

**Understanding the Role of Neutrophils in Female Reproductive Tract (FRT) Epithelial
Barrier Remodeling *In Vivo***

By

Atta Yazdanpanah

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Department of Immunology

University of Manitoba

Winnipeg, Manitoba, Canada

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ABSTRACT

When the mucous membrane of the female reproductive tract (FRT) is compromised, women are more susceptible to pathogens, especially human immunodeficiency virus (HIV), which emphasizes the importance of an intact barrier in female vaginal health. In a large human cohort analysis, previous studies have observed a strong association between neutrophil activation, epithelial barrier damage and HIV infection, supporting the hypothesis that these innate cells are an important modulator of the vaginal mucosal barrier and HIV susceptibility. However, a gap in knowledge is the biological mechanisms that regulate the recruitment of neutrophils and their interaction with the vaginal epithelium. Here, we show, using a mouse model, that neutrophils are recruited into the vaginal mucosa in response to progesterone. Proteomic analysis of the cervicovaginal lavage fluid revealed that pathways involved in epithelial barrier disruption were activated, and that these pathways were significantly reduced after *in vivo* depletion of neutrophils. We show that vaginal neutrophils expressed high levels of CXCR2 that allowed their positioning along the outer layers of the vaginal epithelium. Vaginal inoculation of BSA at steady-state and neutrophil depletion did not assign neutrophils to epithelial disruption. Furthermore, elevated expression of neutrophil-associated mediators MPO, MMP8 and elastase in the diestrus phase were observed. In the presence of anaerobic bacteria that are associated with bacterial vaginosis (BV), expression of neutrophil elastase, MMP8, and MMP9 trended higher, suggesting that different bacteria species can elevate inflammation reactions by activating mucosal neutrophils. These data argue that the mouse model can recapitulate key aspects of neutrophil:epithelium interactions *in vivo*. Our overall goal is to further define the role of mucosal neutrophils, and whether blocking their recruitment/activation can reduce HIV susceptibility.

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DEDICATION

I dedicate this thesis specially to my lovely and supportive wife Maryam, family, and friends whose unbelievable support, motivation and understanding have made this possible.

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LIST OF ABBREVIATIONS

°C	celsius
2M	2 molar
6Ckine	chemokine (C-C motif) ligand-21 (CCL21)
ACK	ammonium-Chloride-Potassium
AMPs	adenosine monophosphates
APCs	antigen presenting cells
BAL	bronchoalveolar lavage
BD	becton dickinson
BLT	bone marrow-liver-thymus
BSA	bovine serum albumin
BV	bacterial vaginosis
Cat	catalogue
CCL2	chemokine (C-C motif) ligand 2
CCL4	chemokine (C-C motif) ligand 4
CCL5	chemokine (C-C motif) ligand 5
CCR5	C-C chemokine receptor-5
CCR7	C-C chemokine receptor-7
CD	cluster of differentiation
cfu	colony-forming unit
CG	cathepsin G
Cl ⁻	chloride anion
CR3	complement receptor 3
CR4	complement receptor 4
CT1	cervicotypes 1
CT4	cervicotypes 4
CVL	cervicovaginal lavage
CXCL	chemokine (C-X-C motif) ligand
CXCR2	CXC chemokine receptor 2
CXCR3	CXC chemokine receptor 3
DCs	dendritic cells
dsRNA	double-strand ribonucleic acid
ECM	extracellular matrix
ECs	epithelial cells
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
Fc	fragment crystallizable
FKN	fractalkine
fMLP	formylmethionyl-leucyl-phenylalanine
FRT	female reproductive tract
FSH	follicle-stimulating hormone
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMPs	guanosine monophosphates
GnRH	gonadotropin-releasing hormone

GPCRs	G protein-coupled receptors
GTPases	hydrolysis of guanosine triphosphate
H ₂ O ₂	hydrogen peroxide
HBD	human β -defensin
HCC-1/4	hepatocellular carcinoma-1/4
HIV	human immunodeficiency virus
HNP1-2-3	human neutrophil defensins 1-2-3
HOCl	hypochlorous acid
HPV	human papillomavirus
HSC	hematopoietic stem cell
HSV-2	herpes simplex virus type-2
i.p	intraperitoneally
IFN γ	interferon alpha
IgG	immunoglobulin G
IgG2a	immunoglobulin Ga2s
IL	interleukin
IL-1 α	interleukin-1 alpha
IL-1 β	interleukin-1 beta
iNOS	inducible nitric oxide synthase
IP-10	interferon gamma-induced protein-10
IPA	ingenuity pathway analysis
ITGB2	integrin beta 2
KO	knockout
LASSO	least absolute shrinkage and selection operator
LFA-1	lymphocyte function-associated antigen-1
LH	luteinizing hormone
LIF	leukemia inhibitory factor
LMPPs	lymphoid-primed multipotent progenitors
LPS	lipopolysaccharide
LSR	liquid silicone rubber
Ly6G	lymphocyte antigen 6 complex locus G6D
MAC-1	macrophage-1
MCP-1	monocyte chemoattractant protein-1
MCP-3	monocyte-chemotactic protein-3
MDC	macrophage-derived chemokine
mg/kg	milligram/kilogram
mg/mL	milligram/millilitre
MIF	macrophage migration inhibitory factor
MIP-1	macrophage inflammatory protein-1
MIP-1 β	macrophage inflammatory protein-1 beta
MIP-1 α	macrophage inflammatory protein-1 alpha
MIP-3 α	macrophage inflammatory protein-3 alpha
mL	milliliter
MMPs	matrix metalloproteinases
MPO	myeloperoxidase
MPP	multipotent progenitor cell

MS	mass spectrometry
N-9	nonoxynol-9
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NF-kB	nuclear Factor kappa-light-chain-enhancer of activated B cells
NGAL	neutrophil gelatinase-associated lipocalin
NKs	natural killer cells
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain-like receptors
NSP4	neutrophil serine protease-4
NSPs	neutrophil serine proteases
O.C.T	optimal cutting temperature
PA-GFP	photoactivatable green fluorescent protein
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PBS/BSA	phosphate-buffered saline/bovine serum albumin
PFA	paraformaldehyde
PR3	proteinase 3
PrEP	Pre-exposure prophylaxis
PRRs	pattern recognition receptors
R&D	research & development
RANTES	regulated on activation, normal T cell expressed and secreted
Ref	reference
ROI	reactive oxygen intermediate
ROSs	reactive oxygen species
RPMI	roswell park memorial institute
SEM	standard error of the mean
SLPI	secretory leukocyte protease inhibitors
STIs	sexually transmitted infections
TAP	tracheal antimicrobial peptide
TER	transepithelial electrical resistance
TGF- β	transforming growth factor beta
Th1	T helper type-1
Th17	T helper type-17
TLP	TRAP-1-like protein
TLRs	toll-like receptors
TNF	tumor necrosis factor
TNF- α	tumor necrosis factor-alpha
TZ	transformation zone
uNKs	uterine natural killer cells
VLA-4	very late antigen-4
ZO-1	zonula occludens-1
ZO-2	zonula occludens-2
ZO-3	zonula occludens-3
$\mu\text{g/mL}$	microgram/milliliter

μL	microliter
$\mu\text{L/mL}$	microliter/milliliter
μm	micrometer

CHAPTER 1 – INTRODUCTION

1. Immunity of the female reproductive tract (FRT)

The mucosal immune system within the female reproductive tract (FRT) acts as the first line of defence against the external environment (Hickey et al., 2011). Immunity of the FRT, which consists of both the innate and adaptive immune compartments, has greatly evolved in order to meet the unique requirements of balancing responses to commensal microorganisms, sexually transmitted infections (STIs) and maintaining embryonic development during pregnancy (Ochiel et al., 2008; Mor and Cardenas., 2010). Furthermore, the FRT poses specific challenges that shapes their characteristics, and the innate and adaptive immune systems must respond to foreign antigens and initiate effective responses. The presence of cells expressing toll-like receptors (TLRs) in the fallopian tubes, uterus, cervix, and vagina, and immune cells (DCs, NKs, and neutrophils) that respond to select pathogen-associated molecular patterns (PAMPs) indicate that the FRT mucosal immune system has adapted to each of these sites to balance response to STIs and to support an immunological distinct fetal placental unit (Pioli t al., 2004; Itoh et al., 2006).

2. Innate and adaptive immunity in the FRT

The innate and adaptive mucosal immune system are precisely regulated by both cytokines/chemokines and sex hormones, estrogen and progesterone, which fluctuate during the menstrual cycle (Ochiel et al., 2008; Wira et al., 2010). The FRT innate immune system is characterized as the first line of defence and plays a key role in preventing and controlling pathogen invasion (Wira et al., 2005). The innate immune system is specialized to recognize foreign substances from self-antigens and depends on receptors that recognize conserved PAMPs found on microorganisms (Akira et al., 2006). TLRs expressed by DCs, NK cells, neutrophils, and

epithelial cells are a group of pattern recognition receptors (PRRs) that recognize PAMPs, and the initiation of complex signalling through TLRs results in recruitment and activation of immune cells. These responses that culminate in the production of intracellular and secreted anti-microbial factors can kill invading pathogens but also bridge innate with adaptive immunity (Schutte and McCray., 2002; Akira and Takeda., 2004; Schaefer et al., 2004; Fazeli et al., 2005; Nasu et al., 2007). The adaptive immune system that are generated after infection within the FRT are important to provide long-lived protection against re-infection. Antigen presenting cells (APCs) initiate the adaptive immune response to pathogens by engulfing and processing antigens and presenting them to T cells, leading to T cell activation and the production of appropriate chemokine and cytokines, cytotoxicity and antibody production. Although the mechanisms that regulate the mucosal immune system in the FRT are still not entirely understood, the last decade of substantial research has provided important insights into the associations between sexually transmitted infections (STIs) and how the vaginal microbiota communities modulate HIV acquisition (Ward and Rönn., 2010). However, a better mechanistic understanding of FRT function, and how genetic and environmental factors can impact HIV susceptibility, is not yet completely understood.

3. Anatomical and biological functions of the FRT

The lower portion of the FRT is the initial site that comes in contact with the external environment and is comprised of four discrete anatomical regions: (1) the vaginal introitus, which is covered by a keratinized stratified squamous epithelium, (2) the vaginal mucosa, which is covered by a non-keratinized stratified squamous epithelium, (3) the ectocervix, covered by mucosal layers similar to that of the vagina, and (4) the endocervix, consisting of single layer of

columnar epithelium. The transformation zone (TZ) exhibits swift transition between ectocervix and endocervix which differs in susceptibility to infectious organisms (Pudney et al., 2005)

3.1 FRT Ovarian cycle

Females undergo complex cycling of reproductive hormones referred to as the ovarian cycle and is described as part of the menstrual cycle with changes in the follicles in the ovaries. It consists of three phases: follicular phase, ovulation phase, and luteal phase.

3.1.1. Follicular phase

This phase typically persists for 12 to 16 days. The start of this phase begins with day 1 of the cycle and ends with ovulation. The growth of the follicles is stimulated by follicle stimulating hormone (FSH) which in turn become the source of estrogen in the cycle. Therefore, the early follicular phase is characterized by high plasma levels of FSH and lower levels of luteinizing hormone (LH), estrogen, and progesterone. FSH and LH utilize a combined effect on the follicles to stimulate secretion of estrogen, which by the middle to the late follicular phase, is secreted by the dominant follicle (Van Look et al., 1980). Estrogen levels in plasma rise by mid-follicular phase and suppress secretion of FSH by action on the pituitary gland. By late follicular phase, the estrogen has increased and utilizes a positive feedback effect on LH by amplifying LH responsiveness to gonadotropin-releasing hormone (GnRH) and increasing its secretion (Van Look et al., 1980; Buffet et al., 1998). Moreover, progesterone at this time of the cycle is also beginning to elevate and applies a positive feedback effect on LH. The ovulatory surge “GnRH-LH-FSH” is induced by the ovarian estrogen-progesterone signal system. While a number of follicles will

develop in the ovary at the same time, only one will go on to become the ovum that is released at ovulation. Other follicles will undergo atresia, or cell death (Buffet et al., 1998).

3.1.2. Ovulation

The ovulation phase is a process by which the mature oocyte (egg) is released from the ovary. At the end of the follicle phase estrogen levels peak, resulting in surge increase of FSH and luteinizing hormone (LH) levels. Elevations in hormones FSH and LH leads to oocyte maturation and follicle wall impairment in the ovary, resulting in the mature follicle to release its secondary oocyte. This secondary oocyte first becomes an ootid (immature ovum) then matures into an ovum (Buffet et al., 1998).

3.1.3. Luteal phase

This phase usually persists for 10 to 16 days and is the final phase of the ovarian cycle. In this phase with elevation of pituitary hormones FSH and LH, the remaining part of the dominate mature follicle transforms into the corpus luteum (Buffet et al., 1998). Production and secretion of estrogen and progesterone in the luteal phase is a result of the corpus luteum. The increased levels of estrogen and progesterone lasts for a number of days after ovulation during which FSH secretion is inhibited and remains low (Buffet et al., 1998). Increase in progesterone levels stimulate the growth of the endometrium which permit development and growth of the fertilized ovum (Buffet et al., 1998). If fertilization does not occur, plasma levels of estrogen and progesterone decline leading to shedding of the endometrium which is seen as menstrual flow. It is at this time that FSH levels begin to rise which initiate the development of a new set of follicles, and the cycle begins all over again (Van Look et al., 1980; Buffet et al., 1998).

3.2 Immune cells of the FRT

3.2.1 Epithelial cells (ECs)

Epithelial cells (ECs) have clearly been shown to be involved in both the innate and adaptive immunity of the FRT. ECs form a mucosal barrier between the vaginal lumen and underlying cells, which prevent opportunistic and pathogenic microbes from entering the body and enable the transport of sperm or ovum to the site of fertilization (Fahey et al., 2005). Furthermore, ECs transport immunoglobulins into the FRT secretions and generate bactericidal and viricidal substances. Through the production of cytokines and chemokines, they can initiate the recruitment and activation of innate and adaptive immune cells from the periphery, thereby participating in the initial stages of the immune response (Schaefer et al., 2004). A unique function of ECs is their ability to respond to fluctuated levels of sex hormones within the FRT. Studies have shown that vaginal epithelial cells can directly respond to estrogen and progesterone levels and indirectly to cytokines/chemokines produced by resident and migratory cells in the FRT. Estrogen and progesterone are known to have distinct effects on leukocyte trafficking and activation, as described in more detail below (Cooke et al., 1997).

3.2.1.1 Epithelial cells provide a physical barrier from the external environment

The vagina is lined with several layers of stratified squamous epithelial cells, while the upper portion of the cervix is lined with a single layer of columnar epithelium (Trifonova et al., 2014). The vaginal epithelial cell integrity is dependent on the presence and maintenance of tight junctions (Blaskewicz et al., 2011) through expression of ZO-1, ZO-2 and ZO-3 and symplekin that function to seal off the lumen from the vaginal cavity (Mitic et al., 1998; Nustrat et al., 2000). However, this epithelial barrier is continuously and dynamically regulated by

cytokines/chemokines, bacterial products and especially sex hormones (Madara., 1990; McKay and Baird., 1999). Disruption to these tight junctions or damage to the epithelial layer can allow pathogens to enter the submucosa and cause disease. In tissue culture, ECs produce a trans-monolayer electrochemical gradient that reflects barrier function (Planchon et al., 1999; Matter et al., 2005), and the tightness of epithelial tight junctions can be measured as trans-epithelial electrical resistance (TER) (Tsukita et al., 2001; Shin et al., 2006). Estradiol concentrations influence epithelial integrity and significantly reduce TER of mouse epithelial cells (Grant and Wira., 2004) which are believed to be mediated by the expression of matrix metalloproteinases (MMPs). In addition, a 25% reduction of TER in ECC-1 cells in the presence of estradiol was reported, while progesterone had no effect on TER (Wira et al., 2010). However, these studies were not able to recapitulate the multilayered squamous epithelium that make up the vaginal epithelial layer. Using a rhesus macaque model, where the anatomical structure of the vagina is similar to those in humans, Carias *et al* intravaginally inoculated macaques with a fluorescently-labeled BSA, and showed that photoactivatable GFP-labeled HIV-1 (PA-GFP HIV-1) can effectively penetrate the intact squamous epithelium in regions where cell-cell junctions are lacking, but not anywhere else, demonstrating that the ECs provide robust protection against pathogens if intact (Carias et al., 2013). Several studies have indicated that nonoxynol-9, a component of spermicides and well-known to disrupt the vaginal epithelium, can increase HIV susceptibility (Fichorova et al., 2001; Van Damme et al., 2002; Su et al., 2011). Repeated use of nonoxynol-9 has been reported to elevate proinflammatory chemokines IL-8, IL-1 α/β and MIP 1 α/β in cervicovaginal secretions, suggestive of neutrophil influx and their possible role in disruption of the epithelium (Fichorova et al., 2001; Iyer and Poddar., 2008; Lozenski et al., 2012).

3.2.1.2 Antigen presentation by epithelial cells

While numerous APCs that are found within the FRT, vaginal epithelial cells have also been shown to present antigens directly to T cells (Fahey et al., 2006), and that sex hormones can also regulate this process. Studies have demonstrated that in rats, the epithelial, stromal and vaginal cells can present antigen to CD4⁺ T cells, which is under hormonal and cytokine regulation (Wira and Rossell., 1995). Furthermore, estradiol is able to enhance antigen presentation by ECs during the time that antigen presentation by uterine and vaginal stromal cells is inhibited (Prabhala and Wira., 1995). Studies in rats have shown higher presentation efficiency in epithelial cells at the peak of estrogen expression compared to other phases of the estrous cycle (Wira and Rossoll., 1995). In contrast, antigen presentation by vaginal epithelial cells differ with stage cycle, where it was the highest at diestrus phase and lowest at proestrus phase (Wira and Rossoll., 1995). These studies demonstrate some abilities of FRT ECs to directly activate CD4⁺ T cells through antigen presentation.

3.2.1.3 Production of antimicrobial molecules by epithelial cells

Vaginal epithelial cells, similar to ECs found at other mucosal sites, produce a wide range of soluble factors with microbicidal effects, including enzymes lysozyme and lactoferrin, defensins, secretory leukocyte protease inhibitors (SLPI), and the tracheal antimicrobial peptide (TAP) (King et al., 2000; Fahey et al., 2006). ECs have been reported to secrete human defensin-5 and human β -defensin-1 (HBD-1) *in situ* (King et al., 2002; Schaefer et al., 2005). In addition, King *et al* reported HBD-3 expression to be the highest during the luteal phase, while HBD-4 expression levels elevated in the follicular phase of the menstrual cycle (King et al., 2003), suggesting that ECs respond directly to sex hormones levels. A study also reported apical

secretions from epithelial cells obtained from women in the follicular and luteal phases of the menstrual cycle, but not from post-menopausal women, have equally effective bacterial killing capabilities (Fahey and Wira., 2001). They also reported that concentrations of SLPI expression in pre-menopausal women were remarkably higher than post-menopausal women, indicating a correlative relation between SLPI and bactericidal activity that is regulated by sex hormones. In addition, studies have indicated higher expression of SLPI levels in tissue in the luteal phase compared to the follicular phase (King et al., 2000; Fleming et al., 2003). Interestingly, endometrial SLPI expression is elevated by estrogen in rats, (Chen et al., 2004) whereas progesterone regulates SLPI levels in rhesus monkey (Ace and Okulicz., 2004) and humans (King et al., 2003). These finding highlight that together with providing a robust barrier, ECs can produce immune factors that are, at least partly, regulated by the menstrual cycle.

3.2.1.4 TLR expression and production of cytokines/chemokines by vaginal epithelial cells

Numerous cytokines/chemokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor-alpha (TNF- α), IL-1, IL-6, IL-8, leukemia inhibitory factor (LIF), and TGF- β have been shown to be produced by epithelial cells in female reproductive tract (FRT) (Wira et al., 2005). FRT epithelial cells express different TLRs to respond to a range of pathogens that include bacteria, viruses and fungi (Darville et al., 2003; Pioli et al., 2004; Schaefer et al., 2004). Primary vaginal and cervical ECs has been shown to express TLR-1, -3, -5 and -6 (Fichorova et al., 2002). After TLR engagement, activation of the nuclear factor-kB (NF-kB) pathway leads to the induction and secretion of pro-inflammatory cytokines and chemokines (Kawai and Akira., 2007). An interesting feature of TLRs is their ability to initiate highly specific signals in response to microbial threats.

Using ECC-1 epithelial cell lines, TLR-2 and -5 agonists zymogen and flagellin, respectively, can stimulate that production of chemokine IL-8, monocyte chemoattractant protein-1 (MCP-1) and IL-6 (Schaefer et al., 2004). In addition, lipopolysaccharide (LPS) from gram-negative bacteria bind to TLR4 homodimers (Hirschfeld et al., 2000), and that TLR4 expression is upregulated during intra-uterine inflammation in mice (Elovitz et al., 2003). In addition, studies have shown that poly (I:C), a synthetic dsRNA that is recognized by TLR3, induces that expression of chemokines IL-8, MCP-1 (CCL2), and MIP-1 β as well as of MIF and HBD-1/HBD-2, and pro-inflammatory cytokines IL-6, G-CSF, GM-CSF, and TNF- α in uterine epithelial cells, (Schaefer et al., 2005).

The production of pro-inflammatory mediators function to attract and stimulate immune cells. For example, chemokines IL-8, MCP-1 (CCL2) and MIP-1 β are chemoattractants for neutrophils, monocyte and T cells, respectively. The chemotaxis activity of IL-8 for neutrophils was demonstrated by the lack of neutrophil recruitment into the FRT in the presence of IL-8 neutralizing antibody. Notably, GM-CSF, a neutrophil chemoattractant, can synergize with IL-8 and enhance neutrophil activation (Gomez-Cambronero et al., 2003; Shen et al., 2004; Meter et al., 2005). Moreover, TNF- α , IL-6, GM-CSF, and G-CSF secreted from immune cells are potent inducers of leukocyte activation (Schaefer et al., 2004). In addition, secreted factors from ECs show multiple functionality towards both innate and adaptive immune cells. Studies have shown the microbicide HBD-2 produced by epithelial cells in culture (Schaefer et al., 2005) is chemotactic for memory T cells, immature DCs (Yang et al., 1999) and neutrophils (Niyonsaba et al., 2004). The chemokine MIP-3 α secreted by ECs in response to LPS (Crane-Godreau and Wira., 2005) has also been reported to have microbicidal activity (Hoover et al., 2002). Notably, Kaysili *et al* highlighted the importance of chemokines IL-8 and MCP-1 (CCL2) in normal uterine physiology

in regard to proliferation, menstruation and implantation (Kaysili et al., 2002). Withdrawal of progesterone results in elevated MCP-1 and IL-8, leading to monocyte and neutrophil chemotaxis and activation, which induces matrix metalloproteinases (MMPs) activation to initiate menstruation. (Critchley et al., 2001). Thus, these studies suggest that sex hormones can also regulate cytokine/chemokine expression, in addition to TLRs.

