Ecological and epithelial regulation of immunity in the female reproductive tract

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Abstract

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Among mucosal tissues, the female reproductive tract has a unique biological susceptibility to viral infection, as tissue-specific immunity requires rapid antimicrobial responses to pathogens, while maintaining tolerance toward commensal organisms, sperm, and fetal products. Over the last twenty-plus years, a number of research groups have demonstrated two factors that significantly alter the likelihood of acquiring a sexually transmitted infection by the vaginal route: (a) the composition of the vaginal microbiome and (b) the predominant hormone in the days leading up to the encounter.

We chose to focus first on women diagnosed with bacterial vaginosis. Because new treatments have not entered the market in decades, it is important that we learn to identify patients likely to experience treatment failure; no criteria currently exist. In our search to identify community signatures associated with treatment failure, we demonstrate that pre-treatment

richness and evenness were significantly lower for women who sustained clearance, and that these women also experienced improved mucosal tissue health.

We next sought to identify factors that make the vagina an opportune niche for pathogens such as Zika virus. Our findings suggest that in conjunction with a rapid antiviral response, the vaginal epithelium induced a non-canonical "activation" of epithelial cells demonstrated by acute upregulation of genes that function in terminal differentiation and desquamation, in an apparent attempt to increase cell turnover and expel virus. These findings led us to investigate the potential breadth of this response. Through re-analysis of publicly available data from the Gene expression omnibus (GEO), we found that this activation could be broadly observed following TLR stimulation by several TLR ligands, suggesting that this mechanism is a conserved epithelial response to PAMP.

Given the essential role of sex hormones in determining immune responses of the FRT, we sought to explore epithelial activation through additional *in vivo* studies of ovariectomized mice under specific hormone conditions. Analysis via flow cytometry and single-cell RNAseq demonstrated that high levels of progesterone and estrogen – as observed during pregnancy - direct epithelial programs in the vagina to restrain T cell activation and recruitment, subsequently exacerbating infection.

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My parents have always supported me tirelessly, but their love during a period of upheaval in my final year at the University of Illinois restored my footing – and my faith in myself. During that time, they helped me develop the ability to enjoy hard work not just as a means to ends, but because working hard and watching something grow is a privilege – and can be a joy. Their enthusiasm for my pursuits in the years that followed – even when the end goal was not obvious – was a constant buoy. And when we lost my dad just before I started at UW, my mom pulled double-duty, always finding new ways to say how proud and excited she was, always finding ways to provide support in unexpected ways, and always reminding me dad would be proud too.

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DEDICATION

To my dad, who always wanted one of these, but put his family first.

To my wife, who bent over backwards to make this possible.

Chapter 1. INTRODUCTION

1.1 HOMEOSTASIS IN THE LOWER FEMALE REPRODUCTIVE TRACT

1.1.1 *Basic anatomy of the FRT*

From a reproductive standpoint, the female reproductive tract (FRT) consists of two functionally distinct compartments. In the lower portion of the FRT, the vagina and ectocervix maintain a dynamic barrier to pathogen entry that must cultivate commensal microbes, facilitate sperm survival, and prevent pathogens from ascending through the cervix into the upper FRT, where together, the endocervix, uterus, fallopian tubes, and ovaries are responsible for egg development, fertilization, and gestation, and menstruation [(Wira et al., 2015)]. Casts of the luminal surface of the lower FRT in reproductive age females have demonstrated a mean surface area of approximately 87cm^2 [(Pendergrass et al., 2003)], and the true value may extend up to 360cm^2 when rugae are included in the measurement [(Boskey et al., 1999)]. With a defensible space that is slightly larger than surface area of a grapefruit, the vaginal lumen is a prime site for pathogen contact and necessitates a multi-tiered defense.

1.1.2 *Chemical barriers to infection*

While the vaginal epithelium is thought to lack classic mucus producing cells, the entire epithelial layer serves as a secretory tissue, with networked channels that navigate between epithelial tight junctions and terminate at the luminal surface as pore-like openings []. These channels enable metabolically active cells deep in the tissue to secrete antimicrobial agents that include the human beta defensins, lactoferrin, calprotectin, lysozyme, hydrogen ions, and secretory leukocyte

protease inhibitor, all of which can be carried by transudate and released through the surface pores as a preemptive chemical barrier [(Farage et al., 2009), (Wira et al., 2010), (Mitchell et al., 2014), (Wira et al., 2015)]. These secreted molecules combine with draining cervical mucus, which itself contains antimicrobial peptides, antibodies, and pathogen trapping mucins, to form the initial barrier to pathogenic interactions [(Wira et al., 2015)].

1.1.3 *Physical barriers to infection*

1.1.3.1 Structure of the stratified squamous epithelium

Beneath the protective mucus layer, the lower FRT is composed primarily of squamous epithelial cells - often reported to be between 24 and 28 layers thick [(Patton et al., 2000)] - with the uppermost layer thought to be shed every four hours on average [(Anderson et al., 2014)]. Scholars have broadly categorized the regions within the vaginal epithelial tissue according to their location (depth) within the tissue. Basal cells in contact with the basement membrane form the foundational layer, wherein a portion of the cells constitutively divide to replenish cells shed at the surface via desquamation; the cell layers above are often referred to as the parabasal, suprabasal, and superficial layers (regions) [(Thompson et al., 2001)]. Additional Latin terminology is often used in the literature to convey that layers within a squamous epithelium attain distinct functions as they are pushed toward the luminal surface, and in the case of the FRT, these terms are often borrowed from studies of the epidermis. Thus, the cells from the basal layer are also known as the *stratum basalis*, and as cells are pushed toward the surface they transition through the *stratum spinosum* (thorny layer), *stratum granulosa* (grainy layer), and finally the *stratum corneum* (horny layer) before being shed [(Anderson et al., 2014)]. Because these terms are not universally used (and their functions are often inferred from the epidermis, rather than demonstrated in the FRT)

they are mentioned here for completeness only. But they do serve to introduce the important concept that cells within the squamous epithelium change as they move toward the surface, in a process known as terminal differentiation.

1.1.3.2 Cell turnover in the squamous epithelium

The basal layer of stratified epithelial tissues is composed of cuboidal cells in contact with the semi-permeable basement membrane. A portion these cells have a demonstrated ability to replicate for extended periods of time, and these stem-like cells are believed to produce two populations of cells. The first, known as transient amplifying cells, are a minor population that replicate for only a short time prior to differentiation; the second are a population of cells that immediately initiate the terminal differentiation pathway and populate the bulk of the tissue [(Alonso et al., 2003)]. While studies of this process in the FRT are limited, bromodeoxyuridine (BrdU) incorporation assays in mice have demonstrated that active DNA synthesis in the FRT is limited to a stable population of cells in the basal compartment under homeostatic conditions [(Ali et al., 2020)]. Like the epidermis, the majority of cells produced by cell-division in the basal layer of the FRT are not intrinsically programmed to divide, but rather, to differentiate.

From a mechanical standpoint, differentiating epithelial cells are responsible for the resistance and flexibility required of the vaginal tissue during intercourse and labor. These mechanical properties are achieved through the extensive use of the epithelial-specific intermediate filaments known as the keratins. As an intermediate filament, keratin networks provide rigidity *and* flexibility for the cell membrane, stability for the nucleus, and mechano-sensing properties that regulate gene expression [(Bragulla et al., 2009)]. Keratins (K) – of which there are 54 known in humans - are broadly divided by their isoelectric point and categorized as either basic or acidic. In the cell, keratin filaments are formed through the accumulation of

heterodimers composed of a basic keratin paired with an acidic keratin, which are present in the cell at equimolar ratios [(Moll et al., 1982)]. Each keratin protein is the product of a unique gene, and the programmed expression of specific keratin pairs shifts during epithelial differentiation. Basal epithelial cells in both the FRT and skin are defined by the paired expression of K5 and K14 [(Moll et al., 1982), (Gimenez-Conti et al., 1994), (Ali et al., 2020), (Byrne et al., 1994)]. As cells in the FRT are pushed toward the luminal surface, the detachment of cell junctions and increasing intracellular calcium levels prompt the cell to shift from the expression of K5/K14 to the differentiation associated keratin pairs K4/K15 (epidermis), K4/K13 (mucosa), and/or K1/K10 (both tissue types) [(Zhang et al., 2019), (Ali et al., 2020), (Gimenez-Conti et al., 1994)]. While these pairings are well supported by the literature, non-canonical pairing has been observed, i.e., keratins have some flexibility regarding partners, so long as the requirement for basic-acidic coupling is fulfilled. While the sequential utilization of unique keratin pairs in stratified epithelia has been observed since the 1980s, the biological necessity for these shifts remained uncertain for several decades.

Early hypotheses posited that the deployment of diverse keratins throughout the cell life cycle was related to cytoskeletal requirements associated with the transition from the cuboidal form in the basal layer to a flattened squame at the surface. Today, keratins are increasingly appreciated for their ability to regulate cellular states. Experimental knock-down of the basal K14 in epithelial cells has shown that it is essential to maintaining a proliferative state [(Alam et al., 2011)], while knockdown of differentiation-associated K10 causes the cell to revert to a mitotically active state [(Reichelt et al., 2004)]. Deeper analysis of molecular changes during these knock-down experiments revealed that the loss of specific keratins significantly altered phosphorylation potential and kinase activity in the cells. Importantly, despite the high levels of genomic similarity,

it was later determined that subtle differences in protein sequences dramatically altered the exposed residues and binding pockets on keratin filaments – and suggested that the keratin cytoskeleton plays an active role in maintaining cellular homeostasis [(Bunick et al., 2017)].

As successive waves of cell division push daughter cells toward the luminal surface, they initiate a controlled cell death program known as cornification [(Levy et al., 2000]. Like apoptosis, this programmed death is non-inflammatory in nature. But unlike apoptosis, the cell is not broken up for phagocytosis; rather, organelle disassembly is thought to occur by autophagic mechanisms that minimize the release of DAMPs, resulting in the controlled transition from a metabolically active sentinel to a scale-like barrier, which is eventually shed [(Koenig et al., 2020), (Eckhart et al., 2018), (Lippens et al., 2005)]. As internal organelles are recycled, the cell orchestrates the construction of the cornified envelope by expressing a suite of genes collectively referred to as the epidermal differentiation complex; the resulting proteins interface with superficial keratins and the plasma membrane to form the cornified envelope [(Kypriotou et al., 2012)].

The purpose of the cornified envelope is to form a superficial barrier that aids in water retention and protects living cells in the tissue below, and the three gene families of the epidermal differentiation complex, which cluster on human chromosome 1q21 (mouse 3F1), likely arose from duplication events during adaption to terrestrial habitats [(Backendorf et al., 1992)]. Unlike the epidermis, however, cornified cells in the vaginal epithelium do not link via corneodesmosomes to form a waterproof barrier; in fact, the cavities between cornified cells are thought to play a role in commensal bacteria cultivation (discussed in greater detail below). The cornified envelope (CE) precursor family includes loricrin, involucrin, several small proline-rich repeat (SPRR) proteins, and the late cornifying envelope proteins (LCE). Involucrin is expressed

during the earliest events of cornification; as intracellular calcium levels rise, transglutaminase I binds involucrin to various plakins attached to the plasma membrane. The resulting heterotetramers form a proteinaceous foundation that anchors additional layers of the developing cornified envelope. While this process is believed to be shared across all terminally differentiating stratified epithelia, the subsequent utilization of the other CE precursors to build up the cornified structure can vary by tissue - and even within tissues according to differentiation status and/or environmental cue [(Niehues et al., 2016)]. For example, while LCEs share strong sequence homology, LCE3 is found predominantly in suprabasal cells of the epidermis (*stratum granulosum*), while the entire LCE2 group is coordinately expressed and present in the superficial layer (*stratum corneum*). Both the LCEs and the SPRRs appear to be inducible during wound healing processes and their expression can also be triggered by inflammatory cues such as interferon-γ [(Candi et al., 2005)]. Another major family of the EDC includes the majority of S100 genes in the genome, which are a set of calcium binding proteins that regulate intracellular, calcium-dependent process, as well as extracellular signaling. Several S100 genes are expressed during homeostatic epithelial differentiation, and their utility can be supplemented by additional S100 genes that are only expressed during cell stress [(Donato et al., 2013)]. The third family, known as the fused-gene family, also possess calcium-binding domains and are expressed throughout differentiation. One example, filaggrin, is produced in a pro-form and is an essential component of the early cornified envelope, and its cleavage product may play a role in controlled nuclear degradation [(Kypriotou et al., 2012)]. Notably, the accumulating evidence of differential EDC expression and utilization suggests that these genes can serve as informative biomarkers about the tissue-level responses to wounding and/or infection. Importantly, cells that initiate terminal differentiation are not permanently dedicated to this path; differentiating epithelial

cells can and do exit the terminal differentiation program in cases of sterile or infectious injury. When this occurs, superficial protein pairs such as K1/K10 are replaced by expression of hyperproliferative keratins (K6/16/17), and because keratins are regulated at the genetic level, detection of their transcripts provides additional insight to cell responses to infection and injury.

1.1.4 *Organelle disassembly and immune regulation during the cornification process*

Throughout the cornification process, organelles including the nucleus are disassembled. Unlike apoptosis, the components are not packaged and released for phagocytosis, but instead recycled intracellularly [(Koenig et al., 2020)]. Importantly, the immune system has evolved sensing mechanisms to recognize several of the components from organelles like mitochondria and the nucleus; known as DAMPs, their detection indicates that cell injury has occurred. Thus, these molecules can serve as a redundant mechanism for pathogen sensing in the absence of their direct detection by PRRs [(Schaefer et al., 2014)]. Importantly, these molecules can also be released by sterile injury when cells are damaged or destroyed without time to complete controlled cell death. Thus, cornifying epithelial cells must tightly regulate disassembly of cell structures to prevent DAMP release [(Eckhart et al., 2018)]. Interestingly, cornifying cells have also been shown to initiate anti-inflammatory programs prior to metabolic shutdown as a mechanism to ward off inappropriate immune activation. Two such mechanisms include the suppression of inflammasome activation through the expression of pyroptosis-adjacent genes lacking effector domains and the expression of IL1 antagonists [(Eckhart et al., 2018)]. Our ongoing research expands upon this notion to include the specific regulation of cytotoxic T cells; findings are discussed in chapter 3.

The complex programs regulating epithelial homeostasis occur in the face of persistent environmental threats. Ultimately, the cornified epithelial cell reaches the luminal surface, devoid of intracellular organelles and incapable of genetic response to stimuli. And yet, while gene expression ceases within the now anucleated shell, the cell is far from inert; rather, defensive peptides and enzymes that were preemptively stockpiled remain active, providing a chemical barrier to pathogen entry that persists even after desquamation.

1.2 PATHOGEN DETECTION IN THE FRT

1.2.1 *Epithelial detection mechanisms and the cell-intrinsic response*

In addition to meeting the mechanical needs of the tissue, epithelial cells in the vagina are capable of active immune surveillance and cell-intrinsic defense against pathogens. Nucleated epithelial cells utilize pattern recognition receptors (PRRs) to detect specific microbial signatures, broadly described as pathogen-associated molecular patterns (PAMPs). Epithelial cells in the FRT monitor the microbial milieu via Toll-like receptors (TLRs) 1, 2, 6, 3, and 5, which can detect bacterial, fungal and viral pathogens through their di- and triacyl lipopeptides, double-stranded RNA (dsRNA), and flagellin, respectively [(Fazeli et al., 2005)]. (Additional studies performed at the transcription level have demonstrated expression of all TLR's in mouse vaginal epithelial cells across the estrous cycle [(Hickey et al., 2013)].) When a TLR ligand binds the appropriate receptor, signaling cascades within the cell result in a robust inflammatory response that signals for leukocyte assistance and facilitates their entry into tissue. Vaginal epithelial cells also monitor the cytoplasm for viral dsRNA via RIG-I-like receptors (RLRs) [(Chow et al., 2018),2 (Chambouleyron et al., 1996)]. Following retinoic acid-inducible gene I (RIG-I) activation, signaling cascades result in IFN production that provides autocrine and paracrine activation of the

JAK-STAT pathway; ultimately, antiviral, proinflammatory, and cell signaling genes, which are collectively referred to as interferon stimulated genes, are induced to control replication and spread [(Kell et al., 2015)]. It should be noted that while the rapid induction of antiviral defense mechanisms is a hallmark of innate immunity, two recent studies have suggested innate immune responses in the FRT may be dampened (by design), due in part to low levels of sensors like RIG-I [(Khan et al., 2016), (Khan et al., 2019)]. Given these studies' reliance on a chemically-induced diestrus state in their mice, additional studies are needed in this area, for reasons discussed further in section 1.4 and chapter 3.

1.2.2 *Leukocytes in the lower FRT*

Epithelial defenses in the lower FRT are supplemented by resident and migratory leukocytes. The most populous are the cervicovaginal Langerhans cells (cvLC), a type of epithelia-specific macrophage first identified in the skin; non-langerin+ macrophages are also present [(Khan et (Zhou et al., 2018)., 2019)]. Interestingly, the transcriptional profile of cvLC more closely resembles vaginal dendritic cells (DC) than Langerhans from the skin – a finding which echoes the tissue-specific gene expression patterns that are observed in epithelial cells across tissues [(Duluc et al., 2014)]. cvLCs, macrophages, and CD14+ DCs are all capable of eliciting type 1 responses, while cvLCs and CD14- DCs are additionally capable of initiating type 2 responses [(Duluc et al., 2014)]. Several populations of T cells are also residents of the vaginal mucosa, with the majority being CD8 resident memory cells [(Khan et al., 2019), (Zhou et al., 2018),]. Like DCs, vaginal T cells surveil both above and below the lamina propria [(Patton et al., 2000)]. Upon activation, resident T cells release IFN-g to initiate inflammation, DC maturation, chemokine gradient formation, recruitment of natural killer (NK) cells. [(Khan et al., 2019), (Zhou et al., 2018)]. And while B cells and plasma cells are virtually absent at baseline, T cell

activation leads to the rapid recruitment of circulating B cells, which serve as a potent source of antibodies against previously encountered pathogens [(Oh et al., 2019)]. While immune cell populations in the FRT are extensively studied, knowledge about the role of epithelial cells in regulating their abundance and activity is limited. As will be discussed below, the hormones progesterone and estrogen are well known for modifying the abundance, location, and activity of immune cells in the FRT, but the mechanisms are not well understood. Our research, covered in Chapter 4, provides new evidence that epithelial cells are a major regulator of immune cells in the FRT, and that these interactions are hormone dependent.

1.3 THE INFLUENCE OF ESTROGEN, PROGESTERONE, AND DMPA ON THE LOWER FRT

1.3.1 *A brief history of birth control*

The awareness of cyclic changes in the FRT - and the ability to shape fertility - have been known by non-western cultures for millenia, yet their "primitive" views and wisdom have been historically excluded from western medicine. It was not until the early 20th century that westernized medicine began to catch up. Beginning with animal models, scientists utilizing glandular secretions began to demonstrate that the cyclical physiology of the female reproductive tract depended on endogenous chemical control and could be manipulated [(Santen et al., 2019)]. With the discovery and purification of progesterone and estrogen, experimental scientists began to unravel the specific mechanisms that facilitated fertilization, gestation, and menstruation. Shortly after their discovery, scientists and clinicians cooperated to demonstrate that these purified chemicals could be utilized as a form of female birth control [(Goldzieher et al., 1993), (Piette et al., 2018)]. After a number of years in use, observational studies noted that harmful side effects could be linked to the relatively high dosages [(Lackie et al., 2016)]; dosages were subsequently

reduced to levels that have been widely considered safe, and to this day the regimen for oral birth control remains largely unchanged. DMPA, a long-acting injectable form of progestin-based birth control, is utilized by approximately half of birth control users in sub-Saharan Africa and is frequently utilized globally in resource-limited settings [(Ayele et al., 2021)]. In the last decade, several studies have indicated that the use of the DMPA is associated with a significantly increased risk of contracting HIV [(Ahmed et al., 2019), (Hapgood et al., 2018), (Ayele et al., 2021)]. In 2016, the World Health Organization (WHO) publicly acknowledged the potential risk associated with the use of the DMPA, but upon the 2019 publication of the ECHO study, the WHO revised its statement and reported that the DMPA was safe for use in all populations [(Ayele et al., 2021)]. Critics of the study argue that it was underpowered to detect meaningful differences, and experimental studies continue to provide evidence that DMPA decreases the ability for cells to respond to viral infection, increases the abundance of HIV target cells, and leads to reduced barrier integrity in the FRT. The following section will discuss the effects of endogenous estrogen and progesterone on epithelial and immune cells in the lower FRT; subsequent sections will summarize findings from clinical studies of exogenous hormone delivery and the impact of hormone treatments on innate defenses as observed through *in vitro* and *in vivo* infection models.

1.3.2 *The tissue-level and cell-intrinsic responses to endogenous hormones during the human menstrual cycle and the murine estrous cycle*

1.3.2.1 Endogenous hormone levels fluctuate during the cycle

The menstrual cycle varies in length, from as short as 17 days to the “classic” 28-day cycle - or even several days longer [(Wira et al., 2015)]. This duration varies across women and is not

fixed within individual women; the strength of the hormone response can also vary month to month. Marking the beginning of the cycle with the onset of menses, the menstruation phase lasts approximately 5 days (in a 28-day calendar). The proliferative (follicular) phase follows menstruation and is defined by increasing levels of estrogen that surge just prior to the ovulatory phase; both follicle stimulating hormone and luteinizing hormone also peak at this time [(Wira et al., 2015)]. The secretory (luteal) phase begins next and lasts until the onset of menstruation. Progesterone levels increase for approximately 10 days and return to low, baseline levels just prior to menstruation; during this time, estrogen levels gently climb and fall along with progesterone changes, but levels are far below the peak achieved prior to ovulation [(Wira et al., 2015)]. During pregnancy, both estrogen and progesterone levels climb steadily, achieving concentrations in the circulation that are 1-2 orders of magnitude greater than levels observed during the menstrual cycle [(Tulchinsky et al., 1972)].

