ESTRADIOL INFLUENCES THE FUNCTION OF VAGINAL DENDRITIC CELLS

INVESTIGATING THE ROLE OF ESTRADIOL AND THE MUCOSAL MICROENVIRONMENT ON T_h17 RESPONSES PRIMED BY DENDRITIC CELLS IN THE FEMALE GENITAL TRACT

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy

DOCTOR OF PHILOSOPHY (PhD) 2015 McMaster University

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TITLE: Investigating the role of Estradiol and the mucosal microenvironment on T_h17 responses primed by Dendritic cells in the female genital tract

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NUMBER OF PAGES: xviii, 301

ABSTRACT:

Clinical and experimental studies have shown that estradiol (E2) can enhance protection against sexually transmitted infections such as HSV-2 and HIV-1. Antigen presenting cells (APCs) such as Dendritic cells (DCs) are critical for generating immune responses against these infections, and it is unclear whether unique factors present in the genital mucosa can influence immune responses by directly modulating the phenotype and function of local APCs. To address this, I hypothesized that sex hormones, such as E2 and innate factors in the local microenvironment can regulate the phenotype and function of vaginal APCs. The work summarized in this thesis addressed this central hypothesis.

In the first section of the thesis, I examined whether vaginal APCs were distinct in their phenotype and function compared to those in other mucosal tissues or spleen. The results show that the vagina was enriched in $CD11c^+$ $CD11b^+$ $MHCII^-$ DCs. Functionally, vaginal tissue cells (TC) and $CD11c^+$ DCs were more potent inducers of T_h17 responses in co-cultures with $CD4^+$ T cells, compared to lung, small intestine or spleen APCs. E2 was critical for the conditioning of vaginal DCs to prime these T_h17 responses through an IL-1-dependent pathway, indicating that sex hormones such as E2 can directly influence the function of vaginal APCs.

In the next section, I determined whether other co-factors in the genital microenvironment such as microflora and innate lymphocytes could also influence vaginal APC functions. We found that while microflora was not

essential, IL-17 produced by innate lymphocytes was critical for the induction of IL-1 from DCs, and consequently for potentiating T_h17 responses.

Finally, I attempted to develop an *in vivo* mouse model where the effect of E2 on vaginal APCs could be examined in the context of genital HSV-2 infection. I tested a 7-day injectable E2 and a 21-day E2 pellet delivery model, and found that both regimes had limitations for examining E2-effects on anti-viral responses. Yet, subsequent to the work done in this thesis, we were able to confirm our observations of E2-conditioned T_h17 responses *in vivo* in an intranasal immunization model utilizing E2 pellet delivery, and thereby addressed the mechanism underlying enhanced anti-viral protection following E2-treatment.

In conclusion, this is the first study to show the effect of E2 on genital tract APCs and their ability to prime T_h17 responses. It provides future avenues to examine whether modulation of this microenvironment can help optimize vaccine-induced immune responses against STIs. On a more fundamental level, it highlights the need to consider the inherent distinctions in APC populations among different mucosal tissues.

ACKNOWLEDGEMENTS

This has been a long time coming. I have been in graduate school since 2008, and during this time, I have faced one of the darkest periods of my life. However, I have been extremely fortunate to have a number of people who have helped me get through these years. This section finally gives me a chance to thank most of them, for I definitely could not have accomplished this work without their support.

First and foremost, my deepest thanks to my dear family who have loved and supported me throughout my life. Words cannot express how much I love you Mom, Dad and bro. I remember the days when we moved to Canada. In spite of the hard times, Mom and Dad, Both of you both have never us down. You have always given us more than we could ever ask for. Amma, I wish I could tell you this in person. Alas! I will never have the opportunity. There is nothing more that I would want in my life, than to spend a few more minutes with you; to hear your sweet voice; to see you smile; to hug you; and be called “kanna” by you. Dad, bro and I miss you very much, and our life has changed forever since we lost you. Not a day goes by, or will forever go by, without us thinking about you. Amma, you are responsible for everything that we are today, and everything that we will ever be. I was foolish to take you for granted and for being arrogant when you used to call me. I used to take out all my anger and frustrations with you, Dad and bro. Despite all that, all of you were patient, and still cared about me. The opportunity to be in this family is the greatest gift God has given me. Any

accomplishment that I have achieved, or will achieve throughout my life, is a reflection of you: Ma, Dad and Big bro. Amma/Shobhi, I know you wanted to be there for my defense. Even at the very end, you said that you would be there. By some divine will, if there is a way that you can read this: Please know that I am very sorry for not having realized how amazing you were. I was wrong to take you for granted. Yet, I will always thank God for giving me the opportunity during those last couple of years to become closer to you Ma. I will miss seeing you on Facetime while I walked to campus; I miss talking and laughing with you; and harkening back to our days in Canada, I miss forcing you guys to come visit pet stores and garden centers with me. At the same time, all of us are glad that its over. You didn't deserve the suffering you went through Dear Shobhi.

I hope I can make you proud one day. I hope that in some way, I will contribute to the world such that others do not have to suffer the pain and suffering you had to go through. That would make my existence worthwhile.

Secondly, I would like to thank my supervisor Dr. Charu Kaushic. Thank you for giving me the chance. You have been extremely supportive and very patient with all my quirks and annoyances. You have taught me not to "loose sight of the forest for the trees" numerous times during this PhD. While I was always bogged down by data, you have re-directed me towards the right path. The completeness of this work would not have been possible without your foresight and direction. You even went the extra mile to help me with my weaker skills. You have spent an unbelievable amount of time correcting my writing. You

gave me the opportunity to do some amazing things, and I will always remember and thank you for all of that. Thank you!

This brings me next to my committee members Dr. Denis Snider & Dr. Yonghong Wan. Thank you very much for teaching me and challenging me. I feel that I have learnt all my critical analysis skills based on our meetings. Both of you have shown me what it takes to think as a researcher. You were never easy on me intellectually. But you have always been patient and understanding of my weaknesses, and gave me ample opportunities to work on them. Thank you very much for taking the time out of your busy schedules to read my imperfect writing and provide useful feedback. Thank you very much!

I would also like to thank Hong Liang. Dr Snider and Hong have been instrumental in teaching me everything about flow cytometery. It is still one of the coolest machines created by man, and I don't think I could achieve the caliber of data seen here without the help both of you have provided. Hong you were a great mentor. I don't think I would have become so comfortable with flow, if it wasn't for your guidance. You are an amazing person, and we are lucky to have you in the facility.

Next, I want to thank all the past, and current members, in the Kaushic lab. These years would have been hard without all your friendship. Firstly, to my dear friends Jess (boo boo) and Victor, you guys have been there from the beginning. We have been through a lot together, and you were the best people that I could ever hope for. You calmed me down from doing something rash or stupid. On top

of that: Jess! in spite of the fact that you were done, you spent a lot of time going through my deplorable first drafts. This thesis would not have been in the current state, or completed in the current timeline without your help. My heartfelt and sincere thanks for everything, and I wish you guys both the very best for your future. Next up Kristy: you are the most efficient person I have ever known. I still cannot get over how great you were with your time management, and how you used to finish like OVX/sacrificing 10 mice in the same time that I would do 4. Together we were a great team. Thank you for all your help in my work, and thank you for being a good friend. Next, Sara: I think I can confidently say that you were the only junior student throughout my PhD whom I could trust with my samples (my fault: my OCD. Haha). I don't think anyone else assisted me with the grunt work in this thesis as much as you did. You are terrific, hardworking, and super careful with everything you do. I don't think I would have done as much as I managed, if it wasn't for you. Thank you very much. Aisha, you have been an amazing mentor in the lab. You have taught me and helped me with my work, and more importantly, you were always there for moral support. Thank you so very much for all that, and for the best cookies on Planet Earth. Kristen, I don't think I know anyone else who was more "by the book/protocol" than you (well, apart from Charu, and now Phil). You are super organized, and were instrumental in getting me the knockout mice and supplies that I was required for this work. Last, but not the least, the newest members of the lab: Philip and Puja. The last year could not have been as easy if it wasn't for both of you. Phil, you amaze me

with your efficiency and your motivation. They say you should never be ashamed to learn from younger people, and I have definitely learnt a thing or two from you. Puja, you are as Martin put it, a perfect replacement for me in the lab. You are hardworking, smart and a fantastic person. Altogether, I cherish all your friendships and help in keeping me grounded. Thank you all very much.

Next are the current and former members of MIRC: Nicole B., you are among my dearest friends. I cherish all the times we spent together. I cherish all the bus rides, and Tim Hortons runs. Thank you for helping me, and giving me the feedback to finish this document. Next are Jeanette B., Dannie B., Rodrigo J. S., Katherine A., Jonathan Pol, Chris V., Derek C., Ryan S., Stephanie S. and Amy G. B. All of you, especially Jeanette and Dannie, have been amazing friends, who walked me through the early stage of my life as a graduate student when there was no one to walk me through my early experiments. Thank you. I would also like to thank all the PIs at MIRC: especially Dr. Martin Stampfli, Dr. Manel Jordana and Dr. Zhou Xing. You guys are awesome, and your collaboration made the work in this thesis a reality. Martin, I especially thank you for being an inspiration. Thank you for teaching me 3IO3, and showing me that a researcher can also be cool. Haha. Also, thank you for taking the time to talk to me, and to check up on me especially during my toughest times.

Last but not least, my dear closest friends or my chosen brothers: Andrew Tse, Faisal K. and Dr. Tony S. I am lucky to have you as my friends. You have allowed me to be myself, and were always there for me to vent my frustrations.

You have kept me sane, and always supported me. Drew, thank you so much for teaching me about life, for being my friend since our undergrad days, and for going through my thesis, fixing my terrible English. I really appreciate it bud. Faisal, bro, you were my first friend in Canada, and have been always there for me. You will always be a brother to me. I love all of you guys. Thank you very much.

Lastly, this may be a bit weird: thank you to my dear carnivorous plants. Haha! My *Nepenthes sp.*, *D. muscipula*, *C. follicularis* and *Utricularia sp.* for being so amazingly awesome, mesmerizing me, and for keeping me busy and sane. *Heliamphora sp.* and *Drosera sp*., I'll get to you one day. Haha! Thank you *Tim Hortons*™ for your ice coffee, and thank you McMaster University for being my home for the last 11 years. Its time for me to move on, but I will definitely miss you.

With that, I will now let you get to the actual science. I hope this work helps humanity in some form.

PREFACE

This thesis has been prepared in the standard format as outlined in the "Guide for the preparation of Master's & Doctoral theses, 2014" from the School of Graduate Studies, Mcmaster University. The first chapter serves as a general introduction. The body of this thesis contains 3 chapters (3.1 to 3.3), each of which is an independent study. The work in chapter 3.1 is currently included in a manuscript that is in communication, and chapter 3.2 will be submitted before the defense of this thesis. All these chapters have been written by the author of this thesis, who is also the primary author of the mentioned manuscripts. The preamble section preceding each chapter describes the contributions of other authors to the multi-authored works. Finally, chapter 4 is the discussion section and summarizes the conclusions of this thesis, and draws out the overall implications.

Table of Contents

LIST OF ABBREVIATIONS

APCs	Antigen presenting cells
BMDCs	Bone marrow derived DCs
CD	Cluster of differentiation
CDPs	Common DC precursor
DCs	Dendritic cells
DMPA	Depot medroxyprogesterone acetate
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
E2	17β-estradiol
ECs	Epithelial cells
ER	Estradiol receptor
ERKO	Estradiol receptor α knockout
FOXP3	Forkhead box P3
GECs	Genital epithelial cells
GF	Germ-free
HIV-1	Human immunodeficiency virus 1
HSV-2	Herpes simplex virus type 2
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
ILCs	Innate lymphoid cells
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factors
ISGs	Interferon-stimulated genes
KO	Knockout
LCs	Langerhans cells
LP	Lamina propria
LTi	Lymphoid tissue-inducer cells
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NO	Nitric oxide
OVA	Chicken ovalbumin

OVX	Ovariectomized
p.i.	Post infection
P4	Progesterone
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDCs	Plasmacytoid DCs
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
ROR-γ	RAR-related orphan receptor gamma
STAT	Signal transducer and activator of transcription
STIs	Sexually transmitted infections
TC	Tissue cells
TC+CD4	Tissue cells + OT-II Tg $CD4^+$ T cells
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TK	Thymidine kinase
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
VALTs	Vagina-associated lymphoid tissue
WT	Wild type

CHAPTER 1

GENERAL INTRODUCTION & OBJECTIVES

1.0 ANTIGEN PRESENTING CELLS:

1.0.1 Background:

Antigen presenting cells (APCs) including Dendritic cells (DCs), macrophages and B cells are specialized cells that serve as the link between innate and adaptive immunity. APCs are known to play a critical part in the initiation of the adaptive immune responses to pathogens such as bacteria, viruses and parasites (Reis e Sousa, 2006; Soloff & Barratt-Boyes, 2010). They recognize pathogens through a variety of pattern recognition receptors (PRRs), and present their antigens in the context of major histocompatibility complexes I or II (MHC I or II) on their surface, to stimulate naïve antigen specific $CD8^+$ or $CD4^+$ T cells, respectively. Upon activation, $CD4^+$ T cells can mature into a variety of T helper (T_h) phenotypes such as T_h1, T_h2, T_h17 and Tregs. The nature of the antigen, and the cytokines produced by the APC, can determine the differentiation profile of $CD4^+$ T cells (Diebold, 2008; Mildner & Jung, 2014). Although, macrophages are also considered as professional APCs that can present antigens to naïve T cells, predominantly, they specialize in the clearance of cellular debris, apoptotic cells and pathogens (Guilliams et al., 2014). The antigen presentation role of macrophages may likely be more important in stimulating memory T cells within peripheral tissues, rather than priming naïve T cells (Hickman et al., 2011; Paul, 2012). In mice, macrophages have been typically identified by high levels of F4/80 (Austyn & Gordon, 1981; Hirsch, Austyn, & Gordon, 1981; Leenen, de Bruijn, Voerman, Campbell, & van Ewijk,

1994): a surface molecule from the epidermal growth factor – transmembrame 7 (EGF-TM7) family, that has been implicated to play an important role in antigen-specific tolerance (van den Berg & Kraal, 2005). On the other hand, DCs, usually distinguished by a stellate morphology (R. M. Steinman & Cohn, 1973), are generally more effective than either B cells or macrophages, at stimulating naïve T cells (R. M. Steinman & Witmer, 1978). In a recent review published in *Annu. Rev. Immunol.*, murine DCs were typically identified as $CD11c^{hi}$ $MHCII^{+}$ cells (Merad, Sathe, Helft, Miller, & Mortha, 2013). DCs and macrophages are widely spread throughout the body and have been classified into a variety of subpopulations (Guilliams et al., 2014; Malissen, Tamoutounour, & Henri, 2014; Merad et al., 2013; Mildner & Jung, 2014).

1.0.2 Dendritic cells:

Over the last decade, immunologists have attempted to classify and standardize DC subsets. These have included attempts to differentiate DCs based on their origin, function, or whether they were resident or migratory (Reis e Sousa, 2006; Rescigno & Di Sabatino, 2009). A review published by Guilliams et al in 2014 is the latest opinion of scientists to classify DCs, macrophages and monocytes based on a new unified nomenclature. They suggest that DCs should be classified using a two-level nomenclature: 1) ontogeny 2) function, location and/or phenotype. Based on these criteria, and their developmental pathways, DCs have been divided into three broad subsets: 1) Basic leucine zipper transcription factor ATF-like 3 (BATF3) dependent classical/conventional type 1

DCs (cDC1s) that include $CD8\alpha^+$ DCs and $CD103^+$ $CD11b^-$ DCs 2) IRF4-dependent classical/conventional type 2 DCs (cDC2s) that are $CD11b^+$ DCs and $CD172a^+$ DCs, and 3) basic helix-loop-helix transcription factor (E-protein) (E2-2/Tcf4)-dependent plasmacytoid DCs (pDCs) (Cisse et al., 2008; Guilliams et al., 2014; Merad et al., 2013).

Although a functional distinction has been observed in that cDC1s prime T_h1 responses, and cDC2s prime T_h2 responses, this is not always exclusive (Guilliams et al., 2014). Each of the three subsets is believed to originate from a common DC precursor (CDPs) in the bone marrow. Given the functional (stage 2 of nomenclature) overlap among the DC, macrophage and monocyte populations, the authors suggest that ontogeny should be the primary criteria for classifying these cells. In the past, Langerhans cells (LCs), typically characterized as $CD4^-$ $CD8^{low}$ $CD205^{hi}$ $CD11b^+$ $CD207^+$ cells, have been defined as DCs. But based on their embryonic origin independent of CDPs, the authors suggest that they should be included in the macrophage family, along with other specialized cells such as microglia, Kupffer cells, alveolar macrophages and spleen red pulp macrophages (Guilliams et al., 2014; Hoeffel et al., 2012).

Functionally, DCs have a few differences compared to macrophages (Mildner & Jung, 2014): 1) Antigen processing: unlike macrophages, cDCs can control lysosomal degradation of antigens to preserve peptides for T cell recognition (Savina et al., 2006). 2) Migration: unlike macrophages, cDCs can detect CCL19 and CCL20, and are more efficient at migrating towards T cell

zones in the local secondary lymphoid organs (Dieu et al., 1998). 3) Stimulating naïve T cells: conditional ablation of cDCs confirmed that cDCs, but not macrophages, are critical for initiating naïve T cell responses (Jung et al., 2002). Other leukocytes such as macrophages may restrictively amplify pre-exisiting memory T cell responses (Mildner & Jung, 2014).

1.0.3 Macrophages

While both macrophages and DCs can originate from hematopoietic stem cells, few subsets of macrophages can also originate early on during embryogenesis from embryonic progenitors (Ginhoux et al., 2010; Guilliams et al., 2014; Hoeffel et al., 2012). During embryonic development, these cells spread into peripheral tissues, and become tissue-resident macrophages that can sustain themselves from local progenitors (Guilliams et al., 2014). Macrophages have been shown to be dependent on macrophage colony stimulating factor 1 (CSF1) and IL-34 for differentiation and survival (Guilliams et al., 2014). They have a wide range of functional abilities, and can either promote or inhibit host anti-microbial responses, anti-tumor responses and inflammatory responses (Ginhoux & Jung, 2014; Malissen et al., 2014; Murray & Wynn, 2011b). Tissue macrophages are professional phagocytes that typically ingest pathogens or debris in order to maintain homeostasis (Murray & Wynn, 2011b). Murine macrophages are typically identified as $CD11b^+$ $F4/80^+$ $CD68^+$ $CD18^+$ cells (Murray & Wynn, 2011b). They have been functionally divided into multiple subtypes: M1 macrophages are classically activated macrophages that mediate

anti-tumor immunity and host defense against bacteria, viruses and protozoa (Murray & Wynn, 2011a, 2011b). M2 macrophages or tumor-associated macrophages are alternatively activated macrophages that secrete large quantities of IL-10 (Sutterwala, Noel, Clynes, & Mosser, 1997), and exhibit immunosuppressive activities to promote wound healing, or attenuate anti-tumor responses (Murray & Wynn, 2011a, 2011b).

1.0.4 Monocytes & monocyte-derived cells:

Similar to DCs and macrophages, monocytes also arise from hematopoietic stem cells. Mouse monocytes have been distinguished into two types: Classical Ly6C^{hi} monocytes, and Non-classical Ly6C^{low} monocytes. Ly6C^{hi} monocytes arise from a common monocyte progenitor, and are typically found circulating in an undifferentiated state in blood, and tissues such as spleen, lymph nodes, skin and lungs. On the other hand, Ly6C^{low} monocytes are typically only found patrolling the endovascular walls (Guilliams et al., 2014). Upon inflammation, many of the Ly6C^{hi} classical monocytes enter inflamed tissues, outnumbering resident DCs and macrophages, and differentiate into monocyte-derived DCs (mDCs), monocyte-derived macrophages, or myeloid-derived suppressor cells (MDSCs) (Ginhoux & Jung, 2014). Similar to cDCs, mDCs can express CD11c, MHCII and can activate naïve T cells. However, similar to macrophages, they are also highly professional phagocytes, and can express F4/80. Although mDCs typically have poor migratory ability compared to cDCs, the relative similarities among mDCs and monocyte-derived macrophages

suggest that both these daughter populations may altogether represent a single highly plastic subset of monocyte-derived cells whose functions are dependent on environmental cues (Guilliams et al., 2014).

1.0.5 Mucosal DCs:

The mucosal surfaces are continually exposed to innocuous environmental agents, commensal microflora and harmful pathogens (Holmgren & Czerkinsky, 2005). Depending on the nature of these environmental challenges, which are distinct for each mucosal surface, the immune system in each mucosa has evolved to respond appropriately with anti- or pro-inflammatory responses. APCs such as DCs and macrophages are sentinels that play a central role in shaping the adaptive immune responses at these mucosal sites (Rescigno & Di Sabatino, 2009). Therefore, populations of APCs that reside in these specialized microenvironments may likely be educated to induce distinct immune responses dependent on antigens specific to each mucosal tissue (Hu & Pasare, 2013).

Multiple subsets of resident and migratory APCs have been defined in the murine gut, respiratory tract and reproductive tract (Iwasaki, 2007; Iwasaki & Kelsall, 1999; Kelsall & Rescigno, 2004; Rescigno & Di Sabatino, 2009). Mucosal DCs are compartmentalized within these tissues in the lumen, epithelial layers and lamina propria (Soloff & Barratt-Boyes, 2010). These populations are continually replenished either by the differentiation of local precursors, or via the influx and differentiation of monocytes. In general, the mucosal tissues in our body have been characterized based on the type of epithelium covering the

tissue: Type 1 tissues include the small intestine, large intestine, nasal cavity, respiratory tract and the upper reproductive tract, and are characterized by a single cell epithelial layer (Iwasaki, 2007). Type 2 tissues include the vagina, cornea, mouth and esophagus, and are covered by a stratified squamous epithelium (Iwasaki, 2007).

Type 1 tissues are predominantly supported by local mucosal associated lymphoid tissues (MALT), and their physiological role is predominantly based on absorption, exchange and respiration. Accordingly, DCs in these tissues have a critical function of maintaining immunogenic balance between tolerant and inflammatory responses. Type 2 tissues on the other hand are predominantly barrier tissues, and these are typically monitored by regional lymph nodes instead of MALTs (Soloff & Barratt-Boyes, 2010). However, this is not necessarily exclusive: previous studies in our lab have provided evidence to suggest the formation of local vagina-associated lymphoid tissues (VALTs) that include $CD11c^+$ DCs, $CD3^+$ T cells and $CD4^+$ T cells (A. E. Gillgrass, Tang, Towarnicki, Rosenthal, & Kaushic, 2005). We have shown that the presence of these VALTs was directly correlated to the quality of protection post-challenge in a mouse model of HSV-2 infection (A. E. Gillgrass, Tang, et al., 2005). DCs in type 2 tissues generally face pathogens, and hence, are conditioned to predominantly prime inflammatory immune responses (Iwasaki, 2007). However, since pathogens can invade either type of mucosa, DCs in all mucosal tissues must be capable at modulating the immune response towards inflammation or tolerance.

While intestinal and lung DC populations have been compared previously (Hu, Troutman, Edukulla, & Pasare, 2011; Persson et al., 2013; Schlitzer et al., 2013), the phenotype and function of vaginal DCs have never been compared to those from other mucosal tissues. In this dissertation, as one of our objectives, we have attempted to address this gap in literature. In chapter 3.1, I have summarized the work where we compared the phenotype and function of DCs from lung, small intestine and vagina.

1.0.5.1 DCs in the small Intestine:

While the gastrointestinal tract can be exposed to a wide variety of pathogens, the antigen burden here is predominantly comprised of commensal bacteria and other microflora (Soloff & Barratt-Boyes, 2010). Hence, intestinal DCs have a two-fold task of maintaining tolerance to environmental, commensal and food antigens, while possessing the ability to respond with inflammation in the context of pathogens. DCs in the small and large intestine can be found in the epithelium, congregated as isolated lymphoid follicles, and compartmentalized within Peyers Patches (small intestine) or Caecal patches (Large intestine) of the lamina propria.

In isolated lymphoid follicles, DCs work alongside specialized M-cells that can transport antigens across the lumen for antigen presentation. Adoptive transfer studies have shown that lamina propria (LP) $CD11c^{hi}$ $CD11b^{hi/low}$ $CD103^{+}$ DCs (Ruane & Lavelle, 2011), but not Peyers patch (PP) DCs, induce regulatory responses (Chirdo, Millington, Beacock-Sharp, & Mowat, 2005; Coombes et al.,

2007; Sun et al., 2007). However, others have shown that a specific subset of LP DCs ($CD11c^{hi}$ $CD11b^{+}$ $TLR5^{+}$) prime T_h17 responses (Atarashi et al., 2008; Denning, Wang, Patel, Williams, & Pulendran, 2007). On the other hand, PP DCs ($CD11c^{low}$ $iNOS^{+}$ $TNF\text{-}\alpha^{+}$) were shown to play an important role in class switching of B cells to produce secretory IgA (Tezuka et al., 2007). This suggests that the heterogeneity of DC subtypes may be an adaptation to adequately address the commensal or pathogenic challenges in this tissue.

Numerous groups have attempted to examine the function of intestinal DC subsets in gut immune responses (Chirdo et al., 2005; Kelsall & Rescigno, 2004; Rescigno & Di Sabatino, 2009). Given that a majority of small intestinal DCs express CD103, a T-epithelial cell associated integrin αEβ7, scientists have attempted to distinguish mucosal DC subsets by CD103 to examine their functions (Ruane & Lavelle, 2011). $CD103^{+}$ DCs have been shown to arise from local progenitors, while the $CD103^{-}$ $CD11b^{+}$ DCs may arise from $Ly6^{hi}$ monocytes (Bogunovic et al., 2009). $CD103^{+}$ DCs were found to be the primary migratory population that travel to the local draining mesenteric lymph nodes to prime T cells (O. Schulz et al., 2009). Altogether, this suggests that the small intestine harbors multiple subsets of cDCs and mDCs that have distinct roles in priming inflammatory or regulatory responses.

1.0.5.2 DCs in the lung:

The respiratory tract is another mucosa that is regularly exposed to both innocuous antigens and pathogens. Hence, DCs in this tissue must be able to

respond appropriately to these stimuli. As the mileau of the upper respiratory tract (presence of commensals and constant exposure to foreign materials) and lower respiratory tract (relatively sterile) can differ drastically, distinct DC networks with specific functions have been shown to exist in the respiratory tract. Conventional DCs (cDCs) in the lung alveolar insterstitium have been shown to express CD11c, MHCII and variable levels of CD11b and CD8α. Plasmacytoid DCs (pDCs) also been shown to exist in the lung and have been phenotyped as $CD11c^{int}$ $Gr\text{-}1^{+}$, $B220^{+}$ and $PDCA\text{-}1^{+}$ cells. In the murine lung, cDCs have been divided into two subsets: $CD103^{+}$ $CD11b^{lo}$ $CX3CR1^{-}$ cells and $CD103^{-}$ $CD11b^{hi}$ $CX3CR1^{+}$ cells (Iwasaki, 2007). While these populations may seem to resemble the previously mentioned (section 1.0.1) conventional cDC1s and cDC2s (Guilliams et al., 2014), selective depletion of CD11c cells followed by reconstitution of $Ly6C^{hi}$ monocytes was able to reconstitute both these DC subsets (Iwasaki, 2007). This suggests that these cells may belong to the mDC family, and not the cDP-derived cDC1s or cDC2s (Guilliams et al., 2014).

Unlike the small intestine that contains a large population of DCs, few DCs reside within the murine lung alveoli under homeostatic conditions (Iwasaki, 2007). Rodent alveolar APCs under steady state are primarily composed of $CD11c^{+}$ $CD11b^{+}$ $Gr\text{-}1^{-}$ $CD205^{+}$ $MHCII^{-}$ alveolar macrophages that maintain immune quiescence to inhaled innocuous antigens, and constitutively inhibit the $CD11c^{+}$ $F4/80^{+}$ $CD11b^{+}$ $CD8\alpha^{-}$ $Gr\text{-}1^{+}$ $CD205^{+}$ $MHCII^{+}$ alveolar DCs, which are recruited upon allergenic exposure (Holt et al., 1993; von Garnier et al., 2005).

This suggests that lung APCs may potentially represent a very different population compared to those in other mucosal tissues such the gut, and may differentially rely on macrophages or DCs to maintain homeostasis.

1.0.5.3 DCs in the vagina

The murine vaginal tract has a unique microenvironment characterized by a low pH and colonization with a variety of bacterial and fungal species (Goldacre et al., 1979). Early on, four groups of Langerhans cells have been characterized in the vagina by immunohistochemistry: I-A^{+} F4/80^{+}, I-A^{+} F4/80^{-}, I-A^{-} CD205^{+} and I-A^{+} CD205^{-} (Parr & Parr, 1991). In a separate study, using flow cytometry, CD11c^{+} MHCII^{+} DCs in the vaginal epithelium have been identified as CD11b^{+} F4/8^{hi}, CD11b^{+} F4/80^{int}, and CD11b^{-} F4/80^{-} subsets (Iijima, Linehan, Saeland, & Iwasaki, 2007). The same group has also described a network of CD11c^{+} CD11b^{+} MHCII^{+} DCs in the lamina propria of the murine genital tract (Zhao et al., 2003).

Unlike lung or intestinal DCs, relatively little is known about vaginal DC subsets; whether specific subsets are specialized to perform distinct functions in the female genital tract is unknown (Iijima, Thompson, & Iwasaki, 2008). Additionally, the role of specific DC subsets in the immune response to pathogens, also remains poorly understood and should be the topic of future investigation. In this dissertation, I have described the phenotype of DC populations in the murine vagina under homeostatic conditions and compared them to other mucosal DCs. Furthermore, I have focused my study on vaginal

DCs, and examined the effects of local factors in the genital tract on their functions.

1.1 ADAPTIVE LYMPHOCYTES IN THE IMMUNE SYSTEM:

1.1.1 Background:

The primary function of APCs is to capture, process and display antigens to lymphocytes, integral components of the immune system. Three populations of lymphocytes originating from hematopoietic progenitors have been described: Thymus-derived lymphocytes (T cells), bone marrow derived lymphocytes (B cells) (Bursa of Fabricius derived cells) (Ribatti, Crivellato, & Vacca, 2006) and natural killer (NK) cells. While T cells and B cells are the primary components of adaptive immune responses, the innate lymphocyte group, which used to be exclusively comprised of NK cells, has seen a number of additions: Gamma delta T cells ($\gamma\delta$), innate lymphoid cells (ILCs) (I, II and III), and lymphoid tissue inducer (LTi) cells (Gasteiger & Rudensky, 2014). Both innate and adaptive lymphocytes arise from a common lymphoid progenitor (CLP) in the bone marrow (Zuniga-Pflucker & Schmitt, 2005). The adaptive T lymphocytes can be primarily divided into two subsets: $CD4^+$ and $CD8^+$ T cells. In this section, I have described how APCs such as DCs interact with these cells, and the role of these lymphocytes in the immune system.

1.1.2 $CD8^+$ T cells:

$CD8^+$ T cells are a key component of cytotoxic response to intracellular pathogenic infections including viruses, protozoa and bacteria (Harty, Tvinnereim,

& White, 2000; N. Zhang & Bevan, 2011). Naïve CD8 T cells are primarily located within the T cell zones of the draining lymph nodes, or are found circulating through the lymphatic system (Bousso & Robey, 2003). They are primed by migratory DCs that present pathogenic peptides, or cross-present antigens from apoptotic, infected cells in the context of MHC I (Germain, 1994). For efficient generation of long-lasting memory responses, $CD8^+$ T cells may require $CD4^+$ T cell help (Novy, Quigley, Huang, & Yang, 2007). Upon activation, $CD8^+$ T cells can induce cytolysis of infected cells through perforin/granzyme or Fas-FasL pathways (Harty et al., 2000). $CD8^+$ T cells can also produce cytokines such as IFN-γ and TNF-α, as well as chemokines such as RANTES, macrophage inflammatory protein 1α (MIP-1α) and MIP-1β (Cocchi et al., 1995) to recruit and activate the microbicidal activities of other effectors such as macrophages and neutrophils (Harty et al., 2000).

1.1.3 $CD4^+$ T cells:

$CD4^+$ T cells are key effectors of the immune system. They are involved in a myriad of roles ranging from the immune responses against bacteria, viruses, fungi and parasites, to self-tolerance, lymphocyte homeostasis, allergies and autoimmunity (Zhu & Paul, 2008). They accomplish these functions by their ability to activate B cells, $CD8^+$ T cells and macrophages, or by recruiting neutrophils, eosinophils and basophils, or via the production of effector cytokines and chemokines (Zhu & Paul, 2008). Naïve $CD4^+$ T cells in the secondary lymphoid organs are activated by DCs that present exogenous antigens in the context of

MHCII (Itano & Jenkins, 2003). Upon activation, $CD4^+$ T cells can differentiate into a multitude of T helper subsets. The differentiation profile depends on the cytokine microenvironment, and to a lesser extent, the strength of the TCR-MHC-peptide interaction (Luckheeram, Zhou, Verma, & Xia, 2012; Tao, Constant, Jorritsma, & Bottomly, 1997). $CD4^+$ T cell effectors include, but are not limited to: T_h1, T_h2, T_h17 and Tregs based on their functional profile.

1.1.3.1 T_h1 cells:

A cytokine microenvironment that includes DC derived IL-12 and TNF-α, and IFN-γ from NK cells, initiates the developmental cascade for the differentiation of T_h1 cells (Trinchieri, Pflanz, & Kastelein, 2003). T-box transcription factor (T-bet), activated through STAT1 and STAT4 pathways, is the master regulator transcription factor for T_h1 cells (Afkarian et al., 2002). It can actively suppress transcription factors for alternative differentiation pathways such as GATA3 and ROR-γ (Lazarevic et al., 2011). T-bet and STAT4 induce the production of IFN-γ in T_h1 cells, and this can act as a positive feedback loop to further increase T-bet expression and IL-12R expression (Lugo-Villarino, Maldonado-Lopez, Possemato, Penaranda, & Glimcher, 2003).

T_h1 cells are the primary effectors in the clearance of intracellular pathogens such as bacteria and viruses. They accomplish these functions through the activation of phagocytes such as macrophages (Melzer, Duffy, Weiss, & Halonen, 2008), and the activation of IFN-γ-responsive genes in infected cells (Boehm, Klamp, Groot, & Howard, 1997). Furthermore, IL-2,

produced by T_h1 cells, provides autocrine/paracrine signaling as a growth factor (Liao, Lin, & Leonard, 2013), and can also facilitate the development of $CD8^+$ T cell responses (Williams, Tyznik, & Bevan, 2006). T_h1 cells producing IFN-γ are the primary effectors in response to viruses such as HSV-2 in the genital tract (Johnson, Nelson, Bird, Chu, & Milligan, 2010; Milligan, Dudley-McClain, Young, & Chu, 2004).

1.1.3.2 T_h2 cells:

A cytokine milieu containing IL-4 and IL-2 during antigen presentation leads to the differentiation of T_h2 effectors. IL-4 induces STAT6 within naïve T cells that can up-regulate the expression of GATA-binding protein (GATA3), the master regulator for T_h2 cells (Glimcher & Murphy, 2000; Zhu, Yamane, Cote-Sierra, Guo, & Paul, 2006). GATA3 can suppress T_h1 differentiation by down-regulating STAT4 (Usui, Nishikomori, Kitani, & Strober, 2003). Other cytokines and transcription factors such as IL-6, STAT5 and STAT3 have also been implicated in T_h2 differentiation pathways (Diehl & Rincon, 2002; Luckheeram et al., 2012). T_h2 cells are primarily involved in responses to extracellular parasites and bacteria. They have also been implicated in allergic diseases such as asthma and rheumatoid arthritis (Del Prete, 1992). T_h2 effector cytokines include IL-4, IL-5, IL-9, IL-10, IL-25 and IL-13 (Luckheeram et al., 2012; Romagnani, 2000). Among these, IL-4 and IL-13 have been shown to be important for class switching of B cells to produce IgE, and for up-regulation of FcεRII (IgE receptor) on mast cells and basophils (Steinke & Borish, 2001; Tomkinson et al., 2001).

This can result in the priming of allergic sensitization pathways (Steinke & Borish, 2001).

1.1.3.3 Tregs:

There are two types of T regulatory cells that have been currently described: Natural Tregs (nTregs) and Induced Tregs (iTregs). While natural T regs develop in the thymus and predominantly play a role in homeostasis, iTregs develop in the periphery (W. Chen et al., 2003), outside the thymus. TGF-β, when it is present in the APC-T cell priming microenvironment, is key for forkhead box P3 transcription factor (FOXP3), the key lineage-specific transcription factor for iTreg differentiation (Fontenot, Gavin, & Rudensky, 2003). While FOXP3 is also expressed by nTregs, their development pathway may be independent of TGF-β (Horwitz, Zheng, & Gray, 2008), and may be more dependent on self-antigen-TCR interactions (Lin et al., 2013).

The criteria distinguishing nTregs from iTregs is still under investigation, but Neuropilin 1 (Nrp1) and Helios have emerged as promising markers to distinguish $FOXP3^+$ nTregs from iTregs (Lin et al., 2013). SMAD2 and SMAD3, activated downstream of TGF-β, are key to the expression of FOXP3 in iTregs (Luckheeram et al., 2012), and can inhibit the expression of ROR-γt, the differentiation factor for T_h17 cells (Martinez et al., 2009; Takimoto et al., 2010). The transcription factor STAT5, downstream of IL-2 signaling, was also found to enhance FOXP3 expression in iTregs (Davidson, DiPaolo, Andersson, & Shevach, 2007).

Both nTregs and iTregs play an important role in maintaining tolerance and/or controlling inflammation after clearance of pathogens (Ouyang, Rutz, Crellin, Valdez, & Hymowitz, 2011; Sakaguchi et al., 2006). However, nTregs and iTregs may have distinct roles in the adaptive immune response (Horwitz et al., 2008). Although the mechanisms are still being studied, both Treg populations may suppress immune responses via contact, granzyme-B-dependent mechanisms, or through the production of soluble factors such as IL-10, IL-35 and TGF-β (Sakaguchi et al., 2006).

1.1.3.4 T_h17 cells:

A cytokine microenvironment containing IL-6, TGF-β, IL-21 and IL-23 during antigen presentation has been shown to be important for T_h17 differentiation (Muranski & Restifo, 2013). The phosphorylation and activation of STAT3 (X. O. Yang et al., 2007), through IL-6 and IL-21 signaling, leads to the transcription of retinoic acid receptor-related orphan gamma-T (ROR-γt), the master regulator of T_h17 cells (Ivanov et al., 2006). Along with ROR-γt, ROR-α (Ivanov, Zhou, & Littman, 2007; X. O. Yang et al., 2008) may also play a synergistic role in the differentiation of human T_h17 cells. More recently, IRF4 and Aryl Hydrocarbon receptor (AHr) were also found to play an important role in T_h17 polarization (Brustle et al., 2007; Veldhoen et al., 2008). While the presence of IL-6 and TGF-β during TCR stimulation with anti-CD3/CD28 beads was required to induce T_h17 differentiation *in vitro*, recent studies have also uncovered IL-6-independent pathways of T_h17 differentiation (Kimura, Naka, & Kishimoto, 2007;

Zhou et al., 2007). Cytokine combinations such as IL-6, IL-1β, TGF-β and IL-23, or IL-21 and TGF-β, or IL-1β, IL-6 and IL-23, can all direct naïve $CD4^+$ T cells to differentiate into a T_h17 phenotype (Ghoreschi et al., 2010; Korn et al., 2007; L. Steinman, 2007; Zhou et al., 2007).

Recent studies showed that IL-1 signaling is critical to the differentiation of T_h17 cells from naïve or pre-differentiated T_h1/Treg phenotypes *in vivo* (Chung et al., 2009; Coccia et al., 2012). However, overexpression of IRF4 and ROR-γt through IL-1 independent pathways may also directly polarize T_h17 cells. This is because IRF4 may act downstream of IL-1β, and can positively regulate IL-21 in an autocrine fashion (Q. Chen et al., 2008; Chung et al., 2009). IL-1 and IL-6 are both pleotropic cytokines that can be induced in response to tissue damage, commensals or pathogens (Kimura et al., 2007; Luheshi, Rothwell, & Brough, 2009). Although further investigation is required to directly compare the functional significance of multiple pathways of T_h17 differentiation, it has been suggested that T_h17 cells induced by specific pathways can have distinct effector functions (Chung et al., 2009): For instance, naïve T cells differentiated in the presence of IL-6, IL-23 and IL-1 can lead to T_h17 cells that produce a higher quantity of IL-22 than T_h17 cells differentiated in the presence of IL-6 and TGF-β alone (Chung et al., 2009). This highlights the incredible plasticity of T_h17 cells, and the significance of considering the local microenvironment to understand the whole spectrum of T_h17 cell effector functions.

T_h17 cells produce IL-17A, IL-17F, IL-22 and IL-21, and play a fundamental role in the resolution of extracellular fungal and bacterial infections (Aujla et al., 2008; Ishigame et al., 2009). However, aberrant T_h17 responses can also lead to autoimmunity or chronic inflammatory diseases. (Dubin & Kolls, 2008; Guglani & Khader, 2010; Harrington et al., 2005; Kolls & Linden, 2004; Park et al., 2005). Their effector response involves the induction of inflammatory mediators such as TNF-α, IL-1, IL-8, G-CSF, and recruitment of neutrophils to the effector site to destroy infected cells, and/or free pathogens (Kolls & Linden, 2004; Pappu, Ramirez-Carrozzi, Ota, Ouyang, & Hu, 2010; Pappu, Ramirez-Carrozzi, & Sambandam, 2011). In the reproductive tract, T_h17 responses were shown to be part of the immune response to *Candida albicans* and *Neisseria gonorrhoeae* infections (Feinen, Jerse, Gaffen, & Russell, 2010; Hernandez-Santos & Gaffen, 2012; Pietrella et al., 2011), However, the role of T_h17 cells in viral infections of the genital tract are less clear.

1.2 INNATE LYMPHOCYTES IN THE IMMUNE SYSTEM:

Similar to adaptive lymphocytes, innate lymphocytes arise from common lymphoid progenitors originating in the bone marrow (Gasteiger & Rudensky, 2014). Innate lymphocytes include NK cells, γδ T cells and innate lymphoid cells (ILCs), and involved primarily in resolving infections, tissue repair and regulating inflammation.

1.2.1 Natural Killer cells:

Conventional NK cells, large granular lymphocytes with cytotoxic and cytokine-inducing effector functions, have been studied extensively (Vivier et al., 2011; Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). They are localized throughout the body in lymphoid and non-lymphoid organs. Type 1 IFNs, IL-12, IL-18 and IL-15 produced by other immune cells such as DCs, macrophages and T cells can activate NK cell effector functions. NK cells utilize conserved PRRs, and a variety of cell surface activating- and inhibitory-receptors for their activities (Long, Kim, Liu, Peterson, & Rajagopalan, 2013). They are primarily cytotoxic innate lymphocytes that can kill infected cells via two mechanisms, both requiring direct cell-cell contact: 1) Perforin/granzyme pathway where perforin disrupts the cell membrane, and granzymes (serine proteases) enter the target cell to induce apoptosis. 2) Caspase-dependent apoptosis where cell death is induced by the engagement of death receptor (Fas on target cell) and cognate ligand (FasL) on NK cell (Smyth et al., 2005). Additionally, NK cells also produce effector cytokines such IFN-γ that can enhance adaptive immune responses against intracellular pathogens (Boehm et al., 1997) (Vivier et al., 2011).

Murine studies show that NK cells play a key role in regulating and controlling tumor cells (Smyth et al., 2005). NK cells are key innate effectors in viral infections such as HSV-2, HSV-1, HIV-1 and influenza (Vivier et al., 2011; Vivier et al., 2008). They also have a fundamental physiological role in remodeling the uterine tissue during pregnancy (Vivier et al., 2011; Vivier et al.,

2008). Their role is being extended and investigated for other immunological conditions including allergies, organ transplantation, parasitic infections, immunopathology and immune regulation (Vivier et al., 2008).

1.2.2 Gamma delta (γδ) T cells:

γδ T cells also originate from the thymus, similar to traditional $CD4^+$ and $C8^+$ T cells. During T cell development from common lymphoid progenitors in the thymus, $CD4^-$ $CD8^-$ thymocytes may rearrange their T cell receptor (TCR) γ and δ genes, or α and β genes to become γδ T cells, or the conventional αβ T cells, respectively (Xiong & Raulet, 2007). γδ T cells are among the first immune cells to develop in a fetus, and provide immunity prior to the generation of the adaptive immune system (Sinkora, Sinkorova, & Holtmeier, 2005). Unlike traditional T lymphocytes that utilize their TCR to interact with MHC-peptide complexes, γδ cells utilize their TCR as a PRR, recognizing patterns of microbial products and cellular damage (Eberl et al., 2002).

γδ T cells may play an important role in the early response to various pathogens such as bacteria, fungi, viruses and parasites (Bonneville, O'Brien, & Born, 2010). They reside predominantly at mucosal sites, and rapidly activate upon exposure to these agents, or pro-inflammatory cytokines released by APCs (Bonneville et al., 2010). γδ cells resolve infection by the production of inflammatory molecules such as IFN-γ and IL-17, and facilitate bacterial clearance by recruiting neutrophils, macrophages and NK cells (S. M. Schulz,

Kohler, Holscher, Iwakura, & Alber, 2008) (Lockhart, Green, & Flynn, 2006; Peng et al., 2008).

γδ lymphocytes were one of the earliest identified IL-17-producing innate lymphocytes. IL-1, IL-6, IL-18, IL-23 and TGF-β have all been implicated to be important for IL-17 production by γδ cells (Sutton, Mielke, & Mills, 2012). *In vitro* studies show that γδ cells are dependent on exogenous cytokines, as they do not appear to produce IL-17 upon stimulation with TLR agonists alone (Lalor et al., 2011). Furthermore, while γδ T cells from IL-6KO mice were shown to produce IL-17 at comparable levels to WT mice, and those from TGF-β KO mice produce attenuated levels of IL-17, γδ cells from IL-1RKO mice fail to secrete any IL-17 in response to IL-23 and TLR agonists (Lalor et al., 2011; Sutton et al., 2009). This suggests a critical role of IL-1 in the production of IL-17 by γδ cells. IL-17$^+$ γδ T cells were found under constitutive conditions in the vagina, and have been implicated to play a pathological role in intravaginal HSV-2 infection (Kim, Cha, Kim, & Kweon, 2012). These observations are currently being further investigated in our lab, and has been outlined in this dissertation.

1.2.3 Innate lymphoid cells (ILCs):

ILCs constitute a separate group of cells that also accumulate at mucosal sites. Broadly, these have been categorized into three separate groups based on the effector cytokines, and their corresponding similarities to CD4$^+$ T helper subsets: Type 1 ILCs produce IFN-γ similar to T_h1 cells, Type 2 ILCs are dependent on RORα, and produce T_h2-associated cytokines such as IL-4, IL-5,

IL-9 and IL-13, and Type 3 ILCs are dependent on ROR-γt, and produce IL-17A, IL-17F and IL-22 (Artis & Spits, 2015; Sutton et al., 2012). All three subsets may play an important role in tissue homeostasis. Unlike γδ T cells, ILCs may not express any known antigen-specific receptors.

In a recent review, these ROR-γt type 3 ILCs have been divided broadly into two subtypes: 1) fetal lymphoid tissue inducer cells (LTi cells) and 2) adult LTi-like cells (Killig, Glatzer, & Romagnani, 2014). The fetal LTis are strictly required for development of lymph nodes and Peyers patches. On the other hand, adult LTi-like cells are present after birth and located predominantly within isolated mucosal lymphoid follicles. Fetal LTis are characterized by the expression of ROR-γt, IL-7R, c-kit, IL-17, IL-22 and LTα1β2. Meanwhile, the adult LTi-like type 3 ILCs have been differentiated into two distinct populations: CCR6$^+$ CD4$^+$ IL-17$^+$ IL-22$^+$ ROR-γt$^+$ cells and CCR6$^-$ CD4$^-$ IL-17$^-$ IL-22+ ROR-γt$^+$ cells. The CCR6$^-$ population may be further divided into subsets that can express NK-cell specific markers such as NKp46, NK1.1 and IFN-γ. Murine type 3 ILCs have been shown to display plasticity, and the NKp46$^+$ type 3 ILCs can altogether lose ROR-γt expression and become indistinguishable from typical mucosal NK cells (Killig et al., 2014).

Type 3 ILCs play an important role in barrier function due to the production of IL-22. IL-22 is an important regulator of epithelial cell homeostasis against pathogens such as viruses or commensal microflora (Sanos, Vonarbourg, Mortha, & Diefenbach, 2011). IL-22 binds to the IL-22R and induces the

production of antimicrobial peptides and proteins such as β-defensins, calgranulins, and mucins such as Muc1, Muc3, and Muc10, through an STAT3 dependent pathway within epithelial cells (Sugimoto et al., 2008). It can also promote wound healing by inducing migration of epithelial cells (reviewed in Killig et al., 2014). Unlike other mucosal tissues, ILCs are relatively uncharacterized in the vagina. In this dissertation, I have attempted to identify populations of type 3 ILCs in the vagina.

1.2.4 Interactions of adaptive and innate lymphocytes

Innate cells such as DCs can produce a variety of cytokines such as IL-12, IL-15 and IL-18 that can influence the polarization and function of NK cells and ILCs (Gasteiger & Rudensky, 2014). However, emerging evidence suggests that adaptive lymphocytes may also help and regulate innate immune responses.

In the context of inflammation, innate cytokines IL-12 and IL-18 can induce the expression of IL-2 receptors on NK cells and ILCs (Lee, Fragoso, & Biron, 2012). As a result, these cells can respond to IL-2 produced by adaptive $CD4^+$ T effectors, and may compete with differentiating $CD4^+$ T cells (Gasteiger & Rudensky, 2014). NK cells can also control immunopathology associated with anti-viral $CD4^+$ T effectors by a TNF-related apoptosis-inducing ligand (TRAIL) pathway (Peppa et al., 2013). In other studies, type 3 ILCs have been shown to process antigens and present them on MHCII molecules to control $CD4^+$ T cell responses against commensal microflora (Hepworth et al., 2013). Furthermore,

the production of cytokines by NK cells or ILCs such as GM-CSF, IL-10, IL-13, IL-4, IL-17 or TGF-β can skew the polarization of adaptive $CD4^+$ effector responses.

Conversely, the cytokines produced by adaptive T cells can instruct innate lymphocyte responses either directly or indirectly through modulation of myeloid cells. Tregs can also regulate the functions of innate lymphocytes by consuming large amounts of IL-2, thereby regulating innate lymphocyte responses (Ghiringhelli et al., 2005). Innate lymphocytes can themselves interact with adaptive T cells via $CD4^+$ TCR–MHCII interactions, and bi-directionally regulate one another (Gasteiger & Rudensky, 2014).

Studies have shown that both innate and adaptive IL-17-producing cells are co-dependent on one another, and co-amplify IL-17 responses to maintain mucosal tissue homeostasis (Do, Visperas, Dong, Baldwin, & Min, 2011; Do, Visperas, O'Brien, & Min, 2012; Hepworth et al., 2013; Qiu et al., 2013; Sutton et al., 2009). Innate lymphocytes may be a relatively small proportion of responding lymphocytes in mucosal tissue infections. Yet, they may be a more potent source of IL-17 compared to activated $CD4^+$ T cells (Lockhart et al., 2006). IL-17 producing γδ cells are the predominant source of IL-17 in patients suffering with pulmonary tuberculosis (Lockhart et al., 2006). In models of EAE, IL-1β and IL-23 induces IL-17 from γδ cells, which can in turn amplify IL-17 production by $CD4^+$ T cells (Sutton et al., 2009). However, in Aryl hydrocarbon receptor KO mice, type 3 ILCs were drastically reduced, and mice were prone to spontaneous colitis with increased segmented filamentous bacteria due to increased T_h17 cells (Qiu et al.,

2013). IL-22 production by the type 3 ILCs also plays a key role in the regulation of IL-17 produced by T_h17 cells thus revealing the intricate balance between ILCs and T_h17 cells in response to commensal microflora. Overall, these studies collectively indicate the complex interplay between innate and adaptive lymphocytes in the overall mucosal immune response.

1.3 HERPES SIMPLEX VIRUSES:

1.3.1 Epidemiology

Genital Herpes, caused predominantly by HSV-2, is one of the most prevalent sexually transmitted infections in the world (Looker, Garnett, & Schmid, 2008). Global estimates of HSV-2 infections indicate that over 517 million people aged 15-49, representing over 15% of the worldwide population in this age group, are infected with HSV-2 (Looker et al., 2015). Among them, over 19.2 million were newly infected individuals in 2012. Africa (32%) has the highest prevalence followed by the Americas (14%). In the United States, a 2005-2008 National Health and Nutrition Survey found that over 16.2% of people between 14-49 years were seropositive for HSV-2 (Centers for Disease & Prevention, 2010). Similarly, in Canada, a Canadian Health Measures Survey between 2009-2011 found that over 13.6% of people aged between 14-59 were seropositive for HSV-2 (Rotermann, Langlois, Severini, & Totten, 2013). Epidemiological trends consistently indicate that women are more susceptible to HSV-2 infection, and represent over two-thirds of the global HSV-2 seropositive population (Looker et al., 2015). These high rates of seroprevalence, and the associated public health

burden, support the need for further study of HSV-2 infection in the female genital tract.

1.3.2 Biology of Herpes Simplex Virus

HSV-2 is spherical, 100-200nm in diameter, and has a typical four layer structure (Subak-Sharpe & Dargan, 1998). The viral core contains the large double-stranded 155-kbp DNA genome encapsulated in an icosahedral capsid. This is surrounded by a group of proteins collectively known as the tegument, and the whole structure is enclosed within a glycoprotein-based lipid bilayer membrane (R. J. Whitley, 1996). Herpes viruses initiate their infection by using their surface glycoproteins (gB and gC in HSV-2) to bind to heparin sulfate, a glycosaminoglycan moiety on epithelial cells (Spear, 2004). Following attachment, glycoprotein gD can bind to its target receptors (Herpes virus entry mediator (HVEM), Nectin-1 and nectin-2), and further interactions by glycoproteins gB, and a gH-gL heterodimer, leads to the formation of the glycoprotein fusion complex (gD, gB and gH-gL heterodimer), which triggers fusion of the viral envelope and cell membrane (Akhtar & Shukla, 2009; Linehan et al., 2004; Spear, 2004). Subsequently, the capsid is uncoated and transported to the nuclear pore, where the viral DNA is released into the nucleus. Due to the ubiquitous nature of their entry receptors, HSV-2 can infect a variety of cells including T cells and DCs, (Jones et al., 2003; Raftery et al., 1999; Spear, 2004), however, the virus primarily infects epithelial cells and neurons.

1.3.3 Pathogenesis of genital herpes infections:

While both HSV-1 and HSV-2 can cause genital herpes, HSV-2 is the most common cause of sexually transmitted genital ulcers (Gupta, Warren, & Wald, 2007). HSV-2 pathogenesis can be divided into three aspects: primary infection, latency and reactivation. Primary infection occurs through contact with an infected person who is symptomatically, or asymptomatically shedding virus at their facial or genital skin, or within their oro-genital secretions (Tronstein et al., 2011). Viral replication occurs locally at the site of infection - largely in the genital, perigenital, or anal sites (Koelle & Corey, 2008; R. Whitley, Kimberlin, & Prober, 2007). Primary infection occurs in epithelial cells (ECs), and it can be asymptomatic in most individuals (Steiner & Benninger, 2013). Upon infection, infected cells fuse to form multinucleated giant, spherical cells with degenerated nuclei. On the exterior, the damage caused to the epithelial layer is seen as fluid-filled blisters containing cellular debris, immune cells and daughter virions (Gupta et al., 2007). Itching and pain precede the lesions for 1-2 days before the symptoms appear. The lesions evolve from pustules to wet ulcers over a 10-day period, and scabs can form over the damaged epithelial tissue. At the mucosal surfaces, ulcers are typically observed instead of blisters (Fields, Knipe, & Howley, 2013). Symptom severity generally tends to be worse and prolonged during primary infection rather than during reactivations. Common sites of symptoms in women include the outer vagina, vaginal mucosa and cervix.

However, the lesions can also extend towards surrounding cutaneous tissue in the buttocks, thighs and perianal mucosa.

Additionally, during primary infection, few virions can be transported retrograde to the sacral ganglia innervating infected epithelial cells. Although HSV can undergo limited replication in neurons, it does not cause cell death. Instead, it establishes latency which and can persist throughout the host's life (Gupta et al., 2007). The mechanism by which the virus establishes latency is not fully understood (Fields et al., 2013). Periodically, under a wide range of stimuli including but not limited to stress, menstruation and ultra violet light, latent virus can reactivate, travel anterograde to the site of primary infection, and induce symptomatic lesions (Gupta et al., 2007; Wald et al., 1997; R. J. Whitley, Kimberlin, & Roizman, 1998). Reactivations are largely asymptomatic, or mild with fewer localized lesions that heal faster than primary infection.

