The Use of Oxytocin in Experimental Models of the Human Female Genital Tract with Implications for HIV Susceptibility

by

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Abstract

Introduction: Oxytocin has been implicated in reductions in inflammation at skin and gut epithelia, as well as improved wound healing outcomes in small animal models and clinical trials of skin wounds. Susceptibility to HIV infection at the female genital tract (FGT) has been associated with increased levels of inflammation and breakdown of the epithelial barrier. I sought to investigate whether oxytocin could 1) reduce inflammation and improve wound healing of FGT epithelial cells and 2) reduce susceptibility to vaginal HIV infection in a humanized mouse model.

Methods: VK2/E6E7 (Vk2), Ect1/E6E7 (Ect1), and End1/E6E7 (End1) cell lines were used to model vaginal, ectocervical, and endocervical cells, respectively. Inflammation was assessed by the levels of pro-inflammatory cytokine/chemokine transcripts and secreted protein. The effect of oxytocin on wound healing was assessed using scratch assay models. Immunologically humanized mice treated longitudinally with oxytocin vaginal gel were challenged with HIV-1 and followed for ≥ 63 days. Cervicovaginal lavage, blood samples, and lymphatic tissues were collected for analysis. Additional analysis of oxytocin receptor (OXTR) expression of the FGT cell lines was performed using qPCR, Western blot, confocal microscopy, mass spectrometry, and calcium flux.

Results: Media formulations for FGT epithelia were found to contain oxytocin. After correction of this factor using alternative culture media, it was found that oxytocin modulated pro-inflammatory cytokine transcript and protein levels in Ect1 and End1 cells and inhibited wound healing in Vk2 and End1 cells. OXTR validation in Vk2, Ect1, and End1 cells revealed non-significant mRNA protein levels, with oxytocin also failing to elicit calcium flux. Intravaginal administration of oxytocin gel had no effect on leukocyte activation or regulation of pro-inflammatory cytokine/chemokine expression in our humanized mice. Control mice did not succumb to HIV-1 infection, preventing conclusions on susceptibility.

Conclusions: Oxytocin was found to alter pro-inflammatory cytokine expression and reduce wound healing in FGT epithelial cells *in vitro*, despite the absence of detectable OXTR expression. Additionally, FGT media formulations were found to confound previous oxytocin studies due to inherent oxytocin levels. Vaginal oxytocin gel did not affect leukocyte activation or cytokine levels in humanized mice, with further work on HIV-1 susceptibility needed.

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Dedication

I dedicate this thesis to the two people who have supported me most emotionally, and with advice, throughout this program. To my mother, Christine Neufeld, you are always there for me and have taught me to never quit even when I desperately want to. Because of you, I have persevered through this thesis and I'm very glad that I have. I don't believe I would have developed the resilience or work ethic that I have if not for your loving direction throughout this program. To my partner, Kendra Kuo, I love you more and more every day and you continue to both support me and push me to greater heights as we go on our journey together through life. Every day with you is a blessing and I cherish each and every one.

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List of Abbreviations

AIDS – Acquired immunodeficiency syndrome

APC – Antigen presenting cell

ATCC – American type culture collection

BCA – Bicinchoninic acid

BPE – Bovine pituitary extract

BSA – Bovine serum albumin

Ca^{2+} – Calcium

$CaCl_2$ – Calcium chloride

Caco2BB – Immortalized gut crypt villi epithelium

cART – Combination antiretroviral therapy

Cat# – Catalogue number

cDNA – Complementary deoxyribonucleic acid

CNS – Central nervous system

CREB – cAMP response-element binding protein

CSF – Cerebrospinal fluid

C_t – Cycle threshold

CVL – Cervicovaginal lavage

D-KSFM – Defined keratinocyte serum free medium

ddH_2O – Double distilled water

DMEM – Dulbecco's modified eagle medium

DNA – Deoxyribonucleic acid

Ect1/E6E7 – Ectocervical keratinocytes immortalized with the HPV E6/E7 gene

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

EMEM – Eagle's minimum essential medium

End1/E6E7 – Endocervical keratinocytes immortalized with the HPV E6/E7 gene

FBS – Fetal bovine serum

FGT – Female genital tract

FSFI – Female sexual function index

G-418 – Geneticin

GFP – Green fluorescent protein

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GPCR – G-protein-coupled receptor

GvHD – Graft-versus-host disease

hBLT – Human bone, liver, and thymus

HEC – Hydroxyethyl-cellulose

HEK 293 – Human embryonic kidney epithelial cell line

HEK 293T – Human embryonic kidney epithelial cell line transformed with the SV40 T antigen

HEPA – High efficiency particulate air

HESN – HIV-exposed seronegative

HIV – Human immunodeficiency virus

HPV – Human papilloma virus

HT-29 – Adenocarcinoma colorectal cell line

IκB – Inhibitor of NF-κB

IL – Interleukin

INI – Integrase inhibitor

IP_3 – Inositol tri-phosphate

IP_3R – Inositol tri-phosphate receptor

KSFM – Keratinocyte serum free medium

LC-MS/MS – Liquid chromatography tandem mass spectrometry

LPS – Lipopolysaccharide

MCP-1 – Monocyte chemoattractant protein 1

MES – 2-(*N*-morpholino)ethanesulfonic acid

MFI – Median fluorescence intensity

NF-κB – Nuclear factor kappa B

NML – National Microbiology Laboratories

NRTI – Nucleoside reverse transcriptase inhibitor

OXTR – Oxytocin receptor

P/S – Penicillin streptomycin

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate buffered saline

PHM1-41 – Uterine myometrial cell line

PI – Protease inhibitor

PKA – Protein kinase A

PLC – Phospholipase C

PVN – Paraventricular nuclei

qPCR – Relative quantitative reverse transcriptase polymerase chain reaction

RANTES – Regulated on activation, normal T cell expressed and secreted

rEGF – Recombinant human epidermal growth factor

RIPA – Radio-immunoprecipitation assay

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT – Reverse transcription

SON – Supraoptic nuclei

SV40 – Simian vacuolating virus 40

$TCID_{50}$ – Tissue culture infectious dose 50%

TKO – Triple knockout

TLR – Toll-like receptor

TRPC – Transient receptor potential-like channel

UCLA – University of California, Los Angeles

VK2/E6E7 – Vaginal keratinocytes immortalized with the HPV E6/E7 gene

Chapter 1: Introduction

1.1 Overview of Oxytocin

1.1.1 Oxytocin production and structure

Oxytocin is a peptide hormone first synthesized as a pro-peptide in the form of pre-pro-oxyphysin and then cleaved into neurophysin I and oxytocin through a series of enzymatic cleavages (Rao et al., 1992; Sheldrick & Flint, 1989). These products accumulate in vesicles of neurons in the paraventricular (PVN), supraoptic (SON), and accessory magnocellular nuclei of the hypothalamus in the brain (**Figure 1.1**). These neurons are able to communicate with both the central nervous system (CNS) via direct axonal connections to the sensory and prefrontal cortices, amygdala, hippocampus, and other structures, as well as into the peripheral circulation through the posterior pituitary (Grinevich & Stoop, 2018; Harvey, 2020). Other organs, such as the testis, ovaries, adrenal glands, pancreas, and thymus have also been shown to secrete oxytocin (Jenkins & Nussey, 1991; Lee et al., 1998; Stadler et al., 2020)—indicating its potential roles throughout the body. Oxytocin shares significant sequence homology to both vasopressin, its closely related water-retention hormone, and its ancestral precursors, for example isotocin and mesotocin, found in birds, reptiles, and bony fishes (Odekunle & Elphick, 2020; Van Kesteren et al., 1992). Indeed, vasopressin and oxytocin are thought to originate from a common gene ancestor and be the product of divergence after a gene duplication event from invertebrates into vertebrates (Donaldson & Young, 2008; Theofanopoulou, 2021). Oxytocin is preserved among all mammals as a nine amino acid cyclic peptide, however, its high degree of sequence similarity to vasopressin—which shares seven of the same nine amino acids (78% sequence identity)—creates the potential for off-target effects whereby both oxytocin and vasopressin can bind each other's receptors with reduced affinity. In humans, oxytocin is able to bind vasopressin receptor V_{1a} with an affinity 1:100 that of vasopressin, while vasopressin is able to bind the oxytocin receptor at an affinity 1:10 that of oxytocin (Kimura et al., 1994; Manning et al., 2012; Taylor et al., 2020).

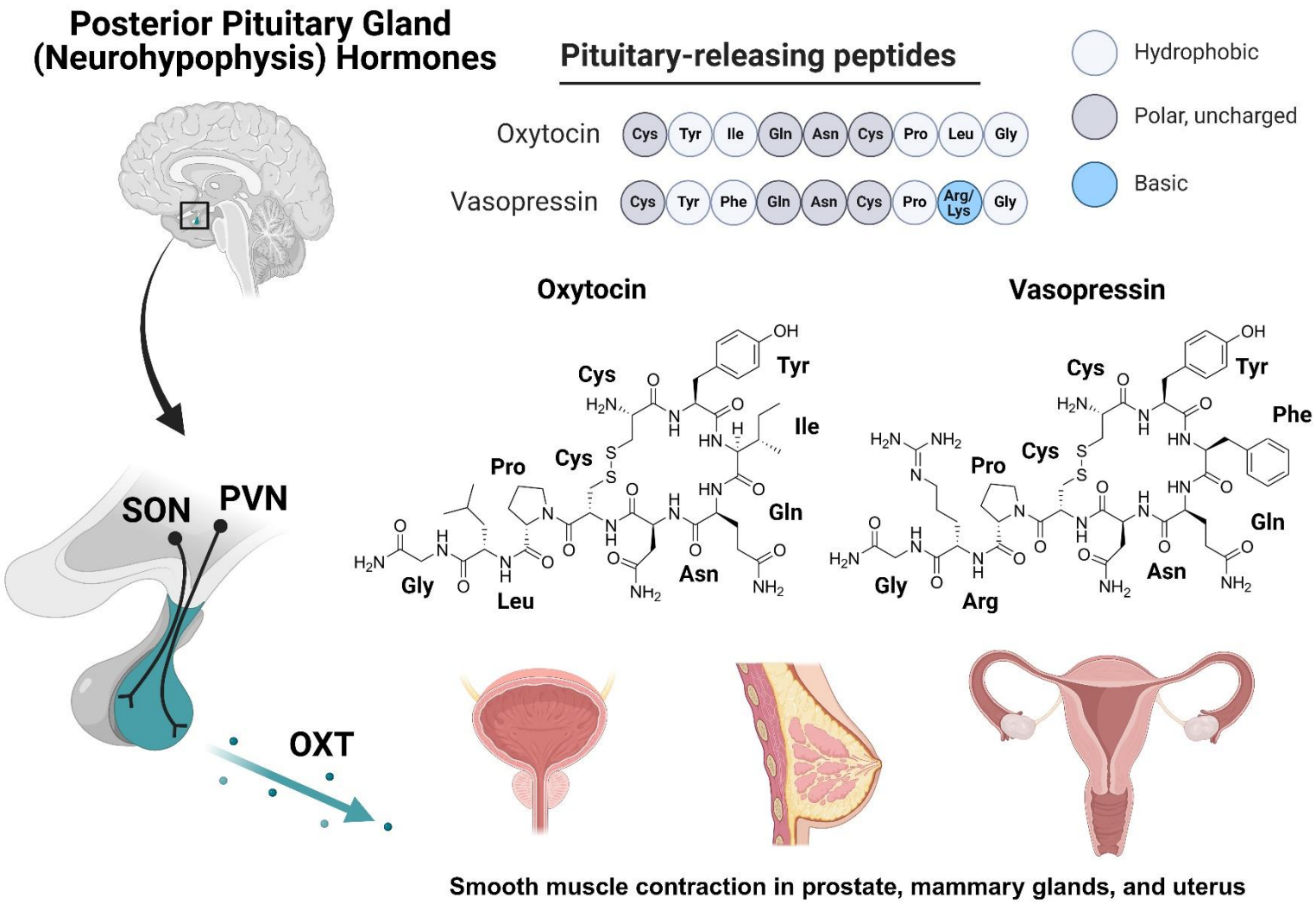


Figure 1.1 Overview of oxytocin production and function. Oxytocin is produced in the SON and PVN of the hypothalamus, and then travels through axons to the posterior pituitary. From there, it enters the peripheral circulation where it primarily effects smooth muscle contraction at sites such as the prostate, breast, and uterus. It has sequence homology with vasopressin, a closely-related posterior pituitary hormone, and the two are able to interact with each other's receptors. Made using Biorender.

Arg – arginine, Asn – asparagine, Cys – cysteine, Gln – glutamine, Gly – glycine, Ile – isoleucine, Leu – leucine, Phe – phenylalanine, Pro – proline, Tyr – tyrosine, oxytocin – oxytocin, PVN – paraventricular nucleus, SON – supraoptic nucleus.

1.1.2 Oxytocin in human physiology

Oxytocin is most well characterized as a neurotransmitter that is released in the brain and strengthens processes such as trust, social bonding, and facial recognition (De Dreu et al., 2011; Heim et al., 2009; Peltola et al., 2018). The peripheral, non-CNS effects of oxytocin are less well studied but include phenomena such as uterine contraction, smooth muscle contraction during orgasm, and even regeneration of heart tissue after myocardial infarction (Roy et al., 2019; Uvnäs-Moberg et al., 2019; Vignozzi et al., 2004; Wasserman et al., 2022; Xiong et al., 2020). Oxytocin is incredibly short-lived in the blood stream with a half-life of 3 to 5 minutes (Rydén & Sjöholm, 1969), although this half-life decreases even further in late pregnancy due to the action of oxytocin-degrading enzymes—oxytocinases. Levels found in the blood tend to be comparable to those seen in the cerebrospinal fluid (CSF), with levels ranging between 1 – 10 pg/mL in normal physiological conditions (Altemus et al., 1999; Heim et al., 2009; Martin et al., 2014). During pregnancy, oxytocin levels can rise to between 50 - 2000 pg/mL in the blood, with values being consistent within but not between individuals (Prevost et al., 2014). Larger meta-analyses have shown values to be more consistently approximating 200 pg/mL in pregnancy, with values reaching double or more at the onset of labour (Uvnäs-Moberg et al., 2019). The exact mechanism of oxytocin increase in pregnancy is unclear, but it is thought to arise from hormonal changes as a consequence of pregnancy itself. In particular, there is evidence showing that the uterus, along with the amnion and chorion of the developing pregnancy, are able to synthesize oxytocin in response to estrogen stimulation (Chibbar et al., 1993). This allows for the uterine environment to experience a greater local oxytocin concentration than that seen in peripheral circulation. Oxytocin is also implicated in the milk ejection reflex after delivery, and during the breastfeeding process instigated and maintained by infant suckling of the breast (Liu et al., 2022).

Separate from its roles in pregnancy, oxytocin has been associated with the neurological disorder autism, where deficiencies in oxytocin or its receptor are correlated to autistic behaviour (Hörnberg et al., 2020; Sikich et al., 2021; Yamasue et al., 2022). This will be discussed in greater depth in a later section (***Section 1.6.2***). It also possesses more general roles in response to tactile stimulation, being connected to both physical touch and the sexual response. In individuals undergoing continuous blood draw, it was found that oxytocin levels peaked during orgasm and

then fell quickly in both men and women from levels of 1 – 2 pg/mL at baseline to 6-7 pg/mL during orgasm with women tending to have higher levels on average than men at both baseline and at orgasm (Carmichael et al., 1987; Caruso et al., 2017; Marazziti et al., 2019; Murphy et al., 1987).

1.1.3 Oxytocin in epithelial inflammation

Oxytocin has been connected to inflammatory abrogation in both gut and skin epithelium, providing models for how it may affect the epithelium of the female genital tract (FGT). In skin it has been shown that oxytocin receptor (OXTR) knockout of keratinocytes led to increased reactive oxygen species (ROS) and increased secretion of IL-6, CCL5, and CXCL10 (Deing et al., 2013). Transgenic rat models, where the oxytocin promoter was under the control of clozapine-N-oxide, showed that induction of peripheral oxytocin in the bloodstream inhibited degranulation of tissue-resident mdast cells (Nishimura et al., 2022). Additionally, a study utilizing the gut crypt villi cell line Caco2BB demonstrated the ability of oxytocin to combat inflammation induced by 400 ng/mL LPS treatment by upregulating IκB—a protein responsible for sequestering the pro-inflammatory transcription factor NF-κB (Klein et al., 2016). Oxytocin was further found to play a role in reducing LPS-induced inflammation within the colorectal gut cell line HT-29 by inhibiting activation of NF-κB. Specifically, oxytocin treatment led to reductions in TSLP, IL-25, and IL-33, which have all been associated with allergy and autoimmune conditions (Yu et al., 2022). This was found to function through an OXTR-dependent mechanism and human cohort study subjects who experienced food allergy, and as a consequence had more inflamed guts, had ~two-fold higher levels of oxytocin in their plasma (Yu et al., 2022). Whether this is a corrective response by the body or simply an association requires further investigation. Enterocytes, as well as enteric neurons, have been shown to express the OXTR and are responsive to oxytocin treatment. These cells were shown to maintain a reduced inflammatory status in a C57BL/6 mouse colitis model, whereby OXTR-knockout mice exhibited exacerbated forms of colitis while wild type mice displayed milder symptoms that could be further reduced with oxytocin treatment (Welch et al., 2014). Interestingly, reductions in induced transcription of TNFα and CCR5—a pro-inflammatory cytokine and the HIV co-receptor, respectively—were seen in the colons of wildtype mice after

oxytocin treatment in experimental colitis. These reductions in TNFα and CCR5 were also found for saline-treated mice who had not undergone experimentally-induced colitis, underscoring the potential for oxytocin to combat inflammation at the gut more generally (Welch et al., 2014). This demonstrates that oxytocin may hold the potential to reduce HIV susceptibility at the gut mucosa through reductions in TNFα and CCR5 expression—regardless of inflammatory status—but how these findings may translate to the FGT is unclear.

1.1.4 Oxytocin's potential link to regulatory T cells and wound healing

Wound healing studies in murine models, utilizing the probiotic lactobacillus *L. reuteri*, demonstrated the dependence of *L. reuteri*'s wound healing improvements on oxytocin and suggest that oxytocin plays a critical role in this process. These studies utilized an oxytocin–deficient mouse model as well as surgically severing the vagal nerve—with consequent inability to produce oxytocin—and showed that these mice lost the inflammatory reduction and improved wound healing mediated by *L. reuteri* ingestion (Poutahidis et al., 2013; Varian et al., 2017). Specifically, *L. reuteri* altered murine hosts regulatory T (T_{reg}) cells via an as-of-yet unknown oxytocin-dependent mechanism. T_{regs} are a regulatory cell type that maintains the balance of immune homeostasis by controlling the extent of immune activation and inflammation within the body. Poutahidis (2013) et al. were able to show that T_{regs} transplanted from *L. reuteri*-treated wildtype mice with the ability to produce oxytocin to mice that were oxytocin-deficient recovered the phenotype of *L. reuteri* ingestion. Treatment with *L. reuteri* was also associated with increased levels of plasma oxytocin and reduced levels of plasma corticosterone—the inflammatory stress hormone known to inhibit wound healing (Ebrecht et al., 2004; Radstake et al., 2023). This suggests that oxytocin, induced by *L. reuteri*-treatment, may affect T_{reg} cells in a persistent way that allows their effect on wound healing to be transferrable. This further suggests that control of inflammation, through targets such as T_{regs}, is potentially a key component of oxytocin's ability to improve wound healing (Varian et al., 2017), and I wished to study this phenomenon further as I believed it may have implication for improving barrier integrity at the female genital tract—a factor associated with risk of heterosexual HIV transmission (Burgener et al., 2015).

1.2 Oxytocin Receptor Signaling Pathways

1.2.1 The oxytocin receptor as a G-protein-coupled receptor

The OXTR is a G-protein-coupled receptor (GPCR) that spans the outer and inner layers of the cell membrane with seven trans-membrane regions, and is able to attach to different intracellular protein subunits to affect separate downstream signaling cascades (Gimpl & Fahrenholz, 2001; Jurek & Neumann, 2018). In line with its previously mentioned effects, it has been shown to be expressed in the nervous system on central and peripheral neurons (Helgers et al., 2021; Nersesyan et al., 2017; Welch et al., 2014), the uterine myometrium and breast epithelium (Ariana et al., 2019; Arrowsmith, 2020; Chung et al., 2010), as well as on human immune cells in monocytes, macrophage, and fibroblasts (Ding et al., 2022; Ross et al., 2013; Szeto et al., 2017). Other less frequently cited locations for the OXTR are cardiomyocytes, renal cells of the kidney, adipose tissue, as well as in gut, skin, and prostate epithelium (Deing et al., 2013; Gimpl & Fahrenholz, 2001; Gould, 2023; Wasserman et al., 2022; Welch et al., 2014). Important to this study, there is also one paper putatively demonstrating its presence in human Vk2/E6E7 vaginal epithelial cells (Kallak & Uvnäs-Moberg, 2017). Since the OXTR is a GPCR, it can have four main alpha subunits that dictate its signaling pathways: $G\alpha_q$, $G\alpha_i$, $G\alpha_s$, and $G\alpha_{12}$ (Busnelli et al., 2012; Syrovatkina et al., 2016). These G-proteins also couple with $\beta\gamma$ subunits that compose a second messenger, but the alpha sub-units are the primarily variable subunit and are given priority in naming conventions i.e., $G\alpha_q$-coupled G-proteins are referred to as G_q.

Different coupling of G-proteins to the OXTR can also lead to altered associations with the membrane: G_q-associated OXTRs occupy caveolin-enriched domains that invaginate into the membrane, whereas OXTR associated with the classically inhibitory G_i subunit are found in caveolin-free areas of the plasma membrane (Gimpl & Fahrenholz, 2001; Williams & Lisanti, 2009). It appears that association with caveolin is what drives G_q rather than G_i association of the OXTR, at least in epithelial cell line models (Guzzi et al., 2002), although further work has suggested that association with caveolin drives the OXTR towards signaling through EGFR and ERK1/2 pathways transiently with mitogenic effects (Rimoldi et al., 2003). This is opposed to the more prolonged EGFR and ERK1/2 activation that occurs when the OXTR is stimulated in

caveolin-free zones (Rimoldi et al., 2003). These results were supported by data seen in prostate tissue from those suffering from benign prostatic hyperplasia, whereby OXTR association with caveolin-enriched domains was associated with proliferative effects (Herbert et al., 2007). Additional studies refined this association and suggested that it is the OXTR's association with caveolin-containing lipid rafts, rather than traditional caveolae—invaginations of the plasma membrane—that drives this association (Gould, 2023).

1.2.2 'Stimulatory' and 'inhibitory' signals for the OXTR

G_q, the most commonly reported G-protein subunit for the OXTR, is associated with cellular growth and division. This effect occurs primarily through the MAPK signaling pathway (S. H. Kim et al., 2017; Van Den Burg & Neumann, 2011). G_q tends to be the stimulatory subunit for OXTR coupling, while the G_i subunit is associated with anti-proliferative or inhibitory effects (**Figure 1.2**). These roles have been known to reverse, however, in both breast and prostate cancer cells (Cassoni et al., 2004; Imanieh et al., 2014). Prostatic cancer cells have been shown to be highly responsive to oxytocin, and oxytocin has been found at high levels in cancerous prostatic tissue indicating a potential positive feedback loop (Xu et al., 2017). These cells display signaling through the MAPK pathway, typically characterized by G_q coupling, when stimulated with oxytocin. Despite this, further studies were able to demonstrate that prostatic cancer cells mediated signaling through the OXTR by means of $G_{i\alpha 2}$ coupling, a G-protein subunit that has been associated with anti-proliferative effects in non-cancerous cell types (Caggia et al., 2018). This indicates that cancerous cells may alter traditional OXTR signaling, although further work is required to confirm this.

Calcium (Ca^{2+}) release/flux signaling pathways show a distinct correlation with proliferative phenotypes, while inhibitory ones are often associated with cyclic adenosine monophosphate (cAMP) (Cassoni et al., 1997, 2001; Tom & Assinder, 2010). Phospholipase C (PLC) and inositol triphosphate (IP_3) are the primary messengers by which Ca^{2+} signaling takes place. IP_3 is able to bind its receptor, the inositol triphosphate receptor (IP_3R), on Ca^{2+} store structures such as the endoplasmic reticulum and Golgi complex to engage Ca^{2+} flux culminating in intracellular Ca^{2+} release (Pinto et al., 2015). cAMP, in contrast, is able to influence downstream

effectors such as protein kinase A (PKA) and cAMP response-element binding protein (CREB). PKA in particular is then able to further act on other kinases downstream that have roles in cell growth and metabolism (Zhang et al., 2020).

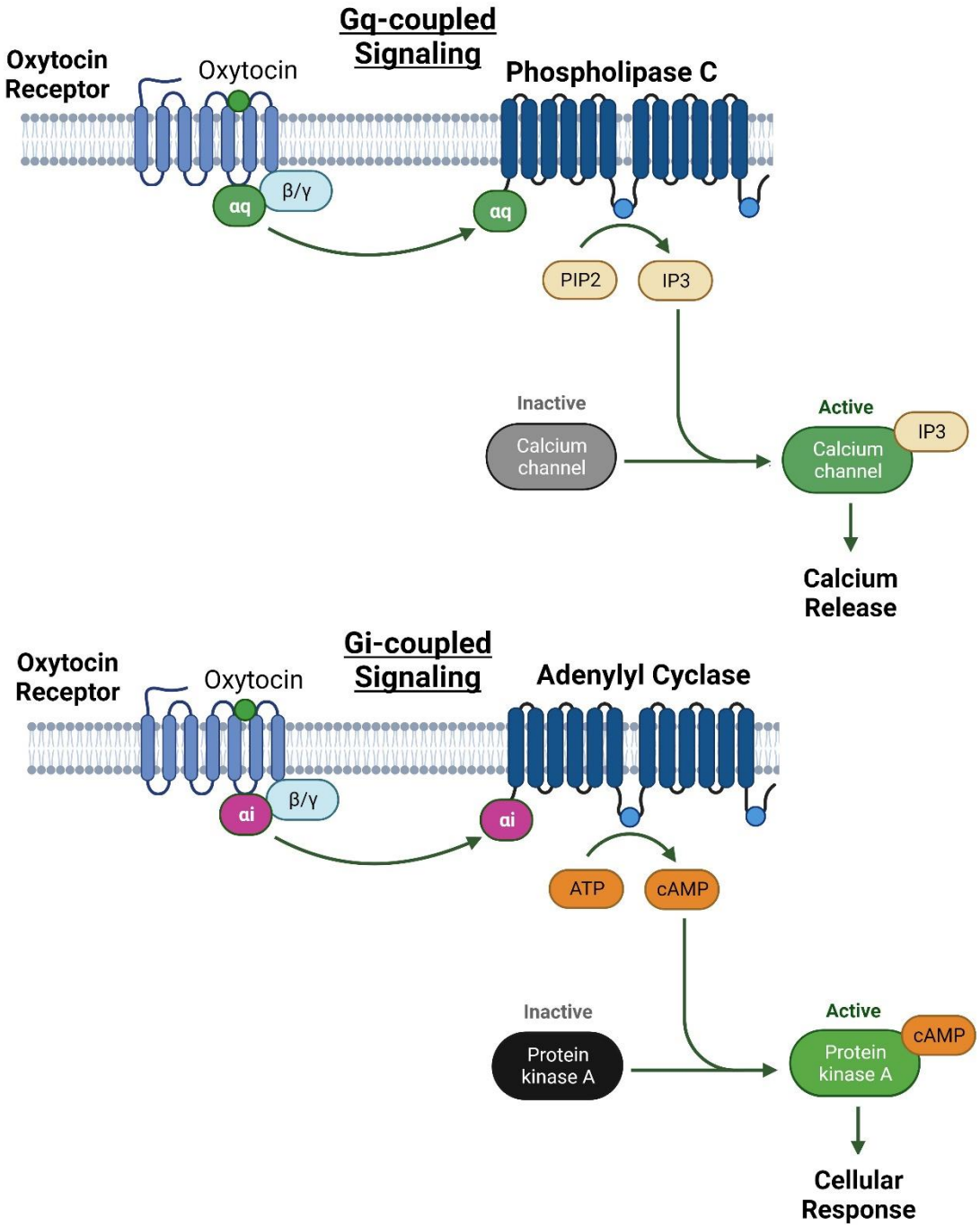


Figure 1.2 Alternative couplings for the oxytocin receptor leads to differential response. The oxytocin receptor can couple to either the G_q or G_i α-subunits, which leads to subsequent alterations in downstream signaling. G_q coupling is associated with activation of phospholipase C and subsequent calcium release, while G_i coupling instigates activation of adenylyl cyclase with incident cAMP production. Calcium release is typically associated with proliferative effects, while cAMP inhibits proliferation. Made using Biorender.

1.2.3 Oxytocin receptor agonists/antagonists and their interaction with the OXTR

Carbetocin is an alternative OXTR agonist that is able to activate classic G_q stimulatory effects and engage in Ca^{2+}-flux related OXTR signaling (Larciprete et al., 2013; Torres et al., 2020). Carbetocin is also able to engage with the receptor in a way that induces receptor internalization by an unknown process that does not utilize the standard β-arrestin dependent method, which further indicates that alternative signaling agonists are capable of altering the way the receptor interacts with the cell and is recycled (Passoni et al., 2016). In contrast with agonists are competitive inhibitors, such as atosiban, which is able to competitively inhibit binding of oxytocin to the OXTR (S. H. Kim et al., 2016, 2019). It accomplishes this by binding to the receptor with a 100-fold higher affinity than oxytocin itself, but without engaging in classical oxytocin signaling. While it is able to inhibit G_q signaling of the OXTR it is itself able to induce signaling down the G_i inhibitory pathway (Reversi et al., 2005). It additionally has been shown to activate G_i-linked signaling pathways that initiate pro-inflammatory responses in myometrial cells (S. H. Kim et al., 2016). This does not lead to receptor internalization as opposed to the G_q PLC-mediated pathway induced by oxytocin binding to the receptor (Reversi et al., 2005). Other OXTR antagonists, such as nalosiban, also display unintended consequences with additional blocking of the vasopressin receptor occurring due to the high degree of similarity between oxytocin and vasopressin (S. H. Kim et al., 2019). Most of these processes have not been studied using *in vivo* systems or endogenously expressed OXTR in cells and instead utilize cell systems that have been engineered to over-express the OXTR. Understanding these systems is core to interpreting these findings and potentially translating them to humans (Kii et al., 2018; Reversi et al., 2005; M. Wang et al., 2022).

1.3 Previous In Vitro Oxytocin Systems

1.3.1 The PHM1-41 uterine myometrium cell line

Oxytocin has been previously studied in PHM1-41 cells, an immortalized cell line derived from uterine myometrium, which has been shown to express the receptor and respond to oxytocin

stimulation through Ca^{2+} signaling (Burghardt et al., 1999; Monga et al., 1996). The cell line was initially isolated from a cesarean section of a patient 38 weeks pregnant at the time of delivery, which is significant as the uterine myometrium does not always express the OXTR at a high level and instead upregulates the receptor as pregnancy nears the end of the third trimester (Fuchs et al., 1984; Kimura et al., 1996).

PHM1-41 cells show significant (~90%) transcriptome similarity to primary human myometrial cells, as well as high similarity regarding Ca^{2+} response after induction with oxytocin (Siricilla et al., 2019). They do, however, display a lower threshold for Ca^{2+} induction by oxytocin than primary cells, indicating that they may be even more sensitive to the hormone. Human uterine smooth muscle cells exhibit receptor, intracellular-store, and diacylglycerol mediated Ca^{2+} influxes (Chung et al., 2010). The transient receptor potential-like channels (TRPCs) used to transport Ca^{2+} are similar between PHM1-41 and primary human myometrial cells and both cells support Ca^{2+} stimulation through these channels (Chung et al., 2010). This further supports the use of PHM1-41 as both a positive responder to oxytocin as well as an accurate proxy for human myometrial tissue. PHM1-41 cells also capture one of the major functions of oxytocin's action outside of the nervous system: smooth muscle contraction. This also allows the model to relate to other smooth muscle such as cardiac muscle cells, muscle cells involved in sexual function/orgasm, and even the smooth muscle cells of the gut (Alqudah et al., 2023; Jankowski et al., 2020; Vignozzi et al., 2004). For these reasons, I chose this cell line as my primary *in vitro* positive control for oxytocin in this thesis.

1.3.2 The HEK 293 kidney epithelial cell line

HEK 293 cells, a commonly used cell line for studying GPCRs by transfection, have also been used to examine the OXTR and oxytocin interactions (Atwood et al., 2011; Malik et al., 2022; Ooi et al., 2016; Pillers et al., 2014). These cells are considered to be negative for a wide array of GPCRs, are easy to culture, are widely used, and are readily available (Chun & Shim, 2015; Namkung et al., 2016; Pampillo & Babwah, 2015). Lack of OXTR expression in HEK 293 cells is supported by negative OXTR staining in confocal microscopy assays (Jasper et al., 1995; Ma et al., 2013). This is further supported in functional assays that have been used to study OXTR

response and show clear differences between *OXTR*-transfected and untransfected HEK 293 cells (Busnelli et al., 2012; Nersesyan et al., 2017). This reinforces the suitability of these cells to study the effects of oxytocin and its receptor, as well as their ability to serve as a negative control for oxytocin response when untransfected. It also allows for exploration of OXTR mutations and potential impacts on oxytocin binding and signaling events (Meyer et al., 2022).

1.4 Animal Models for the Study of Oxytocin

Animal models have also been used to study the effects of oxytocin in *in vivo* systems with rats and mice making up the majority of oxytocin study systems, although guinea pigs have been used to a lesser extent (Ayers et al., 2016; Pisansky et al., 2017; Schellenberg, 1995). These small animal models have primarily targeted questions directed at the microbiome or specific organs—such as the uterus (Danhof et al., 2023; Zhou et al., 2007). Oxytocin has been used to study gastric motility in rat models due to the longitudinal and circular smooth muscle of the stomach being sensitive to oxytocin (Qin et al., 2009). Treatment with oxytocin was seen to increase intragastric pressure, which was able to be blocked by pre-administration of the competitive oxytocin inhibitor atosiban (Qin et al., 2009). Rat myometrium was further able to show that both G_q and G_i signaling are present in the myometrium, and that oxytocin is able to utilize both G_q and G_i pathways in the same cell type/organ (Zhou et al., 2007). Specifically, non-pregnant myometrium was seen to favour G_i coupling of the OXTR, while pregnant myometrium favoured the 'stimulatory' G_q coupling of the receptor (Zhou et al., 2007). This indicates that OXTR signaling may not only be cell-type specific, but environmentally influenced through hormones and other signaling molecules.

1.5 Modelling the Female Genital Tract

1.5.1 The VK2/E6E7, Ect1/E6E7, and End1/E6E7 cell lines

Lower female genital tract (FGT) cell lines can be used to model the vaginal and cervical mucosa through *in vitro* methods. In particular, the VK2/E6E7 (Vk2), Ect1/E6E7 (Ect1), and End1/E6E7 (End1) cell lines derived through transfection of healthy patient biopsies with the E6/E7 oncogenes of HPV16 have been used to model the vaginal, ectocervical, and endocervical epithelium, respectively (Fichorova et al., 1997). These cells maintain their phenotypes up to at least one year in culture, and showed distinct morphological differences to the HeLa cervical adenocarcinoma cell line (Fichorova et al., 1997). As a consequence of this, these cell lines are considered to be immortalized cell lines but not true carcinomas. They are strong candidates for basic science research as they are widely used, able to be kept in culture for extended periods of time, and together fully represent the diversity of the lower FGT epithelium.

Studying the epithelium is of core concern with respect to sexual exposure to pathogens, such as HIV, as this is the first barrier that pathogens must overcome in order to achieve infection (Chambers & Vukmanovic-Stejic, 2020; Nguyen & Soulika, 2019). Ultimately, models of the FGT can be broken down into *in vitro*, *ex vivo*, and *in vivo* models (**Figure 1.3**). All of these systems attempt to capture or model some aspect of the epithelium and/or immunity of the FGT. *In vitro* models can be further broken down into monolayer and multilayer cultures. The most straightforward and widely used method for FGT epithelial cell culture is standard monolayer cell culture in a tissue culture flask or well with media covering the cells (Philippeos et al., 2012). This has been further expanded upon with the advent of transwell culturing which allows for separation of treatments at both the apical and basal layers of the cell by having cells grown on a semi-permeable well-insert (Edwards et al., 2022; Rose et al., 2012).

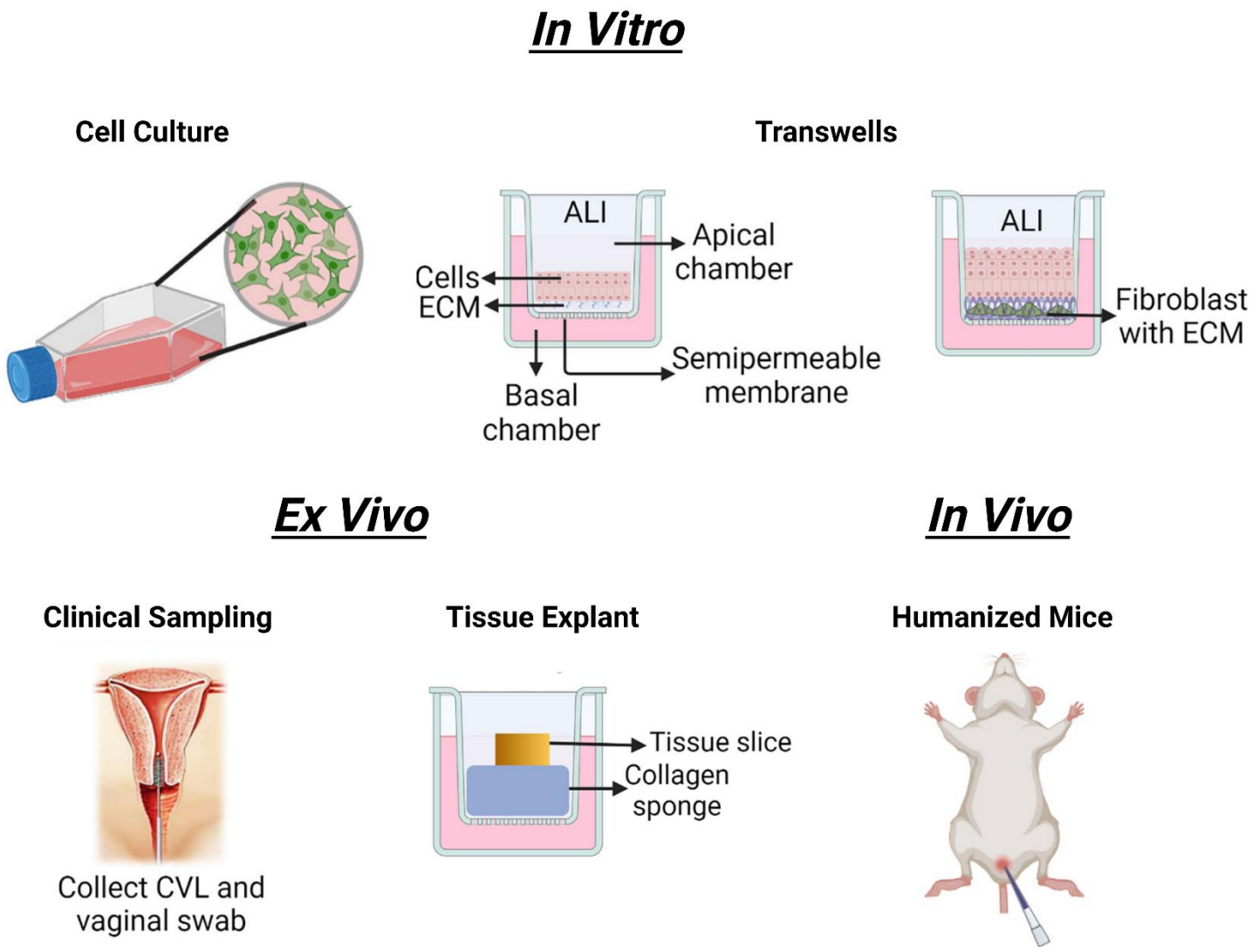


Figure 1.3 Models used in the study of female genital tract epithelium. Models for the study of female genital tract epithelium can generally be broken down into *in vitro*, *ex vivo*, and *in vivo* models. *In vitro* models are able to model the epithelium through the use of 2D cell culture and transwells. Transwells offer the ability to create a 3D environment through the use of a semi-permeable well insert that is supported in a tissue culture dish or plate. *Ex vivo* models are comprised of CVL and vaginal swab collection of epithelial cells, as well as tissue explants of the female genital tract typically maintained in transwell culture. *In vivo* modeling can be achieved through the use of humanized mice whereby effects on female genital tract epithelium can be studied in concert with human immune cells due to these mice having human lymphocytes and/or bone marrow transplanted into their systems. Clinical randomized control trials have notably been excluded here as they are typically not suitable for basic science experiments. Modified from Plesniarski et. al 2021 with permissions. Made using Biorender.

1.5.2 Transwell models

Transwells allow for two different types of media to be used during the cell culture process and produce a more complex modeling of the epithelial layer, with the apical side representing the lumen and the basal side representing the basement membrane of a tissue layer. It is also possible to grow vaginal epithelial cells—a stratified squamous epithelial cell—as multilayers with subsequent differentiation into cornified epithelium (Rose et al., 2012). The other key component of this model is the ability to introduce what is known as an air-liquid interface. This involves leaving the apical portion of the transwell without media and exposed to air (Srikanth et al., 2016; Tata et al., 2013). This allows for epithelial cells to differentiate into terminally-differentiated epithelium, although the cells still receive nourishment from the bottom portion of the basal layer (Tata et al., 2013). Transwell methods further allow for the addition of other cell types to the system, such as immune cells like monocytes or $CD4^+$ T cells, and are effective co-culture systems (Edwards et al., 2022). This allows them to also be used in cellular migration studies, where they can be used to study the effects of chemoattractants from the FGT (Kashem et al., 2020).

Transwell models can be further built into '3D' models that are composed of multilayer epithelium as well as a basolateral matrix (Edwards et al., 2022). These 3D models have also been shown to be amenable to supporting mucus-producing cells and the growth of vaginal microbial flora, both commensal and pathogenic, to allow for microbiome questions to be tested (Edwards et al., 2022). Lactic acid effects on Vk2 and Ect1 cells have been studied using this model with work showing that treatment of these cells under low pH conditions, similar to a *Lactobacillus*-dominated microbiome, led to reductions in inflammation while treatment under higher pH conditions, similar to a bacterial vaginosis microbiome, led to no effect (Delgado-Diaz et al., 2020). This was further expanded on with work showing that lactic acid was significantly associated with an increase in epithelial barrier proteins (Delgado-Diaz et al., 2022); this is a key component in protection from certain STIs, such as HIV, as barrier function has been implicated to play a key role in HIV susceptibility (Burgener et al., 2015). Lactic acid was also shown to reduce IL-6, IL-8, TNFα, RANTES, and MIP3α in Vk2 and Ect1 cells using this model, with similar results being found in primary cervicovaginal epithelium (Hearps et al., 2017). This underscores the ability for this model to provide both barrier function and immunological data.

1.5.3 Tissue explants and cytobrush sampling

Ex vivo models tend to be broken down into tissue explant or patient samplings that involve collecting post-operative samples or samples obtained via swabbing the vaginal vault (Berry et al., 2022; Delgado-Diaz et al., 2022; Introini et al., 2017). Tissue explants are useful models in that they often retain a complete component of basement membrane and associated cells. They can be used either to isolate a specific cell type, such as endometrial cells, from patient tissue (J. C. Chen & Roan, 2015) or the tissue biopsy can be left intact to study effects on full tissue (Berry et al., 2022; Introini et al., 2017). Obtaining cells to study cells in isolation can be useful if wishing to examine specific effects such as cell-cell interactions or response to a particular agonist, while studying tissues can allow for more complete or holistic analysis of a particular site.

Tissue explant samples have been successfully used to study HIV replication and pathogenesis in a full tissue model composed of underlying basal tissue and immune cells (Introini et al., 2018). Challenges exist in maintaining tissue integrity and functional architecture for longer than two weeks, however. Variability between donors also poses a challenge for comparing between multiple treatments and time points using different samples, something which cell lines *in vitro* are able to avoid (Introini et al., 2018; Merbah et al., 2011). This variation can also pose a boon, though, in that it provides a more robust sample set that is more closely representative of the population. Explant models have been useful in illustrating the penetration of HIV into the layers of stratified squamous epithelium at the FGT that were initially thought to pose a significant enough barrier to prevent contact of HIV with immune cells (Carias et al., 2013). It was shown that the columnar epithelium of the FGT—generally thought to be a higher risk for viral entry—had greater than expected protective power due to the presence mucus layers, illustrating the importance of emergent properties in tissues that cannot be captured in *in vitro* culture (Carias et al., 2013).

Collected FGT samples, usually through cytobrush, also allow for examining primary cells, although the cells available tend to be limited (Brown et al., 2023). Primary cells can also be collected by washing the vaginal vault with a liquid to obtain cervicovaginal lavage (CVL), which is useful for collecting a sampling of secretions with the potential to capture primary cells as well (Bradley et al., 2018). Cervical cytobrush sampling provides a low-invasive method with which to

sample the FGT (Mohammadi et al., 2020). It has been demonstrated that cytobrush sampling itself can induce changes in the immune state of the cervicovaginal vault, such as by increasing the levels of $CD4^+$ T cells, antigen presenting cells (APCs), and neutrophils. These changes have been shown to persist for up to at least 48 hrs, and indicate that longitudinal cytobrush sampling needs to control for the immunological impacts of cytobrush sampling itself (Mohammadi et al., 2020). Importantly, these changes did not have an impact on vaginal flora that were detected, and microbiome studies utilizing this sampling method appear to be free of direct confounding. In comparison to other sampling methods, such as vaginal swabs, cytobrushes have been found to perform better at detecting high-diversity microbial community structure in a small number of cases (Mitra et al., 2017). Collection of epithelial cells has been shown to be enhanced by utilizing a cervicovaginal wash after administration of the cytobrush, as has been shown in previous studies comparing the two methods (Goldberg et al., 1989). The cells collected are almost exclusively limited to the lower FGT, as accessing the upper FGT is not possible under most conditions without surgical intervention. This means that samples obtained are often vaginal, ectocervical, and/or endocervical cells that are present at the apical layer of the tissue, or immune cells that have entered the lumen of the lower FGT. The challenges with collecting these cells, however, are that they are often terminally differentiated and near the end of their cellular life cycle (Rocha et al., 2022; C. Tang et al., 2021). This limits what can be done with them, and they do not often persist in culture for greater than 14 days. It is also difficult to ascertain the degree to which the terminally differentiated upper layer of the epithelium best models the overall FGT mucosa, although an argument can be made that these cells are nearest the point of exposure to sexually transmitted infections (STIs) and therefore relevant (Kaushic et al., 2010; Keele & Estes, 2011).

Ultimately, tissue explant and cytobrush sampling methods are effective in providing *ex vivo* models that don't require the same level of ethics or clinical access that longitudinal human cohort or clinical studies would necessitate, but still allow for a representative sample of the FGT to study interactions of microbiota, hormones, drugs, and pathogens with primary cells (Introini et al., 2017; Lantz & Nicol, 2022).