Unlike humans, the murine estrous cycle occurs over just 4-6 days and does not include the shedding of endometrium via menstruation; rather, the murine endothelium is resorbed [(Yip et al., 2013)]. But similar to human females, cycling mice experience the highest levels of estrogen in the first half of the cycle and the highest levels of progesterone in the second half. Additionally, progesterone levels for both humans and mice peak during a period when they are most receptive to fertilization, and in both cases, there is also a second, smaller estrogen peak at this time [(Wood et al., 2007)]. Importantly, while the terms “proliferative” and “secretory” refer to changes in the endometrial lining of the uterus, the effects of estrogen and progesterone impact vaginal tissue as well, including its structure, immune function, immune surveillance, and susceptibility to infection.

1.3.2.2 Tissue-level changes to the FRT structure

A study of ovulating women found a small but significant difference in the total number of cell layers between pre- and post-ovulatory stages of the menstrual cycle [(Patton et al., 2000)]; in the authors' discussion, however, they suggested these differences (28.1 ± 0.6 vs 26.0 ± 0.6) might not be biologically meaningful. Additional studies have validated that the full thickness does not vary by cell layer or measured depth [(Wira et al., 2015), (Edfeldt et al., 2020)]. Individual cells do appear larger when estrogen is elevated, and this change is due to the estrogen-induced accumulation of glycogen, which causes cells to swell [(Dizzell et al., 2019)]. Glycogen is an essential component for the maintenance of an optimal vaginal microbiota, and this is discussed in detail in section 1.5.

Finally, while data suggest the thickness of the vaginal tissue does not vary significantly with cyclic hormone changes, a 2020 study leveraging novel image analysis demonstrated that specific layers within the tissue varied significantly by menstrual stage [(Edfeldt et al., 2020)]. Specifically, they noted that the superficial epithelium thickness was reduced in the luteal (progesterone-dominant) phase, while the loosely connected, metabolically active layer just below was deeper; their findings highlight the complex nature of hormone regulation in a differentiating tissue.

Estrogen is frequently cited in reviews as a proliferative stimulus for vaginal epithelial cells, but these statements often reference murine experiments as the sole support for this statement [see Wira et al., 2015)]. While the use of mouse data to make statements about the human vaginal epithelium is tenuous for reasons discussed below, human studies of aging and hormone supplementation provide clarity about the impact of estrogen on tissue homeostasis. Aging human women experience a slow but steady decrease in estrogen production during the perimenopausal

period, with noted reductions in vaginal epithelial thickness and glycogen retention [(Farage et al., 2006)]. These changes are observed in vulvar tissues as well; in fact, the entire lower FRT and external genitalia experience a loss of epithelial thickness, tissue elasticity, and moisture retention as the period of menstruation comes to a close. Importantly, estrogen replacement therapy has been shown to slow – and even reverse – these changes [(Farage et al., 2009)]. Thus, while the vaginal epithelium in humans may not fluctuate widely during the menstrual cycle, cycling estrogen is essential to maintaining this tissue over time.

As mentioned above, murine models are an essential tool for the study of the FRT homeostasis and immune responses, but unlike human vaginal tissue, the mouse vagina experiences dramatic remodeling over the course the estrous cycle [(Ali et al., 2020), (Horvat et al., 1992)]. The ratio of estrogen to progesterone increases throughout proestrus as the mouse prepares to ovulate, and during this time the thickness of the vaginal epithelium rapidly increases[(Lamb et al., 1978), (Traurig et al., 1971), (Miyagawa et al., 2015)]. The epithelium reaches its maximal depth at estrus when estrogen levels peak and progesterone levels are at their lowest. This ratio begins to invert during metestrus, leading to desquamation of the superficial layers of vaginal epithelium. By diestrus, the ratio is fully inverted, with progesterone peaking and estrogen at its lowest levels; the vaginal epithelium reaches its thinnest state at this time and is composed of just 3-4 layers of cells.

1.3.2.3 Immune cells in the FRT during reproductive cycling

As discussed above, T cells dominate the lower FRT, and these findings are consistent across the menstrual cycle. In fact, multiple studies have shown that all immune populations appear to be maintained at relatively constant levels. But an interesting distinction was made by Edfeldt et al., who provided evidence that while total numbers appear to be maintained, their position relative to

the tissue surface can vary based on hormone status [(Edfeldt et al., 2020)]. Specifically, they showed that during times of elevated estrogen, CD4+ CCR5+ T cells remained nearer to the basal lamina, and that the use of DMPA, a progestin-based birth control, led to these HIV target cells residing closer to the luminal surface. This question of whether hormones play a role in immune cell populations within the lower FRT is of great importance; more specifically, clinicians have noted for years that progesterone and progestin-based birth control appears to increase the risk of contracting HIV and potentially other STIs. As Chapter 3 provides convincing evidence for hormone-based changes in immune cell populations - along with potential mechanisms - the next section will introduce a number of *in vitro* and *in vivo* animal studies that have also demonstrated evidence of and potential mechanisms for this increase. Importantly, while the studies cited above and below are reflective of a general consensus on the impact of hormones, reviews on the subject– and this introduction – rely on surprisingly few primary studies to make assertions and draw conclusions. It is this author's opinion that comprehensive, comparative studies of human and animal cyclic FRT changes are sorely needed and should leverage emerging technologies, with a focus on longitudinal spatial transcriptomic studies.

1.3.3 *The impacts of exogenous hormone*

1.3.3.1 Ovariectomy models of estrogen and progesterone action *in vivo*

Several murine studies have utilized ovariectomy to demonstrate the direct link between estrogen and tissue homeostasis. Ovariectomy involves the removal of ovarian tissue; because the ovaries are the primary source of estrogen and progesterone during the estrous and menstrual cycles, their complete removal virtually eliminates endogenous estrogen. The vaginal epithelium of

ovariectomized (OVX) mice closely resembles the tissue during diestrus, with just 3-4 layers of cells present; the remaining cells possess the capacity for replacement, but the tissue becomes relatively static [(Ali et al., 2020)]. While progesterone supplementation does not lead to gross changes in the tissue, estrogen supplementation leads to the rapid buildup of the vaginal epithelium [(Gillgrass et al., 2005)]. Interestingly, while the vaginal epithelium under control of estrogen alone differentiates into an estrus-like state (deep tissue capped by a keratinized layer), combination treatment with both estrogen and progesterone induces a proestrus-like state that closely mirrors the human vaginal epithelium during the luteal phase [(Hapgood et al., 2018)]. And while it is outside the scope of this introduction to fully investigate cyclic changes in non-human primates, it is relevant to note that the impact of estrogen and progesterone on OVX NHPs have produced similar results [(Slayden et al., 2004), (Parakkal et al., 1972), (Quispe Calla et al., 2020), (Marx et al., 1996)]

1.3.3.2 DMPA in research and clinical settings

DMPA is a synthetic progestin that mimics progesterone and is utilized as a long-acting form of birth control due to its significantly longer half-life [(Archer et al., 1997)]. Importantly, its use in mouse models of intravaginal infection became practically *de rigueur* after a 1994 article reported it induced long-lasting diestrus that made mice consistently infectable (covered in detail in 4.2.2). Thus, a brief discussion of its mechanism of action is warranted.

DMPA functions in the cell by binding to the progesterone receptor, which then dimerizes and translocates to the nucleus, where it binds to progesterone response elements and promotes gene expression [(Dinh et al., 2019)]; ultimately, this activity prevents pregnancy by sustaining natural impacts of progesterone, which include blocking follicular maturation and ovulation [(Rivera et al., 1999)]. Unlike naturally occurring progesterone, DMPA can also bind to androgen and

glucocorticoid receptors with high affinity, resulting in the potential for collateral gene induction far beyond regulation of reproductive processes [(Woods et al., 2021)]. Glucocorticoid receptors in particular are widely (but variably) expressed throughout the body, and activation of these receptors can be immunosuppressive [(Cain et al. ,2017)]. In addition to its role in progesterone signaling, extended MPA use promotes hypoestrogenism and reduces total estrogen receptors [(Zalenskaya et al., 2018)]. DMPA as birth control, however, has become controversial in recent years, as its use has been associated with increased risk of multiple STIs, especially HIV. Yet in 2019, following the publication of the ECHO trial – which many researchers write was underpowered - the WHO withdrew its previously stated concerns regarding the use of DMPA in areas of high HIV incidence and reinstated its full support [(Ahmed et al., 2019), (Hapgood et al., 2018), (Ayele et al., 2021)]. The immunoregulatory potential of DMPA remains of interest to researchers across multiple fields.

The early cross-sectional studies that raised concerns regarding DMPA and STI acquisition have since been expanded to longitudinal trials; at the same time, studies in animals and *in vitro* models have provided a number of potential mechanisms for DMPA risks [(Caine et al., 2019), (Woods et al., 2018), (Gillgrass et al., 2005), (Quispe Calla et al., 2020), (Hughes et al., 2021), (Cotreau et al., 2007), (Ali et al., 2020), (Edfeldt et al., 2020)]. Some of these findings appear intuitive when one considers that DMPA has long been used to increase the chances of successful intravaginal infection in murine and NHP animal models [(Teepe et al., 1990), (Parr et al., 1994), (Kaushic et al., 2003), (Trunova et al., 2006), (Abel et al., 2004)]. Upon the discovery that ZIKV could be transmitted sexually, animal models of intravaginal infection were rapidly developed. DMPA quickly became a standard pretreatment in preparation for intravaginal challenge - including our longitudinal ZIKV infection study, which is covered in Chapter 3. The

pervasive use of DMPA in animal models of intravaginal infection is discussed in length in Chapter 4.

1.4 ZIKV VIRUS

1.4.1 *A brief history of the virus*

Zika virus was discovered in 1947 in a sentinel macaque from the Zika forest in Uganda [(Dick et al., 1952a), (Dick et al., 1952b)]. African lineage Zika virus was later determined to be actively circulating in several African countries following seroprevalence surveys conducted in the 1950s; infections in humans were largely asymptomatic, though flu-like symptoms were occasionally observed. In the 1960s, ZIKV was detected in surveys of Asian mosquito populations in the south Pacific region [(Marchette et al., 1969)], and following the Java outbreak in 1977, seroprevalence surveys revealed ZIKV was broadly circulated throughout the region [(Olsen et al., 1981), (Liu et al., 2019)]. These circulating strains were later deemed the Asian lineage after they were determined to be phylogenetically distinct from the African lineage; a S139N mutation in the membrane protein and V982A mutation in the NS1 protein were noted as the defining substitutions [(Liu et al., 2019)].

In 2013, a notable outbreak of Asian lineage ZIKV occurred in French Polynesia [(Cao-Lormeau et al., 2014)]; for the 1st time, ZIKV infection included neuropathological complications, as evidenced by more than 40 cases of Guillain-Barre syndrome [(Cao-Lormeau et al., 2016)]. This virus retained the S139N substitution but was distinct from earlier Asian strains due its A982V reversion [(Liu et al., 2019)]. The S139N has since been shown to increase neurotropism, while A982V may contribute to increased infection efficiency in mosquitos and

inhibition of IFN-β [(Liu et al., 2019)]; the significance of these findings became painfully clear with the emergence with the 2015 emergence of Zika virus in the Americas.

1.4.2 *Sexual transmissibility of ZIKV and the consequences of in utero infection*

ZIKV is unique among arboviruses in its ability to spread through sexual contact and achieve maternal-fetal transmission [(Miner et al., 2017)]. The first documented case of ZIKV sexual transmission was reported in 2011, which described a Colorado woman who developed a flu-like illness with overt rash in 2008 [(Foy et al., 2011)]. Her symptoms developed just days after her husband – who had recently returned from Senegal - presented with similar symptoms; the couple reported condom-less sex prior to symptom development. While noteworthy at the time, the report's significance grew tremendously upon the discovery of rapidly spreading ZIKV transmission in Brazil during late 2015. ZIKV was quickly declared a global public health threat following the determination that children born to women who were infected during pregnancy were at risk of developing microcephaly [who.int].

In May of 2016, French scientists obtained the first evidence of ZIKV in the FRT, when a 27-year-old with ZIKV symptoms tested positive for the virus; evaluation of vaginal secretions confirmed ZIKV on day 3 post-symptom onset, and follow-up samples remained positive through day 11 [(Prisant et al., 2016)]. Since these first reports, a growing body of epidemiological work has confirmed that sexual transmission during the ZIKV epidemic was dramatically underestimated [(Allard et al., 2017), (Aguilar Ticona et al., 2021), (Coelho et al., 2016)]. Several of these studies have noted that sexual contacts of index cases were far more likely to be seropositive than household contacts, and largescale seroprevalence studies have further demonstrated that women were more likely to be seropositive than men [(Rosenberg et al., 2019), (Magalhaes et al., 2021), (Coelho et al., 2016)]. These findings are in line with data from

previous non-ZIKV epidemics, which show that women consistently face greater health consequences than men during major outbreaks [(Wenham et al., 2020)].

The consequences of in utero infection have unfortunately become more apparent in recent years. While microcephaly was an overt outcome easily detected during the epidemic, rigorous follow up of children born to mothers infected during pregnancy has demonstrated a constellation of less apparent symptoms. Children with and without microcephaly are at greater risk for developmental delays, autism spectrum disorder, and vision and hearing impairments; these findings are often not diagnosed until the second or third year of life in non-microcephalic children [(Walker et al., 2019)]. Epidemiological studies have demonstrated that symptoms in children are likely to be worse when the infection occurred early during pregnancy [(Nielsen-Saines et al., 2019)]. The most common cause of death in children diagnosed with congenital Zika syndrome is pneumonia that is presumed to be secondary to occult aspiration; detailed post-mortem CTs have shown widespread calcifications and hypoplasia [(de Fatima Viana Vasco Aragão et al., 2019), (Adams Waldorf et al., 2018)]

1.4.3 *Cell-intrinsic immune responses to ZIKV infection*

The innate immune response of the ZIKV infected cell is triggered through the RLR/MAVS pathway and plays a major role in controlling ZIKV replication and spread [(Esser-Nobis et al., 2019)]. Specifically, RIG-I activation initiates a signaling cascade that results in the nuclear translocation of phosphorylated transcription factors IRF3 and IRF7, which then facilitate expression of IFN (Types I and III). IFN production results in both autocrine and paracrine signaling through IFN-specific receptor binding, activating the JAK/STAT pathway. This cascade ultimately results in the induction of antiviral, proinflammatory and cell signaling genes, broadly referred to as interferon stimulated genes (ISGs). Through *in vitro* studies in human vaginal

epithelial cells, we have observed STAT1 and STAT2 phosphorylation, followed by robust induction of antiviral ISGs in response to both IFN-B (Type I) and IFN-L (Type III). The activation of the RLR pathway and subsequent ISG induction was also apparent *in vitro* when primary vaginal epithelial cells and the VK2 cell line were grown in an organotypic tissue culture model. Chapter 3 covers the rapid activation and cell and tissue specific nature of this response, as detected by bulk and single-cell RNAseq, and Chapter 4 discusses the optimization of organotypic culture.

1.4.4 *Animal models of ZIKV infection*

The earliest attempts to study ZIKV in small animal models were largely abortive [(Dick et al., 1952b)]. While a successful murine model emerged in the early 1950s through serial passage of infected brain homogenates, the resultant MR766 strain demonstrated an inconsistent phenotype in wild-type mice and has since raised concerns regarding its relevance to circulating ZIKV[(Dick et al., 1952b), (Miner et al., 2017)]. Efforts to reinvigorate murine models rapidly expanded as the extent of the 2015 ZIKV outbreak became clear, and several groups screening immunodeficient mouse lines established that Type I IFN receptor knock out (IFNAR KO) and TYPE I/III IFN KO (AG129) lead to productive infection through subcutaneous, intraperitoneal, and intracranial infection routes [((Dowall et al., 2016), (Lazear et al., 2016), (Rossi et al., 2016), (Tang et al., 2016), (Yockey et al., 2016)]. IFNAR blockade similarly lead to productive infection. During this time, the ZIKV polymerase protein NS5 was identified as an antagonist of human STAT2, which is essential to mounting a response to ZIKV-induced IFN signaling [(Grant et al., 2016)]; importantly, the authors of this study noted that the NS5 protein did not antagonize murine STAT2, thus explaining the need for IFN blockade or knock out to generate productive ZIKV

infections in mice. Since that time, a more complete stat block has been reported, which was suggested to be due to ZIKV NS5 binding to heat shock protein 90 [(Roby et al., 2020)].

In response to the findings regarding STAT2, our collaborators in the Diamond Lab at Washington University developed a transgenic mouse on a C57BL/6 background that expresses the human STAT2 gene in place of the murine STAT2 gene [(Gorman et al., 2018)]. The immunocompetent STAT2 knock-in (S-KI) retains an intact IFN response and is susceptible to productive ZIKV infection, allowing for physiologically relevant *in vivo* examinations of ZIKV pathogenesis. The S-KI is essential to our investigations, as evaluation of our hypothesis that microbiota influence antiviral responses hinges on an intact IFN response. As discussed above (1.3.3.2), a portion of our studies of intravaginal ZIKV leveraged DMPA as a pretreatment to synchronize estrous cycles across all mice into a durable diestrus-like state, and the implications of its use in the context of our findings are discussed in Chapter 4.

1.4.5 *Intravaginal ZIKV infection in mice and NHP*

As discussed above, the mouse model of ZIKV infection has largely depended on immunocompromised mice, and this is true for the bulk of studies published on murine intravaginal infections. Thus, the study of intravaginal infection in mice has to contend with the confounding potential of both estrous stage and immune deficiency. The earliest model utilized progesterone to induce a diestrus-like state and contrasted this against an induced estrus-like state, which was achieved following administration of pregnant mare serum gonadotropin[(Tang et al., 2016)]; the mice in the these studies were all AG129. The authors reported successful infection of mice in the diestrus-like state. Not all early models utilized immunodeficient mice: in a 2016 study, virgin wild-type mice in an induced diestrus were shown to be susceptible. Importantly, this study also demonstrated that infection in early pregnancy resulted in fetal brain infection and fetal growth

restriction [(Yockey et al., 2016)]. Subsequently, studies in AG129 demonstrated that sexual transmission via infected males was "highly efficient", that the sexual route of infection was more efficient than intravaginally pipetted virus, and that pregnant mice experienced increased viral burden in the FRT – whether the challenge occurred subcutaneously or intravaginally [(Duggal et al., 2017), (Duggal et al., 2018)]. In wild-type mice it was also shown that virus-specific T cells were an essential component of infection resolution in the reproductive tract; here again, mice were pre-treated with DMPA [(Scott et al., 2018)]. Perhaps one of the most striking findings in animal models to date are that in an NHP model of intravaginal ZIKV infection, the virus was shown to preferentially replicate in reproductive structures, and that it mediated persistent infection [(Carroll et al., 2017)].

While the above sections demonstrate that the FRT is a highly dynamic environment, they have not addressed the sizable contribution of the microbiome in regulating host immunity. Clinical studies have revealed that the homeostatic mechanisms and luminal defenses discussed above can be supplemented through the cultivation of complex ecological partnerships with beneficial resident microbes. Thus, the following section provides a detailed primer on the vaginal microbiome in health and disease, the implications of a suboptimal microbiome in the context of STI risk, the weakness of current treatment strategies for microbial imbalances, and new approaches for microbiome optimization.

1.5 Establishment and kinetics of the vaginal microbiome

1.5.1 *The optimal microbiome*

Studies investigating the longitudinal stability of the vaginal microbiome have demonstrated that lactic acid producing bacteria– most frequently *Lactobacillus* - colonize the FRT shortly after birth

[Farage et al, 2009]. Their abundance is greatly reduced as levels of circulating maternal hormones decline, but with the onset puberty, endogenous estrogen levels increase, fostering a return of *Lactobacillus* dominance [Farage et al, 2009]. As discussed above, increases in circulating estrogen promote glycogen deposition in human vaginal epithelial cells, and as they differentiate, these cells restructure their keratin networks, build a proteinaceous cornified envelope, reduce tight junctions between cells, and unzip from the layers below (desquamate). Once shed, these cells break down in the vaginal lumen, and their stored glycogen - in conjunction with endogenous α-amylase – supports the nutritional requirements of multiple genera of lactic acid bacteria, *Lactobacillus* species chief among them [(Nasioudis et al., 2015), (Spear et al., 2014)]. Microbial metabolism of host glycogen results in the production of lactic acid and the maintenance of an acidic vaginal space. Lactic acid levels effectively restrict the potential for pathogenic bacteria entry into the FRT, and in recent years, the direct viricidal activity of lactic acid has become increasingly appreciated in the HIV field. This potential for an optimal microbiome to enhance protection against STI has generated considerable interest in the reproductive microbiome.

1.5.2 *The sub-optimal microbiome*

The loss of *Lactobacillus* dominance can result in a polymicrobial condition known clinically as bacterial vaginosis, a state which can promote acquisition of HIV and other STIs. Age, race, geographic location, sexual and hygiene practices, and birth control strategies have all been investigated as potential contributors to BV. Diagnosis has historically relied upon clinical presentation in conjunction with semi-quantitative microscopy, which considers bacteria morphology and shedding of clue cells. Recent advances in genomic characterization have increased the ability to detect and assign BV-associated bacteria (BVAB) that frequently co-occur

during symptomatic episodes. Importantly, increased urogenital microbial surveillance has revealed the presence of these organisms in asymptomatic individuals as well, complicating diagnosis and risk assessments.