Although infected hosts have robust long-term memory responses following primary infection (Gebhardt & Mackay, 2013; Koelle et al., 1998), it is unknown why some individuals can suffer recurring outbreaks. Furthermore, those previously infected with a particular strain of HSV-2 are still susceptible to exogenous re-infection with a different strain of HSV-2 (Fields et al., 2013). It has been hypothesized that this may be the result of impaired T cell responses (Gebhardt & Mackay, 2013). This highlights the need for understanding factors in genital microenvironment that may influence susceptibility and immune responses to HSV-2.

1.3.4 HSV-2 vaccines:

Antivirals are currently the only form of therapy available for resolving the symptoms of HSV-2 infections. However, they cannot clear latent infections or prevent subsequent reactivations (Dasgupta, Chentoufi, Nesburn, Wechsler, & BenMohamed, 2009). Hence, HSV-2 an ideal candidate for vaccine development to potentially prevent acute disease, and/or prevent latency and the incidence of reactivations (Dasgupta et al., 2009).

A variety of HSV-2 vaccines have been explored in clinical trials and some are still under development. Subunit vaccines are among the most frequently studied in clinical trials. These are vaccines utilizing HSV glycoproteins such as gB, a combination of gB and gD (Koelle & Corey, 2008; Roth, Ferreira, & Kaushic, 2013) to generate protective immune responses. As early as 1985-1990, human trials were conducted with a vaccine prepared from purified HSV-2 glycoproteins. While it generated effective humoral and cellular responses, it was unable to protect against HSV-2 acquisition (R. Ashley et al., 1985; Mertz et al., 1990). This was again observed in a later trial with recombinant gB and gD proteins along with a MF59 adjuvant. This vaccine induced both antibody and cellular responses; yet, there were no improvements in protection, duration of first clinical outbreak, or frequency of reactivations. (Corey et al., 1999; Straus et al., 1994) More recently, promising results were observed by GlaxoSmithKline Inc. (GSK) using gD with an aluminium hydroxide (alum), and a 3-deacylated monophosphoryl lipid A (3-dMPL) adjuvant mix. This vaccine induced efficient

antibody and cell-mediated immune responses. While it was not efficacious in men or HSV-1 seropositive women, it reduced disease acquisition by 70% in a subgroup of HSV-1 and HSV-2 seronegative women (Stanberry et al., 2002). Unfortunately, a follow-up study with the same vaccine did not show any efficacy in preventing HSV-2 disease acquisition (Cohen, 2010) in any cohort.

Other alternate vaccine candidates include peptide-based, DNA-based and live attenuated formulations (Johnston, Koelle, & Wald, 2011; Wald et al., 2011). While peptide and DNA based vaccines are also being investigated, live attenuated vaccines hold the most promise for inducing broad and long-lasting immunity (K. Roth et al., 2013). Live attenuated vaccines have been shown to induce limited protection, and have been associated with lower frequency of recurrences. However, safety concerns have stalled the development of these vaccines (K. Roth et al., 2013). At present, this lack of efficacy with a variety of vaccine formulations beckons the need for further understanding HSV-2 anti-viral responses in the reproductive tract.

1.4 IMMUNE RESPONSES TO HSV-2:

The epithelial cell layer lining the lower genital tract is the first line of defense against pathogens such as HSV-2. The virus infects and undergoes productive replication in these ECs; hence, these are the first responders in the immune response to HSV-2 (Kaushic, Nazli, Ferreira, & Kafka, 2011). ECs produce a wide variety of innate factors, cytokines and chemokines that can help initiate and mobilize further innate immunity mediated by leukocytes such as

neutrophils, DCs, macrophages, monocytes and NK cells (Nazli et al., 2009). However, early proteins transcribed during viral replication can interrupt and suppress these innate immune responses (Ferreira, Nazli, Mossman, & Kaushic, 2013; Schiffer & Corey, 2013). As a result of such evasion mechanisms, the virus can partially overcome these innate immune responses (Schiffer & Corey, 2013). Eventually, an adaptive immune response mediated by adaptive lymphocytes is required to control and clear HSV-2 from the genital tract.

1.4.1 Innate immune responses to HSV-2:

The innate immune response is a broad, and early defensive strategy that helps limit the spread of HSV-2 infection, while initiating the cascade of events to generate an HSV-2-specific adaptive immune response. Our laboratory has previously examined the early immune responses mediated by GECs *ex vivo* (Nazli et al., 2009). Pattern recognition receptors (PRRs) that recognize pathogenic patterns, such as Toll-like receptors (TLRs), are surface or intracellular proteins that specifically recognize viral components. Human genital tract ECs can induce the expression of TLRs 1-10 *ex vivo*, and our laboratory has shown that TLR3, 5 and 9, are critical for the HSV-2 antiviral innate immune response.

GECs mediate this innate immune response through the production of biologically active type 1 interferon (IFN) (IFN-β), nitric oxide (NO), IL-6 and TNF-α, via MyD88-dependent (through NFκB activation), and MyD88-independent (through the activation of IRF3) pathways (Nazli et al., 2009). TLR ligand

treatment leads to a significant reduction in HSV-2 replication *ex vivo* in GECs cultures.

Type 1 IFNs induced by TLR activation leads to the induction of interferon stimulated genes (ISGs). These ISGs can amplify the aforementioned type 1 IFN response to effectively create an antiviral state, aiding in the control of viral infection in neighbouring ECs (Akira & Takeda, 2004). Type1 IFN is also important for the activation of other innate immune cells such as Natural Killer (NK) cells, plasmacytoid Dendritic cells (pDCs), and antigen presenting cells such as Dendritic cells (DCs) and macrophages in the genital tract.

1.4.1.1 Role of DCs in the HSV-2 immune response:

In the context of intravaginal HSV-2 infection, intraepithelial LCs, commonly identified as the primary migratory APC subset to prime $CD4^+$ T cell responses (Charles A Janeway, Travers, Walport, & Shlomchik, 2001), have been shown to be destroyed by the virus within 48h post infection (p.i.) (Zhao et al., 2003). Submucosal $CD11c^+$ $CD11b^+$ DCs recruited from the local tissue or peripheral sources, were shown to form foci beneath the infected epithelium 24h p.i., and subsequently migrate to the draining lymph nodes to stimulate antigen specific T cells (Zhao et al., 2003). The observation that epithelial LCs, the typical migratory APCs, may not migrate to the draining LN to prime HSV-2 specific $CD4^+$ T cells, suggests that the type of pathogen, and local microenvironment may selectively influence vaginal anti-viral responses through antigen presentation. While *in vitro* studies have indicated that factors such as sex

hormones can influence DC phenotype and function (Carreras et al., 2010; Mao, Paharkova-Vatchkova, Hardy, Miller, & Kovats, 2005; Paharkova-Vatchkova, Maldonado, & Kovats, 2004), their effect *in vivo,* and in the context of HSV-2 infection is unclear. In this dissertation, I have attempted to address this gap in literature.

1.4.1.2 Role of macrophages in the HSV-2 immune response:

During HSV-2 infection, $CD11c^{-}$ $CD11b^{+}$ $F4/80^{+}$ macrophages have been shown to be recruited and rapidly activated (within 12h of infection) in the vagina (Ellermann-Eriksen, 2005). However, while they may not directly contribute to priming $CD4^{+}$ T cell responses (Ellermann-Eriksen, 2005; Iijima et al., 2008; Iwasaki & Kelsall, 1999), they are involved in the overall anti-viral response. The anti-viral activity of macrophages has been typically divided into two facets: 1) Intrinsic: Macrophages are generally non-permissive to HSV viral replication, and this has been correlated to either spontaneous or constitutive production of autocrine IFN-α/β, and 2) Extrinsic: Macrophages can inactivate free virus, inhibit viral replication, and suppress HSV-induced apoptosis in other cells, by the production of reactive oxygen species (ROS) and iNOS (Ellermann-Eriksen, 2005). Although others have suggested that $CD11b^{+}$ submucosal DCs are the primary APC subset responsible for priming T_h1 responses to HSV-2 (Zhao et al., 2003), it is unknown whether macrophages can influence the profile of these responses. In this dissertation, I have sorted $F4/80^{+}$ macrophages, and also

attempted to examine their role in conditioning the differentiation profile of vaginal $CD4^+$ T cell responses.

1.4.1.3 Role of monocytes in the HSV-2 immune response:

Monocytes may also be involved in the HSV-2 antiviral response. During intravaginal HSV-2 infection, type 1 IFN stimulates CCL2 secretion, leading to the recruitment of $Ly6C^{hi}$ $CCR2^+$ inflammatory monocytes into the genital tract (Iijima, Mattei, & Iwasaki, 2011). Subsequently, these monocytes can differentiate into both $CD11b^+$ $CD11c^+$ DCs and $CD11c^-$ $CD11b^+$ macrophages (Guilliams et al., 2014; Iijima et al., 2011). While the priming, recruitment and effector potential of $CD4^+$ T cells remains intact in $CCR2^{-/-}$ mice (CCR2 is required for the mobilization of monocytes into peripheral tissues), $Ly6^{hi}$ $CCR2^+$ monocyte-derived APCs may be required for re-stimulating effector T_h1 cells to produce anti-viral IFN-γ (Iijima et al., 2011).

1.4.1.4 Role of NK cells in the HSV-2 immune response:

NK cells are one of the early responders in viral infections that are activated by type 1 IFN, and stimulated by IL-15 (Gill & Ashkar, 2007). NK cells can mediate antiviral mechanisms by mediating apoptosis of infected cells through perforin-granzyme mediated mechanisms, and through the production of IFN-γ (Vivier et al., 2008). IFN-γ mediates antiviral functions by activating inducible Nitric oxide synthase (iNOS) which catalyzes the oxidation of nitric oxide (NO) in ECs, DCs, and macrophages to control viral replication within infected cells (Bogdan, 2001). NK cells are the predominant source of IFN-γ for

the first 3 days post infection, and plays an important role in skewing the $CD4^+$ T cell response towards a T_h1 phenotype, that is critical for ultimately clearing virus from the genital tract (Milligan & Bernstein, 1997). The significance of these NK cells was examined using IL-$15^{-/-}$ and RAG-$2^{-/-}/\gamma_c^{-/-}$ mice (mice lacking NK and NKT cells). These mice were 100-fold more susceptible to vaginal HSV-2 infection compared to controls (Ashkar & Rosenthal, 2003). However, in a separate study that examined immune responses in the vagina post primary HSV-2 infection, antibody-mediated depletion of NK cells *in vivo* did not significantly effect the rate of HSV-2 clearance from the vagina (Milligan & Bernstein, 1997). Collectively, this suggests that while NK cells are not essential, they may be additional effectors that play a contributory role to the early innate response in controlling viral replication in the genital tract.

1.4.1.5 Role of pDCs in the HSV-2 immune response:

pDCs are another important source of type1 IFN in response to HSV-1 infection (Hochrein et al., 2004). A low number of pDCs survey the vaginal mucosa under steady state, and more are recruited to the vagina post intravaginal HSV-2 infection. There, they can produce large amounts of type 1 IFNs via a TLR9-dependent pathway. pDCs are not essential to the development of adaptive immune responses, and are strictly innate antiviral effectors useful in controlling the replication of HSV-2 (Lund, Linehan, Iijima, & Iwasaki, 2006).

1.4.2 Adaptive immune responses to HSV-2:

While the innate immune responses are helpful for controlling viral replication, adaptive immune responses are critical for clearing HSV-2 from the genital tract. Both humoral and cell-mediated immune responses are induced in response to HSV-2.

1.4.2.1 Humoral responses

HSV-2 specific IgG and IgA antibodies have been detected in the vaginal secretions of both humans (R. L. Ashley et al., 1992) and mice (McDermott, Brais, & Evelegh, 1990) infected with HSV-2. IgG was induced at higher levels in the vaginal secretions of adult HSV-2 immunized mice than S-IgA. Furthermore, IgG was found to have higher HSV-2 neutralizing activity than S-IgA, and may contribute to immune protection by neutralizing virus in the vaginal lumen (E. L. Parr & M. B. Parr, 1997). Our lab has also shown that lower pathology and viral shedding in E2-treated intranasally immunized mice post intravaginal HSV-2 challenge, could be correlated with high anti-HSV-2 IgG titers (Bhavanam, Snider, & Kaushic, 2008). However, whether antibodies are essential for protection, or just correlates of protection against HSV-2, is still under debate. The induction of high levels of anti-HSV-2 neutralizing antibodies in the serum of patients immunized with a subunit vaccine against HSV-2 gB and gD was not effective in preventing HSV-2 acquisition (Corey et al., 1999). Similarly, passive transfer of anti-HSV-2 monoclonal antibodies failed to prevent murine models against vaginal infection with HSV-2 (McDermott et al., 1990). Yet, later studies

with B-cell-deficient μMT mice showed significantly higher viral shedding post-infection in the absence of B cells (Dudley, Bourne, & Milligan, 2000). The virus was still rapidly cleared in these mice despite a 2-day delay compared to WT controls. This established that antibodies were helpful in limiting viral replication; however, were not crucial for HSV-2 clearance (Dudley et al., 2000; L. A. Morrison, Zhu, & Thebeau, 2001; Parr & Parr, 2000). Further investigation showed that apart from the direct neutralization of free virus, the generation of multiple and diverse antibody effector responses such as antibody-dependent cellular cytotoxicity, may be required for the effective humoral control of HSV-2 (C. F. Chu et al., 2008).

1.4.2.2 Cell-mediated responses:

The adaptive immune response mediated by $CD8^+$ and IFN-γ^+ $CD4^+$ T cells is critical to the resolution of HSV-2 infection. In humans, large populations of $CD8^+$ T cells and $CD4^+$ T cells (Cunningham, Turner, Miller, Para, & Merigan, 1985) have been detected in genital lesions, and have been correlated with viral clearance (Koelle et al., 1998). $CD8^+$ T cells induce apoptosis of infected cells through IFN-γ, perforin and/or Fas-mediated cytolytic mechanisms (Dobbs, Strasser, Chu, Chalk, & Milligan, 2005). Yet, in the mouse model, $CD8^+$ T cells may not play a critical role. While higher viral shedding can be observed in the vagina of $CD8^+$ T cell depleted mice (Parr & Parr, 1998), these mice were still protected against HSV-2 challenge, similar to WT controls, suggesting that $CD4^+$

T cells can compensate for the lack of $CD8^+$ T cells (Gill & Ashkar, 2009; Milligan & Bernstein, 1997).

Protection mediated by IFN-γ-producing $CD4^+$ T_h1 cells is the most important part of the HSV-2 anti-viral immune response in mouse models (Johnson et al., 2010). IFN-γ has multiple mechanisms by which it may directly or indirectly clear HSV-2 (Schroder, Hertzog, Ravasi, & Hume, 2004): 1) Enhance antigen processing and presentation (Epperson et al., 1992; Samuel, 2001), 2) Activate anti-viral genes such as RNA-dependent protein kinase R (PKR) to inhibit viral replication (Dobbs et al., 2005; Samuel, 2001), 3) Degrade viral RNA (Rebouillat & Hovanessian, 1999), 4) Synergise with type 1 IFN to inhibit replication (Sainz & Halford, 2002), and 5) Recruit and activate innate immune cells by the induction of iNOS (Chan & Riches, 2001). Depletion of $CD4^+$ T cells alone induced a significant delay in viral clearance and reduced protection against HSV-2 challenge in immunized mice (Gill & Ashkar, 2009; Milligan & Bernstein, 1997). $CD4^+$ T cells were also shown to be important for mobilizing $CD8^+$ T cells to site of infection (Nakanishi, Lu, Gerard, & Iwasaki, 2009). $CD8^+$ T cell migration to the vagina was dependent on IFN-γ mediated induction of CXCL9 and CXCL10 in the infected tissue, and $CD4^+$ T cells were the major source of anti-viral IFN-γ, as evidenced by a greater decrease in IFN-γ levels upon CD4 depletion, when compared to CD8 depletion alone (Milligan, Bernstein, & Bourne, 1998). $CD4^+$ T cells were also found to be necessary for activating B-lymphocytes, and thereby essential for the development of humoral responses

against HSV-2 (Dudley et al., 2000). Yet, depletion of both CD8 and CD4 subsets led to higher viral burden compared to depletion of either subset alone (Milligan et al., 1998). Overall, this suggests that both $CD4^+$ and $CD8^+$ T cells are important for a balanced HSV-2 anti-viral response, yet $CD4^+$ T cells producing IFN-γ are the most critical correlate of protection against HSV-2.

T_h2 cells may not play a protective role in mouse model of HSV-2 infection (Sin et al., 1999). However, Tregs may play a minor role in the HSV-2 anti-viral immune response. $FoxP3^{DTR}$ mice depleted of $FoxP3^+$ Tregs by DT treatment, succumb more rapidly to primary genital HSV-2 infection than Treg intact controls (Lund, Hsing, Pham, & Rudensky, 2008). The authors also observed significantly higher viral burdens in the vagina and spinal cords of these Treg depleted mice compared to the controls. Furthermore, depletion of these Tregs was also correlated with lower levels of type 1 IFNs in the genital mucosa, and delayed recruitment of pDCs, NK cells and T cells (Lund et al., 2008). Yet, in the context of re-infections, Tregs were found to potentially suppress memory T cell effector responses to HSV-2 (Diaz & Koelle, 2006). Overall, this suggests that while Tregs may facilitate the early innate immune response to HSV-2, a critical balance between inflammatory and regulatory $CD4^+$ T cell responses may be key to effective control of viral replication and disease pathology.

The role of IL-17^+ T_h17 cells in HSV-2 infection is relatively unclear. While few have suggested that T_h17 cells may not play a direct protective role in HSV-2 infection (Johnson et al., 2010; J. O. Kim et al., 2012), our laboratory has been

trying to examine the contribution of these cells to the overall HSV-2 immune response. The studies summarized in this dissertation have directly contributed to novel findings indicating that IL-17^{+} T_h17 responses may be important for the development of effective HSV-2 anti-viral T_h1 responses. Furthermore, their presence coincides with low disease pathology, and better survival as observed in estradiol-treated mice (Bhavanam et al., 2008; A. Gillgrass, Chege, Bhavanam, & Kaushic, 2010) (Anipindi V, Roth K, et al., See appendix A).

1.5 SEX HORMONES IN THE FEMALE GENITAL TRACT

Sex hormones are fat-soluble steroid compounds synthesized from cholesterol. They play a major role in the development of the reproductive system, as well as exerting other wide-ranging effects on bones, biosynthesis in the liver, brain, and the circulatory system.

1.5.1 Menstrual cycle:

The menstrual cycle in humans is generally a 21-35 day cycle, and can be largely divided into three phases depending on events in the ovaries (ovarian cycle: follicular phase, ovulation and luteal phase), or the uterus (uterine cycle: menstruation, proliferative phase and secretory phase) (Marieb & Hoehn, 2012). A conventional cycle begins at menstruation, marked with bleeding, when the uterine wall is sloughed off, and reset to a non-pregnant state. Under the influence of rising levels of estradiol (E2), and the presence of luteinizing hormone (LH) and follicle stimulating hormone (FSH), the ovarian follicles grow (follicular phase), while the uterine lining thickens (proliferative phase). Around

day 14 of the cycle, there is a sudden surge in LH, when the ovum is released from the ovarian follicle (ovulation). Post ovulation, the remains of the follicle, now known as the corpus luteum, starts producing large amounts of progesterone (P4) (luteal phase), and this prepares the endometrium lining of the uterus for implantation (secretory phase). In the absence of implantation over the next two weeks, the corpus luteum degrades to the corpus albicans, leading to a drop in both P4 and E2, which correspondingly leads to the shedding of the uterine lining during menstruation (Sherman & Korenman, 1975).

1.5.2 Murine Estrus cycle

The estrous cycle is the equivalent of the human reproductive cycle (menstrual cycle) in lower mammal species (Westwood, 2008). In rodents, this is correspondingly a 4-5 day cycle divided into four phases: proestrus (P) (12-14h), estrus (E) (25-27h), matestrus (M) (6-8h) and diestrus (D) (55-57h). However, extra days in the cycle corresponding to an extra day of vaginal cornification (seen in E), or an extra day of leukocyte infiltration (diestrus), is a normal occurrence (Westwood, 2008). The vagina and uterus show gross coordinated morphological changes through the stages of the estrous cycle. Starting at D, the vaginal epithelium is the thinnest spanning only 3-7 layers. During this stage, the corpus lutea in the ovaries are generally the largest, and the uterus is small lacking prominent vasculature. There is notable epithelial proliferation at the end of this phase, along with reduced leukocyte infiltration. During P, the vaginal epithelium shows mitotic figures, and there is an appearance of keratinized

epithelial cells along with progressive development of the superficial layers, ending with a fully cornified epithelial layer. At the same time, there is mitosis in the columnar epithelium of the uterus, and the corpus lutea degenerate. This is generally the pre-ovulatory day. Overnight, the LSH and FSH levels surge, culminating in ovulation. The next stage E, is marked with a loss of mitotic figures in the vagina and uterus, and there is progressive shedding of the cornified epithelial layers and debris in the lumen. Finally, the detachment of the complete cornified epithelium marks the start of M, and this is accompanied with progressive leukocyte infiltration (Westwood, 2008). These stages can be visually tracked based on vaginal smears whose contents correspond to the cellular remodeling described above: D is identified by smears rich in mucous containing predominantly leukocytes, P can be identified by nucleated large epithelial cells, and few cornified epithelial cells (Caligioni, 2009), E is distinct with no mucus and a large number of cornified irregular shaped epithelial cells that occur in clusters, and M contains a mix of leukocytes, cornified epithelial cells, and few nucleated epithelial cells (Caligioni, 2009).

These cyclic changes in the estrous cycle are driven by the female sex hormones estradiol (E2) and progesterone (P4). The level of E2 peaks during E (P: 40pg/ml, E: 60pg/ml, M/D: 40 pg/ml), and P4 peaks during D (P: 7.5ng/ml, E: 2ng/ml, M: 7.5ng/ml, D: 18ng/ml) (Wood, Fata, Watson, & Khokha, 2007). The uterine width is positively correlated with E2 serum levels, but negatively correlated with P4 levels (Wood et al., 2007).

1.5.3 Estradiol:

Estrogen is an aromatic compound that can be synthesized from cholesterol and acetate. 17β-estradiol (E2) is the primary bioactive estrogen synthesized by an aromatase from testosterone or converted from estrone. E2 is an important regulator of growth, differentiation and function in both males and females, and plays an important role in a variety of biological systems: male and female reproductive tracts, skeletomuscular system and the cardiovascular system.

Serum concentrations of E2 peak (60pg/ml) during the E phase of the murine estrous cycle (P: 40pg/ml, E: 60pg/ml, M/D: 40 pg/ml) (Wood et al., 2007). Given that E2 is a steroid hormone, it predominantly exerts its effects via two unique, independently encoded intracellular receptors: estradiol receptor-α (ERα) and ERβ (Giguere, Tremblay, & Tremblay, 1998). They share homology at their DNA-binding, and ligand-binding domains. They can bind to E2 or similar DNA-response elements (Giguere et al., 1998). Although, there is evidence supporting overlapping roles of ERα and ERβ *in vitro* for E2 signaling, there is a distinct expression pattern of these receptors in different tissues *in vivo*. ERα is the predominant ER expressed in the uterus, vagina, cervix, breast and other tissues, while ERβ has a limited scope, and is predominantly expressed in ovary, thymus, brain, prostate, testis, spleen, hypothalamus and lung (Kuiper et al., 1997). The distinct expression of these receptors mirrors the functional dysregulation seen in estradiol receptor α knockout (ERKOα) and ERKOβ mice.

ERKOα female mice are infertile due to E2 insensitivity, hyperplaysia in the reproductive tract, and underdeveloped mammary glands. Similarly, males are also infertile due to testicular and epididymal degeneration. On the other hand, ERKOβ female mice are subfertile with inefficient ovarian function, while male mice are fully fertile (Couse & Korach, 1999; Hall, Couse, & Korach, 2001). ERα receptors are expressed on a variety of immune cells including lymphocytes, DCs, NK cells, macrophages, mast cells and monocytes (Pierdominici et al., 2010). ERβ on the other hand, is not expressed ubiquitously on immune cells, but is expressed on splenic B lymphocytes (Kovats, Carreras, & Agrawal, 2010).

Four different pathways have been proposed for E2-mediated responses: 1) classical pathway in which E2 binds to its receptors and activates E2 response elements (EREs) in promoters, leading to up- or down-regulation of target genes 2) ligand-independent pathway in which growth factors can activate ER and corresponding ERE independent of E2 3) DNA-binding independent pathway in which E2-ER complexes can associate with other transcription factors such as AP-1 to control gene expression indirectly, and 4) cell-surface (non-genomic) signaling in which E2 can rapidly induce tissue responses via a putative membrane-associated binding site (Hall et al., 2001). The existence of ER-independent pathways of E2 mediated functions was speculated when E2 effects were incompletely blocked upon use of ER antagonists (Filardo, 2002; Filardo et al., 2006). In 2002, this novel E2-binding receptor was described by Filardo E. J, et al as GPER.

E2 can bind to putative ERα receptors associated in the cell membrane and/or G-protein coupled estrogen receptor 1 (GPER) to activate kinases that can lead to the phosphorylation of multiple transcription factors to indirectly regulate gene expression in response to stimuli (Filardo, 2002; Filardo, Quinn, Frackelton, & Bland, 2002; Hsieh et al., 2007; Meldrum, 2007). These receptors are expressed in the lung, kidney, adrenal glands, liver, ovary, CNS, and a variety of immune cells including macrophages (Hazell et al., 2009; Prossnitz & Hathaway, 2015; Rettew, McCall, & Marriott, 2010). While the absence of ERα and ERβ has shown defects predominantly associated with the reproductive system, GPER has a wide variety of target ligands including natural estrogens, phytoestrogens, vitamins, and selective estrogen receptor modulators. As a result, the effects of GPER-mediated effects of E2 has far reaching consequences in physiology and disease including the CNS, adipose tissues, pancreas, skeletal muscle, liver and immune cells (Barton & Prossnitz, 2015; Prossnitz & Hathaway, 2015).

1.5.4 Progesterone:

Progesterone (P4) is another major steroid hormone that plays an important role in the reproductive system. Similar to E2 and testosterone, it is biosynthesized from cholesterol. In sexually mature females, P4 is synthesized in the corpus luteum before implantation, and both corpus luteum and placenta after implantation. It plays an important role in pregnancy by maintaining implantation, inducing uterine contractions, and labor. However, P4 is also produced to a

smaller extent by glial cells and Schwann cells in the central nervous system (CNS) (Schumacher et al., 2012). P4 also plays an important role in mammary gland development (Arendt & Kuperwasser, 2015). Moreover, it interacts with osteoblasts in a receptor-dependent mechanism to promote bone formation (Prior, 1990).

Plasma P4 concentrations peak during the luteal D stage of the murine estrous cycle (P: 7.5ng/ml, E: 2ng/ml, M: 7.5ng/ml, D: 18ng/ml) (Wood et al., 2007). P4 exerts its effects via two intracellular receptors (PR): progesterone receptor isoform A (PR-A) and PR-B. These isoforms vary in their ability to differentially regulate P4-specific target genes in specific tissues. For instance, PR-A knockouts have no defects in mammary gland or thymus function; yet they may possess severe abnormalities in ovarian and uterine functions. On the other hand, PR-B knockouts have defects in their mammary gland function (Conneely, Mulac-Jericevic, DeMayo, Lydon, & O'Malley, 2002; Gadkar-Sable, Shah, Rosario, Sachdeva, & Puri, 2005; Lydon, DeMayo, Conneely, & O'Malley, 1996). The expression of PR in immune cells is still under investigation. Rodent leukocytes from the thymus, liver and spleen, and most macrophages and BMDCs express PR (Kovats et al., 2010). Upon binding to P4, inactive PR bound and stabilized by heat shock proteins dissociate and act as transcription factors by binding to P4-response elements (PREs) in target regions of promoter sequences. PR expression is known to be regulated by estrogens via the ER (Ing & Tornesi, 1997).

1.6 INFLUENCE OF THE HORMONES ON VAGINAL IMMUNE RESPONSES

Women are more susceptible to STIs such as HIV-1 and HSV-2 compared to men (Kaushic, Roth, Anipindi, & Xiu, 2011). Numerous studies have implicated this discrepancy in STI susceptibility and disease progression to the effect of sex hormones on immune responses in the genital tract (Wira, Rodriguez-Garcia, & Patel, 2015). In humans, the stage of the menstrual cycle, and hormone contraceptives may influence immune responses in the reproductive tract (Kaushic, Roth, et al., 2011). We have previously reviewed a number of studies showing significant differences in systemic and mucosal adaptive immune responses of women at different phases of the cycle (Kaushic, Roth, et al., 2011).

1.6.1 Influence of the hormone cycle on immune responses in the genital tract:

The female reproductive tract is physiologically regulated by cyclic hormonal changes in preparation for fertilization and implantation (Marieb & Hoehn, 2012). Additionally, a number of studies have also indicated that a wide number of resident immune cells, such as APCs, T cells and NK cells, are distributed in the human and rodent female genital tract under homeostatic conditions (Wira et al., 2015). Analysis of both rodent and human tissues showed that the frequency and distribution of these immune cells might vary with the stage of the hormone cycle (Kaushic, Frauendorf, Rossoll, Richardson, & Wira, 1998; Wira et al., 2015). In humans, the levels of IgA and IgG antibodies produced in cervicovaginal secretions were found to vary pre- and post ovulation

(Lu et al., 1999); there is a significant decrease in IgG and IgA in human cervical mucus (Kutteh, Moldoveanu, & Mestecky, 1998), and a corresponding decrease in B cell frequencies in other compartments such as spleen and lymph nodes during the P4-high post ovulation luteal phase (Lu et al., 2002). The expression of CXCR4 and CCR5, well-known co-receptors for HIV-1 infection, was also found to vary with stages of the menstrual cycle (Wira et al., 2010). Additionally, the activity of cytotoxic $CD8^+$ T lymphocytes (CTL) and NK cells was also influenced by the hormonal cycle. During the follicular phase, CTL and NK cell cytolytic activity was higher than the luteal phase (Souza et al., 2001; White et al., 1997). A study examining T_h17 cells in the upper genital tract found a significant decrease in mucosal T_h17 cells in the endometrium of premenopausal women compared to postmenopausal women (Wira et al., 2015). An increase in Treg frequencies and their suppressive function was also observed during the proliferative phase of the cycle (Berbic et al., 2010) (Fish, 2008). E2 was linked to driving the expression of programmed death-1 (PD-1) in Tregs, thereby modulating their suppressive capabilities (Polanczyk, Hopke, Vandenbark, & Offner, 2007). Altogether, these studies indicate that along with driving physiological changes in the reproductive tract, the female sex hormones can also modulate local immune responses. Consequently, they may also influence susceptibility to a variety of sexually transmitted infections.

1.6.2 Hormone effects on immune responses

1.6.2.1 Estrogen can influence immune responses in women:

A number of studies from the field of infectious diseases to autoimmunity have shown that E2 can directly influence immune responses in women. ER are expressed by T cells, B cells, DCs, macrophages, neutrophils, and NK cells (Couse, Lindzey, Grandien, Gustafsson, & Korach, 1997; Pierdominici et al., 2010). In systemic lupus erythematosus (SLE) mouse models, E2-treatment accelerated disease progression and death (Walker, McMurray, Besch-Williford, & Keisler, 1992). E2 increased the activity of autoreactive polyclonal plasma cells in the bone marrow and spleen (Bynoe, Grimaldi, & Diamond, 2000). E2 was also found to increase the frequency of murine splenocytes that secrete IL-6 and IL-10 (L. Yang, Liang, Yao, Chen, & Hou, 2005). In women, E2 treatment increased IgM and IgG levels from human PBMCs cultured *in vitro* (Lahita, 1990; Sthoeger, Chiorazzi, & Lahita, 1988). Meanwhile, while testosterone reduced B cell responses to mitogens, E2 enhanced their activity (Paavonen, 1987; Sthoeger et al., 1988). Since the 1990s, scientists have observed that women were 3-9 fold more prone to SLE, Sjorgren's syndrome, and rheumatoid arthritis than men (Ben-Chetrit & Ben-Chetrit, 1994; Bruce & Laskin, 1997; Folomeev et al., 1992; Verthelyi, 2001). Critically, E2 may have a dose-dependent effect on immune responses. Low levels of E2 enhanced T_h1 differentiation, and higher levels promoted T_h2 differentiation (Maret et al., 2003). Changes in the level of E2 during pregnancy were shown to influence susceptibility to autoimmune

disorders. E2 was also found to decrease TNF-α production by $CD4^+$ T cells (Ito et al., 2001). E2 may also affect innate immune responses. E2 treatment inhibits CD16 expression in monocytes and macrophages leading to lower production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (Kramer, Kramer, & Guan, 2004). E2 may also increase nitric oxide synthase activity in neutrophils (Duran et al., 2000). Altogether, these studies indicate that E2 can have a significant impact on the immune system in women.

1.6.2.2 E2 can influence the differentiation and function of DCs:

APCs such as DCs bridge innate and adaptive immune responses. E2 has also been shown to influence DC differentiation and responses. E2 has been shown to drive IRF4 expression, and preferentially induce the differentiation of $CD11c^+$ $CD11b^+$ DCs from bone marrow progenitors (Carreras et al., 2010; Mao et al., 2005; Paharkova-Vatchkova et al., 2004). Similarly, other groups have shown that E2 can promote the differentiation of BMDCs, and enhance IL-12 and IFN production in splenocytes, in response to TLR ligands (Siracusa, Overstreet, Housseau, Scott, & Klein, 2008). E2 was also shown to upregulate IL-6 and IL-8 expression by immature DCs (Fish, 2008). The role of E2 on vaginal DC responses *in vivo* is relatively unknown. This topic is the primary focus of this dissertation.

Overall, all these studies indicate that E2 can directly influence innate and adaptive immunity, thereby modulating immune responses to pathogens.

1.6.2.3 P4 can influence immune responses

A number of groups have associated P4 with attenuated anti-viral responses in the genital tract. P4 can inhibit CTL activity by blocking perforin expression (Borel et al., 1999; Cherpes, Busch, Sheridan, Harvey, & Hendricks, 2008). While T_h1 responses are a critical component of the immune response to HSV-2, P4 can potentially skew cytokine production favouring the development of a T_h2 response (Piccinni et al., 1995). P4 can also attenuate the infiltration of lymphocytes, NK cells, monocytes and macrophages into the vagina (Arici, Senturk, Seli, Bahtiyar, & Kim, 1999; Yeaman, Collins, Fanger, Wira, & Lydyard, 2001). P4 can also impair NK cell function (Yovel, Shakhar, & Ben-Eliyahu, 2001) and decrease FcγR expression on monocytes (Gomez, Ruiz, Lopez, & Rivera, 2002). Hence, similar to E2, P4 can directly influence innate and adaptive immune responses.

1.6.2.4 P4 can influence the differentiation and function of DCs:

Similar to E2, P4 can also potentially influence the differentiation and function of DCs. P4 and associated contraceptives such as DMPA were found to inhibit pDC mediated anti-viral IFN-α production thereby regulating innate anti-viral immunity (Hughes, Thomas, Li, Kaja, & Clark, 2008). However, unlike E2, P4 was found to inhibit the differentiation and function of DCs from bone marrow progenitors (Butts et al., 2007; Liang, Sun, Wang, & Hou, 2006). The effect of P4 on vaginal DCs *in vivo* in the context of HSV-2 infection is relatively unknown.

Although it was not the primary focus, in this dissertation, we have attempted to compare the effect on E2 and P4 on vaginal DC phenotype and functions.

1.6.3 P4 and P4 based contraceptives can increase susceptibility to STIs

Globally, over 150 million women use oral contraceptives, and over 50 million use P4-based injectable contraceptives such as Depot medroxyprogesterone acetate (DMPA) (C. S. Morrison, Turner, & Jones, 2009). Hormone contraceptives such as DMPA are a highly effective, long lasting, and a preferred cost-effective solution for millions of women around the world (UN, 2003). In a 10 year study, women who used injectable contraceptives such as DMPA were more susceptible to acquiring HIV-1 infection compared to women who did not use contraceptives (Lavreys et al., 2004). Similar observations of increased susceptibility were noted in a more recent study where DMPA users in Africa had a 6% higher risk of acquiring HIV-1 (Leclerc, Dubois-Colas, & Garenne, 2008). This correlation was further strengthened by a recent large prospective study examining HIV-1 transmission among 3790 serodiscordant couples in 7 African countries (Heffron et al., 2012). The use of hormone contraceptives, specifically injectable contraceptives, was associated with a statistically significant increase in the risk of HIV-1 transmission to the seronegative partner (Heffron et al., 2012). Numerous studies have also linked women who use DMPA to display accelerated HIV-1 disease progression and mortality compared to controls (Stringer et al., 2007). It is speculated that P4 and P4-based hormone contraceptives such as DMPA, affect STI susceptibility and

outcomes by directly influencing immune responses in the genital tract. A number of animal studies have linked the use of hormone contraceptives to increased risk of HIV-1 acquisition, faster disease progression and higher mortality (Hel, Stringer, & Mestecky, 2010; Stringer et al., 2007). Similar to observations in human studies, rhesus macaques were more susceptible to intravaginal SIV infection during luteal phase (P4 high) compared to the follicular phase (E2 high) (Sodora, Gettie, Miller, & Marx, 1998). DMPA administration significantly enhanced the risk of SIV-1 acquisition and increased plasma viral loads in macaques (Marx et al., 1996; Trunova et al., 2006). DMPA treatment was found to attenuate the anti-SIV cellular immune response, and increased SIV replication in infected animals (Trunova et al., 2006). In a separate study, DMPA administration also abrogated the protection conferred by a lentivirus-based vaccine against SIV-1 (Abel et al., 2004). While a few studies suggested that P4 influences susceptibility by causing thinning the vaginal epithelium, this remains uncertain (Hild-Petito, Veazey, Larner, Reel, & Blye, 1998; Mauck et al., 1999; Miller et al., 2000). It is hypothesized that another effect of P4 contraceptives is the decreased colonization of peroxide-producing lactobacillus in the vagina post DMPA treatment (Miller et al., 2000). The production of H_2O_2 by these bacteria could directly kill free virus, as suggested by observations of higher HIV-1 acquisition in women without lactobacillus in their vaginal microbiome (Martin et al., 1999). Overall, these studies suggest that the use of P4-based contraceptives may be an important risk factor for susceptibility to STIs.

1.6.4 E2 can have a protective effect against STIs

In contrast to P4, E2 has been linked to having a protective effect against the acquisition of STIs. Implantation of subcutaneous E2 pellets protected ovariectomized (OVX) rhesus macaques against intravaginal SIV-1 infection (S. M. Smith, Baskin, & Marx, 2000). However, this effect was tissue specific as the same animals were susceptible to subcutaneous infection (S. M. Smith et al., 2000). Similar results were observed in a separate study where topical estrogen treatment (Ovestin) protected OVX macaques against intravaginal challenge with SIV (S. M. Smith et al., 2004). Three primary reasons were speculated for the protection in this model: 1) Physical barrier: The vaginal epithelium in most cases is a highly effective innate barrier that can resist pathogenic infections, and sex hormones can significantly affect its physiology. HIV-1 and SIV transmission may primarily occur through damaged vaginal epithelium (Hel et al., 2010). E2 can thicken the vaginal epithelium in women, macaques and mice (Hel et al., 2010). The thick vaginal epithelium can block HIV/SIV access to target cells such as DCs, $CD4^+$ T cells and macrophages in the subepithelial layers. 2) Low pH: E2 also decreases the pH in the cervicovaginal environment (Molander, Milsom, Ekelund, Mellstrom, & Eriksson, 1990; P. Smith, 1993), making it less hospitable for pathogens. 3) Microbiome: A healthy lactobacilli dominated microbiome may play a key role in the prevention of a number of STIs such as HSV-2 and HIV-1. E2 treatment increases glycogen production, which has been correlated with increased lactobacilli colonization in the genital tract (Galhardo et al., 2006;

Mirmonsef et al., 2014). Additional reasons for the protective effect may include E2-effect on immune responses in the vagina. In addition to the aforementioned effects of E2 on immune responses (given in section 1.6.2), E2 can also downregulate the expression of adhesion markers such as ICAM-1, E-selectin and VCAM-1. This decreases the migration of inflammatory T cells and macrophages, targets for HIV-1 infection in the genital tract (Straub, 2007).

1.6.5 E2 and P4 can influence susceptibility in the HSV-2 mouse model

A number of studies have suggested that E2 and P4 can modulate susceptibility to STIs such as HSV-2 (Kaushic, Roth, et al., 2011) (Grabowski et al., 2015). Female mice become susceptible to HSV-2 infection only during the P4-dominant diestrus phase of the estrous cycle, and remain resistant to infection during the E2-dominant estrus phase (Parr & Parr, 2003). We have previously shown that DMPA treatment increased susceptibility of mice by 100-fold compared to untreated mice (Kaushic, Ashkar, Reid, & Rosenthal, 2003). In a separate study, prolonged P4 treatment resulted in poorer mucosal immune responses and greater susceptibility to HSV-2 (A. E. Gillgrass, Ashkar, Rosenthal, & Kaushic, 2003). In later studies, OVX mice were treated with the hormone E2 or P4 to deconstruct their individual effects on immune responses. These studies showed that P4 increased disease pathology and lowered survival compared to E2 in a vaccine model of intravaginal HSV-2 infection (A. E. Gillgrass, Fernandez, Rosenthal, & Kaushic, 2005; A. E. Gillgrass, Tang, et al., 2005). These observations were also replicated in intranasal and subcutaneous

immunization models (Bhavanam et al., 2008). Mice were immunized under the influence of E2 or P4 with an attenuated strain of HSV-2, and challenged intravaginally many weeks later with WT HSV-2. Mice immunized under the influence of E2 showed significantly higher survival rates, reduced pathology, and lower viral shedding, compared to those immunized under the influence of P4 or placebo (Bhavanam et al., 2008). Others have since confirmed these observations, and showed that E2 improves vaccine outcomes using a HSV-2 gD peptide-based formulation (Pennock et al., 2009). The cellular mechanisms by which hormones influence susceptibility to intravaginal HSV-2 infection remain unclear. APCs such as DCs are critical to the generation of HSV-2 anti-viral responses, and E2 and P4 can directly modulate DC differentiation and function. It is critical to examine whether hormones influence anti-viral responses by modulating vaginal APC populations. This was among the primary objectives of the studies described in this dissertation.

1.7 RATIONALE, HYPOTHESIS & AIMS:

Heterosexual intercourse is a major mode of transmission for sexually transmitted infections (STIs) in the female reproductive tract (Kuehn, 2006; UNAIDS, 2008). The stage of menstrual cycle, and contraceptive use can influence susceptibility to STI infections including Candidiasis, gonococcal infections, viral infections such as HSV-2, HIV-1 and Chlamydia in women (Kaushic, Roth, et al., 2011; Wira et al., 2015). Previous research has shown that female mice are only susceptible to HSV-2 infection during the P4-dominant

diestrus phase of the estrous cycle, and resistant to infection during the E2-dominant estrus phase (Parr & Parr, 2003). P4 and P4-based injectable contraceptives such as DMPA further increased this susceptibility 100-fold (Kaushic et al., 2003). Numerous studies have suggested that E2 and P4 can influence susceptibility by modulating immune responses in the genital tract (Wira et al., 2015). Using an OVX mouse model, we and others have shown that intravaginal, subcutaneous or intranasal immunization with an attenuated virus or peptide vaccine, under the influence of E2, induces significantly better outcomes following HSV-2 challenge, such as better survival and lower pathology compared to P4- or mock-treated controls (Bhavanam et al., 2008; A. E. Gillgrass, Fernandez, et al., 2005; A. E. Gillgrass, Tang, et al., 2005; Pennock et al., 2009).

Although both humoral and cellular responses are induced against HSV-2, it is well accepted that IFN-γ mediated T_h1 responses are key for viral clearance in the vagina (C. F. Chu et al., 2008; Dobbs et al., 2005; Johnson et al., 2010; Milligan & Bernstein, 1997; Milligan et al., 1998; Milligan et al., 2004). $CD11c^+$ DCs in the vaginal submucosa were found to be critical in generating these responses (Zhao et al., 2003). We have found that enhanced protection in E2-treated mice against HSV-2 correlates with robust vagina-associated lymphoid tissues (VALTs) containing $CD11c^+$ cells and $CD4^+$ T cells (A. E. Gillgrass, Tang, et al., 2005). Whether E2 and P4 affect HSV-2 disease outcomes by modulating vaginal APCs was unknown. The phenotype of APC populations in the vagina

varies during the different stages of the menstrual cycle (Iijima et al., 2007; Zhao et al., 2003). Additionally, E2 and P4 can directly influence the differentiation and function of dendritic cells from bone marrow precursors *in vitro* (Butts et al., 2007; Carreras et al., 2010; Liang et al., 2006; Mao et al., 2005; Paharkova-Vatchkova et al., 2004). However, the individual effect of E2 and P4 on the phenotype and function of vaginal APCs *in vivo* is unknown. Furthermore, the contribution of other important factors such as IL-17 produced by innate lymphocytes and microflora is also unknown.

Based on this, we postulated the hypothesis that "Estradiol and factors in the local microenvironment such as microflora and innate lymphocytes can regulate the phenotype and function of vaginal APCs."

I addressed this hypothesis in my dissertation through the following three objectives that have been addressed in section 3.1, 3.2 and 3.3.

OBJECTIVE 1: Examine the effect of E2 on the function of vaginal APCs

OBJECTIVE 2: Examine the effect of E2 and innate IL-17 on vaginal T_h17 responses

OBJECTIVE 3: Optimize an E2 delivery regimen to study the effect of E2 on vaginal APC responses *in vivo*

Overall, the goal of this project was to provide critical insights into the underlying mechanism by which the hormonal environment influences anti-viral immune responses in the female reproductive tract. Given the increased susceptibility to HSV-2 in women, and the role of hormones and hormone-

contraceptives in this increased susceptibility, modulating the hormonal microenvironment may prove to be an effective strategy for generating optimal immunity with HSV-2 vaccines.

CHAPTER 2

MATERIALS & METHODS

2.1 Animals & associated treatments:

2.1.1 Animals:

C57BL/6 mice were used for these studies. These were obtained from Charles River laboratories Inc (Saint-Constant, QC, Canada). Chicken ovalbumin (OVA) receptor transgenic (Tg) mice (OT-II Tg) whose $CD4^+$ T cells express TCR specific for the ovalbumin 323-339 ($OVA_{323-339}$) epitopes (Robertson, Jensen, & Evavold, 2000), and IL-6 knockout mice (IL-6 KO) (Kopf et al., 1994), were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). IL-1β KO, IL-1α KO and IL-17 KO mice kindly provided by Dr. Martin Stampfli (acquired with an MTA from Dr. Yoichiro Iwakura) (University of Tokyo, Minato-ku, Tokyo, Japan) (Horai et al., 1998; Nakae et al., 2002), estradiol receptor α knockout mice (ERKO) kindly provided by Prof. P. Chambon (University de Strasbourg, France) (Dupont et al., 2000), and CD11c-DTR mice kindly provided by Dr. Jonathan Bramson (L. Zhang et al., 2013), were bred internally at the Central Animal Facility (CAF) (McMaster University, Hamilton, ON, Canada). IRF4 KO mice (Mittrucker et al., 1997) were kindly provided by Dr. Tak Wah Mak (University Health Network, Princess Margaret Cancer Centre, Toronto, ON), and were maintained internally at the CAF (McMaster University, Hamilton, ON, Canada). Germ-free mice were bred in-house and purchased from the Farncombe Family Axenic-Gnotobiotic Facility (McMaster University, Hamilton, ON). All the animals and protocols used in this study were approved by the Animal Research Ethics Board at McMaster University.

2.1.2: Genotyping:

2.1.2.1 ERKO mice:

Genotyping was conducted according to standard protocols as outlined below.

DNA extraction: Tails from infant mice were obtained from attending CAF technician in charge of maintaining the animals. DNA was extracted from tails by incubation with 12µl/tail proteinase K (10 mg/mL), and 300µl/tail Direct PCR lysis reagent (Cedarlane, Cat# 102-T) at 55°C overnight in a shaking water bath. Next morning, samples were centrifuged at 14000 rpm for 5 min, and supernatant was transferred to fresh tubes. Proteinase K was inactivated by incubation at 85°C for 45-60 min, and DNA was used for PCR amplification.

Component	Vol/rxn
dH2O	36.1ul
10x PCR buffer	5ul
50uM MgCl2	1.5ul
P1 or P3	2.5ul
P2 or P3	2.5ul
10mM dNTP	1ul
5x Rapid load buffer	5ul
Taq polymerase	0.4ul
DNA	1ul

Temperature	Time/cycle	#cycles
94°C	5m	
94°C	1m	
54°C	1m	30
72°C	1m	
72°C	5m	
4°C		

PCR: PCR was conducted using two sets of primers as described by Dupont et al. (Dupont et al., 2000).

Set 1: 30 cycles

P1: 5′-CGGCTGCCACTTACCATGACCA-3′

P2: 5′-GGGGAGCCTGGGAGCTCTCAGAT-3′

Set 2: 30-45 cycles

P3: 5′-ACCATGACCATGACCCTTCACA-3′

P4: 5′-CTCTCAGATCGTGTTGGGGAAG-3′

The PCR mastermix and conditions are given in the adjacent tables.

The program utilized is labelled as “ERKOa” on the instrument in the lab.

Gel: Samples were run on a 2% agarose gel (2g agarose in 100ml TBS) with 5µl red safe dye (Cedarlane, Cat# 21141) for visualization.

2.1.2.2 CD11c DTR mice:

Genotyping of CD11c DTR mice was conducted based on the protocol obtained from Jane Ann, that is a modified version of the protocol described above for ERKO mice. PCR program utilized is labelled as “CD11DTR” in the PCR instrument. The presence of a 600-700bp band in the gel signifies the presence of the DTR transgene, and can be seen in heterozygotes and homozygotes. Both were suitable for experimental use. See the adjacent table for PCR mix components. The primer sequences utilized are as follows:

Component	Quantity/ 1 PCR reaction
dH2O	11.75ul
10x PCR buffer with MgCl2	2.5ul
Fwd primer	1.0ul
Rev primer	1.0ul
2.5mM dNTP	2.0ul
5x Rapid load buffer	5ul
Taq polymerase	0.25ul
DNA	1.5ul

Temperature	Time/cycle	#cycles
94°C	3m	
94°C	30s	
62°C	1m	35
72°C	1m	
72°C	5m	
4°C		

Forward primer: 5’ GGG ACC ATG AGG CTG CTG CCG 3’

Reverse primer: 5’ TCA GTG GGA ATT AGT CAT GCC 3’

2.1.2.3 IRF4 KO mice:

Tail DNA was extracted from infant mice as described for ERKO mice. A PCR mastermix was created based on the component mix as described above for CD11c DTR mice. The PCR program "IRF4KO" utilized for these mice is shown in the adjacent table.

Temperature	Time/cycle	#cycles
95°C	15m	
94°C	30s	
61°C	1m	30
72°C	1:30m	
72°C	10m	
4°C		

Given below are the primers and expected band sizes:

<u>WT PCR</u>

WT Primer GCA ATG GGA AAC TCC GAC AGT

WT Primer CAG CGT CCT CCT CAC GAT TGT

Expected band: ~ 150 bp

<u>Mutant PCR</u>

Mutant Primer CCG GTG CCC TGA ATG AAC TGC

Mutant Primer CAA TAT CAC GGG TAG CCA ACG

Expected band: ~ 500 bp

Samples were run on a 2% agarose gel as described above for ERKOα mice.

2.1.3 Ovariectomies:

Endogenous hormones were depleted by ovariectomies (OVX) according to previously published protocols (A. E. Gillgrass, Tang, et al., 2005). Briefly, mice were given analgesic (temgesic 0.03mg/mL) 30 min prior to surgery. They were then anaesthetized with an IP injection of 120-150µl of Ketamine (0.75ml) +

Xylazine (0.25mL), diluted in 0.9% saline (4ml). An incision was made dorsally on either side below the rib cage to find both the ovaries surrounded by adipose tissue. Clamps were then applied underneath the fallopian tubules, and a scalpel was used to excise ovaries. The peritoneal wall was then closed with dissolvable 4-0 vicryl-braided sutures. Finally, the outer skin was stapled with sterile surgical staples, and mice were allowed to recover for 2 weeks, to deplete circulating hormones, before starting experiments.

2.1.4 Hormone pellets:

To supplement OVX mice with hormones, mice were anaesthetised with an IP injection of anaesthetic (150mg Ketamine/kg body weight + 10mg/xl Xylazine as described above), and a shallow subcutaneous incision was made behind their neck. Hormone pellets purchased from Innovative Research of America (Sarosota, FL, USA): 17β Estradiol (cat# E-121 0.010mg/pellet) and progesterone (cat# P-131 10mg/pellet), were composed of a proprietary matrix containing cholesterol, cellulose, lactose, phosphates, stearates fused with the active product. Pellets were implanted in this pocket, and would release E2 (476 ng/mouse/day) or P4 (476 µg/mouse/day) for a guaranteed period of 21 days. The validity and immunogenic inertness of these pellets have been previously verified in the lab, and utilized in a peer-reviewed publication (Bhavanam et al., 2008).

2.1.5 Subcutaneous hormone injections:

Daily subcutaneous hormone injection protocols were used for the 7 day E2 standardization model (section 3.3.3). Briefly, 0.01g of 17-β Estradiol (Calbiochem cat# 3301) was resuspended in 10ml of 100% ethanol. A 100µl of this stock solution was evaporated in a small Erlenmeyer flask in a chemical lab hood for 1 hour. The resultant E2 preparation is water soluble, could be dissolved in 20ml of sterile 0.9% saline, and administered with a glass syringe subcutaneously at 100µl/mouse. To prepare progesterone (Calbiochem cat#5341), 0.15g of progesterone was homogenized (30-50times/each) continuously with 3 x 10ml of 0.9% sterile saline in a glass homogenizer to obtain a total 30ml of P4 solution. It is administered subcutaneously at 100µl/mouse using a glass syringe. Hormones were stored at 4°C, used for 7 days and discarded at the end of every experiment.

2.1.6 DC depletion in CD11c DTR mice:

CD11c DTR mice were anaesthetised IP as described above (150mg Ketamine/kg body weight + 10mg/xl Xylazine). Diptheria toxin treatment was standardized as described in section 3.1.4. The standardized protocol for vaginal DC depletion was to inject anaesthetized mice with 400ng diphtheria toxin (DT) (Sigma Aldrich cat# D0564) (200ng intraperitoneal injection (in a 100µl volume administered with 1ml BD insulin syringe) + 200ng intravaginal injection (in a 10µl volume administered with p20 pipette)). (DT treatments were conducted by a licensed DT user: Dr. Stephanie Swift, ex-lab personnel from Dr. Jonathan

Bramson's laboratory). Control mice received PBS alone. CD11c cells would remain depleted in treated mice for 18-24h as confirmed by flow cytometry in section 3.1.4.

2.1.7 Infections:

OVX mice were infected with (TK-) HSV-2, HSV-2 333 or HSV-2 OVA as indicated in the appropriate experiments. The infection protocol was adapted based on a previously published protocol (A. E. Gillgrass, Fernandez, et al., 2005; A. E. Gillgrass, Tang, et al., 2005). Briefly, injectable anaesthesia was prepared as described above in section 2.1.3. The stock viral aliquots were thawed from -70°C freezer on ice. Appropriate dilutions were conducted to obtain $1x10^4$ pfu/µl in PBS, and mice were infected with 10µl to obtain an infection at $1x10^5$ pfu/mouse. These infection protocols have been previously standardized and published in peer-reviewed journals (Bhavanam et al., 2008; K. L. Roth et al., 2013).

2.1.8 Vaginal washes:

To monitor viral shedding and replication in the vagina, vaginal washes were collected for 5 days post infection. These were conducted by using 2 x 30µl of cold PBS washes using a p100 pipette. Mice were held up by their tails with all feet resting on the hopper, and washes were conducted gently by pipetting PBS up and down 4-5 times before collection in an Eppendorf tube on ice and stored at -70°C before analysis.

2.1.9 Quantification of shed virus:

To quantify virus shed in the vagina, titrations were conducted with vaginal washes from intravaginally infected mice using previously published protocols (A. E. Gillgrass, Fernandez, et al., 2005; Kaushic et al., 2003). Briefly, 12-well plates were prepared from the required number of confluent vero flasks. Veros were trypsinized, and adequately distributed into 12-well plates according to the number of samples. The plates were ready 24h later, and subsequently, vaginal washes were thawed on ice. A total of 6 serial dilutions were conducted with each sample, and each was added to a well in these 12-well plates (according to the described protocol). Two days later, media was removed, and crystal violet with FAA fixative was used to visualize viral plaques among vero monolayers, and plaques were quantified under a light microscope.

2.1.10 Pathology & survival monitoring:

Post intravaginal infection, mice were monitored daily to monitor virus induced pathology in the genital area. Pathology was scored on a 1-5 scale as indicated in the adjacent

SCORE	PHENOTYPE
0	No apparent infection
1	Slight redness of external vagina
2	Redness and swelling of external vagina
3	Severe redness & swelling of vagina & surrounding tissue
4	Genital ulceration with severe redness & hair loss
5	Severe ulceration extending to surrounding tissues

table. Mice reached endpoint at stage 5. Pathology scores were plotted on an XY graph in Graphpad Prism 5.0. Survival graphs based on percent survival were represented using survival curves created in Graphpad Prism.