3.2.2 Macrophages in the FRT

Macrophages are professional phagocytes that play a critical role in the initiation, maintenance and resolution of host inflammatory responses. They are widely distributed throughout the FRT and account for approximately 10% of the total number of leukocytes in FRT (Givan et al., 1997; Lee et al., 2015). Macrophages are abundant in the endometrial stroma and myometrial connective tissue of the uterus (Hunt., 1994). Previous studies have reported that during the menstrual cycle, steroid hormones can regulate the recruitment uterine macrophages. Fluctuating estrogen and progesterone levels are consistent with migration changes in macrophages within the endometrium (DeLoia et al., 2002; Lavin et al., 2014). Macrophage influx into pre-menstrual endometrial stroma parallels with that of lower estrogen and progesterone levels, which is thought to be through hormone-regulated cytokine and chemokine expression (DeLoia et al., 2002). MCP-1 (CCL2) expression is dramatically inhibited when stromal cells were treated with estradiol and correlates with down regulation of macrophage migration (Arici et al., 1999). Moreover, secretion of MCP-3, FKN and MIP-1 β , which are known macrophage chemoattractants, are elevated peri-menstrually (Jones et al., 2004). In the luteal phase of the menstrual cycle, accumulation of endometrial macrophages is evident (Arici et al., 1999), and consistent with previous data indicating upregulation expression of macrophage chemokines

MDC, MCP-3, FKN, 6Ckine, HCC-1/4, MIP-1 during this phase (Jones et al., 2004). These data clearly indicate that the dynamic interaction of cytokines, chemokines and sex hormones act to collectively modulate macrophage localization within the FRT (DeLoia et al., 2002). Moreover, macrophages are key effector cells of ovarian function. Same with the uterine macrophages, distribution of ovarian macrophages differs with the periodicity of the menstrual cycle (Wu et al., 2004). Ovulation is associated with an inflammation reaction and is subject to tight physiological patrol via immune cells. Ovarian macrophages secrete cytokines TNF- α , IFN- γ , IL-1, IL-6, IL-10, and IL-12 in order to regulate inflammation reactions (Erroi et al., 1989; Merogi et al., 1997; Bukulmez and Arici., 2000).

3.2.3 Dendritic cells (DCs) in the FRT

Dendritic cells bridge innate and adaptive immunity by initiating and modulating T cell activation (Zhao et al., 2003). DCs are present in the stratified squamous epithelial layer and in the submucosal lamina propria in the vagina (Hirbod et al., 2011). DC migration into the epithelial surface is initiated by inflammatory cytokine and chemokines secreted in response to pathogens by epithelial cells (Iijma et al., 2007). Subsequent DC maturation leads to their migration into T cell zones of the draining lymph nodes and engagement with native T cells in an antigen-specific manner (Cella et al., 1996). Production of IL-12 by DCs (Cella et al., 1996) leads to the differentiation of IFN- γ producing Th1 cells to mount an effective cell-mediated immune response (Macatonia et al., 1995). Sex hormones have also been shown to modulate and regulation of DC function (Escribese et al., 2011). Komi *et al* showed that estrogen inhibits the functional differentiation of DCs and can impact the development of inflammatory Th1 response (Komi and

Lassila., 2000). Together, DCs are important APCs that initiates adaptive immunity with their ability to sense vaginal pathogens and migrate to the draining lymph nodes.

3.2.4 Natural killer cells (NK cells) in the FRT

NK cells are key innate immune cells with a variety of potent abilities in eliminating tumors and pathogens by secretion of cytotoxic products (Lee et al., 2011). They are distributed throughout the FRT, with a variation of 10% to 30% in different regions of the FRT in non-pregnant women (Lee et al., 2015). NK cell activity defect is associated with increased herpes viral infections, uterine cancer and endometriosis, while elevated NK cells activity is associated with recurrent pregnancy loss (Thum et al., 2004; Dosiou et al., 2005). They are able to amplify an inflammatory response through the release of IFN- γ , which can further activate macrophages and enhance cytotoxic T cell responses (Lidstrom et al., 2003). Throughout the menstrual cycle, the uterine endometrium is known to be regulated by sex hormones and while the number of NK cells are low in the follicular phase, they increase in number as the menstrual cycle progress and reach a peak at the luteal phase (Givan et al., 1997). Thus, it has been proposed that sex hormones might be able regulate NK cell recruitment and expansion in the endometrium (DeLoia et al., 2002). NK cells within the FRT show different cell surface phenotypes (Hickey et al., 2011). Uterine NK cells (uNKs) have a distinct phenotype of CD56^{bright}CD16^{dim} while peripheral blood NK cells have a CD56^{dim}CD16^{bright} phenotype, and express CD9 and CD69 on their cell surface (Mselle et al., 2007). Studies have shown that uNKs express chemokine receptors CXCR3, CCR5 and CCR7 (Chantakru et al., 2001; Red-Horse et al., 2001). Kitaya *et al* reported the increased expression of CCL4, CXCL9 and CXCL10 during the menstrual cycle which correlated with increase NK cell numbers in the endometrium (Kitaya et al., 2004). In addition, data shows that estrogen and

progesterone can induce CXCL10 (NK chemoattractant) and CXCL11 (chemotactic for activated T cells) expression (Sentman et al., 2004). Finally, uNK cells can express cytokines IFN- γ , GM-CSF, IL-8, IL-10, IL-15, and TGF- β which in combination with PAPMs result in further activation of immune immunity (Cooper et al., 2001; Shang et al., 2014).

3.2.5 Neutrophils in the FRT

3.2.5.1 Role in innate immunity

Neutrophils (CD45⁺CD11b⁺Ly6G⁺) are short-lived effector polymorphonuclear leukocytes of the innate immune system that comprise the first line of host immune defence against invading pathogens and play a major role during acute inflammation (Mantovani et al., 2011). Decrease in neutrophil numbers in human blood can result in severe immunodeficiency (Nathan., 2006; Borregaard., 2010). Neutrophils have high phagocytic activity, and have the ability to secrete granular enzymes, generate reactive oxygen intermediates (ROI) and produce antimicrobial factors (Lambeth et al., 2004). While neutrophils have long been known as a homogenous cell population with a conserved phenotype and function, recent studies highlight heterogeneity in their characteristics and function, especially in the context of inflammation (Silvestre-Roig et al., 2016; Hong., 2017; Tak et al., 2017). Finally, in response to specific stimuli, neutrophils have the ability to produce NETs (neutrophil extracellular traps) where they extrude modified chromatin structures decorated with specific cytoplasmic and granular proteins (Sollberger et al., 2018). NETs may have evolved to defend against infections by trapping and killing microbes, further highlighting neutrophil recruitment as an essential component of innate immunity.

3.2.5.2 Development, lifespan and migration capacity of neutrophils

Neutrophil development and maturation takes place in the bone marrow and is divided into three stages: multiplication, maturation and function, which are regulated by different transcriptional factors (Summers et al., 2010). In the beginning, a self-renewing hematopoietic stem cell (HSC) differentiates into a multipotent progenitor cell (MPP), then transforms into lymphoid-primed multipotent progenitor cells (LMPPs). Finally, LMPPs give rise to granulocyte–monocyte progenitors (GMPs), which under the influence of G-CSF, progress into the promyelocyte, myelocyte, metamyelocyte, and finally band neutrophil stages to become mature neutrophils (Manz and Boettcher., 2014). In normal conditions, an estimated 2×10^{11} mature neutrophils leave the bone marrow and enter the circulation (Furze et al., 2008). In the circulation, neutrophils are the most abundant leukocyte population contributing about 50-70% of all circulating leukocyte in humans and 10-25% in mice (Welch et al., 1989; Doeing et al., 2003). Mature neutrophils have an average diameter of 7-10 μm with a segmented nucleus and a cytoplasm that is enriched with granules and secretory vesicles (Borregaard et al., 2010). Neutrophils were previously considered short lived with a half-life in the circulation of 1.5 hours in mice and 8 hours in humans (Pillay et al., 2010; Galli et al., 2011). However, a more recent study shows that under normal conditions, neutrophil circulatory lifespan in mice is up to 12.5 hours whereas in humans it is 5.4 days (Pillay et al., 2010). Neutrophils are activated by cytokines/chemokines, growth factors and microbial products through TLR stimulation (Kim et al., 2011). Neutrophil lifespan increases dramatically during inflammation that ensures their increased presence at the site of inflammation (Summers et al., 2010) and contribute to the inflammatory response and the generation of adaptive immunity (Kim et al., 2011).

During tissue injury or pathogenic insult, neutrophils are rapidly recruited from peripheral blood, where they first undergo intravascular rolling, adhesion, crawling and firm adhesion onto vascular endothelial cells at the site of inflammation (Phillipson et al., 2006; Voisin et al., 2013). This is facilitated by the expression of L-selectin on the surface of neutrophils and allows “tethering and rolling” along the vascular endothelium (Ley., 2003). This leads to conformational changes of integrins, including VLA-4 (CD49D/CD29), MAC-1 (CD11b/CD18), and LFA-1 (CD11a/CD18) (Furze et al., 2008) and their enhanced affinity enables their firm arrest on endothelial cells under shear flow (DiStasi and Ley., 2009; Evans et al., 2009). Neutrophil extravasation is established through two main routes, either paracellularly or transcellularly. In the paracellular route, leukocytes move through endothelial cell junctions while the transcellular route involves transmigration directly through the endothelial cell body (Voisin and Nourshargh., 2013). Neutrophils travel on the endothelial membrane in order to find a small gap between pericytes which are cells located near microvessels (DiSatasi and Ley., 2009). These cells are in charge of controlling capillary permeability and surround endothelial cells and cover 22-99% of their surface. They are high in pattern recognition receptors (PRRs) that allows them to sense inflammatory activities. Finally, in order to gain access to the inflammatory site and initiate a phagocytic reaction, neutrophils shed their CD18 integrins by their vesicles located on the extended tail on to the sub-endothelial layer (Navarro et al., 2016).

Neutrophil activation is a complex process. It is initiated through the transit of neutrophils within the vascular endothelium at the time of recruitment. Subsequently, response to pro-inflammatory stimuli in the tissue fully activates neutrophils which is characterized by the release of granule proteins, gaining phagocytic abilities, and production of neutrophil extracellular traps (NETs) which all enhance neutrophils’ pathogen destruction magnitude (Doerfler et al., 1989;

Swain et al., 2002). Neutrophils recognize and bind to microbe specific molecules via different cell surface and intracellular receptors. Neutrophil pattern recognition receptors (PRRs) recognize PAMPs on microbial pathogens and initiate an inflammatory response that can contribute to inflammation-induced tissue damage. Dectin-1, a potentially known primary endocytic PRR internalizes and eliminates bacterial and fungal pathogens (Li et al., 2011). In addition, nonphagocytic PRRs such as neutrophil TLRs play an important role in elevating phagocytic capacity, stimulating increased cytokine/chemokine release and neutrophil apoptosis. NOD1 and NOD2 are important cytosolic microbial sensors related to PRRs signaling that can recognize peptidoglycan-related molecules on bacterial and fungal pathogens (Kanneganti et al., 2007). Moreover, essential complement receptors CR3 and CR4 are also important for neutrophil mediated pathogen killing. G protein-coupled receptors (GPCRs) are highly expressed on neutrophils and are important in recognizing bacterial and endogenous molecules during inflammation which enhance neutrophil migration and activation to the site of stimuli (Rabiet et al., 2007; Schymeinsky et al., 2007).

Neutrophils are the first cell type to arrive at the site of microbial infection, where in order to prevent pathogen diffusion exert diverse antimicrobial mechanisms. Three primary components are involved in pathogen killing via neutrophils: (1) production of highly toxic reactive oxygen species (ROS) in the pathogen containing vacuole; (2) fusion of neutrophil granules which contain various antimicrobial mediators to the vacuole; and (3) NETs formation (Amulic et al., 2012).

Neutrophil phagocytosis is a specific form of endocytosis where neutrophils rapidly engulf pathogens, which results in the formation of a defined vacuole referred to as the phagosome. Subsequently the phagosome fuses with the lysosome to form the phagolysosome structure which contains proteolytic enzymes, antimicrobials and highly toxic reactive oxygen species (ROSs)

which are a results of NADPH oxidase, myeloperoxidase (MPO), or nitric oxide (NO) synthetase and initiate microbial killing mechanisms (Roos et al., 2003). NADPH oxidase generate free radicals containing substances that are used to eliminate pathogens in a process referred to as the respiratory burst. Myeloperoxidase (MPO) is a primary granule protein which catalyzes the formation of hypochlorous acid (HOCl), hydrogen peroxide (H₂O₂) and chloride anion (Cl⁻). These oxygen derived enzymes play an important role in the killing of pathogenic bacteria and fungi (Klebano., 2005; Odobasic et al., 2016). Nitric oxide (NO) synthetization is also an important part of neutrophil antimicrobial activity. NO, is a short lived and highly reactive molecule produced by inducible nitric oxide synthase (iNOS), present in the primary granules capable of initiating ROS production by neutrophils to exert antimicrobial functions (Winterbourn et al., 2016).

Neutrophil degranulation is a process used by neutrophils to kill invading pathogens by releasing proteinases and peptidases such as MPO, neutrophil elastase and antimicrobial peptides that are stored in their granules and fuse with the phagosome during pathogen uptake (Borregaard and Cowland., 1997). Neutrophils contain several types of granules based on the content of their membrane protein: (A) azurophilic granule; containing MPO, and a wide spectrum of neutrophil serine proteases (NSPs) including cathepsin G (CG), neutrophil elastase (NE), proteinase 3 (PR3) and neutrophil serine protease-4 (NSP4), all of which contribute to immune protection and killing of pathogenic microbial agents (Stapels et al., 2015) (B) specific granule; containing a wide range of antimicrobial compounds which include calprotectin (S100A), neutrophil collagenase (MMP8), lysozymes, neutrophil gelatinase-associated lipocalin (NGAL), and lactoferrin (Embleton et al., 2013). (C) tertiary granule (also known as gelatinase granule); they lack MPO and lactoferrin. However, they act as a storage location for a number of matrix metalloproteases such as MMP9 and leukolysin which are involved in the breakdown of extracellular matrix in normal

physiological processes such as wound healing and are active in neutrophil migration across the baseline membrane and also neutrophil maturation (Chakrabarti et al., 2006).

In addition to phagocytosis and degranulation, neutrophils also exert antimicrobial activities through the formation of neutrophil extracellular traps (NETs). During NET formation, neutrophils extrude mesh-like chromatin fibers of DNA/histones containing granule-derived antimicrobial peptides and enzymes including neutrophil elastase, MPO, cathepsin G (CG), calprotectin (S100A), proteinase 3 (PR3), lactoferrin, pentraxin 3 and LL-37 in a process referred to as NETosis in order to extracellularly eliminate microbial agents (Brinkmann et al., 2004). Although NETs promote potent antimicrobial activities, they can cause unintended tissue injury and activation of undesirable immune reactions due the presence of proinflammatory molecules (such as chromatin-LL37 complexes) and autoantigens (such as histones) in their structure (Knight and Kaplan., 2012).

3.2.5.3 Regulation of neutrophil influx and function in the FRT

Neutrophils play a crucial role in eliminating pathogens and responding to inflammation (Pillay et al., 2010; Kamp et al., 2012). Under normal conditions, neutrophils are present in all tissues of the FRT (Kunz et al., 1997;) but are highest in the fallopian tubes compared to the lower FRT at steady-state (Givan et al., 1997). While surges in estrogen and progesterone changes the thickness of the vaginal epithelium, neutrophil numbers remained consistent throughout the menstrual cycle in healthy women (Patton et al., 1999). In contrast, the vaginal epithelium is infiltrated with high number of neutrophils in mice following ovulation and during high progesterone levels (diestrus phase). This influx is associated by a surge of CXCL2 (rodent IL-8 homologue) secreted by vaginal epithelial cells, suggesting that neutrophil migration into the

vagina is dependent on CXCL2 expression (Sonoda et al., 1998; Gabrielli et al., 2016; Armstrong et al., 2017; Girbl et al., 2018). Moreover, CXCL1/2 has been shown to signal neutrophils to transmigration into the vaginal lumen, and that estrogen can suppress their expression while progesterone enhances it (Lasarte et al., 2015). While these studies suggest that FRT neutrophils are differentially regulated in humans and mice, women with higher expression of IL-8 in the vaginal tissue display higher neutrophil counts (Cauci et al., 2003). This highlights the fact that neutrophils are highly responsive to changes in IL-8 (or CXCL2 in mice) within the FRT and need this signal to enter the epithelial barrier (Kidney and Proud., 2000). Corresponding to the increased presence of neutrophils during the luteal phase, higher levels of neutrophil-related cytokines such as IL-1 β , IL-12 p40, GM-CSF and chemokine CCL4/MIP-1 β have been reported (Hickey et al., 2013). This is in contrast to cytokines expressed during the estrus phase (estrogen dominant phase) in mice, namely IL-2, IL-3, IL-6, IL-13 and IL-17. Notably, higher expression levels of IL-1 β has been reported in the cervicovaginal lavage in human cohorts (Boily-Larouche et al., 2019) during the luteal phase and mice in the diestrus phase, suggestive of high neutrophil numbers during this progesterone-dominant phase. It is also important to note that standard immunohistochemistry or flow cytometry analyses may not be a sensitive measure of neutrophil numbers within the FRT, since they are very prone to apoptosis or NETosis. Regardless, neutrophil recruitment is an active process that is regulated by the expression of chemoattractant molecules within the FRT.

Neutrophils may also aid in the breakdown of the endometrium by releasing matrix metalloproteinases (MMPs) (Curry and Osteen., 2003; Galant et al., 2004; Page-McCaw et al., 2007). It is generally thought that mucosal neutrophils during menses serves two purposes: (1) to assist in the breakdown of the endometrial tissue by releasing elastase, resulting in matrix-metalloproteinases (MMPs) activation, and: (2) to participate in the innate immune response

during the disruption of epithelial barrier where susceptibility to infections may be high. MMPs are proteolytic enzymes that degrade components of the extracellular matrix. Strong correlative evidence supports a key role of MMPs (MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, and MMP11) expression during menstruation and disruption of the vaginal epithelial barrier in humans and mice (Marbix et al., 1996; Zhang et al., 2002; Brasted et al., 2003; Kaitu'u et al., 2005). Moreover, MMP activity itself may also be under ovarian steroid hormonal control. Uterine remodeling and endometrial shedding involves estrogen-induced changes in MMPs activity (Russo et al., 2009; Selvais et al., 2009), while progesterone may affect MMPs expression and regulate factors for the maintenance of endometrial lesions (Mönckedieck et al., 2009). However, activated neutrophils may contribute to tissue damage during inflammation through the release of matrix modifying mediators such as neutrophil elastase (NE), myeloperoxidase (MPO) and directly lead to tissue injury (Ishii et al., 2010; Papayannopoulo et al., 2010; Carter et al., 2013; Voisin et al., 2019). These data indicate that while neutrophils are important in the homeostatic regulation of the FRT, their abnormal recruitment and aberrant activation can exacerbate tissue damage through release of matrix modifying enzymes and inflammatory factors.

3.3 HIV susceptibility and the role of the FRT immune system

In order for HIV-1 to establish an infection, it must first overcome numerous innate and adaptive immune factors in the FRT. While the majority of HIV-1 infection in women occur as a result of heterosexual intercourse, a mechanistic understanding of sexual HIV transmission in women remain unknown (Carias et al., 2013). While HIV-1 transmission can happen anywhere within the FRT tract, the transformation zone (TZ) has been previously proposed to favor initial infection, due to the fact that a single columnar epithelial layer with a thickness of just 10-30 μm

separates the virus from intraepithelial and submucosal target cells such as CD4⁺ T cells. (Shattock and Moore., 2008; Carias et al., 2013). CD4⁺CCR5⁺ T cells, which are the primary target cells of HIV-1 infection are present in high numbers within the TZ and contribute to the vulnerability of this area of the FRT (Kumamoto and Iwasaki., 2012; Trifonova et al., 2014; Vitali et al., 2017). There are also an abundant presence of APCs including CD14⁺ macrophages and DCs in the cervical transformation zone (Pudney et al., 2005; Trifonova et al., 2014). In addition, dendritic cells (DCs) are also present in the squamous epithelial layers of vagina and ectocervix, which can be infected with HIV-1 and are believed to facilitate the transfer of HIV-1 across the epithelium (Tchou et al., 2001, Pudney et al., 2005; Ballweber et al., 2011). Although the main target of HIV-1 is activated T cells, other cells such as macrophages, DCs and neutrophils can either become infected or can directly or indirectly contribute to HIV-1 susceptibility. Recent studies have shown that the secretion of neutrophil-derived antimicrobial and tissue-modulating enzymes can significantly impact both HIV transmission and pathogenesis (Valore et al., 2002; Ogawa et al., 2013; Cole and Cole., 2017). While peripheral neutrophils show reduced antimicrobial functions during HIV infection, the mechanistic role of neutrophils within the FRT during HIV transmission remains unclear (Hernandez et al., 2015; Prodger et al., 2016).

