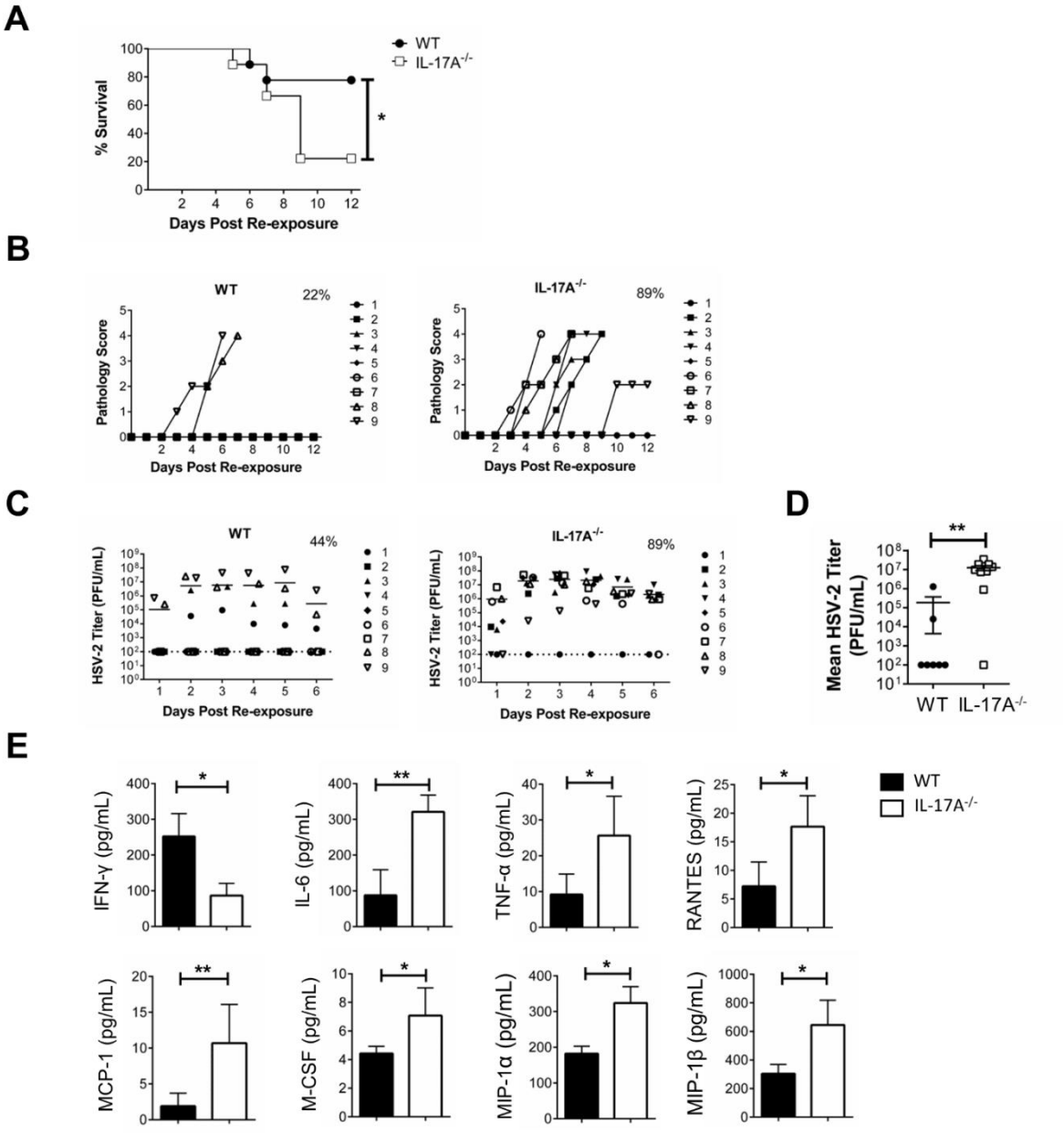

Figure 2: Pre-exposed *IL-17A*$^{-/-}$ mice are more susceptible to intravaginal HSV-2 re-exposure. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=9/group) were intravaginally exposed to WT HSV-2 (10^2 PFU/mouse), and 6 weeks later, re-exposed intravaginally with a higher dose of WT HSV-2 ($5x10^3$ PFU/mouse). Survival was monitored (A) and pathology scores were recorded on a scale of 0 to 5 (B) for 12 days after re-exposure. Significance in difference in survival (A) was calculated using the log rank (Mantel-Cox) test (*, $P < 0.05$). Data points superimposed on the x axes of panel B indicate mice without

genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. Vaginal washes were collected daily for 6 days after re-exposure; HSV-2 viral shedding was calculated using a Vero cell-based assay (C and D), and cytokine and chemokine (IFN-γ, IL-6, TNF-α, RANTES, MCP-1, M-CSF, MIP-1α and MIP-1β) concentrations were measured by multi-analyte assays (E). The bars in panel C indicate mean PFU per milliliter of shed virus. The dotted lines in panel C indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. The percentages in panel C represent maximum numbers of mice that shed virus on any given day. Data shown in panel D represent the viral loads (means ± SEMs) over 6 days. Each symbol represents a single animal. Data shown in panel E represent the means ± SEMs from two independent experiments, done in duplicate (n=4-7/group). Data were analyzed using the unpaired, non-parametric, two-tailed Mann-Whitney test with 95% confidence interval, with the ROUT method used to identify outliers and the Bonferroni correction used to correct for multiple measures. *, $P < 0.05$; **, $P < 0.01$.

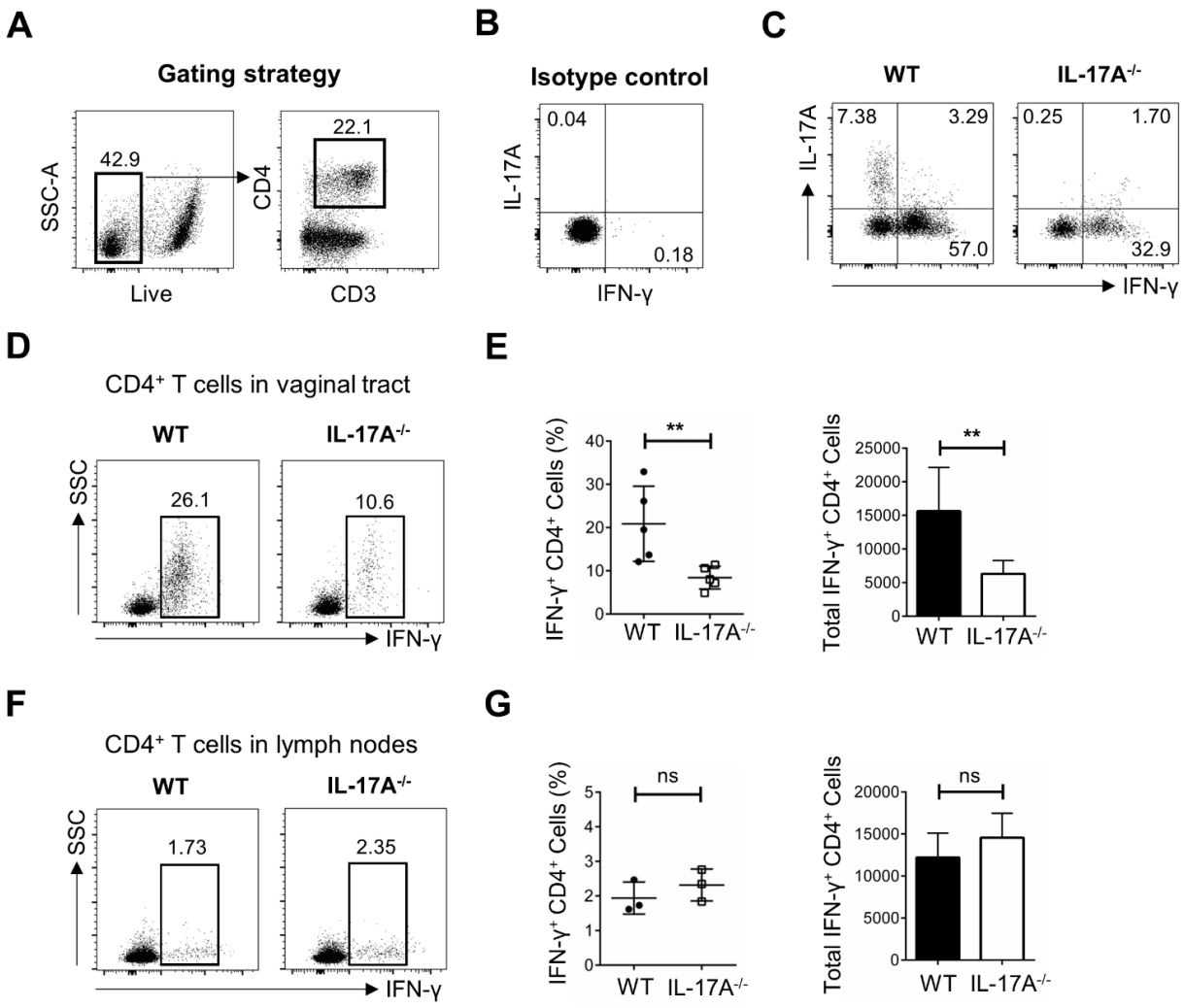

Figure 3: Pre-exposed *IL-17A*$^{-/-}$ mice have lower proportions of IFN-γ$^+$ T$_h$1 cells in the vaginal tract following intravaginal HSV-2 re-exposure. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=4-6/group) were intravaginally exposed to WT HSV-2 (10^2 PFU/mouse), and 6 weeks later, re-exposed intravaginally with a higher dose of WT HSV-2 ($5x10^3$ PFU/mouse). Vaginal tissue and lymph nodes were isolated at day 3 following re-exposure, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. (A) CD4$^+$ T cells were gated among total live CD3$^+$ T cells in the vaginal tissue. (B) Isotype controls for intracellular staining of IL-17A and IFN-γ. (C) Vaginal cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) containing golgi inhibitors and PMA + ionomycin for 16h to detect intracellular staining of IL-17A and IFN-γ. (D) Intracellular staining for IFN-γ was used to examine the *in vivo* response to HSV-2 (without *in vitro* stimulation), and the differentiation of CD4$^+$ T cells into T$_h$1 cells in the vaginal tract. (E) The differences in percentages and total cell number of IFN-γ-producing CD4$^+$ T cells after

HSV-2 re-exposure in the vaginal tract were compared across five independent experiments (n=4-6/group). (F and G) Intracellular staining for IFN-γ (F) and the differences in percentages and total cell number of IFN-γ-producing $CD4^+$ T cells after HSV-2 re-exposure in the lymph nodes (G) was compared across three independent experiments (n=5-6/group). Data shown in panels E and G represent means ± SEMs. Significant difference in IFN-γ expression was calculated using the unpaired, two-tailed t-test with 95% confidence interval. **, $P < 0.01$.

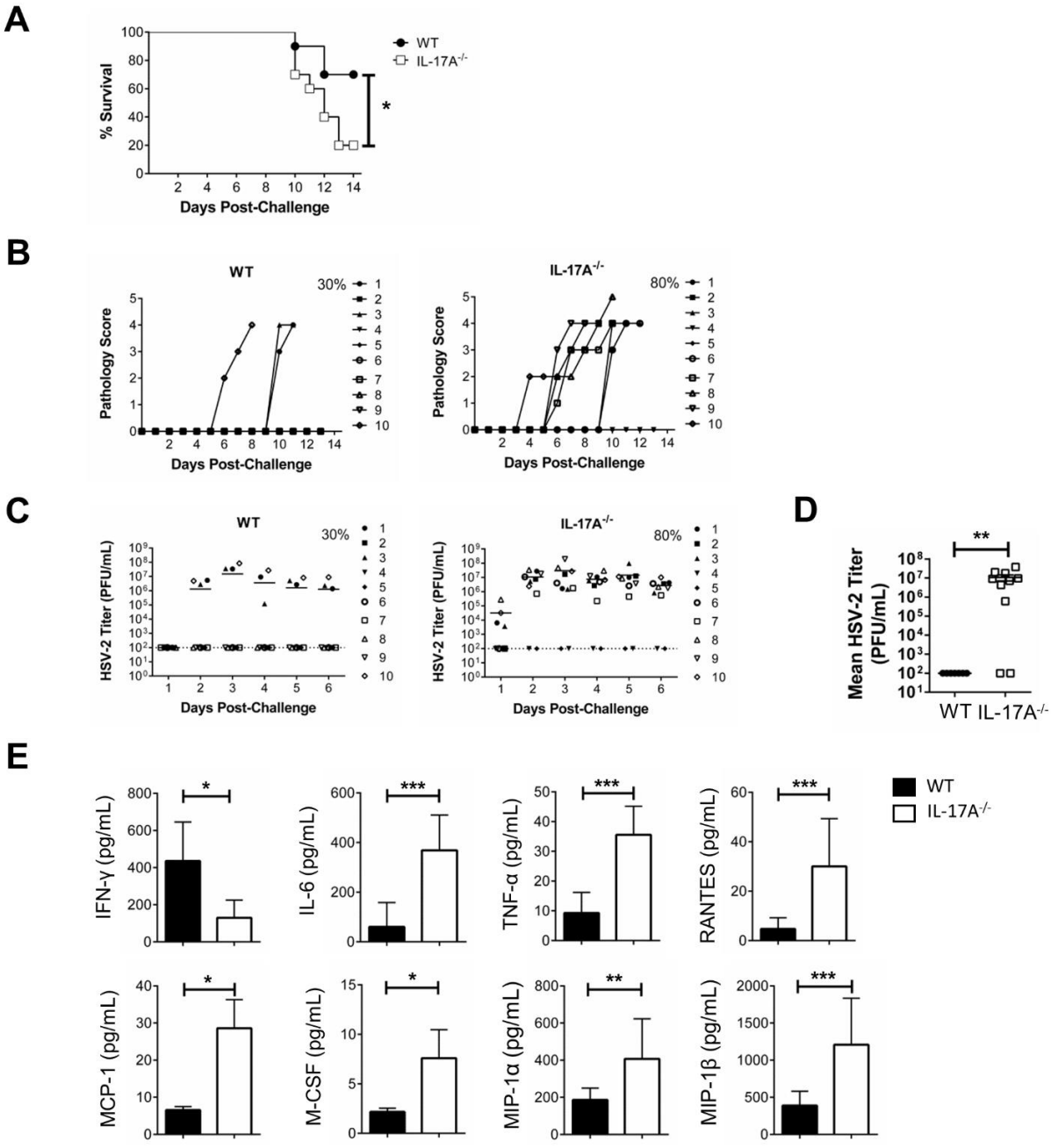

Figure 4: Intranasally immunized *IL-17A*$^{-/-}$ mice are more susceptible to intravaginal HSV-2 challenge. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=10/group) were immunized intranasally with TK^{-} HSV-2 (10^2 PFU/mouse), and 6 weeks later, challenged intravaginally with WT HSV-2 ($5x10^3$ PFU/mouse). Survival was monitored (A) and pathology scores were recorded on a scale of 0 to 5 (B) for 14 days post-exposure. Significance in difference

in survival (A) was calculated using the log rank (Mantel-Cox) test (*, $P < 0.05$). Data points superimposed on the x axes of panel B indicate mice without genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. Vaginal washes were collected daily for 6 days after re-exposure; HSV-2 viral shedding was calculated using a Vero cell-based assay (C and D), and cytokine and chemokine (IFN-γ, IL-6, TNF-α, RANTES, MCP-1, M-CSF, MIP-1α and MIP-1β) concentrations were measured by multi-analyte assays (E). The bars in panel C indicate mean PFU per milliliter of shed virus. The dotted lines in panel C indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. The percentages in panel C represent maximum numbers of mice that shed virus on any given day. Data shown in panel D represent the viral loads (means ± SEMs) over 6 days. Each symbol represents a single animal. Data shown in panel E represent the means ± SEMs from two independent experiments, done in duplicate (n=4-7/group). Data were analyzed using the unpaired, non-parametric, two-tailed Mann-Whitney test with 95% confidence interval, with the ROUT method used to identify outliers and the Bonferroni correction used to correct for multiple measures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

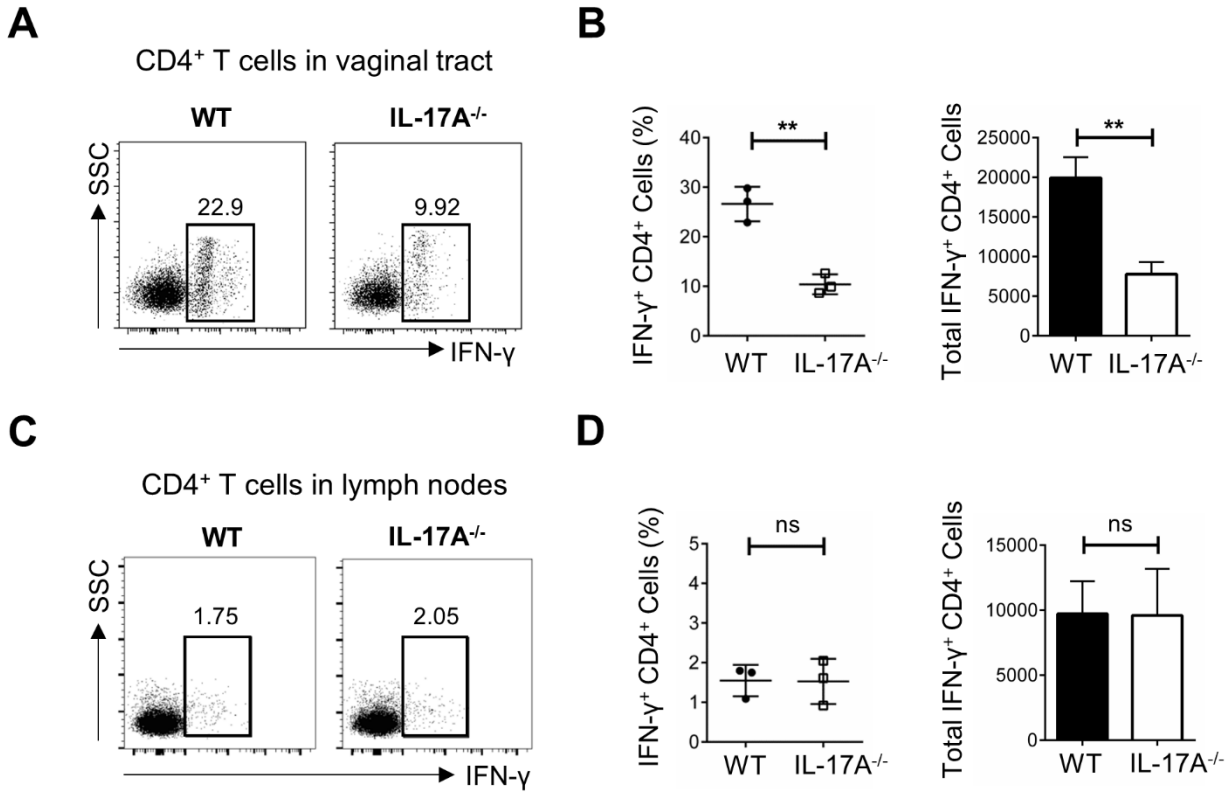

Figure 5: Intranasally immunized *IL-17A*$^{-/-}$ mice have lower proportions of IFN-γ$^+$ T$_h$1 cells in the vaginal tract following intravaginal HSV-2 challenge. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=4-6/group) were immunized intranasally with TK$^-$ HSV-2 (10^2 PFU/mouse), and 6 weeks later, challenged intravaginally with WT HSV-2 ($5x10^3$ PFU/mouse). Vaginal tissues and lymph nodes were isolated at day 3 post-challenge, pooled, processed, stained with a panel of antibodies, and examined by flow cytometry. Intracellular staining for IFN-γ was used to examine the *in vivo* response to HSV-2. (A and B) The differentiation of CD4$^+$ T cells into T$_h$1 cells in the vaginal tract (A) and the differences in percentages and total numbers of IFN-γ-producing CD4$^+$ T cells post-HSV-2 challenge in the vaginal tract (B) were compared across three independent experiments (n=4-6/group). (C and D) The differentiation of CD4$^+$ T cells into T$_h$1 (C) and the differences in percentages and total numbers of IFN-γ-producing CD4$^+$ T cells after HSV-2 challenge in the lymph nodes were compared across three independent experiments (n=4-6/group). Data shown in panels B and D represent means ± SEM. Significant differences in IFN-γ expression was calculated using the unpaired, two-tailed t-test with 95% confidence interval. **, $P < 0.01$. ns, no significance.

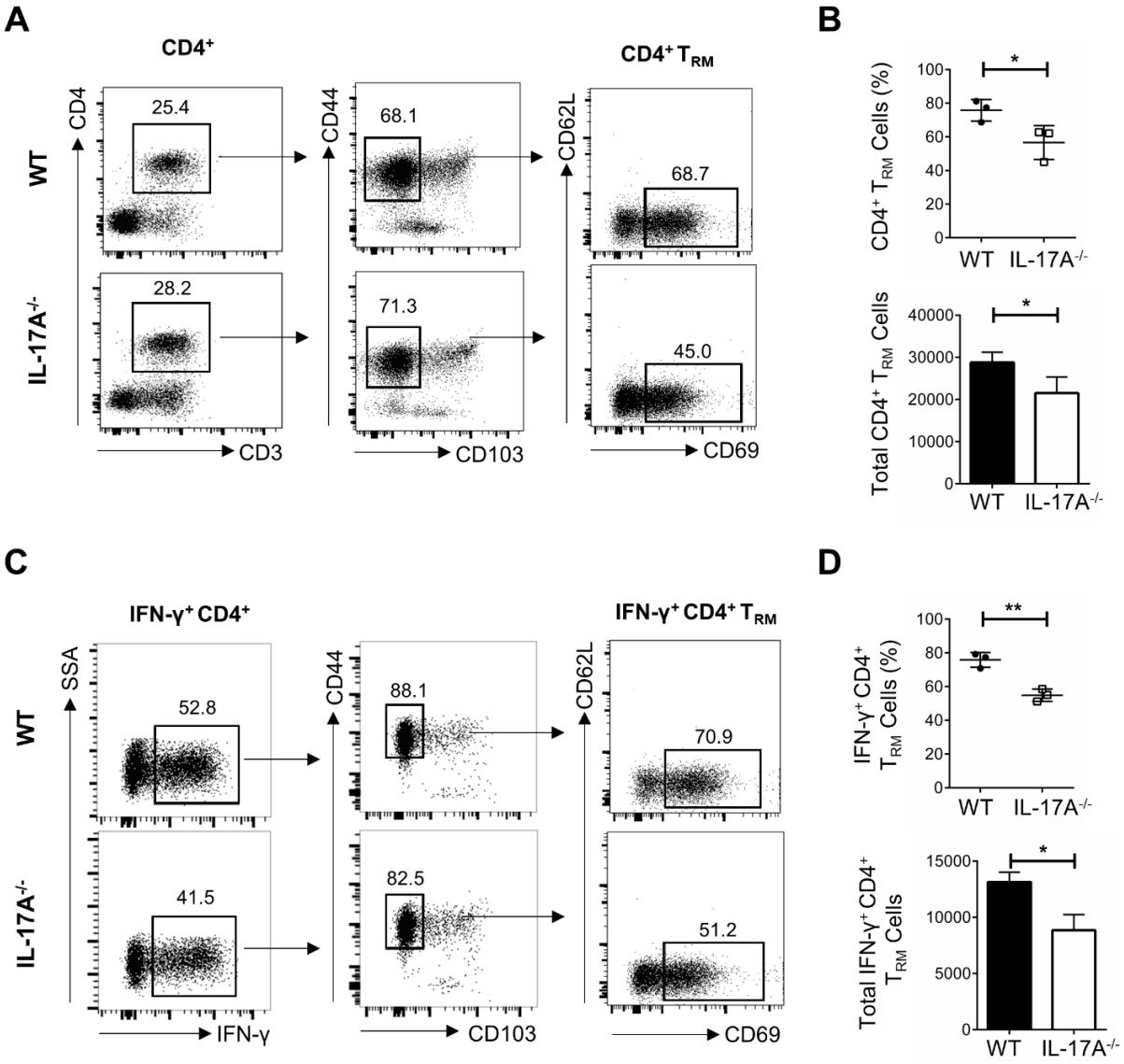

Figure 6: Phenotypic and functional characteristics of CD4$^+$ T_{RM} cells localized in the vaginal tract of WT and *IL-17A*$^{-/-}$ mice following HSV-2 vaccination. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=5-7/group) were vaccinated intravaginally with TK$^-$ HSV-2 (10^4 PFU/mouse), and 3 weeks later, vaginal tissues were collected, pooled and processed. Vaginal cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) containing golgi inhibitors and PMA + ionomycin for 16h, stained with a panel of antibodies, and examined by flow cytometry. (A) CD4$^+$ T cells were gated among total live CD3$^+$ T cells in the vaginal tissue, and CD4$^+$ T_{RM} cells were detected using surface markers CD44, CD103, CD69 and CD62L. CD4$^+$ T_{RM} cells were defined as CD4$^+$ CD44$^+$ CD103$^-$ CD69$^+$ CD62L$^-$. (B) The differences in percentages and total numbers of CD4$^+$ T_{RM} cells

post-immunization in the vaginal tract were compared across three independent experiments (n=5-7/group). (C) IFN-γ^+ $CD4^+$ T_{RM} cells were further gated based on detection of surface markers associated with T_{RM} cells. (D) The differences in percentages and total numbers of IFN-γ^+ $CD4^+$ T_{RM} cells post-immunization in the vaginal tract were compared across three independent experiments (n=5-7/group). Data shown in panels B and D represent mean ± SEMs. Significant difference in IFN-γ expression was calculated using the unpaired, two-tailed t-test with 95% confidence interval. *, $P < 0.05$; **, $P < 0.01$.

TABLE 1 Cumulative pathology scores for HSV-2-re-exposed mice[a]

Group	Pathology score	No. of mice	No. of days	Cumulative pathology	Avg pathology/mouse
WT (5×10^2 PFU)	0	3	15	0	9.6
	4	2	6	48	
IL-17A$^{-/-}$ (5×10^2 PFU)	0	2	15	0	14.4
	4	1	5	20	
	4	1	6	24	
	4	1	7	28	
WT (5×10^3 PFU)	0	7	12	0	5.8
	4	1	7	28	
	4	1	6	24	
IL-17A$^{-/-}$ (5×10^3 PFU)	0	2	0	0	13.3
	4	4	4	64	
	4	2	6	24	
	4	1	8	32	
WT (5×10^4 PFU)	0	4	12	0	0
IL-17A$^{-/-}$ (5×10^4 PFU)	0	2	12	0	7.25
	3	1	3	9	
	4	1	5	20	

[a]Cumulative pathology is calculated by noting the number of mice with their maximum pathology score and the number of days that score was observed. Mice that did not survive the challenge were given highest pathology score for the duration of the experiment to accurately reflect overall pathology for each group. This takes into consideration that each mouse in a group can reach various degrees of pathology through the experiment. The average pathology score per mouse was calculated by dividing the sum of cumulative pathology by total number of mice.

TABLE 2 Cumulative pathology scores for HSV-2 (5 x 10^3 PFU)-challenged mice[a]

Group	Pathology score	No. of mice	No. of days	Cumulative pathology	Avg pathology/mouse
WT (5 × 10^3 PFU)	0	7	12	0	5.2
	4	1	6	24	
	4	1	4	15	
	4	1	3	12	
IL-17A$^{-/-}$ (5 × 10^3 PFU)	0	2	13	0	14.4
	4	2	3	24	
	4	3	4	48	
	4	1	6	24	
	4	1	7	28	
	5	1	4	20	

[a]Cumulative pathology is calculated by noting the number of mice with their maximum pathology score and the number of days that score was observed. Mice that did not survive the challenge were given highest pathology score for the duration of the experiment to accurately reflect overall pathology for each group. This takes into consideration that each mouse in a group can reach various degrees of pathology through the experiment. Average pathology score per mouse was calculated by dividing the sum of cumulative pathology by total number of mice.

CHAPTER 3: IL-17 production by γδ$^+$ T cells is critical for inducing T$_h$17 responses in the female genital tract and regulated by estradiol and microbiota

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In this study, we characterized the primary source of innate or innate-like IL-17 in the FRT, and examined the importance of IL-17 during the induction of adaptive T$_h$17 responses. We found that γδ$^+$ T cells were the predominant source of innate IL-17 in the FRT and that this population was regulated by E2 and the presence of commensal microbiota. Furthermore, vaginal DCs isolated from IL-17A-deficient mice were severely impaired at priming T$_h$17 responses *in vitro*, and this was associated with decreased IL-1β production by vaginal CD11c$^+$ DCs. Overall, in this work we described a novel role for innate-like IL-17 in modulating immunity in the FRT.

Dr. Varun Anipindi and I equally contributed to this work. Drs. Charu Kaushic, Varun Anipindi and I conceived and designed the experiments. Drs. Varun Anipindi and Rodrigo Jiménez-Saiz, Sara Dizzell and I performed the experiments. Drs. Manal Jordana and Martin Stämpfli provided materials and assisted with data analysis. Drs. Charu Kaushic, Varun Anipindi, Denis Snider and I analyzed and interpreted the data, and also wrote and edited the manuscript.

ABSTRACT

IL-17 can be produced by adaptive immune cells such as T_h17 cells, and by immune cells that produce IL-17 without prior priming. This latter category, which we will refer to as 'innate', includes innate cells such as NK cells and innate lymphoid cells (ILCs), and innate-like T cell populations such as NKT cells and gamma-delta ($\gamma\delta^+$) T cells. Studies in mucosal tissues have shown that the induction of T_h17 immunity is amplified by innate IL-17 produced within those tissues. However, the role of innate IL-17 and its effect on T_h17 induction in the female genital tract (FGT) is largely unknown. Here, we characterize the primary source of IL-17-secreting vaginal cells and show that innate IL-17 plays a critical role in priming adaptive T_h17 responses in the FGT. Under homeostatic conditions, $\gamma\delta^+$ T cells were the predominant source of innate IL-17 in the murine FGT and this population was modulated by both the sex hormone estradiol (E2) and the presence of commensal microbiota. Compared to wild-type (WT) C57BL/6 mice, vaginal APCs isolated from IL-17A-deficient (*IL-17A*$^{-/-}$) mice were severely impaired at priming T_h17 responses in APC-T cell co-cultures. Furthermore, the defect in T_h17 induction in the absence of innate IL-17 was associated with impairment of IL-1β production by vaginal $CD11c^+$ dendritic cells (DCs). Overall, our study describes a novel role for IL-17 in the FGT and further demonstrates the importance of factors in the vaginal microenvironment that can influence adaptive immune responses.

INTRODUCTION

Interleukin-17 belongs to a family of cytokines that consists of six related ligands (IL-17A to IL-17F) (1). IL-17 is primarily secreted by $CD4^+$ T cells that differentiate into T_h17 effector cells under the influence of cytokine combinations comprised of IL-6, IL-1β, TGF-β, IL-21, and IL-23 (2-5). The effector response of IL-17 involves the induction of inflammatory mediators such as TNF-α, IL-1, IL-8, G-CSF, and the recruitment of neutrophils for pathogen clearance (6-8). T_h17 immunity and the production of IL-17 have been shown to play a significant role in the resolution of bacterial and fungal infections in mucosal tissues (9, 10), while demonstrating a pathological role in autoimmune and chronic inflammatory diseases (6, 11-13).

Within the female genital tract (FGT), a mucosal site exposed to diverse pathogens, IL-17 has been shown to play an important role during bacterial and fungal infections, including *Neisseria gonorrhoeae*, *Candida albicans*, and *Chlamydia trachomatis* (14-19). However, the role of IL-17 during genital viral infections has not been fully elucidated. We recently described a novel anti-viral role for IL-17 in the FGT using a mouse model of genital herpes infection (20, 21). Genital herpes, predominantly caused by HSV-type 2 (HSV-2), is one of the most prevalent sexually transmitted viral infections globally and affects more women than men (22). The primary protective response against HSV-2 involves the production of IFN-γ by $CD4^+$ T cells in the FGT (23-25). However, we recently showed that enhanced protection against vaginal HSV-2 infection also coincided with increased levels of IL-17 production by $CD4^+$ T cells (21). We found that mice treated with the female sex hormone, estradiol (E2), were completely protected against genital

HSV-2 challenge and protection in these animals was related to increased T_h17 immunity (21). Along with augmented T_h17 responses, mice also demonstrated enhanced T_h1 responses (21). Furthermore, in the absence of T_h17 responses, vaccinated IL-17A-deficient mice (*IL-17A*$^{-/-}$) had impaired IFN-γ^+ CD4$^+$ T cell responses as well as poor disease outcomes against HSV-2 challenge, compared to wild-type (WT) C57BL/6 mice (20). Collectively, these studies suggest a significant role for T_h17 cells in the immune response to viral infections in the FGT.

Interestingly, although T_h17 cells are often associated with IL-17 production, other immune cells can also produce IL-17 without any prior priming. This production of IL-17, which we will refer to as 'innate' IL-17, comes from sources such as innate cells (NK cells and innate lymphoid cells (ILCs)), and innate-like T cell populations (NKT cells and gamma-delta ($\gamma\delta^+$) T cells) (26). Though innate and innate-like cells represent a relatively small proportion of the responding lymphocytes to mucosal infections, they are more potent at producing IL-17 compared to T_h17 cells (27). Specifically, innate-like $\gamma\delta^+$ T cells are one of the earliest identified sources of IL-17 (28), and have been described as the major source of IL-17 under homeostatic conditions in the FGT (29). They develop early in the fetus and provide immunity prior to the generation of the adaptive immune system (30), and unlike traditional T lymphocytes, $\gamma\delta^+$ T cells utilize their TCR directly as pattern recognition receptors to respond to pathogens (31, 32). $\gamma\delta^+$ T cells are predominantly localized in mucosal tissues, and rapidly activate upon exposure to pathogens and pro-inflammatory cytokines released by APCs (32). IL-1, IL-6, IL-18, IL-23 and TGF-β have all been shown to be important for IL-17 production by $\gamma\delta^+$ T cells (28).

Production of innate IL-17 under homeostatic conditions has been implicated in the amplification of T_h17 responses in mucosal tissues (33-37). In models of autoimmune encephalomyelitis (37) and colitis (33), IL-17^+ $\gamma\delta^+$ T cells promoted T_h17 differentiation and amplified IL-17 production by T_h17 cells. In our own studies, we have observed that vaginal cells can also constitutively produce IL-17, without any additional stimulation *in vitro* (21). This suggests that innate IL-17 may be an important component of the vaginal microenvironment and can potentially effect induction of T_h17 responses. These findings prompted us to further determine the role of IL-17 in the FGT.

In the current study, we examined the importance of innate IL-17 during the induction of adaptive T_h17 responses in the FGT. Similar to previous studies (20, 21), we found that in the absence of IL-17, mice were unable to initiate protective anti-viral immunity and could not effectively clear HSV-2 virus *in vivo*. We then examined the ability of *IL-17A*$^{-/-}$ mice to induce an adaptive T_h17 response *in vitro* and found that vaginal dendritic cells (DCs) from *IL-17A*$^{-/-}$ mice were significantly impaired at priming T_h17 responses, and this was related to attenuated IL-1β production. We further characterized the innate IL-17-secreting cells and found that under homeostatic conditions, $\gamma\delta^+$ T cells were the predominant source of IL-17 in the FGT. Finally, we demonstrated that IL-17 production by $\gamma\delta^+$ T cells was modulated by the presence of female sex hormone, E2, and commensal microflora. These data suggest that production of IL-17 in the vaginal tract under homeostatic conditions is critical for inducing T_h17 responses, thereby amplifying the overall IL-17 response in the FGT.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Charles River Laboratories Inc. (Saint-Constant, Quebec, Canada). *IL-17A*$^{-/-}$ mice were kindly provided by Dr. Martin Stämpfli (acquired with a Material Transfer Agreement from Dr. Yoichiro Iwakura) (University of Tokyo, Japan) (38, 39), and estradiol receptor α knockout mice (ERKO) were kindly provided by Dr. Pierre Chambon (University de Strasbourg, France) (40) and bred in the Central Animal Facility (McMaster University, Hamilton, Canada). Germ-free (GF) mice were bred in-house and purchased from the Farncombe Family Axenic-Gnotobiotic Facility (McMaster University). OVA receptor transgenic (Tg) mice (OT-II Tg) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) (41). All mice were maintained under specific pathogen-free and standard temperature-controlled conditions which followed a 12-hour light/dark cycle. Routine quality assurance was done by serology and PCR to ensure mice remained specific pathogen-free, and included testing dirty bedding sentinels, direct resident animals and exhaust air duct samples of racks. All animal studies were approved by, and in compliance with, the Animal Research Ethics Board at McMaster University.