2.2 Tissue isolation and processing:

2.3.1 Vagina:

Vaginal tissues were isolated and processed using a standard protocol (K. L. Roth et al., 2013). Briefly, mice were euthanized by cervical dislocation, and an incision was made ventrally below the diaphragm to expose the reproductive tract. The vagina including the cervix, but excluding the uterine horns was removed. The urethra was detached by firmly pulling it away from the vaginal tract, and discarded. Vaginal tissues from each group of mice were pooled, cut in half, washed to remove mucous, and minced into pieces in a petri dish with 2ml of RPMI 1640 media. They were then transferred into a small beaker with enzymes (15ml of 0.00157g/mL collagenase A) (Roche Life Science, USA), and stirred with a stir bar on a magnetic plate at 37°C for 1h. The supernatant was removed and stored on ice, before repeating enzymatic digestion with another 15ml of fresh enzymes for one more hour. At the end of digestion, supernatant was removed, and remaining tissue was pressed through a 40µm filter, and added to the previously collected supernatants (BD Biosciences, USA). All collected liquid content was centrifuged for 10min at 4°C and 1200rpm. Supernatant was decanted, and cell pellets were resuspended in 1ml of RPMI media, and counted before further usage.

2.3.2 Lungs:

Lung tissues were extracted, digested and prepared by a former student Christopher R. Shaler (PhD) from Dr. Zhou Xing's lab. The protocol used was based on previously published articles (Horvath, Shaler, Jeyanathan, Zganiacz, & Xing, 2012). Briefly, lungs were perfused with HANKS buffer, and cut into small pieces that were digested in 150U/mL of collagenase I (Sigma Aldrich, Canada) for 1h at 37C. The digested cell suspension was filtered through a 100µm filter to exclude debris, and resultant pellets after centrifugation were resuspended in RPMI media for further use. Cells were characterized by flow cytometry and shown in section 3.1.1.

2.3.3 Small intestines:

Intestinal tissues were isolated, processed and provided by personnel from Dr. Manel Jordana's laboratory: Derek Chu (MD-PhD) and Rodrigo Jiménez-Saiz (PhD). The protocols utilized were outlined in previously published articles (Carlens et al., 2009; D. K. Chu et al., 2014). Briefly, small intestines were removed, and peyers patches were discarded before opening these tissues longitudinally and washing them in ice cold PBS to remove the fecal matter. Tissues were cut into small pieces and incubated in 1mM DTT in PBS (Sigma Aldrich, Canada) for 15min. They were then digested with three rounds of incubation (15min/round) in 10% FBS and 2mM EDTA, before digestion in 0.239 mg/mL Collagenase A (Roche Life Science, USA) with DNAse for 60min before percoll purification (GE Healthcare separation). Samples were finally filtered

through a 40µm filter to obtain a single cell suspension and characterized by flow cytometry to examine APC populations as shown in section 3.1.1.

2.3.4 Lymph nodes and spleen:

The lymph nodes and spleen were mechanically disrupted in 1ml of PBS inside a 6-well plate that has been scarred with a needle, using the rubber-end of a 3ml syringe. Contents were transferred to a 15ml falcon tube and centrifuged at 1500rpm for 5 min. Red blood cells were lysed in spleen preparations with 2ml/spleen ACK lysis buffer (Sigma Aldrich, Canada) for 2 min at room temperature, and centrifuged with 3 x ACK buffer volume in PBS. Final pellets were resuspended in 1ml PBS (lymph nodes) or 5ml PBS (per spleen) and counted before use in further experiments.

2.4 Co-cultures:

2.4.1 Lymphocyte culture media:

Culture media utilized in all our experiments outlined in this dissertation was based on a recipe provided by former student Jeanette Boudreau (PhD) from Dr. Yonghong Wan's lab. The recipe has been previously published in a peer-reviewed article (Boudreau, Koshy, Cummings, & Wan, 2008). The media components utilized are outlined in the adjacent table.

Component	**volume**
RPMI 1640 media	500mL
10% FBS	50mL
100 IU/mL penicillin	5mL of
100 ug/mL streptomycin	P/S
1% L-glutamate	5mL
1 x non-essential amino acids	5mL
1 x sodium pyruvate	5mL
0.1% 2-mercaptoethanol	0.5mL

2.4.2 OVA peptide stock:

Chicken ovalbumin peptide (323-339) (Biomer technology, Pleasanton, CA, USA) with sequence ISQAVHAAHAEINEAGR was purchased as a crude preparation in a lyophilized form. The powder was dissolved in sterile DMSO at 10mg/mL, and aliquoted into multiple aliquots of 50-500µl and stored at -70°C until use. Stocks were diluted to 1/10 in culture media, and cells were stimulated with OVA at 5µg/well. Diluted stocks could be re-used within the next 2-3 months.

2.4.3 CFSE stock:

5(6) – Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma Aldrich, Canada) was purchased as a 25mg lyophilized formulation. Based on previously published protocols (Parish, Glidden, Quah, & Warren, 2009), it was dissolved in 0.896mL of sterile DMSO to obtain a stock solution at a concentration of 50mM that could be stored as 25-100µl aliquots at -20C. Fresh aliquots were used for individual experiments.

2.4.5 Peptide pulse:

$5x10^4$ - $1x10^5$ cells were plated per well in a U-bottom 96-well plate in 100µl of culture media/well. For optimal results and statistical analysis, 4-6 replicates were conducted for each sample group. 5µl of the 1/10 diluted OVA peptide stock (as described above in 2.4.2) was added to each well, and plates were tapped gently to evenly disperse the peptide, before incubation at 37°C for 12-18h. At the end of this incubation step, 100µl of fresh culture media was added to each well, plates were centrifuged at 1200rpm for 10 min. Supernatants

containing excess peptide was decanted, and cells were resuspended with 200µl of fresh culture media per well.

2.4.6 MACS $CD4^+$ T cell isolation & CFSE staining:

Spleens from OT-II Tg mice were isolated using the aforementioned protocol (section 2.3.4), and processed into a cell suspension. $CD4^+$ T cells were magnetically sorted using CD L3TE microbeads (Miltenyi Biotec, Auburn, CA, USA), according to manufacturer protocols. Briefly, splenocytes in ice-cold MACS buffer (filter-sterilized pH 7, 0.5% BSA and 2mM EDTA in PBS) were counted to identify the appropriate volume of beads required per sample. Cells were labelled with microbeads by incubating both splenocytes and beads at 4°C for exactly 15min. Cells were washed twice with PBS to remove unbound beads, before resuspension in MACS buffer. The sample was then eluted through a MS or LS column (Miltenyi Biotec, USA) that was mounted on a miniMACS or octoMACS magnet (Miltenyi Biotec, USA). The viability of purified $CD4^+$ T cells was confirmed by trypan blue staining, and live cells were counted using a hemocytometer.

2.4.7 CFSE staining:

$CD4^+$ T cells sorted by the above protocol were stained with CFSE (Sigma Aldrich, St. Louis, MO, USA) according to previously published protocols (Parish et al., 2009). Briefly, $CD4^+$ T cells were resuspended in ice-cold PBS at $10x10^6$ cells/mL, and stained with 50µM CFSE (diluted from CFSE stock 5mM aliquots in PBS), at room temperature for 5 minutes. Stained cells were washed thrice with

10 times the volume of ice-cold PBS (compared to the quantity of CFSE), and pellets were re-suspended at $10x10^6$ cells/mL in complete RPMI media.

2.4.7 Co-cultures:

Total tissue cells, or sorted APCs, pulsed with peptide were co-cultured with CFSE-stained splenic OT-II Tg $CD4^+$ T cells in a 1:1 ratio, or the ratio induced in the figure legends, for 4 days at 37°C. In some experiments, 40ng/mL rIL-6, 250pg/mL rIL-17 or 100ng/mL rIL-1β (R&D systems, MN, USA) was added on the first day of co-culture. Co-culture supernatants were frozen for cytokine analysis, and $CD4^+$ T cell proliferation, and intracellular cytokine expression was examined using flow cytometry on a BD LSRII flow cytometer (BD Biosciences, Canada).

2.4.8 Cytokine analysis:

Co-culture supernatants were assayed using Quantakine or DuoSet ELISA kits to measure IL-17, IL-23, IL-22 and TGF-β (R&D Systems, MN, USA), as per the manufacturer's protocols. In some experiments, a custom MSD multiplex kit (Meso Scale Discovery, Rockville, MD, USA) was utilized to measure TNF-α, IL-12, IFN-γ, IL-6, IL-17, IL-2, IL-4 and IL-10, as per manufacturer's protocols. MSD plates were analyzed on a Sector Imager 2400 (Meso Scale Discovery, Rockville, MD, USA), and ELISA plates were measured using a plate reader available in the McMaster Immunology Research Centre (MIRC) facility.

2.5 Flow cytometry:

2.5.1 Surface staining:

The protocol used for flow staining is based on the one outlined in Laboratory protocol directory under “Staining for flow cytometry”. Briefly, mononuclear tissue preparations were resuspended in 100-200µl of FACS buffer (0.2% BSA in PBS) at 1-3 x 10^6 cells/tube. 2µl of FC block (BD Biosciences, Canada) was added to block non-specific Fc receptor staining for 5-10min before proceeding with staining protocol. A cocktail of antibodies [CD11c PE-Cy7, Gr-1 AF700, F4/80 APC, CD3 AF700 (eBioscience, San Diego, CA, USA), CD11b PE-CF594, I-A^b FITC (BD Biosciences, Canada) CD3 BV785, CD4 BV421 (BioLegend, San Diego, CA, USA) at concentrations based on manufacturer specification sheets, was standardized and prepared in FACS buffer, and cells were stained with 50µl of cocktail/tube for 30 min on ice. Cells are washed twice by centrifugation at 1500rpm for 5 min in FACS buffer, and pellets were resuspended in 350µl of FACS buffer for analysis on a BD LSR II flow cytometer.

2.5.2 Intracellular staining:

For intracellular staining, tissue cells or co-culture cells on day 2 of culture were treated with 2µl/mL Cell Stimulation Cocktail (This is a cocktail of phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin), (eBioscience, San Diego, CA, USA) for 12-16 h, permeabilized and fixed with BD Pharmingen™ Transcription Factor Buffer Set (BD Biosciences, Canada) using manufacturer protocols. Intracellular staining was used to identify expression of

cytokines and transcription factors (IL-17 APC, IL-6 PE, BD Biosciences; ROR-γ PE, BD Biosciences; IRF4 PE, eBioscience). The validity of intracellular staining was verified by fluorescence minus one (FMO) controls, and/or appropriate isotype controls. Data was acquired on a BD LSRII flow cytometer (BD Biosciences, Canada), and analyzed with FlowJo software (Treestar, Ashland, OR, USA).

2.5.3 FACS sorting:

Flow sorting to purify $CD11c^+$ APCs was conducted using a BD FACSAria™ III flow sorter by Hong Liang. Vaginal or lung cells were isolated from 12-15 mice, pooled to obtain 3-5 samples containing 5-10 x 10^6 cells/tube in MACS buffer (as described in section 2.4.6) and stored on ice until use. Cells were analyzed and initially gated on forward and side scatter parameters to select total cells excluding debris or aggregates. Singlet events were selected based on forward scatter area, height and width parameters. $CD11c^+$ cells, or other APC populations, were gated, and samples were sorted in culture media. Sorting required 8-12h for completion, and the viability of sorted populations was verified by trypan blue staining before experimental use. Samples were centrifuged at 1000rpm for 10 min, and cells were resuspended in culture media for use in co-cultures.

2.5.4 Gating strategy:

Flow data was analyzed in Flowjo (Treestar, Ashland, USA) software for windows or mac. The target lymphocyte population was identified by FSC-A and

SSC-A plot. This was further gated to exclude doublets and aggregates using a FSC-A and FSC-H plot, and further refined to exclude dead cells (in indicated experiments) using DAPI (Sigma Aldrich, USA) or AF-ef780 viability dye (eBioscience, USA), according to manufacturer protocols. $CD3^+$ $CD4^+$ T cells were selected, and IL-17^+ cells were gated to examine T_h17 populations. DCs and macrophages were gated based on their expression of CD11c, CD11b, F4/80, Gr-1 and MHCII as described within the results section (See section 3.1.5).

2.6 Statistical analysis:

Data was analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA), and represented as mean ± standard deviation over an XY or grouped analysis plot. Significance was calculated by comparing the means by one-way or two-way analysis of variance (ANOVA), or t-tests as indicated in individual figure legends. p values less than 0.05 were considered significant. The range of p values observed in our analysis has been indicated in each figure legend.

CHAPTER 3.1

Vaginal CD11c^{+} dendritic cells are potent inducers of T_h17 responses through an IL-1-dependent mechanism

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Dr. Charu Kaushic, and I were responsible for the design and interpretation of all the experiments in this work. I performed all experiments, and generated and analyzed the data. Christopher R. Shaler provided lung isolates. Derek and Rodrigo provided small intestinal isolates. Hong conducted flow sorting. Sara, Jessica and Aisha helped with ELISAs. Stephanie administered DT injections to DTR mice. Dr. Bramson, Dr. Xing, Dr. Jordana and Dr. Stampfli were our primary collaborators in this work. Dr. Snider and Dr. Wan helped with data analysis and provided valuable feedback to this work. I wrote this chapter with feedback and suggestions from Dr. Kaushic.

3.1.0 OBJECTIVE

To address the overall hypothesis outlined in section 1.7, my first objective was to examine the mechanism by which E2 can influence the function of vaginal APCs. Sex hormone receptors are expressed to varying degrees in different tissues and on a wide variety of cell types including APCs. Previous studies have examined hormone effects on APC phenotype and function, using cells generated from bone marrow precursors, or cells isolated from draining lymph nodes and the spleen (Butts et al., 2007; Carreras et al., 2010; Liang et al., 2006; Mao et al., 2005; Paharkova-Vatchkova et al., 2004). In the absence of the tissue microenvironment, it is unknown whether these responses are representative of hormonal effects on APC populations *in vivo* in the vagina. Additionally, APCs such as DCs and macrophages play a central role in shaping immune responses at mucosal sites (Rescigno & Di Sabatino, 2009). Given that the different mucosal tissues may face unique antigens, it is likely that mucosal APCs residing in these tissues prime distinct immune responses to address their respective antigenic challenges. Hence, for this part of the study, we hypothesized that: ***"E2 can directly influence the function of vaginal APCs in priming $CD4^+$ T cell responses. Furthermore, vaginal APCs will have a unique phenotype and function compared to APCs from other mucosal tissues."*** This hypothesis was addressed through the following sub-objectives:

1) Characterize the phenotype of APCs from mucosal tissues (vagina, lung and small intestine) and associated lymphoid organs (Spleen, iliac, medestinal and mesenteric lymph nodes).
2) Examine functional differences in APC populations from these different tissues.
3) Examine the effect of E2, and the mechanism by which E2 can drive vaginal APC responses

3.1.1 The vaginal tract contains a resident APC population enriched in myeloid $CD11c^+$ $CD11b^+$ $MHCII^-$ DCs under homeostatic conditions

In order to compare mucosal APC populations under homeostatic conditions, the lungs, small intestine, and vagina were collected from normal, C57Bl/6 mice. A panel of antibodies against cell-surface markers (CD11c, CD11b, MHCII, F4/80 and Gr-1) that were commonly used in other studies (Iijima et al., 2007; Iwasaki, 2007; Zhao et al., 2003) was used to identify subsets of APCs in these tissues. CD11c was used as a broad surface marker to define DCs, while macrophages were defined as $CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$ cells. We found three subsets of $CD11c^+$ DCs in the lung, small intestine and vagina: $CD11b^+$ $MHCII^+$ cells, $CD11b^+$ $MHCII^-$ cells and $CD11b^-$ $MHCII^-$ cells (Fig. 1). $CD11c^+$ $CD11b^+$ $MHCII^-$ DCs were the dominant DC subset in the vagina (53%) compared to the lung (4%) or small intestine (16%). In contrast to the vagina, $CD11c^+$ $CD11b^-$ $MHCII^-$ DCs were the dominant DC subset in the lung (78%), while the small intestine contained $CD11c^+$ $CD11b^+$ $MHCII^+$ and $CD11c^+$ $CD11b^-$ $MHCII^-$ cells in similar proportions (~30%), and a smaller population of (16%) of $CD11c^+$ $CD11b^+$ $MHCII^-$ cells. The lung, small intestine and vagina also contained $CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$ macrophages, but there were no significant differences among the vagina, lungs or small intestine, when normalized to total tissue yield (Table 1). A significant population of $CD11c^-$ $CD11b^+$ $F4/80^-$ $Gr\text{-}1^+$ cells, consistent with a neutrophil phenotype, was also seen in vaginal tract (Fig. 1). In comparison to the mucosal tissues, there were no

dramatic differences among DC subsets in the medestinal, mesenteric and iliac lymph nodes, the local draining lymph nodes for the lung, small intestine and vagina, respectively (Fig. 2, Table 1). Overall, these results indicate that mucosal tissues contain distinct APC populations, whereas their draining lymph nodes contain APC populations that are similar to each other, under homeostatic conditions.

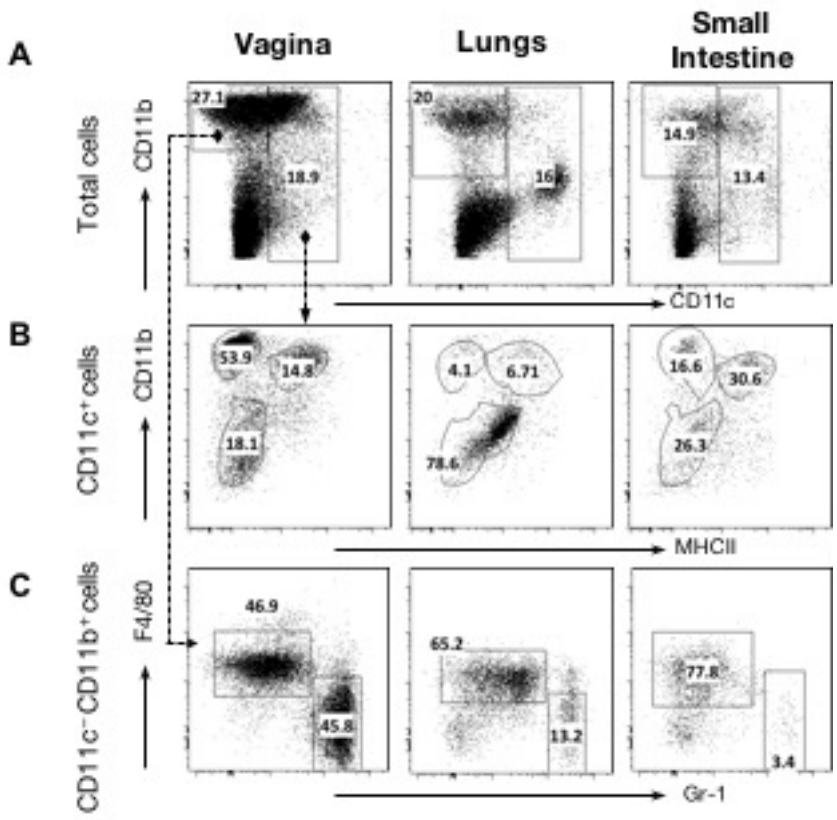


Figure 1: Antigen presenting cell populations in vagina, lungs and small intestine. The vagina, lungs and small intestine from C57BL/6 mice were processed and stained with a variety of APC cell surface markers. Dead cells were excluded using DAPI, and A) live cells were differentially gated based on their CD11c expression. B) Dendritic cells ($CD11c^+$ cells) were classified based on their MHCII and CD11b expression, C) while $CD11b^+$ $CD11c^-$ cells were further separated into macrophages ($CD11b^+$ $CD11c^-$ $F4/80^+$ $Gr\text{-}1^-$ cells) and neutrophils ($CD11b^+$ $CD11c^-$ $F4/80^-$ $Gr\text{-}1^+$ cells). Results are a representative from 3 separate experiments with similar trends.

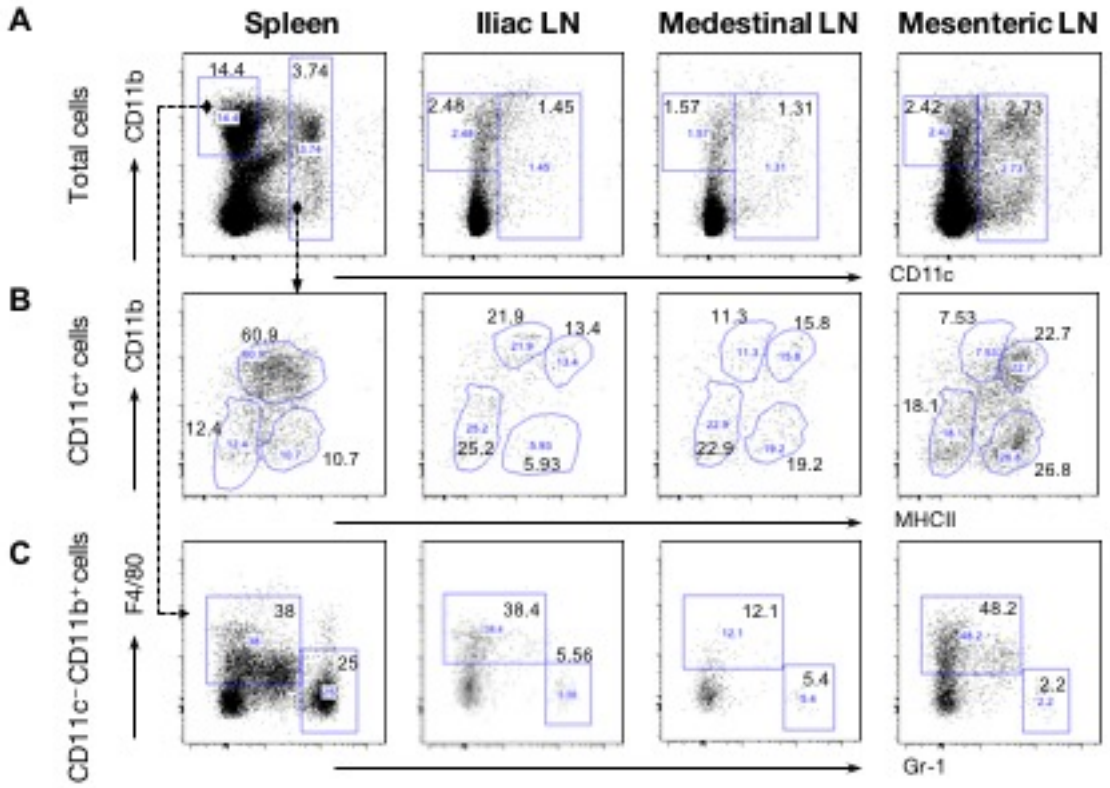


Figure 2: Antigen presenting cell populations in spleen and mucosal draining LN. The spleen, Iliac, medestinal, and mesenteric LN from C57BL/6 mice were processed and stained with a variety of APC cell surface markers. Dead cells were excluded using DAPI, and A) live cells were differentially gated based on their CD11c expression. B) Dendritic cells ($CD11c^+$ cells) were classified based on their MHCII and CD11b expression, C) while $CD11b^+$ $CD11c^-$ cells were further separated into macrophages ($CD11b^+$ $CD11c^-$ $F4/80^+$ $Gr\text{-}1^-$ cells) and neutrophils ($CD11b^+$ $CD11c^-$ $F4/80^-$ $Gr\text{-}1^+$ cells). Results are a representative from 3 separate experiments with similar trends.

Table 1: Phenotype of APC populations within mucosal tissues and their draining lymph nodes

Tissue type	Dendritic cells ($CD11c^+$ cells)			Macrophages ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$)	Neutrophils ($CD11c^-$ $CD11b^+$ $F4/80^-$ $Gr\text{-}1^+$)
	$CD11b^+$ $MHCII^-$	$CD11b^+$ $MHCII^+$	$CD11b^-$ $MHCII^-$		
Vaginal tract	10.29	2.5	3.9	12.9	12.9
Lungs	0.64	0.84	14.4	13	2.6
Small intestine	2.2	3.9	3.38	10.8	0.42
Iliac LN	0.22	0.17	0.32	0.71	0.08
Medestinal LN	0.39	0.26	0.15	0.22	0.08
Mesenteric LN	-	0.677	0.51	0.71	0.04
Spleen	-	2.07	0.38	5.32	3.5

Data is presented as % of total tissue single cells.
LN: Lymph nodes; iliac LN correspond to vagina, medestinal LN correspond to lung and mesenteric LN correspond to small intestine. Data is a representative from one of 3 separate experiments with similar trends.

3.1.2: Optimization of a chicken ovalbumin peptide based co-culture model to examine differences in mucosal APC functions

APCs such as DCs uptake antigens and present them to $CD4^+$ T cells to initiate adaptive immune responses. We wanted to standardize an *in vitro* model to examine and compare antigen presentation by APCs from various mucosal tissues. We decided to utilize a previously well-described chicken ovalbumin (OVA) peptide based model (Robertson et al., 2000). According to this model, APCs can be pulsed or incubated with OVA peptide, and co-cultured with CFSE-stained OVA specific $CD4^+$ T cells from OT-II transgenic mice (OT-II Tg $CD4^+$ T cells) for 3-4 days. Intracellular staining, and cytokines analysis within co-culture supernatants, could be used for examining $CD4^+$ T cell responses.

The focus of our study was to examine vaginal APC responses; hence, we first standardized the co-culture conditions using vaginal tissue cells. OT-II Tg $CD4^+$ T cells from the spleen were purified using CD4 L3TE microbeads (Miltenyi Biotec, USA), according to manufacturer protocols, and verified by flow cytometry (Fig. 3A). This kit allowed us to consistently obtain OT-II Tg $CD4^+$ T cells at purities above 94% in all our experiments (Fig. 3A). We then optimized the co-culture conditions to show that vaginal tissue cells (TC) co-cultured with purified OT-II Tg $CD4^+$ T cells, did not stimulate $CD4^+$ T cell responses in the absence of OVA peptide stimulation (Fig. 3B: TC+CD4 NO PEPTIDE). Furthermore, CFSE stained OT-II Tg $CD4^+$ T cells cultured alone in the presence of OVA peptide ($CD4_{cfse}$ + OVA) were also incapable of proliferating/responding (Fig. 3B),

suggesting that there was no contamination of splenic APCs in the purified OT-II Tg $CD4^+$ T cell fractions (Fig. 3B: $CD4_{cfse}$ + peptide).

The next step in the optimization process was to determine the ratio of vaginal TC:CD4 cells per well for optimal $CD4^+$ T cell responses in co-cultures. Vaginal tissue cells from ovariectomized (OVX) mice were pulsed or incubated with OVA peptide (protocols described below), and co-cultured with CFSE-stained OT-II Tg $CD4^+$ T cells at the indicated ratios. $CD4^+$ T cell proliferation was only observed at a ratio of 1:1 (Fig. 3C). This indicated that 1:1 was the minimum ratio of TC:CD4 required for optimal $CD4^+$ T cell responses in our conditions.

Lastly, we also standardized the peptide stimulation aspect of these co-cultures. We wanted to examine whether peptide pulse, where TC are transiently exposed to OVA peptide before co-culture with T cells, or peptide incubation along with TC and $CD4^+$ T cells throughout the co-culture, was the optimal protocol for inducing T cell responses. To accomplish this, vaginal TC co-cultures were conducted utilizing both methods, and $CD4^+$ T cell proliferation was compared after 3.5 days of co-culture. For peptide pulse, vaginal TC where incubated with OVA peptide at 4µg/mL or 8µg/mL for 12-24h, before conducting a media wash (to remove excess peptide), and co-cultured with OT-II Tg $CD4^+$ T cells. For peptide incubation, vaginal TC were co-cultured with OT-II Tg CD4+ T cells, and OVA peptide at 4µg/mL or 8µg/mL, was added at the beginning of co-culture. Our results indicated that the peptide pulse was able to induce $CD4^+$ T cell proliferation at comparable levels to those observed with peptide incubation

throughout the co-culture (Fig. 3D). Furthermore, there were no differences in $CD4^+$ T cell proliferation with 4µg/mL or 8µg/mL OVA peptide (Fig. 3D). Therefore, the peptide pulse protocol with 5µg/mL OVA peptide was used as the standard stimulation protocol throughout all subsequent experiments.

Overall, with the experiments described above, we were able to optimize the conditions for the vaginal APC-T cell co-cultures, which were used thereafter throughout this study to examine functional differences in mucosal APC populations.

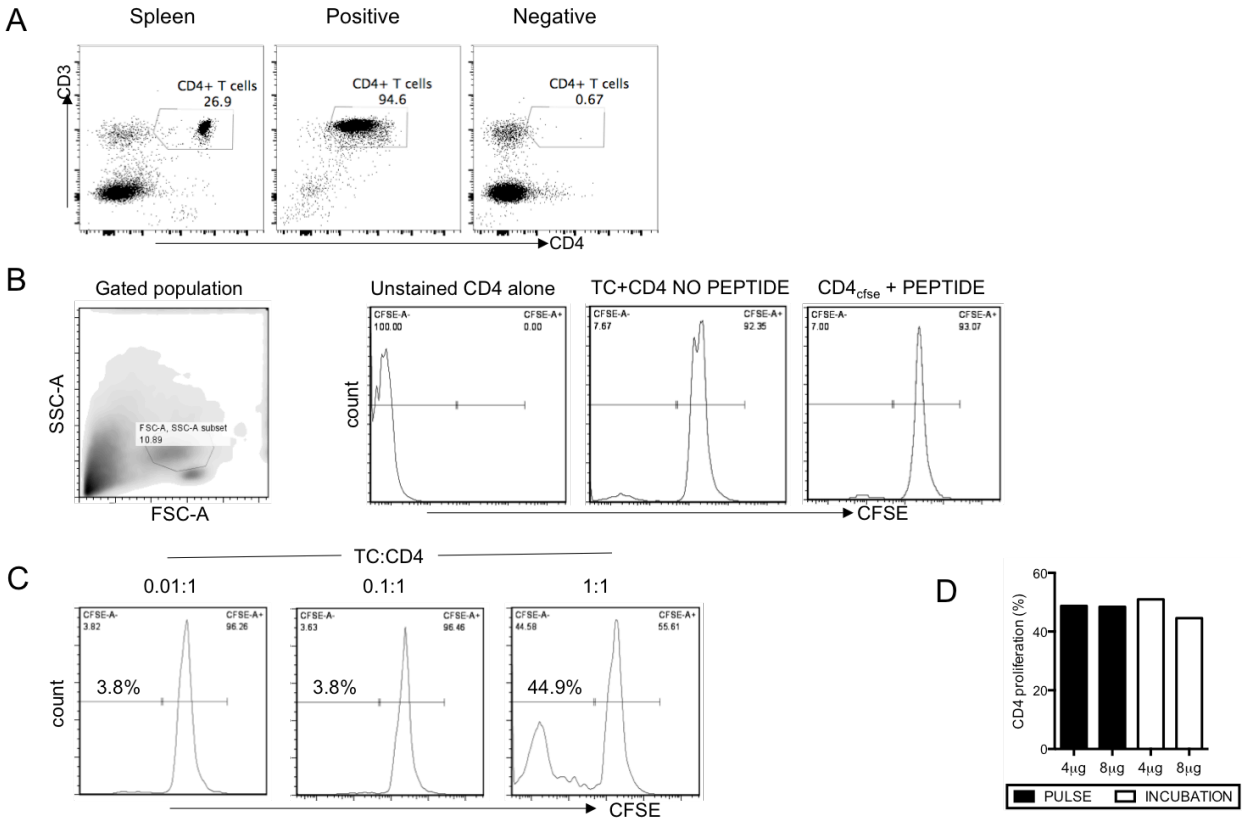


Figure 3: Standardization of an OVA peptide based co-culture model.

A) Spleen from OT-II Tg mice were excised and $CD4^+$ T cells were purified by MACS. Purity of $CD4^+$ T cells was verified by examining proportion of $CD4^+$ T cells in positive and negative fractions by flow cytometry. B) To standardize co-culture conditions, purified OT-II $CD4^+$ T cells stained with CFSE were incubated with OVA peptide for 3.5 days; and vaginal tissue cells (TC) were co-cultured in the absence of peptide with unstained CD4+ T cells (TC+CD4 NO PEPTIDE); CFSE-stained CD4+ T cells were cultured alone with 8µg/mL OVA peptide ($CD4_{cfse}$ + PEPTIDE), for 3.5 days. C) Vaginal TC from OVX mice were incubated or pulsed with 4µg/mL or 8µg/mL OVA peptide for 12-24h, and co-cultured with CFSE-stained OT-II Tg $CD4^+$ T cells at various ratios (TC:CD4 = 0.01:1; 0.1:1; 1:1), and proliferation of $CD4^+$ T cells was examined by flow cytometry. D) $CD4^+$ T cell proliferation levels were compared to examine the effect of OVA peptide doses (4µg/mL or 8µg/mL) and peptide pulse/incubation protocols.

3.1.3 Vaginal tissue cells are potent inducers of T_h17 responses

Having standardized APC-T cell co-cultures with vaginal cells (described in section 3.1.2), in order to examine functional differences among APCs from different mucosal tissues at inducing T-helper responses, tissue cells (TC) from each mucosa were isolated, pulsed with 5μg/mL OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells (TC + CD4) at a 1:1 ratio of TC:CD4 for 3.5 days. The tissue preparations contained mononuclear cells including all APCs such as DCs, and macrophages, tissue fibroblasts and other structural cells, present under homeostatic conditions in each mucosa after enzymatic digestion. A similar profile of $CD4^+$ T cell proliferation was observed in co-cultures of TC + CD4 from all three mucosal tissues and their draining lymph nodes (Fig. 5A). Levels of IL-4, IL-10, IL-12 and IFN-γ levels were comparable in all mucosal TC + CD4 co-culture supernatants (Fig. 4A). However, intestinal and lung co-cultures contained 2-fold and 7-fold higher levels of IL-2 compared to vaginal co-cultures, respectively. Furthermore, lung co-cultures contained close to 2-fold higher TNF-α levels compared to vaginal co-cultures, but there was no difference in TNF-α among vaginal and intestinal co-cultures.

Of note, vaginal TC+CD4 co-cultures contained over 15-fold and 4-fold higher levels of IL-17 and IL-22, respectively, compared to lung or intestinal tissue co-cultures (Fig. 4B). Intracellular staining on day 2 (D2) of co-culture showed that 70% of proliferating CFSE-stained OT-II Tg $CD4^+$ T cells expressed ROR-γt, the master-regulator transcription factor for T_h17 cells (Muranski &

Restifo, 2013), and IL-17, indicating that vaginal TCs were potent inducers of T_h17 responses (Fig. 4D). Vaginal tissue cells (TC) cultured alone without $CD4^+$ T cells also produced approximately 271 ± 110 pg/mL of IL-17 constitutively, an observation not seen in lung or intestinal tissue cells cultured alone (Fig. 4B). Intracellular staining showed that this IL-17 was constitutively produced in the vagina by three $CD11c^-$ $CD11b^-$ cell populations with size and granularity consistent with innate lymphocytes: $CD3^+$ $\gamma\delta^+$, $CD3^-$ $\gamma\delta^+$ and $CD3^-$ $\gamma\delta^-$ cells (Fig. 4C). Interestingly, there were no differences in IFN-γ, IL-12, TNF-α, IL-2, IL-4, IL-17, IL-6, IL-23, TGF-β or IL-10 levels among TC+CD4 co-cultures from lymph nodes draining these three mucosa (Fig. 5B & C). The lymph node isolates include lymphocytes such as B cells, T cells and APCs such as follicular DCs and lymph node macrophages. Overall, these results indicate that when these three mucosal tissues were compared within the context of their resident microenvironments, vaginal tissue cells were more potent inducers of IL-17 responses compared to lung and intestinal cells.

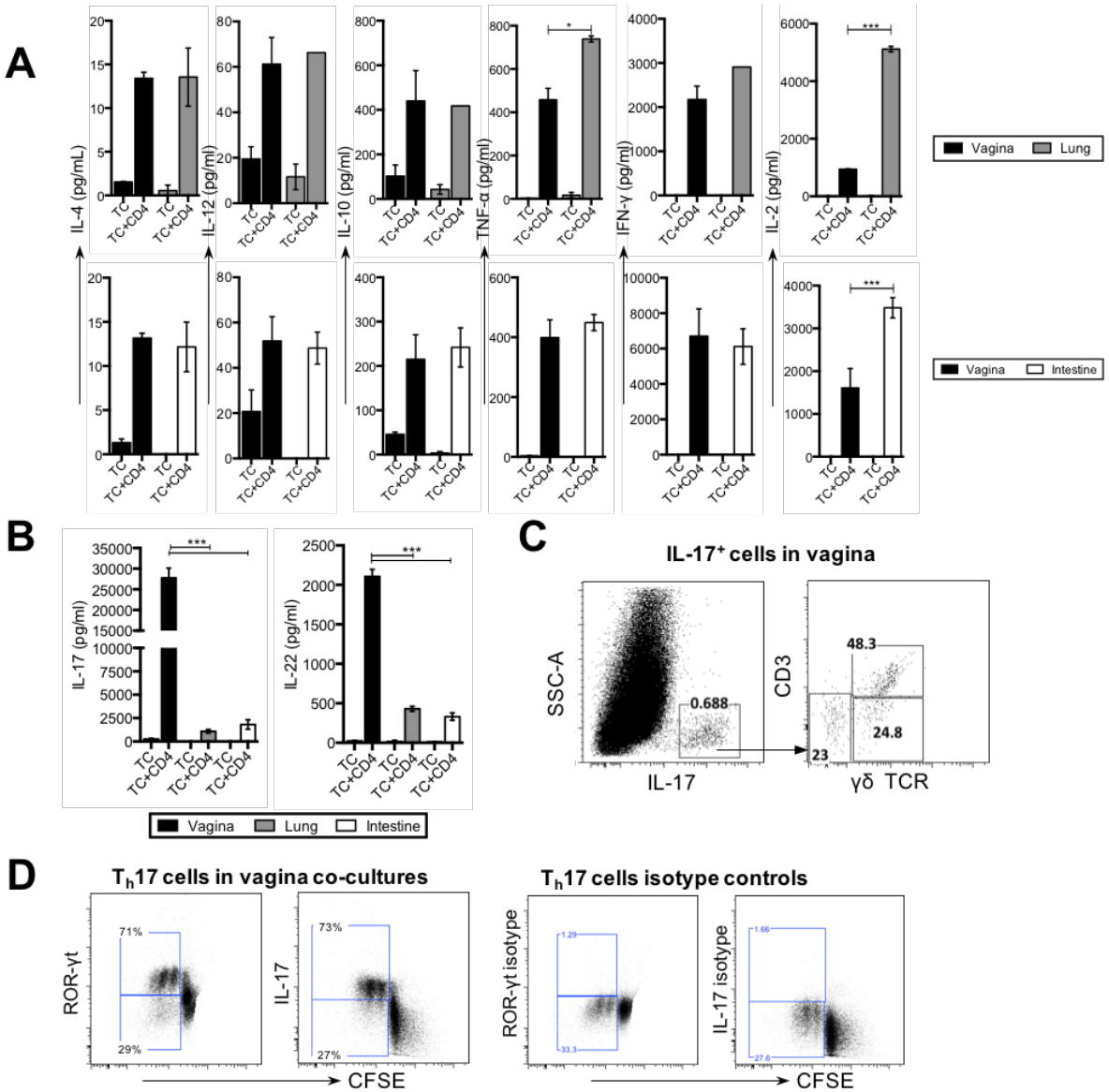

A
IL-4 (pg/mL)
IL-12 (pg/ml)
IL-10 (pg/ml)
TNF-α (pg/ml)
IFN-γ (pg/ml)
IL-2 (pg/ml)
TC
TC+CD4
Vagina
Lung
Intestine
B
IL-17 (pg/ml)
IL-22 (pg/ml)
***
C
IL-17+ cells in vagina
SSC-A
IL-17
CD3
γδ TCR
0.688
48.3
24.8
23
D
Th17 cells in vagina co-cultures
Th17 cells isotype controls
ROR-γt
IL-17
ROR-γt isotype
IL-17 isotype
CFSE
71%
29%
73%
27%

Figure 4: Vaginal tissue cells are more potent inducers of T_h17 responses compared to intestine or lung tissue cells. Tissue cells (TC) from the vagina, small intestines and lungs were isolated, and $1x10^5$ cells/well were pulsed with OVA peptide for 12-24h, and co-cultured with CFSE-stained OT-II Tg $CD4^+$ T cells (TC+CD4) at a 1:1 ratio, as described in 3.1.2. Four days post co-culture, A) T_h1 and T_h2 cytokines in the supernatants were compared among tissue cell co-cultures using MSD multiplex assays. B) T_h17 cytokines IL-17 and IL-22 were examined in vaginal, lung and intestine culture supernatants using ELISA. On D2 of co-culture, cells were intracellular stained to examine C) IL-17 production in vagina TC alone, and D) IL-17 and ROR-γ expression in vaginal TC+CD4 co-cultures. Cytokine data is mean±SD of 3 separate co-culture wells from one of 3 separate experiments with similar trends, and significance was calculated by two-way ANOVA. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

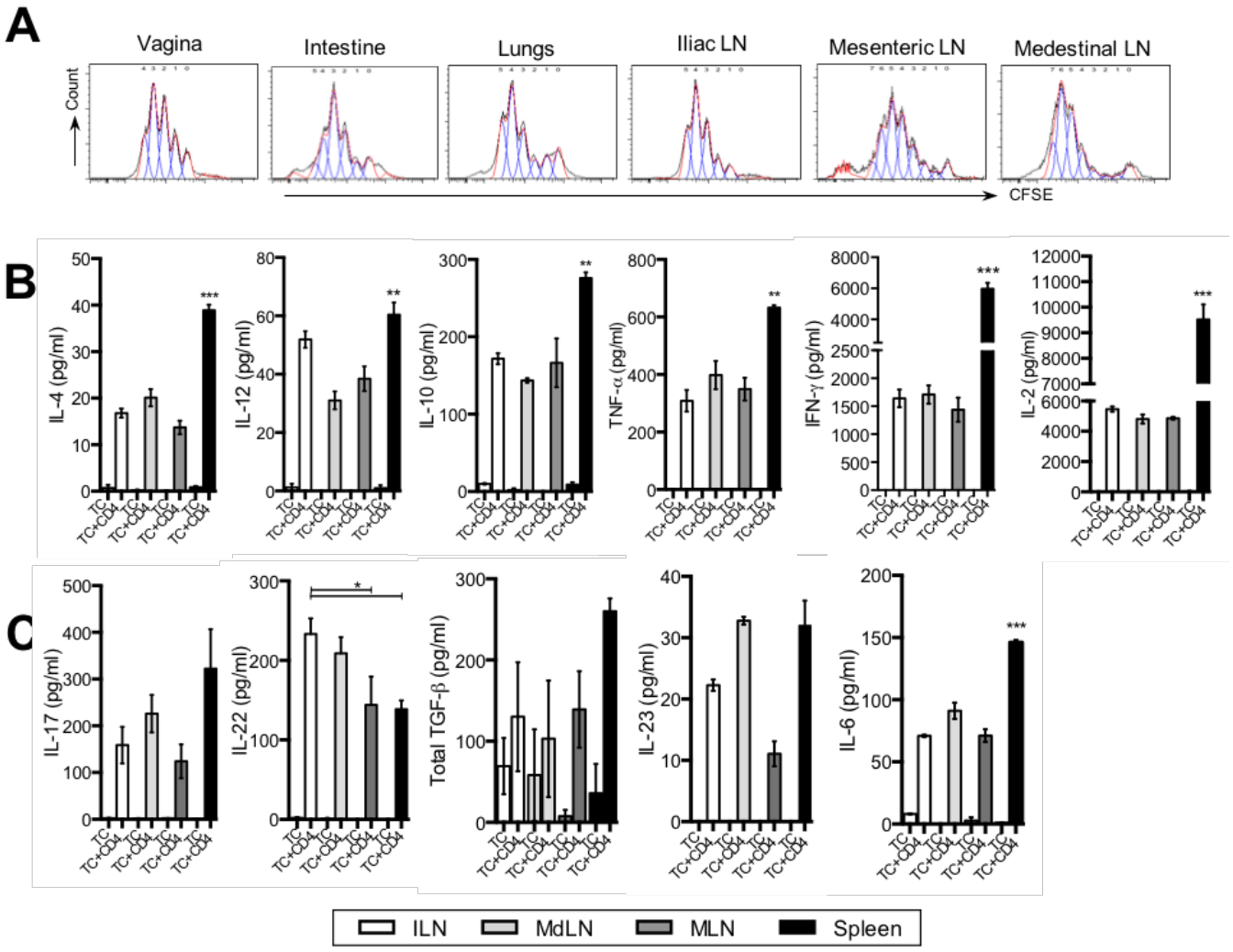

A
Vagina
Intestine
Lungs
Iliac LN
Mesenteric LN
Medestinal LN
Count
CFSE
B
IL-4 (pg/ml)
IL-12 (pg/ml)
IL-10 (pg/ml)
TNF-α (pg/ml)
IFN-γ (pg/ml)
IL-2 (pg/ml)
C
IL-17 (pg/ml)
IL-22 (pg/ml)
Total TGF-β (pg/ml)
IL-23 (pg/ml)
IL-6 (pg/ml)
TC
TC+CD4
ILN
MdLN
MLN
Spleen

Figure 5: Co-cultures conducted with tissue cells from lymph nodes draining mucosal tissues induced similar T_h1, T_h2 and T_h17 responses. Tissue cells (TC) from the vagina, small intestines, lungs and their draining lymph nodes (ILN: iliac lymph nodes that drain the vagina, MdLN: Medestinal lymph nodes draining the lungs, & MLN: Mesenteric lymph nodes that drain the small intestine) were isolated, pulsed with OVA peptide, and co-cultured using protocols standardized in 3.1.2. A) Three and a half days later, $CD4^+$ T cell proliferation was examined by flow cytometry. B) T_h1, T_h2 and C) T_h17 associated cytokines in LN co-culture supernatants was examined by MSD multiplex assays or ELISA. Data is mean±SD of 3 separate co-culture wells from a representative of one of 4 separate experiments with similar trends, and significance was calculated by two-way ANOVA comparing cytokine levels between each lymph node and spleen co-culture. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.1.4: Optimization of a CD11c depletion model to confirm the role of $CD11c^+$ APCs in vaginal T_h17 responses

Antigen presenting cells such as $CD11c^+$ DCs play an essential role in the development of anti-viral responses against HSV-2. To examine the role of $CD11c^+$ DCs in priming $CD4^+$ T cell responses, a transgenic CD11c DTR (Diptheria toxin receptor) mouse model has been previously established and commonly used in most studies (Bar-On & Jung, 2010; Jung et al., 2002). .Diptheria toxin (DT) mediated cytotoxicity is strictly dependent on receptor mediated endocytosis using the DTx receptor, a heparin-binding EGF-like growth factor (hbEGF) (Bar-On & Jung, 2010). Endogenous DTx receptor in mice is highly resistant to toxin. However, expression of primate/human DTx hBEGF within these cells can render them susceptible to toxin cytotoxicity (Jung et al., 2002). Since, CD11c DTR mice express human DTx only under the CD11c promoter, this renders $CD11c^+$ DCs selectively susceptible to toxin, allowing for their conditional depletion *in vivo*.

To standardize the depletion of $CD11c^+$ DCs in the vagina for our studies, CD11c DTR mice were injected with 100ng DT intraperitoneally (IP) or 400 ng DT (200ng IP + 200ng intravaginally (IVAG)), while controls received PBS alone. CD11c DC depletion was verified within the vagina and spleen by flow cytometry (Fig. 6A). As an alternative approach, CD11c DTR vagina tissue cells were cultured in the presence of 100ng DT *in vitro* (Fig. 6A). While IP injection of 100ng of DT was sufficient for the depletion of over 90% of spleen $CD11c^+$ cells,

depletion in the vagina was very low, possibly due to low bioavailability of DT within the vagina (49%). This issue was effectively addressed when mice were administered 200ng DT IVAG + 200ng DT IP (Fig. 6A). With this method, over 78% of vaginal DCs could be depleted (PBS controls: 2.54%; DT treated: 0.58%) (Fig. 6A). On the other hand, depletion *in vitro* was not an effective solution, as there were no differences in $CD11c^+$ cell proportions 18h post DT treatment *in vitro* (Fig. 6A). Overall, these observations enabled us to choose 400ng DT treatment protocol (200ng IP + 200ng IVAG) an effective regimen to deplete DCs in the vagina.

In order to examine the role of DCs in $CD4^+$ T cell proliferation within vagina tissue cell co-cultures, CD11c DTR mice were either treated with 400ng DT or PBS as described above, and 18h later, vaginal tissue cells (TC) were isolated, pulsed with OVA peptide and co-cultured with CFSE-stained OT-II Tg $CD4^+$ T cells at various ratios of TC:CD4 T cells as indicated in the graph in Fig. 6C. A representative of co-culture data at a 1:1 ratio of TC:CD4 shown in Fig. 6B. Depletion of $CD11c^+$ DCs led to a significant reduction in $CD4^+$ T cell proliferation at all ratios of TC:CD4 (Fig. 6B & C). Furthermore, unlike PBS control co-cultures, increasing the ratio of TC:CD4 in DT treatment group did not significantly affect $CD4^+$ T cell proliferation (Fig. 6C). Altogether, these results indicate that $CD11c^+$ DCs play a critical role in inducing $CD4^+$ T cell responses in vaginal tissue cell-CD4 T cell co-cultures.

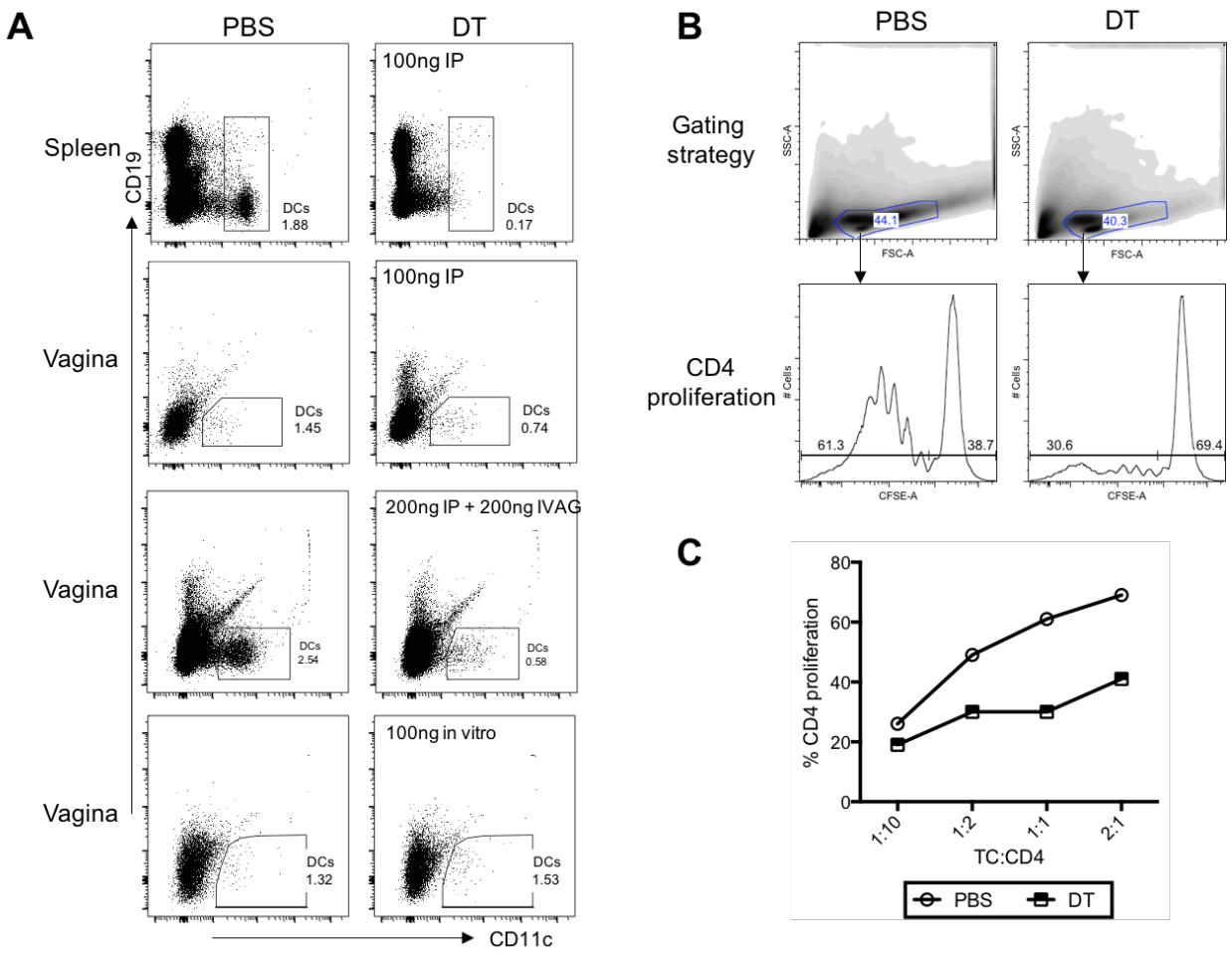


Fig 6: Optimization of CD11c+ DC depletion in the CD11c DTR model. CD11c DTR mice were treated with DT (100ng IP, 400ng (200ng IP + 200ng IVAG), or PBS as a control. For in vitro treatment, vaginal tissue cells were extracted from CD11c DTR mice and cultured for 18h in the presence of 100ng DT or PBS as a control. A) Depletion of $CD11c^+$ DCs in the vagina or spleen was verified by flow cytometry 18h post treatment. To examine the effect of $CD11c^+$ DCs in vagina co-cultures, vagina tissue cells (TC) from 400ng DT treated animals and PBS controls were pulsed with OVA peptide, and co-cultured at various ratios of TC:CD4 (1:10, 1:2, 1:1 and 2:1) with CFSE-stained OT-II Tg $CD4^+$ T cells. B) A representative of proliferation data at TC:CD4 of 1:1 is shown. C) Graph summarizing proliferation levels at various ratios of TC:CD4 in co-cultures with vaginal TC from DT and PBS treated animals.

3.1.5 Depletion of $CD11c^+$ cells significantly reduces T_h17 responses primed by vaginal cells

We had previously shown in section 3.1.3 that vaginal tissue cells were potent inducers of T_h17 responses. Following these observations, we wanted to identify the APC populations in the vagina in priming these $CD4^+$ T cell responses. To examine whether vaginal $CD11c^+$ DCs were the primary inducers of T_h17 responses in our co-cultures, $CD11c^+$ cells were depleted *in vivo* using the previously standardized CD11c DTR model (shown in section 3.1.4), and vaginal cells were isolated, pulsed with OVA peptide and co-cultured with OT-II Tg $CD4^+$ T cells. Vaginal TC from CD11c-depleted mice (DTR group) induced 3.5-fold lower levels of IL-17, and 2-fold lower levels of IL-22 compared to TC from CD11c-intact mice (PBS group) (Fig. 7).

In order to examine the effect of $CD11c^+$ cell depletion in other tissues, we also examined IL-17 levels in small intestine and spleen TC co-cultures from these mice. Consistent with a previous report (Denning et al., 2007), we observed a reduction in IL-17 levels within intestinal co-cultures from CD11c-depleted animals (Fig. 7). However, overall IL-17 levels within intestinal co-cultures was significantly lower compared to vaginal co-cultures in both DT- and PBS-treated experimental groups (Fig. 7). Interestingly, splenic co-cultures from CD11c-depleted mice showed a significant increase in IL-17 (Fig. 7), suggesting tissue specific differences in the ability of APCs to induce $CD4^+$ T cell responses.

Overall, this suggests that CD11c$^+$ cells may be critical to the T_h17 responses observed in mucosal tissue cell co-cultures, particularly in the vagina.

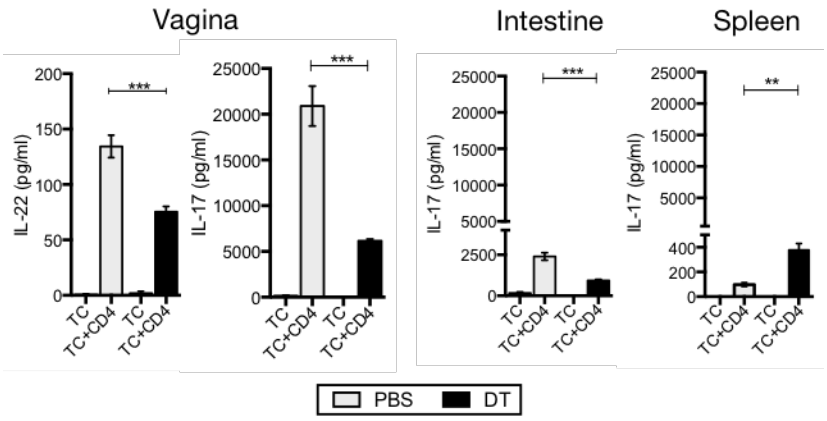


Fig. 7: Depletion of CD11c+ DCs significantly reduces mucosal T_h17 responses: CD11c$^+$ DCs were depleted in CD11c DTR mice by treating them with 400ng DT (200ng IVAG + 200ng IP), or PBS-treated in controls, and 18h later, the indicated tissues were isolated. 1x10^5 Tissue cells (TC) were pulsed with OVA peptide, and co-cultured with OT-II Tg CD4$^+$ T cells in a 1:1 ratio for 3.5 days, and IL-17 and IL-22 levels in vaginal co-cultures, and IL-17 levels among small intestine and spleen tissue co-cultures from DT-treated vs PBS-control CD11c-DTR mice were compared. Data is represented as mean±SD of 3 separate culture wells from one of two separate experiments with similar trends, and significance was calculated by two-way ANOVA (** $p<0.01$, *** $p<0.001$).