While it is important to note that a number of complex factors are likely required to initiate a shift and establish a high diversity, BVAB community, the loss of *Lactobacillus* sometimes observed around menses provides a basis for understanding how a shift away from Lactobacillus dominance may occur. Specifically, studies investigating the longitudinal stability of the vaginal microbiome have demonstrated that Lactobacillus dominance can be disrupted around menses, when increasing iron levels permit outgrowth of opportunistic BVAB such as *Gardnerella vaginalis* [(Srinivasan et al., 2010)]. The relative abundance of *Lactobacillus* typically recovers with the return to the follicular phase, yet for some women, the increase in BVAB such as *G. vaginalis* can lead to a stable shift in community structure [(Srinivasan et al., 2010), (Gajer et al., 2012)]. When dominant, *G. vaginalis*-produced biofilms may act as a scaffold for a succession of BVAB genera, including *Atopobium, Prevotella, Sneathia, BVAB1-3,* and a number of anaerobic cocci known to be short chain fatty acid producers. In the elevated pH environment associated with BV, these genera are thought to collectively stabilize the polymicrobial state through a number of approaches that converge on epithelial barrier degradation, which in turn promotes adhesion and nutrient acquisition. Specifically, *Prevotella spp*. production of polyamines promotes mucin degradation and, along with sialidases and prolidases, also promotes epithelial exfoliation [(Aldunate et al., 2015)]. This breakdown of the epithelial barrier coincides with increasing ammonia production, which maintains an elevated pH and may support other BVAB [(Aldunate et al., 2015)]. *Sneathia* virulence factors include mucolytic agents, as well as adhesins, invasins, and endopeptidases; additionally, their production of superoxide dismutase may afford

protection from H2O2 [(Onderdonk et al., 2016)]. And anaerobic cocci such as *Finegoldia, Parvimonas, Anaerococcus, Peptoniphilus,* and *Megasphaera* produce butyric acid, which is thought to promote host chromatin remodeling. The majority of prevalent BVAB are also known to promote sub-clinical inflammation through the production of short-chain fatty acids levels in the FRT, which promotes sub-clinical inflammation [(Thurman et al., 2015)].

Chapter 2. RECURRENT BV FOLLOWING METRONIDAZOLE TREATMENT ASSOCIATED WITH MICROBIOTA RICHNESS AT DIAGNOSIS

2.1 INTRODUCTION

Bacterial Vaginosis (BV) is a polymicrobial condition in the female reproductive tract (FRT) that is known to increase the risk of sexually transmitted infection (STI) acquisition and reproductive sequelae[(Anahtar et al., 2015), (Brotman et al., 2010), (Cherpes et al., 2006), (Gosmann et al., 2017), (Lennard et al., 2018), (Thurman et al., 2015)]. BV rates among women globally vary widely; recent estimates suggest a 30% prevalence in the US, while rates in sub-Saharan Africa may exceed 50% [(Kenyon et al., 2013),(McKinnon et al., 2019)]. Despite this high prevalence, no single causative agent of BV is known.

The most universal feature of BV is the loss of *Lactobacillus* dominance in the vaginal ecosystem, which is accompanied by an increase in the proportion of polymicrobial anaerobic taxa [(McKinnon et al., 2019)]. The disruption of *Lactobacillus* dominance increases pH levels and nutrient availability, enabling the outgrowth of bacterial vaginosis-associated bacteria (BVAB) such as *Gardnerella vaginalis* [(Gajer et al., 2012), (Srinivasan et al., 2010)]. Persistent colonization by *Gardnerella vaginalis* is associated with the development of biofilms that support

other BVAB, including *Atopobium, Prevotella, Sneathia,* and *Shuttleworthia* [(Mars et al., 2012), (Swidsinski et al., 2008), (Swidsinski et al., 2013), (Verstraelen et al., 2013)].

The largescale availability of marker gene sequencing data has enabled the stratification of vaginal communities into discrete categories defined by their composition – frequently referred to as cervicotype (CT) [(Anahtar et al., 2015)] or community state types (CSTs) [(Ravel et al., 2011)]. Dominance by *Lactobacillus crispatus* (CT1) is considered an optimal state and is linked to a marked decrease in STI susceptibility. CT2 is dominated by *Lactobacillus iners* and is frequently considered a transitional phenotype to CT3 and CT4 - collectively referred to as "molecular-BV" [(Gajer et al., 2012), (Srinivasan et al., 2010)]. These communities, which are dominated by *Gardnerella vaginalis* or a highly diverse BVAB population (CT3 and CT4, respectively), strongly correlate with an increase of inflammatory factors, reproductive sequelae, and STI susceptibility [(Anahtar et al., 2015), (Brotman et al., 2010), (Cherpes et al., 2006), (Gosmann et al., 2017), (Lennard et al., 2018), (Thurman et al., 2015)].

Despite these known risks, BV recurrence rates remain above 70% within 12 months of treatment [(Bostwick et al., 2016)]. Current CDC guidelines recommend metronidazole as a first line treatment [CDC.org] As a pro-drug, metronidazole is reduced to its active state under anaerobic conditions – a mechanism that spares beneficial *Lactobacillus*. However, the complete removal of BVAB is likely complicated by the presence of biofilms, which shield BVAB by preventing metronidazole penetration.

Here, we build upon an examination of women with symptomatic BV who enrolled in a clinical study (CONRAD 115, ClinicalTrials.gov NCT01347632) [(Thurman et al., 2015)]. The initial analysis identified several BV-associated changes; these included increased inflammatory mediators and HIV target cells in the FRT, and reduced antimicrobial activity of the CVL

(Thurman et al., 2015)]. We hypothesized that 16S ribosomal RNA (rRNA) gene analysis would identify keystone taxa or community structures that were present at diagnosis and that contribute to BV recurrence or treatment failure; our primary objective was to leverage these data toward the development of a molecular predictor of treatment failure.

2.2 RESULTS

2.2.1 *Lactobacillus recovery varies following metronidazole treatment*

Twenty-eight of 33 women were confirmed to have molecular BV by 16S rRNA sequencing, while five patients found to have *Lactobacillus*-dominant microbiomes pre-treatment were removed from further analyses. At diagnosis, mean *Lactobacillus* abundance was 4.4% (Fig. 1a,e). Known BVAB, such as *Shuttleworthia*, *Sneathia, Prevotella*, and *Gardnerella,* were the most likely to be the dominant genus in a given sample at baseline (Fig. S1a,b). Several other highly prevalent bacterial genera frequently associated with BV diagnosis were observed at mean abundances of less than 5%, including *Megasphaera, Atopobium, Clostridium*, *Parvimonas*, *Dialister*, *Peptonophilus*, and *Gemella* (Fig. 1d,e).

At visit 2 one-week post-treatment, *Lactobacillus* mean relative abundance reached 70.1%, while several genera that were dominant at baseline decreased significantly (Fig. 1b,e). At visit 3, approximately one-month post-treatment, mean *Lactobacillus* abundance was down to 24.7% across all samples, while *Sneathia*, *Prevotella*, and *Shuttleworthia* increased on average (Fig. 1c,e). Mean values were found to obscure divergent treatment outcomes; these are discussed in detail below.

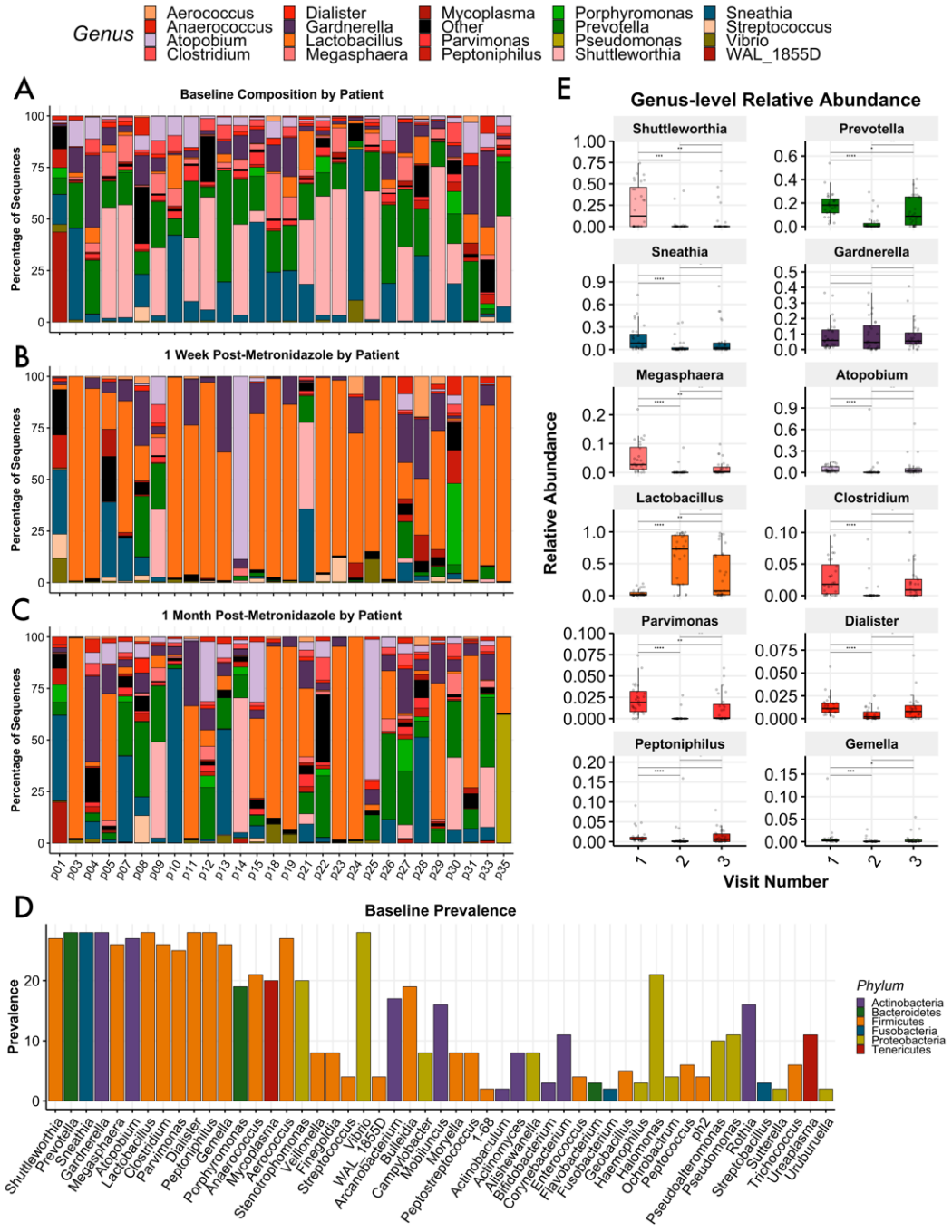

Figure 2.1. Baseline and posttreatment vaginal microbiota profiles of women diagnosed with Nugent-BV. Relative abundance taxonomic plots of cervicovaginal lavage assessed by 16S ribosomal RNA gene profiling; women were sampled at baseline on BV diagnosis (A) 7e10 days after conclusion of 10-day course of metronidazole (B) and again at 1 month posttreatment (C) A

histogram demonstrates most of the genera considered BV-associated were universally prevalent at baseline, including Prevotella, Sneathia, Gardnerella, Lactobacillus, Dialister, and Peptonophilus; Shuttleworthia and Atopobium were present in 27 of 28 analyzed samples. Several rare taxa were also highly prevalent (D). Box-and- whisker plots define median and interquartile ranges; all BV-associated bacteria detected in this study were significantly reduced at visit 2 following metronidazole treatment, though the mean levels rose again at visit 3. Lactobacillus was significantly increased at visits 2 and 3 (E).

The alpha diversity measurements of richness and evenness assess the number of observed taxa per sample and the degree of parity amongst taxa, respectively, while inverse Simpson suggests the complexity of a sample by estimating the likelihood that two randomly chosen reads from a sample would identify the same taxa. In the context of BV treatment, positive therapeutic responses include reducing the observed diversity and increasing dominance by *Lactobacillus*. While all measurements of diversity were significantly decreased *at the level of the cohort* for visit 2, a number of individuals actually experienced increases in richness and diversity (Fig. 2a). Further analysis revealed that a subset of women who did develop *Lactobacillus iners* dominance by visit 2 failed to retain these communities at visit 3, indicating BV recurrence. The variable response to treatment can be measured by Beta diversity, which estimates the degree off difference between two communities; these differences can be visualized using dimensionality reduction techniques such as Principal Coordinates Analysis (PCoA), where an increasing distance between any two samples represents decreasing similarity. In the present study, while patients clustered together at baseline, community composition and structure varied widely following treatment (Fig. 2b). It should be noted that patients were not found to cluster by age, BMI, cycle day, contraception method, or self-reported race(Fig.S6a-e).

Figure 2

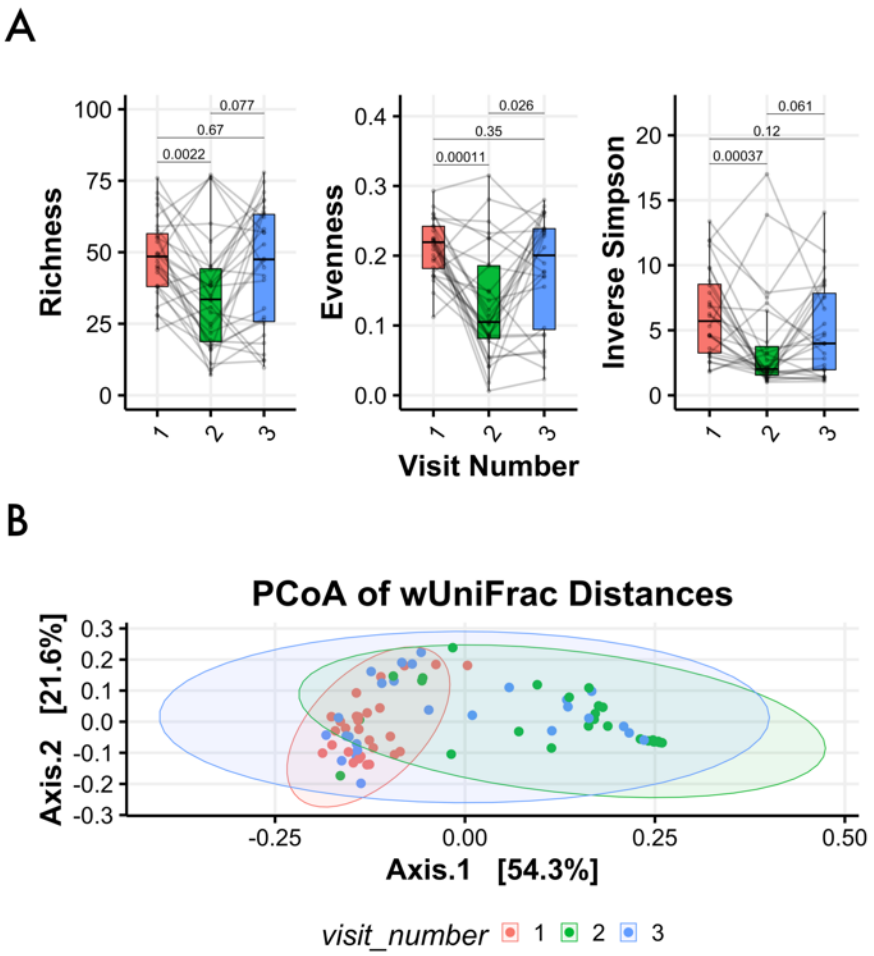

Figure 2.2. Alpha and beta diversity assessment by visit. Box-and-whisker plots define the median and interquartile ranges for measures of alpha diversity such as richness, evenness, and the inverse Simpson index, which were significantly decreased 1 week posttreatment; only evenness remained significantly reduced at visit 3 (A). A principal coor- dinate analysis of weighted UniFrac distances with samples (dots) colored here by visit: visit 1 (red), 2 (green), and 3 (blue); shaded ellipses represent 95% confidence intervals for cervicotype distribution. The plot illustrates samples clustered at visit 1 before treatment whereas the samples were considerably more varied at visits 2 and 3, reflecting divergent responses to metronidazole therapy (B).

2.2.2 *Soluble immune factors in FRT respond to microbiota shift*

Given the diverse patterns of change in microbial structure following treatment for BV, we cervicotyped patients at each visit. We elected to use the classification strategy described in Anahtar *et al, 2015*., which utilizes 4 CTs assigned according to dominant organism, with CT1 dominated by *Lactobacillus crispatus*, CT2 by *Lactobacillus iners*, CT3 by *Gardnerella vaginalis*, and CT4 by any of a number of diverse anaerobes associated with BV. We first validated the distinct composition of CTs through wUNIFRAC assessment (Fig. 3a), finding significant differences between each CT pairwise (2v3: adj.*p*=0.003,2v4: adj.*p*=0.003,3v4:adj.*p*=0.027); CT1 was not observed in the current study. We next assessed vaginal pH levels across these groupings and found that women supporting CT2 had a significantly lower pH at the time of sampling than women in either CT3 and CT4 (Fig. 3b*;* *p*=0.001; *p*=3.9e-12). In addition to differences in vaginal pH, we identified significantly higher levels of SLPI, GRO*a*, and MIP3*a* in CVL from women in CT2 as compared to CT4(Fig. 3c); these women also had significantly lower levels of ICAM-1 (*p*=2e-4) and a trend toward lower IL-1B (*p*=0.067). CD4+ cells from the lamina propria as assessed by microscopy were significantly higher in women categorized as CT4(*p*=0.034), in agreement with recent reports [(Gosmann et al., 2017), (Thurman et al., 2015)].

We hypothesized that immune profiles generated by combining all measured soluble factors would demonstrate an alignment between CT and immune response for each sample collected. Dimensionality reduction of the log-transformed values demonstrated that immune profiles clustered by CT (Fig. S3A; adj.*p*=0.003). Importantly, the clusters generated through immune profile analysis strongly resemble clusters generated using metataxonomic data (Fig. S3B). When combined using Procrustes rotation - a technique to compare ordinations by

minimizing the residual sum of squares between plots - these data demonstrate that a shift in CT was consistently associated with a shift in immune profile (Fig. 3d).

2.2.3 *Community structure at treatment initiation impacts response to metronidazole*

Assignment of CT for each patient by visit identified patients who experienced failed, transient, delayed, or sustained BV clearance as determined by 16S rRNA gene analysis. Twenty-five percent of patients failed to clear BV (did not improve to CT2 at any visit); 35.7% demonstrated a transient BV clearance - shifting to CT2 at visit 2 only; 7.1% demonstrated delayed clearance - reaching CT2 at the final visit only; and 32.1% of patients experienced sustained BV clearance - maintaining a shift to CT2 at both visit 2 and 3. Both the transient and sustained BV clearance groups experienced significant community reorganization following metronidazole treatment; specifically, *Lactobacillus* relative abundance swelled at visit 2 (Fig. 4a), with reciprocal decreases to *Shuttleworthia*, *Prevotella*, *Sneathia*, and *Atopobium* (Fig. 4b). For women that failed to clear BV, relative abundance of BV-associated taxa such as *Shuttleworthia* and

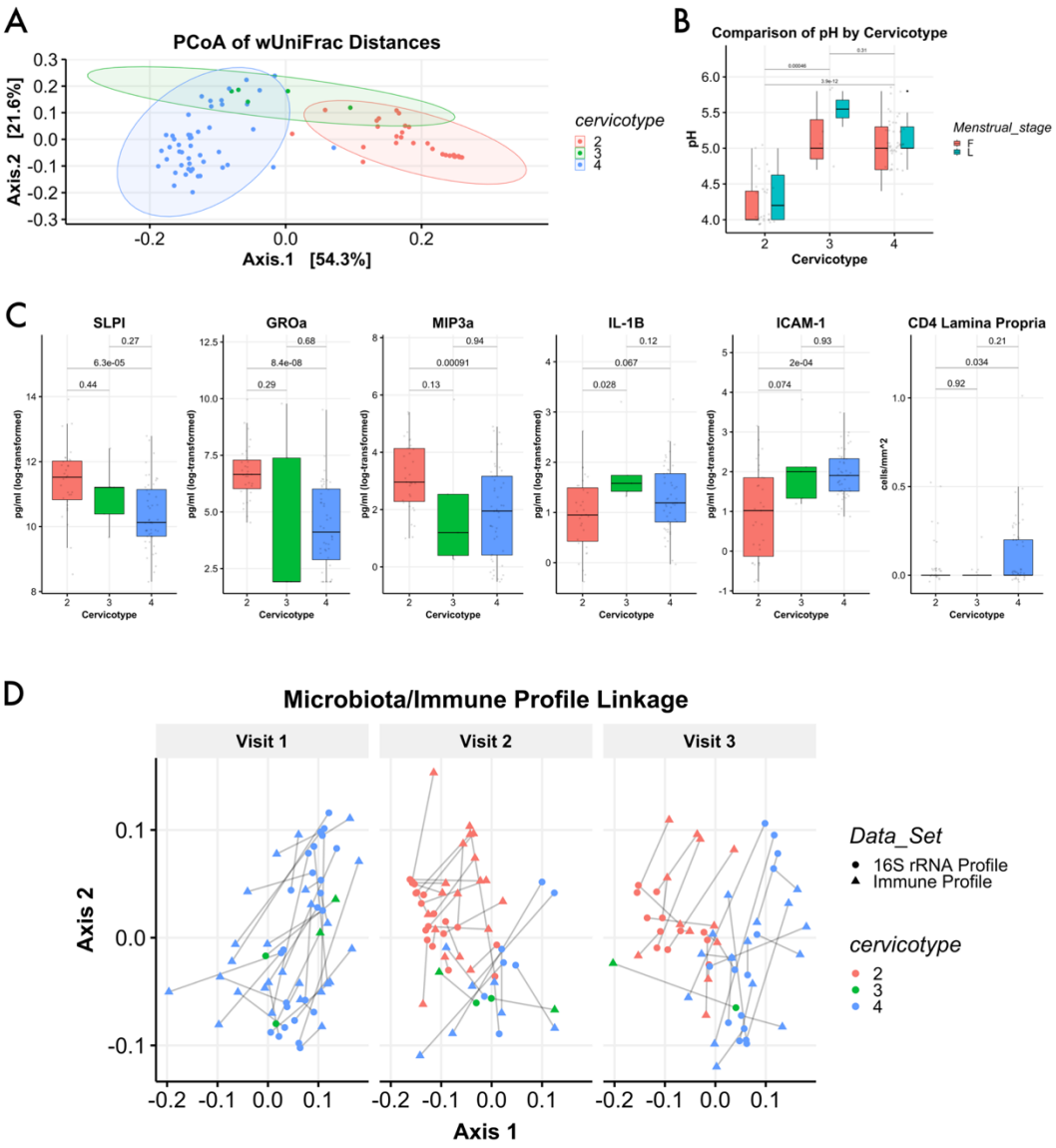

Figure 2.3. Immune profiles segregate by cervicotypes. Principal coordinate analysis based on weighted UniFrac distances demonstrated the clustering of samples by cervictoype. The samples (dots) are colored here by cerivcotype (CT): CT2 in red; CT3 in green, and CT4 in blue. CT1 was not observed in this study. Shaded ellipses represent 95% confidence intervals for cervicotype distribution (A). Assessment of sample pH based on the assigned cervicotype shows significant elevations in pH for women with cervicotype 3 or 4 (B). Several soluble factors analyzed from cervicovaginal lavage showed signficant differences between CTs 2, 3, and 4; in addition, CD4þ cells in the lamina propria were significantly elevated for women in CT4 (C). Vaginal immune profiles were generated through the measurement of select soluble factors present in CVL. The samples were then compared based on the aggregated measurements through Bray-Curtis dissimilarity and visualized through principal coordinate analysis, with the plot split into visits 1, 2, and 3. 16S ribosomal RNA gene data were visualized in the same plot through procrustes rotation. By visit 2, women who transitioned to Lactobacillus dominance possessed a statistically

distinct immune profile (P.adjusted1⁄4.003 by permanova), and this distinction remained significant at visit 3 1 month posttreatment (P.adjusted1⁄4.003 by permanova). The emergence of a distinct immune profile for women with Lactobacillus dominance is paralleled by the emergence of distinct bacterial community compositions associated with a shift to CT2 (P.adjusted1⁄4.006 at visit 2 and visit 3 by permanova) (D).