Hormone treatment

For hormone experiments, mice were ovariectomized (OVX) to deplete endogenous hormones according to previously published protocols (42, 43). Briefly, mice were administrated an injectable anesthetic preparation of Ketamine and Xylazine

intraperitoneally. Ovaries were removed by making two bilateral incisions, followed by small incisions through the peritoneal wall, and excised through the incisions. Incisions were closed using surgical clips and mice recovered for 14 days before the start of experiments. Two weeks later, OVX mice were anesthetised with injectable anesthetic (Ketamine and Xylazine) and implanted subcutaneously with 21-day release 17β-Estradiol (476 ng/mouse/day), or placebo (mock) pellets (Innovative Research of America, Sarasota, FL), according to previously published protocols (43). The level of serum E2 resulting from the pellets has previously been shown to correspond to that measured during the estrus cycle (44).

Viral infection

OVX mice were immunized intranasally with an attenuated strain of HSV: thymidine kinase-deficient (TK^-) HSV-2. Briefly, mice were anesthetized using isoflurane and then inoculated with 5 μl of TK^- HSV-2 (10^2 PFU/mouse) into each nare with a micropipette. Six weeks later, mice were challenged intravaginally with WT HSV-2 ($5x10^3$ PFU/mouse). Vaginal washes were collected for 6 days post-challenge by pipetting 30 μl of PBS into the vagina 5-6 times and repeated to collect a total volume of 60 μl.

Genital pathology scoring

Genital pathology was monitored daily based on a five-point scale, as described previously (20, 21): no infection (0), slight redness of external vagina (1), swelling and redness of vagina (2), severe swelling and redness of vagina and surrounding tissues (3), genital ulceration with severe redness and hair loss (4) and severe ulceration extending to

surrounding tissues, ruffled hair, and lethargy (5). Animals were sacrificed before they reached stage 5.

Viral titration

Viral shedding in vaginal washes was determined by conducting viral plaque assays on Vero cell (ATCC, Manassas, VA) monolayers. Vero cells were grown in α-MEM (Gibco Laboratories, Burlington, ON, Canada) supplemented with 5% FBS (Gibco Laboratories), 1% penicillin-streptomycin (Invitrogen, Burlington, ON, Canada), L-glutamate (BioShop Canada Inc., Burlington, ON, Canada), and 1% HEPES (Invitrogen). Cells were grown to confluency in 12-well plates, vaginal washes were diluted in α-MEM and then added to monolayers. Infected monolayers were incubated at 37°C for 2 h. Infected monolayers were then overlaid with α-MEM, and infection was allowed to occur for 48 h at 37°C. Cells were then fixed and stained with crystal violet, and viral plaques were enumerated under a microscope. The number of PFU/ml was calculated by taking a plaque count for every sample and accounting for the dilution factor.

Tissue isolation and co-cultures

Vaginal tissue was enzymatically 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 for 2 h and filtered through a 40 μm filter, to obtain a total tissue cell (TC) suspension (45). OT-II Tg spleens were mechanically disrupted and Ammonium-Chloride-Potassium lysing buffer (Sigma Aldrich, St. Louis, MO) was used to lyse blood cells. Mononuclear cells were counted, and $CD4^+$ T cells were magnetically sorted using CD4 L3TE microbeads

(Miltenyi Biotec, Auburn, CA) based on manufacturer protocols. Sorted $CD4^+$ T cells were stained with 50 μM CFSE (Sigma Aldrich) according to published protocols (46). Vaginal TC ($5x10^5$ cells/ml) were incubated in a 96-well plate pulsed with 5 μg/well OVA peptide (323-339) (Biomer Technology, Pleasanton, CA, USA) with sequence ISQAVHAAHAEINEAGR for 16-18 h. CFSE-stained OT-II Tg $CD4^+$ T cells were added at a 1:1 ratio and co-cultured for 3.5 days at 37°C in complete RPMI media (47), based on previously standardized protocols (48). During certain experiments, 100 ng/ml recombinant IL-1β (rIL-1β) or 250 ng/ml recombinant IL-17 (rIL-17) (R&D systems, Minneapolis, MN) was added during the peptide-pulse stage or on the first day of co-culture.

Flow cytometry

For IL-17A or IL-1β intracellular staining, freshly isolated vaginal tissue cells or 2-day-old co-cultures were treated with 2 μl/ml of cell stimulation cocktail plus 500X protein transport inhibitors: a cocktail of PMA, ionomycin, brefeldin A and monensin (eBioscience, San Diego, CA, USA) for 16 h at 37°C. Cells were stained with allophycocyanin (APC)-ef780 viability dye (eBioscience) for 30 min to exclude dead cells, and subsequently, stained with surface antibodies: CD3 BV785 (BioLegend, San Diego, CA, USA), CD4 BV421 (BioLegend), TCR γδ PE (eBioscience), NKp46 PE-cf594 (eBioscience), CD11c PE-Cy7 (BD Biosciences, Mississauga, ON, Canada), or CD11b PE-cf594 (BD Biosciences). Cells were then permeabilized and fixed with BD Pharmingen Transcription Factor Buffer Set (BD Biosciences) according to manufacturer protocols, and stained with intracellular antibodies: IL-17A APC (eBioscience), IL-1β PE (R&D Systems)

and pro-IL-1β PerCP-ef710 (eBioscience), or control Rat IgG1 APC (eBioscience), Rat IgG1 PE (R&D Systems) and Rat IgG1 PerCP-ef710 (eBioscience). Data was acquired on a BD LSRII Flow Cytometer Systems (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR).

Cytokine analysis

IL-17 levels in co-cultures supernatants were measured using the Duoset ELISA kit (R&D Systems), according to manufacturer protocols, and plates were analyzed on a Sector Imager 2400 (Meso Scale Discovery, Rockville, MD).

Statistics

Statistical analysis and graphical representations were performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). Data are expressed as mean ± SEM, typically derived from n=3 replicates. Significance was calculated by comparing means using one-way or two-way ANOVA or t-tests, as indicated in the figure legends. Statistical significance was defined as *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

Ethics statement

All animal use was conducted in accordance to protocols approved by the McMaster University Animal Research Ethics Board as per Animal Utilization Protocol #14-09-40 in accordance with Canadian Council of Animal Care guidelines.

RESULTS

Innate IL-17 in the FGT plays an important role in inducing T_h17 responses

While studies in other mucosal sites, such as the gut, have shown that innate and innate-like sources of IL-17 can help amplify the overall IL-17 response by enhancing T_h17 responses (33-37), a similar role for IL-17 in the FGT has not been yet reported. Therefore, we wanted to examine the effect of innate IL-17 on T_h17 cell priming by vaginal DCs.

First, to confirm the *in vivo* significance of vaginal IL-17 in the context of a viral infection, we used a mouse-model of HSV-2 immunization and challenge to examine the survival of mice in the absence of IL-17, similar to our previous studies (20, 21). *IL-17A*$^{-/-}$ mice and WT C57BL/6 controls were OVX, immunized with an attenuated strain of TK^{-} HSV-2, and then challenged intravaginally with WT HSV-2. Survival and genital pathology were monitored daily, and vaginal washes were collected to measure viral shedding and determine disease severity. Consistent with our previous observations (20, 21), following genital HSV-2 challenge, *IL-17A*$^{-/-}$ mice had poor disease outcomes and demonstrated greater mortality (Fig. 1). Only 17% of *IL-17A*$^{-/-}$ mice survived the viral challenge, compared to 83% of WT controls by day 12 (Fig. 1A). All mice that reached endpoint developed genital pathology (*IL-17A*$^{-/-}$: 5/6 mice; WT: 1/6 mice) (Fig. 1B). Similarly, viral shedding was also significantly higher in *IL-17A*$^{-/-}$ animals (p=0.0281) (Fig. 1C), suggesting that IL-17 plays an important role in modulating anti-viral responses in the FRT, and confirming our previous findings (20, 21).

Next, we wanted to determine the effect of innate IL-17 production on the induction of antigen-specific T_h17 responses in the FGT. We used a previously well-described, *in vitro* chicken OVA peptide co-culture model with OVA-specific OT-II Tg $CD4^+$ T cells (41). Vaginal tissue cells (TC) including APCs were isolated from *IL-17A*$^{-/-}$ mice and WT C57BL/6 controls, cultured in medium alone or pulsed with OVA peptide, and co-cultured with CFSE-labelled OT-II Tg $CD4^+$ T cells (TC+CD4) for 3.5 days, as previously described (21). Using this model, we measured IL-17 levels in TC-alone cultures and found that WT mice produced innate IL-17 (100 ± 43 pg/ml), while IL-17 was below detection in TC cultures from *IL-17A*$^{-/-}$ mice (p=0.003) (Fig. 2A). To determine the role of innate IL-17 on T_h17 differentiation, IL-17 levels were measured in TC+CD4 co-culture supernatants. Cultures containing TC from *IL-17A*$^{-/-}$ mice contained 30-fold lower IL-17 compared with those with TC from WT mice (p=0.001) (Fig. 2A). In addition, we conducted intracellular cytokine staining on day 2 of the co-cultures and found that a lower proportion of proliferating CFSE-stained OT-II Tg $CD4^+$ T cells expressed IL-17 in *IL-17A*$^{-/-}$ TC+CD4 co-cultures (39%) compared to WT controls (55%) (Fig. 2B). These observations provide further evidence that the absence of innate IL-17 results in diminished T_h17 responses. The small population of $CFSE^-$ cells within WT TC+CD4 co-cultures (3.87%) likely represented the innate IL-17 population in the FGT that was entirely absent in *IL-17A*$^{-/-}$ co-cultures (Fig. 2B). The innate IL-17 population had a ~30-fold higher median fluorescence intensity (MFI) compared to the $CFSE^+$ T_h17 cells (p=0.040), indicating that the $CFSE^-$ population of IL-17-secreting cells was a more potent source of IL-17 than T_h17 cells, on a per cell basis (Fig. 2C).

Given the attenuated priming of T_h17 responses demonstrated by *IL-17A*$^{-/-}$ vaginal TCs (Fig. 2A), we wanted to examine whether exogenous addition of rIL-17 could rescue the ability of DCs within the *IL-17A*$^{-/-}$ vaginal TCs to induce T_h17 responses. Vaginal cells from *IL-17A*$^{-/-}$ mice and WT mice were isolated, pulsed with OVA peptide, and co-cultured with OT-II Tg CD4$^+$ T cells. To determine whether IL-17 is only required transiently as a pre-treatment, 250 pg/ml of rIL-17 (a level consistent with that observed in cultures of vaginal cells from WT controls alone (Fig. 2A)) was added during the OVA peptide pulse, and removed 12 h later by washing, before co-culturing with OT-II Tg CD4$^+$ T cells. Alternatively, to examine whether pro-longed exposure of IL-17 is required during the process of T cell differentiation, rIL-17 was added at day 1 of the co-culture and remained in culture for the duration of the experiment. In both conditions, rIL-17 was unable to significantly influence T_h17 responses in *IL-17A*$^{-/-}$ co-cultures (Fig. 2D), suggesting that short-term restoration of IL-17 may not be sufficient to restore the ability of *IL-17A*$^{-/-}$ vaginal APCs to prime T_h17 responses. Taken together, these results affirm the importance of IL-17 in the anti-viral response in the FGT and show that innate IL-17 within the FGT is important for the induction of T_h17 responses.

Innate IL-17 induces vaginal DCs to prime T_h17 responses via an IL-1β-dependent pathway

Various cytokines have been implicated for IL-17 production (28). In our own studies, we showed that within the FGT, adaptive IL-17 production by T_h17 cells was primed by CD11c$^+$ DCs via an IL-1-dependent pathway (21). Given the attenuated T_h17 responses observed in co-cultures containing vaginal APCs from *IL-17A*$^{-/-}$ mice (Fig. 2),

we wanted to examine whether innate IL-17 plays a role in programming vaginal APCs to produce IL-1.

Vaginal TCs from *IL-17A*$^{-/-}$ mice and WT controls were isolated and cultured overnight (12 h), prior to staining with antibodies against CD11c, CD11b and IL-1β. Dead cells were excluded using a fixable viability dye, CD11c^{+} DCs were gated (Fig. 3A), and intracellular IL-1β expression was compared by flow cytometry. We found that *IL-17A*$^{-/-}$ mice contained a significantly lower proportion of IL-1β-producing CD11c^{+} DCs in the FGT compared to WT mice (p=0.04) (Fig. 3B, 3C). In addition, CD11c^{+} DCs from *IL-17A*$^{-/-}$ mice also contained reduced amounts of IL-1β per cell compared to WT controls, based on MFI (Fig. 3D), although not statistically significant (p=0.400).

Given this observation, we wanted to examine whether the addition of exogenous IL-1β would restore T_h17 responses in *IL-17A*$^{-/-}$ co-cultures. Addition of rIL-1β to *IL-17A*$^{-/-}$ co-cultures on day 1 (TC+CD4+IL-1β) was able to significantly (p=0.0005) enhance T_h17 responses almost 10-fold compared to cultures without IL-1β (Fig. 3E). However, IL-17 levels were still significantly lower than those seen in cultures with WT vaginal TCs (Fig. 3E).

Overall, these results showcase an inherent deficit in the ability of vaginal DCs from *IL-17A*$^{-/-}$ mice to produce IL-1β, thereby suggesting that the priming of T_h17 responses by innate IL-17 is dependent on IL-1β. Even with the addition of exogenous IL-1β, there was only a partial rescue of T_h17 responses primed by *IL-17A*$^{-/-}$ vaginal DCs. Together, this

suggests that innate IL-17 is important for programming vaginal DCs to produce IL-1β, which in turn is critical for priming vaginal T_h17 responses in the FGT.

$\gamma\delta^+$ T cells are the predominant source of innate IL-17 in the FGT

Having established an important role for innate IL-17 in the priming of T_h17 responses in the FGT, we next wanted to characterize the primary sources of IL-17 production. IL-17 in mucosal tissues can be produced by a variety of innate lymphocytes (NK cells and ILCs) (26), innate-like lymphocytes (NKT and $\gamma\delta^+$ T cells) (26) and adaptive sources (T_h17 cells) (49), and so we focused on examining these lymphocyte populations.

Vaginal cells isolated from C57BL/6 mice were stained with a panel of antibodies against CD3, CD4, TCR γδ, IL-17 and NKp46 to identify these lymphocyte populations (29, 50). Dead cells were excluded using a fixable viability dye, and single cells were gated to identify total IL-17^+ populations using flow cytometry (Fig. 4A). We found that IL-17^+ cells did not express NKp46 (Fig. 4A) and could instead be divided into 2 major populations based on their CD3 and CD4 expression ($CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$) (Fig. 4B). These were further separated based on TCR γδ expression (Fig. 4B), and we observed that $\gamma\delta^+$ T cells ($CD3^+$ $CD4^-$ $\gamma\delta^+$) were the primary source of IL-17 (Fig. 4C, 4D). The $\gamma\delta^+$ T cell population accounted for 75% of total IL-17-producing cells, which was significantly higher than both $CD4^+$ T cells (21%) and other sources (4%) of IL-17 (Fig. 4C, 4D).

We also examined TCR γδ expression by vaginal cells from *IL-17A*$^{-/-}$ mice and found frequencies of $\gamma\delta^+$ cells comparable to those in WT animals (Fig. 4E) (not significant; p=0.243), suggesting no inherent defect in the development of the $\gamma\delta^+$ population in the

absence of IL-17. Similar to previous reports (29), these findings confirm that innate-like $\gamma\delta^+$ T cells represent the primary source of IL-17 in the FGT, under homeostatic conditions.

E2 enhances IL-17 production by $\gamma\delta^+$ T cells in the FGT

The FGT is a complex tissue, regulated by a variety of factors within the local microenvironment. One major component which influences immune cell populations and responses in the FGT is the presence of female sex hormones. Specifically, we have shown that the female sex hormone E2 is protective against viral infections in the FGT and can enhance T_h17 responses primed by vaginal $CD11c^+$ DCs (21). Therefore, we wanted to examine whether E2 can also influence innate IL-17 populations in the FGT.

To determine the effect of E2 on the primary IL-17-secreting cell population ($\gamma\delta^+$ T cells), C57BL/6 mice were OVX to remove endogenous sources of reproductive hormones and implanted with prolonged release E2 or placebo (mock) pellets. Two weeks later, vaginal cells were isolated and stained for flow cytometry. We found that E2 treatment consistently and significantly (p=0.05) induced greater proportions of IL-17^+ cells in the FGT compared to mock-treated or ERKO controls (Fig. 5A, 5B). Furthermore, IL-17^+ cells from E2-treated mice produced significantly greater IL-17 per cell (p=0.007) compared to controls, based on MFI levels (Fig. 5C). When we characterized the overall IL-17^+ population based on CD3 and CD4 expression, and further separated these populations based on TCR γδ expression as done previously (Fig. 4), we found that the predominant source of IL-17 in E2-treated animals was the $\gamma\delta^+$ T cell population (Fig. 5D-5F) described earlier (Fig. 4). However, this population was largely absent or diminished in mock-treated mice (E2: 64±12%; Mock: 16±6%; p=0.02) (Fig. 5F).

Taken together, these findings demonstrate that factors in the microenvironment of the FGT, such as the presence of E2, can influence innate levels of IL-17. Specifically, E2 plays a critical role in up-regulating IL-17 production by $\gamma\delta^+$ T cells.

Commensal microbiota enhances IL-17 production by $\gamma\delta^+$ T cells in the FGT

Another factor shown to influence IL-17 production in mucosal tissue is the presence of the commensal microflora. In the gut, the local microbiota has been reported to drive IL-17 production by both $\gamma\delta^+$ T cells (51, 52) and T_h17 cells (53). Similarly, the vaginal microflora has also been shown to regulate immune responses in the FGT (54). As a result, we investigated whether the vaginal microbiota could also influence innate IL-17 in the FGT.

To determine the effect of the microbiota on the primary IL-17-secreting cell population ($\gamma\delta^+$ T cells), vaginal cells from hormone-cycle matched C57BL/6 GF mice and WT mice were isolated, and IL-17 production was compared by flow cytometry. We consistently found that GF mice had significantly lower proportions of total IL-17^+ cells compared to WT controls (p=0.028) (Fig. 6A, 6B), as well as lower IL-17 produced per cell (p=0.002) (Fig. 6C). Subsequently, a distribution of IL-17-producing cell subtypes based on CD3 and CD4 expression (Fig. 6D), followed by TCR γδ expression (Fig. 6E), showed that IL-17^+ $\gamma\delta^+$ T cells were significantly decreased in GF mice (GF: 26±4%; WT: 54±8%; p=0.02) (Fig. 6F). Overall, these results demonstrate that presence of microbiota enhances IL-17 production by $\gamma\delta^+$ T cells in the FGT.

DISCUSSION

Although IL-17 is commonly referred to as a $CD4^+$ T cell-secreted cytokine, the majority of IL-17 released constitutively under homeostatic conditions is produced by $CD4^-$ innate and innate-like immune cells. The presence of innate IL-17 in mucosal tissues has been shown to be important for potentiating T_h17 immunity, and thereby, playing an important role in protecting against pathogen exposure and disease (33-37). In the FGT, T_h17 responses have shown to be protective against bacterial, fungal and viral infections (14-19). Recent work from our lab has focused on the role of T_h17 responses during genital HSV-2 infection (20, 21). We have shown that vaginal DCs are critical for priming T_h17 responses, which leads to enhanced T_h1 responses (21), and that IL-17 plays a critical role in mediating T_h1 responses during *in vivo* HSV-2 challenge (20, 21). However, the significance of IL-17 produced by innate or innate-like sources in the FGT and its influence on T_h17 immunity is less understood. In the current study, we investigated the role of innate IL-17 in the induction of T_h17 responses in the FGT and found that innate IL-17 was important for inducing vaginal T_h17 responses. This was evident as vaginal APCs from *IL-17A*$^{-/-}$ mice were severely impaired at priming T_h17 responses in APC-T cell co-cultures (Fig. 2). In addition, vaginal DCs from these mice also produced significantly lower levels of IL-1β (Fig. 3), which is critical for priming T_h17 responses in the FGT (21). We further characterized the IL-17-secreting cells in the FGT and discovered that the predominant source was innate-like $\gamma\delta^+$ T cells (Fig. 4). Furthermore, IL-17 production by $\gamma\delta^+$ T cells was shown to be influenced by factors such as sex hormones (E2) and the microbiota (Fig. 5, 6). To the best of our knowledge, this is the first study demonstrating that innate IL-17

plays a critical role in potentiating T_h17 immunity in the FGT and examining the factors that regulate it.

Numerous studies have shown that innate and innate-like lymphocytes can influence the differentiation and function of adaptive lymphocytes (55). We previously observed that vaginal cells can produce modest amounts of IL-17 in culture without any stimulation (21), and we wanted to further examine the influence of this innate IL-17 on the priming of T_h17 responses by vaginal DCs. We consistently found that vaginal cells from *IL-17A*$^{-/-}$ mice were impaired at priming T_h17 responses *in vitro* (Fig. 2A, 2D), suggesting that the presence of innate IL-17 can influence adaptive immunity primed by vaginal APCs. Interestingly, rIL-17 (250 pg/ml) did not restore the functional ability of the DCs in priming these responses (Fig. 2D). This is consistent with the observations made in a study by *Kumar et al*., which provided evidence demonstrating that T_h17 differentiation is not directly dependent on IL-17RA signaling (56), and thereby suggests that IL-17 affects T_h17 differentiation in an indirect manner. However, other *in vivo* studies suggest that the absence of IL-17 may lead to inherent defects in DC-mediated priming of $CD4^+$ T cell responses (57). *Bai et al.* demonstrated, using a lung *C. muridarum* infection model, that neutralization of IL-17 significantly impaired DC function including lower levels of IL-12 production and lower expression of MHC class II and CD40 (57). Furthermore, the mice produced higher levels of IL-10 and IL-4, and skewed the immune responses toward a T_h2 phenotype, instead of the typical T_h1 response seen in controls (57). Likewise, we have previously shown that in the absence of IL-17 (*IL-17A*$^{-/-}$ mice), there was a 7-fold lower T_h1 response in the FGT compared to WT mice (20, 21). Our current findings further

expand on the idea that under homeostatic conditions, IL-17 plays a critical role in priming $CD4^+$ T cell responses in the FGT.

The production of IL-17 has been shown to depend on different cytokines including IL-1, IL-6, IL-21, IL-23 and TGF-β (2-5, ,28). However, while $\gamma\delta^+$ T cells from IL-6 knockout (KO) and TGF-β KO mice produced comparable or attenuated levels of IL-17, respectively, those from IL-1R KO mice failed to secrete any IL-17 in response to IL-23 and TLR agonists (37, 58). This suggests that IL-1 may play a critical role in the production of IL-17 by $\gamma\delta^+$ T cells. Likewise, we previously showed that IL-1β, but not IL-6, production by vaginal $CD11c^+$ DCs is critical for priming T_h17 responses in the FGT (21). Therefore, we wanted to examine whether the attenuated T_h17 responses seen in *IL-17A*$^{-/-}$ co-cultures was due to a defect in the ability of *IL-17A*$^{-/-}$ mice to produce IL-1β. We found that vaginal DCs from *IL-17A*$^{-/-}$ mice produced lower amounts of IL-1β (Fig. 3B, 3C), compared to WT DCs. These observations are directly consistent with prior literature indicating that IL-17 can induce IL-1β production in mucosal APCs (59, 60). *Nakae et al.* observed lower IL-1β and impaired $CD4^+$ T cell responses by Langerhans cells from *IL-17A*$^{-/-}$ mice (39). Furthermore, while the addition of rIL-1β increased T_h17 differentiation in *IL-17A*$^{-/-}$ co-cultures (Fig. 3E), these levels were still significantly lower than those observed in WT cultures. Given that there are numerous stimulatory and inhibitory factors involved in priming T_h17 responses (61), we suggest that there may be other intrinsic defects in *IL-17A*$^{-/-}$ DCs related to signal transduction, activation markers (56) or cytokine processing (39), which cannot be restored by the transient addition of rIL-1β. Overall, our findings demonstrate that IL-17 in the FGT microenvironment may have an important

effect on the function of vaginal DCs and their ability to prime adaptive T_h17 responses via an IL-1β-dependent pathway.

While innate IL-17 can be produced by a mixed population of lymphocytes under homeostatic conditions, consistent with a previous report by *Kim et al.* (29), we found that $\gamma\delta^+$ cells are the primary source of innate IL-17 in the FGT (Fig. 4D). Additionally, we looked at $\gamma\delta^+$ cells in the FGT of *IL-17A*$^{-/-}$ mice to determine if there was a defect in the $\gamma\delta^+$ T cell population in the absence of IL-17 and found no significant difference, as compared to WT mice (Fig. 4E). We also identified other sources of innate IL-17- producing cells. While the $CD3^+$ $CD4^+$ $IL\text{-}17^+$ cells are typically identified as T_h17 cells, sub-populations in this subset can also represent innate cell types such as fetal lymphoid tissue inducer cells or NK cell receptor negative type 3 ILCs (50). Due to the lack of CD4 expression in $CD3^+$ $CD4^-$ $\gamma\delta^-$ $IL\text{-}17^+$ cells, these may represent NK cell receptor negative type 3 ILCs. However, given the previous observations by *Serafini et al.* showing that GATA3 is important for type 3 ILCs (63), further studies with GATA3 KO mice may be required to conclusively identify these cells in the FGT. Irrespective of these other sources of IL-17, our findings demonstrate that $\gamma\delta^+$ T cells are the primary source of innate IL-17 in the FGT.

Having identified the primary source of IL-17-producing cells critical for inducing adaptive T_h17 responses, we examined how two important factors in the local FGT microenvironment, sex hormones and the microbiota, can influence the presence and function of these cells. We have previously shown that E2 can enhance T_h1 and T_h17 responses in the FGT (20, 21). However, the effect of E2 on levels of innate IL-17 has not been reported. We found that E2-treated mice contained a higher proportion of overall IL-

17^+ cells in the FGT compared to mock or ERKO control animals (Fig. 5A-C). Also, the primary source of this IL-17 in the FGT, $\gamma\delta^+$ T cells, was almost completely absent in E2-depleted mice (Fig. 5D-F). These results suggest that E2 is involved in modulating and enhancing the production of innate IL-17.

There is also cross-talk between the microbiota and hormones present in the FGT, and E2 has been shown to be important for a healthy vaginal microflora (64, 65). Interestingly, the gut microbiota has been linked to IL-1β-mediated T_h17 responses primed by mucosal DCs (66), and the microbiome has also been linked to IL-17 production by innate and innate-like lymphocytes in mucosal tissues (36, 67); however, the influence of the vaginal microbiota on IL-17 production is largely unknown. We examined whether the microbiota is important for IL-17 production in the FGT by using GF mice devoid of commensal bacteria, and additionally, we used stage-matched animals to ensure there was limited influence of hormones on the immune responses measured. We observed a significant reduction in the overall proportion of IL-17^+ cells in the FGT of GF mice, compared to WT controls (Fig. 6A-C), and there was also a marked decrease in the proportion of $\gamma\delta^+$ T cells in these mice (Fig. 6E, 6F). These findings are in contrast to a previous report by *Kim et al.* (29), where they concluded that the vaginal microflora does not affect IL-17 production by genital $\gamma\delta^+$ T cells. However, they used antibiotics via drinking water to deplete commensal bacteria, while we used GF mice which were completely devoid of microflora. The effects of the temporary depletion compared to complete absence of bacteria during development of mucosal immunity may explain the discrepancy between our findings. Together, our findings in regard to the effects of E2 and

microbiota demonstrate the degree to which the microenvironment can influence the induction of immune responses and the development, self-renewal, or maintenance of IL-17-producing $\gamma\delta^+$ T cells in the FGT.

In summary, our study expands on previous work regarding the importance of anti-viral T_h17 immunity in the FGT (20, 21) and provides insight regarding a novel role for IL-17 in the vaginal microenvironment. We have shown that innate IL-17 is primarily secreted by $\gamma\delta^+$ T cells in the FGT and is modulated by both E2 and the microbiota. This IL-17-rich environment conditions vaginal DCs to prime potent T_h17 responses, likely via an IL-1β-dependent pathway. To our knowledge, this is the first report to show the importance of IL-17 produced by $\gamma\delta^+$ T cells in the induction of T_h17 immunity in the FGT, as well as demonstrate an association between E2, microbiota and IL-17-producing $\gamma\delta^+$ T cells. Our findings showcase the complexity of the vaginal microenvironment, and highlight the importance of understanding how endogenous factors (IL-17, hormones, microbiota) can regulate the priming of adaptive immune responses by vaginal DCs, and thereby, influence overall immunity in the FGT.

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FIGURES

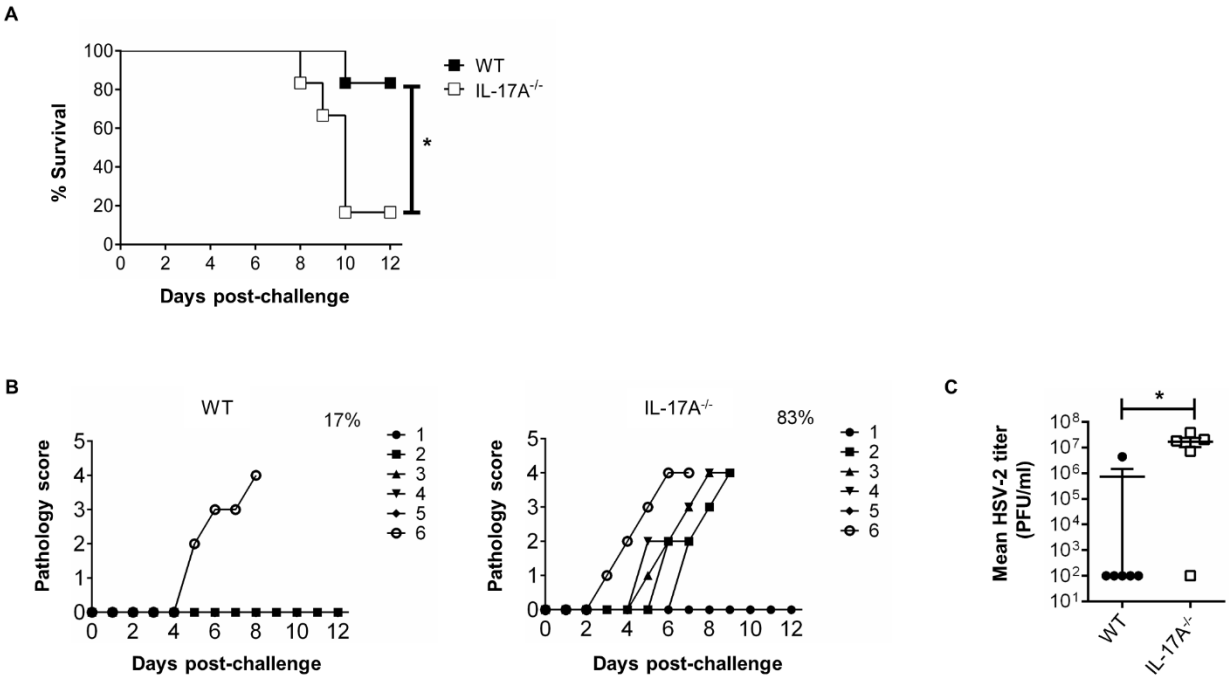

Figure 1: *IL-17A*$^{-/-}$ mice are more susceptible to intravaginal HSV-2 challenge following intranasal immunization. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=6/group) were immunized intranasally with TK^{-} HSV-2 (10^2 PFU/mouse) and 6 weeks later, challenged intravaginally with WT HSV-2 ($5x10^3$ PFU/mouse). Survival and genital pathology was monitored, and vaginal washes were collected daily for 6 days post-challenge. (A) Significance in difference in survival was calculated using the log rank (Mantel-Cox) test (*$p<0.05$). (B) Genital pathology scores were recorded on a scale of 0 to 5. Data points superimposed on the x axes of panel B indicate mice without genital pathology, and the percentages represent maximum numbers of mice that demonstrated pathology. Each symbol represents a single animal. (C) HSV-2 shedding was calculated using a Vero cell-based plaque assay, and data represents the viral loads (means ± SEMs) over 6 days. Data were analyzed using an unpaired t-test with 95% confidence interval (*$p<0.05$). Data shown is representative of three independent experiments with similar results.

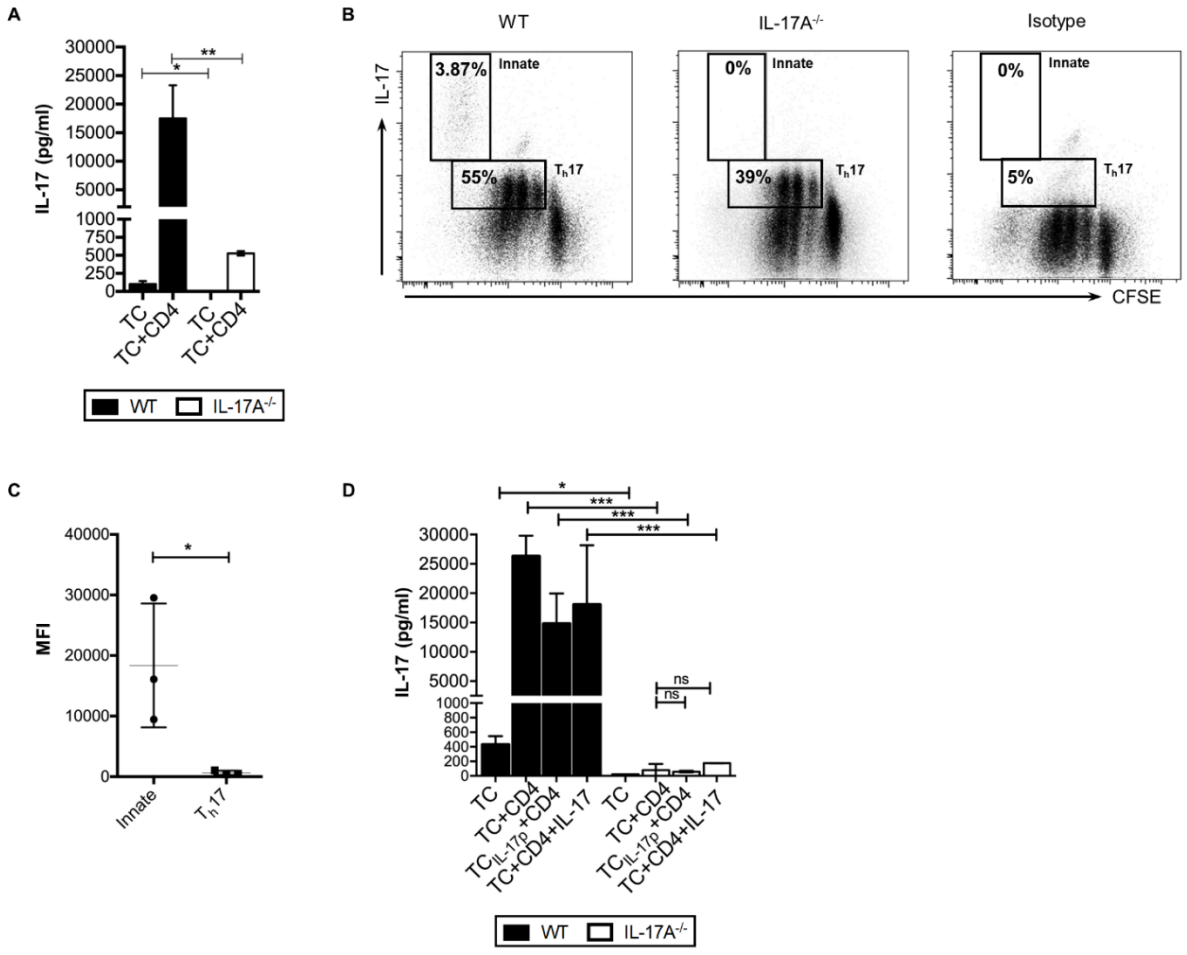

Figure 2: IL-17 produced by innate sources in the FGT is critical for potentiating T_h17 responses primed by vaginal APCs. Vaginal cells from hormone cycle-matched WT (C57BL/6) or *IL-17A*$^{-/-}$ mice (n=5) were pooled, pulsed with chicken OVA peptide, and cultured alone (TC) or co-cultured at a 1:1 ratio with CFSE-stained OT-II Tg CD4$^+$ T cells (TC+CD4) for 3.5 days. (A) IL-17 levels in culture supernatants were measured by ELISA. (B) On day 2 of co-culture, a cell stimulation cocktail containing Golgi inhibitors and PMA plus ionomycin was added, and 18 h later, co-cultures were stained with antibodies against CD3, CD4 and IL-17, and analyzed by flow cytometry to identify IL-17$^+$ cells and measure median fluorescence intensity (MFI). Upper box indicates CFSE$^-$, IL-17-producing cells in culture. The lower box indicates CFSE$^+$, IL-17-producing CD4$^+$ T cells, with a lower cut-off which excludes 95% of isotype control CSFE$^+$ cells. (C) The difference in MFI was compared between innate and T_h17 sources of IL-17 (n=3 independent experiments). Data were analyzed using an unpaired t-test with 95% confidence interval (*$p<0.05$). **(**D**)** Similar co-cultures were conducted, with 250 ng/ml of recombinant IL-17 (rIL-17) added during the peptide pulse stage ($TC_{IL\text{-}17p}$+CD4) and washed away before co-culture, or added on day 1 of co-culture (TC+CD4+IL-17) and remained present throughout the duration of the

experiment, as indicated on the x-axis. IL-17 levels in culture supernatants were measured by ELISA. Data in (A) and (D) is mean ± SEM of three individual wells per co-culture condition. Data is representative of three independent experiments with similar results. Significance was calculated by two-way ANOVA (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ns=no significance).