3.3.1 Dual role of FRT neutrophils on HIV acquisition and pathogenesis

The association between elevated risk of HIV acquisition and fewer peripheral blood neutrophils *in vivo* have been reported, which emphasizes their important role against HIV-1 infection (Ramsuran et al., 2011; Kourtis et al., 2012). In a study conducted on South African high-risk female sex workers, a 3-fold increased risk of HIV acquisition in individuals with neutropenia compared to healthy controls was reported (Ramsuran et al., 2011). Similarly, another cohort study

of South African women noted that higher neutrophil counts in the mother and infant were associated with a 11% reduced risk of perinatal HIV infection (Kourtis et al., 2012). Moreover, *in vitro* analysis reported that α -defensins (also known as human neutrophil peptides or HNP), and in particular HNP1-2-3, can directly inactivate or block HIV replication by altering target cell signaling pathways (Chang et al., 2005; Klotman and Chang., 2006; Demirkhanyan et al., 2012). Interestingly, the release of myeloperoxidase (MPO) by neutrophils, which produces the antimicrobial factor hypochlorous acid, has been reported to have direct viricidal activity to HIV-1 *in vitro* (Saitoh et al., 2012). Finally, NET formation by human genital neutrophils after HIV exposure prevented infection of susceptible cells through irreversible viral inactivation (Barr et al., 2018). However, in contrast to these studies, several studies have also implicated genital neutrophils in enhancing HIV acquisition through a number of possible mechanisms. A study conducted by Levinson *et al* of Kenyan female sex workers showed that elevated levels of α -defensins HNP1-3 and LL-37 in CVL secretion were associated with subsequent HIV-1 acquisition (Levinson et al., 2009). In addition, increased neutrophil-attractant chemokine IL-8 secretion in CVL was reported in South African women who acquired HIV infection compared to those that did not (Masson et al., 2015). These studies suggest that the presence of neutrophils in the FRT can either directly or indirectly increase HIV risk. One possibility is that neutrophils secrete a number of potent proteases (e.g. MMPs, neutrophil elastase) that facilitate their migration and penetration through tissue and their release into the extracellular space can cause host tissue damage by degrading the structural proteins of the vaginal epithelial cell barrier (Gursoy et al., 2013; Kolaczowska and Kubes., 2013). This may result in increased vaginal barrier permeability, and access to target cells by HIV. CVL cytokine/chemokine analysis from Kenyan female sex workers have revealed a strong correlation between elevated neutrophil-associated protease levels

and high proinflammatory cytokine production including GM-CSF, MIP-3 α , IL-1 β , IL-8, and IL-17 (Arnold et al., 2016), suggesting that the presence of vaginal neutrophils may exacerbate genital inflammation. Elevated IL-17 expression correlates with neutrophil protease expression, which highlights the importance of the Th17/neutrophil axis in HIV acquisition since Th17 cells are optimal HIV target cells in the FRT (McKinnon et al., 2011; Rodriguez-Garcia et al., 2014; Arnold et al., 2016; Stieh et al., 2016). These studies strongly suggest that FRT neutrophils can increase HIV transmission rate by directly affecting the vaginal epithelium or indirectly by amplifying genital inflammation and recruiting susceptible T cell populations. However, these functions have not been demonstrated mechanistically, and what regulates their recruitment and activation in the FRT are unknown.

3.3.2 Epithelial barrier disruption increases HIV risk

Many other factors contribute to HIV-1 protection, since the frequency rate of infection is considered low at 1 in 200 to 1 in 2000 per act of unprotected receptive vaginal intercourse (Hladik and McElrath., 2008). The lower FRT is composed of several layers of squamous epithelium that comes in contact with infected seminal fluid and comprises the majority of the exposed surface of the FRT that serves as a major access site for HIV-1. As discussed earlier, epithelial cells in the lower FRT express a wide array of PRRs including TLRs and NOD-like receptors and recognize potent pathogen through PAMPs expressed on microorganisms (Pioli et al., 2004; Herbst-Kralovetz et al., 2008; Ghosh et al., 2013). Pro-inflammatory cytokines are produced in response to activated signalling of the transcription factor NF- κ B (Fichorova et al., 2002; Nguyen et al., 2014). Vaginal epithelial cells can induce TLP pathway and produce pro-inflammatory cytokines IL-6, IL-8, TNF- α , MIP-1 α , and MIP-1 β (Fichorova et al., 2015) which can subsequently lead to

the activation and migration of inflammatory immune cell such as macrophages and neutrophils to the site of inflammation and contribute to vaginal epithelial junction disruption (Nazli et al., 2010; Ferreira et al., 2013). Thus, disruption of vaginal epithelial tight junctions can significantly enhance HIV-1 susceptibility. The result of micro-abrasions during heterosexual intercourse can cause vaginal epithelial barrier disruption, which activate local inflammation and wound healing processes and can promote infiltration of inflammatory immune cells to the site of disruption and subsequently amplify the population of HIV-1 target cells (Miller and Shattock., 2003). Studies have reported that women with high vaginal epithelium disruption are at the highest risk of HIV-1 acquisition in comparison to women without compromised epithelial barriers (Mesquita et al., 2009; Abbai et al., 2016). Additionally, HIV-1 envelope glycoprotein gp120 can directly cause disruption in tight junction proteins such as ZO-1 and occludin in cultured primary epithelial cells lines (Nazli et al., 2013). In addition, hormonal and microbial composition fluctuation have been reported to influence protease levels in the FRT, which may impact the integrity of the vaginal epithelial barrier (Birse et al., 2015; Arnold et al., 2016; Borgdorff et al., 2016). Vaginal pH can also play an important role in HIV-1 susceptibility. Although a low pH provides an extra layer of protection against potent pathogens, an alkaline pH of the semen can elevate vaginal pH which in result can prevent HIV-1 from neutralization via the acidic cervicovaginal mucus (Lai et al., 2009). Thus, physical disruption of the vaginal epithelium during intercourse and virally-derived molecules can impact HIV acquisition by allowing more virus to penetrate the epithelial barrier (Figure 1).

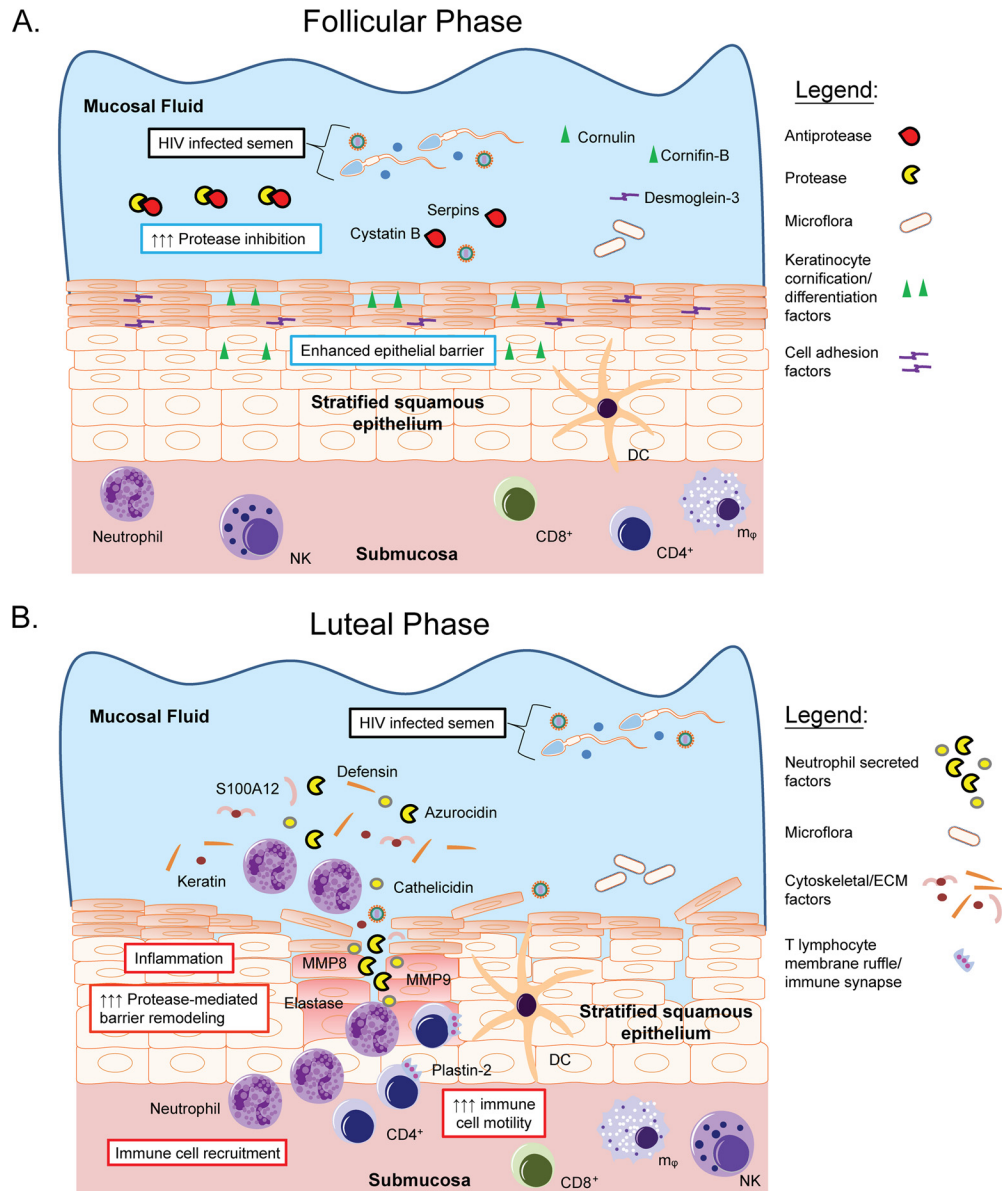


Figure 1. Immunological changes during different stages of the menstrual cycle modulate potent mechanisms that enhance susceptibility to HIV infection. (A) The follicular phase (estrogen dominant phase) is associated with increased levels of protease inhibitors such as serpins and cystatin B, and elevated expression of cell to cell adhesion factors such as desmoglein 3 and cornification/epidermal differentiation factors (cornulin and cornifin b) which enhance epithelial

barrier integrity and resembles mucosal surface less vulnerable to HIV infection. **(B)** The luteal phase (progesterone dominant phase) is manifested by an increase in immune cell recruitment and elevated protease-mediated epithelial barrier remodeling and disruption, which is associated with enhanced levels of neutrophil secreted factors such as S100A12, defensin, azurocidin, cathelicidin, MMPs, and elastase. The presence of HIV target cells may contribute to physical barrier degranulation and enhancing HIV infection (Figure adapted from Birse et al., 2015).

3.3.3 Role of STIs, inflammation and sex hormones in HIV acquisition

In addition to epithelial barrier disruption, any condition resulting in immune cell activation or recruitment leading to inflammation activities can significantly increase HIV-1 susceptibility. Several studies have reported the importance of sexually transmitted infections (STIs) at key risk factors for HIV-1 acquisition; both herpes simplex virus 2 (HSV-2) and human papillomavirus (HPV) have been associated with 2 to 3 folds increased risk of HIV-1 acquisition. (Freeman et al., 2006; Houlihan et al., 2012). Although the precise mechanisms associated with these risk factors are unclear, investigators suggest that the increased risk is due to an elevated inflammatory status in the FRT characterized by high inflammatory cytokine profiles present at the time of infection (Ferreira et al., 2015). Moreover, Masson *et al* recently reported a 3-fold increase in risk of HIV-1 acquisition in women with vaginal inflammation, which were defined by elevated HIV-1 cell recruiting chemokine and inflammatory cytokines (Masson et al., 2015). This is consistent with studies by Lajoie *et al* that showed that HIV exposed but seronegative (HESN) individuals had significantly reduced expression of inflammatory cytokines IFN- γ , IFN- γ induced protein 10 (IP-10), and IL-1 α in their genital mucosa (Lajoie et al., 2012). These important observations strongly

suggest that HESN women exhibit a specific pattern of mucosal cytokine/chemokine expression that reduces cell tracking/activation in the vaginal mucosa and reduces HIV-1 susceptibility.

Female sex hormones estradiol and progesterone can also regulate and influence immune cell phenotype and function in the FRT (Kaushic., 2011; Dunbar et al., 2012; Nguyen et al., 2014; Vitali et al., 2017). Trifonova *et al* reported a significantly higher presence of CD4⁺, CD8⁺, and B cells in the ectocervix than in the endocervix of women (Trifonova et al., 2014). In addition, the ectocervix of women composed of higher numbers of CD4⁺ T cells during vaginal and cervical inflammation (Pudney et al., 2005). Wieser *et al* also reported increased number of CD1a⁺ Langerhans cells important in HIV-1 transfer along the epithelium in women when treated intravaginally with exogenous progesterone (Wieser et al., 2001). Elevated secretion of AMPs including defensins, trappin-2/elafin and SLPI is correlated with increased levels of progesterone expression in the luteal phase (Dunbar et al., 2012). Similarly, Birse *et al* showed using a proteomics approach that a number of inflammatory signature pathways are upregulated during the luteal phase, some that implicate vaginal neutrophils, further demonstrating that the sex hormones can impact the inflammatory status in healthy women (Birse et al., 2015). This is further strengthened by the fact that PRRs and NF- κ B signalling are modulated by estradiol. (Ghisletti et al., 2005; Schaefer et al., 2005). Wagner and Johnson reported the expression of inflammatory cytokines IL-1 α , IL-6, IL-8 and TNF- α in vaginal epithelial cell lines were inhibited via estradiol (Wagner and Johnson., 2012). Collectively, both estrogen and progesterone can modulate FRT immunity and impact HIV susceptibility, possibly through recruitment and activation of immune cells during the menstrual cycle (Wira et al., 2010; Saba et al., 2013).

3.4 Vaginal microbiota composition impacts HIV susceptibility

The microbiological flora lining the mucosal tissues in the FRT (known as the vaginal microbiome) is a dynamic and complex microbial colonization that modulate the physiology and immunity at this mucosal site (Ma et al., 2012). The commensal microflora of the lower FRT has been increasingly recognized as an important mucosal defense system against STIs (Nunn et al., 2015). *Lactobacillus* species maintain a healthy vaginal environment by producing antimicrobial agents and decreasing vaginal mucosa pH by lactic acid production (Linhares et al., 2011; Ma et al., 2012). While a mechanistic understanding of these bacterial communities remain unknown, the development in high-throughput sequencing technologies that can characterize the human microbiome have improved dramatically in recent years, which has increased our understanding of the role of vaginal microbiome in inflammation, STI infections and HIV-1 susceptibility (Handley et al., 2016; Monaco et al., 2016). A normal vaginal microbiota is described as vaginal fluid smears indicating gram positive rods, with cultures revealing a predominance of *Lactobacillus* species, particularly *L. crispatus* and *L. jensenii*. (Vallor et al., 2001; Vasquez et al., 2002). It is believed that lactobacilli promote a healthy environment by producing lactic acid, hydrogen peroxide and bacteriocins that exclude pathogens from the FRT niche (Aroutcheva et al., 2001). For this reason, their dominance in the vaginal microbiota are often described as an “optimal” bacterial community (McKinnon et al., 2019). However, the change from a *Lactobacillus* dominant vaginal microbiota to an anaerobic and facultative bacterial dominance results in bacterial vaginosis (BV) (Atashii et al., 2008; Srinivasan et al., 2010; Klatt et al., 2017). BV is the most commonly reported microbiological syndrome in women in their reproductive years and is associated with a wide range of health issues, such as preterm delivery, pelvis inflammatory disease, elevated acquisition or transmission of sexually transmitted infections

(STIs), and increased susceptibility to HIV infection (Laxmi et al., 2012; Durugbo et al., 2015; Nasioudis et al., 2016). BV is largely associated with a dominance of bacteria of the *Gardnerella*, *Atopobium*, *Prevotella*, *Peptostreptococcus*, *Mobiluncus*, *Sneathia*, *Leptotrichia*, *Mycoplasma* genera (Atashili et al., 2008; Donders., 2010; Marrazzo et al., 2010) and a loss of many *Lactobacillus* species. (Sobel., 2000; Fredricks et al., 2005). Vaginal fluid smears from women with BV demonstrate a loss of Gram-positive rods replaced by Gram-negative/variable cocci and rods (Nugent et al., 1991). Notably, the disruption of the vaginal microbiota has been reported to be associated with increased risk of STIs including HIV-1 infection (Rottingen et al., 2001; Atashili et al., 2008). Studies conducted in Thailand, Malawi, and Uganda reported a positive association between the clinical diagnosis of BV and HIV-1 seropositivity (Cohen et al., 1995; Sewankambo et al., 1997; Taha et al., 1999). Bacterial vaginosis has been reported to significantly increase HIV-1 infection up to 60% (Atashili et al., 2008). Interestingly, Gosmann *et al* reported that women with diverse vaginal bacterial communities dominated by anaerobes other than *Gardnerella* (CT4) were at significantly higher (over 4-fold) risk of acquiring HIV, which was manifested with elevated numbers of activated mucosal CD4⁺ T cells in comparison to women with *Lactobacillus* dominant (CT1) communities (Gosmann et al., 2017). Furthermore, the main mechanism by which vaginal bacteria are able to elevate HIV acquisition is due to alteration of local inflammation and recruitment of CD4⁺ T cell population (HIV-1 target cells) (Gosmann et al., 2017; Lennard et al., 2018). A “non-optimal” bacterial community leads to elevated levels of proinflammatory cytokines IL-1 α , IL-1 β , TNF- α , IL-8 and CCL5 (RANTES) by activating cellular TLRs (Boomsma et al., 2010; Thurman et al., 2015; Zevin et al., 2016; Gosmann et al., 2017). These proinflammatory signatures were not induced in the presence of *Lactobacillus* species (Fichorova et al., 2011; Anahtar et al., 2015). Numerous clinical studies have also reported

a direct association between non-*Lactobacillus*-dominant vaginal microbiota and elevated levels vaginal inflammation, including IL-1 α , IL-1 β , IL-8, IL-10, IL-17, IL-23, TNF- α , IFN- γ , MIP-1 α and MIP-1 β (Anahtar et al., 2015; Birse et al., 2015; Arnold et al., 2016; Gosmann et al., 2017). Moreover, comparison of phenotypic and transcriptional analyses of genital APCs obtained from women with highly diverse vaginal microbiota indicated the contribution of APCs to genital inflammation by responding via TLR4, and activating NF-kB signalling pathways, which result in activation and recruitment of HIV-1 target cells. Thus, the vaginal epithelium and APCs are key players in modulating HIV-1 acquisition risk by responding to changes in the vaginal microbiota (Anahtar et al., 2015).

3.5 Regulation of the vaginal microbiome by sex hormones

The potential impact of fluctuating levels of sex hormones on the vaginal microbiota composition and FRT immunity has implications during STIs. Although the vaginal microbiota is relatively stable throughout the menstrual cycle, the extensive hormonal shifts during puberty and menopause dramatically change its composition from a mainly anaerobic bacteria to a *Lactobacillus* dominant microbiome (Alvarez-Olmos et al., 2004; Vitali et al., 2017). Estrogen levels significantly decline during menopause in women, and the vaginal microbiota is less likely to be dominated by *Lactobacillus* species in comparison to pre- and peri-menopausal women. Thus, colonization by diverse bacteria in post-menopausal women suggests that estrogen may play a role in regulating the vaginal microbiota towards *Lactobacillus* dominance. (Brotman et al., 2014; Shen et al., 2016). Moreover, Silva *et al* reported elevated adhesion of lactobacilli to epithelial cells is mediated by estrogen (Silva et al., 2004). Farage and Maibach reported estrogen elevate glycogen removal in the vaginal epithelium (Farage and Maibach., 2006). Glycogen is an

essential nutrient for lactobacilli (Mirmonsef et al., 2014), and can be synthesized and released into the vaginal mucus via epithelial cells. Nevertheless, the recruitment and activation of inflammatory immune cells, elevated expression of inflammatory cytokines, changes in sex hormones, and the presence of a “non-optimal” vaginal microbiota can all contribute to increased HIV-1 susceptibility. These studies highlight the need for a better mechanistic understanding of how these biological changes converge to increase HIV risk.

4. Overall Rationale, Hypothesis, Objectives, Significance, and Impact

4.1 Rationale

Neutrophils abundantly migrate into the vaginal mucosa during the progesterone dominant phase (luteal phase in humans; diestrus phase in mice) of the menstrual cycle and estrous cycle of humans and mice respectively (Sonoda et al., 1998; Hickey et al., 2013; Schaefer et al., 2014), but whether they aid in epithelial barrier disruption is unclear. Their ability to penetrate the squamous epithelium and express degradative enzymes (e.g. MMPs and neutrophil elastase) and proinflammatory cytokines/chemokines suggests that they can actively remodel the barrier after ovulation (Al Harthi et al., 2001; Carter et al., 2013; Hickey et al., 2013; Birse et al., 2015; Arnold et al., 2016; Voisin et al., 2019). In addition, the presence of anaerobic bacteria (e.g. *Mobiluncus*) can alter signalling, activation and recruitment of immune cells, such as neutrophils, to the site of stimuli and facilitate vaginal epithelial barrier disruption, which can potentially increase HIV susceptibility (Atashili et al., 2008; Masson et al., 2014; Zevin et al., 2016).

4.2 Hypothesis

Female reproductive tract (FRT) neutrophils are directly involved in the breakdown of the vaginal epithelial barrier and are regulated by cytokines/chemokines, sex hormones, and bacterial communities of the vaginal lumen.

4.3 Objectives

To assign a mechanistic function to FRT neutrophils and determine their role in epithelial barrier regulation during changes in sex hormone and vaginal microbiome.

4.4 Significance and Impact

Previous human cohort studies have highlighted the importance of mucosal neutrophils in FRT barrier function regulation, however a clear mechanistic understanding of their role in vaginal health is still incomplete. Our research used an *in vivo* mouse model to study the interplay between neutrophils and FRT barrier function in response to changes in sex hormone levels and vaginal commensal composition. Our approach will begin to assign a biological function to FRT neutrophils, and the results may also allow us to uncover the fundamental role of neutrophils in vaginal barrier integrity and reveal their actual contribution in elevating sexual HIV susceptibility and acquisition.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Isolation, purification and cell surface staining of mouse neutrophils

2.1.1 Cervicovaginal lavage (CVL)

Cervicovaginal lavage (CVL) was collected from naïve virgin female BALB/c mice (8-12 weeks old) maintained at the Central Animal Care Services, University of Manitoba. Mice were anesthetized with isoflurane using a holding induction chamber, and the CVL collected by pipetting 70 µL of sterile PBS up-and-down the vaginal cavity five times and transferred to a 1.5 mL Eppendorf tube on ice. The mouse estrous cycle stage was determined by vaginal cytology based on the presence or absence of leukocytes, cornified squamous epithelial, and nucleated epithelial cells (Caligioni., 2009). A total of 5 µL of CVL was added to 5 µL of trypan blue on a glass slide and examined under a light microscope using a 10x objective. The CVL was then centrifuged at 400 x g for 10 minutes and either the pelleted cells were used for flow cytometry or the supernatant collected for further analysis.

2.1.2 Peripheral blood

Mice were physically restraint in a proper manner and approximately 200 µL of blood was collect from the facial vein and transferred to BD Microtainer® blood collection tubes (Becton Dickinson, USA) on ice. The red blood cells were lysed by adding 5 mL ACK Lysing Buffer (Quality Biological, USA) and incubating at room temperature for 5 minutes. The white blood cells were collected by centrifuging the cells at 300 x g for 5 minutes at room temperature. The supernatant was then aspirated, and the cells were resuspended with 1 mL sterile PBS for a final concentration of 1×10^6 cells/mL.

2.1.3 Spleen

Mice spleens were surgically removed and transferred to a Falcon tube containing sterile PBS on ice. The spleens were placed on a 40 μ m cell strainer in a 6 well plate containing 1 mL sterile PBS. Splenocytes were obtained by mechanically disrupting the spleens using a plunger from a 1 mL syringe. The cell suspension was filtered through a cell strainer and transferred to a 50 mL Falcon tube and centrifuged at 400 x g for 5 minutes. The red blood cells from the splenocyte suspension were lysed by adding 5 mL ACK lysing buffer and the cells collected by centrifugation.