1.6 Therapeutic Uses of Oxytocin

1.6.1 Oxytocin in labour and delivery

Oxytocin is broadly used in the clinical setting as a uterotonic to induce uterine contractions during labour and delivery (Uvnäs-Moberg et al., 2019; Walter et al., 2021). This is most relevant in situations where labour contractions have stopped, or assistance is needed to finish the birthing process. Oxytocin, or its pharmacological equivalent pitocin, has also been used to halt uterine hemorrhage after delivery (Anderson & Etches, 2007; Litorp et al., 2021). This is not without its risks, however, as oxytocin administration also carries the potential risk of uterine rupture (Cahill et al., 2006; Zhang et al., 2021). Use of oxytocin was also found to increase the rate of cesarean sections, epidural anesthesia, and intrapartum maternal fever in first time and repeat mothers (Hidalgo-Lopezosa et al., 2016). Labour augmentation and/or induction, along with its use in halting post-partum hemorrhage, have led to oxytocin being a broadly utilized clinical staple recommended for all countries by the World Health Organization (WHO) (Lambert et al., 2020; WHO, 2020). This widespread availability, combined with relatively low production costs, positions oxytocin to be an affordable and widely available drug suitable as an adjuvant or treatment for other purposes, such as HIV prevention (Lambert et al., 2020).

1.6.2 Oxytocin in autism

The other widely studied use for oxytocin is in its classical role as a neurotransmitter, where it is often administered through nasal spray (Yamasue & Domes, 2018; Yatawara et al., 2016). Prior work has also suggested use of oxytocin as an antidepressant with potential in treating depression, anxiety disorders, schizophrenia, and autism (Matsuzaki et al., 2012). This has been built upon with work in autistic individuals, as well as those undergoing marriage counselling, to affect an improvement in sociability and strengthen relationships (Hörnberg et al., 2020; Wudarczyk et al., 2013; Yamasue et al., 2022). Significant associations have been found between OXTR single nucleotide polymorphisms (SNPs) and autism spectrum disorder (LoParo & Waldman, 2014), with the SNPs rs7632287, rs237887, rs2268491, and rs2254298 being

significantly associated with autism. These findings were discovered after metanalyses of 16 OXTR SNPs among 3941 autistic individuals (LoParo & Waldman, 2014). This further strengthens the connection between the oxytocinergic system and autism spectrum disorder. In autistic individuals, oxytocin is found to improve social responsiveness, as rated by caregivers, and improve autistic individuals' ability to interact and communicate with others when the drug is administered at a young age (Yamasue & Domes, 2018; Yatawara et al., 2016). Since autism falls on a spectrum, it can be difficult to categorize the applicability of oxytocin sprays across the varying degrees of autism, although studies show an effect across degrees of autism with those who are higher-functioning showing more favourable outcomes (Yamasue et al., 2022; Yamasue & Domes, 2018; Yatawara et al., 2016).

1.6.3 Oxytocin in sexual functioning

Oxytocin has been further explored for potential roles in erectile function due to the interaction of oxytocin with smooth muscle (Bratzu et al., 2019; Melis & Argiolas, 2021; Oti et al., 2021). While results have been promising in rats, an association with human subjects is still yet to be found (Melis & Argiolas, 2021). Rare case studies in women have found an enhancement of sexual drive, specifically during either high estrogen or progesterone states, as is often the case during estrogenic birth control or under an alternative co-incident therapy (Anderson-Hunt & Dennerstein, 1994). This raises the potential for oxytocin to be used as an aid for female hypoactive sexual desire disorder and/or sexual dysfunction—an area much less supported in the current drug market than that of male sexual dysfunction (Cera et al., 2021; Mesbahi et al., 2022). Studies have not shown strong evidence that oxytocin on its own is able to enhance female desire as a nasal spray, but studies examining other aspects of sexual function—particularly in women undergoing menopause—have shown stronger results.

1.7 Oxytocin as a Therapeutic at the Lower Female Genital Tract

1.7.1 Oxytocin and the OXTR in the VK2/E6E7 cell line

Oxytocin has been studied for its effects directly at the FGT both *in vitro* and in clinical settings. A singular *in vitro* study has been performed testing the effect of oxytocin on Vk2 cells, which demonstrated that oxytocin was able to increase rates of Vk2 proliferation (Kallak & Uvnäs-Moberg, 2017). The confocal microscopy receptor staining performed by Kallak & Unväs-Moberg (2017), however, showed OXTR staining throughout the cell rather than at the plasma membrane (Kallak & Uvnäs-Moberg, 2017). OXTR staining is expected to be present directly at the cell membrane (Jasper et al., 1995; Ma et al., 2013), although anomalies have been seen such as the OXTR associating with the nucleus after treatment with oxytocin in murine osteoblasts (Di Benedetto et al., 2014). There have also been multiple sizes of OXTR reported in the literature through western blot, with sizes typically occupying the range of 40-46 kDa or 65-70 kDa (Lee et al., 1998; Z. Li et al., 2018; Szeto et al., 2017; Whittington et al., 2004). These likely correspond to unglycosylated, and glycosylated forms of the OXTR but studies do not often characterize the receptor's glycosylation status (Ivell et al., 2001; Kimura et al., 1997). This implies that *in vitro* work needs to be repeated with more robust OXTR detection methods to better characterize OXTR expression in these cells.

1.7.2 Oxytocin in treatment of menopausal vaginal atrophy

Clinical work, primarily investigating the potential of oxytocin vaginal gels as an alternative therapy to estrogen creams, suggests that oxytocin vaginal gels have a clinically significant impact on relieving menopausal vaginal symptoms (Al-Saqi et al., 2015; Moussa et al., 2023). Direct comparisons of epithelial biopsies taken from the vaginal introitus before and after treatment with oxytocin vaginal gel demonstrated reductions in atrophy by histological evaluation and increased numbers of superficial, or 'mature', cells (Al-Saqi et al., 2016). Together with patient reports of decreased dryness, pain, and irritation, oxytocin vaginal gel may provide an alternative to topical estrogen creams in cases where patients complain of side effects or are

contraindicated towards estrogen for other reasons. The main mechanism by which oxytocin acts appears to be through increasing the number of cell layers, increasing the number of matured/superficial cells, and increasing cell size. These experiments have been repeated across multiple cohorts with diverse demographic data and similar findings (Abedi et al., 2020; Moussa et al., 2023; Torky et al., 2018; Zohrabi et al., 2020). Oxytocin has been further compared to promestriene, a synthetic estrogen compound, for treatment of vaginal atrophy and improvement in epithelial barrier thickness (Santos et al., 2022). Both compounds showed a significant increase in vaginal epithelial thickness over baseline in a prospective, double-blind, pilot study (Santos et al., 2022). Promestriene did, however, show a greater increase in vaginal epithelial thickness than oxytocin treatment. This provides further evidence that oxytocin is able to increase the maturation and thickness of vaginal epithelium when used as a vaginal gel, however, these results have only been tested and demonstrated in those suffering from vaginal atrophy and not those without menopausal conditions.

It is worth reporting that a larger meta-analysis has been done examining the potential for a placebo effect in studies treating women's sexual functioning and menopausal symptoms, which are potentially correlated with improvements in the vaginal epithelium, with findings indicating that nearly 70% of improvement seen—reduced dryness, pain during intercourse, and increased lubrication—can be accounted for by placebo (Weinberger et al., 2018). This was done using a metric known as the Female Sexual Function Index (FSFI), which captures overall satisfaction in sex life rather than directly measuring the state of the vaginal epithelium, however. This does not indicate that oxytocin vaginal gel therapy's effects on vaginal epithelium can be explained by placebo effect, but that subjective experiences in improvement of intercourse may. Regardless, indications that oxytocin vaginal gels improve the maturation and thickness of the epithelium suggest it may enhance barrier function of the FGT, a system that involves epithelial cell barriers, mucus, and immunoglobulins (**Figure 1.4**)—all implicated in protection from HIV transmission (Burgener et al., 2015; Carias & Hope, 2019).

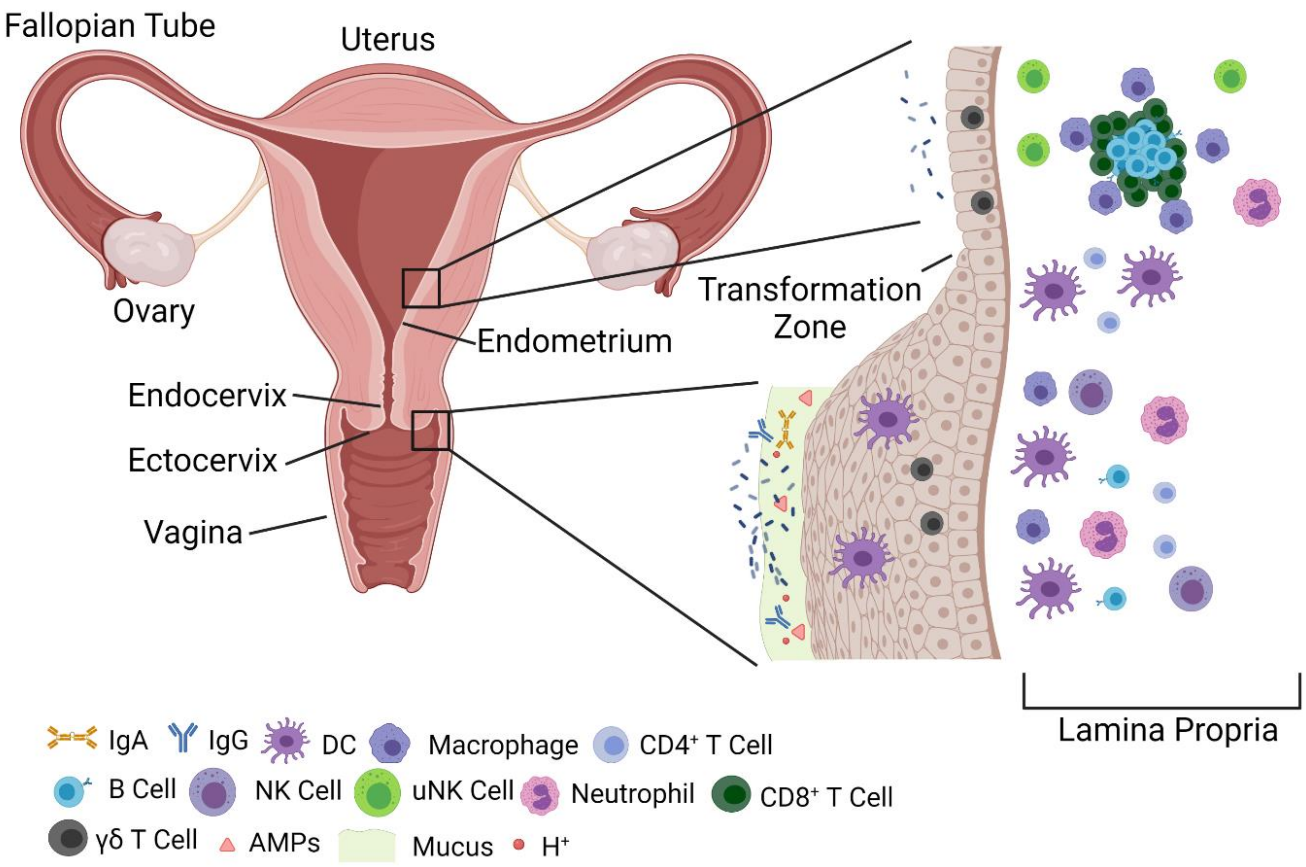


Figure 1.4 Overview of FGT anatomy and components of barrier function. The FGT is divided generally into an upper (endometrium and endocervix) and a lower (vagina and ectocervix) tract. The lower FGT is considered the primary point of contact for pathogens, and harbours multiple innate immune mechanisms, such as AMPs, mucus, and immunoglobulins that prevent transmission across the epithelial barrier. The epithelium itself is composed of a stratified squamous epithelium that arises from a basement membrane and terminates in fully keratinized, senescent, cells. Microbiota, primarily *Lactobacillus*, can be found in a high abundance within the lower FGT, but have also been shown to be resident within the upper FGT in lower numbers. As the epithelium progresses to the upper FGT it changes to a single columnar epithelium at a junction known as the 'transformation zone' between the ectocervix and endocervix. The upper FGT harbours unique uNK populations, as well as lymphoid aggregates consisting of B cells surrounded by $CD8^+$ T cells and macrophage. These immune gatekeepers balance immune surveillance and response with the need to maintain a fertile environment for pregnancy. Abbreviations: FGT: Female genital tract; IgA: Immunoglobulin A; IgG: Immunoglobulin G; DC: Dendritic cell; NK: Natural killer; uNK: Uterine natural killer; AMP: Antimicrobial peptide. Taken from Plesniarski et al., 2021 with perimssions. Made using Biorender.

1.8 Human Immunodeficiency Virus (HIV)

1.8.1 HIV and acquired immunodeficiency syndrome (AIDS)

HIV, the causative agent of AIDS, is a lentivirus from the family Retroviridae. It contains a viral envelope composed of glycoproteins gp120 and gp41 that encapsulate a protein capsid composed of p24 subunits. The genome for HIV contains the *env*, *pol*, *gag*, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* genes, which encode for the viral envelope, the viral polymerase, structural proteins, and a host of other accessory and viral infectivity proteins (Engelman & Cherepanov, 2012; Rossi et al., 2021). HIV's main target of infection are host immune cells, primarily $CD4^+$ T cells (Sabin & Lundgren, 2013). Once $CD4^+$ T cells drop to a level of approximately 200 cells/mL of blood from the typical levels of 500 – 1500 cells/mL (Garcia & Guzman, 2022), patients are considered to be at risk of progression to AIDS with subsequent susceptibility to opportunistic infections (**Figure 1.5**). The rate at which HIV infection progresses to AIDS—HIV disease progression—is controlled by many factors, including age, access to treatment, and set-point viral load (Goodreau et al., 2018, 2022; Lavreys et al., 2006). Set-point viral load describes the variation in the level of HIV viremia that stabilizes between HIV patients after the initial spike during acute infection (**Figure 1.5**). This value, as well as the number of HIV copies present before a patient begins and antiretroviral regimen, are correlated with remaining $CD4^+$ T cell levels and general immune function throughout treatment. This stresses the importance of identifying and treating HIV infection as soon as possible to reduce future sequalae and complications during viral suppression. HIV infection is typically dominated by R5-tropic viruses early on in infection that target CCR5-expressing $CD4^+$ T cells and macrophage (Joseph et al., 2015) and infection is somewhat tailored for the mucosa i.e. heterosexual transmission. After infection has progressed and $CCR5^+$ cells become limited as targets for the virus, HIV tends to switch to a predominantly CXCR4-favouring, or an X4-tropic, phenotype (Joseph & Swanstrom, 2018), and the virus becomes more tailored for blood to blood transmission.

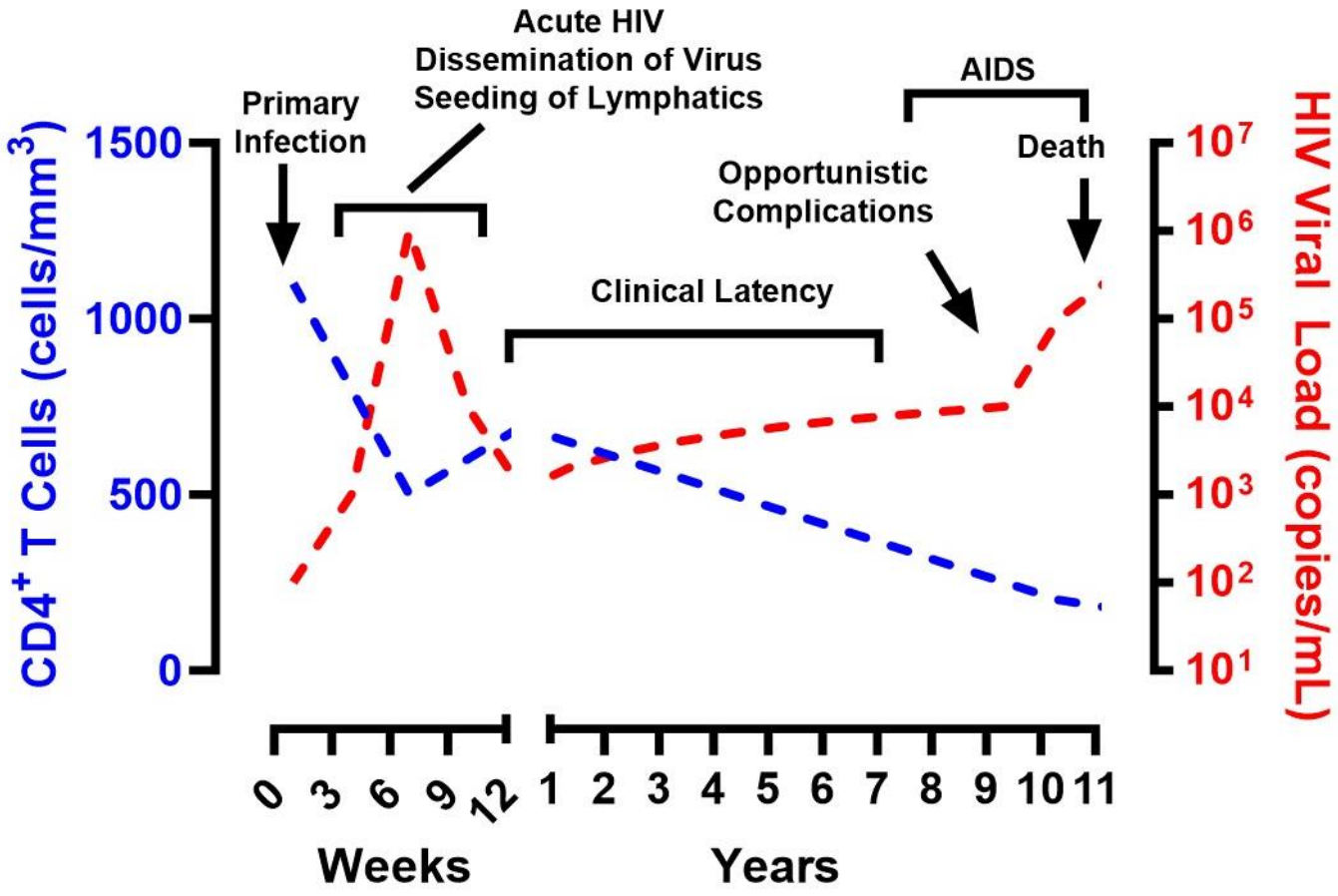


Figure 1.5 HIV disease progression to AIDS as measured by CD4$^+$ T cell count and HIV viremia. CD4$^+$ T cell levels in the blood are indicated in blue from the point of primary/initial infection, with a small recovery before the establishment of clinical latency and progression to AIDS. HIV plasma viral load is shown in red with an initial spike proceeding primary infection and subsequent control by the host immune system. Throughout clinical latency HIV slowly replicates until replication grows exponentially preceding and during AIDS. Time is listed on the x-axis dividing HIV infection into short-term (weeks) and long-term (years) timeframes. Adapted from (Amherst & Giorgi, 2011; Epstein et al., 1993; Fauci et al., 1991, 1996). Made using Biorender.

1.8.2 Challenges in HIV eradication and epidemiology

What makes HIV incredibly difficult to prevent through vaccination is the hyper-mutative nature of the virus (Cuevas et al., 2015; Yeo et al., 2020). It has been demonstrated that HIV has a mutation rate of ~4.1—5.2x10^{-3} bases/cell (Cuevas et al., 2015; Rowland-Jones & Andrews, 2017) or 10^{-5}-10^{-3} errors/base-pair/cycle (Yeo et al., 2020). Most antibody- or cell immunity-based vaccines do not generate a response against an epitope of the virus stable enough to generate stable immunity against both the various clades of HIV as well as its mutation escape variants (Bonsignori et al., 2017; Isitman et al., 2012; Mann & Ndung'u, 2015). In addition to this, true cure of HIV from the body is nearly impossible due to the establishment of latent reservoirs in resting memory T cells (Vanhamel et al., 2019). These latent reservoirs cannot be targeted by therapeutics unless they undergo activation and become transcriptionally active to allow for either lysis by cytolysis or detection and clearance by cellular immunity (Warren et al., 2020). This has led to a 'shock and kill' strategy whereby patients are given latency-reversing agents in order to activate cells containing latent HIV reservoirs in concert with a combination antiretroviral therapy (cART) regimen to prevent further spread of the virus with hopes of viral clearance (Jimmy Yeh & Ho, 2021; Y. Kim et al., 2018). To date there have been no successful cases of true HIV cure using this method, however.

Rare instances of HIV cure have been achieved when patients have received bone marrow transplants from donors who are CCR5-Δ32 homozygous—containing a deletion within the co-receptor of many HIV strains—and the patients have had their own immune cells targeted with radiation and chemotherapy treatments (Allers et al., 2011; Hütter et al., 2009). This has happened in only a handful of rare cases, and due to the lack of availability of bone marrow transplants, and the risks involved in both donation and in receiving the transplant, this does not present a tenable option for HIV treatment. Despite the fact that HIV is no longer an untreatable disease (Weichseldorfer et al., 2021), it's inability to be cured has led to a degree of endemicity—particularly within developing or resource limited communities and nations (Assefa & Gilks, 2020). This means that HIV, while broadly under control globally, has stabilized with ~1.3 million new infections per year and just under 39 million infected persons living with HIV (*Global HIV & AIDS Statistics — Fact Sheet | UNAIDS*, 2024). Given the slow rate of decline, this implies that

HIV eradication may not be seen for decades, or potentially even longer, given that the change in new infections may plateau or approach zero.

The HIV pandemic has led to a global dissemination of the virus, but distributions of infected persons are not equal. In particular, the African continent accounts for over half of all women and girls living with HIV, where heterosexual transmission remains the major route of exposure to HIV (*20.2 Million Girls and Women Living with HIV | UNAIDS*, 2024). The high concentration of infected persons in Africa is likely a consequence of prevailing economic disadvantages, inequalities for women and girls with regards to dictating sexual activity, and social stigma and challenges surrounding HIV (McKinnon & Karim, 2016). The initial pandemic has been linked to HIV's origins within the African continent, thought to be a result of bush meat hunting and eating practices in Western Africa of incidentally SIV-infected chimpanzees (Sharp & Hahn, 2011).

The ability to study SIV as an HIV analogue in non-human primate systems, as well as the ability to form SIV/HIV chimeras for studying HIV, have led to non-human primate models being the cornerstone of studying viral dynamics and HIV retroviral therapies (Apetrei et al., 2012; Veazey & Lackner, 2017). While these animals provide useful models for studying HIV dynamics, other models have been developed to allow for housing a greater number of subjects and study of HIV directly using reconstituted human immune cells.

1.8.3 Humanized mice as an HIV model

The above issues are now being addressed with the advent of humanized mice as an alternative small animal model that can be used to model HIV infection (Gillgrass et al., 2021; Marsden, 2020). Humanized mice were initially designed using a non-obese diabetic severe combined immunodeficiency disease IL-2γ chain knockout (NOD-SCID-IL2γc$^{-/-}$) murine background. These mice were then further injected with human hematopoietic stem cells to effect the reconstitution of leukocytes, including T cells, B cells, and myeloid cells (Ishikawa et al., 2005; Martinov et al., 2021; Pearson et al., 2008). Successful reconstitution is seen as a % of human (CD45^{+}) cells in the range of 20-50% within the blood, and even up to 80% in the bone marrow

(Martinov et al., 2021; Pearson et al., 2008). Additionally, CD4:CD8 ratios for T cells should see $CD4^+$ cells being approximately 1.5-2.5 fold higher than $CD8^+$ T cells in circulation (Xia et al., 2019). Models reconstituting lymphocytes by injecting adult human peripheral blood mononucleocytes (PBMCs) into mice (hu-PMBC mice) tend to see the highest reconstitutions, but B cell and myeloid cell populations suffer in terms of response with B cells being unable to engage in a primary immune response to antigens they have not yet encountered (J. Chen et al., 2022). Hematopoietic stem cell models of humanized mice (hu-HSC) utilizing $CD34^+$ stem cells from bone marrow or umbilical cord blood typically take longer to reconstitute than hu-PBMC mice, with levels of $CD45^+$ human cells in the periphery not reaching 25-60% until four weeks after transplant (J. Chen et al., 2022).

These models have been expanded, however, to incorporate injection of human fetal bone-derived hematopoietic stem cells along with transplantation of fetal liver and thymus (hBLT) to more robustly reconstitute human adaptive and innate cells within a humanized mouse model (Karpel et al., 2015). This provides further benefits in that hBLT mice display mucosal immunity and secondary lymphoid tissue along with the ability to mount an adaptive immune response, although there is a reduction in lymph nodes and lack of B cell foci which limits translation to human subjects (J. Chen et al., 2022). This makes them excellent candidates for use in models studying mucosal transmission of pathogens, such as HIV. One of the major challenges for humanized mice in general is that because the mice undergo a xenograft of human immune cells, they are highly susceptible to graft versus host disease (GvHD) (Hess et al., 2021; Tsuzuki et al., 2021). While the occurrence of GvHD is a boon in studies specifically examining it (Bézie et al., 2018; Elhage et al., 2022), in the context of HIV animal models this provides a major limitation to the usefulness of humanized mice for longitudinal studies. This is particularly important in the context of examining long-term HIV infection and latency—latency being, as previously discussed (*Section 1.8.2*), the main mechanism by which HIV evades eradication. It also raises challenges for treating mice with cART therapy in order to study the effects of viral suppression and rebound—a phenomenon which will be described in the following section.

Typical hBLT mice can show GvHD approximately 20 weeks after reconstitution (J. Chen et al., 2022). Because of this, further developments have been made to the hBLT mouse model with the advent of triple knockout (TKO) mice (Di & Lavender, 2022; Lavender et al., 2013, 2018).

These TKO-hBLT mice contain an additional mutation that knocks out CD47 expression (Lavender et al., 2013). This deletion allows the murine host cells to avoid detection by the human xenografted immune cells—specifically by avoiding the recognition of macrophage—and reduces both the rate of onset and burden of GvHD (Lavender et al., 2013). Specifically, these mice have been able to persist for up to 45 weeks without signs of GvHD (J. Chen et al., 2022). This allows for examining HIV latency and/or antiretroviral treatment, since these mice are able to survive long enough to achieve viral suppression on cART as well as allow for rebound after cessation of therapy (Di & Lavender, 2022; Sutter et al., 2019). Using these small animal models, it is now possible to carry out larger longitudinal *in vivo* HIV-treatment or immunology studies without having to establish clinical cohorts or secure funds and space for expensive non-human primate models. While cART therapy remains one of the most powerful tools in controlling HIV viremia and preventing the spread of new infections, new technologies are always required to circumvent barriers to accessing treatment, stigma, and potential viral resistance.

1.9 Current HIV Therapies and the Potential of Oxytocin

1.9.1 Current treatments for HIV

With the advent of antiretroviral therapy, HIV has, for most, become a chronic manageable condition rather than a terminal illness (Weichseldorfer et al., 2021). There is the potential for escape mutations from the virus, especially in the case of monotherapy with any given antiretroviral drug (Batorsky et al., 2014; Collier et al., 2019), although this has been largely addressed by the advent of cART which utilizes multiple antiretrovirals simultaneously (Y. Liang et al., 2022; Pau & George, 2014; van Welzen et al., 2021). Because there is often a fitness cost associated with each HIV escape mutation, cART therapy creates an environment whereby HIV is unable to generate an escape mutant sufficient to propagate under the combined genetic pressure of the combination regimen (Kühnert et al., 2018; Theys et al., 2018; Zanini et al., 2017). These regimens are often composed of a nucleoside reverse transcriptase inhibitor (NRTI), integrase inhibitor (INI), and protease inhibitor (PI) that target viral transcription, integration into the host genome, and protein processing, respectively (Y. Liang et al., 2022; Pau & George, 2014).

Further developments in the field have led to the development of dual therapies, generally incorporating an NRTI and an INI, with these strategies offering the potential to limit side-effects over tri-therapies while having no apparent reduction in efficacy (Maggiolo et al., 2022; van Welzen et al., 2021). Despite this, side-effects persist with complications such as skin discolouration, neurological effects, and even suicidality (Bengtson et al., 2017; Pau & George, 2014). This leads to reductions in adherence, and ultimately leads to breakthrough infections that allow for transmission of HIV virus to uninfected individuals. Studies examining barriers to HIV care and testing often deal with small or specific populations. Parallels can be drawn between studies, however, with many citing fear, distrust of the medical system, and maintaining an official status as HIV-negative, as reasons for not engaging in care (Ahmed et al., 2022; Jaafari et al., 2022; Mey et al., 2017). Practical barriers such as finances and inability or difficulty reaching health care facilities are also commonly cited as barriers to access (Dombrowski et al., 2015; Posse et al., 2008). Programs tackling certain aspects of this, such as by eliminating travel to health care facilities and/or reminding patients to take their medication, have proven fruitful (Barnabas et al., 2022). Specifically, in South Africa, there is a service whereby patients may have their HIV drugs delivered directly to their homes for a one-time payment of $2-6 USD, which has shown high acceptability and improvements in viral suppression among people living with HIV (Barnabas et al., 2022).

Adherence, combined with lack of access to treatment and stigma, presents one of the largest roadblocks to reaching the 95/95/95 goals set out by the UNAIDS commission (Frescura et al., 2022; Heath et al., 2021). These goals seek to have 95% of HIV-positive individuals diagnosed, 95% of diagnosed people given access to antiretroviral therapy, and 95% of those on therapy achieve viral suppression by 2030 (Frescura et al., 2022; Heath et al., 2021). Overall, coverage globally has been quite successful with similar 90/90/90 targets reached broadly for 2020, except in low-mid prevalence countries (HIV prevalence < 12.1%) (Heath et al., 2021). This is likely a factor of cost related to how much low-mid prevalence countries may be willing or able to spend on antiretroviral therapy interventions. Key populations, such as commercial sex workers, men who have sex with men, and transgender individuals, see lower rates of viral suppression and linkage to care generally as compared to the rest of the public, and this is thought to be primarily a result of social stigmatization and discriminatory practices and policies (Frescura et al., 2022). In order to circumvent potential financial barriers, as well as provide non-stigmatizing and better-

tolerated HIV prevention options, efforts have been made to develop new and/or adjuvant therapies for preventing HIV infections. This is particularly important for young women in the African continent who represent more than half of women living with HIV, with 63% of new infections in women and girls occurring in Sub-Saharan Africa (*20.2 Million Girls and Women Living with HIV | UNAIDS*, 2024; *Global HIV & AIDS Statistics — Fact Sheet | UNAIDS*, 2024).

1.9.2 The immunoquiesence hypothesis and oxytocin

One endogenous immune component that has been found to correlate with protection from HIV acquisition is a reduction in the level of pro-inflammatory cytokines and activated cells at both the systemic and mucosal levels (Fowke et al., 2012; Ossa-Giraldo et al., 2022; Su et al., 2015). In particular, direct molecular evidence has shown that reducing IRF-1 levels—a regulator of anti-viral gene expression—in susceptible $CD4^+$ T cells restricted HIV replication (Su et al., 2015). This paradoxical effect was associated not with a reduction in pro-inflammatory cytokines per-se, but a reduction in the duration of pro-inflammatory response seen in these cells as a result of HIV infection. Additional studies have also demonstrated that increased levels of pro-inflammatory cytokines, as measured in CVL, were associated with reductions of efficacy of a vaginal gel containing the HIV anti-retroviral tenofovir—indicating that inflammation not only is associated with susceptibility but reduces the efficacy of preventative measures (McKinnon et al., 2018). These observations together have led to the 'immunoquiesence hypothesis', which summarizes the idea that activated $CD4^+$ T cells provide optimal targets for HIV infection and that by reducing both levels of activation and inflammatory cytokines, both systemically and at the mucosa, fewer target cells are recruited to the site of infection and the risk of HIV transmission is reduced (Card et al., 2013). This discovery is founded across multiple cohorts of HIV-exposed seronegative (HESN) individuals, including female sex workers in Africa where the rate of heterosexual transmission is at its highest (Blondin-Ladrie et al., 2021; Fourcade et al., 2018; Gluchowski et al., 2022; Röhl et al., 2021; Yao et al., 2014). These women were classified as HESN based on working continually within the sex trade for greater than seven years while remaining HIV-negative (Card et al., 2009; Lajoie et al., 2012; Yao et al., 2014).

These correlates and potential mechanisms of protection identified within the immunoquiesence hypothesis have led to a push to utilize other FDA-approved treatments that are, importantly, not stigmatized or recognized as HIV treatments as alternative or supportive therapies against HIV infection, for example aspirin and hydroxychloroquine (Lajoie et al., 2018, 2021; Mwakyandile et al., 2021). Aspirin trials in particular showed a direct correlation between the levels of aspirin found within a woman's blood and reduction of $CD4^+$ T cells expressing the HIV coreceptor CCR5, as well as a reduction in the expression of the activation marker HLA-DR in the highly inflammatory Th_{17} T-cell subset (Lajoie et al., 2021). While these results present promising indications towards using over-the-counter anti-inflammatory compounds to induce correlates of protection associated with the immunoquiesence hypothesis, they have yet to develop a method that can be used as a vaginal gel or applied to the mucosa directly.

Oxytocin presents a unique opportunity in that it is globally available, FDA-approved, and has shown evidence towards reducing inflammation in atopic dermatitis and LPS-induced inflammation in gut epithelial cells, macrophage, and systemically (Clodi et al., 2008; Deing et al., 2013; Garrido-Urbani et al., 2018; Klein et al., 2016). It possesses multiple routes of administration, notably as both a nasal spray and topically as a vaginal gel (Al-Saqi et al., 2016; Ochiai et al., 2021; Yamasue et al., 2022), and its use as a vaginal gel in the treatment of menopausal vaginal atrophy provides guidelines for its safe use more generally (Abedi et al., 2020; Al-Saqi et al., 2016; Mesbahi et al., 2022; Moussa et al., 2023). Its association with improved wound healing outcomes also suggests it may help to limit or quickly repair epithelial damage (Poutahidis et al., 2013; Varian et al., 2017), with the epithelial barrier being implicated in protection from HIV transmission at the FGT mucosa (Burgener et al., 2015; Carias et al., 2013; Carias & Hope, 2019). There are also few side-effects reported with oxytocin administration, with nasal sprays showing limited reports of headaches and thirst and vaginal gels showing rare cases of vaginal burning or cramping. These symptoms generally seem to be comparable to what is seen in the placebo arms of these studies, with the rarity and general nature of them suggesting feasibility for use in animal or even clinical trials (Guastella et al., 2022; Mesbahi et al., 2022; Yatawara et al., 2016). For these reasons I sought to explore the use of oxytocin as a potential anti-HIV prophylactic at the FGT.

1.10 Gaps of Knowledge

It is unclear if oxytocin is able to reduce inflammation induced in epithelial cells of the FGT, or reduce secretion of pro-inflammatory cytokines as is seen in gut crypt villi Caco2BB cells with LPS stimulation (Klein et al., 2016). Further work examining skin keratinocytes suggests a key role of the OXTR in controlling IL-6, CCL5, and CXCL10 pro-inflammatory cytokine/chemokine secretion, but how this occurs and if it extends to the epithelium of the FGT is unknown (Deing et al., 2013). To date, only the vaginal Vk2 cell line has been examined for effects of oxytocin treatment with results suggesting increased proliferation at doses within physiological ranges (Kallak & Uvnäs-Moberg, 2017). This potentially explains increases in epithelial barrier thickness seen in oxytocin vaginal gel treatments for vaginal atrophy (Santos et al., 2022), but it remains unclear if oxytocin is acting directly or indirectly *in vivo*. It is also unknown how oxytocin vaginal gel might affect the FGT of women who are not experiencing menopausal vaginal atrophy symptoms. Ectocervical and endocervical cells remain unstudied in this area, despite being relevant components of the FGT epithelial barrier. Work implicating oxytocin in the wound-healing enhancement of *L. reuteri* in C57BL/6 mice (Poutahidis et al., 2013) and human subjects (Varian et al., 2017) suggests that oxytocin plays a role in accelerating wound healing, but what remains to be shown is if oxytocin is acting directly to carry out these effects and, if so, on what cell types. Further hints that oxytocin may be acting indirectly on immune cells by an as-of-yet unknown mechanism have been illustrated by intravenous oxytocin administration in healthy men, whereby reductions in LPS-induced TNFα and IL-6 secretion were seen in plasma but not when similar experiments were conducted on *ex vivo* PMBCs and monocytes (Clodi et al., 2008). Further work examining *ex vivo* monocytes and macrophage from human subjects showed no effect of oxytocin in altering LPS-induced inflammation (Ross et al., 2013), suggesting that if oxytocin interacts with immune cells—and perhaps for the process of wound healing as well—that it does so indirectly. Further work is required to determine what cell types oxytocin may be acting on, and the mechanisms by which these effects are taking place.

1.11 Rationale for the study

This is the first study to examine the effects of oxytocin on the immunology of cells from the FGT. The FGT is a major site for the transmission of STIs, such as HIV, and oxytocin is a hormone both involved in the sexual response and implicated in inducing reductions in inflammation and improvements in wound healing—both factors associated with protection from HIV. The ways in which oxytocin regulates innate immunity at the female reproductive tract remain unknown, and the effects of oxytocin on cellular susceptibility to HIV are still uncharacterised. The limited research available on oxytocin showing its anti-inflammatory effects in epithelium have been conducted primarily on gut and skin epithelial cells (Deing et al., 2013; Poutahidis et al., 2013; Welch et al., 2014). It would then follow that it may affect similarly the epithelium of the FGT, with potential additional effects in influencing the activation state of resident immune cells and the mucosal immune milieu. Lowering activation of resident immune cells through a reduction in pro-inflammatory cytokine levels may be one mechanism by which oxytocin is able to exert an anti-inflammatory effect at local immune sites. Since HIV preferentially infects activated $CD4^+$ T cells, a reduction in activation—either directly or indirectly—by oxytocin may reduce susceptibility in these cells. In order to determine if oxytocin has any effect on HIV susceptibility at the FGT it is crucial to utilize a humanized mouse model to enable treatment of human $CD4^+$ T cells within an *in vivo* model.

1.12 Hypothesis

Oxytocin will reduce the expression of pro-inflammatory cytokines and chemokines in cell lines of the lower FGT, improve their ability to heal from wounds, and reduce susceptibility of TKO-hBLT mice to vaginal HIV infection when applied as a vaginal gel.

1.13 Objectives

1. Assess oxytocin's effect on pro-inflammatory cytokines and chemokines produced by FGT epithelium.
2. Determine oxytocin's ability to accelerate wound healing of FGT epithelium *in vitro*.
3. Test oxytocin's ability to prevent vaginal HIV transmission at the female genital tract in a TKO-hBLT humanized mouse model.

Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Cell lines used

The cell lines used to model FGT epithelium in this thesis are VK2/E6E7 (ATCC; Cat# CRL-2616), Ect1/E6E7 (ATCC; Cat# CRL-2614), and End1/E6E7 (ATCC; Cat# CRL-2615) to model vaginal, ectocervical, and endocervical epithelium, respectively. The uterine myometrial cell line PHM1-41 (ATCC; Cat# CRL-3046) was used as a positive control in OXTR validation and signaling studies. Human embryonic kidney (HEK 293) cells (ATCC; Cat# CRL-1573), and their SV40 T antigen transformed derivative (HEK 293T) (ATCC; Cat# CRL-3216), were used likewise as negative controls. Caco2BB (ATCC; Cat# CRL-2102), Jurkat (ATCC; Cat# TIB-152), and MCF-7 (ATCC; Cat# HTB-22) cell lines were used to compare to previously published literature. FGT cell lines were used up to a passage number of 30. All other cell lines were used up to a passage number of 15.

2.1.2 Standard cell passaging procedures and growth conditions for cell lines

All cell lines were grown to a confluency of 80-90% before passaging to new vessels. The media used to culture each cell type is outlined in **Table 2.1**.

Table 2.1. Cell culture media for all cell types used *in vitro*

Cell Type	Culture Medium
Vk2	BPE-containing K-SFM (ThermoFisher; Cat# 17005042) supplemented with 50 µg/mL BPE (ThermoFisher; Cat# 13028014), 0.1 ng/mL rEGF (ThermoFisher; Cat# 10450013), and 0.4 mM $CaCl_2$ **or** BPE-free 'defined' KSFM (ThermoFisher: Cat# 10744019) supplemented with provided proprietary supplement mix
Ect1	
End1	
PHM1-41	DMEM (ATCC; Cat# 30-2002) supplemented with 0.1 mg/mL G-418 (InvivoGen; Cat# ant-gn-1), 2 mM glutamine (ThermoFisher; Cat# 25030081), and 10% FBS (Sigma; Cat# F1051-500ML)
HEK 293	EMEM (ATCC; Cat# 30-2003) supplemented with 10% FBS and 1% P/S (ThermoFisher; Cat# 15140122)
HEK 293T	DMEM supplemented with 10% FBS and 1% P/S
Caco2BB	DMEM supplemented with 0.0.1 mg/mL human transferrin (Sigma; Cat# T0665-50MG), 10% FBS, and 1% P/S
Jurkat	RPMI (ThermoFisher; Cat# 11875093) supplemented with 10% FBS and 1% P/S
PBMCs	
MCF7	EMEM supplemented with 10% FBS, 1% P/S, and 0.01 mg/mL human recombinant insulin (Sigma-Aldrich; Cat# 91077C)

2.1.3 Stimulation of epithelial cells and PHM1-41 cells for inflammatory studies

FGT and HEK 293 epithelial cell lines, as well as PHM1-41 cells, were grown to 80-90% confluence in either T25 flasks (Corning; Cat# 430639) or 6-well plates (Corning; Cat# 3516). For FGT cells the media used was KSFM for experiments that contained BPE, and D-KSFM (ThermoFisher; Cat# 17044019) for those that did not. HEK 293 and PHM1-41 cell lines were cultured in given culture media (**Table 2.1**) for all experiments. Poly(I:C) (HMW)/LyoVec (InvivoGen; Cat# tlrl-piclv) was added directly to the culture vessel for stimulations, and before oxytocin (Sigma-Aldrich; Cat# O3251) in the case of co-treatments. PHM1-41 cells underwent an additional stimulation with LPS (Sigma-Aldrich; Cat# L3024) at 100 ng/mL, and before oxytocin in the case of co-treatments. 'Media' controls were treated with equivalent volumes of PBS (NML Media Services). RNA was collected from cells treated for three hours, while supernatants were collected from separate cultures at twenty-four hours post-treatment to be used in Milliplex (Millipore-Sigma; Cat# HCYTOMAG-60K) cytokine/chemokine assays. Both RNA lysate and supernatants were stored at -80°C until further processing.

2.2 Molecular Work

2.2.1 RNA isolation and cDNA synthesis

RNA was isolated from cells using the Qiagen RNeasy Mini Kit (Qiagen; Cat# 74104) as follows. Cell cultures were washed with PBS, and then lysed following manufacturer's instructions with the addition of 2-mercaptoethanol (BioRad; Cat# 1610710). Lysates were then either stored at -80°C for future analysis, or immediately processed using the following RNeasy protocol. RNA was then quantified using the Take 3 plate (BioTek; Cat# TAKE3) for the BioTek Synergy H1 plate reader, and stored at -80°C for future cDNA synthesis. cDNA synthesis was carried out using the aforementioned isolated RNA and the Qiagen QuantiTect Reverse Transcription Kit following manufacturer protocols (Qiagen; Cat# 205313). cDNA was stored at -80°C for future use in qPCR experiments.

2.2.2 qPCR

qPCR was performed using the LightCycler 96 (Roche; Cat# 05815916001) in LightCycler 480 Multiwell Plates (Roche; Cat# 04729692001). For each gene, 2 µL of cDNA was mixed with 10 µL of SYBR Green Mastermix (Qiagen; Cat# 204145), 1 µL of 10 µM combined forward and reverse primer, and 7 µL of RNAse-free H_2O. 18s rRNA reactions were carried out similarly, but, instead of 1 µL of combined forward and reverse primer and 7 µL of RNAse-free H_2O, 2 µL of 18s rRNA Primer Assay (Qiagen; Cat# 249900) and 6 µL of RNAse-free H_2O was added. Plates were sealed with provided Roche plate covers, and then spun down at 2000 x g for one minute to collect all volumes in the bottom of the wells. qPCR was performed with a fifteen-minute 95°C denaturation, forty-five cycles of 94°C for fifteen seconds followed by 60°C for thirty seconds followed by 72°C for thirty seconds, and then a melt curve analysis of 95°C for ten seconds followed by 65°C for sixty seconds followed by a final hold at 97°C for one second. Relative expression was calculated by subtracting the C_t value of the house keeping gene, 18s rRNA, from a given target gene, and raising 2 to the power of that as a negative exponent:

$$2^{-(C_t \text{ Gene of Interest} - C_t \text{ 18s rRNA})} = \text{Relative expression of gene of interest}$$

Values of expression were then normalized to the media control, for unstimulated samples, or the Poly(I:C) (HMW)/LyoVec control, for stimulated samples, by dividing all relative expression values by the relative expression of the control:

$$\frac{\text{Relative expression of sample}}{\text{Relative expression of control}} = \text{Normalized relative expression of sample}$$

Primer sequences for all given primers are outlined in **Table 2.2**.

Table 2.2. 5' to 3' primer sequences for qPCR

Gene	Forward	Reverse
OXTR	GATTCCAACCGAGGCTCCAG	TCCGCAGGCGAACCTAAAG
IL-1β	CTGTACCTGTCCTGCGTGTT	AGACGGGCATGTTTTCTGCT
IL-6	GAACTCCTTCTCCACAAGCG	ATCTTCTCCTGGGGGTACTGG
IL-8	GAACTGAGAGTGATTGAGAGTGGA	CTCTTCAAAAACTTCTCCACAACC
18s rRNA	Proprietary (Qiagen; Cat# 249900)	Proprietary (Qiagen; Cat# 249900)

2.2.3 Milliplex cytokine/chemokine assays

Milliplex cytokine/chemokine assays were designed to target the analytes GM-CSF, IL-1α, IL-1β, IL-2, IL-6, IL-8, MCP-1, and RANTES (Sigma-Aldrich; Cat# HCYTOMAG-60K). The assays were carried out according to manufacturer protocols, and then the plate was washed with a Bio-Plex Pro II Wash Station (BIO-RAD; Cat# 30034376) through aspiration, and then two rounds of 200 µL wash buffer soaking followed by aspiration. 25 µL of the Detection Antibodies were added to each well, and the plate was incubated at room temperature for one hour, covered in foil, at 300 rpm. 25 µL Streptavidin-Phycoerythrin was then added to each well, and the plate was incubated a further thirty minutes as above. The plate was then washed once more using the same protocol of the Wash Station, and then 150 µL of the Sheath Fluid from the Bio-Plex 200 (BIO-RAD; Cat# 171000205) was added to each well. The plate was incubated for five minutes on a plate shaker at 300 rpm, and then run on the Bio-Plex 200 to quantify fluorescence.