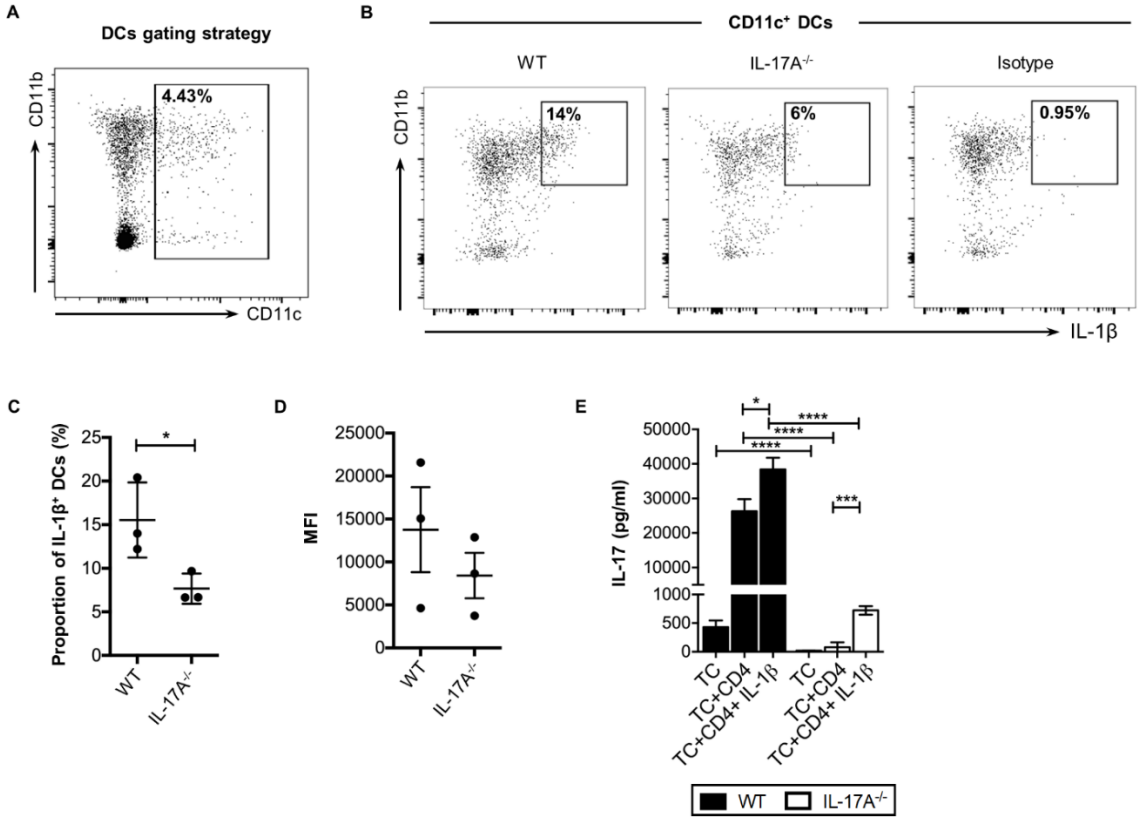

Figure 3: Vaginal CD11c$^+$ DCs from *IL-17A*$^{-/-}$ mice are impaired in potentiating T$_h$17 responses due to diminished IL-1β production. Vaginal cells from hormone cycle-matched WT or *IL-17A*$^{-/-}$ mice (n=5) were pooled, cultured overnight without any additional stimulation, stained with antibodies against CD11c, CD11b and IL-1β, and analyzed by flow cytometry to identify IL-1β$^+$ cells, with dead cells excluded. (A) CD11c$^+$ DCs were gated as shown, and (B) IL-1β expression by vaginal CD11c$^+$ CD11b$^+$ DCs from WT and *IL-17A*$^{-/-}$ mice was compared with isotype control. (C) The differences in percentage and (D) median fluorescence intensity (MFI) of IL-1β$^+$ DCs were compared between vaginal cells from WT and *IL-17A*$^{-/-}$ mice (n=3 independent experiments). Data were analyzed using an unpaired t-test with 95% confidence interval (*p<0.05). (E) Vaginal cells from hormone cycle-matched WT or *IL-17A*$^{-/-}$ mice (n=5) were pooled, pulsed with chicken OVA peptide, and cultured alone (TC) or co-cultured at a 1:1 ratio with OT-II Tg CD4$^+$ T cells (TC+CD4) for 3.5 days. Alternately, 100 ng/ml of recombinant IL-1β (rIL-1β) was added on day 1 of co-culture (TC+CD4+IL-1β). IL-17 levels in culture supernatants were measured by ELISA. Data is mean ± SEM of three individual wells per co-culture condition. Data is representative of three independent experiments with similar results. Significance was calculated by two-way ANOVA (*p<0.05, ***p<0.001, ****p<0.0001).

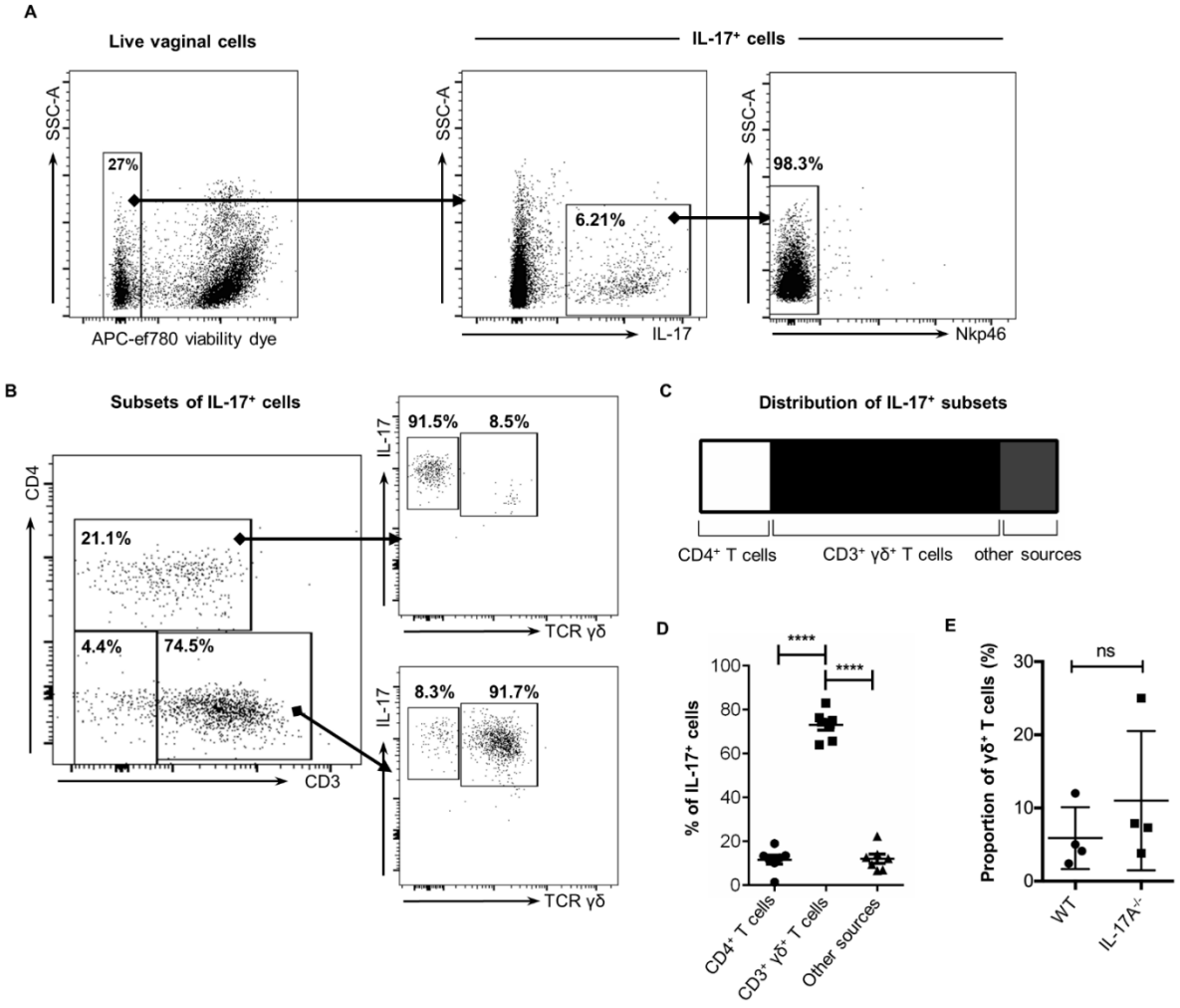

Figure 4: IL-17 in the FGT is predominantly produced by γδ⁺ T cells. Vaginal cells from hormone cycle-matched WT (C57BL/6) or *IL-17A*$^{-/-}$ mice (n=5-7) were isolated, pooled, and stimulated with a cell stimulation cocktail containing Golgi inhibitors and PMA plus ionomycin for 18 h. Cells were stained with antibodies against CD3, CD4, NKp46, TCR γδ and IL-17 and analyzed by flow cytometry to identify IL-17^{+} cells. (A) Dead cells were excluded and total IL-17^{+} cells were gated. (B) Subsets of IL-17^{+} cells were further gated based on CD3 and CD4 expression, and each subset was examined for TCR γδ expression. (C) The distribution of cell subsets among all IL-17-producing cells were examined, and (D) quantified in five independent experiments. Data is mean ± SEM and significance was calculated by one-way ANOVA (****p<0.0001). (E) The difference in percentage of γδ^{+} cells was compared between WT and *IL-17A*$^{-/-}$ mice from four independent experiments. Data were analyzed using an unpaired t-test with 95% confidence interval (ns=no significance). Data is representative of four independent experiments with similar results.

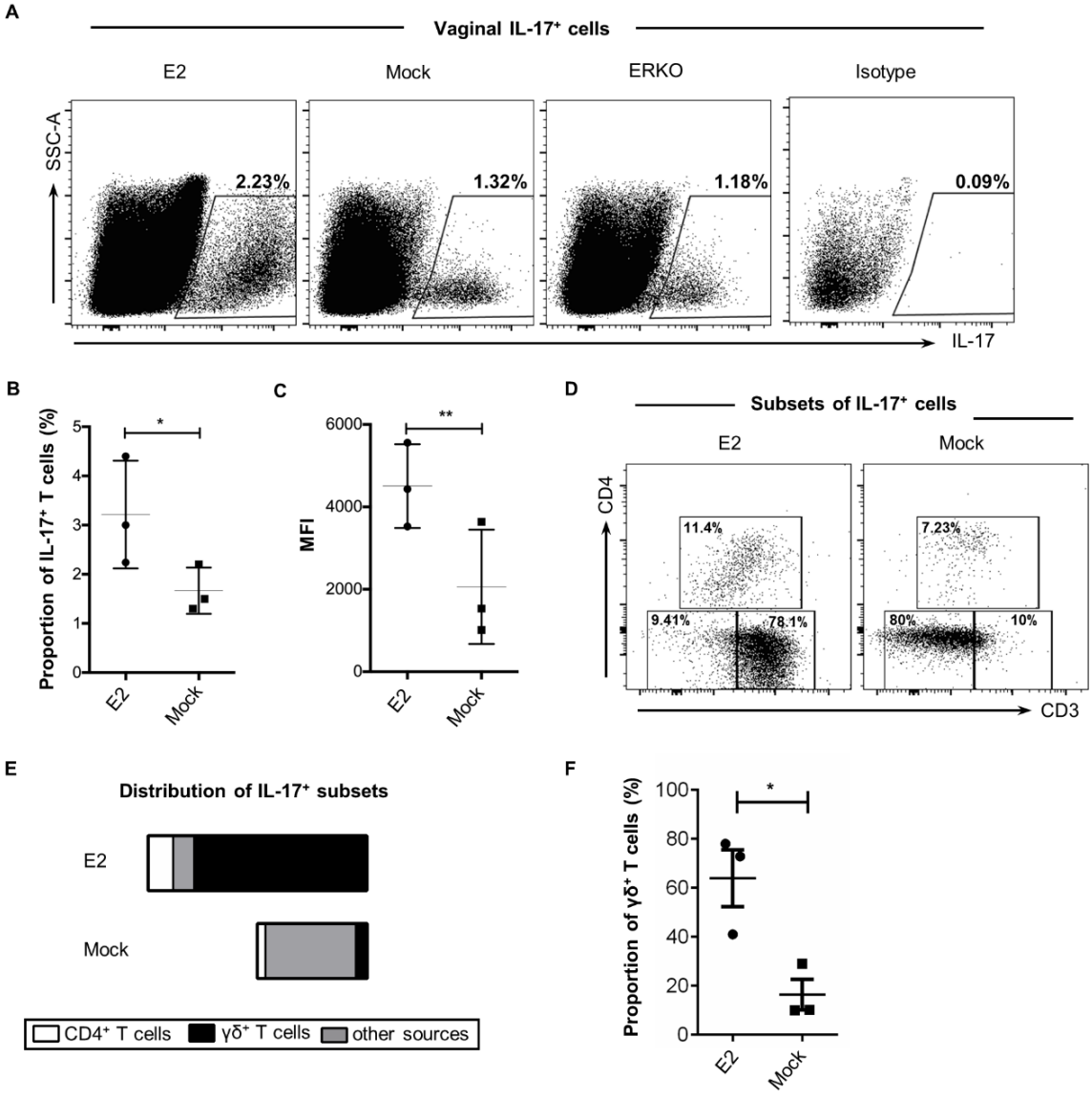

Figure 5: E2 induces IL-17-producing γδ$^+$ T cells in the FGT. Vaginal cells from OVX WT (C57BL/6) mice treated with estradiol (E2) or placebo (mock) pellets (n=5) were isolated, pooled, and stimulated with a cell stimulation cocktail containing Golgi inhibitors and PMA plus ionomycin for 18 h. (A) Cells were stained with antibodies against CD3, CD4 and IL-17, and analyzed by flow cytometry. ERKO mice were used as additional controls. Dead cells were excluded and total IL-17$^+$ cells were gated. (B) The differences in percentage and (C) median fluorescence intensity (MFI) of IL-17$^+$ cells were compared between E2 and mock mice from three independent experiments. Data were analyzed using the unpaired t-test with 95% confidence interval (*p<0.05, **p<0.01). (D) Subsets of IL-17$^+$ cells were further identified based on CD3 and CD4 expression. (E) The distributions

of the cell subsets among all IL-17-producing cells comparing E2-treated and mock-treated mice. (F) The difference in percentage of IL-17-producing $\gamma\delta^+$ cells was compared between E2 and mock mice from three independent experiments. Data is mean ± SEM and significance was calculated using an unpaired t-test with 95% confidence interval (*$p<0.05$). Data is representative of three independent experiments with similar results.

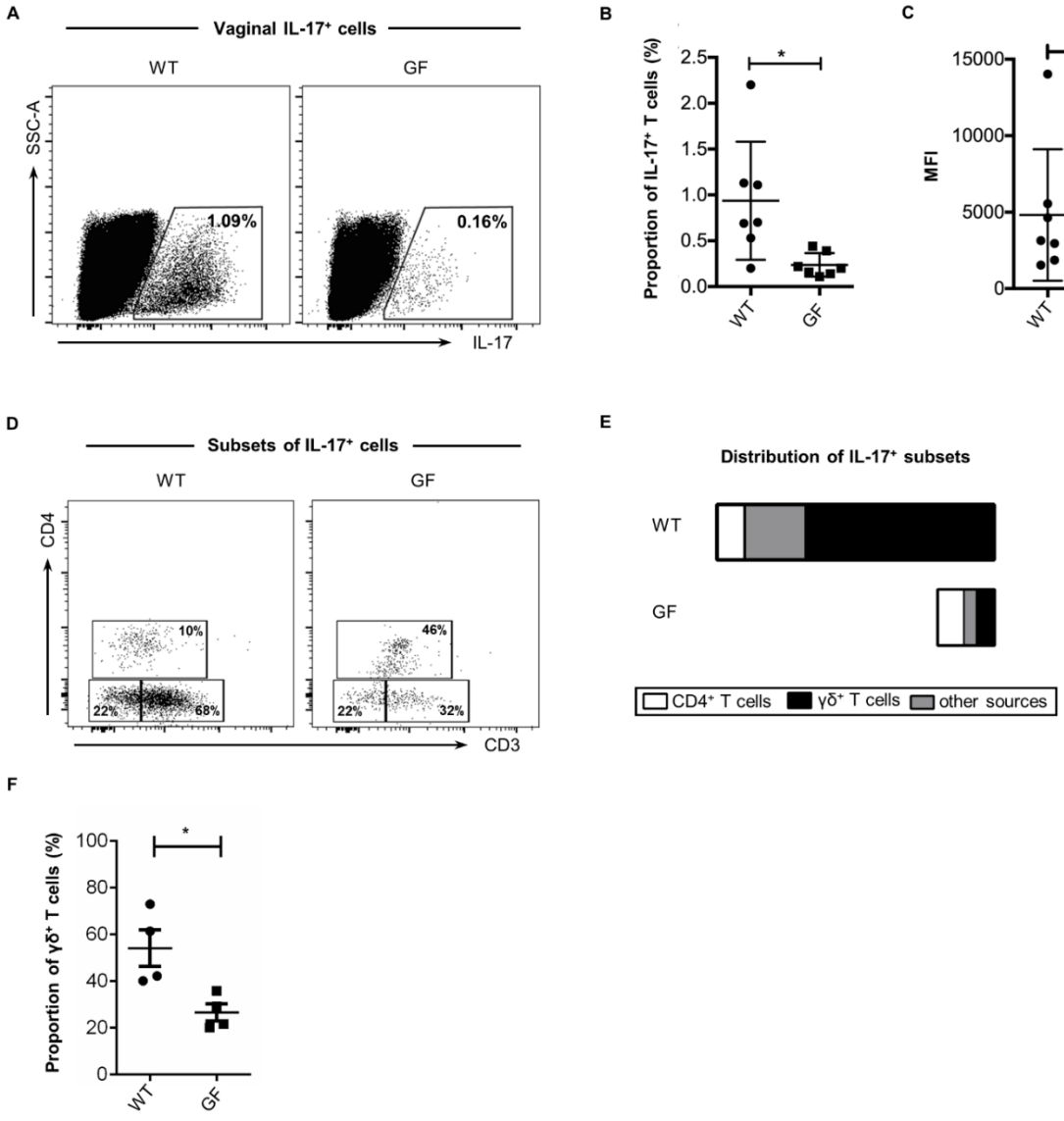

Figure 6: Microbiota induces IL-17-producing γδ⁺ T cells. Vaginal cells from hormone cycle-matched WT (C57BL/6) or germ-free (GF) mice (n=5) were isolated, pooled, and stimulated with a cell stimulation cocktail containing Golgi inhibitors and PMA plus ionomycin for 18 h. (A) Cells were stained with antibodies against CD3, CD4 and IL-17, and analyzed by flow cytometry. Dead cells were excluded and total IL-17$^+$ cells were gated. (B) The differences in percentage and (C) median fluorescence intensity (MFI) of IL-17$^+$ cells were compared between GF and WT mice from seven independent experiments. Data were analyzed using the unpaired t-test with 95% confidence interval, with the ROUT method used to identify outliers (*$p<0.05$, **$p<0.01$). (D) Subsets of IL-17$^+$ cells were further identified based on CD3 and CD4 expression. (E) The distributions of the cell subsets among all IL-17-producing cells comparing WT and GF mice. (F) The

difference in percentage of IL-17-producing $\gamma\delta^+$ cells was compared between WT and GF mice from four independent experiments. Data is mean ± SEM and significance was calculated using an unpaired t-test with 95% confidence interval (*$p<0.05$). Data is representative of four independent experiments with similar results.

CHAPTER 4: Estradiol enhances anti-viral CD4$^+$ tissue-resident memory T cell responses following mucosal vaccination through an IL-17-mediated pathway

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In this study, we examined the protective effects of E2 following mucosal immunization. We found that post-IN immunization with HSV-2, E2 treatment resulted in greater establishment of CD4$^+$ memory T cells in the URT, as well as enhanced establishment of CD4$^+$ T_{RM} cells in the FRT. The anti-viral CD4$^+$ T_{RM} cells in E2-treated mice were able to protect against subsequent genital HSV-2 challenge. Furthermore, IFN-γ^+ CD4$^+$ T_{RM} cells observed in E2-treated mice were found to be enhanced through an IL-17-dependent pathway. Overall, in this work we described a novel role for E2 in improving CD4$^+$ memory T cell responses.

Dr. Charu Kaushic and I conceived and designed the experiments. Ramtin Ghasemi, Joshua McGrath, Danya Thayaparan, Emma Yu and I performed the experiments. Drs. Andrew Brooks and Martin Stämpfli provided materials and helped with analysis. Dr. Charu Kaushic and I analyzed and interpreted the data, and wrote and edited the manuscript.

ABSTRACT

It is well known that hormones modulate immunity, thereby affecting susceptibility to infections and impacting vaccine efficacy. Estradiol (E2) is a female sex hormone which has shown to be protective against viral infections, including herpes simplex virus type 2 (HSV-2). However, few studies have examined the underlying mechanisms by which this occurs. Here, we investigated the effect of E2 on the establishment of memory T cell responses following mucosal vaccination. $CD4^+$ T cell responses were compared between E2-treated and placebo-treated female mice following intranasal (IN) immunization with an attenuated strain of HSV-2. Immediately following immunization, E2-treated mice had greater and earlier IL-17^+ $CD4^+$ T cell responses in the upper respiratory tract (URT), which preceded augmented IFN-γ responses. These enhanced memory populations persisted in E2-treated mice for up to 4 weeks post-immunization. IN immunization also resulted in the establishment of $CD4^+$ memory T cells in the female reproductive tract, and E2-treated mice had greater vaginal IFN-γ^+ and IL-17^+ $CD4^+$ tissue-resident memory T cells (T_{RM} cells) compared to untreated mice. The $CD4^+$ T_{RM} cells in E2-treated mice were sufficient for protection against subsequent genital HSV-2 challenge, even in the absence of migrating effector T cells. Finally, the enhanced vaginal IFN-γ^+ $CD4^+$ T_{RM} cells were found to be modulated through an IL-17-dependent pathway, as E2-treated IL-17A deficient mice had impaired establishment of these cells. Overall, this study describes a novel role for E2 in enhancing $CD4^+$ memory T cell responses in mucosal tissues, and provides insight on effective strategies for generating optimal immunity during vaccination.

INTRODUCTION

One of the major biological factors that influences susceptibility to infection in women is the presence of female sex hormones. Sex hormones play a major role in the development of the reproductive system by exerting profound effects on cell growth, development, differentiation and homeostasis (1). The natural presence of endogenous hormones, such as estrogen (E2) and progesterone (P4), as well exogenous administration of hormonal contraceptives, have been shown to regulate susceptibility to sexually transmitted infections (STIs) such as herpes simplex virus type 2 (HSV-2). Hormones can directly influence immune responses in the female reproductive tract (FRT), the primary site of heterosexual transmission of most STIs, as both innate and adaptive immune cells express receptors for E2 and P4 (2-4). Interestingly, we and others have shown that while P4 and P4-based hormonal contraceptives are associated with increased susceptibility and transmission of sexually transmitted viruses, E2 is generally considered protective (5-12). In early studies, we showed that mice immunized under the influence of E2 were completely protected from subsequent genital HSV-2 challenge, with limited viral shedding and genital pathology (10, 11). Others have also reported a protective role for E2 *in vivo* when examining susceptibility to viral infections (13-17). However, the underlying mechanisms by which E2 mediates protection remain unclear. Better understanding of these mechanisms can provide greater insight on how to protect against HSV-2, which is critical because there is currently no cure or viable vaccine available for HSV-2.

HSV-2, the virus which causes genital herpes, is one of the most common STIs in the world, with over 400 million individuals infected globally and approximately 17 million

new infections occurring each year (18). Following initial infection, HSV-2 establishes life-long latency within the dorsal root ganglia, and can be reactivated throughout the lifetime of the infected individual (19). Similar to other STIs, rates of HSV-2 infection are disproportionally higher in women compared to men; globally, approximately 14.8% of women are infected compared to 8.0% of men (18). While previous attempts at HSV-2 vaccine development have primarily focused on eliciting antibody responses for protection through systemic routes of immunization, limited success has been observed in clinical trials using these strategies (20, 21). Instead, studies have shown that T helper 1 (T_h1) immunity and the production of interferon gamma (IFN-γ) are critical for protection (22-24), and that compared to systemic immunization, mucosal vaccination generates more balanced immunity in mucosal sites (25-28). In the context of HSV-2, studies have shown that intranasal (IN) immunization provides better protection against subsequent challenge in the FRT compared to systemic immunization, and similar protection as observed following intravaginal (IVAG) immunization (25-28). While local immunization induces the strongest immune responses, IN immunization can also generate protection at distal sites such as the FRT (29). Additionally, IN immunization is considered a more clinically relevant method of vaccination, as it more feasible and less invasive compared to other routes (30). Limited studies examining the kinetics of T cell priming and dissemination following IN immunization have shown that initial priming and activation of T cells occurs in the upper respiratory tract (URT), which includes the nasal-associated lymphoid tissue (NALT) and cervical lymph nodes (cLNs) (31-36), and then the resulting antigen-specific T cells migrate to the FRT and the iliac LNs (iLNs) which drain the FRT. However, the

precise mechanism by which IN immunization results in protection in the FRT is not yet fully understood.

Based on these findings, preventative strategies against HSV-2 which induce strong mucosal T_h1 cell immunity in the FRT should be explored. Interestingly, we recently showed that E2 treatment induced vaginal dendritic cells (DCs) to prime robust T_h17 responses following IN immunization with HSV-2, and this coincided with greater accumulation of IFN-γ^+ $CD4^+$ effector T cells post-challenge (37). Consequently, E2-treated mice were completely protected against HSV-2 challenge. This study was the first to suggest that T_h17 cells are also involved in protective anti-HSV-2 immunity, and that E2 can enhance T_h1 immunity in the FRT. In follow-up studies examining the anti-viral role of IL-17, we found that IL-17 is critical for the induction of optimal T_h1 cell responses and overall protection against genital HSV-2 challenge (38). Following HSV-2 challenge, IL-17A deficient mice (*IL-17A*$^{-/-}$ mice) had impaired T_h1 cell responses, with significantly lower proportions of vaginal IFN-γ^+ $CD4^+$ T cells, and this related to significantly greater mortality and disease severity compared to wildtype (WT) mice. Furthermore, the impaired T_h1 response in *IL-17A*$^{-/-}$ mice also coincided with smaller populations of vaginal $CD4^+$ tissue-resident memory T cells (T_{RM} cells) following IVAG immunization, suggesting that IL-17 is also involved in generating optimal T_h1 memory responses in the FRT.

T_{RM} cells are a newly described subset of non-circulating memory T cells which are retained in non-lymphoid tissues long-term (39, 40). Due to their close proximity to the site of pathogen exposure, T_{RM} cells can rapidly respond to infection and thus, represent a critical population which can be targeted to improve vaccination outcomes. While $CD8^+$

T_{RM} cells have been extensively studied, less is known about $CD4^+$ T_{RM} cells, especially in the FRT (41-44). This is partly due to the fact that unlike $CD8^+$ T_{RM} cells, which can be distinguished based on the high expression of CD103, there is no definitive marker identified for $CD4^+$ T_{RM} cells (45). Iijima and Iwasaki recently described a protective role for $CD4^+$ T_{RM} cells in the FRT against lethal HSV-2 infection, where immunized mice relying only on circulating memory T cells succumbed to infection, while those with $CD4^+$ T_{RM} cells were completely protected (46). Interestingly, these $CD4^+$ T_{RM} cells, were found to be absent following IN immunization. However, in a separate study, an IFN-γ^+ $CD4^+$ T cell population was observed in the FRT up to 3 weeks post-IN immunization (27); albeit, these cells were not characterized as T_{RM} cells. As such, further work is required to confirm the establishment of vaginal $CD4^+$ T_{RM} cells following IN immunization.

The purpose of the current study was to examine if E2-mediated protection against HSV-2 could be attributed to greater establishment of $CD4^+$ memory T cell responses post-immunization. To begin with, we monitored the kinetic of the $CD4^+$ T cell response in E2-treated and placebo-treated ovariectomized (OVX) female mice following IN vaccination with an attenuated strain of HSV-2. At early time points immediately following immunization (day 3), E2-treated mice first demonstrated greater IL-17^+ $CD4^+$ T cell responses in the URT, which preceded enhanced IFN-γ responses observed shortly thereafter (day 7). Similar results were observed in the FRT, where E2 treatment appeared to only enhance IL-17^+ $CD4^+$ responses (day 7), and this coincided with greater IFN-γ responses observed at a later time (day 28). We next examined the establishment of $CD4^+$ memory T cells at 4 weeks post-immunization in these tissues, and found greater

frequencies of $CD4^+$ memory T cells in the URT and FRT of E2-treated mice. These populations were further characterized based on the expression of several memory markers and we found that IN immunization resulted in the establishment of T_h1 and T_h17 T_{RM} cells in the FRT, which were enhanced in the presence of E2. Interestingly, the enhanced IFN-γ^+ $CD4^+$ T_{RM} cells appeared to be dependent on IL-17, as E2-treated *IL-17A*$^{-/-}$ mice had impaired T_h1 T_{RM} cell populations in the FRT. Finally, we tested the anti-viral efficacy of $CD4^+$ T_{RM} cells by treating immunized mice with the drug Fingolimod (FTY720), which prevented the recruitment of memory T cells from lymphoid organs into the FRT prior to IVAG challenge with WT HSV-2. Even without the help of other migrating memory T cells, E2-treated mice were completely protected against HSV-2 challenge, thus demonstrating that $CD4^+$ T_{RM} cells in the FRT were sufficient for protection. Overall, our data suggests that E2 can enhance $CD4^+$ memory T cell responses, and our findings showcase a novel immunological mechanism by which E2 mediates greater protective outcomes during vaccination.

MATERIALS AND METHODS

Mice

C57BL/6 female mice were obtained from Charles River Laboratories Inc. (Saint-Constant, Quebec, Canada). *IL-17A*$^{-/-}$ mice (C57BL/6 background) were generated by Dr. Yoichiro Iwakura (University of Tokyo, Tokyo, Japan) and gD-II transgenic mice were kindly provided by Dr. Andrew G. Brooks (University of Melbourne, Australia), and bred in the Central Animal Facility (McMaster University, Hamilton, Canada). The gD-II mice express transgenes encoding for the T cell antigen receptor (Vα3.2/Vβ2) specifically recognizing the HSV gD-derived epitope, $gD_{315-327}$ (47-49). All mice were maintained under specific pathogen-free and standard temperature-controlled conditions that followed a 12h light/dark cycle. To ensure that mice remained specific pathogen-free, routine quality assurance was done by serology and PCR and this included testing dirty bedding sentinels, direct resident animals and exhaust air duct samples of racks. All animal studies were approved by, and in compliance with, the Animal Research Ethics Board at McMaster University in accordance with the Canadian Council of Animal Care guidelines.

Ovariectomy surgeries

Endogenous hormones in mice were depleted by OVX, according to previously published protocols (11). Briefly, mice were administrated an injectable anesthetic preparation of Ketamine and Xylazine intraperitoneally. Ovaries were removed by making two bilateral incisions, followed by small incisions through the peritoneal wall, and excised through the incisions. Incisions were closed using surgical clips and mice recovered for 10-14 days before the start of experiments.

Hormone treatment

To examine the effect of E2, mice were treated with 17β-estradiol hormone pellets (0.010 mg/pellet) produced by Innovative Research of America (Sarosota, FL, USA), according to previously published protocols (11). These hormone pellets are designed to release 476 ng/day/mouse for 21 days. Briefly, two weeks following OVX surgery, mice were anesthetised with injectable anesthetic (Ketamine and Xylazine) and implanted subcutaneously with 17β-estradiol or placebo (mock) pellets. The level of serum E2 resulting from the pellets has previously been shown to correspond to that measured during the estrous cycle (50).

Intranasal immunization

OVX mice were intranasally immunized with an attenuated strain of HSV: thymidine kinase-deficient (TK^-) HSV-2. Mice were lightly anesthetized using isoflurane and then inoculated with 5 μl of TK^- HSV-2 (10^4 PFU/mouse) into each nare with a micropipette, for a total of 10 μl.

FTY720 treatment

FTY720 treatment was used to prevent T cell circulation into the FRT. FTY720 (Sigma-Aldrich, St. Louis, MO) was administered via drinking water pre- and post-HSV-2 challenge. Briefly, three weeks following IN immunization with TK^- HSV-2, mice were given drinking water containing 4 μg/mL of FTY720 or PBS daily for 10 days prior to IVAG challenge with WT HSV-2. FTY720 treatment was then continued post-challenge, for the duration of the experiment.

Intravaginal challenge

In FTY720 experiments, immunized mice were challenged intravaginally with WT HSV-2. Briefly, 4 weeks after IN immunization and following initiation of FTY720 treatment, mice were anaesthetized intraperitoneally and intravaginally infected with 10 µl of WT HSV-2 strain 333 ($5x10^3$ PFU/mouse). After inoculation, mice were placed on their backs for approximately 30-45 minutes to allow for the inoculum to infect the vaginal tract.

Collection of vaginal washes

Vaginal washes were collected for up to 6 consecutive days post-challenge by pipetting 30 µl of phosphate-buffered saline (PBS) into and out of the vagina 5-6 times. This was repeated twice to collect a total volume of 60 µl, and stored at -80ºC until required.

Genital pathology scoring

Genital pathology was monitored daily post-challenge based on a five-point scale, as described previously (38): no infection (0), slight redness of external vagina (1), swelling and redness of vagina (2), severe swelling and redness of vagina and surrounding tissues (3), genital ulceration with severe redness and hair loss (4), and severe ulceration extending to surrounding tissues, ruffled hair, and lethargy (5). Animals were sacrificed before they reached stage 5.

Viral titration

Viral shedding in vaginal washes was determined by conducting viral plaque assays on Vero cell (ATCC, Manassas, VA) monolayers, as described previously (38). Vero cells were grown in α-MEM (Gibco Laboratories, Burlington, ON, Canada) supplemented with

5% FBS (Gibco Laboratories), 1% penicillin-streptomycin (Invitrogen, Burlington, ON, Canada), L-glutamate (BioShop Canada Inc., Burlington, ON, Canada) and 1% HEPES (Invitrogen). Cells were grown to confluency in 12-well plates, and washes were diluted in α-MEM and then added to monolayers. Infected monolayers were incubated at 37°C for 2h. Infected monolayers were then overlaid with α-MEM, and infection was allowed to occur for 48h at 37°C. Cells were then fixed and stained with crystal violet, and viral plaques were enumerated under a microscope. The number of PFU/ml was calculated by taking a plaque count for every sample and accounting for the dilution factor.

Whole blood analysis

For peripheral blood analysis, whole blood samples were collected, processed using Ammonium-Chloride-Potassium (ACK) lysing buffer (Sigma Aldrich), and stained for cell surface markers using the following antibodies at concentrations based on manufacturer specification sheets: CD4 BV421 (BioLegend, San Diego, CA, USA), CD3 PE-cf594 (BD Biosciences, Mississauga, ON, Canada) and CD45 BV786 (BD Biosciences). Cells were incubated with these antibodies for 30 minutes, before being analyzed by flow cytometry.

Single-cell preparation and cultures

For iLN and cLN analysis, samples were collected, and a single cell suspension was prepared by mechanically disrupting the tissue. For NALT samples, tissues were collected and pooled per group, and cell suspensions were prepared by gently teasing the tissue between frosted glass slides followed by filtration through a 40 μm filter (Small Parts, Miami Lakes, USA) (51, 52). The supernatants containing single cells were collected and centrifuged for 5 minutes (1500 rpm) at 4°C. For vaginal tissue analysis, vaginal tracts were

removed, pooled, cut lengthwise, washed to remove mucous and minced into 2-4 mm pieces. The vaginal tissue pieces were enzymatically 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 for 2h and filtered through a 40 μm filter, to obtain a total tissue cell suspension (53). Vaginal cell samples were then centrifuged for 10 minutes (1200 rpm) at 4°C.

Cells were then re-suspended in 1-5 mL of RPMI 1640 media supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% L-glutamine, 0.1% 2-mercaptoethanol, 1x non-essential amino acids and 1x sodium pyruvate (Gibco Life Technologies, Burlington, ON, Canada). Finally, mononuclear cells were counted and cell preparations were seeded in a 24-well plate at $3x10^6$ cells/well for spleen and LN samples, and $5x10^5$-$1x10^6$ cells/well for vaginal and NALT samples, and the total volume in the well was topped up to 1 mL with previously described supplemented RPMI 1640 media. Cells were either left unstimulated (brefeldin A and monensin) at 37°C or underwent *in vitro* stimulation with: 1) 2 μl/mL of cell stimulation cocktail (CSC) plus protein transport inhibitors (500X) (cocktail of PMA, ionomycin, brefeldin A and monensin (eBioscience, San Diego, CA, USA)) for 15h, 2) heat inactivated HSV-2 ($5x10^4$ PFU) for 12h, or 3) 25 μg/well of gD peptide (315-327) (Biomer Technology, Pleasanton, CA, USA) with sequence IPPNWHIPSIQDV for 12h.