2.1.4 Bone Marrow

The mouse femurs were surgically removed and transferred to a Falcon tube containing sterile PBS on ice. Bone marrow was flushed out into a 50 mL Falcon tube by inserting a 26-gauge needle attached to a 1 mL syringe filled with sterile PBS. Remaining clumps and debris were removed by passing cell suspension through a 40 μ m cell strainer. The cell suspensions were centrifuged at 300 x g for 5 minutes and red blood cells lysed with ACK lysing buffer. Cells were resuspended with 1 mL sterile PBS for a final concentration of 1×10^6 cells/mL.

2.1.5 Female reproductive tract (FRT)

The mouse lower FRT were surgically removed and placed in cold PBS, then placed in 1 mL PBS containing 500 μ L Collagenase D [50mg/mL] (Sigma-Aldrich, USA, Cat # 11088866001) and 20 μ L of 2M Calcium Chloride. The tissues were minced with a scissor and placed in a 37 °C water bath for 30 minutes. The FRT tissue and supernatant were filtered with a 40 μ m mesh nylon strainer which was placed on top of a Falcon tube. Single-cell suspension was

collected and centrifuged at 400 x g for 5 minutes and resuspend 1 mL PBS for final concentration of 1×10^6 cells/mL.

2.2 Flow cytometry analysis

Cell suspensions from CVL, peripheral blood, spleen, bone marrow and FRT were stained for cell surface markers using conjugated dyes/antibodies listed in Table 1. Cells were pretreated with 5 μ L/mL Fc-blocker (anti-mouse CD16/32, BioLegend, USA, Cat # 101301) for 10 minutes at 4 °C, and then incubated with 1 μ L of the antibody for 20 minutes at 4 °C. Cells were then washed by adding 2 mL FACS buffer and centrifuging at 400 x g for 5 minutes. The supernatant was discarded, and cells were resuspended in 350 μ L FACS buffer on ice. Cell acquisition was performed on the LSR II (BD Biosciences, USA) flow cytometer using FACSDiva software. The data was analyzed using FlowJo software (version 10.3).

Table 1. Summary of cell surface antibodies.

Number	Antibody	Catalogue #	Clone	Company
1	Fixable Viability Stain 700	564997	–	BD, Biosciences, NJ, USA
2	Anti-mouse CD3 Pacific Blue	100214	17A2	BioLegend, San Diego, CA, USA
3	Anti-mouse CD45 APC	103112	30-F11	BioLegend, San Diego, CA, USA
4	Anti-mouse Ly6G Alexa Fluor® 488	127626	1A8	BioLegend, San Diego, CA, USA

5	Anti-mouse/human CD11b - APC/Cy7	101226	M1/70	BioLegend, San Diego, CA, USA
6	Anti-mouse CD182 (CXCR2) - PE	149304	SA044G4	BioLegend, San Diego, CA, USA

2.3 Tissue Immunohistochemistry

The mouse FRT was surgically removed and placed in 4% paraformaldehyde (PFA) (Fisher Scientific, USA, Cat # 3190-32) for 24 hours. The FRT was first placed in 10% sucrose solution for 1 hour at 4 °C, then transferred to 20% sucrose for 1 hour, and finally in 30% sucrose for 1 hour. Tissues were washed with PBS and embedded in O.C.T compound (Thermo Fisher Scientific, USA). Each sample was placed in a separate Cryomold (Sakura, Japan) and stored in -80 °C for 24 hours. The tissue samples were sectioned using a cryostat at a thickness of 10 microns and mounted onto glass slides. Once thawed, slides were washed with PBS/Triton X-100 buffer (Sigma-Aldrich, USA, # T8787) two times for 5 minutes. The slides were air dried and with a Pap Pen a line was drawn around the tissue(s). Tissue sections were pretreated with 50 µl Fc Receptor Blocker (Innovex Biosciences Inc, USA, # NB309-5S) for 30 minutes at room temperature. The Fc Receptor Blocker was then discarded, and tissue samples were incubated with 250 µl of 2% PBS/BSA buffer containing bovine serum albumin and normal mouse serum (ImmunoReagents Inc, USA, # SP-002-VX5) and normal donkey serum (ImmunoReagents Inc, USA, # SP-072-VX10) for 1 hour at room temperature. The blocking buffer was then removed and 200 µL of primary antibody anti-Ly6G (Abcam, USA, Cat # ab25377; 1:100 dilution), anti-Human/Mouse E-Cadherin (R&D Systems, USA, Cat # AF748; 10 µg/mL) or anti-myeloperoxidase (Abcam, USA, Cat # ab188211; 1:2000) diluted in 2% PBS/BSA buffer was placed on top and incubated

overnight at 4 °C. Tissues were washed with PBS/Triton X-100 buffer three times for 10 minutes. The slides were then incubated for 1 hour at room temperature with 200 µL of secondary antibody (mouse anti-rat IgG2b Alexa Fluor 488) (Abcam, USA, Cat # 172334; 1:100), donkey anti-goat IgG H&L (Alexa Fluor 568; 1:200) or goat anti-rabbit IgG (Alexa Fluor 488) (Thermo Fisher Scientific, USA, Cat # A21245; 1:100) in 2% PBS/BSA buffer. Tissues were washed with PBS/Triton X-100 buffer three times for 10 minutes. The slides were incubated for 15 minutes in the dark with Hoechst 33342 (Thermo Fisher Scientific, USA, Cat # H1399) at a 1:2000 dilution in PBS. Slides were washed, air-dried and mounted with 50 µL of ProLong Gold antifade mounting media (Thermo Fisher Scientific, USA, Cat # P36934). Vaginal tissue samples were imaged using Zeiss Axio Observer Inverted Microscope Z1 and analyzed with ZEN 2 Imaging Software (Oberkochen, Germany).

2.4 Intravaginal bacterial inoculation

Naïve female BALB/c mice (8-12 weeks old) were subcutaneously injected with 0.5 mg 17β-estradiol (Cayman Chemical, USA, # 10006315) three days prior to bacterial challenge. For bacterial inoculations, mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and intravaginally dispensed with 20 µL of either sterile PBS, 10⁸ cfu *Lactobacillus crispatus* or 10⁸ cfu *Mobiluncus mulieris*. A second inoculation with PBS or bacteria was repeated after four days. CVL was collected on days 0, 4, 7, and 9.

2.5 Intravaginal bovine serum albumin (BSA) inoculation

Mice were vaginally inoculated with 50 µL of Alexa Fluor 647 conjugated BSA (5 mg/mL (Thermo Fisher Scientific, USA, Ref # A34785) and sacrificed after 6 hours, where the lower FRT

was extracted and analysed by immunohistochemistry. In some experiments, mice were intravaginally inoculated with 50 μ L 1% Nonoxynol-9 (N-9) (Abcam, USA, # 143673) solution, 24 hours prior to BSA application.

2.6 In vivo neutrophil depletion

Mice were intraperitoneally (i.p) injected with 500 μ g of either isotype IgG2a, clone 2A3 (Bio X Cell, USA, Cat # BE0089) or anti-Ly6G, clone 1A8 (Bio X Cell, USA, Cat # BE0075) monoclonal antibodies. Neutrophil depletion was confirmed in the blood, spleen, and FRT after 48 hours of antibody injection. Cell suspensions from blood, spleen, and FRT were stained specifically for neutrophil cell surface markers using anti-mouse Ly6G/Ly6C (Gr-1) clone RB6-8C5 (BioLegend, USA, Cat # 108417) and other conjugated dyes/antibodies listed in Table 1.

2.7 Intravaginal neutrophil adoptive transfer

CVL neutrophils from mice in the diestrus phase were stained with 1 μ L of CellTracker™ Orange CMTMR Dye (Thermo Fisher Scientific, USA, Cat # C2927) for 15 minutes at 37 °C. Cells were underlaid with 2 mL of 100% FBS centrifuged at 400 x g for 5 minutes, and the supernatant was gently discarded. Cells were resuspended in 1 mL complete RPMI media (1×10^6 cell/mL). Mice in the estrus or diestrus phase were intravaginally inoculated with 25 μ L of labeled neutrophils, and the vaginal tissues removed after 2 and 5 hours for analysis by immunohistochemistry.

2.8 Mass Spectrometry

CVL samples were processed using label-free trypsin digestion method overnight and were analyzed using Q Exactive™ Quadrupole Orbitrap Mass Spectrometer (ThermoFisher, USA). Due to the low sample volume available there was no protein quantification prior to processing, and each sample had an equal volume of CVL digested with trypsin overnight. Data was normalized via peptide quantification prior to being loaded on to the mass spectrometer. Raw data were processed by Progenesis QI (Nonlinear Dynamics, USA) and Mascot (Matrix Science Inc, USA) using the Swiss-Prot human and bacterial protein database in 2013. All proteomic analysis was performed by Dr. Adam Burgener's group at the JC Wilt Center for Infectious Diseases (Birse et al., 2015).

2.9 ELISA (enzyme-linked immunosorbent assay)

Elastase expression in CVLs were analyzed using a commercially available kit (Mouse Neutrophil Elastase/ELA2, R&D Systems, USA, Cat # DY4517-05). All other cytokines/chemokines were measured by multiplex cytokine arrays by Eve Technologies (Calgary, Canada).

2.10 Statistical analyses

GraphPad Prism (software version 7.0d) was used for data analyses. Unpaired two-tailed t-test was used in two-group comparison data. A p-value of <0.05 was expressed as statistically significant.

CHAPTER 3 – RESULTS

3.1 Regulation of neutrophil migration and localization within the FRT

Previous studies have demonstrated that protein signatures involved in leukocyte migration, epithelial barrier remodeling and a general immune activation were highly upregulated during the luteal phase in healthy human cohorts (Birse et al., 2015). Notably, a strong enrichment for neutrophil-related protein signatures were reported, suggesting that neutrophils are actively recruited into the vaginal tissue and are a major source for inflammatory mediators in the FRT. To provide a mechanistic understanding of mucosal neutrophils in the FRT, we enumerated the number of neutrophils in the cervicovaginal lavage (CVL) of mice across the different murine estrous cycle (Figure 2). We observed that while CD4⁺ T cell numbers in CVL did not significantly change during the transition from the estrus to the diestrus phase (Figure 3A), neutrophil numbers (CD45⁺CD3⁻CD11b⁺Ly6G⁺) were significantly increased during the metestrus and diestrus phases (Figure 3B). Next, we visualized the presence of neutrophils in the vaginal tissue and observed that high numbers of Ly6G⁺ neutrophils were observed in the metestrus and diestrus phases compared to no infiltration in the estrus phase (Figure 4A), consistent with previous reports (Sonoda et al., 1998; Schaefer et al., 2014). Image quantification from vaginal tissue sections confirmed the significantly higher percentage of vaginal epithelium and lamina propria tissue occupied by Ly6G⁺ neutrophils in the metestrus and diestrus compared to the estrus phase ($p < 0.001$) (Figure 4B). Thus, the data clearly showed that neutrophils are highly abundant in mouse CVL and vaginal tissues, and that they responded to elevated progesterone levels during diestrus.

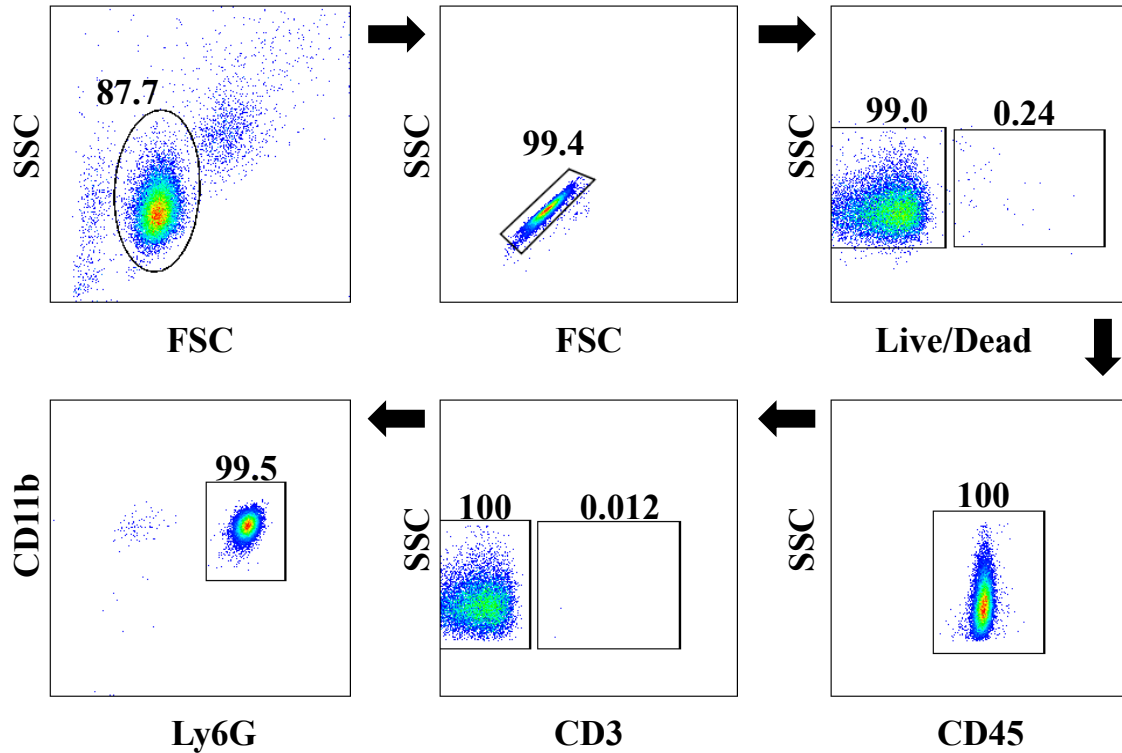


Figure 2. Gating strategy to identify neutrophils in mouse cervicovaginal lavage (CVL). CVL was taken from naïve virgin female BALB/c mice (8-12 weeks old) in the diestrus phase of the estrous cycle and prepared for FACS staining. The CVL cells of interest were gated onto a forward scatter (x-axis) versus side scatter (y-axis) plot, singlet cells identified and gated on live cells using the live/dead indicator dye. CD45 was then used to distinguish viable and single hematopoietic cells within the live cell population. CD3 negative and positive cells were sorted from the CD45 positive population. From the CD3 negative cell population Ly6G positive (x-axis) and CD11b positive (y-axis) neutrophils were identified to describe CVL neutrophils as (CD45⁺CD3⁻CD11b⁺Ly6G⁺).

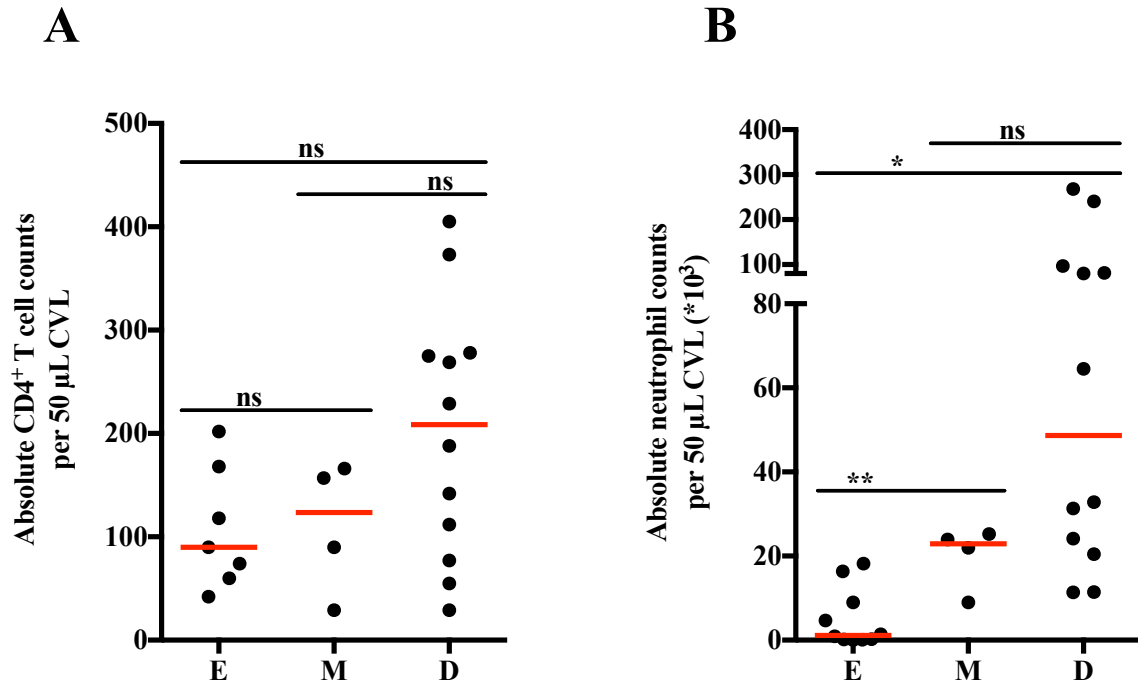


Figure 3. Absolute CD4⁺ T cell and neutrophil counts in CVL of mice at different stages of the estrous cycle. CVL from naïve virgin female BALB/c mice (8-12 weeks old) at different estrous stages were collected and prepared for flow cytometry. **(A)** Absolute CD4⁺ T cell counts (CD45⁺CD3⁺CD4⁺) and **(B)** Absolute neutrophil counts (CD45⁺CD11b⁺Ly6G⁺) in each stage of the estrous cycle are shown (E, estrus; M, metestrus; D, diestrus). Red lines indicate median values. Data are pooled from six experiments and were analyzed using unpaired two-tailed t-test (*, **, $p < 0.05$; ns; non-significant).

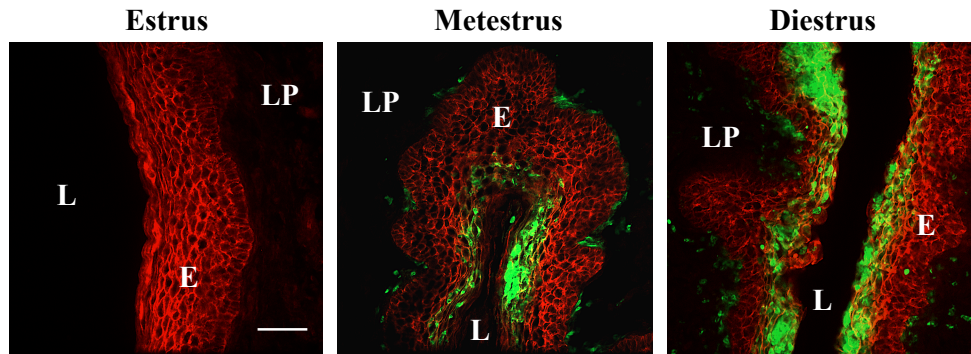
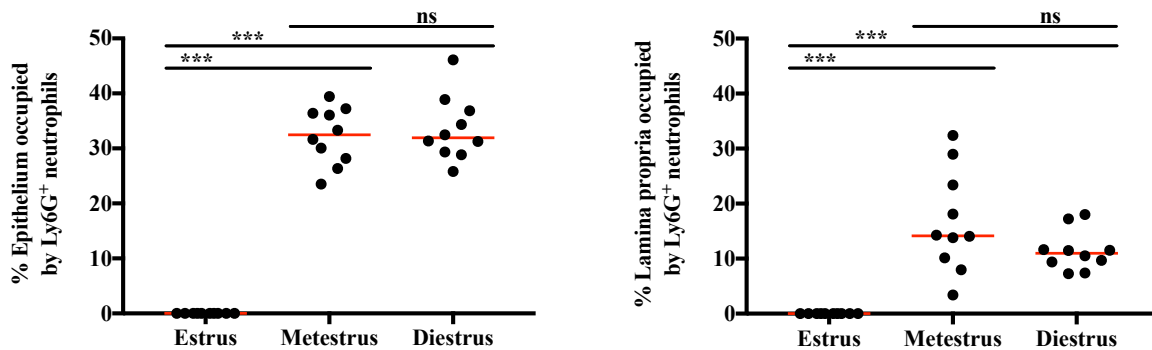
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Figure 4. Distribution of vaginal neutrophils in mice at different stages of the estrous cycle. (A) Vaginal tissue sections were taken from naïve virgin female BALB/c mice (8-12 weeks old) at the indicated stage of the estrous cycle and stained for E-cadherin (epithelium; red) and Ly6G⁺ (neutrophils; green). E, epithelium; L, lumen; LP, lamina propria. Images were taken at 20x magnification. Scale bar = 50 μ m. (B) Percentage of vaginal epithelium or lamina propria occupied by Ly6G⁺ neutrophils at each stage of the estrous cycle are shown. Data were pooled from five independent experiments. Red lines indicate median values. Analyses of Ly6G⁺ distribution in the epithelium and lamina propria compartments were performed by using unpaired two-tailed t-test (***, $p < 0.001$; ns; non-significant).

3.2 FRT neutrophils express CXCR2 and respond to ligand CXCL2

Previous studies have shown that mucosal neutrophil recruitment is dependent on CXCR2 expression (Sonoda et al., 1998; Sadik et al., 2011; Mishra et al., 2015). To confirm this, we analyzed the expression of cell surface CXCR2 expression of neutrophils in different compartments. We observed high CXCR2 expression on neutrophils in cervicovaginal lavage (CVL), blood and spleen and low expression in bone marrow (Figure 5A). Furthermore, to assess the functionality of the CXCR2 receptor, CVL neutrophils from mice in the diestrus phase were collected, stained with CellTracker™ Green CMTMR Dye and embedded into 3D collagen chambers in the presence of either media or 10 ng/mL CXCL2, the ligand for CXCR2. Live-cell imaging studies revealed that CXCL2 significantly increased neutrophil migration speeds and displacement compare to media controls, suggesting that CVL neutrophils are viable and responsive to chemokine (Figure 5B-D). These observations showed that CVL neutrophils retain their motility and expressed high levels of CXCR2 that allowed their positioning along the outer layers of the vaginal epithelium.