Prevotella were moderately reduced at visit 2, while *Atopobium* levels were increased. In cases of transient BV clearance, BVAB levels at visit 3 returned to - or exceeded – levels detected at BV diagnosis.

To evaluate our hypothesis that elements of the community structure at the time of treatment initiation impact outcome, we first assessed distinctions in alpha diversity metrics between clearance profiles. Women who failed to clear BV demonstrated the highest median levels of richness, evenness, and diversity at visit 1 (Fig. 4c). Importantly, both richness and evenness were significantly lower for women that sustained BV clearance versus those women who failed to clear BV (Fig 4c). All groups were indistinguishable at baseline by beta diversity assessment, and this finding suggests that the accumulation of rare or low-abundance taxa may play a larger role than dominant taxa in determining treatment outcome. Accordingly, analysis of differential abundance to identify keystone taxa that were significantly enriched or notably absent failed to identify meaningful pre-treatment differences between any of the 4 clearance groups. Finally, we asked if the occurrence or duration of CT shifts following metronidazole treatment could be due to an immunological predisposition and found that immune profiles for the 4 response groups were indistinguishable at baseline (Fig. 4d). Importantly, analyses of post-treatment visits demonstrated distinct patterns of change that stratified according to clearance profile (Fig. 4e).

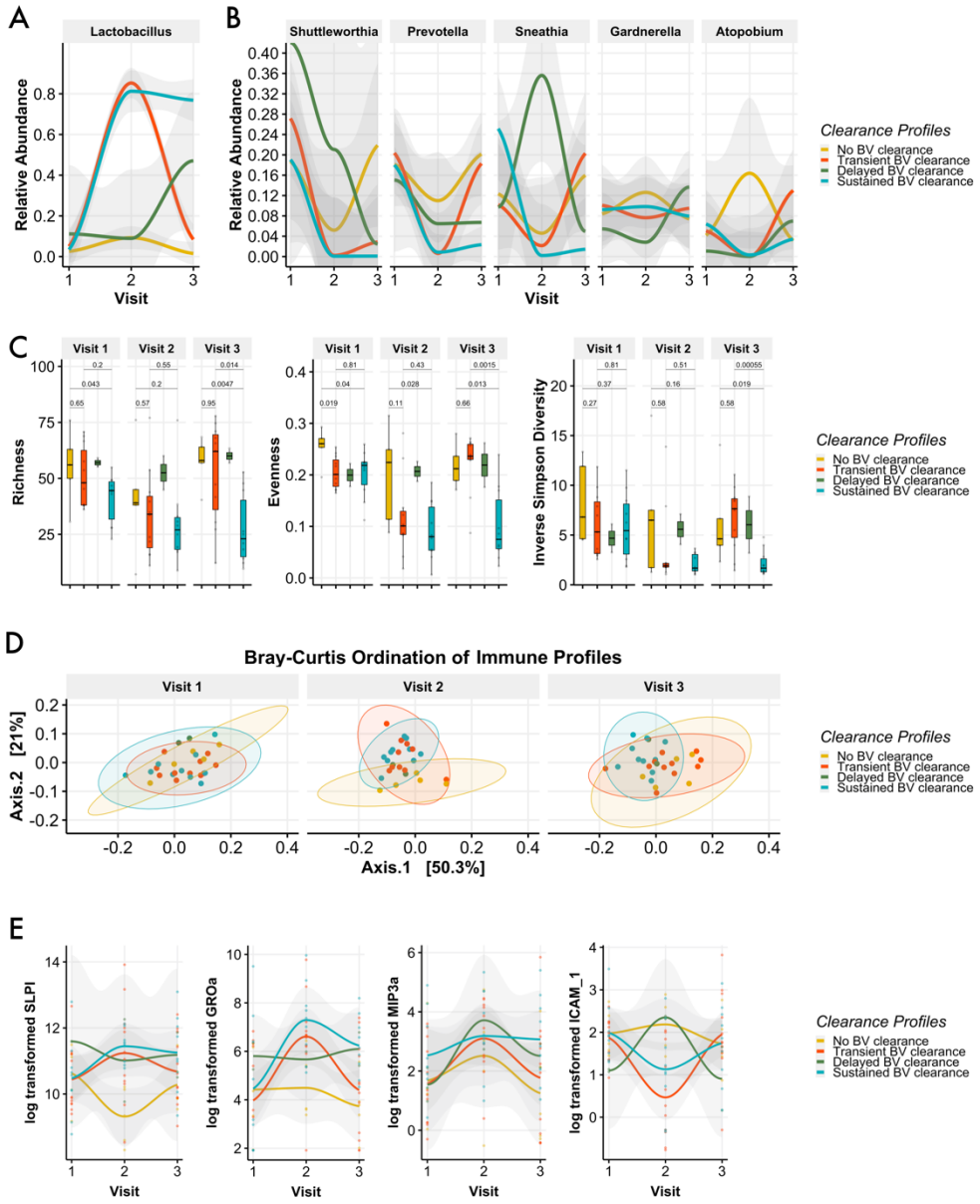

Figure 2.4. Posttreatment shifts to Lactobacillus dominance vary in timing and duration. Only a portion of women experienced significant increases in Lactobacillus levels following treatment; of these women who were cleared of SeqBV at visit 2, only a portion remained clear at visit 3 (A). The relative abundance levels of several bacterial-vaginosis-associated bacteria seen here were inversely related to the gains in Lactobacillus; the levels of these bacteria were at or above baseline level for many women at the final visit (B) Women who sustained SeqBV clearance following treatment had significantly lower levels of observed taxa and measured evenness before treatment initiation (C) SeqBV clearance patterns were not predicted by immune profile at baseline (D) Smoothed plot of select soluble factors over time that were shown to differ significantly by CT (E).

2.3 DISCUSSION

2.3.1 *Principle findings*

Here, a significant increase in the prevalence of a diverse collection of low-abundance taxa was associated with metronidazole treatment failure. Equally important, women who experienced total treatment failure or BV recurrence experienced a subsequent increase in prevalence of these taxa above their baseline levels. While markers of mucosal health improved with a shift to *Lactobacillus* dominance, these changes were transient in women who experienced recurrence.

2.3.2 *Results in the Context of What is Known*

In this study, we stratified metronidazole treatment outcomes according to the occurrence and duration of vaginal microbiota shifts to *Lactobacillus*-dominated CT2. Importantly, not a single woman in this study shifted to CT1, the most optimal vaginal microbial structure. This approach also allowed us to ask if community characteristics prior to treatment initiation contributed to treatment outcome. We found that the microbiota of women with a durable shift to *Lactobacillu*s-dominance were significantly less complex at diagnosis than those women who failed to shift to CT2. These findings suggest that metronidazole treatment is more likely to be effective when community richness is lower. As niche space is subdivided within an ecosystem, functional resistance to environmental stressors is often increased [(Baert et al., 2016), (Pennekamp et al., 2018)], and thus, the development of a high complexity vaginal microbiota may increase compositional stability. Indeed, research into the stability of vaginal microbiota across health and

disease has demonstrated that women who shift to a higher, more complex CT are unlikely to return to a lower complexity state [(Anahtar et al., 2018)].

Within the context of antibiotic pressure, highly diverse communities may benefit polymicrobial anaerobic assemblies by providing the collective capacity to interfere with environmental metronidazole activity; this notion is strongly supported by a growing body of research that has demonstrated a role for both gut and vaginal microbes to metabolize, alter, and sequester drugs [(Klatt et al., 2017), (Zimmermann et al., 2019), (Lee et al., 2020)]. Alternatively, highly diverse communities may simply reflect well-established scaffolds known to play a role in relapsing BV; specifically, biofilms produced by genera such as *Gardnerella vaginalis* provide barriers to antibiotics and can serve to protect other taxa as well; *Atopobium* specifically have been shown to comprise as much as 40% of BV-associated biofilms [(Swidsinski et al., 2008)]. Given the significant role that community richness may play in treatment failure, it is important to note that women in this study who experienced transient shifts to CT2 following treatment concluded the study with elevations in mean diversity, not reductions. These data suggest that ineffective treatment may predispose women to future treatment failure and facilitate development of more intractable BV. Indeed, we found that in cases of treatment failure or relapse, the biofilm-associated genera *Atopobium* and *Sneathia* generally increased after antibiotic exposure(Fig. 4b), a finding in agreement with research that has shown *Atopobium* to be a strong contributor to BV relapse [(Swidsinski et al., 2013)].

In addition to these findings, our work adds to the growing body of evidence demonstrating microbiota-induced modification of antimicrobial responses and immunity in the vaginal mucosa. Our finding that immune profiles clustered by CT at the time of sampling suggests that patients undergoing treatment for BV experience microbiome-mediated immune shifts as community

composition changes. This strongly supports the growing body of work that indicates a direct role of the microbiome in modifying mucosal immune health in the FRT [(Anahtar et al., 2015)]. These immune shifts were likely advantageous for women who shifted to *Lactobacillus*-dominated CT2, as they experienced significant increases in levels of SLPI -– which is both antimicrobial and known to protect mucosal barrier tissues from damage caused by neutrophil elastase [(Thurman et al., 2015)]. Importantly, increased levels of SLPI are associated with reduced risk of HIV acquisition. Women who shifted to CT2 also demonstrated a significant reduction in ICAM-1 – a biomarker for FRT inflammation – which was further supported by a trend toward reduced IL-1B, a strongly pro-inflammatory cytokine [(Thurman et al., 2015)].

2.3.3 *Clinical Implications*

Taken together, our data demonstrate that treatment failure or BV recurrence following metronidazole therapy may actually strengthen factors associated with intractable BV. Treating all patients diagnosed with BV as a single, homogenous condition may place women at risk. Sequence-based assessments that would identify these established, resistant communities are currently not accessible in the clinical setting. This indicates an intense need to develop bedside tools capable of staging BV prior to treatment. It should be noted that while Nugent score was positively correlated with microbiota richness by all metrics, there was some discordance between patients deemed BV+ by Nugent score and those categorized as BV- by sequencing analysis.

The conspicuous finding that none of the women observed here shifted to CT1, which is dominated by *Lactobacillus crispatus*, suggests that ongoing efforts to develop biotherapeutics that supply active cultures of *L. crispatus* may be an essential aspect of future treatment. Several ongoing safety and efficacy trials should be monitored closely for clinically viable therapeutics.

2.3.4 *Research Implications*

While presence/absence approaches such as 16S rRNA gene analysis and shotgun metagenomics have provided novel insights to the complex makeup of the vaginal microbiome, understanding of the functional activities of the frequently unculturable taxa it contains should be sought in the form of transcription-based assays. Because DNA-based approaches utilize genomic material that may persist following treatment, early timepoints may identify taxa as present despite their death due to treatment. Additionally, these assays fail to provide mechanistic responses to therapy; the identification of transcriptional changes in response to metronidazole could provide insights into community vulnerabilities that translate into new therapeutic approaches.

2.3.5 *Strengths and limitations*

A major strength of our assessment is that it provides previously unavailable insights into factors associated with metronidazole treatment failure. Several previous studies have investigated the most dominant BVAB through PCR-based methods and found that their levels do not determine therapeutic outcomes. Here, our unbiased approach allowed us to identify that increased diversity of "minor" BVAB may play a larger role in treatment outcomes than previously expected. Additionally, our work expands upon previous cross-sectional studies by showing that improvements to mucosal immune health fluctuate in real time with improvements in microbiota composition – and that these changes are transient when BV recurs.

Caveats include an absence of measurements for antibiotic levels, though all patients self-reported taking metronidazole as prescribed. Future studies would be improved through the collection of BV recurrence data; this could enable a comparative assessment of microbial communities between women experiencing their first episode of BV treatment and those women

who have been treated for BV previously. While a larger sample size in a similar microbiome marker study would lend additional strength to our findings, it is notable that the data collected from the 28 enrolled participants were sufficient to discriminate between cervicotypes and response profiles - and ultimately detect significant differences in (a) microbiota complexity prior to treatment and (b) mucosal immune profiles as community profiles shifted. Additionally, while the sample size of 28 is modest, the ability to track the sequenced-microbiome prior to and following therapeutic treatment with antibiotics is a major strength of the work – and one that is currently lacking in the literature.

Additionally, shotgun sequencing approaches would enhance the ability to detect antimicrobial resistance (AMR) genes – a study that is lacking in the field at this time. Finally, keystone bacteria such as *Lactobacillus iners* are known to alter transcriptional profiles substantially in the context of increased diversity; a metatranscriptional approach could resolve how bacterial responses stratify according to treatment outcome.

2.3.6 *Conclusions*

Current approaches to managing BV do not adequately assess the risk of treatment failure, which is demonstrated by the unacceptably high levels of relapse experienced by women receiving the standard of care. The presence or absence of individual taxa seems unlikely to be a direct contributor to treatment outcome, meaning clinicians continue to lack a prognostic biomarker. Our data suggest complex community dynamics govern the collective response to treatment. Therefore, while one arm of research must aim to provide clinicians with bedside staging strategies that anticipate difficult-to-treat cases, another must examine the mechanistic underpinnings of BV persistence toward the goal of providing them with targeted therapeutics.

Chapter 3. PATHOGEN DETECTION IN THE FEMALE REPRODUCTIVE TRACT TRIGGERS NON-CANONICAL DEFENSES THROUGH EPITHELIAL ACTIVATION IN A HORMONE-DEPENDENT MANNER

3.1 INTRODUCTION

The physical and emotional risks of sexual reproduction are borne largely by women and children[https://www.unfpa.org/maternal-health]. A portion of this burden is due to the elevated acquisition rates for sexually transmitted infections (STI), which benefit from the restrained immune responses of the female reproductive tract (FRT) [(Khan et al., 2016), (Khan et al., 2019)]. This susceptibility is a compromise designed to promote successful fertilization. Despite these known risks, the ZIKV epidemic illustrated that our understanding of immune responses to viral infection in the FRT remains inadequate. Recent studies have demonstrated non-canonical IFN induction in the FRT following intravaginal viral challenge[(Khan et al., 2016), (Miner et al., 2017)], and adjacent studies have suggested that the maternal microbiota may further modulate antiviral programs [(Anahtar et al., 2015), (Gosmann et al., 2017), (Hensley-McBain et al., 2016)]. Thus, reducing the global burden of sexually transmitted viral infections likely requires identification of maternal factors that modulate cell-intrinsic immunity within the context of tissue-specific immune pathways.

While the global response to the emergence of Zika virus (ZIKV) in 2015 focused primarily on mitigating vector borne transmission, the risks of sexual transmission were far greater than early calculations suggested [(Magalhaes et al., 2021), (Linde Arias et al., 2020), (Allard et al., 2017)]. ZIKV is unique among flavivirus in its ability to spread sexually [(Musso et al.,

2015)]. Recent reports demonstrate a tropism for reproductive structures in both men and women, and persistent infection of these tissues is now well documented [(Carroll et al., 2017), (Oliveira et al., 2018), (Duggal et al., 2017)]. Importantly, vertical transmission has been shown to promote a constellation of developmental impairments in the developing fetus, and in vivo studies have demonstrated higher rates of fetal infection following sexual transmission [(Duggal et al., 2018)]. The mechanisms that make the FRT a favorable niche for ZIKV are not well understood. The FRT is a mucosal tissue lined with a squamous epithelium, and these cells provide the initial target population for intravaginally acquired ZIKV. Macaque studies have demonstrated that ZIKV can replicate to high titers in the vaginal epithelium before disseminating systemically [(Carroll et al., 2017)], and thus, a better understanding of epithelial defenses is foundational to developing preventative strategies for ZIKV and other STI. Studies of the epidermis demonstrate that homeostatic cell turnover in squamous epithelia is the central mechanism for maintaining physical and chemical barriers to infection [(Anderson et al., 2014)], but the potential for these mechanisms to mitigate ZIKV infection are not known.

Importantly, the standard model for mouse and macaque studies of intravaginal ZIKV utilize depot medroxyprogesterone acetate (DMPA) [(Kaushic et al., 2003), (Tang et al., 2016), (Scott et al., 2018), (Carroll et al., 2017)]. While the use of DMPA in these studies as a tool to promote successful infection is well documented (and standard practice in other STI models), the mechanisms that increase susceptibility remain debated [(Hapgood et al., 2020)]. Large scale human and animal studies have demonstrated progesterone-based birth control can increase STI risks, and in vitro tissue culture studies have shown that progesterone can reduce innate immune functions in vaginal epithelial cells [(Vitali et al., 2017), (Wessels et al., 2019), (Bosinger et al., 2018), (Lee et al., 2016), (Quispe Calla et al., 2020)]. In contrast, increased estrogen levels

are associated with an optimized, lactobacillus-dominant microbiome, which has been shown to decrease risk of HIV/SIV acquisition [(Anahtar et al., 2015)], and animal models and *in vitro* work have provided evidence estrogen can directly decrease HIV acquisition and increase innate immune responses to viral challenge [(Hughes et al., 2021), (Gillgrass et al., 2005)].

To gain insight into the mechanisms that constitute tissue specific defenses in the LRT, we first examined antiviral immunity in a longitudinal study of intravaginal infection using the immunocompetent mouse model known as the Stat2 knock-in (STAT2-KI); this mouse is made susceptible to ZIKV antagonization of JAK/STAT signaling through the replacement of murine Stat2 with the human analogue [(Gorman et al., 2018)]. Our findings suggest that in conjunction with a rapid antiviral response, the vaginal epithelium induced a non-canonical "activation" of epithelial cells demonstrated by acute upregulation of genes that function in terminal differentiation and desquamation, in an apparent attempt to increase cell turnover and expel virus.

These findings led us to investigate the potential breadth of this response. Through re-analysis of publicly available data from the Gene expression omnibus (GEO), we found that this activation could be broadly observed following TLR stimulation by several TLR ligands, suggesting that this mechanism is a conserved response to PAMP. Given the essential role of sex hormones in determining immune responses of the FRT, we sought to explore these findings through additional in vivo studies of ovariectomized mice under specific hormone conditions. Importantly, while flow cytometry and single-cell analyses revealed that estrogen is essential to this response, our findings also suggest that estrogen can direct epithelial programs to restrain the adaptive immune response during intravaginal infection and exacerbate infection.

3.2 RESULTS

3.2.1 *ZIKV disseminates rapidly, widely following intravaginal infection*

To investigate the local and disseminated immune response to intravaginal ZIKV infection, we utilized the fully immunocompetent, human STAT2-knock-in (hSTAT2-KI) mouse model [(Gorman et al., 2018)]; these mice were developed on a BL6 background and made susceptible to ZIKV antagonization of the host interferon response through insertion of the human STAT2 gene. Seven-week-old hSTAT2-KI mice were synchronized in the diestrus stage through subcutaneous administration of depot medroxyprogesterone acetate (DMPA) 5 days prior to intravaginal challenge[ref] (Fig.1a). These mice were intravaginally challenged at day 0 with 10^6 pfu of a mouse-adapted strain of ZIKV Dakar[(Gorman et al., 2018)]; mice (n = 3/timepoint) were subsequently necropsied at 1- ,4- , and 8-days post-infection (dpi) to assess for infectious virion by plaque assay. A second study was conducted identically with timepoints at 1-, 2-, 4-, 6-, and 8dpi to assess tissues by qPCR and RNAseq.

We did not detect overt signs of clinical illness during these infections as assessed by grooming, posture, movement, sensation, or social behavior. However, the infected mice, which at 7 weeks of age are still growing, failed to gain weight at the same rate as uninfected mice (Fig.1b). Upon necropsy, we collected the lower and upper reproductive tracts (LRT and URT, respectively), which were divided just caudal to the bifurcation of the uterine horns. Lumbar lymph nodes were collected as pairs, and the spleen was collected as a whole tissue.