3.1.6 Vaginal $CD11c^+$ DCs are the primary inducers of Th17 responses

In section 3.1.5, we demonstrated that depletion of $CD11c^+$ cells significantly diminished the T_h17 responses primed by vaginal tissue cells *in vitro*. However, in order to identify the specific APC populations within the vagina that are critical for priming these T_h17 responses, DCs ($CD11c^+$), macrophages ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$), neutrophils ($CD11c^-$ $CD11b^+$ $F4/80^-$ $Gr\text{-}1^+$), monocytes ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^+$) and other cells ($CD11c^-$ $CD11b^-$) were sorted by FACS, pulsed with peptide and co-cultured in different ratios with $1x10^5$ OT-II Tg $CD4^+$ T cells (Fig. 8B). Both vaginal DCs (DC+CD4) and macrophages (m+CD4) induced a similar degree of proliferation of CFSE labeled OT-II Tg $CD4^+$ T cells (Fig. 8A). However, cytokine analysis showed that $CD11c^+$ DCs were the primary inducers of IL-17 (1331 ± 276 pg/mL) in co-cultures (Fig. 8B). Macrophages also induced IL-17, albeit at 10-fold lower level (170 ± 125 pg/mL) than DCs. Neutrophils, monocytes and other cells did not induce any detectable IL-17 in co-cultures.

In order to compare the T_h17 priming abilities of vaginal and lung $CD11c^+$ DCs directly, $CD11c^+$ and $CD11c^-$ cells from the lungs and vagina of wild type (WT) C57BL/6 mice were sorted and co-cultured with OT-II Tg $CD4^+$ T cells (Fig. 8C). When $CD11c^+$ DCs from the vagina were compared to those from the lung, vaginal $CD11c^+$ DCs induced 10-fold higher IL-17 levels in DC-T cell co-cultures compared to same number of lung $CD11c^+$ DCs (Fig. 8C). In order to examine the role of $CD11c^-$ cells in conditioning the T_h17 priming capabilities of lung and

vaginal $CD11c^+$ cells, $CD11c^+$ cells from each mucosa were co-cultured with $CD11c^-$ tissue cells from the heterologous mucosa. However, $CD11c^-$ cells did not influence the ability of vaginal or lung DCs to prime T_h17 responses as seen by experiments where vaginal and lung mucosal $CD11c^+$ and $CD11c^-$ were mixed in co-cultures with T cells (Fig. 8C). Vaginal and lung $CD11c^+$ DCs retained their respective ability to induce T_h17 responses, irrespective of whether they were mixed with heterologous $CD11c^-$ cells. This suggests that mucosal DCs are likely pre-programmed within their respective tissue microenvironment, and a short-term co-culture with cells from other tissues is not sufficient to change this propensity. These results indicate that $CD11c^+$ DCs in the vagina are more potent inducers of T_h17 responses, compared to other mucosal DCs.

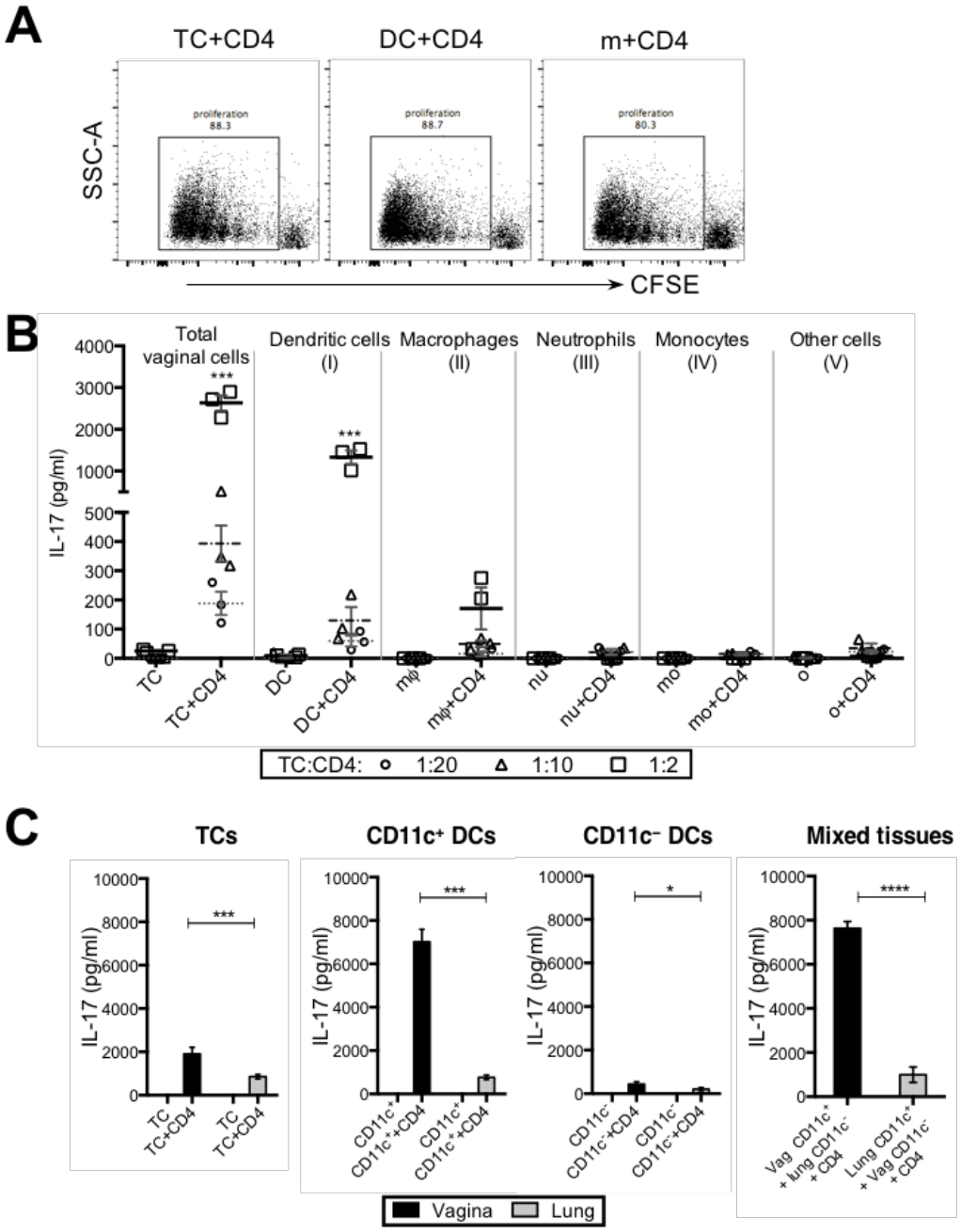

A
TC+CD4
DC+CD4
m+CD4
SSC-A
proliferation
88.3
proliferation
88.7
proliferation
80.3
CFSE
B
Total vaginal cells
Dendritic cells (I)
Macrophages (II)
Neutrophils (III)
Monocytes (IV)
Other cells (V)
IL-17 (pg/ml)
TC
TC+CD4
DC
DC+CD4
mφ
mφ+CD4
nu
nu+CD4
mo
mo+CD4
o
o+CD4
TC:CD4: 1:20 1:10 1:2
C
TCs
CD11c+ DCs
CD11c− DCs
Mixed tissues
IL-17 (pg/ml)
Vagina
Lung

Figure 8: Vaginal CD11c$^+$ DCs were the primary inducers of T_h17 responses, and are more potent inducers than other mucosal DCs. Vaginal cells from WT mice (n=13) were pooled and sorted by FACS, and total vaginal cells as well as sorted populations were pulsed with OVA peptide and co-cultured with 5x10^5 cells/ml OT-II Tg CD4$^+$ T cells at the indicated ratios. A) CD4$^+$ T cell proliferation in total vaginal tissue cell co-cultures, CD11c+ DC co-cultures and macrophage co-cultures. B) IL-17 in co-culture supernatants was measured by ELISA and represented as mean±SD of 3 separate wells per co-culture condition. Statistical analysis was done by one-way ANOVA, to calculate significant differences in IL-17 levels between total vaginal co-cultures and indicated cell-specific co-cultures at each given ratio of APCs:T cells. C) CD11c$^+$ DCs and CD11c$^-$ cells were sorted by FACS from the lungs and vagina of WT mice, and total tissue cells (1x10^5 cells/ml), or purified cells (5x10^4 cells/ml) were OVA peptide pulsed and co-cultured with OT-II Tg CD4$^+$ T cells (1x10^5 cells/ml). For heterologous mixed co-cultures, CD11c$^+$ cells (5x10^4 cells/ml) from the vagina or lung were mixed with CD11c$^-$ cells (5x10^4 cells/ml) from the other tissue, pulsed with OVA peptide, and co-cultured with CD4$^+$ T cells (1x10^5 cells/ml). IL-17 levels in supernatants were measured by ELISA. Significance was calculated by comparing mean±SD of 3 separate co-culture wells for each sample, by one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Data is a representative from 2 separate experiments.

3.1.7 Estradiol plays a critical role in vaginal T_h17 responses

Next, we wanted to examine factors that were responsible for the potent T_h17 responses primed by vaginal DCs. Previously, we and others have shown that sex hormones such as E2 and P4 can influence immune responses to STIs in the genital tract (Bhavanam et al., 2008; Brotman, Ravel, Bavoil, Gravitt, & Ghanem, 2014; Heffron et al., 2012; Wira et al., 2015). E2 can directly influence the differentiation and function of bone-marrow derived $CD11c^+$ DCs (Mao et al., 2005; Paharkova-Vatchkova et al., 2004). More critically, studies conducted in E2-treated mice demonstrated accelerated and higher IFN-γ^+ and IL-17^+ T cell responses (PhD thesis, Roth K., 2015) post challenge in an intranasal immunization model (Anipindi V, Roth K, et al., See appendix A). Therefore, we hypothesized that E2 treatment must condition vaginal APCs to induce the differentiation of T_h1 and T_h17 cells.

To test this hypothesis, OVX mice were implanted with E2 or P4 pellets, while mock controls received placebo pellets. Two weeks later, total vaginal tissue cells (TC) were isolated, pulsed with OVA peptide and co-cultured with CFSE-stained OT-II Tg $CD4^+$ T cells (TC+CD4) for 3.5 days at a 1:1 ratio. T_h1 and T_h17 differentiation was compared among co-cultures conducted with vaginal TC from hormone-treated mice by ELISA by measuring levels of IFN-γ and IL-17, respectively . Co-cultures with TC from E2-treated mice contained over 10-fold higher IL-17 and IFN-γ compared to co-cultures with TC from P4-treated mice or mock controls (Fig. 9A).

In order to examine if such potent T_h17 responses could be observed in the vagina of normal mice during different phases of the reproductive cycle, co-cultures were conducted with TC isolated from mice in the E2-dominant phase (estrus) or P4-dominant phase (diestrus) of the reproductive cycle. Both E2 and P4 are continually present, albeit in different ratios through the different phases of the reproductive cycle. Vaginal TCs from mice in estrus induced over 3-fold higher IL-17 production from T cells compared to TCs from diestrus or OVX-control mice (Fig. 9B). To confirm the role of E2, IL-17 levels were also compared among co-cultures containing TC from ERKO mice, and a group of pooled WT mice at different stages of the reproductive cycle. Vaginal cells from ERKO mice induced 10-fold lower IL-17 levels compared to WT controls. Interestingly, this decrease was not observed in ERKO spleen co-cultures, suggesting that the E2-dependent T_h17 responses may be specific to the vagina (Fig. 9C). Overall, this indicates that E2 can specifically condition vaginal DCs to become potent inducers of T_h17 responses. Furthermore, as the E2 effect on DCs seems to be effective even in mice with intact ovaries and normal reproductive cycle, it allowed us to use regular animals without OVX in further experiments.

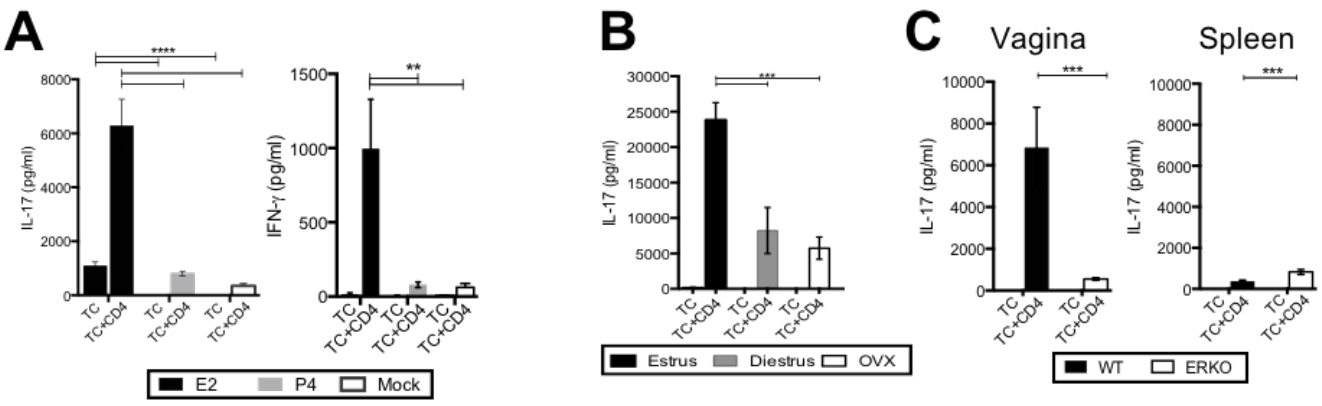


Figure 9: E2 can influence the differentiation of CD4$^+$ T cells in vaginal APC-T cell co-cultures. A) WT OVX mice were implanted with E2-, or P4-, or placebo pellets (mock). Vaginal cells (TC) were isolated, pulsed with OVA peptide and co-cultured with OT-II Tg CD4$^+$ T cells (TC+CD4). IL-17 and IFN-γ levels in co-culture supernatants were measured by ELISA. B) Vaginal cells from mice with intact ovaries were grouped based on the stage of their reproductive cycle: E2-dominant: Estrus, and P4-dominant: Diestrus, and OVX controls, were co-cultured with CD4$^+$ T cells. IL-17 levels within co-culture supernatants. C) IL-17 levels compared among WT and ERKO vaginal and spleen tissue co-cultures. Data is mean±SD of 3 co-culture wells from one of 3 separate experiments, and significance was calculated by two-way ANOVA (** $p<0.01$, *** $p<0.001$).

3.1.8 E2 conditioned vaginal DCs induce T_h17 responses through an IL-1-dependent, but IL-6-independent mechanism

A cytokine microenvironment containing IL-6, TGF-β and IL-23 is considered essential for priming canonical T_h17 responses (Iwakura, Ishigame, Saijo, & Nakae, 2011). However, an alternative pathway involving IL-1 signaling in combination with IL-6, IL-21 and IL-23 has been described (Chung et al., 2009). To determine the key factors for vaginal DC induced T_h17 responses, IL-6, IL-23 and TGF-β levels were measured in supernatants of APC-T cell co-cultures from the vagina, and compared to lung and intestine co-cultures (Fig. 10A). There were no significant differences in IL-23 or total TGF-β levels among all three mucosal TC+CD4 co-cultures (Fig. 10A). However, unlike intestinal or lung TC, vaginal TC themselves constitutively produced 4755 ± 1223 pg/mL of IL-6 (Fig. 7A), and this was further enhanced in vaginal TC+CD4 co-cultures (14407 ± 1602 pg/mL). These levels of IL-6 were not observed in either lung or intestinal co-cultures. Despite multiple attempts, we were unable to detect soluble IL-1β in the co-culture supernatants by ELISA. However, intracellular staining showed that $CD11c^+$ DCs were the primary source of IL-6 and IL-1β in the vagina (Fig. 10B).

In order to examine the role of IL-6 in T_h17 responses primed by vaginal tissue cells, vagina TC (Fig. 10C) and/or purified $CD11c^+$ cells (Fig. 10E) from IL-6 KO mice and WT controls were co-cultured with OT-II Tg $CD4^+$ T cells. Vaginal TC and purified DCs from IL-6 KO mice (Fig. 10C & E) were fully capable of priming T_h17 responses in co-cultures, and addition of exogenous rIL-6 did not

influence IL-17 levels (Fig. 10E). These results were also confirmed by blocking the activity of IL-6 using anti-IL-6 and/or anti-IL-6R antibodies in co-cultures with vaginal cells from WT or IL-6-intact mice (Fig. 10D). The neutralization of IL-6 and/or IL-6R in co-cultures with vaginal TC from WT mice did not influence IL-17 levels (Fig. 10D).

To examine the role of IL-1 in vaginal DC-primed T_h17 responses, we conducted co-cultures with total vaginal TC (Fig. 10C, F and G) and/or $CD11c^+$ DCs (Fig. 10F) from IL-1β KO mice. Vaginal TC and DCs from IL-1β KO mice were significantly impaired (90% decrease) in inducing T_h17 responses (Fig. 10C & F). This effect was reversible when 100ng/mL of exogenous rIL-1β was added to co-cultures (Fig. 10F). Furthermore, given that IL-1β can signal through IL-1R, which is also the receptor for IL-1α, we examined T_h17 responses primed by vaginal TC from IL-1α KO mice. Similar to the observations with vaginal TC from IL-1β KO mice (Fig. 10F), vaginal TC from IL-1α KO mice were also significantly impaired at priming T_h17 responses compared to WT controls (Fig. 10G), and this effect was again reversible by the addition of 100ng/mL rIL-1β. Overall, this indicates that IL-1 signaling was critical for the T_h17 responses primed by vaginal DCs.

In order to determine the link between E2 and IL-1β production by vaginal DCs, OVX mice were implanted with E2, P4 or placebo (mock) pellets (according to the protocols described in materials & methods section 2.1.4). Fourteen days later, vaginal tissue cells were obtained and ICS was conducted to identify

whether E2 treatment induces IL-1β production within vaginal DCs. As expected based on observations in sections 3.1.7, E2 treatment induced a unique, IL-1β^{high} $CD11c^{+}$ DC population that was absent in P4-treated and placebo-treated mock controls (Fig. 10H). Overall, these are novel observations indicating that E2 can condition vaginal $CD11c^{+}$ DCs to produce IL-1β, and this IL-1 plays a critical role in priming T_h17 responses in vaginal co-cultures.

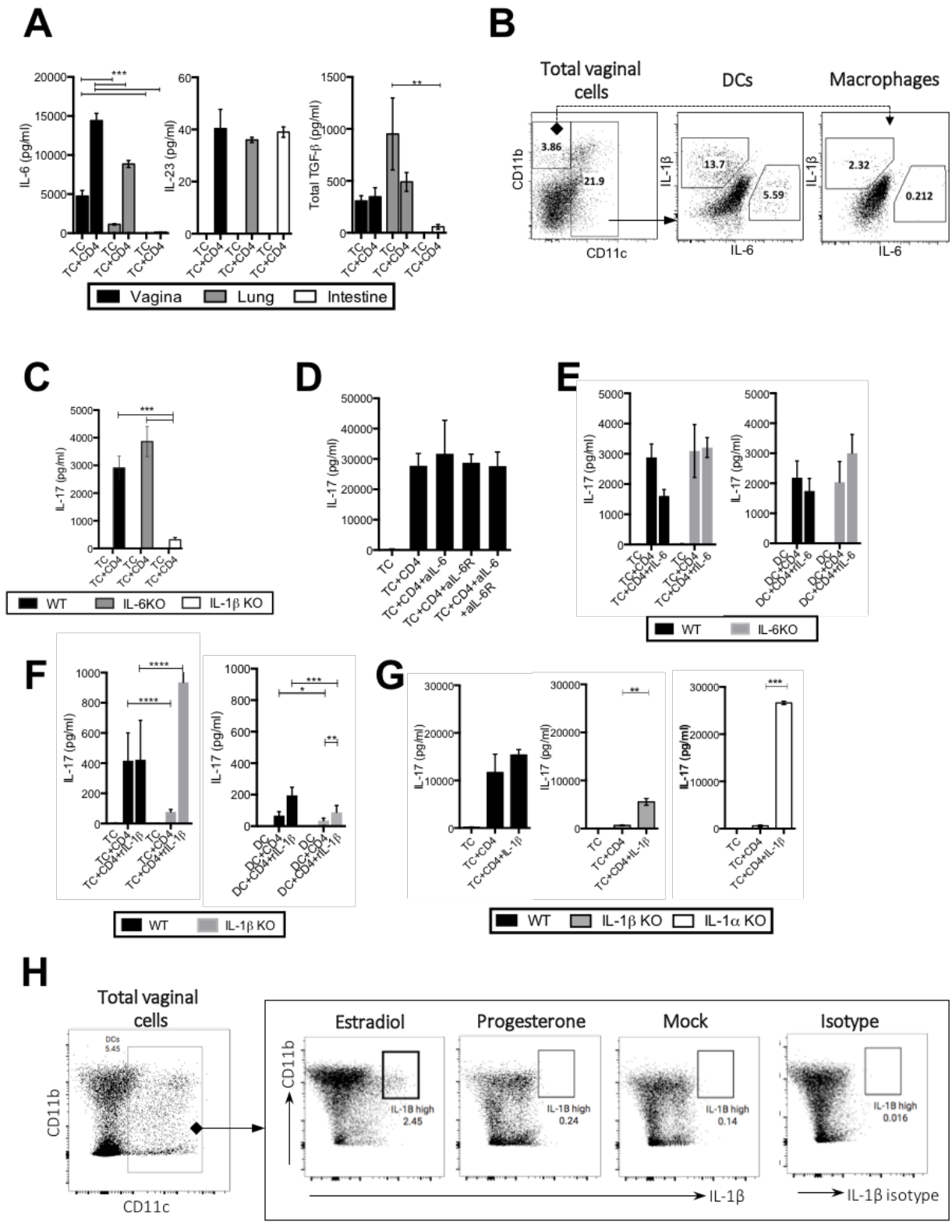

A
IL-6 (pg/ml)
IL-23 (pg/ml)
Total TGF-β (pg/ml)
TC
TC+CD4
Vagina
Lung
Intestine
B
Total vaginal cells
DCs
Macrophages
CD11b
CD11c
3.86
21.9
IL-1β
IL-6
13.7
5.59
2.32
0.212
C
IL-17 (pg/ml)
WT
IL-6KO
IL-1β KO
D
TC
TC+CD4
TC+CD4+aIL-6
TC+CD4+aIL-6R
TC+CD4+aIL-6 +aIL-6R
E
IL-17 (pg/ml)
TC+CD4+rIL-6
DC
DC+CD4
DC+CD4+rIL-6
WT
IL-6KO
F
IL-17 (pg/ml)
TC+CD4+rIL-1β
DC+CD4+rIL-1β
WT
IL-1β KO
G
IL-17 (pg/ml)
TC+CD4+IL-1β
WT
IL-1β KO
IL-1α KO
H
Total vaginal cells
DCs
5.45
CD11b
CD11c
Estradiol
Progesterone
Mock
Isotype
IL-1B high
2.45
0.24
0.14
0.016
IL-1β
IL-1β isotype

Figure 10: Vaginal DCs induce T_h17 responses through an IL-1-dependent pathway. A) Total cells (TC) from vagina, lung and intestine were pulsed with OVA peptide and co-cultured with $CD4^+$ T cells (TC+CD4). IL-6, IL-23 and TGF-β was measured in co-culture supernatants by ELISA. B) Intracellular staining of vagina co-cultures on day 2 of co-culture to examine IL-1β and IL-6 production by vaginal DCs ($CD11c^+$ cells) and macrophages ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$). C) IL-17 levels compared among vaginal TC co-cultures from IL-6 KO, IL-1β KO and WT control mice. D) IL-6 and/or its receptors were neutralized by within vaginal co-cultures by utilizing aIL-6 and/or aIL-6R neutralizing antibodies, and IL-17 was compared with vaginal TC+CD4 co-cultures. E) IL-17 levels compared in co-cultures conducted with TC or $CD11c^+$ DCs purified from the vagina of IL-6 KO mice and WT controls. Forty ng/ml of rIL-6 was added to co-cultures as indicated on X-axis. F) IL-17 levels were compared in co-cultures conducted with TC or $CD11c^+$ DCs purified from the vagina of IL-1β KO mice and WT controls. G) IL-17 levels were compared in co-cultures conducted with vaginal TC from WT, IL-1β KO and IL-1α KO mice. 100 ng/ml rIL-1β was added to co-cultures as indicated on X-axis. H) Vaginal cells were cultured overnight without any stimulation, and intracellular staining conducted to identify IL-1β production among $CD11c^+$ DCs from OVX mice implanted with E2, P4 or placebo (mock) pellets. Data for all cytokine measurements is represented as mean±SD of three separate intraexperimental co-culture replicates. Data is representative of at least 2 separate experiments with similar results, and significance was calculated by two-way ANOVA. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

3.1.9 IRF4 is not essential for the generation of vaginal T_h17 responses

Other groups have shown that human and mouse mucosal DCs that express IRF4 play a central role in T_h17 differentiation (Persson et al., 2013; Schlitzer et al., 2013). Furthermore, E2 has been shown to induce IRF4 expression in bone-marrow-derived DCs *in vitro* (Carreras et al., 2010). Therefore, we wanted to examine whether IRF4 is involved in the pathway of E2 conditioned vaginal T_h17 responses.

OVX mice were implanted with E2, P4 or placebo (mock) pellets, and 14 days later, vaginal cells were isolated, and ICS was conducted based on protocols detailed in the materials and methods section. IRF4 expression was examined in total vaginal tissue cells and vaginal $CD11c^+$ DCs. Consistent with the previously published data on *in vitro* BMDCs (Carreras et al., 2010), E2 treatment led to 2-fold higher IRF4 expression in total vaginal cells, and purified $CD11c^+$ DCs, compared to mock controls, or P4-treated mice (Fig. 8A). Data from ERKO mice also showed similar frequency of IRF4-expressing DCs as mock controls, confirming the role of E2 in vaginal DC IRF4 expression (Fig. 11A).

In order to examine the role of IRF4 in priming T_h17 responses in vaginal co-cultures, vaginal tissue cells from IRF4KO mice were utilized for co-cultures. The IRF4 KO phenotype in these mice was confirmed by ICS of spleen $CD4^+$ T cells which can constitutively express IRF4 (Fig. 11B). However, upon conducting co-cultures with vaginal TC from both IRF4KO mice and their WT littermates, we did not observe any significant differences in IL-17 levels between co-culture

supernatants from both groups (Fig. 11C). Overall, this shows that although E2 can up regulate IRF4 in vaginal DCs, it might not be critical for vaginal DC-mediated T_h17 responses.

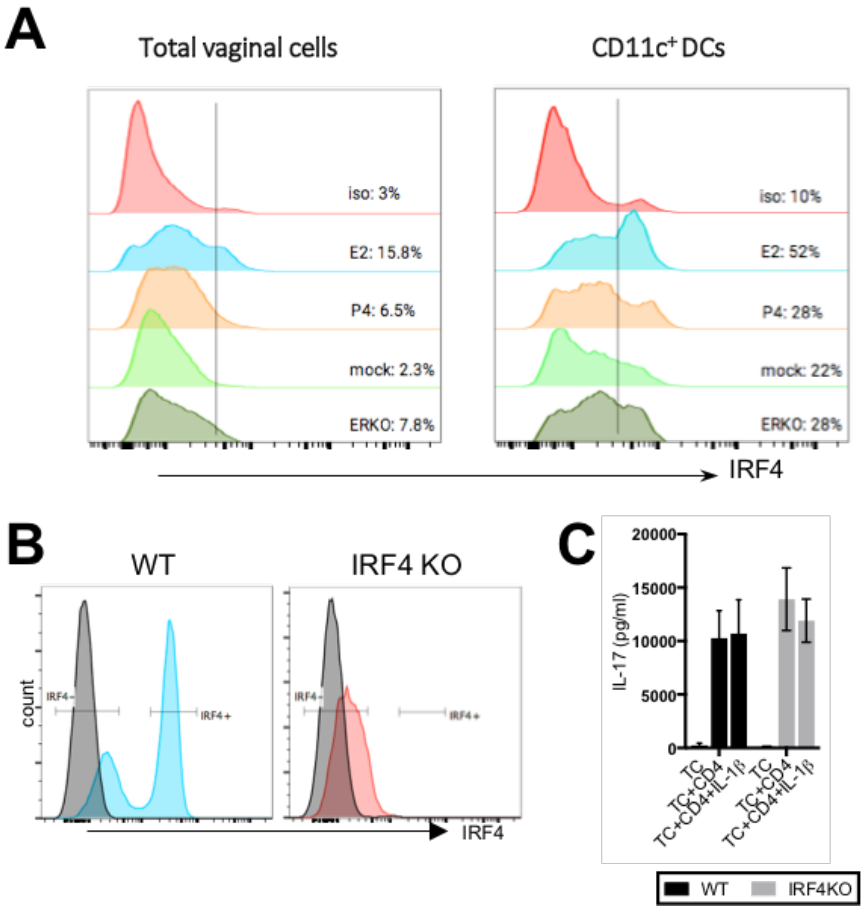


Figure 11: IRF4 expression is not critical for E2-mediated priming of vaginal T_h17 responses. A) Vaginal cells from WT OVX mice implanted with E2, P4 or placebo (mock) pellets for 14 days were cultured in media overnight without any stimulation (12h), and stained for antibodies against IRF4 and DCs (CD11c, CD11b). ICS was conducted according to protocols in materials and methods to identify IRF4 expression in total vaginal cells and vaginal $CD11c^+$ DCs. B) Spleen from IRF4KO mice and their WT littermates were isolated, and cultured overnight. IRF4 expression in CD4+ T cells was compared by ICS. C) Vaginal cells from reproductive cycle stage matched WT and IRF4 KO mice were pulsed with OVA peptide, and co-cultured with OT-II Tg CD4+ T cells for 3.5 days. IL-17 levels in co-cultures was measured by ELISA and expressed as mean±SD of 3 replicate wells from one of two different experiments. Analysis was conducted by two-way ANOVA.

3.1.10 Summary:

In summary, in section 3.1, we showed that under homeostatic conditions, APCs in the vaginal tract have a distinct phenotype and function compared to lung or intestinal APCs. Vaginal $CD11c^+$ DCs were more potent inducers of T_h17 responses in co-cultures with $CD4^+$ T cells, compared to lung, small intestine or spleen tissue cells. By using ERKO mice and WT mice at different stages of their reproductive cycle, we showed that E2 is critical for the conditioning of vaginal DCs in priming these T_h17 responses. Finally, we have also demonstrated the mechanism and shown that E2 conditioned vaginal DCs prime T_h17 responses through an IL-1-dependent pathway. Furthermore, unlike previous reports that indicated that lung and intestinal $IRF4^+$ DCs were critical for priming T_h17 responses (Persson et al., 2013; Schlitzer et al., 2013), IRF4 expression was not critical for T_h17 responses primed in vaginal co-cultures. Overall, our results support our hypothesis proposed in section 1.7.1, in that vaginal APCs are a unique population compared to those resident in other tissues, and sex hormones such as E2 can critically influence their functions.

CHAPTER 3.2

IL-17 producing innate lymphocytes play a critical role in T_h17 responses primed by vaginal DCs

Varun C. Anipindi, Sara Dizzell, Rodrigo Jiménez-Saiz, Kristy L. Roth and Charu Kaushic

Dr. Charu Kaushic, and I were responsible for the design and interpretation of these experiments. I performed all the experiments, generated and analyzed the data. Sara provided assistance with ELISAs. Rodrigo provided the GF mice in some of these experiments. Kristy provided some of the hormone-treated mice. I wrote this chapter with feedback and suggestions from Dr. Kaushic.

3.2.0 OBJECTIVE

Upon addressing the first part of the overall hypothesis in section 3.1, we found that E2 primes $CD11c^+$ DCs to drive potent T_h17 responses in vagina through an IL-1-dependent pathway. Yet, we were aware that innate sources of IL-17 such as γδ T cells and type 3 ILCs, may be a more potent source of IL-17 than conventional T_h17 cells (Do et al., 2011; Lockhart et al., 2006; Sutton et al., 2009). Innate and adaptive IL-17 sources can be co-dependent on one another, and can co-amplify overall IL-17 responses in mucosal tissues in response to microflora (Do et al., 2011; Do et al., 2012; Hepworth et al., 2013; Qiu et al., 2013; Sutton et al., 2009). Others have also linked E2 to the composition of the vaginal microflora (Brotman et al., 2014; Goldacre et al., 1979; Hill et al., 2005; Martin et al., 1999), which can potentially drive mucosal T_h17 responses (Shaw, Kamada, Kim, & Nunez, 2012). Therefore, we wanted to examine the role of these other microenvironmental factors such as microflora and innate IL-17 on E2-primed T_h17 responses in the vagina.

Hence, for this part of the study, we hypothesized: **"*The local vaginal microenvironment including IL-17 produced by innate sources, and genital microflora is integral for conditioning vaginal DC-primed T_h17 responses that were induced by E2.*"** This hypothesis was addressed through the following objectives:

1) Characterize the endogenous $IL\text{-}17^+$ populations in the vagina under homeostatic conditions

2) Examine the effect of E2 and microflora on innate IL-17 producers in the vagina
3) Examine whether the absence of IL-17 can influence the function of vaginal DCs at priming T_h17 responses.

3.2.1 Characterization of innate IL-17 producers in the vagina:

IL-17 in mucosal tissues can also be produced by innate sources (Artis & Spits, 2015; Sutton et al., 2012), and previous studies have shown that these innate IL-17 producing cells may be more potent sources of IL-17 than conventional T_h17 cells (Lockhart et al., 2006; S. M. Schulz et al., 2008; Sutton et al., 2012).). We and others (J. O. Kim et al., 2012) have observed innate populations of IL-17-producing cells in the vagina (Section 3.1.3). While γδ T cells producing IL-17 have been previously reported in the vagina (J. O. Kim et al., 2012), other populations of innate lymphoid cells (ILCs) are relatively unknown. We wanted to characterize these populations based on markers summarized in a recent review, to identify murine IL-17 producing type 3 ILCs (Killig et al., 2014; J. O. Kim et al., 2012).

A panel of antibodies against CD3, CD4, γδ, IL-17 and NKp46, was used to identify γδ T cells and populations of type 3 ILCs in the vagina (Killig et al., 2014; J. O. Kim et al., 2012). Dead cells were excluded, and single cells were differentially gated on their CD3 and CD4 expression to examine lymphocyte populations (Fig. 12A & B). We observed 5 distinct populations of IL-17^+ cells in the vagina. Consistent with a previous report, TCR $\gamma\delta^+$ T cells (these include $CD3^+$ $CD4^-$ $\gamma\delta^+$ T cells (population II in Fig. 12B), and $CD3^-$ $CD4^-$ $\gamma\delta^+$ T cells (population IV in Fig. 12B) were the dominant source of IL-17 in the vagina (J. O. Kim et al., 2012). $CD3^+$ $CD4^+$ $\gamma\delta^-$ cells were second major source of innate IL-17 (population I in Fig. 12B), with smaller populations of $CD3^+$ $CD4^-$ $\gamma\delta^-$ cells

(population III in Fig. 12B), and a relatively minor $CD3^-$ $CD4^-$ $\gamma\delta^-$ subset (population V in Fig. 12B) rounding up the remaining sources of IL-17 in the vagina (Table 2). None of these populations expressed NKp46, suggesting that these were consistent with NK cell receptor NK cell receptors negative (NCR^-) type 3 ILCs or fetal LTi cells (Killig et al., 2014).

In order to understand the effect of this innate IL-17 on T_h17 responses primed by vaginal DCs, we first needed to examine the status of these specific populations in IL-17 KO mice. Even in the absence of IL-17, based on the expression of CD3, CD4 and γδ, it appears that the aforementioned innate populations (Fig. 12B) may be intact in IL-17 KO mice (Fig. 12C). This suggests that the development and presence of these populations in the vagina may likely be independent of IL-17 expression (Fig. 12C). Overall, we have shown that innate IL-17 in the vagina is produced by a heterogeneous population of lymphocytes.

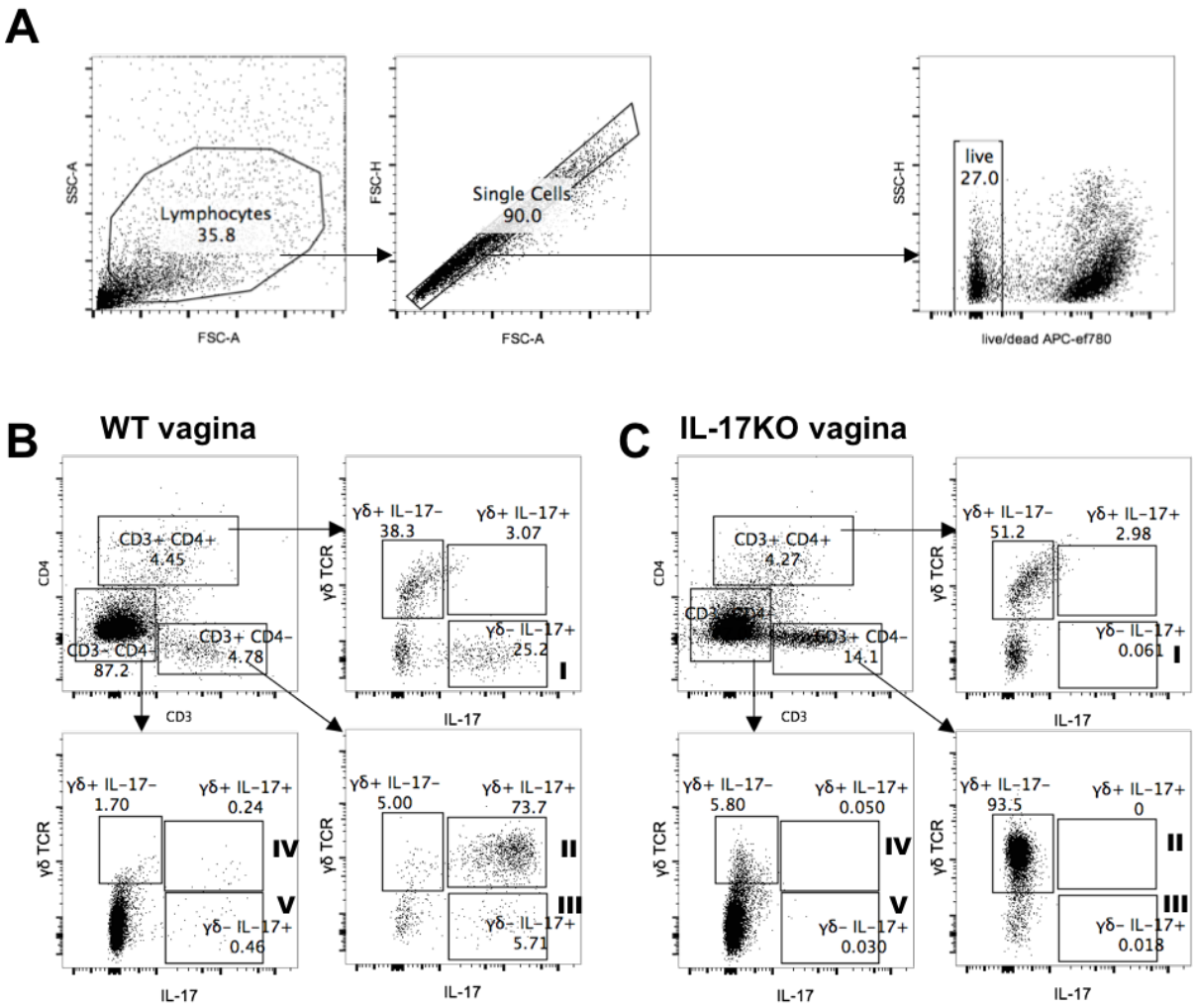


Fig. 12: Endogenous IL-17 within the vagina is produced by a heterogeneous population of innate lymphocytes. Vaginal cells from WT & IL-17 KO mice were isolated and cultured for 2 days. For the last 18h, cell stimulation cocktail with PMA and ionomycin along with protein transport inhibitors was added, and cells were stained with a panel of surface and intracellular markers before analysis on a BD LSR II flow cytometer. A) Live cells were examined, and B) CD3+ CD4-, CD3+ CD4+ and CD3- CD4- populations were differentially gated to identify multiple populations of ILC3s and γδ T cells in the vagina. Data is a single representative from 3 separate experiments.

Table 2: Phenotype of IL-17^{+} ILCs in the vagina.

	I (25.2%)	II (73.7%)	III (5. 71%)	IV (0.24%)	V (0.46%)
Population	CD3+ CD4+ γδ−	CD3+ CD4− γδ+	CD3+ CD4− γδ−	CD3− CD4− γδ+	CD3− CD4− γδ−
% of vaginal tissue cells	1.1	3.6	0.27	0.21	0.38
IL-17	+	++	+	+	+
γδ TCR	−	+	−	+	−
CD3	+	+	+	−	−
CD4	+	−	−	−	−
NKp46	−	−	−	−	−

% in the table header indicates cell proportions as noted in Fig. 12.
+ indicates expression, ++ indicates high expression, − indicates no expression.
I – V in the table header indicate specific populations as denoted in Fig. 12

3.2.2 Commensal microflora can influence overall proportions of innate IL-17 producers in the vagina:

Studies have shown that factors such as microflora can drive IL-17 production from γδ T cells and other innate lymphocytes (Duan, Chung, Troy, & Kasper, 2010; Sanos et al., 2011). Others have also suggested that innate IL-17 producers can modulate the expansion of microflora (Sonnenberg et al., 2012), and certain subsets among these cells, specifically LTi-like type 3 ILCs, are dependent on interactions with commensal bacteria for their development (Sonnenberg & Artis, 2012). Hence, we wanted to examine whether microflora regulates the innate IL-17 producers in the vagina. Vaginal cells isolated from hormone-cycle matched germ-free (GF) mice and WT controls were compared by ICS. While we were unable to find consistent differences in the populations of IL-17^+ cells between the vagina of GF mice and WT controls, GF mice consistently contained a significantly lower proportion of overall IL-17^+ cells compared to WT controls (Fig. 13A & B). This suggests that microflora may play an important role in the endogenous IL-17 responses mediated by lymphocytes in the vagina.

Next, given that gut microflora may also directly induce homeostatic T_h17 responses within the intestine (Niess, Leithauser, Adler, & Reimann, 2008), we wanted to examine whether microflora can influence T_h17 responses primed by vaginal DCs (described previously in section 3.1.3). Vaginal tissue cells (TC) from hormone-cycle matched GF mice and WT controls were pulsed with chicken ovalbumin (OVA) peptide for 12-24h, and co-cultured with OT-II Tg $CD4^+$ T cells

for 3.5 days, according to protocols outlined in the materials and methods section. Consistently in 3 separate experiments, there were no significant differences in IL-17 levels in between GF and WT co-culture supernatants (Fig. 13C). This suggested that microflora might not be a critical factor for conditioning T_h17 responses primed by vaginal TCs.

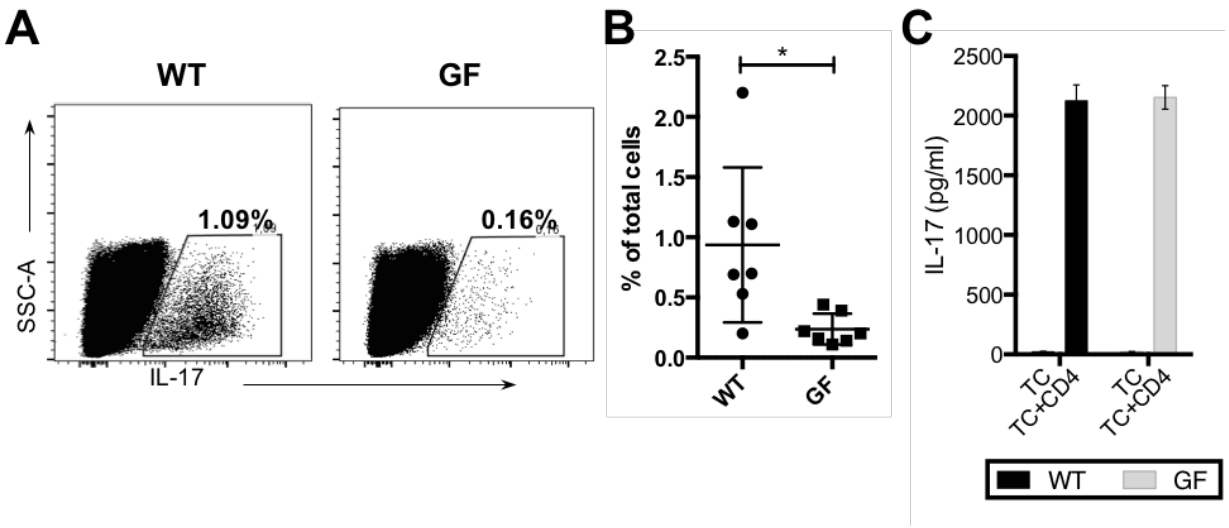


Fig. 13: Microflora can influence overall proportion of IL-17-producing cells in the vagina. Vaginal tissues from WT and GF mice (n=3-5 /group) were isolated, pooled, and cultured in cRPMI media for 48h. For the last 18h, cell stimulation cocktail containing PMA + ionomycin along with protein transport inhibitors was added, and cells were stained with antibodies against IL-17, CD3, CD4 and γδ, and analyzed on a BD LSR II flow cytometer. A) Total IL-17$^+$ cell populations compared between WT and GF mice. Data is a single representative from the 7 independent experiments outlined as a graph in B. B) Graphical summary showing median±SEM of total vaginal IL-17$^+$ cells compared between WT and GF mice from 7 independent experiments. Each point represents proportion of IL-17$^+$ cells from each experiment. Significance was calculated by a two-tailed un-paired t-test. (* $p<0.05$). C) Vaginal tissue cells (TC) from WT and GF mice were pulsed with OVA-peptide for 12-24h, and co-cultured with OT-II Tg CD4$^+$ T cells for 3.5 days. IL-17 levels in culture supernatants were measured by ELISA. Data shown here is mean±SD of 3 sepearate co-culture wells from one of 3 unique experiments.

3.2.3 E2 can influence overall proportions of innate IL-17 producers in the vagina:

Previously, we have shown that E2 directly conditions vaginal DCs to induce vigorous T_h17 responses (Section 3.1 in this dissertation, & Fig. 14A). We have already shown above (section 3.2.2) that microflora can influence innate IL-17 producing cells in the vagina. Other groups have shown that E2 can influence the microflora community in the vagina (Galhardo et al., 2006; Mirmonsef et al., 2014). Hence, we wanted to examine whether E2 can also influence innate IL-17-producing cells in the vagina.

OVX mice were implanted with prolonged release E2 or placebo (mock) pellets, and two weeks later, vaginal TC were isolated and cultured alone, or pulsed with OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells for 3.5 days. Consistent with previous observations (shown in section 3.1.7 of this dissertation), E2 induced significantly greater T_h17 responses in co-cultures (TC+CD4), compared to mock controls (Fig. 14A). As seen in those previous experiments, E2 treatment was also associated with 195 ± 41 pg/mL of IL-17 in vaginal TC cultured alone that was completely absent in mock controls (4 ± 0.5 pg/ml) (Fig. 14A).

To examine whether E2 directly influences the population of $IL\text{-}17^+$ cells in the vagina, vaginal TCs were cultured alone overnight in the presence of cell stimulation cocktail and stained with antibodies against IL-17, CD3, CD4 and γδ. We were unable to find differences in the subpopulations of $IL\text{-}17^+$ cells. However, consistently, E2 treated mice contained approximately 2-fold higher

proportion of total IL-17^{+} cells compared to mock controls and estradiol-receptor α knockout mice (Fig. 14B & C). Furthermore, absolute IL-17 production per cell, as evaluated by median fluorescence intensity (MFI), was also significantly enhanced in E2 treated mice compared to mock controls (Fig. 14C). Overall, these results suggest that E2 may directly enhance the quantity of IL-17 produced, and proportion of IL-17^{+} innate lymphocytes in the vagina.

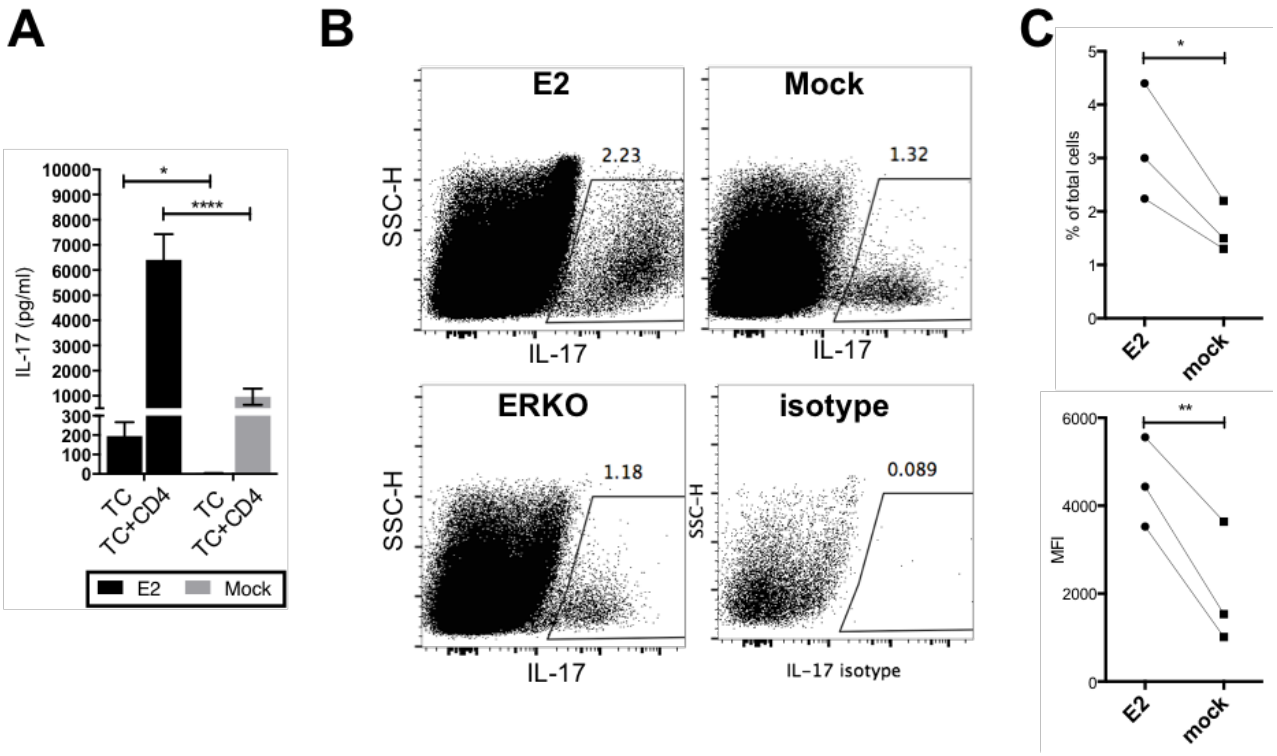


Fig. 14: E2 can influence innate IL-17 responses in the vagina. OVX mice were implanted with E2 or placebo (mock) pellets. A) Vaginal TC were cultured alone, or pulsed with OVA peptide, and co-cultured with OT-II Tg CD4+ T cells for 3.5 days. IL-17 levels produced in vaginal TC cultured alone and co-cultures (TC+CD4), were measured by ELISA. Data is mean±SD of 3 co-culture wells from one of 3 separate experiments with similar trends. Significance was calculated by two-way ANOVA (* $p<0.05$, *** $p<0.0001$). Vaginal cells from E2, placebo (mock) treated mice and ERKO mice were cultured in media for 48h. For the last 18h, cells were stimulated with cell stimulation cocktail, and stained with antibodies against IL-17, CD3, CD4 and γδ, and analyzed on a BD LSR II flow cytometer. B) IL-17$^+$ cell proportions compared between vaginal cells from E2, mock and ERKO mice. Graphical summary showing C) total vaginal IL-17$^+$ cells, and MFI of IL-17 staining, compared between E2 and mock treated mice from 3 independent experiments. Each line represents paired data from each experiment. Significance was calculated by a paired t-test. (* $p=0.02$, ** $p=0.006$).

3.2.4 IL-17 produced by innate lymphocytes in the vagina is important for T_h17 responses primed by vaginal DCs:

Previously, in section 3.1 of this dissertation, we have shown that vaginal tissue cells, specifically vaginal $CD11c^+$ DCs, were potent inducers of T_h17 responses. Multiple groups have shown that both innate and adaptive sources of IL-17 in the gut are co-dependent on each other, and amplify local IL-17 responses to maintain tissue homeostasis (Do et al., 2011; Do et al., 2012; Hepworth et al., 2013; Qiu et al., 2013; Sutton et al., 2009). Therefore, we wanted to examine whether IL-17 produced by innate lymphocytes in the vagina, can influence T_h17 responses primed by vaginal APCs.

Vaginal tissue cells (TC) from hormone-cycle matched IL-17KO and WT mice were isolated, pulsed with OVA-peptide and co-cultured with CFSE-stained OT-II Tg $CD4^+$ T cells (TC+CD4) for 3.5 days. T_h17 differentiation was examined by ICS, and IL-17 and IFN-γ levels in co-culture supernatants were measured by ELISA. Supernatants of co-cultures (TC+CD4) with TC from IL-17 KO mice contained 30-fold lower IL-17 and 7-fold lower IFN-γ compared to WT controls (Fig. 15A). Intracellular staining was conducted on day 2 to identify the sources of IL-17 in these co-cultures (Fig. 15B). A small population (3.89%) of $CFSE^-$ cells representing IL-17 produced by innate lymphocytes in the vagina, was observed in WT co-cultures, but absent in IL-17 KO co-cultures. This confirmed the lack of endogenous IL-17 production by vaginal innate lymphocytes in IL-17 KO mice (Fig. 15B). Furthermore, while 55% of proliferating $CD4^+$ T cells expressed IL-17

in WT control co-cultures, only 39% of proliferating cells in IL-17 KO co-cultures expressed IL-17 (Fig. 15B). Overall, these results (Fig. 15) suggest that IL-17 produced by innate sources in the vagina is important for maximal potentiation of vaginal APCs in priming T cell responses.

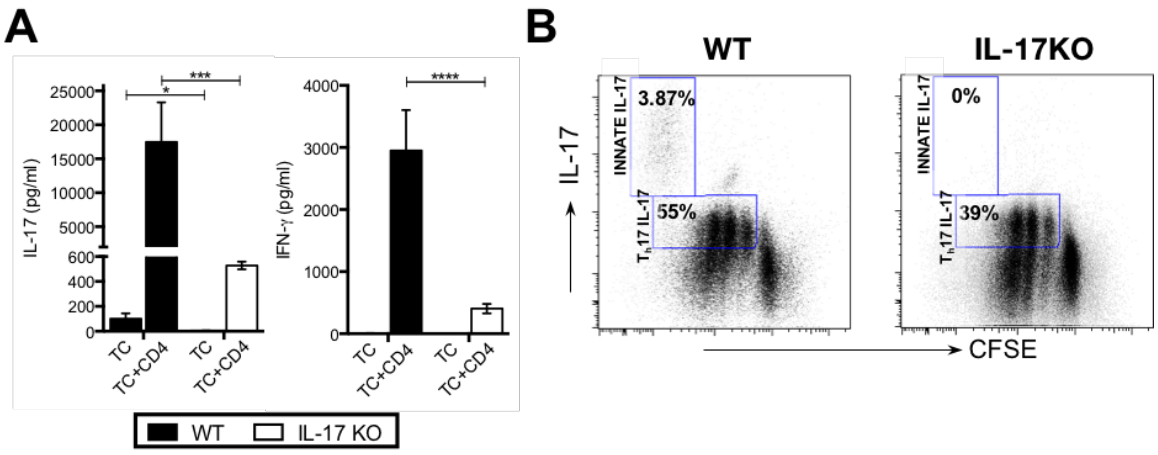


Fig. 15: IL-17 produced by innate lymphocytes plays an important role in vaginal DC primed T_h17 responses. Vaginal tissues from stage-matched WT and IL-17KO mice were isolated, pulsed with OVA peptide and co-cultured for 3.5 days with CFSE-stained OT-II Tg $CD4^+$ T cells. A) IL-17 and IFN-γ levels produced by vaginal tissue cells alone (TC) and co-cultures (TC+CD4) was measured by ELISA. Data is represented as mean±SD of 3 co-culture wells, and is a representative of 3 separate experiments (IL-17 levels. IFN-γ levels were examined in only one of these 3 experiments) with similar trends. Significance was calculated by two-way ANOVA (* p=0.03, *** p=0.0007 & **** p<0.0001). B) To identify IL-17-producing cells within co-cultures (TC+CD4), cell stimulation cocktail was added on D2 of co-culture. Sixteen hours later, ICS was conducted based on protocols outlined in the materials and methods section. “Innate IL-17” indicates IL-17 produced by innate lymphocytes in vaginal TC. “T_h17 IL-17” indicates proportion of dividing CFSE-stained OT-II Tg $CD4^+$ T cells that differentiate into T_h17 cells.