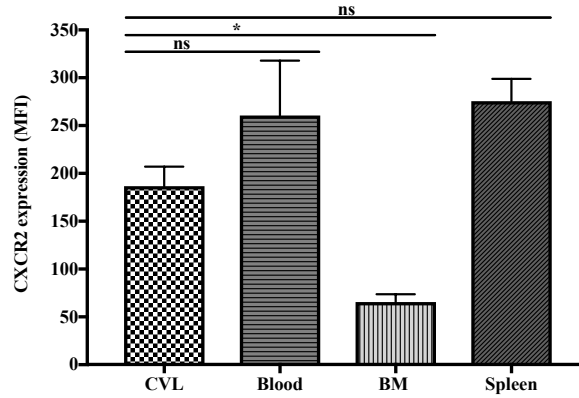
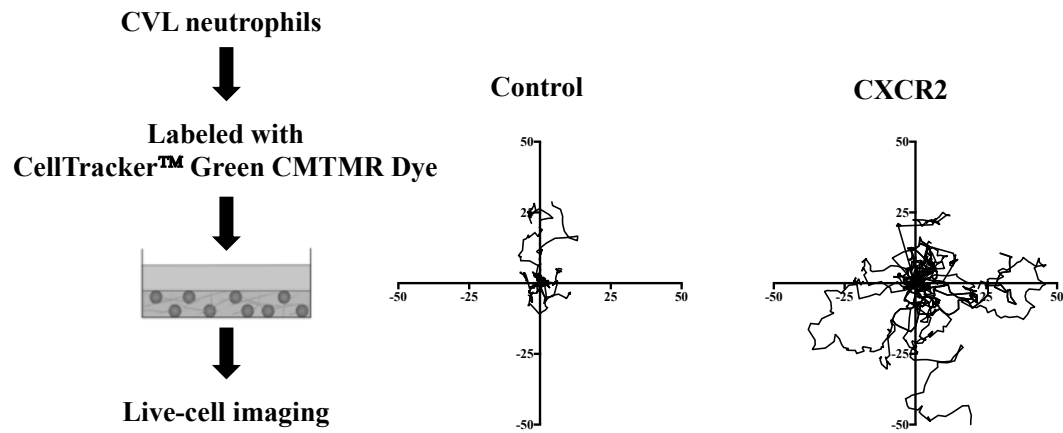
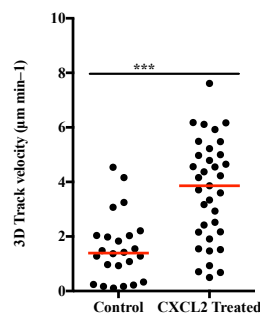
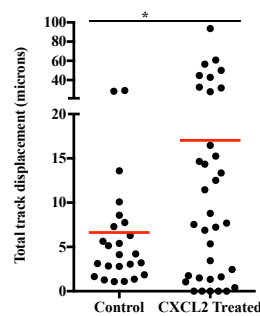
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Figure 5. CVL neutrophils are viable and respond to CXCL2. (A) Median fluorescence intensity (MFI) analyses of neutrophils isolated from different compartments stained with antibody specific for mice CXC chemokine receptor 2 (CXCR2). BM; bone marrow. (B) Cervicovaginal lavage (CVL) neutrophils from diestrus BALB/c mice (8-12 weeks old) were stained with CellTracker™ Green CMTMR Dye and imbedded into 3D collagen chambers in the presence of

either media (control) or with 10 ng/mL CXCL2. Time-lapse videos were analyzed using ImageJ. **(C)** 3D Track velocity and **(D)** Total track displacement of individual neutrophils are shown. Data are representative of two independent experiments and were analysed using unpaired two-tailed t-test. Red line indicates median values (*, $p < 0.05$; ***, $p < 0.001$; ns; non-significant).

3.3 CVL neutrophils do not penetrate into the epithelium from the vaginal lumen

We postulated, given the large number of neutrophils in the vaginal canal and its localization near the superficial layers of the vaginal epithelium, that neutrophils derived from the upper FRT, such as the uterus, would migrate from the vaginal lumen and into the epithelium. To test this possibility, CVL neutrophils were labeled with CellTracker™ Orange CMTMR Dye and adoptively transferred intravaginally into female BALB/c mice in the estrus and diestrus phases to determine whether neutrophil had the ability to enter the vaginal epithelium from the luminal space (Figure 6A). Mice were sacrificed after 2 and 5 hours and vaginal tissue sections were analyzed by immunohistochemistry (Figure 6B). We observed that while rare transferred neutrophils were localized within the outermost layer of the epithelium, they were never found in the basolateral layers or lamina propria. These data suggested that vaginal neutrophils are not recruited from the luminal space, but rather from peripheral blood, given their abundance in the lamina propria (Figure 4).

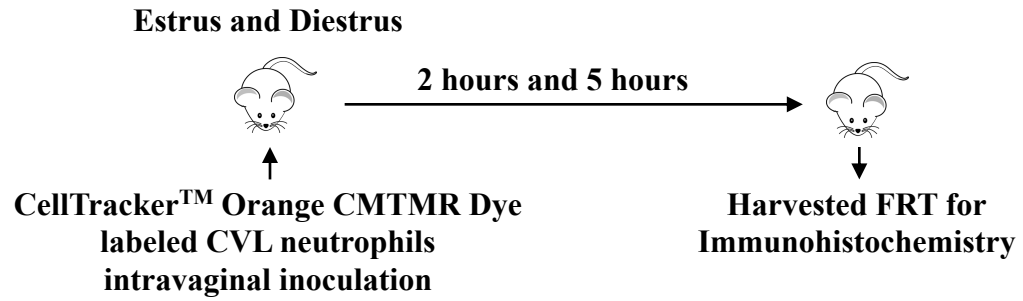
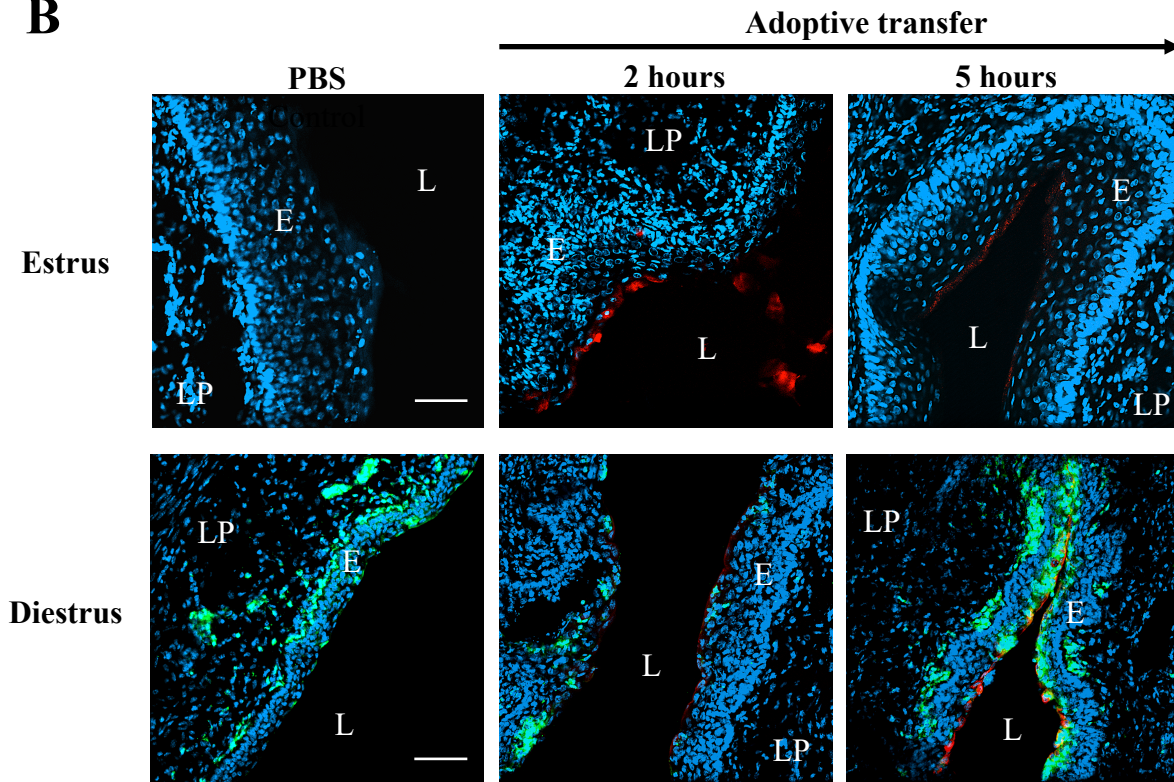
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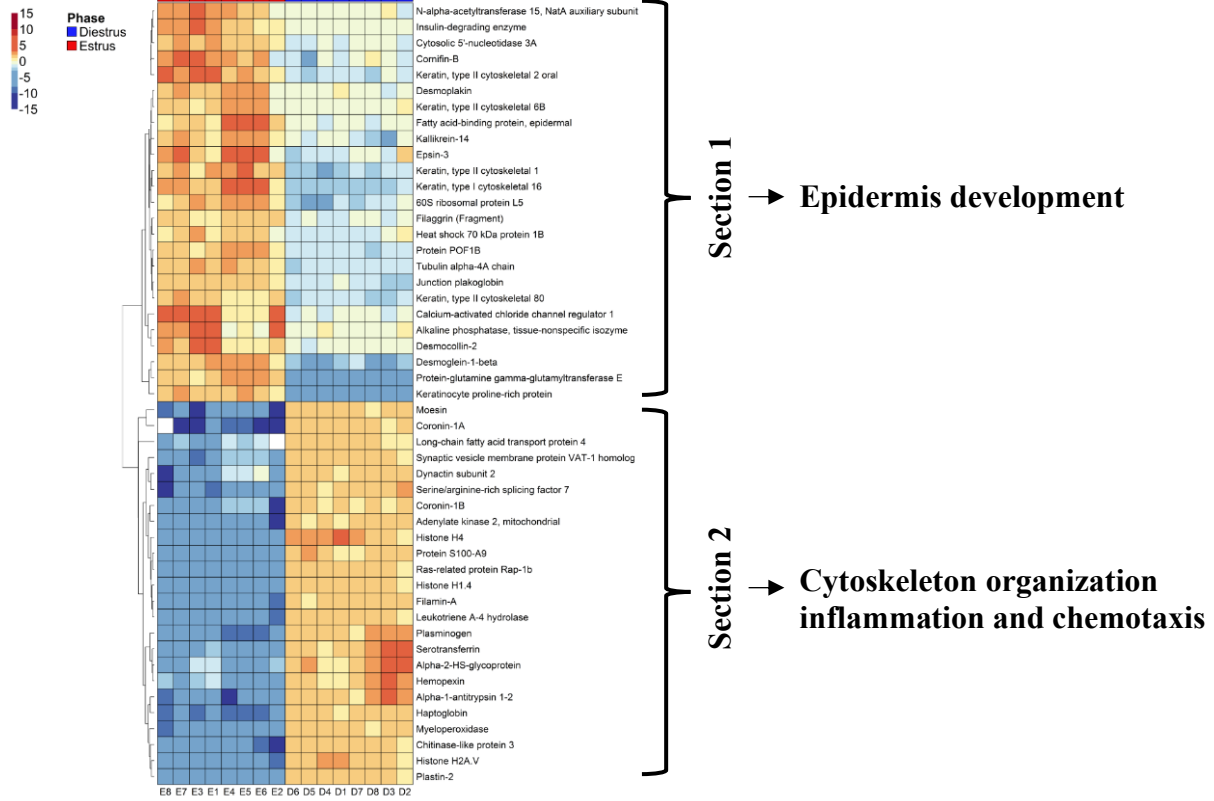
Figure 6. Adoptively transferred CVL neutrophils from the lumen do not penetrate into the vaginal epithelium. (A) Cervicovaginal lavage (CVL) neutrophils (1×10^6) labeled with CellTracker™ Orange (CMTMR; red) Dye were adoptively transferred intravaginally into naïve virgin female BALB/c mice (8-12 weeks old) at the indicated estrous cycle. (B) Vaginal tissue sections were taken after 2 hours and 5 hours and stained for Ly6G⁺ (neutrophils; green) and cell

nucleus (DAPI, blue). CMTMR labeled neutrophils are shown in red. E, epithelium; L, lumen; LP, lamina propria. Images were taken at 20x magnification. Scale bar = 50 μ m.

3.4 Proteomic analysis of mouse CVL reveals an increase in inflammation, wound healing and neutrophil-related pathways in the diestrus phase

We next performed mass spectrometry (MS)-based proteomic analyses of murine CVL in order to gain an unbiased view of the molecular pathway signatures that are differentially regulated between the estrus and diestrus phase. MS analysis revealed a total of 568 unique proteins of which 50 proteins were found to be differentially abundant between the two phases (Figure 7A). Ingenuity Pathway Analysis (IPA) was used to determine the biological function associated with the unique proteins. Canonical pathways significantly enhanced during the estrus or diestrus phases were also analyzed (Figure 7B) and revealed that biological processes related to peptidase activity and epidermis development were upregulated during the estrus phase. In contrast, biological processes that are associated with cytoskeleton organization, inflammatory responses, leukocyte chemotaxis and wound repair were highly associated with the diestrus phase, suggestive of extensive tissue remodeling and leukocyte infiltration/activation.

A



B

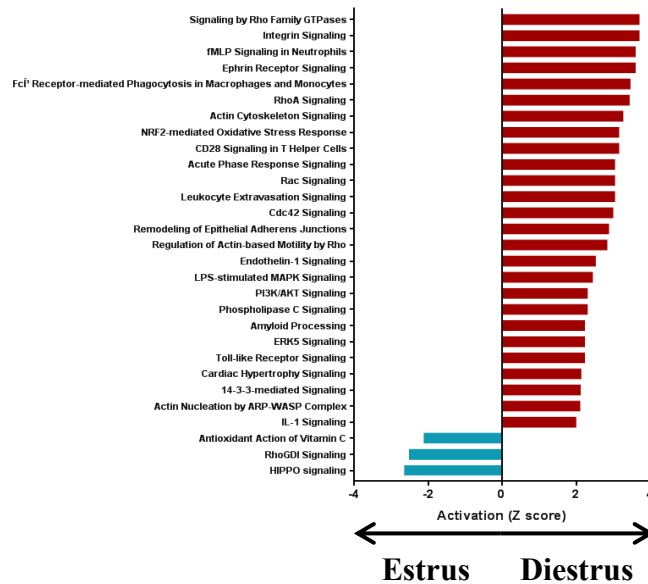


Figure 7. Significantly differentially abundant proteins and associated biological functions and pathways between the estrus and diestrus phases. Unique proteomes of mice CVL in the estrus and diestrus phases were characterized using Q-Exactive mass spectrometry analysis. **(A)** A total of 568 unique proteins were identified and 50 significant proteins differentially abundant between the two phases were analyzed. Clear phase-specific differences in protein abundance is exhibited. Underabundant proteins are presented in blue heat map and overabundant proteins are presented in red. Elevated protein expressions in estrus phase with biological processes related to epidermis development (Section 1) and diestrus phase (Section 2) related to immune cell recruitment factors are shown. **(B)** Canonical pathways significantly enhanced during estrus phase (blue) and diestrus phase (red). Data analyses for proteomes and canonical pathways were performed by using Mann-Whitney U test and right-tailed Fisher exact test respectively ($p < 0.05$, ≥ 2 -fold change).

3.5 Neutrophil depletion in vivo reduces inflammatory and wound healing signaling pathways

Next, we addressed to what extent vaginal neutrophils contributed to the increased inflammatory and wound healing pathways observed during diestrus, where neutrophil numbers in the FRT are high. To evaluate this, female BALB/c mice were either injected with isotype or anti-Ly6G monoclonal antibody (Figure 8A) and depletion of neutrophil numbers confirmed in peripheral blood and vaginal tissue by flow cytometry (Figure 8B). Vaginal tissue sections analyzed by immunohistochemistry also confirmed the absence of vaginal neutrophils (Figure 8C). Image quantification showed that the epithelium and lamina propria were significantly ($p < 0.001$) occupied by Ly6G⁺ neutrophils in the isotype injected mice but not after anti-Ly6G injection (Figure 8D). MS analysis of CVL after neutrophil depletion identified a total of 78 unique proteins

differentially abundant in isotype and anti-Ly6G injected mice (Figure 9A). Increased biological processes involved in epithelial morphogenesis, cell-cell junction and cellular macromolecular catabolism were associated with neutrophil depletion. In contrast, the absence of FRT neutrophils reduced protein signatures that associate with innate immune responses, cell signaling and immune activation (Figure 9B), demonstrating that neutrophils can regulate the FRT landscape as they entered and exited the vaginal tissue.

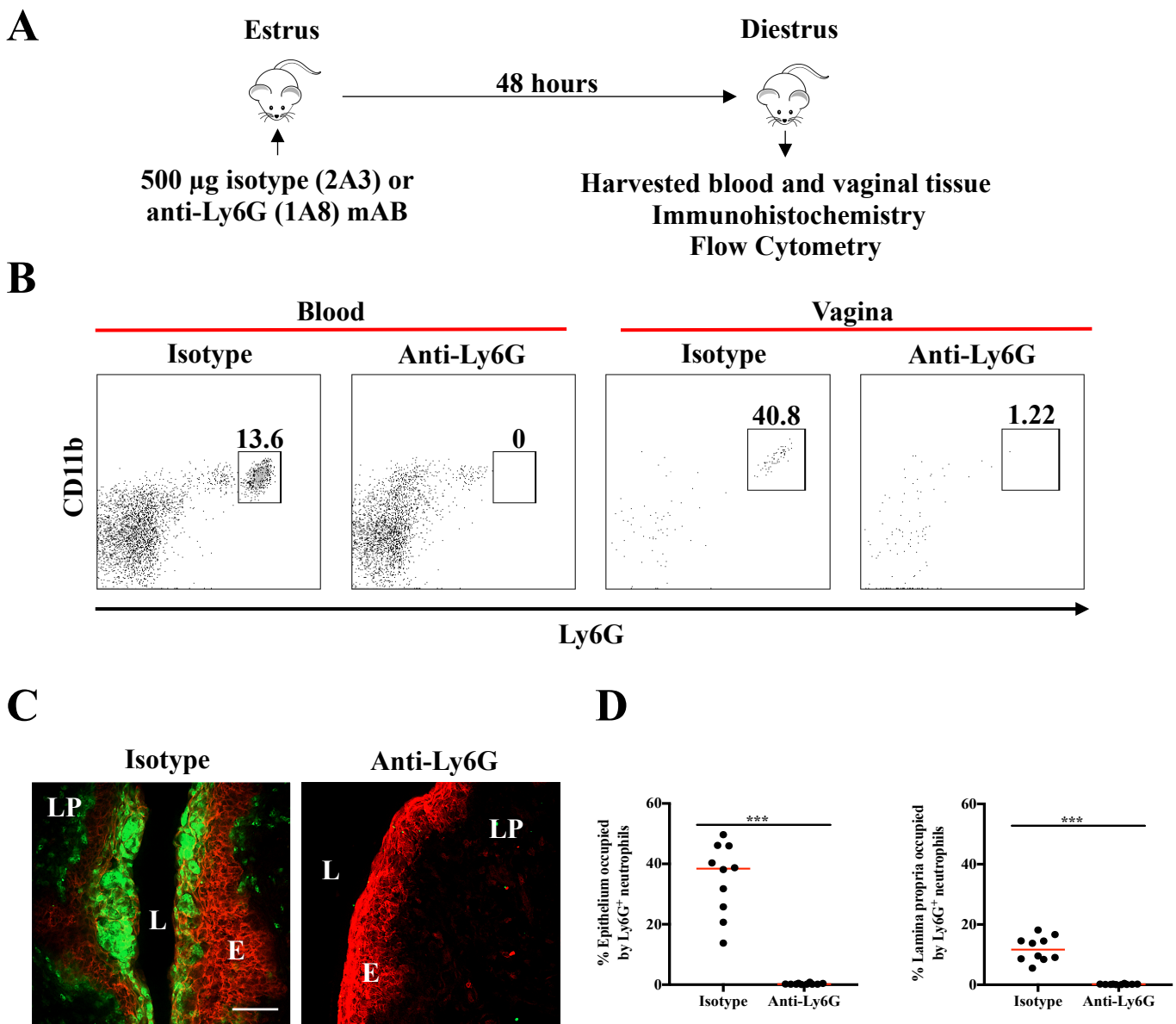


Figure 8. *In vivo* depletion of vaginal neutrophils. (A) Female BALB/c mice were intraperitoneally (i.p) injected with 500 µg of either isotype (2A3) or anti-Ly6G (1A8) monoclonal antibodies. After 48 hours, peripheral blood and the lower FRT were prepared for flow cytometry analysis and immunohistochemistry. (B) Percentage of CD45⁺CD11b⁺Ly6G⁺ neutrophils in both blood and vagina are shown. (C) Micrographs of vaginal tissue stained for E-cadherin (epithelium; red) and Ly6G⁺ (RB6-8C5; green). E, epithelium; L, lumen; LP, lamina propria. Images were taken at 20x magnification. Scale bar = 50 µm. (D) Percentage of vaginal epithelium or lamina propria occupied by Ly6G⁺ neutrophils after isotype or depleting antibody injection. Red lines indicate median values. Data were analysed using unpaired two-tailed t test (***, $p < 0.001$).

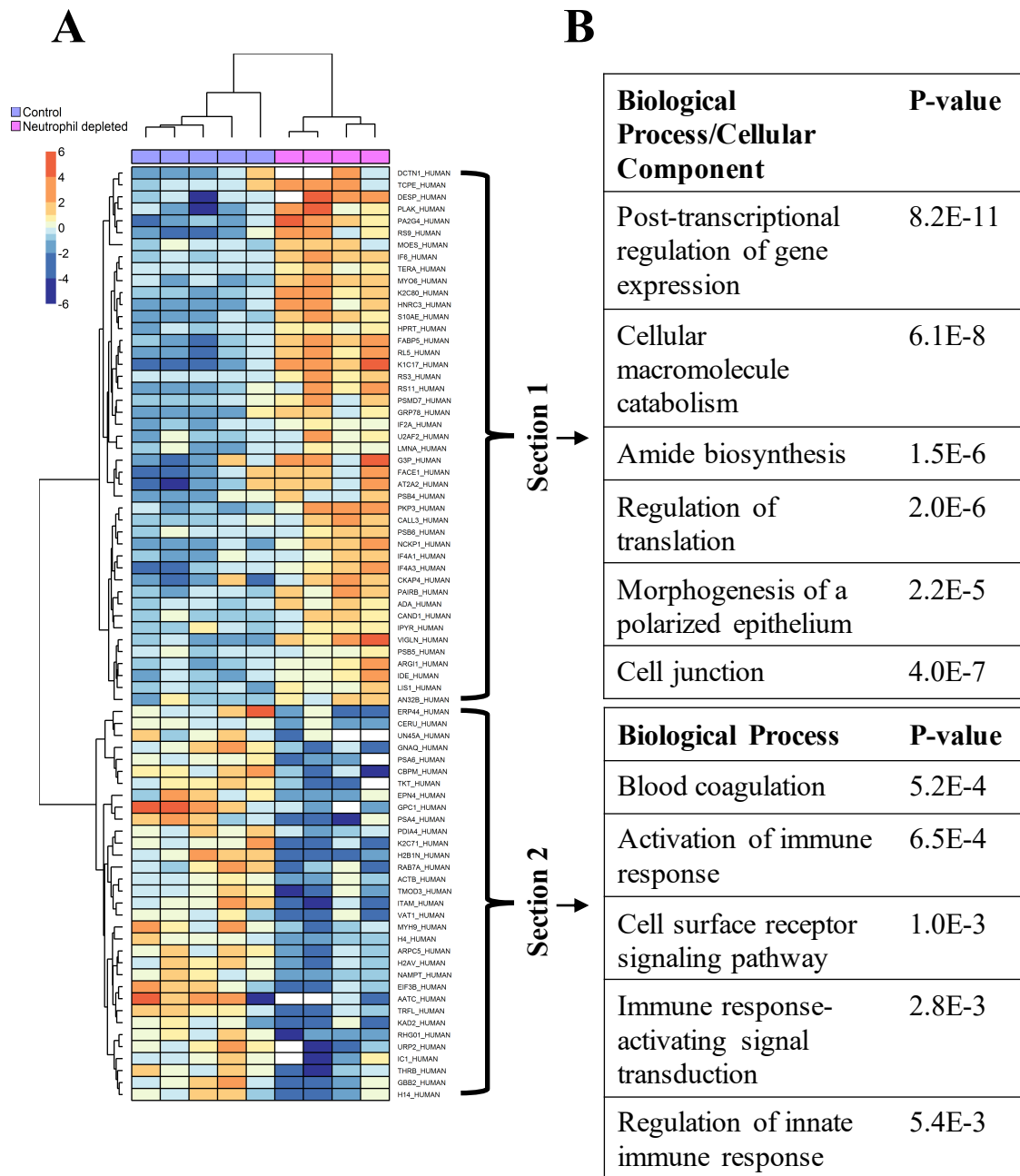


Figure 9. Importantly distinctive abundant proteins expressed in neutrophil depleted mice.

