2013

Characterization Of Innate Immunity In The Female Reproductive Tract For The Prevention Of Hiv Acquisition

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CHARACTERIZATION OF INNATE IMMUNITY IN THE FEMALE REPRODUCTIVE TRACT FOR THE PREVENTION OF HIV ACQUISITION

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Burnett School of Biomedical Sciences
in the College of Graduate Studies
at the University of Central Florida
Orlando, Florida

Spring Term
2013

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ABSTRACT

Human immunodeficiency virus (HIV) infects 30 million people worldwide. In sub-Saharan Africa, the region most affected by HIV, women comprise 60% of the infected population. Heterosexual transmission is a major mode of viral acquisition, mandating further research of the process and prevention of HIV acquisition via the female reproductive tract (FRT). The FRT is a dynamic environment, protected by host immune mechanisms and commensal microbes. The disruption of either of these elements can increase susceptibility to HIV. Accordingly, one common risk factor for HIV acquisition is the microbial shift condition known as bacterial vaginosis (BV), which is characterized by the displacement of healthy lactobacilli by an overgrowth of pathogenic bacteria. As the bacteria responsible for BV pathogenicity and their interactions with host immunity are not understood, we sought to evaluate the effects of BV-associated bacteria on reproductive epithelia.

Here we have characterized the interaction between BV-associated bacteria and the female reproductive tract by measuring cytokine and defensin induction in FRT epithelial cells following bacterial inoculation. Four BV-associated bacteria were evaluated alongside six lactobacilli for a comparative assessment. Our model showed good agreement with clinical BV trends; we observed a distinct cytokine and human β-defensin-2 response to BV-associated bacteria, especially *Atopobium vaginae*, compared to most lactobacilli. One lactobacillus species, *Lactobacillus vaginalis*, induced an immune response similar to that elicited by BV-associated bacteria. These

data provide an important prioritization of BV-associated bacteria and support further characterization of reproductive bacteria and their interactions with host epithelia.

We next evaluated the effect of this interaction on HIV infection by investigating the soluble effectors secreted when FRT epithelial cells were cocultured with *A. vaginae*. We observed increased proviral activity mediated by secreted low molecular weight effectors, and determined that this activity was not likely mediated by cytokine responses. Instead, we identified a complex mixture containing several upregulated host proteins. Selected individual proteins from the mixture exhibited HIV-enhancing activity only when applied with the complex mixture of proviral factors, suggesting that HIV enhancement might be mediated by synergistic effects.

In addition to characterizing the immune interactions that mediate the enhanced HIV acquisition associated with BV, we also evaluated the safety and efficacy of RC-101, a candidate vaginal microbicide being developed for the prevention of HIV transmission. RC-101 has been effective and well tolerated in preliminary cell culture and macaque models. However, the effect of RC-101 on primary vaginal tissues and resident vaginal microflora requires further evaluation. Here, we treated primary vaginal tissues and vaginal bacteria, both pathogenic and commensal, with RC-101 to investigate compatibility of this microbicide with FRT tissue and microflora. RC-101 was well tolerated by host tissues and commensal vaginal bacteria, while BV-associated bacteria were inhibited by RC-101. By establishing vaginal microflora, the specific antibacterial activity of RC-101 may provide a dual mechanism of HIV protection.

ACKNOWLEDGMENTS

First and foremost, many thanks to my labmates who provided stimulating discussion and endless entertainment over the years: Matthew P. Wood, Gowrishankar Muthukrishnan, Ryan P. Lamers, Nicole A. Cowan, Nitya Venkataraman, Julie A. Martellini, Vanathy Paramanandam, Christine Chong, and Karthikeyan Sivaraman. I would like to extend my sincere gratitude to Camila Diaz for her assistance, patience and company throughout the course of my studies. I would also like to thank my committee, Annette Khaled, Ph.D., Saleh Naser, Ph.D., and Travis Jewett, Ph.D., for their guidance. Finally, I would like to acknowledge my advisors, Alexander M. Cole, Ph.D., and Amy L. Cole, Ph.D., for all they've taught me throughout the years.

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1. GENERAL INTRODUCTION

1.1 HIV Infection via the Female Reproductive Tract

More than 30 million people worldwide are infected with HIV, and every year another two million individuals will become newly infected [1]. Sub-Saharan Africa bears the majority of this epidemiological burden, harboring an estimated 68% of infected individuals. In this region, women make up nearly 60% of the infected population, with sexual transmission being a main mode of transmission [1].

The female reproductive tract (FRT) is a primary site of heterosexual HIV acquisition [2]. For male-to-female HIV transmission to occur during heterosexual coitus, infectious virus must be introduced to the FRT via male ejaculate, either as cell-free virions, or as intracellular virus contained within infected seminal leukocytes [3]. The virus must then infect susceptible target cells utilizing specific cell surface receptors; the majority of founder HIV strains (those that cause initial infection) utilize the cell surface receptor CD4 and the coreceptor CCR5 in order to bind and fuse to host cells [4, 5]. These receptors can be found coexpressed on CD4+ T-cells and dendritic cells, which are present in the FRT mucosa, either as intraepithelial immune surveyors, or as infiltrating responders, recruited upon chemotactic stimulation [2].

It is critical to prevent initial infection of these target cells at the mucosa, and toward this aim, the FRT possesses multiple mechanisms of innate defense, including molecular effectors and physical barriers to halt HIV transmission. The foremost of these barriers is the protective epithelial cell layer that lines the FRT, and represents the first point of contact for invading pathogens.

1.2 Epithelial Cells and Innate Immunity in the Female Reproductive Tract

As the outermost cell layer, FRT epithelia represent the primary barrier between potential pathogens and susceptible underlying host cells [5]. In addition to acting as a physical barrier, the epithelia of the FRT also perform a critical role in immune surveillance. Pathogens introduced to the FRT will first encounter the epithelia, and these cells are equipped with immune sensory mechanisms to detect invaders and respond accordingly [6]. The epithelial response, largely mediated by the release of soluble cytokines and antimicrobial effectors, initiates an appropriate host immune response.

The dynamic environment of the FRT is also maintained by regular turnover of epithelial layers [7]. The removal of sloughed epithelia assists in pathogen clearance, and is facilitated by the presence of cervicovaginal fluid, a complex fluid comprised of epithelial secretions, cervical mucus and plasma transudate [8]. This protein-rich fluid contains high concentrations of innate immune effectors, such as host defense peptides exhibiting broad-spectrum antimicrobial activity, including potent anti-HIV activity [9]. Importantly, the anti-HIV activity of cervicovaginal fluid is not mediated by individual effectors; instead, the antimicrobial factors exhibit synergistic activity to achieve a potent antiviral effect [10].

Together, innate immune barriers and antimicrobial effectors protect the host from invading pathogens. Yet not all microbes pose a threat; the healthy FRT is host to

a dense population of commensal bacteria that actually contributes to its inherent resilience.

1.3 Protective Lactobacilli Are Displaced by Bacterial Vaginosis

The healthy human vagina is guarded by a complex population of commensal bacteria. Comprised almost entirely of lactobacilli, these microbial inhabitants colonize the vaginal lumen at densities reaching 10^9 bacteria per milliliter [11]. Commensal lactobacilli are thought to protect the FRT mucosa from invading pathogens by secreting antimicrobial compounds, including bacteriocins, hydrogen peroxide and lactic acid [12]. The lactic acid produced by vaginal bacteria maintains the healthy vagina at a pH less than 4.5 [13], with higher pH indicating displacement or disruption of lactobacilli, a common occurrence known as bacterial vaginosis (BV) [14].

BV is a microbial shift condition, characterized by the displacement of commensal vaginal lactobacilli and the overgrowth of mixed pathogenic bacterial populations [15, 16]. Neither the presence nor absence of any single bacterial species is sufficient for diagnosis, but instead multifactorial clinical and microbiological criteria are used to diagnose BV [17, 18]. BV affects between 20-60% of women worldwide, and can pose serious immediate and long-term sequelae [19, 20, 21]. Women who have BV are at a higher risk of developing pelvic inflammatory disease, and pregnant women experiencing BV are significantly more likely to encounter complications, including preterm birth [22]. Furthermore, BV increases a woman's chance of acquiring sexually transmitted infections, including HIV [20].

The mechanism by which BV increases acquisition of HIV remains enigmatic. The displacement of lactobacilli, which otherwise prevent infections by secreting lactic acid and hydrogen peroxide, weakens inherent mucosal defenses to reproductive pathogens. At the same time, intruding pathogenic bacteria induce an inflammatory host response that is thought to initiate pathogenic sequelae [23]. Combined, these effects of BV result in a 60% increased susceptibility to HIV acquisition [20]. The serious clinical consequences of BV, combined with its high prevalence, make this condition of immediate priority. In consideration of these factors, drug development for FRT application must demonstrate safety not only for host tissue, but also for the commensal FRT bacteria.

1.4 RC-101 Is a Promising Candidate Microbicide

Together, host and microbial immune barriers ward off infection at the FRT mucosa. Still, HIV infection continues to spread at a rate of two million individuals per year [1], mandating a more effective prophylactic to halt heterosexual viral transmission. Vaginal microbicides have become an attractive option for antiviral protection, primarily because they allow susceptible women the opportunity to control their protective regimen. Especially in Sub-Saharan Africa, where sex is often non-negotiable for women, it is critical that antiviral prophylaxis be in place prior to viral exposure [24]. Microbicides offer this opportunity, as many formulations can be applied according to a routine regimen, and confer stable prophylaxis in the case of HIV exposure [25].

Despite the promising attributes of microbicide formulations, the search for a suitable antiviral agent has been complicated by unforeseen inconsistencies between the bench and the clinic. Early microbicide formulations consisted of sulfated polymers or polyanionic surfactants [26]. For these compounds, promising results obtained in early testing were not recapitulated in clinical trials. Such inconsistencies result from poor modeling of *in vivo* environments during initial characterization of the candidate microbicide, as the disruption of either host tissues or bacterial inhabitants can actually increase susceptibility to HIV. The former was exemplified by the well-publicized nonoxonyl-9 clinical trials, wherein antiviral effects observed in cell culture were reversed in clinical trials due to the uncharacterized damaging effects of the surfactant microbicide on FRT tissues [27]. Similarly, disruption of healthy FRT microflora can also render women more susceptible to HIV acquisition, as is demonstrated in BV [28]. Therefore, in order to prevent side effects that counter antiviral activity, anti-HIV vaginal microbicide candidates must demonstrate compatibility with FRT tissues and microflora prior to clinical trials.

In consideration of these requirements, host defense peptides have arisen as promising microbicide candidates. Host defense peptides are expressed across diverse phylogenetic lineages, including plants, animals, fungi and bacteria, and comprise an ancient form of innate immunity [26]. Research has revealed a promising microbicide candidate, the cyclic peptide RC-101, which has thus far demonstrated a desirable safety and therapeutic profile [27]. RC-101 is an analogue of the retrocyclin RC-100, a cyclic theta-defensin whose expression was lost over the course of primate evolution; retrocyclins are encoded in the human genome, but not expressed due to a premature

stop codon in the peptide proregion [28]. Retrocyclins have been synthetically recreated by solid-phase synthesis, and the resulting peptides exhibit broad-spectrum antimicrobial activity, including anti-HIV and antibacterial activity [29, 30]. RC-100, the peptide encoded by the human retrocyclin pseudogene, inhibits HIV at IC_{50} as low as 1.0 μg/mL.

RC-101 is nearly identical to the retrocyclin RC-100, excepting a single arginine to lysine mutation, and exhibits better activity, with reported IC_{50} as low as 0.19 μg/mL [26, 31, 32]. Ongoing research seeks to restore translation of endogenous RC-100 to the FRT using premature termination codon readthrough agents [33], while RC-101 has become the primary focus of microbicide development. As such, it is essential to characterize the interactions between RC-101 and the FRT, including both host and microbial inhabitants, to ensure that mucosal immunity remains intact in the presence of this candidate antiviral.

In these studies, we investigated the complex environment of the FRT in order to characterize innate immunity and HIV susceptibility at the female reproductive mucosa. We describe the interactions between host epithelia and resident microbes, prioritizing BV-associated pathogens and revealing unexpected immune interactions. We additionally describe the innate immune response initiated by FRT epithelia in response to pathogenic bacteria, and elucidate novel immune response mechanisms that likely contribute to HIV susceptibility. Finally, we characterize the promising antiviral microbicide candidate, RC-101, demonstrating its compatibility with FRT tissues and healthy microflora. It is hoped that the studies performed herein will contribute to a

better understanding of the dynamic FRT environment, and lead to new approaches for preventing HIV acquisition via the FRT.