2.2.4 Oxytocin enzyme-linked immunosorbent assays

Oxytocin enzyme-linked immunosorbent assays (ELISAs) were performed using pre-made oxytocin detection ELISAs from Enzo Life Sciences (Cat# ADI-900-153A-153) according to manufacturer protocols. Results were read using a BioTek Synergy H1 plate reader (Cat# H1MG) at 405 nm. Lower limit and upper limits of detection for the assay were 15 and 1050 pg/mL oxytocin, respectively. Non-BPE components were added to the assay at the following

concentrations: epithelial growth factor (EGF) was added at a concentration of 0.15 µg/mL, calcium chloride ($CaCl_2$) was added at a concentration of 0.04 mM, fetal bovine serum (FBS) was added at a concentration of 10% v/v, and penicillin-streptomycin (P/S) was added at a concentration of 50 U/mL. The proprietary supplement mix provided for the alternative BPE-free 'defined' KSFM media formulation was added in its entirety when tested.

2.3 Protein Work

2.3.1 Protein isolation

Protein lysates were made by lysing cells, after washing with PBS, using RIPA buffer of the following formula:

150 mM sodium chloride (NML Media Services)

1% NP-40 (Sigma-Aldrich; Cat# I8896-50ML)

0.5% sodium deoxycholate (Sigma-Aldrich; Cat# D6750-50G)

0.1% SDS (Sigma-Aldrich; Cat# 71725-100G)

50 mM Tris, pH 8.0 (NML Media Services)

ddH_2O to fill total volume

The cOmplete, mini, EDTA-free Protease Inhibitor Cocktail (Millipore-Sigma; Cat# 11836170001) and PhosSTOP (Millipore-Sigma; Cat# 4906837001) tablets were added to the RIPA buffer to inhibit general and phosphatase proteases, respectively. RIPA was added to cells in a ratio of 1 mL RIPA to 1.0 x 10^7 cells. Lysates were then incubated at 4°C with rotation for thirty minutes, and cell debris were removed by centrifugation at 12 000 x g for twenty minutes at 4°C. Supernatants were then aliquoted into separate microcentrifuge tubes, and protein concentration was determined with BCA assay (Millipore-Sigma; Cat# 71285). Protein lysates were stored at -80°C until further use.

2.3.2 Western blots

To perform western blots, protein lysates were thawed on ice, mixed with NuPAGE LDS Sample Buffer (4X) (ThermoFisher; Cat# NP0007), NuPAGE Sample Reducing Agent (10X) (ThermoFisher; Cat# NP0009), and ddH_2O in appropriate volumes to effect a final volume of 15 µL. Samples were then incubated at 70°C for ten minutes in a heating block to denature the protein, and then cooled at 4°C, as per Abcam's protocol (*Sample Preparation for Western Blot | Abcam*, 2024). The ladder for the ECL-based westerns was generated using 5 µL of Prism Ultra Protein Ladder (Abcam; Cat# ab116027), while for Licor-based westerns it was generated using 2 µL of Odyssey One-Colour Protein Molecular Weight Marker (Licor; Cat# 928-40000). Samples and ladders were then run on NuPAGE 4-12% Bis-Tris gels (ThermoFisher; Cat# NP0321BOX) with a 2-(*N*-morpholino)ethanesulfonic acid (MES) running buffer (ThermoFisher; Cat# NP0002) at a constant voltage of 200V for thirty minutes. After the gel had been run it was transferred to a nitrocellulose membrane using the iBlot (ThermoFisher; Cat# IB1001) and associated iBlot Transfer Kit (ThermoFisher; Cat# IB301002) using default settings.

Membranes were then blocked in PBS-T (0.1% Tween-20 [FisherScientific; Cat# BP337-500]) supplemented with 5% skim milk powder for one hour at room temperature. Primary antibody incubations were then performed overnight at 4°C and 300 rpm on a plate shaker in the dark. All ECL-based westerns utilized a probing buffer of PBS-T + 5% skim milk powder, while Licor-based westerns utilized PBS-T + 0.25% BSA (Sigma-Aldrich; Cat# A9085-25G). The primary antibodies used, and their relevant parameters, are listed in **Table 2.3**.

Table 2.3. Primary antibodies used for Western blots

Target	Host Species	Catalogue Number	Concentration	Clonal Status
OXTR	Rabbit	23045-1-AP	1:1000	Polyclonal
OXTR	Rabbit	ab181077	1:1500	Monoclonal
OXTR	Rabbit	ab217212	1:500	Polyclonal
OXTR	Mouse	sc-515809	1:1000	Monoclonal
β-Actin	Mouse	sc-1616	1:2000	Monoclonal

After overnight incubation, blots were washed with PBS-T three times for five minutes each. Then, blots were probed with secondary antibodies against their respective host species appropriate for the detection type, and incubated for one hour at room temperature at 300 rpm on a plate shaker. Licor-based westerns were protected from light with aluminum foil. The secondary antibodies used, and their relevant parameters, are outlined in **Table 2.4**.

Table 2.4. Secondary antibodies used for Western blots

Target	Host Species	Catalogue Number	Concentration	Detection Method
Rabbit	Mouse	sc-2357	1:5000	ECL
Mouse	N/A	sc-516102	1:5000	ECL

N/A – Not applicable

After incubation, blots were again washed with PBS-T three times for five minutes each, and then developed according to their detection method:

ECL-based blots were incubated with a 1:1 mixture of Reagent A and B from the Novex ECL Chemiluminescent Substrate Kit (ThermoFisher; Cat# WP20005) and imaged on the BIO-RAD Universal Hood II Gel Doc (BIO-RAD; Cat# 170-8170), while Licor-based blots were imaged on the Licor Odyssey CLx (Licor; 9140) through the 700 and 800 nm fluorescent channels.

2.3.3 Confocal Microscopy

Confocal microscopy was performed by growing cell lines on autoclaved coverslips (Corning; Cat# 2850-18) in 6-well plates until confluent or near-confluent. Cells were then washed with ice cold PBS before being fixed with 4% paraformaldehyde (Electron Microscopy Sciences; Cat# 15713-S) for ten minutes at room temperature. Coverslips were then washed three times with

PBS-T (0.1% Tween-20), before being blocked in PBS-T + 1% BSA for thirty minutes at room temperature. After blocking, coverslips were incubated with primary antibody in PBS-T + 1% BSA overnight at 4°C. The primary antibodies used are outlined in **Table 2.5**.

Table 2.5. Primary antibodies used for confocal microscopy

Target	Host Species	Catalogue Number	Concentration	Clonal Status
OXTR	Rabbit	23045-1-AP	1:200	Polyclonal
Isotype Control	Rabbit	02-6102	1:2000	Polyclonal

After incubation the coverslips were washed three times with PBS-T, and then incubated with secondary antibody appropriate to the host species in PBS-T + 1% BSA for one hour at room temperature protected from light. The secondary antibodies used for confocal microscopy are outlined in **Table 2.6**. When used, wheat germ agglutinin conjugated to Alexa Fluor 488 (ThermoFisher; Cat# W11261) was included alongside secondary antibody staining at a concentration of 10 ng/mL.

Table 2.6. Secondary antibodies used for confocal microscopy

Target	Host Species	Catalogue Number	Concentration	Fluorophore
Rabbit	Donkey	D-301-C-ABS2	1:1000	APC
Rabbit	Donkey	406421	1:1000	PE

APC = Allophycocyanin; PE = Phycoerythrin

Post-incubation all slides were washed three times with PBS-T and mounted on microscope slides (Globe; Cat# 1324) using mountant containing NucBlue as a nuclear stain (ThermoFisher; Cat# P36981). Slides were then left to cure overnight at room temperature in the dark, and then stored at 4°C in the dark until acquisition.

Slides were imaged using an LSM 700 Confocal Microscope, and concomitant Zen Black 2009 analysis software, from Zeiss.

2.3.4 Peptide binding assays

Peptide binding assays were performed by growing cells to confluency in 6-well plates on coverslips as described for *2.3.3*. After reaching confluency, cells were blocked in 500 µL 2% bovine serum albumin (BSA)-PBS solution for 30 minutes at room temperature. Following blocking biotin-tagged peptide was added at a concentration of 10 ng/mL in 2% BSA-PBS solution for 30 minutes at room temperature. Peptides used are outlined in **Table 2.7**. The plate was placed on ice and each well was washed twice with 500 µL ice cold PBS. After washing, wells were fixed by first adding 150 µL ice cold PBS followed by 150 µL of 2% paraformaldehyde (PFA) (Electron Microscopy Sciences; 15710-S) solution with 30 minutes of fixation allowed on ice. Wells were again washed twice with 500 µL ice cold PBS and then 5 ng/mL streptavidin-PE conjugate was added to each well in 2% BSA-PBS for 20 minutes at room temperature in the dark. Following this, wells were again washed twice with 500 µL ice cold PBS. Coverslips were then mounted on microscope slides using a NucBlue-containing mountant and left to cure overnight at room temperature.

The following day the slides were imaged using an LSM 700 Confocal Microscope, and concomitant Zen Black 2009 analysis software, from Zeiss.

Table 2.7. Peptides used for peptide bindings assays

Target	Peptide	Catalogue Number	Concentration	Fluorophore
OXTR	Oxytocin	AS-23994	10 ng/mL	N/A
Non-specific	Hemagglutinin	HY-P74038	10 ng/mL	N/A
Biotin	PE-Streptavidin	12-4317-87	5 ng/mL	PE

N/A – Not applicable; PE = Phycoerythrin

2.3.5 Mass spectrometry of the oxytocin receptor

Mass spectrometry was carried out on protein lysates, prepared as indicated above, by the NML Mass Spectrometry and Proteomics Core Facility using a triple quadrupole mass spectrometer (TQMS). Protease and phosphatase inhibitors were not added to protein lysates before submission to the mass spectrometry core. Before injection into the mass spectrometer protein lysates underwent trypsin digestion and liquid chromatography purification.

Mass spectrometry runs utilized an untagged peptide identification approach utilizing recombinant OXTR (MyBiosource; Cat# MBS2009192) as the initial marker for identification of OXTR peptide fragments. Cutoff values for peptide identification were at least two fragments detected per run with 99% confidence. The number of spectra matching the OXTR sequence was then used to quantitate OXTR abundance relative to the other samples run.

2.4 Cell Culture Assays

2.4.1 Scratch assays

Scratch assays involved cells being grown to complete confluence in 24-well plates (Corning; Cat# 3524). For those assays that excluded BPE, KSFM media was replaced with D-KSFM and cells were allowed to acclimatize for one day before performance of scratch assays. After cells had reached confluence, scratches were made by purposefully dragging a p200 pipette tip (RAININ; Cat# 30389266) vertically up a given well. Media was then removed and replaced with fresh media, and agonist was added to each well at the concentration indicated. Progress of scratch closure was monitored through phase contrast images taken via an AMG EVOS microscope. Images were analyzed through hand measurement of average scratch gap size in ImageJ software, taken as the average of ten measurements along the scratch.

2.4.2 Ca^{2+} flux assays

Ca^{2+} flux assays were carried out by growing cells to confluence in 6-well plates, and processing them through Abcam's 520 AM dye-based kit (Abcam; Cat# ab233472). After reaching confluence, cells had their media replaced with 0.5 mL of Assay Buffer. 1 µL of 520 AM dye stock solution was added to each well, and the plates were incubated at 37°C for thirty minutes in the dark. Assay Buffer was removed and replaced with 0.4 mL of provided HBSS buffer. Treatments were prepared at 5X concentration in HBSS buffer to the final concentration indicated, and 100 µL was added to a given well to elicit the recorded response. All fluorescent images were captured using the GFP filter channel of an AMG EVOS microscope five seconds post-treatment.

2.5 Humanized Mouse Work

2.5.1 Humanized hBLT mouse model for studying vaginal HIV-infection

Female TKO (C57BL/6 *Rag2*$^{-/-}$*γc*$^{-/-}$*CD47*$^{-/-}$) mice, purchased by the UCLA Humanized Mouse Core, were irradiated to remove murine immune cells and grafted with human hemopoietic stem cells, in collaboration with Dr. Scott Kitchen. Reconstitution of human immune cells was validated at 12-16 weeks and 16-18 weeks post-reconstitution using flow cytometric analysis for this study. These mice were humanized through injection of fetal bone and liver hematopoietic stem cells alongside implantation of fetal human thymus (hBLT). All animal handling protocols were approved by the animal care committee of the NML, and NML veterinary technicians were responsible for all handling of live mice and tissue collection. Weight loss greater than 15% starting weight, as well as signs of hair loss suspected to be a consequence of GvHD, were grounds for early sacrifice. Mice were housed in HEPA-filtered cages in the CL2+ animal care facility of the NML and fed a diet of Uniprim mouse chow (Envigo; Cat# TD.06596).

The design of the humanized mouse treatment strategy is outlined in **Figure 2.1**.

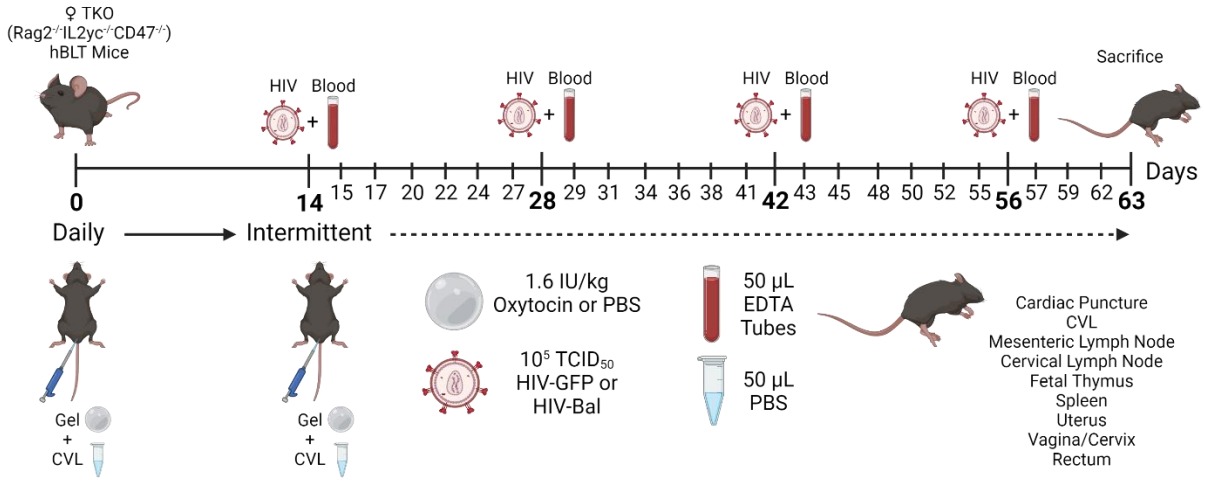


Figure 2.1. Treatment strategy of humanized mice for testing oxytocin vaginal gel as an HIV prophylactic. Female TKO ($Rag2^{-/-}\gamma c^{-/-}CD47^{-/-}$) hBLT mice housed at the NML were treated daily with 20 µL of either a PBS or 1.6 IU/kg oxytocin vaginal 1% HEC gel. Before gel administration, CVL samples were collected by washing the vaginal canal with 50 µL of PBS. Beginning on Day 14, and at every bolded day until Day 56, mice had a 50 µL baseline blood sample taken into EDTA-coated tubes via retro-orbital bleed and were vaginally challenged with either 20 µL of 10^5 $TCID_{50}$ HIV-GFP, 10^5 $TCID_{50}$ HIV-Bal, or a PBS sham infection. All infections were performed under the influence of isoflurane anesthesia. Mice continued to be treated with vaginal gel and CVL collection on the non-bolded days post-Day 14. Sacrifice was scheduled for Day 63 but could be carried out earlier in the case of either a positive HIV test by flow cytometry, weight loss ≥ 15%, or hair loss indicating potential GvHD. Sacrifice was carried out by cervical dislocation under the influence of isoflurane anesthesia. At sacrifice, a CVL was taken, cardiac puncture was performed to exsanguinate, and fetal thymus, mesenteric and cervical lymph node, spleen, uterus, vagina/cervix, and rectal tissue were all collected. In rare cases mice were taken beyond the Day 63 endpoint to try and achieve HIV infection.

2.5.2 Vaginal gel treatments and HIV infection for humanized mice

Gels for vaginal treatment used a vehicle of 1% HEC (Sigma-Aldrich; Cat# 09368-100G) dissolved in ddH_2O and contained either 1.6 IU/kg oxytocin or PBS. Mice were treated with 20 µL of gel daily up until Day 14 where they were then treated on an intermittent schedule as outlined in **Figure 2.1**.

HIV infection was performed vaginally with 20 µL 10^5 $TCID_{50}$ HIV-GFP, 10^5 $TCID_{50}$ HIV-Bal, or PBS beginning on Day 14 after blood draw, and at each other indicated day in **Figure 2.1**. These dosages were consistent with other vaginal infections performed in the literature (Denton et al., 2008; Deruaz et al., 2017; J. Kim et al., 2017). All infections were performed under the influence of isoflurane anesthesia. Presence of HIV positivity was determined by p24 detection and/or GFP positivity in $CD45^+CD4^+$ cells via flow cytometry (below) and was grounds for early sacrifice if detected.

2.5.3 Sample collection for humanized mice

Cervicovaginal lavage (CVL) and blood samples were collected from the mice via wash of the vaginal canal with 50 µL of PBS and 50 µL retro-orbital bleed, respectively. Blood was collected into EDTA-coated microtainer tubes (BD; Cat# 365974) with EDTA-coated minivettes (Starstedt; Cat# 17.2113.050) to prevent coagulation. After collection, CVL and blood were spun at 450 x g for ten minutes at room temperature. Supernatants were stored at -80°C for further analysis. CVL pellets were resuspended in 80 µL flow cytometry staining buffer (PBS + 2 mM EDTA + 2% FBS) and kept on ice until processed through the flow cytometry protocol below. Blood pellets were treated with 500 µL of ACK Lysing Buffer (ThermoFisher; Cat# A1049201) at room temperature for five minutes, and then spun at 300 x g for five minutes. Supernatant was removed, and the pellet was resuspended with 250 µL of PBS. Tubes were then spun at 300 x g, and, after removal of supernatant, pellets were resuspended in 100 µL of flow cytometry staining buffer and kept on ice until flow cytometry staining.

At sacrifice, performed by cervical dislocation under isoflurane anesthetic, a final CVL was collected, exsanguination was performed by cardiac puncture, and fetal thymus, mesenteric and cervical lymph nodes, spleen, uterus, vagina/cervix, and rectal tissue was collected. CVL was processed as above. Blood from exsanguination was treated as above to obtain plasma supernatant for later analysis, but, instead of being treated with ACK lysis buffer, was resuspended in 1 mL of flow cytometry staining buffer and transferred to a 15 mL conical tube (FisherScientific; Cat# 50-809-220). The blood collection tube was then washed twice with 1 mL of flow cytometry staining buffer, and the total was pooled with the initial wash at room temperature until further processing.

Fetal thymus, spleen, lymph node, uterus, vagina/cervix, and rectal tissues were minced with mincing scissors to turn gross tissues into a fine paste. Uterus, vagina/cervix, and rectal tissues were treated with digestive enzymes to further digest tissue samples before further processing. All tissue samples were then resuspended in 1 mL of flow cytometry staining buffer and passed through 40 µm cell strainers (Corning; Cat # 431750) in petri dishes (FisherScientific; Cat# 07-202-011). Cell scrapers (FisherScientific; Cat# 08-100-241) were used to press tissue samples against the cell strainer to further break up tissue pieces and aid in filtration. Strained sample for spleen and thymus was transferred to 15 mL conical tubes, while for the other tissue samples it was transferred to 5 mL polypropylene tubes (FisherScientific; Cat# 14-959-11A). This process was repeated until 5 mL of strained sample was collected. Spleen and thymus samples were brought up to 14 mL of volume with flow cytometry staining buffer and split equally between two 15 mL conical tubes.

The blood, spleen, and thymus samples then had 4 mL of Lymphoprep (Stemcell; Cat# 07851) underlaid below their volumes, and were spun at 1400 rpm for twenty five minutes with no brake. After centrifugation, buffy coats were collected with transfer pipettes (FisherScientific; Cat# 13-711-7M) into new 15 mL conical tubes containing 6 mL of flow cytometry staining buffer. Tubes were brought up to 14 mL with flow cytometry staining buffer, and then mixed through inversion. After mixing, tubes were spun at 1600 rpm for seven minutes, supernatant was removed, and cells were resuspended in flow cytometry staining buffer for further downstream staining. For lymph node, uterus, vagina/cervix, and rectal tissue, the 5 mL polypropylene tubes were spun at 1250 rpm for eight minutes, supernatant was discarded, and pellets were resuspended in flow cytometry staining buffer for further downstream staining.

2.5.4 Flow cytometry for humanized mice

Flow cytometry was performed using the FOXP3 staining buffer set from eBioscience (ThermoFisher; Cat# 00-5523-00). Cells isolated from either blood or tissue were put through the following protocol. Cells were placed into 5 mL polystyrene tubes (Corning; Cat# 352054), washed with 2 mL flow cytometry staining buffer, and then spun at 1200 x g for 8 minutes. Supernatants were discarded, and cells were then mixed with a 1:1 ratio of human (Biolegend; Cat# 422302) and mouse (Biolegend; Cat# 101320) Fc receptor blockers for 10 minutes at room temperature. After incubation cells were treated with the surface stain panel indicated in **Table 2.8** for 30 minutes in the dark at 4°C.

Table 2.8. Surface stain panel for humanized mouse flow cytometry

Target	Catalogue Number	Volume per Sample (µL)	Fluorophore
Live/Dead	L34957	1	V500
CD45	563792	0.625	BUV395
CD4	300556	1.25	BV605
CD8	301042	1	BV650
CCR5	565000	5	BV421
CD69	310942	1.25	PE/Dazzle
HLA-DR	307642	1.25	BV786
CD38	612946	5	BUV496
CD25	557753	5	APC-Cy7

After incubation with the surface stain panel, the cells were washed once again with 2 mL of flow cytometry staining buffer, and spun at 1200 x g for 8 minutes. After discarding supernatants, all tubes were fixed by adding 1 mL of FOXP3 Fixation/Permeabilization Buffer (ThermoFisher; Cat# 00-5123) pre-diluted with FOXP3 Fixation/Permeabilization Diluent (ThermoFisher; Cat# 00-5223) (1 part buffer:3 parts diluent). All samples were incubated for one

hour at room temperature in the dark, and then washed with 2 mL of diluted Permeabilization Buffer (ThermoFisher; Cat# 00-8333) with a spin at 1200 x g for 8 minutes. Supernatants were discarded, and cells were stained with the following intracellular staining panel in **Table 2.9**.

Table 2.9. Intracellular staining panel for humanized mouse flow cytometry

Target	Catalogue Number	Volume per Sample (µL)	Fluorophore
Helios	563951	5	AF647
FOXP3	25-4776-42	2.5	PE-Cy7
p24	6604667	1.25	PE

Cells were incubated for 30 minutes at room temperature in the dark, and then washed by adding 2 mL of Permeabilization Buffer and spinning at 1200 x g for 8 minutes. Cells were washed one more time with the same procedure as above, and then resuspended in 300 µL of Permeabilization Buffer before being read on an LSR II Flow Cytometer (BD; Cat# 640735Q1).

Gating was performed by selecting for single cells through size exclusion comparing FSC-H and FSC-A, and then leukocytes were gated out through comparing SSC-A to FSC-A. Cells were then selected for those simultaneously negative for live/dead staining and positive for hCD45 to gate out dead and murine cells.

2.5.5 Eve Technologies analysis of TKO-hBLT serum and CVL

To analyze both TKO-hBLT humanized mouse serum and CVL from the longitudinal study outlined in *2.5.1*, samples were shipped to Eve Technologies Corporation after inactivation. Both serum and CVL were aliquoted into two 25 µL aliquots and then inactivated with 25 µL 1:5 TritonX-100 (Merck; Cat# X100-100ML) diluted in PBS for one hour at room temperature. Samples were then frozen at -80°C and shipped on dry ice to Eve Technologies Corporation for

Milliplex analysis. Serum samples underwent a further 1:10 dilution, and CVL samples underwent a further 1:2 dilution, before being run by the company which is included in the analysis.

Mouse cytokines and chemokines were detected using the Mouse Cytokine Proinflammatory Focused 10-Plex Discovery Assay Array (Eve Technologies Corporation; Cat# MDF10), while human cytokines and chemokines were detected using the Human Cytokine Proinflammatory Focused 15-Plex Discovery Assay Array (Eve Technologies Corporation; Cat# HDF15).

2.6 Data Analysis

Statistical analyses were performed using GraphPad Prism (version 9). Normality tests were performed using Shapiro-Wilks normality tests. Datasets that followed non-normal distributions were analyzed through non-parametric methods, while those following a normal distribution were analyzed parametrically. If one group within a dataset followed a non-normal distribution then all data for that set of experiments was analyzed non-parametrically.

Parametric comparisons between two groups were carried out utilizing standard t-test analysis, while non-parametric comparisons were made using Mann-Whitney U tests, at $\alpha = 0.05$. Parametric comparisons between more than one group were conducted with analysis of variance (ANOVA) analysis with post-hoc multiple comparisons at $\alpha = 0.05$ and corrections for multiple comparisons. In the case of non-normal data, Kruskal-Wallis tests, with similar post-hoc multiple comparisons and corrections were performed. Longitudinal analysis was analyzed through two-way ANOVA for parametric data, and Mixed-Methods models for non-parametric data, with similar post-hoc analysis and corrections as for comparisons made between more than one group. Mixed-effects models were chosen as they allow for computing two-way ANOVAs for longitudinal datasets while allowing for missing values, which was necessary in analyzing the TKO-hBLT data (***Section 3.6***). In the case of macroscopic analysis, for example confocal staining of the OXTR, results were analyzed on positive or negative signal rather than through direct quantitative analysis.

Chapter 3: Results

3.1 Standard media systems for studying FGT epithelium contain inherent oxytocin

Previous work studying oxytocin at the female genital tract *in vitro* has only been done through one study examining the Vk2 vaginal cell line (Kallak & Uvnäs-Moberg, 2017). This prior work utilized the standard bovine pituitary extract (BPE) containing media conditions recommended by the supplier of the Vk2 cell line, but throughout the course of this thesis I found the BPE additive within this formulation to contain high levels of intrinsic oxytocin. Because of this, I later transitioned my *in vitro* experiments to utilize an alternative BPE-free 'defined' KSFM media rather than the standard BPE-containing KSFM formulation. Typical levels of oxytocin within the body vary from 1 – 50 pg/mL oxytocin, so even small additions of oxytocin may skew experiments replicating physiological conditions (Altemus et al., 1999; Heim et al., 2009; Liu et al., 2022; Martin et al., 2014). This also has pointed implications for studying oxytocin *in vitro*, as it confounds expected oxytocin doses. Given that oxytocin has been used in previous studies in the range of 1 – 100 000 pg/mL (D. Chen et al., 2015; Klein et al., 2014, 2016; Yu et al., 2022), it was necessary to quantify the level of oxytocin contributed by BPE to the media formulation. To accomplish this, oxytocin ELISAs were used to measure oxytocin in BPE-containing and BPE-free KSFM media, as well as in other culture media used throughout this thesis in order to ensure there was no unintentional exposure of the cells to oxytocin.

3.1.1 Measurement of oxytocin in bovine pituitary extract and other media additives

Oxytocin has been previously studied for its effects on epithelial cells *in vitro* (D. Chen et al., 2015; Klein et al., 2014, 2016; Yu et al., 2022), however, without measuring the levels of oxytocin within specific epithelial medias the true dose of oxytocin in these experiments may be confounded. Quantifying this pre-existing oxytocin allows for consideration of this confounding variable when designing and interpreting experiments. I hypothesized that BPE from KSFM

medium would contain detectable levels of oxytocin within the range of previously performed *in vitro* experiments in the literature (1 – 100 000 pg/mL) cells (D. Chen et al., 2015; Klein et al., 2014, 2016; Yu et al., 2022).

In order to measure the levels of oxytocin in each type of media, with and without their particular supplements, oxytocin ELISAs were performed. BPE was added at a concentration of 25 µg/mL, or the indicated relative dilution, as this is the concentration recommended by the manufacturer. Other components were added as detailed in *Section 2.2.4*. Comparisons were made using one-way ANOVA and Holm-Sidak's multiple comparisons test at an $\alpha = 0.05$.

Dilution of BPE to 1:100 that of manufacturer concentration indicated a true concentration of 7410 pg/mL oxytocin in KSFM media with BPE supplement (**KSFM + 1:100 BPE**: 74.1 pg/mL vs. **PBS**: 0 pg/mL, $p < 0.0001$) (**Figure 3.1**). The level of oxytocin found in KSFM alone was not found to be statistically significant (**KSFM No Supplements**: 21.2 pg/mL vs. **PBS**: 0 pg/mL, $p = 0.34$) (**Figure 3.1**). This was also true for KSFM with either EGF or $CaCl_2$ (**KSFM + EGF**: 14.5 pg/mL vs. **PBS**: 0 pg/mL, $p = 0.76$; **KSFM + $CaCl_2$**: 14.4 pg/mL vs. **PBS**: 0 pg/mL, $p = 0.68$) (**Figure 3.1**).

The level of oxytocin found in the alternative BPE-free 'defined' KSFM with or without its supplements was not statistically significant (**Defined KSFM All Supplements**: 12.1 pg/mL vs. **PBS**: 0 pg/mL, $p = 0.96$; **Defined KSFM No Supplements**: 8.5 pg/mL vs. **PBS**: 0 pg/mL, $p = 0.99$) (**Figure 3.1**).

DMEM:F-12, DMEM, EMEM, and their supplements of FBS and P/S showed no detectable level of oxytocin (p-values > 0.05) (**Figure 3.1**).

In summary, oxytocin was detected in KSFM media supplemented with BPE at a concentration of 7410 pg/mL oxytocin. This level of oxytocin is within the range used for *in vitro* experiments (1 – 100 000 pg/mL) studying oxytocin's effects in epithelial cells (D. Chen et al., 2015; Klein et al., 2014, 2016; Yu et al., 2022). This supports the hypothesis that KSFM media supplemented with BPE contains detectable levels of oxytocin within the range used in several *in vitro* studies.

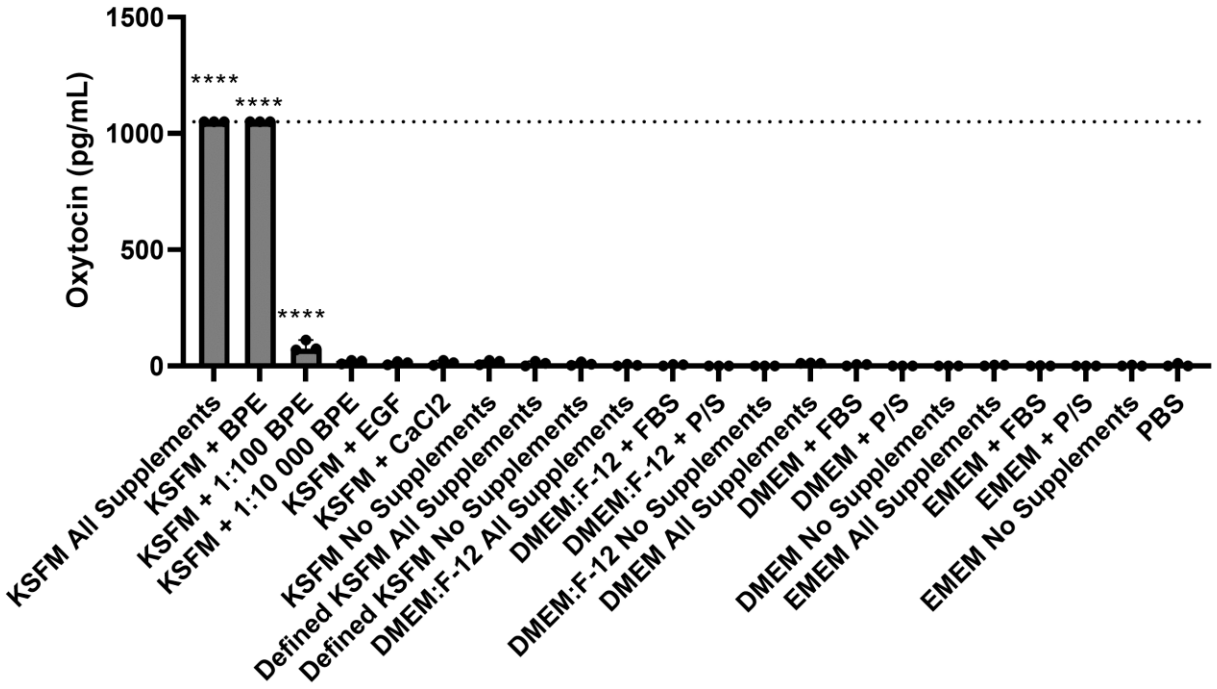


Figure 3.1 Measurement of oxytocin concentration in bovine pituitary extract (BPE), a supplement used in keratinocyte serum-free medium (KSFM) for epithelial culture through ELISA. Various media with and without supplements were tested for levels of oxytocin. The dashed line represents the upper limit of detection of the ELISA. Concentrations of additives: BPE (undiluted) – 25 000 ng/mL; EGF – 0.1 ng/mL; $CaCl_2$ – 0.4 mM; FBS – 10% vol/vol; P/S – 10 U/mL penicillin, 10 µg/mL streptomycin. 'All supplements' for Defined KSFM used the entire supplement tube provided with the media. Comparisons made using one-way ANOVA and Holm-Sidak's multiple comparisons test with an $\alpha = 0.05$. n = 3. **** $p < 0.0001$

3.2 Oxytocin has limited effects on modulation of cervical pro-inflammatory cytokine transcription and secretion

Based on published evidence supporting oxytocin as an anti-inflammatory immune mediator in both gut and skin epithelium (Deing et al., 2013; Klein et al., 2016), I studied its role in influencing the inflammatory status of epithelium from the lower female genital tract (FGT). The epithelia of the lower FGT are among the first host cells that encounter HIV during heterosexual mucosal exposure. As a pro-inflammatory environment at the vaginal mucosa is associated with increased risk of HIV-1 infection, I aimed to determine if exposure of FGT epithelium to oxytocin could reduce pro-inflammatory cytokine gene transcription and decrease pro-inflammatory cytokine secretion in these cells. This was done by measuring changes in the production of the select inflammatory cytokines/chemokines IL-1α, IL-1β, IL-2, IL-6, CXCL8/IL-8, GM-CSF, MCP-1, and RANTES.

Vk2, Ect1, and End1 cells, derived from normal tissues of the lower FGT, were used to model vaginal, ectocervical, and endocervical epithelium, respectively. Vk2, Ect1 and End1 cells were exposed to titrating concentrations of oxytocin, and three- or twenty-four-hours following oxytocin treatment cells and culture supernatants were collected for measurement of RNA transcripts and cytokine secretion, respectively. Three-hour treatment was chosen for studying RNA transcript levels based on preliminary experiments indicating the greatest potential effect of oxytocin, with twenty-four hours being chosen for cytokine production and secretion based on similar grounds.

To determine whether oxytocin could modulate the immune responsiveness of FGT epithelium to an immune stimulus, high molecular weight poly(I:C)/LyoVec was used. Poly(I:C)/LyoVec is a synthetic dsRNA polymer complexed with LyoVec, a transfecting reagent. Unlike naked poly(I:C), which is recognized by TLR3, poly(I:C)/LyoVec is sensed by RIG-I/MDA-5 (Gitlin et al., 2006; Kato et al., 2005). I chose poly(I:C)/LyoVec because it was shown to successfully elicit inflammatory responses in Vk2, Ect1, and End1 cells in preliminary experiments, and because RIG-I receptor utilized by this compound has also been implicated in the response to HIV-1 infection (Berg et al., 2012; P. Li et al., 2016; Y. Wang et al., 2013). HEK 293, an immortalized human embryonic kidney cell line, was included as a negative control for

oxytocin response as HEK 293 cells are known to be negative for the oxytocin receptor (OXTR) (Jasper et al., 1995; Ma et al., 2013). PHM1-41, an immortalized myometrial fibroblast cell line, was included as an experimental positive control for the assay due to the fact that it is known to express the receptor and respond to oxytocin (Burghardt et al., 1999; Monga et al., 1996, 1999). PHM1-41 cells, unfortunately, do not have published literature detailing a response to poly(I:C)/LyoVec or changes in pro-inflammatory cytokines in response to oxytocin treatment. As this is the first study of its kind to examine the effect of oxytocin treatment on pro-inflammatory cytokine transcription and secretion in epithelial cells, these cells were used to control the experiment as well as possible. PHM1-41 cells have been shown to respond to lipopolysaccharide (LPS) and, for this reason, LPS was included as a stimulation condition for PHM1-41 cells exclusively. (Hutchinson et al., 2014; Mishra & Hirsch, 2020; Monga et al., 1996; Rajagopal et al., 2015). Because Vk2, Ect1, and End1 cells do not express the receptor necessary to respond to LPS—toll-like receptor 4 (TLR4)—this stimulation condition could not also be used for these cells.

3.2.1 Oxytocin's effect on female genital tract epithelium pro-inflammatory cytokine transcription

To examine the effect of oxytocin on the transcription of pro-inflammatory cytokine genes, the RNA transcript levels of IL-1β, IL-6, and CXCL8 in Vk2, Ect1, and End1 cells were assessed using relative quantitative real time polymerase chain reaction (qPCR). IL-1β, IL-6, and CXCL8 were chosen as the focus of this study, because these three pro-inflammatory genes have been shown to be constitutively expressed at the female genital tract (B. Basso et al., 2005; Fichorova et al., 2004; Tuddenham et al., 2021; Hebisch et al., 2004) as well as other epithelial sites such as ocular and oral epithelium (F. G. Basso et al., 2016; Santacruz et al., 2015). I hypothesized, given oxytocin's previous anti-inflammatory actions in gut and skin epithelium, that oxytocin would decrease expression of IL-1β, IL-6, and CXCL8 in all three FGT cell lines.

Vk2, Ect1, and End1 cells grown as monolayers in bovine pituitary extract (BPE)-containing or BPE-free 'defined' KSFM media were treated with oxytocin and/or poly(I:C)/LyoVec when they reached 80% confluency. Oxytocin dosage was titrated 0, 10, 100,

1000, and 10 000 pg/mL doses for work in BPE-containing media, and at 0, 1, 10, 100, 1000, and 10 000 pg/mL in work performed in BPE-free media. CXCL8 analysis, as well as HEK 293 and PHM1-41 controls, were not added until the experiments utilizing BPE-free media. Poly(I:C)/LyoVec and LPS stimulations were carried out at 1 µg/mL and 100 ng/mL, respectively, based on prior in-house work for poly(I:C)/LyoVec and published literature for LPS (Hutchinson et al., 2014; Rajagopal et al., 2015). All expression values are normalized to the housekeeping gene 18S rRNA. Experiments were carried out in duplicate within an assay and three separate experiments were performed, except in the case of HEK 293 cells where five experiments were done. Analysis of oxytocin dose-effects was performed using Kruskal-Wallis tests with Dunn's multiple comparisons. Effects of stimulation were analyzed by comparing the 0 pg/mL oxytocin dose of the unstimulated condition to the 0 pg/mL oxytocin dose of the stimulated condition through unpaired t-tests at $\alpha = 0.05$.

Work utilizing BPE-containing KSFM media showed no significant effect of oxytocin on any of the three cell lines at any dose of oxytocin for IL-1β or IL-6 (p-values > 0.05) (**Figure 3.2**). No significant effect of poly(I:C)/LyoVec stimulation was seen for all three cell lines (p-values > 0.05) (**Figure 3.2**).

In agreement, work utilizing BPE-free KSFM showed that oxytocin had no significant impact on IL-1β, IL-6, or CXCL8 transcript levels in all three FGT cell lines (p-values > 0.05) (**Figure 3.3**). Significant increases in IL-6 transcript levels in Ect1 cells and CXCL8 transcript levels in End1 cells were observed in response to poly(I:C)/LyoVec stimulation, indicating successful response to stimulation in these genes (Ect1: IL-6: **Stimulated**: 8.8×10^{-5} vs. **Unstimulated**: 2.2×10^{-5}, p = 0.014; End1: CXCL8: **Stimulated**: 3.2×10^{-4} vs. **Unstimulated**: 1.2×10^{-4}, p = 0.047) (**Figure 3.3**). In agreement with the literature, the level of IL-1β, IL-6, and CXCL8 transcripts were significantly increased in PHM1-41 cells following LPS-stimulation (PHM1-41: IL-1β: **Stimulated**: 2.0×10^{-4} vs. **Unstimulated**: 1.7×10^{-5}, p = 0.0001; PHM1-41: IL-6: **Stimulated**: 1.2×10^{-3} vs. **Unstimulated**: 2.3×10^{-4}, p = 0.027; PHM1-41: CXCL8: **Stimulated**: 7.0×10^{-3} vs. **Unstimulated**: 1.0×10^{-3}, p = 0.0045) (**Figure 3.3**) (Hutchinson et al., 2014; Mishra & Hirsch, 2020; Rajagopal et al., 2015). Oxytocin had no effect on IL-6 or CXCL8 transcript levels in stimulated Ect1 or End1 cells, respectively (p-values > 0.05) (**Figure 3.3**). Surprisingly,

oxytocin also showed no effect on IL-1β, IL-6, or CXCL8 transcript levels in PHM1-41 cells after either poly(I:C)/LyoVec or LPS treatment (p-values > 0.05) (**Figure 3.3**).

Fold change analysis was then performed, relative to the 0 pg/mL oxytocin treatment for each stimulation, in order to control for potential inter-run variability that may have limited the power to detect oxytocin's effects. After this conversion, work utilizing BPE-containing KSFM media identified a reduction in IL-1β transcripts in Ect1 cells at an oxytocin dose of 1000 pg/mL and 10 000 pg/mL (Ect1: IL-1β: **1000 pg/mL**: 0.63 fold vs. **0 pg/mL**: 1 fold, p = 0.049; Ect1: IL-1β: **10 000 pg/mL**: 0.59 fold vs. **0 pg/mL**: 1 fold, p = 0.01) (**Figure 3.4**). End1 cells also showed reductions in IL-1β and IL-6 transcripts at doses of 1000 and 10 000 pg/mL, respectively (End1: IL-1β: **1000 pg/mL**: 0.48 fold vs. **0 pg/mL**: 1 fold, p =0.049; End1: IL-6: **10 000 pg/mL**: 0.50 fold vs. **0 pg/mL**: 1 fold, p = 0.025) (**Figure 3.4**).

In BPE-free media, there remained no significant effect of oxytocin on IL-1β, IL-6, or CXCL8 transcription in any of the three FGT epithelial cell lines (p > 0.05) (**Figure 3.5**).

Together, this shows that in BPE-containing media oxytocin reduced the fold change transcript levels of IL-1β in both Ect1 and End1 cells, and of IL-6 in End1 cells only. These effects were not replicated in BPE-free 'defined' KSFM, however. This supports the hypothesis that oxytocin is able to downregulate pro-inflammatory gene transcription in FGT epithelial cells but must be interpreted with caution given the lack of response to oxytocin seen in PHM1-41 positive control cells.

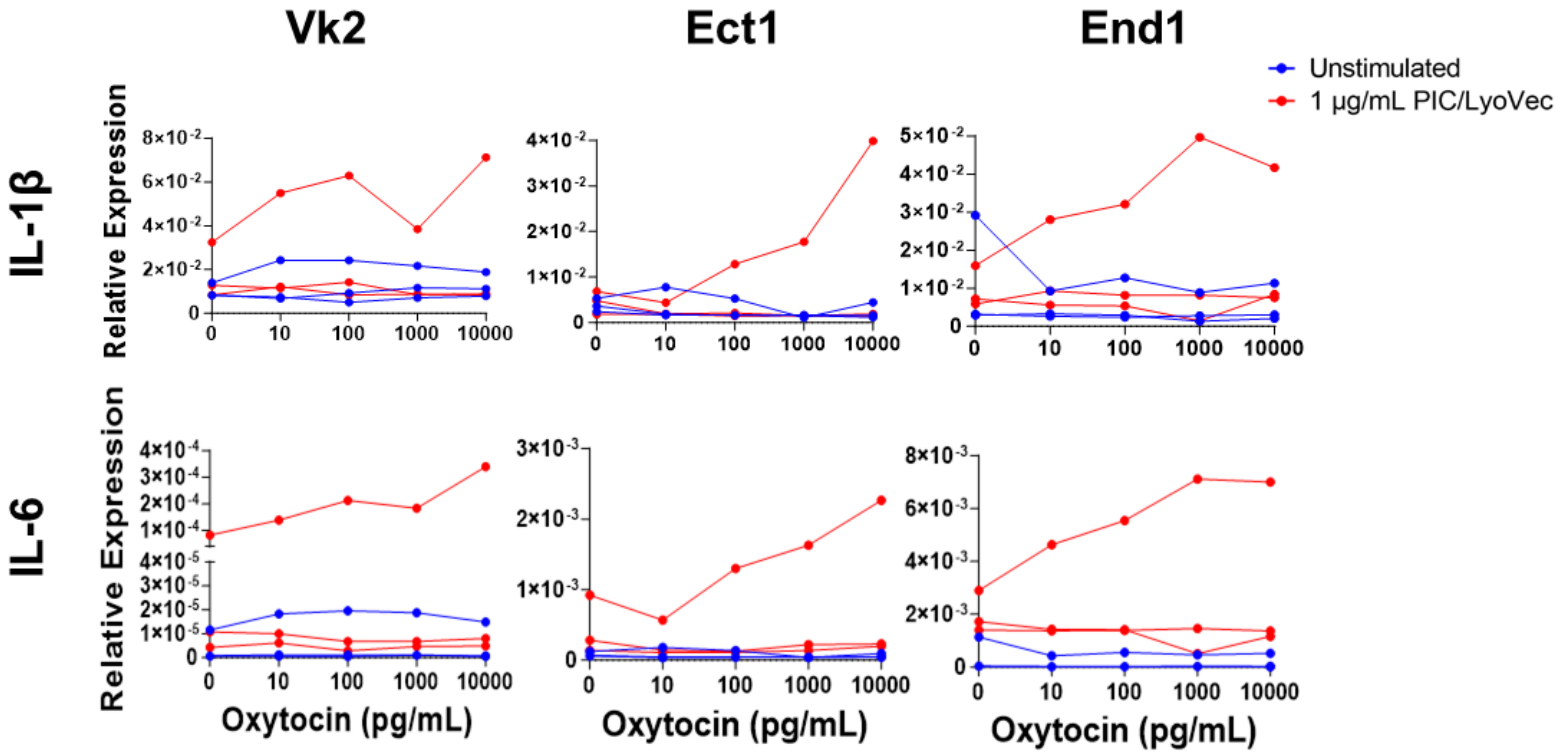


Figure 3.2 Oxytocin does not reduce relative gene expression of pro-inflammatory cytokines in epithelial cell lines of the female genital tract grown in BPE-containing KSFM media as measured through qPCR. Vk2, Ect1, and End1 cell lines of the female genital tract were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 10, 100, 1000, and 10 000 pg/mL doses. Gene expression is represented as relative expression of a gene normalized to 18S rRNA expression. Graphs represent individual experiments connected through lines. Effect of stimulation tested through unpaired t-test with the PIC/LyoVec condition compared to the Unstimulated condition at 0 pg/mL oxytocin. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation. n = 3.