(A) Exclusive proteomes were identified in CVL from mice injected with either isotype or anti-Ly6G monoclonal antibodies using Q-Exactive mass spectrometry analysis. A total of 78 unique proteins differentially abundant were characterized between isotype and anti-Ly6G injected mice.

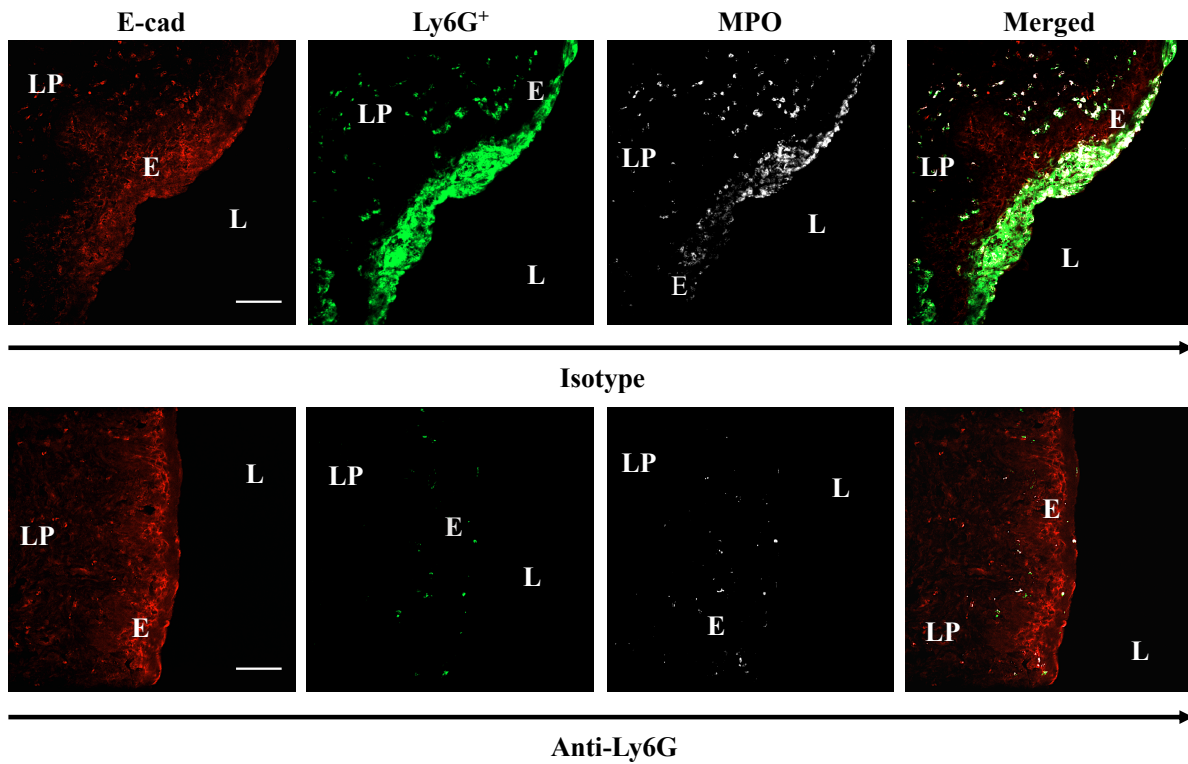
Clear phase-specific differences in protein abundance is exhibited. Underabundant proteins are presented in blue heat map and overabundant proteins are presented in red. **(B)** Primary biological processes estimated to be activated in isotype and anti-Ly6G depleted mice along with each process associative protein are presented (Section 1 and Section 2). Data were analyzed using unpaired two-tailed t-test ($p < 0.05$).

3.6 Evaluation of neutrophil-derived inflammatory mediators in the FRT in vivo

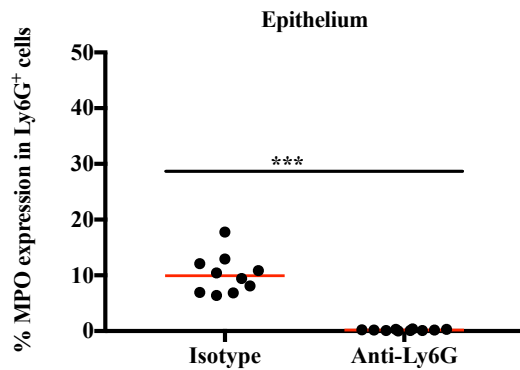
In order to assess neutrophil activation, expression of myeloperoxidase (MPO) which is considered a key component of neutrophil's cytotoxicity and is a known neutrophil activation marker (Lau et al., 2005; Odobasic et al., 2016) was evaluated in the estrus and diestrus phases using immunohistochemistry analysis (Figure 10A). Image quantification results (Figure 10B and 10C) clearly revealed significantly lower percentage of MPO expression of Ly6G⁺ neutrophils present in the epithelium and lamina propria in the estrus phase ($p < 0.001$) when compared to the diestrus phase. Moreover, immunohistochemistry vaginal tissue analyses from neutrophil depleted mice revealed higher expression of MPO in isotype injected mice compared to anti-Ly6G mice (Figure 11A). Image quantification clearly showed a significant decrease of MPO expression in the vaginal epithelium and lamina propria of depleted mice compared to isotype injected mice ($p < 0.001$) (Figure 11B and 11C).

Scale bar = 50 μ m. Percentage of MPO expressed in **(B)** Epithelium and **(C)** Lamina propria in mice in the estrus and diestrus phase. Red lines indicate median values. Data analyses were performed by using unpaired two-tailed t-test (***, $p < 0.001$).

A



B



C

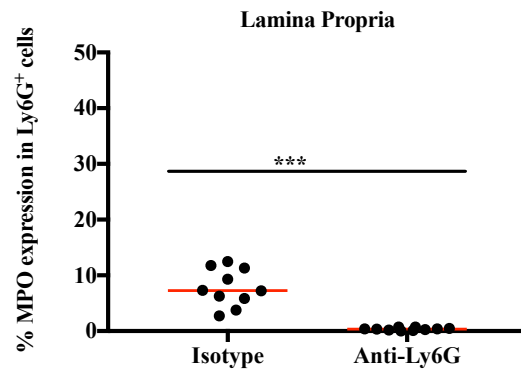


Figure 11. Decreased myeloperoxidase (MPO) expression in the vagina of neutrophil-depleted mice. (A) Vaginal tissue sections of mice, 48 hours after intraperitoneal injection with 500 μ g of either isotype or anti-Ly6G antibody and stained for E-cadherin (epithelium; red), Ly6G⁺ (neutrophils; green) and MPO (white). E, epithelium; L, lumen; LP, lamina propria. Images were taken at 20x magnification. Scale bar = 50 μ m. Percentage of MPO expressed in Ly6G⁺ cells in **(B)** Epithelium and **(C)** Lamina propria of mice injected with isotype and anti-Ly6G antibodies. Red lines indicate median values. Data analyses were performed by using unpaired two-tailed t-test (***, $p < 0.001$).

To further assess the relation between matrix metalloproteinases (MMPs) expression which have been reported to be involved in various inflammatory and repair process regulations (Parks et al., 2004) we evaluated the expression of matrix metalloproteinases (MMPs) 2, 3 and 8 reported (Manicore and McGuire., 2008; Ong et al., 2015; Medeiros et al., 2017) to be related to neutrophil activation using ELISA. Expression of MMP2, 3 and 8 in the CVLs of mice in the estrus phase were significantly lower compared to the metestrus and diestrus phases ($p < 0.05$ and $p < 0.001$) (Figure 12A-C). Moreover, MMP2 and 3 expression levels in anti-Ly6G injected mice did not show significantly lower expression levels compared to isotype injected mice (Figure 13A-B). However, MMP8 expression in depleted mice was evidently lower than isotype injected mice ($p < 0.05$) (Figure 13C). Similarly, we assessed the expression of neutrophil elastase, a known serine proteinase involved in neutrophil activation and recruitment which is expressed during inflammation (Emboriadou et al., 2007; Korkmaz et al., 2010) by ELISA. Results revealed a significantly higher level of neutrophil elastase expression in the metestrus phase compared to mice in the estrus phase ($p < 0.05$) (Figure 12D). Similar results were observed in isotype injected

mice where the expression of neutrophil elastase was shown to be crucially higher compared to anti-Ly6G injected mice ($p < 0.05$) (Figure 13D).

In addition to MMPs and neutrophil elastase expression, we further assessed a panel of cytokine expression that played a role in neutrophil recruitment, activation and function, such as granulocyte colony-stimulating factor (G-CSF) (Eyles et al., 2008; Barjami et al., 2016), IL-12 p40 (Bliss et al., 2000; Moreno et al., 2006), CCL2 (Goodyear et al., 2010; Jin et al., 2018) and CCL5 (Pan et al., 2000; McDonald and Kubes., 2010). Results obtained from ELISA analysis showed higher expression of these cytokines in the metestrus and diestrus phases compared to the estrus phase (Figure 12E-H) and confirmed that their expression was suppressed when neutrophils were depleted *in vivo* (Figure 13E-H). These data clearly indicated that vaginal neutrophils expressed a number of inflammatory mediators such as MPO, MMP8 and neutrophil elastase at steady-state, and that these proteins were highly upregulated during the diestrus phase, suggesting a possible role of neutrophils in modulating the vaginal epithelial barrier function and regulating the status of genital inflammation.

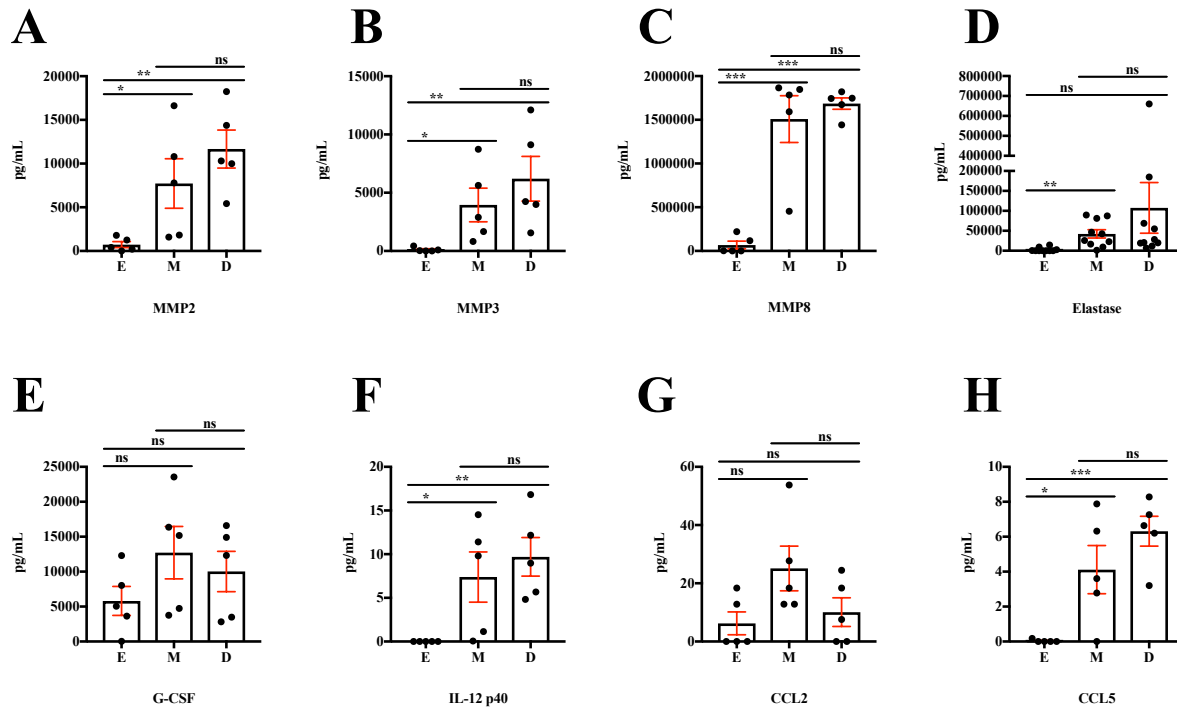


Figure 12. Increased expression of inflammatory mediators associated with CVL neutrophils in mouse estrous cycle. Secretion levels of (A) MMP2 (B) MMP3 (C) MMP8 (D) Neutrophil elastase (E) Granulocyte-colony stimulating factor (G-CSF). (F) Interlukin-12 p40 (IL-12 p40). Chemokine (C-C motif) ligand (G) CCL2 and (H) CCL5 in cervicovaginal lavage (CVL) from female BALB/c mice in different stages of the estrous cycle are presented. E, estrus; M, metestrus; D, diestrus. Red bars represent mean $\pm$ SEM. Data were analyzed using unpaired two-tailed t-test (*, **, $p < 0.05$; ***, $p < 0.001$; ns; non-significant).

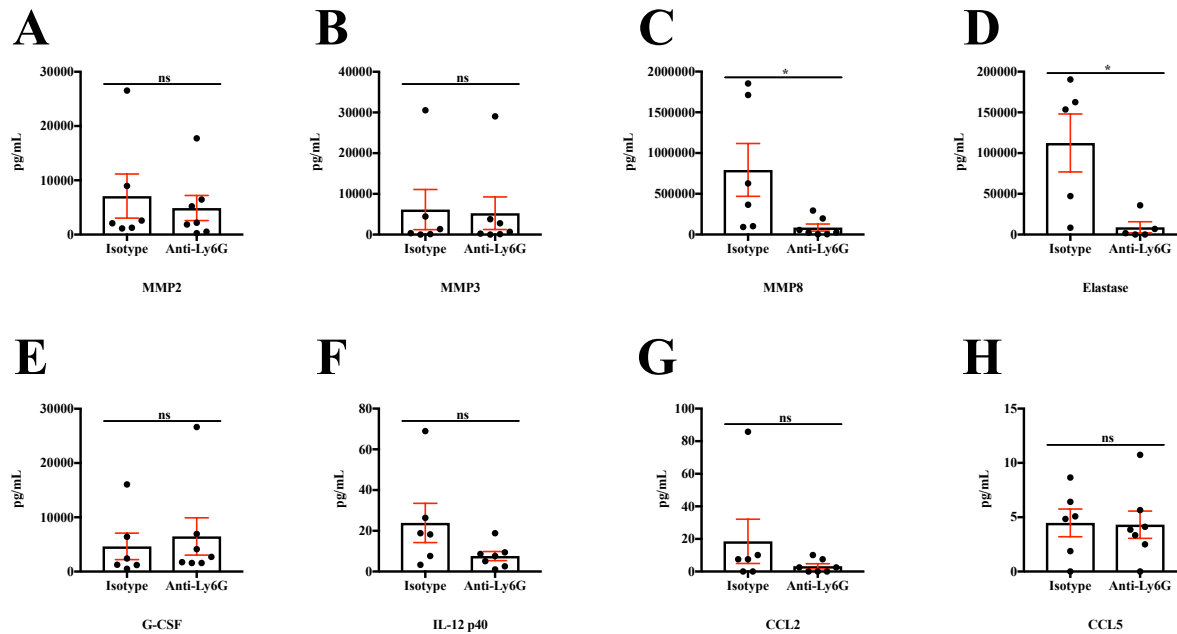


Figure 13. Expression of inflammatory mediators associated with CVL neutrophils decrease with neutrophil depletion. Secretion levels of (A) MMP2 (B) MMP3 (C) MMP8 (D) Neutrophil elastase (E) Granulocyte-colony stimulating factor (G-CSF). (F) Interlukin-12 p40 (IL-12 p40). Chemokine (C-C motif) ligand (G) CCL2 and (H) CCL5 in cervicovaginal lavage (CVL) from female BALB/c mice in different stages of the estrous cycle are presented. E, estrus; M, metestrus; D, diestrus. Red bars represent mean $\pm$ SEM. Data were analyzed using unpaired two-tailed t-test (*, $p < 0.05$; ns; non-significant).

3.7 Vaginal epithelial barrier integrity is not compromised by neutrophils at steady-state

The large infiltration of neutrophils suggested that they may modulate the vaginal epithelium. To directly measure vaginal barrier integrity, we intravaginally inoculated Alexa Fluor 647-labeled bovine serum albumin (AF647-BSA) as a fluid-phase marker since it is small enough (~66kDa) to enter water permeable spaces (Carias et al., 2013). At 6 hours post-inoculation, the

FRT was removed and vaginal tissue sections were analyzed by immunohistochemistry (Figure 14A). Results revealed a significant percentage of BSA penetration into the vaginal epithelium in the metestrus phase in comparison to the estrus and diestrus phases ($p < 0.05$) (Figure 14B). Moreover, studies in the past have shown that Nonxynol-9 (N-9), a surfactant used in spermicide, can cause inflammation and epithelial disruption (Catalone et al., 2005; Lozenski et al., 2012). When diestrus mice were inoculated with 1% N-9 for 24 hours prior to intravaginal challenge with BSA for 6 hours (Figure 14A), we observed increased BSA penetration ($p < 0.05$) into the vaginal epithelium, demonstrating the utility of BSA as a fluid-phase marker to assess epithelial barrier integrity *in vivo*. Finally, we assessed the role of neutrophil recruitment on the vaginal epithelial barrier at steady-state through *in vivo* depletion of neutrophils using anti-Ly6G antibodies. Mice were subsequently intravaginally inoculated with AF647-conjugated BSA after 48 hours of depletion and were sacrificed after 6 hours (Figure 15A). Image quantification showed no significant difference in the percentage of BSA penetration into the vaginal epithelium between the isotype and anti-Ly6G antibody treated groups (Figure 15B). Overall, these data indicated that there is no obvious disruption of FRT epithelial barrier integrity during the mass recruitment of neutrophils during diestrus.

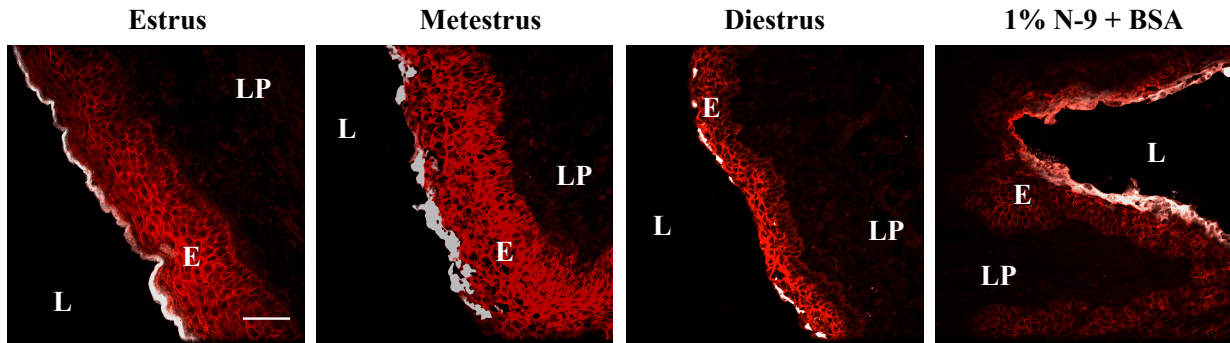
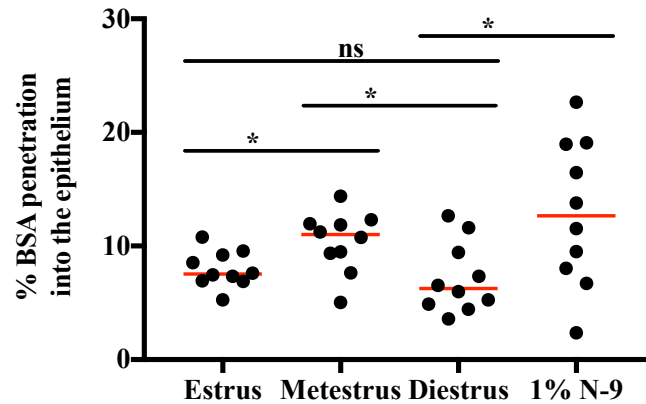
A**B**

Figure 14. Percent BSA penetration into the vaginal epithelium. Female BALB/c mice at different stages of the estrous cycle were intravaginally inoculated with 50 μ L AF647-conjugated bovine serum albumin (BSA; 5 mg/ml) and the lower FRT was extracted after 6 hours. **(A)** Vaginal tissue sections were stained for E-cadherin (epithelium; red). BSA is shown in white. Mice in the diestrus phase were also vaginally inoculated with 50 μ L 1% nonoxynol-9 (N-9) for 24 hours prior to BSA treatment. E, epithelium; L, lumen; LP, lamina propria. Images were taken at 20x magnification. Scale bar = 50 μ m. **(B)** Percent BSA penetration into the vaginal epithelium is shown for each estrous stage and 1% nonoxynol-9 (N-9) inoculated mice in the diestrus phase. Red lines indicate median values. Data were analysed using unpaired two-tailed t-test (*, $p < 0.05$; ns; non-significant).