Flow cytometry

Following 12-15h of incubation at 37°C, cells were collected and stained with APC-ef780 viability dye (eBioscience) for 30 minutes. Cells were washed and incubated for 5-10 minutes with 2 μl of Fc block (anti-mouse CD16/32; eBioscience) to reduce nonspecific

Fc receptor staining. Cells were then stained for cell surface markers using the following antibodies at concentrations based on manufacturer specification sheets: CD4 BV421, CD3 PE-cf594, CD8 PE-Cy7 (BD Biosciences), CD103 BV510 (BD Biosciences), CD44 AF700 (BioLegend), CD62L BB515 (BD Biosciences) and CD69 PE (BD Biosciences). Cells were incubated with these antibodies for 30 minutes, and then permeabilized and fixed using the Transcription Factor Buffer Set (BD Biosciences) following manufacturer's protocol. Cells were then stained for intracellular markers using the following antibodies: IFN-γ FITC or FITC-IgG1 isotype control, and IL-17A APC or APC-IgG2 isotype control (eBioscience). The validity of staining was verified by fluorescence minus one (FMO) controls, and/or appropriate isotype controls. Data was collected by flow cytometric analysis using a BD LSRII Flow Cytometer System (BD Bioscience Pharmigen) and results were analyzed using FlowJo software.

Statistical analysis

Statistical analysis and graphical representation was performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). The Mantel-Cox log-rank test was used to calculate significant differences in survival. Data are expressed as mean ± standard error of mean (SEM), typically derived from minimum n=3 replicates. Significance was calculated by comparing means using one-way or two-way ANOVA or t-tests, with appropriate additional tests, as indicated in the figure legends. Statistical significance was defined as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

RESULTS

E2 treatment leads to enhanced IL-17 levels following IN immunization, which precedes greater IFN-γ responses

While our previous work has examined the effect of E2 on T cell responses generated post-HSV-2 challenge, here, we were interested in understanding the effect of E2 during immunization. As mentioned previously, following IN immunization, T cell priming occurs in the URT (NALT and cLNs) and this is followed by the induction of T cell responses in the genital mucosa (31-36). Thus, to determine the effect of E2 on T cell responses following IN immunization with attenuated HSV-2 and the kinetics of that T cell response, we examined the cytokine characteristics of T cells taken at various times post-immunization from both the URT and FRT of immunized mice.

E2-treated and mock-treated mice were immunized intranasally with TK^- HSV-2 (10^4 PFU) and 3 or 7 days later, $CD4^+$ T cell responses were examined using flow cytometric analysis of cells at the site of immunization (NALT and cLNs), as well as distal tissue sites of protection (FRT and iLNs). Cells were first gated on the total lymphocyte population, followed by single cells, and finally gated on the live population (Fig. 1A). We then examined the expression of CD3 and CD4 to identify the $CD4^+$ T cells present, and functional differences were also compared based on intracellular IFN-γ and IL-17 expression. Overall, at day 3 post-immunization, the $CD4^+$ T cell responses observed were quite limited and mainly restricted to the lymph nodes (Fig. 1B and C). Studies have shown that immune responses following IN immunization are initiated in the cLNs (31-36), which

explains why there was not much of a $CD4^+$ T cell response detected in the NALT at the earlier time point (data not shown). Interestingly, a unique phenomenon was observed following E2 treatment. We found that E2 first enhanced the IL-17 response in the cLNs at day 3 (Fig. 1C), and this preceded enhanced IFN-γ responses in the cLNs observed at day 7 (Fig. 1D). Additionally, the greater IL-17 production in the cLNs was maintained at day 7 (Fig. 1E). In contrast, cells of the iLNs from E2-treated mice did not have enhanced IL-17 production compared to mock-treated mice at day 3 (Fig. 1B and C), but did show enhanced IL-17 at day 7 (Fig. 1C and E). Finally, by day 7, significant IL-17 responses from $CD4^+$ T cells were observed in the NALT and FRT. Thus, by day 7, E2 treatment appears to only enhance the IL-17 response in the FRT, and not the IFN-γ response (Fig. 1F and G). Additional experiments were then performed to determine the longer-term responses in the URT. By day 28, there were enhanced IL-17 and IFN-γ responses in the NALT and cLNs (Fig. 2B-C and 3B-C) of E2-treated mice relative to mock-treated controls.

Overall, these findings show that E2 first enhances T_h17 responses in the local cLNs and then NALT tissues, and this T_h17 response precedes an increased IFN-γ response observed at a later time. This demonstrates a kinetic of the $CD4^+$ T cell response following IN immunization in the presence of E2, starting from the draining lymph nodes at the site of immunization (cLN) to the distal tissue site of protection (FRT).

E2 enhances $CD4^+$ memory T cells in the URT following IN immunization

Since an effective vaccine strategy should result in long-term immunity, we next wanted to determine if the presence of E2 during IN immunization would affect memory T cell responses in the URT. While we have previously shown that E2 influences T cell responses in the FRT (37, 54), little is known about $CD4^+$ memory T cell populations in the URT, as well as the effect of E2 on T cell responses in these tissues.

In order to first characterize memory T cells in the URT, we looked at the expression of known memory T cell markers and examined memory T cell populations in E2-treated and mock-treated mice 4 weeks following IN immunization. In the URT, we found that prior treatment with E2 significantly enhanced the percentages and total numbers of IL-17^+ and IFN-γ^+ producing $CD4^+$ T cells in the NALT (Fig. 2A-C), as well as the cLNs (Fig. 3A-C). These populations were almost completely absent in mock-treated mice. We further examined the effect of E2 on $CD4^+$ memory T cell populations using CD44 and CD103 as memory cell markers. Again, we found that E2 enhanced the proportion and total number of both $CD44^+$ and $CD103^+$ expressing $CD4^+$ T cells in the NALT (Fig. 2D and E) and cLNs (Fig. 3D and E). Since others have shown a critical anti-viral role for $CD8^+$ memory T cells in the URT, we also looked at the effect of E2 on the total $CD8^+$ T cell population, and the IFN-γ-producing $CD8^+$ T cells. We found that E2 enhances these cells in the cLNs, but not the NALT (Fig. 2F and 3F). Taken together, these findings demonstrate that IN immunization under the influence of E2 leads to long-lasting $CD4^+$ memory T cell responses in the URT.

E2 enhances the establishment of $CD4^+$ T_{RM} cells in the FRT following IN immunization

The important result we next wanted to determine was if IN immunization with TK$^-$ HSV-2 would lead to the establishment of long-lasting CD4$^+$ memory T cells, T_{RM} cells, at the target site of protection, the FRT. This would further validate the use of IN vaccination as an effective route of immunization against HSV-2, since the CD4$^+$ T_{RM} cell population has been shown to be critical for mediating protection following immunization (27). Interestingly, previous studies have reported conflicting results regarding the generation of vaginal CD4$^+$ T_{RM} cells following IN immunization (27, 46). Concurrently, we wanted to understand if E2 treatment would enhance the establishment of anti-viral memory T cells, thus suggesting a potential mechanism by which E2 leads to better protection against HSV-2 in the FRT.

We first looked at the total CD4$^+$ T cell population in the FRT 4 weeks post-immunization and found that E2 enhanced both the percentage and total number of CD4$^+$ T cells (Fig. 4A and B). Next, we specifically examined T_{RM} cells using an extensive panel of markers and a gating strategy that has been published by others, as well as our own group (CD44$^+$ CD103$^-$ CD69$^+$ CD62L$^-$) (Fig. 4C and D) (38, 46). We found that there were greater proportions and total numbers of CD4$^+$ T_{RM} cells in the FRT of E2-treated mice compared to mock controls (Fig. 4D and E). Our gating strategy for CD4$^+$ T_{RM} cells is based on the combination of specific markers known to be present or absent on memory cells. However, as this is a relatively new field, there is still some debate regarding the importance of CD103 expression (45). At some anatomical sites, CD103 expression is a hallmark of T_{RM} cells, while other studies have shown that vaginal CD4$^+$ T_{RM} cells do not express CD103 (45). As such, we also looked separately at CD103 expression by CD4$^+$ T cells and found that

E2 also enhances the $CD103^+$ $CD4^+$ T cell population in the FRT (Fig. 4F). Similarly, as most literature surrounding T_{RM} cells in the FRT has focused on the importance of $CD8^+$ T cells, we also examined the population of $CD8^+$ T cells present. We found that $CD4^+$ T cells constitute approximately a 7-fold higher frequency of the $CD3^+$ T cell population compared to $CD8^+$ T cells in the response observed in the FRT (Fig. 4G). Additionally, there was no notable effect of E2 treatment on the small population of $CD8^+$ T cells (data not shown). Finally, for comparison we examined $CD4^+$ T cell responses in the spleen (Fig. 4H) and iLNs which drain the FRT (Fig. 4I), and found that E2 did not significantly impact the frequency of $CD4^+$ T cells at these sites, suggesting that the effect of E2 on $CD4^+$ memory T cells is tissue-specific. Overall, these results suggest that IN immunization in the presence of E2 leads to the establishment of $CD4^+$ T_{RM} cells in the FRT.

E2 increases HSV-2-specific $CD4^+$ T cells expressing IL-17 and IFN-γ and the frequency of T_h1 and T_h17 T_{RM} cells in the FRT following IN immunization

To further characterize the $CD4^+$ T_{RM} cells that develop after vaccination in the presence of E2, we examined the HSV-2 antigen-specificity and phenotype of the FRT $CD4^+$ T cells responding to HSV-2 antigen. Four weeks following IN immunization, we isolated FRT cells and stimulated them *in vitro* to induce cytokine production. To look at antigen-specific responses, we first conducted *in vitro* stimulation of the FRT cells using heat inactivated HSV-2. Although the frequency of $CD4^+$ T cells producing IFN-γ or IL-17 increased somewhat after stimulation with heat inactivated HSV-2 and was enhanced in E2-treated mice, the numbers of cytokine-producing $CD4^+$ T cells were not high enough to further characterize these memory cells based on the expression of T_{RM} markers (Fig. 5A).

We therefore used a second model involving IN immunization of gD-II transgenic mice whose $CD4^+$ T cells express transgenes encoding for the T cell receptor specific for the HSV gD-derived epitope ($gD_{315-327}$), to look at antigen-specific $CD4^+$ T cell responses (47-49). Four weeks post-IN immunization, FRT cells from gD-II mice were stimulated *in vitro* with the corresponding gD peptide. Again, there was an antigen-specific response of IFN-γ^+ or IL-17^+ producing $CD4^+$ T cells that was enhanced in E2-treated mice. However, we were unable to do any further phenotypic analysis with markers related to T_{RM} cells due to the small numbers of antigen-specific T cells (Fig. 5A). Finally, we used a cell stimulation cocktail (CSC) consisting of PMA and ionomycin to stimulate the cells, and this resulted in significant production of the target cytokines. We have previously used this method to examine T cell responses in other published work (37, 38, 54). To validate that the CSC-stimulated T cell responses resulted from the immunization with HSV-2, we also stimulated FRT cells from non-immunized (naïve) mice with CSC to measure any non-specific responses. The comparison of the cytokine response by $CD4^+$ T cells from the FRT of immunized and non-immunized mice indicated much higher frequencies (2 to 5 fold) of IL-17^+ and IFN-γ^+ producing cells from immunized mice (Fig. 5A), suggesting that CSC-stimulated cells in immunized mice mostly represent an antigen-specific response. We then used the CSC *in vitro* stimulation strategy to determine what frequency of FRT $CD4^+$ T_{RM} cells that expressed IL-17 or IFN-γ were present in immunized mice, comparing E2-treated and mock-treated mice. We found that E2-treated mice had greater frequencies and total numbers of both IL-17^+ and IFN-γ^+ producing $CD4^+$ T_{RM} cells in the FRT compared to

mock-treated mice (Fig. 5B-E). These findings indicate that E2 enhances antigen-specific T_h1 and T_h17 T_{RM} cells in the FRT following IN immunization.

E2 augments T_h1 T_{RM} cells in an IL-17-dependent manner

We have previously shown that E2-mediated protection coincides with greater T_h17 responses in the FRT, and that T_h1 responses in the FRT are significantly reduced in the absence of IL-17 (37, 38). Here, we have seen that in E2-treated mice, greater induction of T_h17 responses preceded enhanced T_h1 immunity. Therefore, to understand the importance of IL-17 in the establishment of T_h1 T_{RM} cells, we examined the $CD4^+$ T cell responses in *IL-17A*$^{-/-}$ mice to see if the absence of IL-17 would impact the establishment of IFN-γ^+ T_{RM} cells.

First, we determined if E2 specifically enhanced IL-17^+ $CD4^+$ memory T cells in the FRT. We looked at the total population of IL-17-secreting $CD4^+$ T cells in the FRT of E2-treated WT mice 4 weeks following IN immunization, and gated for CD44, the primary T_{RM} cell marker used in the analysis of these memory cells. We found that approximately 64% of all IL-17^+ producing $CD4^+$ T cells were $CD44^+$ $CD103^-$ (Fig. 6A) memory cells, suggesting that the remaining 36% of the T_h17 cells were not part of the typical population of T_{RM} cells. However, when we included the $CD44^+$ T cells which also expressed $CD103^+$ in a separate analysis, 80% of the cells expressed $CD44^+$ and $CD103^+$ (Fig. 6B). Overall, it appears that majority of the T_h17 cells found in the FRT of E2-treated mice following IN immunization are memory cells, and only a small proportion of these cells are non-specifically enhanced in the presence of E2. To determine the importance of these E2-

mediated enhanced IL-17$^+$ cells in promoting greater T_h1 responses, we examined IFN-γ^+ CD4$^+$ T cells in the FRT of immunized E2-treated *IL-17A*$^{-/-}$ mice. We found that T_h1 cells were greatly reduced in these mice, compared to E2-treated WT mice (Fig. 6C). Similarly, when we looked specifically at IFN-γ^+ T_{RM} cells (CD4$^+$ CD44$^+$ CD103$^-$ CD69$^+$ CD62L$^-$), we found that the frequency of IFN-γ^+ T_{RM} cells was significantly decreased in E2-treated *IL-17A*$^{-/-}$ mice, compared to control mice (Fig. 6D). These findings suggest that IL-17 is important for E2-mediated enhancement of T_h1 T_{RM} cells in the FRT.

E2-enhanced CD4$^+$ T_{RM} cells are sufficient for protection against IVAG HSV-2 challenge

Finally, we wanted to test the anti-viral efficacy of the CD4$^+$ T_{RM} cells generated in the FRT following IN immunization and examine the ability of these cells to mediate protection against lethal IVAG HSV-2 challenge. We wanted to determine the anti-viral efficacy of these T_{RM} cells independently, without the help of any circulating memory T cells which might migrate to the tissue upon HSV-2 challenge. To accomplish this, we used the drug Fingolimod, an antagonist of sphingosine-1-phosphate receptor 1 (S1PR1), which blocks lymphocyte egress from lymph nodes (55). FTY720 binds to S1PRs on lymphocytes causing the receptors to internalize and degrade, which results in accumulation of lymphocytes in secondary lymphoid organs, and leads to depletion of lymphocytes from circulation. Thus, FTY720 treatment prevents the circulation of T cells, and allows us to study the ability of T_{RM} vaginal cells to respond to HSV-2 challenge independently of migrating subsets of memory T cells.

Intranasally immunized mice were administered FTY720 or PBS daily via drinking water for 10 days prior to IVAG challenge with WT HSV-2 ($5x10^3$ PFU). To ensure circulating memory T cells were no longer present, peripheral blood was collected on day 9 of FTY720 treatment, one day prior to challenge. To verify the effectiveness of the drug, we examined the number of $CD4^+$ T cells present in the blood. Mice administered FTY720 had significantly depleted $CD4^+$ T cells in circulation compared to mice which received PBS (Fig. 7A). Both the frequencies and total numbers of $CD4^+$ T cells were significantly lower in FTY720-treated animals, compared to PBS-treated controls (Fig. 7B). Once we confirmed the effectiveness of the drug, mice were challenged intravaginally with WT HSV-2 and daily FTY720 treatment continued, while survival, viral shedding and genital pathology were monitored. We found that similar to E2-PBS control mice, E2-FTY720 mice, which must rely primarily on T_{RM} cells for protection, were completely protected against HSV-2 challenge (100% survival) (Fig. 7C). E2-treated mice administered FTY720 or PBS also had limited genital pathology (Fig. 7D) and significantly lower viral shedding (Fig. 7E and F), compared to mock-treated groups. Both pathology and viral shedding were comparable in both E2-treated groups. Likewise, both mock-FTY720 and mock-PBS groups had similar disease outcomes as well; both groups succumbed to HSV-2 challenge by day 12 (Fig. 7C), all mice developed genital pathology (Fig. 7D), and both groups had similar levels of viral shedding (Fig. 7E and 7F). Overall, both E2-treated groups were better protected against HSV-2 challenge compared to mock groups. These results demonstrate that in the presence of E2, even without the help of any of migrating memory T cells (E2-FTY720), $CD4^+$ T_{RM} cells generated in the FRT following IN HSV-2

immunization are sufficient for protection against subsequent IVAG HSV-2 challenge. This suggests that the enhanced establishment of vaginal $CD4^+$ T_{RM} cells observed in E2-treated mice may contribute to E2-mediated protection against HSV-2.

DISCUSSION

Following immunization, the establishment of $CD4^+$ T_{RM} cells helps generate rapid protection against pathogen re-exposure. In the context of HSV-2 infection, the generation of long-lasting, mucosal T_h1 memory responses is especially critical for effective vaccine strategies. Interestingly, we recently demonstrated that E2 is able to protect against genital HSV-2 infection by differentially priming $CD4^+$ T cells and inducing greater T_h1 cells in the FRT (37). In the current study, we wanted to further investigate the mechanisms underlying the protective effects of E2, and so we examined the effect of E2 on the establishment of memory T cell responses following IN immunization with TK^- HSV-2. We found that E2 first enhanced IL-17^+ $CD4^+$ T cells in the LNs as early as 3 days post-immunization, which was followed by greater IFN-γ^+ $CD4^+$ T cells observed by day 7. Similar observations were also made in the NALT and FRT. When examining $CD4^+$ T cells in the URT and FRT at the 4 week timepoint, we found E2 treatment resulted in greater frequencies of IL-17^+ and IFN-γ^+ producing $CD4^+$ memory T cells in the URT, as well as enhanced establishment of antigen-specific $CD4^+$ T_{RM} cells in the FRT. Furthermore, IFN-γ^+ $CD4^+$ T_{RM} cells observed in E2-treated mice were enhanced through an IL-17-dependent pathway, as E2-treated *IL-17A*$^{-/-}$ mice had impaired establishment of T_h1 T_{RM} cells. Finally, the $CD4^+$ T_{RM} cells in E2-treated mice were able to protect against subsequent IVAG HSV-2 challenge, without help from any additional migrating memory T cells; thus, demonstrating that the establishment of $CD4^+$ T_{RM} cells post-IN immunization is sufficient for protection against HSV-2. To our knowledge, this is the first study to demonstrate that E2 enhances $CD4^+$ T cell memory populations.

The primary aim of this study was to better understand how E2 treatment mediates better protection against HSV-2 infection in the FRT. In our previous work, we have examined the effect of E2 on $CD4^+$ T cell responses generated following IVAG challenge (37). Here, we wanted to examine how E2 influences T cell responses during immunization. We hypothesized that E2 establishes enhanced memory responses post-immunization, which then provide greater protection against subsequent HSV-2 challenge. First, we found that following IN immunization, E2 treatment enhanced IL-17 production by $CD4^+$ T cells in the URT, and this was followed by greater IFN-γ^+ $CD4^+$ T cell responses observed at a later timepoint (Fig. 1). This was also observed in the FRT, and overall, these findings suggest that greater levels of IL-17 present in the tissue play a role in enhancing IFN-γ production by $CD4^+$ T cells. Khader et al. (56) similarly showed that in a pulmonary *Mycobacterium tuberculosis* vaccination model, IL-17^+ $CD4^+$ T cells accumulated in the lungs at earlier timepoints compared to IFN-γ^+ $CD4^+$ T cells. The influence of IL-17 on T_h1 responses has also been reported by others in the both the lungs and FRT (57-60). We showed *in vitro* that vaginal DCs cells isolated from *IL-17A*$^{-/-}$ mice were significantly impaired at inducing IFN-γ production by $CD4^+$ T cells, compared to those from WT mice; thus, suggesting there is an intrinsic impairment in the priming of T_h1 cell responses by vaginal DCs in the absence of IL-17 (37). This was confirmed *in vivo*, where we found there was a significant decrease in IFN-γ production by vaginal $CD4^+$ T cells in *IL-17A*$^{-/-}$ mice post-HSV-2 challenge (38). Likewise, Bai et al. (2009) demonstrated that mice treated with anti-IL-17 antibodies had significantly delayed clearance of *Chlamydia muridarum* in the lungs, which corresponded with significantly reduced T_h1 responses (57). Furthermore,

they also showed that DCs isolated from IL-17-neutralized mice induced lower levels of IFN-γ production by $CD4^+$ T cells. These findings suggest that a similar phenomenon may also be occurring in the URT following IN immunization, where E2 may be differentially priming T cells and inducing greater T_h17 responses, which then leads to enhanced T_h1 cells. Further work is required to determine the effect of E2 on T cell priming by nasal DCs.

While we have previously shown that E2 treatment induces greater T_h17 responses, which coincides with increased proportions of IFN-γ^+ $CD4^+$ cells in the FRT post-HSV-2 challenge (37), a direct correlation between enhanced IL-17 levels and greater IFN-γ responses was not established. Here, we also show greater establishment of IFN-γ^+ and IL-17^+ producing $CD4^+$ T_{RM} cells in the FRT of E2-treated mice post-IN immunization (Fig. 4 and 5). To confirm the importance of IL-17 in the mechanism by which E2 enhances T_h1 immunity, we examined $CD4^+$ memory T cells in immunized *IL-17A*$^{-/-}$ mice treated with E2. We found that E2-treated *IL-17A*$^{-/-}$ mice did not have enhanced IFN-γ^+ $CD4^+$ T_{RM} cells present in the FRT (Fig. 6C and 6D), suggesting that the mechanism by which E2 enhances T_h1 immunity in the FRT is dependent on IL-17. The importance of IL-17 in enhancing memory T_h1 responses was also shown in our previous study, where post-IVAG immunization, *IL-17A*$^{-/-}$ mice had significantly smaller populations of IFN-γ^+ $CD4^+$ T_{RM} cells in the FRT compared to WT mice (38). The precise mechanism by which IL-17 influences T_h1 memory responses is still unclear, and further work is required to elucidate this mechanism. Additionally, we showed that E2 specifically enhances T_h17 T_{RM} cells, as the majority of T_h17 cells found in the FRT following IN immunization expressed tissue-resident markers (Fig. 6A and 6B). Only a small proportion of the vaginal T_h17 cells were

non-specifically enhanced in the presence of E2. Overall, this suggests that E2 specifically induces greater T_h17 T_{RM} cells in the FRT following immunization, which are involved in enhancing T_h1 T_{RM} cells.

With no viable vaccine available for HSV-2, vaccine strategies should focus on generating balanced, long-lasting immunity in the FRT, and studies have shown that mucosal vaccination is an effective method to accomplish this (25-28). Additionally, interest in vaccine strategies which establish substantial T_{RM} cell populations has grown considerably over the past few years. Unlike other memory T cells, such as effector memory T cells and central memory T cells, T_{RM} cells respond more efficiently and rapidly to infection, primarily due to their anatomical location within non-lymphoid tissues (45). As such, vaccine strategies which establish T_{RM} cells are considered more favourable for eliciting superior protection. In this work, we used an IN immunization model for vaccination, which is a more clinically relevant route of mucosal vaccination compared to IVAG immunization. However, a limitation cited by others has been the inability of IN immunization to establish substantial, long-term $CD4^+$ T cell immunity in the FRT (46). In particular, it has been shown that vaginal $CD4^+$ T_{RM} cells are established in extremely low numbers post-IN immunization, making them especially difficult to detect and characterize. Conversely, others have reported the presence of IFN-γ-producing $CD4^+$ T cells in the FRT for up to 3 weeks post-IN immunization, although they did not characterize the subtypes of $CD4^+$ memory T cells in depth, or investigate the protective efficacy of the memory T cell population (27). In the current study, we show that mice treated with E2 establish a substantial population of antigen-specific vaginal $CD4^+$ T_{RM} cells post-IN immunization

(Fig. 4 and 5), which are able to provide protection against subsequent IVAG HSV-2 challenge (Fig. 7). These findings are the first to demonstrate the establishment of $CD4^+$ T_{RM} cells in the FRT following IN immunization, and suggest that E2 can be used to improve vaccination outcomes during IN immunization. This work also provides further support towards using IN immunization as a viable vaccination route for generating protection against infections in the FRT.

Most T_{RM} studies have focused on $CD8^+$ T_{RM} cells, however, recent studies have also started to identify subsets of $CD4^+$ T_{RM} cells, including T_h17 T_{RM} cells, which have been reported to be present in many tissues including the nose, skin and lungs (42, 44, 61-64). For instance, an elegant study by Vesely et al. (44) used IL-17A tracking-fate mouse models to show that majority of airway $CD4^+$ T_{RM} cells found during bacterial infection are derived from T_h17 cells, and that these cells are critical for protection against bacterial infection. In the context of the URT, Wilk et al. (62) identified both IL-17 and IFN-γ-secreting $CD69^+$ $CD4^+$ T_{RM} cells in the nasal tissue of mice following bacterial infection with *Bordetella pertussis*. Similarly, Allen et al. (42) found that IN immunization with a novel vaccine against *Bordetella pertussis* led to the establishment of T_h17 T_{RM} cells in the nose, which conferred long-term protection against nasal colonization and lung infection. We have now also shown that E2 can induce long-term memory populations of T_h17 cells in the URT (Fig. 2 and 3). Similarly, E2 treatment also enhanced these cells in the FRT (Fig. 5). To our knowledge, this is the first study to report the presence of T_h17 T_{RM} cells in the FRT. T_h17 cells have been shown to influence disease outcomes following bacterial, fungal and viral infection in the FRT (37, 38, 65-70). Based on the protective role of T_h17

cells observed against pathogens including HSV-2, *Neisseria gonorrhoeae* and *Candida albicans*, the ability of E2 to enhance T_h17 T_{RM} cells in the FRT should theoretically mediate better protection against these infections.

Very few studies have reported the influence of hormones on memory T cells. Within the FRT, Rodriguez-Garcia et al. (71) examined the effect of aging and changes in hormone levels on $CD8^+$ memory T cells in the endometrium and cervix. They found that with aging, there was a loss of $CD103^+$ $CD8^+$ T_{RM} cells in the cervix, while these populations were increased in the endometrium. Swaims-Kohlmeier et al. (72) examined $CCR7^{hi}$ $CD4^+$ memory T cell levels in vaginal lavage samples from healthy women, and found that proportions of these memory cells positively correlated with P4 levels; thus, suggesting that P4 enhances $CD4^+$ memory T cell populations. However, these cells were not characterized as T_{RM} cells. Conversely, Hall et al. (73) reported that P4 treatment in mice resulted in significantly reduced numbers and activity of antigen-specific $CD8^+$ T_{RM} cells in the lungs. Here, we demonstrate that E2 increases $CD4^+$ memory T cells in multiple tissues. Evidently, further work is required to understand the influence of hormones on memory T cell populations, as well as to determine the cellular mechanisms involved.

Although our work has focused on examining the influence of E2 on susceptibility to pathogens in the FRT, E2 has also been shown to be protective against viral infections in the lower respiratory tract, such as influenza infection in the lungs. Studies have shown that E2 mediates protection against influenza by supressing inflammatory responses (16), as well as by recruiting neutrophils and increasing $CD8^+$ T cell responses in the lungs (15). These studies were done in the context of primary infection of the lungs, and mainly

focused on $CD8^+$ T cell responses. However, the effect of E2 on $CD4^+$ T cell responses during influenza infection, particularly in the URT, has not been reported. Here, we show that E2 treatment leads to greater IL-17^+ and IFN-γ^+ $CD4^+$ cells in the NALT and cLNs following IN immunization (Fig. 1 and 2), suggesting that a similar phenomenon may also occur in other viral disease models. As such, it would be beneficial to determine if enhanced $CD4^+$ memory T cells generated in the URT can help prevent dissemination of pathogens from the URT into the lungs. This was shown in a study by Pizzolla et al., where influenza-specific $CD8^+$ T_{RM} cells ($CD103^+$ $CD69^+$) established in the URT following IN immunization were able to block the transmission of the virus into the lungs (74). Our findings suggest that E2 treatment can help enhance $CD4^+$ T_{RM} cells in the URT and have a similar protective effect.

In summary, our study expands on previous work regarding the mechanisms by which E2 protects against viral infections and demonstrates a novel role for E2 in enhancing $CD4^+$ memory T cell responses. We have shown that in an IN vaccination model, E2 enhances the establishment of immediate and memory IFN-γ^+ and IL-17^+ $CD4^+$ T cell responses in the URT. E2 treatment also results in greater antigen-specific $CD4^+$ T_{RM} cells in the FRT post-IN immunization, which are sufficient for protection against subsequent IVAG HSV-2 challenge. Furthermore, E2-mediated enhancement of T_h1 T_{RM} cells was shown to be dependent on IL-17. Overall, these findings demonstrate a novel mechanism by which E2 protects against HSV-2 infection, where the presence of E2 during IN immunization leads to greater establishment of memory T cells, including $CD4^+$ T_{RM} cells, in the FRT. As such, when mice are then exposed to HSV-2 in the FRT, there is a rapid and

robust $CD4^+$ T cell response which protects against infection. This work has important implications in terms of vaccine development, as an effective vaccination strategy should aim to generate rapid, long-lasting immunity. We propose that HSV-2 vaccine strategies which incorporate mucosal immunization in conjunction with E2, may promote effective T_h1 memory responses and generate better overall vaccine efficacy.

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FIGURES

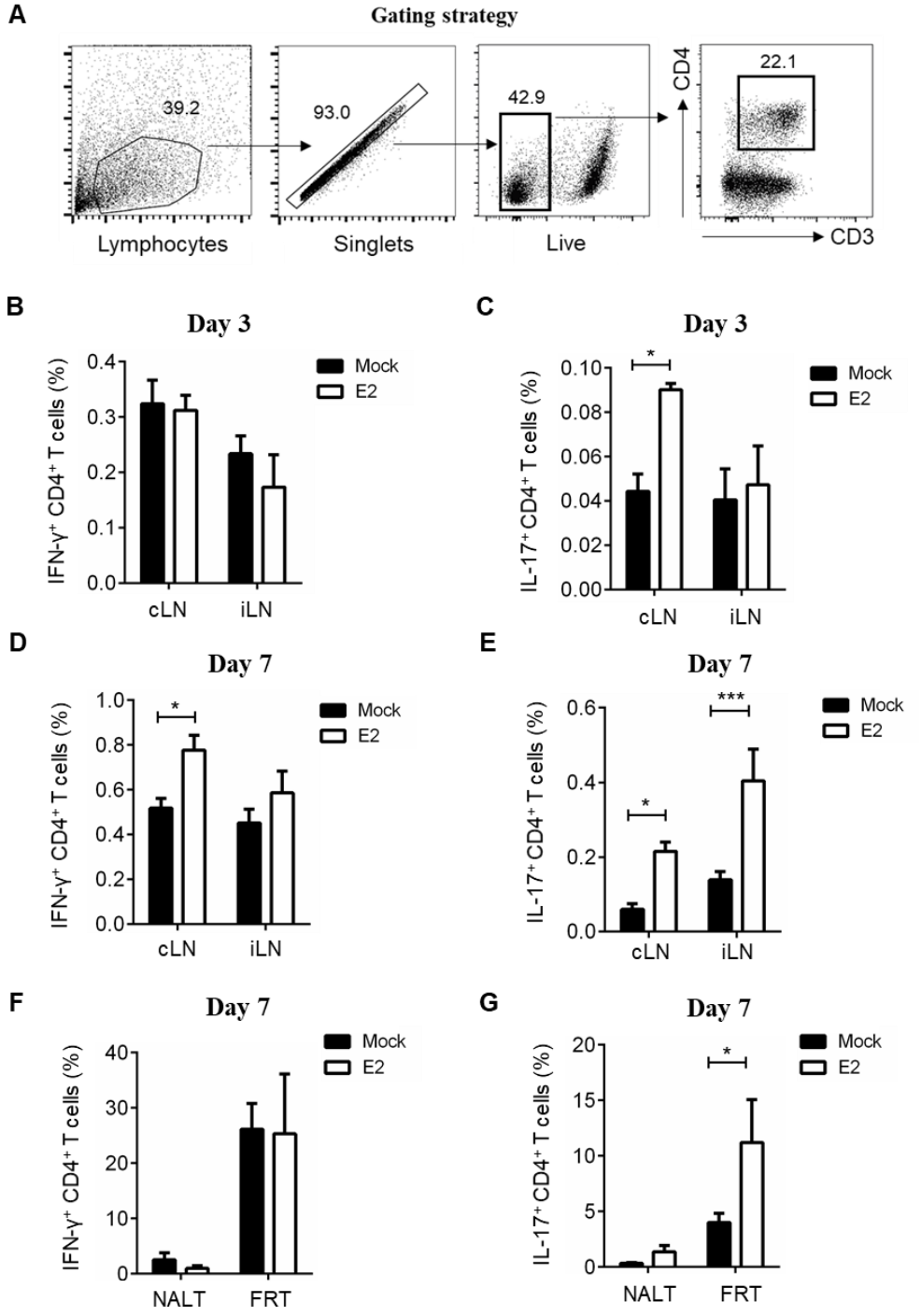
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Figure 1: Enhanced IL-17 levels precede IFN-γ response in E2-treated mice following IN immunization. OVX WT (C57BL/6) mice (n=5-10/group) were implanted with E2 or placebo (mock) pellets, and immunized intranasally 2 weeks later with TK^- HSV-2 (10^4 PFU/mouse). 3 and 7 days post-immunization, cervical lymph nodes (cLN), iliac lymph nodes (iLN), nasal-associated lymphoid tissue (NALT) and vaginal tissue (FRT) were collected and processed. NALT or FRT tissue were pooled within groups. Cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) for 15h, and then stained with a panel of antibodies (CD3, CD4, IL-17 and IFN-γ), and examined by flow cytometry. (A) Cells were gated based on the lymphocyte population, followed by single cells and then $CD4^+$ T cells were gated among total live $CD3^+$ T cells. The differences in the percentages of $CD3^+$ $CD4^+$ cells that were IFN-γ^+ (B and D) and IL-17^+ (C and E) in the cLN and iLN were compared between E2-treated and mock-treated mice at day 3 and (D and E) day 7 post-immunization. The differences in the percentages of $CD3^+$ $CD4^+$ cells that were IFN-γ^+ (F) and IL-17^+ (G) in the NALT and FRT were compared between E2-treated and mock-treated mice at day 7 post-immunization. Data shown in panels B-G represent means ± SEMs. Data were analyzed using the unpaired, two-tailed t test with 95% confidence interval. *, $P < 0.05$; ***, $P < 0.001$. Data represents 3 independent experiments with similar results.