2. IDENTIFICATION AND CHARACTERIZATION OF BACTERIAL VAGINOSIS-ASSOCIATED PATHOGENS USING A COMPREHENSIVE CERVICAL-VAGINAL EPITHELIAL COCULTURE ASSAY

2.1 Introduction

Bacterial vaginosis (BV) is the most common disorder of the female reproductive tract (FRT) for which clinical intervention is sought [15]. In BV, commensal vaginal lactobacilli are displaced by mixed populations of pathogenic bacteria, including *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus curtisii* and *Prevotella bivia* [34, 35]. This microbial shift condition predisposes women to pathogenic sequelae, including increased HIV acquisition.

Research has just begun to elucidate the mechanisms of BV pathogenicity. It is apparent that bacterial pathogens elicit an immune response in the FRT, characterized by an upregulation of proinflammatory cytokines, such as IL-8, IL-1α, and IL-1β, yet the host cells responsible for pathogen recognition and immune response remain uncharacterized [36, 37, 38, 39]. It has been postulated that increased inflammation could increase HIV susceptibility by two mechanisms: first, increased chemotactic recruitment of CD4+/CCR5+ immune cells could provide additional susceptible target cells for HIV to establish an initial infection [40], or second, increased concentrations of inflammatory cytokines could activate NF-κB, a major transcription factor driving viral RNA transcription and resulting HIV genomic replication [41].

Other aspects of the host innate immune response to BV are less clear; there is conflicting evidence regarding regulation of antimicrobial effector proteins, such as the human β-defensins (hBDs). This family includes antibacterial peptides that are reported

to be induced by bacterial stimuli [42], yet studies of hBD regulation in the context of BV have had conflicting results, with some studies showing a significant increase in hBD levels in the FRT coincident with BV, and others reporting a significant decrease [43, 44]. Furthermore, with dozens of bacterial species associated with the microbial shift that defines BV, researchers have yet to characterize the pool of candidate pathogens and elucidate their immunostimulatory properties [45, 46, 47, 48].

With insufficient characterization of pathogens, little can be done to streamline BV treatment. Prior studies have been insufficient in comparing FRT bacteria on account of the limited number of species evaluated within a consistent model [44, 49]. Furthermore, variations between these studies make it impossible to compare host-bacterial interactions between reports. Here, we developed a coculture model to characterize the response of various FRT epithelial cells (the frontline in FRT mucosal defense) to a comprehensive collection of vaginal bacteria, including both commensal lactobacilli and BV-associated bacteria (BVAB). We evaluated a total of ten bacteria on three separate epithelial cell types, monitoring host response under consistent coculture conditions. In doing so, we observed distinct differences in immune responses between the three types of reproductive epithelia, as measured by cytokine and defensin induction. These responses demonstrated good agreement of our model with clinical BV samples. We also found that only a select few of the tested bacterial species elicited an immune response from host cells. Surprisingly, not all BVAB elicited potent immune responses, whereas one *Lactobacillus spp.* did stimulate significant cytokine and defensin induction in FRT epithelia. Thus, in addition to developing a model for immune interactions in the FRT, we also report unexpected trends in bacterial-host interactions,

emphasizing the utility of this approach for understanding host-pathogen interactions in the FRT.

2.2 Materials and Methods

2.2.1 Reagents and Materials

Trizol and keratinocyte serum free media (KSFM) with supplements were from Invitrogen, while RNA Storage Solution and DNAse I kit were purchased from Ambion (both of Life Technologies, Carlsbad, CA, USA). Bio-Rad (Hercules, CA, USA) iScript and Sybr Green Supermix were used for RTqPCR experiments. RPMI1640, DPBS, and DMEM/F12 were from MediaTech, Inc., while collagen-coated Transwells were from Corning Life Sciences (both of Corning Inc, Corning, NY, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (West Sacramento, CA, USA).

2.2.2 Epithelial and Bacterial Cultures

The following human epithelia were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA): End1 (CRL-2615) from endocervix; Ect1 (CRL-2614) from ectocervix; VK2 (CRL-2616) from vagina. These were maintained according to ATCC instructions. Briefly, cells were grown in KSFM supplemented with additional calcium chloride, recombinant epidermal growth factor, and bovine pituitary extract. For maintenance, cultures were grown to 50-70% confluence before splitting. For Transwell experiments, cells were seeded at confluence (1.6×10^6 End1 cells, 1.1×10^6 Ect1 cells or

1.0×10^6 VK2 cells, seeding varies according to cell kinetics). For all other experiments, cultures were grown to confluence on tissue culture-treated plates (Techno Plastic Products, Trasadingen, Switzerland). The following bacteria were purchased from ATCC as common representatives of commensal flora [12, 50, 51, 52, 53, 54, 55, 56, 57]: *Lactobacillus crispatus* (33197); *Lactobacillus acidophilus* (4356); *Lactobacillus johnsonii* (11506); *Lactobacillus jensenii* (25258); *Lactobacillus gasseri* (9857); *Lactobacillus vaginalis* (49540). The following bacteria were purchased from ATCC as representatives of BVAB [58, 59]: *Gardnerella vaginalis* (49145); *Atopobium vaginae* (BAA-55); *Mobiluncus curtisii* (35241); *Prevotella bivia* (29303). Lactobacilli were grown in MRS broth or plates at 37°C/5% CO_2. *G. vaginalis*, *A. vaginae*, *M. curtisii*, and *P. bivia* were grown in tryptic soy broth (TSB) with 5% defibrinated rabbit blood (all media from Becton, Dickinson and Company, Franklin Lakes, NJ, USA), or on equivalent agar plates. *G. vaginalis* was grown at 37°C/5% CO_2, while the other 3 BVAB were grown in anaerobic GasPaks (Becton, Dickinson and Company) at 37°C. To achieve consistency in bacterial preparations, maintenance cultures of each species were aliquoted and snap frozen by submerging in liquid nitrogen for 2 hr, then transferred to -80°C until use.

To prepare inocula for experiments, snap-frozen aliquots of aerobic bacteria were thawed and inoculated into prewarmed, pregassed MRS (for lactobacilli) or TSB (for *G. vaginalis*) for 2 hr to allow for recovery prior to coculturing. Desired volumes of cultures were then centrifuged at 4000xg for 10 min, supernatants were aspirated, and bacteria were resuspended in KSFM. Snap-frozen aliquots of anaerobic bacteria were thawed, and desired volumes were centrifuged. Supernatants were aspirated, and

bacteria were resuspended in distilled water for one minute (to lyse erythrocytes carried over from maintenance media), and then diluted with four volumes KSFM (to restore osmolarity). This distilled water wash did not affect bacterial viability. The bacteria were centrifuged again, the supernatent was removed, and the bacteria were resuspended in KSFM.

In all experiments, the multiplicity of infection (MOI) was calculated as the number of bacterial colony forming units (CFUs) divided by the number of epithelial cells in a given coculture condition. The number of bacterial CFUs was determined by serially diluting the inocula and plating on appropriate media for back-calculation of inocula density. The final MOIs used were in agreement with reports of clinical bacterial load in the FRT, and with other published coculture models [49, 60, 61]. In bacterial-epithelial cocultures where bacterial stimulation of epithelia was compared, BVAB or *L. vaginalis* were applied at a lower MOI than nonstimulatory lactobacilli, to preclude concerns about small MOI differences effecting significant immune response differences. Coculture kinetics were monitored at the assay endpoint, and Appendix B: Figure B.1 and Figure B.2 demonstrate bacterial and epithelial viability after coculture. Additionally, heat-killed inocula were evaluated for stimulatory BVAB to determine whether bacterial viability was required for epithelial stimulation. These data are presented in Appendix B: Figure B.3.

2.2.3 Bio-plex Analysis of Transwell Underlay

For Transwell cocultures, epithelial cells were seeded on 24 mm collagen-coated 0.4 μm Transwells. The next day, excess apical media and unattached cells were removed, basal media was changed, and cells were maintained at the air-liquid interface. Twenty-four hr after transitioning to air-liquid interface, underlay media was changed and cell monolayers were inoculated apically with 100 μL bacterial inoculum. Epithelia were coincubated with bacteria for 24 hr, then media underlay was collected and frozen at -20°C until analysis. For analysis, media underlay were clarified and analyzed by Bio-Rad Bio-plex multiplex cytokine array. Experimental analysis was performed according to manufacturer's instructions.

2.2.4 Bio-plex Analysis of Cervicovaginal Lavage Samples

CVLs were provided by HIV-negative participants in the Bronx/Manhattan consortium of the Women's Interagency HIV Study (WIHS), a longitudinal observational cohort study of HIV-positive and HIV-negative women, at their routine semi-annual WIHS visits. Written informed consent was obtained from all participants, and samples were collected in accordance with protocols approved by the Institutional Review Board (IRB) of Montefiore Medical Center for this study. CVLs were obtained by irrigating the cervix and vaginal wall with 10mL sterile saline as previously described [62] and cryopreserved at -80ºC. BV was assessed by Amsel criteria [63] with the presence of at least three (of four) criteria indicating the presence of BV. For this study, the BV-negative group was comprised of samples that demonstrated the absence of all four

criteria. Cervicovaginal lavage fluid was clarified before Bio-plex analysis. Experimental analysis was performed according to manufacturer's instructions.

2.2.5 Primary Lymphocyte Isolation

Venous blood was drawn from adult volunteers who provided written consent, and samples were obtained in accordance with a UCF IRB approved protocol for this study. Blood was drawn into acid citrate dextrose vacutainers (Becton, Dickinson and Company), and peripheral blood mononuclear cells (PBMCs) were separated within an hour of the donation. To separate PBMCs, whole blood was diluted in an equal volume of DPBS, manually overlaid on lymphocyte separation media (LSM, MP Biomedicals, Santa Ana, CA, USA), then centrifuged at 400xg for 30 min. PBMCs were isolated from LSM density gradients, and were washed twice with DPBS, then resuspended in RPMI containing 10% FBS and plated on tissue culture-treated plates (Techno Plastic Products). Plates were incubated at 37°C/5% CO_2 for 2 hr before isolating non-adherent lymphocytes from adherent monocytes. Lymphocytes were maintained in RPMI with 10% FBS, and used within 24 hr of isolation.

2.2.6 Chemotaxis of Primary Lymphocytes

Peripheral blood lymphocytes were resuspended in RPMI supplemented with 1% FBS at a density of two million cells per mL. Serial dilutions of recombinant human β-defensin 2 (hBD2, Peprotech, Rocky Hill, NJ, USA) or equivalent vehicle control were prepared in the same media. hBD2 and vehicle dilutions were plated in a ChemoTX plate (Neuroprobe, Gaithersburg, MD, USA), alongside media alone controls. The ChemoTX filter was attached to the plate, and 50 µL lymphocyte suspension was applied to the surface of each well. The ChemoTX plate was incubated at 37°C/5% CO_2 for 3 hr. To compare migrated cells, media above the filter was removed, and apical surface of filter was washed once in DPBS with 5 mM EDTA, then incubated with the same wash for 30 min at 4°C. This second wash was removed, the ChemoTX plate was centrifuged at 400xg for 5 min, and the filter was removed. Cells in the lower chamber were resuspended in a volume of 100 µL, and the CytoTox Glo assay (Promega, Fitchburg, WI, USA) was used to compare total cell number according to manufacturer's instructions. A standard curve of known cell numbers was used to calculate the number of migrated cells from relative light units.

2.2.7 hBD2 Acid-Urea (AU) Western

Epithelial cells were harvested and lysed by scraping into 10% acetic acid. These cell lysates were vortexed 30 min at room temperature to extract protein. Soluble extracts were clarified and concentrated, then resolved on an acid-urea polyacrylamide gel electrophoresis (AU-PAGE). A standard of recombinant hBD2 was

run alongside cell extracts on each gel. Gels were transferred to PVDF membranes and blotted with a goat polyclonal antibody against hBD2 (Peprotech).

2.2.8 ELISA

Cell culture supernatants from treated epithelial cells were clarified and subjected to ELISA quantification using the Peprotech hBD2 ELISA Development Kit and Becton, Dickinson and Company OptEIA IL-6 and IL-8 Kits. Assays were performed according to suppliers' instructions.

2.2.9 Real-Time Quantitative Polymerase Chain Reaction (RTqPCR)

To isolate RNA, epithelial cells were rinsed in cold DPBS, harvested in Trizol reagent, and stored at -80°C until extraction. RNA was then precipitated, treated with DNAse I, and reverse transcribed. cDNAs were analyzed by RTqPCR using the following primer pairs: hBD2_F atctcctcttctcgttcctcttc; hBD2_R ccacaggtgccaatttgtttatac; hBD3_F cttctgtttgctttgctcttcc; hBD3_R cacttgccgatctgttcctc; GAPDH_F tggtatcgtggaaggactc; GAPDH_R agtagaggcagggatgatg. All cycle thresholds were averaged from duplicate reactions. Cycle thresholds for hBD amplicons were normalized to the GAPDH standard, and fold expression was calculated using the delta-deltaCt method. All fold expressions were reported as increases compared to a 0 hr control.