ZIKV infectious titer has been shown to correlate with viral detection by qPCR[(Yockey et al., 2016)]; as such, we utilized a qPCR assay targeting ZIKV prM/E to track infection across tissues for an 8-day time course. We detected virus in the lower reproductive tract in all mice at

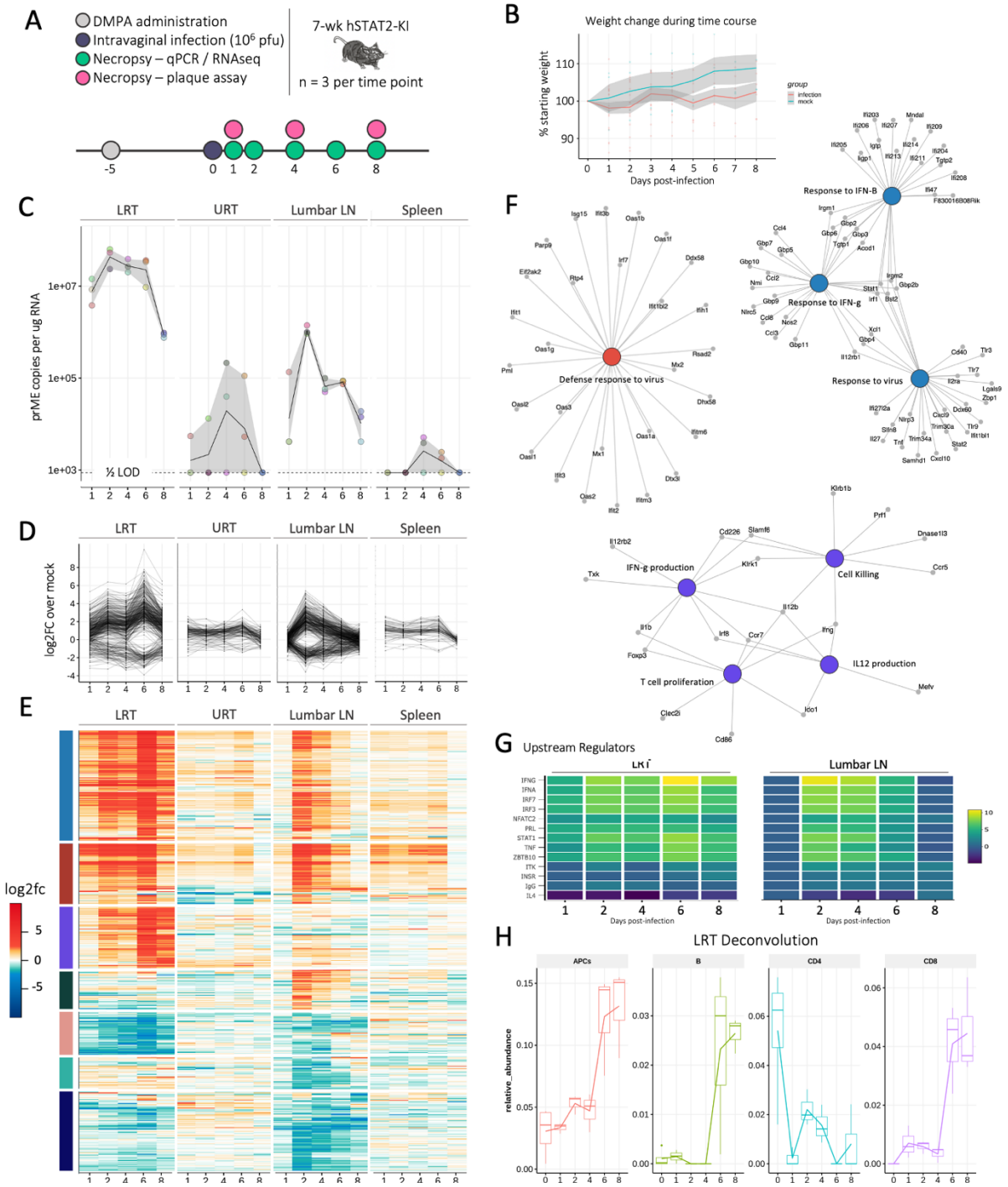

Figure 3.1 Intravaginal ZIKV progresses to systemic infection and initiates innate immune programs in tissue specific manner. Study design (A). Relative weight change (B). Viral load as measured by qPCR (C). CEMiTool coexpression gene clusters enriched for innate immune processes; expression over time (D). Heatmap of al unique innate genes detected across all tissues (E). ORA analysis of clusters with high expression in the LRT (F). IPA predicted upstream regulators in LRT and Lumbar LN (G). Cibersort deconvolution predictions of cell type changes over time (H).

all timepoints, Intravaginal with viral loads peaking at 2dpi (Fig.1c). Virus in the URT was

detected less consistently, with only 1/3 mice at 1 and 2dpi exceeding the limit of detection(Fig.1c). Virus in the URT peaked at 4dpi (Fig.1c) while LRT levels remained high, and virus was no longer detectable at 8dpi by qPCR. ZIKV was detected in the lumbar lymph nodes in 1/3 mice at 1dpi, with peak levels observed at 2dpi. Virus levels steadily declined following this peak but remained detectable in all mice through 8 dpi. Finally, ZIKV was first detectable in the spleen at 4dpi (2/3 mice) and remained detectable at 6dpi; ZIKV was not subsequently detected. Collectively, ZIKV was seen to disseminate from the rapidly following intravaginal challenge, and ZIKV remained detectable at high levels in the LRT and lumbar lymph nodes throughout the 8-day study. Thus, our findings agree with recent findings that the vaginal mucosa is a favorable niche for ZIKV replication.

3.2.2 *RIG-I and IFN signaling establish overlapping antiviral programs in diverse ZIKV-positive tissues.*

We next sought to identify the kinetics of innate immune induction following intravaginal infection and the degree to which innate immune programs at the site of primary infection were shared by sites of disseminated infection. To address this question, we performed bulk RNA sequencing on the sample set described above. Data sets for each tissue were assessed independently, and genes that remained in the data set following quality control were analyzed for coexpression using the CEMiTool platform [(Russo et al., 2018)]. This unsupervised approach first selects genes with the highest variation in expression levels, and subsequently clusters those genes hierarchically to determine distinct coexpression modules. We analyzed each coexpression module by overrepresentation analysis (ORA) [(Boyle et al., 2004)] to identify significantly enriched biological processes and found that each tissue contained one module that was highly enriched for antiviral mechanisms and interferon signaling processes; we assigned these as "innate response"

modules. The log2 fold-change over mock (log2fc) were maximal for genes in the LRT and draining lymph nodes, where viral detection was also highest (Fig.1d). Interestingly, peak transcription of innate genes precipitated a reduction in viral detection in both tissues, but while this occurred rapidly in the draining lymph nodes (2dpi), viral loads in the LRT plateaued for several days until a day 6 spike in transcription appeared to predicate reduced viral loads by 8dpi.

To determine the degree to which specific innate immune programs were utilized across tissue types, we combined the genes from each innate coexpression module into a master list; genes that were not detectable in all four tissues were removed and are considered later in their tissue-specific context. The remaining genes (#) were clustered according to their expression pattern across tissues (Fig.1e), and then assessed for functional enrichment. Our analyses identified two clusters that were broadly upregulated in all tissues, with Ingenuity results broadly indicative of antiviral processes. Specifically, upregulated genes in the red module were enriched for cell-intrinsic, antiviral defenses, including several genes from the Oas, Ifit, and Ifitm families, and pattern recognition receptor RIG-I; and transcription factor IRF7 (Fig.1f). This list of genes suggested cytosolic detection of viral nucleic acid by RIG-I and downstream phosphorylation of IRF3, and this observation was substantiated by Ingenuity analysis of upstream regulators (Fig.1g). The blue module was similarly upregulated across all tissues and was enriched for antiviral defense closely associated with a response to interferon (Fig.1f). Coexpressed genes in this module, included STAT1 and STAT2, as well the PRRs TLR3, 7, 9 and ZBP1; several canonical ISGs were also upregulated, including RSAD2, CXCL10, and multiple guanylate binding proteins (GBPs). To confirm the relevance of these results in human cells, we grew a vaginal epithelial cell line (VK2) at an air-liquid interface (ALI) to induce cell layering akin to the squamous epithelia of the LRT [(Lee et al., 2016)]. Cells were grown at ALI for 10 days, infected at an MOI of 5, and

harvested at 24- and 48-hours post-infection. Cells demonstrated productive infection by qPCR and immunofluorescent microscopy (data not shown). Importantly, analysis of vaginal cell RNA by a custom NanoString panel recapitulated findings from *in vivo* intravaginal infection in immunocompetent mice (data not shown), demonstrating that findings in mice were physiologically reflective of the cell-intrinsic immune programs of the human vaginal epithelium.

3.2.3 *Cytotoxic T cell signature precedes reduction of viral burden in both LRT and dLN*

Unlike genes in the red and blue modules, genes in the purple module were predominantly upregulated in the LRT. ORA enrichments included the biological processes of Il-12 production, T cell proliferation, IFNg production, and cell killing. Examination of this cluster suggested that a large portion of upregulated genes were likely due to tissue infiltration by specific cell types, including Cd86 by antigen presenting cells (APCs), Foxp3 by regulatory T cells, and Ccr7 by dendritic cells (DCs) homing to lymph node. This concurrent increase of leukocyte markers and IFNg transcripts in the LRT peaked just prior to a decline in viral load when IPA predicted peak IFNg activity. To further establish this connection, we utilized Cibersort deconvolution to estimate changes in LRT tissue cellularity based on transcriptional markers [(Newman et al., 2015)]. As predicted, Cibersort deconvolution estimated 3-5 fold increases in APCs, B cells and CD8 T cells beginning at 6dpi (Fig.1I, see Methods). From these findings, we concluded that the induction of canonical antiviral programs occurs in mice following intravaginal ZIKV in a manner consistent with *in vitro* findings from human vaginal epithelial cells. Our transcriptional analysis also agrees with existing literature that supports a role for cytotoxic T cells in ZIKV clearance following intravaginal infection [(Scott et al., 2018)]. Collectively, these findings reiterate the physiological relevance and utility of the immunocompetent STAT2-KI model in intravaginal infection studies, and provide the first comprehensive transcriptional analysis in this model.

3.2.4 *Epithelial differentiation upregulated following intravaginal infection*

During our initial assessment of transcriptional activity across tissues, we were struck by several epithelial specific genes known as small proline rich repeats (Sprr) that were clustered within the innate coexpression modules of the FRT (Sprr2a3, Sprr2f, and Sprr2g). The Sprr gene family is a component of the epidermal differentiation complex (EDC) – a suite of genes on human chromosome 1q21 (mouse, 3q) that are required during the formation of the cornified cell envelope [(Kypriotou et al., 2012)]. This process of terminal differentiation is a form of programmed cell death that is specific to squamous epithelial tissues and essential to homeostatic cell turnover. Thus, we focused on two prominent coexpression modules (Krt.A, Krt.B) that were upregulated following infection and significantly enriched for functions associated with epithelial cell terminal differentiation, also known as cornification (Fig.2a,b). Module Krt.A contained 316 coexpressed genes with peak expression at 1dpi and included 21 of the 57 genes found in the EDC. Krt.B contained 59 coexpressed genes, and expression levels were elevated throughout the 8-day time course (Fig.2a). In addition to the upregulation of late-stage cornification genes, these modules were enriched for protease inhibitors such as secretory leukocyte protease inhibitor (Slpi) and genes from the serpin gene superfamily, which participate in homeostatic cell renewal in

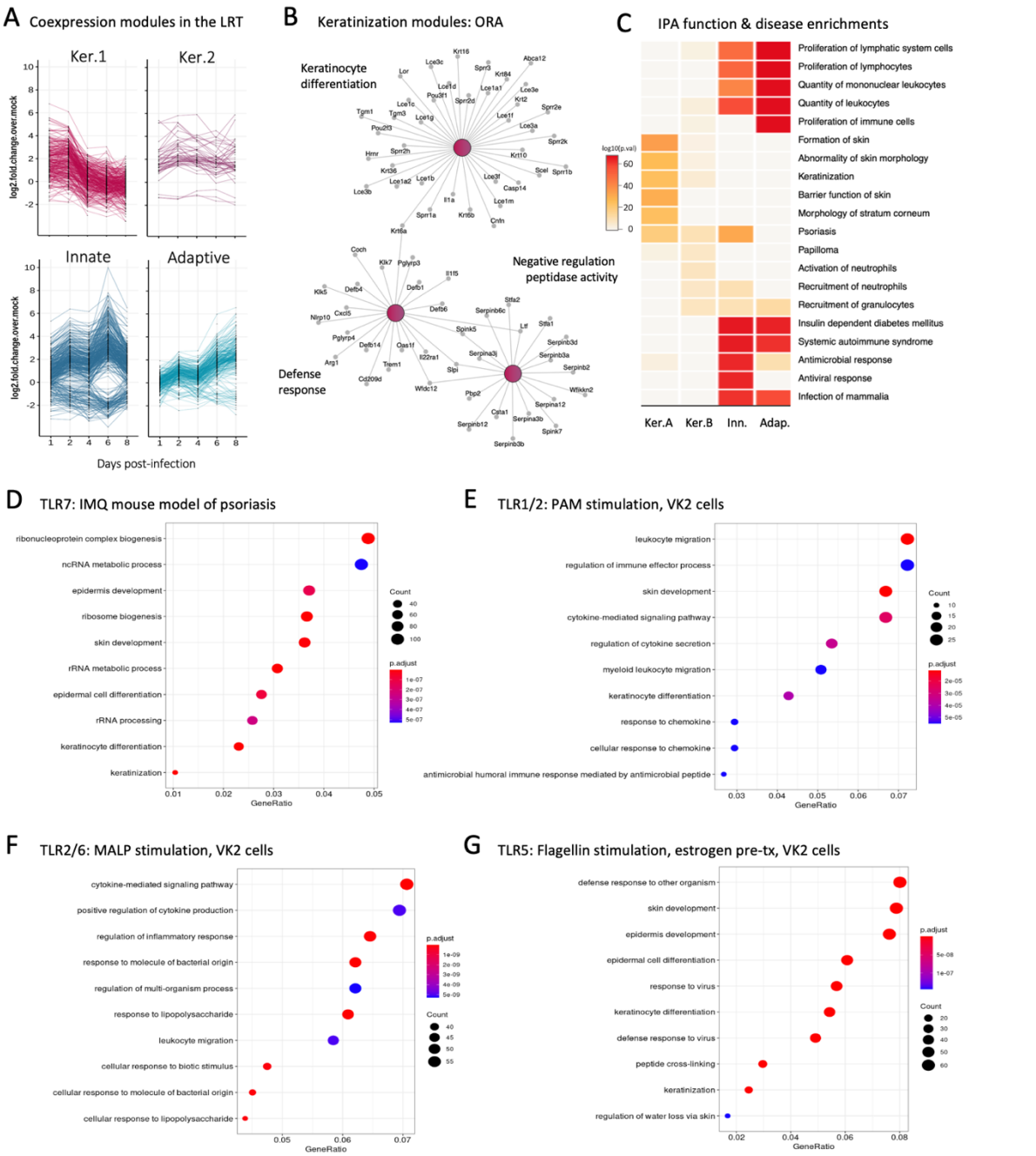

Figure 3.2. LRT upregulates epithelial differentiation upon ZIKV infection; response shared by other PRR pathways. Dominant CEMiTool coexpression modules from LRT; expression over time (A). ORA analysis of keratin-specific modules (B). IPA generated enrichments for function and disease for each coexpression module (C). ORA of public data sets for IMQ stimulation in mice (D), and PAM, MALP, and flagellin stimulation in VK2 cells (E-G).

squamous tissues by mitigating tissue damage and downregulating immune signaling that typically occurs when excessive protein breakdown is detected (a form of damage-associated molecular

patterns (DAMPs)) [(Eckhart et al., 2018)]. Several secreted antimicrobial peptides (AMPs) and proteins were also coexpressed, including multiple defensins (Defb), peptidoglycan recognition proteins (Pglyrp), and kallikreins (Klk), which are a family of (chymo)tryptic-like serine proteases shown to play a role in desquamation and the activation of AMPs [(Yamasaki et al., 2006)]. Several genes in these modules are known to participate in both homeostasis and defense, including the iron-binding molecule lactotransferrin (Ltf) and Krt6a, which have been shown to have antimicrobial functions [(Carvalho et al., 2017), (Tam et al., 2012)]. Cumulatively, these findings suggest that upon ZIKV infection, the vaginal epithelium accelerates homeostatic mechanisms of cell turnover that are designed to fortify the physical and chemical properties of the luminal surface while shedding infected cells through desquamation; this coordinated activity increases physical and chemical defenses in concert with activation of canonical, cell-intrinsic antiviral mechanisms.

3.2.5 *ZIKV-induced epithelial activation response parallels PRR activation in diverse model systems*

To gain insights into the epithelial programs that were activated during intravaginal ZIKV infection, we asked if the transcriptional signature resembled known responses to infection or disease. To do so, we utilized IPA to search for conditions that included activation of innate immunity in conjunction with epithelial differentiation (Fig.2c, see Methods). Results indicated that our modules for innate immune responses and keratinization significantly overlapped with expression data from experimental and clinical psoriasis samples. Psoriasis is classified as an immune-mediated inflammatory diseases (IMID) wherein chronic immune induction manifests through dysregulated epithelial proliferation, differentiation, and cell death [(De Simone et al., 2019)]. And while psoriasis is a multifactorial disease in humans, we were interested to learn that

in the murine model of psoriasis, hyperplasia is initiated through the topical application of the TLR7 agonist imiquimod (IMQ) [(Kjær et al., 2015)]. Like RIG-I, TLR7 is a key PAMP sensor known to restrict viral infections, including ZIKV [(Yockey et al., 2016)]. Thus, we sought public data from this model of psoriasis to compare the pattern of TLR7-induced epithelial activation with the activation signature identified in our intravaginal ZIKV data. We identified GSE63684, which utilized a mouse model to contrast IMQ-induced psoriasis with healthy mouse skin [(Kjær et al., 2015)]. Raw counts from this data set were analyzed to identify differentially expressed genes (adjusted p. value <0.1; log2fc > 0.25, see Methods). ORA of upregulated genes in mouse psoriasis included enrichment of several GO terms also identified from our intravaginal ZIKV analysis, and importantly, both data sets were enriched for keratinocyte differentiation (Fig.2d). Specifically, we identified 23 genes involved in late-stage cornification that were upregulated by both intravaginal ZIKV infection and topical TLR7 stimulation in murine skin). The identification of this shared upregulation of EDC genes led us to hypothesize that PRR detection in the LRT could initiate a defensive activation of the epithelium that was not limited to ZIKV.

To test this hypothesis more broadly, we searched the Gene Expression Omnibus (GEO) for data sets examining PAMP stimulation of human vaginal epithelial cells. While GEO lacked data sets examining viral infection in human vaginal epithelial cells, we did identify two studies that spanned a diverse spectrum of TLR stimulations. The first, by Zalenskaya et al., GSE68182 included array-based data from TLR stimulation of VK2 cells [(Zalenskaya et al., 2015)],. Specifically, the authors used the TLR ligands IMQ, macrophage-activating lipopeptide of 2 kDa (MALP-2) and Pam3CSK4 (PAM), which are sensed through TLR7, the TLR2:6 heterodimer, and the TLR1:2 heterodimer, respectively. We reanalyzed this public data set according to the methods

discussed above, and then investigated the resultant DEG lists by ORA. Unlike the mouse model of psoriasis, IMQ was a poor inducer of keratinocyte differentiation in vaginal epithelial cells; of note, it also failed to induce a gene signature enriched for antimicrobial responses. MALP treatment, however, significantly increased gene expression associated with keratinocyte differentiation (Fig.2e), and PAM stimulation induced upregulation of both EDC genes and an antimicrobial response (Fig2f).

The cell line being stimulated – known as VK2 cells - are an immortalized cell line developed from a healthy stratified squamous vaginal epithelium [(Fichorova et al., 1997)]. Cell programs in epithelial cells are tissue dependent [(Tseng et al., 1982), (Chatterjee et al., 2019)], and expression patterns in the reproductive epithelium have been shown by our group and others to be differentially regulated by endogenous and experimentally applied sex hormones, such as estrogen and progesterone [(Caine et al., 2019), (Woods et al., 2018), (Gillgrass et al., 2005), (Quispe Calla et al., 2020), (Hughes et al., 2021), (Cotreau et al., 2007), (Ali et al., 2020)]. Thus, GSE135583 by Stanton et al., which investigated filaggrin stimulation of TLR5 in the presence or absence of estrogen pre-treatment, provided a more physiologically relevant opportunity to compare our ZIKV-induced epithelial activation signature to PRR stimulation of human vaginal cells [(Quispe Calla et al., 2020)]. We reanalyzed this data set as above, and found that TLR5 stimulation also lead to a significant upregulation for keratinocyte differentiation, as was reported by the authors. Importantly, estrogen pre-treatment prior to flagellin stimulation resulted in a massive upregulation of keratinocyte differentiation genes that nearly replicated our intravaginal ZIKV infection results (Fig.2g). Taken as a whole, our analyses suggested that accelerated epithelial differentiation in the LRT was not constrained to ZIKV, but rather, a broad,

strategic defense activated upon PAMP sensing in the LRT; importantly, previously published data by our group and others suggested this response could be estrogen dependent.

3.2.6 *ZIKV titers significantly increased in lower and upper reproductive tracts when estrogen present*

Based on the upregulation of differentiation-associated genes seen in our intravaginal infection study - and the detection of similar patterns across multiple PRRs - we hypothesized that ZIKV-induced immune signaling leveraged homeostatic mechanisms of cell turnover to accelerate pathogen expulsion. Because previously published data from our group and others suggested this functionality could be estrogen dependent, we designed an experiment utilizing 6-week-old, ovariectomized (OVX) STAT2-KI mice that received hormone replacement after recovering from OVX surgery (Fig.3a). Hormones were delivered via long-acting injection, with mice that received progesterone alone (PRG) entering a fixed diestrus-like state, while mice that received both estrogen and progesterone in combination (E+P) were fixed in a proestrus-like state (Fig.3c, top row). After 21 days of hormone treatment, mice were intravaginally infected with 10^6 PFU ZIKV Dakar MA and harvested at 5dpi to span both innate and adaptive immune responses.