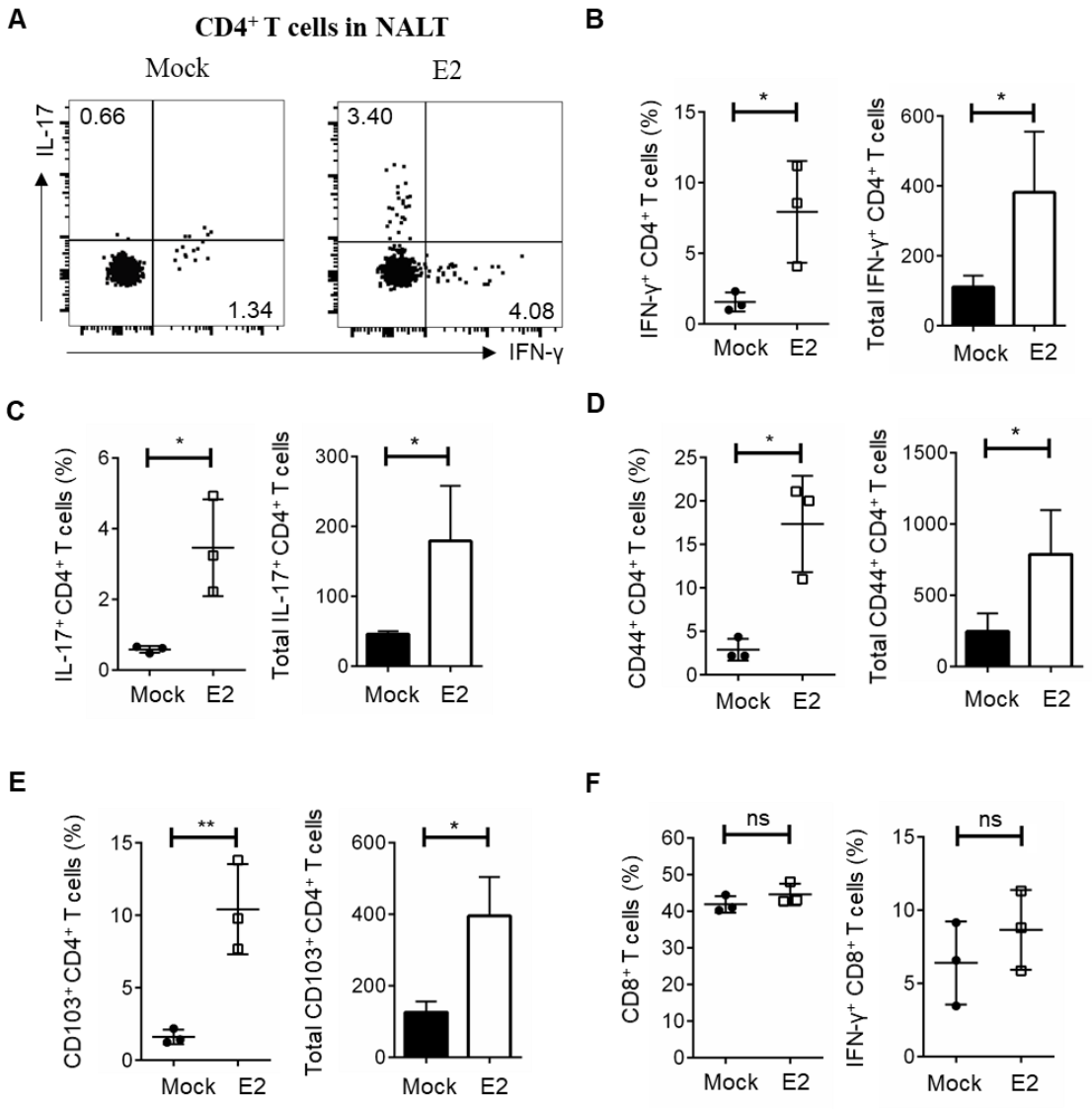


Figure 2: E2 enhances IFN-γ$^+$ and IL-17$^+$ CD4$^+$ memory T cells in the NALT following IN immunization. OVX WT (C57BL/6) mice (n=5-10/group) were implanted with E2 or placebo (mock) pellets, and immunized intranasally 2 weeks later with TK$^-$ HSV-2 (10^4 PFU/mouse). 4 weeks post-immunization, nasal-associated lymphoid tissue (NALT) was collected, pooled and processed. Cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) for 15h, stained with a panel of antibodies (CD3, CD4, IL-17, IFN-γ, CD44 and CD103), and examined by flow cytometry. Cells were gated based on the lymphocyte population, followed by single cells and then CD4$^+$ T cells were gated among total live CD3$^+$ T cells. (A) IL-17 and IFN-γ expression by CD4$^+$ T cells was examined, and the differences in the percentages and total numbers of CD3$^+$ CD4$^+$ cells that were IFN-γ$^+$ (B), IL-17$^+$ (C), CD44$^+$ (D) and CD103$^+$ (E) were compared between E2-treated and mock-treated mice in 3 independent experiments. The differences in the percentages of CD3$^+$

$CD8^+$ cells and $CD3^+ CD8^+$ cells that were IFN-γ^+ (F) were compared between E2-treated and mock-treated mice in 3 independent experiments. Data shown represent means ± SEMs. Data were analyzed using the unpaired, two-tailed t test with 95% confidence interval, with the ROUT method used to identify outliers. *, $P < 0.05$; **, $P < 0.01$. ns, no significance.

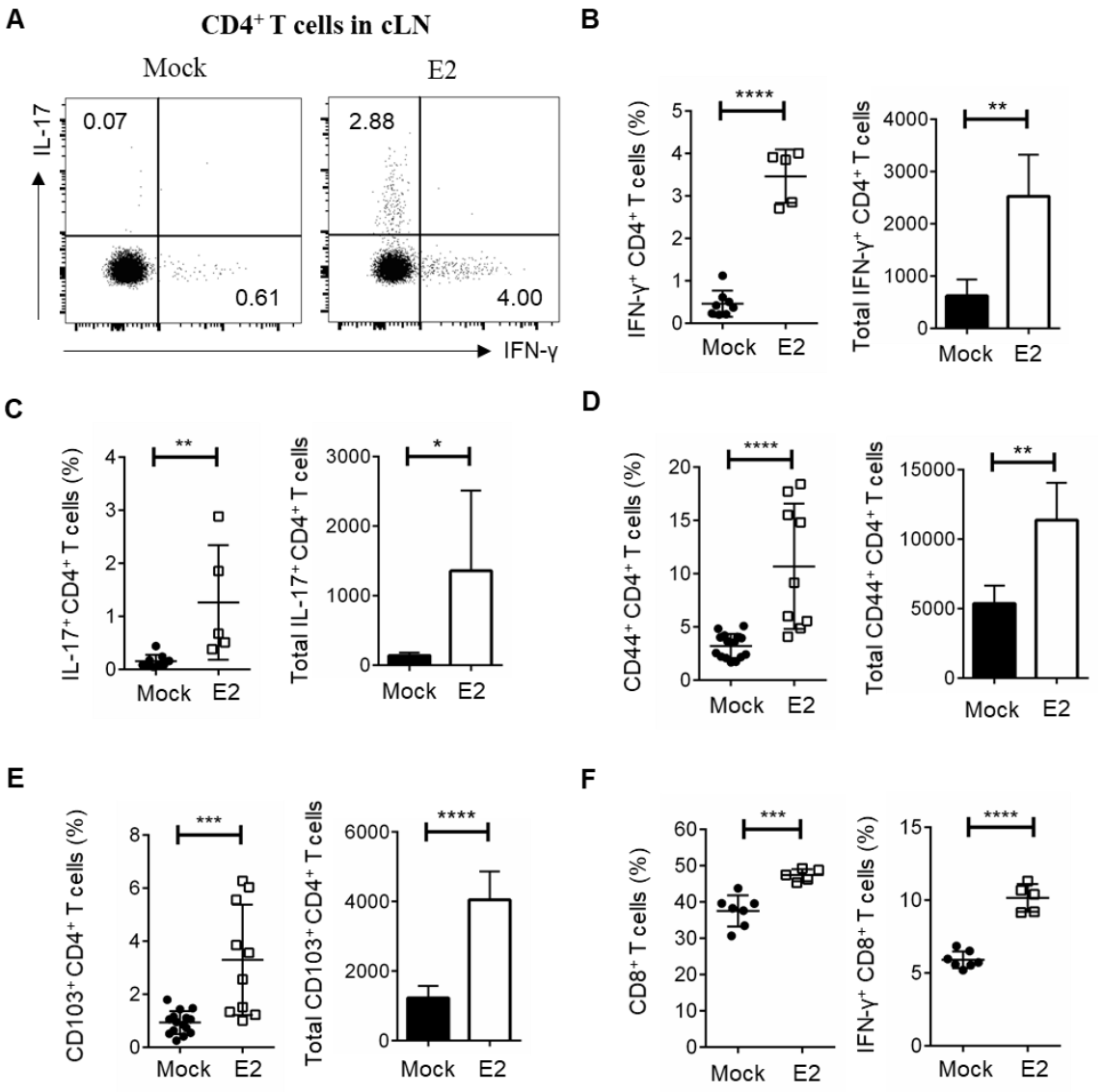


Figure 3: E2 enhances IFN-γ$^+$ and IL-17$^+$ CD4$^+$ memory T cells in the cLNs following IN immunization. OVX WT (C57BL/6) mice (n=5-10/group) were implanted with E2 or placebo (mock) pellets, and immunized intranasally 2 weeks later with TK$^-$ HSV-2 (10^4 PFU/mouse). 4 weeks post-immunization, cervical lymph nodes (cLN) were collected and processed. Cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) for 15h, stained with a panel of antibodies (CD3, CD4, IL-17, IFN-γ, CD44 and CD103), and examined by flow cytometry. Cells were gated based on the lymphocyte population, followed by single cells and then CD4$^+$ T cells were gated among total live CD3$^+$ T cells. (A) IL-17 and IFN-γ expression by CD4$^+$ T cells was examined, and the differences in the percentages and total numbers of CD3$^+$ CD4$^+$ cells that were IFN-γ$^+$ (B), IL-17$^+$ (C), CD44$^+$ (D) and CD103$^+$ (E) were compared between E2-treated and mock-treated mice in 3 independent experiments. The differences in the percentages of CD3$^+$ CD8$^+$ cells and CD3$^+$

$CD8^+$ cells that were IFN-γ^+ (F) were compared between E2-treated and mock-treated mice in 3 independent experiments. Data shown represent means ± SEMs. Data were analyzed using the unpaired, two-tailed t test with 95% confidence interval, with the ROUT method used to identify outliers. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

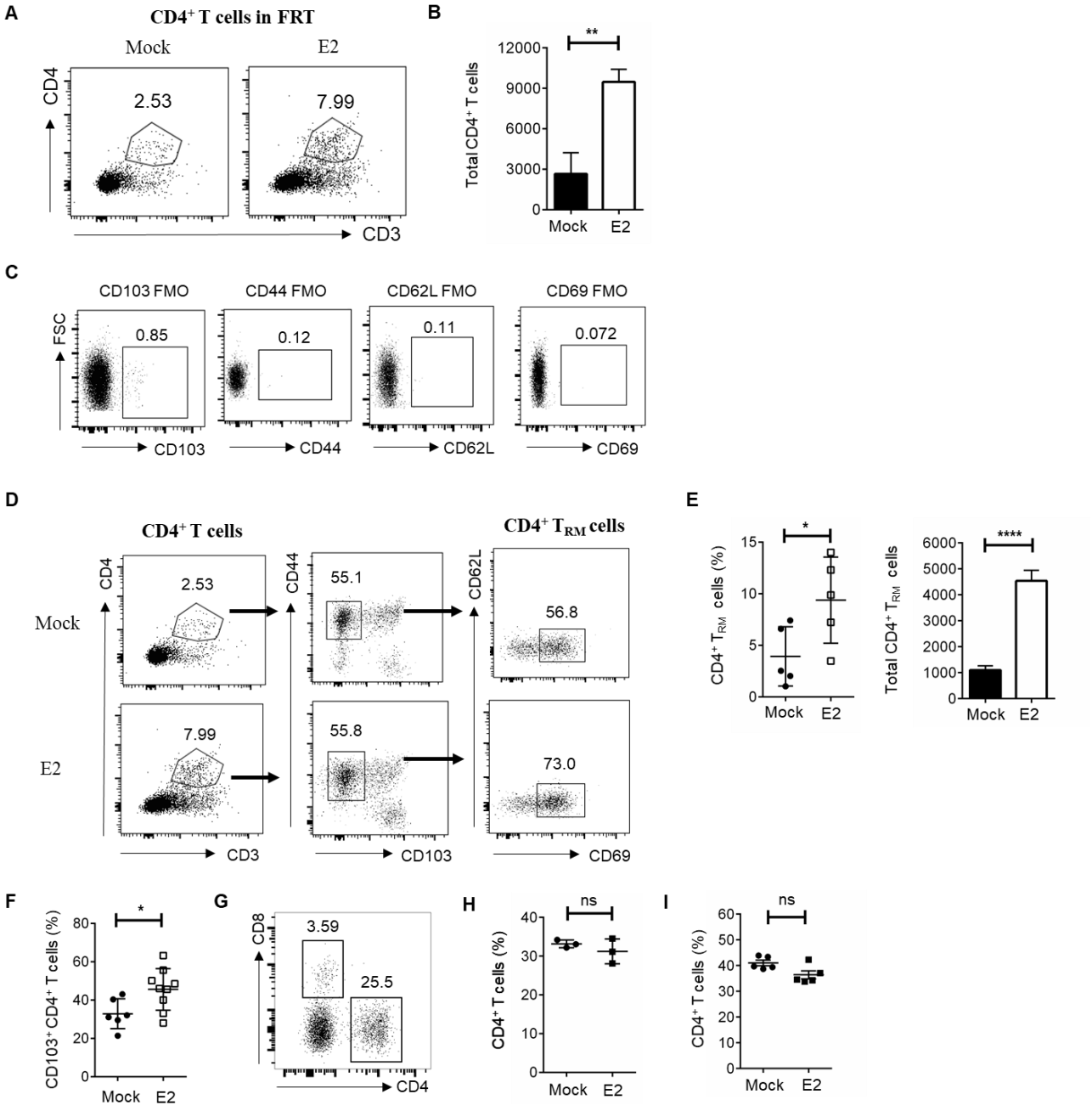


Figure 4: E2 enhances the establishment of CD4$^+$ T$_{RM}$ cells in the FRT following IN immunization. OVX WT (C57BL/6) mice (n=5-10/group) were implanted with E2 or placebo (mock) pellets, and immunized intranasally 2 weeks later with TK$^-$ HSV-2 (10^4 PFU/mouse). 4 weeks post-immunization, vaginal tissue was collected, pooled and processed. Cells were stimulated *in vitro* with a cell stimulation cocktail (CSC) for 15h, stained with a panel of antibodies (CD3, CD4, IL-17, IFN-γ, CD44, CD103, CD69L and CD69), and examined by flow cytometry. Cells were gated based on the lymphocyte

population, followed by single cells and then $CD4^+$ T cells were gated among total live $CD3^+$ T cells. The differences in the percentages (A) and total numbers (B) of $CD3^+$ $CD4^+$ T cells post-immunization in the female reproductive tract (FRT) were compared between E2-treated and mock-treated mice. (C) Fluorescence minus one (FMO) controls for tissue-resident memory T cell (T_{RM}) surface markers CD103, CD44, CD62L and CD69. $CD4^+$ T cells were gated among total live $CD3^+$ T cells in the FRT, and $CD4^+$ T_{RM} cells were detected using surface markers CD44, CD103, CD69 and CD62L (D). $CD4^+$ T_{RM} cells were defined as $CD4^+$ $CD44^+$ $CD103^-$ $CD69^+$ $CD62L^-$. The differences in the percentages and total numbers of $CD4^+$ T_{RM} cells in the FRT were compared between E2-treated and mock-treated mice in 5 independent experiments (E). The differences in the percentages of $CD3^+$ $CD4^+$ cells that were $CD103^+$ in the FRT were compared between E2-treated and mock-treated mice (F). (G) The percentage of $CD3^+$ $CD8^+$ T cells in the FRT of E2-treated mice. The differences in the percentages of $CD3^+$ $CD4^+$ T cells in the (H) spleen and (I) iliac lymph nodes were compared between E2-treated and mock-treated mice. Data shown represent means ± SEMs. Data were analyzed using the unpaired, two-tailed t test with 95% confidence interval. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. ns, no significance.

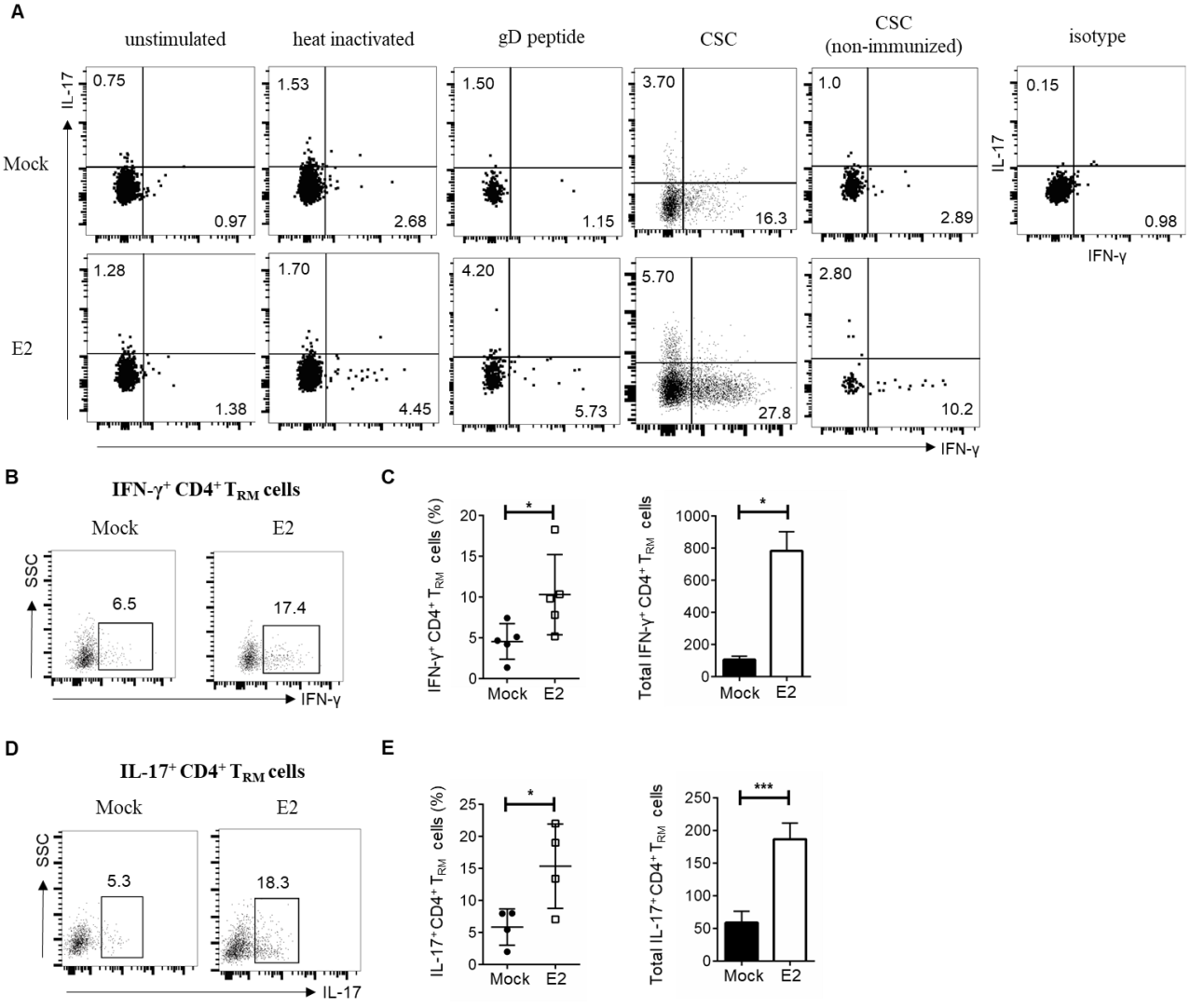


Figure 5: HSV-2 specific CD4$^+$ T cells expressing IL-17 and IFN-γ and the frequency of T_h1 and T_h17 T_{RM} cells in the FRT are enhanced by E2 following IN immunization. OVX WT (C57BL/6) or gD-II mice (n=5-10/group) were implanted with E2 or placebo (mock) pellets, and immunized intranasally 2 weeks later with TK$^-$ HSV-2 (10^4 PFU/mouse) or PBS (non-immunized). 4 weeks post-immunization, vaginal tissue was collected, pooled and processed. (A) Cells were either left unstimulated or stimulated *in vitro* for 12h with heat inactivated HSV-2, gD peptide (315-327) or a cell stimulation cocktail (CSC). Cells were stained with a panel of antibodies (CD3, CD4, IL-17 and IFN-γ), and examined by flow cytometry. Cells were gated based on the lymphocyte population, followed by single cells and then CD4$^+$ T cells were gated among total live CD3$^+$ T cells. (A) The percentage of IFN-γ$^+$ and IL-17$^+$ producing CD4$^+$ T cells from the female reproductive tract (FRT) responding to heat inactivated HSV-2, gD peptide or CSC was compared between E2- and mock-treated mice. The isotype control for IFN-γ and IL-17

staining is included (far right), along with CSC-stimulated cells from non-immunized mice for comparison. (B) $CD4^+$ T cells were then gated among total live $CD3^+$ T cells, and $CD4^+$ tissue-resident memory T cells (T_{RM}) were defined as $CD4^+$ $CD44^+$ $CD103^-$ $CD69^+$ $CD62L^-$. $CD4^+$ T_{RM} cells were further gated based on IFN-γ and IL-17 expression. The differences in the percentages and total numbers of IFN-γ^+ (B and C) and IL-17^+ (D and E) $CD4^+$ T_{RM} cells in the FRT were compared between E2-treated and mock-treated mice in 5 independent experiments. Data shown represent means ± SEMs. Data were analyzed using the unpaired, two-tailed t test with 95% confidence interval, with the ROUT method used to identify outliers. *, $P < 0.05$; ***, $P < 0.001$.

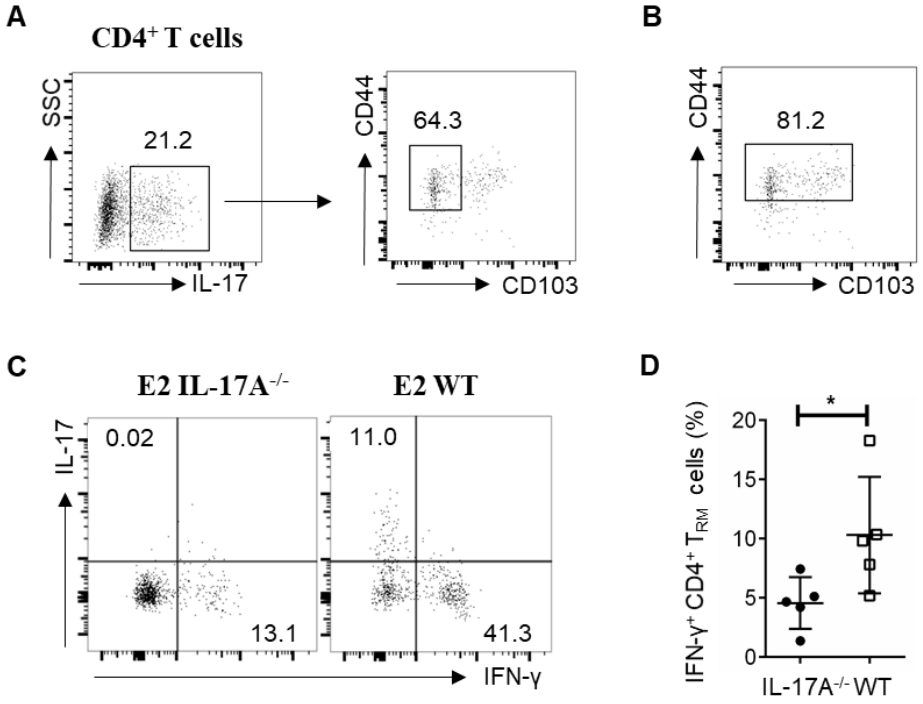


Figure 6: E2-mediated enhancement of T_h1 T_{RM} cells is significantly diminished in the absence of IL-17. OVX WT (C57BL/6) and *IL-17A*$^{-/-}$ mice (n=5-10/group) were implanted with E2 or placebo (mock) pellets, and immunized intranasally 2 weeks later with TK$^-$ HSV-2 (10^4 PFU/mouse). 4 weeks post-immunization, vaginal tissue was collected, pooled and processed. Cells were stimulated *in vitro* for 15h with a cell stimulation cocktail (CSC), stained with a panel of antibodies (CD3, CD4, IL-17, IFN-γ, CD44, CD103, CD62L and CD69) and examined by flow cytometry. Cells were gated based on the lymphocyte population, followed by single cells and then CD4$^+$ T cells were gated among total live CD3$^+$ T cells. The total population of IL-17-secreting CD4$^+$ T cells in the female reproductive tract (FRT) of E2-treated WT mice was examined for CD44$^+$ CD103$^-$ (A) and CD44$^+$ CD103$^+$ IL-17-secreting CD4$^+$ T cells (B). (C) The differences in IFN-γ$^+$ and IL-17$^+$ CD4$^+$ T cells were compared between E2-treated *IL-17A*$^{-/-}$ and WT mice. CD4$^+$ T cells were then gated based on tissue-resident memory T cell (T_{RM}) markers (CD44$^+$ CD103$^-$ CD69$^+$ CD62L$^-$), and further gated based on IFN-γ expression. (D) The differences in the percentages of IFN-γ$^+$ CD4$^+$ T_{RM} cells in the FRT were compared between E2-treated *IL-17A*$^{-/-}$ and WT mice across 5 independent experiments. Data shown represent means ± SEMs. Data were analyzed using the unpaired, two-tailed t test with 95% confidence interval. *, $P < 0.05$.

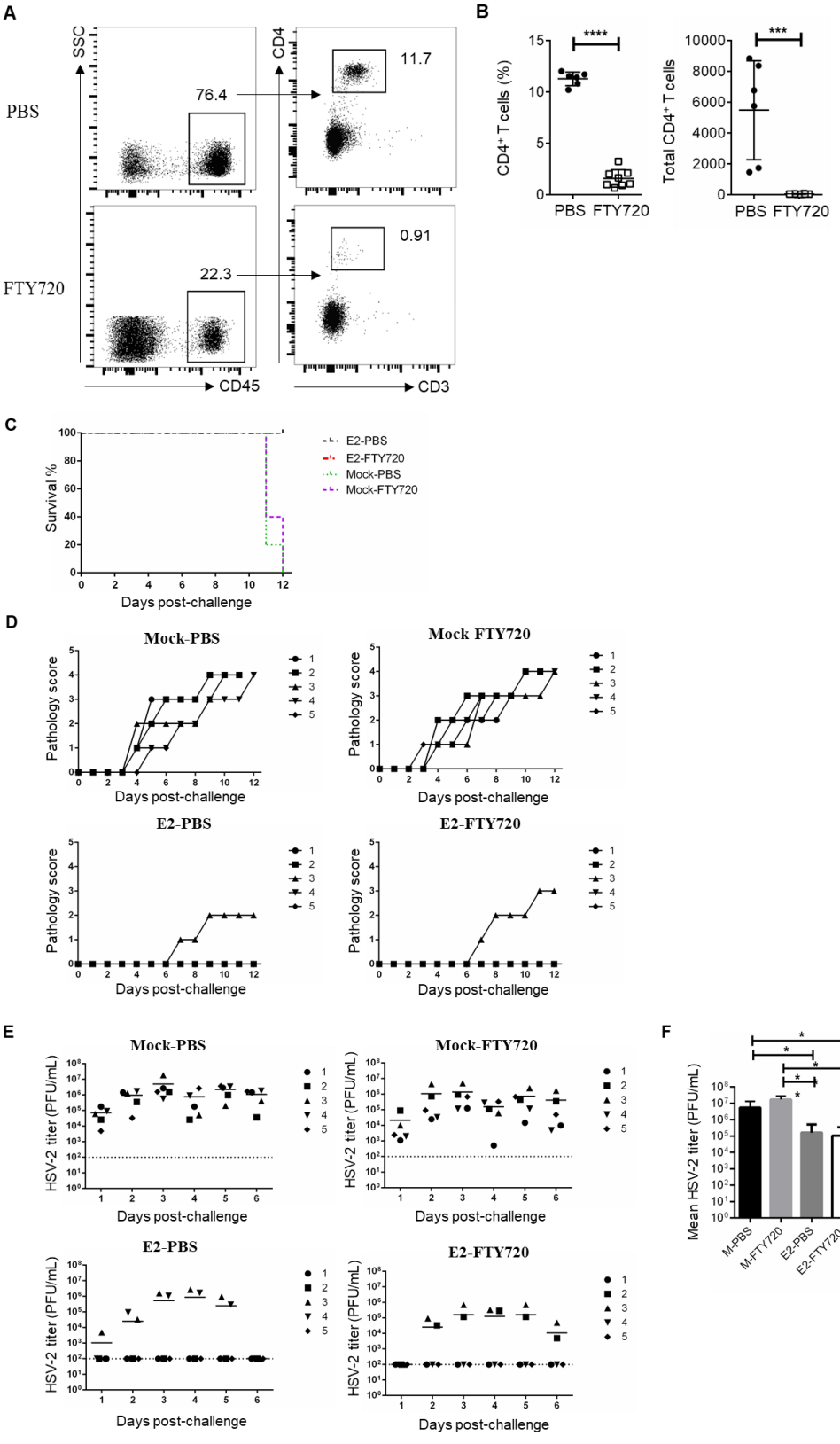
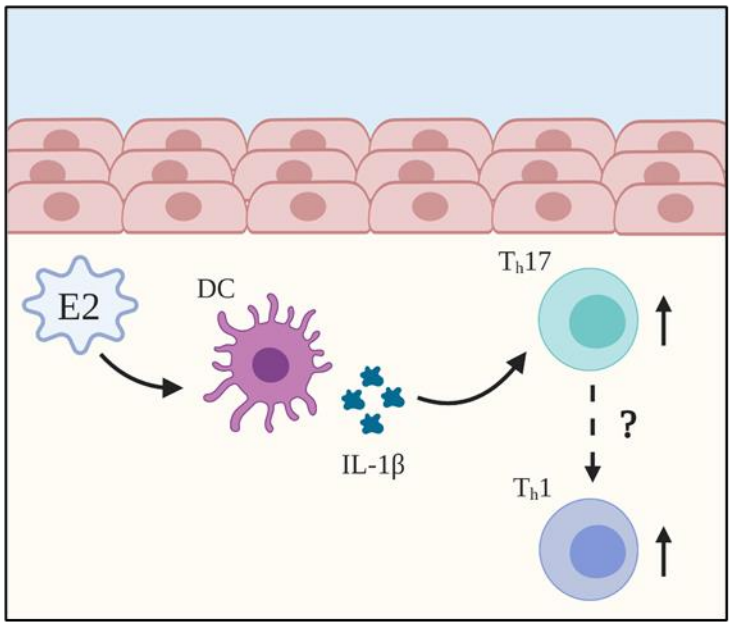

A
SSC
CD4
PBS
76.4
11.7
FTY720
22.3
0.91
CD45
CD3
B
CD4+ T cells (%)
****
PBS FTY720
Total CD4+ T cells
***
PBS FTY720
C
Survival %
E2-PBS
E2-FTY720
Mock-PBS
Mock-FTY720
Days post-challenge
D
Mock-PBS
Mock-FTY720
E2-PBS
E2-FTY720
Pathology score
Days post-challenge
E
Mock-PBS
Mock-FTY720
E2-PBS
E2-FTY720
HSV-2 titer (PFU/mL)
Days post-challenge
F
Mean HSV-2 titer (PFU/mL)
M-PBS
M-FTY720
E2-PBS
E2-FTY720

Figure 7: E2-enhanced $CD4^+$ T_{RM} cells are sufficient for protection against IVAG HSV-2 challenge. OVX WT (C57BL/6) mice (n=5-10/group) were implanted with E2 or placebo (mock) pellets, and immunized intranasally 2 weeks later with TK^- HSV-2 (10^4 PFU/mouse). 3 weeks post-immunization, mice were given drinking water containing 4 μg/ml of FTY720, or PBS for the control groups, for ten days prior to intravaginal (IVAG) challenge with WT HSV-2 ($5x10^3$ PFU/mouse). Mice continued to receive treatment throughout the duration of the experiment. (A) On day 9 of FTY720 or PBS treatment and prior to IVAG HSV-2 challenge, blood was collected, processed, stained with a panel of antibodies, and examined by flow cytometry to confirm depletion of $CD4^+$ T cells in circulation. Cells were gated based on the lymphocyte population, followed by single cells and then $CD3^+$ $CD4^+$ T cells were gated among total live $CD45^+$ T cells. (B) The differences in the percentages and total numbers of $CD4^+$ T cells in the blood were compared between FTY720-treated and PBS-treated mice across two independent experiments. Data shown in panel B represent means ± SEMs. Data were analyzed using the unpaired, two-tailed t test with 95% confidence interval. Post-IVAG challenge, survival was monitored (C) and pathology scores were recorded on a scale of 0-5 (D) for 12 days. Significance in difference in survival (C) was calculated using the log rank (Mantel-Cox) test (***, $P < 0.001$). Data points superimposed on the x axes of panel D indicate mice without genital pathology. Vaginal washes were collected daily for 6 days post-challenge and HSV-2 shedding was calculated using a Vero cell-based assay (E and F). Each symbol represents a single animal. The dotted lines in panel E indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. Data shown in panel F represent the viral loads (means ± SEMs) over 6 days. Data were analyzed using a one-way ANOVA with Tukey's multiple comparisons test. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

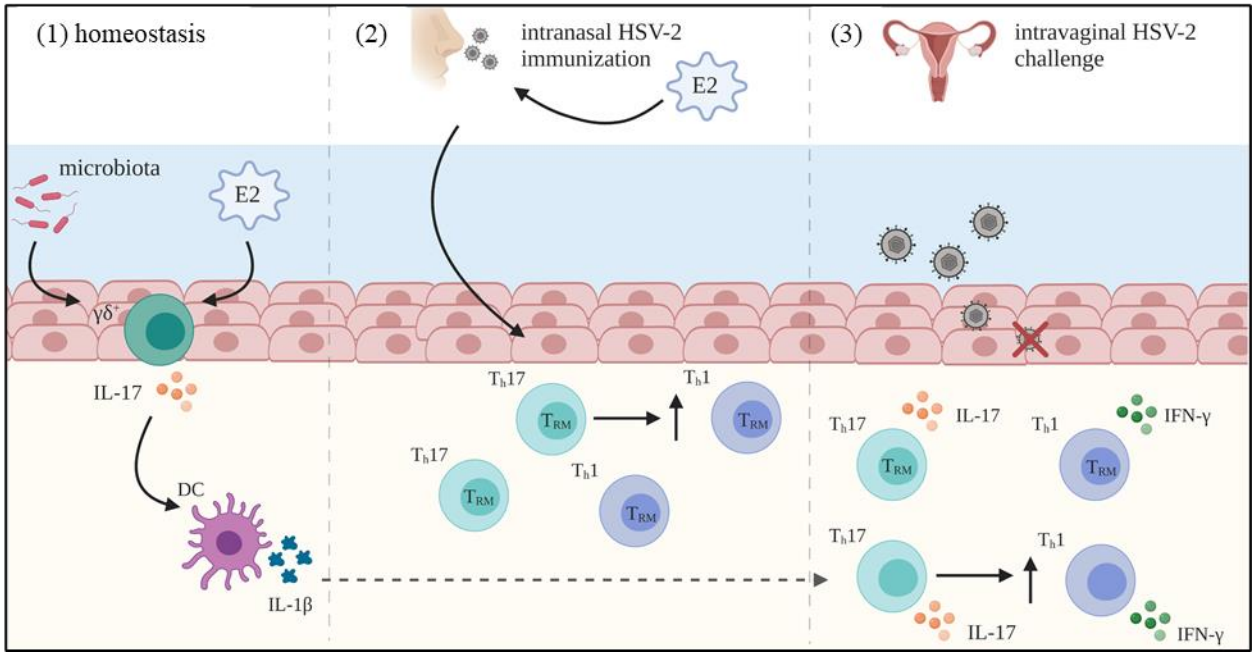
CHAPTER 5: Discussion

A major biological factor that influences susceptibility to STIs is the presence of sex hormones, and a primary focus in our lab for over a decade has been trying to understand how hormones modulate immunity in the FRT and thereby, affect susceptibility to infection. While early studies in our lab showed that E2 was protective against HSV-2 infection (371), the mechanism by which this occurred was not completely understood. Recently, our lab published a study examining the underlying mechanism of E2-mediated protection against HSV-2 infection (341). The findings from this study demonstrated that E2 treatment induced potent T_h17 responses via an IL-1-dependent mechanism in the FRT and this coincided with an earlier, and greater, accumulation of mucosal IL-17^+ and IFN-γ^+ $CD4^+$ effector T cells post-challenge (Fig. 5.1A) (341). Consequently, E2-treated mice were completely protected against HSV-2 challenge. The results from this study suggested that protection observed in E2-treated mice was related to enhanced T_h17 responses, which appeared to augment T_h1 immunity, as well as greater $CD4^+$ effector T cell responses following HSV-2 challenge. While the protective anti-HSV-2 role of T_h1 cells is well described in literature, little has been published on the importance of anti-viral T_h17 immunity. Thus, the primary objective of this dissertation was to further explore these findings by investigating the role of IL-17 in the FRT during HSV-2 infection and to determine if IL-17 is important for mediating efficient anti-viral protection in the vaginal mucosa. We hypothesized that IL-17 enhances anti-viral protection against HSV-2 infection by mediating more efficient $CD4^+$ T cell immunity in the FRT.