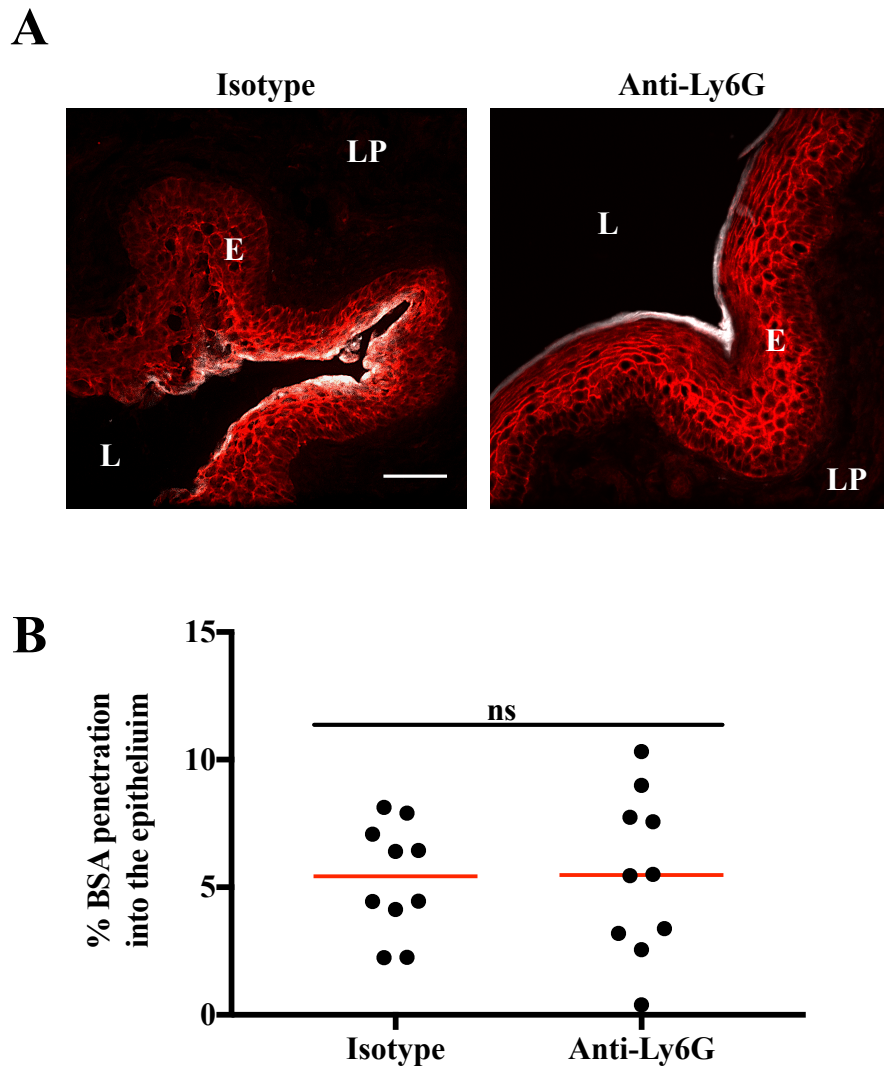


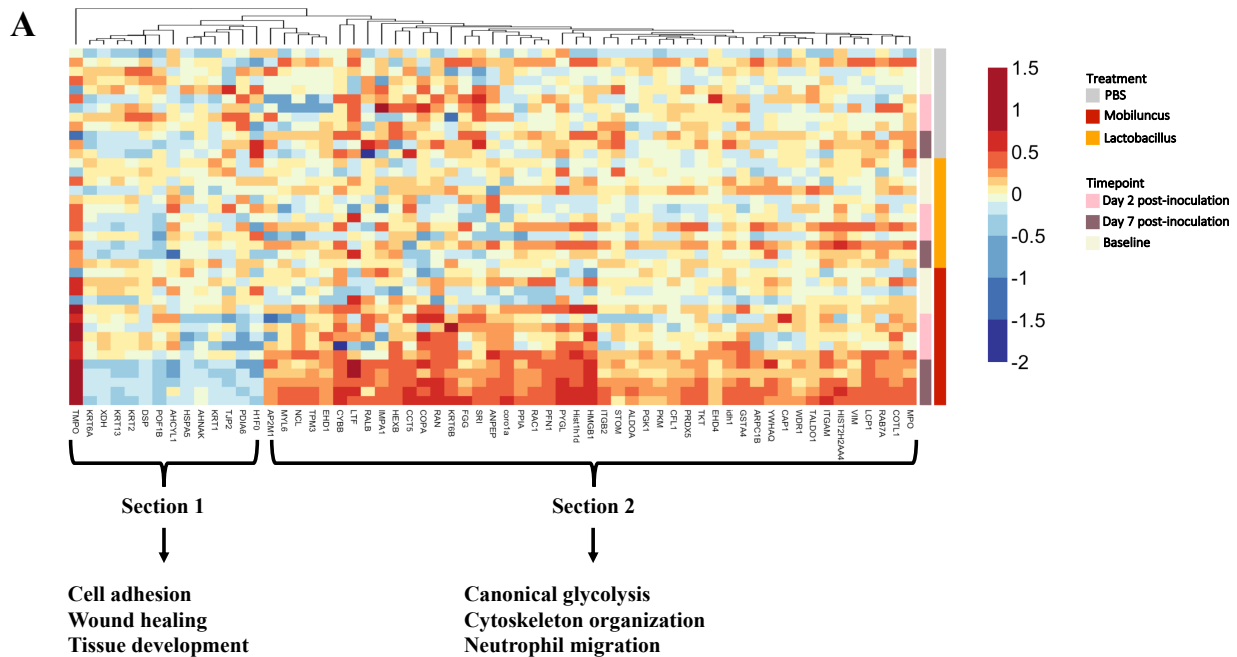
Figure 15. Mucosal neutrophils do not compromise vaginal epithelial barrier integrity at steady-state. (A) Female BALB/c mice were intraperitoneally injected with 500 μ g of either isotype or anti-Ly6G antibody. 48 hours later, mice were intravaginally inoculated with 50 μ L of AF647-conjugated BSA for 6 hours and the vaginal tissue sections were stained for E-cadherin (epithelium; red). BSA is depicted in white. E, epithelium; L, lumen; LP, lamina propria. Images were taken at 20x magnification. Scale bar = 50 μ m. **(B)** Percent BSA penetration into the vaginal

epithelium is shown for both groups. Red lines indicate median values. Data were analyzed using unpaired two-tailed t-test (ns; non-significant).

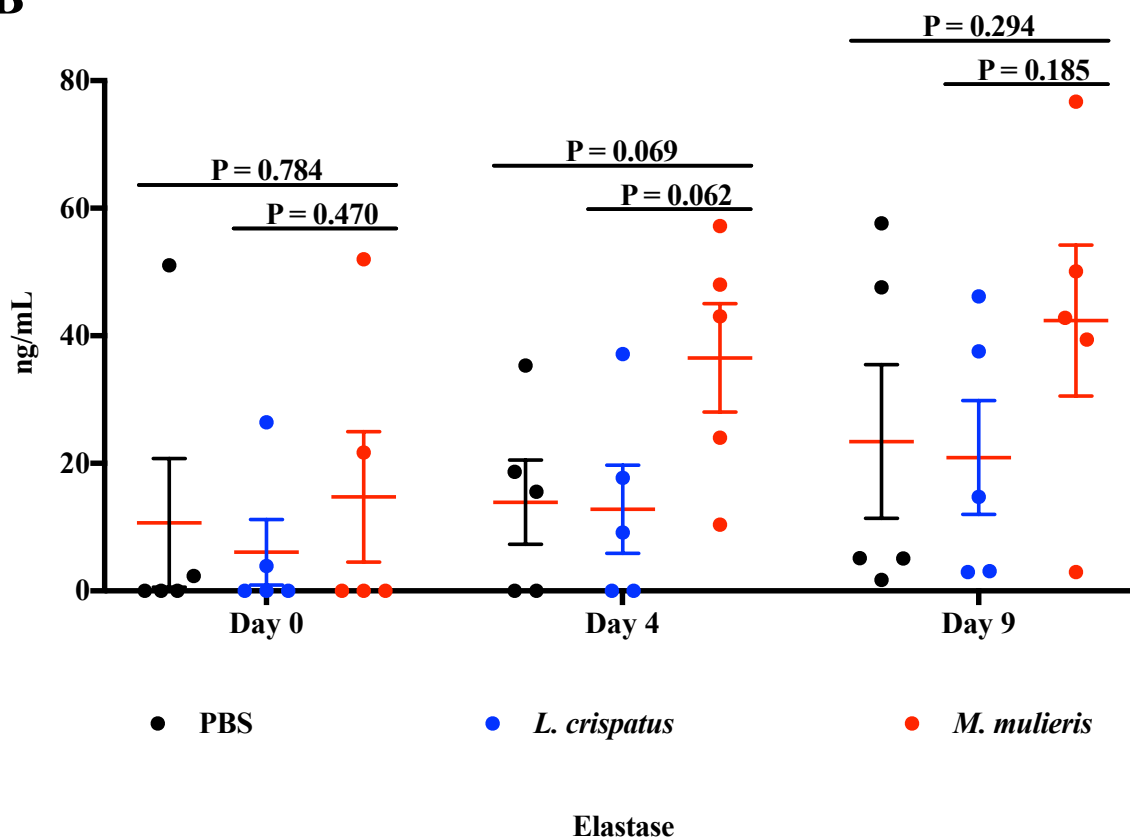
3.8 Vaginal inoculation with *Mobiluncus mulieris* induces high expression of neutrophil-related inflammatory mediators

To demonstrate whether the presence of different bacterial species leads to the recruitment and activation of neutrophils within the FRT, we intravaginally inoculated mice with either PBS alone, *Lactobacillus crispatus* or *Mobiluncus mulieris* at different time points. Mass spectrometry analysis of mice CVL revealed a total of 418 common proteins in mouse samples across all three inoculated groups. A 3-5% of difference in protein expression in the first (Day 0) and second inoculation (Day 4) with either PBS and *L. crispatus* was observed. In contrast, while mice inoculated with *M. mulieris* showed minor changes in specific protein expression after the first inoculation, a significant increase (15%; 63 specific protein) in protein expression was detected after the second inoculation. Functional protein analysis revealed a significant increase in biological pathways associated with canonical glycolysis, cytoskeleton organization and neutrophils migration in response to *M. mulieris* compared to the PBS and *L. crispatus* groups. In contrast, a reduction in protein signatures associated with cell-cell adhesion and tissue development was observed after *M. mulieris* inoculation compared to *L. crispatus* (Figure 16A). Furthermore, to assess whether neutrophil activation is linked to alteration of vaginal microbiome bacteria, we next assessed the expression of neutrophil elastase, a key inflammatory mediator that modulates the ECM, in the CVL in response to vaginal inoculation with either PBS alone, *L. crispatus* and *M. mulieris*. ELISA analyses revealed a trend towards increased neutrophil elastase expression in the presence of *M. mulieris* after the first and second inoculations compared to PBS

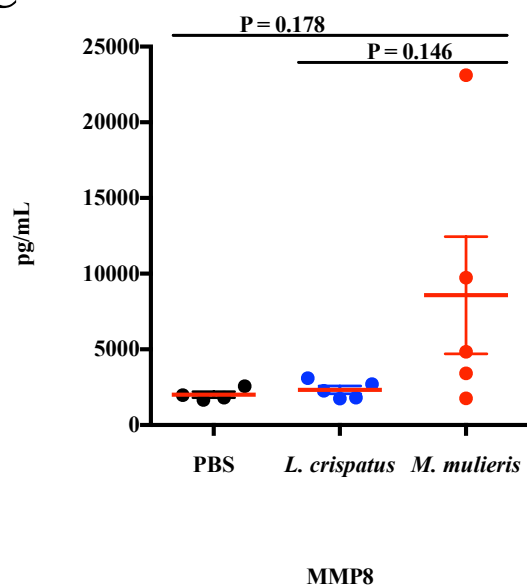
and *L. crispatus* inoculated mice in different time points (Figure 16B). In addition, elevated expression of MMP8 and MMP9 important neutrophil activators were observed at day 4 of post inoculation with *M. mulieris* (Figure 16C and 16D). The data supports the notion that vaginal neutrophil respond to BV-associated *M. mulieris*, but not *L. crispatus*, by initiating a heightened state of inflammation and increased pathway signatures that associate with cell-cell disruption and cytoskeletal reorganization.



B



C



D

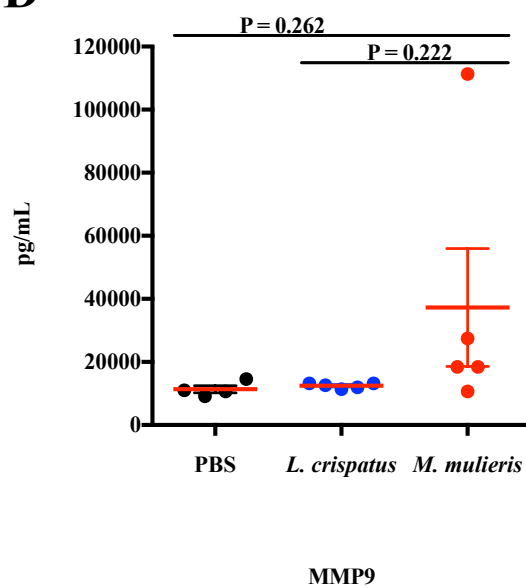


Figure 16. Significantly differentially abundant proteins and associated biological functions and pathways after intravaginal inoculation with different bacterial species associated with optimal or non-optimal vaginal microbiome. Distinctive proteomes of CVL from mice inoculated with PBS, *L. crispatus* or *M. mulieris* at different time points were identified using Q-Exactive mass spectrometry analysis. **(A)** A total 63 unique proteins were upregulated and recognized in the second inoculation. Clear phase-specific differences in protein abundance is exhibited. Underabundant proteins are presented in blue heat map and overabundant proteins are presented in red. Decreased (Section 1) and elevated (Section 2) neutrophil related protein expressions along with their main associated biological processes are shown. Data analyses for proteomes was performed by using Mann-Whitney U test ($p < 0.05$, ≥ 2 -fold change). **(B)** Neutrophil elastase expression levels at different time points of bacterial inoculation. Expression levels of **(C)** MMP8 and **(D)** MMP9 at day 4 of bacterial inoculation. Red bars represent mean $\pm$ SEM. Data were analyzed using unpaired two-tailed t-test.

CHAPTER 4 – DISCUSSION

Epithelial cells and mucus are major components of the mucosal barrier of the female reproductive tract (FRT) (Hickey et al., 2011) and provides a potent physical and immunological barrier to protect the host from the external environment (Carias et al., 2013). Whenever this epithelial barrier is compromised, women are more susceptible to pathogens, especially human immunodeficiency virus (HIV) (Hickey et al., 2011; Anderson et al., 2016). The integrity of the vaginal epithelium can be challenged under the impact of inflammation, hormones and infection. Immune cells tightly occupy the FRT and are important mediators of mucosal immunity but can cause pathology through the release of inflammatory mediators, which can lead to epithelial barrier breakdown and prevention of wound healing (Hickey et al., 2011). The tight relation between immune cell activation and epithelial barrier function has been reported in previous human cohort studies, where it was suggested that immune cells are important modulators of the vaginal mucosal barrier (Burgener et al., 2015; Arnold et al., 2016). Moreover, during the menstrual cycle, alterations in ovarian sex hormones, estradiol and progesterone, can influence epithelial barrier thickness and mucus viscosity, which can elevate the risk of HIV acquisition (Birse et al., 2015). Arnold *et al* identified 16 inflammatory mediators that were highly associated with HIV susceptibility, of which 3 cytokines were neutrophil-derived proteases (Arnold et al., 2016). Additionally, Birse *et al* reported that the luteal phase was strongly associated with immune activation, barrier remodeling and neutrophil activation through MS-based proteomic analyses, which has also been shown to be the phase of enhanced HIV susceptibility (Birse et al., 2015). These studies suggest that neutrophil recruitment is associated with immune activation and modulation of the vaginal epithelial integrity. However, the molecular underpinning of the role of neutrophils on FRT biology has not been studied in detail. In this research thesis, we utilized a

mouse model to assign a mechanistic function to FRT neutrophils and to examine their recruitment dynamics in response to sex hormones and different vaginal microbial species. We demonstrated, using tandem mass spectrometry analyses, that similar immune activation, innate immunity and neutrophil-related pathways are highly upregulated during the diestrus phase, which is equivalent to the progesterone-high luteal phase in humans. We confirmed that neutrophils played a dominant role in these processes, as *in vivo* neutrophil depletion led to reduction in biological signatures related to immune activation and wound healing. We showed that FRT neutrophils, while expressing some levels of extracellular matrix (ECM) modulating proteases such as collagenase, elastase and MPO at baseline, did not measurably disrupt epithelial barrier function *in vivo*. However, upon vaginal inoculation with microbial species associated with bacterial vaginosis (*Mobiluncus mulieris*), proteomic analysis revealed significantly elevated activation of inflammatory, leukocyte migration and cell-cell junction disruption pathways compared to vaginal inoculation with *Lactobacillus crispatus*, widely considered a part of an ‘optimal’ microbiome. Elevated levels of elastase were also detected after vaginal inoculation with *Mobiluncus mulieris*, suggesting that neutrophils can rapidly respond to changes in vaginal microbial communities and directly remodel the FRT landscape (Figure 17). Thus, our data demonstrates that FRT neutrophils are important players in mucosal immunity and are regulated by sex hormones, which if mis-directed, can orchestrate a cascade of events that lead to immune activation and leukocyte infiltration, possibly contributing to elevated HIV risk.

4.1 Regulation of neutrophil recruitment and activation by sex hormones

During the menstrual cycle, the vaginal epithelium faces substantial remodelling in response to the fluctuation of ovarian sex hormones estradiol and progesterone (Bouman et al.,

2005). Previous studies have shown neutrophil influx into the human endometrium during the luteal phase where progesterone levels are dominant (Sonoda et al., 1998; Birse et al., 2015; Arnold et al., 2016). We first confirmed previous work in mice by measuring neutrophil influx dynamics using flow cytometry analysis (Figures 1 and 2). Our results are in accordance with other studies (Sasaki et al., 2009; Schaefer et al., 2014) that showed that the majority of cells present in mouse CVL in the diestrus phase (progesterone-dominant phase) are Ly6G⁺ neutrophils. We confirmed neutrophil infiltration into vaginal tissue and observed that the majority of recruited neutrophils in the diestrus phase were localized within the stratum corneum, (Sasaki et al., 2009; Schaefer et al., 2014). Neutrophil recruitment into the vaginal tissue in humans has been reported to be associated with a surge of IL-8 secretion by the uterine endometrium (Arici et al., 1998; Critchley et al., 1999). Similar to humans, neutrophil influx into the vaginal tissue of mice is known to be regulated by elevated expression of the chemokine (C-X-C motif) ligand 2 (CXCL2), a rodent IL-8 homologue, that is expressed by vaginal epithelial cells (Sonoda et al., 1998; Lindqvist et al., 2009; Hickey et al., 2011; Schaefer et al., 2014; Lasarte et al., 2015). We confirmed expression of CXCR2, the chemokine receptor for CXCL2, on CVL neutrophils (Khandaker et al., 1998; Pham., 2006; Alves-Filho et al., 2010; Eash et al., 2010; Sadik et al., 2011; Zenobia et al., 2013; Lasarte et al., 2015) and that they were responsive to CXCL2 stimulation by increasing their migration in 3D collagen matrix. These data show that the mouse model can be used to perform detailed analyses into the role of neutrophils on FRT biology *in vivo*.

4.2 Comprehensive proteomic analysis of changes in the mucosal immunological environment

Previous proteomic analysis of cervicovaginal lavage fluid from healthy women reported a number of biological changes that define the luteal phase, with some overlapping with pathways

that are associated with HIV susceptibility (Birse et al., 2015; Arnold et al., 2016). Using a multivariate model based on LASSO-selected biomarkers, they reported 18 proteins that were able to accurately discriminate between the two stages, detailing profound proteome changes that shapes the FRT landscape during surges of different sex hormones. In our study, we further extended these findings to determine whether similar bio-functional pathways were associated with the estrus (estrogen-dominant) or diestrus (progesterone-dominant) phases in mice and is the first comprehensive analysis of the mouse mucosal proteome. Consistent with the human data, biological processes related to epidermis development were up-regulated in the estrus phase, as expected. Overabundance of proteins such as cornifin B and keratin type II cytoskeletal 2 oral, which are involved in generating a dense keratinocyte barrier within the epithelium were upregulated during the estrus phase, is consistent with what has been reported in the human studies. (Contzler et al., 2005; Brocklehurst and Philpott., 2013; Hartlieb et al., 2013; Birse et al., 2015). In contrast, CVL proteomic analysis from mice in the diestrus phase revealed overabundance in biofunctions related to actin cytoskeleton re-organization (actin nucleation by ARP-WASP complex), inflammation (IL-1 signaling, toll-like receptor signaling), and chemotaxis (fMLP signaling in neutrophils, Rho signaling family of GTPases, integrin signaling). Overabundance of myeloperoxidase (MPO) and plastin-2, both associated with neutrophil activation, is consistent with the large influx of neutrophils into the vaginal mucosa during this phase. Together, we demonstrated profound estrous phase-related changes in the overall abundance of proteins and highlight key overlap with similar proteomic studies in human and pig-tailed macaques (Birse et al., 2015; Vishwanathan et al., 2015). Additionally, neutrophil-related pathways are abundant during the diestrus phase, allowing us to manipulate the mouse model to better study their contribution to FRT biology and their possible role in HIV susceptibility (Levinson et al., 2009;

Ogawa et al., 2013; Hirbod et al., 2014; Mackelprang et al., 2014; Birse et al., 2015; Burgener et al., 2015).

4.3 Neutrophils are recruited from the blood and not the upper FRT

Generally, neutrophils are recruited into inflamed tissue by extravasating from blood and do not station within healthy peripheral tissues (Witko-Sarsat et al., 2000; Miyabe et al., 2017). Previous studies have highlighted that neutrophils are found within the FRT in response to inflammation and infection (Morre-Connors et al., 2013; Carey et al., 2014). Interestingly, neutrophil recruitment into the uterus before the onset of menses has been observed in humans (Salamonsen and Woolley., 1999) and largely assumed that neutrophils enter the endometrium from the circulation in response to IL-8 to directly regulate vascular breakdown to facilitate shedding of the endometrium (Arici et al., 1996; Amsalem et al., 2014). In mice, the establishment of CXCL1 gradient was required for neutrophil positioning within the vaginal epithelium, and that disruption of CXCL1 gradients compromises neutrophil responses to challenge (Lasarte et al., 2015). We tested the hypothesis that vaginal neutrophils are derived from the uterus, reach the lower FRT during menses and actively migrate into the vaginal mucosa. The fact that intravaginal transfer of neutrophils into diestrus mice, when high CXCL2 expression is secreted by vaginal epithelial cells, did not result in an influx of luminal neutrophils into the vaginal mucosa, confirmed that these cells are derived from the peripheral blood.