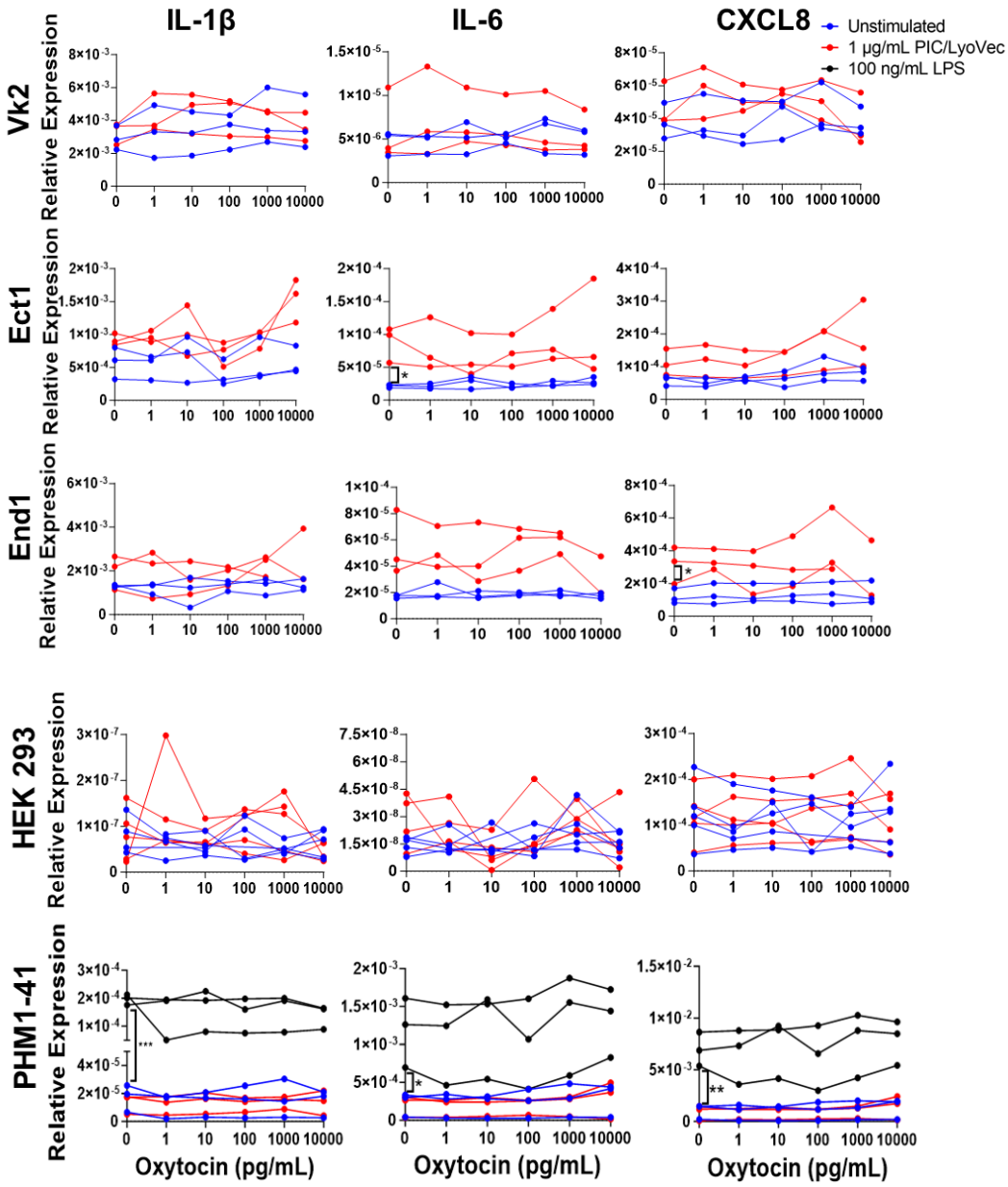


Figure 3.3 Oxytocin does not reduce relative expression of pro-inflammatory cytokines in epithelial cell lines of the female genital tract grown in BPE-free defined media as measured through qPCR. Vk2, Ect1, and End1 cell lines of the female genital tract were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 1, 10, 100, 1000, and 10 000 pg/mL doses. PHM1-41 cells underwent similar treatment, with an additional 100 ng/mL LPS stimulation (•) for PHM1-41 cells. Gene expression is represented as relative expression of a gene normalized to 18S rRNA expression Graphs represent individual experiments connected through lines. Effect of stimulation tested through unpaired t-test (vertical significance bars) with PIC/LyoVec or LPS conditions compared to Unstimulated at 0 pg/mL oxytocin. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation (horizontal significance bars). n = 3 for all samples except for HEK 293 n =5. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

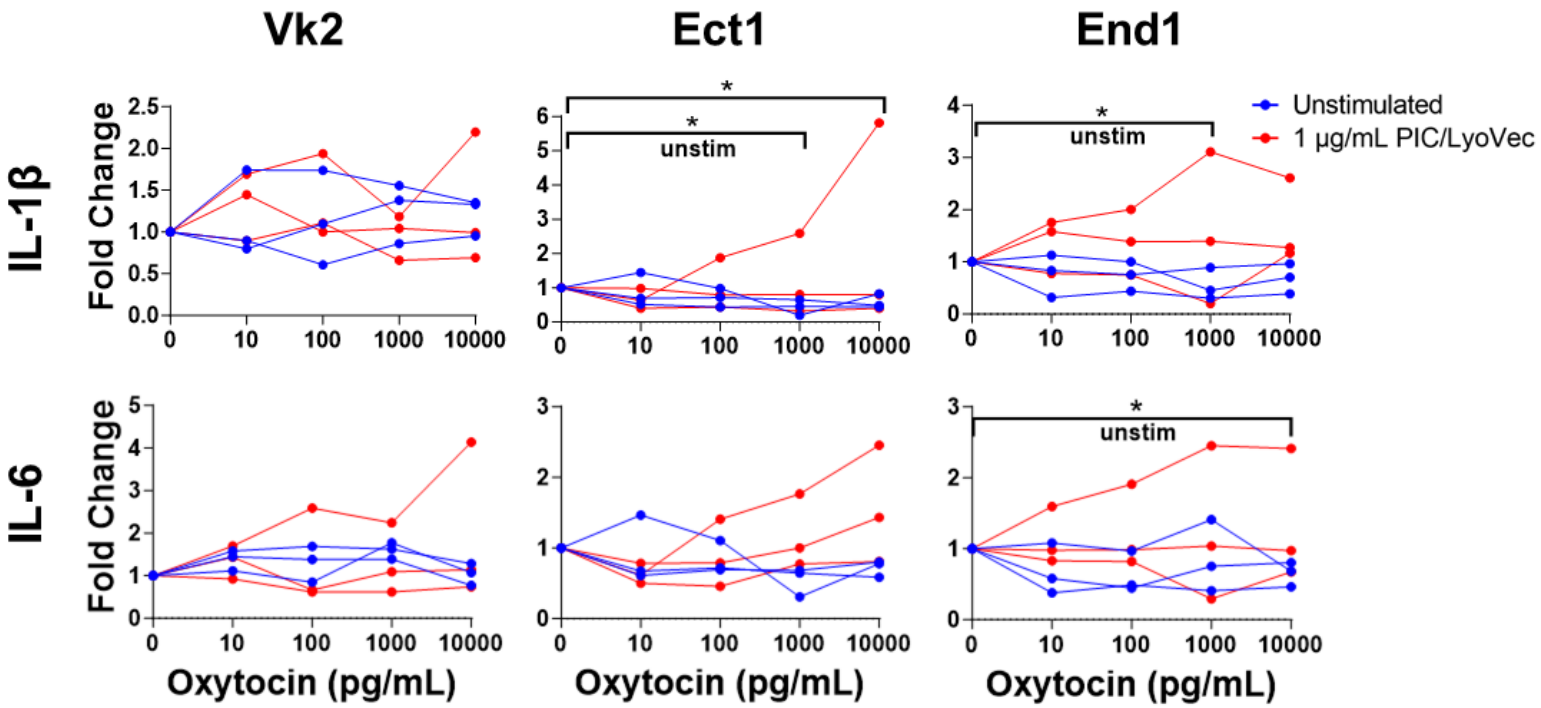


Figure 3.4 Oxytocin reduces fold change expression of pro-inflammatory cytokines in epithelial cell lines of the female genital tract grown in BPE-containing KSFM media as measured through qPCR (transformed from Figure 3.2). Vk2, Ect1, and End1 cell lines were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 10, 100, 1000, and 10 000 pg/mL doses. Gene expression is represented as fold change relative to 0 pg/mL oxytocin for each stimulation condition. All values are normalized to 18s rRNA expression. Graphs represent individual experiments connected through lines. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation. n = 3. * $p < 0.05$

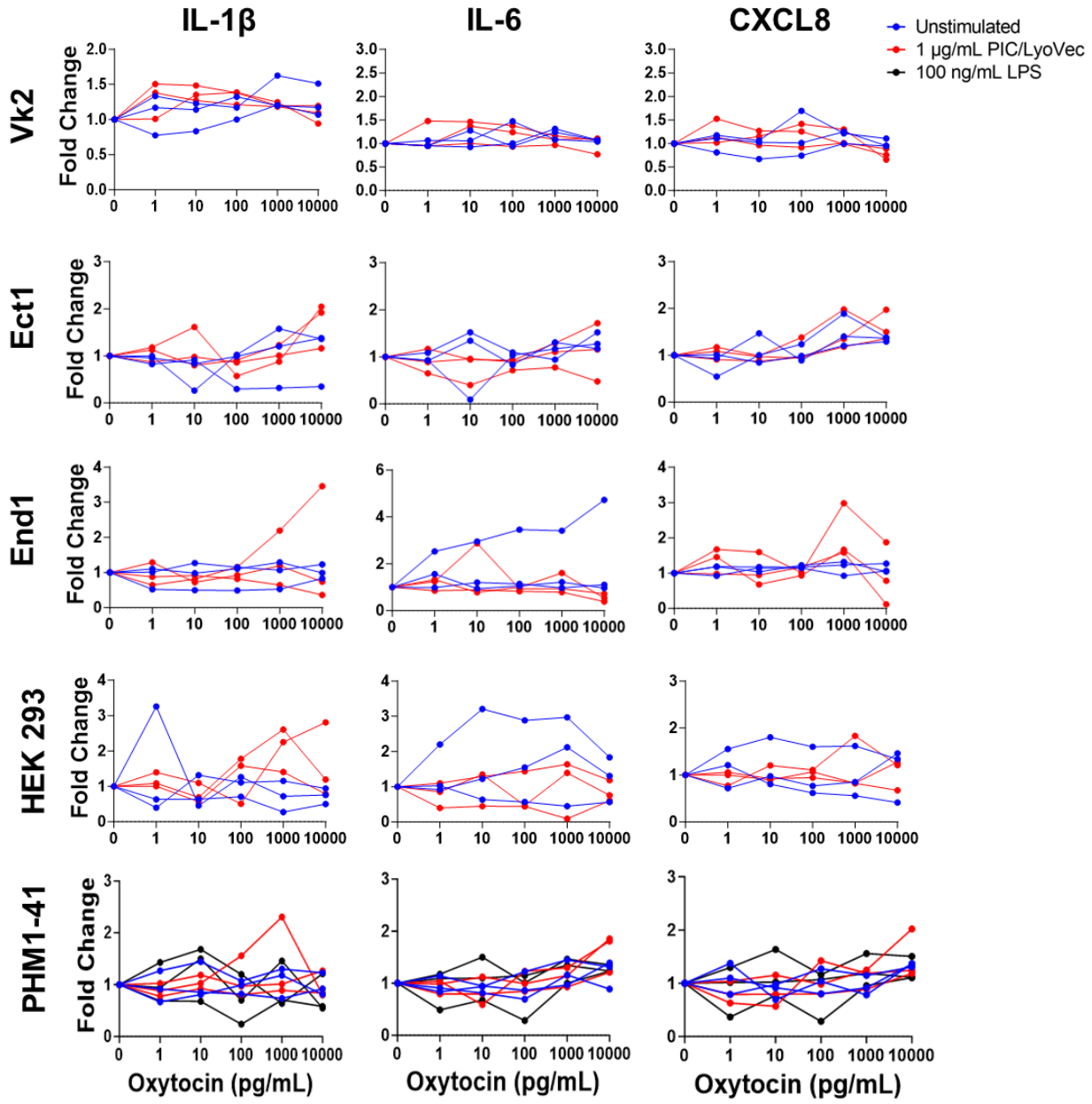


Figure 3.5 The effect of oxytocin on the fold change expression of pro-inflammatory cytokines in epithelial cell lines of the female genital tract grown in BPE-free defined media as measured through qPCR (transformed from Figure 3.3). Vk2, Ect1, and End1 cell lines of the female genital tract were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 1, 10, 100, 1000, and 10 000 pg/mL doses. PHM1-41 cells underwent similar treatment, with an additional 100 ng/mL LPS stimulation (•) for PHM1-41 cells. Gene expression is represented as fold change relative to 0 pg/mL oxytocin for each experimental condition. All values are normalized to 18s rRNA expression. Graphs represent individual experiments connected through lines. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation. n = 3 for all samples except for HEK 293 n =5.

3.2.2 Oxytocin's effect on female genital tract epithelium pro-inflammatory cytokine/chemokine secretion

To examine oxytocin's ability to modulate pro-inflammatory cytokine/chemokine production at the protein level in epithelial cells of the lower FGT, multiplexed ELISA was performed to quantify cytokine secretion. Due to potential incongruences between RNA transcription and protein secretion, it is necessary to capture the effects of oxytocin on both to sufficiently characterize its effects on Vk2, Ect1, and End1 pro-inflammatory cytokine/chemokine production. Based on previous reports of oxytocin's associations with reductions in inflammation for both gut and skin (Deing et al., 2013; Klein et al., 2016) epithelial cells, I hypothesized that Vk2, Ect1, and End1 cells treated with oxytocin would show reduced secretion of GM-CSF, IL-1α, IL-1β, IL-2, IL-6, IL-8, MCP-1, and RANTES. These cytokines/chemokines were chosen because they were shown to be expressed by at least one of the Vk2, Ect1, or End1 cell lines and were associated with inflammation at the mucosa (Fichorova et al., 2002; Govender et al., 2014; Kouokam et al., 2011; Sathe & Reddy, 2014; Sharkey et al., 2007, 2012; Zalenskaya et al., 2015).

Vk2, Ect1, and End1 cells were grown in monolayer liquid culture in either BPE-containing KSFM or BPE-free 'defined' KSFM and treated with oxytocin and/or poly(I:C)/LyoVec when they reached 80% confluency. Oxytocin dosage was titrated 0, 10, 100, 1000, and 10 000 pg/mL doses for work in BPE-containing media, and at 0, 1, 10, 100, 1000, and 10 000 pg/mL in work performed in BPE-free media. HEK 293 and PHM1-41 controls were not added until the experiments utilizing BPE-free media. Poly(I:C)/LyoVec and LPS stimulations were carried out at 1 μg/mL and 100 ng/mL, respectively, based on prior in-house work for poly(I:C)/LyoVec and published literature for LPS (Hutchinson et al., 2014; Rajagopal et al., 2015). After twenty-four hours, cell supernatants were collected and spun down to eliminate cellular debris/free-floating cells. Supernatants were then analyzed using Milliplex multiplexed cytokine assays and values were interpolated from standard curves. Fold changes represent values as a fraction of the untreated value for a given stimulation condition. The Milliplex assay has a range of detection for each cytokine/chemokine of 3.2 – 10 000 pg/mL. Analysis of oxytocin treatment effect was performed using Kruskal-Wallis tests with Dunn's multiple comparisons at $\alpha = 0.05$. Effect of stimulation

was analyzed by comparing the 0 pg/mL oxytocin dose of the unstimulated condition with the 0 pg/mL oxytocin dose of the stimulated condition through unpaired t-tests at an $\alpha = 0.05$.

Oxytocin treatment in BPE-containing KSFM had no effect on any of the tested cytokines for any of the three FGT epithelial cell lines (p-values > 0.05) (**Figure 3.6**). Poly(I:C)/LyoVec stimulation did increase GM-CSF and IL-1α in Vk2 cells, GM-CSF and RANTES in Ect1 cells, and GM-CSF, IL-1α, IL-6, IL-8, and RANTES in End1 cells (Vk2: GM-CSF: **Stimulated**: 33.0 pg/mL vs **Unstimulated**: 4.9 pg/mL, p = 0.026; Vk2: IL-1 α**: Stimulated**: 197.4 pg/mL vs. **Unstimulated**: 15.5 pg/mL, p = 0.0049) (**Figure 3.6A**); (Ect1: GM-CSF: **Stimulated**: 76.0 pg/mL vs. **Unstimulated**: 9.6 pg/mL, p = 0.033; Ect1: RANTES: **Stimulated**: 665.7 pg/mL vs. **Unstimulated**: 3.7 pg/mL, p = 0.0079) (**Figure 3.6B**); (End1: GM-CSF: **Stimulated**: 127.9 pg/mL vs. **Unstimulated**: 5.4 pg/mL, p = 0.031; End1: IL-1α: **Stimulated**: 557.2 pg/mL vs. **Unstimulated**: 67.5 pg/mL, p = 0.015; End1: IL-6: **Stimulated**: 3350.5 vs. **Unstimulated**: 71.7 pg/mL, p = 0.043; End1: IL-8: **Stimulated**: 5620.1 pg/mL vs. **Unstimulated**: 376.2 pg/mL, p = 0.019; End1: RANTES: **Stimulated**: 5940.5 pg/mL vs. **Unstimulated**: 418.2 pg/mL, p = 0.0035) (**Figure 3.6C**). For each of these upregulated cytokines/chemokines no effect of oxytocin was seen on cytokine/chemokine secretion at any dose (p-values > 0.05) (**Figure 3.6**).

Work in BPE-free 'defined' KSFM showed similar results with a general lack of response to oxytocin (p-values > 0.05) (**Figure 3.7**), except in the case of reduced End1 IL-1α in response to 1 pg/mL oxytocin treatment (**1 pg/mL**: 7.6 pg/mL vs. **0 pg/mL**: 11.9 pg/mL, p = 0.04) (**Figure 3.7C**). Stimulation with poly(I:C)/LyoVec resulted in increased secretion of IL-1α in Ect1 cells, and IL-6 in End1 cells (Ect1: IL-1α: **Stimulated**: 10.8 pg/mL vs. **Unstimulated**: 3.9 pg/mL, p = 0.04) (**Figure 3.7B**); (End1: IL-6: **Stimulated**: 62.8 pg/mL vs. **Unstimulated**: 17.9 pg/mL, p = 0.01) (**Figure 3.7C**). No effect of oxytocin was seen on either IL-1α or IL-6 of these stimulated cytokines/chemokines (p-values > 0.05) (**Figure 3.7B and C**). Negative control HEK 293 cells showed no response to oxytocin, as expected (p-values > 0.05) (**Figure 3.7D**), but unexpectedly PHM1-41 positive control cells also showed no response (p-values > 0.05) (**Figure 3.7E**). This was also true after LPS-induced stimulation of IL-6, IL-8, MCP-1, and RANTES in PHM1-41 cells (p-values > 0.05) (**Figure 3.7E**).

Data was then transformed to fold change relative to the 0 pg/mL oxytocin treatment for each stimulation to control for potential inter-experimental variability that may reduce the power

to detect an effect of oxytocin treatment. After conversion to fold change for experiments performed in BPE-containing KSFM, a mild increase in Ect1 IL-8 secretion was seen for 100 pg/mL and 10 000 pg/mL oxytocin doses (Ect1: IL-8: **100 pg/mL**: 1.1 fold vs. **0 pg/mL**: 1 fold, p = 0.037; Ect1: IL-8: **10 000 pg/mL**: 1.1 fold vs. **0 pg/mL**: 1 fold, p = 0.048) (**Figure 3.8B**).

In BPE-free 'defined' KSFM media, fold change analysis again showed a reduction in End1 IL-1α at both 1 pg/mL and 10 000 pg/mL oxytocin (End1: IL-1α: **1 pg/mL**: 0.7 fold vs. **0 pg/mL**: 1 fold, p = 0.04; End1: IL-1α **10 000 pg/mL**: 0.7 fold vs. **0 pg/mL**: 1 fold, p = 0.02) (**Figure 3.9C**).

In summary, oxytocin was shown to increase the fold change of IL-8 secretion in Ect1 cells cultured in BPE-containing KSFM, and reduce both absolute levels and fold change of IL-1α secretion in End1 cells cultured in BPE-free 'defined' KSFM. These results, however, must be interpreted in the context of a lack of response in positive control PHM1-41 cells. Therefore, this data rejects the hypothesis that oxytocin reduces pro-inflammatory cytokine secretion of FGT epithelial cells, and instead supports a variable effect based on cell type and media conditions.

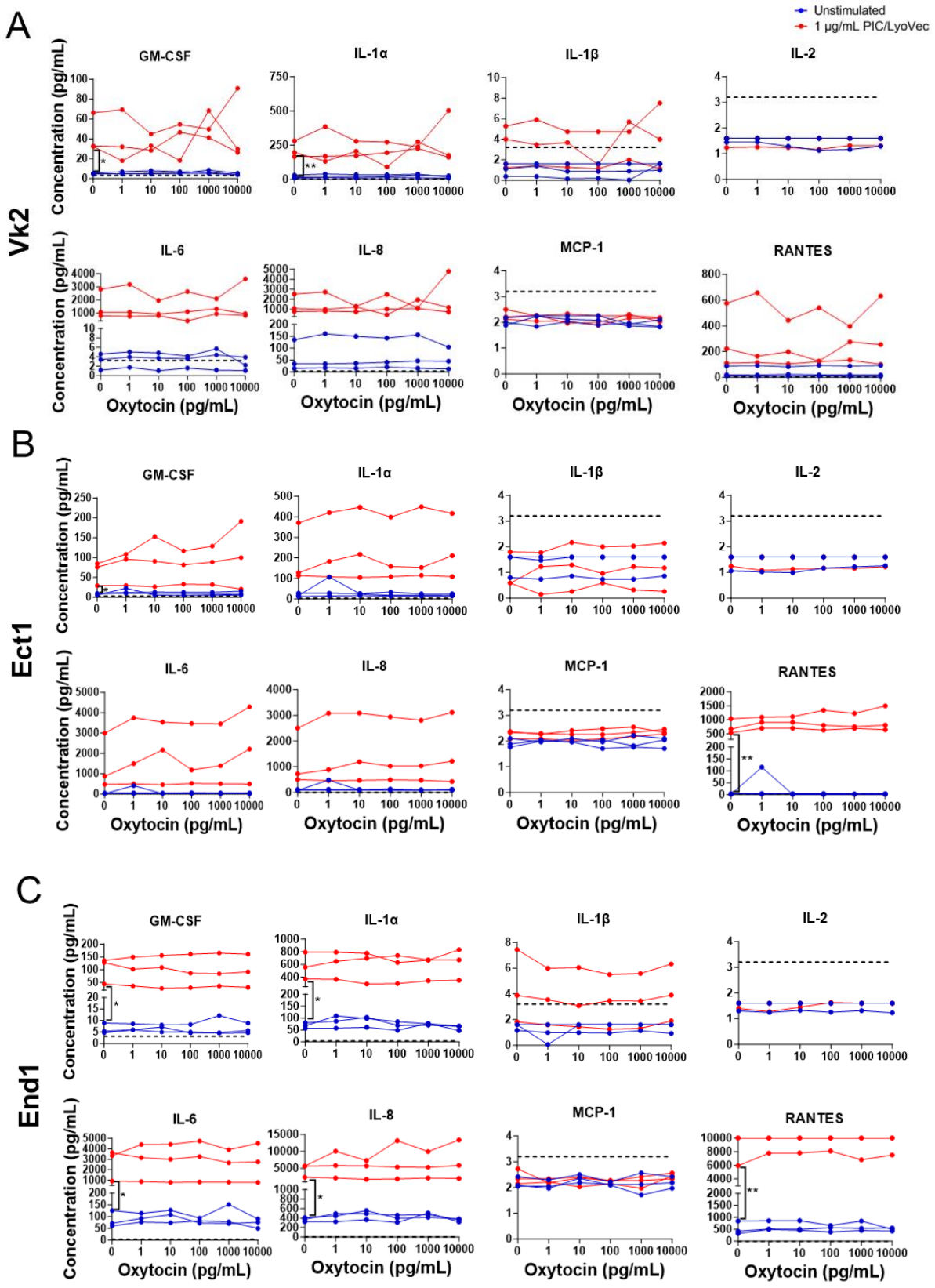
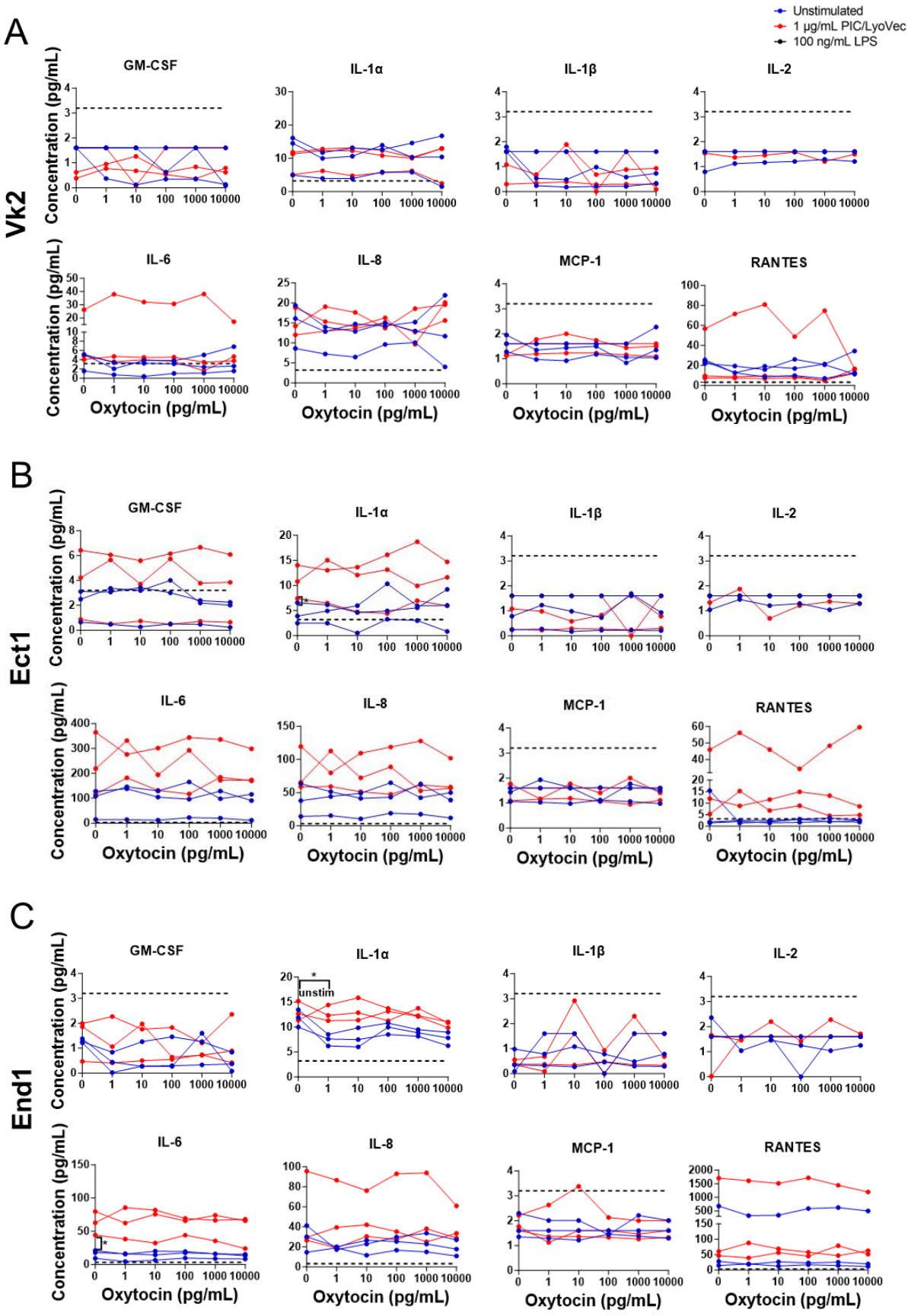

A
Vk2
B
Ect1
C
End1
Unstimulated
1 µg/mL PIC/LyoVec
GM-CSF
IL-1α
IL-1β
IL-2
IL-6
IL-8
MCP-1
RANTES
Concentration (pg/mL)
Oxytocin (pg/mL)

Figure 3.6 Oxytocin does not modulate absolute cytokine secretion in female genital tract epithelial cell lines grown in BPE-containing KSFM media as measured through multiplexed ELISA. Vk2 (A), Ect1 (B), End1 (C) cell lines were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 1, 10, 100, 1000, and 10 000 pg/mL doses. Graphs represent individual experiments connected through lines. Effect of stimulation tested through unpaired t-test (vertical significance bars) with PIC/LyoVec compared to Unstimulated at 0 pg/mL oxytocin. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation (horizontal significance bars). Dashed lines (···) indicate the lower limit of detection - 3.2 pg/mL, with values below this threshold obtained by extrapolation. n = 3. * $p < 0.05$ ** $p < 0.01$


A
Vk2
Unstimulated
1 µg/mL PIC/LyoVec
100 ng/mL LPS
GM-CSF
IL-1α
IL-1β
IL-2
IL-6
IL-8
MCP-1
RANTES
Concentration (pg/mL)
Oxytocin (pg/mL)
B
Ect1
C
End1
unstim
*

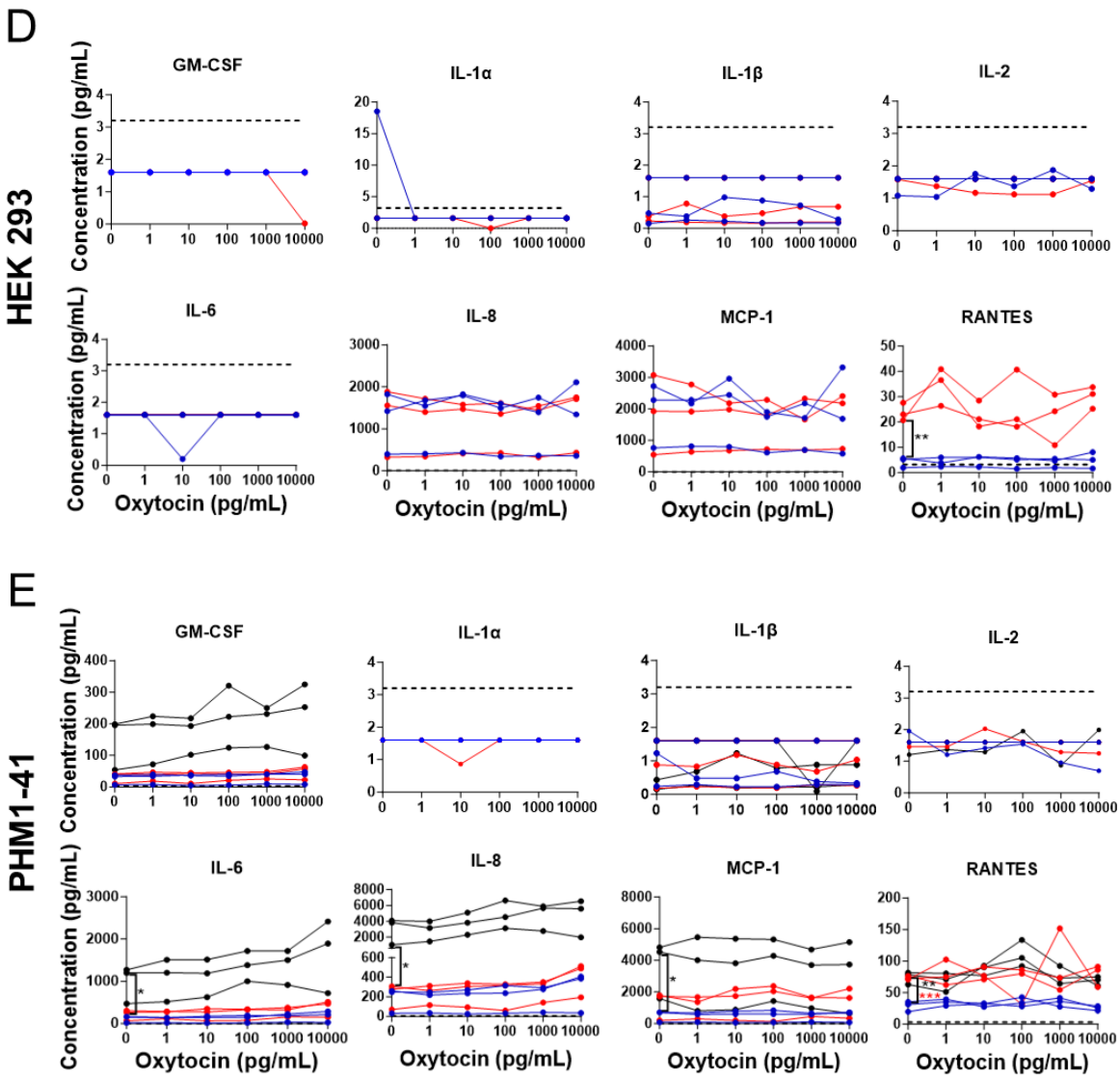


Figure 3.7 Effect of oxytocin on absolute cytokine secretion in female genital tract epithelial cell lines grown in BPE-free defined media as measured through multiplexed ELISA. Vk2 (A), Ect1 (B), End1 (C), HEK 293 (D), and PHM1-41 (E) cell lines were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 1, 10, 100, 1000, and 10 000 pg/mL doses. PHM1-41 cells underwent an additional 100 ng/mL LPS stimulation (•) with oxytocin co-treatment. Concentrations listed are not normalized. Treatment group linked to significant changes are denoted below asterisk bars or colour-coded through asterisks, where applicable. Graphs represent individual experiments connected through lines. Effect of stimulation tested through unpaired t-test (vertical significance bars) with PIC/LyoVec or LPS conditions compared to Unstimulated at 0 pg/mL oxytocin. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation (horizontal significance bars). Dashed lines (···) indicate the lower limit of detection - 3.2 pg/mL, with values below this threshold obtained by extrapolation. n = 3. * $p < 0.05$

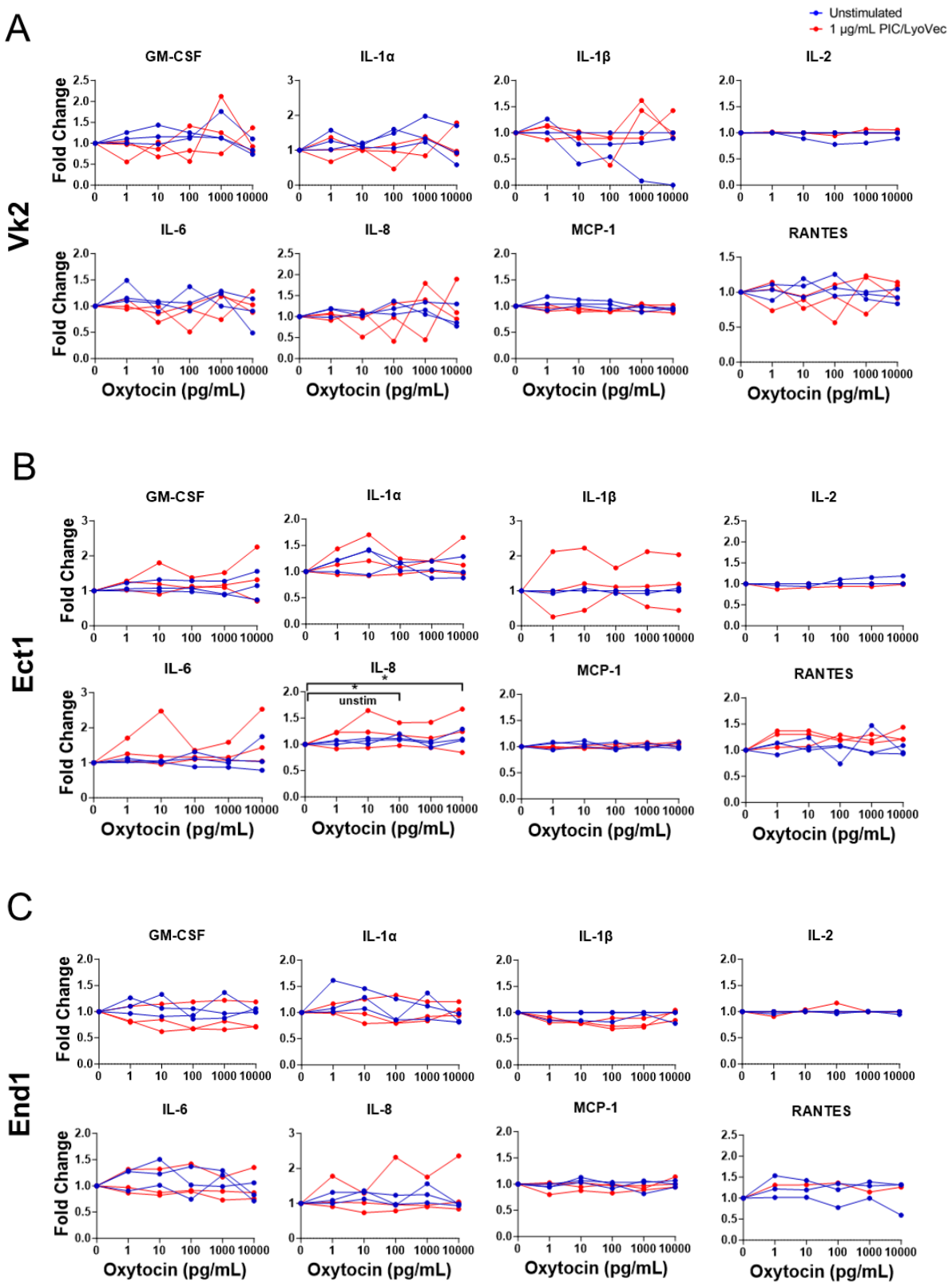
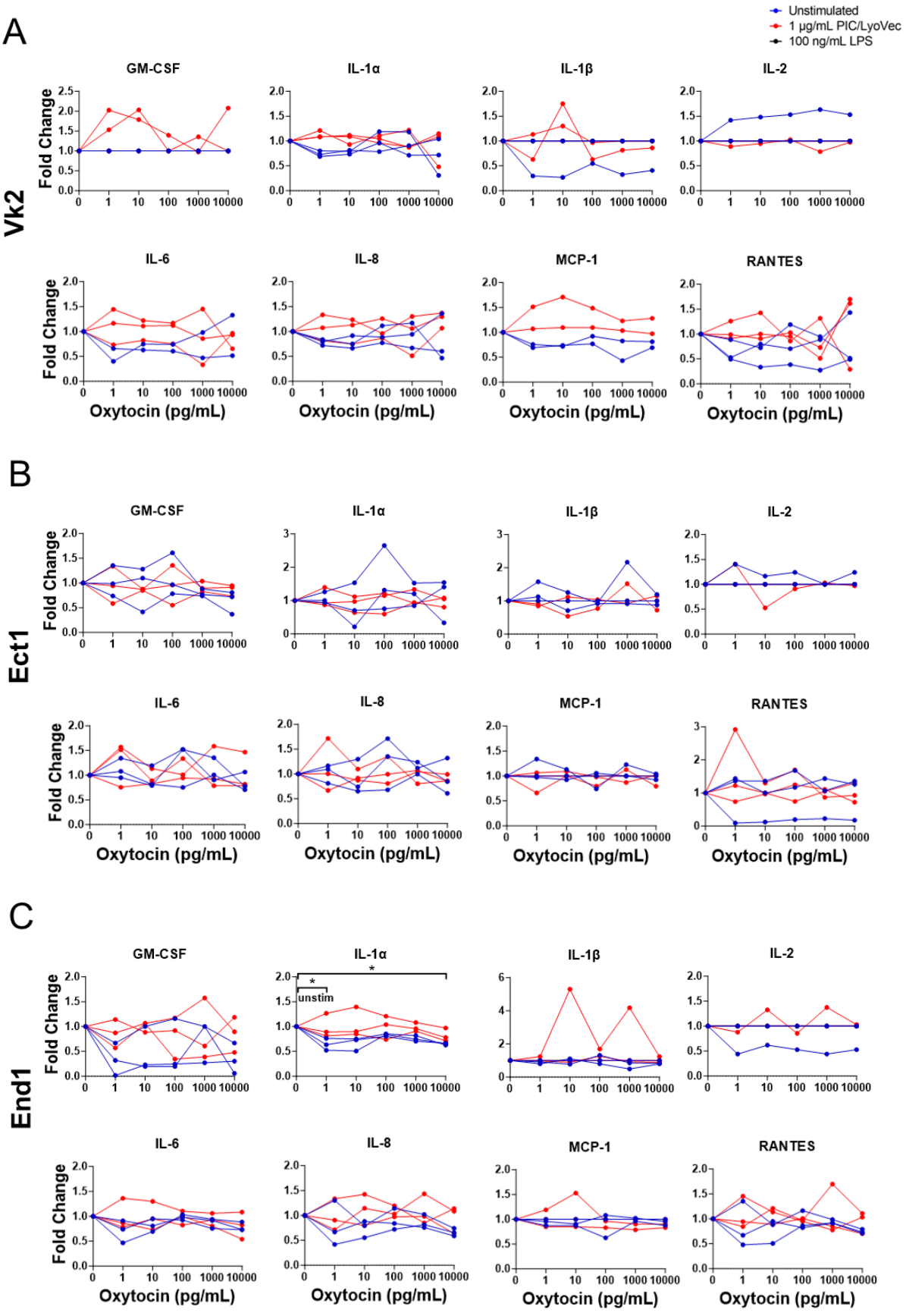

A
Unstimulated
1 µg/mL PIC/LyoVec
Vk2
GM-CSF
IL-1α
IL-1β
IL-2
IL-6
IL-8
MCP-1
RANTES
Fold Change
Oxytocin (pg/mL)
B
Ect1
unstim
*
C
End1

Figure 3.8 Oxytocin modulates fold change of cytokine secretion in female genital tract epithelial cell lines grown in BPE-containing KSFM media as measured through multiplexed ELISA (transformed from Figure 3.6). Vk2 (A), Ect1 (B), End1 (C) cell lines were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 1, 10, 100, 1000, and 10 000 pg/mL doses. Fold change is relative to the 0 pg/mL oxytocin unstimulated condition for each cell line. Treatment group linked to significant changes are denoted below asterisk bars. Graphs represent individual experiments connected through lines. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation (horizontal significance bars). n = 3. * $p < 0.05$


A
Vk2
Unstimulated
1 µg/mL PIC/LyoVec
100 ng/mL LPS
GM-CSF
IL-1α
IL-1β
IL-2
IL-6
IL-8
MCP-1
RANTES
Fold Change
Oxytocin (pg/mL)
B
Ect1
C
End1
unstim
*

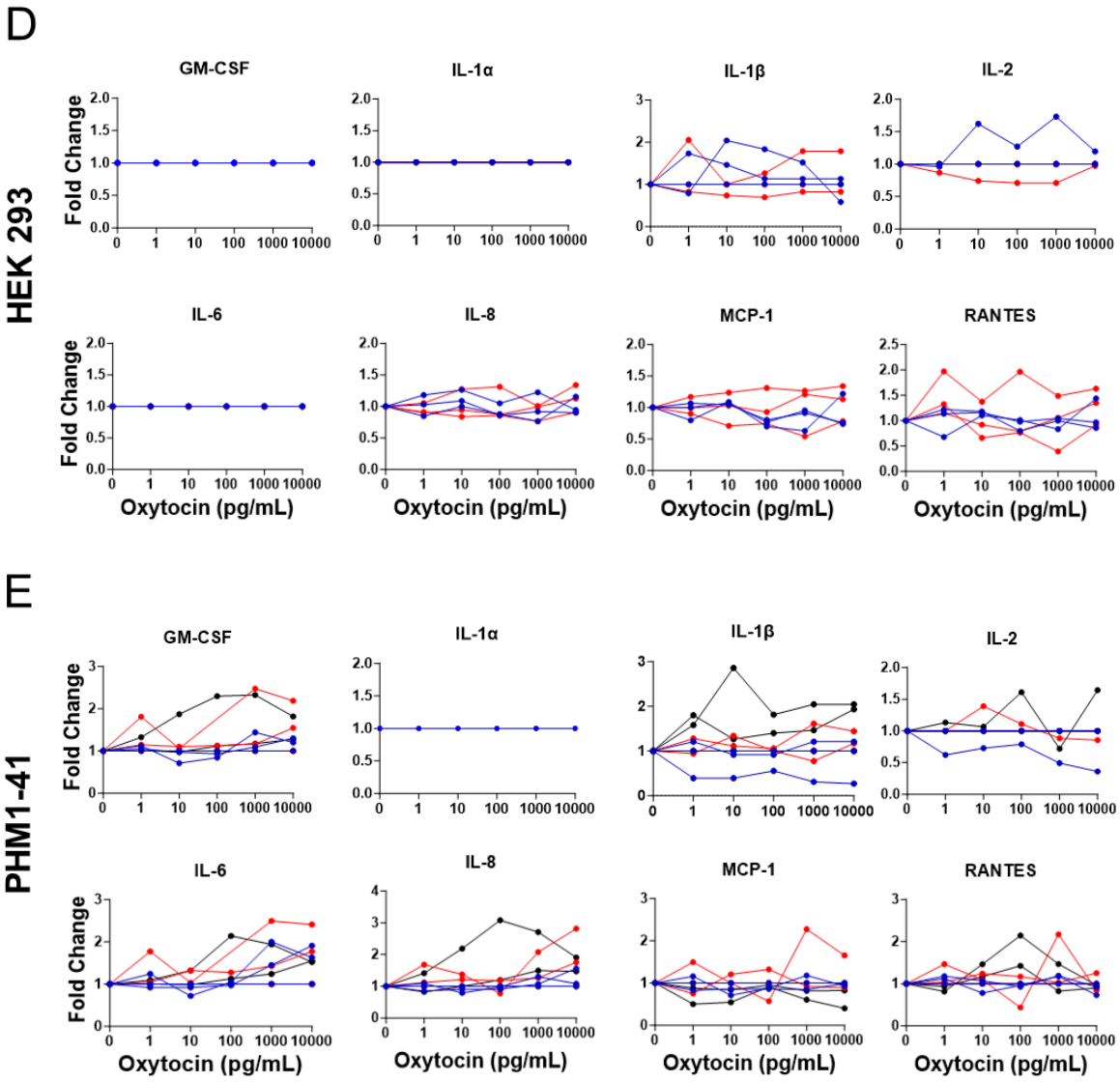


Figure 3.9 Effect of oxytocin on fold change cytokine secretion in female genital tract epithelial cell lines grown in BPE-free defined media as measured through multiplexed ELISA (transformed from Figure 3.7). Vk2 (A), Ect1 (B), End1 (C), HEK 293 (D), and PHM1-41 (E) cell lines were either unstimulated (•) or stimulated with 1 µg/mL poly(I:C)/LyoVec (•) and co-treated with oxytocin at 0, 10, 100, 1000, and 10 000 pg/mL doses. PHM1-41 cells underwent an additional 100 ng/mL LPS stimulation (•) with oxytocin co-treatment. Fold change is relative to the 0 pg/mL oxytocin unstimulated condition for each cell line. Treatment group linked to significant changes are denoted below asterisk bars. Graphs represent individual experiments connected through lines. Comparisons for the effect of oxytocin are made using Kruskal-Wallis tests with Dunn's multiple comparisons relative to the 0 pg/mL condition for a stimulation (horizontal significance bars). n = 3.