2.2.10 Statistical Analyses

All statistical analyses were carried out in Microsoft Excel or GraphPad Prism. For Bio-plex cytokine data, raw values were log-transformed and two-way ANOVA with Bonferroni post test was used to compare bacterial and mock conditions from three independent experiments. For RTqPCR data, fold expression values for each condition were calculated relative to 0 hr. Fold expression of mock versus treated conditions was compared using one-way ANOVA for endpoint analysis, or two-way ANOVA for timecourse analysis, with Bonferroni post test. For chemotaxis experiments, cell numbers were normalized to media-only (control treatment) wells, and increase in hBD2 over matched vehicle wells was compared by two-way ANOVA with Bonferroni post tests. For IL-6, IL-8 and hBD2 ELISAs, fold expression was calculated compared to mock-treated cells, and one-way ANOVA with Tukey-Kramer post test was performed to compare *L. vaginalis* to other lactobacilli, or to BVAB. For figures in which only two conditions are directly compared, Student's t-test was used.

2.3 Results

2.3.1 BV-Associated Bacteria Induce a Cytokine Response From Reproductive Epithelial Cells

The pathogenic sequelae resulting from BV may be attributed to the host inflammatory response to pathogenic BV-associated bacteria (BVAB). We hypothesized that this inflammatory response is mediated by the epithelial cells that line FRT and represent the initial point of contact for bacteria invading the FRT. In order to measure

the contribution of FRT epithelial cells to BV-associated inflammation, we developed a coculture model to measure host epithelial response to BVAB. Epithelial cells derived from the vagina (VK2), ectocervix (Ect1), or endocervix (End1) have been previously used as a model to evaluate FRT microbicide tolerance and immune response [44, 49]. In our initial characterization, we sought to identify the cytokine repertoire that these epithelia are capable of producing in response to bacterial stimulation. Epithelia were seeded on transwells and inoculated at the air-liquid interface with a high MOI of either commensal bacteria (*Lactobacillus johnsonii*) or BVAB (*Gardnerella vaginalis* or *Atopobium vaginae*). After a 24 hr coculture, media underlay were analyzed in order to obtain a cytokine response profile for each epithelial type. Many of the analytes quantified were below the limit of detection (IL-2, IL2-Rα, IL-3, IL-4, IL-5, IL-9, IL-12p40, IL-15, IL-16, IL-17, IL-18, eotaxin, IFN-α2, FGF-β, GM-CSF, MCP-1, MCP-3, MIG, Mip-1α, SCF, SCGF-β, TNF-α, TNF-β, TRAIL, and HGF) and were not analyzed further. Others cytokines were below 50 pg/mL in all conditions (IL-1β, IL-7, IL-10, and IL-13), suggesting that the epithelia analyzed do not produce considerable concentrations of these cytokines under basal conditions or as part of their immune response to BVAB (Figure 2.1).

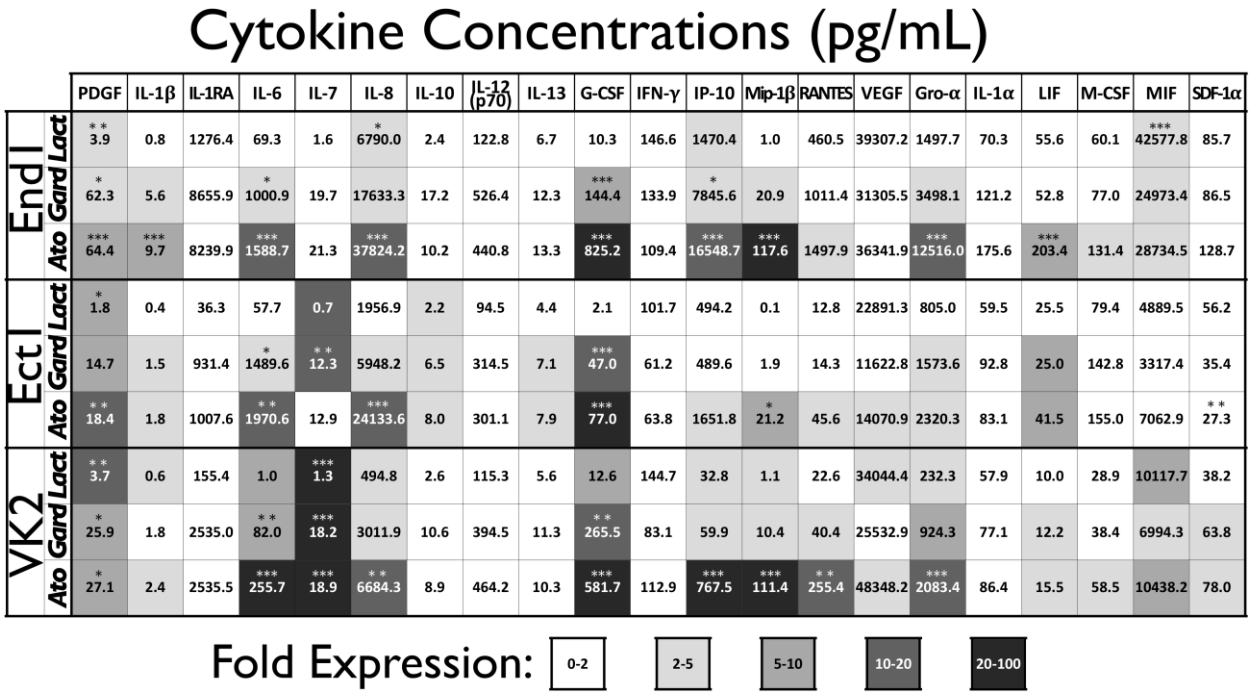


Figure 2.1. BVAB Induce an Innate Cytokine Response in Female Reproductive Epithelia.

Transwell monolayers of each epithelial line were inoculated with indicated bacteria (Lact = *L. johnsonii*, average MOI = 33, Gard = *G. vaginalis*, average MOI = 15, Ato = *A. vaginae,* average MOI = 13) and 24 hr post-inoculation conditioned media were analyzed by multiplex cytokine bead array. Cytokine values are averaged from three independent experiments. Relative induction, represented here as fold expression and shaded accordingly, was calculated as the average increase in cytokine concentration compared to mock-inoculated control cells from three independent experiments. Significant differences in cytokine concentrations between bacteria-inoculated and mock condition are indicated by one ($p<0.05$), two ($p<0.01$) or three ($p<0.001$) asterisks.

On the other hand, the analytes IL-1RA, IL-6, IL-8, IP-10, RANTES, VEGF, Gro-α and MIF were recovered in nanogram/mL concentrations from some conditions, indicating that these cytokines are primary components of baseline or stimulated epithelial cytokine production. We compared the concentrations of these analytes in *L. johnsonii* condition versus BVAB conditions, and found that overall the BVAB induced greater cytokine responses from FRT epithelium than the commensal lactobacillus strain. The analytes IL-6, IL-8, G-CSF, IP-10, Mip-1β, RANTES, and Gro-α were upregulated in all three epithelial lines by BVAB more than by commensal lactobacilli, as indicated by the fold expression shading. Furthermore, the BVAB *A. vaginae* elicited more robust cytokine responses from each epithelial cell type compared to the other bacteria tested, with some chemokines achieving nanogram/mL concentrations in *A. vaginae*-stimulated conditions. Of note, we also observed greater cytokine production from End1 epithelial cells compared to the other two reproductive cell types.

To demonstrate the relevance of our epithelial-bacteria coculture model, we compared the cytokine responses we observed in our epithelial model of BV with the cytokine trends of cervicovaginal lavage (CVL) samples obtained from a representative cohort of women with or without BV. Previous studies have reported increased concentrations of IL-1β, IL-6 and IL-8 in BV-positive CVL compared to BV-negative samples [36,37,38,39]. Here, we use a small cohort exhibiting trends consistent with the literature for comparison to our model. The cytokine concentrations in CVL were quantified, and fold increases in each cytokine between BV-positive and BV-negative women were compared to the cytokine regulation between *A. vaginae*- and *L. johnsonii*-inoculated conditions in our FRT model (Figure 2.2).

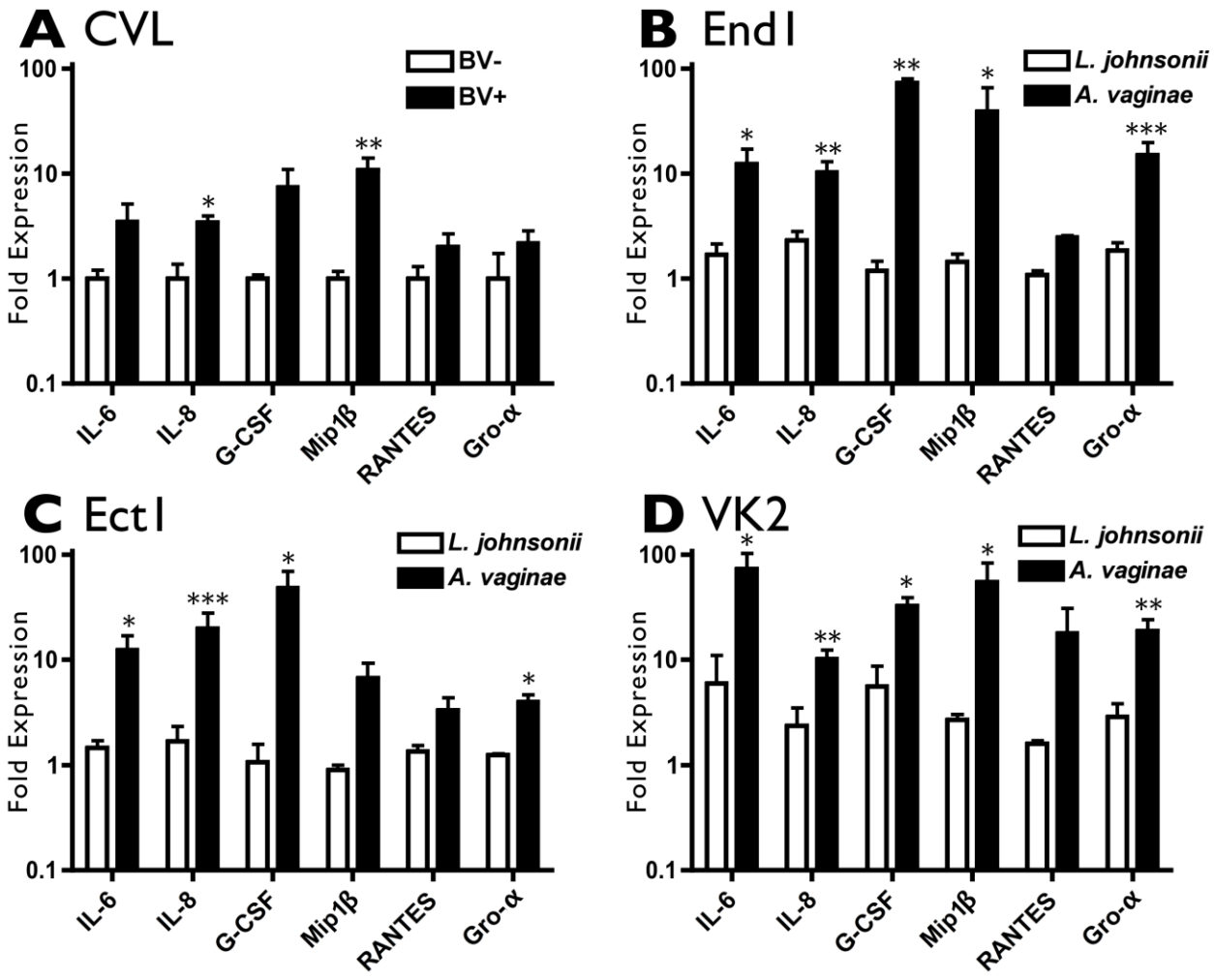


Figure 2.2. Epithelial Coculture Mirrors In Vivo Cytokine Response.

A) Cervicovaginal lavage samples from BV-negative (n=5) or BV-positive (n=13) women were analyzed by multiplex cytokine bead array. Fold expression for each cytokine was calculated relative to the average value of the BV-negative samples. One ($p<0.05$) or two ($p<0.01$) asterisks indicate a significant increase in cytokine concentration for the BV-positive samples over the BV-negative samples. B-D) Cytokine induction in each epithelial line B) End1, C) Ect1, and D) VK2 in response to *L. johnsonii* (average MOI = 33) and *A. vaginae* (average MOI = 13) normalized to their paired mock-inoculated conditions. One ($p<0.05$), two ($p<0.01$), or three ($p<0.001$) asterisks indicate a

significant increase in cytokine concentration for the *A. vaginae*-inoculated conditions over the *L. johnsonii*-inoculated conditions.