3.2.5 Vaginal DCs from IL-17 KO mice are impaired at producing IL-1β.

Previously, we have shown that T_h17 responses in vagina co-cultures were primed by vaginal DCs through an IL-1-dependent pathway (shown in section 3.1.8 of this dissertation). Given the observation of attenuated T_h17 responses in IL-17 KO co-cultures, we wanted to examine whether these diminished responses could be due to impaired IL-1β production by vaginal DCs in IL-17 KO mice.

To examine IL-1β from vaginal DCs, tissue cells from IL-17KO and WT controls were isolated and cultured overnight prior to staining with antibodies against CD11c, CD11b, pro IL-1β and IL-1β. Myeloid sources of IL-1β such as DCs synthesize IL-1β in its pro form that accumulates in the cytosol (Lopez-Castejon & Brough, 2011). Upon inflammasome activation, this IL-1β is cleaved into its active form by enzymes such as Caspase 1, and secreted outside the cell (Lopez-Castejon & Brough, 2011; Martinon, Burns, & Tschopp, 2002). We examined both the active and pro forms of IL-1β in vaginal cells from WT and IL-17 KO mice. DCs were gated ($CD11c^+$ cells) (Fig. 16A), and intracellular staining for both pro IL-1β (clone NJTEN3) and IL-1β (clone 166931) was conducted to compare $IL\text{-}1\beta^+$ cells between both groups of mice. IL-17KO mice contained 50% lower proportions of pro IL-1β and IL-1β producing DCs in compared to WT controls (Fig. 16B). Additionally, while it did not reach statistical significance, IL-17 KO $CD11c^+$ DCs also produced about 30% lower IL-1β per cell, based on MFI levels (Fig. 16C), compared to WT controls. Overall, these results (Fig. 16)

suggest that IL-17 produced by innate lymphocytes may be important for conditioning vaginal DCs to produce IL-1β.

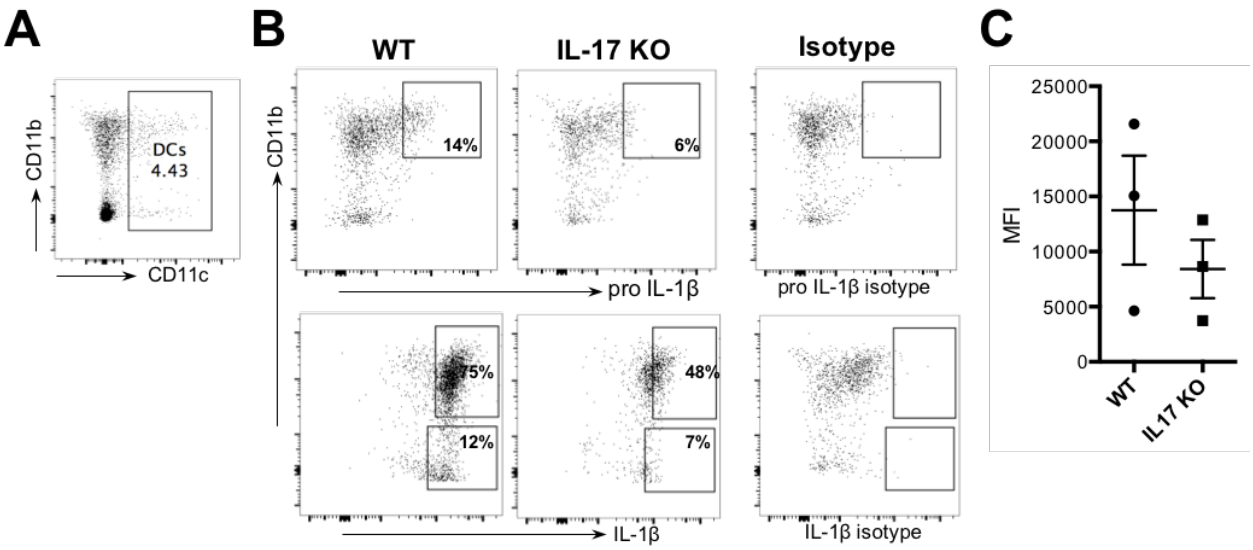


Fig. 16: IL-17 produced by innate lymphocytes in the vagina condition vaginal DC to produce IL-1β. Vaginal tissues from stage-matched WT and IL-17 KO mice were isolated and cultured overnight without any stimulation, before staining them with antibodies against DC surface markers CD11c and CD11b, and intracellular markers pro-IL-1β and IL-1β. A) $CD11c^+$ cells were gated, and B) the expression of pro-IL-1β and IL-1β was compared between $CD11c^+$ DCs from WT or IL-17 KO mice. C) To compare per-cell level differences in IL-1β expression, MFI levels of IL-1β-expressing cells were compared among WT and IL-17 KO mice. Data is a representative from 2 separate experiments with similar trends.

3.2.6 Addition of rIL-1β, but not rIL-17, is able to enhance Th17 responses primed by vaginal APCs from IL-17 KO mice

Having observed impaired IL-1β production in vaginal DCs from IL-17 KO mice (shown above in section 3.2.5), we wanted to examine whether addition of rIL-17 could restore the ability of these DCs to overcome this limiting factor, and prime T_h17 responses at levels comparable to those seen in WT control co-cultures.

Vaginal cells (TC) from IL-17 KO mice and WT controls were isolated, pulsed with OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells. To examine whether IL-17 is only required during the antigen pulse stage: 250pg/ml of rIL-17, representing levels consistent with that observed in cultures of vaginal cells from WT controls, was added along with OVA peptide, and 12h later, washed away before conducting co-cultures with OT-II Tg CD4+ T cells ($TC_{IL\text{-}17p}$+CD4). In separate experiments, we also examined whether IL-17 is required during the co-culture. In these experiments vaginal cells underwent regular OVA peptide pulse, and 250pg/mL of rIL-17 was added at D1 of co-culture with OT-II Tg CD4+ T cells (TC+CD4+IL-17). Interestingly, in both conditions, rIL-17 was unable to influence T_h17 responses primed by IL-17 KO vaginal APCs (Fig. 17A). This suggests that short-term restoration of IL-17 levels may not be sufficient to influence the function of vaginal APCs and their propensity for priming T_h17 responses.

We also wanted to examine whether addition of rIL-1β can influence T_h17 responses in IL-17 KO co-cultures. The direct addition of 100 ng/mL rIL-1β on D1

of co-culture (TC+CD4+IL-1β) was able to enhance T_h17 responses 10-fold. Despite this, IL-17 did not reach levels comparable to those seen in WT co-cultures (Fig. 17B). While the dosage of rIL-1β might require optimization, an alternate explanation could be that other defects may exist in IL-17 KO vaginal DCs that could explain the relative lower levels of in IL-17 produced when compared to control co-cultures. Overall, this confirmed the notion that IL-1β production may be dysregulated in IL-17KO vaginal DCs, and a transient addition of rIL-17 is not sufficient to change their propensity to prime T_h17 responses

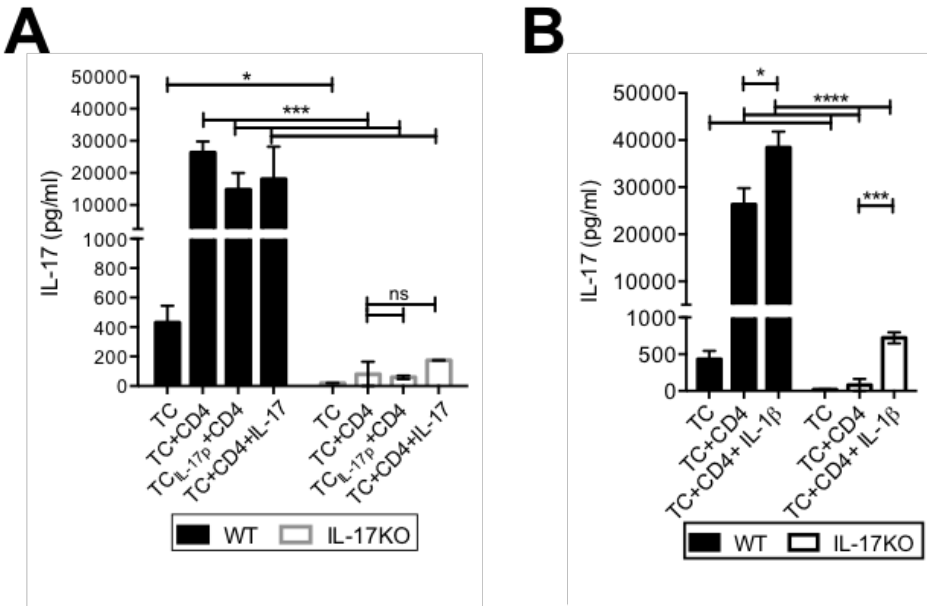


Fig. 17: rIL-1β addition, but not rIL-17 in IL-17 KO co-cultures enhances IL-17 in vaginal co-cultures. Vaginal tissues from stage-matched WT and IL-17 KO mice were isolated, pulsed with OVA peptide and co-cultured for 3.5 days with CFSE-stained OT-II Tg $CD4^+$ T cells. IL-17 levels produced by vaginal tissue cells alone (TC), and co-cultures (TC+CD4) was measured by ELISA. A) To examine whether exogenous IL-17 can influence Th17 differentiation, co-cultures were either pulsed with rIL-17 (250 pg/ml) during OVA peptide pulse ($TC_{IL\text{-}17p}$ + CD4) or added to co-cultures of IL-17 (250 pg/ml) (TC+CD4+IL-17) B) To identify whether rIL-1β can improve Th17 differentiation, 100ng/ml rIL-1β was added as indicated (TC+CD4+IL-1β). Data is representative of two separate experiments. Data is represented as mean±SD of 3 replicate co-culture wells from one of three separate experiments, and significance was calculated by 2-way ANOVA (* $p<0.05$, ** $p<0.005$, *** $p<0.001$ & **** $p<0.0001$).

3.2.6 Summary:

In summary, we have shown that IL-17 is produced by a heterogeneous population of innate lymphocytes in the vagina, and factors such as microflora, and E2, can directly influence the overall proportion of these innate lymphocytes. Using IL-17KO mice, we have shown that IL-17 produced by innate lymphocytes is important for potentiating T_h17 responses primed by vaginal DCs. Furthermore, we have explored the mechanism and shown that this IL-17 may be important for inducing IL-1β production from vaginal DCs, thereby influencing their ability to prime T_h17 responses.

CHAPTER 3.3

Optimizing an E2 delivery regimen to test the effect of E2 on DC responses in an HSV-2 mouse model

Varun C. Anipindi, Kristy L. Roth, Nicole Barra and Charu Kaushic

Dr Charu Kaushic and I were responsible for the design and interpretation of these experiments. I was responsible for the generation and analysis of this data. Kristy helped me with the design, execution and analysis of the *in vivo* HSV-2 OVA experiment in 3.3.9, (Fig. 26). Nicole conducted all the adoptive transfers in 3.3.8 and 3.3.9. I wrote this chapter with feedback and suggestions from Dr. Charu Kaushic

3.3.0 OBJECTIVE

Our observations addressing the overall hypothesis as stated in section 1.7 led to the conclusion that sex hormones such as E2 (section 3.1), and the local microenvironment factors (section 3.2) can influence the function of vaginal APCs. Next, we wanted to extend our observations *in vivo* to the HSV-2 mouse model. We wanted to examine whether the potent T_h17 responses induced by E2 can influence anti-viral responses in the context of HSV-2 infection. However, the thickening and keratinization of the vaginal epithelium induced by E2 treatment precludes intravaginal infection in these mice (Parr & Parr, 2003). This makes it extremely challenging to study E2's effect *in vivo* in the context of anti-viral responses. Therefore, we first needed to modify pre-existing models such that we could study the effect of E2 while mice were susceptible to intravaginal HSV-2 infection. Previous studies in our lab have shown that sex hormones such as E2 and P4 can influence anti-viral immune responses, and specifically, P4 can increase susceptibility, to HSV-2 infection *in vivo* (Bhavanam et al., 2008; A. Gillgrass et al., 2010; A. E. Gillgrass, Fernandez, et al., 2005; Kaushic et al., 2003). Given these observations, we also included a group of P4-treated mice in all experiments associated with section 3.3.

For this part of the study, we hypothesized that ***"Following E2 treatment, there should be a window of time when OVX mice are susceptible to HSV-2 infection, while their vaginal APCs are still under the effect of E2."*** This hypothesis was addressed through the following objectives:

1) Elucidate the effect of hormones on APC populations in the vagina
2) Optimize an *in vivo* model where the effect of E2 treatment on APCs can be observed while the mice are susceptible to HSV-2 infection
3) Using the above model, examine functional differences in APCs from E2 and P4 treated mice following HSV-2 infection.

3.3.1: E2 directly influences APC populations in the vagina

Previously, we and others have shown that the frequency of lymphocyte populations in the vagina can vary during the stages of the reproductive cycle (Kaushic et al., 1998; Wira et al., 2015). We wanted to examine whether sex hormones such as E2 and P4 can directly influence the phenotype of vaginal APCs. OVX mice (n=6/group) were implanted with 21-day prolonged release E2, P4 or placebo (mock) pellets (see materials and methods section 2.1.4 for protocol). Seven, fourteen and twenty-one days after pellets were implanted, vaginal tissues from each group of mice were pooled, processed, and stained with a panel of antibodies against cell surface markers: CD11c, CD11b, F4/80 and MHCII, according to protocols outlined in materials and methods section 2.5. Dead cells were excluded with DAPI, and single cells in the vagina were gated based on their CD11c expression to identify DC populations (Fig. 18A). $CD11c^+$ DCs were further characterized into subsets based on their expression of CD11b and MHCII (Fig. 18B). Macrophages were identified as $CD11c^-$ $CD11b^+$ $F4/80^+$ cells (Fig. 18B). The absolute number of total $CD11c^+$ DCs, $CD11c^+$ $CD11b^+$ $MHCII^-$ DCs and $CD11c^-$ $CD11b^+$ $F4/80^+$ macrophages, per $1x10^6$ vaginal TCs were examined at all three time points (Fig. 18C).

We found that E2 significantly enhanced the proportion (Fig. 18B) and absolute number (Fig. 18C) of $CD11c^+$ $CD11b^+$ $MHCII^-$ DCs in the vagina. This was apparent at all three time points: 7,14 and 21 days following implantation of hormone pellets. Similar to DC populations, $CD11c^-$ $CD11b^+$ $F4/80^+$

macrophages were also enhanced in the vagina of E2 treated mice at all three time points (Fig. 18B & C). Interestingly, the contrast in the proportions of $CD11c^{+}$ $CD11b^{+}$ $MHCII^{-}$ DCs and macrophages seemed to be more pronounced after 14 and 21 days compared to 7 days.

Altogether, these results suggest that E2 can enhance the presence of $CD11b^{+}$ $MHCII^{-}$ DCs and macrophages in the vagina, and this phenotype becomes more evident following prolonged hormone treatment.

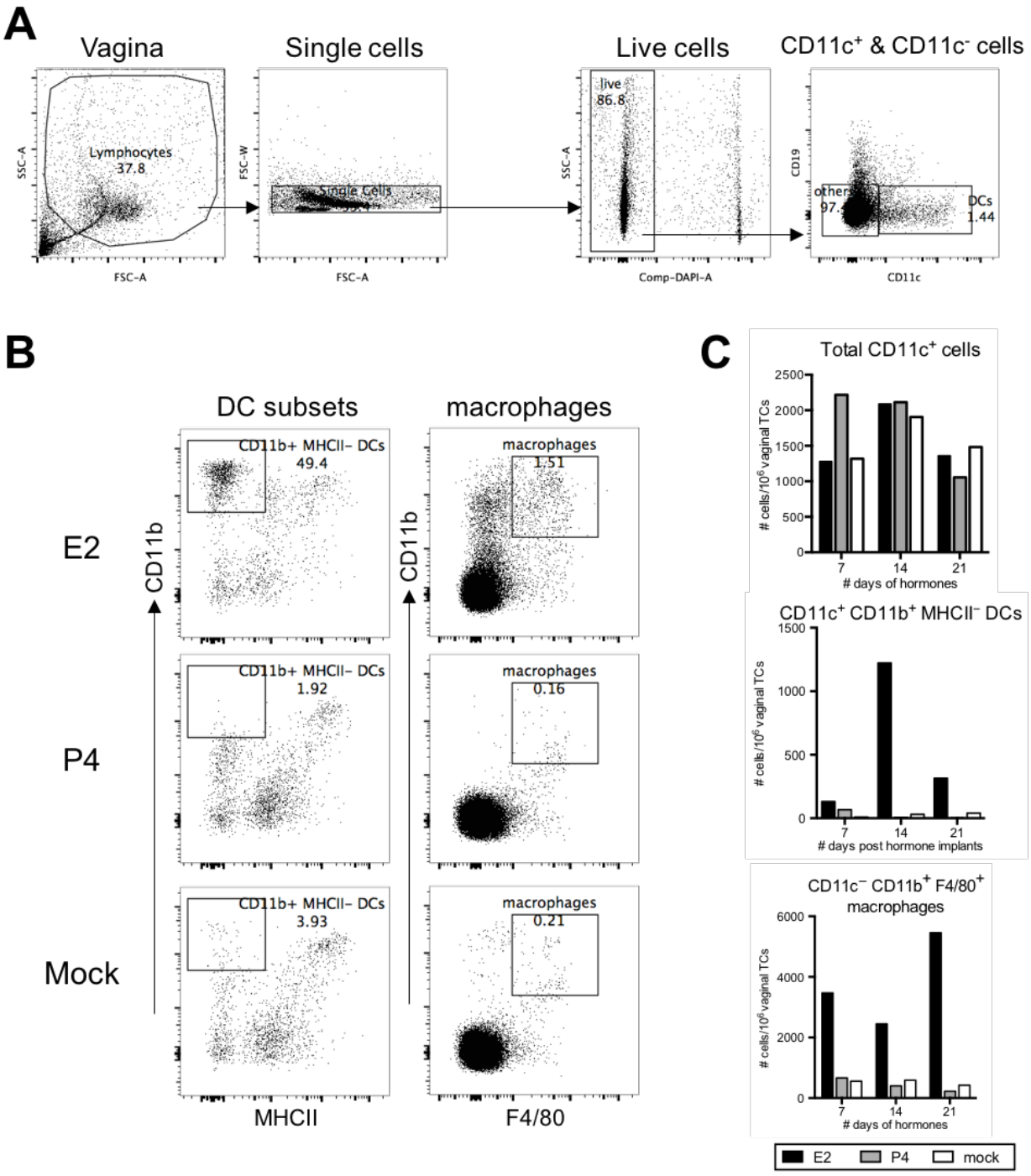

A
Vagina
Single cells
Live cells
CD11c+ & CD11c- cells
Lymphocytes
37.8
SSC-A
FSC-A
FSC-W
Single Cells
live
86.8
Comp-DAPI-A
CD19
others
97.
DCs
1.44
CD11c
B
DC subsets
macrophages
E2
P4
Mock
CD11b
CD11b+ MHCII- DCs
49.4
1.92
3.93
macrophages
1.51
0.16
0.21
MHCII
F4/80
C
Total CD11c+ cells
cells/10^6 vaginal TCs
days of hormones
CD11c+ CD11b+ MHCII- DCs
days post hormone implants
CD11c- CD11b+ F4/80+
macrophages
E2
P4
mock

Fig. 18: Prolonged E2 treatment polarizes APCs towards a CD11b^{+} DC and macrophages in the vagina. Vaginal tissues from OVX mice implanted with E2, P4 or placebo (mock) pellets were isolated, stained with a panel of antibodies against markers CD11c, CD11b, MHCII and F4/80, and APC populations among hormone treatment groups were compared by flow cytometry. A) Gating strategy: Dead cells were excluded with DAPI, and cells were gated on CD11c and CD19. B) CD11c^{+} cells (Dendritic cells) were further characterized by CD11b and MHCII expression, and macrophages were identified as CD11c^{-} CD11b^{+} F4/80^{+} cells. Representative of data from the 14-day group. C) Proportions of DC and macrophage populations were extrapolated to calculate and compare absolute cell counts among hormone treated mice.

3.3.2: E2 treated mice are not susceptible to HSV-2 infection

Previously, others (Parr & Parr, 2003) and we (A. Gillgrass et al., 2010; A. E. Gillgrass, Fernandez, et al., 2005) have shown that E2 treatment renders mice resistant to intravaginal HSV-2 infection. Physiologically, E2 induces proliferation and keratinization of the vaginal epithelium (Goldman, Murr, & Cooper, 2007), making it an impenetrable barrier against viral infection. We wanted to develop a model where this physical barrier could be bypassed, and E2-treated mice could be infected intravaginally with HSV-2 to study E2's effects on anti-viral immunity *in vivo*.

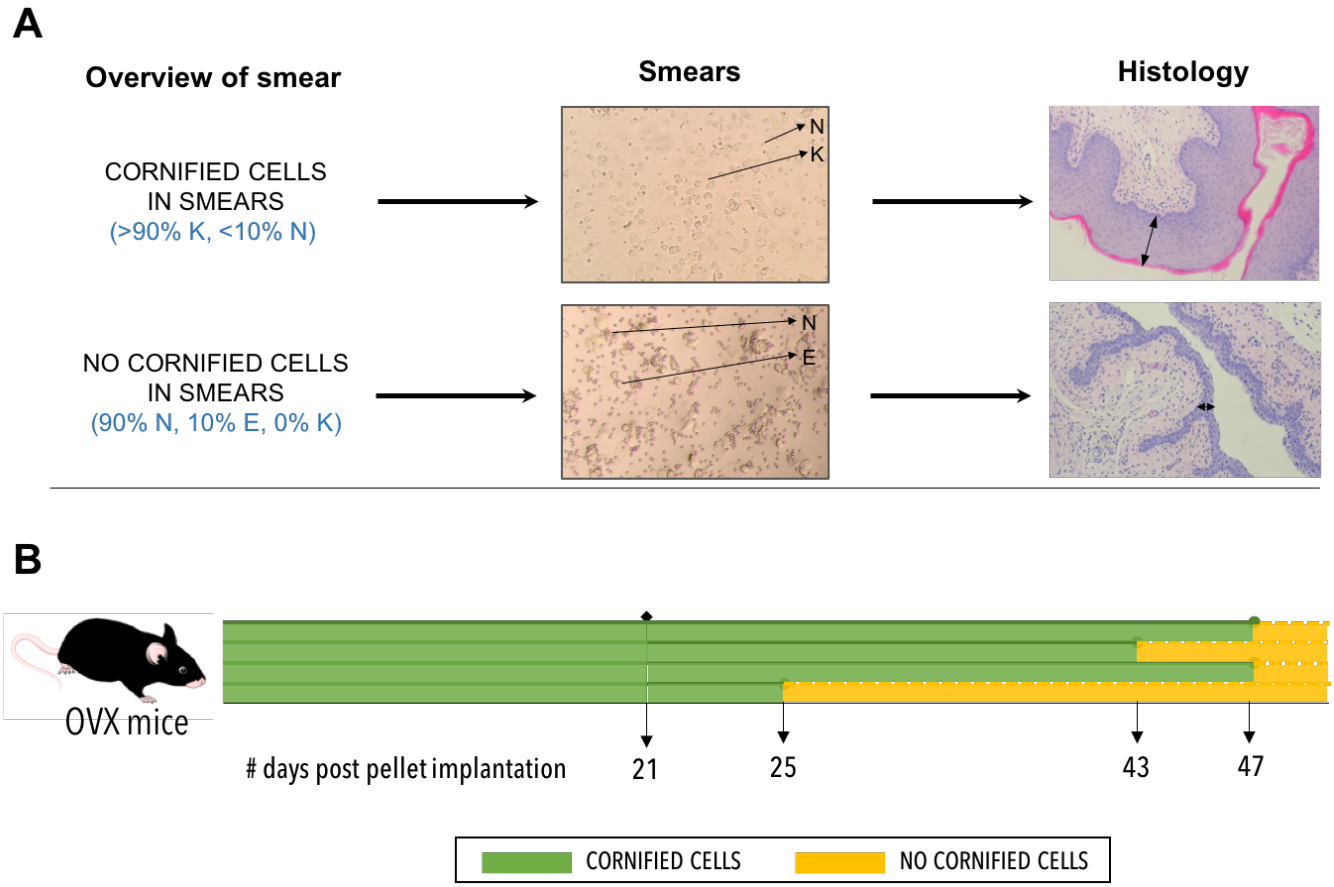
Previously, we have reported that mice treated with an injectable formulation of E2 (500ng) for 3 days, are susceptible 5 days after last hormone treatment (A. Gillgrass et al., 2010). The use of E2 pellets in section 3.1.7 and 3.2,3, and above in 3.3.1 demonstrated that prolonged hormone treatment can polarize APC phenotypes; therefore, we first attempted to develop an intravaginal infection model with E2 pellets. OVX mice (n=4) were implanted with E2 pellets according to protocols detailed in the materials and methods section 2.1.4. These pellets were designed by Innovative Research of America (Sarosota, FL, USA) for a sustained release E2 over the course of 21 days. The physiological effect of E2 on the vaginal epithelium was monitored by tracking the stages of the estrous cycle (see Introduction section 1.5.2 for explanation). Briefly, the impenetrable vaginal epithelium induced by E2 corresponds to the estrus stage, which is characterized by the presence of cornified/keratinized epithelial cells in vaginal

smears. Hence, from day 22, vaginal smears (using protocols outlined in (Kaushic et al., 2003)) were used to monitor the earliest time point when cornified epithelial cells (ECs) were no longer observable, an indication that the epithelial barrier could be susceptible to viral infection.

Vaginal smears from 3 out of the 4 mice implanted with E2 pellets contained cornified ECs until D43-47 post pellet implantation (Fig. 19B). However, the vaginal smears of 1 out of 4 mice stopped containing cornified ECs by D25. We related the cellular composition (Fig. 19A overview of smear) of vaginal smears (Fig. 19A middle panels) to the thickness of the vaginal epithelium, by histological staining in a separate group of mice (Fig. 19A right most panels). E2 pellet implanted mice whose vaginal smears contained cornified cells, and those that did not, were sacrificed, and vaginal tissues were fixed in formaldehyde, and stained with hemotoxylin & eosin by standard protocols. As expected, mice whose smears contained cornified ECs showed a thick epithelium (indicated by the length of double-sided black arrow), while mice whose smears did not contain these cells, showed a thin vaginal epithelium (length of black arrow) (Fig. 19A: Histology).

Overall, these results indicate that the biological effect of E2 pellets, observed by monitoring its direct effect on the vaginal epithelium, may be cleared at different rates in individual mice. This raises significant concerns with regards to infecting a large number of mice intravaginally at once in order to obtain sufficient n numbers for statistical analysis. Hence, given this practical concern

with the inconsistent clearance of hormone pellets, we decided to develop an alternate model where E2 could be delivered by subcutaneous injections, to examine E2 effects on APC responses to HSV-2 infection..



Visual estimate of cells in vaginal smear K: cornified ECs, N: Neutrophils, & E: Epithelial cells

Fig. 19: The variability in the biological effect of E2 in 21 day E2 pellet treated mice. OVX mice (n=4) were implanted with E2 pellets. Vaginal smears were conducted starting from D22 to and examined using a light microscope (Smears: A middle panel), and cellular composition was noted (A: overview of smears). In a separate group of 2 OVX mice implanted with E2 pellets, vaginal tissues were collected from mice corresponding to the presence or absence of cornified cells. Tissues were fixed in formalin for 1 week before embedding in paraffin for H&E staining using standard protocols (A: Histology). B) An overview of appearance of cornified cells in vaginal smears of mice (n=4) after E2 pellet implantation. Each bar represents smears from a single mouse.

3.3.3: Optimizing a 7 day injectable E2 treatment model for studying hormone effects on HSV-2 infection

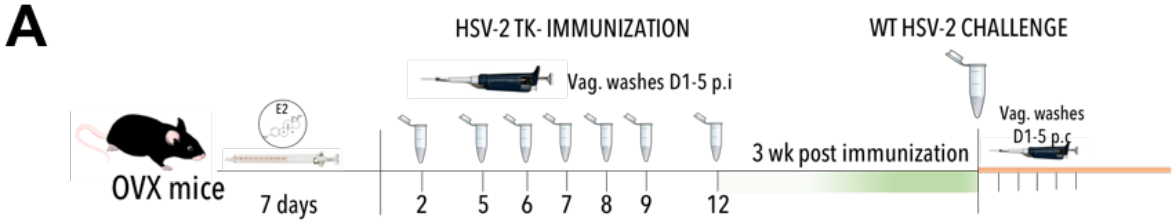
The practical issues related to using the 21-day hormone pellets as described in the previous section (section 3.3.2), required us to develop an intravaginal infection model using injectable E2 treatments. Previously, we have shown that mice treated with injectable E2 for 3 days were susceptible to intravaginal HSV-2 infection 5 days post hormone treatment (A. Gillgrass et al., 2010). However, in section 3.3.1, we have demonstrated that the effect of E2 on APC populations, as seen by changes in their APC phenotype, became clearly evident at least 7 days following hormone pellet implantation (Section 3.3.1) Hence, we wanted to develop a new 7 day injectable E2 model, so that the effects on DCs could be easily identified, but at the same time, all E2-treated mice would be susceptible to HSV-2 infection.

OVX mice were injected with E2 (500ng/mouse) via subcutaneous injection for 7 days at 24-hour intervals (outlined in Fig. 20A). Two, five, six, seven, eight, nine and twelve days post last injection, mice were immunized in groups (n=2-3/group) intravaginally with $1x10^5$ pfu/mouse (TK−) HSV-2 (An attenuated strain of HSV-2 that is deficient in viral Thymidine kinase). Vaginal washes were conducted by flushing the vaginal canal with 2 x 30µl of PBS for the next 5 days and titrated to examine viral shedding. At three weeks post immunization, mice were challenged intravaginally with $1x10^5$ pfu/mouse WT HSV-2 333. Vaginal washes were collected for 5 days post-challenge to examine

WT HSV-2 viral shedding in the vagina. Survival and protection were monitored for 2 weeks post challenge (Fig. 20A).

Mice immunized at D2, D5, and D6 (with the exception of one mouse) post last E2 injection, did not show any viral shedding in post-immunization vaginal washes (Fig. 20B: Immunization titers), that may likely indicate unsuccessful immunization. However, mice inoculated at D7, D8, D9 and D12 post E2 treatment, all showed significant viral shedding in their vaginal washes, indicating successful immunization. The effectiveness of this immunization was confirmed by lethal intravaginal WT HSV-2 challenge, 3 weeks later. Mice immunized at D2, D5 and D6 (all mice) showed high levels of viral shedding post challenge, indicating an unsuccessful immunization, and rampant WT HSV-2 replication in the vagina (Fig. 20B: Challenge titers). On the other hand, few, but not all mice in groups D7, D8, D9, and D12 showed limited viral shedding post-challenge, but all mice in these groups survived, confirming that immunization was successful in these groups.

Overall, these results indicate that mice treated with injectable E2 for 7 days are susceptible to intravaginal HSV-2 infection as early as 7 days after hormone treatment. Since, this day was the earliest time point following E2 treatment at which all E2-treated mice became consistently susceptible to intravaginal infection, we chose day 14 (after D1-7 of E2 treatment) to infect OVX mice treated with E2, in the following experiments described in this chapter.


A
HSV-2 TK- IMMUNIZATION
WT HSV-2 CHALLENGE
Vag. washes D1-5 p.i
Vag. washes
D1-5 p.c
3 wk post immunization
OVX mice
7 days
2
5
6
7
8
9
12

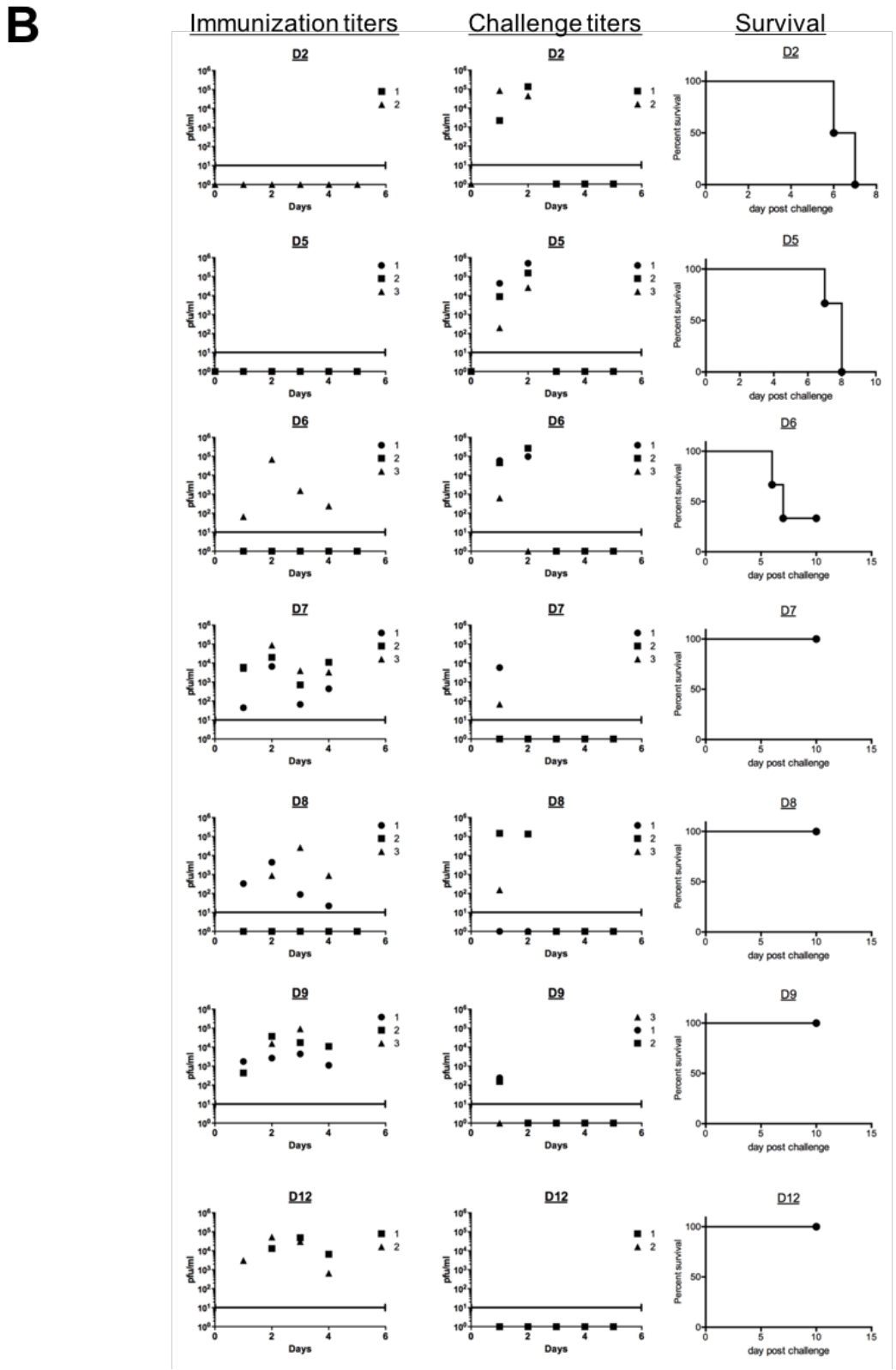

B
Immunization titers
Challenge titers
Survival
D2
D5
D6
D7
D8
D9
D12
pfu/ml
Days
Percent survival
day post challenge

Fig. 20: Standardization of the 7 day injectable E2 model for intravaginal HSV-2 infection. A) Experimental plan to standardize the 7 day injectable model. OVX mice were injected with 500ng E2 daily for 7 days subcutaneously. At days 2, 5, 6, 7, 8, 9 and 12 post hormone treatment, mice were divided into groups and infected with $1x10^5$ pfu/mouse (TK−) HSV-2, and vaginal washes were collected for 5 days post-immunization. Three weeks post immunization, all groups of mice were challenged with $1x10^5$ pfu/mouse WT HSV-2. Vaginal washes were collected for 5 days post challenge, and survival was monitored. Viral titrations were conducted, and B) post-immunization and post-challenge shedding and survival was plotted.

3.3.4: Phenotype of vaginal APCs from 7 day hormone-treated mice before and after immunization with HSV-2 TK-.

In the previous section, we optimized a 7 day regime which could enable us to consistently conduct intravaginal infections in E2 treated mice. Next, we wanted to use this model to examine whether sex hormones such as E2 and P4 influence anti-viral responses by modulating the phenotype of vaginal APCs. Given that P4 treated mice are immediately susceptible to intravaginal HSV-2 infection, we had to stagger our hormone treatments: E2 treatment group received 500ng of injectable E2 for 7 days (D1-7); P4 and mock control groups received 500ug of injectable P4 or 100ul of sterile saline (mock control) for the next seven days (D7-13). Finally, on D14, vaginal tissues from hormone-treated mice were isolated to examine APC phenotype and function by flow cytometry.

First, we examined whether the 7-day injectable E2 regime could reproduce the phenotypic differences observed in the pellet treatment model (section 3.3.1). Vaginal tissue cells from hormone treated mice were isolated and stained with antibodies against surface markers: CD19, CD11c, CD11b and MHCII, and intracellular markers IL-12 and TNF-α (T_h1 associated cytokines that are important for an anti-viral HSV-2 T_h1 response (Milligan et al., 1998; Milligan et al., 2004)), using standard protocols without any *in vitro* stimulation (see materials and methods section 2.5 for detailed protocols).

E2-treated mice contained a higher proportion of $CD11c^{+}$ $CD11b^{hi}$ $MHCII^{-}$ DCs, and $CD11c^{-}$ $CD11b^{+}$ $SSC\text{-}A^{low}$ cells (a phenotype similar to $CD11c^{-}$ $CD11b^{+}$

F4/80^{+} macrophages or monocytes (Hastings, 2014)) compared to P4 or mock controls (Fig. 21A). This was consistent with the observations in the 21-day pellet model (seen in section 3.3.1). Intracellular staining showed no significant differences in the expression of TNF-α or IL-12 (Fig. 21B) in vaginal DCs or macrophages between E2, P4 or mock treated mice.

Overall, these results show that the 7-day E2 regime is a suitable alternative to the 21-day pellet model (section 3.3.1), because it enables us to consistently infect a large group of mice, while preserving the expected phenotype of increased CD11b^{+} myeloid DCs and macrophages in E2 treated mice compared to P4 or mock controls.

Next, we wanted to examine the influence of hormones on vaginal APCs in the context of HSV-2 infection. Mice treated with the 7 day hormone regime, as described above, were infected intravaginally with 1x10^{5} pfu/mouse (TK-) HSV-2, 24h after the last hormone injection (D14). One-day post infection (D15), vaginal tissue cells were isolated, stained with the same panel of surface and intracellular markers as described above, and characterized by flow cytometry. Unlike the observations made in uninfected mice (described above, Fig. 21A), 24h post-infection, there were no differences in the proportions of CD11c^{+} CD11b^{hi} MHCII^{-} DCs, or CD11c^{-} CD11b^{+} SSC-A^{low} macrophages (Fig. 21C). However, intracellular staining for TNF-α and IL-12 showed that CD11c^{+} DC and CD11c^{-} CD11b^{+} SSC-A^{low} macrophages from P4-treated mice expressed higher levels of

TNF-α compared to E2 or mock controls (Fig. 21D). There were no differences in IL-12 expression between all three groups of mice.

These results indicate that post intravaginal HSV-2 infection, while there were no differences in the proportion of resident populations among E2 or P4 treated mice, P4 may induce a more potent inflammatory TNF-α^+ phenotype in vaginal DCs and macrophages.

Overall, these results indicate that the sex hormones such as E2 and P4 may influence the phenotype of vaginal APC populations.

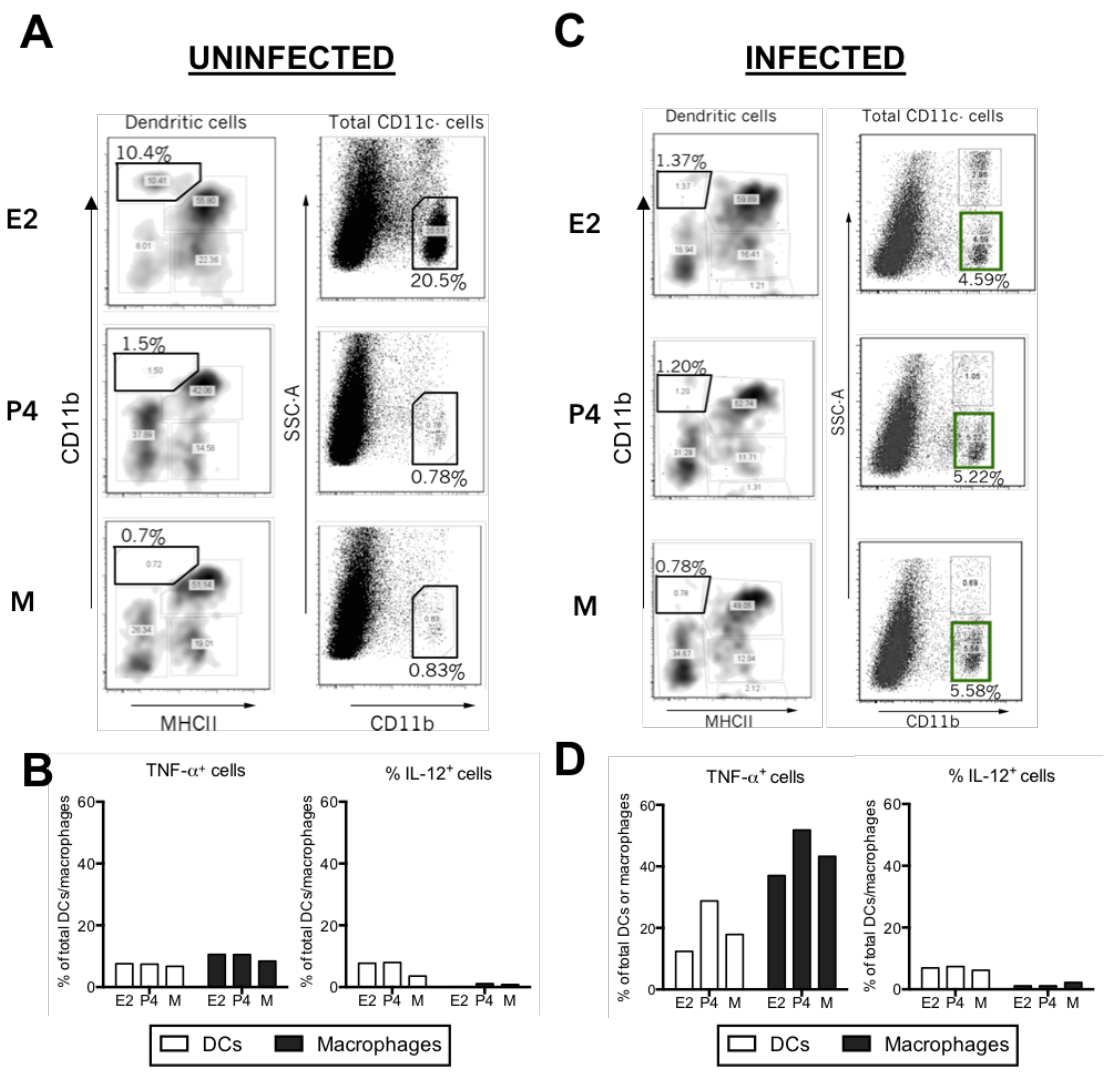

A
UNINFECTED
Dendritic cells
Total CD11c· cells
E2
P4
M
10.4%
20.5%
1.5%
0.78%
0.7%
0.83%
CD11b
SSC-A
MHCII
CD11b
C
INFECTED
Dendritic cells
Total CD11c· cells
1.37%
4.59%
1.20%
5.22%
0.78%
5.58%
B
TNF-α+ cells
% IL-12+ cells
% of total DCs/macrophages
E2 P4 M E2 P4 M
DCs
Macrophages
D
TNF-α+ cells
% IL-12+ cells
% of total DCs or macrophages
% of total DCs/macrophages

Fig. 21: The effect of sex hormones E2 and P4 on the phenotype of vaginal APC populations in uninfected and infected mice. A) OVX mice were divided into three groups: Group 1 (n=6) was treated with 500ng injectable E2 for 7 days. Starting on the last day, group 2 (n=6) and 3 (n=6) of OVX mice were injected with 500ug P4 or 100ul saline from D7-D13. On day 14, vaginal tissues were isolated, blocked with Brefeldin-A for 6h, and stained with surface antibodies against CD11c, CD11b, MHCII and CD19. They were then fixed and stained with intracellular antibodies against TNF-α and IL-12, before characterization on a BD LSR II flow cytometer to identify $CD11c^{+}$ $CD11b^{+}$ $MHCII^{-}$ DC populations and $CD11c^{-}$ $CD11b^{+}$ SSC-A^{low} macrophages. B) Graph representing TNF-α and IL-12 expression in total CD11c+ DCs and macrophages. C) In a separate experiment, OVX mice were treated with hormones as described above, and 24h after the final hormone injection, infected intravaginally with $1x10^{5}$ pfu/mouse (TK-) HSV-2. One day later, vaginal tissue cells were isolated, stained and characterized as described above. D) Graph representing TNF-α and IL-12 expression in total CD11c+ DCs and macrophages from mice 24h post infection. Data is representative of a single experiment with pooled tissues from n=6 mice per group.

3.3.5: Total vaginal cells and CD11c$^+$ DCs from E2, P4 or mock-treated mice induce similar levels of CD4$^+$ T cell differentiation.

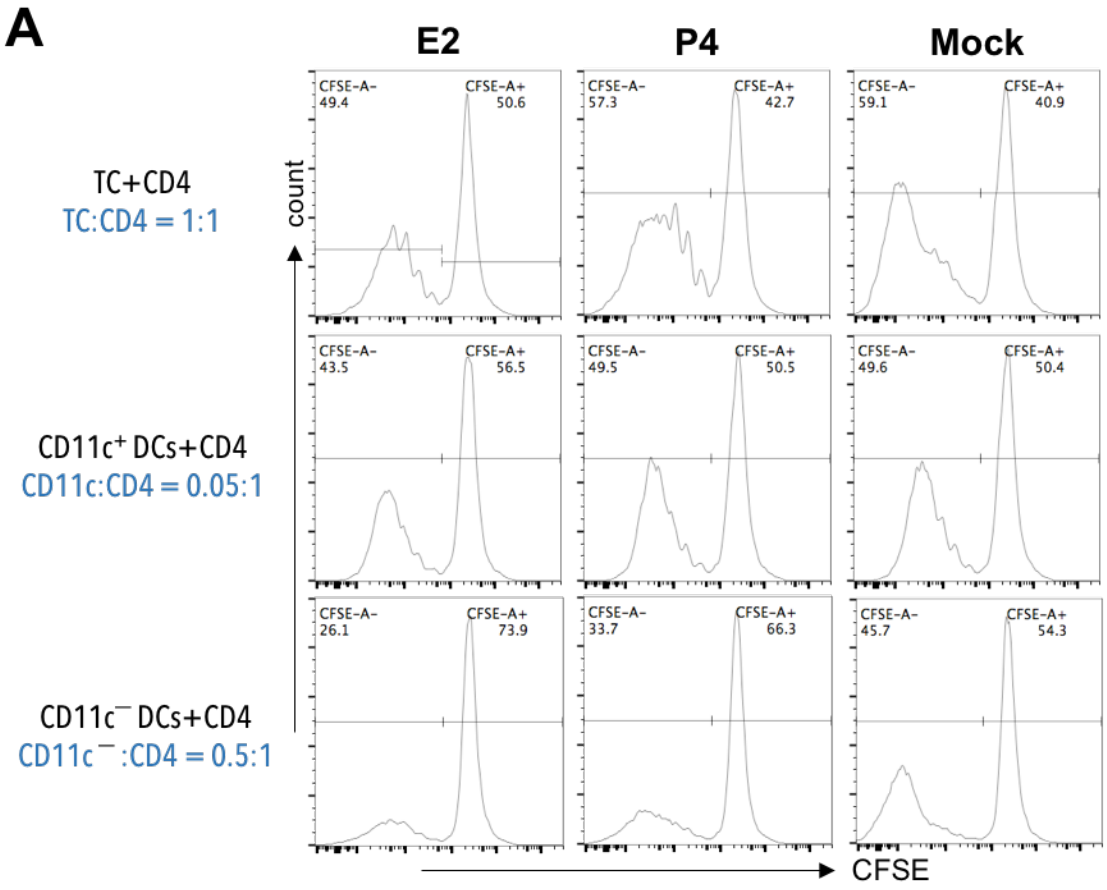
In the previous section, we showed that the sex hormones E2 and P4 could influence the phenotype of vaginal APCs. The next step was to examine whether there were differences in the functional abilities of these cells at inducing CD4$^+$ T cell responses. We used the previously standardized *in vitro* co-culture model to directly examine and compare the functional capabilities of vaginal APCs (section 3.1.2).

OVX mice were treated with E2, P4 or saline (mock) for 7 days (as described above in section 3.3.4), and vaginal tissue cells (TC) were isolated 24h post last hormone treatment. CD11c$^+$ DCs were magnetically sorted using CD11c microbeads (Miltenyi Biotec, USA), according to manufacturer protocols. Vaginal TCs or CD11c$^+$ DCs were pulsed with OVA-peptide, and co-cultured with OT-II Tg CD4$^+$ T cells at the TC:CD4 or CD11c:CD4 ratios indicated in Fig. 22, for 3.5 days. CD4$^+$ T cell responses were compared among co-cultures by flow cytometry.

There were no significant differences in the level of CD4$^+$ T cell proliferation among co-cultures conducted with TC from E2- or P4- or placebo-treated (mock) mice (Fig. 22A). Furthermore, CD11c$^+$ DCs at a CD11c:CD4 ratio of 0.05:1, were able to induce similar levels of T cell proliferation seen in total cell co-cultures at a TC:CD4 ratio of 1:1; while CD11c$^-$ cells induced significantly lower CD4$^+$ T cell proliferation at a CD11c$^-$:CD4 ratio of 0.5:1 compared to total

cells or CD11c co-cultures (Fig. 22A & B). This showed that $CD11c^+$ DCs were the primary APC population that can induce $CD4^+$ T cell responses in these co-cultures. This was consistent with the observations made in section 3.1.4 and section 3.1.5 of this dissertation.

Overall, the results of this section suggest that $CD11c^+$ DCs from mice treated with E2 or P4 using the 7-day regime, are equally capable at inducing $CD4^+$ T cell differentiation.


A
E2
P4
Mock
TC+CD4
TC:CD4 = 1:1
CD11c+ DCs+CD4
CD11c:CD4 = 0.05:1
CD11c− DCs+CD4
CD11c−:CD4 = 0.5:1
count
CFSE
CFSE-A-
CFSE-A+
49.4
50.6
57.3
42.7
59.1
40.9
43.5
56.5
49.5
50.5
49.6
50.4
26.1
73.9
33.7
66.3
45.7
54.3

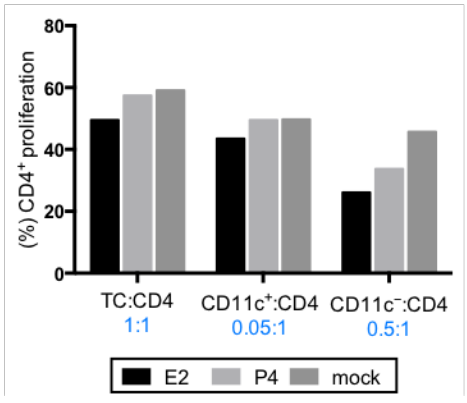

B
(%) CD4+ proliferation
80
60
40
20
0
TC:CD4
1:1
CD11c+:CD4
0.05:1
CD11c−:CD4
0.5:1
E2
P4
mock

Fig. 22: Comparison of $CD4^+$ T cell proliferation induced by total vaginal cells (TC) or $CD11c^+$ DCs from hormone treated mice. OVX mice were treated for 7 days with injectable E2, P4 or saline (mock), as described in section 3.3.4. Vaginal tissue cells (TC) were isolated, $CD11c^+$ DCs were purified by MACS, and TC, or $CD11c^+$ cells, or $CD11c^-$ cells, were pulsed with OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells for 3.5 days. A) CD4+ T cell proliferation was quantified by examining dilution of CFSE dye in CD4+ T cells. B) Graph outlining CD4+ T cell proliferation in TC+CD4, $CD11c^+$+CD4 and $CD11c^-$+CD4 co-cultures from E2, P4 or mock treated mice.

3.3.6: Vaginal APCs from HSV-2 infected mice induce inflammatory IFN-γ and TNF-α responses in co-cultures

In section 3.3.5, we had shown that TC from E2 and P4 treated mice were equally capable at inducing $CD4^+$ T cell proliferation in co-cultures. However, given that $CD4^+$ T cells can differentiate into a variety of effector phenotypes (Luckheeram et al., 2012), the examination of cytokines in these co-culture supernatants was essential for comparing the functional propensities of APCs from hormone-treated mice. But first, we wanted to identify the optimal time point post infection (p.i.) to examine functional differences in DC responses between E2 and P4 treated mice in the context of HSV-2 infection. Hence, we first utilized vaginal cells from OVX mice that were isolated 24h and 48h post (TK-) HSV-2 infection for these experiments; tissue cells from uninfected OVX mice were used as controls.

Vaginal tissue cells (TC) were pulsed with OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells (TC+CD4) for 3.5 days according to previously described protocols (3.3.5). The functional propensity of vaginal APCs from uninfected and infected mice, were compared by measuring TNF-α, IL-12, IL-6, IL-10, IFN-γ and IL-17 levels in co-culture supernatants using a multiplex assay from MSD.

Vaginal TC from uninfected OVX mice produced TNF-α (40 ± 0.2 pg/mL), IL-12 (211 ± 77 pg/mL), IL-10 (839 ± 585_pg/mL), IFN-γ (521 ± 37 pg/mL), IL-6 (6978 ± 300_pg/mL) and IL-17 (24617 ± 3348 pg/mL), in co-cultures (Fig. 23). In

co-cultures conducted with TC from infected mice 24h p.i., there was an apparent induction in TNF-α (121 ± 20 pg/mL) and IFN-γ (6602 ± 977 pg/mL), and a reduction in IL-6 (3996 ± 83 pg/mL) and IL-17 (20105 ± 263 pg/mL). However, there were no discernable differences in IL-10 (1122 ± 596 pg/ml) or IL-12 (278 ± 38 pg/ml) levels. This suggests that intravaginal infection with HSV-2 can influence and direct the function of vaginal APCs in priming $CD4^+$ T cells towards a potent anti-viral T_h1 phenotype with high levels of TNF-α and IFN-γ.

Interestingly, vaginal TCs from infected mice 48h p.i. showed a marked decrease in IL-12 (51 ± 5 pg/mL), IL-10 (457 ± 72_pg/mL), IFN-γ (5085 ± 87 pg/mL) and IL-17 (7609 ± 623_pg/mL) in co-cultures, compared to those isolated at the 24h time point. However, there was a further increase in TNF-α (219 ± 96 pg/mL), while IL-6 (4667 ± 3353 pg/mL) remained relatively similar to levels seen in cultures with TC from the 24h time point. Given that APCs such as DCs migrate to the draining lymph nodes 24-48h post infection, and the reduction in cytokine responses, this suggests that 24h p.i. may be the ideal time point to examine vaginal DC responses to HSV-2 infection.

We also examined cytokine responses in co-cultures with iliac lymph node tissue cells (TC) isolated from these groups of mice. Largely, these co-cultures produced lower levels of all these cytokines in comparison to vaginal tissue co-cultures (Fig. 23: right panel LYMPH NODES). However, there was a slight increase in TNF-α, IL-12, IL-10, IFN-γ and IL-17 levels in co-cultures conducted with TC from the 48h p.i. group, compared to those in the 24h p.i. or control

uninfected groups. Statistical analysis could not be performed in these experiments due to the lack of sufficient replicates (n=2 independent wells/condition).

Overall, these results suggest that intravaginal HSV-2 infection can directly influence the function of vaginal APCs to induce potent inflammatory IFN-γ and TNF-α responses, and 24h p.i. may be the ideal time point to compare functional differences in APC responses between E2 and P4 treated mice.