A


E2
DC
IL-1β
T_h17
?
T_h1

B


(1) homeostasis
(2)
intranasal HSV-2 immunization
E2
(3)
intravaginal HSV-2 challenge
microbiota
E2
γδ
IL-17
DC
IL-1β
T_h17
T_h1
T_{RM}
IL-17
IFN-γ

C

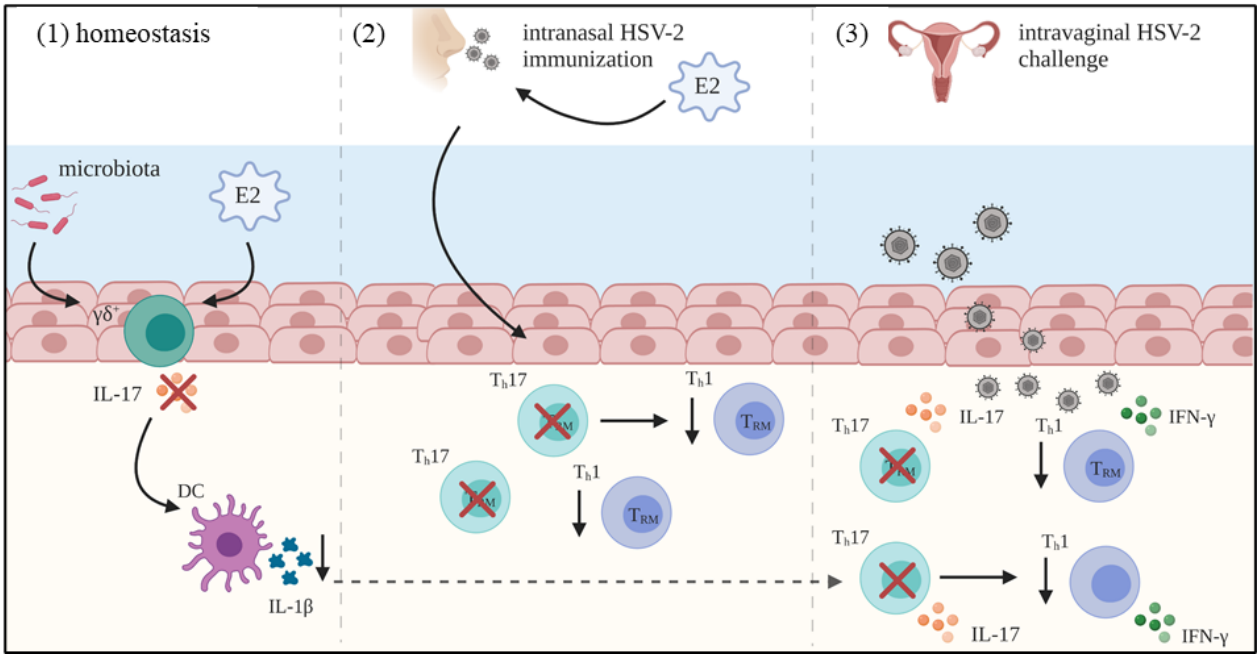


Figure 5.1: Summary of IL-17 mediated anti-viral immunity in the FRT. (A) In previous work, we showed that E2 treatment induced vaginal dendritic cells (DCs) to prime potent T_h17 responses via an IL-1-dependent mechanism. As such, upon intravaginal HSV-2 challenge, there was a robust T_h17 response, which coincided with a greater T_h1 response post-challenge and mice were better protected. However, it was unclear if the enhanced IFN-γ^+ $CD4^+$ T cell response was due to the robust IL-17^+ $CD4^+$ T cell response. (B) In the current dissertation, we propose the following role of IL-17 in the FRT: (1) During periods of homeostasis, innate IL-17 is constitutively produced by innate lymphocyte populations, primarily gamma delta ($\gamma\delta^+$) T cells, and regulated by E2 and commensal microbiota. This innate IL-17 is important for the induction of T_h17 responses primed by vaginal DCs (Chapter 3). (2) Following intranasal HSV-2 immunization under the influence of E2, IL-17 is important for the establishment of T_h1 T_{RM} cells in the FRT (Chapter 4). (3) These T_h17 and T_h1 T_{RM} cells are then able to protect against subsequent HSV-2 challenge. Additionally, IL-17 is critical for inducing efficient IFN-γ^+ $CD4^+$ T cell recall responses in the FRT post-challenge (Chapter 2). (C) In the absence of IL-17, there is reduced T_h17 immunity generated (1), and overall anti-viral T_h1 responses are significantly lowered both post-immunization (2) and post-challenge (3), even in the presence of E2. As a result, there is less protection generated against HSV-2 infection in the FRT. Created with BioRender.

First, we wanted to establish if IL-17 plays an important anti-viral role in the FRT (Chapter 2). To address this, we infected *IL-17A*$^{-/-}$ and WT mice intravaginally with WT HSV-2 and examined disease outcomes. Initially, we infected mice at three different doses and examined survival, genital pathology and viral shedding following primary infection, and found no significant differences between *IL-17A*$^{-/-}$ and WT mice. However, when we re-exposed the mice to higher doses of HSV-2, the absence of IL-17 resulted in significantly worse disease outcomes. *IL-17A*$^{-/-}$ mice had lower rates of survival, increased viral shedding in vaginal washes and greater genital pathology following re-exposure, as compared to WT mice. The same was observed when we repeated these experiments in the IN immunization model using TK^{-} HSV-2, followed by IVAG challenge with WT virus. The lack of protection in *IL-17A*$^{-/-}$ mice was associated with lower levels of IFN-γ in vaginal secretions, as well as an increased pro-inflammatory cytokine environment, as seen by higher levels of MIP-1, MCP-1, M-CSF, IL-6, RANTES and TNF-α. Additionally, the absence of IL-17 also coincided with lower T_h1 responses compared to WT mice, with significantly less IFN-γ production by vaginal CD4^{+} T cells. We also examined the establishment of vaginal CD4^{+} T_{RM} cells following IVAG immunization in *IL-17A*$^{-/-}$ and WT mice and found that in the absence of IL-17, mice had smaller populations of CD4^{+} and IFN-γ^{+} CD4^{+} T_{RM} cells. In summary, while it is well established that anti-viral protection against HSV-2 is largely mediated by T_h1 responses, this study is the first to suggest that IL-17 potentiates T_h1 immunity in the FRT, and may be contributing to anti-viral immunity against HSV-2.

Second, having established the importance of T_h17 immunity in the FRT, we wanted to determine the importance of innate IL-17 in mediating immune responses in the vaginal mucosa (Chapter 3). This was based on the fact that IL-17 released constitutively under homeostatic conditions in different mucosal tissues is predominately produced by innate and innate-like immune cells (382), and in a previous study from our lab, we found that vaginal cells induced IL-17 production in the absence of any antigenic stimulation (341). Hence, we specifically wanted to investigate the effect of innate IL-17 on the induction of T_h17 immunity in the FRT. We first conducted APC-T cell co-cultures using vaginal APCs isolated from *IL-17A*$^{-/-}$ and WT mice, to determine how the absence of innate IL-17 would influence the induction of T cell immunity. Compared to APCs from WT mice, vaginal APCs isolated from *IL-17A*$^{-/-}$ mice were severely impaired at priming T_h17 responses in APC-T cell co-cultures; thus, demonstrating that innate IL-17 is important for potentiating adaptive T_h17 responses in the FRT. This defect in T_h17 induction was further associated with impairment of IL-1 production, as vaginal DCs from *IL-17A*$^{-/-}$ mice also produced significantly lower levels of IL-1β, a cytokine shown to be critical for priming T_h17 responses in the FRT (341). We also characterized the primary source of IL-17-secreting vaginal cells, and consistent with previous reports (145), found that $\gamma\delta^+$ T cells were the predominant source of innate IL-17 production in the FRT. We then examined how factors in the vaginal microenvironment, such as E2 and commensal microbiota, modulated this source of innate IL-17. We found enriched populations of $\gamma\delta^+$ T cells in E2-treated mice that also produced greater amounts of IL-17, and found that the absence of microbiota in GF mice led to significantly lower proportions of these same IL-17$^+$ $\gamma\delta^+$ T cells. In

summary, this work demonstrated that IL-17 in the FRT is induced by a variety of factors. It is also the first study to demonstrate that innate IL-17 production under homeostatic conditions in the vaginal mucosa is critical for inducing T_h17 responses, thereby amplifying the overall IL-17 response in the FRT, and that this innate IL-17 is modulated by E2 and microbiota.

Third, having thoroughly examined the influence of IL-17 at the primary phase of the immune response generated in the FRT, we next wanted to determine if IL-17, along with E2, is related to greater establishment of memory T cells following immunization (Chapter 4). Specifically, our aim was to examine the effect of E2 on the establishment of a local reservoir of vaginal T_{RM} cells post-IN immunization and determine the role of IL-17. We found that following IN immunization with TK^- HSV-2, E2-treated mice primed higher frequencies of T_h1 and T_h17 cells at the primary site of immunization (URT), which were found to migrate to the FRT by day 7 and establish residency. Upon further characterization of the cells which remained in the FRT up to 4 weeks later, the $CD4^+$ T cells were identified as T_{RM} cells. Furthermore, the enhanced IFN-γ^+ $CD4^+$ T_{RM} cells in the vaginal mucosa appeared to be dependent on IL-17, as E2-treated *IL-17A*$^{-/-}$ mice had impaired establishment of T_h1 T_{RM} cell populations in the FRT. Next, we tested the anti-viral efficacy of the local $CD4^+$ T_{RM} cells and found that in E2-treated mice which were administered FTY720 in order to prevent T cell migration, $CD4^+$ T_{RM} cells were sufficient for mediating protection against subsequent genital HSV-2 challenge. Since we used an IN immunization model, we also examined the effect of E2 on memory T cells in the URT and found that E2-treated mice had greater memory T_h1 and T_h17 responses in the URT as well.

In summary, this is a novel study which suggests a link between E2, IL-17 and the enhancement of $CD4^+$ memory T cells.

The results summarized in this dissertation provide significant insight regarding how IL-17 modulates critical anti-viral T cell responses in the FRT (Fig. 5.1). Firstly, while it is well established that anti-viral protection against HSV-2 is largely mediated by T_h1 responses, our findings are the first to show that T_h17 responses are also important in the anti-viral immune response against HSV-2. Although the role of IL-17 in the FRT during fungal and bacterial pathogens has been well described, the role of IL-17 during viral infections in the FRT is less clear. To the best of our knowledge, only one study has previously reported an association between IL-17 and disease outcomes to HSV-2. Kim et al. (145) reported that in the absence of IL-17, mice had delayed mortality following primary infection with a lethal dose of HSV-2, suggesting a pathogenic role for IL-17. However, there were several limitations to the model used by Kim et al., as explained previously (Chapter 1.3.6.2). Our findings show that while IL-17 does not play a critical role during primary infection with lower doses of virus, the impact of IL-17 is observed when examining secondary immune responses. This is similar to what has been shown in other studies investigating the role of IL-17 during infection with intracellular pathogens such as *M. tuberculosis*. Khader et al. demonstrated that although IL-17 is dispensable during primary infection, it appears to play a critical role in inducing T_h1 immunity following vaccination (383, 384). Our work here also shows that IL-17 is critical for inducing an efficient T_h1 immune response following HSV-2 immunization. The contribution of IL-17 to the development of T_h1 immunity against pathogens has been

similarly described by others (383-387); however, our work is the first to show this role of IL-17 during HSV-2 infection. Our findings also support, and further expand on, previous work from our lab which showed *in vitro* that vaginal APCs from *IL-17A*$^{-/-}$ mice were significantly impaired at inducing IFN-γ production by CD4^{+} T cells in APC-T cell co-cultures (341). Overall, our work investigating the importance of IL-17 during HSV-2 infection proposes a novel anti-viral role for IL-17, in which IL-17 improves mucosal vaccination efficacy by mediating greater T_h1 immunity. Furthermore, to the best of our knowledge, our work is the first to suggest a protective role for IL-17 during HSV-2 challenge and suggests that instead of focusing just on IFN-γ as the primary mediator of protection, IL-17 production should be considered as well.

Similar to demonstrating an important role for T_h17 immunity in the FRT, we have also described a novel role for innate IL-17 in influencing immunity in the vaginal mucosa. While the presence of innate IL-17 has been shown to be important for potentiating T_h17 immunity in other mucosa such as the gut and lungs (388-392), the significance of IL-17 produced by innate or innate-like sources in the FRT and its influence on T_h17 immunity in the vaginal mucosa is less understood. Our findings support results seen in other mucosa and demonstrate that innate IL-17 produced in the FRT is also important for inducing T_h17 responses. Furthermore, we propose a mechanism by which this occurs, as vaginal APCs from *IL-17A*$^{-/-}$ mice produced lower amounts of IL-1β compared to WT DCs; thus, suggesting that innate IL-17 induces vaginal DCs to prime T_h17 responses via an IL-1β-dependent pathway. Additionally, consistent with previous findings by Kim et al. (145), we show that γδ^{+} cells are the primary source of innate IL-17 in the FRT under homeostatic

conditions. We extend these findings to showcase multiple factors found within the vaginal microenvironment, such as E2 and microbiota, which influence innate IL-17 production by $\gamma\delta^+$ cells. To our knowledge, this is the first study to show that E2 enhances $\gamma\delta^+$ cells in the FRT and results in greater IL-17 production by these cells. Likewise, while commensal bacteria have been associated with IL-17 production by innate and innate-like lymphocytes in the gut (391, 393), the influence of the vaginal microbiota on IL-17 production was largely unknown. Our work shows that the vaginal microbiome may also influence the presence of $\gamma\delta^+$ cells and innate IL-17 production. Overall, this is the first report to show the importance of IL-17 produced by $\gamma\delta^+$ T cells in the induction of T_h17 immunity in the FRT, as well as demonstrate an association between factors such as E2, microbiota and IL-17-producing $\gamma\delta^+$ T cells, and priming of adaptive immunity in the vaginal mucosa.

Finally, this work expands on what is known regarding the protective effects of E2 in the FRT during HSV-2 infection and presents a novel mechanism to explain why E2 treatment leads to better protection against infection. While previous studies have shown that E2-treated mice are better protected against HSV-2 challenge, the immunological correlates of protection have been less understood. Here, we show that E2 enhances the establishment of $CD4^+$ memory T cells in the FRT following IN immunization, suggesting that better protection observed under E2 treatment could be the result of greater memory responses generated during immunization. This is the first report to show that E2 influences $CD4^+$ memory T cell responses in the FRT. While other studies have failed to demonstrate the establishment of $CD4^+$ T_{RM} cells in the FRT following IN immunization (261), we show that IN immunization under the influence of E2 results in the generation of long-lasting

vaginal $CD4^+$ T_{RM} cells which are able to protect against subsequent IVAG HSV-2 challenge. As such, our findings demonstrate that IN immunization can be used as an effective route of vaccination to establish protective memory responses in the FRT. We also suggest that E2-mediated enhancement of $CD4^+$ T_{RM} cells occurs through an IL-17-mediated pathway, further demonstrating the importance of IL-17 in generating more efficient anti-viral immunity in the FRT. Additionally, our novel findings demonstrating greater T_h1 and T_h17 memory responses in the NALT and cLNs of E2-treated mice show that E2 can be used to enhance memory T cell responses in the URT as well, which has implications regarding a protective role for E2 against nasal pathogens. Taken together, this work presents a novel mechanism underlying E2-mediated anti-viral protection, where E2 may enhance memory T cells in various mucosal tissues following immunization.

5.1 Clinical and therapeutic implications

The work in this dissertation largely focuses on understanding mechanisms which may promote anti-viral immunity against HSV-2. As discussed earlier (Chapter 1.3.3), several HSV-2 candidate vaccines have failed to demonstrate adequate protection in human clinical trials (172, 394); thus, emphasizing the need for novel approaches in vaccine development. An effective HSV-2 vaccine needs to induce balanced immunity at the primary site of infection, i.e. the FRT. To accomplish this, several factors must be taken into consideration including the type of vaccine, the route of immunization, the use of specific adjuvants and factors such sex hormones; all of which can influence vaccine efficacy.

Most vaccines currently approved and in market are administered through non-mucosal routes, mainly through parenteral routes such as subcutaneous or intramuscular injections. Although they stimulate systemic immune responses, parenteral routes of administration often fail to induce mucosal immunity. In contrast, mucosal immunization is known to establish better protection in mucosal tissues compared to systemic immunization (187, 190, 191, 206, 395). Since most pathogens enter the body through mucosal surfaces, it is critical to induce local mucosal immunity in order to generate the most effective, rapid immune response at the site of pathogen exposure. In this regard, we and others have focused on mucosal immunization routes including IVAG and IN immunization. Studies have shown that IN immunization provides better protection in the FRT compared to systemic immunization, and similar protection as observed following IVAG immunization (191, 205, 206). Both IVAG and IN immunization with TK^- HSV-2

leads to comparable protection against subsequent genital HSV-2 challenge, and this is mediated primarily by $CD4^+$ T cell production of IFN-γ (191, 207, 208). However, a limitation of IN immunization cited by others studies was the failure to establish substantial, long-lasting $CD4^+$ memory T cells in the FRT (396). The work summarized in this dissertation (Chapter 4), demonstrates that IN immunization following E2 treatment results in the establishment of a significant population of long-lasting $CD4^+$ T_{RM} cells which are able to provide protection against subsequent HSV-2 challenge (Chapter 4). This suggests that IN immunization in the context of E2 can help overcome lack of $CD4^+$ memory T cells observed by others. Our findings further illustrate the protective benefits of mucosal vaccination and provide strong support for IN vaccination in combination with E2 to be considered in HSV-2 vaccine development. While IN immunization is considered a more feasible and clinically relevant method of vaccination, there are currently very limited mucosal vaccines approved for human use (195). Some disadvantages associated with IN immunization include the need for live viruses, higher doses of antigen and the use of adjuvants (195). For instance, the live attenuated influenza vaccine, FluMist, is one of the few approved nasal vaccines which is offered as an alternative to the traditional injectable flu shot; however, FluMist has been reported to lack efficacy in certain populations (397). As such, further work is required to develop effective IN vaccines. Our findings show that it would be beneficial to continue to explore IN vaccines and demonstrate promising results for the use of IN immunization for protection against non-respiratory pathogens as well.

Another factor which should be taken into consideration during vaccine design and implementation is the hormonal microenvironment. The findings in this dissertation, along with many other studies conducted by our group over the past decade, demonstrate the importance of the hormonal microenvironment in mediating immunity in mucosal tissues and dictating susceptibility to STIs (200, 341, 368-371, 377). Overall, we have found that E2 treatment provides protection against IVAG HSV-2 challenge following immunization (341, 371), while DMPA or P4 treatment in mice increases susceptibility to infection (200, 368). These findings have been confirmed by others, where E2 was shown to improve vaccine outcomes in an animal study looking at an HSV-2 gD peptide-based vaccine formulation (374). HIV-1 studies have also shown that susceptibility is greatest during the P4-high secretory phase of the menstrual cycle (398), and this was associated with increased frequencies of HIV-1 target cells (399). Similarly, in a study examining productive HIV-1 infection of cervicovaginal tissue explants *ex vivo*, only tissues from women in the secretory phase of the cycle, but not in the proliferative phase, were able to support productive viral replication (400). Conversely, E2 has been associated with protection against HIV-1 infection. In animal studies, vaginal E2 treatment in non-human primates was shown to decrease risk of simian immunodeficiency virus infection (401). Furthermore, E2 is known to establish an anti-inflammatory environment in the FRT by downregulating the expression of pro-inflammatory mediators (48, 329, 330, 402, 403), and so fewer HIV-1 target cells are present during E2-high phases of the cycle. Interestingly, the effect of hormones in human HSV-2 infection and viral shedding is not well defined. One study looking at HSV shedding in cervical swabs from women co-

infected with HSV and HIV-1 found that the use of HCs (oral contraceptives containing both E2 and P4 vs. P4-only contraceptives) did not increase HSV shedding (404), while a separate study examining HIV-1 shedding in co-infected women reported that the highest levels of HIV-1 shedding occurred during the follicular phase, as compared to the periovulatory and luteal phases (405). Additionally, a recent study by Micks et al. (364) demonstrated that HSV-2 seropositive women not using any form of HC had higher frequency of HSV-2 shedding during the follicular phase of the cycle, while no differences were observed in genital lesions. They also found that the use of HC did not affect genital HSV-2 detection or lesions. With varying, conflicting results and lack of substantial studies in this area, it is important for more research to focus on the impact of sex hormones on immune responses to infections. The work detailed in this dissertation further adds to this body of literature by demonstrating the protective effects of E2 during immunization and showcasing the ability of E2 to generate greater memory responses which can mediate better protection against HSV-2 infection (Chapter 4).

While all the studies described above highly suggest that the immune response generated during vaccination can be influenced by the phase of the menstrual cycle, limited studies have actually examined the impact of sex hormones on immune responses to vaccines in humans. A few studies examining vaccine candidates for STIs have looked at the effect of hormone levels on antibody responses. In a study examining an HPV vaccine candidate, IgG and IgA titers were measured in the cervical secretions of ovulating women post-immunization and the effect of the menstrual cycle was examined (342). They found that the cervical titers of specific IgG, as well as total IgG and IgA levels, were highest

during the follicular phase (E2-dominant), decreased significantly during ovulation, and increased during the luteal phase (P4-dominant). However, this study was quite small (n=11), the vaccine was administered intramuscularly, and the stage of the cycle was not controlled for at the time of immunization. Another study investigated the influence of exogenous hormones on local and systemic levels of IgA and IgG antibodies in cervical and vaginal secretions following IVAG immunization with an inactivated cholera vaccine (406). Interestingly, they found no differences in antibody responses between the different groups of women who were either using P4-containing intrauterine devices, oral contraceptives or were not using any HC at the time. A separate study also investigating the induction of immune responses following immunization with a cholera vaccine, reported that women who received the vaccine IVAG during the follicular phase had greater antigen-specific IgA responses compared to women immunized during the luteal phase (407). Although the effect of female sex hormones on vaccine outcomes is severely understudied, and cellular immunity has not been examined, there have been some studies done examining sex-specific immune responses to vaccines. In general, females elicit greater antibody and cell-mediated immune responses to antigenic stimulation, vaccination and infection, compared to males (408). For instance, protective antibody responses can be up to twice as high in females than males following vaccination for influenza, yellow fever, rubella, measles, mumps, hepatitis A and B, HSV-2, rabies, smallpox and dengue viruses (409). Clinical studies have shown that females have higher $CD3^+$ and $CD4^+$ T cell counts, greater T cell proliferation and activation and exhibit greater cytotoxic T cell activity (410, 411). Females also have upregulated expression of anti-viral and pro-inflammatory genes

(412). These differences can be attributed to a multitude of factors including an inherent imbalance in the expression of genes encoded on the X and Y chromosomes (408), as well as immune regulation by sex hormones. Concentrations of sex hormones including testosterone, E2 and P4 differ between the sexes throughout the lifespan, especially during the years between puberty and reproductive senescence (409). Sex hormones can directly influence immune cells by binding to receptors expressed in many immune cells. As sex steroids can readily diffuse across the cell membrane, most sex hormone receptors are located in the cytoplasm. Once the hormone binds to the receptor, the resulting complex translocates to the nucleus, where it binds to specific hormone response elements found on DNA (408). This results in direct influence of signalling pathways, resulting in differential production of cytokines and chemokines (413). Sex hormones can also affect the migration, proliferation and activity of $CD4^+$ and $CD8^+$ T cells, as well as antibody production (408). For example, higher antibody production in women following immunization can be the direct result of the ability of E2 to stimulate antibody production by B cells (414). In a study examining the effect of menopause and E2 therapy on immune responses to influenza vaccination, Engelmann et al. (415) found a direct correlation between plasma E2 levels and IgG titers, where higher E2 concentrations were associated with enhanced IgG antibody responses. However, some vaccines such as tetanus and diphtheria elicit greater antibody responses in males compared to females (411). The lack of consistency observed in sex-specific antibody responses following vaccination demonstrates the need for more studies to analyze data with sex-related differences in mind. This idea was illustrated in a clinical study using a recombinant gD-based HSV-2 vaccine, where there was a sex-bias observed

in efficacy (416). The overall efficacy of the vaccine was 38–42% in HSV-1 seronegative women, while no efficacy was observed in men or HSV-1 seropositive women. Interestingly, there were no sex differences in the immune responses to the vaccine, suggesting that while immunogenicity may be equivalent, efficacy can still vary between sexes, which further adds to the complexity of the issue. These differences in immune responses between males and females can affect pathogenesis of infectious diseases. Although females have better immune responses to vaccines, side effects from vaccination such as fever, pain and inflammation are also more frequent and severe in females compared to males (411, 417). It is theorized that while heightened immunity in females can help reduce viral load and enhance pathogen clearance, these enhanced responses can also result in increased symptoms of disease if they become too robust or remain elevated for too long; thus, helping explain why disease outcomes are more severe in females compared to males (408, 411). Sexual dimorphism in immunity is a topic which has recently generated great interest amongst the research community. Many funding agencies, including the National Institutes of Health and the Canadian Institute of Health and Research, now have policies in place which state that sex must be accounted for as a biological variable in research. Our findings and those discussed above, all of which show clear impacts of hormones on the induction of immune responses, suggest that it would be highly beneficial for studies to be designed such that there are balanced numbers of males and females (human and animal studies), for clinical data to be analyzed for sex-specific variation and for vaccine studies to record the hormonal status of women at the time of vaccination. The current approach towards vaccines is a “one size fits all” strategy, which

is not ideal considering the biological differences in immunity described throughout this dissertation. For instance, since women have greater immune responses following vaccination, which have also been associated with adverse effects, perhaps females require lower doses of certain vaccines to provide equivalent protection (411). We need to consider these additional factors and adjust things such as optimal dosing and scheduling of vaccines accordingly.

Along with implications regarding hormones and vaccine responses, our data demonstrating enhanced protection against HSV-2 in the presence of E2 also underlines the importance of considering the composition of HCs and how they can also influence susceptibility to infections. Specifically, the association between the use of certain HCs and increased susceptibility to STIs has been an important area of discussion over the past few years. Studies have shown that compared to other HCs, DMPA use increases HIV-1 acquisition by 1.4-fold (356). This has especially been a major issue in sub-Saharan Africa, where DMPA is the most common form of HC and coincidently, rates of HIV-1 infection are the highest compared to any other region in the world, especially in young women and girls (357, 358). While the use of DMPA has been linked to increased HIV-1 susceptibility (14, 15, 418-420), the exact underlying biological mechanisms by which this occurs remains largely unknown. Animal studies have shown that administration of DMPA results in dramatic thinning of the vaginal epithelium (367), which is linked to increased susceptibility, but this has not been the case in studies looking at women (421). In humans, DMPA use has been linked to greater expression of RANTES and decreased expression of certain AMPs, which have been implicated in HIV-1 seroconversion (422). More recently,

it has also been suggested that DMPA increases susceptibility by enhancing the frequency of $CCR5^+$ $CD4^+$ T cells, which are considered HIV-1 target cells (423, 424). Women using progestin-only contraceptives had ~4 times the frequency of cervical $CCR5^+$ $CD4^+$ T cells compared to women not using HC and who were in the naturally occurring secretory phase of the menstrual cycle (423). However, the increased frequency of HIV-1 target cells is not consistently observed in women using DMPA (425, 426). Other potential mechanisms that have been linked to increased risk of HIV-1 transmission with DMPA use include disruption of the epithelial barrier, immune suppression in the FRT and changes in the vaginal microbiome (19, 427, 428). Similarly, progestin-based HCs such as DMPA and levonorgestrel have been shown to diminish the genital mucosal barrier in mice, leading to increased mucosal epithelial permeability and subsequently, increased susceptibility to HSV-2 (375, 376). Interestingly, the effect of HCs in human HSV-2 infection and viral shedding is less clear, as some studies have linked DMPA with increased susceptibility, while others report no effect of HC use on genital HSV-2 detection or lesions in infected women (360-363). There is also some controversy regarding the association between DMPA use and increased risk of HIV-1 acquisition. While several studies have shown 40-50% increased risk of HIV-1 acquisition with DMPA use, a recent clinical trial conducted across four countries demonstrated no differences in risk of HIV-1 infection among women using DMPA compared to those using copper intrauterine devices or levonorgestrel implants (14-16, 419, 429, 430). As such, further research understanding how DMPA and other HCs modulate immune responses is required. Overall, our work here suggests that

HCs which incorporate E2 may be a better contraceptive option as compared to P4-based HCs, especially in key populations which are more likely to acquire STIs.

Since we showed that immunization in the presence of E2 resulted in increased $CD4^+$ T_{RM} cells (Chapter 4), and that E2-mediated protection was also associated with greater IL-17 responses, which were shown to be important for mediating efficient anti-viral immunity in the FRT (Chapter 2 and 3) (Figure 5.1B and C), vaccine strategies should focus on inducing similar responses in the vaginal mucosa. While most vaccines focus on eliciting antibody responses in hopes of generating sterile immunity, a more practical goal for an HSV-2 vaccine would be to generate potent T cell responses which can kill virally infected cells and prevent viral replication and dissemination, leading to a "functional cure". Here, we have shown that E2 enhances anti-viral T cell immunity and establishes long-lasting memory responses. Therefore, as discussed previously, the administration of vaccines during the E2-high phase of the menstrual cycle may help provide optimal protection against STIs such as HSV-2. Similarly, a vaccine formulation incorporating E2 may also improve protective immune responses in women. However, the concept of administrating hormones during vaccination is quite complicated, as E2 is also associated with increased susceptibility to some infections such as chlamydia and candida (431-434). Alternatively, factors such as adjuvants or cytokines which induce similar protective responses against infection could be incorporated into vaccine design instead. For instance, one mechanism of E2-mediated protection is through enhanced production of IL-17, and so factors which drive T_h17 polarizing cytokines, such as IL-1 and IL-6, can be used to directly enhance T_h17 responses in the mucosa (435). Recently, a study which used a T_h17-

inducing adjuvant to try and improve protection against acellular pertussis (whooping cough) found that IN immunization in conjunction with the adjuvant resulted in the generation of persistent IgA levels, as well as both T_h17 and T_{RM} cells (436). Cationic Adjuvant Formulation no. 1 (CAF01), the adjuvant used in the study, is a cationic liposome composed of glycolipid trehalose 6,6'-dibehenate and dimethyldioctadecylammonium, and has been shown to induce T_h1 and T_h17 responses (437). A recent study actually used this same adjuvant (CAF01) in a chlamydia vaccine formulation to help provide protection against transcervical infection by enhancing T_h1 and T_h17 immunity (438). They found that following parenteral immunization, a pool of tissue resident T_h1 and T_h17 cells ($CD69^+$ $CD103^-$ $CD49d^-$ $CCR7^-$) was generated and helped protect against subsequent re-infection (438). This demonstrates that adjuvants could be used to enhance protective T_{RM} cell responses in mucosal tissues, including the FRT. It is, however, important to recognize that a fine balance must be maintained between generating protective T_h17 responses as opposed to pathogenic responses, since IL-17 is associated with pro-inflammatory conditions. Upstream factors which play a critical role in IL-17 production, such as IL-1β (Chapter 3), can also be used to enhance T_h17 immunity. Interestingly, a recent study demonstrated that IL-1β may act as an adjuvant for the induction of $CD8^+$ T_{RM} cells in the lungs following IN immunization (439). Mice were immunized with a novel recombinant adenoviral vector vaccine for influenza with vector-encoded IL-1β, and the addition of IL-1β promoted the generation of $CD103^+$ $CD69^+$ T_{RM} cells, which mediated protection against influenza infection. While they did not look specifically at the effect of IL-1β on IL-17 production, the fact that T_{RM} cells were enhanced is promising and suggests a similar

strategy should be considered in HSV-2 vaccine development. It also suggests that IL-1β can influence the generation of long-term T cell immunity. The generation of T_{RM} cells in vaccine design is quite promising, and is the focus of many current vaccine studies (440). Our work further highlights the importance of T_{RM} cells in vaccination, as T_{RM} cells established following immunization were shown to be effective for eliciting protection against HSV-2 challenge (Chapter 4). Furthermore, we have also demonstrated that E2 can be used to augment T_{RM} cells in tissues (Chapter 4), and as such, the incorporation of E2 in vaccine development can help strengthen the establishment of T_{RM} cells.

5.2 Study limitations

While the findings in this dissertation are novel and provide significant insights regarding mucosal immunity, there are limitations which must be considered, especially when extrapolating these results and suggesting direct implications for human health.

To begin with, these studies were conducted using an animal model. While the mouse model recapitulates several aspects of human disease as discussed previously (Chapter 1.3.4), there are considerable differences in immune responses and tissue physiology between humans and mice. For instance, while the mouse model of HSV-2 infection that we use is preferred compared to other animal models available, it does not perfectly mimic elements of human disease and there are challenges associated with this model. As discussed previously (Chapter 1.3.4), mice are not naturally susceptible to genital HSV-2 infection (204). Instead, mice can only be infected during the diestrus stage of the hormonal cycle, when P4 levels are highest and the murine epithelium is significantly thinner compared to other stages. In contrast, women can contract HSV-2 during any point of the menstrual cycle. Thus, mice must either be treated with Depo, which thins the epithelium to allow viral entry to occur, or have their ovaries removed to deplete endogenous sex hormone levels, which makes them susceptible to infection (200, 201). Additionally, not all aspects of infection in mice mirror human conditions. For instance, while HSV-2 often re-activates in humans, mice do not experience periodic episodes of reactivation (196). However, it would be difficult to understand the role of specific factors which affect susceptibility without the use of animal models, and the mouse model is an excellent research tool. Elements related to HSV-2 pathogenesis such as tissue infection,

viral replication within the FRT and viral dissemination, are very similar between mice and humans, and so the animal model has been critical for understanding this information. Therefore, while there are numerous aspects of disease which differ between both species, the mouse model still provides critical insights about HSV-2 from which we can learn from and apply towards better understanding this disease in humans.

Similarly, our study examining the effect of E2 on immune responses following immunization (Chapter 4) was done in an experimental setting, where all other endogenous sex hormones present were depleted through the process of OVX. Although this study design allows us to specifically examine the direct effects of E2, it does not simulate conditions normally found in women, where both E2 and P4 are present at various concentrations throughout the menstrual cycle. By examining the isolated role of E2, there may be outcomes that only occur when different hormones interact together, which are being overlooked in our model system. To mitigate this as best as possible, we work with E2 treatments that closely mimic physiological concentrations found in women. However, these levels are based on serum hormone levels and not active concentrations found in FRT tissues, and so again, our results may not completely recapitulate what occurs in cycling women. To confirm the effectiveness of E2 in enhancing anti-viral immunity during vaccination, it would be beneficial to conduct studies where E2 is present in conjunction with P4. Furthermore, future human studies should examine the effectiveness of vaccination during different phases of the menstrual cycle in women to determine if immunization during the E2-high phase of the cycle coincides with improved vaccine efficacy.

The HSV-2 "vaccine" used in our work is a live attenuated strain of the HSV-2 virus. TK$^-$ HSV-2 lacks thymidine kinase, which is required for viral replication specifically in neurons (204). Although attenuated viruses are weakened forms of the virus, they are not considered ideal vaccine candidates as there is a possibility of the virus mutating and reverting to its virulent form. In the case of TK$^-$ HSV-2, even without resulting in latency, IVAG inoculation still leads to the establishment of primary infection and can cause mild pathology in mice. While this makes TK$^-$ HSV-2 unacceptable for use in humans, there are other attenuated strains of HSV-2 which are considered more clinically relevant and can be used instead. For instance, the HSV-2 replication-defective vaccine (*dl*5-29/HSV529) is a similar attenuated formulation that has been tested in phase 1 clinical trials and demonstrated promising early results (178). For our purposes, TK$^-$ HSV-2 is an effective tool to study T cell-mediated mechanisms of viral clearance in the FRT. While this strain replicates similarly to WT HSV-2 in genital ECs, due to the deficiency of replication in neurons, infection with TK$^-$ HSV-2 will initiate immune responses for viral clearance without the concern of mortality (204). Most importantly, the protective immune response generated includes all critical factors of adaptive immunity including T and B cells, as well as memory cells to protect against subsequent infections. The use of live attenuated vaccines also generates long-lasting, durable immunity and these viruses are less likely to require the use of frequent boosters to maintain protection (195). These features allow us to study the role of T cells and memory responses following immunization. Future studies using alternate HSV-2 vaccine formulations which more closely resemble vaccine candidates being tested in clinical trials, can be used to validate our findings and ensure our

results are more applicable to human vaccine development. For example, Pennock et al. (374) tested the subunit gD/AS04 vaccine formulation in the mouse model and found that E2-treated mice had improved disease prevention, thus confirming our previous findings using TK^- HSV-2 (371).