4.4 Expression of neutrophil-related protein mediators and their role in shaping the FRT

The immune response within the FRT is largely regulated by the spatiotemporal expression of cytokines and chemokines (Schaefer et al., 2005; Hickey et al., 2011; Machado et al., 2014).

Measures of key inflammatory cytokines and chemokines that are upregulated in response to infection and play an important role in immune cell activation and recruitment into the mucosa have been described as biomarkers of FRT inflammation (Schwartz et al., 2006, McKinnon et al., 2018). Recent studies have outlined the relationship between inflammatory mediator expression and fluctuations in sex hormones during the follicular and luteal phases, and their potential role in increasing HIV susceptibility (Mannan et al., 2011; Aboud et al., 2014; Buve et al., 2014; Vishwanathan et al., 2015). Recent studies by McKinnon *et al* have identified a panel of inflammatory mediators that are highly associated with HIV infection and that these women are not protected from 1% tenofovir gel as PrEP (Pre-exposure prophylaxis) (McKinnon et al., 2018). While these and other studies collectively suggest that vaginal inflammation is a strong predictor of HIV acquisition, the molecular underpinning of this increase in HIV risk is not clear. Given that neutrophil-related pathways were associated with HIV infection (Arnold et al., 2016), and that a number of neutrophil-derived proteins were readily detected in the CVL during diestrus, we examined the potential for neutrophils to contribute to FRT remodeling, either through their recruitment alone, or through their expression of inflammatory mediators. Proteomic and multiplex ELISA analyses demonstrated that MPO, MMP8 and elastase were all upregulated during the diestrus phase and confirmed that they were predominantly expressed by neutrophils while G-CSF and CCL2 cytokines remained unchanged between the estrus and diestrus phase. We confirmed that these inflammatory mediators were derived predominantly by FRT neutrophils, as their *in vivo* depletion led to a marked reduction in their expression in the CVL. Here, we discuss these mediators in detail and their implications on regulating FRT biology.

4.4.1 Myeloperoxidase (MPO)

Myeloperoxidase (MPO) is an abundant PMN-derived heme protein, and facilitates neutrophil recruitment during inflammation (Reumaux et al., 2003; McMillen et al., 2005; Machado et al., 2014). However, the role of MPO in the reproductive mechanisms of women is less studied. It is now well-established that the menstrual cycle is associated with changes in the inflammatory state and is modulated via sex hormones and recruitment of leukocytes (Wira et al., 2015; Moncla et al., 2016). Bekesi *et al* reported that MPO activity was lower in postmenopausal compared to perimenopausal woman, suggesting a relation between MPO and the menstrual cycle. A normal menstrual cycle is in relation with ovulation, thus, MPO may also be associated with ovulation (Bekesi et al., 2001). In this study, we indicated that in the abundant neutrophil influx in the diestrus phase (progesterone dominant), MPO expression was significantly elevated. In contrast, in the estrus phase (estrogen dominant) MPO expression dramatically decreased due to the lack of neutrophil presences. Our findings are consistent with Vishwanathan *et al* who reported high MPO expression during the luteal phase of the menstrual cycle in pig-tailed macaques (Vishwanathan et al., 2015). Several studies have reported decreased levels of neutrophil infiltration in inflamed tissue in MPO deficient mice. MPO^{-/-} mice showed significantly decreased levels of neutrophil influx and inflammation in response to LPS exposure which was reported to be mainly due to the decreased migration capacity of MPO^{-/-} neutrophils (Aratani et al., 2006; Haegens et al., 2009; Klinke et al. 2011; Shen et al., 2017). In contrast, Kremserova *et al* showed that MPO deficient mice had significant increase of neutrophil influx in lung airways compared to WT mice during lung inflammation, which was followed by higher levels of CCL5 (RANTES) in BAL fluid from MPO deficient mice (Kremserova et al., 2016). Nevertheless, in this study we revealed that anti-Ly6G injected mice showed significantly lower levels of MPO expression in

vaginal tissue in comparison to isotype injected mice. Therefore, neutrophils are the dominant producers of MPO within the vaginal mucosa of mice. Thus, based on the fact that in many chronic inflammatory diseases such as rheumatoid arthritis and cardiovascular diseases, high MPO expression, through the generation of oxidants, can cause host tissue damage (Klebano et al., 2013; Nauseef., 2014), and that MPO plays an important role in inducing MMP8 (collagenase, a collagen cleaving enzyme) activation during disease progression (Franco et al., 2017). These data suggested that MPO expression could also possibly have an impact on vaginal epithelial integrity during the large neutrophil influx during diestrus.

4.4.2 Neutrophil elastase (NE)

Neutrophil elastase secreted by activated neutrophils is a potent mediator known to be involved in inflammation and tissue injury (Zeihner et al., 2002; Meyer-Hoffert and Wiedow, 2011). It is involved in the pathogenesis of various acute and chronic tissue-destructive diseases (Owen et al., 1997; Sandhaus and Turino., 2013), which is characterized by disrupted epithelium integrity and sloughing of epithelial cells (Sansing et al., 2011; Semple et al., 2015). Therefore, in this study we evaluated neutrophil elastase expression levels in different phases of the mouse estrous cycle and observed high expression levels of neutrophil elastase in the diestrus compared to the estrus phase. Our results are in line with other studies reporting elevated inflammatory cytokines in the luteal phase to be associated with increased expression levels of neutrophil-associated proteases (Birse et al., 2015; Arnold et al., 2016). We confirmed that the majority of elastase expression found in the CVL at steady-state are derived from FRT neutrophils, since their depletion abolished elastase levels compared to isotype controls. However, we did not observe any measurable changes in BSA penetration into the vaginal epithelium with or without neutrophils, suggesting that at

steady-state, the expression of elastase by neutrophils situated within the stratum corneum does not lead to increased disruption of the epithelium. These findings are consistent with previous studies indicating that neutrophil elastase is not essential for basement membrane transmigration of neutrophils to inflamed sites (Hirche et al., 2004; Benabid et al., 2012). However, it is worth noting that there is still debate regarding the key role of neutrophil elastase in neutrophil recruitment activities (Pham., 2006). Several studies suggest that other proteases expressed by neutrophils in the vicinity might be involved in neutrophil recruitment to inflamed sites (Walker et al., 1995; Wang and Doerschuk., 2002; Hirche et al., 2008). Overall, our data suggest that, at steady-state, the expression of elastase does not affect the vaginal epithelial barrier integrity, and that vaginal epithelial shedding proceeds normally even in the absence of neutrophils. We postulate that neutrophil elastase plays an important role in modulating FRT inflammatory responses that can lead to damage of endothelial cells (Young et al., 2004) and cleave cell associated adhesion molecules (Korkmaz et al., 2008).

4.4.3 Matrix metalloproteinases (MMPs)

We also evaluated expression levels of matrix metalloproteinases (MMPs) including MMP2, MMP3 and MMP8 in mouse CVL, which are known proteases involved in proteolytic degradation of elastic fibers and collagens of vaginal tissue (Stamenkovic., 2003; Nerusu et al., 2007; Lin et al., 2008; Medeiros et al., 2017). Our results are consistent with previous studies that showed a correlation between higher MMP levels in the presence of neutrophils in response to fluctuating sex hormones levels (Diaz-Cueto et al., 2006; Rahkonen et al., 2009; Ong et al., 2015). We observed high expression levels of MMP2, MMP3 and MMP8 in the CVL of mouse in diestrus compared to estrus. However, after neutrophil depletion, we did not observe significant changes

in MMP2 and MMP3 levels. Geraghty *et al* showed that neutrophil elastase can potentially activate and induce MMP2 expression in KO mice tissue, suggesting that neutrophil elastase expression in MMP2^{-/-} is the result of receptor-mediated degranulation responses in neutrophils (Geraghty et al., 2007). Moreover, Savarin *et al* showed that neutrophil depletion in mice had no effect on MMP3 expression, suggesting MMP3 expression is distinct from neutrophil-secreted proteases such as TNF and IL-1 β and is more related to the active role of resident cells participating and potentially collaborating with infiltrating cells in regulating MMP permeability (Savarin et al., 2011). Similar to previous studies, MMP8 expression in anti-Ly6G depleted mice showed lower levels of expression in CVL compared to isotype, suggesting that MMP8 secretion is a neutrophil-related function and subsequently can modulate inflammation in the vaginal tissue (Eliason et al., 2005; Pino-Tamayo et al., 2016).

4.4.4 Fluctuations of other inflammatory mediators

Vaginal cell-mediated immunity plays an important role in anti-HIV responses as well as HIV transmission (Hickey et al., 2013). It is well-known that the innate immune response is regulated by sex hormones through the expression of mucosal pro-inflammatory cytokines that can directly modulate vaginal epithelial integrity and leukocyte recruitment into the FRT (Wood et al., 2007; Wira et al., 2010). We performed multiplex cytokine analyses on a panel of cytokines/chemokines to assess how they were differentially regulated by the estrous cycle. We observed that G-CSF, a known neutrophil regulator of chemotaxis, was unchanged throughout the estrous cycle and not expressed by neutrophils, as their levels were not altered after neutrophil depletion (Kim et al., 2006; Eash et al., 2010; Campbell et al., 2016). Similarly, we measured CCL2 (MCP-1) levels, as it has been previously reported to be expressed in response to elevated

levels of progesterone in the diestrus phase, and activate innate immune responses (Kayisli et al., 2002). Blocking CCL2 using inhibitors decreased neutrophil numbers and inflammation in the colon and lung (Bhatia et al., 2008; Balamayooran et al., 2011). We observed no changes in CCL2 expression throughout the estrous cycle and after neutrophil depletion, suggesting that they did not play a role in mucosal neutrophil recruitment in our hands.

Several studies have emphasized the importance of interleukin-12 (IL-12) in neutrophil recruitment and activation in the presence of sex hormones (Bebo et al., 1999; Sun et al., 2007). In agreement with these studies, we observed that IL-12 was significantly up-regulated during diestrus (Lopez and Stanley., 2005). Finally, CCL5 (RANTES) is a known potent neutrophil attractant and is involved in neutrophil recruitment activities (Pan et al., 2000; Ajuebor et al., 2001). Neutrophil extracellular traps (NETs) have been shown to be regulated by CCL5 expression. Interestingly, Al Harthi *et al* showed elevated expression levels of CCL5 (RANTES) in CVL from examined in HIV-positive women in the luteal phase compared to the follicular phase. They stated that this increase correlated with increased levels of progesterone during the luteal phase of the menstrual cycle, where abundant neutrophils are present in the vaginal tissue (Al Harthi et al., 2001). In line with their study, in our mouse model, we also observed significantly higher levels of CCL5 expression in CVL in the diestrus phase compared to the estrus phase. CCL5 levels were unchanged after neutrophil depletion, indicating that their expression was not by neutrophils, but possibly be due to the secretion of by vaginal epithelial cell (Appay and Rowland-Jones., 2001). Together, we have reported inflammatory mediators that were upregulated or downregulated in response to changes in the estrous cycle, and highlight those that are directly regulated by neutrophils, paving the way to use these as biomarkers of neutrophil activation in response to the vaginal microbiota.

4.5 Does the presence of neutrophils modulate the vaginal epithelium at steady state?

The mucosal barrier of the lower female reproductive tract provides a defensive physical and immunological structure against foreign pathogens. The FRT structure consists of multi-layered squamous epithelial cells with tight junctions comprised of an intermembrane fibril matrix of transmembrane proteins (Langbein et al., 2002; Marchiando et al., 2010). However, the integrity of vaginal tissue cell junctions can be compromised by various growth factors, inflammatory mediators and pathogen invasion (Nusrat et al., 2000; Gorodeski., 2007; Shen et al., 2008). However, only a few reports have studied the importance of intercellular junctions in cervicovaginal tissue function (Bouschbacher et al., 2008; Nazli et al., 2010). Therefore, in this study, we visualized the integrity of the vaginal epithelial barrier by intravaginally inoculating mice with bovine serum albumin (BSA), a fluid phase marker. Our results revealed that in the estrus phase (follicular phase in humans) where the estrogen levels are at its highest and the epithelial layer is the thickest (Poonia et al., 2006; Kersh et al., 2014), BSA was able to only penetrate into the apical cellular layers of the vaginal epithelium and the stratified squamous epithelium layer provided robust protection to BSA penetration. Moreover, in the absence of neutrophils, percentage of BSA penetration into the vaginal epithelium was similar to steady-state, suggesting no correlation between neutrophils and vaginal epithelial barrier disruption. These results are in line with our proteomics data which revealed biological factors that are regulators of epidermis development in the estrus phase (Birse et al., 2015). Our results are also in accordance with Blaskewicz *et al* who assessed the ectocervical epithelium permeability by tracking the penetration of fluorescent immunoglobulin G (IgG) and reported that the endocervical epithelium provided a robust barrier to penetration of Cy3-tagged IgG (Blaskewicz et al., 2011). Moreover, Carias *et al* reported that fluorescent BSA could only penetrate into the superficial layers of the

squamous epithelium of macaques (Carias et al., 2013). Interestingly, in our study, the diestus phase, where the vaginal epithelium is at its thinnest (Poonia et al., 2006; Blaskewicz et al., 2011) showed no significant difference in percent BSA penetration compared to the estrus phase. As a control, pre-treatment with nonoxynol-9, a well-known microbicide that induces vaginal inflammation (Cone et al., 2006) that has been clinically linked to increased HIV risk (Quinn et al., 2000; Catalone et al., 2005) caused higher BSA penetration into the vaginal epithelium. Fichorovo *et al* reported that three doses of N-9 application can lead to neutrophil influx into the vaginal tissue, which subsequently led to increase levels of inflammatory mediators IL-1 α/β , MIP-1 β , IL-8 and neutrophil elastase (Fichorovo et al., 2001). Using a functional measure of epithelial barrier function *in vivo*, we show that while neutrophils express inflammatory mediators such as MPO, MMP8 and elastase at steady-state, they did not measurable causes epithelial barrier disruption, which is consistent with the fact that neutrophils are only responsive to pathogenic insults. These data provided us with baseline measures of vaginal epithelial barrier function, and whether vaginal microbial species can negatively impact this barrier through neutrophil activation.

4.6 Elevated expression neutrophil-derived inflammatory mediators in response to bacterial species associated with bacterial vaginosis (BV)

Bacterial vaginosis (BV) is a common vaginal condition in women of reproductive age associated with serious urogenital and reproductive health outcomes, including HIV acquisition (Atashili et al., 2008; Brotman., 2011; Fichorova et al., 2015; Haddad et al., 2018; McKinnon 2019). BV is characterized by decreased presence of *Lactobacillus* species and an increased presence of anaerobic gram-negative rods, such as *Garnderella vaginalis* and *Mobiluncus* species (Ravel et al., 2011; Onderdonk et al., 2016). The fact that BV is associated with risked HIV

acquisition risk makes a strong case for the cervicovaginal microbiota in modulating the function and immunological responses of the FRT. BV leads to elevated expression levels of inflammatory mediators in the vaginal mucosae (Hedges et al., 2006; Boomsma et al., 2010; Campos et al., 2012; Anahta et al., 2015), which can increase HIV infection directly by recruiting target immune cells, or indirectly by affecting epithelial barrier function (Sturm-Ramirez et al., 2000; Steinhandler et al., 2002; Thurman et al., 2011; Mirmonsef et al., 2012). Only few studies have addressed the role of neutrophil during bacterial vaginosis (Giraldo et al., 2012; Gilbert et al., 2013; Beghini et al., 2015). Yudin *et al* reported that BV and the presence of vaginal neutrophils were highly predictive of histological endometritis, suggesting that vaginal neutrophils are highly inflammatory (Yudin et al., 2003). Heightened immune activation and decreased barrier function in women with *Gardnerella vaginalis*-dominated bacterial profiles has been shown (Zevin et al., 2016), and increased neutrophil accumulation and activation proteomic signatures were associated with women with BV (Adam Burgener, personal communication). Here, we evaluated whether neutrophils can directly respond to changes in the vaginal microbiome after inoculation with either PBS, *Lactobacillus crispatus* (dominant in an “optimal” microbiome) or *Mobiluncus mulieris* (part of a “non-optimal” microbiome). CVL proteomic analysis revealed increased signatures related to neutrophil migration (coro1a, ITGB2), leukocyte extravasation signaling and integrin signaling in response to *M. mulieris* compared to *L. crispatus* and PBS controls, suggesting that there was an influx of neutrophils in response to *Mobiluncus*. Elevated expression of MPO after *M. mulieris* was clearly observed, indicating the release of neutrophil-related inflammatory mediators. In addition, similar results were obtained in our neutrophil elastase analysis, where a higher trend in neutrophil elastase expression in the CVL of mice inoculated with *M. mulieris* was evident compared to those inoculated with *L. crispatus*. Moreover, increased expression of MMP8 and

MMP9 in the presence of *M. mulieris* was observed at day 4 of bacterial inoculation. Our results are in accordance with Arnold *et al* who identified elevated expression of neutrophil related proteases MMP8 and MMP9 in HIV-uninfected female sex workers who presented increased levels of proinflammatory FRT cytokines. Thus, our findings highlight, for the first time, that neutrophils are directly responsive to the presence of *M. mulieris* and not *L. crispatus* in the vaginal canal, and that through expression of neutrophil related mediators such as MPO and elastase *in vivo*, can initiate an acute inflammatory response (Sahoo et al., 2014). Whether *Mobiluncus*-associated neutrophil activation leads to decreased vaginal epithelial barrier integrity and impaired wound-healing responses needs to be tested *in vivo*. However, previous studies have established an association between highly diverse anaerobic bacterial communities with epithelial barrier disruption and elevated immune activation, and that lower diversity *Lactobacillus* species communities to be associated with expression of important mucosal proteins involved in maintaining epithelial integrity (Zevin et al., 2016), and that these have direct implications on HIV susceptibility (Masson et al., 2015; Arnold et al., 2016; Borgdorff et al., 2016). Together, our studies provide a foundation where the mechanistic interplay between vaginal microbial species, mucosal neutrophils and the FRT can be studied *in vivo*. In future studies, we will test whether HIV susceptibility is directly affected by the presence of vaginal microbial communities.

CHAPTER 5 – SIGNIFICANCE AND FUTURE DIRECTION

Our work is the first to report a comprehensive proteomic analysis of biological changes that occur during the estrus and diestrus phase, and that many of these signatures correlate with that in human studies. In addition, we established an *in vivo* mouse model to study the functional role of neutrophils in the FRT, either at steady-state or in response to vaginal microbes. While all previous studies have only addressed their presence, but not their function as modulators of the FRT. Our research is also the first to demonstrate that different vaginal microbes can change the inflammatory status of the mouse vagina.

While we have shown that neutrophil influx did not alter the vaginal epithelial barrier integrity at steady-state, we have not tested whether neutrophils can disrupt barrier function in response to different bacterial challenges. An interesting experiment will be to test for barrier integrity using BSA after intravaginal challenge with *Lactobacillus* vs *Mobiluncus*. We can evaluate the contribution of neutrophils by performing *in vivo* depletion with the presence of bacteria in order to assess whether epithelial barrier disruption is caused by either neutrophils or non-optimal bacteria.

Studies showing upregulation of neutrophil-related pathways and increased HIV risk are correlative, and there is not data showing a direct cause. Using a humanized BLT mouse model of HIV infection, future studies will entail bacterial inoculations with intravaginal HIV challenge experiments. Neutrophil depletion studies will reveal whether the recruitment/activation of neutrophils in response to BV-associated anaerobic bacterial species are a pre-requisite for increased HIV susceptibility, or whether they are dispensable.

In conclusion, our *in vivo* mouse model can recapitulate similar key biological processes observed in human and is a step forward in better understanding how immune cells, specially

neutrophils can modulate FRT barrier function in relation to sexual HIV susceptibility and acquisition.

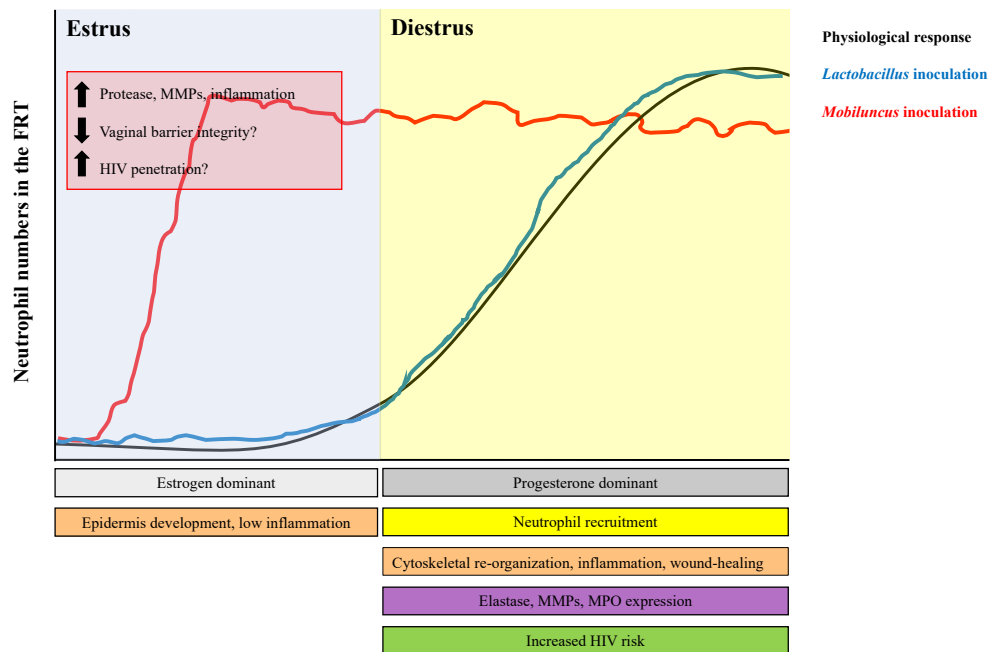


Figure 17. Graphic illustration of physiological and non-physiological neutrophil recruitment and associated effects on the FRT. Physiological neutrophil influx into the FRT is regulated by the estrous cycle. Higher HIV risk is associated with the progesterone-dominant luteal phase (diestrus in mice). Proteomic analysis reveal that the estrus phase is associated with epidermis development, cell-cell junction and low inflammatory pathways. The diestrus phase is associated with high neutrophil recruitment, expression of inflammatory mediators and initiation of wound-healing pathways. Vaginal inoculation with *Mobiluncus mulieris* induces activation of inflammatory, neutrophil recruitment and vaginal epithelial disruption pathways, whereas *Lactobacillus crispatus* does not. Future studies will assess whether *Mobiluncus* can directly disrupt the vaginal epithelium or whether this is through the recruitment of activated neutrophils.

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