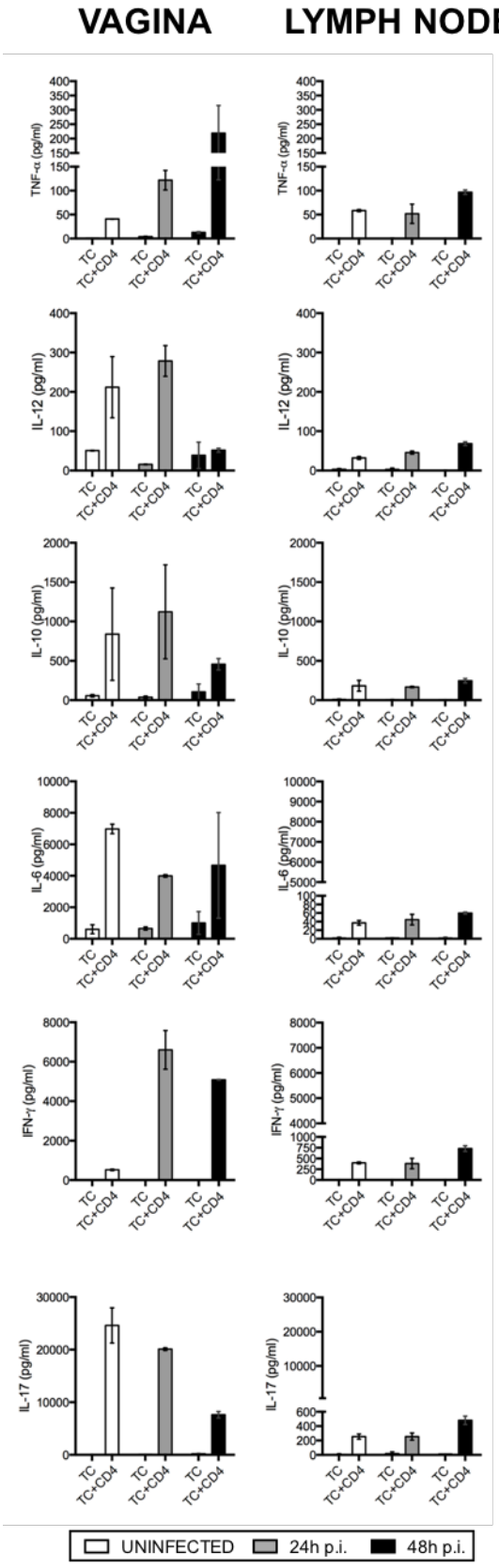


Fig. 23: Cytokine responses in co-culture conducted with vaginal APCs fror uninfected and (TK-) HSV-2 infected mice OVX mice were infected with $1x10^5$ pfu/mous (TK-) HSV-2 (n=12), and vaginal tissues an their draining iliac lymph nodes were isolated 2 hours (24h p.i.) (n=6) or 48h (48h p.i.) (n=6 post infection. Tissue cells (TC) were peptide pulsed, and co-cultured with OT-II Tg $CD4^+$ cells for 3.5 days in duplicate wells pe condition. Cytokine responses in co-cultur supernatants were measured by MSD multiple assays. Data is represented as the mean±SD c 2 replicates per culture condition.

3.3.7: E2 conditioning of vaginal DCs is absent in mice treated with E2 using the 7-day treatment regime

The optimizations conducted in sections 3.3.2 to 3.3.6 had enabled us to develop a model to directly compare the functional responses of vaginal APCs in HSV-2 infected E2 and P4-treated mice. Previous studies in our lab where mice were immunized under the influence of hormones correlated E2 with protection, and P4 with inflammation, post WT HSV-2 challenge (Bhavanam et al., 2008; A. Gillgrass et al., 2010). In these *in vivo* infection models, the event of immunization under the influence of either E2 or P4, is the only difference in these groups of mice. The 7-day regime developed and outlined above in section 3.3.3 to 3.3.6, appeared to be a promising model to examine whether differences in protection quality post HSV-2 challenge in hormone-treated mice, were related to functional differences in APC populations.

OVX mice were treated with E2, P4 or saline (mock) using the 7-day regime (n=6/group) developed in section 3.3.4. Twenty-four hours after the last hormone injection (Day 14: as described in section 3.3.4), all three groups of mice were immunized intravaginally with $1x10^5$ pfu/mouse (TK-) HSV-2. One day later (24h p.i.), vaginal tissue cells (TC) from each group of mice were processed and pooled, peptide-pulsed and co-cultured with OT-II Tg $CD4^+$ T cells for 3.5 days. TC from uninfected hormone-treated mice were used as controls. Cytokine responses in co-culture supernatants were measured by MSD multiplex assays.

Vaginal TC from infected mice irrespective of hormone-treatment induced higher levels of TNF-α compared to uninfected controls (Fig. 24). We also observed a decrease in IL-6 and IL-17 24h p.i., while IL-12, IL-10 and IFN-γ was similar between co-cultures with TC from uninfected and infected mice. These results were consistent with observations made in section 3.3.6.

Upon examination of cytokine levels between hormone treatments in uninfected mice, E2 induced higher levels of IL-6 (14452 ± 1040 pg/mL) compared to P4 (5210 ±_1844 pg/mL) or mock (6232 ± 673 pg/mL) treatments (Fig. 24: Uninfected). However, there were no clear differences in TNF-α, IL-12, IL-10, and IL-17 (Fig. 24: Uninfected). This enhancement in IL-6 levels associated with E2, was also observable in co-cultures with TC from HSV-2 infected mice. However, there were no differences in all other cytokines between co-cultures from E2, P4 or mock treated mice (Fig. 24: 24h p.i.). Since these measurements were conducted using duplicates, we were unable to perform statistical analysis

The lack of any differences in IL-17 levels is contradictory to our previous observations in section 3.1.7. In those experiments, we had established that E2 conditioned vaginal DCs to induce significantly more potent T_h17 responses than P4 or placebo controls (Fig. 9: Section 3.1.7). This suggests that the hormone effects on APC functions seen in the pellet model may not be replicated in the 7-day regime where APC function is examined 7 days after E2 treatment. It is likely that the biological effects of E2 on vaginal APC functions may have waned during

this 7-day waiting period. Overall, this suggests that the 7-day E2 regime may not be ideal for obtaining a complete understanding of E2's effects on the function of vaginal APC populations.

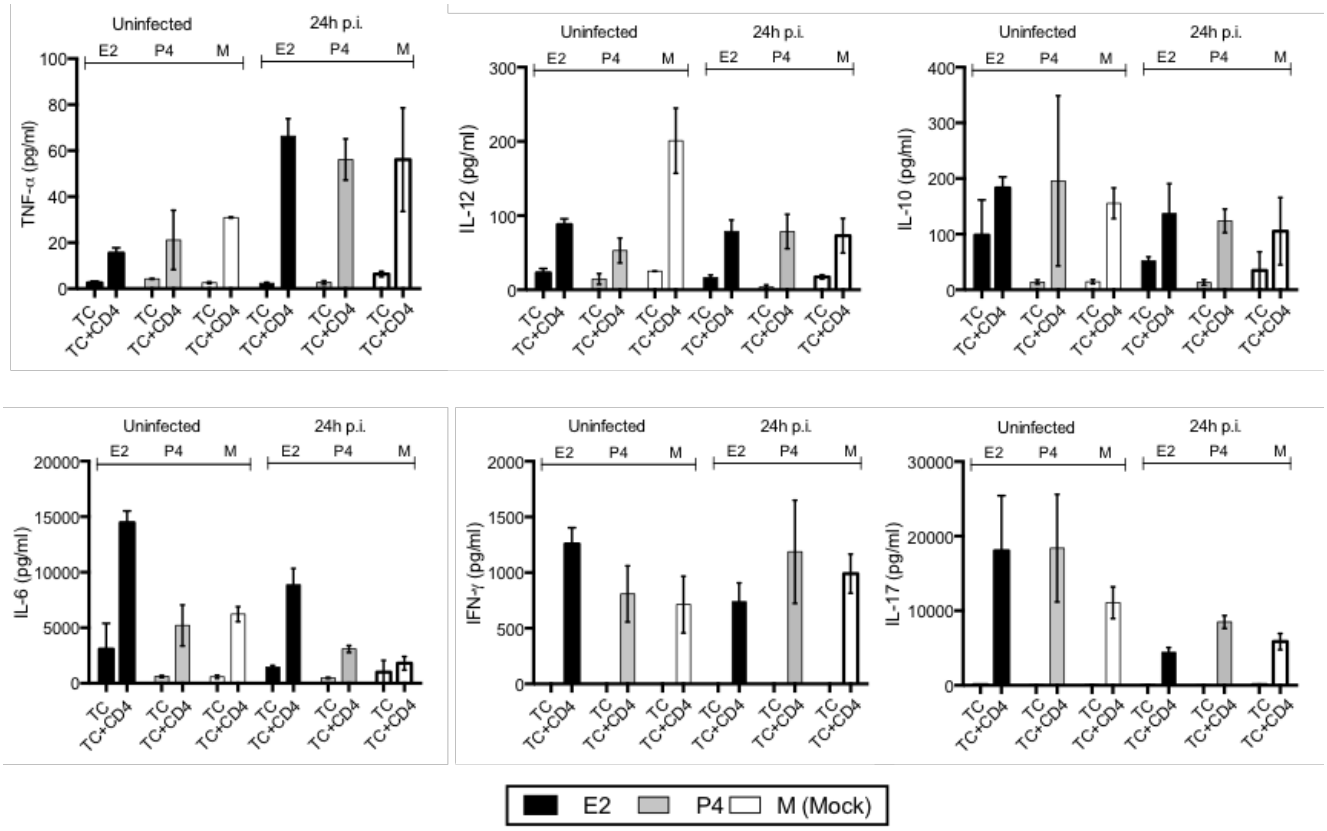


Fig. 24: Comparison of CD4$^+$ T cell responses in co-cultures conducted with vaginal APCs from hormone-treated, uninfected or (TK-) HSV-2 infected mice. OVX mice (n=6/group) were treated with E2, P4 or saline (mock) according to the previously described 7-day regime (see section 3.3.5), and 24h after the last injection, were intravaginally infected with 1x10^5 pfu/mouse (TK-) HSV-2. One day later (24h p.i.), vaginal tissue cells (TC) from each group were pooled, processed, peptide-pulsed and co-cultured with OT-II Tg CD4$^+$ T cells in duplicate wells per culture condition for 3.5 days. A separate group of hormone-treated mice (n=6/group) were used as uninfected controls. Cytokines in co-culture supernatants were measured by MSD multiplex assays. Data presented is mean±SD of duplicates. Statistical analysis was not performed due to the lack of sufficient replicates.

3.3.8: Standardization of a HSV-2 OVA infection model to examine CD4 responses *in vivo*.

The observations made in section 3.3.7 suggest that the 7-day E2 regime may not be optimal for examining hormone effects on vaginal APC function. Hence, we wanted to develop an alternative *in vivo* model to compare hormone effects on the function of vaginal APCs, by examining differences in HSV-2-specific $CD4^+$ T cell responses in infected mice. An HSV-2 specific CD4 transgenic mouse whose $CD4^+$ T cells express TCR specific for HSV-2 proteins would be an ideal choice for our purposes. Previously, others have utilized mice with transgenic $CD8^+$ T cells specific to HSV-2 gB (van Lint et al., 2005; Wallace, Keating, Heath, & Carbone, 1999) to examine cytotoxic T cell responses in the context of HSV-2 infection. However, due to the lack of HSV-2 transgenic mice to precisely examine the virus specific CD4 T cell response, we adopted an infection model with transgenic HSV-2 OVA that has been used by others for examining HSV-2 specific responses (Dobbs et al., 2005). This model facilitates use of OVA-specific OT-II Tg $CD4^+$ T cells, which can then be used to examine and compare virus specific $CD4^+$ T cell responses between hormone-treated mice.

The first step was to validate OVA expression and optimize the adoptive transfer model. To validate OVA expression by HSV-2 OVA, vero cells were infected with HSV-2 OVA at $1x10^7$ pfu/250mL vero flask. Two other flasks: one infected with $1x10^7$ pfu (TK-) HSV-2, and the other uninfected, we used as

controls. Two hours post infection, golgi inhibitors were added to all three flasks, and 18h later, vero cells were collected, and stained with AF-488 Rabbit anti-OVA polyclonal antibody to examine OVA expression by flow cytometry. Over 70% of vero cells infected with HSV-2 OVA expressed were positive for OVA protein (Fig. 25A). Veros from (TK-) HSV-2 infected, and uninfected groups were both negative for OVA expression. This result validated OVA expression by HSV-2 OVA.

Next, we standardized the adoptive transfer model to examine HSV-2 OVA specific CD4+ T cell *in vivo*. CFSE-stained OT-II Tg $CD4^+$ T cells were adoptively transferred by tail-vein intravenous injection (2 x 10^6 cells/mouse) (n=6 mice). Twenty-four hours later, mice were infected with 1x10^6 pfu/mouse HSV-2 OVA (n=3), while controls received PBS (n=3). Three days post infection, the iliac lymph nodes were isolated, processed and stained with antibodies against CD3 and CD4 before analysis on a flow cytometer. Lymph nodes from both groups of mice showed $CFSE^+$ cells confirming the engraftment of OT-II Tg $CD4^+$ T cells (Fig. 25B). Additionally, lymph nodes from HSV-2 OVA infected mice showed differentiation of these transgenic $CD4^+$ T cells evident by the dilution of CFSE dye in the subsequent generations of proliferating CD4+ T cells. This proliferation was absent in controls, confirming that it is a directed response to HSV-2 OVA infection.

Overall, these results validate the use of HSV-2 OVA to examine hormone effects on APC function by monitoring $CD4^+$ T cell responses *in vivo*.

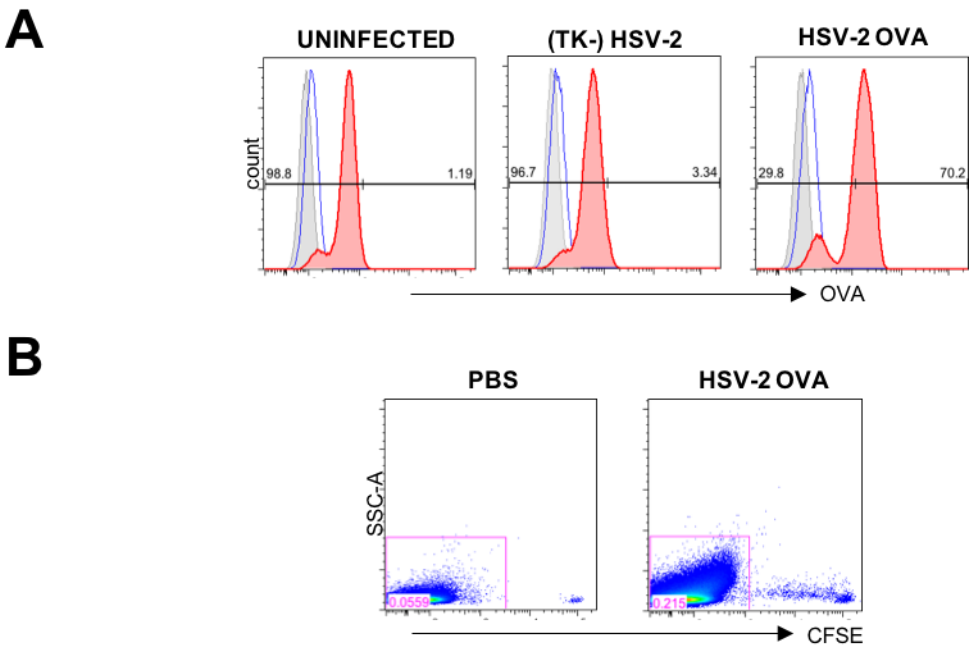


Fig. 25: Optimization of HSV-2 OVA infection model to examine HSV-2 specific CD4+ T cell responses *in vivo*. A) A flask of vero cells was infected with $1x10^7$ pfu HSV-2 OVA. Separate flasks infected with $1x10^7$ pfu (TK-) HSV-2 or uninfected, were used as controls. Two hours post infection, golgi inhibitors were added to all flasks, and 18h later, vero cells were detached with a scraper, and stained with rabbit anti-OVA antibody before analysis on a BD LSR II flow cytometer. A) OVA expression in veros infected with HSV-2 OVA B) CFSE-stained OT-II Tg $CD4^+$ T cells ($2x10^6$ cells/mouse) were adoptively transferred to OVX mice (n=6), and 24h later, 3 mice were infected with $1x10^6$ pfu/mouse HSV-2 OVA, while controls (n=3) received PBS alone. Three days later, iliac lymph node cells were isolated, stained with antibodies against CD3 and CD4, and analyzed by flow cytometry. CFSE expression in $CD3^+$ $CD4^+$ T cells was examined.

3.3.9: HSV-2 OVA model can be used to examine virus specific $CD4^+$ T cell responses *in vivo*

In the previous section (3.3.8), we optimized the HSV-2 OVA infection model to examine virus specific $CD4^+$ T cell responses *in vivo* in the lymph nodes draining the vagina. As a proof of concept, we extended these observations and identify whether E2 can influence anti-viral $CD4^+$ T cell responses in the vagina by influencing local APC functions.

OVX mice (n=5/group) were treated with E2 or saline (mock) using the 7-day regime as described earlier (section 3.3.5). Twenty-four hours after the last saline injection (day 14), CFSE-stained OT-II Tg $CD4^+$ T cells ($2x10^6$ cells/mouse) were adoptively transferred as described above (section 3.3.8), to these mice, and one day later, mice were infected with $1x10^6$ pfu/mouse HSV-2 OVA. Three days later, vagina and draining lymph nodes were isolated, cultured overnight in the presence of golgi inhibitors, and stained with antibodies against CD3, CD4, IFN-γ and IL-17, before analysis on a flow cytometer (Fig. 26A).

We detected a large population (9% (E2) to 11% (Mock)) of IFN-γ^+, and a relatively minor (0.5%) IL-17^+ $CD4^+$ T cell subsets among total $CD3^+$ $CD4^+$ T cells in the lymph nodes (Fig. 26B: Lymph nodes). To identify HSV-2 OVA specific responses, $CFSE^+$ cells were gated within the total $CD3^+$ $CD4^+$ population, and cytokine responses were examined. Remarkably, over 30% of $CFSE^+$ OT-II Tg $CD3^+$ $CD4^+$ T cells in the lymph nodes expressed IL-17, while only 3% expressed

IFN-γ. As expected, based on observations in section 3.3.7, there were no discernable differences between E2 and mock treatment groups.

The examination of virus specific $CD4^+$ T cell responses in the vagina was significantly more difficult, relative to the observations in the lymph nodes (Fig. 26B: Vagina). While we detected overall, and $CFSE^+$ OT-II Tg, $CD3^+$ $CD4^+$ T cells, we could not distinctly identify $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells in the vagina. Further optimization of this protocol may be required to clearly examine anti-viral T cell responses in the vagina.

Overall, these results suggest that the HSV-2 OVA infection model may be useful to examine anti-viral responses of vaginal DCs *in vivo*.

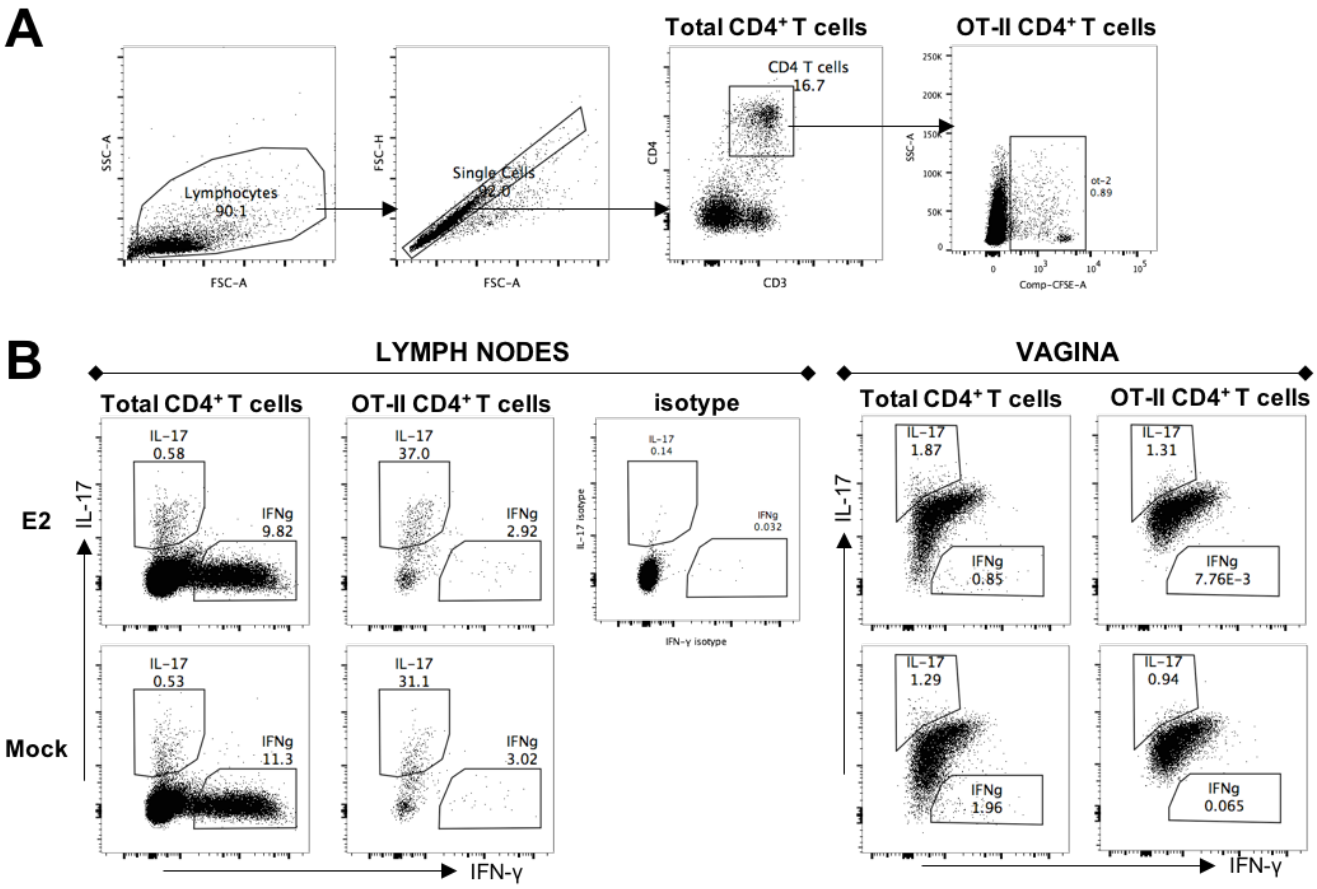


Fig. 26: HSV-2 OVA model to examine HSV-2 specific CD4$^+$ T cell responses *in vivo*. OVX mice were treated with E2 or saline (mock) using the previously described 7-day regime. On day 13 CFSE-stained OT-II Tg CD4+ T cells were adoptively transferred (2x10^6 cells/mouse) to these mice, and 24h later, mice were infected with 1x10^6 pfu/mouse HSV-2 OVA. Three days post infection, vagina and draining iliac lymph nodes were isolated, cultured overnight in the presence of cell stimulation cocktail, and stained with antibodies against CD3, CD4, IFN-γ and IL-17. A) Gating strategy to examine total and OT-II Tg CD3$^+$ CD4$^+$ T cells in the lymph nodes and vagina. B) IL-17 and IFN-γ expression in total and transgenic CD4$^+$ T cells in the draining lymph nodes and vagina.

3.3.10 Summary:

Overall, the results in section 3.3 outline our attempts to standardize an effective model to examine the effect of E2 on anti-viral responses in the vagina. We have confirmed the *in vitro* observations made in section 3.1 *in vivo*, and showed that sex hormones can influence the phenotype of vaginal APCs. However, we have also shown that intravaginal infection models using the 21-day or 7-day treatment regimes may not be effective strategies to examine hormone effects on anti-viral responses. Alternative models such as the previously described intranasal immunization model (Bhavanam et al., 2008) may serve as feasible approaches to address our hypothesis. While not described in this dissertation, we have utilized this model and confirmed our *in vitro* observations, and shown that E2 induced T_h17 responses may be linked with increased efficiency of anti-viral T_h1 responses in the vagina (Anipindi V, Roth K, et al., See appendix A). Finally, the early observations in the HSV-2 OVA infection model highlight the promising possibilities of using this transgenic virus as an effective tool to examine HSV-2-specific responses in the reproductive tract.

CHAPTER 4

DISCUSSION

4.1 Overall Summary:

In this body of work, we have shown that factors in the local mucosal microenvironment of female genital tract, in particular, the sex hormones E2 and P4, commensal microflora, and IL-17, produced by innate and adaptive lymphocytes, can influence vaginal immune responses (Fig. 27). We have shown that E2 can influence the phenotype of DCs and macrophages in the vagina. Furthermore, *in vitro* we have shown that E2 conditioned vaginal DCs to become potent inducers of T_h17 responses in APC-T cell co-cultures. This conditioning was dependent on the production of IL-1β by vaginal DCs. The induction of T_h17 responses, under the influence of E2 is a novel and unique observation that is specific to vaginal DCs. DCs from the spleen or other mucosal tissues did not produce as robust T_h17 responses as those seen in vaginal cell co-cultures. Furthermore, the phenotype and function of APCs present in the draining lymph nodes of mucosal tissues did not reflect that observed in their respective mucosa. This suggests that under homeostatic conditions, mucosal APCs are unique and distinct from those in their draining lymph nodes or other distal tissues (i.e., spleen).

We have also shown that *in vivo* in the vagina, under homeostatic conditions, IL-17 is produced by a heterogeneous population of innate lymphocytes. In addition to E2, factors in the local microenvironment such as the microflora, and these innate lymphocytes, play an important role in the IL-1-dependent T_h17 responses primed by vaginal DCs.

In order to verify these *in vitro* results, we optimized an *in vivo* model where mice treated with E2 for 7 days, could subsequently be infected intravaginally with HSV-2, one week following hormone treatment. However, *in vitro* co-cultures conducted with vaginal cells from the 7-day E2-treated mice, infected with HSV-2, did not show any differences in T_h17 responses compared to mock controls, suggesting that a different *in vivo* model may be required to differentiate E2's effects on anti-viral immune responses.

Based on our previous studies, we subsequently utilized an intranasal immunization model (Bhavanam et al., 2008) to examine the role of E2-conditioned T_h17 responses in HSV-2 anti-viral immunity. While not described in this dissertation, the observations made in this model have allowed us to link T_h17 responses to the increased efficiency of anti-viral T_h1 responses seen in E2-treated mice (Fig. 27). Some of these observations have been described in the dissertation published by K.L Roth, and a manuscript discussing this data is currently under review in Mucosal Immunology, Nature Publishing Group (Anipindi V, Roth K, et al., See appendix A).

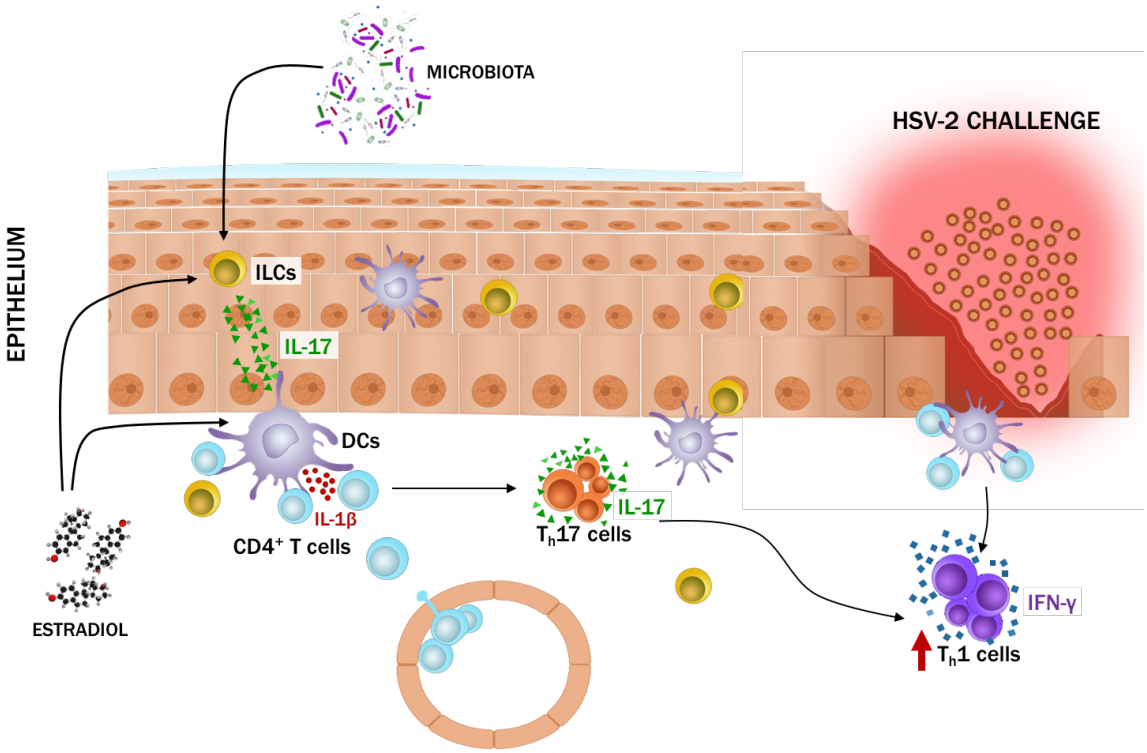


Fig. 27: Summary: The effect of E2 and local microenvironment on vaginal DCs. DCs in the vaginal tract play a critical role in mediating anti-viral responses against genital HSV-2 infection. Based on the observations made in during this dissertation, we propose that *in vivo*, E2 can condition vaginal DCs to produce IL-1β and thereby, induce T_h17 responses (section 3.1). Furthermore, E2 and microflora can regulate constitutive IL-17 producing innate lymphocyte populations, and IL-17 produced by these innate cells is important for the potentiation of the aforementioned T_h17 responses primed by vaginal DCs (section 3.2). In the context of intravaginal HSV-2 challenge, the presence of these T_h17 responses was associated with earlier and more robust IFN-γ^+ T_h1 responses (attached manuscript Appendix A).

4.2 E2 conditions vaginal DCs to become potent inducers of Th17 responses through an IL-1-dependent pathway.

Sex hormone receptors are differentially expressed in various tissues (Couse et al., 1997; Kuiper et al., 1997), and on immune cells including APCs like DCs and macrophages (Kovats et al., 2010; Verthelyi, 2001, 2006). Studies conducted with bone marrow precursor derived cultures (BMDCs) demonstrate that hormones can influence the differentiation and function of DCs and macrophages (Kovats, 2015). However, given the fundamental differences in antigens encountered by the immune system in different mucosal tissues, it is likely that their resident APCs may have adopted distinct phenotypes and/or functions to address these challenges. While few studies have compared APC populations between the lung and intestinal mucosa (Lefrancois & Puddington, 2006; Persson et al., 2013), vaginal APCs have never been compared to other mucosal APCs. Hence, we first addressed this by comparing the phenotype and function of vaginal APC populations with other tissues.

In our studies, we examined DC populations by gating $CD11c^+$ cells, and macrophages by gating $CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$ cells in the lung, vagina, and small intestine. Others who have investigated DCs in mucosal tissues have defined these cells based on markers such as CD11c, MHCII, CD8α, CD103, F4/80 and CD205 (Chirdo et al., 2005; Coombes et al., 2007; Iijima et al., 2007; Iwasaki, 2007; Jaensson et al., 2008; von Garnier et al., 2005; Zhao et al., 2003). We chose CD11c as a broad marker to define mucosal DC populations in all

tissues in order to make equivalent comparisons. However, this strategy to identify DCs using such a broad marker does have its drawbacks. For example, other studies examining $CD11c^+$ DCs in the lung (von Garnier et al., 2005) have found that under steady state conditions, $CD11b^-$ $MHCII^-$ alveolar macrophages also express CD11c, and may suppress infiltration of migratory DC populations into the lung lamina propria (LP) (Holt et al., 1993; Jakubzick, Tacke, Llodra, van Rooijen, & Randolph, 2006). Thus, these alveolar macrophages may likely represent the primary $CD11c^+$ population (78% of total $CD11c^+$ cells) that we have observed in the lung. However, given that we primarily used total tissue cells in our co-cultures, using CD11c as a distinguishing marker for DCs does not deter us from examining the total potential of APCs in any specific mucosa.

Our phenotypic comparison of mucosal APCs showed a large population of neutrophils present in the vagina during steady state (Fig. 1). The infiltration of neutrophils into the vagina is dependent on the stage of the estrous cycle (Sasaki, Nagata, & Kobayashi, 2009). Although neutrophils are not a professional APC, upon co-incubation with OT-II Tg $CD4^+$ T cells, they can up-regulate MHCII and CD86 on their surface, and present OVA antigen to $CD4^+$ T cells to induce T_h1 and T_h17 responses (Abi Abdallah, Egan, Butcher, & Denkers, 2011). With this in mind, we also sorted vaginal $CD11c^-$ $CD11b^+$ $F4/80^-$ $Gr\text{-}1^+$ neutrophils, and co-cultured them with OT-II Tg $CD4^+$ T cells. Evidenced by the lack of IL-17 induction in neutrophil co-cultures, neutrophils did not directly play a role in vaginal T_h17 responses (Fig. 8). However, given the role of IL-17 in the induction

of neutrophilia, their presence may be functionally important in the vaginal microenvironment, such as for immune responses post intravaginal HSV-2 infection (Milligan, 1999).

In order to compare the functional characteristics of APCs from various mucosal tissues, we optimized a previously described chicken ovalbumin peptide based co-culture model (Parish et al., 2009; Robertson et al., 2000). By using a consistent population of transgenic antigen-specific $CD4^+$ T cells, this model allowed us to compare the function of various APC populations across different mucosal tissues. We did not observe any significant differences in $CD4^+$ T cell proliferation among co-cultures from any of the mucosal or non-mucosal tissues (Fig. 5A). Given that $CD4^+$ T cells differentiate into a variety of T helper subtypes, we next examined cytokines within these culture supernatants (Zhu & Paul, 2010). While others have suggested that there may be a T_h2 bias to immune responses primed by APCs in the lung and small intestine (Kjerrulf, Grdic, Kopf, & Lycke, 1998), we did not observe such differences in T_h1 or T_h2 cytokine responses between lung, intestine or vaginal co-culture supernatants. However, the most distinct observation was the presence of significantly higher T_h17 responses (IL-17 and IL-22 levels) in vaginal co-cultures compared to other mucosal tissue co-cultures, indicating that under homeostatic conditions, vaginal APCs were potent inducers of T_h17 differentiation compared to other mucosal APCs.

Although intracellular staining showed that OT-II Tg $CD4^+$ T cells were the primary source of IL-17 in these co-cultures, $\gamma\delta^+$ $CD4^-$ T cells may also contribute to the total IL-17 levels. This is consistent with published reports, which have shown that $\gamma\delta^+$ $CD4^-$ T cells are the primary producers of IL-17 under steady state *in vivo* in the genital tract (J. O. Kim et al., 2012). $\gamma\delta^+$ TCR T cells regulate tissue remodeling, and are among the early innate barriers against extracellular pathogens in mucosal tissues (Gladiator, Wangler, Trautwein-Weidner, & LeibundGut-Landmann, 2013; Khader, Gaffen, & Kolls, 2009; Spits & Di Santo, 2011; Sutton et al., 2012). In chapter 3.2, we characterized innate lymphocytes in the vagina that produce IL-17, and examined the role of IL-17 produced by these cells on T_h17 responses primed by $CD11c^+$ DCs. Our observations have been addressed in section 4.3 of this Discussion.

Next we identified the subsets of vaginal APCs critical for these robust T_h17 responses. $CD11c^-$ cells such as macrophages and other non-DCs were responsible for less than 10% of the T_h17 response (Fig. 8B & 8C), while $CD11c^+$ DCs were the primary APC subset responsible for over 90% of T_h17 differentiation in vaginal co-cultures (Fig. 7, 8B & 8C). We employed two distinct approaches to assess the requirement of $CD11c^+$ cells to T_h17 responses: 1) Conditional $CD11c^+$ DC ablation model: A CD11c DTR mouse model was standardized to deplete over 70% of $CD11c^+$ DCs in the vagina. Although, there are inherent limitations to using this model (as it uses CD11c, a broad marker to define DCs (van Blijswijk, Schraml, & Reis e Sousa, 2013)), it allows us to

determine the effect of depleting $CD11c^+$ cells *in vivo* in hormone-treated mice. 2) Fluorescence-activated cell sorting (FACS) allowed us to selectively examine the function of $CD11c^+$ cells. However, this technique is also associated with its own limitations: i) Low cell yield: Since DCs are relatively rare in the vagina, a large number of mice (n=10 to 15) were required to obtain a total of $5x10^5$ DCs to carry out these experiments with sufficient replicates. ii) Low viability: $CD11c^+$ DCs are highly sensitive to electrical charges that are used to deflect cells into collection tubes during sorting. Hence, the extensive tissue processing, and FACS sorting procedures can lead to cell death prior to conducting co-cultures iii) Duration of cell-sorting: The sorter requires up to 3 hours per sample to sort APC subsets. These cells are stored in collection tubes on ice until the entire sorting process is completed. The prolonged wait time increases the likelihood of cell death making it extremely challenging to collect live purified vaginal $CD11c^+$ cells. Despite this, we are able to obtain highly pure (>98%) $CD11c^+$ DCs in sufficient numbers to discern the role of APC subsets in mucosal T helper (T_h) differentiation.

While others have shown that $CD11c^+$ DCs in the vagina (Hervouet et al., 2010), lungs (Fei et al., 2011) and intestine (Denning et al., 2007) can prime T_h17 responses, our study is the first to show that vaginal $CD11c^+$ DCs were significantly more potent inducers of T_h17 responses than $CD11c^+$ DCs from the lungs or small intestine. Multiple pathways have been attributed to the differentiation of T_h17 responses by DCs (Muranski & Restifo, 2013). These pathways involve IL-6, IL-23, TGF-β, IL-1β and IL-23 among others. Our results

indicate that $CD11c^+$ DCs induced T_h17 responses in vaginal tissue co-cultures via an IL-1-dependent mechanism (Fig. 10). While high levels of IL-6 were also observed in vaginal co-cultures (Fig. 10A), these T_h17 responses were induced independently of IL-6 as seen by: 1) T_h17 responses in vaginal co-cultures conducted with cells isolated from IL-6KO mice (Fig. 10C & E), and 2) using anti-IL-6 and anti-IL-6R neutralization antibodies to block the effect of IL-6 *in vitro* (Fig. 10D). With both these approaches, there was no change/difference in T_h17 responses in co-cultures.

While IL-6, along with IL-23 and TGF-β, is required for the canonical pathway of T_h17 differentiation (Muranski & Restifo, 2013), IL-6-independent T_h17 responses have also been identified in mucosal tissues (Chung et al., 2009; Kimura et al., 2007). For instance, in the LP of the small intestine, the IL-1β-IL-1R pathway was essential for the differentiation of steady state T_h17 cells in response to the microflora, independent of IL-6 (Shaw et al., 2012). In the context of a pathogenic infection, while IL-6-dependent T_h17 responses were critical for the clearance of *Citrobacter rodentium* (Li et al., 2014), IL-6-independent T_h17 cell responses were important for the resolution of *Heligmosomoides polygyrus* infection (K. A. Smith & Maizels, 2014). Collectively, these observations suggest that the type of antigen and/or the local cytokine milieu can determine the pathway of T_h17 differentiation. We found that while IL-1β and IL-6 were both produced by vaginal DCs, only IL-1β was critical for vaginal T_h17 responses (Fig. 10C & F). However, constitutive IL-6 production in the vagina may play a role in

the T_h17 responses mediated by macrophages (Fig. 8B). Further experiments focused on the function of macrophages are needed to confirm the role of IL-6 in macrophage-induced T_h17 responses.

Given that sex hormones such as E2 and P4 can influence the differentiation and function of $CD11c^+$ cells (Butts et al., 2007; Carreras et al., 2010; Liang et al., 2006; Mao et al., 2005; Paharkova-Vatchkova et al., 2004), we examined the role of hormones in generating vaginal T_h17 responses (Fig. 9). By using ovariectomized (OVX) mice, ovary-intact mice grouped into either the E2-high estrus phase or the P4-high diestrus phase, and ERKO mice, we were able to show that T_h17 responses primed by vaginal DCs were critically dependent on the interaction between E2 and ERα (Fig. 9). We were also able to link E2 to IL-1β-mediated induction of vaginal T_h17 responses; E2 primed $CD11c^+$ DCs expressed significantly higher levels of IL-1β, compared to controls (Fig. 10H). In order to examine the pathway/s that link E2 to IL-1 production and T_h17 differentiation, we examined intracellular factors that are induced by E2 and are involved in T_h17 differentiation.

IRF4 is among the intracellular factors expressed in lung and intestine DCs that has been implicated to play an important role in priming T_h17 responses (Persson et al., 2013; Schlitzer et al., 2013). Additionally, E2 can induce IRF4 expression in DCs generated from bone marrow precursor cultures (Carreras et al., 2010). Hence, we sought to examine whether IRF4 was involved in the induction of T_h17 responses by E2-primed vaginal DCs. While we were able to

show that E2 did indeed upregulate IRF4 expression in vaginal DCs *in vivo* (Fig. 11A), IRF4 was dispensable for T_h17 differentiation *in vitro,* since vaginal TC from IRF4 KO mice were intact in their ability to prime T_h17 responses (Fig. 11C). This is in stark contrast to lung and intestinal DCs, which have been reported to be critically dependent on IRF4 for priming T_h17 responses (Persson et al., 2013; Schlitzer et al., 2013). These observations further support our hypothesis that vaginal DCs are unique compared to other mucosal DCs. IRFs are pleiotropic transcription factors expressed by many immune cells such as DCs, and IRF4 specifically is involved in the differentiation of lymphocytes including $CD4^+$ T cells, $CD8^+$ T cells and B cells (Huber & Lohoff, 2014; Ozato, Tailor, & Kubota, 2007). It is likely that other IRFs or alternative factors may be compensating for the lack of IRF4 in DCs from IRF4 KO mice (Huber & Lohoff, 2014). Alternatively, E2-dependent IL-1β induction in vaginal DCs may occur through a TLR4-dependent pathway, independent of IRF4 (Calippe et al., 2010). Further studies are necessary to examine the associated mechanisms by which E2 induces IL-1β and corresponding T_h17 responses.

While others have examined the functional relevance of T_h17 responses in reproductive tract infections, its role under homeostatic conditions had not been examined prior to this work. IL-17 has been shown to be an important part of the immune response to *N. gonorrheae* and *C. albicans* infections (Feinen et al., 2010; Hernandez-Santos & Gaffen, 2012; Relloso et al., 2012). Vulvovaginal fungal infections affect 70-75% of women, and susceptibility to these infections

has been predominantly correlated to the E2-dominant phase of the reproductive cycle (Jerse, 1999; Relloso et al., 2012; Tarry, Fisher, Shen, & Mawhinney, 2005). Therefore, a pre-programmed T_h17 response under the influence of E2 may represent a pre-existing adaptation for protecting the female genital tract against these pathogens. However, the role of T_h17 responses in viral infections is less clear. Two previous studies have suggested that IL-17 does not have a direct protective role in vaginal HSV-2 infection (Johnson et al., 2010; J. O. Kim et al., 2012). However, both of these studies utilized MPA, a P4 derivative, to make mice susceptible to HSV-2. Previous studies, including our own, have shown that MPA can significantly downregulate endogenous hormones such as E2, and attenuate mucosal anti-viral responses to HSV-2 (A. E. Gillgrass et al., 2003; Jeppsson, Gershagen, Johansson, & Rannevik, 1982; Kaushic et al., 2003). This may have precluded an accurate assessment of the role of T_h17 cells to anti-viral immunity in these studies.

While not included in this dissertation, we subsequently extended the *in vitro* observations described in this dissertation to examine E2-mediated effects *in vivo* in the HSV-2 mouse model. Better protection in E2-treated immunized mice was correlated with rapid and greater recruitment of T_h17 and T_h1 cells in the vagina, post-challenge (See Appendix). Furthermore, we validated the significance of IL-17 using IL-17KO mice, and showed that IL-17KO mice were more susceptible to re-infection with HSV-2, and their vaginal DCs were defective at inducing IFN-γ production from $CD4^+$ T cells. Our results are analogous with

the paradigm described in a *M. tuberculosis* mouse model (Gopal et al., 2012; Khader et al., 2007). In these studies, immunization with a BCG vaccine elicited potent T_h17 responses in the lung, and these T_h17 responses were correlated with increased expression of $CD4^+$ T cell chemo-attractants such as CXCL9 and CXCL10. Correspondingly, the T_h17 responses were also associated with a more efficient T_h1 response that was essential for clearing bacteria post-challenge (Khader et al., 2007). A similar mechanism may be involved in our *in vivo* HSV-2 model, where E2-mediated T_h17 responses lead to early and more efficient T_h1 responses to clear HSV-2 infection post intravaginal challenge, when compared to placebo-treated controls (Anipindi V., Roth K., et al, MI, in revision, appendix A).

In summary, the results described in section 3.1 highlight intrinsic differences in the APC populations among various mucosal tissues. The vaginal mucosa, under homeostatic conditions, has a strong bias for constitutive IL-17 as seen with the presence of tissue-resident innate lymphocytes that produce IL-17, and $CD11c^+$ DCs that are potent inducers of T_h17 differentiation. This phenomenon is dependent on E2, and its effect may be specific to the vagina. In comparison to vaginal DCs, those from lung, spleen and intestine could only induce modest T_h17 responses. Overall, our observations indicate that sex hormones such as E2 have a significant impact on the function of tissue-resident APC populations. This highlights the importance of considering hormonal status

when designing vaccines for the generation of effective genital tract immune responses against STIs.

4.3: IL-17 produced by innate lymphocytes in the vagina is important for vaginal Th17 responses.

In the previous section, we have shown that E2 conditions vaginal $CD11c^+$ DCs to prime T_h17 responses. However, the contribution of other factors in the vaginal microenvironment such as IL-17 produced by innate sources, and the effect of microflora, on E2-primed T_h17 responses was not explored. E2 has been linked to healthy microflora in the vagina (Galhardo et al., 2006; Mirmonsef et al., 2014), and others have linked gut commensal microflora to IL-1β-mediated T_h17 responses primed by DCs in the small intestinal LP (Shaw et al., 2012). Others (J. O. Kim et al., 2012) and we (described in section 3.1.3) have observed innate lymphocytes in the vagina that produce IL-17, and these innate lymphocytes have been linked to the generation of mucosal T_h17 responses (Do et al., 2011; Do et al., 2012; Hepworth et al., 2013; Qiu et al., 2013; Sutton et al., 2009). Hence, we sought to examine the role of both these factors: microflora and innate IL-17 producers on T_h17 responses primed by vaginal APCs.

A number of studies have shown that innate cells are a more potent source of IL-17 *in vivo* under steady-state conditions compared to T_h17 cells (Lockhart et al., 2006; S. M. Schulz et al., 2008; Sutton et al., 2012). These innate cells may play a critical role in maintaining tissue homeostasis (Sawa et al., 2011). Therefore, we first characterized the innate populations that produce

IL-17 in the vagina under homeostatic conditions. We chose 5 markers to identify IL-17 producers in the vagina (CD3, CD4, γδ, IL-17 and NKp46) based on published literature (Killig et al., 2014), and found 5 distinct cell populations (Fig. 12B). Consistent with previous reports (J. O. Kim et al., 2012), $\gamma\delta^+$ cells were the primary source of IL-17. There were two populations of $\gamma\delta^+$ cells in the vagina: the major **$CD3^+$ $\gamma\delta^+$ $IL\text{-}17^+$** cells (population II), and minor **$CD3^-$ $\gamma\delta^+$ $IL\text{-}17^+$** cells (population IV). Both these populations are consistent with $\gamma\delta^+$ T cells in various organs (K. Sato, Ohtsuka, Watanabe, Asakura, & Abo, 1993). However, we found 3 other populations: 1) **$CD3^{low}$ $CD4^+$ $\gamma\delta^-$ $IL\text{-}17^+$** cells (population I), 2) **$CD3^+$ $CD4^-$ $\gamma\delta^-$ $IL\text{-}17^+$** cells (population III), and 3) **$CD3^-$ $CD4^-$ $\gamma\delta^-$ $IL\text{-}17^+$** cells (population V). Killig M. et al have published a review highlighting markers expressed by type 3 innate lymphoid cells (type 3 ILCs) that can also produce IL-17 (Killig et al., 2014). Based on their criteria (Killig et al., 2014), since neither of these 3 subsets expressed Nkp46, we can identify population 1 as either fetal lymphoid tissue inducer cells (LTis), or NK cell receptors negative (NCR^-) type 3 ILCs. Due to the lack of CD4 expression in population 2 and 3, these can only represent NCR^- type 3 ILCs. Unfortunately, given the limited markers, and the high degree of overlap among both fetal LTis and NCR^- type 3 ILCs (Killig et al., 2014), it is difficult to conclusively identify these three populations. Furthermore, the expression of CD4 in population 1 ($CD3^{-/low}$ $CD4^+$ $\gamma\delta^-$ $IL\text{-}17^+$ cells) also complicates distinguishing these cells from tissue-resident prototypic T_h17 lymphocytes. In the future, two possible avenues can be followed to resolve this

ambiguity: 1) These subsets can be examined in the vagina of athymic nude mice, which can help us exclude any conventional T_h17 cells. 2) Innate sources of IL-17 can be examined in the vagina of GATA3$^{-/-}$ mice. Recently, Serafini R. et al. have shown that GATA3$^{-/-}$ mice lack all intestinal type 3 ILC subsets (Serafini et al., 2014). Since T_h17 cells are not dependent on GATA3 for their development, population 1 can be identified more confidently. We have also used IL-17KO mice, and showed that all the endogenous IL-17 producing populations ($\gamma\delta^+$ and $\gamma\delta^-$ subsets) appear to be intact in IL-17KO mice (Fig. 12C). This shows that the presence of IL-17 itself is not required for seeding these innate lymphocytes in the vaginal mucosa.

Previously, others have shown that type 3 ILCs play a critical role in maintaining mucosal homeostasis against commensal microflora (Qiu et al., 2013; Sawa et al., 2010; Sawa et al., 2011; Sonnenberg et al., 2012; van de Pavert & Mebius, 2010). Additionally, this microflora may have an important role in the development of T_h17 cells under steady state (Shaw et al., 2012). Thus, we examined the role of microflora on IL-17 produced by innate sources in the vagina, and T_h17 differentiation in vaginal co-cultures. The proportion of IL-17$^+$ vaginal cells was compared between germ-free mice and microflora-intact WT controls. While we were unable to find consistent differences among the various IL-17$^+$ subsets between these mice, we observed a significant reduction in the proportion of total IL-17$^+$ cells in the vagina of germ-free mice, compared to WT controls (Fig. 13A & B). This can be related to reports that have suggested that

commensal microflora may be essential for some (LTi-like type 3 ILCs and NCR^+ type 3 ILCs), but not all NCR^- type 3 ILCs (Sonnenberg & Artis, 2012). Further experiments may be needed to confirm whether these subsets are missing in the vagina of GF mice. The regulation of microflora on type 3 ILC populations is controversial and still under debate. However, it is clear that signals from microflora can influence the function of ILCs (Qiu et al., 2013; Sonnenberg & Artis, 2012). To date, we are not aware of any information on the role of microflora in regulating vaginal IL-17 responses by $\gamma\delta^-$ cells. Kim et al. performed antibiotic treatments in regular mice and suggested that microflora may not play a role in IL-17 responses by $\gamma\delta^+$ cells (J. O. Kim et al., 2012). However, our report is the first to use germ-free mice and shows that microbiota may have a significant impact on the presence of innate IL-17^+ cells under homeostatic conditions in the vagina. Furthermore, we also examined whether the microflora can influence the induction of DC-primed vaginal T_h17 responses (Fig. 13C). Vaginal DCs from germ-free mice were fully capable of inducing T_h17 responses in co-cultures equivalent to those observed in WT microflora-intact control co-cultures. This suggests that the microflora itself may not directly influence the function of vaginal DCs. Overall, we have shown that microflora may play a role in IL-17 responses mediated by innate lymphocytes under homeostatic conditions, but it does not significantly influence the inherent ability of vaginal DCs to prime T_h17 responses.

The first part of this dissertation (section 3.1) showed that E2 can influence T_h17 responses generated by vaginal DCs (section 3.1.7). However, E2 can also influence the microflora community in the vagina. E2 treatment increases glycogen production in the vaginal tract, and this has been correlated with increased *Lactobacillus sp.* colonization (Mirmonsef et al., 2014; Walmer, Wrona, Hughes, & Nelson, 1992). Given that E2 can influence vaginal microflora, and our observations in section 3.2.2 indicating that microflora can influence IL-17 produced by innate lymphocytes, we wanted to examine whether E2 can also influence innate IL-17 producers in the vagina, independent of its effects on vaginal DCs (Results section 3.2.3). Our results showed that E2-treatment was associated with significantly increased IL-17 levels in supernatants from vaginal tissue cells (TC) cultured alone *ex vivo,* compared to placebo treated OVX controls (Fig. 14A). To determine the cause of lower IL-17 responses in the absence of E2, we conducted intracellular staining to examine IL-17 production in vaginal tissues from OVX E2, OVX placebo (mock) treated and ERKO mice. While we were unable to identify consistent differences in the specific populations outlined in section 3.2.1, the vagina of E2 treated mice showed significantly higher proportions of total IL-17^+ cells compared to mock or ERKO mice (Fig. 14B & C). Furthermore, we also observed a significant increase in the MFI of IL-17^+ cells in E2 compared to mock controls (Fig. 14C), suggesting that E2 induced IL-17 production by vaginal innate lymphocytes. Overall, these results indicated that E2, either through its interaction with the microflora community, or directly by its

influence on innate IL-17$^+$ cells, could influence homeostatic IL-17 responses in the vagina.

To further dissect the contribution of microflora and E2 on IL-17 responses, experiments would need to be conducted in germ-free ERKO mice. However, to the best of our knowledge, these are not available. Phenotypic analysis did not show any consistent differences in the innate IL-17$^+$ lymphocyte subpopulations (characterized in section 3.2.1) between E2 treated mice and controls. The effect of E2 on the development, self-renewal and maintenance of γδ$^+$, γδ$^-$ IL-17$^+$ innate lymphocytes in the vagina is unknown, and requires further investigation.

Next, we wanted to examine whether IL-17 primed by innate vaginal lymphocytes can influence vaginal DC functions. Numerous studies have shown that innate lymphocytes can influence the differentiation and function of adaptive lymphocytes (Gasteiger & Rudensky, 2014). We compared IL-17 levels in co-cultures conducted with vaginal cells from IL-17 KO mice and WT controls, and found that endogenous IL-17 responses were critical for the induction of T_h17 responses primed by vaginal TCs in co-cultures (Fig. 15A). IL-17 was reduced 30-fold in co-cultures conducted with vaginal cells from IL-17KO mice compared to WT controls. Intracellular staining shows that only 39% of the proliferating CFSE-stained OT-II Tg CD4$^+$ T cells were differentiating into T_h17 cells in IL-17KO co-cultures, compared to the 55% in WT control co-cultures (Fig. 15B). Given that IL-1 plays an important role in vaginal T_h17 responses, we next

determined whether vaginal DCs from IL-17KO mice were deficient in producing IL-1β.

IL-1β is a multifunctional cytokine. It is synthesized by myeloid cells, such as macrophages and DCs, as a 35-kd precursor (pro-IL-1β) lacking biological activity (Lopez-Castejon & Brough, 2011). A number of non-microbial factors such as stress, danger signals such as collagen and clotting factors, as well as microbial factors, can induce the synthesis of pro-IL-1β (Dinarello, 1996). IL-1β-converting enzyme (ICE) or enzymes such as Cathepsin G, Chymotrypsin and Granzyme A can cleave pro-IL-1β into a 17-Kd bioactive IL-1β molecule, which is secreted out of the cell via a non-classical pathway that avoids the ER-golgi apparatus (Dinarello, 1996; Rubartelli, Cozzolino, Talio, & Sitia, 1990). The synthesis and release of bioactive IL-1β are two separate events in DCs. For instance, while monocytes stimulated with LPS can readily secrete inactive pro-IL-1β and bioactive IL-1β, immature DCs do not produce pro-IL-1β under homeostatic conditions (Gardella et al., 2000), and produce pro-IL-1β only upon maturation with microbial stimuli, or interaction with antigen-specific $CD4^+$ T cells via CD40-CD40L interactions (Gardella et al., 2000). However, this only leads to the intracellular accumulation of pro-IL-1β in DCs and not its release or conversion to IL-1β. The second event, which is the activation and release of active IL-1β, is independent of these interactions and it is still a topic under investigation. Upon stimulation of DC PRRs by a variety of PAMPs and DAMPS, the PRRs can form inflammasomes, leading to caspase-1 mediated activation

and release of bioactive IL-1β (Lopez-Castejon & Brough, 2011; Martinon et al., 2002). We have shown two separate populations of $CD11c^+$ DCs in freshly isolated vaginal cells that can potentially produce IL-1β without any *ex vivo* stimulation (Fig. 16B): 1) $CD11c^+$ $CD11b^+$ DCs were the only source of pro-IL-1β, and 2) Both $CD11c^+$ $CD11b^+$ cells and $CD11c^+$ $CD11b^-$ cells produced active IL-1β. The proportion of both these cells was markedly reduced in vaginal cells from IL-17KO mice. There was also a discernable, although statistically not significant, reduction in the MFI (indicator of amount of IL-1β or pro-IL-1β produced by each cell) in IL-17KO mice (Fig. 16C). This suggests that IL-17 produced by innate lymphocytes in the vagina can influence $CD11c^+$ DC IL-1β synthesis and processing. Our observations are consistent with prior studies showing that IL-17 can stimulate the production of IL-1β by APCs (Jovanovic et al., 1998; M. S. Wilson et al., 2010). Hence, this led us to question whether the defect in T_h17 responses primed by IL-17KO vaginal DCs could be remediated by addition of rIL-17 or rIL-1β.