3.3 Oxytocin demonstrates inhibitory effects on FGT epithelial wound healing

3.3.1 Oxytocin's effect on female genital tract epithelium wound healing in vitro

Barrier disruption has been associated with increased risk of HIV acquisition (Burgener et al., 2015; Carias & Hope, 2019), as displayed in previous HIV vaginal gel prophylaxis trials (Van Damme et al., 2002; D. Wilkinson et al., 2002). The role of oxytocin in promoting wound healing of FGT epithelium remains to be studied as a potential adjuvant therapy for promoting a healthy and robust epithelial barrier. Oxytocin has been shown to enhance wound healing effects in gut and skin epithelium (D. Chen et al., 2015; Gouin et al., 2010; Işeri et al., 2008), and so I wished to study the effects of oxytocin in assisting wound healing in FGT epithelium. Analysis of wound healing *in vitro* can be performed through scratch assays that measure the migration of cells into a wound created in monolayer culture (Bobadilla et al., 2019; C. C. Liang et al., 2007). For this reason, I carried out scratch assays on each of the FGT epithelial cell lines and hypothesized that oxytocin would promote improved wound healing in *in vitro* scratch assays.

To assess whether oxytocin had direct effects on wound healing of FGT epithelium, Vk2, Ect1, and End1 cells were grown in monolayer culture to confluency and then scratched with a p200 pipette tip to create a wound. The cells were then rinsed with PBS to remove cellular debris and given fresh media containing oxytocin. Work in BPE-containing media only utilized a dose of 10 000 pg/mL oxytocin, while work in BPE-free 'defined' media utilized doses of 1 pg/mL, 10 pg/mL, 100 pg/mL, 1000 pg/mL, 10 000 pg/mL, and 10 µg/mL oxytocin. 100 ng/mL TGF-β1 was also used as a positive control for wound healing, as TGF-β1 has been used in epithelial scratch assays to promote wound healing previously (Governa et al., 2019; L. Wang et al., 2011). Experiments in BPE-containing media utilized time points of 0 hrs, 3 hrs, 8 hrs, and 12 hrs due to increased growth kinetics, while experiments in BPE-free 'defined' media utilized time points of 0 hrs, 8 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs, and 120 hrs. Images were taken on a light microscope and then analyzed through ImageJ, with scratch areas being measured by hand and converted to a relative decimal fraction of the initial scratch size. Comparisons were made between treatments and the untreated conditions using a mixed-effects model with Dunnet's multiple comparisons at $\alpha = 0.05$.

Representative light microscopy images have been shown to visualize wound closure in experiments with (**Figure 3.10A**) and without (**Figure 3.11**) BPE. Gross analysis of light microscope images in BPE-free work revealed that TGF-β1-treated cells reached full, or near-full, closure at experimental end points, indicating that Vk2, Ect1, and End1 cells responded positively to a wound-healing stimulus (**Figure 3.11**). Vk2 and End1 cell lines that were treated with TGF-β1 took 120 hrs to reach near-full wound closure, while TGF-β1-treated Ect1 cells took 48-72 hrs (**Figure 3.11**).

Work in BPE-containing media revealed that oxytocin did not have any significant effect on wound healing in Vk2, Ect1, or End1 cells (Vk2: 8 hrs: **10 000 pg/mL**: 73 AU vs. **0 pg/mL**: 62 AU, p = 0.33; Ect1: 8 hrs: **10 000 pg/mL**: 142 AU vs. **0 pg/mL**: 140 AU, p = 0.91; End1: 12 hrs: **10 000 pg/mL**: 144 AU vs. **0 pg/mL**: 151 AU, p = 0.71) (**Figure 3.10**). Work in BPE-free 'defined' KSFM demonstrated that Vk2 cells treated with 10 000 pg/mL oxytocin showed significantly larger wound sizes at 72 hrs, 96 hrs, and 120 hrs (Vk2: 72 hrs: **10 000 pg/mL**: 90% vs. **0 pg/mL**: 51% wound size, p = 0.005; Vk2: 96 hrs: **10 000 pg/mL**: 85% vs. **0 pg/mL**: 53% wound size, p = 0.02; Vk2: 120 hrs: **10 000 pg/mL**: 85% vs. **0 pg/mL**: 57% wound size, p = 0.03) (**Figure 3.11A**). End1 cells also showed significant decreases in wound healing with 10 pg/mL and 10 µg/mL oxytocin doses at 96 hrs and 120 hrs (End1: 96 hrs: **10 µg/mL**: 68% vs. **0 pg/mL**: 45% wound size, p = 0.04; End1: 120 hrs: **10 pg/mL**: 63% vs. **0 pg/mL**: 38% wound size, p = 0.02; End1: 120 hrs: **10 µg/mL**: 62% vs. **0 pg/mL**: 38% wound size, p = 0.04) (**Figure 3.11C**). No effect of oxytocin was seen on Ect1 wound healing (p-values > 0.05) (**Figure 3.11B**). As expected, Vk2 and End1 cells showed reduced wound sizes in response to TGF-β1 treatment (Vk2: 120 hrs: **100 ng/mL TGF**: 9% vs. **0 pg/mL**: 54% wound size, p = 0.0003; End1: 96 hrs: **100 ng/mL TGF**: 21% vs. **0 pg/mL**: 45% wound size, p = 0.03) (**Figure 3.11A and C**), but no effect was seen in Ect1 cells (p-values > 0.05) (**Figure 3.11B**).

In summary, oxytocin had no effect on wound healing in Vk2, Ect1, and End1 cells grown in BPE-containing KSFM, but showed inhibition of wound healing in Vk2 and End1 cells cultured in BPE-free 'defined' KSFM. This data rejects my hypothesis that oxytocin treatment leads to improved *in vitro* wound healing of FGT epithelium, and instead suggests that oxytocin inhibits wound healing in FGT epithelium in a dose- and media-dependent manner.

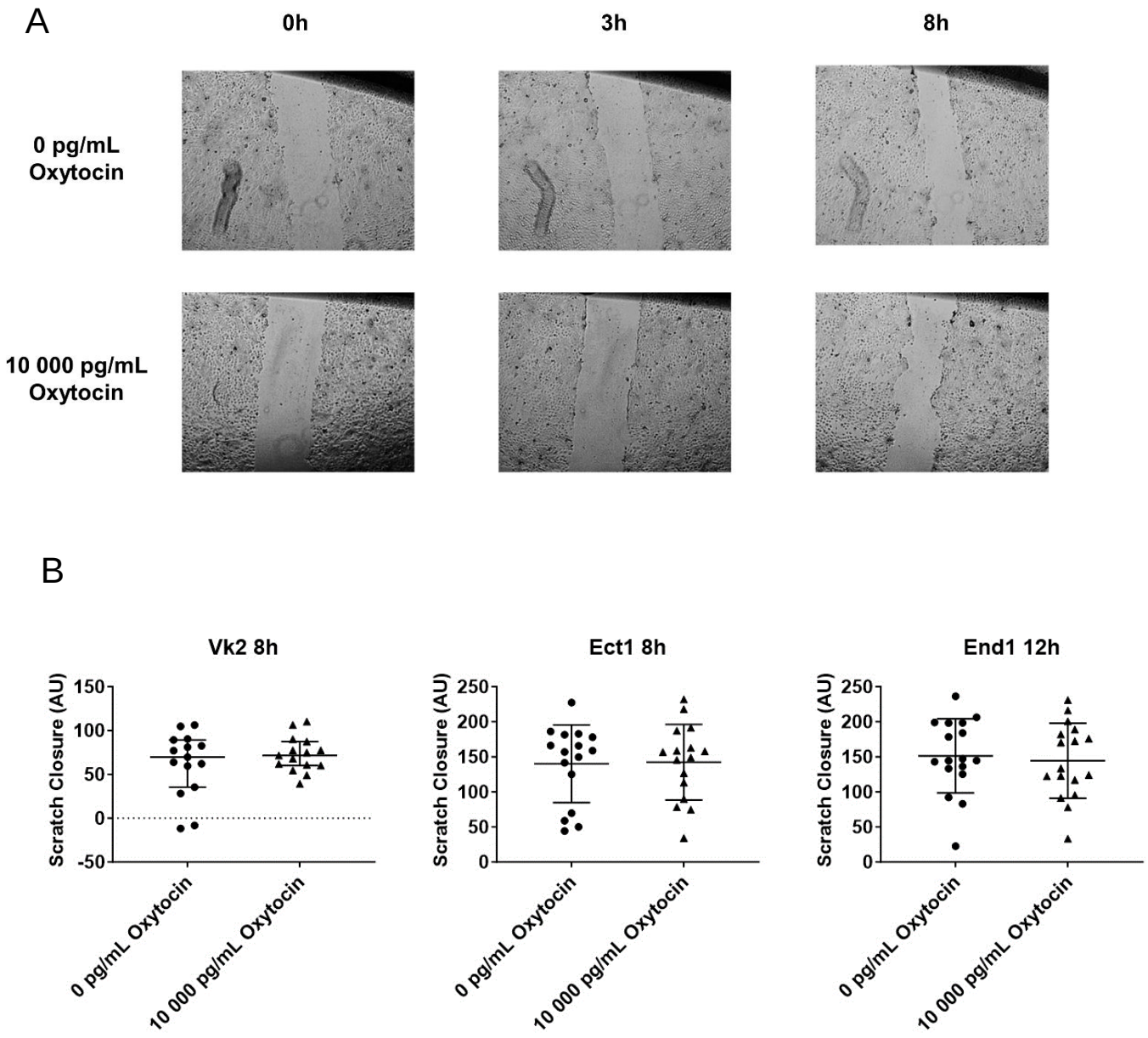


Figure 3.10 Oxytocin had no effects on the rate of FGT wound healing in BPE-containing media as measured in scratch assay. (A) Representative light microscopy images of Vk2 cells undergoing the wound healing process at 0, 3, and 8 hours for both oxytocin-treated (10 000 pg/mL) and untreated control (0 pg/mL) conditions. (B) Quantitative wound healing of Vk2, Ect1, and End1 cells as measured in arbitrary units (AU) after significant change in the wound front had occurred for each cell line in hours (h). Comparisons made using an unpaired two-tailed t-test with an $\alpha = 0.05$. $n = 15$

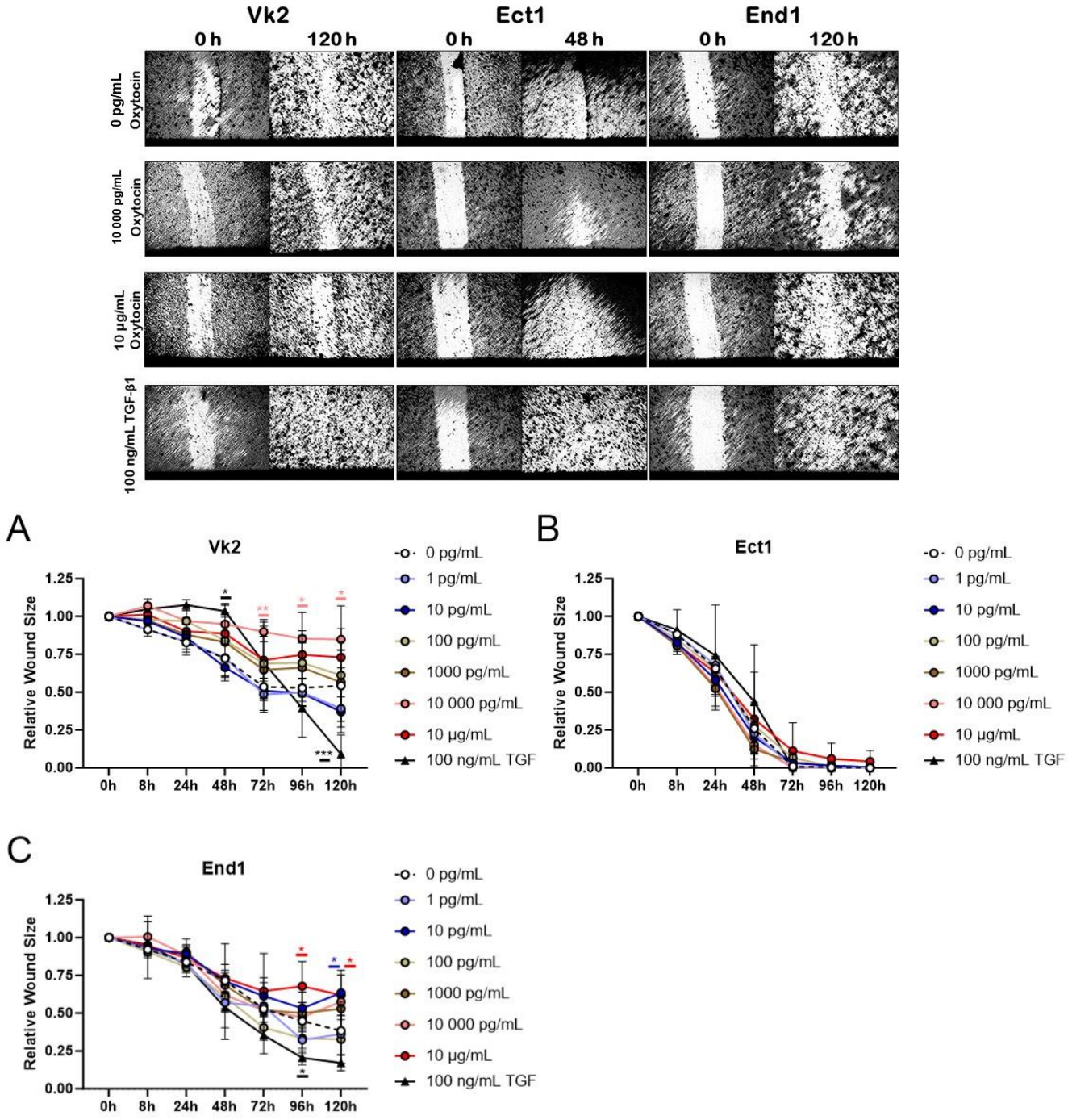


Figure 3.11 Oxytocin impedes wound healing in Vk2 and End1 cell lines grown in BPE-free defined media as measured in scratch assay. Oxytocin was used to treat (A) Vk2, (B) Ect1, and (C) End1 cells at listed concentrations. TGF-β1 was used as a positive control at 100 ng/mL, while PBS was used as a vehicle for the 0 pg/mL oxytocin treatment. Relative wound size was measured as the area of the scratch at a given time point relative to its initial size. Light microscopy images depict representative images with the '0 h' taken immediately after scratch and the '48/120 h' images taken at listed endpoint. Comparisons made using a mixed-effects model with Dunnet's multiple comparisons at $\alpha = 0.05$. n = 3. * $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$

3.4 Biologically active oxytocin does not trigger Ca^{2+} flux in FGT epithelium

3.4.1 Oxytocin-induced Ca^{2+} flux in PHM1-41 cells and Vk2, Ect1, and End1 cells

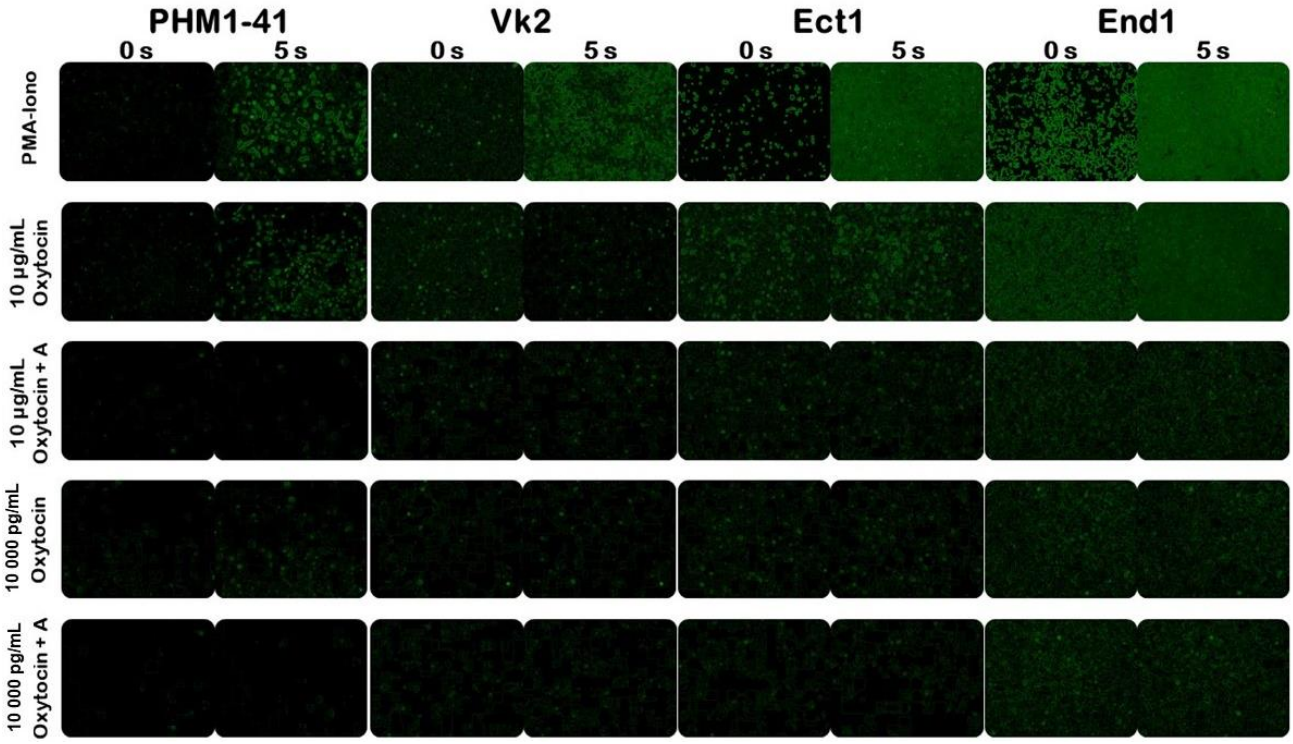
Based upon the somewhat limited response to oxytocin in FGT epithelium as well as the lack of a clear response to oxytocin in PHM1-41 cells in the previous two sections (***Sections 3.2–3.3***), I sought to test the ability of oxytocin to elicit a response through direct cell signaling in these cell lines. Oxytocin has been shown to stimulate Ca^{2+} release in target cells as the most common pathway of oxytocin signaling (S. H. Kim et al., 2017; Van Den Burg & Neumann, 2011), and Ca^{2+} flux assays allow for measurement of intracellular cell signaling utilizing Ca^{2+} imaging techniques. These techniques can quantify the amount of Ca^{2+} released after binding with an agonist, which can then be used to infer general responsiveness (Liao et al., 2021; Moon & Park, 2020). PHM1-41 cells have previously been demonstrated to respond to oxytocin treatment through Ca^{2+} release, and so I determined this would be a definitive way to test if the oxytocin used in this study was biologically active or if the PHM1-41 cells were not responding as expected (Burghardt et al., 1999; Monga et al., 1996, 1999). Based on prior associations with the literature, I hypothesized that PHM1-41 cells would show Ca^{2+} release in response to the study oxytocin, while Vk2, Ect1, and End1 cells would show limited Ca^{2+} release in line with their modest responses in previous sections (***Sections 3.2–3.3***).

In order to assess the bioactivity of the oxytocin used within the study, PHM1-41, Vk2, Ect1, and End1 cells were grown to confluency and incubated with the green-fluorescing 520 AM dye. Cells were then treated with either 0 pg/mL, 10 000 pg/mL, or 10 µg/mL oxytocin and visualized under a fluorescent microscope. Atosiban was used as an inhibitor of the OXTR to show receptor-dependency by pretreating cells with an equivalent amount of Atosiban prior to oxytocin treatment. Images were captured five seconds after the addition of oxytocin agonist to allow for maximum intensity. Cell fluorescence was then quantified using ImageJ, with values indicating the change in intensity before and after oxytocin addition in arbitrary units (AU). Simultaneous treatment with 50 ng/mL phorbol myristate acetate (PMA) and 100 ng/mL ionomycin was used as a positive control for Ca^{2+} flux, as it leads to an indiscriminate influx of Ca^{2+} into the cells.

Comparisons were made between treatments within a cell line using one-way ANOVA followed by Dunnett's multiple comparisons test at $\alpha = 0.05$.

As expected, PHM1-41 cells showed detectable Ca^{2+} flux signaling at an oxytocin dose of 10 µg/mL (PHM1-41: **10 µg/mL**: 4146 AU vs. **0 pg/mL**: 171 AU, p = 0.03) (**Figure 3.12A**). Furthermore, this Ca^{2+} release was found to be inhibited by the competitive inhibitor atosiban (PHM1-41: **10 µg/mL + Atosiban**: 1088 AU vs. **0 µg/mL oxytocin**: 171 AU, p = 0.8) (**Figure 3.12A**). Vk2, Ect1, and End1 cells did not show significant responses to oxytocin at either 10 pg/mL or 10 µg/mL oxytocin (p-values > 0.05) (**Figure 3.12B-D**). All cell lines showed significant responses to PMA-ionomycin treatment, as expected (PHM1-41: **PMA/I**: 8879 AU vs. **0 pg/mL**: -171 AU, p = 0.0001 ; Vk2: **PMA/I**: 25680 AU vs. **0 pg/mL**: -403 AU, p < 0.0001; End1: **PMA/I**: 38983 AU vs. **0 pg/mL**: -998 AU, p < 0.0001; End1: **PMA/I**: 35748 AU vs. **0 pg/mL**: -1550 AU, p < 0.0001) (**Figure 3.12**).

In summary, PHM1-41 control cells responded as expected, and study oxytocin was found to be biologically active. Vk2, Ect1, and End1 cells did not respond to oxytocin with Ca^{2+} flux. This supports the hypothesis that PHM1-41 cells would respond to oxytocin with Ca^{2+} release and Vk2, Ect1, and End1 cells would show a limited response to oxytocin.


PHM1-41
Vk2
Ect1
End1
0 s
5 s
PMA-Iono
10 µg/mL Oxytocin
10 µg/mL Oxytocin + A
10 000 pg/mL Oxytocin
10 000 pg/mL Oxytocin + A

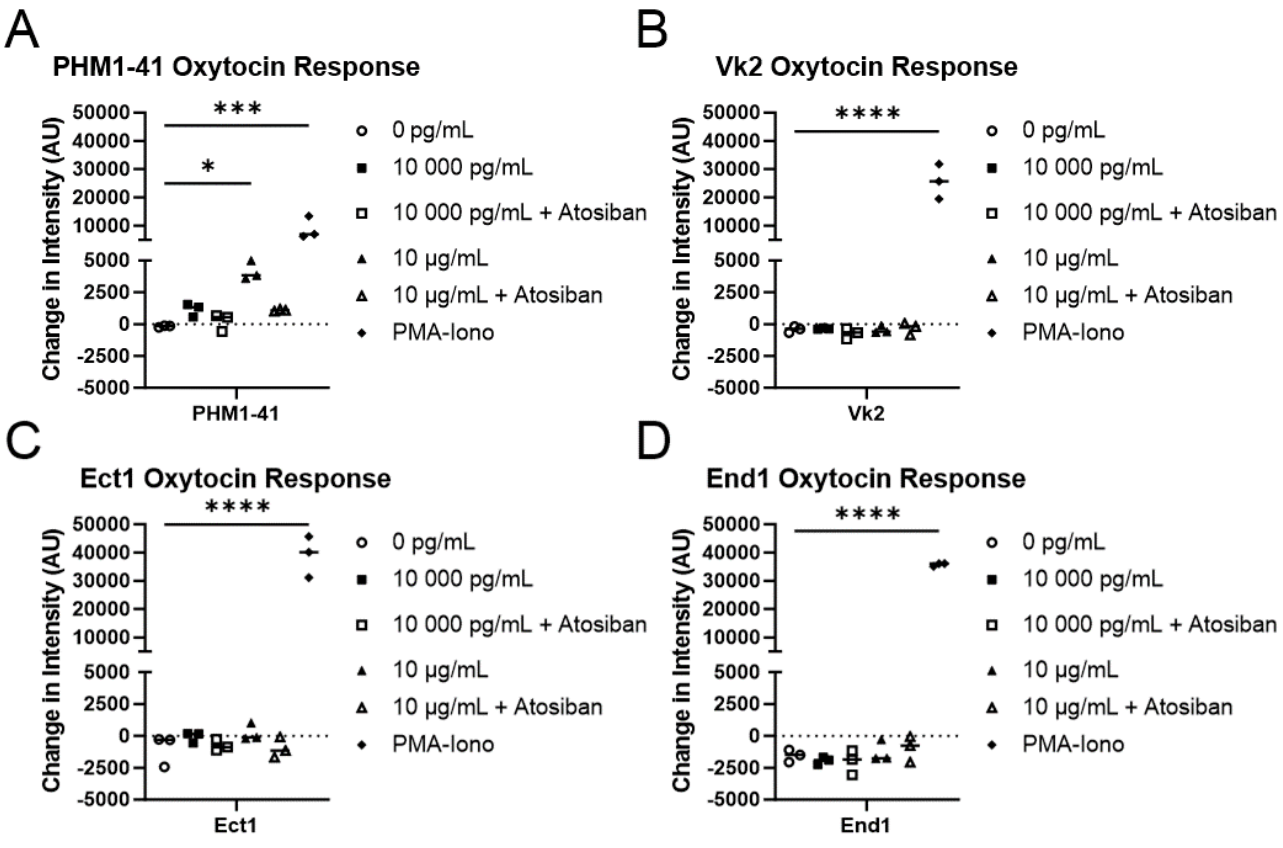

A
PHM1-41 Oxytocin Response
B
Vk2 Oxytocin Response
C
Ect1 Oxytocin Response
D
End1 Oxytocin Response
Change in Intensity (AU)
0 pg/mL
10 000 pg/mL
10 000 pg/mL + Atosiban
10 µg/mL
10 µg/mL + Atosiban
PMA-Iono
PHM1-41
Vk2
Ect1
End1

Figure 3.12 Immortalized epithelial cell lines from the female genital tract do not show Ca^{2+} release upon treatment with oxytocin as measured in Ca^{2+} flux assays. Fluorescent microscopy images depict representative images with the '0 s' images taken immediately before agonist addition and the '5 s' images taken five seconds after agonist addition. Oxytocin was used at 10 000 pg/mL or 10 µg/mL concentrations with or without an equivalent amount of atosiban competitive inhibitor (+A) on (A) PHM1-41, (B) Vk2, (C) Ect1, and (D) End1 cells. PMA (50 ng/mL)-ionomycin (100 ng/mL) treatment (PMA-Iono) was used as a positive control for Ca^{2+} release. Changes in intensity are the differential of fluorescence before and after agonist addition as measured in arbitrary units (AU). Dashed line indicates the cut-off for no change in intensity. Green fluorescence above baseline indicated intracellular Ca^{2+} release. Comparisons made by one-way ANOVA using Dunnett's multiple comparisons test at $\alpha = 0.05$. n = 3. * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$

3.5 Vk2, Ect1, and End1 cells display insignificant levels of OXTR expression

Early in the thesis I had performed confirmatory OXTR testing in each FGT epithelial cell line to ensure each cell line would be suitable for treatment with oxytocin. These preliminary experiments were positive (**Figures 3.13A and 3.15**), and so I designed and carried out the rest of my experiments. After discovering that oxytocin had a generally limited impact on FGT epithelial cell cytokine/chemokine production and wound healing (***Sections 3.2–3.3***), as well as a lack of Ca^{2+} flux in response to oxytocin treatment (***Section 3.4***), I revisited the analysis of the OXTR in each cell line. The effect oxytocin can have on a cell or cell line is partially dependent on the prevalence and expression of its receptor (P. Wang & Slaughter, 2007). The OXTR exists in both glycosylated and unglycosylated forms, and so can typically vary in size between 42 and 70 kDA (Ivell et al., 2001; Kimura et al., 1997; McKay & Counts, 2020). Previously, only one study had examined OXTR expression within Vk2 epithelial cells (Kallak & Uvnäs-Moberg, 2017), and no studies to date have examined OXTR expression within Ect1 or End1 cell lines.

To aid in determining OXTR expression in each of the three FGT cell lines, I overexpressed the OXTR with a green fluorescent protein (GFP) tag in HEK 293 cells. I chose this cell line as it is widely used in studies of OXTR overexpression (Chruścicka et al., 2019; Kii et al., 2018; Pillers et al., 2014). I additionally included PHM1-41, Caco2/Caco2BB, and Jurkat cell lines as naturally-expressing OXTR positive control cell lines, due to their evidence of OXTR expression either through published literature or use in anti-OXTR antibody company datasheets (Klein et al., 2014, 2016; *OXTR Antibody (23045-1-AP) | Proteintech*, 2024).

3.5.1 Detection of the OXTR through antibody and peptide binding

Characterization of receptor protein expression often employs Western blot, ELISA, confocal microscopy, and flow cytometry. While the study by Kallak and Uvnäs-Moberg (2017) showed Vk2 cells to express the OXTR by confocal microscopy, the OXTR staining was cytoplasmic and there was a lack of specific enrichment at the cell membrane. This is counter to

the nature of the OXTR as a transmembrane receptor (Gimpl & Fahrenholz, 2001; Jurek & Neumann, 2018). Based on this, I suspected that the previous staining by Kallak and Uvnäs-Moberg (2017) may have been eroneous, and hypothesized that the Vk2, Ect1, and End1 cell lines expressed either no, or very minimal, OXTR.

Western blots were performed with listed antibodies (***Section 2.3.2)*** that were specific for the OXTR or for GFP (for use in targeting GFP-tagged OXTR). β-Actin was additionally blotted as a loading control for total protein. Secondary antibodies conjugated to horseradish peroxidase (HRP) were used in conjunction with chemiluminescent detection. Confocal microscopy and peptide binding assays were performed using monolayers that had grown to 80% confluency on glass microscope slides. Confocal staining was conducted using listed anti-OXTR and isotype antibodies (***Section 2.3.3)***, with fluorophore-conjugated secondary antibodies utilized for detection. Peptide binding assays were carried out using listed biotin-conjugated peptides (***Section 2.3.4***) with streptavidin-PE conjugates for detection. Both confocal and peptide binding methods utilized DAPI nuclear stain present in the mountant preparation to stain nuclei. Western blots were analyzed using Quantity One software, while confocal and peptide binding images were analyzed using Zeiss confocal imaging software. All images were analyzed qualitatively for OXTR presence.

Initial Western blot experiments early on in the thesis indicated positive OXTR staining at ~43 kDa—corresponding to the expected size of the unglycosylated receptor—for each of Vk2, Ect1, and End1 (**Figure 3.13A**). Faint bands were also seen in the positive controls Jurkat, Caco2, and PHM1-41, with no bands seen in the HEK 293T and $CD4^+$ T cell negative controls (**Figure 3.13A**). This Western utilized the anti-OXTR antibody ab181077, which was discontinued at the time of revisiting OXTR expression, and so direct retesting with the same antibody was not possible. As a result, alternative anti-OXTR antibodies were used in testing OXTR presence.

Anti-OXTR antibody ab217212 showed a strong band for the PHM1-41 positive control at ~69kDa—corresponding to the expected size of the glycosylated OXTR—but bands also appeared in the negative control lanes for HEK 293 and HEK 293T cells (**Figure 3.13B**). Binding was not observed for the truncated recombinant OXTR, but the targeted epitope of ab217212 was absent from the recombinant protein and this was expected (**Figure 3.13**). No bands at expected OXTR

sizes were seen for Vk2, Ect1, or End1 cells, but because of the mixed binding with the negative control these results had to be considered inconclusive (**Figure 3.13B**).

Antibody 23045-1-AP displayed expected staining of the truncated recombinant OXTR protein at ~37 kDa, with a faint band at ~67 kDa in PHM1-41 cells corresponding to the expected size for glycosylated OXTR (**Figure 3.13C**). No bands were seen in the Vk2, Ect1, End1, or negative control lanes (**Figure 3.13C**).

Anti-OXTR antibody sc515809 produced bands for the recombinant OXTR at ~37 kDa, but was unable to show positive staining for the PHM1-41 positive control (**Figure 3.13D**). No bands were seen for Vk2, Ect1, End1, or negative control HEK 293 and HEK 293T cell lines (**Figure 3.13D**).

In order to test if the non-specific binding of antibody ab217212 in HEK 293 and HEK 293T cells was due to OXTR binding, HEK 293 *OXTR*-transfected cells were blotted alongside mock- and vector-only transfected controls. Antibody ab217212 bound to all HEK 293 variants equally with a band at ~43 kDa, corresponding to the expected size of the unglycosylated OXTR (**Figure 3.13E**). A faint band at ~43 kDa was also seen in the PHM1-41 control lane (**Figure 3.13E**).

In summary, ab217212 was seen to bind non-specifically to HEK 293 cells and could not be considered conclusive for OXTR staining. Anti-OXTR antibodies 23045-1-AP and sc-515809 both demonstrated a lack of OXTR in Vk2, Ect1, and End1 cells, but PHM1-41 positive control staining was absent for antibody sc-515809 and weak for antibody 23045-1-AP.

Given the promising results from antibody 23045-1-AP, it was utilized for follow-up testing of protein lysate supernatant and insoluble fractions to test for possible loss of OXTR to the insoluble fraction during protein isolation. Examining protein supernatant fractions of both 1% octylglucoside (**Figure 3.14A**) and RIPA (**Figure 3.14B**) lysis buffers revealed bands at ~60 kDa in the HEK 293 *OXTR*-transfected positive control lane when blotted with either antibody 23045-1-AP or an anti-GFP antibody. Vk2, Ect1, and End1 cells did not show any detectable OXTR in supernatant, but bands were also absent from PHM1-41 and Jurkat positive controls.

Analysis of the insoluble protein lysate fraction revealed strong bands for HEK 293 *OXTR*-transfected cells in both 1% octylglucoside (**Figure 3.14C**) and RIPA (**Figure 3.14D**) buffer

corresponding to the expected size of GFP-tagged OXTR at ~60 kDa. Vk2 cells grown in BPE-free KSFM displayed bands in both lysis buffers, but they were outside the expected ~43 kDa size of the unglycosyalted OXTR (**Figure 3.14C and D)**. Vk2 cells, likewise, displayed an erroneous band in RIPA buffer outside the expected ~43 kDa of the OXTR (**Figure 3.14D**). No bands were seen in either PHM1-41 or Jurkat positive controls (**Figure 3.14C and D**).

In summary, antibody 23045-1-AP detected OXTR in both the supernatant and insoluble fractions of HEK 293 *OXTR*-transfected lysates, but not in Vk2, Ect1, or End1 lysates. Lack of PHM1-41 and Jurkat positive control staining means the results must be interpreted with caution.

Attempting to test for the OXTR using an alternative method, confocal staining using anti-OXTR antibody 23045-1-AP was performed. Initial experiments early on in the thesis with this antibody revealed positive expression of the OXTR in Vk2, Ect1, and End1 cells (**Figure 3.15**). Furthermore, this staining was associated with the cell membrane as shown through co-staining with wheat germ agglutinin membrane dye, and supported by positive staining in PHM1-41 cells and a lack of strong signal in OXTR-negative $CD4^+$ T cells (**Figure 3.15**). Repeating this work using the same antibody but utilizing an adherent cell negative control (HEK 293 cells) revealed non-specific binding of the antibody (**Figure 3.16**). Follow-up study using a biotin-labelled oxytocin peptide to circumvent antibody non-specificity yielded similar issues with non-specific binding and background, whereby PHM1-41 and *OXTR*-transfected HEK 293 cells, as well as mock- and vector-transfected HEK 293 cells, showed positive signal for oxytocin binding (**Figure 3.17**).

In conclusion, Vk2, Ect1, and End1 cell lines displayed OXTR staining through both Western blot and confocal experiments early on in the thesis, but follow-up testing found these results to be either non-reproducible or non-specific. Results from anti-OXTR antibody 23045-1-AP suggest a lack of OXTR presence in Vk2, Ect1, and End1 cells, but this antibody's inconsistent detection of PHM1-41 and Jurkat positive control cell lines warrant further confirmation of these findings. These findings tentatively support the hypothesis that Vk2, Ect1, and End1 cells express minimal or no OXTR.

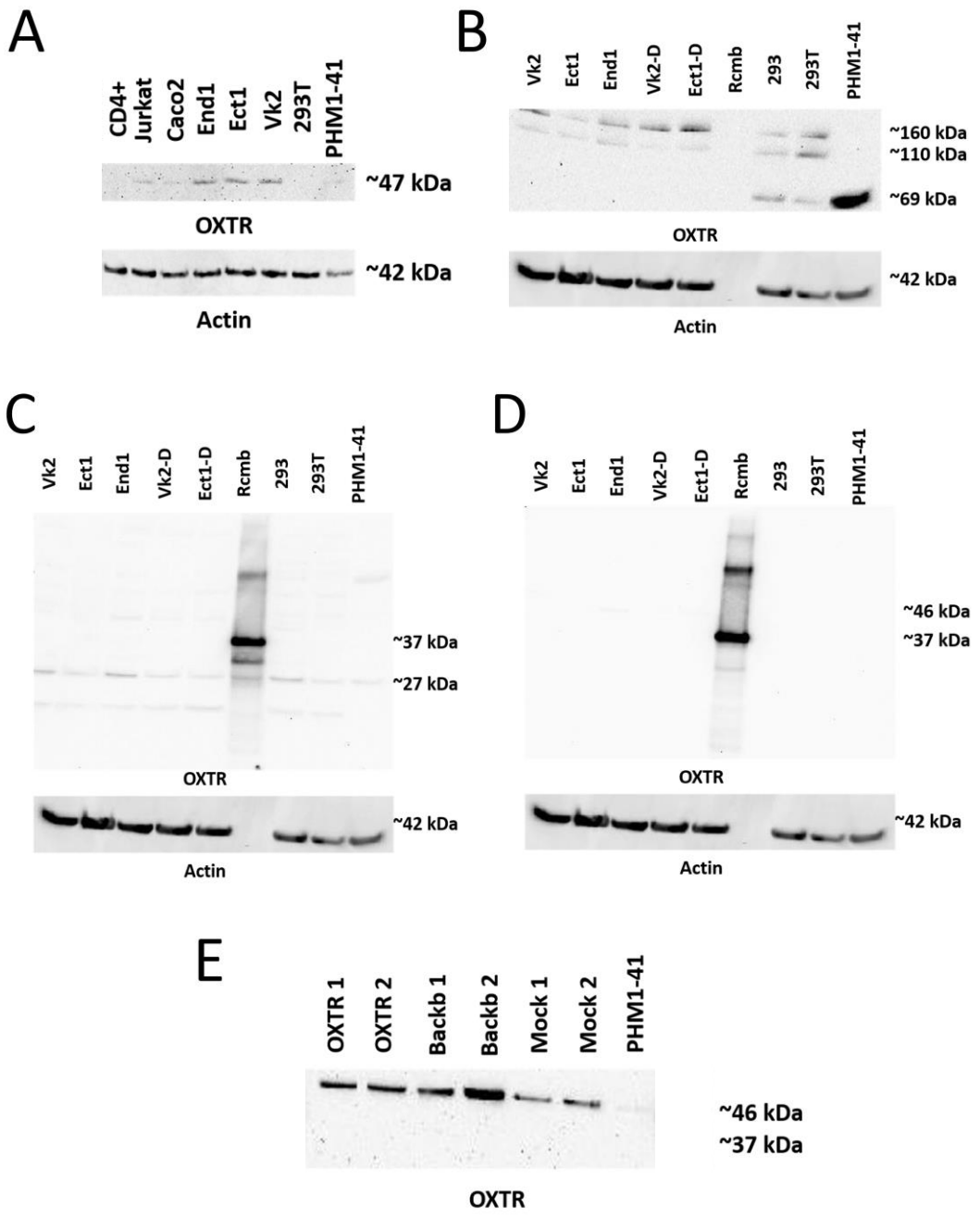


Figure 3.13 Western blots give variable results as to the presence of OXTR for Vk2, Ect1, and End1 cell lines. Western blots against OXTR are shown for (A) ab181077, (B and E) ab217212, (C) 23045-1-AP, and (D) sc-515809. β-Actin used as a loading control. OXTR expected between 22 and 65 kDa. β-Actin expected at 43 kDa. PHM1-41, Caco2, Jurkat, OXTR recombinant (Rcmb), *OXTR*-transfected HEK 293s (OXTR 1 and 2) cells included as positive controls, while $CD4^+$ T cells, HEK 293s (293), HEK 293Ts (293T), vector-only (Backb 1 and 2) and mock-transfected (Mock 1 and 2) HEK 293s included as negative controls. Vk2 and Ect1 cells grown in BPE-free defined media denoted '-D'.

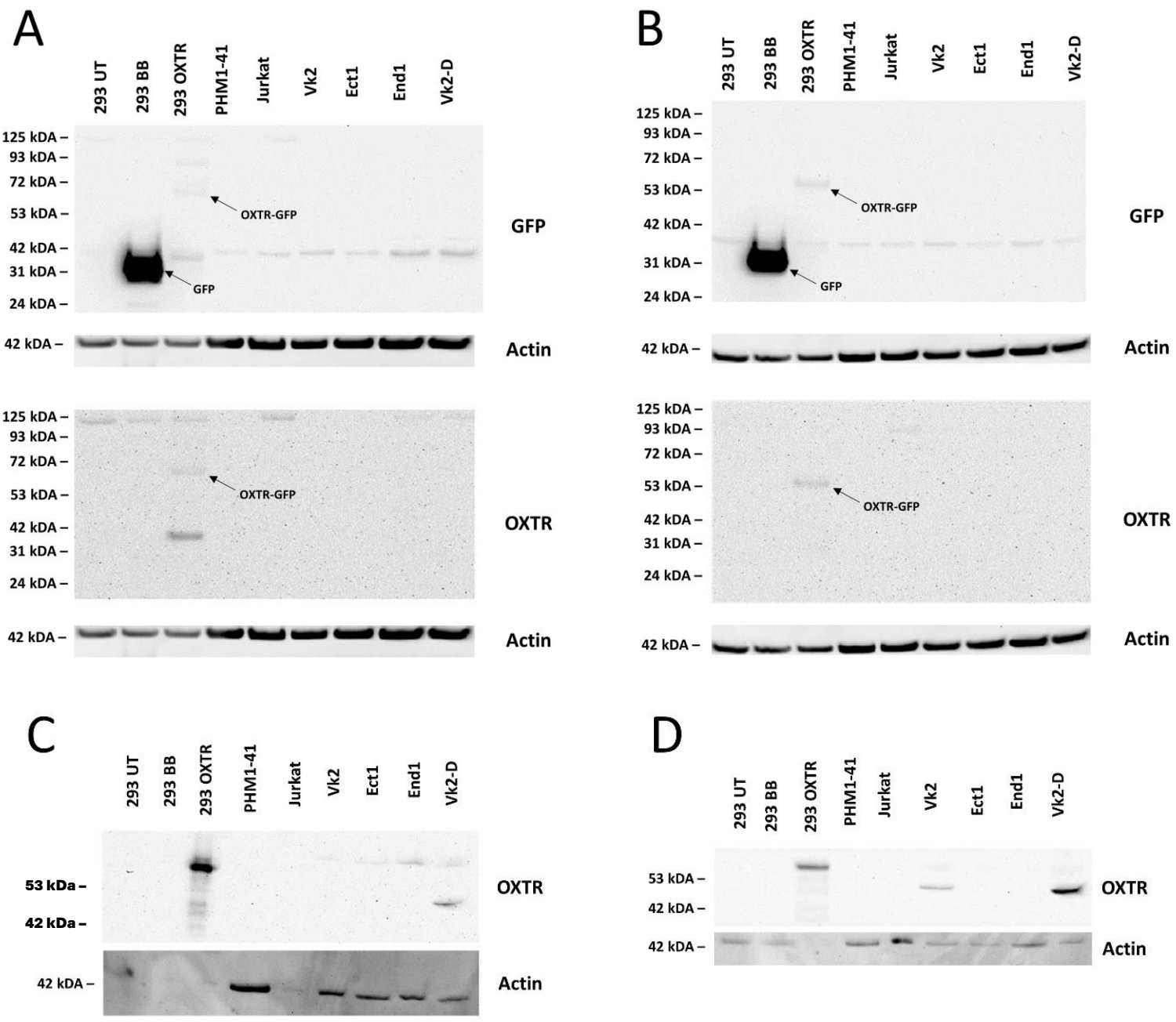


Figure 3.14 Western blot shows no detection of OXTR in insoluble fractions of Vk2, Ect1, and End1 protein lysates. Protein lysates of HEK 293 untransfected (293 UT), HEK 293 Vector-only transfected (293 BB), HEK GFP-tagged-*OXTR*-transfected (293 OXTR), PHM1-41, Jurkat, Vk2, Ect1, End1, and Vk2 grown in BPE-free defined media (Vk2-D) were lysed with 1% octylglucoside (A,C) or RIPA buffer (B,D). These fractions were further separated into soluble lysis fractions (A,B) or insoluble lysis fractions that underwent further sonication before blotting (C,D). GFP – 24 kDa, OXTR – 47 kDa (90 kDa glycosylated), OXTR-GFP – 70 kDa. β–Actin – 43 kDa – was used as a loading control.