Along with the formulation of the vaccine being used, the route of immunization is also a study limitation to be considered. Compared to distal routes of immunization, local IVAG immunization results in the greatest level of protection against subsequent challenge with HSV-2 (188, 205). In our model, IVAG immunization is not used because of the physical barrier to infection caused by keratinization of ECs under the influence of E2. Instead, we use an alternate mucosal route of vaccination, IN immunization, which also generates an immune response in the FRT and confers substantial protection against IVAG HSV-2 challenge (191, 205, 206). However, studying memory subsets of T cells is more difficult following IN immunization. The FRT is an immunologically restrictive tissue that prevents entry of activated T cells in the absence of inflammation or infection (266). This makes it difficult to induce a large enough population of vaginal T cells post-IN immunization to be able to conduct extensive phenotypic analysis or do any cell-sorting/isolation experiments. Since the parent populations of memory T cells are already rare in the absence of active infection, further studying smaller subsets, such as IFN-γ- and IL-17-secreting $CD4^+$ T_{RM} cells, becomes more complex. For this reason, in our studies, we stimulated cells *in vitro* with PMA and ionomycin to assess maximum cytokine production by T_{RM} cells. Although this stimulation process may have resulted in T cell responses which are not strictly HSV-2-specific, it allowed us to measure the maximum

capability of the vaginal T cells to induce cytokine responses, which closely mimics the overall T cell response produced following HSV-2 infection. To ensure most of the responses observed were antigen-specific, we stimulated non-immunized cells and found that only cells from immunized mice responded in a robust manner to PMA and ionomycin treatment. We also used *in vitro* stimulation with heat inactivated HSV-2 to confirm antigen-specificity. Even with this stimulation, the percentages of $CD4^+$ T cells we can identify in the FRT are quite low, especially in mock-treated mice. To try and increase the number of $CD4^+$ T cells post-immunization, we acquired HSV-specific $CD4^+$ transgenic mice from Dr. Andrew G. Brooks (the University of Melbourne). The gDT-II transgenic mice express transgenes encoding for the TCR specific for the HSV gD-derived epitope ($gD_{315-327}$) (441-443), and should theoretically allow us to study HSV-2-specific $CD4^+$ T cell responses at much higher numbers. The idea was to not only increase the number of $CD4^+$ T cells, but also use this model to examine antigen-specific $CD4^+$ T cells. Although we did see an antigen-specific response following *in vitro* stimulation with the corresponding gD peptide, which was enhanced in E2-treated mice, we were unable to do any further phenotypic analysis of markers related to T_{RM} cells due to again, small numbers of antigen-specific T cells (Chapter 4). Therefore, a better model is required to unequivocally examine antigen-specificity. Similarly, additional steps may be required to mitigate issues around low numbers of $CD4^+$ T_{RM} cells. For example, Shin and Iwasaki (186) have developed a vaccine strategy called “prime and pull” in order to overcome this barrier and establish local T_{RM} cells at the site of potential viral exposure. The “prime” step refers to vaccination with HSV-2 to elicit a systemic T cell response, and this is followed

by a "pull" step in order to recruit activated T cells to the site of potential viral exposure. They topically applied chemokines CXCL9 and CXCL10 to the vaginal cavity of subcutaneously immunized mice to "pull" activated T cells into the vaginal tissue. Utilizing a similar prime and pull strategy may help increase recruitment of effector T cells to the vaginal tract of E2- and mock-treated mice, thus better allowing us to study the effect of E2 on the establishment of T_{RM} cells.

Interestingly, there is still some debate regarding the characterization of $CD4^+$ T_{RM} cells. The flow cytometric experiments conducted in our studies were carefully designed to capture the expression of known vaginal T_{RM} cell markers. An extensive panel was used to include key markers, and appropriate measures such as isotype controls and FMO controls were used to ensure adequate phenotypic analysis of this memory subset. However, since this is a new area of investigation, especially in the FRT, it is possible that the phenotypic description of this population will continue to change. As such, additional markers such as CD44 and CD11a (396) can be added to the proposed marker panel in future experiments to ensure this population is being thoroughly captured. Furthermore, rather than marker expression, true tissue residency is determined based on lack of cell mobility. In our work, we paired flow analysis with *in vivo* treatment of FTY720 in order to test the protective capabilities of the T_{RM} cell population. While FTY720 is commonly used to examine T_{RM} cell populations in various tissues and was adequate for our purposes, alternative strategies such as parabiosis and *in vivo* cell labelling can also be used to examine these cells more closely. For instance, while the use of FTY720 allows us to study the role of T_{RM} cells in the absence of any circulating cells, this does not allow us to study the role of just

circulating memory T cells. Ideally, it would be best to compare the ability of T_{RM} cells to mediate protection against the ability of circulating memory T cells, both in isolation. Parabiotic combinations can be generated such that some mice rely entirely on circulating memory cells from their conjoined partner, which would allow us to make the comparison between circulating memory T cells and T_{RM} cells. As such, further work examining the modulation of memory responses by E2 may benefit from the use of such tools and techniques.

Similar to the limitation of defining $CD4^+$ T_{RM} cells, we also have limited our characterization of vaginal DCs as being primarily $CD11c^+$. CD11c is broadly expressed by a number of cell types, including macrophages and monocytes (444, 445), and the expression of this marker varies depending on the location of the DCs. Based on previously published work (341), we used CD11c as a broad marker to represent the vaginal DC population (Chapter 3). While we did not further resolve this rare population of cells within the FRT using additional markers, we felt that using CD11c as the main marker was sufficient, as we have shown that $CD11c^+$ DCs are the primary inducers of T_h17 responses in the FRT (341). In the previous study conducted in our lab, different sources of APCs were sorted from the FRT, including DCs, macrophages, neutrophils and monocytes, and only T cell co-cultures conducted with $CD11c^+$ DCs resulted in IL-17 production (341). Since vaginal $CD11c^+$ DCs were shown to be the primary source of APCs responsible for stimulating T cells, we feel our characterization of this population was adequate for our purposes. Additionally, the OVA-peptide co-culture model was used to examine immune responses by vaginal APCs under steady state conditions, in the presence or absence of IL-

17. It is likely that within this system, the APCs may be stimulating a broad spectrum of immune responses which do not accurately mimic what would happen in the context of a viral infection. We also faced a similar limitation when identifying innate lymphocytes which secrete IL-17 in the vaginal mucosa under homeostatic conditions. In our study, we found that $\gamma\delta^+$ T cells were the primary source of innate IL-17 (Chapter 3). However, there were other minor populations of IL-17-secreting cells which we would have liked to further characterize (data not shown) but were unable to due to low cell yields, and lack of distinct markers used to describe innate lymphoid populations. In future studies, further characterization with a larger panel of markers is warranted to identify these populations of innate IL-17-producing cells.

5.3 Future directions

Throughout this work, we have demonstrated several potential mechanisms through which IL-17 and E2 enhance protection against HSV-2 in the FRT (Figure 5.1). While we have shown that the presence of E2 often coincides with greater protective immune responses such as enhanced levels of IL-17 and IFN-γ, and greater establishment of $CD4^+$ T_{RM} cells, there is limited data to demonstrate a direct cause/effect relationship between these factors and greater protection post-HSV-2 challenge. More elaborate studies are required to unequivocally determine if the protection observed in E2-treated mice is a direct result of these effects. For instance, $CD4^+$ T cells can be isolated from E2-treated mice following IN immunization and adoptively transferred to mock-treated, non-immunized control mice prior to IVAG challenge. Following the transfer, T cell responses in the FRT of the recipient mice can be examined to see if there are greater T_h1 and T_h17 responses observed, and if the mice demonstrate similar protection as seen in E2-treated mice. Similarly, it is still unclear if the protection observed in E2-treated mice is a direct result of enhanced T_h17 responses on their own, or if enhanced T_h17 responses indirectly mediate greater protection by enhancing T_h1 immunity. To this end, it would be worthwhile to treat IFN-γ KO animals with E2 and examine if there is still enhanced T_h17 immunity following immunization, and if the mice are better protected against HSV-2 challenge as a result. Additionally, more extensive studies can be conducted to better understand how the presence of E2 results in greater establishment of $CD4^+$ T_{RM} cells in the FRT post-IN immunization. There are still several questions surrounding the role of E2 in the context of T cell memory. It is unclear if E2 is enhancing the trafficking of memory cells into the FRT,

enabling their survival once they are localized within the FRT, or if the effect of E2 is related to $CD4^+$ T_{RM} cell retention. For instance, we can examine the effect of E2 on the priming of HSV-2-specific $CD4^+$ cells by nasal DCs, and assess whether E2 enhances the trafficking of these cells to the FRT. Nasal DCs can be isolated from E2- and mock-treated mice and co-cultured with $CD4^+$ T cells isolated from gDT-II Tg mice *in vitro,* with the use of inactivated HSV-2 to pulse the cells. The $CD4^+$ T cells can then be isolated from the cultures and adoptively transferred into non-immunized control mice, and we can examine if a greater number of HSV-2-specific $CD4^+$ T cells from the E2 co-cultures traffic to the FRT following adoptive transfer. Experiments like these can provide better mechanistic insights about how E2 mediates enhanced anti-viral immunity *in vivo*.

A potential area of exploration, which comes as a result of *in vitro* studies done in our lab, is to examine the upstream factors involved in T_h17 responses. As previously mentioned, a cytokine milieu of IL-6, TGF-β and IL-23 is considered essential for priming T_h17 responses (446). However, alternative pathways involving IL-1 signalling in combination with IL-6, IL-21 and IL-23 have also been described (108). While we have examined the role of IL-1β in the induction of innate IL-17 in this work (Chapter 3), we have also showed similar results in a previous publication, where T_h17 responses were significantly impaired in co-cultures containing $CD11c^+$ DCs from IL-1β KO mice (341). When exogenous rIL-1β was added, this effect was partially reversed. Taken together, these studies show that IL-1 expression by vaginal DCs is essential for the induction of IL-17. Based on this work, it would be interesting to test the *in vivo* response to HSV-2 in KO animal strains where some of these upstream factors are absent. For example, preliminary

experiments were done using IL-1β KO mice, where mice were pre-exposed to lower doses of WT HSV-2 virus (IVAG) and then re-exposed to higher doses six weeks later (Appendix II). There were some differences in susceptibility observed when comparing IL-1β KO mice to WT controls, with IL-1β KO mice demonstrating greater mortality, genital pathology and viral shedding at certain doses. However, these experiments would have to be repeated and extended further to examine this in more detail. We would need to repeat this work using the IN immunization model, with greater animal numbers. Additionally, the effect of IL-1β on the establishment of memory T cells can also be studied using these mice. Thus, using KO strains of mice would further confirm the critical role of factors upstream of IL-17 induction which are contributing to anti-viral immunity in the FRT.

Another avenue to be explored in terms of understanding the anti-viral role of IL-17 in the FRT is to examine if IL-17 mediates more efficacious IFN-γ responses by modulating chemokine production. In the pulmonary *M. tuberculosis* vaccination model, Khader et al. (384) found that T_h17 responses induced in the lung following immunization played a key role in accelerating T_h1 responses, and that the induction of these responses resulted in the rapid resolution of bacterial infection post-challenge. In the absence of IL-17, IFN-γ responses and clearance of the bacteria were significantly delayed, whereas addition of IL-17 restored the IFN-γ recall response. This is similar to what we observed in our studies (Chapter 2), where IFN-γ responses were significantly diminished in *IL-17A*$^{-/-}$ mice and resulted in greater susceptibility to HSV-2 challenge. In the study by Khader et al., they showed that the T_h17 response post-challenge correlated with a concurrent CXCL9, CXCL10 and CXCL11 chemokine response, which was essential for the

accumulation of IFN-γ^+ $CD4^+$ T cells in the lungs (384). Other studies have shown the role of these CXCR3 chemokine ligands in the context of genital HSV-2 infection. For example, CXCL9 and CXCL10 expression induced by IFN-γ has been shown to be important for recruiting effector $CD8^+$ T cells to infected tissue via CXCR3 post-challenge (92). Likewise, another study observed that topical application of chemokines CXCL9 and CXCL10 was sufficient to recruit effector T cells to the vagina, even in the absence of infection (186). As such, it would be worthwhile to study the role of these chemokines in the context of T_h17-mediated acceleration of $CD4^+$ T cell anti-viral immunity in the FRT. This can be done by comparing the induction of these chemokine transcripts in the vaginal tracts of WT and *IL-17A*$^{-/-}$ mice post-challenge.

Since the work in this dissertation has suggested multiple mechanisms through which E2 modulates immunity, ultimately, understanding the effects of E2 on a molecular level will allow us to better understand the precise cellular pathways involved. This could be useful in determining specific gene targets that can be manipulated to enhance anti-viral immunity or regulate susceptibility. As mentioned earlier, we have previously shown that E2 primes vaginal DCs to induce a greater T_h17 response, and that this response greatly coincides with a greater T_h1 response; thus, demonstrating that E2 influences DC priming in the FRT (341). Additionally, in Chapter 4 we demonstrate that E2 treatment leads to greater establishment of $CD4^+$ T_{RM} cells post-immunization, which consequently, results in greater protection against viral challenge. These findings suggest that E2 is likely modulating the local tissue microenvironment within the FRT and influencing vaginal DC programming to induce greater T_h17 responses. Therefore, we wanted to examine how

treatment with E2 might alter the expression of genes in whole vaginal tissue, as well as vaginal DCs. As such, we conducted a microarray analysis using the Clariom S platform (Applied Biosystems, ThermoFisher Scientific) to provide a comprehensive transcriptional profile of whole murine vaginal tissue (Appendix III), as well as $CD11c^+$ vaginal DCs (Appendix IV), following *in vivo* E2 treatment. This type of analysis provides a broader understanding of genes and pathways that may be upregulated by E2 and can further help decipher the impact of E2 in mediating protective immunity in the FRT.

Following differential expression analysis, a list of ~4000 genes were identified which were differentially regulated due to E2 treatment (either up- or downregulated) in whole vaginal tissues (adj. $P < 0.05$), compared to OVX mice (Appendix III). Many of the genes identified were related to structural integrity of the FRT, including cell to cell junctions (desmoglein genes) and keratin-related genes. This is similar to what others in the lab have found in microarray studies using vaginal cell lines and primary cells (unpublished), and is not surprising given E2 has broad effects on the structural composition of the FRT. We also found that genes related to IL-1 were differentially expressed by E2 treatment, which supports our work demonstrating that E2 treatment mediates greater T_h17 responses through an IL-1-dependent pathway (341) (Chapter 3), and suggests that the role of IL-1 should be examined more closely. We also looked at the influence of E2 treatment on protein networks, by conducting a KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis to identify pathways enriched within the list of differentially expressed genes. Again, T_h17 cell differentiation was one of the primary pathways enriched, supporting our findings. Interestingly, many of the other

pathways were related to metabolic processes involved in the survival of T_{RM} cells. Memory T cells require additional energy, and pathways related to oxidative phosphorylation, metabolism and fatty acid metabolism, as well as HIF-1-, FoxO-, mTOR- and sphingolipid-signalling pathways have been shown to be important for long term survival and residency of T_{RM} cells (447-449). For instance, recent studies have shown that $CD8^+$ T_{RM} cells found in the skin following viral infection highly express several molecules that mediate lipid uptake and intracellular transport, including fatty-acid binding proteins (450). In the absence of these proteins, the persistence of $CD8^+$ T_{RM} cells was strongly diminished and less protection was observed following subsequent viral challenge; thus, demonstrating that these fatty-acid binding proteins play an important role in the maintenance, longevity and function of $CD8^+$ T_{RM} cells. Similarly, another study looking at the importance of metabolism on memory T cell generation showed that blocking mTOR inhibited the formation of memory $CD8^+$ T cells in the intestinal and vaginal mucosa (451). These pathways have not been examined in the context of $CD4^+$ T_{RM} cells in the FRT, but could potentially be playing similar roles in supporting the formation and maintenance of these cells. Additionally, further clustering analysis also demonstrated that genes in these pathways are not only differentially expressed by E2, but are upregulated following E2 treatment. These findings suggest that E2 might be regulating pathways important for T_{RM} cell survival and retention in the FRT. However, as this analysis was done using whole tissue, further studies examining T cells specifically are required. Overall, while further work is required, the initial microarray analysis conducted on whole vaginal tissue confirms our findings regarding the upregulation of T_h17-related pathways by E2 and also provides

insight regarding the modulation of T_{RM} cells by E2. These findings suggest that future studies should focus on specific pathways to further understand the influence of E2 on T_{RM} cell metabolism and survival.

We also looked at the differential expression of genes in $CD11c^+$ vaginal DCs isolated from E2- and mock-treated mice (Appendix IV). Not surprisingly, since we were examining such a specific subtype of immune cells, a smaller list of ~60 genes were identified which were differentially regulated due to E2 treatment (either up- or downregulated). KEGG enrichment and protein-protein analysis demonstrated that T_h17 cell differentiation and IL-17 signalling pathways were both enriched amongst the gene list, supporting current findings. This data suggests that E2 influences the programming of vaginal DCs, and thereby, modulates T cell priming. Interestingly, one of the top nodes identified with numerous protein interactions was NLRP3 (NLR Family Pyrin Domain Containing 3). NLRP3 plays an important role in innate immunity and inflammation and initiates the formation of the inflammasome polymeric complex (452). Recruitment of pro-caspase 1 (CASP1) promotes its activation, and leads to maturation and secretion of IL-1β and IL-18 (452). As we have shown that E2 induces T_h17 and IL-17 responses via an IL-1-dependent pathway (341) (Chapter 3), upregulation of NLRP3 suggests that this may be the upstream pathway through which E2 regulates IL-1β induction and consequently, IL-17 production. By using NLRP3 KO mice and conducting similar *in vitro* co-culture and *in vivo* experiments described throughout our work, we can examine whether E2 leads to the induction of inflammasome pathways in vaginal DCs and if this is the pathway through which E2 enhances T_h17 and IL-17 responses. Overall, the microarray studies conducted

provide further evidence to support the mechanisms of E2-mediated protection in the FRT which we have previously published and presented throughout this thesis. Furthermore, this work provides further insight into other potential mechanisms through which E2 modulates immunity in the FRT.

With the processes involved in microarray analysis now well optimized, ultimately, the goal is to do similar analysis using a variety of FRT cells, including DCs and $CD4^+$ T cells collected from mice under different conditions (i.e. E2 treatment, no hormone, in the absence of IL-17, in the presence and absence of HSV-2), to identify the effect of E2 on relevant genes pathways. For example, we are very interested in understanding how E2 might be regulating T_{RM} cells in the FRT. Gene analysis will allow for the detection of transcriptional factors in the FRT (i.e. T-bet, S1P1, KLF2, Hobit, Blimp1, EOMES, TCF1, Bcl6, Id3) which have been shown to play an important role in the localization and maintenance of T_{RM} cells (61, 62). By sorting vaginal $CD4^+$ T cells post-IN immunization, we can examine the effect of E2 on the expression of factors related to T_{RM} cell survival and retention. Additionally, effector T cells are programmed to home specifically to certain tissues, and tissue-specific homing is mediated through the expression of various integrins and chemokine receptors. For instance, CCR5 and CXCR3 expression by T cells has been implicated in FRT-homing following IVAG immunization with TK^- HSV-2 (92, 186, 219). Similarly, both chemokine receptors have shown to be involved with T cell trafficking to the FRT during chlamydial infection (65), along with expression of integrins VLA-4 ($\alpha4\beta1$) and LFA-1 (453, 454). However, it has yet to be determined if these are the same markers responsible for $CD4^+$ T cell homing to the FRT post-IN immunization. Using this

microarray approach, we will examine the transcriptional profile of vaginal $CD4^+$ T_{RM} cells post-IN immunization to determine which integrins and chemokine receptors are expressed and how this expression is modulated by E2. Finally, T cell homing into the FRT also involves the expression of corresponding chemokines and adhesion molecules. Interestingly, female sex hormones including E2 are known to regulate vaginal tissue expression of these factors (315), suggesting a critical role for the hormonal microenvironment in T cell homing. Unlike the gut and skin, where expression of distinct homing chemokines and adhesion molecules is well characterized, little is known regarding the expression of homing molecules by the FRT. Studies have shown a role for vaginal tissue expression of CCL5 in $CD4^+$ T cell retention (261), and CXCL9 and CXCL10 in $CD8^+$ T cell recruitment (16, 63) following genital HSV-2 infection. Expression of VCAM-1 and ICAM-1 by the FRT has also been implicated in attracting T cells into the tissue following genital chlamydial and HSV-2 infection (454, 455). However, the roles of these chemokines/adhesion molecules and others in $CD4^+$ T cell homing following IN immunization have not been described. Therefore, we can examine the effect of E2 on the expression of these chemokines and adhesion molecules in the FRT. This approach for understanding the effect of E2 on a molecular level would generate extensive data, which can then be used to generate novel hypothesis-driven questions.

Finally, based on our findings regarding the effect of E2 on T cell responses in the URT, it would be beneficial to replicate similar studies in respiratory disease models such as influenza. Infections with seasonal influenza viruses are initially established in the URT; although, progression to the lungs is generally essential for the development of more severe

pulmonary disease (456). As such, most of what is known regarding immune responses following influenza infection is based on studies done in the lower respiratory tract (lungs), where protective immunity has been shown to be correlated with the persistence of $CD8^+$ T_{RM} cells (456). However, if the virus can be prevented from being transmitted from the URT into the lungs, this could prevent the development of more severe disease. Specifically, the establishment of influenza-specific T_{RM} cells in the URT could help prevent dissemination of the virus into the lungs. While there have been reports about the protective effects of E2 against influenza infection in the lungs, the influence of E2 on T cell responses in the URT remains largely unknown. Studies led by Dr. Sabra Klein have shown that E2 mediates protection against influenza infection in the lungs by supressing inflammatory responses (457), as well as by recruiting neutrophils and increasing $CD8^+$ T cell responses (458). These studies were all done in the context of primary infection of the lungs and mainly focused on $CD8^+$ T cell responses. Interestingly, very little is known about T_{RM} cells in the URT. To our knowledge, there is only one published study that looked specifically at $CD8^+$ T_{RM} cells in the context of influenza infection and showed that following IN immunization, influenza-specific $CD8^+$ T_{RM} cells ($CD103^+$ $CD69^+$) were established in the URT and were able to block the transmission of the virus into the lungs (456). Other studies examining nasal bacterial infection with *Bordetella pertussis* described IFN-γ^+ and IL-17^+ $CD4^+$ T_{RM} cells ($CD44^+$ $CD69^+$) in the nasal tissue (459), which conferred long-term protection against nasal colonization and lung infection (286). We propose to examine the effect of E2 on the establishment of T_{RM} cells in the URT following IN immunization with influenza. Post-immunization, influenza-specific $CD4^+$ and $CD8^+$

T_{RM} cells can be characterized in the URT, along with functional T cell responses (IFN-γ and IL-17). We expect that compared to mock-treated mice, E2 treatment will enhance these T cell populations in the URT. Furthermore, we will test if the enhancement of T_{RM} cells observed in the presence of E2 will lead to better protection against influenza challenge, by conducting survival studies and examining protection in the lungs. These studies will have important implications regarding strategies to help augment protective immune responses induced following respiratory vaccination.

5.4 Concluding remarks

In conclusion, this dissertation summarizes the original contributions and work completed during the last five years to understand numerous ways IL-17 influences anti-viral protection in the FRT (Figure 5.1). We have provided a comprehensive analysis of the role of IL-17 in the vaginal mucosa by examining both innate and adaptive sources of this cytokine, and demonstrated a novel anti-viral role for T_h17 immunity in the FRT, where IL-17 appears to mediate more efficient T_h1 immunity. We have also shown that factors in the local microenvironment of the FRT, such as E2, the vaginal microflora and innate lymphocytes, can modulate these IL-17 responses. Furthermore, this work provides further understanding of the protective, anti-viral mechanisms of E2, which appears to mediate protection through an IL-17-dependent pathway and plays an important role in establishing greater memory T cell responses in mucosal tissues. However, there is still a gap in our understanding of the underlying pathways being modulated by factors in the FRT which affect immunity, and so further studies are warranted to better understand the regulation of immune responses at a molecular level. Overall, the knowledge generated from this dissertation provides further insight on effective strategies to improve mucosal vaccines and generate more efficient protection against viral infections such as HSV-2.

CHAPTER 6: References

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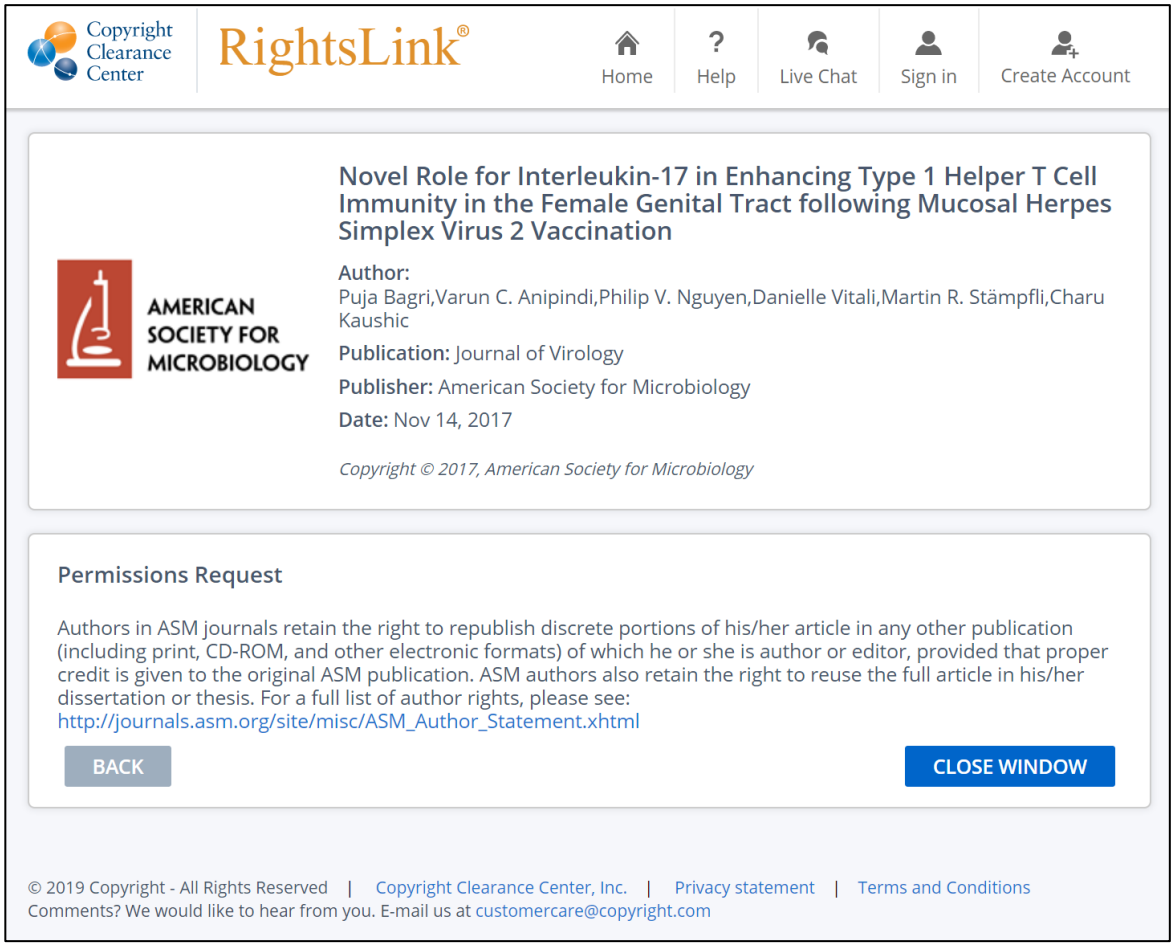

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APPENDIX II: IL-1β experiments

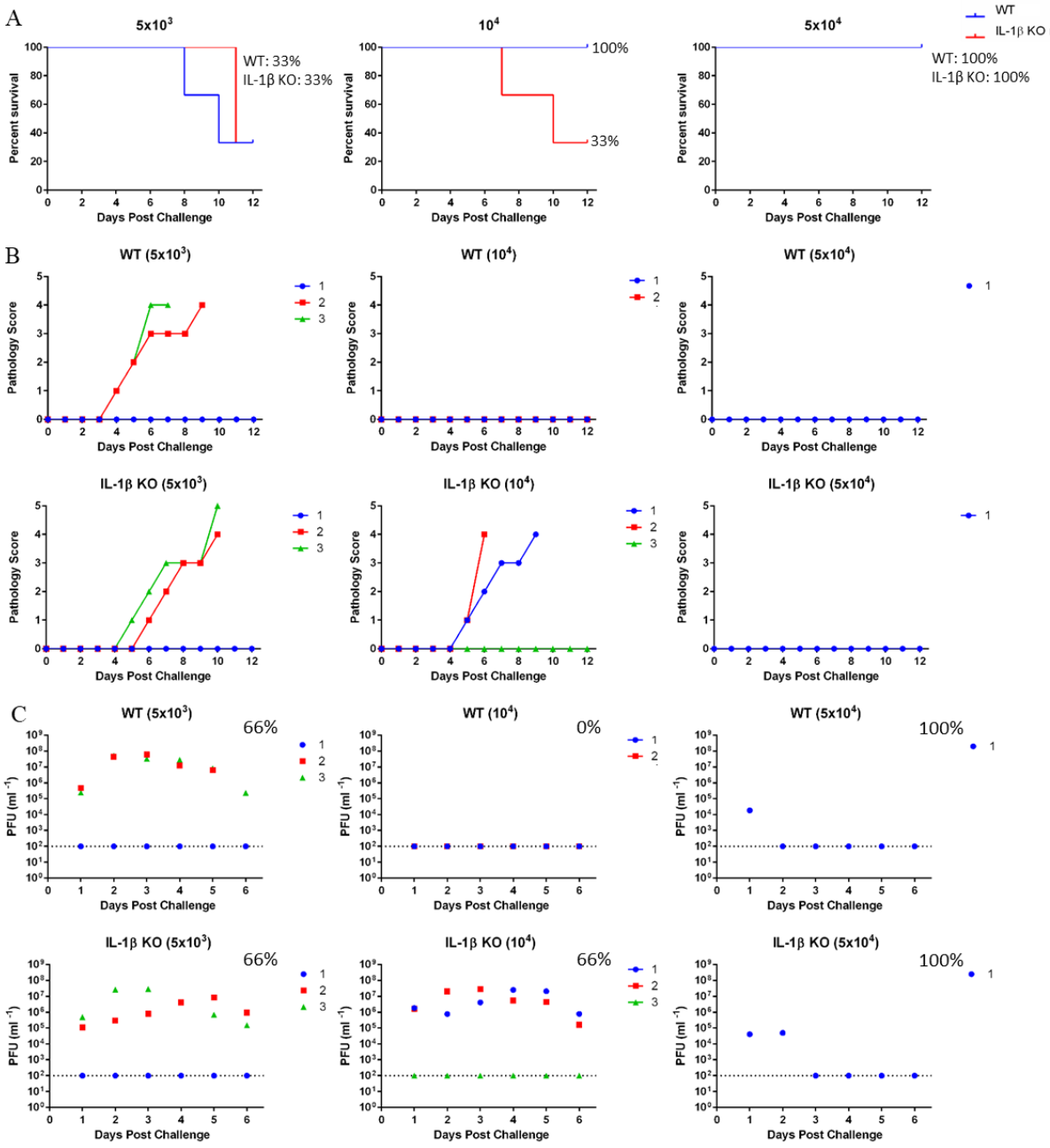

Figure 1: Disease outcomes in IL-1β KO mice following HSV-2 challenge. OVX WT (n=3-4/group) and IL-1β KO (n=3-4/group) mice were intravaginally exposed to sub-lethal doses of WT HSV-2 (10^2, $5x10^2$ and 10^3 pfu/mouse), and 6 weeks later, intravaginally re-exposed to a higher dose of WT HSV-2 ($5x10^3$, 10^4 and $5x10^4$ pfu/mouse). Survival was monitored (A) and pathology scores were recorded (B) for 12 days post re-exposure. Data points superimposed on the x axes of panel B indicate mice without genital pathology. (C) Vaginal washes were collected daily for 6 days post re-exposure and viral plaque assays were done. Plaques were counted and viral titers were expressed as pfu/mL. Each symbol represents a single animal. The dotted lines in panel C indicate the lower detection limit of the assay, and data points on this line indicate undetectable viral shedding. The percentages in panel C represent maximum numbers of mice that shed virus on any given day.

APPENDIX III: Microarray analysis of whole vaginal tissue

Here, we have used microarray analysis to provide a comprehensive transcriptional profile of changes in murine vaginal tissue following *in vivo* E2 treatment. To examine the effects of E2 on the overall gene expression of the vaginal tissue, OVX mice were treated with E2 or mock-pellets and two weeks later, tissue was collected. Cervical tissue was dissociated and removed, and only vaginal tissue was used. Each mouse was processed individually, with a total of 3 mice per group. Vaginal tissue was homogenized using mechanical disruption via metal beads (Bullet Blender, Next Advance Inc., Troy, NY, USA) and RNA was extracted from whole tissue lysate using the RNeasy Plus Mini Kit (QIAGEN Inc., Toronto, ON, Canada). Bioanalysis of RNA samples was completed at the McMaster Genome Facility (Hamilton, ON, Canada) to ensure high-quality RNA samples were run on the microarray. The average RNA integrity value (RIN) for all samples was >8, which is indicative of high RNA quality. Microarray chip hybridization and processing were performed by the Genetic and Molecular Epidemiology Laboratory facility at the David Braley Cardiovascular and Stroke Research Institute (Hamilton, ON, Canada). For whole tissue analysis, the Clariom S Plus Mouse GeneChip (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) was used as per the manufacturer's instructions. Data was normalized and initially analyzed by bioinformatics specialist, Dr. Christopher P. Verschoor. All pre-processing steps and differential expression analyses were performed in R v3.3.2 (The R Foundation for Statistical Computing). All raw data were loaded using the package "oligo" and background correction and normalization was performed using robust multiarray average (RMA). Array batch and sample effects were

removed using the ComBat function in the package "sva" and the following probe sets were removed: spike-in, positive and negative housekeeping, and background control and reporter probe sets. The probe sets, whose intensity was below the 95th percentile of both anti-genomic and intronic probe sets of housekeeping genes in more than 25% of chips, were analyzed. The final probe set count for the whole tissue analysis was 7590. Adjusted P-values were used to account for multiple testing using Benjamini-Hochberg's procedure for controlling false discovery rate (FDR). Further analysis was done using the NetworkAnalyst platform (v3.61) (http://www.networkanalyst.ca) to identify biological networks changed by E2 treatment. NetworkAnalyst is a visual analytics platform for comprehensive gene expression profiling and meta-analysis. First-order protein-protein interaction (PPI) networks were generated using the STRING interactome database with a confidence score cut off of 900.

Following pre-processing steps and differential expression analysis, a list of ~4000 genes were identified which were differentially regulated due to E2 treatment (either upregulated or downregulated) and met an adjusted P value < 0.05 (adj. P < 0.05). Table 1 highlights the top 20 genes differentially regulated by E2 treatment, based on foldchange. As seen in this table, many genes are related to structural integrity of the FRT, including cell-cell junctions (desmoglein genes) and keratin-related genes. This is similar to what we have seen in transcriptomic studies using vaginal cell lines and primary cells (unpublished), and is not surprising as E2 has great effects on the structural composition of the FRT. Interestingly, genes related to IL-1 are also shown to be differentially expressed by E2

treatment. We have shown in previous work that E2 treatment induces greater T_h17 responses through an IL-1-dependent pathway (1, 2), and these findings support this.

To identify which biological gene pathways are differentially regulated due to E2 treatment, data was analyzed using NetworkAnalyst. NetworkAnalyst was used to determine the potential influence E2 treatment has on protein networks within vaginal tissue. The differentially expressed transcripts from E2-treated mice (adj. $P < 0.05$) were uploaded into NetworkAnalyst to examine the potential effect proteins encoded from these transcripts have on cellular protein networks. We did an initial KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis to identify pathways enriched within the list of differentially expressed genes. There were 121 different pathways which were highly associated with the differentiated genes (adj. $P < 0.05$), some of which are highlighted in Table 2. Again, T_h17 cell differentiation was one of the primary pathways enriched, supporting previous findings (1, 2). Interestingly, many of the pathways highlighted are related to metabolic pathways involved in the survival of T_{RM} cells. Memory T cells require additional energy, and pathways related to oxidative phosphorylation, metabolism and fatty acid metabolism, as well as HIF-1-, FoxO-, mTOR- and sphingolipid-signalling pathways have been shown to be important for long term survival and residency of T_{RM} cells (3-5). This suggests that E2 may be regulating pathways important for T_{RM} survival and retention in the FRT.

Due to the large volume of genes and pathways identified which were differentially expressed following E2 treatment, we analyzed the data based on clustering of genes according to a heat map (Fig. 1). Genes in cluster 1 (red) and 3 (green) were significantly

upregulated by E2 treatment, while genes in cluster 2 (cyan) and 4 (purple) were downregulated. When looking at the two clusters upregulated by E2, 34 different pathways were found to be associated with genes in cluster 1 (Table 3), while 29 different pathways were related to the genes in cluster 3 (Table 5). Several pathways mentioned previously (oxidative phosphorylation, metabolic pathways, fatty acid metabolism, HIF-1-, FoxO-, mTOR- and sphingolipid-signalling) could be found within clusters 1 and 3, showcasing that not only are genes related to these pathways differentially expressed by E2, but they are upregulated following E2 treatment. Next, the biological significance of the first-order PPIs was determined by examining gene enrichment within the Reactome database (Table 4 and 6). Ontological analysis demonstrated that E2 treatment results in the upregulation of transcripts that encode proteins that interact with and modulate other proteins involved in inflammatory pathways such as NF-kappa B and TNF signalling pathways, IL-17 signalling and T_h17 cell differentiation, apoptosis and FoxO signalling (Table 4 and 6). When looking at the two clusters downregulated by E2, only 3 different pathways were found to be associated with genes in cluster 2 (Table 7), while 51 different pathways were related to the genes in cluster 4 (Table 9). Some interesting pathways associated with downregulated genes following E2 treatment include cancer pathways, herpes simplex virus and ribosome.