Shown are six cytokines, IL-6, IL-8, G-CSF, Mip-1 β, RANTES and Gro-α, that are elevated in clinical BV samples and similarly increased in our coculture model after inoculation with *A. vaginae*, but not with the commensal lactobacillus species *L. johnsonii*. Remaining cytokine data are included in Appendix B: Figure B.4. Thus, our epithelial model aptly recapitulates the cytokine changes that occur in the reproductive tract as a result of BV. This comparison illustrates the contribution of FRT epithelia to the proinflammatory cytokine milieu that characterizes BV, and provides a convenient model for evaluating host-bacterial interactions in the reproductive tract. In addition to evaluating cytokines, we also investigated the induction of other innate immune effector proteins in response to BVAB.

2.3.2 Reproductive Epithelia Upregulate hBD2, a Lymphocyte Chemoattractant, in Response to BVAB

Human defensins are important mediators of innate immunity, as they exhibit both antibacterial activity and chemotactic recruitment of immune cells, yet their role in BV has been studied with conflicting results [43, 44, 64]. To elucidate the host defensin response to BVAB, we measured hBD2 and hBD3 responses of reproductive epithelia at the transcript and protein levels following exposure to lactobacilli or BVAB. Epithelial cells were inoculated with commensal *L. johnsonii*, or pathogenic bacteria *G. vaginalis* or *A. vaginae,* and gene expression was measured by RTqPCR. In agreement with

cytokine trends, hBD2 gene upregulation for each epithelial line was greatest in response to the pathogen *A. vaginae* (Figure 2.3).

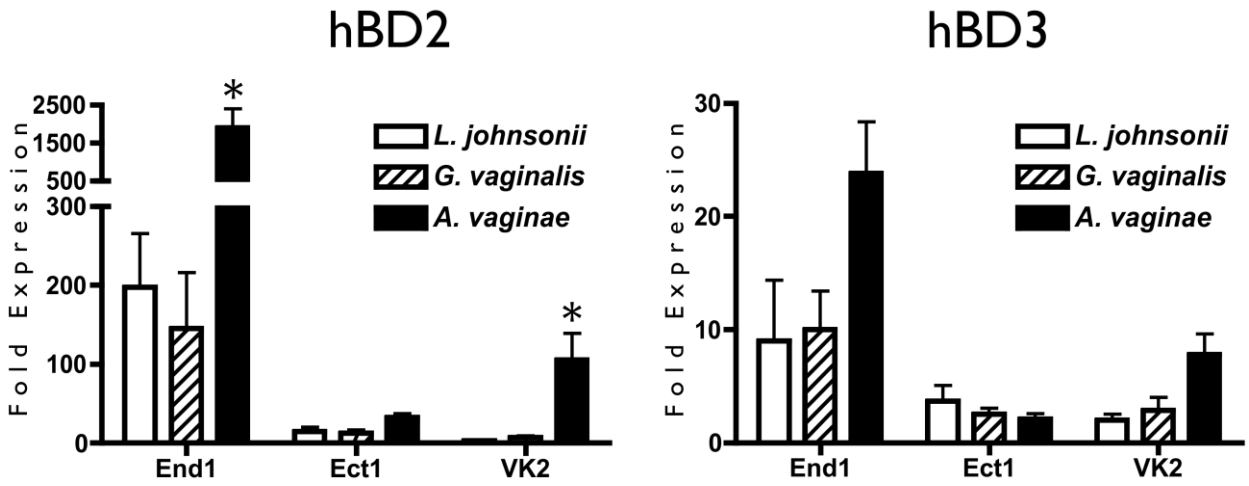


Figure 2.3. Human β-Defensin Gene Expression Is Upregulated in Reproductive Epithelia in Response to BVAB.

Confluent monolayers of epithelial cells were inoculated with bacteria and coincubated for 48 hr, then analyzed by RTqPCR for hBD2 and hBD3 expression. Transcript expression is reported relative to 0 hr cells, and is shown as the average of three independent experiments. Average MOI are: 10 for *L. johnsonii*, 5.8 for *G. vaginalis*, and 5.6 for *A. vaginae*. Asterisks indicate a significant ($p<0.05$) increase over the *L. johnsonii*-treated condition.

This upregulation was significant for End1 and VK2 cells, with End1 epithelia showed the greatest hBD2 response to bacterial stimulation compared to the other two epithelia. This is emphasized by a >1500-fold increase in hBD2 gene expression by End1 cells after inoculation with the BVAB *A. vaginae*. End1 and VK2 cells exhibited

trends of hBD3 upregulation in response to *A. vaginae*, whereas the Ect1 cell type did not respond, indicating a difference in immune response to BVAB between cell types of the FRT.

To confirm the functional production of hBD2, we performed an ELISA to measure soluble hBD2 protein secreted by epithelia in response to bacterial exposure. hBD2 was quantified in conditioned media from epithelial cells cocultured with either commensal or pathogenic bacteria. In correspondence with transcript regulation, inoculation with BVAB resulted in a more robust upregulation of hBD2 protein by End1 cells compared to inoculation with commensal bacteria (Figure 2.4 panel *A*). However, neither Ect1 nor VK2 cells secreted significantly more hBD2 in response to BVAB than mock-inoculation.

In addition to measuring soluble hBD2, we considered that ELISA might not account for additional protein that remained cell-associated. To address this possibility, we performed AU-PAGE western blot to probe for cell-associated hBD2 in epithelia stimulated with *A. vaginae*. hBD2 protein was detected in End1 cell extracts after stimulation with BVAB *A. vaginae*, while neither Ect1 nor VK2 showed similar cell-associated protein (Figure 2.4 panel *B*). The cell-associated hBD2 protein recovered from *A. vaginae*-inoculated End1 cells appeared consistent over the three-day timecourse, and based on the internal protein standard, was calculated to contribute an additional 1.6 ng per 100 mm dish, compared to the 2.0 ng soluble protein per dish as measured by ELISA.

Previous reports have demonstrated that hBD2 protein stimulates chemotaxis of memory T cells and dendritic cells through the chemokine receptor CCR6. Having seen

that hBD2 was significantly upregulated by reproductive epithelia in response to pathogenic bacteria, we sought to verify the ability of this chemoattractant to recruit primary lymphocytes. In agreement with previous reports [64], we observed dose-dependent increases in cell migration of unstimulated primary lymphocytes toward a recombinant hBD2 protein gradient (Figure 2.4 panel *C*). Importantly, our observation of hBD2-mediated chemotaxis of peripheral lymphocytes suggests that these cells can be recruited to tissues expressing increased levels of hBD2.

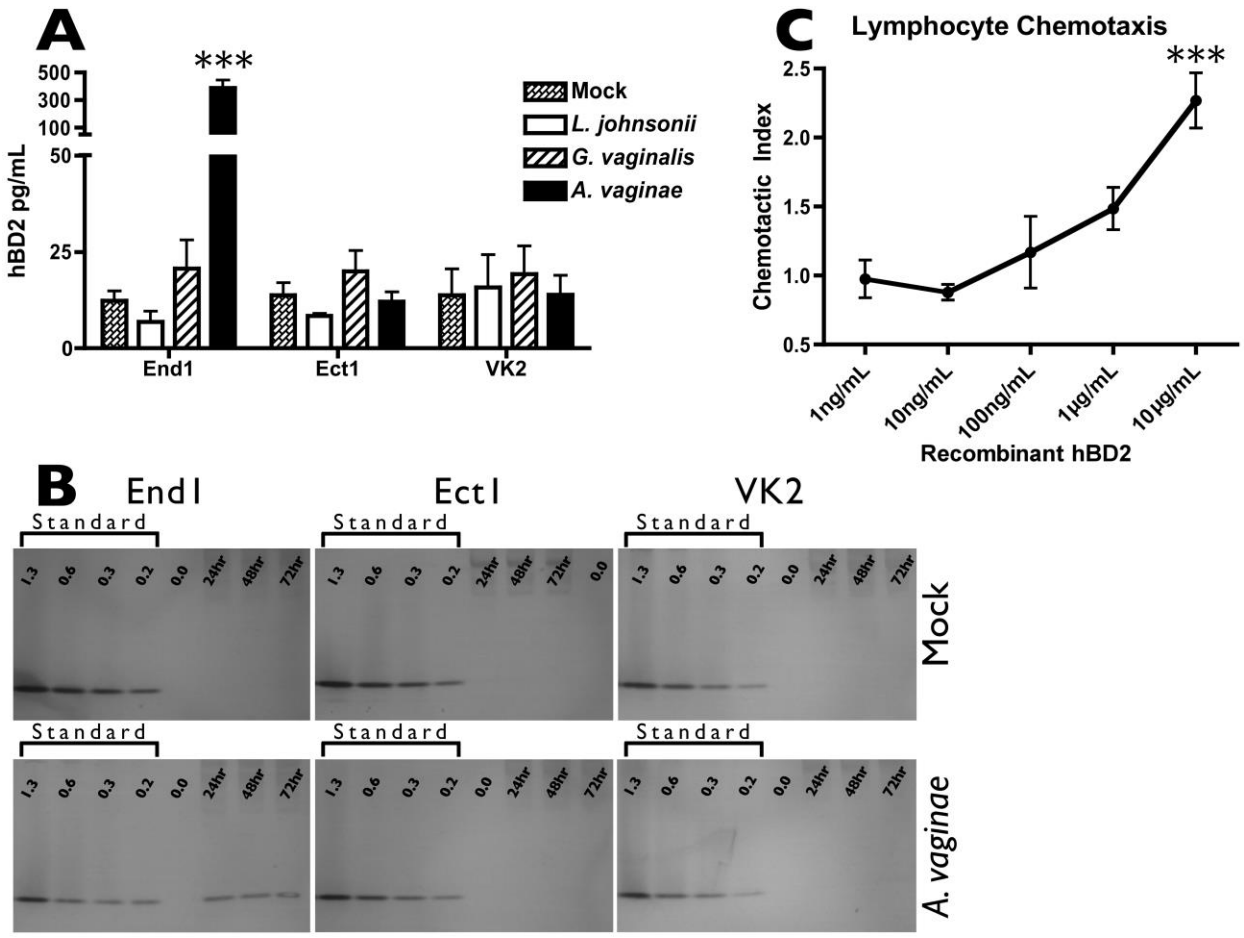


Figure 2.4. A. vaginae Induces Epithelial Expression of Soluble and Cell-Associated hBD2, a Protein That Attracts Primary Lymphocytes.

A) Confluent monolayers of epithelial cells were inoculated with bacteria. Twenty-four hr post-inoculation, conditioned media were collected, clarified, and analyzed by ELISA to quantify concentrations of soluble hBD2 protein. Average MOI are: 5.8 for *L. johnsonii*, 2.5 for *G. vaginalis*, and 1.4 for *A. vaginae*. Results are averaged from 3 independent experiments, and asterisks indicate significant ($p<0.001$) increase over mock-treated condition. B) Confluent monolayers were inoculated with *A. vaginae*, or mock-inoculated. 24, 48, and 72 hr post-inoculation, cell monolayers were acid-extracted and analyzed by AU-PAGE western to quantify cell-associated hBD2

protein. A recombinant hBD2 protein standard (shown in ng per lane) was run alongside cell extracts for semi-quantitative comparison. Average MOI is 21. Shown is one example of three independent experiments. C) Unstimulated primary lymphocytes were isolated and evaluated for chemotaxis toward recombinant hBD2 protein. Chemotactic index is the ratio of migrated cells in hBD2-containing wells over vehicle-control wells, and significant increases over the matched vehicle condition are shown by three ($p<0.001$) asterisks.

Having determined that reproductive epithelia provide a relevant model for characterizing host-pathogen interactions in the reproductive tract, we next sought to employ our model to explore the host response to a variety of reproductive bacteria, both commensal and pathogenic in nature.

2.3.3 *Lactobacillus vaginalis* Induces an Innate Immune Response

Since epithelial hBD2 response mirrored cytokine induction, we first used hBD gene expression as a predictive gauge of BVAB stimulatory capacity. We extended our analysis to a total of six lactobacillus strains (*Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensenii, Lactobacillus johnsonii,* and *Lactobacillus vaginalis*) and four BVAB (*Atopobium vaginae, Garderella vaginalis, Mobiluncus curtisii* and *Prevotella bivia*). In agreement with our previous analyses, we observed that *A. vaginae* induced the most robust hBD2 gene upregulation (>100-fold increase in all three cell types), with *M. curtisii* and *P. bivia* eliciting considerable

responses as well (Figure 2.5). Surprisingly, the lactobacillus species *L. vaginalis* also induced significant hBD2 gene upregulation from End1 (>400-fold) and VK2 cells (>60-fold), in contrast to its perceived commensal classification.