Contrary to our hypothesis that estrogen would enhance antiviral protections, we observed infectious viral loads in the vaginal tissue that were approximately 2 log higher in mice that received E+P compared to mice receiving PRG alone (focus forming unit (FFU) assay, Fig.3b). Importantly, this elevated viral titer in the vagina was associated with significantly higher viral loads in the cervix and uterus as well. *In situ* hybridization (ISH) targeting ZIKV revealed that detectable virus was constrained to the thin vaginal epithelial layer in vehicle and PRG mice, while mice receiving E+P therapy exhibited considerable viral penetration into the underlying lamina

propria (Fig.3c). Because these findings complicated our understanding of the potential roles of estrogen during intravaginal infection, we sought to refine our understanding of cell-intrinsic and tissue-level responses of the LRT through single-cell analysis.

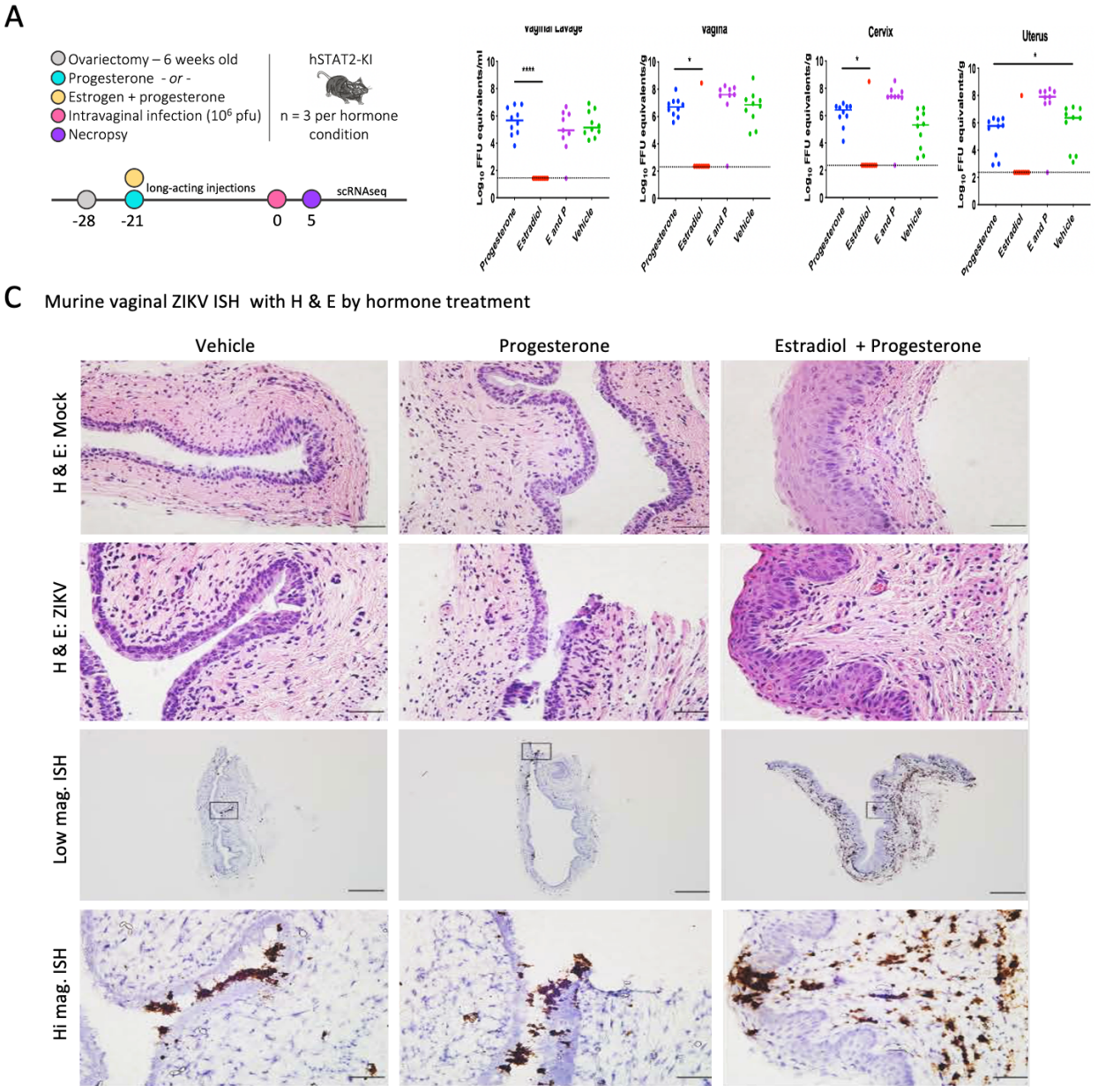

Figure 3.3. ZIKV viral loads and dissemination are increased in the presence of Estrogen. Study design (A). Viral loads in FRT samples across hormone conditions at 5 dpi (B). H&E and ZIKV ISH in the LRT across hormone treatments (C).

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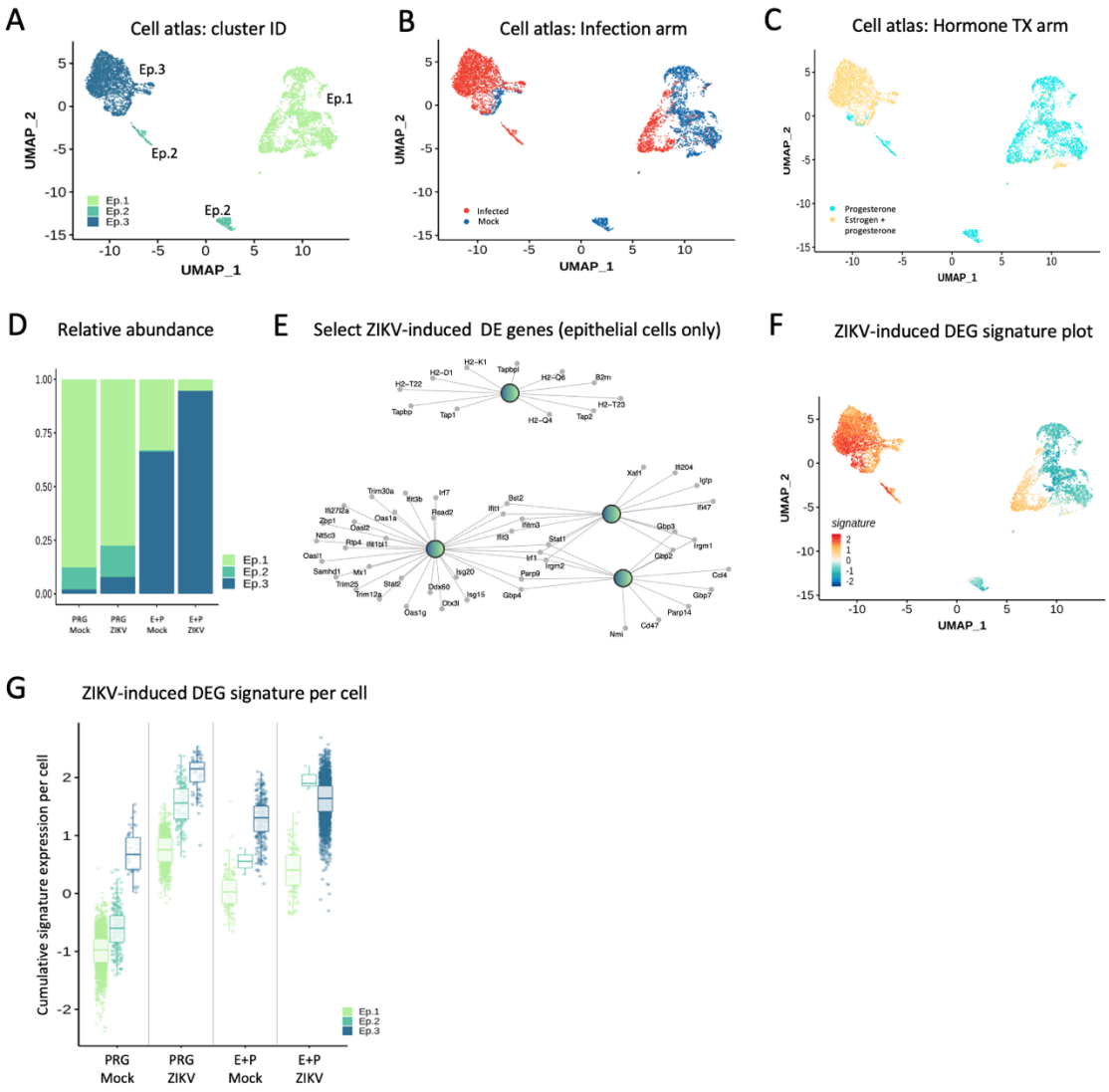

Figure3.4 Estrogen promotes baseline antiviral state in epithelial cells of the LRT. UMAP of epithelial cells by cluster assignment (A), infection status (B), and hormone treatment (C). Relative abundance of epithelial cell clusters across treatment arms (D). ZIKV-induced DE genes in epithelial cells (E). ZIKV-induced gene signature per cell by UMAP (F) and by treatment arm (G).

3.2.7 *Epithelial cells of the LRT display two dominant phenotypes and their proportional abundance depends on estrogen availability*

Using the same model outlined above (Fig.3a), 3 mice for each experimental condition were harvested 5dpi and processed individually; tissues were processed into single cell suspensions and then combined prior to library preparation; thus, the sequenced "samples" for each condition are the pooled results of 3 mice. Data sets for each sample were assessed independently for quality and ambient RNA contamination (see Methods); cells that remained (n = 11,043) were merged across conditions to generate a single-cell transcriptional atlas of the LRT that spanned hormone treatments and infection status.

Because data from our longitudinal study indicated a prominent role for epithelial cells in the acute response to ZIKV, we focused our initial analyses on three clusters with near ubiquitous expression of Krt18/19. Following their *in silico* isolation from non-epithelial cells, we performed additional clustering and began to assess the relative differences between epithelial populations (Fig.4a, see Methods). Preliminary analysis demonstrated that while cells were not clustering by infection status (Fig.4b), hormone treatment appeared to be a primary driver of cluster separation (Fig.4c). Relative abundance analysis confirmed that the epithelium of PRG animals were primarily composed of cells clustering in Ep.1 and Ep.2, while animals receiving E+P were largely composed of cells from Ep.3 (Fig.4d). Marker gene analysis of uninfected epithelial cells demonstrated that cells from Ep.1 were defined by expression of Krt4/15, while cells from Ep.3 were defined by Krt13/23. These findings suggested that cells from Ep.1 and Ep.3 were structurally unique, and because their distribution across treatment arms was uneven, we were motivated to evaluate these clusters for potential functional distinctions as well.

3.2.8 *Epithelial cells exhibit higher baseline antiviral gene expression in presence of estrogen*

Due to the elevated ZIKV titers in mice receiving combined E+P treatment, we hypothesized that estrogen might lead to unanticipated defects in antiviral detection that would foster higher levels of replication. To test if hormones influenced baseline PRR expression in epithelial cells, we performed DEG analysis on uninfected cells and found no significant differences in transcripts for RIG-I-like receptors (RLRs) or TLRs between PRG and E+P-treated mice. We next asked if the level or breadth of antiviral gene induction differed by hormone treatment. We identified a core set of genes (n = 143) that were upregulated following infection in both hormone treatment arms and were highly enriched for antiviral processes; the genes induced by infection mirrored antiviral gene induction observed during the intravaginal infection time course discussed above(Fig.4e). To assess if expression levels of these genes varied by treatment arm or epithelial cluster, we first visualized the antiviral gene signature on a per-cell basis within the epithelial UMAP (Fig.4f, see Methods). This visualization appeared to indicate that the antiviral gene signature was expressed at higher levels in Ep.3; interestingly, it also indicated that baseline expression levels were elevated in uninfected Ep.3 cells. To develop a more intuitive depiction of how antiviral gene expression was shaped by epithelial cluster, hormone treatment, and infection status, we viewed the per cell values as points, grouped by sample type and then split by epithelial cluster (Fig.4g). The results indicated that expression of antiviral genes was higher at baseline in mice receiving E+P. Additionally, we noted that within each group, cells from Ep.3 consistently expressed the highest levels of antiviral genes. When assessed for statistical significance by differential expression analysis, we found that 100% of the shared antiviral response genes were significantly elevated in cluster Ep.3 at baseline, - the dominant epithelial cell type in mice receiving E+P. Cumulatively, the results suggest that known estrogen-induced differences in LRT (i.e., barrier thickness) are

more than structural – and importantly – that the dominant cell type in proestrus stage mice (E+P) constitutively express higher levels of antiviral genes.

3.2.9 *Krt13 epithelial cells upregulate physical and chemical barriers in conjunction with immunomodulatory genes*

Thus far, our data confirmed the expected thickening of the vaginal epithelium during E+P treatment (Fig.3c,top row), and relative abundance analysis demonstrated this tissue was dominated by cells clustering in Ep.3(Fig.4d). Marker gene analysis established that these cells expressed high levels of Krt13, while this marker was virtually absent in cells from Ep.1(Fig.5a). These single-cell data agreed with coexpression patterns in our longitudinal ZIKV study, which showed that Krt13 expression was correlated with late-stage EDC genes (Fig5a). Taken together, these data indicated that E+P mice have a large number of Krt13+ epithelial cells supporting continuous cell turnover at the luminal surface of the vagina. Our analysis of epithelial defenses above also indicated that the cells in this thickened tissue expressed higher levels of antiviral genes than did non-Krt13 cells. These findings broadly agree with data from other epithelia that show squamous tissues upregulate antimicrobial genes during terminal differentiation to reinforce the chemical barrier[(Ali et al., 2020), (McCormick et al., 2010)]. Why then, were animals with a thickened epithelial barrier that was preemptively antiviral *more* susceptible to ZIKV replication and dissemination? As migrating squamous epithelial cells differentiate, they undergo cornification to become metabolically inactive barrier cells, but in order to prevent an inappropriate immune response to this programmed form of cell death, the differentiating cells disassemble organelles and nuclear material in a manner that limits inappropriate immune activation [(Eckhart et al., 2018)]. We hypothesized that homeostatic mechanisms meant to limit inappropriate immune activation could be detrimental during the course of an infection and provide

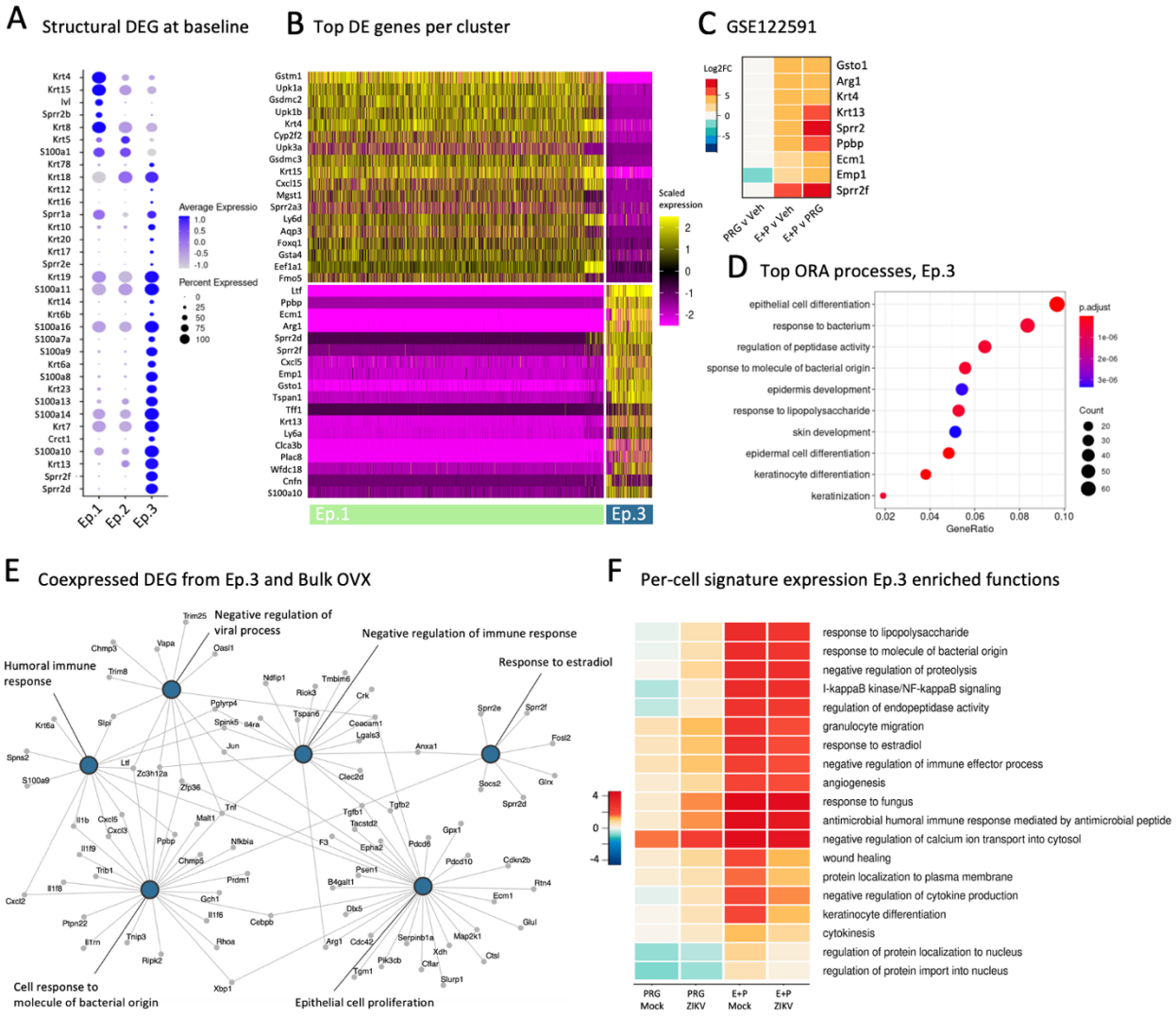

Figure 3.5. Estrogen promotes luminal barrier reinforcement while reducing adaptive immune potential. Differentially expressed keratins and EDC genes across epithelial clusters in uninfected treatment arms (A). Top DEG in epithelial clusters 1 and 3 (B). Expression heatmap of Ep.3 marker genes from bulk sequencing (C). ORA analysis for Ep.3 (D). Shared ORA processes and genes from single-cell Ep.3 and bulk RNAseeq analysis (E). Heatmap of expression levels per cell for specific functions that are enriched in Ep.3 (F).

a favorable environment for ZIKV replication in E+P mice. We thus sought to define programs associated with Krt13+ epithelial cells that could explain the higher viral titers observed in these animals.

To begin, we noted that our data contained an imbalance in the total number of uninfected epithelial cells between treatment arms (PRG, n = 2138; E+P, n = 411), which extended to an imbalance in cluster size(Ep.1, n = 2008;Ep.3, n= 317). To confirm that that our analyses of epithelial cells at the single-cell level were robust to these differences, we incorporated a new analysis of bulkRNAseq data previously published by our group in an identical model (GSE122591) [(Caine et al., 2019)]. To do so, we first identified single-cell marker genes for Ep.1 and Ep.3 (Fig.5b); we then then compared these genes to top DEG from bulk analysis contrasting hormone treatments in uninfected mice. Results indicated that despite the sparsity of single-cell count matrices, the markers genes identified in epithelial cluster Ep.3 were consistent with bulk analysis of uninfected E+P mice (Fig.5c).

We thus transitioned to functional analysis comparing Ep.1 and Ep.3. Our results demonstrated that compositional differences in the epithelial layer of the LRT (Fig.4d) result in dramatically different functional profiles as well. PRG-only mice were dominated by Ep.1 cells, which express high levels of genes associated with ribosomal proteins (Rpl, Rps); the functional enrichments for Ep.1 were dominated by metabolic and biogenesis processes (Fig.Sx). These results were common to both bulk and single-cell data sets, indicating this was not an artefact of the single-cell sample processing or quality control. In contrast, mice receiving E+P were enriched for the response to estradiol, epithelial differentiation, and epithelial proliferation - as was inferred from upregulation of the late-stage EDC genes in the Sprr family (Fig.5a-c,e); this analysis also indicated Krt13+ cells from Ep.3 elevated transcription for programs associated with terminal

differentiation, such as autophagy, antimicrobial defenses, and the regulation of DAMP signaling. Importantly, these cells were also enriched for a set of genes associated with regulation of the immune response (Fig.5e).

When we examined the genes associated with immune modulation, we realized that Krt13+ epithelial cells expressed several genes with the ability to regulate T cell recruitment, activation, and proliferation. Importantly, several of these genes, including Arg1 and transforming growth factor beta 1 and 2 (Tgfb1/2), were also associated with epithelial proliferation. Arg1 is best known for being secreted by M2 polarized macrophages, which can prevent T cell activation and proliferation. We found that Arg1 was a prominent cell marker for Krt13+ cells in our single cell analysis. Arg1 was also coexpressed with keratinization-associated genes from our longitudinal ZIKV infection (Fig.2b) and our re-analysis of data from OVX, hormone-treated animals. Additionally, Arg1 expression was significantly upregulated by topical TLR7 stimulation in mice and *in vitro* stimulation of TLR7 and TLR5 in vaginal epithelial cells. These findings demonstrate a programmed role for Arg1 in terminally differentiating epithelial cells and suggest Krt13 cells may possess immunomodulatory properties. Tgfb1 and Tgfb2 were also prominent markers of Krt13+ cells in our single-cell data, and their coexpression with Krt13 and Sprr2a-i genes was also confirmed in the bulk OVX data set. These molecules are frequently associated with regulatory T cells (Tregs) and are well known inhibitors of effector T cell activation[ref]. We also identified coexpression of Clec2d in Krt13+ epithelial cells, which has been shown in glioblastoma models to bind the inhibitory T cell receptor CD161, leading to decreased cytokine signaling and cytotoxicity by bound T cells. Finally, Zc3h12a codes for an RNase that regulates immune signaling by degrading mRNA for interleukin-6 (Il-6), a molecule shown to promote T cell expansion. Taken together, these findings suggested that as Krt13+ epithelial cells terminally

differentiate, they initiate several major homeostatic programs that include: a) making structural changes to enforce the physical barrier of the vaginal lumen; b) upregulating antimicrobial genes to reinforce the chemical barrier (Slpi, Ltf); c) minimizing DAMP-related immune signaling through the regulation of proteolysis; and d) limiting inappropriate T cell activation. This delicate balance likely evolved to prevent microbial invasion while also preserving fertilization potential. However, given findings from our longitudinal study that supported a need for a cytotoxic T cell response to control intravaginal ZIKV, these findings suggested a potential vulnerability that could explain elevated viral titers in E+P mice.