Interestingly, rIL-17 (250pg/mL) added either during OVA peptide priming of vaginal isolates (TC), or during the 3.5-day co-culture with OT-II Tg $CD4^+$ T cells (TC+CD4), was incapable of completely restoring the impaired T_h17 responses in IL-17KO vaginal co-cultures (Fig. 17A). Although we did not optimize the dose of rIL-17 used in this experiment, it is more likely that the transient presence of IL-17 during co-culture was just insufficient to restore the normal function of vaginal APCs. An alternative approach might involve the

prolonged injection of IL-17KO mice with rIL-17 *in vivo* prior to tissue collection, and vaginal co-cultures. However, it is also likely that DCs from IL-17KO mice may be inherently defective in their ability to prime $CD4^+$ T cells. In a lung *C. muridarum* infection model, neutralization of IL-17 significantly impaired DC functions (Bai et al., 2009). DCs from IL-17-neutralized mice produced lower levels of IL-12 and expressed lower MHCII and CD40. Furthermore, they produced higher levels of IL-10, induced higher IL-4, and skewed immune responses toward a T_h2 phenotype instead of typical T_h1 response in controls (Bai et al., 2009). This was consistent with our observations in IL-17 KO co-cultures where we noticed 30-fold lower T_h17 and 7-fold lower T_h1 responses (Fig. 15A). Overall, this suggests that DCs from IL-17 KO mice may be impaired in their ability to prime $CD4^+$ T cell responses.

A number of stimulatory and inhibitory factors exist for priming T_h17 responses (Muranski & Restifo, 2013). Previously, Dr. Yoichiro Iwakura (source of our IL-17KO mice), also observed lower IL-1β responses, and impaired $CD4^+$ T cell responses with Langerhans cells from IL-17KO mice (Nakae et al., 2002). This led us to question whether the addition of rIL-1β itself can restore T_h17 differentiation in IL-17KO co-cultures. While the addition of 100ng/ml rIL-1β did significantly increase T_h17 differentiation in IL-17KO co-cultures, the levels of IL-17 were still not comparable to WT controls. This suggests that other intrinsic defects in IL-17KO DCs such as signaling pathways, or activation markers, may exist which may not be restored by the transient addition of rIL-1β addition during

co-culture. Overall, these results indicate that IL-17 produced by innate lymphocytes in the vagina may have an important role in DC-primed T_h17 responses.

In summary, we have identified that E2 can influence both innate and adaptive IL-17 responses in the vagina. E2 significantly increases the population of IL-17$^+$ innate lymphocytes in the vagina compared to OVX or ERKO controls. We also observed a significant reduction in IL-17 producing innate lymphocytes in the vagina of germ-free mice. This suggests that some, but not all, populations of IL-17-producing innate lymphocytes in the vagina are dependent on hormones and microflora. Finally, we demonstrated that vaginal APCs from IL-17 KO mice were impaired in inducing T_h1 and T_h17 responses, and neither rIL-17 nor rIL-1β, was able to completely restore T_h17 differentiation. Overall, our work shows that the vaginal tract is a complex microenvironment where multiple factors such as sex hormones, microflora and innate lymphocytes can regulate APC functions.

4.4: Optimizing an E2 delivery regimen to test the effect of E2 on DC responses in an HSV-2 mouse model

The results summarized in sections 3.1 and 3.2 of this dissertation demonstrated that the mucosal microenvironment can influence the function of vaginal APC populations. Specifically, we found that E2 could condition vaginal DCs to become potent inducers of T_h17 responses. The final goal of this work was to relate these observations *in vivo* in the HSV-2 experimental mouse model, to examine whether better protection quality in E2-treated mice corresponded with vaginal DCs that could prime T_h17 responses post HSV-2 infection.

We first determined whether there were phenotypic differences between E2 and P4 treated mice that correlated with functional differences *in vivo*. We compared $CD11c^+$ DCs and $CD11c^-$ $CD11b^+$ $F4/80^+$ macrophages among mice treated with 21-day release E2 or placebo pellets. Given our previous studies where we have found that P4 can increase susceptibility to HSV-2 infection *in vivo*, we also included a group of P4 treated mice in this chapter. E2 treatment induced a higher proportion of myeloid $CD11c^+$ $CD11b^{hi}$ $MHCII^-$ DCs and $CD11b^+$ $F4/80^+$ macrophages compared to P4 or mock controls (Fig. 18). Longer hormone treatment (14 or 21 days versus 7 days), increased the proportions of myeloid DCs and macrophages (Fig. 18C), demonstrating that prolonged exposure to hormones might lead to more polarized differences in vaginal APC phenotype. Numerous studies have shown that sex hormones can influence the phenotype of APCs in the vagina (Iijima et al., 2007; Wira, Rossoll, & Kaushic,

2000; Zhao et al., 2003). *Zhao et al.* described three distinct populations of DCs in the vaginal epithelium, and these were more numerous during the P4-dominant diestrus phase compared to the E2-dominant estrus phase (Zhao et al., 2003). In contrast, $CD11c^+$ DC proportions were dominant under E2 treatment in our study. However, they had defined DCs as strictly $CD11c^+$ $MHCII^+$ cells, while we had also included $CD11c^+$ $MHCII^-$ cells among DCs. In a later publication by the same group, they reported $F4/80^+$ $MHCII^-$ $CD11b^+$ monocytes were enhanced during the E2-dominant estrus phase (Iijima et al., 2008), which appear to be consistent with our observation of increased myeloid $CD11c^+$ $CD11b^{hi}$ $MHCII^-$ populations under E2 treatment.

MHCII expression is regulated in immature DCs by ubiquitination, thereby leaving immature DCs with low levels of MHCII expression under steady state conditions (Ma, Platt, Eastham-Anderson, Shin, & Mellman, 2012; Shin et al., 2006; N. S. Wilson, El-Sukkari, & Villadangos, 2004). However, upon stimulation with PAMPs and maturation, MHCII ubiquitination in these immature DCs is downregulated, and its expression is dramatically increased on the surface of mature DCs (Roche & Furuta, 2015; Shin et al., 2006; Villadangos et al., 2001; N. S. Wilson et al., 2004). On the other hand, monocytes are classically $MHCII^-$ $F4/80^{low}$ cells that can express CD11c, and can be divided into two populations based on their Ly6C expression (Rose, Misharin, & Perlman, 2012). While these $MHCII^-$ monocytes may be part of the populations that were enhanced in E2-treated mice, it is important to consider that monocytes can still differentiate into

DCs or macrophages in peripheral tissues (Guilliams et al., 2014). Thus, $CD11c^{+}$ $CD11b^{hi}$ $MHCII^{-}$ cells in the vagina of E2 treated mice may represent DC precursors, and E2 may be directly enhancing the development of mDCs in the vagina. Further investigation is required to confirm this theory. To identify the role of these $MHCII^{-}$ cells in vaginal T_h17 responses, FACS could be used to sort E2-induced $CD11c^{+}$ $CD11b^{hi}$ $MHCII^{-}$ cells, and they could be co-cultured with OT-II Tg $CD4^{+}$ T cells. In summary, regardless of our phenotypic definition of DCs, we have shown that E2 could significantly influence the phenotype of APC populations in the vagina.

Next, we wanted to examine how these differences in APCs translate to anti-viral responses *in vivo*. Twenty four hours post intravaginal infection is the ideal timeframe to examine APC responses in the vagina, as submucosal DCs acquire HSV-2 antigens and mature, and migrate to the draining lymph nodes to prime $CD4^{+}$ T cells 48-72h post infection (Zhao et al., 2003). However, the biological effect of E2 on the genital tract that leads to proliferation and keratinization of vaginal epithelium renders mice resistant to intravaginal infection (Buchanan et al., 1998; Parr et al., 1994; M. B. Parr & E. L. Parr, 1997; Parr & Parr, 2003). We have previously described a 3-day E2 treatment regime with an injectable formulation of E2 (500ng/mouse), where mice were susceptible to HSV-2 infection 5 days after last hormone treatment (A. Gillgrass et al., 2010). However, given our observations where prolonged hormone treatment was associated with more pronounced differences in APC phenotypes (section 3.3.1

Fig. 18), we wanted to standardize an infection protocol with the 21-day E2 pellets. The biological effect of E2 pellets on the vaginal epithelium was observed to last between 25-47 days in a group of 4 mice (section 3.3.2, Fig. 19). This presented a practical problem of consistency, and in obtaining a large enough group of mice that could be infected intravaginally on the same day to obtain sufficient experimental *n* numbers for statistical analysis. We have previously used these 21-day pellets in an intranasal and subcutaneous immunization model (Bhavanam et al., 2008) where E2-treated mice were challenged intravaginally with WT HSV-2, 7 weeks (49 days) after E2-pellet treatment. Hence, an alternative 7-day injectable regime was developed where E2 treated mice could be consistently infected intravaginally, 14 days after the start of hormone treatment (section 3.3.3, Fig. 20).

Having standardized the E2 7-day regime, we examined whether E2 and P4 influence antiviral responses by modulating APC phenotype and function. The vagina of uninfected E2-treated mice contained a higher proportion of $CD11c^{+}$ $CD11b^{hi}$ $MHCII^{-}$ APCs, and $CD11c^{-}$ $CD11b^{+}$ $SSC\text{-}A^{low}$ cells (a phenotype similar to $CD11c^{-}$ $CD11b^{+}$ $F4/80^{+}$ macrophages or monocytes (Hastings, 2014) compared to P4 or mock controls (section 3.3.4, Fig. 21). This was consistent with the observations in the 21-day pellet model (section 3.3.1), and suggested that the effect of E2 on vaginal APC populations likely extends beyond the effect on the vaginal epithelium. Intracellular staining for T_h1 polarizing cytokines IL-12 and TNF-α (critical for the HSV-2 anti viral response), in vaginal APCs from these

uninfected mice, showed that there were no significant differences in the production of these cytokines by DCs or macrophages between the hormone groups. However, 24h post immunization (primary infection) with (TK-) HSV-2, there was a dramatic change in the vaginal APC phenotype of E2 treated mice. The E2-mediated enhancement of $CD11c^{+}$ $CD11b^{hi}$ $MHCII^{-}$ DCs and $CD11c^{-}$ $CD11b^{+}$ SSC-A^{low} macrophages in uninfected mice was not seen post-infection, and the APC phenotype was remarkably similar among the three groups (Fig. 21A & C). However, lower proportions of $CD11c^{+}$ DCs and $CD11c^{-}$ $CD11b^{+}$ SSC-A^{low} macrophages from E2 treated mice expressed TNF-α compared to P4 or mock controls (Fig. 21). Yet, there were no differences in IL-12 between APCs from all three hormone treatment groups. Previously, *in vitro* studies have shown that P4-treatment can downregulate APC function by lowering MHCII and CD80 expression, and can suppress pro-inflammatory cytokines including TNF-α and IL-1β from bone marrow derived DCs (Butts et al., 2008; Butts et al., 2007), and macrophage cell lines *in vitro* (Miller & Hunt, 1998). Similarly, in a study conducted with PBMC from patients immunized against *Human Papillomavirus*, both E2 and P4 significantly decreased production of pro-inflammatory cytokines such as IL-12 and TNF-α (Marks et al., 2010). However, the P4-induced TNF-α increase in our study is consistent with HIV-1 studies in the human female genital tract, where P4 or P4-based contraceptives were associated with increased pro-inflammatory cells in cervico-vaginal lavages (Ghanem et al., 2005). As suggested earlier in this discussion (section 4.2), the differences in our

observations compared to some of these studies could be explained by the tissue-specific effects of sex hormones. Studies showing P4's anti-inflammatory effects on DC responses have utilized BMDC cultures, or cells derived from non-mucosal compartments such as PBMCs or splenic DCs. However, studies utilizing vaginal tissues show that P4 may be associated with pro-inflammatory responses. Furthermore, our studies in the HSV-2 mouse model have shown increased vaginal pathology post-challenge in P4-treated mice (Bhavanam et al., 2008; A. E. Gillgrass, Fernandez, et al., 2005; Kaushic et al., 2003). Lung *Mycobacterium sp.* infection models have related excessive TNF-α and dysregulated T_h1 responses as one of the primary causes for immunopathology (Bekker et al., 2000). Further studies will be required to relate the observed increase in TNF-α expression (Fig. 21), and greater immunopathology in P4-treated animals. Overall, these results indicate that we could successfully develop an E2-treatment model to compare the effect of hormones on anti-viral responses *in vivo*.

Next, to examine whether these phenotypic differences can translate into functional differences at priming anti-viral $CD4^+$ T cell responses, co-cultures were optimized with vaginal TCs from no-hormone OVX uninfected and infected mice. The primary observation was that intravaginal HSV-2 infection directed vaginal TCs (24h p.i) to prime potent inflammatory IFN-γ (10-fold induction compared to uninfected controls) and TNF-α (3-fold induction compared to uninfected controls) responses in co-cultures (section 3.3.6 & Fig. 23). A marked

decrease in T_h1 associated cytokines such as IFN-γ (24h: 6602 ±_977 pg/mL ; 48h: 5085 ± 87 pg/mL) and IL-12 (24h: 278 ±_38 pg/ml ; 48h: 51 ± 5 pg/mL) in the 48h p.i group (Fig. 23), may represent the migration of vaginal DCs from vagina to the draining lymph nodes at 48h post HSV-2 infection (Zhao et al., 2003). Hence, this suggested that the 24h p.i. may likely be better than 48 p.i.to compare vaginal APC responses among hormone-treated mice.

Among uninfected mice themselves, E2–treatment was associated with enhanced IL-6 (14452 ± 1040 pg/mL) compared to P4 (5210 ±_1844 pg/mL) or mock (6232 ± 673 pg/mL) treatments (Fig. 24: Uninfected). Comparing uninfected and 24h (TK-) HSV-2 infected mice, vaginal TC from infected mice regardless of the hormone-treatment, induced higher levels of TNF-α compared to uninfected controls (Fig. 24). There were no significant differences among other cytokines including IL-17 and IFN-γ (Fig. 24). This was contrary to the potent induction in IL-6, IL-17 and IFN-γ levels seen in section 3.1.7, and the induction of IFN-γ levels seen in section 3.3.6. In addition to the inconsistency in these results, only 2 replicate co-culture wells per condition were used in these particular experiments. This prevented us from conducting statistical analysis, and it precludes drawing any conclusions from these results. However, the key observation from these 7-day E2 treatment co-cultures (section 3.3.7) was the loss of E2-mediated conditioning on vaginal DCs to prime potent T_h17 responses. This is in contrast to our observations in section 3.1.7 (Fig. 9). This may indicate that the effect of E2 on vaginal APCs is transient, and may wane by day 14 when

mice become susceptible to intravaginal HSV-2 infection. Hence, the 7-day regime may not be ideal for examining the effect of E2 on APC function *in vivo*.

However, given that our co-cultures utilize OT-II Tg $CD4^+$ T cells in the context of OVA peptide stimulation, it is also possible that we may be loosing E2-specific differences, which could be better represented in a model with viral stimulation and HSV-2-specific $CD4^+$ T cells. Hence, we developed an alternate *in vivo* model with HSV-2 OVA infection to compare hormone effects on HSV-2 OVA specific $CD4^+$ T cell responses between hormone-treated mice. We first validated the expression of OVA by the virus (section 3.3.8 & Fig. 25A), and proceeded to standardize an adoptive transfer model that enabled us to detect the responses of virus specific $CD4^+$ T cells post intravaginal infection (Fig. 25B).

In a preliminary experiment to examine the effect of E2 on anti-viral responses, CFSE-stained OT-II Tg $CD4^+$ T cells were adoptively transferred into mice treated with E2 using the 7-day regime or placebo and infected with HSV-2 OVA (section 3.3.9). Three days post-infection, while proliferation and cytokine responses of total and virus-specific OT-II Tg $CD4^+$ T cells was clearly visible in the draining lymph nodes, it was much more difficult to identify T cell responses in vaginal tract (Fig. 26B). Furthermore, no clear differences in T_h1 or T_h17 cell populations were observed between E2 and placebo controls. We concluded that most likely, based on similar results in section 3.3.7, E2 conditioning of APCs might have waned in the 7 day waiting period following hormone treatment.

While these early observations highlight the promising possibilities of using the HSV-2 OVA model to examine virus specific responses, the lack of sufficient resolution to identify OT-II Tg $CD4^+$ T cells in the vagina is a major concern. This could potentially be attributed to: 1) Delay between priming and trafficking: The responder populations of transgenic $CD4^+$ T cells are recruited to the vagina days 4 to 5 post infection. Hence, 3 days post-infection may be too early to examine $CD4^+$ T cell responses in the vagina. 2) Excessive proliferation: Since vaginal cells have increased autofluorescence, we could only effectively resolve 3 to 4 generations of CFSE-stained cells due to the dilution of the dye and its signal intensity. Therefore, while this is conceptually a valid model to examine HSV-2 specific responses, improvements may be needed to better resolve OT-II Tg $CD4^+$ T cells. One such approach would be the use of congenic mice to distinguish endogenous and adoptively transferred OT-II Tg $CD4^+$ T cells. Alternatively, an antibody specific to the DO11.10 TCR complex could to specifically identify OT-II Tg $CD4^+$ T cells during flow cytometry. However, this still would not address the concern that the 7-day E2 regime may not be an optimal model to examine hormone effects on vaginal APCs. An alternative E2 treatment model would be required to effectively utilize the HSV-2 OVA model to examine hormone effects on anti-viral responses *in vivo*.

We have previously described an experimental model where mice were immunized intranasally with attenuated HSV-2 under the influence of E2 or P4, and differences in protection quality were observed post intravaginal challenge

(Bhavanam et al., 2008). Although this model does not allow us to examine vaginal APC response post-immunization, the nasal mucosa is an effective inductive site for priming and imprinting homing markers on anti-viral $CD4^+$ T cells, that home to the vaginal tract post intravaginal challenge (Bergquist, Johansson, Lagergard, Holmgren, & Rudin, 1997; A. Sato et al., 2014). After a number of unsuccessful attempts to optimize a model which would allow us to directly examine DC-mediated priming of T cells in E2 treated mice (summarized above), we concluded that the intranasal immunization followed by vaginal challenge may be the most effective strategy to compare the effect of E2 or P4 on APCs. The intranasal immunization model was being tested in parallel with the experiments described here, for examining T cell responses *in vivo* by another PhD student (Kristy Roth, PhD thesis). Experiments using this model demonstrated that immunization under the influence of E2 induced earlier and higher mucosal T_h17 and T_h1 responses in the vagina post-challenge, compared to placebo treated controls (Anipindi V, Roth K, et al., See appendix A). These observations complemented the *in vitro* studies shown in section 3.1 of this dissertation. Collectively, our observations prove that sex hormones and the microenvironment of the vagina can directly influence APC responses *in vitro* and *in vivo*.

4.5 Study Implications:

4.5.1 Novelty of mucosal tissue-specific responses:

This is the first significant body of research to show that multiple factors in the mucosal microenvironment of the vaginal tract can influence the phenotype and functions of APCs under homeostatic conditions. We have shown that sex hormones, commensal microflora and resident lymphocyte populations can directly condition vaginal APCs to prime T_h17 responses. Every mucosal tissue is faced with its own environmental challenges, and has evolved to respond appropriately with inflammatory or tolerant immune responses. Much of this is accomplished by APCs such as DCs and macrophages (Chirdo et al., 2005). Previously, others have examined APC populations in the lung, small intestine and vagina (Iwasaki, 2007; Iwasaki & Kelsall, 1999; Kelsall & Rescigno, 2004; Rescigno & Di Sabatino, 2009). While a few studies have compared lung and intestinal DC populations (Persson et al., 2013; Schlitzer et al., 2013; Takenaka, McCormick, Safroneeva, Xing, & Gauldie, 2009), vaginal APCs have not been compared to those from other tissues. We have directly compared APCs from all these tissues, and shown that vaginal APCs are distinct from those in either the lung or small intestine. Furthermore, previous studies used lymph node DCs (Zhao et al., 2003), splenocytes (Butts et al., 2007) or BMDCs (Carreras et al., 2010; Mao et al., 2005; Paharkova-Vatchkova et al., 2004), to examine the effect of hormones on APCs phenotype or function *in vitro*. Our study is the first to show that hormones can induce tissue-specific responses in mucosal APCs. Vaginal

APCs from E2 treated mice were more potent inducers of T_h17 responses, compared to lung or small intestine APCs. Given that IL-17 can enhance cell mediated (Gopal et al., 2012; Lockhart et al., 2006) and humoral responses in mucosal tissues (Wang et al., 2011), modulating the mucosal microenvironment of the vagina could be a valuable tool to manipulate genital tract immunity in order to make women more resistant to STIs.

Our work also highlights concerns regarding the use of lymph node DCs to understand the role of genital tract DCs in immune responses to STIs (Zhao et al., 2003). For example, *Zhao et al.* have suggested that submucosal DCs are the primarily responsible for priming HSV-2 specific T_h1 responses in the draining lymph nodes (Zhao et al., 2003). However, much of their work was conducted with DCs isolated from lymph nodes or spleen, and they showed that these were potent inducers of T_h1 responses. Our work shows that DCs in the lymph node are not representative of APC sub-populations in the vagina. The range of vaginal immune responses against HSV-2 may be quite complex requiring a balance between T_h17 and T_h1 responses primed by infiltrating and resident APCs. Hence, while utilizing spleen or lymph node may be a practical approach to obtain sufficient DCs, their use for studying the function of mucosal APCs may not be ideal.

4.5.2 Vaginal DCs, T_h17 responses and susceptibility to STIs in the genital tract:

Our study provides a mechanistic perspective on how sex hormones can potentially influence HIV-1 susceptibility in the genital tract. T_h17 cells have been

implicated in numerous studies to be preferential targets for HIV-1 infection (El Hed et al., 2010; Klatt & Brenchley, 2010; Rodriguez-Garcia, Barr, Crist, Fahey, & Wira, 2014). Our work suggests that E2 can directly influence $CD11c^+$ DCs to amplify innate and adaptive IL-17 responses in the genital tract, thereby increasing potential targets for HIV-1 infection. This appears to contradict the studies suggesting that E2 is protective against HIV-1 acquisition (Rodriguez-Garcia et al., 2013; Russo et al., 2005; Tasker et al., 2014), it is important to consider that E2 may have distinct effects on different cells. E2 may directly act on $CD4^+$ T cells to reduce HIV-1 entry (Rodriguez-Garcia et al., 2013), and also suppress infection of macrophages by the induction of type 1 IFN (Tasker et al., 2014). The sum of E2's effects may be different than its effects on individual cells; therefore, it may have an overall protective effect, which may not be obvious when examining individual cells. Other factors can also skew these responses and potentially alter the balance of protection and susceptibility to STIs. For instance, tissue resident T_h17 cells producing IL-22 induced by E2 may have a protective effect on the mucosal EC barrier against STIs (C. J. Kim et al., 2012); however, upon breach of this barrier, the presence of these cells could be detrimental and enhance HIV replication.

4.5.3 Implications for Mucosal Vaccine Development:

Our observations highlighting the importance of microenvironment on APC function may have important implications for vaccine development. Factors such as inoculation route and sex hormones should be an important consideration for

the development of mucosal vaccines against STIs. While many of the currently approved vaccines against infectious diseases are administered via a non-mucosal route as subcutaneous or intramuscular inoculations, in recent times, there has been increasing interest in the development of mucosal vaccines (De Magistris, 2006; Ogra, Faden, & Welliver, 2001). The mucosal surfaces of the respiratory, gastrointestinal and reproductive tracts are the primary portals of entry for most pathogens. Mucosal immune responses are believed to be compartmentalized with unique humoral (s-IgA and s-IgG) and cell mediated responses ($CD8^+$ and $CD4^+$ T cell responses), and these may not be adequately primed when vaccines are administered via other non-mucosal routes (De Magistris, 2006; Ogra et al., 2001). Studies have suggested that mucosal vaccination in various tissues such as the genital, oral and nasal mucosa may be key to the induction of a broad spectrum immunity against mucosal pathogens such as HIV-1, HSV-2, *Mycobacterium tuberculosis* and HPV (Belyakov & Ahlers, 2009; Giri, Sable, Verma, & Khuller, 2005). The observations made using the OVA-peptide co-culture model in our work suggests that under homeostatic conditions, baseline immune responses in mucosal tissues against most peptide-based vaccines may be specific to the inoculation site. Hence, vaccine routes and adjuvant formulations should be manipulated to effectively induce appropriate protective immune responses against mucosal pathogens.

4.5.4 The influence of sex hormones on immune responses and vaccine development:

Numerous studies have shown that sex hormones can influence susceptibility to STIs such as HSV-2 and HIV-1 (Kaushic, Roth, et al., 2011). Hormones may accomplish this by directly influencing immune responses in the genital tract (Wira et al., 2015). In the HSV-2 mouse model, we and others have found that intravaginal challenge under the influence of E2 leads to better protection with lower pathology compared to P4 (Bhavanam et al., 2008; A. Gillgrass et al., 2010; A. E. Gillgrass, Tang, et al., 2005; Kaushic et al., 2003; Pennock et al., 2009). However, the mechanism by which E2 regulates immune responses in the vagina post HSV-2 infection was largely unknown. In this body of work, we have shown one possible mechanism by which E2 can directly influence anti-viral immune responses. E2 conditioned vaginal DCs into more inducers of T_h17 and T_h1 responses compared to placebo controls. While others have shown that E2 can also influence DC differentiation and function *in vitro* from BM precursors (Carreras et al., 2010; Mao et al., 2005; Paharkova-Vatchkova et al., 2004), our study is the first to relate hormone effects to the function of vaginal DCs.

Hence, based on our observations, manipulating the hormonal microenvironment during vaccination may be an important approach to generate optimal immune responses in the genital tract of women. However, we are aware that E2 can be associated with both susceptibility (Jerse, 1999; Lasarte et al.,

2013; Relloso et al., 2012; Tarry et al., 2005; X. Zhang, Essmann, Burt, & Larsen, 2000), and protection (Bhavanam et al., 2008; A. Gillgrass et al., 2010; A. E. Gillgrass, Fernandez, et al., 2005; Pennock et al., 2009; S. M. Smith et al., 2000; S. M. Smith et al., 2004; Sodora et al., 1998) against a variety of STIs. The administration of vaccines during the E2 dominant phase of the menstrual cycle, or a vaccine formulation including a small dose of E2 may offer optimal protection in women against specific STIs such as HIV-1 and HSV-2.

4.5.5 The importance of IL-17 in the HSV-2 anti-viral response:

While others have studied the role of IL-17 produced by innate lymphocytes in the gut, the literature pertaining to these subsets in the genital tract is relatively sparse. One study by *Kim et al.* showed that $\gamma\delta^+$ T cells were the primary source of IL-17 in the vagina (J. O. Kim et al., 2012), however they did not further characterize the IL-17-producing $\gamma\delta^-$ cells. Furthermore, they suggested that innate sources of IL-17 might play a relatively minor and pathological role in the HSV-2 immune response. While our results agree with their findings in that $\gamma\delta^+$ T cells were the primary source of IL-17 in the vagina, we have also characterized the minor $\gamma\delta^-$ populations. Furthermore, while *Kim et al.* only used survival as their correlate to identify the role of IL-17 in HSV-2 antiviral responses, our work provides more mechanistic evidence to suggest that IL-17 produced by innate lymphocytes may play an important part of the vaginal microenvironment. In the absence of this IL-17, vaginal DCs may exhibit impaired IL-1β expression, and may be inefficient at priming T_h1 or T_h17 responses. In

related studies, not described here, using IL-17KO mice we have found that IL-17 in the vagina may be important for the development of adequate mucosal memory responses post challenge in the HSV-2 mouse model (Anipindi V, Roth K, et al., See appendix A).

4.6 Study Limitations:

While our observations are unique and provide insights to the mechanism by which E2 can influence DC functions, there are specific limitations of our work.

4.6.1 Identity of DCs

One of the primary limitations in our study is the definition of DCs and our usage of CD11c as the primary marker. A number of cell types can express CD11c (Merad et al., 2013; Reis e Sousa, 2006; van Blijswijk et al., 2013). This makes it very difficult to make an accurate phenotypic comparison among DCs from different mucosal tissues. Practically, using CD11c as a broad marker was the only viable option to obtain adequate cell numbers from the vagina for co-cultures. Furthermore, the use of total cell isolates in co-cultures allows us to make a broad comparison of the function of $CD11c^+$ and $CD11c^-$ cells in mucosal tissues. Therefore, despite the lack of resolution of DC subsets, our observations remain valid with the caveat that they are based on $CD11c^+$ cells in mucosal tissues.

4.6.2 Identity of innate lymphocytes:

A similar limitation exists with the identification of innate lymphocytes that produce IL-17 under steady state in the vagina. While our observations are

consistent with earlier studies that indicate TCR γδ T cells are the primary source of IL-17 in the vagina (J. O. Kim et al., 2012), we have also described three other novel vaginal lymphocyte populations that produce IL-17. However, the great degree of overlap among LTIs and NCR^- type 3 ILCs makes it difficult to conclusively distinguish these populations as LTis, type 3 ILCs or steady-state T_h17 cells. In the future, further characterization with additional markers is essential to positively identify these cells.

4.6.3 Co-culture model:

The OVA-peptide co-culture model is a system that can be used to examine APC responses in the absence of inflammation under steady state conditions. In this context, the APC populations may be stimulating $CD4^+$ T cells to induce a broad spectrum of immune responses that may not accurately represent the responses in the context of a viral infection. Early observations in the intranasal immunization model have shown that E2 also induces T_h17 responses *in vivo* in the vagina post HSV-2 challenge (Anipindi V, Roth K, et al., See appendix A). However, T_h1 responses still appear to be the prominent $CD4^+$ T cell response unlike the prominent T_h17 responses seen in co-cultures. This suggests that the balance of T_h17 and T_h1 responses in the co-culture model may not accurately reflect the *in vivo* immune responses in the context of STIs.

4.6.4 Use of OT-II Tg $CD4^+$ T cells:

There have been concerns with using OT-II Tg mice, as they have been documented to prime impaired T_h2 responses, and more prominent T_h1

responses, compared to normal C57BL/6 mice (Leung, Smith, Myc, Morry, & Baker, 2013). The polarized T_h17 responses in our model were also similar to T_h17 bias seen in a lung model of allergic OVA sensitization (Lemaire et al., 2011). It is important to consider strain specific differences in immune responses, and we are aware that our observations may not translate exactly to other mouse strains or human studies. This needs to be a consideration when extrapolating our work to human genital tract APCs.

4.6.5 Dissimilarities with E2 effect on DC functions in other studies:

Our study highlights novel observations indicating that E2 enhances IL-17 from both innate and adaptive lymphocytes in the genital tract. We are aware of previous studies where others have implicated E2 to impair T_h17 responses. One such study showed that splenic DCs treated with E2 were less efficient at migrating, up-regulating antigen processing pathways, producing IL-23, and triggering the T_h17 immune response (Relloso et al., 2012). In a separate study using mouse splenocytes, E2 was also found to regulate T_h17 differentiation by inhibiting ROR-γ through an ERα dependent mechanism (R. Y. Chen et al., 2015). Another study found that E2 downregulated T_h17 responses to sperm and *C. albicans* (Lasarte et al., 2013). All these studies utilized spleen or lymph node-derived DCs.

Our study clearly shows that vaginal DCs are distinct from spleen or LN DCs, and are more potent inducers of T_h17 responses. In agreement with some of these other studies, we also found that spleen DCs from ERKO mice primed

greater T_h17 responses compared to WT controls (Fig. 9). In a separate study with human tissues, vaginal DC subsets were not potent inducers of T_h17 responses (Duluc et al., 2013). While, this may partly be attributed to the vastly dissimilar allogenic $CD4^+$ T cell co-culture model used in this last study, compared to the OVA-peptide model in our study, it also highlights species-specific dissimilarities in APCs. Future studies should ascertain whether human vaginal DC subsets are conditioned by sex hormones, similar to our observations in the rodent model.

4.6.6 Intravaginal infection in E2 treated mice:

Our work highlights the challenges of examining the effect of E2 on anti-viral responses in the vagina. Others have previously documented the biological effect of E2 on the vaginal epithelium, and resorted to using P4 or medroxy progesterone acetate to make mice susceptible to HSV-2. However, our studies highlight the importance of considering the hormonal microenvironment for examining anti-viral responses in the vagina. We attempted to standardize the 21-day pellet treatment model and a 7-day injectable E2 regime for intravaginal HSV-2 infection. However, the 7-day regime was unable to successfully replicate the E2 effect on vaginal DCs. This is an important limitation in examining anti-viral responses under the influence of E2 in the mouse model. The biological effect of E2 on the vaginal epithelium may or may not be directly linked to the effect on DC functions. Further investigation, and alternate models using chimeric

ERKO mice may be required to disconnect the barrier effect and E2's effects *in vivo* in the context of viral infection.

4.6.7 Mechanism for the induction of IL-1β

Although we have shown that E2 and local IL-17 in the vagina can regulate IL-1β production from vaginal DCs, we have not identified any intracellular pathways. IRF4 was found to be critical for the induction of lung and gut T_h17 responses. However, our study with $IRF4^{-/-}$ mice has shown that IRF4 may not play a critical role in T_h17 responses primed by vaginal TCs. It is unknown whether compensatory mechanisms exist in $IRF4^{-/-}$ mice, which may explain the lack of any differences in co-cultures between $IRF4^{-/-}$ and WT mice. Further investigation into the pathway of IL-1β production is required to understand the mechanism by which E2 influences vaginal DCs.

4.7 Future directions:

4.7.1 Relevance of IL-17 in HSV-2 antiviral immunity:

The next step should be an examination of the role of E2-induced T_h17 responses in HSV-2 antiviral immunity. By using IL-17KO mice, we can identify whether the lack of T_h17 responses post HSV-2 challenge in the vagina can compromise anti-viral T_h1 responses. While not described in this dissertation, we are currently in the process of revising a manuscript indicating that E2 treatment leads to earlier recruitment, and more efficient T_h1 and T_h17 immune responses post HSV-2 challenge *(Mucosal Immunoloy, In revision).* CXCL9 and CXCL10 have been implicated to play an important role in mediating infiltration of memory $CD4^+$ T cells to the genital tract post challenge (Lockhart et al., 2006). We can examine whether IL-17 directly or indirectly leads to the upregulation of these chemokines, thereby influencing anti-viral immunity post challenge.

Furthermore, the significance of IL-1β to the HSV-2 anti-viral response is another important consideration. While IL-1β is a cytokine with a broad array of functions, its role in the HSV-2 anti-viral immune response is unknown. By using IL-$1\beta^{-/-}$ mice, we can examine whether IL-1 is a critical factor in E2-mediated modulation of antiviral responses in the vagina.

4.7.2 The transferability of E2-mediated protection against HSV-2:

We can examine whether the protection induced by E2 in the form of greater T_h17 responses can be transferred to placebo controls. $CD4^+$ T cells can be isolated from the lymphoid tissues of mice immunized intranasally under the

influence of E2, and adoptively transferred to no-hormone/placebo controls prior to intravaginal challenge. Furthermore, in the context of vaccine development, we can examine whether the protective effect provided by E2 can be imparted by the local delivery of E2 within the vaccine formulation. This will allow us to identify whether modifying the hormonal microenvironment during vaccination can generate effective long-term immune responses against STIs such as HSV-2.

4.7.3 Mechanism of E2-conditioned vaginal T_h17 responses:

While we have identified that E2 induces T_h17 responses through an IL-1-dependent pathway, further studies are required to understand the intracellular mechanism for the induction of IL-1. Sex hormones such as E2 can influence a wide variety of genes. Hence, transcriptome analysis of vaginal DCs from E2 treated mice will be helpful to narrow down pathways by which E2 induces IL-1β production in vaginal DCs. It has also been indicated that the NLRP3 inflammasome, and caspase 1 may be involved in the pathway for IL-1β production by DCs, and their subsequent priming of T_h17 resposnes (Mills, Dungan, Jones, & Harris, 2013). We can examine whether E2 leads to the induction of inflammasome pathways in vaginal DCs.

Conclusion:

Overall, this entire body of work provides a comprehensive insight to show that the local microenvironment can influence the phenotype and function of mucosal APCs. Sex hormones such as E2, and local factors such as microflora, and innate lymphocytes, can modulate vaginal IL-17 responses. Furthermore, we have identified that IL-1 is an important part of the mechanism by which E2 conditions T_h17 responses primed by vaginal DCs. This work provides avenues to examine whether modulation of this microenvironment can help optimize vaccine-induced immune responses against STIs. On a more fundamental level, It highlights the need to consider the inherent distinctions in APC populations among different mucosal tissues.

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APPENDIX A

Estradiol enhances $CD4^+$ T-cell anti-viral immunity by priming vaginal DCs to induce T_h17 responses via an IL-1-dependent pathway.

Estradiol primes vaginal DCs to induce potent T_h17 responses

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Abstract:

Clinical and experimental studies have shown that estradiol (E2) confers protection against HIV and other sexually transmitted infections. Here, we investigated the underlying mechanism. Better protection in E2-treated mice, immunized against genital HSV-2, coincided with earlier recruitment and higher proportions of T_h1 and T_h17 effector cells in the vagina, compared to placebo-treated controls. Vaginal APCs isolated from E2-treated mice induced 10-fold higher T_h17 and T_h1 responses, compared to APCs from progesterone-treated, placebo-treated, and estradiol-receptor knockout mice in APC-T cell co-cultures. $CD11c^+$ DCs in the vagina were the predominant APC population responsible for priming these T_h17 responses, and a potent source of IL-6 and IL-1β, important factors for T_h17 differentiation. T_h17 responses were abrogated in APC-T cell co-cultures containing IL-1β KO, but not IL-6 KO vaginal DCs, showing that IL-1β is a critical factor for T_h17 induction in the genital tract. E2 treatment *in vivo* directly induced high expression of IL-1β in vaginal DCs, and addition of IL-1β restored T_h17 induction by IL-1β KO APCs in co-cultures. Finally, we examined the role of IL-17 in anti-HSV-2 memory T cell responses. IL-17 KO mice were more susceptible to intravaginal HSV-2 challenge, compared to WT controls, and vaginal DCs from these mice were defective at priming efficient T_h1 responses *in vitro*, indicating that IL-17 is important for the generation of efficient anti-viral memory responses. We conclude that the genital mucosa has a unique microenvironment whereby E2 enhances $CD4^+$ T anti-viral immunity by priming vaginal DCs to induce T_h17 responses through an IL-1-dependent pathway.

Author Summary

Female sex hormones can affect susceptibility and immune responses to infections. While a number of previous studies, including our own, have shown that progesterone and progesterone-derived hormonal contraceptives increase susceptibility and impair immune responses, estradiol protects against sexually transmitted infections. The reason why estradiol is protective remains unknown. In this study, we investigated the effect of estradiol on dendritic cells, specialized immune cells that determine what type of anti-viral cellular immune responses will be mounted following infection with a sexually transmitted virus, HSV-2. Our studies show that estradiol influences dendritic cells in the vaginal tract of mice to initiate special anti-viral T cell immunity that results in better protection against genital HSV-2 infection. This type of T cell response is unique to the vaginal tract and not found in any other mucosal lining of the body. This is the first study to show directly that estradiol, a female sex hormone, can determine how well the immune system will combat a sexually transmitted viral infection. The information from this study will be very important in understanding what can protect women from sexually transmitted infections and how we can use this to develop better methods, such as vaccines.

Introduction:

The female sex hormones estradiol (E2) and progesterone (P4) play a key role in controlling development and function of the reproductive tract, but can also regulate susceptibility and immunity to sexually transmitted infections (STIs) [1-3]. A number of clinical and experimental studies have shown that the menstrual cycle, hormonal contraceptives, and exogenous hormones can determine susceptibility to HIV-1, HSV-2 and *C. trachomatis* [2, 4-7]. While P4 and P4-based hormonal contraceptives appear to increase susceptibility and transmission to sexually transmitted viruses, E2 is generally considered protective. Studies in macaque models demonstrated that while medroxyprogesterone acetate (MPA), a P4-based contraceptive, enhanced susceptibility to simian immunodeficiency virus (SIV), E2-treatment protected animals against infection [8, 9]. Studies, including our own, have shown that E2, P4 and hormone contraceptives influence the anti-viral immune responses and protection outcomes, in a murine model of HSV-2 infection [10-15]. Although the mechanism underlying increased susceptibility to HIV-1 in women using hormonal contraceptives has gained much attention, the protective effect of E2 remains under-investigated.

HSV-2 is the predominant cause of genital herpes, one of the most prevalent sexually transmitted infections in the world. Over 530 million people worldwide are seropositive for HSV-2 [16], and genital herpes is a known co-factor in the acquisition and transmission of HIV-1 [16]. Currently, there is no known vaccine for HSV-2, and anti-viral formulations only reduce the incidence and symptoms of recurrences. Attempts to develop vaccines against HSV-2 have failed since the 1980s [7]. The last large-scale clinical trial of a glycoprotein D based vaccine showed no efficacy, except for partial protection in a sub-group of women sero-negative for HSV-1 and HSV-2 [17, 18]. These studies emphasize the need to better understand sex-specific immune responses in the

reproductive mucosa, in order to develop effective vaccines against sexually transmitted infections.

A number of studies have examined factors that affect anti-viral immunity in the female reproductive tract [2, 19]. Our own studies have demonstrated that intranasal, subcutaneous or intravaginal immunization with live attenuated thymidine kinase deficient (TK^-) HSV-2, in the presence of P4, led to protection accompanied by excessive genital inflammation and pathology post-challenge [13, 14]. However, immunization in the presence of E2 led to significantly better protection outcomes: better survival without pathology [13-15]. This protective effect of E2 was verified by others, using an HSV-2 subunit-based glycoprotein gD vaccine candidate [10]. Based on these studies, we hypothesized that the differences in protection quality may be due to the influence of sex hormones on the function of antigen presenting cells (APCs), such as dendritic cells (DCs) in the female genital tract. Previous studies have shown that vaginal DCs may be key to the development of $CD4^+$ T cell responses against HSV-2 [20], and both E2 and P4, can modulate DC phenotype and functions [21, 22]. It is well documented that alterations in DC functions can shape $CD4^+$ T cell-mediated adaptive immune responses [23, 24]. For example, IL-12, IL-15, and TNF-α produced by DCs can bias T_h0 cells towards T_h1 effectors, while TSLP, IL-33, and IL-25 can lead to T_h2 responses. Similarly, TGF-β, IL-10, retinoic acid, and the expression of PDL-1 by DCs can prime T regulatory cells, while IL-6, TGF-β, IL-1 and IL-23 can induce T_h17 differentiation [24, 25]. Therefore, we examined whether E2 can directly influence vaginal DCs to direct the differentiation of $CD4^+$ T cells, and consequently alter the profile of anti-viral T cell responses.

The role of T_h1 effectors in HSV-2 anti-viral immunity has been well-described [26]. In brief, IFN-γ-producing T_h1 cells are critical, as demonstrated by studies where the depletion of $CD4^+$ T cells and neutralization of IFN-γ, compromised protection against HSV-2 [27]; the administration of exogenous IFN-γ restored protection to $CD4^+$ T cell deficient mice [28]. T_h2 cells and Tregs may lack a direct anti-viral role in the HSV-2 mouse model [29], but the latter have been implicated in facilitating the efficient influx of immune cells such as NK cells, DCs and T cells to the vagina post-primary infection [30]. IL-17 primarily produced by T_h17 cells is a normal response of the immune system to *C. albicans* and *N. gonorrheae* infections in the vagina [31, 32]. However, the role of T_h17 effector responses in viral infections of the genital mucosa has not been clearly defined.

In the current study, we examined the mechanism underlying the enhanced protection outcomes seen under the influence of E2 in the HSV-2 vaccine model. We observed earlier recruitment, and increased proportions of T_h17 and T_h1 effector cells post-challenge in the vagina of E2-treated immunized mice. E2-treatment directly conditioned vaginal $CD11c^+$ APCs to induce T_h17 responses through an IL-1-dependent, but IL-6-independent pathway. Furthermore, the ability of vaginal $CD11c^+$ APCs to induce predominantly T_h17 responses was distinct compared to APCs from spleen and other mucosa, suggesting that the hormonal conditioning of APCs is unique to the genital mucosa.

Results:

E2 treatment enhances protection against WT genital HSV-2 challenge

We have previously shown that intranasal immunization of ovariectomized (OVX) E2-treated mice with live attenuated TK$^-$ HSV-2, leads to optimal protection with minimal pathology post-challenge, compared to hormone-naïve OVX controls [14]. We wanted to determine whether enhanced protection would also be seen in E2-treated mice immunized with non-live virus vaccine formulations such as an HSV-2 glycoprotein subunit (gD), or a heat-inactivated (HI) HSV-2. OVX mice implanted with 21-day release E2 pellets, were intranasally immunized with HSV-2 gD + CpG, HI HSV-2 + CpG, or live attenuated TK$^-$ HSV-2, and 6 weeks later, were challenged intravaginally (IVAG) with a lethal dose of wild type (WT) HSV-2 333. Survival, genital pathology and viral shedding were monitored post-challenge. The control group of OVX mice was implanted with placebo pellets (mock), but underwent similar immunization and challenge. E2-treatment was associated with 80% survival against lethal HSV-2 challenge in the TK$^-$ HSV-2 vaccine group, and 75% survival in the gD + CpG and HI HSV-2 + CpG vaccine groups (Fig 1A). However, only 30% of mock controls survived lethal challenge in the TK$^-$ HSV-2 vaccine group, while none survived in the gD + CpG or HI HSV-2 + CpG vaccine groups (Fig 1A). Better survival in E2-treated mice corresponded with lower cumulative pathology scores (Table 1), and fewer mice showing viral shedding on any given day post-challenge (Fig 1C), compared to mock controls (Fig 1B and C). Overall, consistent with our previous report [14], these results show that immunization under the influence of E2 enhanced protection by improving survival, and diminishing disease pathology and viral shedding post-challenge, regardless of the vaccine formulation.

Table 1: Cumulative pathology scores for HSV-2 pre-exposed WT and IL-17 KO mice challenged with WT HSV-2.

Treatment Group (total # of mice)	Pathology Score	# of mice	# of days	Cumulative Pathology	Avg. Pathology per Mouse
gD+CpG (n=10)	5	2	9	90	28
	5	1	8	40	
	5	1	7	35	
	5	1	6	30	
	5	3	5	75	
	5	2	1	10	
E2 gD+CpG (n=4)	0	3	14	0	6.25
	5	1	5	25	
HI+CpG (n=5)	5	3	4	60	16
	5	2	2	20	
E2 HI+CpG (n=8)	0	6	10	0	4.38
	5	1	1	5	
	5	1	6	30	
TK- (n=5)	0	2	20	0	28
	5	1	14	70	
	5	1	13	65	
	5	1	1	5	
E2 TK- (n=5)	0	3	20	0	11.2
	3	1	2	6	
	5	1	10	50	

Cumulative pathology is calculated by denoting the number of mice with their maximum pathology score and the average number of days that score was observed for each group. This takes into consideration that each mouse in a group can reach varying degrees of pathology through the experiment. Average pathology scores per mouse was calculated by dividing the sum of cumulative pathology by total number of mice.

E2 treatment leads to earlier recruitment and higher proportion of T_h1 and T_h17 cells in the vagina

HSV-2–specific IFN-γ-producing T_h1 $CD4^+$ T cells are known to play a critical role in the resolution of intravaginal HSV2 infection in the mouse model [27, 28]. We wanted to examine whether improved protection in E2-treated mice was related to enhanced T_h1 responses in the vagina, post-challenge. OVX mice treated with E2 or placebo (mock) pellets were immunized intranasally with TK^- HSV-2, and challenged 6-weeks later, intravaginally, with WT HSV-2. Vaginal tissue from each group of mice was pooled, and the phenotype of $CD4^+$ T cells was examined on days 1, 3 and 5 post-challenge (p.c.). $CD4^+$ T cells were gated based on total $CD3^+$ cells in the vagina (Fig 2A), and the profile of mucosal memory $CD4^+$ T cells ($CD44^+$ $CD103^+$) was compared between E2 and mock treatment groups. Vaginal tissue from E2-treated mice contained higher proportions of mucosal memory $CD4^+$ T cells at all three time points compared to mock controls (Fig 2B). To compare functional differences in the $CD4^+$ T cells between these groups, IFN-γ and IL-17 expression in these cells was examined by intracellular staining (ICS). E2-treated mice showed a higher proportion of IFN-γ^+ T_h1 and IL-17^+ T_h17 cells at earlier time points (days 1 and 3 p.c.) (Fig 2C & Table 2). On day 5 p.c., while a higher proportion of T_h17 cells were still present in the vagina of E2-treated mice compared to the mock controls, fewer T_h1 cells were observed in E2-treated mice, likely due to earlier clearance of virus. Following *in vitro* stimulation with PMA and ionomycin, a higher proportion of total T_h1 and T_h17 cells was seen in E2-treated mice compared to mock controls at all three time points (D1, D3 and D5 p.c.) (Table 2). Overall, these observations suggest that E2 treatment augments anti-viral responses in the female genital tract by accelerating, and enhancing, T_h1 and T_h17 responses post-challenge.

Table 2: Cytokine production by $CD4^+$ T cells in the vagina post intravaginal HSV-2 challenge.

		D1pc		D3pc		D5pc	
Treatments	**Cytokine**	**Mock**	**E2**	**Mock**	**E2**	**Mock**	**E2**
***In vivo* challenge alone**	**IL-17$^+$**	0.9	2.1	0.9	5.3	0.2	2.8
	IFN-γ^+	7.7	17.8	19.9	29.9	14.1	9.8
	IL-17$^+$ IFN-γ^+	0.5	1.8	0.8	4.5	0.06	1.7
***In vivo* challenge + *In vitro* stimulation**	**IL-17$^+$**	6.5	16.7	3.3	12.9	2.7	20.9
	IFN-γ^+	25.0	39.8	34.7	54.3	30.7	51.7
	IL-17$^+$ IFN-γ^+	1.2	4.4	1.1	2.9	1.0	6.9

Mock: Placebo-treated mice, **E2:** E2-treated mice (n=5-10 mice/group, pooled tissue), **pc:** post challenge; ***In vivo* challenge:** Cytokine-producing cells (% of total vaginal CD3+ CD4+ cells) at various time points post WT HSV-2 challenge, blocked with golgi inhibitors for 16h without any additional stimulation; ***In vivo* challenge + *in vitro* stimulation:** Cytokine producing cells (% of total vaginal CD3+ CD4+ cells) at various time points post challenge after *in vitro* stimulation with cell stimulation cocktail containing golgi inhibitors + PMA + ionomycin for 16h. Data representative of two separate experiments with similar results.

Vaginal cells from E2-treated mice induce IL-17 and IFN-γ from T cells in APC-T cell co-cultures

Since E2-treated mice demonstrated accelerated, and greater T_h1 and T_h17 responses, we next wanted to examine whether E2 influences $CD4^+$ T cell responses by conditioning vaginal APCs. OVX mice were implanted with E2, P4, or placebo (mock) pellets, and two-weeks later, vaginal tissue cells (TC) containing all local APCs were isolated, pulsed with OVA peptide, and co-cultured with CFSE-stained OT-II Tg $CD4^+$ T cells (TC+CD4), as detailed in the Materials and Methods. To examine T_h1 and T_h17 differentiation in these co-cultures, IFN-γ and IL-17 levels (mean ± SD) in culture supernatants were measured by ELISA. While there were no differences in $CD4^+$ T cell proliferation between E2, P4 or mock cultures (Fig 3A), the supernatants of co-cultures with TC from E2-treated mice contained over 7 to 18-fold higher IL-17 and 12-fold higher IFN-γ levels, compared to P4 or placebo co-cultures (Fig 3B) (E2: 6297 ± 974 pg/mL; P4: 805.6 ± 82 pg/mL; mock: 349 ± 76 pg/mL); IFN-γ in TC+CD4: (E2: 996 ± 331 pg/mL; P4: 79 ± 19 pg/mL; mock: 61 ± 26 pg/mL)). Intracellular staining (ICS) on day 2 of co-cultures showed that over 70% of proliferating CFSE-stained OT-II Tg $CD4^+$ T cells in E2-treated vaginal TC co-cultures expressed IL-17 and ROR-γt, the master-regulator transcription factor for T_h17 cells (Fig 3C), showing that T_h17 cells were the primary source of IL-17 in these co-cultures.

The E2-dependent induction of T_h17 responses by vaginal tissue cells has not been described previously. Given this unique observation, we examined hormone-dependent T_h17 differentiation further. Both E2 and P4 are continually present in the reproductive tract, albeit in different ratios throughout the different phases of the reproductive cycle [33]. We wanted to examine if the differential conditioning of vaginal APCs to induce T_h17 responses could be observed in the vagina of mice during normal reproductive cycle. Co-cultures were conducted with vaginal TC isolated from mice in estrus (E2-dominant) or diestrus (P4-dominant) stages, while vagina TC from OVX mice served as a control. Vaginal TCs from mice in estrus induced over 3 to 4-fold higher IL-17 levels in co-cultures compared to TCs from mice in diestrus or OVX controls (Fig. 3D) (Estrus: 23827 ± 2452 pg/mL; Diestrus: 8248 ± 3244 pg/mL; OVX: 5744 ± 1573 pg/mL). This suggests that although E2 is present throughout the reproductive cycle, changes in E2 levels during estrus cycle may be sufficient to condition vaginal APCs to prime differential T_h17 responses.

To confirm the role of E2, IL-17 levels were compared between co-cultures conducted with vaginal TC from estrogen receptor knockout (ERKO) mice, and a pooled group of WT mice at different stages of the reproductive cycle. Vaginal cells from ERKO mice induced 12-fold lower IL-17 levels compared to WT controls (WT: 6837 ± 1938 pg/mL; ERKO: 559 ± 58 pg/mL), confirming that E2 is critical for priming of T_h17 responses by vaginal TCs (Fig 3E). Interestingly, ERKO spleen TC co-cultures contained significantly higher IL-17 levels compared to WT controls (WT: 364 ± 61 pg/mL; ERKO: 828 ± 121 pg/mL) (Fig 3E). This suggests that the E2 conditioning of APCs to induce T_h17 responses may be limited to the vagina.

Overall, these results indicate that E2 conditioned vaginal cells induce the differentiation of $CD4^+$ T cells into IL-17-producing T_h17 cells. Furthermore, endogenous

levels of E2 throughout the reproductive cycle appeared sufficient to prime vaginal APCs for induction of T_h17 responses. Therefore, in further studies we pooled mice from all stages of normal reproductive cycles so we could conduct experiments with larger numbers of mice.

Vaginal $CD11c^+$ DCs are potent inducers of T_h17 responses

Next, we wanted to identify the specific APC populations in the vagina that were responsible for priming these T_h17 responses. DCs, broadly classified as $CD11c^+$ cells, macrophages ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$), neutrophils ($CD11c^-$ $CD11b^+$ $F4/80^-$ $Gr\text{-}1^+$), monocytes ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^+$) and other cells ($CD11c^-$ $CD11b^-$) were sorted by FACS, pulsed with OVA peptide, and co-cultured in different ratios with $1x10^5$ OT-II Tg $CD4^+$ T cells. Vaginal TC, $CD11c^+$ DCs, and macrophages induced a similar degree of $CD4^+$ T cell proliferation in co-cultures (Fig 4A). However, cytokine analysis showed that vaginal $CD11c^+$ DCs were the primary inducers of IL-17 from T cells in co-cultures (1331 ± 276 pg/mL at 1:2 ratio, Fig 4B). Macrophages also induced IL-17 levels, albeit 8-fold less in magnitude (170 ± 125 pg/mL at 1:2 ratio) compared to DCs. Neutrophils, monocytes, and other cells did not induce any detectable IL-17, showing that DCs and macrophages may be the sole inducers of T_h17 differentiation *in vitro*.

To confirm that $CD11c^+$ DCs were the primary inducers of T_h17 responses in genital tract, $CD11c^+$ cells were depleted in the vagina by injecting CD11c-DTR mice with 400ng (200ng IP + 200ng intravaginally) of diphtheria toxin (DT); a separate group of CD11c-DTR mice treated with PBS were used as controls. Vaginal TC from both these groups were pulsed with OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells. Since T_h17 cells are known to produce IL-22 in addition to IL-17 [34], we measured both, in co-culture supernatants. Co-cultures with TC from CD11c-depleted mice (DT group) contained approximately 3.5-fold lower levels of IL-17 (PBS: 20895 ± 3766 pg/mL; DT: 6152 ± 341 pg/mL), and 2-fold lower levels of IL-22 (PBS: 134 ± 17 pg/mL; DT: 75 ± 8 pg/mL) compared to TC from CD11c-intact mice (PBS group) (Fig 4C). Similar to the observations in Fig 3E, co-cultures with splenocytes from CD11c-depleted mice showed an increase in IL-17 compared to controls.

These findings confirm the role of vaginal $CD11c^+$ DCs in priming T_h17 responses (Fig 4B), and indicate tissue-specific differences in the propensity of APC populations to prime T_h17 $CD4^+$ T cell responses.