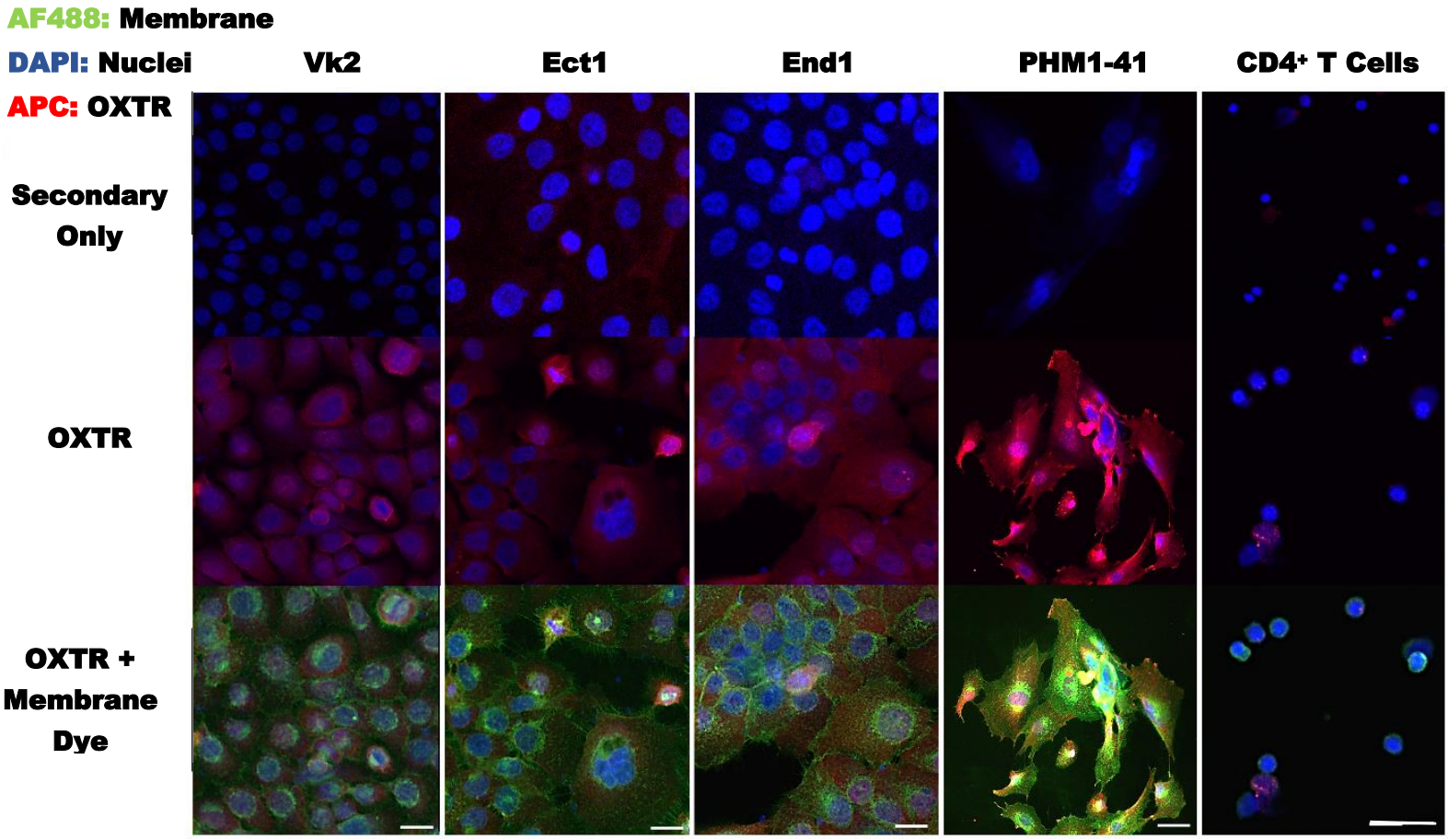


Figure 3.15 Confocal microscopy of the OXTR in Vk2, Ect1, and End1 cells using a non-epithelial negative control. Vk2, Ect1, and End1 cells were assessed for OXTR expression through confocal microscopy. PHM1-41 is provided as a positive control for OXTR staining, and $CD4^+$ T cells are provided as a negative control. 'Secondary only' represents background signal, while 'OXTR' and 'OXTR + Membrane Dye' represent OXTR-Ab staining without or with accompanying membrane stain to illustrate co-localization of receptor staining with the membrane. Blue = nuclei DAPI staining. Red = OXTR APC staining. Green = wheat germ agglutinin AF488-tagged membrane dye. Scale bar represent 20 µm. Images taken at 20x magnification, or 40x in the case of $CD4^+$ T cells.

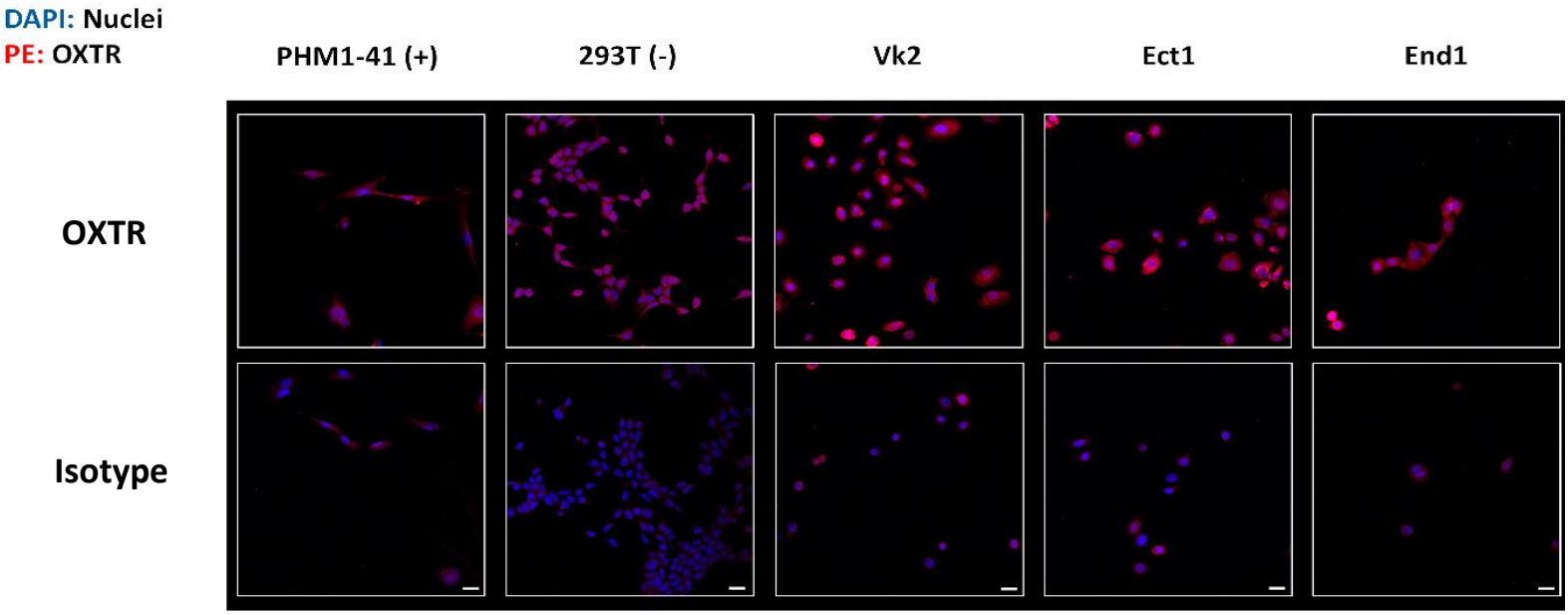


Figure 3.16 Confocal microscopy of the OXTR in Vk2, Ect1, and End1 cells using an epithelial negative control. Vk2, Ect1, and End1 cells were assessed for OXTR expression through confocal microscopy. PHM1-41 is provided as a positive control for OXTR staining, and HEK 293T cells (293T) are provided as an epithelial negative control. 'OXTR' represents OXTR-Ab staining, while 'Isotype' indicates staining with a generic rabbit IgG control antibody. Blue = nuclei DAPI staining. Red = OXTR PE staining. Scale bar represent 20 µm. Images taken at 20x magnification.

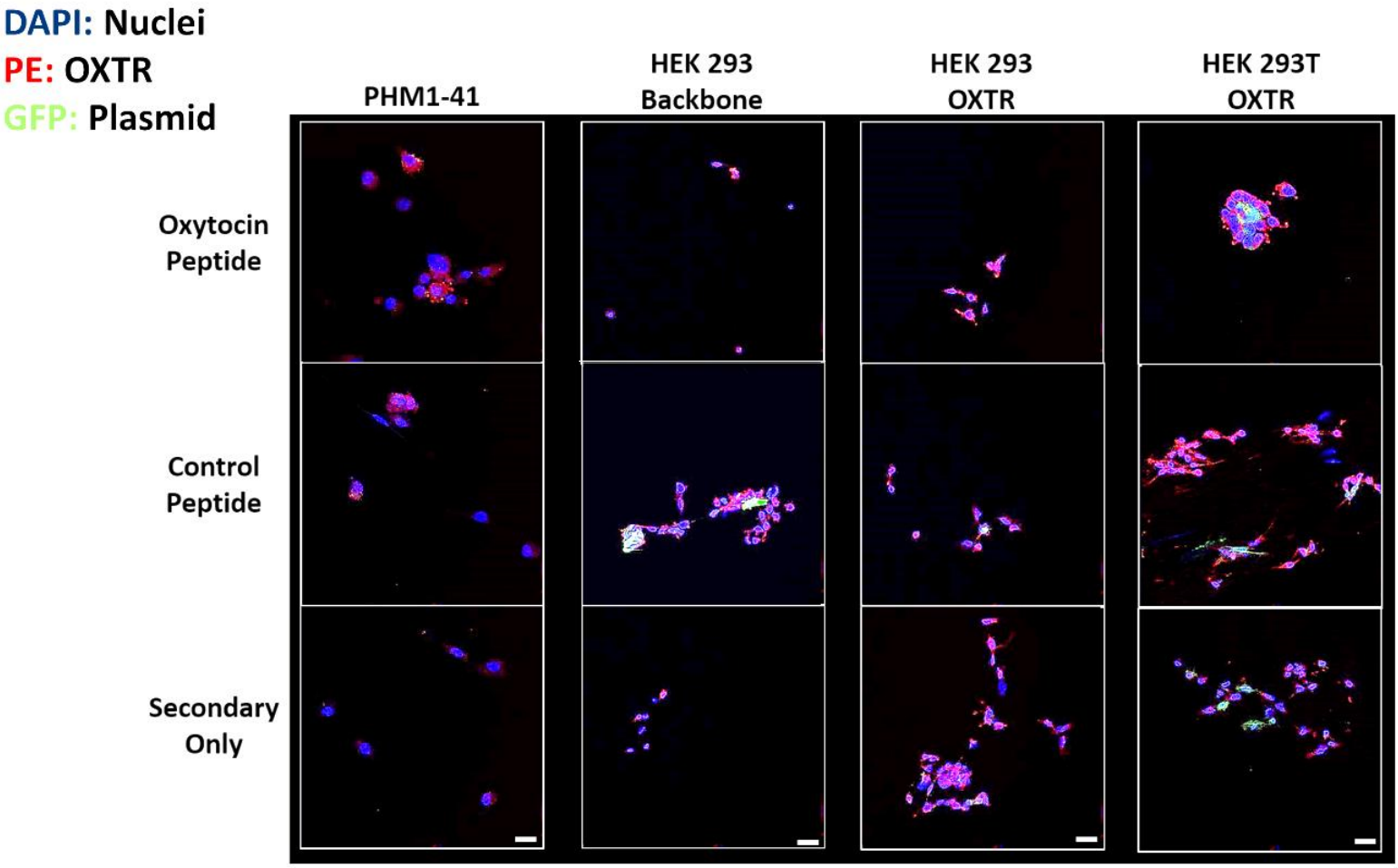


Figure 3.17 Oxytocin peptide binding assays display non-specific binding with epithelial cell negative controls. Peptide binding assay using biotin-labelled oxytocin (Oxytocin Peptide), biotin-labelled HA tag (Control Peptide), and streptavidin-PE conjugate alone (Secondary Only). Representative images of cells grown on cover glasses and treated with peptide are shown for PHM1-41, HEK 293 vector-only transfected cells (HEK 293 Backbone), HEK 293 *OXTR*-plasmid transfected cells, and HEK 293T *OXTR*-plasmid transfected cells. Nuclei are stained with NucBlue, and GFP is a marker of transfected cells. OXTR signal is measured through the PE channel. Scale bars represent 20 µm. All pictures are taken at 20x magnification. n = 3

3.5.2 Micro RNA and proteomics-based detection of the OXTR

Given the somewhat inconclusive nature of the antibody and peptide-binding experiments (***Section 3.5.1***), nucleic acid amplification was used to provide a highly sensitive detection method independent of antibody-specificity to detect the OXTR (Arrowsmith, 2021; Füeg et al., 2019; Ludwig et al., 2022). In tandem with this, a proteomics approach utilizing mass spectrometry was performed in order to capture OXTR protein presence without the use of antibody- or peptide-based binding assays (Angel et al., 2012; Macklin et al., 2020). I hypothesized that qPCR and mass spectrometry would conclusively show a lack of OXTR expression in Vk2, Ect1, and End1 cells.

Quantitative polymerase-chain-reaction for *OXTR* mRNA presence was conducted with RNA isolated from epithelial cells grown in monolayer culture in BPE-containing KSFM medium. All expression values are normalized to 18S rRNA transcription. Comparisons between groups were made with Brown-Forsythe and Welch ANOVA, due to unequal variances, using Dunnett's T3 multiple comparisons at $\alpha = 0.05$. For mass spectrometry, protein lysates of Vk2, Ect1, and End1 cells grown in BPE-containing KSFM or BPE-free 'defined' KSFM (-D) underwent trypsin digestion before being run through unlabeled LC-MS/MS mass spectrometry. Positive OXTR signal was identified with a minimum cut-off of 2 unique peptides at a confidence of 95% for each individual peptide spectra and 99% confidence for the overall protein call. Comparisons were made between groups by Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison analysis at $\alpha = 0.05$.

Analysis of relative *OXTR* mRNA expression revealed that Vk2 and Ect1 cells did not show higher expression levels than HEK 293T negative control cells (**Vk2**: 9.5×10^{-7} vs. **HEK 293T**: 3.3×10^{-6}, $p > 0.99$; **Ect1**: 6.8×10^{-6} vs. **HEK 293T**: 3.3×10^{-6}, $p = 0.11$; **End1**: 1.3×10^{-5} vs. **HEK 293T**: 3.3×10^{-6}, $p = 0.054$) (**Figure 3.18**). As expected, PHM1-41 cells displayed robust levels of *OXTR* mRNA relative to the HEK 293T negative control (**PHM1-41**: 3.7×10^{-3} vs**. HEK 293T**: 3.3×10^{-6}, $p < 0.0001$) (**Figure 3.18**). In summary, none of the FGT epithelial cell lines showed significantly detectable *OXTR* mRNA compared against negative HEK 293T control cells.

Proteomic detection of OXTR indicated a lack of detectable OXTR spectra for Vk2, Ect1, and End1 cells when compared to vector-only transfected HEK 293 controls (**Vk2**: 0 spectra vs. **HEK Vector**: 0 spectra, $p > 0.99$; **Ect1**: 0 spectra vs. **HEK Vector**: 0 spectra, $p > 0.99$; **End1**: 0

spectra vs. **HEK Vector:** 0 spectra, p > 0.99) (**Figure 3.19**). This association remained when Vk2, Ect1, and End1 cells were cultured in BPE-free KSFM (**Vk2-D**: 0 spectra vs. **HEK Vector**: 0 spectra, p > 0.99; **Ect1-D**: 0 spectra vs. **HEK Vector**: 0 spectra, p > 0.99; **End1-D**: 0 spectra vs. **HEK Vector**: 0 spectra, p > 0.99) (**Figure 3.19**). As expected, a significant number of OXTR spectra were detected in a sample of recombinant OXTR and *OXTR*-transfected HEK 293 cells (**Rcmb OXTR**: 33 spectra vs. **HEK Vector**: 0 spectra, p = 0.0098; **HEK OXTR**: 5 spectra vs. **HEK Vector**: 0 spectra, p = 0.0091) (**Figure 3.18**). Surprisingly, PHM1-41 cells did not reach a statistically significant level of detected OXTR spectra (**PHM1-41**: 1 spectrum vs. **HEK Vector**: 0 spectra, p = 0.86) (**Figure 3.19**). The additional positive control cell lines Caco2BB and Jurkat also did not show significantly detectable OXTR peptide levels (**Caco2BB**: 0 spectra vs. **HEK Vector**: 0 spectra, p > 0.99; **Jurkat**: 0 spectra vs. **HEK Vector**: 0 spectra, p > 0.99) (**Figure 3.19**). In summary, no OXTR peptide spectra were detected for Vk2, Ect1, and End1 cells cultured in either BPE-free or BPE-containing media.

In conclusion, qPCR analysis revealed that neither Vk2, Ect1, nor End1 cells showed statistically significant expression of *OXTR* mRNA. In agreement, mass spectrometry analysis demonstrated a lack of OXTR peptide spectra in Vk2, Ect1, and End1 cells, although a lack of significantly detectable spectra in the PHM1-41, Jurkat, and Caco2BB positive control cell lines indicates a potential lack of sensitivity. These data support a lack of significant OXTR expression in Vk2, Ect1, and End1 cells at the mRNA, but not protein, level.

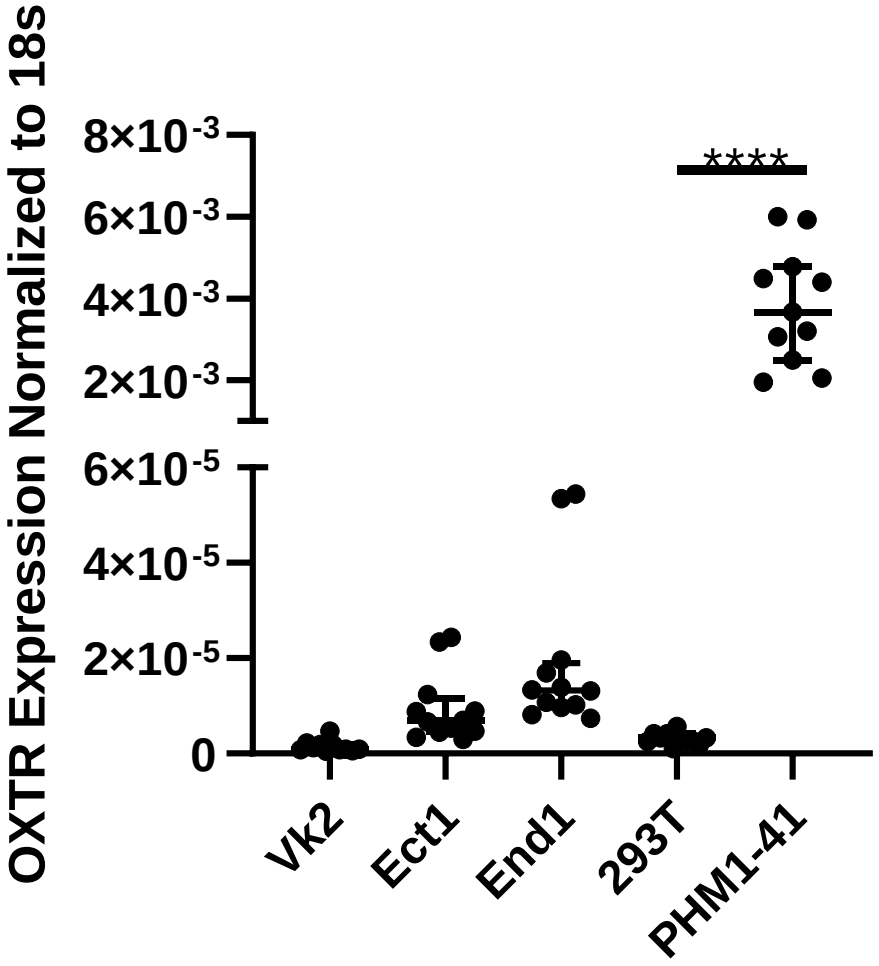


Figure 3.18 RNA expression of the OXTR gene in cell lines from the FGT and associated controls as measured through qPCR. Epithelial cell lines of the FGT (Vk2, Ect1, and End1) show comparable or lower OXTR gene expression, normalized with the housekeeping gene 18s rRNA, to a HEK 293T (293T) negative control cell line. The positive control uterine myometrium cell line PHM1-41 shows OXTR gene expression greater than two orders of magnitude above the other cell lines after normalization with 18s rRNA gene expression. Ect1, End1 n = 12; Vk2, 293T, PHM1-41 n = 11. Comparisons made with Brown-Forsythe and Welch ANOVA using Dunnett's T3 multiple comparisons. **** $p < 0.0001$.

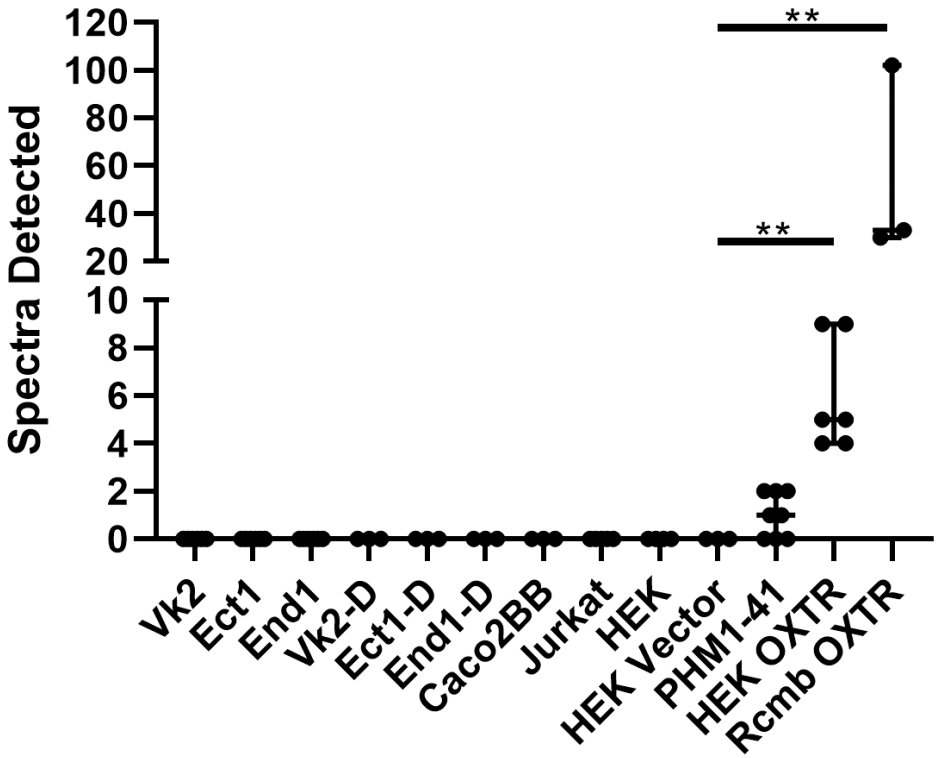


Figure 3.19 Peptide spectra for the OXTR protein are undetectable in immortalized epithelial cell lines of the female genital tract using mass spectrometry. Cell lines with '-D' denote cell lines grown in BPE-free, defined, media. Centre lines indicate medians with error bars denoting interquartile ranges. *OXTR*-transfected HEK 293's (HEK OXTR) and recombinant OXTR (Rcmb OXTR) included as positive controls. HEK Vector denotes empty-plasmid transfected HEK 293's. Comparisons made by Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test with a p-value of 0.05 considered significant. n = 3 for '–D' samples, Caco2BB, Jurkat, HEK Vector, and Rcmb OXTR samples. n = 6 for all others. ** $p < 0.01$.

3.6 Vaginal oxytocin gel treatment does not significantly reduce the expression of leukocyte activation markers or pro-inflammatory cytokines/chemokines in TKO-hBLT mice

Despite the lack of strong response from oxytocin on FGT epithelial pro-inflammatory response *in vitro* (***Section 3.2***), many prior studies have demonstrated an effect of oxytocin in *in vivo* systems (Clodi et al., 2008; Imami et al., 2020; Yussof et al., 2012). Additionally, previous work has demonstrated that effects seen *in vivo* cannot be replicated through *ex vivo* experiments after cells are removed from the body (Ross et al., 2013). Many studies utilizing oxytocin as a vaginal gel have shown promising effects on combating dryness, increasing vaginal wall thickness, and increasing the maturation profile of vaginal epithelial cells (Jonasson et al., 2011; Moussa et al., 2023; Torky et al., 2018; Zohrabi et al., 2020). As a result, I sought to examine the effect of oxytocin vaginal gel on the ability to prevent HIV infection in a humanized TKO-hBLT mouse model (***Section 1.8.3***). This model has been known to be suitable for longitudinal HIV infection studies due to its resistance to graft versus host disease (GvHD) (Lavender et al., 2013, 2018), a typical complication of humanized mice that leads to early sacrifice. A 1.6 IU/kg oxytocin dose was chosen for vaginal gel treatments based on previous literature examining oxytocin nasal spray treatments, as well as preliminary experiments performed in the mice (Guoynes et al., 2018; Guoynes & Marler, 2021; Razo et al., 2022). This arm of the thesis was initially meant to test the impact of oxytocin vaginal gel in preventing mucosal HIV infection, but none of the control mice became infected during the course of the study, preventing this analysis. As a result, an examination of oxytocin vaginal gel's effects on leukocyte activation and pro-inflammatory cytokine/chemokine secretion—without a corresponding inflammatory stimulus (HIV infection)—was performed.

To assess mucosal inflammation I first examined the CVL of our TKO-hBLT mice, however, sufficient leukocytes were collected from only a few mice despite collecting during the diestrus phase—a phase of the estrus cycle where leukocytes infiltrate the vaginal lumen (Ajayi & Akhigbe, 2020; Byers et al., 2012; Cora et al., 2015; McLean et al., 2012). Leukocytes were sufficiently abundant in peripheral blood, which allowed for statistical analysis of systemic inflammation. To expand on this, both CVL and serum were assayed for pro-inflammatory cytokines and chemokines of both murine and human origin, since the TKO-hBLT humanized mouse model is

able to express both. In addition, lymphatic tissues were collected to determine if oxytocin vaginal gel was able to influence leukocytes that reside in, or traffic to, the lymphatic sites of the cervical and mesenteric lymph nodes, the spleen, and the thymus. The experimental treatment schedule for the mice is outlined in ***Section 1.83*** (**Figure 2.1**).

To examine the activation status of leukocytes, the expression of the cellular markers CCR5, CD38, HLA-DR, and CD69 were chosen to measure activation status using flow cytometry (Cibrián & Sánchez-Madrid, 2017; Glaría & Valledor, 2020; Picton et al., 2012; Revenfeld et al., 2016). CCR5 is a co-receptor for HIV-1 infection, specifically R5-tropic HIV typical of mucosal transmission (Grivel et al., 2011; Wu, 2008), and also increases its expression on activated T cells (Picton et al., 2012). CD38, HLA-DR, and CD69 are acute markers of inflammation, with CD38 and HLA-DR having been shown to persist beyond initial activating stimuli and potentially represent chronic, or long-term, activation (Glaría & Valledor, 2020; Revenfeld et al., 2016). These markers allow for capture of both short-term ($CD69^+$) and long-term ($CD69^-$) activation (Cibrián & Sánchez-Madrid, 2017). hCD45 was additionally included as a marker to differentiate murine and human cells, as it is specific to human leukocytes (Baird et al., 2016). CD4 and CD8 markers were used to identify $CD4^+$ T helper cell ($CD4^+/CD8^-$), $CD8^+$ cytotoxic T cell ($CD4^-/CD8^+$), and double positive T cell ($CD4^+/CD8^+$) subgroups of leukocytes (Overgaard et al., 2015; Pennock et al., 2013; Sainz et al., 2013).

3.6.1 Analysis of $hCD45^+$ leukocyte activation in the cervicovaginal lavage of TKO-hBLT humanized mice

Previous studies, particularly those in HIV-exposed seronegative (HESN) women—women repeatedly exposed to HIV but who remain uninfected—have shown that reductions in inflammation at the vaginal mucosa is a key predictor of resistance to HIV infection (Cromarty & Archary, 2020; Lajoie et al., 2012, 2017). Given oxytocin's prior associations with the downregulation of inflammation in macrophage treated with LPS (Inoue et al., 2019; Karten et al., 2021; Yuan et al., 2016), as well as at the gut mucosa (Y. Tang et al., 2019a; Welch et al., 2014; Yu et al., 2022), I hypothesized that longitudinal oxytocin vaginal gel would downregulate

expression of CCR5, HLA-DR, CD38, and CD69 in $CD4^+/CD8^-$, $CD4^-/CD8^+$, and $CD4^+/CD8^+$ leukocytes obtained from the cervicovaginal vault of TKO-hBLT mice.

CVL was obtained from mice undergoing bi-daily treatment with either PBS or 1.6 IU/kg oxytocin vaginal gels, and then centrifuged to separate the pellet and supernatant. The supernatant was removed for later cytokine/chemokine analysis, and the pellet was then used for flow cytometry after fixation and staining for hCD45, CD4, CD8, CCR5, CD38, HLA-DR, CD69 and a live/dead dye. Gating was performed by first excluding $hCD45^-$ cells, and then subdividing cells into $CD4^+/CD8^-$ cells, indicative of $CD4^+$ T helper cells, $CD4^-/CD8^+$ cells, indicative of $CD8^+$ cytotoxic T cells, and $CD4^+/CD8^+$ cells, indicative of double-positive T cells. These subsets were then measured for the percent of cells expressing CCR5, HLA-DR, CD38, or CD69, as well as the intensity of the expression in those cells as measured by median fluorescence intensity (MFI). Comparisons were made between treated and untreated groups using a mixed-effects model with Sidak's multiple comparisons test at $\alpha = 0.05$.

$CD4^+/CD8^-$, $CD4^-/CD8^+$, and $CD4^+/CD8^+$ cells showed no significant differences between PBS and oxytocin treated mice with respect to the % of cells expressing CCR5, HLA-DR, CD38, or CD69 in CVL (p-values > 0.05) (**Figure 3.20A**). MFI analysis yielded similar results for CCR5, CD38, and CD69 in $CD4^+/CD8^-$ cells (p-values > 0.05) (**Figure 3.20B**). Not enough data was available to conduct mixed-methods analysis for HLA-DR MFI in $CD4^+/CD8^-$ cells or CCR5, HLA-DR, CD38, and CD69 MFI in both $CD4^-/CD8^+$ and $CD4^+/CD8^+$ cells (**Figure 3.20B**).

In summary, intravaginal administration of 1.6 IU/kg oxytocin vaginal gel did not significantly alter the % of mucosal leukocytes expressing CCR5, HLA-DR, CD38, or CD69 in any of the $CD4^+/CD8^-$, $CD4^-/CD8^+$, or $CD4^+/CD8^+$ subsets. Effects on the MFI of HLA-DR in $CD4^+/CD8^-$ cells, as well as the MFI of CCR5, HLA-DR, CD38, and CD69 in $CD4^-/CD8^+$ and $CD4^+/CD8^+$ cells could not be analyzed due to missing data. These data do not support the hypothesis that longitudinal oxytocin vaginal gel reduces the expression of CCR5, HLA-DR, CD38, and CD69 in mucosal leukocytes of TKO-hBLT mice.

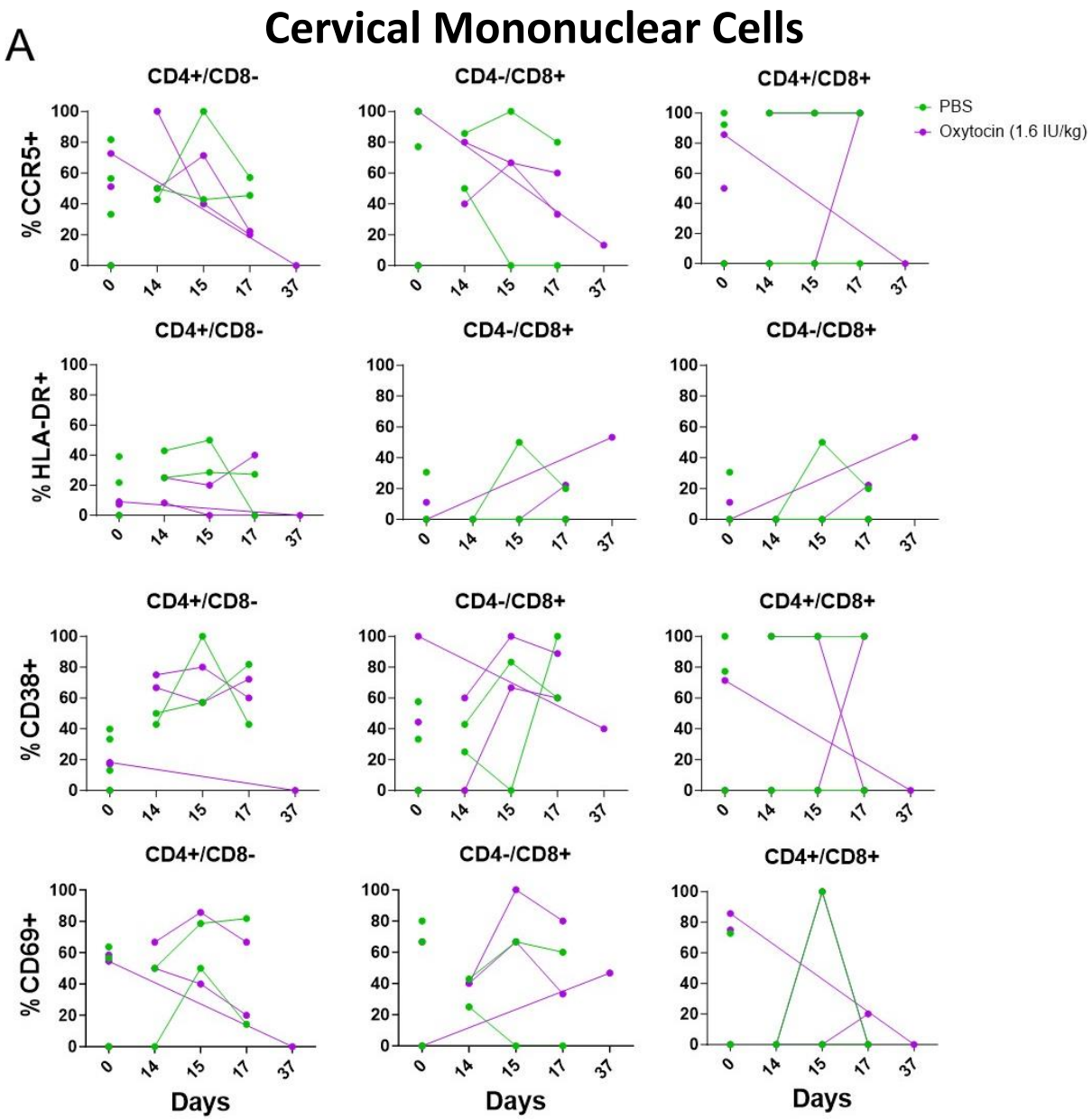


Figure 3.20 (1/2) Intravaginal administration of oxytocin-containing HEC gel does not modulate baseline immune activation status of human leukocytes in the CVL of humanized mice as measured through flow cytometry. Percentage (%) expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured longitudinally in the CVL of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg of mouse weight) or control PBS 1% HEC gel. Comparisons were made using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test with a cut-off of $p < 0.05$ for time points that had both oxytocin and PBS data points. PBS-treated (n = 6) and oxytocin-treated mice (n=5) were analyzed.

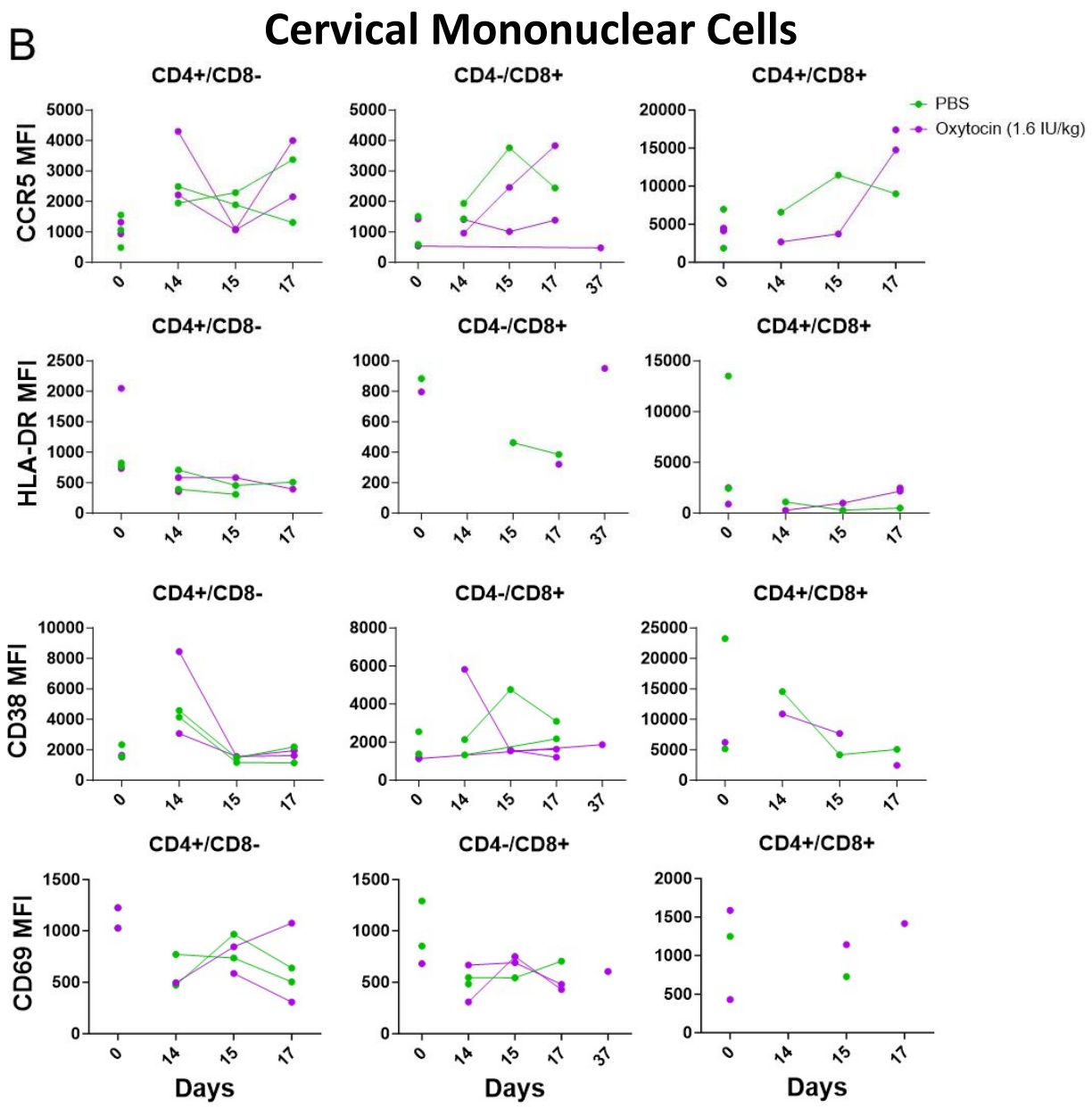


Figure 3.20 (2/2) Intravaginal administration of oxytocin-containing HEC gel does not modulate baseline immune activation status of human leukocytes in the CVL of humanized mice as measured through flow cytometry. Percentage (%) expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured longitudinally in the CVL of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg of mouse weight) or control PBS 1% HEC gel. Comparisons were made using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test with a cut-off of $p < 0.05$ for time points that had both oxytocin and PBS data points. PBS-treated (n = 6) and oxytocin-treated mice (n=5) were analyzed.

3.6.2 Analysis of hCD45$^+$ leukocyte activation in systemic circulation of TKO-hBLT humanized mice

Given the association of increased plasma and blood inflammatory biomarkers with HIV susceptibility and infection risk (Fulcher et al., 2022; McInally et al., 2021), I sought to determine if oxytocin vaginal gel was able to modulate cellular activation of systemic T cells within TKO-hBLT mice. This was further supported by oxytocin's property of modulating biomarkers, such as pro-inflammatory cytokines, within systemic circulation (Imami et al., 2020; Nishimura et al., 2022). I aimed to analyze the systemic impact of oxytocin on CCR5, HLA-DR, CD38, and CD69 expression in peripheral blood leukocytes in TKO-hBLT mice after vaginal oxytocin gel administration. I hypothesized that longitudinal intravaginal oxytocin gel would reduce the expression of CCR5, HLA-DR, CD38, and CD69 in circulating hCD45$^+$ immune cells in the peripheral blood of TKO-hBLT mice.

Blood samples were obtained from mice undergoing bi-daily treatment with either PBS or 1.6 IU/kg oxytocin vaginal gel treatment and then spun down to separate plasma from cells. The cell pellet was then treated to remove red blood cells, and this suspension was then used for flow cytometry after fixation and staining for hCD45, CD4, CD8, CCR5, CD38, HLA-DR, CD69 and a live/dead dye. Gating was performed by first excluding hCD45$^-$ cells, and then subdividing cells into CD4$^+$/CD8$^-$ cells, indicative of CD4$^+$ T helper cells, CD4$^-$/CD8$^+$ cells, indicative of CD8$^+$ cytotoxic T cells, and CD4$^+$/CD8$^+$ cells, indicative of double-positive T cells. These subsets were then measured for the percent of cells expressing CCR5, HLA-DR, CD38, or CD69, as well as the intensity of the expression in those cells as measured by median fluorescence intensity (MFI). Comparisons were made between treated and untreated groups using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test at $\alpha = 0.05$.

CD4$^+$/CD8$^-$ cells showed no differences between oxytocin and PBS treated mice with respect to % of cells expressing CCR5, HLA-DR, CD38, or CD69 (p-values > 0.05) (**Figure 3.21A**). Analysis of CD4$^-$/CD8$^+$ cells showed significant differences between PBS and oxytocin treatment groups for both % of cells expressing CCR5 ($p = 0.0085$) and HLA-DR ($p = 0.0079$) (**Figure 3.21A**), however, post-hoc analysis using Sidak's multiple comparisons test did not reveal specific time points of significant difference between the two groups for either CCR5 or HLA-DR

(p-values > 0.05). No significant differences were found for CD38 or CD69 % expression (p-values > 0.05) (**Figure 3.21A**). $CD4^+/CD8^+$ cells showed a significant difference between PBS and oxytocin treatments in HLA-DR % expression (p = 0.049), but not CCR5, CD38, or CD69 % expression (p-values > 0.05) (**Figure 3.21A**). Multiple comparisons post-hoc analysis for HLA-DR % expression did not indicate a specific time point of significant difference between the two groups (p-values > 0.05).

$CD4^+/CD8^-$ cells showed no significant differences between oxytocin and PBS treated mice in MFI analysis for CCR5, HLA-DR, or CD38 (p-values > 0.05) (**Figure 3.21B)**. CD69 MFI was found to be significantly different between oxytocin and PBS treated mice over the duration of the study (p = 0.0085) (**Figure 3.21B**), however, subsequent post-hoc analysis using Sidak's multiple comparisons test failed to indicate a particular time point responsible for the effect (p-values > 0.05). $CD4^-/CD8^+$ MFI measurements did not reveal significant differences for CCR5, HLA-DR, CD38, or CD69 (p-values > 0.05) (**Figure 3.21B**). In $CD4^+/CD8^+$ cells, CD38 MFI levels were found to be significantly different between oxytocin and PBS treated mice, with one oxytocin-treated mouse showing elevated increases above all other mice between days 56 – 70 (p = 0.0081) (**Figure 3.21B**). Multiple comparisons analysis for CD38 MFI in $CD4^+/CD8^+$ cells did not reveal significant differences between the two groups at any one time point (p-values > 0.05). No differences were detected for CCR5, HLA-DR, or CD69 MFI between the two groups (p-values > 0.05) (**Figure 3.21B**).

In summary, differences were detected between oxytocin and PBS treated mice when analyzed across the duration of the entire study for $CD4^+/CD8^-$ CD69 MFI, $CD4^-/CD8^+$ CCR5 and HLA-DR % expression, and $CD4^+/CD8^+$ HLA-DR % expression and CD38 MFI. Post-hoc analyses using Sidak's multiple comparisons of these findings did not indicate significant differences at any particular timepoint, however. Taken together, this data suggests that oxytocin vaginal gel has impacts on leukocyte activation in systemic circulation, but these effects lack the power to be detected by multiple comparisons analysis. These data do not support the hypothesis that longitudinal oxytocin vaginal gel reduces CCR5, HLA-DR, CD38, and CD69 expression in systemic leukocytes of TKO-hBLT mice.

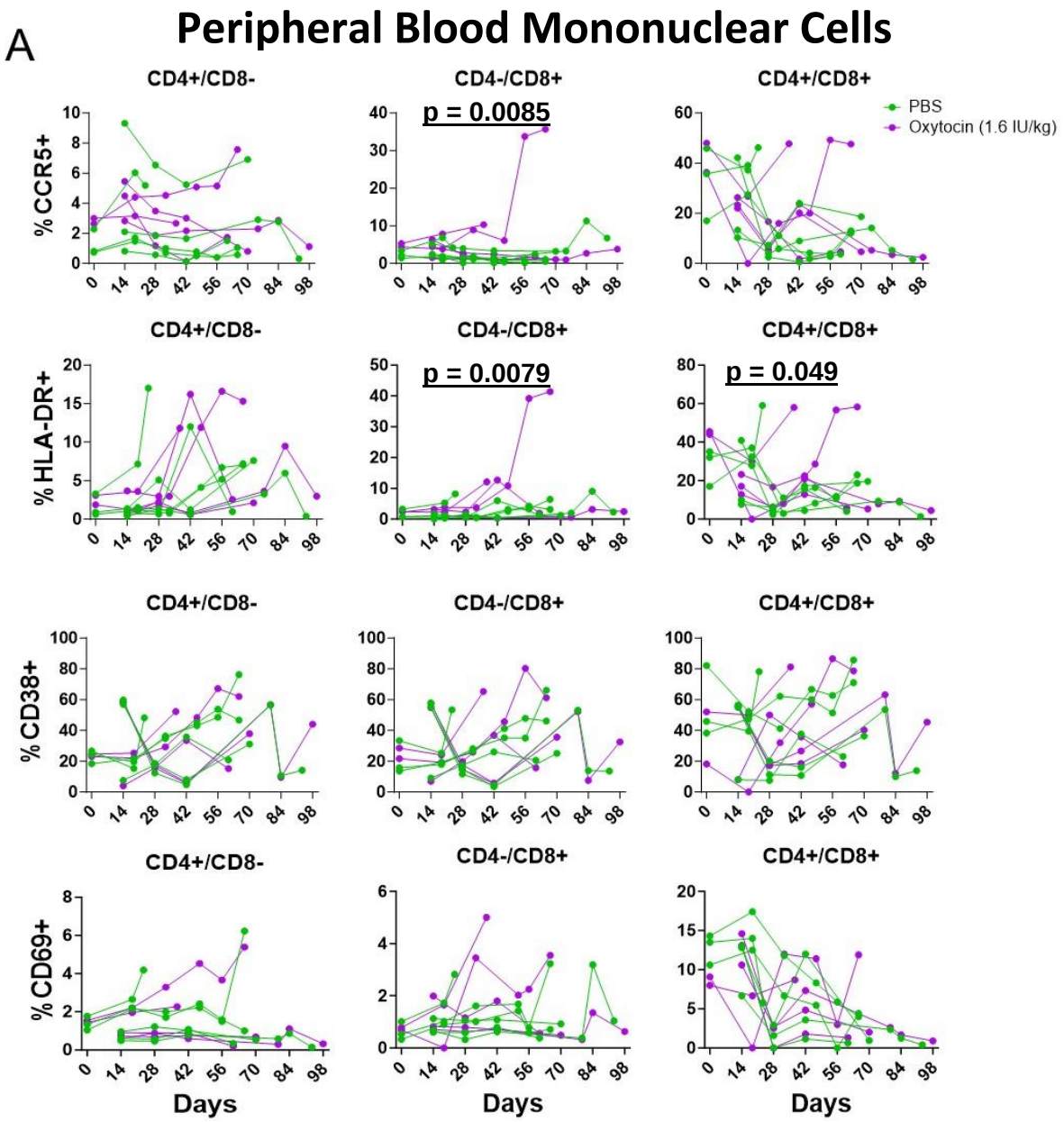


Figure 3.21 (1/2) Oxytocin vaginal gel treatment had no effect on the baseline immune activation status of the circulating human leukocytes in the peripheral blood of the humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of the activation markers CCR5, HLA-DR, CD38, and CD69 were measured longitudinally in the blood of humanized mice that were treated bi-daily with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Comparisons were made using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test with a cut-off of $p < 0.05$. n = 8 PBS-treated and 6 oxytocin-treated mice.