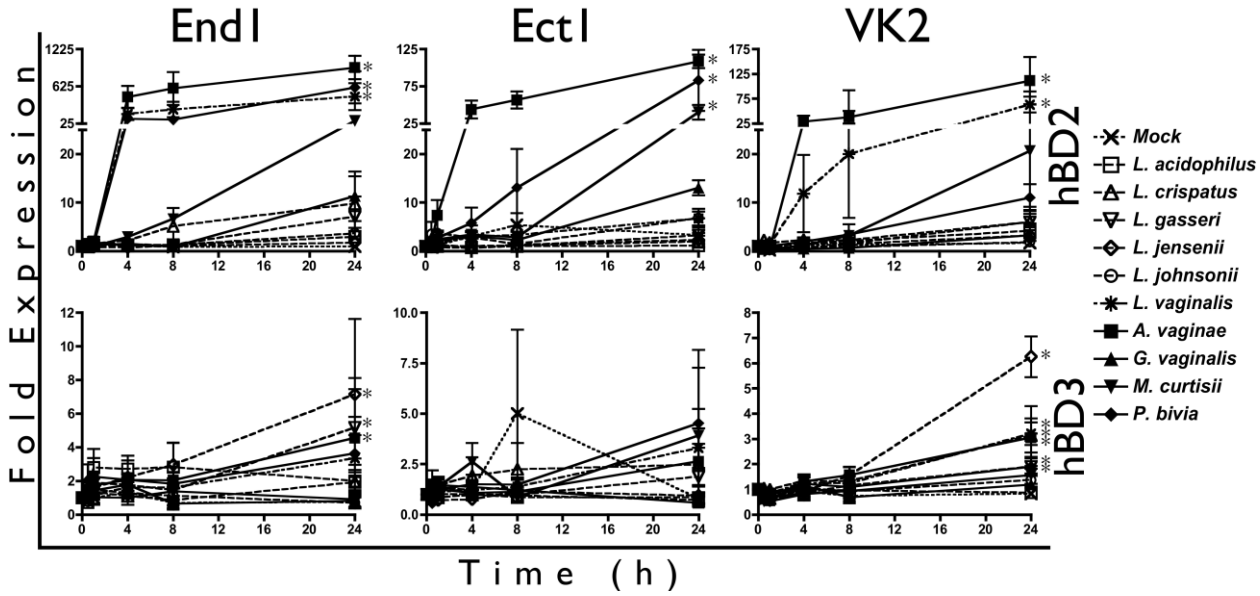


Figure 2.5. *L. vaginalis* Elicits hBD2 Gene Induction from Reproductive Epithelia. Confluent monolayers of epithelial cells were inoculated with bacteria and coincubated for up to 24 hr, then analyzed by RTqPCR for hBD2 and hBD3 expression. Transcript expression was normalized to mock-inoculated cells, and is shown as the average of three or four independent experiments. Average MOI are: 6.9 for *L. acidophilus,* 7.3 for *L. crispatus,* 9.7 for *L. gasseri,* 7.3 for *L. jensenii,* 7.3 for *L. johnsonii,* 3.4 for *L. vaginalis,* 3.7 for *A. vaginae,* 3.1 for *G. vaginalis,* 3.6 for *M. curtisii,* and 3.9 for *P. bivia*. Asterisks indicate a significant ($p<0.05$) increase in expression over mock-treated cells for at least one timepoint.

To confirm the ability of *L. vaginalis* to induce an innate immune response similar to BVAB, we utilized ELISA to measure soluble IL-6, IL-8, and hBD2 proteins secreted by epithelia after bacterial inoculation. In comparison to the other five lactobacillus strains, *L. vaginalis* consistently induced a heightened response (Figure 2.6).

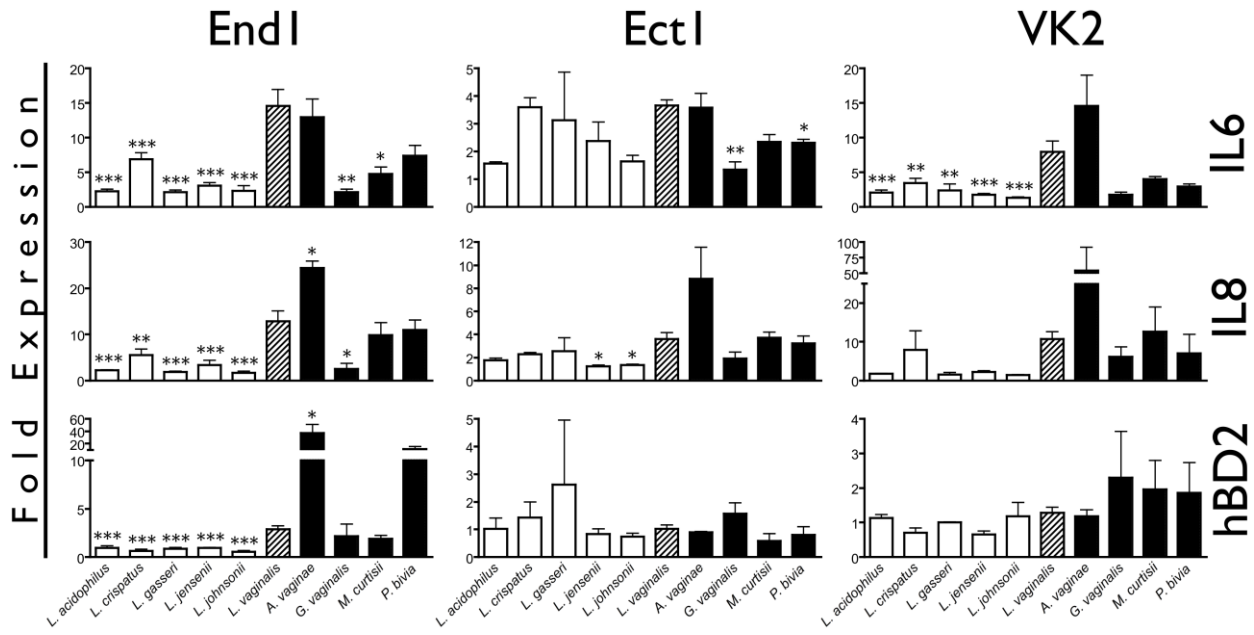


Figure 2.6. *L. vaginalis* Induces a Greater Immune Response than Other Vaginal Lactobacilli.

Confluent monolayers of reproductive epithelia were inoculated with commensal lactobacilli or BVAB, and after 24 hr conditioned media was analyzed for IL-6, IL-8 and hBD2 protein. BVAB are filled black bars, *L. vaginalis* is hatched, and all other lactobacilli are white bars. MOI are the same as in Figure 5. Protein is shown as fold expression compared to a mock-treated condition, and one, two, or three asterisks indicate values that are significantly ($p<0.05$, $p<0.01$, or $p<0.001$, respectively) different from the *L. vaginalis*-treated condition.

In End1 cells, *L. vaginalis* stimulated >10-fold increase in both IL-6 and IL-8, which was significantly higher than all other lactobacilli tested. In agreement with our previous results, End1 cells were the only epithelia to produce considerable amounts of soluble hBD2 protein (picograms/mL) after coculture with bacteria, with *L. vaginalis* inducing significantly higher hBD2 levels than the other *Lactobacillus spp*.

Finally, we performed Bio-Plex analysis to determine whether *L. vaginalis* promoted a cytokine environment similar to that observed clinically and in our coculture model of known BVAB (Figure 2.7).

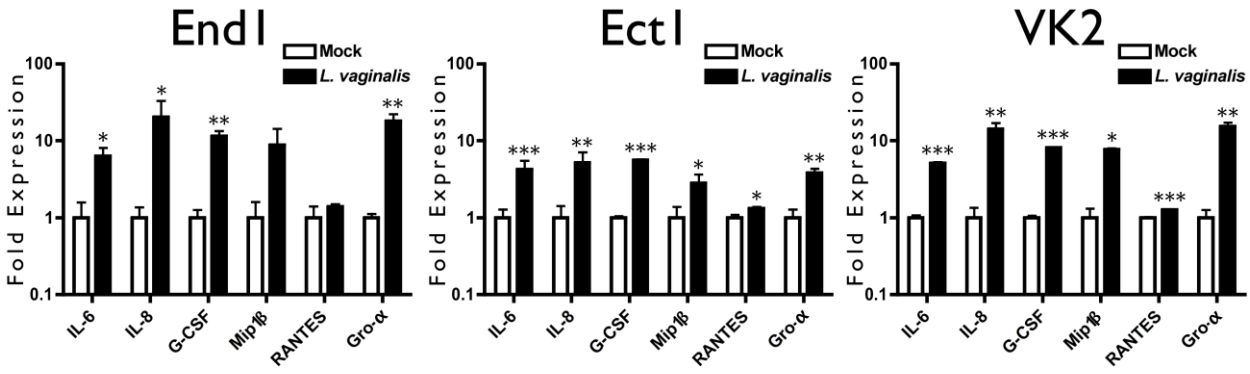


Figure 2.7. *L. vaginalis* Initiates an Innate Immune Response from FRT Epithelia. Confluent monolayers of epithelial cells were inoculated with bacteria and coincubated for 24 hr. Conditioned media were collected, clarified and ELISA was used to quantify hBD2, IL-6 and IL-8 concentrations. MOI is the same as in Figure Five. Analyte concentrations are shown as fold induction compared to mock condition, and are the average of three independent experiments where one ($p<0.05$), two ($p<0.01$), or three ($p<0.001$) asterisks indicate a significant increase over the mock-treated condition.

We observed that *L. vaginalis* stimulated significant upregulation of cytokines compared to mock-treated conditions. Analytes IL-6 and IL-8 were recovered at picogram/mL concentrations, corresponding to the magnitude recovered from BVAB cocultures. In agreement with other indicators, the cytokine trends elicited by *L. vaginalis* mirrored cytokine responses to BVAB and clinical BV trends, suggesting that this lactobacillus strain induces an innate immune response in reproductive epithelia.

2.4 Discussion

In this study, we evaluated the immune response initiated by female reproductive epithelial cells in response to a comprehensive panel of ten bacteria, both commensal and pathogenic in nature, and showed that the overall cytokine response to bacterial pathogens aptly reflected the heightened cytokine environment that characterizes BV. Our side-by-side comparison of cytokine upregulation between our model and clinical BV samples demonstrated the appropriate recapitulation of physiological trends by our coculture method.

Like clinical CVLs, our epithelial coculture demonstrated upregulation of proinflammatory cytokines in response to pathogenic bacteria. While our model did reflect the heightened inflammatory environment created by IL-8, Gro-α and hBD2, other cytokines that are known to be upregulated in BV, including IL-1α and IL-1β [39], were upregulated in epithelia by stimulatory bacteria, but were not produced at appreciable levels. This is in agreement with the literature, which reports monocytic cell

lineages as the major producers of IL-1 [65], thereby suggesting that other cell types in the reproductive tract likely contribute these factors to the BV milieu.

By using three different epithelial cell types, we demonstrated distinct differences in the immune responsiveness of epithelia along the reproductive tract. We consistently observed a heightened immune response from endocervical epithelia compared to ectocervical or vaginal cells. Our results suggest that the naturally colonized epithelia of the vagina and ectocervix display an attenuated immune response, perhaps in order to minimize excessive inflammatory recruitment triggered by transient changes in the dynamic microflora of the vagina. In line with this hypothesis, Ect1 and VK2 cells exhibited a markedly less robust hBD2 response to stimulatory bacteria (Figure 2.3 and Figure 2.4). On the other hand, the endocervix acts as a transition zone to the sterile upper reproductive tract, and is not densely colonized by bacteria. Accordingly, bacterial contact results in a considerable increase in cytokine and defensin protein production; an appropriate response considering that pathogenic bacterial contact could threaten the sterility of the upper female reproductive tract [66,67]. These findings demonstrate the utility of this model in characterizing epithelial function and behavior in the FRT.

In addition to evaluating three types of host epithelia, we also characterized relative stimulatory activity of ten FRT bacterial species, the most thorough comparison reported. We designed our cocultures so that BVAB were applied at a lower MOI than lactobacilli, and additionally found that they were recovered at lower density than lactobacilli at the end of the coculture. Still, BVAB induced an immune response greater than that induced by lactobacilli. It is likely that *in vivo* this difference in stimulatory

activity is greater yet, considering that in BV pathogenic bacteria actively outgrow commensal lactobacilli. This implied difference in metabolic and proliferative capacity suggest that our report, though demonstrating considerable stimulation by BVAB, could be a conservative representation compared to *in vivo* stimulatory magnitude.