Given the distinct differences in T_h17 responses between vagina and spleen co-cultures, we examined whether the potential of vaginal TCs or $CD11c^+$ DCs to prime T_h17 responses is comparable to total cells or $CD11c^+$ DCs from other mucosal tissues, such as the lung or small intestine. Vagina, lung and small intestine TC (containing all their respective APC populations), were pulsed with OVA peptide, co-cultured with OT-II Tg $CD4^+$ T cells, and T_h17 cytokines were examined in co-culture supernatants. There were no differences in $CD4^+$ T cell proliferation among co-cultures of TC+CD4 from all three mucosal tissues (data not shown); however, vaginal co-cultures contained over 15-fold higher levels of IL-17 (Vagina: 27780 ± 4051 pg/mL; Lung: 1086 ± 326 pg/mL; Intestine: 1827 ± 878 pg/mL) and 4-fold higher levels of IL-22 (Vagina: 2105 ± 157 pg/mL; Lung: 430 ± 54 pg/mL; Intestine: 331 ± 81 pg/mL) compared to lung or intestine

TC co-cultures (Fig 4D), suggesting mucosal tissue-specific differences in the ability of the respective APC populations to prime $CD4^+$ T cell responses.

To directly compare the abilities of vagina and lung $CD11c^+$ DCs to prime T_h17 responses, $CD11c^+$ and $CD11c^-$ cells from both these tissues were sorted, peptide pulsed, and co-cultured with OT-II Tg $CD4^+$ T cells at a 1:2 ratio of APC:CD4. Vaginal $CD11c^+$ DCs induced 10-fold higher IL-17 levels compared to lung $CD11c^+$ DCs (vagina: 7017 ± 577 pg/mL; lung: 764 ± 105 pg/mL) (Fig 4E). Vaginal total cells (vagina: 1901 ± 315 pg/mL; lung: 858 ± 102 pg/mL) and $CD11c^-$ cells (vagina: 436 ± 109 pg/mL; lung: 206 ± 70 pg/mL) also induced significantly higher levels of IL-17, although not as dramatic as $CD11c^+$ DCs. In order to examine the role of $CD11c^-$ cells in conditioning the lung and vaginal $CD11c^+$ cells, $CD11c^+$ cells from each mucosa were mixed with $CD11c^-$ cells from the heterologous mucosa in APC:T cell co-cultures. We found that $CD11c^-$ cells did not influence the ability of vaginal or lung $CD11c^+$ cells to prime T_h17 responses (Fig 4E: Mixed tissues). Vaginal and lung $CD11c^+$ DCs retained their respective ability to induce T_h17 responses, regardless of the tissue source of $CD11c^-$ cells (vagina $CD11c^+$ + lung $CD11c^-$: 7633 ± 307 pg/mL; lung $CD11c^+$ + vagina $CD11c^-$: 994 ± 351 pg/mL). This suggests that mucosal DCs are programmed within their respective tissue microenvironment, and a short-term co-culture with cells from other tissues is not sufficient to change their propensity.

Overall, these results indicate that $CD11c^+$ cells in the vagina are the primary inducers of T_h17 responses, and this ability is distinct compared to other mucosal $CD11c^+$ cells.

E2 influences vaginal DCs to induce T_h17 responses through an IL-1-dependent, but IL-6-independent mechanism

Next, we wanted to examine the factors responsible for priming T_h17 responses in vaginal tissue co-cultures. A cytokine microenvironment containing IL-6, TGF-β and IL-23 is considered essential for priming canonical T_h17 responses [35]. However, alternative pathways involving IL-1 signalling in combination with IL-6, IL-21 and IL-23 have also been described [36]. To determine the key factors for T_h17 responses induced by vaginal $CD11c^+$ DCs, IL-6, IL-23, TGF-β and IL-1β levels were measured in supernatants of TC+CD4 co-cultures from vagina, lung and intestines (Fig 5A). There were no significant differences in IL-23 and TGF-β levels were highest in lung co-cultures (Fig 5A). However vaginal TC alone constitutively produced high levels (4755 ± 1223 pg/mL) of IL-6 (Fig 5A), and this was further enhanced in vaginal TC+CD4 co-cultures (14407 ± 1602 pg/mL). Both lung (8857 ± 766 pg/mL) and intestinal (136 ± 9 pg/mL) co-cultures produced significantly lower levels of IL-6. While we were unable to detect IL-1β secreted into co-culture supernatants by ELISA, intracellular staining showed that IL-1β and IL-6 were both produced by vaginal $CD11c^+$ DCs and to a much less extent by macrophages (Fig 5B), suggesting that either one or both cytokines may play an important role in induction of T_h17 responses.

To determine the role of IL-6 and IL-1β in induction of T_h17 responses, vaginal TC (Fig 5C) or $CD11c^+$ cells sorted from reproductive-cycle matched WT controls, IL-6 KO (Fig 5D) and IL-1β KO (Fig 5E) were co-cultured with OT-II Tg $CD4^+$ T cells. Vaginal TC and $CD11c^+$ DCs from IL-6 KO mice (Fig 5C & D) were fully capable of

priming T_h17 responses in co-cultures, and the addition of exogenous rIL-6 did not significantly affect IL-17 levels in co-cultures (Fig 5D). However, T_h17 responses were significantly impaired in co-cultures containing vagina TC or $CD11c^+$ DCs from IL-1β KO mice (Fig 5C & E), and this effect was reversed by the addition of exogenous rIL-1β (Fig 5E). These results show that IL-1, but not IL-6 signalling, was essential in vaginal DCs for induction of T_h17 responses.

Next, we wanted to determine the link between IL-1 and E2 in vaginal DC conditioning. Vaginal TC were isolated from OVX mice treated with E2, P4, or placebo, and ICS was used to examine whether E2 induced IL-1β production within vaginal DCs. E2 treatment induced a unique, $IL\text{-}1\beta^{high}$ $CD11c^+$ DC population that was absent in both P4-treated and placebo-treated (mock) controls (Fig 5F).

Overall, these results show that E2 can directly condition vaginal DCs to become potent inducers of T_h17 responses, through an IL-1-dependent pathway.

IRF4 is not essential for vaginal T_h17 responses

Previously, others have shown that human and mouse mucosal DCs expressing IRF4, may play a central role in mucosal T_h17 differentiation [37, 38]; furthermore, *in vitro,* E2 could induce IRF4 expression in bone-marrow-derived DCs (BMDCs) [39]. Therefore, we wanted to examine whether IRF4 was critical for induction of T_h17 responses primed by vaginal tissue cells. To examine whether E2 directly induced IRF4 expression in vaginal DCs *in vivo*, IRF4 expression in the vagina of OVX mice treated with E2, P4 or placebo was examined. E2 treatment led to approximately 2-fold higher IRF4 expression in freshly isolated total vaginal cells (E2: 15.8%; P4: 6.5%; mock: 2.3%), and $CD11c^+$ DCs (E2: 52%; P4: 28%; mock: 22%), compared to P4- or placebo-(mock) treatments (Fig 6A). ERKO mice showed a similar frequency of IRF4-expressing total cells and DCs compared to placebo-treated mock controls (Fig 6A). This indicates that in agreement with previous *in vitro* studies [39], E2 can directly induce IRF4 expression in vaginal DCs *in vivo.*

Next, we examined whether IRF4 played a critical role in T_h17 responses primed by vaginal DCs by conducting APC-T cells co-cultures with vaginal TCs from IRF4 knockout (KO) mice and comparing them with WT control mice. Somewhat surprisingly, there were no significant differences in IL-17 levels between co-cultures with vaginal cells from IRF4 KO or WT controls (Fig 6C). IRF4 KO phenotype was confirmed by ICS to rule out technical issues with IRF4 KO mice (Fig 6B).

Overall, this shows that while E2 can directly upregulate IRF4 in vaginal DCs *in vivo*, it does not appear to play a critical role in DC priming of T_h17 responses.

IL-17 KO mice are more susceptible to intravaginal HSV-2 re-exposure due to lower IFN-γ responses

Since our results demonstrated that better protection in E2-treated mice post-challenge (Fig 1 and 2) coincided with enhanced T_h17 responses and E2 treatment conditioned DCs to prime T_h17 responses (Fig 3), we wanted to examine whether IL-17 played a role in anti-viral immunity against HSV-2. Based on vaccine models against lung *M. tuberculosis* [40], we predicted that HSV-2 exposed IL-17 KO mice would display a compromised recall memory T cell response, and would be unable to protect

against subsequent intravaginal exposure to HSV-2. OVX IL-17 KO and WT mice were infected intravaginally with a sub-lethal dose (10^2pfu/mouse) of WT HSV-2 333. Following this primary exposure, there were no differences in the survival, pathology or viral shedding, indicating no change in susceptibility or anti-viral responses between IL-17 KO and WT mice to primary infection (data not shown). We then sought to examine whether these HSV-2 pre-exposed IL-17 KO mice would show compromised anti-viral responses following re-exposure, since this would test the efficacy of recall memory T_h1 effector cells. Pre-exposed IL-17 KO and WT OVX mice were re-exposed, intravaginally, to a lethal dose ($5x10^3$ pfu/mouse) of WT virus. Over 60% of WT control mice survived the lethal challenge, while only 25% of mice survived in the IL-17 KO group (Fig 7A). IL-17 KO mice also showed greater cumulative pathology (18.5), compared to WT controls (9) (Table 3, Fig 7B). Furthermore, 100% of IL-17 KO mice shed virus compared to 40% of WT controls (Fig 7C). These results support our hypothesis and show that IL-17 KO mice are more susceptible to HSV-2 re-exposure due to decreased efficiency of recall HSV-2 anti-viral responses compared to WT mice. Similar results were obtained with mice pre-exposed to two other sub-lethal doses of HSV-2 followed by challenge (data not shown).

Next, we wanted to examine whether the decreased efficiency of memory recall responses in IL-17 KO mice corresponded to an intrinsic impairment in priming T_h1 responses by vaginal APCs. Vaginal TCs from estrus cycle stage-matched IL-17 KO mice and WT controls were pulsed with OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells; IFN-γ levels were measured in co-culture supernatants after 3.5 days. Vaginal TCs from IL-17 KO mice were significantly impaired at inducing IFN-γ^+ in co-cultures compared to WT TCs (WT: 2951 ± 650 pg/mL; IL-17 KO: 404 ± 77 pg/mL) (Fig 7D). These results indicate that IL-17 may indeed enhance anti-viral T_h1 responses in the genital tract.

Table 3: Cumulative pathology scores for HSV-2 pre-exposed WT and IL-17 KO mice challenged with WT HSV-2.

Treatment Group (total # of mice)	Pathology Score	# of mice	# of days	Cumulative Pathology	Avg. pathology per Mouse
WT (n=5)	0	3	11	0	9
	5	1	4	20	
	5	1	5	25	
IL-17 KO (n=4)	2	1	2	4	18.5
	5	2	4	40	
	5	1	6	30	

Cumulative pathology is calculated by denoting the number of mice with their maximum pathology score and the average number of days that score was observed for each group. This takes into consideration that each mouse in a group can reach varying 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.
Data representative of three separate experiments with similar results.

Discussion:

In the current study, we provide evidence for a novel mechanism whereby E2 enhances anti-viral responses in the genital tract by modulating the functions of vaginal DCs. We demonstrated that better protection in E2-treated mice coincided with accelerated and greater T_h1 and T_h17 responses in the vagina post-intravaginal HSV-2 challenge. E2 treatment directly conditioned vaginal DCs to become potent inducers of T_h17 responses, and this ability of E2 to direct T_h17 responses was dependent on the induction of IL-1β in vaginal $CD11c^+$ DCs. Furthermore, this novel mechanism of E2-mediated conditioning was specific to vaginal DCs, as it was not observed in DCs isolated from spleen or other mucosal tissues including the intestine and lung. To the best of our knowledge, this is the first study demonstrating that E2 can directly regulate T-cell mediated adaptive anti-viral immunity in the female genital tract by modulating DC functions.

While others and we have previously reported that the presence of E2 during immunization can improve anti-viral protection in HSV-2 vaccine models [10, 12, 14, 15], the underlying mechanism has remained largely unknown. The current study was designed to address this, and shows that the unique feature of the E2-mediated enhanced protection against HSV-2 was through the induction of T_h17 responses in the genital tract. While the contribution of IFN-γ^+ $CD4^+$ T_h1 cells to HSV-2 anti-viral responses is well understood, this is the first report indicating that T_h17 cells may augment the HSV-2 anti-viral T_h1 responses. The efficiency of T-cell immunity against HSV-2 is best tested in recall responses post-challenge, and this effect was clearly seen in IL-17 KO mice pre-exposed to HSV-2, that were impaired in resolving intravaginal viral challenge compared to WT controls (Fig 7). The molecular mechanism of how IL-17 could enhance IFN-γ^+ $CD4^+$ T_h1 remains to be elucidated, nevertheless our data suggests that the generation of

efficient T_h17 responses may be important for efficient anti-viral memory responses in the vagina.

The T_h17 responses shown in our study were induced by vaginal DCs through an IL-1-dependent, but IL-6-independent pathway (Fig 5C, D and E). While IL-6, along with IL-23 and TGF-β, is required for the canonical pathway of T_h17 differentiation [34], IL-6-independent T_h17 responses have also been identified in mucosal tissues [36, 41]. In the lamina propria of the small intestine, the IL-1β-IL-1R pathway, but not IL-6, has been shown to be essential for the differentiation of steady state T_h17 cells in response to the microflora [42]. Furthermore, while IL-6-dependent T_h17 responses were critical for the clearance of *C. rodentium* [43], IL-6-independent T_h17 cell responses were important for the resolution of *H. polygyrus* infection [44]. This suggests that the nature of antigens, and/or the local cytokine milieu, can determine the pathways of $CD4^+$ T cell differentiation. Our study showed that while IL-1β and IL-6 were both produced by vaginal DCs, IL-1β was required, while IL-6 was dispensable, for vaginal T_h17 responses. Furthermore, our results showed that these T_h17 responses were induced by direct conditioning of vaginal DCs by E2 to express high levels of IL-1β. In order to examine the pathway/s that link E2 to IL-1β production and T_h17 differentiation, we examined intracellular factors that are induced by E2 and involved in T_h17 differentiation. Others have shown that IRF4 expression in DCs was critical for the generation of lung and intestinal T_h17 responses [37, 38]. Additionally, E2 was found to directly induce IRF4 expression in bone marrow DCs [39]. Hence, we sought to examine whether IRF4 is integral to the pathway of T_h17 responses primed by vaginal DCs. While E2 did indeed upregulate IRF4 expression in vaginal DCs *in vivo* (Fig 6A), unlike the observations in lung or intestinal DCs [37, 38], IRF4 was dispensable for T_h17 differentiation, as evident by intact IL-17 levels in vaginal TC co-cultures conducted with IRF4 KO and WT mice (Fig 6C). While, it is likely that other IRFs may compensate for IRF4 in vaginal DCs [45], E2 could also upregulate inflammatory mediators, including IL-1β in DCs required for induction of Th17 responses through a TLR-dependent pathway [46]. Further studies are necessary to examine these mechanisms. Based on the results from our study, it is likely that vaginal DCs upregulate T_h17 responses through a distinct pathway, compared to other mucosal DCs.

Although a number of groups have examined the functional relevance of T_h17 responses in reproductive tract infections, its role under homeostatic conditions has not been examined. IL-17 has been shown to be an important part of the immune response to *N. gonhorreae* and *C. albicans* infections [31, 32, 47]. Vulvovaginal fungal infections affect 70-75% of women, and these infections have been correlated with the E2-dominant phase of the reproductive cycle [47-49]. Therefore, pre-programming of DCs to induce a T_h17 response under the influence of E2 may represent an evolutionary adaptation for protecting the reproductive tract against these infections. The role of T_h17 responses in viral infections is relatively unclear. Two previous studies have suggested that IL-17 may not have a direct protective role in vaginal HSV-2 infection [50, 51]. However, both of these studies utilized MPA, a P4 derivative, to make mice susceptible to HSV-2. Previous studies, including our own, have shown that MPA can significantly downregulate endogenous hormone levels, including E2, and decrease mucosal anti-viral responses to

HSV-2 [11, 52]. This may have precluded an accurate assessment of the contribution of T_h17 cells in HSV-2 anti-viral responses. As seen in the current study, the hormonal environment can have a profound effect on the induction of adaptive immune responses. Therefore, anti-viral immune responses in the female genital tract need to be examined under clearly defined hormonal conditions.

Although our study showed that T_h17 responses coincide with augmented anti-viral immunity in E2-treated mice, further studies are needed to demonstrate the underlying mechanism. In a pulmonary *M. tuberculosis* vaccination model where, like anti-HSV-2 immunity, IFN-γ produced by $CD4^+$ T cells is integral to the protective immune response, the presence of IL-17 correlated with accelerated $CD4^+$ T cell responses and early resolution of bacteria post-challenge [40, 53]. The T_h17 response post-challenge was correlated with a concurrent CXCL9, CXCL10 and CXCL11 chemokine response, which was essential for the accumulation of $CD4^+$ IFN-γ^+ T cells in the lung [40]. Our observations were very similar in that E2-induced T_h17 responses coincided with earlier and greater proportions of $CD4^+$ IFN-γ^+ T cells in the vagina post-challenge (Fig 2 & Table 2). We also showed that the presence of IL-17 was critical for priming efficient T_h1 responses *in vitro* (Fig 7). Thus, like the *M. tuberculosis* study, IL-17 is likely responsible for facilitating the rapid infiltration of memory T_h1 cells through chemokine induction in the vaginal tract. Further studies examining the chemokines and T cell subsets in the vagina post-challenge are ongoing to examine this possibility.

In summary, our study describes for the first time a mechanism by which E2 enhances anti-viral protection following vaccination in the genital HSV-2 mouse model. E2-priming resulted in vaginal APCs becoming potent inducers of T_h17 responses, and this coincided with earlier recruitment and a greater accumulation of IFN-γ^+ $CD4^+$ T cells post-challenge. Furthermore, we demonstrated that $CD11c^+$ cells in the vagina were the primary inducers of T_h17 responses, and E2 was the critical factor that upregulated IL-1β, required for induction of T_h17 responses. Overall, our study provides insight into a potential mechanism by which the hormonal microenvironment during immunization can regulate the induction of mucosal anti-viral T cell immune responses in the female genital tract. Hence, hormonal status should be an important consideration in the development of mucosal vaccines against sexually transmitted pathogens, to assess whether the modulation of hormonal microenvironment can potentially optimize vaccine-mediated immune responses against STIs in the female genital tract.

Materials and Methods:

Animals:

C57BL/6 mice were obtained from Charles River Laboratories Inc (Saint-Constant, QC, Canada). Chicken ovalbumin (OVA) receptor transgenic (Tg) mice (OT-II) whose $CD4^+$ T cells express TCR specific for the ovalbumin 323-339 ($OVA_{323-339}$) epitopes [54], and IL-6 knockout mice (IL-6 KO) [55], were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). IL-1β KO and IL-17 KO mice kindly provided by Dr. Yoichiro Iwakura (University of Tokyo, Minato-ku, Tokyo, Japan) [56, 57], estradiol receptor α knockout mice (ERKO) kindly provided by Prof. P. Chambon (University de Strasbourg, France), and CD11c-DTR mice [58] were bred internally (McMaster

University, Hamilton, ON, Canada). IRF4 KO mice [59] were kindly provided by Dr. Tak Wah Mak (University Health Network, Princess Margaret Cancer Centre, Toronto, ON), and bred internally (McMaster University, Hamilton, ON, Canada).

Surgeries & treatments:

Endogenous hormones were depleted by ovariectomies (OVX) according to previously published protocols [13]. Briefly, OVX mice were anaesthetised with injectable anaesthetic (150mg Ketamine/kg + 10mg Xylazine/kg body weight) and subcutaneously implanted with either 21-day release E2 (476 ng/mouse/day), or P4 (476 µg/mouse/day), or placebo pellets, purchased from Innovative Research of America (Sarasota, Florida, USA) using previously published protocols [14]. DCs were depleted in CD11c-DTR mice using 400ng diphtheria toxin (DT) (Sigma Aldrich, St. Louis, MO, USA) (200ng IP + 200ng intravaginal injections) treatment 18h before tissue retrieval.

Infections:

One week after implanting hormone pellets, OVX mice were immunized intranasally with $1x10^3$ TK^- HSV-2 or 5µg HSV-2 gD + 30µg CpG or $1x10^4$ pfu (plaque forming units) HI HSV-2 333 + 30µg CpG. The immunization was repeated two weeks later, and mice were challenged intravaginally with $5x10^3$ pfu/mouse WT HSV-2 333 according to previously published protocols [11]. Vaginal washes were collected daily post-challenge and frozen down until use at -80°C. To quantify shed virus within these washes, plaque assays were conducted on VERO cells, as described before [11]. Survival and genital pathology was monitored on a five-point scale. 0: no infection, 1: slight redness of external vagina, 2: swelling and redness of vagina, 3: severe swelling and redness of vagina and surrounding tissues, 4: genital ulceration with severe redness and hair loss, and 5: severe ulceration extending to surrounding tissues, ruffled hair, hunched back and lethargy. Animals were sacrificed before they reach stage 5.

Tissue isolation and co-cultures:

Mucosal tissues were enzymatically digested (Lung: collagenase I 150U/mL, intestine: collagenase A 0.239mg/mL and DNase I 20U/mL, vagina: collagenase A 150U/mL; (Roche Diagnostics, Mississauga, ON, Canada)) at 37°C for 1-2h as previously described [60-62]. Spleen was mechanically disrupted and ACK buffer (Sigma Aldrich, St. Louis, MO, USA) was used to lyse blood cells. Mononuclear cells were counted and cell preparations were seeded in a 96-well plate, at $5x10^5$ cells/mL or $2.5x10^5$ DCs/mL, in 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). Cells were pulsed with ovalbumin 323-339 (OVA) peptide (Biomer technology, Pleasanton, CA, USA) for 6-18h. $CD4^+$ T cells were magnetically sorted using CD L3TE microbeads (Miltenyi Biotec, Auburn, CA, USA) from the spleen of OT-II mice, and stained with 50µM CFSE (Sigma Aldrich, St. Louis, MO, USA) according to published protocols [63]. Peptide-pulsed tissue cells were co-cultured with CFSE-stained splenic OT-II Tg $CD4^+$ T cells at a 1:1 ratio for 3.5 days at 37°C based on previously published protocols [64]. In some experiments as indicated in figure legend, 40 ng/mL rIL-6 (R&D systems, Minneapolis, MN, USA) or 100 ng/mL rIL-1β (R&D systems, Minneapolis, MN, USA)

was added on the first day of co-culture. Co-culture supernatants were frozen for cytokine analysis and cells were phenotypically characterized for $CD4^+$ T cell proliferation and intracellular cytokine detection by flow cytometry.

Flow cytometry:

Mononuclear tissue cells or cell fractions from co-cultures were stained with a cocktail of antibodies: [CD11c PE-Cy7, Gr-1 AF700, F4/80 APC, CD3 AF700 (eBioscience, San Diego, CA, USA), CD11b PE-CF594, I-Ab FITC (BD Biosciences, San Jose, CA, USA) CD3 BV785, CD4 BV421 (BioLegend, San Diego, CA, USA)]. For intracellular staining, on day 2 of co-culture, cells were treated with 2µl/mL Cell Stimulation Cocktail plus protein transport inhibitors (500X) (cocktail of phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin) (eBioscience, San Diego, CA, USA) for 12-16 h, permeabilized and fixed with BD Pharmingen™ Transcription Factor Buffer Set (BD Biosciences, San Jose, CA, USA), and stained with intracellular antibodies against cytokines (IL-17 APC, IL-6 PE, (BD Biosciences, San Jose, CA, USA); ROR-γ PE, IRF4 PE, (eBioscience, San Diego, CA, USA)). Data was acquired on a BD LSRII flow cytometer (BD Biosciences, Canada) and analyzed with FlowJo software (Treestar, Ashland, OR, USA). Flow sorting was conducted using a BD FACSAria™ III (BD Biosciences, San Jose, CA, USA) flow sorter to isolate the following populations: DCs ($CD11c^+$ cells); macrophages ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$ cells); monocytes ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^+$ cells), neutrophils ($CD11c^-$ $CD11b^+$ $F4/80^-$ $Gr\text{-}1^+$ cells) and others ($CD11c^-$ $CD11b^-$ cells). Purity was verified by flow analysis of purified fractions on the BD FACSAria™ III, and was consistently found to be over 95%. Cells were analyzed and initially gated on forward and side scatter parameters to select total cells excluding debris or aggregates. Singlet events were selected based on forward scatter area, height and width parameters. $CD3^+$ $CD4^+$ T cells were selected, and IFN-γ^+ or IL-17^+ cells were gated to examine T_h1 and T_h17 populations.

Cytokine analysis:

Co-culture supernatants were assayed using DuoSet ELISA kits to measure IL-17, IL-23, IL-22 and TGF-β (R&D systems, Minneapolis, MN, USA). In some experiments, a custom MSD multiplex kit was used to measure TNF-α, IL-12, IFN-γ, IL-6, IL-17, IL-2, IL-4 and IL-10 and plates were analyzed on a Sector Imager 2400 (Meso Scale Discovery, Rockville, MD, USA).

Statistics:

Data was analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA), and represented as mean ± standard deviation and significance was calculated by comparing the means by one-way or two-way analysis of variance (ANOVA).

Ethics Statement:

All animals in this study were housed at the McMaster Central Animal facility, and the protocols used were approved by the McMaster University Animal Research Ethics Board (AREB) as per AUP # 14-09-40 in accordance with Canadian Council of Animal Care (CCAC) guidelines.

Acknowledgements:

The authors would like to thank Kristen Mueller, Victor Ferreira, Philip Nguyen and Chris P. Verschoor for providing technical assistance.

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FIGURE LEGENDS:

Figure 1: E2 pre-treatment enhances protection against genital HSV-2 challenge in intranasally immunized mice.

WT OVX mice treated with E2 or placebo pellets, were intranasally immunized 1 wk and 3 wk later with $1x10^3$ TK^- HSV-2, or 5µg HSV-2 gD + 30µg CpG, or $1x10^4$ pfu HI HSV-2 333 + 30µg CpG (n=5-10 mice/hormone group for each vaccine formulation). Five weeks following the second immunization, all groups of mice were intravaginally challenged with $5x10^3$ pfu/mouse WT HSV-2 333. (A) Survival curves showing the proportion of mice that survived WT HSV-2 challenge in all vaccine formulations. (B) Pathology scores in these mice graded on a 1-5 scale as described in the materials and methods section, was plotted. Data points superimposed on X-axis indicate mice without genital pathology (C) Vaginal washes were conducted for 5 days post challenge, and HSV-2 viral shedding (bar indicates mean pfu/mL of shed virus) was calculated by conducting viral titrations with a vero-cell based assay. Dashed line indicates the lower detection limit of this assay, and data points on this line indicate un-detectable viral shedding. The % indicates maximum number of mice that shed virus on any given day post challenge. Each symbol in B and C represents a single animal and data has been pooled from 2 separate experiments with similar results.

Figure 2: E2 pre-treatment enhances the recruitment of $CD103^+$ $CD44^+$ $CD4^+$ T cells in the vagina, and is related to increased proportions of T_h1 and T_h17 cells, post challenge.

WT OVX mice were implanted with E2 pellets, or placebo pellets (mock) (n=5-10 mice/group in all three time points: D1, D3 and D5), were immunized intranasally with $1x10^3$ pfu/mouse HSV-2 TK^-, and five weeks later, were challenged intravaginally with $5x10^3$ pfu WT HSV-2 333. Vaginal tissues isolated at D1, D3 and D5 post challenge (p.c.), from each group were pooled, processed and stained with a panel of antibodies against CD3, CD4, CD8, CD44, CD103, IL-17 and IFN-γ according to protocols detailed in the materials and methods section, and examined by flow cytometry. (A) $CD8^-$ $CD4^+$ T cells were gated among total $CD3^+$ T cells in the vagina. (B) The proportion of mucosal memory $CD103^+$ $CD44^+$ T cells from tissues isolated on days 1, 3 and 5 p.c. were compared between E2-mice and mock controls. (C) For intracellular staining of IL-17 and IFN-γ, vaginal cells pooled from n=5 mice per group, at days 1, 3 and 5 p.c, were incubated in the presence of golgi inhibitors alone to examine the *in vivo* response to HSV-2 challenge, or stimulated *in vitro* with cell stimulation cocktail (CSC) containing golgi inhibitors and PMA + ionomycin, for 18h. Intracellular staining for IL-17 and IFN-γ was used to examine the differentiation of $CD4^+$ T cells into T_h17 and T_h1 cells, respectively. A representative of this data from day 3 p.c. is shown. Data is representative of two experiments.

Figure 3: E2 can influence the differentiation of $CD4^+$ T cells in vaginal APC-T cell co-cultures.

OVX WT mice (n=6/group) were implanted with E2-, P4-, or placebo pellets (mock), and two weeks later, vaginal tissues from each group were pooled, and $1x10^5$ vagina tissue cells (TC) were pulsed with OVA peptide and co-cultured with $1x10^5$ OT-II Tg $CD4^+$ T cells (TC+CD4) for 3.5 days. (A) Proliferation of $CD4^+$ T cells was compared among co-

cultures conducted with TC from E2, P4 or placebo treated mice. (B) IL-17 and IFN-γ levels in co-culture supernatants were measured by ELISA. Data is mean±SD of 3 individual co-culture wells from one of 3 separate experiments with similar trends, and significance was calculated by 2-way ANOVA (* $p<0.05$, **** $p<0.0001$). (C) Intracellular staining of vaginal co-cultures to identify the cellular source of IL-17. On day 2 of co-culture, 2ul/mL of CSC was added, and 18h later, co-cultures were stained with antibodies against CD3, CD4, IL-17 and IFN-γ, and analyzed on a flow cytometer. (D) Vaginal tissues from ovary-intact mice were pooled depending on the stage of their reproductive cycle (n=6/stage): E2-dominant: Estrus, and P4-dominant: Diestrus, and OVX controls, pulsed with OVA-peptide, and co-cultured with OT-II Tg $CD4^+$ T cells for 3.5 days. IL-17 levels in co-culture supernatants were measured by ELISA. Data is mean±SD of 3 individual co-culture wells, representative from one of 3 separate experiments with similar results, and significance was calculated by 2-way ANOVA (**** $p<0.0001$). (E) IL-17 levels compared among WT and ERKO vaginal and spleen tissue co-cultures. Data is representative of 2 separate experiments with similar results, and significance was calculated by 2-way ANOVA (*** $p=0.0005$).

Figure 4: Vaginal $CD11c^+$ DCs are the primary inducers of T_h17 responses, and are more potent inducers than other mucosal DCs.

Vaginal cells from WT mice (n=13) were pooled and sorted by FACS, and total vaginal cells as well as sorted populations were pulsed with OVA peptide and co-cultured with $5x10^5$ cells/ml OT-II Tg $CD4^+$ T cells at the indicated ratios. (A) $CD4^+$ T cell proliferation in total vaginal tissue cell co-cultures, $CD11c^+$ DC co-cultures and macrophage co-cultures. (B) IL-17 levels in co-culture supernatants was measured by ELISA and represented as mean±SD of 3 separate wells per co-culture condition. Statistical analysis was done by one-way ANOVA, to calculate significant differences in IL-17 levels between total vaginal co-cultures and the indicated cell-specific co-cultures at each given ratio of APCs:T cells. Data is representative of two separate experiments with similar results (C) CD11c DTR mice (n=5/group) were treated with 400ng DT (200ng IVAG + 200ng IP) or PBS, and 18h later, vagina and spleen from each group were pooled, and $1x10^5$ tissue cells (TC) were pulsed with OVA peptide, and co-cultured with OT-II Tg $CD4^+$ T cells in a 1:1 ratio for 3.5 days. IL-17 and IL-22 levels in vaginal co-cultures, and IL-17 levels in spleen tissue co-cultures were compared between DT-treated and PBS-control groups. Data is represented as mean±SD of 3 separate culture wells from one of two separate experiments with similar trends, and significance was calculated by 2-way ANOVA (** $p<0.01$, *** $p<0.001$). (D) $CD11c^+$ DCs and $CD11c^-$ cells were sorted by FACS from the lungs and vagina of WT mice, and total tissue cells ($1x10^5$ cells/ml), or purified cells ($5x10^4$ cells/ml), were OVA peptide pulsed and co-cultured with OT-II Tg $CD4^+$ T cells ($1x10^5$ cells/ml). For heterologous mixed co-cultures, $CD11c^+$ cells ($5x10^4$ cells/ml) from the vagina or lung were mixed with $CD11c^-$ cells ($5x10^4$ cells/ml) from the other tissue, pulsed with OVA peptide, and co-cultured with $CD4^+$ T cells ($1x10^5$ cells/ml). IL-17 levels in supernatants was measured by ELISA. Significance was calculated by comparing mean±SD of 3 separate co-culture wells per condition, by one-

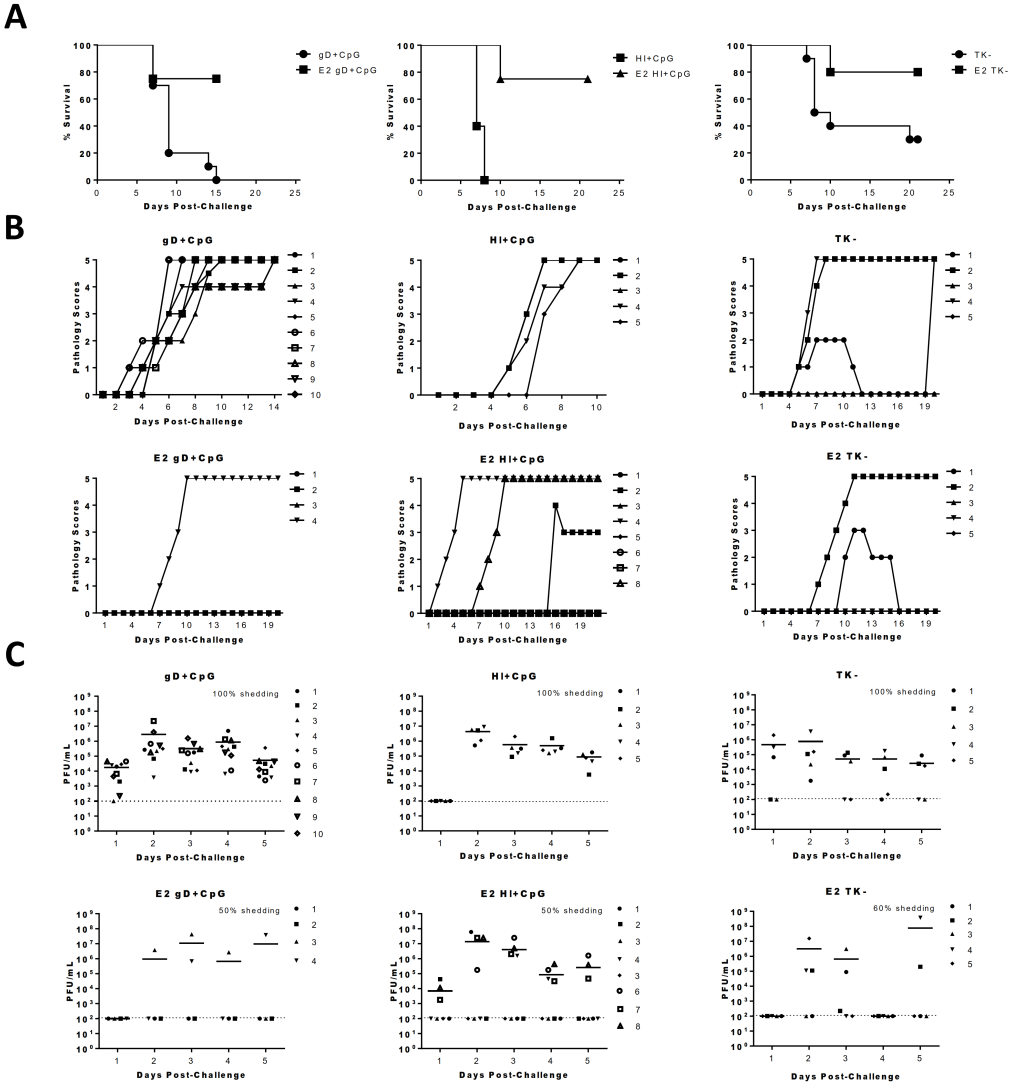
way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Data is a representative from 2 separate experiments with similar trends.

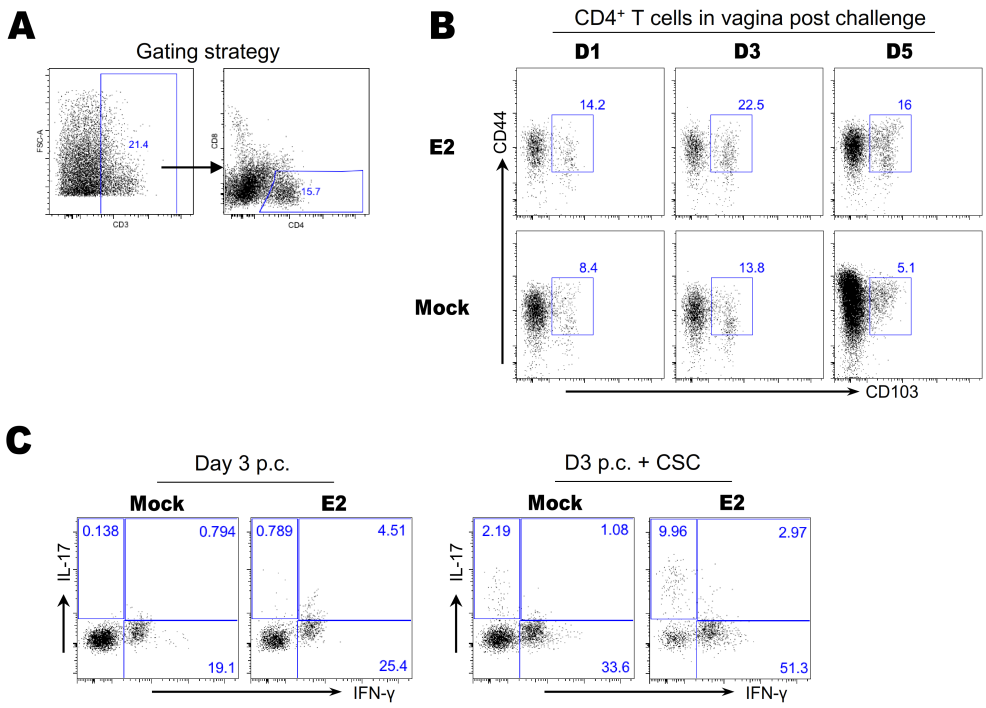
Figure 5: E2 conditions vaginal DCs to induce T_h17 responses through an IL-1-dependent pathway. (A) Total cells (TC) from vagina, lung and intestine were pulsed with OVA peptide and co-cultured with $CD4^+$ T cells (TC+CD4). IL-6, IL-23 and TGF-β was measured in co-culture supernatants by ELISA. (B) Intracellular staining of vagina co-cultures on day 2 of co-culture to examine IL-1β and IL-6 production by vaginal DCs ($CD11c^+$ cells) and macrophages ($CD11c^-$ $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$). (C) IL-17 levels compared among vaginal TC co-cultures from IL-6 KO, IL-1β KO and WT control mice. (D) IL-17 levels compared in co-cultures conducted with TC or $CD11c^+$ DCs purified from the vagina of IL-6 KO mice and WT controls. Forty ng/ml of rIL-6 was added to co-cultures as indicated on X-axis. (E) IL-17 levels were compared in co-cultures conducted with TC or $CD11c^+$ DCs purified from the vagina of IL-1β KO mice and WT controls. Hundred ng/ml rIL-1β was added to co-cultures as indicated on X-axis. (F) Vaginal cells were cultured overnight without any stimulation, and intracellular staining conducted to identify IL-1β production in $CD11c^+$ DCs from OVX mice implanted with E2, P4 or placebo (mock) pellets. Data for all cytokine measurements is represented as mean±SD of 3 separate co-culture wells. Data is a representative of at least 2 separate experiments with similar results, and significance was calculated by two-way ANOVA. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

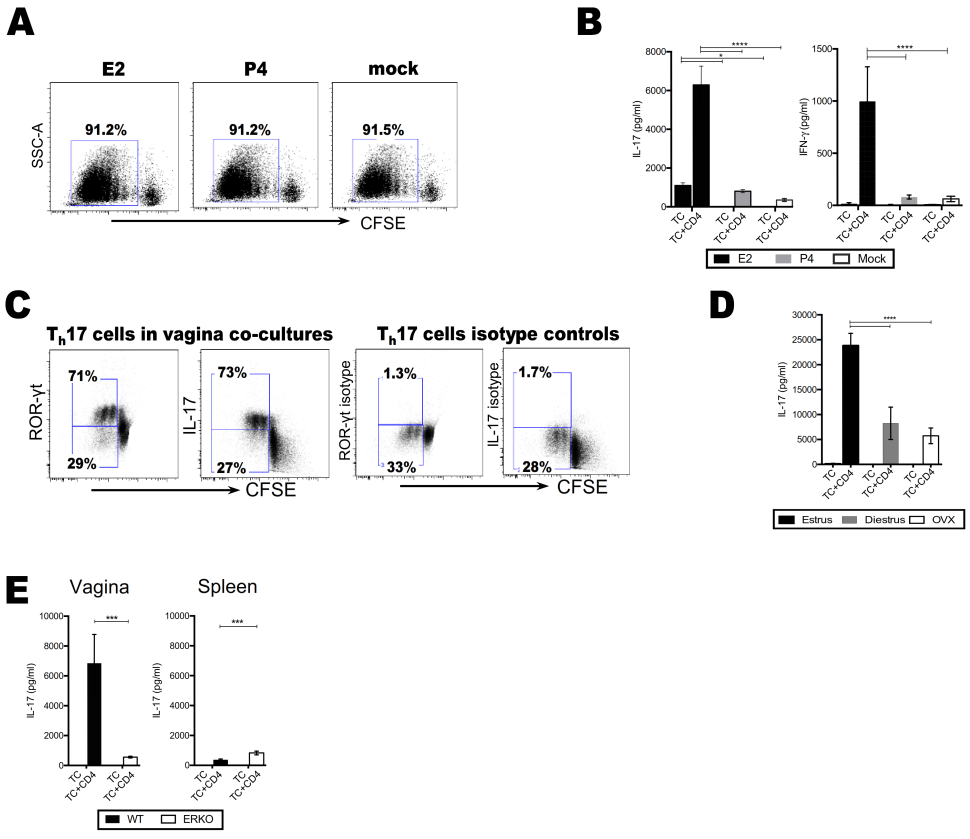
Figure 6: IRF4 expression is not critical for E2-mediated priming of vaginal T_h17 responses. (A) Vaginal cells from WT OVX mice implanted with E2, P4 or placebo (mock) pellets for 14 days were cultured in media overnight without any stimulation (12h), and stained for antibodies against IRF4 and DCs (CD11c, CD11b). ICS was conducted according to protocols in materials and methods to identify IRF4 expression in total vaginal cells and vaginal $CD11c^+$ DCs. (B) Spleen from IRF4KO mice and their WT littermates was isolated, and cultured overnight without stimulation. IRF4 expression in $CD4^+$ T cells was compared between IRF4 KO mice and WT littermates by ICS. (C) Vaginal cells from reproductive cycle stage matched WT and IRF4 KO mice were pulsed with OVA peptide, and co-cultured with OT-II Tg CD4+ T cells for 3.5 days. IL-17 levels in co-cultures was measured by ELISA, and expressed as mean±SD of 3 replicate wells from one of two different experiments. Analysis was conducted by 2-way ANOVA.

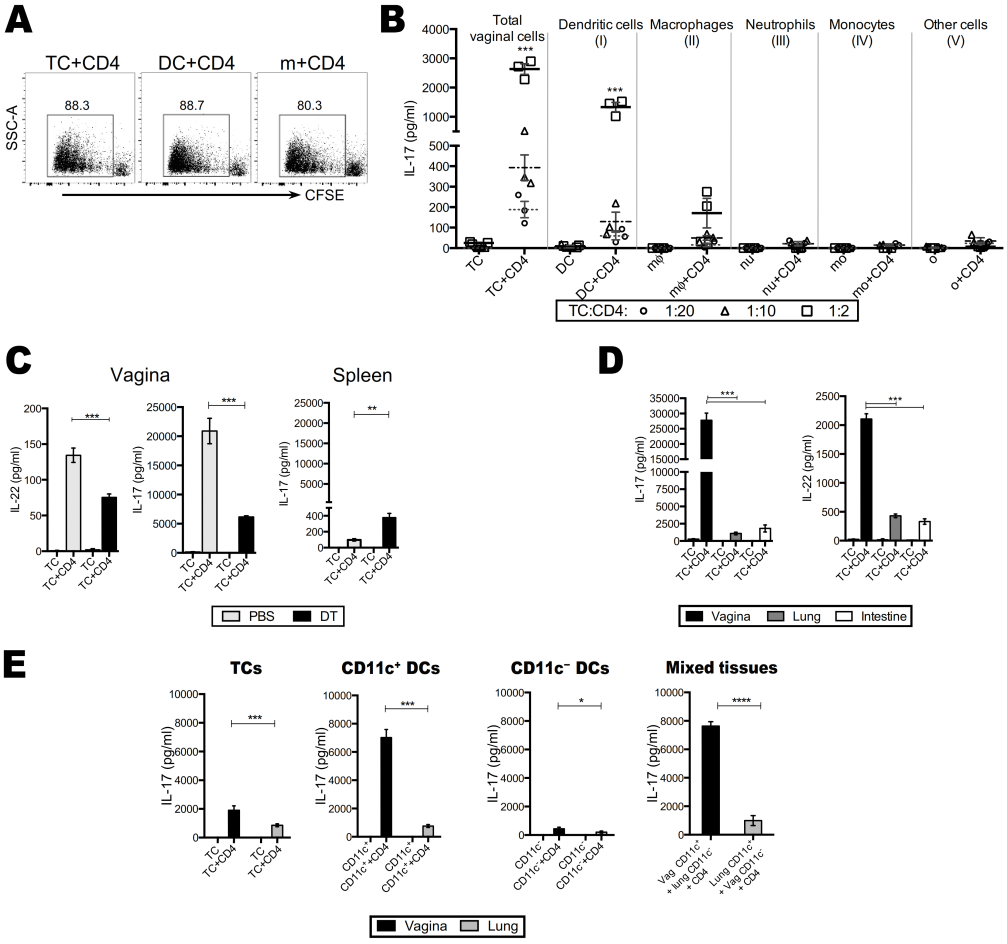
Figure 7: IL-17 KO mice were more susceptible to intravaginal HSV-2 challenge. OVX IL-17 KO (n=4) and WT mice (n=5) were intravaginally exposed to a sub-lethal dose of HSV-2 333 (10^2 pfu/mouse), and 2 months later, intravaginally challenged with a lethal dose of HSV-2 ($5x10^3$ pfu/mouse). (A) Survival curves for IL-17 KO and WT mice showing proportion of mice that survived challenge (B) Genital pathology graded on a 1-5 scale for both groups of mice for 12 days post challenge. (C) HSV-2 viral shedding (pfu/mL) in vaginal washes conducted for 1 week post challenge identified by conducting viral titers with vero cells. The dashed line indicates the lower detection limit of this assay. Each symbol represents a single animal, and data points on the lower limit indicate mice that do not show detectable viral shedding in vaginal washes. The survival curves, pathology and viral titers are from a single representative of 3 separate experiments with

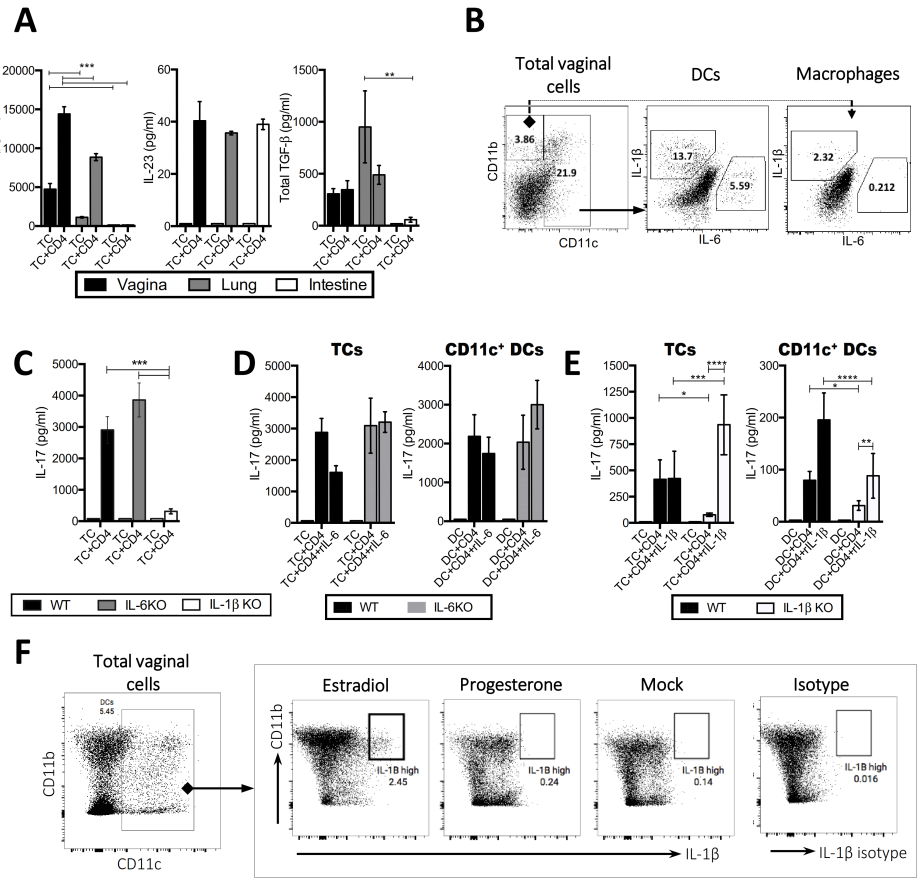
similar results. (D) Vaginal tissues from stage-matched WT and IL-17KO mice were isolated, pulsed with OVA peptide and co-cultured for 3.5 days with OT-II Tg $CD4^+$ T cells. IFN-γ levels produced by vaginal tissue cells alone (TC) and co-cultures (TC+CD4) was measured by ELISA. Data is represented as mean±SD of 3 co-culture wells, and is a representative of 3 separate experiments with similar trends. Significance was calculated by two-way ANOVA (**** $p<0.0001$).

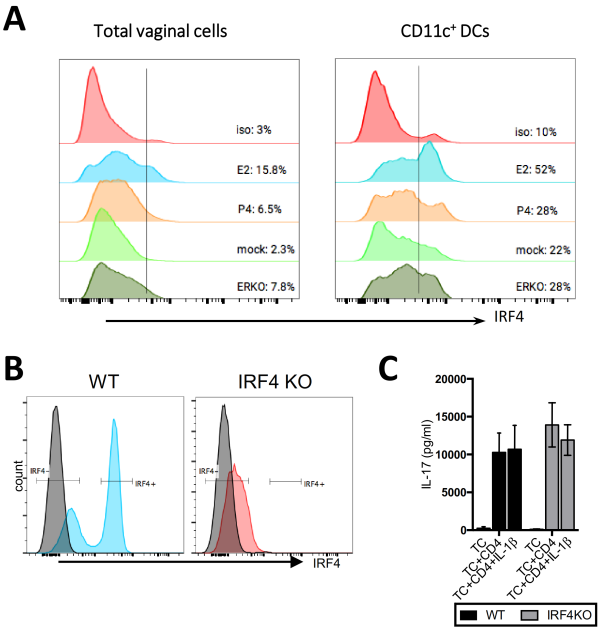

A
% Survival
Days Post-Challenge
gD+CpG
E2 gD+CpG
HI+CpG
E2 HI+CpG
TK-
E2 TK-
B
Pathology Scores
E2 gD+CpG
E2 HI+CpG
E2 TK-
C
PFU/mL
100% shedding
50% shedding
60% shedding

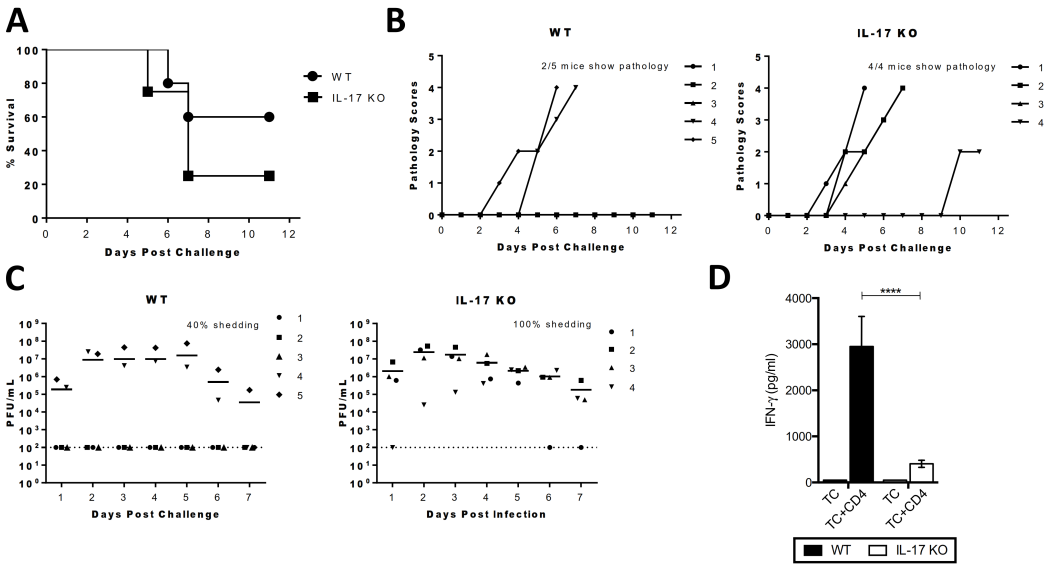

A
Gating strategy
21.4
15.7
CD3
CD4
B
CD4+ T cells in vagina post challenge
D1
D3
D5
E2
Mock
14.2
22.5
16
8.4
13.8
5.1
CD44
CD103
C
Day 3 p.c.
D3 p.c. + CSC
Mock
E2
0.138
0.794
19.1
0.789
4.51
25.4
2.19
1.08
33.6
9.96
2.97
51.3
IL-17
IFN-γ


A
E2
P4
mock
SSC-A
91.2%
91.2%
91.5%
CFSE
B
IL-17 (pg/ml)
IFN-γ (pg/ml)
TC
TC+CD4
E2
P4
Mock
C
Th17 cells in vagina co-cultures
Th17 cells isotype controls
ROR-γt
IL-17
71%
29%
73%
27%
ROR-γt isotype
IL-17 isotype
1.3%
33%
1.7%
28%
CFSE
D
IL-17 (pg/ml)
Estrus
Diestrus
OVX
E
Vagina
Spleen
IL-17 (pg/ml)
WT
ERKO


A
TC+CD4
DC+CD4
m+CD4
88.3
88.7
80.3
SSC-A
CFSE
B
Total vaginal cells
Dendritic cells (I)
Macrophages (II)
Neutrophils (III)
Monocytes (IV)
Other cells (V)
IL-17 (pg/ml)
TC
TC+CD4
DC
DC+CD4
mϕ
mϕ+CD4
nu
nu+CD4
mo
mo+CD4
o
o+CD4
TC:CD4: 1:20 1:10 1:2
C
Vagina
Spleen
IL-22 (pg/ml)
IL-17 (pg/ml)
PBS
DT
D
IL-17 (pg/ml)
IL-22 (pg/ml)
Vagina
Lung
Intestine
E
TCs
CD11c+ DCs
CD11c− DCs
Mixed tissues
IL-17 (pg/ml)
Vagina
Lung


A
B
C
D
E
F
IL-6 (pg/ml)
IL-23 (pg/ml)
Total TGF-β (pg/ml)
TC
TC+CD4
Vagina
Lung
Intestine
Total vaginal cells
DCs
Macrophages
CD11b
CD11c
IL-1β
IL-6
3.86
21.9
13.7
5.59
2.32
0.212
IL-17 (pg/ml)
TCs
CD11c⁺ DCs
TC+CD4+IL-6
DC+CD4
DC+CD4+IL-6
WT
IL-6KO
IL-1β KO
TC+CD4+IL-1β
DC+CD4+IL-1β
Estradiol
Progesterone
Mock
Isotype
DCs 5.45
IL-1B high 2.45
IL-1B high 0.24
IL-1B high 0.14
IL-1B high 0.016
IL-1β isotype


A
Total vaginal cells
CD11c+ DCs
iso: 3%
E2: 15.8%
P4: 6.5%
mock: 2.3%
ERKO: 7.8%
iso: 10%
E2: 52%
P4: 28%
mock: 22%
ERKO: 28%
IRF4
B
WT
IRF4 KO
count
IRF4-
IRF4+
IRF4
C
IL-17 (pg/ml)
20000
15000
10000
5000
0
TC
TC+CD4
TC+CD4+IL-1β
WT
IRF4KO


A
% Survival
Days Post Challenge
WT
IL-17 KO
B
WT
2/5 mice show pathology
Pathology Scores
Days Post Challenge
IL-17 KO
4/4 mice show pathology
C
WT
40% shedding
PFU/mL
Days Post Challenge
IL-17 KO
100% shedding
Days Post Infection
D
IFN-γ (pg/ml)
****
TC
TC+CD4
WT
IL-17 KO