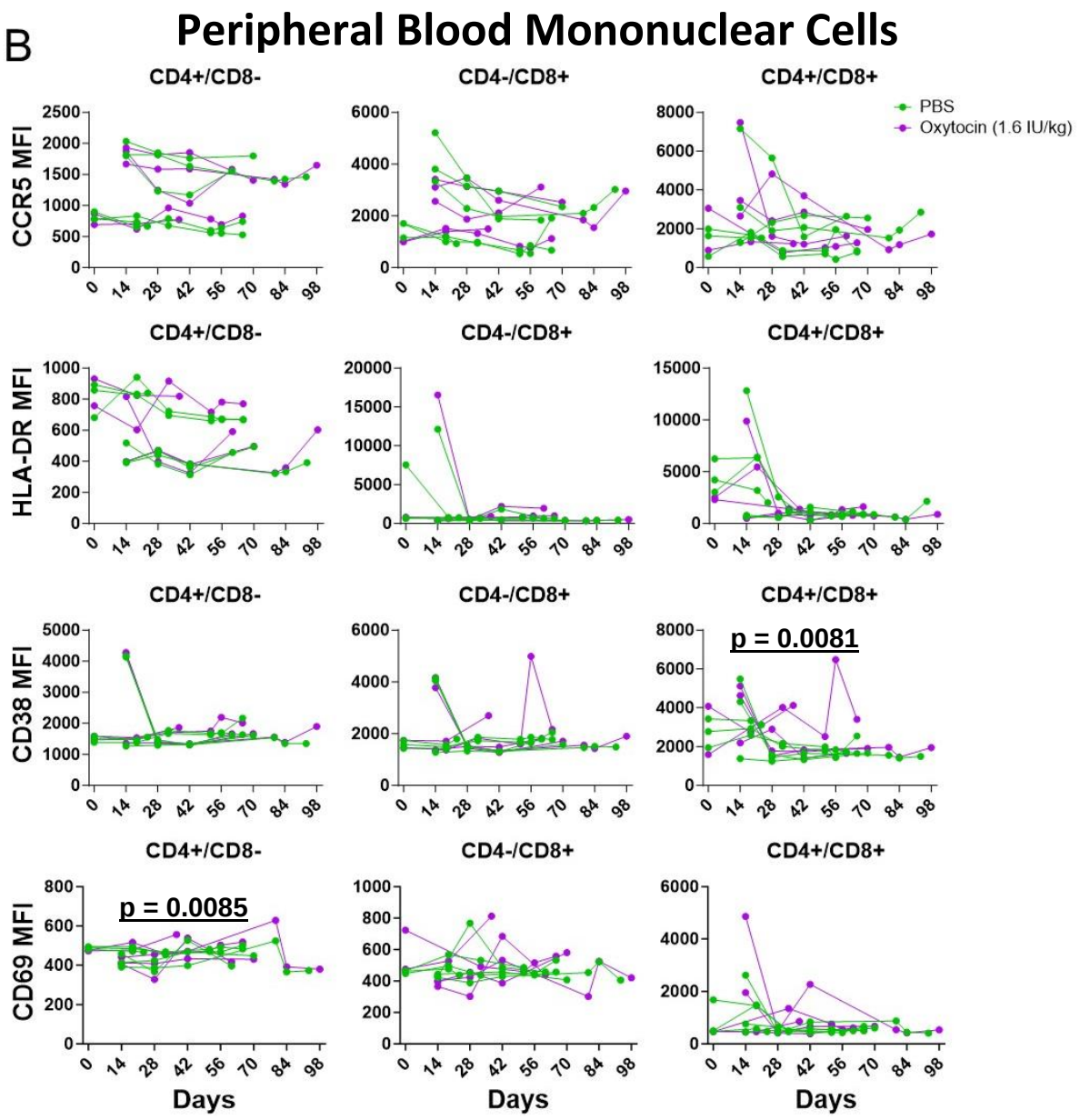


Figure 3.21 (2/2) Oxytocin vaginal gel treatment had no effect on the baseline immune activation status of the circulating human leukocytes in the peripheral blood of the humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of the activation markers CCR5, HLA-DR, CD38, and CD69 were measured longitudinally in the blood of humanized mice that were treated bi-daily with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Comparisons were made using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test with a cut-off of $p < 0.05$. n = 8 PBS-treated and 6 oxytocin-treated mice.

3.6.3 The effect of oxytocin vaginal gel on mucosal and systemic pro-inflammatory cytokine/chemokine levels in TKO-hBLT humanized mice

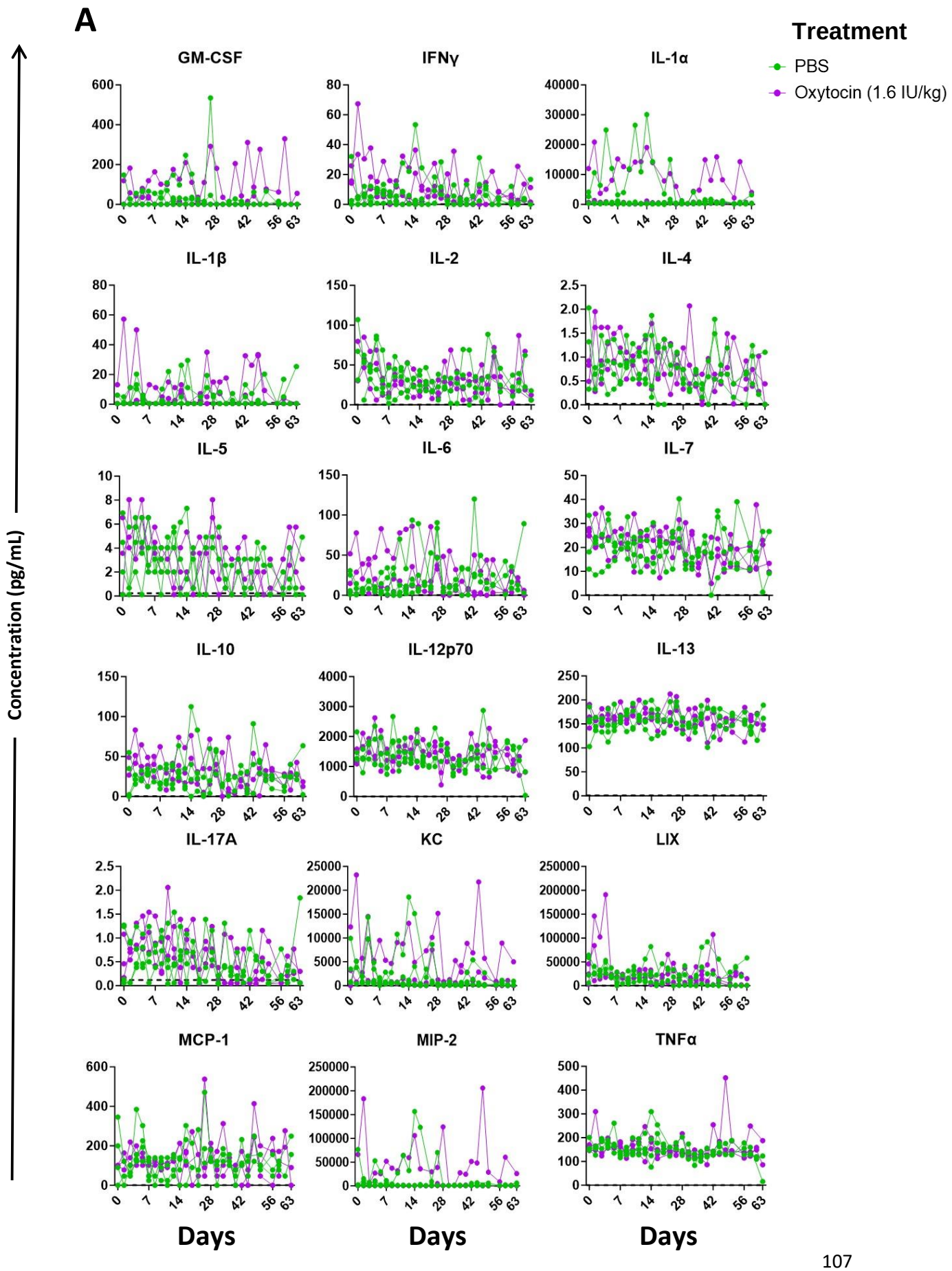
Quantifying pro-inflammatory cytokines and chemokines is useful in predicting the overall inflammatory state at both mucosal and systemic sites (Kany et al., 2019; Koelman et al., 2019). Given the association of HIV susceptibility with increased levels of inflammation (Cromarty & Archary, 2020; Mtshali et al., 2021), I sought to characterize oxytocin vaginal gel's ability to modulate pro-inflammatory cytokine/chemokine levels both in the blood and at cervicovaginal mucosa. Due to the TKO-hBLT model being a chimeric model containing both murine epithelial cells and human leukocytes (Karpel et al., 2015; Lavender et al., 2013, 2018), it is necessary to quantify both murine and human cytokines to capture a complete immunological profile. This is also important given that oxytocin is shared between the two species and is able to bind to both human and mouse OXTRs. Given oxytocin's previous associations with reductions in inflammation at mucosal sites and in immune cells (Imami et al., 2020; Mehdi et al., 2022; Y. Tang et al., 2019b; Welch et al., 2014), I hypothesized that longitudinal oxytocin vaginal gel treatment would reduce the levels of both murine and human pro-inflammatory cytokines/chemokines in the CVL and serum of TKO-hBLT mice.

In order to assay pro-inflammatory cytokines and chemokines in both CVL and serum, CVL and blood from mice undergoing bi-daily treatment with either PBS or 1.6 IU/kg oxytocin vaginal gel were centrifuged to produce purified CVL supernatant and serum. Both murine and human assays were conducted using high-sensitivity assays with variable lower limits of detection for each cytokine/chemokine. Signals that were deemed out of the lowest range of the standard curve for each cytokine/chemokine were presented as ½ the lower limit of detection. Comparisons were made between oxytocin and PBS groups using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test at $\alpha = 0.05$.

Murine pro-inflammatory cytokine/chemokine analysis of TKO-hBLT CVL revealed that there were no significant effects of oxytocin on GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, KC, LIX, MCP-1, MIP-2, or TNFα levels (p-values > 0.05) (**Figure 3.22A**). Similar results were seen for the effects of oxytocin treatment in TKO-hBLT serum (p-values > 0.05) (**Figure 3.22B**). Measurement of human pro-inflammatory

cytokine/chemokines in TKO-hBLT CVL indicated no significant differences for GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, IL-23, and TNFα between oxytocin and PBS treatment groups (**Figure 3.23A**). Serum analysis mirrored results seen in CVL analysis, with no significant effect of oxytocin found on cytokine/chemokine levels (**Figure 3.23B**).

In summary, oxytocin vaginal gel treatment did not significantly alter the levels of pro-inflammatory murine cytokines/chemokines in either TKO-hBLT CVL or serum. Similar findings showed no difference between PBS or oxytocin vaginal gel treatment conditions with respect to human pro-inflammatory cytokines/chemokines in TKO-hBLT CVL and serum. Together, this data does not support the hypothesis that longitudinal oxytocin vaginal gel treatment reduces levels of human or murine mucosal and systemic pro-inflammatory cytokines/chemokines in TKO-hBLT mice.


A
Treatment
PBS
Oxytocin (1.6 IU/kg)
GM-CSF
IFNγ
IL-1α
IL-1β
IL-2
IL-4
IL-5
IL-6
IL-7
IL-10
IL-12p70
IL-13
IL-17A
KC
LIX
MCP-1
MIP-2
TNFα
Concentration (pg/mL)
Days
Days
Days

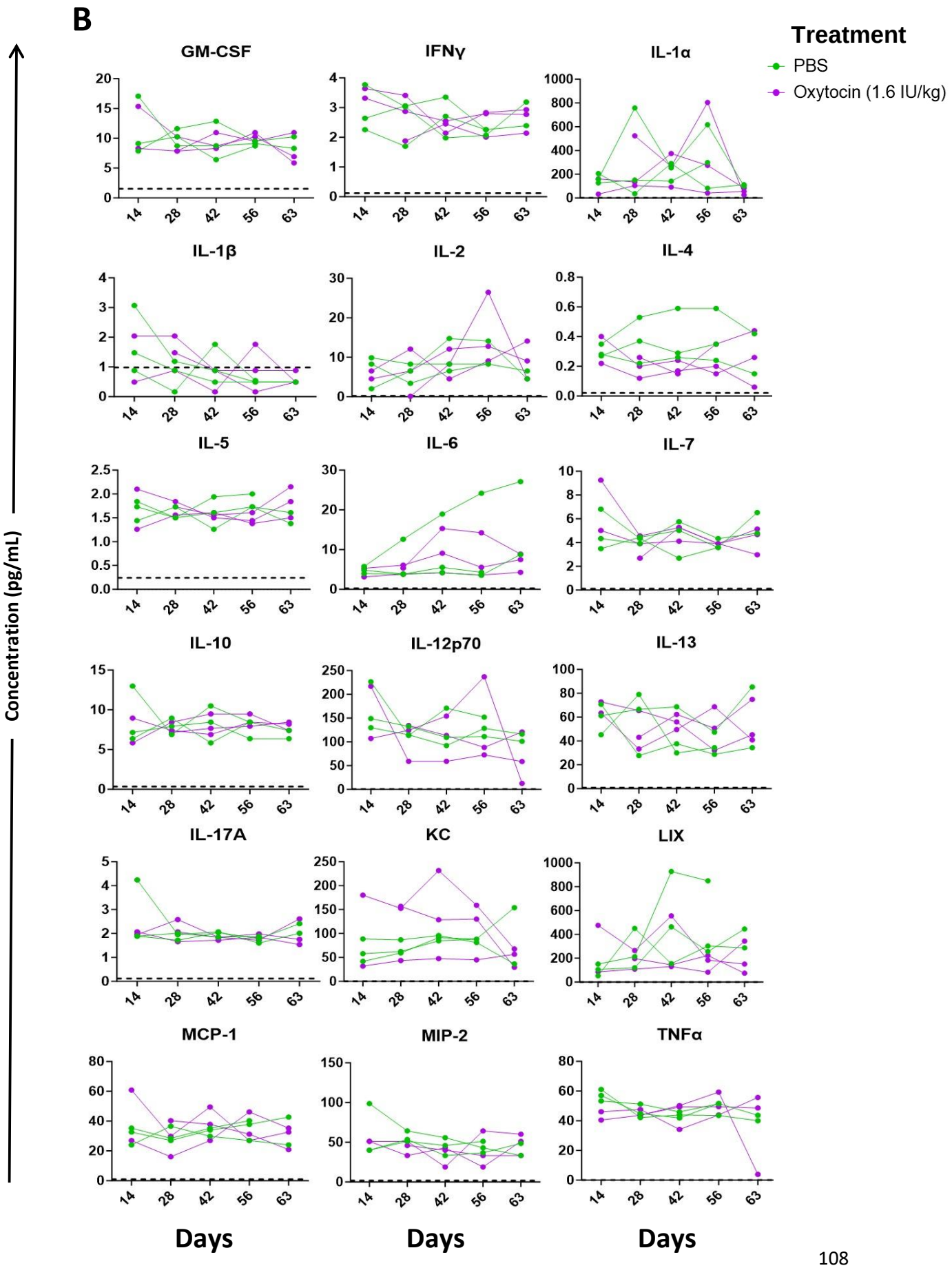
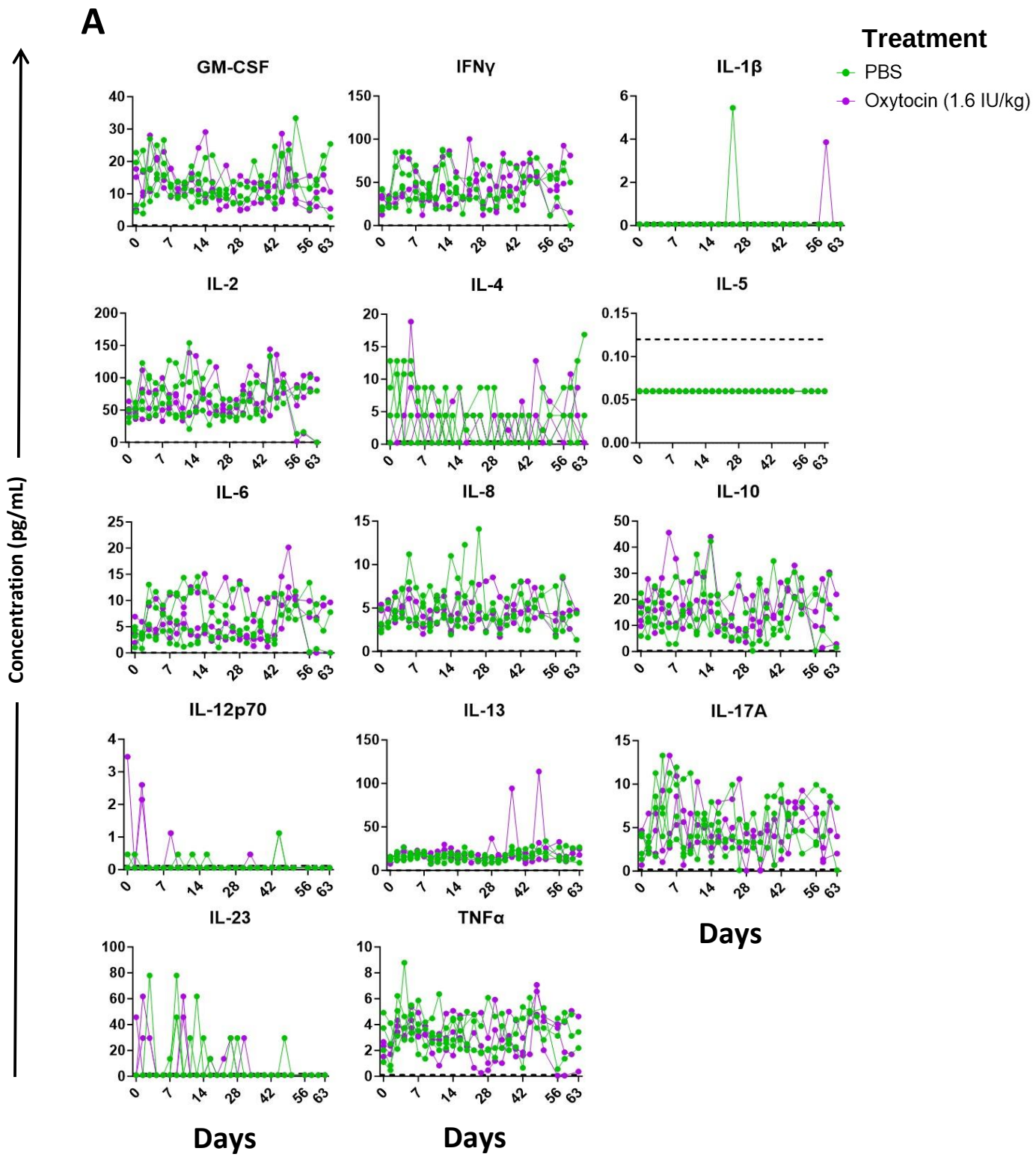

B
Treatment
PBS
Oxytocin (1.6 IU/kg)
Concentration (pg/mL)
GM-CSF
IFNγ
IL-1α
IL-1β
IL-2
IL-4
IL-5
IL-6
IL-7
IL-10
IL-12p70
IL-13
IL-17A
KC
LIX
MCP-1
MIP-2
TNFα
Days
Days
Days

Figure 3.22 Oxytocin vaginal gel treatment does not modulate levels of murine pro-inflammatory cytokines in CVL or serum of humanized mice as measured through multiplexed ELISA. CVL (A) and serum (B) murine pro-inflammatory cytokines were measured longitudinally from humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Comparisons were made using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test with a cut-off of $p < 0.05$. Dashed lines (···) indicate the lower limit of detection, with values below this threshold obtained by extrapolation. Undetectable datapoints estimated at ½ the lower limit of detection. n = 3 PBS-treated and 4 oxytocin-treated mice.


A
Treatment
PBS
Oxytocin (1.6 IU/kg)
GM-CSF
IFNγ
IL-1β
IL-2
IL-4
IL-5
IL-6
IL-8
IL-10
IL-12p70
IL-13
IL-17A
IL-23
TNFα
Concentration (pg/mL)
Days
Days
Days

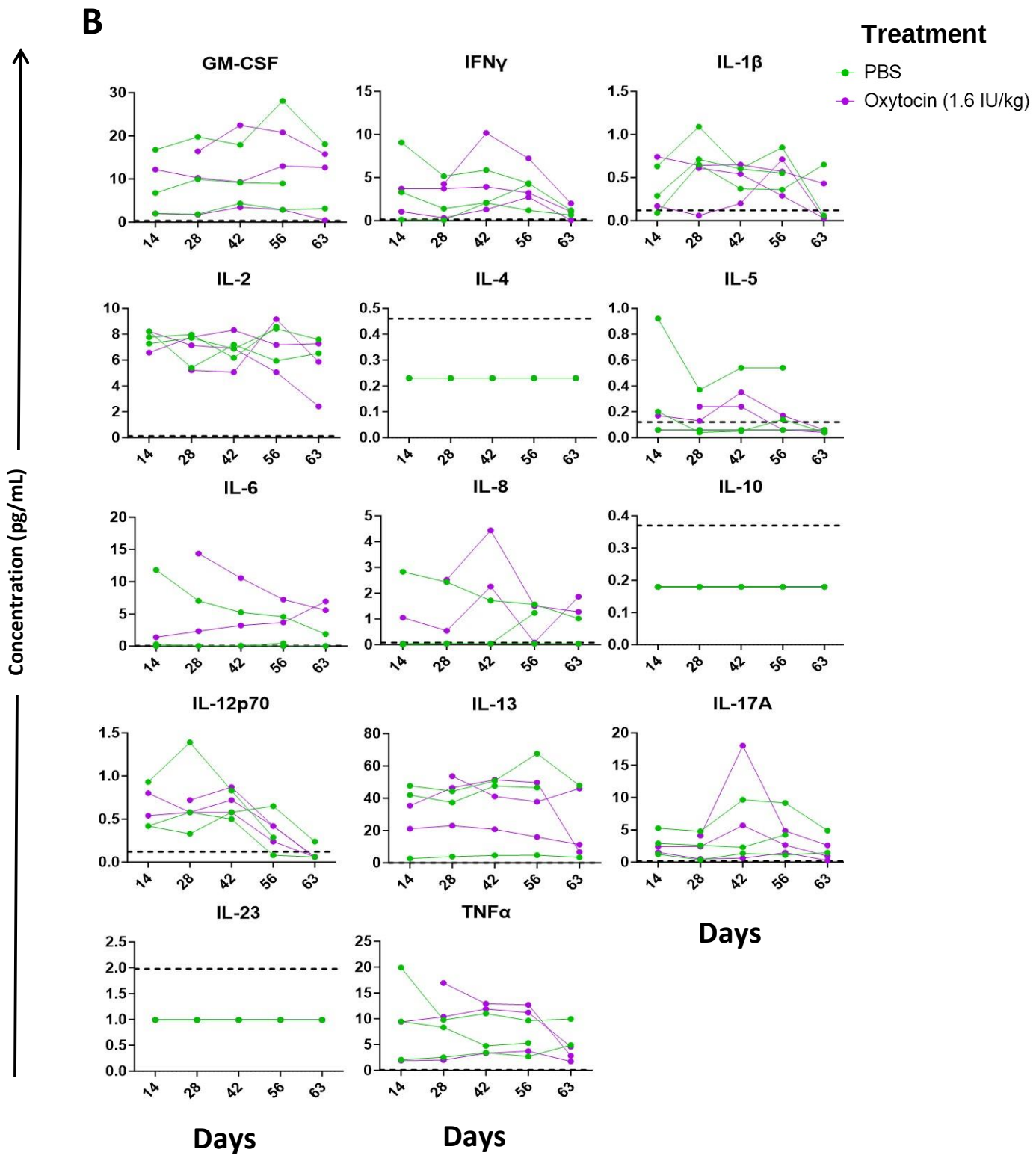

B
Treatment
PBS
Oxytocin (1.6 IU/kg)
GM-CSF
IFNγ
IL-1β
IL-2
IL-4
IL-5
IL-6
IL-8
IL-10
IL-12p70
IL-13
IL-17A
IL-23
TNFα
Concentration (pg/mL)
Days
Days
Days

Figure 3.23 Oxytocin vaginal gel treatment does not modulate levels of human pro-inflammatory cytokines in CVL or serum of humanized mice as measured through multiplexed ELISA. CVL (A) and serum (B) human pro-inflammatory cytokines were measured longitudinally from humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Comparisons were made using a mixed-effects model with Geisser-Greenhouse correction and Sidak's multiple comparisons test with a cut-off of $p < 0.05$. Dashed lines (···) indicate the lower limit of detection, with values below this threshold obtained by extrapolation. Undetectable datapoints estimated at ½ the lower limit of detection. n = 3 PBS-treated and 4 oxytocin-treated mice.

3.6.4 Analysis of hCD45$^+$ leukocyte activation in central lymphatic tissues of TKO-hBLT humanized mice

Tissues represent important immunological niches within the body, with the lymphatic system being responsible for draining these tissue sites. Leukocytes also traffic from peripheral blood to the lymph nodes, where antigens from tissue are brought to activate T and B cells (Bousso, 2008; Kallikourdis et al., 2015). This allows for the potential of HIV transmission and amplification within these lymphatic sites (Lopez et al., 2019; Rinaldo, 2013), as well as the modulation of the activation status of T cells (Castell et al., 2019; Saxena et al., 2019). For this reason, I sought to quantify the degree to which oxytocin vaginal gel was able to modulate the expression of the activation markers CCR5, HLA-DR, CD38, and CD69 in T cell subsets in lymphatic tissue, given the associations of oxytocin with reductions in inflammation (Deing et al., 2013; Imami et al., 2020; Klein et al., 2014, 2016; Nishimura et al., 2022). I hypothesized that longitudinal oxytocin vaginal gel treatment would reduce the levels of expression of CCR5, HLA-DR, CD38, and CD69 in hCD45$^+$ immune cells in the cervical lymph node, mesenteric lymph node, spleen, and thymus of TKO-hBLT mice.

Tissue samples of cervical lymph node, mesenteric lymph node, spleen, and thymus from mice undergoing bi-daily treatment with either PBS or 1.6 IU/kg oxytocin vaginal gel treatments were homogenized to effect single cell suspensions at mouse sacrifice. Single cell suspensions were then filtered to remove remaining tissue. This filtrate was then used for flow cytometry after fixation and staining for hCD45, CD4, CD8, CCR5, CD38, HLA-DR, CD69 and a live/dead dye. Gating was performed by first excluding hCD45$^-$ cells, and then subdividing cells into CD4$^+$/CD8$^-$ cells, indicative of CD4$^+$ T helper cells, CD4$^-$/CD8$^+$ cells, indicative of CD8$^+$ cytotoxic T cells, and CD4$^+$/CD8$^+$ cells, indicative of double-positive T cells. These subsets were then measured for the percent of cells expressing CCR5, HLA-DR, CD38, or CD69, as well as the intensity of the expression in those cells as measured by median fluorescence intensity (MFI). Comparisons were made between treated and untreated groups using Mann-Whitney tests at an $\alpha = 0.05$.

In the cervical lymph node, no significant difference was found between oxytocin and PBS treated mice for % expression of CCR5, HLA-DR, CD38, or CD69 (p-values > 0.05) in any of the subsets, although HLA-DR (**Oxytocin**: 24% vs **PBS**: 7.0%, p = 0.059) and CD69 (**Oxytocin**: 38%

vs **PBS**: 6.9%, p = 0.053) % expression trended towards higher expression in oxytocin-treated mice for $CD4^-/CD8^+$ cells (**Figure 3.24A**). MFI analysis did not reveal any changes in CCR5, HLA-DR, CD38, or CD69 with oxytocin treatment (p-values > 0.05) (**Figure 3.24B**). The mesenteric lymph node showed similar expression levels for CCR5, HLA-DR, CD38, and CD69 in oxytocin and PBS treated mice, with neither % expression (**Figure 3.25A**) nor MFI (**Figure 3.25B**) displaying any significant changes in oxytocin-treated mice (p-values > 0.05) (**Figure 3.25A**). Both spleen (**Figure 3.26**) and thymus (**Figure 3.27**) tissues showed no changes in CCR5, HLA-DR, CD38, or CD69 expression in oxytocin-treated mice (p-values > 0.05).

In summary, oxytocin vaginal gel led to trends in increased expression of % HLA-DR and CD69 in $CD4^-/CD8^+$ cells of the cervical lymph node, as well as increased HLA-DR MFI in $CD4^-/CD8^+$ cells of the mesenteric lymph node. These associations did not reach statistical significance, however. This indicates that oxytocin vaginal gel does not significantly modulate CCR5, HLA-DR, CD38, or CD69 expression in the cervical lymph node, mesenteric lymph node, spleen, or thymus of TKO-hBLT mice, although follow-up studies may be warranted to explore further associations of potential associations of oxytocin treatment with increases in activation markers in cervical lymph node $CD4^-/CD8^+$ cells. These data do not support the hypothesis that longitudinal oxytocin vaginal gel treatment reduces the expression of CCR5, HLA-DR, CD38, and CD69 in leukocytes of the cervical lymph node, mesenteric lymph node, spleen, and thymus of TKO-hBLT humanized mice.

Together, with prior TKO-hBLT data (***Sections 3.6.1–3.6.3)***, these findings suggest that longitudinal oxytocin vaginal gel is insufficient to affect TKO-hBLT $CD4^+$ and $CD8^+$ cell activation or reduce levels of mucosal and systemic pro-inflammatory cytokines/chemokines in TKO-hBLT humanized mice.

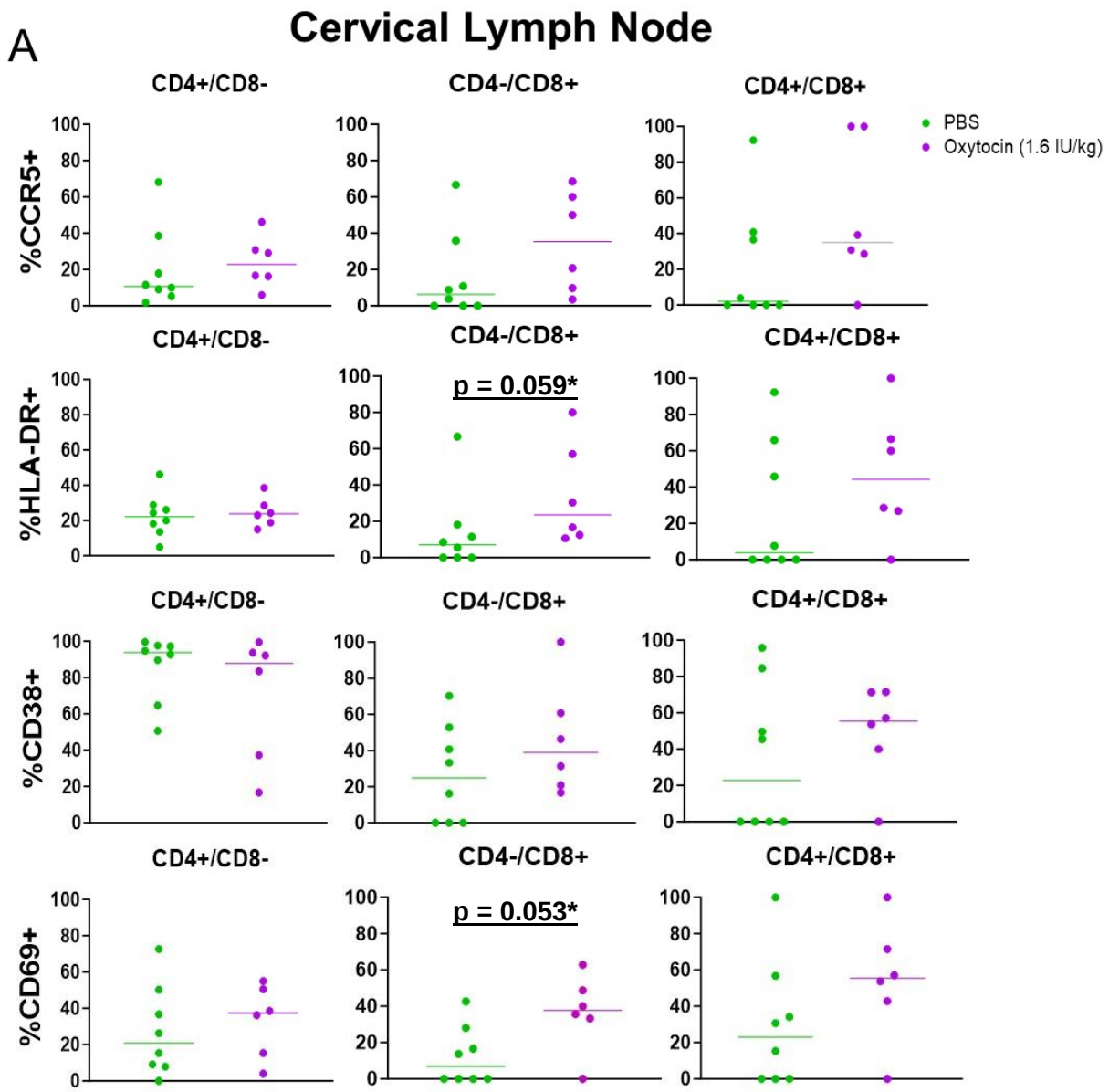


Figure 3.24 (1/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in the cervical lymph node (cLN) of humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in cLN tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

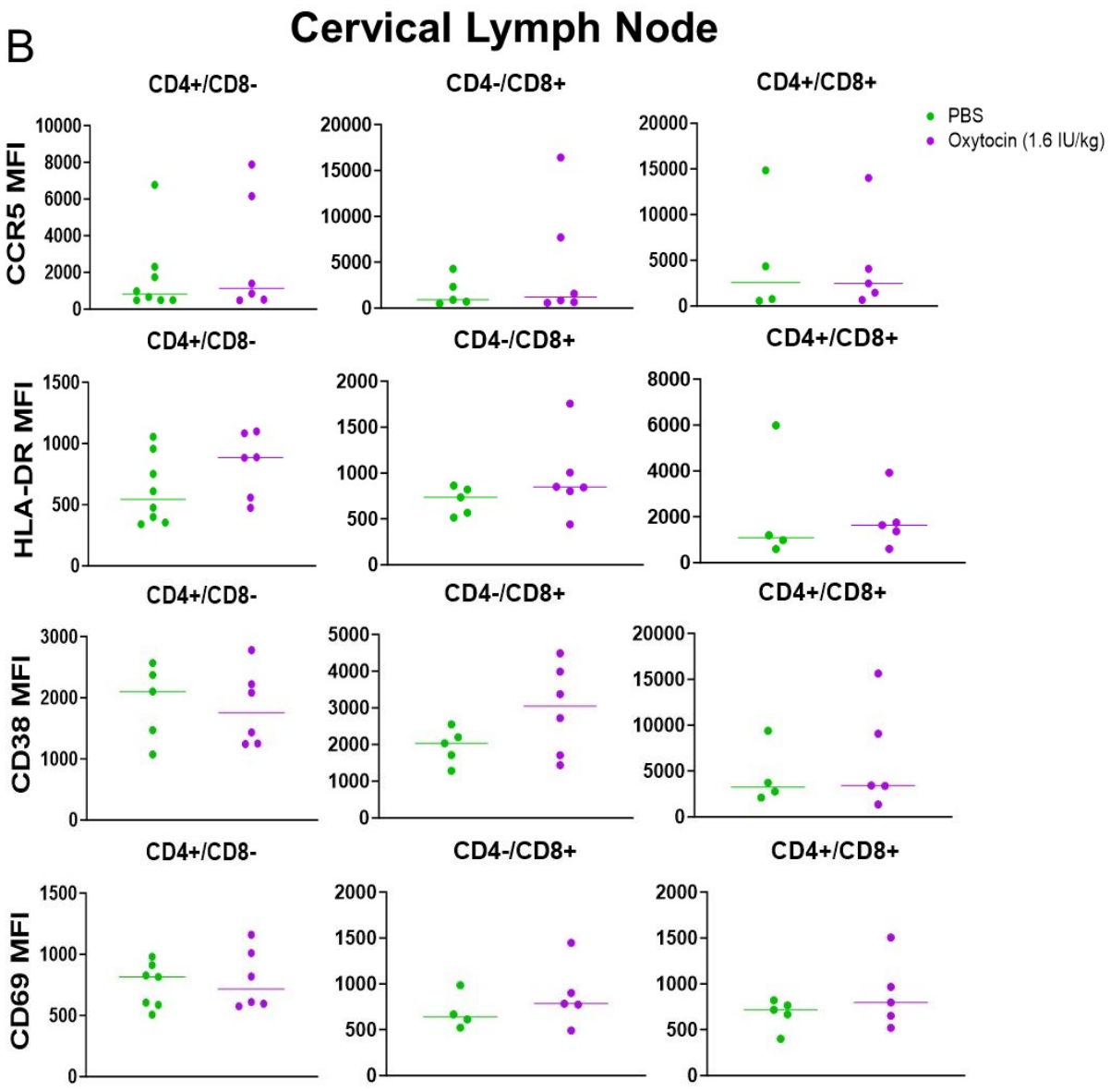


Figure 3.24 (2/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in the cervical lymph node (cLN) of humanized mice s measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in cLN tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

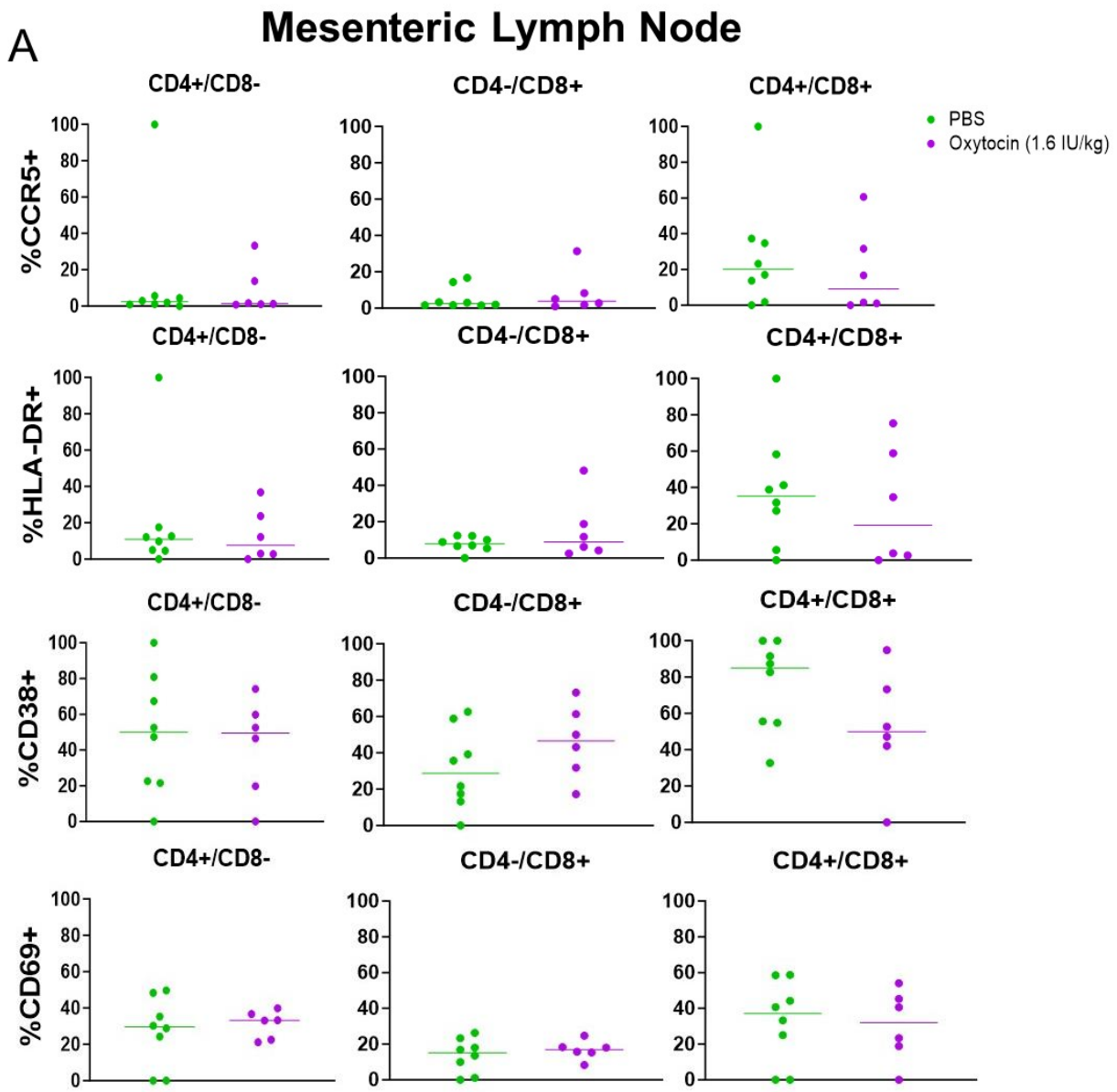


Figure 3.25 (1/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in the mesenteric lymph node (mLN) of humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in mLN tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

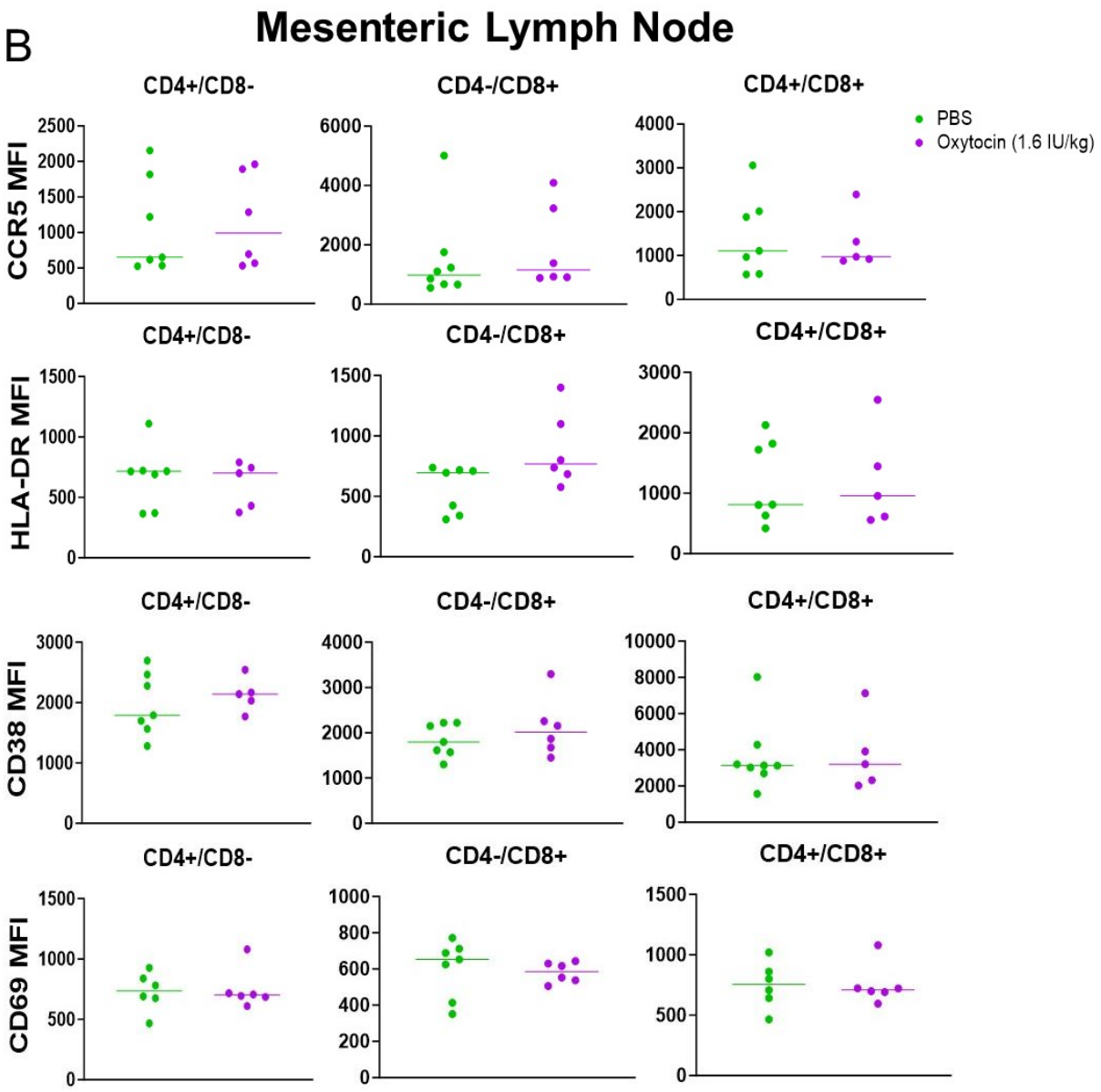


Figure 3.25 (2/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in the mesenteric lymph node (mLN) of humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in mLN tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

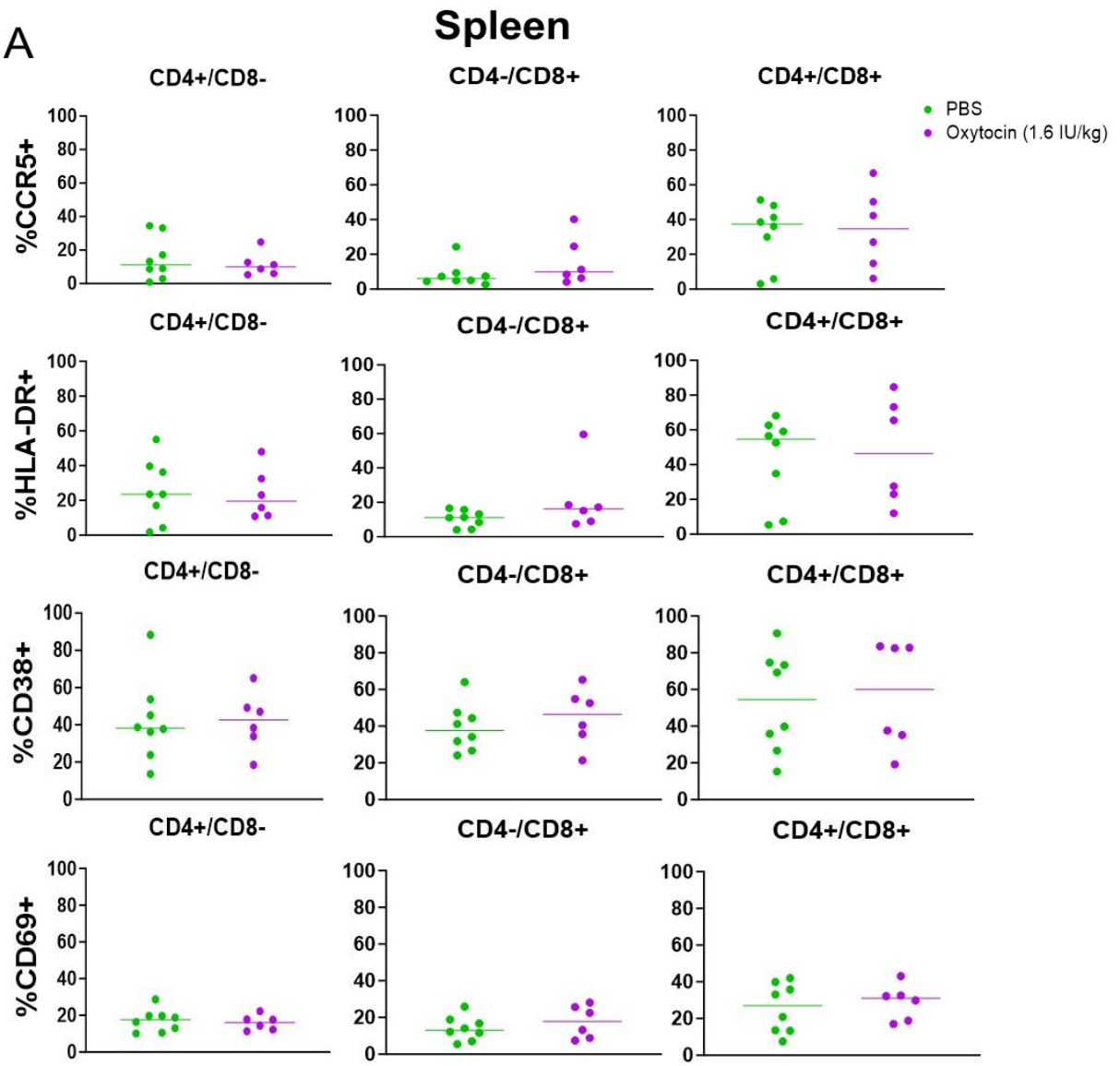


Figure 3.26 (1/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in splenic tissues of humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in splenic tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