This initial analysis is preliminary in nature and requires further work. For instance, to confirm the microarray data, RNA levels of the top differentially expressed genes will be verified using quantitative real time PCR. The pathway analysis will also be explored in more detail, and functional assays will be conducted to identify key mechanisms. In future

studies, we are also interested in examining how the changes we see in E2-treated tissue are altered in the presence of infection. As such, next steps include doing similar experiments and analysis using tissue collected from E2-treated mice following immunization with HSV-2. For instance, we are interested in better understanding how IN immunization with HSV-2 under the influence of E2 leads to greater establishment of T_{RM} cells in the FRT. As such, we can examine the gene expression of different cytokines and chemokines involved in T cell trafficking, and investigate the effect of E2. Additionally, we could sort T cells and conduct microarray studies to specifically look at the effect of E2 on different subsets of cells. Overall, the pathways identified by our whole vaginal tissue microarray analysis can help supplement the findings discussed throughout this dissertation. Furthermore, this data will generate hypothesis-driven questions which will provide insight on the cellular mechanisms by which E2 mediates protection against infections.

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Figure 1: Heat map showcasing clustering of differentially expressed genes in vaginal tissue following E2 treatment (adj. P < 0.05).

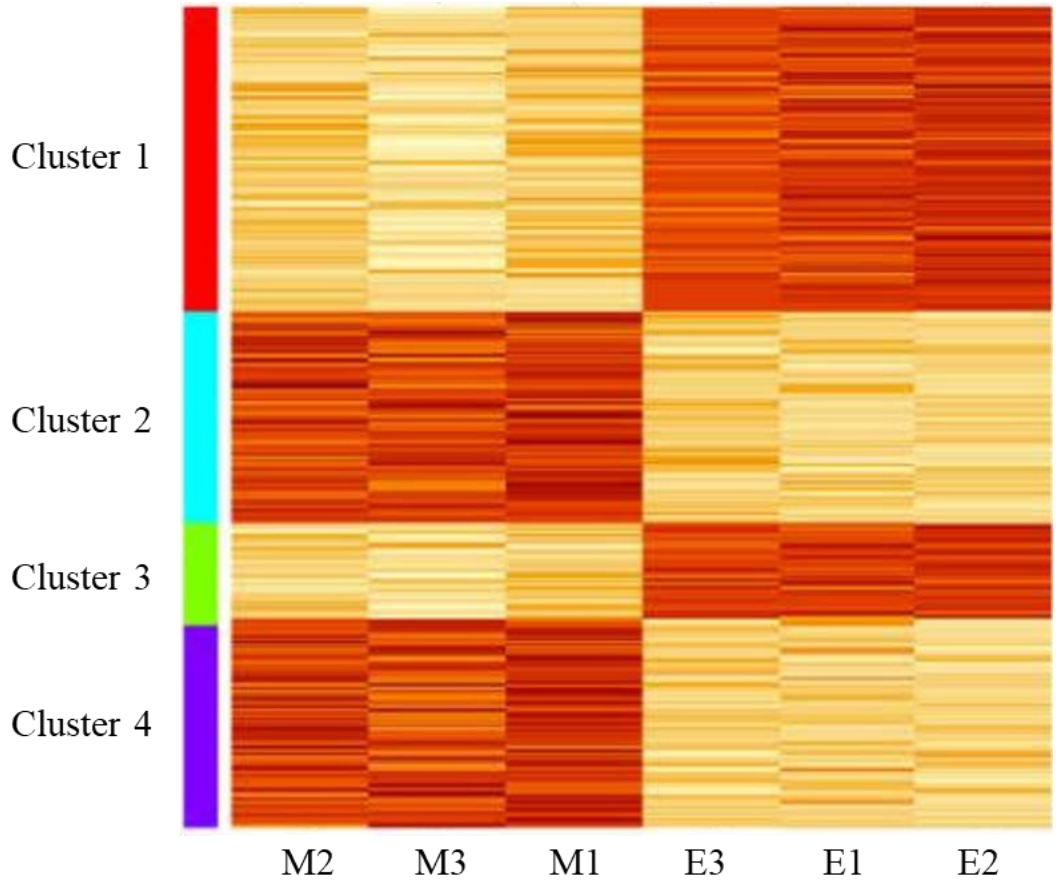

Table 1: Differentially expressed genes in vaginal tissue following E2 treatment (adj. P < 0.05)

Gene	Name	Log F.C.	P value	Adj. P
Hrnr	hornerin	8.095816	3.31E-09	2.82E-07
Dsg1a	desmoglein 1 alpha	7.989674	2.32E-11	2.78E-08
Dsg1b	desmoglein 1 beta	7.523895	1.96E-07	5.15E-06
Kprp	keratinocyte expressed, proline-rich	7.381738	1.07E-11	2.78E-08
Lce3f	late cornified envelope 3F	7.330733	2.38E-10	5.32E-08
Krt76	keratin 76	7.249602	2.69E-12	2.04E-08
Il1f6	interleukin 1 family, member 6	6.942353	5.62E-11	2.78E-08
Lce1a1	late cornified envelope 1A1	6.764353	1.55E-11	2.78E-08
Sprr3	small proline-rich protein 3	6.493348	4.12E-06	4.83E-05
Gm94	predicted gene 94	6.371526	2.23E-08	1.16E-06
Elovl4	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	6.352415	1.72E-09	1.93E-07
Teddm3	transmembrane epididymal family member 3	6.327748	4.38E-06	5.06E-05
Gm6551	predicted gene 6551	6.319165	7.73E-08	2.70E-06
Dsc1	desmocollin 1	6.313479	4.94E-08	2.02E-06
Il1f8	interleukin 1 family, member 8	6.28028	4.69E-10	8.89E-08
Cdsn	corneodesmosin	6.168993	3.16E-11	2.78E-08
Pla2g2e	phospholipase A2, group IIE	6.101471	6.96E-11	2.78E-08
Fabp4	fatty acid binding protein 4, adipocyte	6.042222	4.10E-11	2.78E-08
Serpinb3a	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 3A	5.989606	0.000648	0.002234
Fetub	fetuin beta	5.919193	1.30E-09	1.64E-07

Table 2: Functional annotation clustering using KEGG enrichment of the differentially expressed genes in vaginal tissue following E2 treatment (adj. P < 0.05)

Pathway	P value	Adj. P
Endocytosis	5.43E-12	1.70E-09
Ribosome	1.24E-10	1.94E-08
Proteasome	1.99E-10	2.08E-08
Lysosome	2.69E-10	2.11E-08
Oxidative phosphorylation	1.15E-09	7.20E-08
Pathways in cancer	7.28E-09	3.24E-07
Epstein-Barr virus infection	8.14E-09	3.24E-07
Fluid shear stress and atherosclerosis	8.35E-09	3.24E-07
Metabolic pathways	9.27E-09	3.24E-07
Proteoglycans in cancer	2.82E-08	8.86E-07
Toxoplasmosis	2.08E-07	5.75E-06
Neurotrophin signaling pathway	2.20E-07	5.75E-06
Alzheimer's disease	2.53E-07	6.12E-06
Prostate cancer	3.65E-07	8.19E-06
HIF-1 signaling pathway	6.39E-07	1.24E-05
EGFR tyrosine kinase inhibitor resistance	6.58E-07	1.24E-05
Cellular senescence	6.73E-07	1.24E-05
Estrogen signaling pathway	1.41E-06	2.42E-05
Glutathione metabolism	1.54E-06	2.42E-05
Central carbon metabolism in cancer	1.54E-06	2.42E-05
FoxO signaling pathway	2.04E-06	3.02E-05
mTOR signaling pathway	3.77E-06	4.93E-05
Sphingolipid signaling pathway	2.08E-05	0.000187
TGF-beta signaling pathway	0.000268	0.00156
Toll-like receptor signaling pathway	0.000478	0.0025
TNF signaling pathway	0.00105	0.0049
Fatty acid metabolism	0.00574	0.0194
Fatty acid elongation	0.0124	0.0362
Th17 cell differentiation	0.0126	0.0362
Fatty acid degradation	0.0132	0.0373
PI3K-Akt signaling pathway	0.0166	0.0442

Table 3: Functional annotation clustering using KEGG enrichment of highly upregulated genes in vaginal tissue (cluster 1) following E2 treatment (adj. P < 0.05)

Pathway	P value	Adj. P
Metabolic pathways	1.12E-12	3.52E-10
Oxidative phosphorylation	1.39E-10	2.19E-08
Proteasome	9.59E-09	1.00E-06
Endocytosis	1.84E-08	1.45E-06
Collecting duct acid secretion	5.78E-08	3.63E-06
Steroid biosynthesis	8.10E-06	0.000424
mTOR signaling pathway	2.74E-05	0.00123
Rheumatoid arthritis	4.73E-05	0.00186
Protein processing in endoplasmic reticulum	7.17E-05	0.00241
Non-small cell lung cancer	7.68E-05	0.00241
Glycerophospholipid metabolism	0.000121	0.00344
Autophagy - animal	0.000165	0.00431
Parkinson's disease	0.000329	0.00754
Ether lipid metabolism	0.000358	0.00754
Terpenoid backbone biosynthesis	0.00036	0.00754
Ubiquitin mediated proteolysis	0.000467	0.00916
Synaptic vesicle cycle	0.000515	0.00952
Viral carcinogenesis	0.000775	0.0135
Mineral absorption	0.00093	0.0154
Pancreatic cancer	0.00116	0.0182

Table 4: PPIs of highly upregulated genes in cluster 1 (adj. P < 0.05)

Pathway	P value	Adj. P
NF-kappa B signaling pathway	1.40E-26	5.48E-25
TNF signaling pathway	2.64E-26	8.27E-25
Toll-like receptor signaling pathway	1.59E-25	4.53E-24
Cell cycle	4.42E-23	9.92E-22
T cell receptor signaling pathway	3.87E-22	6.39E-21
Th17 cell differentiation	6.89E-22	1.08E-20
FoxO signaling pathway	3.61E-21	5.15E-20
IL-17 signaling pathway	4.96E-21	6.77E-20
Apoptosis	1.29E-19	1.57E-18
MAPK signaling pathway	3.73E-19	3.82E-18
Prolactin signaling pathway	1.16E-17	1.11E-16
mTOR signaling pathway	3.76E-17	3.37E-16

Table 5: Functional annotation clustering using KEGG enrichment of highly upregulated genes in vaginal tissue (cluster 3) following E2 treatment (adj. P < 0.05)

Pathway	P value	Adj. P
Alzheimer's disease	1.18E-14	3.71E-12
Oxidative phosphorylation	9.81E-13	1.54E-10
Ribosome	2.99E-12	3.13E-10
Parkinson's disease	5.59E-12	4.39E-10
Huntington's disease	1.05E-09	6.58E-08
Non-alcoholic fatty liver disease (NAFLD)	3.24E-09	1.70E-07
Endocytosis	7.10E-09	3.18E-07
Proteasome	3.84E-06	0.000151
Protein processing in endoplasmic reticulum	1.97E-05	0.000686
Central carbon metabolism in cancer	8.07E-05	0.00231
HIF-1 signaling pathway	8.79E-05	0.00231
Alcoholism	8.82E-05	0.00231
Carbon metabolism	0.000341	0.00777
Apoptosis	0.000347	0.00777
Salmonella infection	0.000431	0.00902
Tight junction	0.000922	0.0181
Adherens junction	0.000999	0.0185
Bacterial invasion of epithelial cells	0.00122	0.0213
Lysosome	0.00156	0.0253
Mitophagy - animal	0.00171	0.0253

Table 6: PPIs of highly upregulated genes in cluster 3 (adj. P < 0.05)

Pathway	P value	Adj. P
Apoptosis	4.16E-19	1.86E-17
HIF-1 signaling pathway	3.62E-18	9.47E-17
IL-17 signaling pathway	5.17E-18	1.25E-16
Proteasome	2.80E-16	4.63E-15
Estrogen signaling pathway	3.22E-16	5.06E-15
TNF signaling pathway	1.15E-15	1.72E-14
T cell receptor signaling pathway	2.13E-15	3.04E-14
FoxO signaling pathway	3.16E-14	3.55E-13
Tight junction	4.03E-13	3.51E-12
Th17 cell differentiation	7.58E-13	6.27E-12

Table 7: Functional annotation clustering using KEGG enrichment of highly downregulated genes in vaginal tissue (cluster 2) following E2 treatment (adj. P < 0.05)

Pathway	P value	Adj. P
Herpes simplex infection	3.71E-05	0.0117
Pathways in cancer	0.00031	0.0366
Insulin signaling pathway	0.000349	0.0366

Table 8: PPIs of highly downregulated genes in cluster 2 (adj. P < 0.05)

Pathway	P value	Adj. P
Pathways in cancer	1.91E-08	5.99E-06
MAPK signaling pathway	9.09E-08	1.43E-05
Signaling pathways regulating pluripotency of stem cells	3.44E-06	0.00036
Osteoclast differentiation	5.04E-05	0.00365
Epstein-Barr virus infection	5.81E-05	0.00365
Transcriptional misregulation in cancer	7.73E-05	0.00405
Kaposi's sarcoma-associated herpesvirus infection	0.000116	0.0052
Fluid shear stress and atherosclerosis	0.000138	0.00541
NF-kappa B signaling pathway	0.000333	0.0116
Pertussis	0.000804	0.0216
Toll-like receptor signaling pathway	0.000824	0.0216
Circadian entrainment	0.000824	0.0216

Table 9: Functional annotation clustering using KEGG enrichment of highly downregulated genes in vaginal tissue (cluster 4) following E2 treatment (adj. P < 0.05)

Pathway	P value	Adj. P
Ribosome	2.52E-12	7.92E-10
Pathways in cancer	2.48E-08	3.89E-06
Transcriptional misregulation in cancer	1.56E-07	1.63E-05
Cellular senescence	2.25E-07	1.77E-05
Prostate cancer	4.63E-07	2.91E-05
HTLV-I infection	1.49E-06	7.79E-05
Focal adhesion	3.07E-06	0.000138
Endocrine resistance	4.53E-06	0.000178
Fluid shear stress and atherosclerosis	6.20E-06	0.000216
Epstein-Barr virus infection	7.38E-06	0.000232
TGF-beta signaling pathway	1.84E-05	0.000526
Chronic myeloid leukemia	2.21E-05	0.000568
Estrogen signaling pathway	2.35E-05	0.000568
Hippo signaling pathway	6.52E-05	0.0014
Spliceosome	6.71E-05	0.0014
Proteoglycans in cancer	0.000105	0.00206
Neurotrophin signaling pathway	0.000177	0.00327
Breast cancer	0.000272	0.00475
Thyroid hormone signaling pathway	0.000287	0.00475
Rap1 signaling pathway	0.000384	0.00584

Table 10: PPIs of highly downregulated genes in cluster 4 (adj. P < 0.05)

Pathway	P value	Adj. P
Pathways in cancer	4.29E-10	9.89E-08
Ribosome	6.30E-10	9.89E-08
Epstein-Barr virus infection	1.78E-09	1.86E-07
Endocrine resistance	1.91E-08	1.50E-06
Focal adhesion	4.07E-08	2.40E-06
Hippo signaling pathway	4.60E-08	2.40E-06
Breast cancer	1.09E-07	4.89E-06
Prostate cancer	2.33E-07	8.46E-06
Cellular senescence	2.43E-07	8.46E-06
Chronic myeloid leukemia	4.55E-07	1.43E-05

APPENDIX IV: Microarray analysis of vaginal DCs

Here, we have used microarray analysis to provide a comprehensive transcriptional profile of changes observed in vaginal DCs following *in vivo* E2 treatment. To examine the effects of E2 on the gene expression of vaginal DCs, OVX mice were treated with E2 or mock-pellets and two weeks later, tissue was collected. Mice were pooled for each group, with a total of 10-22 mice per treatment group. Whole tissue was enzymatically digested as previously described (1), and processed into single cell suspensions. In initial experiments, vaginal $CD11c^+$ DCs were directly sorted using the BD FACSAria III flow sorter (BD Biosciences, San Jose, CA, USA). However, this process of sorting was problematic. It took approximately 7 hours to sort the cells, after which the quality of the RNA was quite poor. Also, due to certain properties of the vaginal tissue, such as stickiness of cells, extensive debris and cornified cells, the sorting process was quite difficult and prone to errors (i.e. clogging of the machine, clogging of filters, slow event rate). The RNA was extracted using either the QIAGEN RNeasy Plus Mini Kit or the QIAGEN RNeasy Plus Micro Kit (QIAGEN Inc., Toronto, ON, Canada). Both kits were tested to determine what the best option was. Samples were then sent to the Center for Applied Genomics facility (The Sick Kids Hospital, Toronto, ON, Canada). Bioanalysis of RNA samples was completed, and the samples did not pass the quality control measures in place. The samples were contaminated with large quantities of genomic DNA, and so the RNA was treated with the DNA-free™ DNA Removal Kit (Thermo Fisher Scientific Inc, Waltham, MA USA). However, this did not resolve the poor RNA quality.

To mitigate these issues, we switched to a two-step sorting process. Following tissue digestion, vaginal $CD11c^+$ DCs were first magnetically sorted using the EasySep Mouse CD11c Positive Selection Kit II (STEMCELL Technologies Canada Inc., Vancouver, BC, Canada), and stained with CD11c (PE) and CD11b (PerCP) antibodies (BioLegend, San Diego, CA, USA). $CD11c^+$ populations were then sorted from the pre-enriched fractions using the BD FACSAria III flow sorter (BD Biosciences, San Jose, CA, USA). DAPI (Sigma-Aldrich Canada Co., Oakville, ON, Canada) was used to exclude dead cells, and purity of sorted populations was verified by flow analysis and consistently found to be over 95%. Cells were sorted directly into the RNA extraction buffer (RL buffer), which has been demonstrated to help stabilize RNA and prevent degradation during the sorting process. Finally, RNA was extracted from sorted cells using the Single Cell RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada). This kit was selected as the best option for these samples after testing the other kits mentioned above. Through this optimized process we were able to sort, on average, 10-20 000 DCs from E2-treated mice (n=10-13) and 30-40 000 DCs from mock-treated mice (n=18-22). Following RNA extraction, bioanalysis was conducted as previously described (Appendix III), and the Clariom S Pico Mouse GeneChip (Applied Biosystems) was used as per the manufacturer's instructions. The Pico GeneChip was used as the quantity of RNA was quite low (7-40 ng). Data was analyzed as described previously (Appendix III). The final probe set count for the DC analysis was 3020.

Following pre-processing steps and differential expression analysis, a list of ~60 genes were identified which were differentially regulated due to E2 treatment (either

upregulated or downregulated). Due to the limited scope of genes, the cut-off for inclusion was genes which met an adjusted P value < 0.15. Table 1 highlights the top 20 genes upregulated by E2 treatment. We did an initial KEGG enrichment analysis to examine the top gene pathways associated with all of the differentially expressed genes. There were 11 different pathways which were associated with the genes, although only the lysosome pathway met the statistical cut-off (adj. P < 0.15) (not shown). We then looked at PPI KEGG pathways, and identified ~50 pathways (adj. P < 0.15) associated with the differentially regulated genes, some of which are highlighted in Table 2. T_h17 cell differentiation and IL-17 signalling pathways were both enriched amongst the gene list, supporting previous findings (2, 3).

Interestingly, a first-order network was generated (Fig. 1) and one of the top nodes that acted as the largest hub with numerous protein interactions was NLRP3. NLRP3 (NLR Family Pyrin Domain Containing 3) plays an important role in innate immunity and inflammation, and was also one of the top genes differentially regulated by E2 (Table 1). NLRP3 initiates the formation of the inflammasome polymeric complex, which consists of NLRP3, PYCARD and CASP1 (4). Recruitment of proCASP1 promotes its activation and leads to maturation and secretion of IL-1β and IL-18 (4). In our previous work, we have shown that E2 induces T_h17 and IL-17 responses via an IL-1-dependent pathway (2, 3). Upregulation of NLRP3 suggests that this might be the upstream pathway through which E2 regulates IL-1β induction and consequently, IL-17 production.

Due to the limited number of genes identified, we used a p value of < 0.05 instead of an adjusted p value, and analyzed the data based on clustering of genes according to a

heat map (Fig. 2). Genes in cluster 1 (red) and 3 (yellow) were significantly upregulated by E2 treatment, while genes in cluster 2 (blue) were downregulated. Genes in cluster 4 (green) and 5 (purple) were both only significantly related to ribosome pathways, and so these clusters were not further analyzed. When looking at the two clusters upregulated by E2, 5 different pathways were found to be associated with genes in cluster 1 (Table 3) and in cluster 3 (Table 5). Next, the biological significance of the first-order PPIs was determined by examining gene enrichment within the Reactome database (Table 4 and 6). Ontological analysis demonstrated that E2 treatment results in the upregulation of transcripts that encode proteins which interact with and modulate other proteins involved in inflammatory pathways such as NF-kappa B and TNF signalling pathways, IL-17 signalling and T_h17 cell differentiation, and T_h1 and T_h2 differentiation. These are similar pathways to the ones discussed in the whole tissue analysis (Appendix III). When looking at the cluster downregulated by E2, 10 different pathways were found to be associated with genes in cluster 2 (Table 7), although, none met the statistical cut-off (adj. $p < 0.15$). Likewise, none of the PPI analysis demonstrated required statistical cut-offs (Table 8).

Again, this is preliminary analysis and the microarray data will be verified using quantitative real time PCR. In future studies, we are interested in further examining the role of NLRP3 and functional assays will be conducted to determine if E2-mediated enhancement of IL-1β and IL-17 production occurs through NLRP3. For instance, we can treat NLRP3 deficient mice with E2, and examine how this affects the ability of vaginal DC to induce T_h17 and T_h1 responses. Overall, this work can provide insight on how E2 influences the programming of vaginal DCs.

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Figure 1: Protein network analysis of differentially expressed genes in vaginal DCs from E2-treated mice.

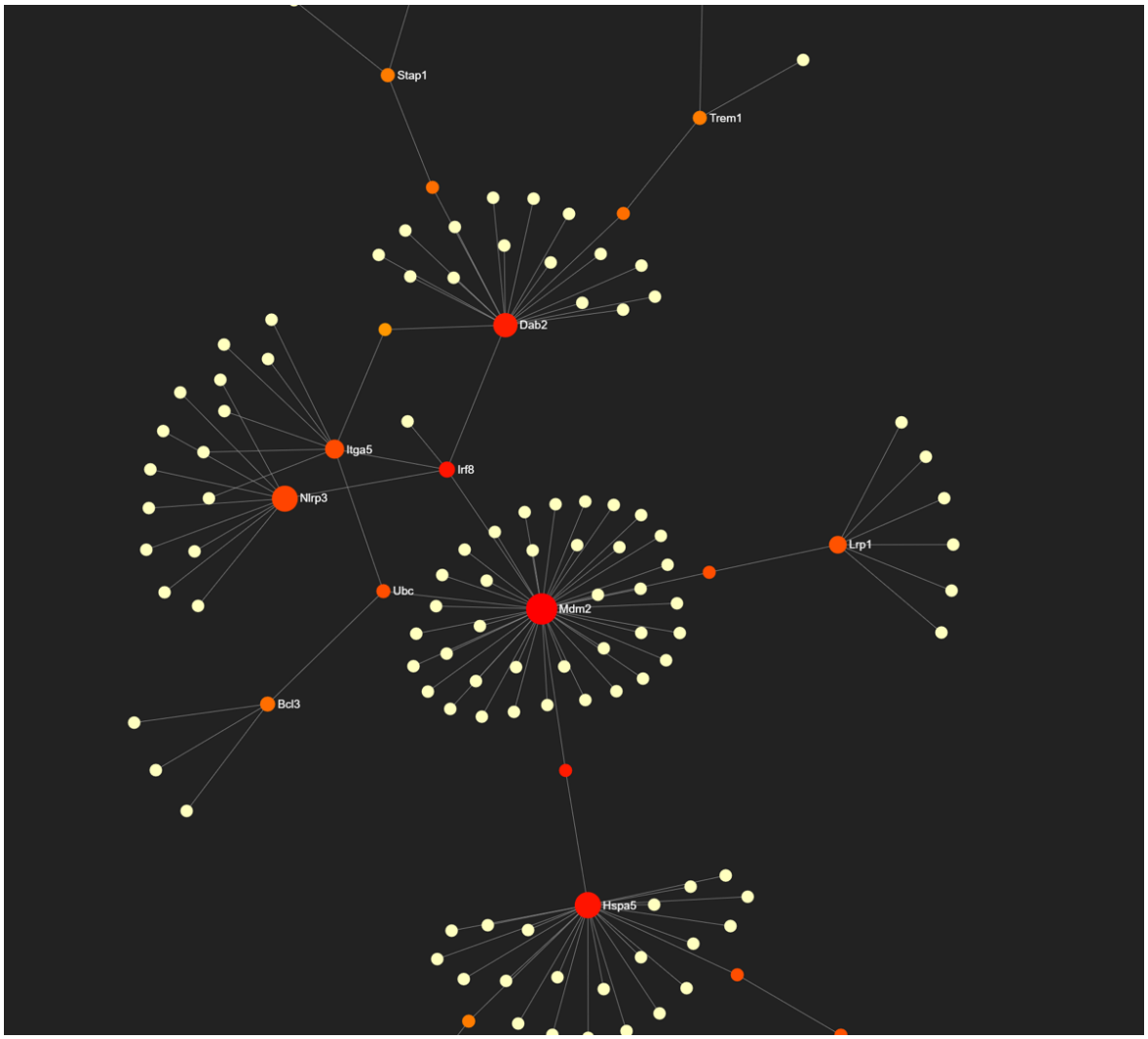

Figure 2: Heat map showcasing clustering of differentially expressed genes in vaginal DCs from E2-treated mice (adj. P < 0.15).

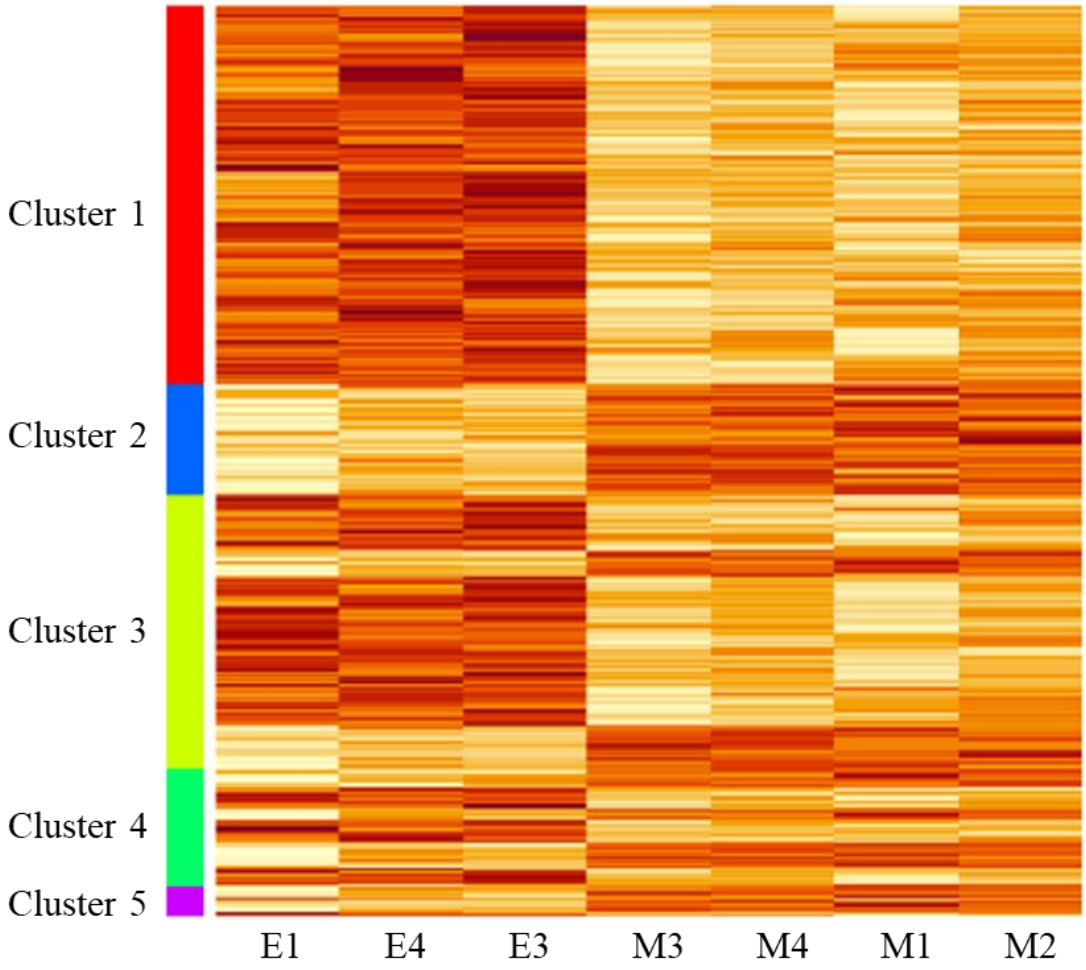

Table 1: Differentially expressed genes in vaginal DCs from E2-treated mice (adj. P < 0.15)

Gene	Name	Log F.C.	P value	Adj. P
Thbs1	thrombospondin 1	1.775743234	0.000106699	0.074682446
Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	1.669926046	0.000265091	0.074682446
B3gnt5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	1.484302579	2.79E-05	0.052133629
Mmp19	matrix metallopeptidase 19	1.426022862	0.000180242	0.074682446
Helz2	helicase with zinc finger 2, transcriptional coactivator	1.253039435	0.001155379	0.116308186
Tgm2	transglutaminase 2, C polypeptide	1.231540145	0.000727524	0.116308186
Itgam	integrin alpha M	1.152374371	0.001413323	0.128030864
Sp110	Sp110 nuclear body protein	0.963442451	4.99E-05	0.052133629
Plk2	polo like kinase 2	0.940897353	0.001590474	0.128030864
Tmem106a	transmembrane protein 106A	0.933175142	0.001006644	0.116308186
Emilin2	elastin microfibril interfacer 2	0.932048204	0.001439626	0.128030864
Mdm2	transformed mouse 3T3 cell double minute 2	0.890016785	0.000343797	0.074682446
Vat1	vesicle amine transport 1	0.860894333	0.00038354	0.077219458
Sipa1l1	signal-induced proliferation-associated 1 like 1	0.854659091	0.002018904	0.129725318
B4galt5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	0.848397752	0.002431277	0.135971443
Nlrp3	NLR family, pyrin domain containing 3	0.842336251	0.001029467	0.116308186
Itga5	integrin alpha 5 (fibronectin receptor alpha)	0.823170421	0.00080655	0.116308186
Eif4e	eukaryotic translation initiation factor 4E	0.792317712	0.002346948	0.135278788
Bcl3	B cell leukemia/lymphoma 3	0.786852586	0.002374098	0.135278788
Sp140	Sp140 nuclear body protein	0.752997301	0.002254513	0.135278788

Table 2: Functional annotation clustering using KEGG enrichment of the differentially expressed genes in vaginal DCs from E2-treated mice (adj. P < 0.15)

Pathway	P value	Adj. P
Pathways in cancer	6.36E-09	2.00E-06
Pertussis	1.47E-08	2.30E-06
Osteoclast differentiation	2.47E-08	2.58E-06
MAPK signaling pathway	2.37E-07	1.86E-05
Hepatitis B	3.61E-07	2.27E-05
PI3K-Akt signaling pathway	6.63E-07	3.47E-05
Measles	7.94E-07	3.56E-05
Breast cancer	9.76E-07	3.83E-05
Prostate cancer	1.68E-06	5.85E-05
NOD-like receptor signaling pathway	4.34E-06	0.000117
NF-kappa B signaling pathway	4.46E-06	0.000117
Prolactin signaling pathway	1.87E-05	0.000345
Cellular senescence	5.71E-05	0.000896
IL-17 signaling pathway	8.56E-05	0.00112
T cell receptor signaling pathway	0.000165	0.00173
Th17 cell differentiation	0.00663	0.0315

Table 3: Functional annotation clustering using KEGG enrichment of highly upregulated genes in vaginal DCs (cluster 1) from E2-treated mice (adj. P < 0.15)

Pathway	P value	Adj. P
Ferroptosis	0.000115	0.036
Viral carcinogenesis	4.00E-04	0.0627
Tuberculosis	0.00122	0.119
Systemic lupus erythematosus	0.00152	0.119
Pertussis	0.00203	0.128

Table 4: PPIs of highly upregulated genes in cluster 1 (adj. P < 0.15)

Pathway	P value	Adj. P
Pathways in cancer	5.83E-26	1.83E-23
PI3K-Akt signaling pathway	7.72E-20	8.08E-18
Prolactin signaling pathway	5.35E-19	3.36E-17
Osteoclast differentiation	3.74E-17	1.68E-15
Jak-STAT signaling pathway	1.51E-16	5.91E-15
Toll-like receptor signaling pathway	2.58E-16	9.01E-15
NF-kappa B signaling pathway	3.30E-15	8.83E-14
Th17 cell differentiation	5.80E-15	1.30E-13
MAPK signaling pathway	7.70E-14	1.51E-12
IL-17 signaling pathway	3.56E-13	6.21E-12
FoxO signaling pathway	4.26E-13	7.03E-12
TNF signaling pathway	2.73E-12	3.89E-11
Apoptosis	4.12E-11	4.79E-10
NOD-like receptor signaling pathway	5.56E-10	5.63E-09
HIF-1 signaling pathway	3.05E-09	2.40E-08
Cell cycle	8.05E-09	5.88E-08
Th1 and Th2 cell differentiation	4.37E-08	2.86E-07
T cell receptor signaling pathway	6.48E-08	4.07E-07

Table 5: Functional annotation clustering using KEGG enrichment of highly upregulated genes in vaginal DCs (cluster 3) from E2-treated mice (adj. P < 0.15)

Pathway	P value	Adj. P
NF-kappa B signaling pathway	0.000354	0.0609
B cell receptor signaling pathway	0.000388	0.0609
MAPK signaling pathway	0.000963	0.0789
PI3K-Akt signaling pathway	0.00101	0.0789
Focal adhesion	0.00166	0.104

Table 6: PPIs of highly upregulated genes in cluster 3 (adj. P < 0.15)

Pathway	P value	Adj. P
Pathways in cancer	1.67E-28	5.24E-26
Epstein-Barr virus infection	9.08E-27	1.43E-24
TNF signaling pathway	2.82E-23	2.21E-21
NF-kappa B signaling pathway	2.85E-22	1.49E-20
Toll-like receptor signaling pathway	4.07E-19	1.28E-17
Osteoclast differentiation	7.10E-18	1.59E-16
RIG-I-like receptor signaling pathway	7.62E-18	1.59E-16
Kaposi's sarcoma-associated herpesvirus infection	9.55E-18	1.87E-16
MAPK signaling pathway	3.47E-16	6.42E-15
Apoptosis	3.98E-16	6.93E-15
T cell receptor signaling pathway	1.17E-14	1.84E-13
B cell receptor signaling pathway	8.29E-14	1.20E-12
PI3K-Akt signaling pathway	8.42E-14	1.20E-12
Neurotrophin signaling pathway	1.02E-13	1.34E-12
IL-17 signaling pathway	1.07E-13	1.34E-12
Viral carcinogenesis	1.22E-13	1.47E-12
Pertussis	2.57E-13	2.99E-12
Focal adhesion	1.99E-12	2.23E-11
Jak-STAT signaling pathway	3.73E-12	3.94E-11
NOD-like receptor signaling pathway	2.70E-11	2.65E-10
Th17 cell differentiation	4.82E-09	3.36E-08

Table 7: Functional annotation clustering using KEGG enrichment of highly downregulated genes in vaginal DCs (cluster 2) from E2-treated mice (adj. P < 0.15)

Pathway	P value	Adj. P
Oxidative phosphorylation	0.00765	1
Parkinson's disease	0.00931	1
Alzheimer's disease	0.0158	1
Phagosome	0.0172	1
Huntington's disease	0.0207	1
Metabolic pathways	0.0278	1
Ubiquinone and other terpenoid-quinone biosynthesis	0.0332	1
Leukocyte transendothelial migration	0.0478	1
Cytokine-cytokine receptor interaction	0.0603	1
Proximal tubule bicarbonate reclamation	0.0653	1

Table 8: PPIs of highly downregulated genes in cluster 2 (adj. P < 0.15)

Pathway	P value	Adj. P
Leukocyte transendothelial migration	0.00107	0.324
Hepatitis C	0.00206	0.324
Thyroid cancer	0.0173	1
Endometrial cancer	0.027	1
Basal cell carcinoma	0.0293	1
Adherens junction	0.0335	1
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.0335	1
Bacterial invasion of epithelial cells	0.0344	1
Colorectal cancer	0.0408	1
Prostate cancer	0.0449	1
Melanogenesis	0.0463	1