We observed that the BVAB, *A. vaginae,* induced the most robust response from all three epithelia as determined by cytokine and defensin upregulation. This is in concordance with recent research that shows *A. vaginae* as a more specific marker of clinical BV symptoms, and a stronger inducer of immune response than *G. vaginalis* [44, 68, 69]. In fact, while *G. vaginalis* was the first pathogen associated with BV [22], more thorough microbiome studies report the frequent isolation of *G. vaginalis* from BV-negative women [70], and in our analyses this bacterium induced responses similar in magnitude to the majority of commensal lactobacilli. This finding emphasizes the value of basic coculture studies to assist in identifying BVAB of clinical significance, and provides a framework in which additional strains may be evaluated for relative stimulatory activity.

When we expanded our analysis to characterize the β-defensin response of End1 cells, we observed significant upregulation of the inducible effector hBD2 that was both freely soluble and cell-associated. This is the first report to quantify cell-associated reservoirs of hBD2 in FRT epithelia, and these findings shed light on conflicting clinical data, which have reported both significant increases and significant decreases in hBD2 protein concentrations in the context of BV. Our data suggest that while hBD2 transcription is significantly upregulated in response to stimulatory bacteria, recovered soluble protein may not accurately depict this induction. *In vivo*, this may be partially

due to dilution of total vaginal protein as a result of increased vaginal fluid discharge (a hallmark symptom of BV), but might also be attributed to retention of hBD2 protein as cell-associated protein. Furthermore, we demonstrated a considerable difference in the hBD2 protein production by different types of reproductive epithelia. In considering these factors, differences in sample method and sample site might contribute to the variation observed in hBD protein recovery from the FRT.

In total, we recovered on average 3.6 ng hBD2 per 100 mm dish, with 1.6 ng remaining cell-associated and 2 ng being freely secreted. The soluble portion of hBD2 alone is unlikely to reach antimicrobial concentrations (μg/mL levels) when secreted lumenally into the vaginal canal and diluted in vaginal fluid. However, it is likely that hBD2, secreted toward the basal submucosa, would reach chemotactic concentrations (30 ng/mL when the total protein is divided by cell monolayer volume). hBD2 is even more likely to achieve these levels at the basal cell surface if maintained in concentrated extracellular domains [71]. Extracellular stores of hBD2 may thus provide haptotactic stimuli for migrating lymphocytes, suggesting that hBD2 is not antibacterial, but rather chemotactic, in the setting of BV [72]. In line with our findings, an increased percent of CD4-positive lymphocytes has been reported in BV-positive vaginal fluid [73]. This may contribute to the increased HIV susceptibility that is associated with BV, as increased CD4-positive target cells concentrated in reproductive tissues may facilitate initial HIV infection. The chemotactic potential of defensin induction may represent an unfortunate host mediator of pathogenic processes.

Finally we used regulation of hBD2, IL-6 and IL-8 to evaluate the relative immune stimulation elicited by ten different vaginal bacteria. By all readouts, epithelial response

to *L. vaginalis* was generally higher than the response to other lactobacilli. This was especially clear when analyzing the response from End1 epithelium, the most sensitive of the three cell types. The immune response elicited by *L. vaginalis* extended to the characteristic cytokine profile we observed for BVAB and for clinical CVL patterns, suggesting that this bacteria, unlike the other lactobacilli evaluated, induces an immune response from host cells that mimics a pathogen-triggered reaction. Clinical evaluation of *L. vaginalis* in reproductive afflictions is sparse, as few studies discern between different species of lactobacilli to obtain species-specific microbiome data [45, 46, 58]. However a recent study demonstrated that within a small sample group, *L. vaginalis* was cultured from 30% of 'normal' FRT individuals, and 50% of 'disturbed' FRT individuals (i.e. women with frequent BV-like vaginal microflora) [54], suggesting that this species may indeed play a role in FRT pathogenesis.

While the complexity of the FRT microbiome is just recently being appreciated, associations between individual bacteria and pathogenic sequelae remain uncharacterized. A recent report demonstrated associations between individual bacterial inhabitants and specific Amsel's criteria [50]. Likewise, it stands to reason that the cytokine and defensin responses observed in BV are associated with certain specific bacterial subsets. Our results support this hypothesis, by demonstrating significant differences between the stimulatory capacities of individual BVAB. Combined with the growing appreciation of FRT microbiome diversity, our observations support reevaluation of FRT bacteria by coculture techniques in order to distinguish stimulatory bacterial strains from inert inhabitants.

3. HIV-ENHANCING EFFECTORS ARE SECRETED UPON REPRODUCTIVE EPITHELIA INOCULATION WITH BACTERIAL VAGINOSIS-ASSOCIATED BACTERIA

3.1 Introduction

FRT innate immunity is highly dependent on the physical and immunological barrier functions of the FRT epithelia. Any perturbation of the epithelium can lead to increased susceptibility to infection, including HIV acquisition. While physical damage (such as microabrasion or lesion), or altered immune function (such as coinfection) are in some instances contributing risk factors, in the case of BV, it is likely the *increased* immune response to pathogenic bacteria that contributes to heightened HIV susceptibility [74].

Immunologically, the FRT epithelia contribute a steady input of antimicrobial effectors, including host defense peptides, to the cervicovaginal fluid. Among these host defense peptides are the alpha- and beta- defensins (e.g. HNP5 and hBD2) [75, 76], and peptides belonging to the whey acid protein family (e.g. SLPI and trappin-2/elafin) [77, 78]. As a result, cervicovaginal fluid exhibits potent antibacterial and antiviral activity, which is accomplished by the synergistic contribution of each individual effector [10]. In addition to basal immune functions, FRT epithelial cells are equipped with Toll-Like Receptors (TLRs) that recognize pathogen-associated molecular patterns, including the conserved molecular motifs present in bacteria and other pathogens [79]. Upon pathogen stimulation, TLRs initiate a signaling cascade that upregulates effector production, including host defense peptides and cytokines [80]. These small proteins can exhibit inherent antimicrobial activity to combat pathogens, or might serve to recruit

additional immune cells to the threatened FRT tissue [40].

The immune response initiated in FRT epithelia upon stimulation with BVAB is characterized by increased secretion of host defense peptides and cytokines [40], and this response is implicated in increasing downstream HIV infection by multiple mechanisms. First, hBD2 upregulation in FRT epithelium is implicated in recruitment of lymphocytes, target cells for HIV infection [40]. Second, intruding BV-associated bacteria induce an inflammatory host response that could increase HIV susceptibility by activating NF-κB, a major transcription factor driving HIV genomic replication [80, 81]. Each of these host immune responses represents a possible mechanism by which BV enhances HIV susceptibility, making it of high priority to characterize the interactions between BV-associated pathogens and host immunity.

We previously evaluated the immune interactions between three types of FRT epithelia and 10 FRT bacteria. We observed that one FRT epithelial cell type, End1, is highly responsive to BVAB, and can be used as a sensitive indicator of pathogenic interactions. At the same time, we demonstrated that of the 10 tested bacterial species, one in particular was a potent pathogen: *Atopobium vaginae*. In this report, we extend these observations to describe how these host-pathogen immune interactions affect downstream HIV infection.

As BVAB induce an immune response in FRT epithelia, we hypothesized the soluble effectors secreted by epithelia in response to pathogenic bacteria might increase downstream HIV infection of target cells. To test this hypothesis, we cocultured End1 epithelia with the pathogenic BVAB *A. vaginae*. We then recovered secreted low molecular weight effectors, and found that they increased HIV infection.

These effectors were identified by a multi-faceted proteomic approach, and select components were assayed individually for proviral activity, but were unable to restore the activity of the complete mixture. However, when select proteins were assayed in the presence of the active CM fraction, each exhibited HIV-enhancing activity. This report suggests that just as antiviral host peptides synergize to inhibit HIV, proviral effectors may work in concert to increase HIV susceptibility as part of the immune response to BV-associated bacteria.

3.2 Materials and Methods

3.2.1 Reagents and Materials

Keratinocyte serum free media (KSFM) with supplements were from Invitrogen Life Technologies. EDTA/Trypsin (0.25% and 0.05%), DMEM, DMEM/F12, Ham's F12 and DPBS were from MediaTech, Inc. Fetal bovine serum (FBS) was from Gemini Bio-Products. Amicon Filters were purchased from Millipore.

3.2.2 Epithelial Cultures

The following human epithelia were purchased from American Type Culture Collection (ATCC): End1 (CRL-2615) from endocervix; Ect1 (CRL-2614) from ectocervix; VK2 (CRL-2616) from vagina. These were maintained according to ATCC instructions. TZM-bl cells (Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.) were acquired from the National Institutes of Health AIDS Research and Reference

Reagent Program, and were maintained in DMEM 10%FBS.

3.2.3 Bacterial Cultures

The bacterial culture *Atopobium vaginae* (BAA-55) was purchased from ATCC. *A. vaginae* was grown in tryptic soy broth (TSB) with 5% defibrinated rabbit blood (Becton, Dickinson and Company), or on equivalent agar plates in anaerobic GasPaks (Becton, Dickinson and Company) at 37°C. To achieve consistency in bacterial preparations, maintenance cultures of each species were aliquoted and snap frozen by submerging in liquid nitrogen for 2 hr, then transferred to -80°C until use.

3.2.4 Inoculation of Epithelia with *A. vaginae*

Epithelia were grown to confluency on 100 mm tissue culture-treated plates (Techno Plastic Products) in KSFM with bovine pituitary extract, recombinant epidermal growth factor, and calcium chloride supplements as instructed by ATCC. To prepare inocula for experiments, snap-frozen aliquots of *A. vaginae* were thawed, and desired volumes were centrifuged. Supernatants were aspirated, and bacteria were resuspended in Ham's F12 media. The maintenance KSFM was aspirated from each 100 mm plate of epithelia, and 8 mL of the resuspended inoculum was applied to each plate. Plates were returned to 37°C/5% CO_2 for the duration of the experiment. In all experiments, epithelia were inoculated at a multiplicity of infection (MOI) of 3.3, which was calculated as the number of bacterial colony forming units (CFUs) divided by the number of epithelial cells in a given coculture condition. The number of bacterial CFUs

was determined by serially diluting the inocula and plating on appropriate media for back-calculation density.

3.2.5 Conditioned Media Preparation

At the experimental endpoint, 24 mL conditioned media (CM) from three 100 mm dishes were pooled and centrifuged at 4,000X g for 10 min at 4°C to clarify. Clarified CM were stored at -20°C until fractionation. To fractionate CM, the pooled CM was subjected to filtration through an Amicon 30 KDa molecular weight cut off (MWCO) filter. The filter retentate was subjected to repeated concentration by filtration then dilution with water in order to desalt. This procedure was repeated until the final salt concentration was negligible (0.01% of the original Ham's F12 composition). The retentate, which contained proteins >30 KDa, was equilibrated to 1 mL with water and stored at -20°C for subsequent experiments. The flowthrough from this filtration was also subjected to similar desalting on an Amicon 3 KDa MWCO filter. Desalting was repeated until salt concentrations were 0.01% of the original composition, and this 3-30 KDa fraction was equilibrated to 1 mL in water and stored at -20°C for subsequent experiments. For downstream cell culture experiments, equal volumes of these 24X desalted CM were combined with 2X DMEM to achieve 1X DMEM equivalent in cell culture. For Bio-plex analysis, 24X CM fractions were diluted 1:10 in sterile DPBS and assayed according to manufacturer's instructions.

3.2.6 HIV Enhancement Assay

TZM-bl cells were seeded at 5,000 cells per well in a 96-well black wall, clear bottom plate. Cells were grown for 48 hr in DMEM 10% FBS prior to experiment. For treatment, media were aspirated from each well, and replaced with 20 µL of 5X FBS diluted in DMEM, 70 µL of CM fraction or recombinant protein prepared in DMEM (all CM treatments equilibrated to 1X DMEM prior to addition) and 10 µL of 10X viral inoculum. Final conditioned were 1%, 5% or 10% FBS, with 2X, 4X or 8X CM (relative to original media volume) and HIV-1 inocula equivalent to 4 ng/mL of p24 antigen. Treatments were gently pipetted to mix, and incubated at 37°C/5%CO_2 for 4 hrs. After 4 hr, wells were aspirated, and treatment media were replaced with identical FBS and CM or recombinant protein conditions, but without HIV. These cultures were incubated for an additional 20 hr at 37°C/5%CO_2. At 24 hr post-infection, media were aspirated from all wells, and 100 µL Glo Lysis Buffer was applied to each well. Plates were sealed and stored at -80°C. Plates were later thawed at room temperature, then 100 µL BrightGlo Reagent was added to each well. Luciferase activity was immediately quantified on a Luminex 96 well plate reader with a 5 s integration time per well. An uninfected control well was also measured, and the background relative light units (RLUs) subtracted from all experimental wells.