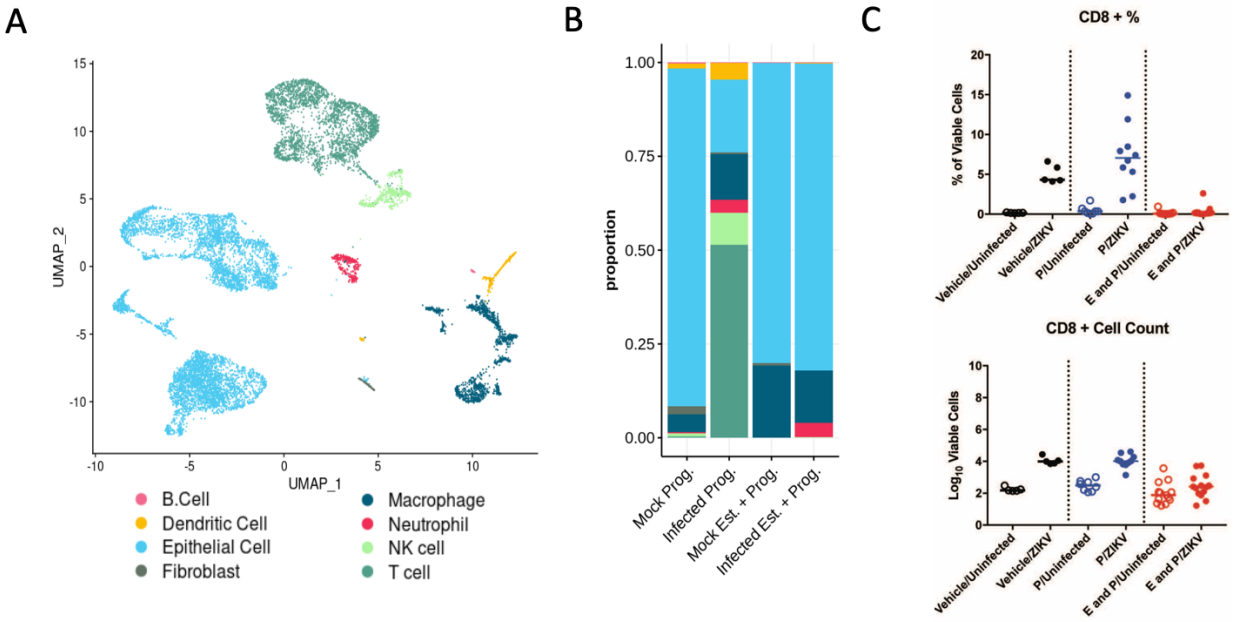

Figure 3.6. T cell response to LRT drastically reduced in presence of estrogen. UMAP of all cells identified by scRNAseq (A). Relative abundance of cell types across treatment arms (B). CD8 as % of viable cells and total viable cells across treatment arms (C).

3.2.10 *T cell response to intravaginal infection is absent at 5dpi in E+P treated mice*

To ask if T cell responses were playing a role in the elevated viral titers observed in E+P mice, we returned to our single cell atlas. In addition to epithelial cells, our conservatively labeled atlas

detected T cells, NK cells, neutrophils, dendritic cells, macrophages, B cells and fibroblasts (Fig.6a). We next calculated the relative abundance of each cell population for all treatment groups (Fig.6b), and found that compared to mock PRG mice, ZIKV-infected PRG mice exhibited a dramatic increase in T cells – both Cd4+ and Cd8+. In sharp contrast, E+P mice showed no evidence for a T cell response to ZIKV infection at 5dpi. The stark nature of these findings prompted additional flow cytometry-based analyses, which recapitulated the nature of our single cell findings; specifically, detection of CD8 T cells were modest in all mock groups, and while PRG mice exhibited a significant increase in CD8 T cells at 5dpi, no increase occurred in E+P mice (Fig.6c). These results appeared to unify our two major findings, suggesting that while epithelial tissues in the LRT can upregulate Krt13+ as part of a proliferation strategy to expel pathogens, tissues dominated by Krt13+ have the capacity to prevent T cell activity, and likely explains the significantly higher viral titers in E+P mice whose LRT were dominated by this cell type.

3.3 DISCUSSION

Our findings suggest that upon pathogen detection, the epithelial layer of the LRT initiates canonical antiviral defenses that are supplemented by accelerated cell turnover at the luminal surface. Yet the data also suggest that an overabundance of these Krt13+ proliferating cells – which are most abundant when estrogen is present – can prevent the T cell responses that are essential to control of ZIKV in the LRT. Thus, this tissue-specific immune response appears to prioritize pathogen expulsion over cell killing. These findings suggest a sensible approach to optimizing fertilization chances: as estrogen levels reach their peak just prior to ovulation, epithelial cells actively prevent T cell accumulation and activation. But they also point to a clear

vulnerability: if infection occurs when estrogen levels are high – and pathogen expulsion is incomplete – then the virus may replicate to high titers locally and increase chances of systemic infection. This notion also invites speculation that infections initiated during pregnancy may encounter a more favorable landscape for replication and spread, as estrogen and progesterone levels increase steadily throughout gestation [(Tulchinsky et al., 1972)].

Beyond ZIKV, the modulation of adaptive immunity by epithelial cells could have broad implications. An established body of literature has investigated the impact of sex hormones on immune responses in the reproductive tract. Despite the 2019 revision of risks associated with progesterone-based birth controls by WHO, findings from clinical cohorts, animal models and *in vitro* experiments tend to converge on an increased susceptibility to STIs when progesterone is elevated [(Caine et al., 2019), (Woods et al., 2018), (Gillgrass et al., 2005), (Quispe Calla et al., 2020), (Hughes et al., 2021), (Cotreau et al., 2007), (Ali et al., 2020), (Edfeldt et al., 2020)]. Depot medroxyprogesterone acetate (DMPA) is a long-acting form of progesterone-based birth control that is most widely utilized in settings where health-resources are limited and HIV incidence is high [(Edfeldt et al., 2020)]. While clinical studies cite higher inflammation rates, increased relative abundance of T cells, and a sub-optimal microbiome as risk correlates of DMPA use, the mechanisms responsible for increased HIV acquisition are not well defined [(Vitali et al., 2017)]. Because DMPA can reduce both estrogen levels and expression of the estrogen receptor, we hypothesize its use could prevent the natural barrier to T cell infiltration that we have observed in E+P treated mice and estrogen-treated vaginal epithelial cells.

Krt13 gene expression levels have also been found to be elevated in solid tumors [(Gouin et al., 2021)]. Of special interest, a recent paper examining bladder cancer noted that Krt13 gene expression was significantly elevated in so-called “cold” tumors, which are tumors that go

relatively unnoticed by typical immune surveillance [(Gouin et al., 2021)]. Importantly, the authors utilized spatial transcriptomics to demonstrate that neighborhoods with high levels of Krt13 expression were significantly associated with an absence of T cells – a finding the authors termed an "immune desert". We examined the differentially expressed genes identified in their cold tumor samples and found that Arg1, Tgfb1, Tgfb2, and Zc3h12b were all significantly elevated along with Krt13. Whether or not our hypothesized mechanism for T cell suppression by Krt13+ epithelial cells can be supported by future studies, the phenomenon of T cell modulation by Krt13+ epithelial cells appears to transcend species, tissues, and disease states.

To return to the FRT, increasing attention is being paid to the development of mucosally applied vaccines, which have the benefit of recruiting immune cells to the site of potential pathogen encounter. Because safety and efficacy trials will be first conducted in small animal models, it is essential that the results account for estrous stage. Trials conducted in DMPA treated animals – which would likely be used to ensure productive infection – could provide misleading evidence regarding efficacy in mechanism due to the over-abundance of T cells. Alternatively – and more hopefully - mucosally applied vaccines that rely on a robust T cell response could influence the tissue composition ahead of vaccine delivery; through the short term use progesterone - or agents that block T cell inhibiting molecules such as Arg1, Tgfb1, and Tgfb2 – the efficacy of the vaccine may be increased.

Finally, our findings cast the dogma of DMPA use prior to intravaginal infection in a new light. While the vaginal epithelium is made susceptible to ZIKV and HSV2 by DMPA pre-treatment, several studies have reported that a diestrous-like state induced by DMPA was *essential* to productive infection. Importantly, these conclusions were made in comparison to estrogen-only treatment, which induces an estrus-like state that includes development of a thick, keratinized

surface layer in mice, rendering the tissue non-susceptible. In contrast, we find that a combination of estrogen and progesterone prior to infection remodels the vaginal epithelium into a proestrus-like state that more closely replicates the epithelial barrier of the human vagina. And as reported above, this pre-treatment resulted in an increased infection burden, both locally and at disseminated sites. Future studies of intravaginal infection will continue to rely on the mouse model. We believe our findings indicate a need to balance these studies of intravaginal infection by challenging independent cohorts of mice in both diestrus and proestrus.

While the ZIKV epidemic waned quickly, its initially explosive spread and devastating sequelae serve as a warning for its eventual recurrence – and the emergence of future, novel STIs with teratogenic capability. Our studies suggest that hormonally regulated epithelial cells can dramatically influence the immune response to ZIKV and determine the severity infection. Importantly, these findings shed light the reproductive tract as a fertile niche for ZIKV replication and persistence. The mechanisms that allow Krt13+ epithelial cells to regulate immune responses to infection warrant further consideration, and future studies must also consider how these findings pertain to hormone-based contraceptives, HIV, and pregnancy. Because infections of the vaginal mucosa induce tissue-specific responses involving cell turnover, the use of spatial technologies to resolve the kinetics of these responses will provide meaningful insights into the central mechanisms and consequences of epithelial activation.

Chapter 4. DISCUSSION

4.1 THE MICROBIOME

4.1.1 *Toward a better understanding of the suboptimal microbiome*

Despite the growing awareness amongst researchers and providers that a suboptimal vaginal microbiota increases the risk of STI and a number of secondary conditions, the clinical management of BV has stagnated; recurrence rates remain above 60 - 70% within 12 months of initial treatment [(Petrina et al., 2017)]. CDC guidelines recommend metronidazole as a front-line drug, administered as an oral or topical preparation. Metronidazole targets anaerobic taxa that reduce the pro-drug to its active state, which promotes DNA damage that ultimately kills the bacterium [(Weir et al., 2021)]; this mechanism of action largely spares beneficial Lactobacillus. While metronidazole treatment has been shown to reduce total bacterial load, problematic BVAB may be shielded within biofilms, by entering a quiescent state during treatment, or moving into host cells. While these examples suggest mechanisms for BVAB persistence following treatment, a cohesive mechanism for BV recurrence remains unknown.

Clinical studies have demonstrated that antibiotic therapies utilizing metronidazole work quickly – but often briefly. In a study focused on the most common BVAB, qPCR assays determined that although higher bacterial loads slowed clearance, suppression of problematic taxa still typically occurred in less than 4 days [(Mayer et al., 2015)]. G. vaginalis and A. vaginae were shown to have the highest rates of return following treatment cessation, and these findings are in line with studies demonstrating a high degree of correlation between these taxa, especially when associated with biofilms [(Castro et al., 202020)]. Due to high rates of relapse, the degree to which FRT bacterial isolates are susceptible to metronidazole and the second-line treatment, clindamycin,

have been investigated extensively. In one large study evaluating over 700 clinical isolates, key anaerobic gram-negative rods and anaerobic gram- positive cocci were largely susceptible to metronidazole, while clindamycin resistance was observed in 20-38% of isolates [(Petrina et al., 2017)]. Conversely, anaerobic gram-positive and facultative gram-variable rods were generally susceptible to clindamycin, while nearly half demonstrated metronidazole resistance. Importantly, while Lactobacillus isolates are commonly viewed as metronidazole resistant due to its mechanism of action, they are broadly susceptible to clindamycin. Despite the ability to target the majority of problematic taxa through combination therapies, BV recurrence is common. A major question then, is whether recurrence following antibiotic treatment is due to antimicrobial resistance (AMR) itself, or a function of the vaginal ecology. Importantly, an unbiased, comprehensive search for antimicrobial resistance elements has not been published to date. While shotgun metagenomic approaches are poised to address this question, sequencing of samples from the FRT suffers from a heavy burden of host associated reads, which necessitates increased sequencing depth that is both monetarily and computationally expensive. Due to the intimate connection between host and microbiota at mucosal surfaces, RNAseq approaches may offer an avenue for exploring the question of AMR genes while also capturing high quality information about the host response [(Bullman et al., 2017)]. Importantly, transcriptional assessment of host-associated microbiota can provide a "real time" snapshot of active bacteria, while 16S rRNA gene analysis includes information of organisms that may or may not be alive; this is especially relevant to studies aimed at assessing the elimination of taxa via antibiotics and the uptake of probiotic species. An illustration of these competing measurements was observed in a study that utilized both 16S rRNA gene analysis and metatranscriptomics of the vaginal microbiota during metronidazole treatment, finding that while G. vaginalis accounted for only 5% of the 16S rRNA gene reads, they accounted

for nearly 47% of all transcriptional reads [(Deng et al., 2018)]. Importantly, this approach also enabled the detection of upregulated Cas gene expression in G. vaginalis among patients refractory to treatment; the authors suggest Cas gene function can include DNA repair, which could be an important survival mechanism under pressure from DNA-damaging agents such as metronidazole. In a separate analysis, these data were subsequently analyzed in the context of a more comprehensive G. vaginalis taxonomy that defined between 8 and 14 distinct Gardnerella genomospecies [(Potter et al., 2019)]. As in the original transcriptomics work, however, the authors found no association between specific genomospecies and metronidazole resistance. The broad persistence of Gardnerella and other BVAB despite antibiotic treatment has encouraged several groups to think outside of cell intrinsic AMR and turn their attention toward ecological mechanisms of collective resistance.

Biofilm formation by Gardnerella and others is now widely viewed as a central mechanism of BVAB resilience. Interestingly, while the Amsel criteria established in 1983 that "clue cells" (vaginal epithelial cells with adherent gram-negative rods) were a major feature of BV, the notion that biofilms were at work was not clearly articulated until 2005, when Swidsinski et al. utilized fluorescence in situ hybridization to prove that non-planktonic G. vaginalis were adhered to the vaginal epithelium through the production of biofilms [(Swidsinski et al., 2005)]. Biofilms are complex extracellular habitats that are produced through the collective efforts of a homogenous community or a polymicrobial assemblage. As the biofilm ecosystem matures, niche space can become divided as nutrient, pH, and oxygen gradients develop [(Hardy et al., 2017)], and importantly, increased diversity in an ecological space is typically thought to increase resistance to environmental stressors for its inhabitants [(Baert et al., 2016), (Pennekamp et al., 2018)]. Bet hedging is one mechanism for increasing tolerance to stress within a community, and indeed,

distinct patterns of gene expression have been observed across genetically identical populations within biofilms [(Hardy et al., 2017)]. Biofilm structures offer an important barrier against host-derived antimicrobial compounds and immune surveillance. This enables resident bacteria to slow metabolism during suboptimal growth conditions without a need for active host evasion; importantly, this ability to hibernate also confers protection during antibiotic therapy. In this way, antibiotic treatment likely selects for biofilm-associated taxa, and suggests that Gardnerella may thus serve as a keystone genus in the etiology of BV. In this role, Gardnerella may stand to benefit as well, as co-habitation with specific BVAB has been shown to upregulate virulence factors associated with attachment and nutrient retrieval [(Castro et al., 2019)].

Other mechanisms of collective resistance to antibiotics have become evident in recent years. The Klatt group has demonstrated that in addition to the metabolization of key PrEP drugs [(Cheu et al., 2020)], the vaginal microbiota are capable of sequestering antibiotics in non-intuitive fashions. Specifically, we observed through co-culture with G. vaginalis that higher levels of Lactobacillus iners (the one problematic Lactobacillus) reduces environmentally available metronidazole, ultimately enhancing G. vaginalis growth [(Lee et al., 2020)]. ODE modeling suggests that Lactobacillus sequesters metronidazole, acting as a sink for the antibiotic; this leads to the paradoxical prediction that higher levels of G. vaginalis actually facilitates metronidazole action against it. Subsequent analyses of 16S rRNA gene data sets from clinical cohorts confirmed that higher pretreatment ratios of L. iners to BVAB was associated with significantly higher rates of recurrence [(Lee et al., 2020)]. These findings may then explain an earlier study examining the role of total bacterial burden on treatment failure, which found that women with sustained recovery of Lactobacillus dominance actually possessed significantly more G. vaginalis at treatment initiation [28792372].

4.1.2 *BV beyond antibiotics*

In order to circumvent the impediments associated with antibiotic treatment, strategies are emerging to specifically target problematic taxa by leveraging information in their genomes. Bacteriophages are viruses that specifically infect bacteria, and their use against bacterial infections predates the antibiotic era. Bacteriophage typically possess a narrow host range, meaning that their clinical application leads to targeted lysis that spares the community at large. In many encounters, however, environmental cues trigger the "phage" to insert their genome into the host bacterium, becoming a prophage. And while no lytic phage against *Gardnerella* have been cultivated in the lab or identified environmentally, prophage sequences have been observed through genome sequencing [(Malki et al., 2016), (Landlinger et al., 2021)]. In some cases, these prophage elements contain genes encoding lethal proteins, which can be engineered into therapeutics. In one such example, a recent study identified and improved upon a prophage endolysin, which is an enzyme expressed late in bacteriophage infection to facilitate bacterial cell wall lysis and subsequent release [(Malki et al., 2016)]. In this study, an engineered form of the endolysin possessed 10-fold greater activity against *G. vaginalis*, resulting in low inhibitory concentrations. Importantly, this "enzobiotic" was also capable of dissolving biofilms recovered from women experiencing BV. Recent research has also turned to alternative host-associated tissues to find novel compounds to fight BVAB. As mentioned briefly above, *L. iners* is unlike most other *Lactobacillus* in that it is associated with progression to BV. In order to target *L. iners* without collateral damage to the beneficial *Lactobacilli*, research in the human gut identified a *Lactobacillus* whose bacteriocins inhibited 100% of *L. iners* while inhibiting essential *Lactobacillus* less than 20% [(Nilsen et al., 2020)]. The study's authors note that the safe elimination of *L. iners* without the use of standard antibiotic therapy could enable simultaneous

supplementation of beneficial *Lactobacillus* and improve chances of colonization by increasing niche space.

This notion of increasing beneficial taxa in order to suppress BVAB has been investigated in recent years from the perspective of both oral supplementation as well as vaginal administration. We have shown that daily oral probiotic consumption can improve mucosal immune health in the GI tract of non-human primates [(Manuzak et al., 2016)], but currently, the ability for oral probiotics to modify vaginal health are still unclear. A primary reason for this uncertainty is the lack of consensus around primary endpoints. One randomized, double-blinded trial examined the ability of *Lactobacillus* strains to reduce symptoms associated with BV, and found a significant reduction in odors, discharge and itching, but this study did not demonstrate a reduction in BVAB by molecular assessment, making interpretation of the results difficult. Other attempts to optimize the vaginal microbiome through oral intake have been investigated following metronidazole therapy. In another randomized, double-blind clinical trial, women experiencing BV were provided standard metronidazole therapy for 7 days in conjunction with a *Lactobacillus*-fortified yogurt and compared to a placebo acidified milk product [(Laue et al., 2018)]. Results of the study indicated a reduction in qualities associated with a BVAB-dominant microbiota, including reduced Amsel score, discharge and odor; during the 4 week intervention Nugent score also decreased significantly in the probiotic arm, suggesting a change in microbial composition. The primary endpoint - a reduction in BV related symptoms - was only observed after 4 weeks, limiting conclusions regarding concurrence. While the significant reductions in symptoms in the treatment arm suggest oral supplementation may improve upon standard antibiotic therapy, this study also did not address molecular BV by qPCR or 16S rRNA gene analysis, meaning that underlying causes of BV cannot be assessed here.

Biotherapeutic strategies have also begun to enlist the help of beneficial yeast in the context of antibiotic therapies and concurrent bacterial supplementation. *S. cerevisiae,* a yeast, has been shown to limit the adherence of *G. vaginalis* to epithelial cells in a murine model [(Sabbatini et al., 2018)], and when applied in combination with *Lactobacillus rhamnosis,* dissolved preformed *G. vaginalis* biofilms, while also reducing total *G. vaginalis* burden [(Sabbatini et al., 2020)]. While encouraging, the application of this therapy will likely need to be direct application to the vaginal surface and require further study in placebo controlled human trials for safety and efficacy.