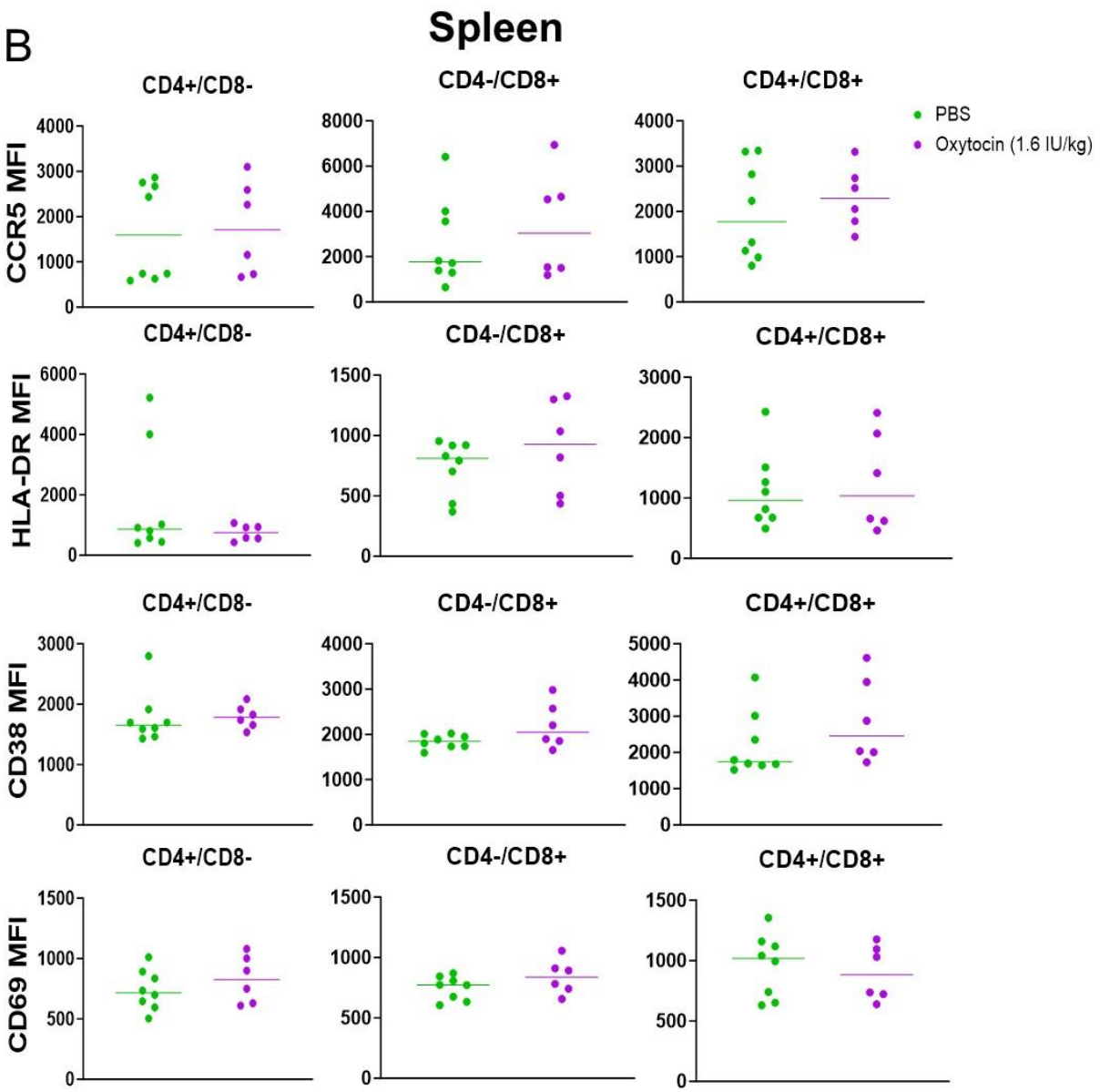


Figure 3.26 (2/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in splenic tissues of humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in splenic tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

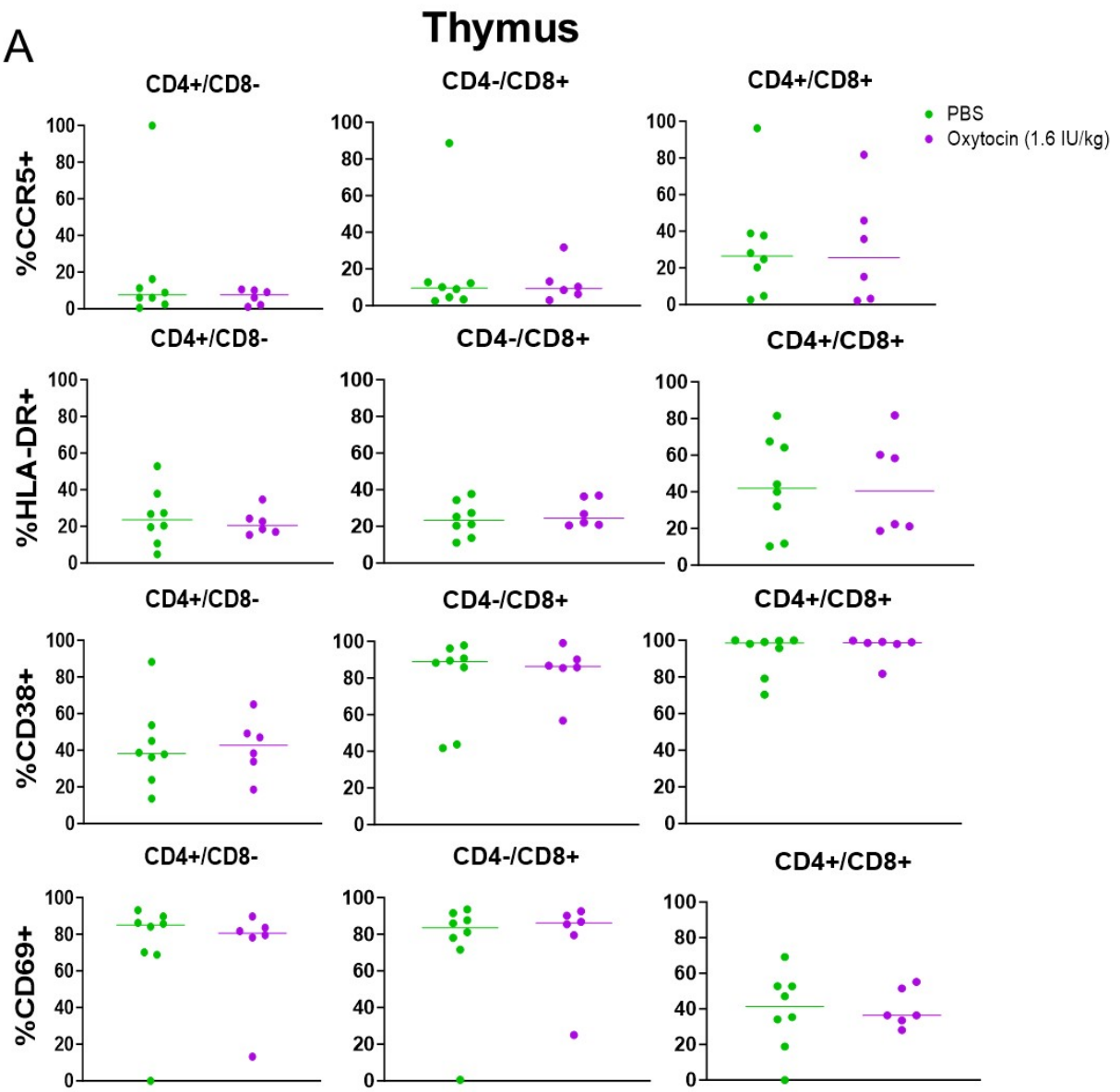


Figure 3.27 (1/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in fetal thymic tissues of humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in fetal thymic tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

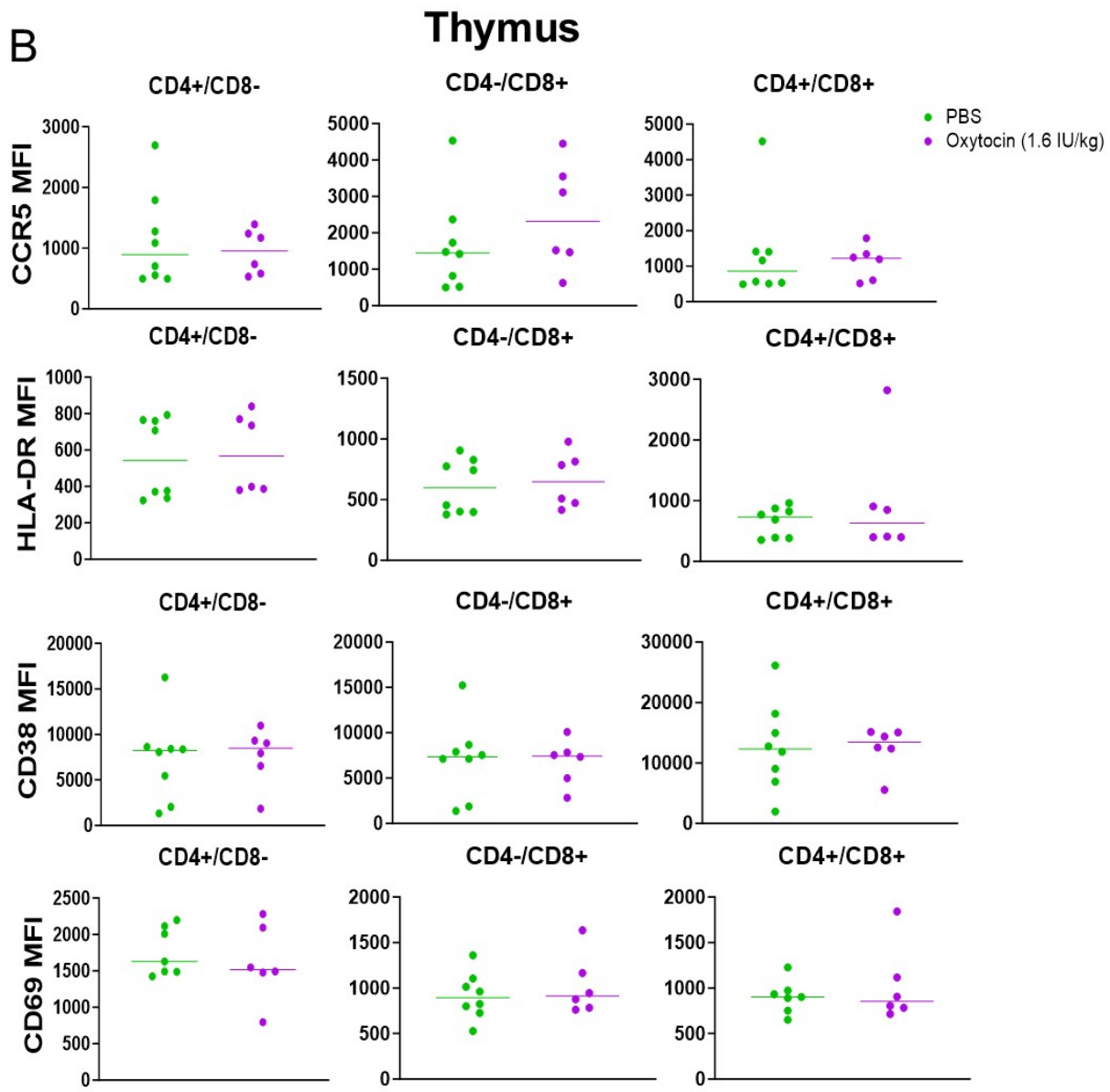


Figure 3.27 (2/2) Oxytocin vaginal gel treatment does not modulate baseline immune activation status of human leukocytes in fetal thymic tissues of humanized mice as measured through flow cytometry. % expression (A) and MFI (B) of activation markers CCR5, HLA-DR, CD38, and CD69 were measured at sacrifice in fetal thymic tissues of humanized mice undergoing repeated bi-daily vaginal gel administration with either an oxytocin-containing (1.6 IU/kg) or control PBS 1% HEC gel. Cells were sub-divided into CD4/CD8 subgroups after selection for the human leukocyte marker CD45. Comparisons were made using a Mann-Whitney test with an $\alpha = 0.05$. Centre lines represent medians. n = 8 PBS-treated and 6 oxytocin-treated mice.

Chapter 4: Discussion

Oxytocin's effect at the female genital tract is an understudied area with important implications for the transmission of sexually transmitted infections, such as HIV. This thesis aimed to determine the impact of oxytocin in directly modulating pro-inflammatory cytokine/chemokine expression and wound healing at the FGT—both key components in impacting HIV susceptibility (Burgener et al., 2015; McInally et al., 2021). My hypothesis was that oxytocin would reduce the expression of pro-inflammatory cytokines/chemokines under basal and stimulatory conditions for FGT epithelium and improve the speed at which the FGT epithelium recovers from wounds in *in vitro* scratch assays. This addresses the gap of knowledge wherein studies have examined oxytocin vaginal gels for treating vaginal atrophy in menopause (Ghorbani & Mirghafourvand, 2021; Moussa et al., 2023; Santos et al., 2022; Torky et al., 2018), but have not examined the impact of oxytocin on inflammatory cytokines/chemokines or wound healing at the FGT. I tested this by examining the effect of oxytocin on cytokine/chemokine expression and wound healing in FGT epithelium directly. To further build on this with *in vivo* work, I expanded the study to include the use of the TKO-hBLT humanized mouse model to characterize the effect of vaginal oxytocin gel on local and systemic cytokine/chemokine levels, as well as leukocyte activation marker expression. I hypothesized that oxytocin would reduce the expression of pro-inflammatory cytokines/chemokines and leukocyte activation markers at both the mucosal and systemic level, leading to potentially reduced HIV susceptibility. Additional findings were made surrounding oxytocin present in standard formulations of FGT epithelial media, as well as a lack of significant OXTR expression and oxytocin-induced Ca^{2+} flux in FGT epithelial cells. What follows is an in-depth discussion of the findings in this thesis regarding oxytocin's role as an immunomodulator of FGT epithelium, as well as the potential implications for its role in protecting against HIV susceptibility.

4.1 Bovine pituitary extract is a confounding source of oxytocin that must be considered in future in vitro studies

I demonstrated that KSFM media supplemented with bovine pituitary extract (BPE) contains ~7410 pg/mL oxytocin (**Figure 3.1**), well within the range used for other *in vitro* studies of oxytocin (Kallak & Uvnäs-Moberg, 2017; Klein et al., 2014, 2016). Most studies culturing Vk2, Ect1, and End1 epithelium use KSFM medium that contains this BPE supplement (Eade et al., 2012; Hijazi et al., 2015; Sharkey et al., 2007). Kallak & Uvnäs-Moberg (2017), in particular, used this media—with double the normal level of BPE—to study the effect of oxytocin on proliferation in Vk2 epithelial cells, where cells treated with 1000 – 10 000 000 pg/mL oxytocin showed increases in proliferation after twenty-four hours of treatment. While 10 000 000 pg/mL is well above being significantly affected by a dose of 14 820 pg/mL—assuming consistent oxytocin levels between lots of BPE—a reported dose of 1000 pg/mL oxytocin treatment is in actuality a dose of 15 820 pg/mL oxytocin being compared against a 'media' control of 14 820 pg/mL oxytocin. This heavily skews the interpretation of these findings. Perhaps more alarmingly, significant findings of increased proliferation were found at doses of 10 and 100 pg/mL oxytocin after seventy-two hours of treatment (Kallak & Uvnäs-Moberg, 2017). In actuality these are dose comparisons of potentially 14 830 pg/mL vs. 14 820 pg/mL and 14 920 pg/mL vs. 14 820 pg/mL oxytocin, implying that the actual effects seen are highly unlikely to be a product of oxytocin treatment and are much more likely to be the result of random chance.

In my own data, significant findings in BPE-containing media tend to be present at the higher end of the dose curve, with Ect1 and End1 cells, for example, showing reductions in IL-1β and IL-6 transcription at 1000 pg/mL and 10 000 pg/mL oxytocin after transformation to fold change (**Figure 3.3**). In actuality, these are comparisons of 8410 pg/mL vs. 7410 pg/mL and 17 410 pg/mL vs. 7410 pg/mL oxytocin. It is not to dismiss these results entirely, as there may very well be a threshold or critical stimulus limit that must be met in order to induce an effect, given the potentially low or absent level of OXTR in FGT epithelium (**Figures 3.18 and 3.19**), but these results must be interpreted in context.

Ideally, BPE would simply not be used in experiments studying oxytocin in epithelial cells, which I was able to accomplish by switching to BPE-free 'defined' KSFM media (**Figures 3.4-**

3.5, 3.8-3.9, 3.11), but it is unclear how removing BPE and the other components of the standard KSFM media formulation may affect FGT epithelium and the ability to compare to previously published literature. Of note, cells grown in standard BPE-containing KSFM grow much faster than those cultured in BPE-free 'defined' KSFM, which was borne out in the decreased timeline for wound healing in BPE-containing (**Figure 3.10**) vs. BPE-free (**Figure 3.11**) media. This is also supported by prior work showing that the immortalized keratinocyte cell line N/TERT2G displays a reduced proliferation capacity when grown in BPE-free vs. BPE-containing KSFM media (Moran et al., 2022). In addition, cells grown in 'defined' KSFM show increased levels of tight junction proteins and epithelial barrier thickness when grown as a multilayer (Moran et al., 2022). For this reason, 'defined' KSFM media should be used in *in vitro* experimental systems studying oxytocin, but further studies are necessary to fully characterize how it may impact other parameters—such as pro-inflammatory cytokine response—in epithelial cells for future studies.

4.2 Oxytocin effects on FGT epithelial cells are limited to cervical cells, indicating a potential biological niche

With regards to effects on pro-inflammatory cytokine/chemokine transcription, oxytocin was found to downregulate IL-1β and IL-6 mRNA transcription in unstimulated Ect1 (IL-1β only) and End1 (IL-1β and IL-6) cells, but not in Vk2 cells in BPE-containing media (**Figure 3.3**). These results are in contrast to previous reports showing that endogenous oxytocin levels were not correlated with IL-6 mRNA levels in ovarian carcinoma cell lines (Cuneo et al., 2019), although oxytocin as an exogenous stimulus likely has different effects than paracrine or endogenous oxytocin signalling. My results indicate that FGT cervical epithelium is more receptive to oxytocin-induced pro-inflammatory transcriptional regulation than either vaginal or ovarian epithelium—indicating this epithelium as a potentially unique target for oxytocin. After removing BPE from the culture system (**Figure 3.5**), oxytocin no longer had these effects on unstimulated Ect1 and End1 cells.

This anti-inflammatory relationship in cervical cells changes when examining pro-inflammatory cytokine secretion, with increased IL-8 secretion in Ect1 cells cultured in BPE-containing media (**Figure 3.7**) and decreased IL-1α secretion in End1 cells cultured in BPE-free

media (**Figure 3.9**). This increase in IL-8 is novel in that no known literature has reported oxytocin's effect in *enhancing* pro-inflammatory cytokine protein secretion. A study examining uterine myometrial tissue, however, has shown a putatively pro-inflammatory effect for oxytocin in activating the transcription factor NF-κB leading to subsequent increases in IL-6 and IL-8 mRNA transcripts (S. H. Kim et al., 2015). Whether increased IL-6 and IL-8 transcripts directly translate to increases in pro-inflammatory cytokine secretion remains to be seen, however, there are grounds in the work performed by Cuneo (2019) et al. that IL-6 mRNA levels were not reflected directly in IL-6 protein levels, supporting this concern. This may indicate that ectocervical cells are uniquely responsive at the FGT in that they experience an upregulation of pro-inflammatory cytokines in contrast to endocervical cells, where a decrease in IL-1α was witnessed (**Figure 3.9**). This decrease seen in endocervical cells agrees with previous reports in aforementioned ovarian carcinoma cell lines, whereby IL-6 protein secretion was directly reduced by oxytocin treatment (Cuneo et al., 2019). Further protein evidence for this down-regulating effect is found in skin epithelium with work by Deing (2013) et al. showing that dermal keratinocytes after OXTR knockout secreted higher levels of IL-6, CCL5, and CXCL10 into culture supernatant. Further work by Klein (2016) et al. demonstrated that the gut epithelial cell line Caco2BB down regulated LPS-induced inflammation as measured by NF-κB phosphorylation following oxytocin treatment. This indicates that oxytocin treatment of the FGT may need to be targeted to the endocervix in order to achieve the greatest potential for reductions in inflammation.

4.3 Oxytocin impedes wound healing of FGT cell lines despite previous associations

Both Vk2 and End1 epithelial cells cultured in BPE-free KSFM showed slower closure of wounds in scratch assay after treatment with oxytocin (**Figure 3.11**). While oxytocin seems to slow the healing of FGT epithelium, clinical studies show associations of endogenous oxytocin levels with improvements in healing using suction blisters (Gouin et al., 2010). The difference between these results and mine may suggest that the effect of oxytocin on wound healing depends on tissue type, e.g. FGT vs skin epithelium. Wound healing in a scratch assay typically involves increased proliferation of cells adjacent to the wound/scratch to close the scratched gap (Bobadilla et al., 2019; Vang Mouritzen & Jenssen, 2018). When investigating whether oxytocin could

increase cellular proliferation of FGT epithelial cells, Kallak & Uvnäs-Moberg (2017) demonstrated that oxytocin treatment increased the proliferation of Vk2 cells twenty-four and seventy-two hours after treatment. This would appear to disagree with my finding that oxytocin inhibits the rate of wound healing in Vk2 cells (**Figure 3.11**), but it is important to note that wound healing rates do not only depend on cellular proliferation and also involve mechanisms such as cell migration (Bobadilla et al., 2019; Vang Mouritzen & Jenssen, 2018). Oxytocin treatment also did not have observable effects on Ect1 wound healing (**Figure 3.11**), although this may have been a result of the increased wound healing speed in Ect1 cells relative to Vk2 and End1 cells—potentially leading to a smaller window in which to detect oxytocin's effects. It is also possible Ect1 cells are non-responsive, or resistant, to exogenous wound healing stimuli, as they also showed no significant improvement in wound healing as a result of TGF-β1 treatment. Conversely, Vk2 and End1 cells both exhibited significant improvements in wound healing at 120 hrs and 96 hrs post-TFG-β1 treatment, respectively, suggesting epithelial cells from different sites of the FGT may have greater or lesser capacities to respond to wound healing agonists.

Oxytocin has been shown to be a required component of a *L. reuteri*-induced wound healing mechanism through both clinical and animal trials—specifically looking at skin-punch biopsies and dermal keratinocytes (Poutahidis et al., 2013; Varian et al., 2017). In particular, *L. reuteri* was shown to improve wound healing by 20% in those using a *L. reuteri* nasal spray and undergoing skin punch biopsies (Varian et al., 2017). In this study oxytocin was not studied directly, and so it is unclear what mechanism might be utilizing oxytocin and what cellular or other factors are involved in this improvement in wound healing. This work was preceded by experiments demonstrating that improvements in wound healing with *L. reuteri* were associated with a ~2-fold increase in plasma oxytocin concentrations (Poutahidis et al., 2013). Mice with this elevated oxytocin phenotype were able to transfer their improved wound healing outcomes to untreated mice through adoptive transfer of regulatory T (T_{reg}) cells. Together with my data, this suggests that if oxytocin is to improve wound healing at the FGT, it is likely necessary to achieve this through indirect routes or cellular intermediaries, such as T_{reg} cells, rather than through direct treatment of the epithelium.

4.4 Female genital tract epithelial cells show no oxytocin-induced Ca^{2+} response, suggesting a lack of both a functional G_q-coupled OXTR and V_{1a} vasopressin receptor

In an attempt to verify the oxytocin used within this study, as well as the PHM1-41 control cell line used throughout the *in vitro* experiments, Vk2, Ect1, and End1 cells were found to be non-responsive to oxytocin treatment through Ca^{2+} flux (**Figure 3.12**). Ca^{2+} is a nearly ubiquitous second messenger in cell signaling, and one of the most common downstream effectors of receptor binding (Cheng et al., 2006; Clapham, 2007). The OXTR utilizes this pathway for its most common GPCR conformation—the G_q coupling—typically associated with cell growth and phospholipase C (PLC) activation in most cell types (Reversi et al., 2005; Zhong et al., 2003). This indicates that Vk2, Ect1, and End1 cells may utilize a cAMP—G_i—coupled version of the OXTR, but further work is needed to test that association. This pathway is typically associated with neoplasms such as prostate and breast cancer (Ariana et al., 2019; Imanieh et al., 2014; Xu et al., 2017). While Vk2, Ect1, and End1 cells are not neoplasms, they are immortalized cell lines and may have altered coupling to the OXTR as a consequence. This would be in potential agreement with the finding that oxytocin *inhibits* wound healing in Vk2 and End1 cells treated with oxytocin rather than improves it (**Figure 3.11**).

The other alternative to the G_i-coupled cAMP pathway of the OXTR is the possibility that oxytocin acts on Vk2, Ect1, and End1 cells through an alternative receptor, such as the vasopressin receptor, which is known to cross-react with oxytocin (Kimura et al., 1994; Manning et al., 2012; Taylor et al., 2020). Oxytocin does bind the V_{1a} vasopressin receptor—the form of the vasopressin receptor most common outside of the kidneys and CNS—with an affinity 1:100 that of vasopressin (Oh, 2008; Taylor et al., 2020; Treschan & Peters, 2006). This may explain why effects that are seen in *in vitro* experiments within this thesis trend towards the high end of the oxytocin dose response curve. Supporting this theory is that V_{1a} receptor has been shown to be present in the vaginal tract of rabbits, although this has never been demonstrated in humans (Maggi et al., 1988). Work has also been done to demonstrate the presence of V_{1a} in the uterus, however, it is unlikely this receptor presence extends to the ecto- and endocervices (Maggi et al., 1990, 1991; Tence et al., 1990). Counter to this possibility is the data indicating that Vk2, Ect1, and End1 cells do not respond to oxytocin with Ca^{2+} flux (**Figure 3.12**), as V_{1a} vasopressin receptors utilize the same

Ca^{2+} flux signaling pathway that G_q-coupled OXTRs do (Omura et al., 1999; Treschan & Peters, 2006). It is further possible, but highly unlikely that the other vasopressin receptors—V_2 and V_{1b}—are present, but they tend to be localized to the kidneys and central nervous system, respectively, with little evidence showing their widespread distribution throughout the body (Oh, 2008; Treschan & Peters, 2006). Of note, the V_2 vasopressin receptor utilizes a cAMP pathway, similar to G_i-coupled OXTRs, and a lack of Ca^{2+} flux in response to oxytocin treatment is not necessarily indicative of its absence (Omura et al., 1999; Treschan & Peters, 2006) (**Figure 3.12**).

4.5 Female genital tract epithelium is characterized by either a lack of, or low level of, OXTR expression

When specific nucleic acid amplification was used to detect OXTR transcripts in Vk2, Ect1, and End1 cells, all three FGT cell lines had levels of OXTR mRNA that were not significantly above the levels seen in HEK 293T negative controls (**Figure 3.18**). This agrees with the literature in that OXTR mRNA signal has been detected previously in Vk2 cells (Kallak & Uvnäs-Moberg, 2017), and HEK 293 cells have shown OXTR mRNA signal through hybridization microarray assay (Atwood et al., 2011). The levels of OXTR mRNA signal observed in HEK 293 cells have been considered below the significance threshold for microarray analysis (Atwood et al., 2011). Importantly, these PCR products have never been sequenced—either in the literature or through this study—and this remains a gap in the identification of these amplicons. In addition, HEK 293 cells have been verified as OXTR-negative numerous times through confocal microscopy (Ma et al., 2013; Mitre et al., 2022), so it is unclear what sequence the OXTR primers may be amplifying.

Challenges arose in protein identification of the receptor, with antibody-based approaches such as Western blot and confocal microscopy being largely plagued by non-specificity (**Figures 3.13–3.17**). Western blots for OXTR in the literature have been performed with a number of antibodies giving a size of either ~43 kDa or ~67 kDa depending on putative glycosylation status. A study using ab181077—now discontinued—by Szeto (2017) et al. shows convincing staining for the OXTR in macrophage with a knockout control to validate their findings. This agrees with

my results showing positive staining in PHM1-41 cells and negative staining in HEK 293T cells using this antibody (**Figure 3.13A**). Interestingly, this is the only antibody that showed positive OXTR staining in the lysates of all three FGT cell lines, but, given the inability to repeat these experiments and the preponderance of evidence suggesting low or absent OXTR expression, these results are highly suspect given the strong bands for Vk2, Ect1, and End1 cells while PHM1-41, Jurkat, and Caco2 cell lines all display faint bands for the OXTR (**Figure 3.13A**).

Antibodies ab217212, sc-515809, and 23045-1-AP all showed inconsistent staining for the OXTR with false-negative staining in PHM1-41 positive control cells and/or false-positive staining in HEK 293 negative control cells (**Figure 3.13–3.14**). Arguably antibody 23045-1-AP shows the most internally consistent blot, with both recombinant OXTR and PHM1-41 cells being detected, while HEK 293 negative controls are not (**Figure 3.13C**). PHM1-41 staining for this antibody is incredibly weak, however, and follow-up experiments utilizing a HEK 293 *OXTR*-transfected construct continued to show a lack of ability to detect naturally expressed levels of OXTR (**Figure 3.14**).

Literature reports using ab217212 have been primarily limited to rodent samples using it for immunohistochemistry (Aydoğan & Bingöl, 2018; Helgers et al., 2021), where it is able to stain the Leydig cells of the testes—a known OXTR expressor (Aydoğan & Bingöl, 2018). There is a lack of Western blot data to support its ability to bind denatured OXTR epitope, however. The antibody is marketed as primarily targeting murine OXTR—which, unlike oxytocin, does not share 100% sequence identity with the human form. There is a singular study examining human expression of the OXTR using this antibody in uterine tissue of women undergoing hysterectomy, where it is used to show positive staining of OXTR in the myometrium (Orazov et al., 2020). This implies that it is able to bind OXTR target in human tissue, but data for its use in Western blot is still lacking.

Antibody sc-515809 only shows reports of staining in murine cells in the literature, despite the fact that its datasheet advertises reactivity to the human OXTR (Althammer et al., 2022; Huang et al., 2021; Li et al., 2021), which potentially explains why it shows binding to only the recombinant form of the OXTR rather than any human cell lysate (**Figure 3.13D**).

Polyclonal 23045-1-AP has seen use in Western blots for human colonic adenocarcinoma cell lines with targeted knockdown of the OXTR present as controls (Sun et al., 2021), giving this

antibody the strongest literature and experimental base to give reliable anti-OXTR staining (**Figure 3.13C**).

Using antibody 23045-1-AP in confocal microscopy, I observed positive staining of the OXTR in all three FGT cell lines (**Figure 3.15**), which was shown to be non-specific when HEK 293T cells were used as a negative control (**Figure 3.16**). This agrees with previous work by Kallak & Unväs-Moberg (2017) that demonstrated OXTR binding in Vk2 cells, but showed localization to the nucleus where it was potentially the consequence of erroneous staining. This prior study, using anti-OXTR antibody ab87312, failed to show proper controls for OXTR binding, and as a result the findings remain suspect. Previous experiments examining OXTR expression in HEK 293 cells through confocal microscopy have shown no signal, giving confidence in its use as a negative control for OXTR staining (Ma et al., 2013; Mitre et al., 2022). For this reason, I believe my experiments are better controlled for false-positives and can conclusively say that antibody 23045-1-AP is non-specific for the OXTR via confocal microscopy (**Figure 3.16**).

Final attempts at protein identification of the OXTR through mass spectrometry were able to show a lack of OXTR peptide spectra in Vk2, Ect1, and End1 cells (**Figure 3.19**), but that lack of significantly detectable OXTR peptide spectra in PHM1-41 positive control cells calls into question the sensitivity of the assay for samples that are not overexpressing the receptor through a plasmid or purified protein of the OXTR. As a consequence, the Vk2, Ect1, and End1 cell lines cannot be said to be verified negative for OXTR protein, but it can be said that they do not express levels of OXTR protein significantly detectable by either Western blot (antibody 23045-1-AP) or mass spectrometry.

4.6 Oxytocin vaginal gel may require an inflammatory state to affect leukocyte activation and inflammation in TKO-hBLT mice

Treatment with oxytocin vaginal gel had no impact on the leukocyte activation markers tested in our TKO-hBLT mouse model (**Figures 3.20, 3.21, 3.24-3.27**), indicating that oxytocin was either unable to reach or affect these cells to alter their activation status. CVL data showed that $hCD45^+$ leukocytes were rare in vaginal washes, with many sample collection days yielding

insufficient cells for analysis (**Figure 3.20**). This contrasts with a prior study showing the presence of $hCD45^+$ cells within the FGT of humanized mice (Allam et al., 2015), although these experiments were carried out in 'DRAG' mice that use a separate matched hematopoietic stem cell reconstitution method, as opposed to the fetal liver and thymus method used in our TKO-hBLT mice. Allam et al. (2015) also utilized whole organ homogenate, while I instead performed CVL washes to collect $hCD45^+$ cells at multiple time points throughout the longitudinal course of treatment. The lack of sufficient mucosal leukocyte collection from CVL washes makes definitive analysis of oxytocin vaginal gel's effects on mucosal leukocytes challenging, and future studies may need to elect for collection of mucosal samples through one time point at sacrifice to achieve enough cells.

Oxytocin vaginal gel also showed no impact on the levels of human or murine pro-inflammatory cytokines/chemokines in either the serum or CVL of TKO-hBLT humanized mice in this study (**Figures 3.22-3.23**), again suggesting a lack of effect for oxytocin or that a higher level of inflammation may be necessary to see effects.

Previous associations with oxytocin have shown abrogation of inflammation in rat gut trials (Chen et al., 2015; Tang et al., 2019a; Welch et al., 2014; Yu et al., 2022) and prevention of myocardial infarction (Jankowski et al., 2010; Xiong et al., 2020), but this is the first time an intravaginal route has been used to deliver oxytocin to affect immune activation. Associations with immunity have also been examined in the context of intravenous oxytocin administration in the case of LPS-induced systemic inflammation (Inoue et al., 2019; Szeto et al., 2017; Yuan et al., 2016), and in *ex vivo* studies comparing macrophage inflammatory capacity with or without oxytocin treatment (Garrido-Urbani et al., 2018; Shi et al., 2021). This indicates that intravenous oxytocin administration may be necessary to affect immune activation at the mucosa or systemically, rather than an oxytocin vaginal gel. Work examining oxytocin's effects on inflammation more generally demonstrated that peripherally administered oxytocin at a rate of 1 pmol/kg/min was only able to show an effect in the context of a 20 IU/kg LPS-induced system-wide inflammatory response, with reductions seen in TNFα, IL-6, and MCP-1 (Clodi et al., 2008).

As this work was initially designed to test oxytocin vaginal gel's effect on HIV transmission at the FGT, HIV infection was intended to be the immune stimulus that would allow for activation and inflammation to be abrogated by oxytocin vaginal gel. Future studies examining

immune activation directly in TKO-hBLT mice may be best served by incorporating an LPS-based immune challenge, due to its well-published interaction with oxytocin (Clodi et al., 2008; Inoue et al., 2019; Klein et al., 2016; Ross et al., 2013), as well as an intraperitoneal route of oxytocin administration to circumvent potential challenges in delivering oxytocin through a vaginal gel vehicle.

4.7 Limitations

The work performed in Vk2, Ect1, and End1 cells must be interpreted in the context of their status as transformed cell lines, which have undergone transformation with the E6/E7 HPV oncogene. These cells have been determined to be morphologically-similar to their primary counterparts (Fichorova et al., 1997), but it is unclear how transformation may alter both OXTR expression and oxytocin responsiveness in these cell lines. Previous work examining these cells has characterized their cytokine and chemokine secretion, but not how it is altered from primary vaginal, ectocervical, and endocervical tissue (Fichorova & Anderson, 1999). For this reason, these results need to be validated in primary tissue to ensure similar responses and interaction with oxytocin.

Additionally, wound healing was also measured in the context of scratch assays focusing on isolated epithelium, but lacking the supporting fibroblasts, neutrophils, and macrophage present in *in vivo* wound repair (Rodrigues et al., 2019; H. N. Wilkinson & Hardman, 2020; Yussof et al., 2012). Utilizing scratch assays to measure wound healing allowed for direct examination of oxytocin's effects on FGT epithelium, but it is important to note that actual effects *in vivo* may differ greatly due to the presence of additional cell types and complex tissue structure. Future studies may consider making use of '3D' or multi-cellular transwell models to better model the complexity of the wound tissue site to address this concern.

The discovery of BPE as a confounding factor with respect to the study of oxytocin remains unclear in whether BPE-containing media alters the response of Vk2, Ect1, and End1 pro-inflammatory cytokine production and wound healing as a consequence of the additional oxytocin added to the media or other factor(s) that are present in the standard KSFM formulation versus the

alternative BPE-free defined KSFM formulation. It is also challenging to characterize the BPE supplement, as it is an undefined mix of bovine pituitary homogenate and protected by commercial intellectual property laws. Continued work to understand which components of KSFM media alter these responses is necessary in order to better control these changes without potentially losing the preferred growth conditions of the standard BPE-containing KSFM formula, as well as to understand potential inadvertent effects that may be introduced to cultured cells through the media.

Compounded by the confounding factor of BPE in the media of BPE-containing experiments is the interpretation of oxytocin's effects in Vk2, Ect1, and End1 cells where no clear OXTR is present. As a consequence, it is not directly clear how oxytocin may be exerting effects upon these cells, and despite corrections for multiple comparisons it is a real possibility that, as in the study by Kallak & Unväs-Moberg (2017), the effects being seen in Vk2, Ect1, and End1 cells are random chance events rather than true effects. This is exacerbated by the fact that many significance values for oxytocin's effects in the *in vitro* work are close to the cut-off for type 1 error of $\alpha = 0.05$. For this reason, any significant findings of oxytocin's effects in Vk2, Ect1, or End1 cells must be interpreted with a modest degree of caution, especially until a definitive mechanism by which oxytocin is acting on FGT epithelium can be identified.

While TKO-hBLT mice are highly resistant to GvHD, a condition where transplanted cells attack the host, the mice had instances of GvHD requiring euthanasia near the end of the 63 day treatment plan. As a consequence, it is unclear if GvHD may have altered immune activation status and masked potential decreases in cellular activation as a consequence of oxytocin vaginal gel. This is compounded by the fact that humanized mice, while containing circulating human leukocytes, lack sufficient generation of B cell germinal centres and integration of B and T cell response, which ultimately leads to somewhat blunted immune responses (Di & Lavender, 2022; Gillgrass et al., 2021; Karpel et al., 2015; Pearson et al., 2008). This limits the power of these models to detect changes and effects in activation status. Additionally, oxytocin vaginal gel is a novel method of oxytocin administration for examining effects on both systemic and mucosal immunity, and while the oxytocin used in this study was found to be biologically active, it is not clear if the oxytocin within the vaginal gels was able to affect the mice. The lack of a direct control for positive response of oxytocin vaginal gel in the TKO-hBLT model suggests a dire need for future studies to either pursue a previously established peritoneal/intravenous route of oxytocin

administration route, or to pioneer a method by which oxytocin vaginal gel can be monitored for its efficacy throughout the experiment.

4.8 Contribution to the Field

The as-of-yet unreported finding of high (~7410 pg/mL) levels of oxytocin within the BPE additive of KSFM media has significant implications for its use in studies examining oxytocin. Popularization and publication of this fact is necessary in properly interpreting previously published literature (Kallak & Uvnäs-Moberg, 2017), as well as planning future experiments.

Additional work surrounding the testing of multiple anti-OXTR antibodies, as well as characterizing—through multiple methods—the OXTR expression of Vk2, Ect1, and End1 epithelial cells reveals the non-specificity, or at the very least lack of sensitivity, for these methods. Future work utilizing these tools can be built upon the knowledge gained from the in-depth pursuit of this topic.

This study also pioneered the use of an oxytocin vaginal gel in the humanized TKO-hBLT mouse model, indicating that vaginal administration of oxytocin may be insufficient to affect systemic, or even mucosal, immune activation and inflammation. Future studies can use this work to either approach the question of oxytocin immunomodulation from a different treatment route, or to be aware of the need to modify dosing and inflammatory status to observe a potential effect through an intravaginal route.

In vitro work tentatively demonstrated that both Ect1 and End1 cells are responsive to immunomodulation by oxytocin, while Vk2 and End1 cells have their wound healing inhibited by oxytocin treatment. Alongside the exploration of how Vk2, Ect1, and End1 cells respond to oxytocin, this opens up the path for future analysis of both a potential cAMP pathway in these FGT epithelial cells or an alternative pathway through an as-of-yet unknown mechanism.

4.9 Conclusions and Future Directions

4.9.1 Conclusions

This study demonstrates that oxytocin is able to influence pro-inflammatory cytokine expression within cervical epithelium of the FGT, but not that of the vagina. Given that oxytocin showed both anti- and pro-inflammatory indications, based on culture conditions, it is unclear how this may translate to altered HIV susceptibility at the FGT. Further work showing oxytocin impeded wound healing in vaginal and endocervical epithelium indicates that oxytocin vaginal gels may have negative consequences for HIV prophylaxis overall. This provides support against the hypothesis that oxytocin would reduce pro-inflammatory cytokine/chemokine expression and improve wound healing of FGT epithelium. Further work examining oxytocin vaginal gel *in vivo* demonstrated no significant effect of oxytocin on the modulation of pro-inflammatory cytokine/chemokine expression within CVL or plasma of TKO-hBLT humanized mice. Examination of leukocyte activation in CVL washes, the blood, and lymphatic organs revealed similar findings. Together, this data suggests that there is little evidence to promote oxytocin vaginal gel as a potential HIV prophylaxis tool.

Characterization of OXTR expression in Vk2, Ect1, and End1 cell lines using either antibody- or peptide-based assays yielded generally inconclusive results. Follow up experiments examining OXTR through alternative methods revealed that these cells express non-significant levels of OXTR mRNA, with proteomic methods lacking the sensitivity to definitively show a complete lack of OXTR protein expression. Signaling assays further indicated a lack of Ca^{2+} flux induced after oxytocin treatment in Vk2, Ect1, and End1 cells, suggesting that effects seen from oxytocin operate either through a cAMP-based pathway or an as-of-yet unknown mechanism. Future studies are required to elucidate this relationship and what pathways oxytocin may be utilizing in these FGT epithelial cells.

4.9.2 Future Directions

Future work should focus on examination of the cAMP pathway in Vk2, Ect1, and End1 cells in response to oxytocin, sequencing of OXTR qPCR transcripts among the host of cell types used in this study, and blotting for the vasopressin receptors in FGT epithelium.

Once assays for OXTR and vasopressin receptor presence and response have been validated in Vk2, Ect1, and End1 cells, obtaining primary samples will allow for comparisons with these cell lines to ensure that any findings, or lack thereof, are not an artifact of their transformation into cell lines. Achieving these sample types will be challenging, as biopsies for lower FGT epithelium are often not done alongside medical procedures, and rather are performed for pure research purposes. Sufficient rationale and evidence will need to be established in order to justify an ethics submission for acquiring primary samples, and so work may need to be performed alongside current clinical trials that allow for biopsies of the FGT. Work on menopausal women is currently being performed that has taken vaginal biopsies for assessments of vaginal thickness and atrophy-reversal (Santos et al., 2022). Collaborating with these studies allows for access to primary lower FGT samples that might otherwise be unavailable. Additionally, establishing these collaborations will allow for examining pro-inflammatory cytokine/chemokine levels, as well as leukocyte activation, in both the CVL and blood of the women participating. This would be best performed initially in menopausal women to correlate with prior studies examining oxytocin's effects on vaginal barrier thickness and relief of vaginal dryness (Ghorbani & Mirghafourvand, 2021; Moussa et al., 2023; Pitsouni et al., 2018; Santos et al., 2022), but would ideally be repeated in non-menopausal women to provide data in younger women—a cohort most at risk for heterosexual HIV infection (McKinnon & Karim, 2016).

The question of which components exactly alter oxytocin's effects on FGT epithelium in BPE-containing KSFM remains unclear. This question could be answered by repeating these experiments in a standard BPE-containing KSFM formula, but with isolating each additive component. These experiments would be used to determine how these components affect oxytocin's impact on both pro-inflammatory cytokine/chemokine production and wound healing in FGT epithelium.

This could be expanded upon to address the gap in the *in vitro* wound healing data presented here, which lacks the supporting elements of connective tissue, fibroblasts, neutrophils, and macrophage. Adding these additional components through the use of a 3D culture system will allow for a more complete picture of oxytocin's potential wound healing effects at the FGT. This is particularly important given that oxytocin is known to influence both fibroblasts and macrophage (Deing et al., 2013; Kinsey et al., 2007; Shi et al., 2021; Szeto et al., 2017), and both of these cell types provide future targets to study in the context of oxytocin and wound healing.

Work examining HIV infection in TKO-hBLT mice needs to be repeated using an intraperitoneal route, as these mice proved refractory to vaginal HIV challenge in our hands. Utilizing both intraperitoneal HIV infection, as well as intravenous oxytocin administration, will allow for a systemic-level analysis of oxytocin's ability to inhibit HIV infection. This does prevent a mucosal route of administration from being tested, and lessens relevance towards heterosexual HIV transmission, but expands the focus of oxytocin towards blood-based routes such as intravenous drug use. This opens the door for other immune cell subsets to be studied as well, and macrophage and monocytes present likely candidates for oxytocin modulation when treatment is given intravenously (Garrido-Urbani et al., 2018; Ross et al., 2013; Szeto et al., 2017). Given the variable impact oxytocin has had on the inflammatory status of monocytes and macrophage in previous studies, it would be crucial to clarify how oxytocin—when given systemically—influences both the course of HIV infection as well as the activation of these cells.

Chapter 5: References

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