3.2.7 Trypan Blue Viability Assay

TZM-bl cells were seeded at 5,000 cells per well in a clear 96-well plate and grown for 48 hr in DMEM 10% FBS prior to experiment. For treatment, media were

aspirated from each well, and replaced with treatments identical to those in the HIV Enhancement Assay, except that 1X DMEM was added in place of viral inoculum. After 4 hr, media were aspirated, and treatment media were replaced with identical FBS and CM conditions. These cultures were incubated for an additional 20 hr at 37°C/5%CO_2. At 24 hr post-treatment, media were aspirated from all wells. Wells were washed with 70 µL warmed PBS, then 30 µL warm 0.05% trypsin with EDTA was applied to each well. The plate was incubated for 5 min at 37°C/5%CO_2, then 120 µL warm DMEM 10%FBS was added to each well. Wells were resuspended by pipetting, and cells were transferred to a round-bottom plate, which was spun at 300X g for 5 min to pellet the cells. 130 µL of the culture supernatent was aspirated, and the cells were resuspended in the remaining 20 µL. An equal volume of 50% trypan blue in PBS was added to each well, and samples were thoroughly resuspended. 10 µL of this resuspension was loaded onto a hemacytometer, and live cells were identified by their exclusion of trypan blue.

3.2.8 Tricine-SDS PAGE for MS Analysis

420 µL of 24X desalted CM (3-30 KDa fraction) was concentrated and resolved by Tricine-SDS PAGE. Proteins were visualized by silver stain method. Bands were excised and stored at -20°C until analysis by mass spectrometry.

3.2.9 Tricine-SDS PAGE for Immunoblotting

Desired volume of 24X desalted CM (3-30 KDa fraction) was concentrated and resolved by Tricine-SDS PAGE. The following recombinant proteins were used as blotting standards: Lipocalin-2 with histidine tag (ab95007) from Abcam; Cyclophilin-A with histidine tag (ab86219) from Abcam; Trappin-2 with histidine tag (CA39) from Novoprotein; HE4 with histidine tag (C550) from Novoprotein. Gels were transferred to PVDF membranes and immunoblotted with the following rabbit polyclonal antibodies: anti-Lipocalin-2 (ab41105) from Abcam; anti-Cyclophilin-A (07-313) from Millipore; anti-Trappin-2 (sc-20637) from Santa Cruz Biotechnology; anti-HE4 (ab85179) from Abcam.

3.2.10 Mass Spectrometry

For analysis of desalted CM (3-30 KDa fraction), 1 mL of 24X CM was concentrated and brought to final volume 50 µL in 50 mM NH_4CO_3. Subsequent reduction, alkylation, quenching and enzyme reagents were also prepared in 50 mM NH_4CO_3; DTT was added to final concentration of 10 mM, and sample was heated to 95°C, then allowed to cool slowly to reduce. Iodoacetamide was added to final concentration 75 mM, and sample was incubated 45 min at ambient temperature protected from light to alkylate. Alkylation was quenched by the addition of excess DTT to final concentration 50 mM, and sample was incubated an additional 45 min room ambient temperature. 1 µg trypsin, prepared in 50 mM NH_4CO_3, was added to the sample, and allowed to digest for 14 hr at 37°C. Sample clean-up was performed on MacroSpin Silica C18 Column (The Nest Group, Southborough, MA, USA) according to

manufacturer's instructions. Eluted proteins were analyzed on a QSTAR-ELITE quadrupole TOF, and resulting MS/MS spectra were searched against Mascot IPI_human database.

3.2.11 Statistical Analyses

For viral enhancement assays of CM at different concentrations, two-way ANOVA with Bonferroni posttests were used to compare fold infection in each epithelial condition to the no epithelial condition, or to compare RLUs in the mock condition to the *Atopobium* condition. For cytokine analysis, paired t-tests were used to directly compare mock and *A. vaginae*-inoculated conditions for each cytokine. For TZM-bl trypan assays, unpaired t-tests were used to compare viability in treated conditions to media controls. For recombinant protein viral enhancement assays, one-tailed upaired t-tests were used to compare treated conditions to untreated controls.

3.3 Results

3.3.1 *A. vaginae* Stimulation Enhances Proviral Activity of FRT Epithelial Conditioned Media

Women experiencing BV are known to have a 60% increased risk of acquiring HIV via heterosexual exposure [20]. Since FRT epithelia are the primary cells contacting invading pathogens in the FRT, we hypothesized that BVAB would induce a response in reproductive epithelia that would enhance HIV infection. Further, we hypothesized that the proviral effect would be mediated by soluble, low molecular

weight effectors, since many small cytokines and antimicrobial peptides regulated by the epithelial innate immune response also alter HIV susceptibility [81, 82]. Therefore, we sought to characterize the effect of these factors on HIV infection.

To isolate the secreted, low molecular weight factors produced by reproductive epithelial cells in response to the BVAB, we inoculated End1, Ect1, or VK2 cells, or a no epithelial control condition with the BVAB *Atopobium vaginae*. Matched mock-inoculated conditions received a media change. After a 24 hr coincubation, the conditioned media (CM) was collected and clarified. The CM was then subjected to sequential filtration to obtain 3-30 KDa and >30 KDa fractions, which were assayed for proviral activity using the HIV reporter line TZM-bl cells. Figure 3.1 demonstrates the proviral effect of CM fractions collected from *A. vaginae*-inoculated epithelia compared to mock-inoculated epithelia.

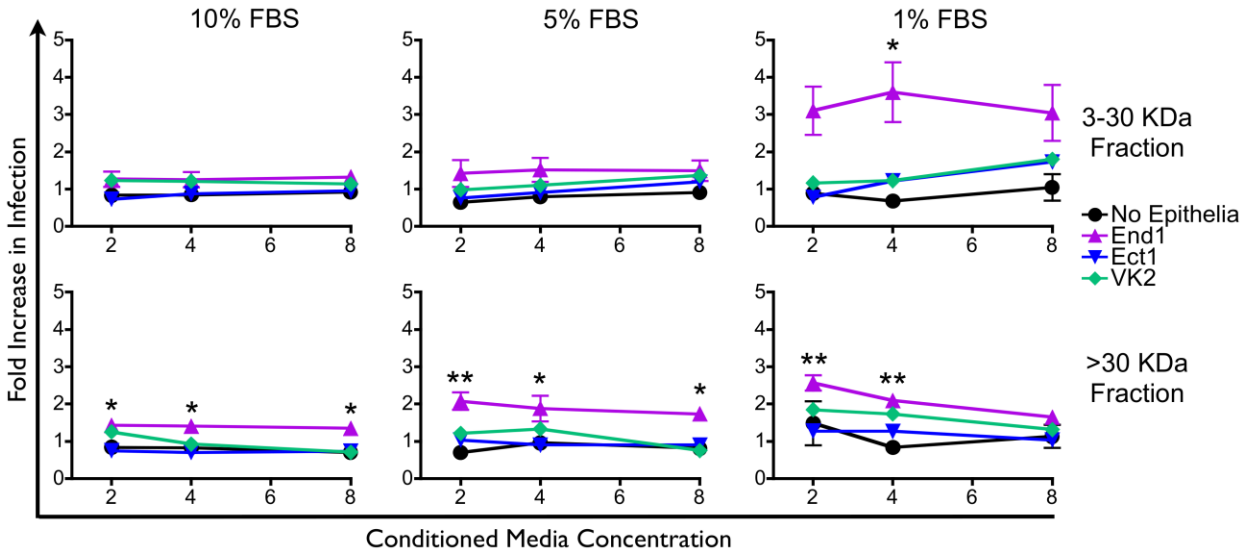


Figure 3.1. *A. vaginae* Stimulation Significantly Enhances the Proviral Activity of Endocervical Epithelial Conditioned Media Fractions.

TZM-bl cells were infected with HIV-1 BaL in the presence of 1-10% FBS and CM fractions from mock- or *A. vaginae*-inoculated epithelial cells. CM fractions of 3-30 KDa or >30 KDa were applied at final concentrations of 2-8X original concentration. Fold increase in infection was obtained by dividing the *Atopobium*-treated CM condition by the mock-treated CM condition. n=2 for all conditions except for End1 conditions, which are n = 3-8, and No Epithelia 1% FBS conditions, which are n = 3. One, two or three asterisks indicate a significant ($p<0.05$, $p<0.01$ or $p<0.001$, respectively) increase in infection of the End1 condition compared to No Epithelia condition.

Importantly, we found that decreasing the FBS concentration from 10% to 1% during the reporter assay incubation improved sensitivity, and revealed significant proviral effects of both the 3-30 KDa and >30 KDa CM fractions generated from End1 cells inoculated with *A. vaginae* CM condition compared to the mock CM condition.

While the >30 KDa fraction demonstrated a more significant increase in infection (compared to the no epithelial control), the 3-30 KDa exhibited a greater fold increase in infection. Importantly, in neither fraction did we observe considerable increased infection for the *A. vaginae*-inoculated no epithelia control condition, indicating that CM fractions obtained from *A. vaginae* incubation alone did not increase HIV infection.

To further evaluate this trend, we examined the raw data obtained by assaying each End1 CM fraction separately, or by combining and assaying them together. Figure 3.2 panel A compares the fold increase in infection mediated by *A. vaginae*-inoculated End1 CM over mock-inoculated End1 CM for the 3-30 KDa fraction, the >30 KDa fraction, and the two fractions combined. The combined fractions exhibited a fold increase trend similar to that observed for the >30 KDa fraction alone.

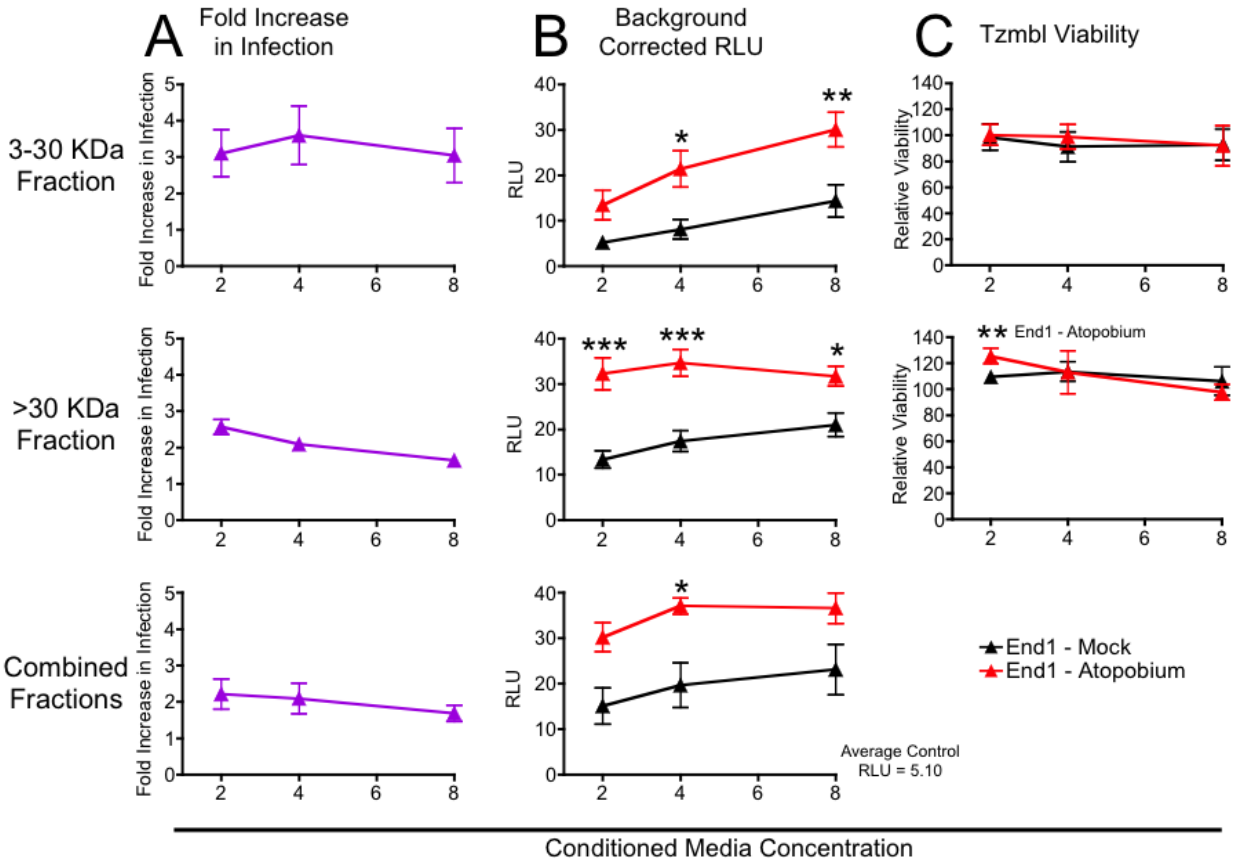


Figure 3.2. 3-30 KDa CM Fraction from End1 Epithelia Exhibits Specific Proviral Activity. TZM-bl cells were treated and infected in the presence of indicated 3-30 KDa fraction, >30KDa fraction, or the two fractions combined at 2-8X concentrations in the presence of 1% FBS. A) Viral enhancement by *Atopobium,* obtained by dividing the *Atopobium*-treated CM condition by the mock-treated CM condition. B) Background-corrected RLU readings. One, two or three asterisks indicate a significant ($p<0.05$, $p<0.01$, and $p<0.001$, respectively) difference between mock and *Atopobium* conditions. n=8 for individual fraction conditions and n=3 for combined condition. C) Matched wells treated with CM fractions from End1 cells or No Epithelia condition were treated in parallel, but not infected with virus, and viability at experiment endpoint was quantified by trypan blue. Viability was normalized to a media-only control condition. Two asterisks indicate

a significant ($p<0.01$) increase compared to media-only control condition. n=3-4 for each condition.