Topical supplementation of the vaginal microbiota with beneficial organisms through inserted capsules provides a more direct and mechanistically intuitive form of intervention, and several trials have begun to study their efficacy in the context of BV. One early biotherapeutic contained a strain of vaginal Lactobacillus jensenii that was genetically modified to express an HIV-1 entry inhibitor; trials were conducted in Rhesus macaques [(Lagenaur et al., 2015)]. Single-dose therapies resulted in colonization in 67% of macaques 14 days post-treatment, and 83% at 21 days. That the *Lactobacillus* could be sustained in the rhesus model is impressive given the high degree of keratinization present in the macaque lower reproductive tract. More recently, several human trials have explored the use of vaginally-delivered probiotics in humans. One such study of non-pregnant Rwandan BV patients followed metronidazole treatment with either behavioral counseling or courses of off the shelf vaginal probiotics [(van de Wijgert et al., 2020)]. While one supplement resulted in significantly higher levels of *Lactobacillus* and a corresponding reduction in BVAB, results demonstrated these results were not durable following the cessation of treatment [(Russo et al., 2019)]. Lactin-V is a *Lactobacillus crispatus biotherapeutic applied by prefilled applicator*. Encouragingly, this strain was still detected in 79%

of patients at week 12, and the risk ratio for recurrence among treated patients was 0.66 and 0.73 compared to placebo at weeks 12 and 24, respectively. Along with the clinical isolation of strong *Lactobacillus* candidates, methods to engineer taxa to support HIV prevention efforts are also ongoing. And in another prong of the approaches currently in development, recombinant *Lactobacillus plantarum* were recently generated that are capable of expressing an algal lectin scytovirin originally identified in a cyanobacterium [(Janahi et al., 2018)]. Measurements of culture supernatants from these modified organisms were shown to bind HIV-1 gp160, effectively reducing observed cytopathic effects in cell culture as low as 56.7%. While the use these bacteria are untested in humans to date, they, along with biofilm-busting agents and antibiotic therapy, may point toward near-term therapies that provide lasting solutions to BV's resilient phenotype. Finally, our group has noted that BV recurrence following metronidazole treatment may actually increase the diversity of the vaginal microbiota, predisposing patients to future failures[Gustin et al, 2021]. One avenue meant to address previously uncurable BV - and provide hope for women experiencing it - was demonstrated in a first of its kind study evaluating vaginal microbiome transplantation (VMT) in 5 recipients [(Myhrer et al., 1992)]. Four of the five women in this study demonstrated successful, long-term shifts to a *Lactobacillus*-dominated microbiota, although repeat transplants were needed in some women. While the scalability of this approach is not currently feasible for addressing the tremendous needs of women experiencing BV around the globe, VMT may provide insights into the components required to overcome chronic, recurrent BV. Information gleaned from downstream analyses of successes and failures in VMT studies should point toward the necessary mechanisms.

4.2 Modeling Host-Microbe Interactions in FRT

4.2.1 Antibiotic-treatment models of microbiome disruption

Work in murine models suggests that depletion of the gut microbiome through broad-spectrum antibiotics leads to chromatin remodeling in the gastrointestinal epithelium, which restricts access to promoters of interferon (IFN) stimulated response elements and limits induction of IFN stimulated genes [(Ganal et al., 2012)]. Other groups utilizing this approach have shown that the loss of systemic bacteria reduces the antiviral activity of macrophages, impairs antigen presenting cell (APC) migration, and limits CD8+ activation [(Abt et al., 2012), (Ganal et al., 2012), (Ichinohe et al., 2011), (Kennedy et al., 2018)]. While these studies highlight the integral role microbes play in immune homeostasis and activation, they all use a broad-spectrum oral antibiotic approach. Because the findings appear to demonstrate global shifts in immune activation and host metabolism, it seems reasonable that these changes would confound any assessment of host-microbiota impact on infection dynamics in the FRT. Nevertheless, the model is widely accepted, and we sought to establish it as a basis for understanding the impact of antibiotic treatment in a mouse model.

Our initial studies utilized an established model of broad-spectrum oral antibiotic treatment. In this model, mice receive the VANM cocktail: vancomycin (0.35g/L), ampicillin(1.0g/L), neomycin(1.0g/L), and metronidazole(1.0g/L) in drinking water that is sweetened to enhance palatability. This cocktail is also used to soak food pellets. During the study, systemic treatment led to early, significant weight loss compared to mock-treated mice, with some mice losing as much as 30% of their starting weight through 10 days of treatment. As weight stabilized between days 6 – 8, mice presented with GI distention sufficient to alter their gait, and

one mouse was euthanized at day 7 after exhibiting labored breathing. Upon necropsy of VANM treated mice, we noted significant cecal distention that displaced thoracic organs caudally, suggesting a mechanism for impaired breathing observed in one mouse. Systemically treated mice also experienced gallbladder enlargement, near total loss of fat pads, and most surprisingly, reduction in ovary size. VANM antibiotics are sufficient to interrupt microbial metabolism in the GI tract [(Kennedy et al., 2018)], and this disruption is thought to impair breakdown of insoluble dietary fiber, which then accumulates in the cecum, leading to an increase in osmotic absorption of water in the GI tract. So while these findings were not entirely unanticipated, their global impact and severity led us to conclude that a systemic antibiotic treatment model precludes meaningful assessment of host-microbe interactions at a local level, specifically the FRT.

Published reports have demonstrated that systemic VANM antibiotic treatment disrupts gut microbiome composition and correlates with enhanced severity of multiple subcutaneous flavivirus infections [(Thackray et al., 2018)]. Because of our observation that VANM treatment severely alters murine homeostasis, we believe the model confounds studies that aim to directly link host-microbiota interactions to altered innate immunity in the female reproductive tract. To address this deficit, we developed a novel murine model of local microbiome disruption, which employs daily application of clinically available antibiotic creams provided at human-equivalent doses; (clindamycin 0.28mg, metronidazole 0.04mg). In two independent experiments, mice receiving this treatment appeared ruffled on day 3 of treatment but responded well throughout the rest of treatment. Importantly, these mice experienced subtle weight gain in line with mock-treated animals, and upon necropsy, demonstrated none of the tissue dysfunction associated with systemic antibiotic treatment.

We conducted a small pilot study to assess the impact of microbiome disruption on ZIKV burden at 5 days post-infection. Following 10 days of antibiotic treatment, 6-8-week-old virgin mice were intravaginally infected with $1x10^6$ PFU ZIKV Dakar MA. Surprisingly, measurements of vRNA in the lower and upper reproductive tracts were consistent across treatments, but importantly, we observed significant elevations of vRNA at disseminated sites in both local and systemic antibiotic treatment cohorts. These results suggested the local antibiotic-mediated disruption was sufficient to increase ZIKV dissemination following intravaginal infection. While in-depth analysis of ZIKV-induced immune responses eventually took precedent, the results of this pilot study warrant future studies.

4.2.2 DMPA-treated infection models

The single-cell data presented in Chapter 3 demonstrates that the opposed states of diestrus and proestrus result in dramatically different responses to intravaginal infection, and these findings raise questions about the primacy of the DMPA model of intravaginal infection. So why did DMPA become the standard model for intravaginal viral infections? The story in begins in 1984 with the development of an attenuated strain of HSV-2, which enabled extended study of intravaginal infection in mice (wild-type HSV-2 is rapidly lethal in mice) [(McDermott et al., 1984)]. This model was widely adopted and used in a 1990 study that examined infection efficiency across the estrous cycle, and infection rates for proestrus (33%), estrus (16%), and metestrus (9%) were relatively low compared to diestrus-staged mice (75%) [(Teepe et al., 1990)]. A 1994 subsequently found that DMPA induced a durable diestrus-like state that lasted up to 4 weeks; importantly, the authors found that because DMPA treatment synchronized the estrous cycles of mice, entire cohorts were easily infectable – all without the need for manual staging of cycles by visual inspection and microscopy [(Parr et al., 1994)]. DMPA models were

subsequently compared to exogenous progesterone and estrogen therapies in OVX mice; the study revealed that DMPA-treated mice were 10x more infectable than progesterone-treated mice [(Kaushic et al., 2003)]. How does this relate to intravaginal ZIKV infection? As discussed above (1.4.5), the first model of intravaginal ZIKV infection found that while immunocompromised mice in an induced-diestrus were far more susceptible than induced-estrus mice. Subsequent studies appear to have taken these data into account, and along with the history of HSV-2 infection models, adopted DMPA pre-treatment as the standard for intravaginal infection models.

This is fascinating in a way, because as detailed above, the mouse vaginal epithelium in diestrus most closely resembles that of a post-menopausal woman, when dramatic reductions in estrogen thin the vaginal epithelium to just a few layers. Meanwhile, the lower FRT in proestrus-stage mice closely resembles reproductive-aged vaginal epithelium in humans, and mice are infectable at this stage. While its easily understood that an overt, highly dependable infection was a research priority during the early days of the ZIKV epidemic, it is time to reconsider DMPA-treatment as the only option for intravaginal infection. Our data presented in Chapter 3 displays a dramatic divergence in phenotype in mice induced to diestrus (via progesterone in OVX mice) versus those in proestrus (via estrogen + progesterone in OVX mice). Importantly, at this time it is very difficult to say which stage most closely recapitulates the immunology of the human FRT. While the visual similarities between proestrus mice and humans are obvious, we do not have the appropriate transcriptional data sets to make a determination at this time; given the importance of physiologically relevant models, such studies should be pursued aggressively.

4.2.3 *Organotypic* infection models

From a systems biology perspective, the ability to model infection in animals is an exquisite privilege that provides a depth of information that is simply not accessible using *in vitro* models.

That said, tissue culture models are an essential platform for refining hypotheses developed using animal models. In order to ensure that the information collected from *in vitro* infections has a fair degree of translational potential, tissue models should emulate the tissues most likely to face infection. In the case of intravaginal ZIKV infection then, vaginal epithelial cells are an excellent starting point.

The VK2 vaginal epithelial cell line was established in 1996 using healthy vaginal tissue that was subsequently purified for epithelial cells and transformed using HPV E6/E7. The long-lived cell line was faithful to protein expression of vaginal epithelial cells *in vivo* (to the extent that the authors looked), and the line has become the most widely used model of vaginal epithelial cells *in vitro*. Because the vaginal epithelium is a stratified tissue, pioneering labs have taken cues from respiratory labs to develop air-liquid interface (ALI) models of tissue culture. The fundamental principal behind this technique is that air stress induces the VK2 cells to form layers, which after the course of 10 days take on a stratified, tissue-like appearance.

We were able to recapitulate these findings using the VK2 cell line – and thanks to a generous gift from the Hladik Lab at University of Washington – we were able to compare their structure to tissues comprised of primary vaginal human vaginal epithelial cells (PVEC) grown via the same ALI protocol. The results were rather striking; while both the VK2 and PVEC showed evidence of cell-stacking by light and immunofluorescent microscopy, only the PVEC ALI appeared to differentiate. More specifically, VK2 grown at ALI formed spires, and cells at the peaks of these towers appeared round and consistently contained nuclei when viewed by immunofluorescence. PVEC grown at ALI, however, developed uniformly thick layers with flattened, anucleated squames at the surface. While we have not optimized the staining protocol for immunofluorescence, the drastically different appearances suggest that while VK2 do stack up

under ALI conditions, they do not fully differentiate; PVEC, however, appear to differentiate completely. Given our current hypotheses regarding the role of homeostatic cell turnover in determining immune responses of stratified epithelial layers, validation of PVEC differentiation will mark a significant stride toward a physiologically relevant *in vitro* model. Subsequent steps should include the incorporation of stromal cells, as graft experiments in mice have demonstrated that estrogen action on stromal cells is essential for appropriate epithelial differentiation [(Buchanan et al., 1998)].

4.3 Lessons Learned

4.3.1 *The microbiome*

The microbiome work presented above represents a small fraction of studies that we conducted or collaborated on over the last 5 years. Because we have always had in-house sequencing capacity, we had the opportunity to work on a diverse range of tissues and sample types, and across infectious disease and chronic illness. A few themes developed over that time that poised a project to produce meaningful data. Perhaps one of the most crucial oversights in projects that were not easily interpretable was the lack of baseline sampling prior to infection or treatment. For example, if one is interested in how an infection impacts the microbiome, then the natural variance within the system – and the technical variation associated with sequencing – must be quantified. A related point is that the sample sizes must be calculated by a power analysis prior to the experiment. While one might think these points go without saying, in a field where animals are expensive to purchase and maintain, it can be tempting to limit the observation period and/or total number of animals. But 5 years of experience have demonstrated that alterations in the microbiome following an experimental perturbation, like infection for example, tend to be subtle; maximizing the number

of sample points – before and after the perturbation - greatly extends the ability to make conclusions. Outside of appropriate baseline sampling and sample size, the most fundamental aspect of a sequencing project is absolutely the collection of detailed metadata. Analysis of a large microbiome project with a limited number of ancillary qualitative and quantitative measurements places all the eggs in one basket, as it were. Understanding just these few basic points make it much easier to design useful microbiome studies, and it also facilitates setting expectations for collaborators who may not be familiar with microbiome analysis.

4.3.2 *Public data*

The Gene Expression Omnibus (GEO), which collects sequencing data from transcriptional experiments, is an absolute embarrassment of riches; unfortunately, its bounty remains unknown and out of reach for most research labs due to the technical hurdles for accessing it. A tool within GEO (GEO2R) attempts to make basic sample assessment accessible and rapid by providing a graphical user interface (GUI) that accepts inputs from a user that are subsequently executed by R in the background. For a systems biologist, however, the data contained in the GEO is astounding. Several revelations discussed in Chapter 3 were due to the ability to test a hypothesis regarding the breadth of epithelial activation in response to PRR stimulation; the convergence of public data sets with our personal observations made the findings much easier to believe and interpret.

As a personal observation, it seems that much of scientific research today aims to identify phenomena that are highly reproducible, but only under tightly controlled conditions. While the merits and translational achievements of this approach are not debated, these successes do not inform us about all that we are *not* seeing by the intentional removal of heterogeneity. In other words, we don't know what we're missing. Public data is by its nature the work product of different labs, different hands, environments, plans, and mistakes. Rather than being a liability,

this is to me its greatest strength. Agreement between data sets under these "messy" circumstances provoke a great deal of confidence that the results are describing a robust phenomenon. In order to leverage the potential for heterogenous validation like this, the powers that be must fund transformative technology development to revolutionize the way this priceless public good is stored, accessed, and utilized. Because in my experience, one well-designed sequencing project contains thousands of potential experiments.

Chapter 5. MATERIALS AND METHODS

Human and nonhuman experimentation: enrollment and sampling (Chapter 2)

The longitudinal, open-label study was approved by the Chesapeake Institutional Review Board (IRB) (Pro #00006122) with a waiver of oversight from the Eastern Virginia Medical School and was registered in ClinicalTrials.gov (#NCT01347632). Thirty-three women seeking treatment for symptomatic vaginal discharge with or without atypical vaginal odor were enrolled upon Nugent scoring of 4 or higher; Amsel criteria were not utilized in this study. All participants were non-pregnant, free of reproductive tract infections, and denied use of antibiotics within the last 14 days. Upon enrollment, vaginal swabs, cervico-vaginal tissue biopsies and cervicovaginal lavage (CVL) were collected. Following collection of biological samples, all women were prescribed fourteen 500mg metronidazole tablets, with one tablet to be taken twice daily; patient adherence was self-reported. Biological samples were collected again between 7 and 10 days following the conclusion of treatment (visit 2), and again 28-32 days post-treatment (visit 3). Biological samples included vaginal pH testing, and CVL was collected through administration of 10cc of normal saline. Three full thickness cervico-vaginal biopsies were obtained under topical anesthesia at the conclusion of CVL collection.

Soluble factor analysis (Chapter 2)

CVL SLPI was measured by ELISA (R&D Systems, Minneapolis, MN) using a victor2 reader (Perkin Elmer Life Sciences, Boston, MA). Interleukin (IL)-1b, IL-6, IL-8, IL- 10, ICAM-1, and tumor necrosis factor alpha were measured in undiluted CVLs by a multiplex electro-chemiluminescence assay (Meso Scale Discovery, Gaithersburg, MD). All measurements were performed in duplicate.

DNA extraction (Chapter 2)

Bacteria from cervicovaginal lavage samples were pelleted by centrifugation and resuspended in 200µl of lysis buffer (30mM Tris-HCl, 10mM EDTA, 200mM sucrose, pH 8.2). Samples were heated at 65°C for 10 minutes prior to the addition of 100mg/ml lysozyme solution to a final concentration of 10mg/ml. Samples were then incubated for 1 hour at 37°C. 5% SDS was then added to a final concentration of 1% w/v and incubated at 56°C for 10 minutes. DNA extractions were then performed using Qiagen's DNeasy Blood and Tissue Kit.

16S rRNA sequencing (Chapter 2)

Extracted DNA was amplified following the Earth Microbiome Protocol for 16S Illumina sequencing utilizing 515F-806R primers originally described by Caporaso et al. [34] to target the V3-V4 region of the 16S SSU rRNA. This broadly-applicable – and widely utilized - primer set was chosen to maximize the comparability of this data set with existing and future marker gene sequencing of the vaginal microbiome. Amplicon concentrations were normalized, pooled, and cleaned prior to KAPA quantification. The pooled library was sequenced using a 2x150 bp Illumina MiSeq run.

Metataxonomic analyses (Chapter 2)

16S rRNA amplicon sequencing reads were demultiplexed in Illumina Basespace. A demultiplexed read object was generated in qiime2-2018.2. Amplicon Sequence Variants (ASVs) were generated using the dada2 denoise-single with 0 trim-left and trunc at 145. Phylogeny was determined using mafft-fastree option and ASVs were assigned taxonomy using the 99% Greengenes 515-806 classifier. Qiime2 objects were exported for downstream analysis in R. All fastq files were deposited in SRA under ascension PRJNA691964.

Viruses (Chapter 3)

A mouse- adapted variant of Dakar 41525 was generously provided by the Diamond Lab at Washington University in St. Louis; the virus has been described previously (Gormann et al., 2018).

Mouse studies (Chapter 3)

For experiments performed at the University of Washington, Seattle, we utilized six-week-old hSTAT2-KI, originally a gift from the Diamond Lab at Washington University in St. Louis; mice were bred in a pathogen-free facility at the University of Washington; the mice have been previously described (Gormann et al., 2018).

For experiments performed at Washington University, six-week-old hSTAT2-KI mice were bred in a pathogen-free facility at the Washington University School of Medicine. Mice were ovariectomized as described in Caine et al., 2019.

Hormone treatment (Chapter 3)

For mice in the longitudinal ZIKV infection performed at University of Washington, Seattle, mice were injected subcutaneously with 2mg medroxyprogesterone in 100ul saline.

For mice utilized in single cell/flow cytometry, which were given estrogen, progesterone, or a combination, the methods were outlined in Caine et al., 2019.

Intravaginal infection (Chapter 3)

For the longitudinal infection, mice were challenged with 10^6 pfu ZIKV Dakar (mouse adapated) in 11.4 ul saline by inserting a 20ul pipette tip into the vagina. Mice were held supine during the inoculation and for 60 seconds afterward.

For intravaginal infection in single cell/flow cytometry mice, mice were infected as described in Caine et al., 2019.

Human vaginal epithelial cell experiments (Chapter 3)

VK2 cells were expanded in VK2 media (per ATCC guide) and seeded onto transwells, Cells were grown at 37C with 5% CO2. Once on transwells, they remained submerged until confluence. Once confluent, the apical media was removed. Cells were allowed to mature for 10 days prior to infection. Challenge took place with apical virus application in 200ul at an MOI of 5. Cells and virus were rocked for 2 hours at 37C with 5% CO2; inoculant was then removed. Cells were harvested for RNA at subsequent timepoints.

Histology and RNA ISH (Chapter 3)

Imaging studies were conducted as described in Caine et al., 2019.

RNA extraction (Chapter 3)

For vaginal epithelial cells in culture, cells were lysed in RLT buffer and stored at -80C. RNA extractions were performed using RNeasy by Qiagen according to manufacturer's instructions.

For tissues from the longitudinal experiment, mice were necropsied following pericardial infusion of saline. Organs were placed immediately in RNAlater and stored until extraction. Tissues were homogenized using Percellys beads prior to extraction. Lymph nodes were processed using the Ribopure RNA purification kit; all other tissues were processed using RNeasy by Qiagen. Mouse tissues for single-cell and flow were homogenized as in Caine et al 2019.

ZIKV qPCR (Chapter 3)

Extracted RNA from tissues was normalized and utilized for cDNA generation using the iScript kit. cDNA was analyzed via qPCR using ZIKV prME-specific primers.

Nanostring

Extracted RNA was normalized and hybridized to nanostring probes per manufacturers recommendations. A custom probe set was utilized to assess RIG-I activation, IRF3 signalling cascades, and IFN-stimulated gene induction.

RNAseq on mouse tissues (Chapter 3)

RNAseq libraries were prepared using RNA extracted as above; Quality was determined by Lab hip and quantity by Qubit. Libraries were made using the KAPA mRNA sequencing kit; Quality was assessed by tape station. Samples were sequenced 2x75 on an illumine novaseq.

Single-cell RNAseq on vaginal tissue

RNAseq data analysis (Chapter 3)

All bulk sequencing data were analyzed in R using a pipeline previously described in Stone et al., 2019 to determine DEG. Subsequent analysis was performed using ORA, CEMiTool and custom scripts. All single-cell sequencing data were analyzed in R using Seurat and custom scripts. Code for all analyses will be made publicly available upon publication of data described in chapter 3.

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VITA

Andrew Gustin was born in Decatur, Illinois in 1983. He attended Lincoln Way High School in New Lenox, IL. He received his B.A. in English and Rhetoric in 2005 from The University of Illinois, Champaign-Urbana. After earning his emergency medical technician license in 2008, Andrew was hired by Sumner County EMS in Gallatin, TN, where he worked until 2016. During that time, he received his A.A.S. in Paramedicine from Volunteer State Community College in Gallatin, TN (2009), was certified as a critical care Paramedic by the state of Tennessee (2011), and was promoted to lieutenant (2013). While working as a paramedic, Andrew studied at Nashville State Community College from 2012-2014, and went on to earn his B.S. in Microbiology at Middle Tennessee State University in 2015. During his time at MTSU, Andrew was mentored by the virologist Dr. Stephen Wright. Andrew began the Pathobiology PhD program at the University of Washington in 2016. He officially joined the laboratory of Dr. Nichole Klatt at the Washington National Primate Research Center in 2017, where he trained as a bioinformatician investigating the intersection of host immunity, viral infections, and the microbiome, with a focus on the mucosal tissues of the female reproductive tract. Upon Dr. Klatt's departure for a new position at the University of Miami, Andrew took up residence as a guest in the lab of Dr. Michael Gale, Jr., while maintaining Dr. Klatt as his primary mentor.