This phenomenon is better interpreted by analyzing the corrected RLU values shown in Figure 3.2 panel B. For both the >30 KDa fraction and the combined fractions, even the mock-inoculated CM condition exhibited proviral activity; at 2X concentration, these treatments resulted in >10 RLU, a >2-fold increase when compared to the average of the media-only control well (5.10 RLU). Though the matched *A. vaginae*-inoculated CM treatments increased RLU readouts to approximately 30, the heightened baseline of the mock-inoculated CM treatments resulted in fold increased infection values of less than three. Further, the RLU measurements of these mock-inoculated CM treatments increased steadily with increased treatment concentration, whereas the *A. vaginae*-inoculated condition exhibited a plateau in RLU readouts, resulting in the decreasing fold increase in infection observed for these fractions. These observations are clarified by Figure 3.2 panel C, which reveals significant changes in TZM-bl cell viability upon treatment with the *A. vaginae*-inoculated >30 KDa CM fraction. At 2X concentration, this fraction induced significant growth of the TZM-bl cell population, likely contributing to the calculated increased fold infection. As treatment concentration increased, viability steadily decreased, which coincided with the plateau in measured RLU.

Oppositely, the 3-30 KDa fraction induced lower background RLU when the mock-inoculated CM condition was applied at 2X concentration (RLU <10). Though increasing concentrations of this mock condition treatment did result in increased RLU

readouts, the *A. vaginae*-inoculated treatment induced higher infection at all concentrations, and steadily increased in a dose-dependent fashion. This resulted in a greater fold increase in infection at all concentrations tested. Further, this 3-30 KDa fraction did not induce changes in TZM-bl viability, suggesting that the observed trends were likely on account of differences in infection, rather than merely assay viability conditions. Therefore, we chose to pursue the *A. vaginae*-induced proviral factors in this 3-30 KDa fraction.

3.3.2 Cytokines Do Not Partition to the Proviral 3-30 KDa CM Fraction

Since inflammatory cytokines are known to be upregulated in epithelia in response to BVAB (including *A. vaginae*), and furthermore are proposed to enhance HIV infection, we hypothesized that cytokines might be upregulated in the *A. vaginae*-inoculated 3-30 KDa CM fraction, and contribute to the observed viral enhancement. To evaluate this possibility, we utilized Bio-Plex cytokine array to quantify 37 cytokines and growth factors in both the 3-30 KDa fraction and the higher molecular weight >30 KDa fraction. To our surprise, nearly the entirety of the secreted cytokines partitioned to the >30 KDa fraction, as shown in Figure 3.3.

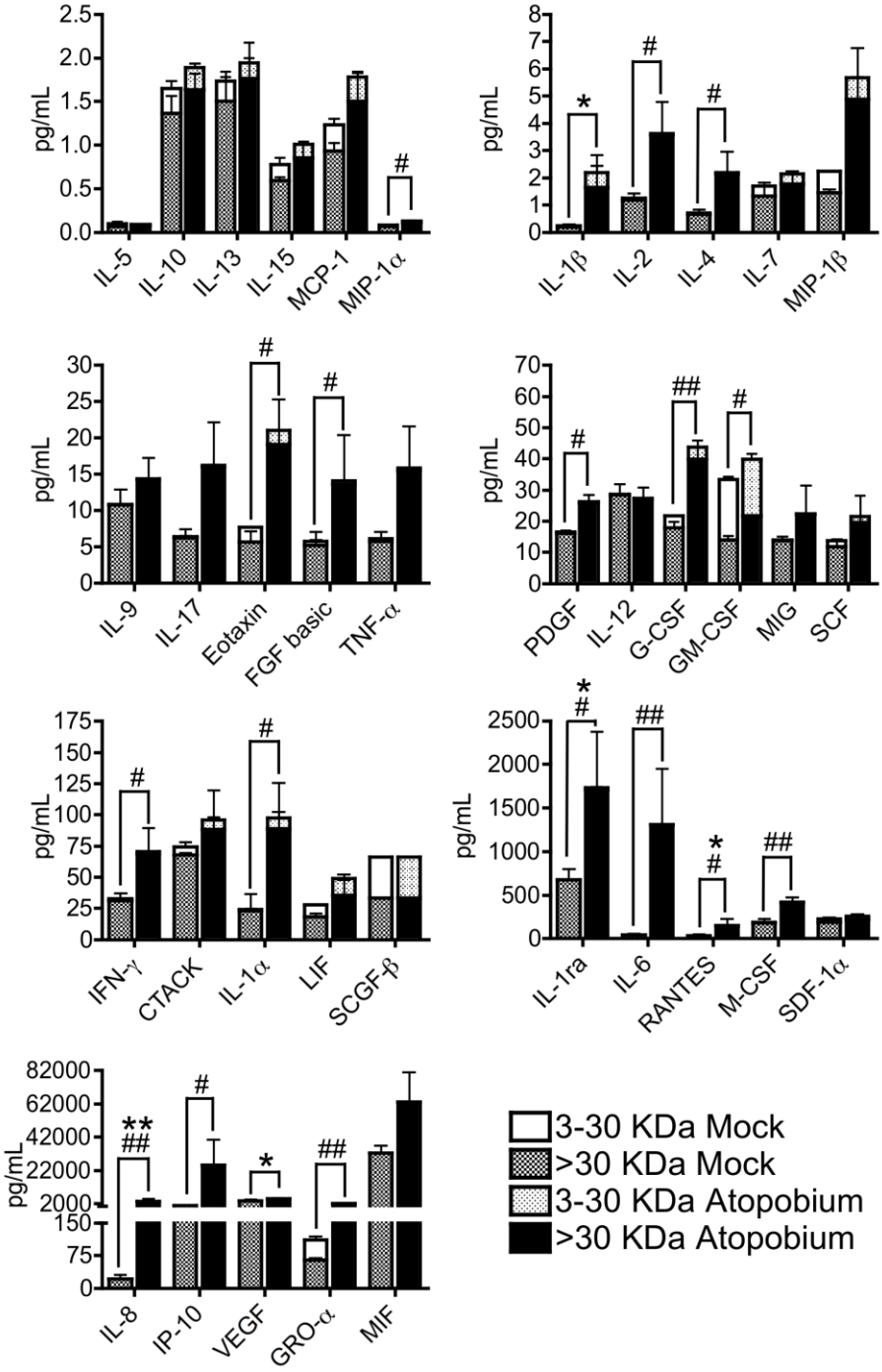


Figure 3.3. Proviral Conditioned Media Fraction Does Not Contain Proviral Cytokines. 24X CM fractions from mock or *A. vaginae*-inoculated End1 cells were analyzed by Bio-plex cytokine array. Each bar demonstrates cytokine recovery for 3-30 KDa fraction

stacked above matched >30 KDa fraction for either mock or *A. vaginae* conditions. One or two asterisks indicate significant ($p<0.05$ or $p<0.01$, respectively) differences in cytokine concentration between mock- and *Atopobium*-inoculated 3-30 KDa fraction, while one or two pound signs indicate significant ($p<0.05$ or $p<0.01$, respectively) differences in >30 KDa fraction. n = 3 for all panels.

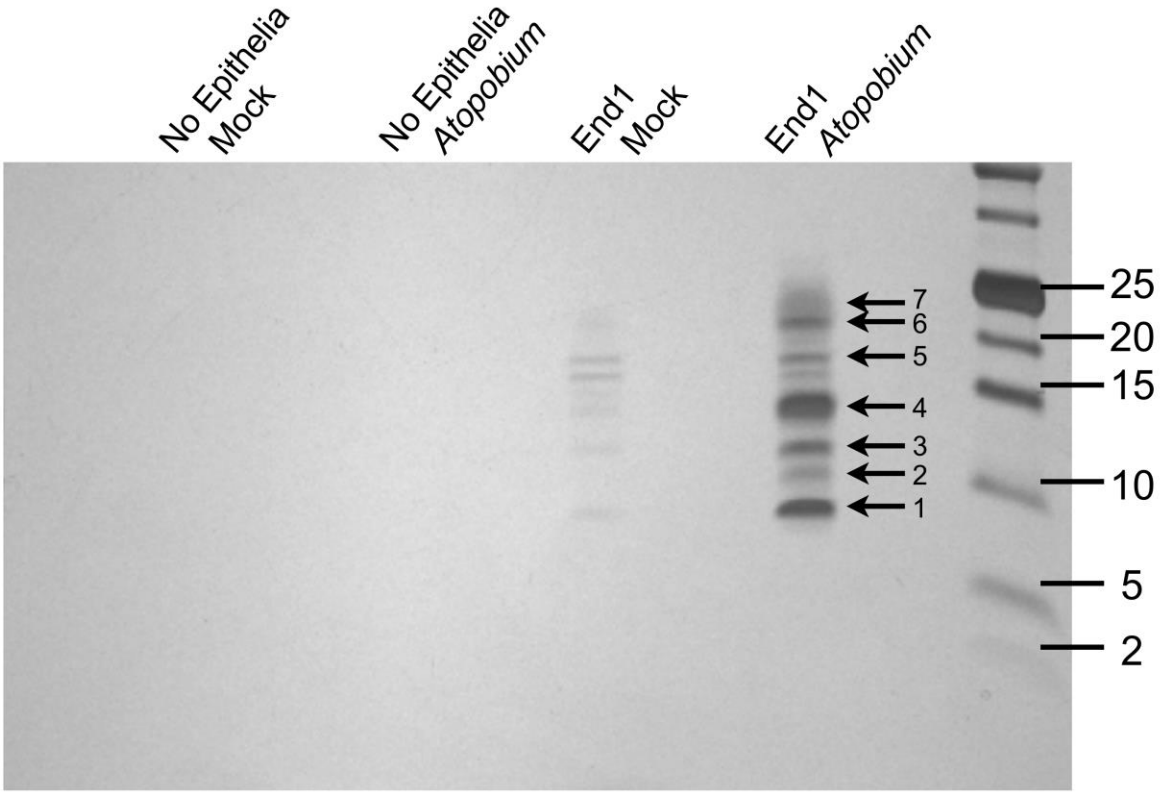
Though a few cytokines showed significant upregulation in the *A. vaginae*-inoculated 3-30 KDa CM fraction, the majority were measured at low pg/mL concentrations (for IL-1β, IL-1ra, RANTES and VEGF, all values were <25 pg/mL). One cytokine, IL-8, was increased from 0.06 pg/mL to 200 pg/mL in the 3-30 KDa fraction derived from *A. vaginae*-inoculated cells, but this concentration appeared to be a minor flowthrough compared to the 3140 pg/mL concentration that partitioned to the matched >30 KDa fraction. These observations suggested that most cytokines did not partition to the 3-30 KDa CM fraction, and therefore were not likely to mediate the observed HIV enhancement observed upon *A. vaginae* inoculation.

3.3.3 Mass Spectrometry Reveals Over 60 Protein Components in the Proviral Fraction

We next pursued a proteomic approach to identify the proviral factors in the stimulated 3-30 KDa CM fraction. The *A. vaginae*-inoculated End1 3-30 KDa CM fraction was concentrated and subjected to ESI-QUAD-TOF analysis, and referenced against the Mascot IPI_human database. Over 60 protein identities were returned (Appendix C: Table C.1). In accordance with our previous results, none of the cytokines

assayed by Bio-Plex cytokine array were identified in the active fraction by mass spectrometry. Additionally, many of the returned identities could be assigned as residual carry over from maintenance media (including insulin and bovine pituitary proteins). To narrow the remaining entries, we utilized a second proteomic approach, in which the 3-30 KDa CM fraction was resolved by PAGE and visualized by silver stain. Figure 3.4 panel A demonstrates proteins recovered in the 3-30 KDa CM fraction from media alone, *A. vaginae* alone, End1 cells alone, or End1 cells inoculated with *A. vaginae*.

A



B

Protein	Sequence
Cyclophilin A	MVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHRIIPGFMCQGGD FTRHNGTGGKSIYGEKFEDENFILKHTGPGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKV KEGMNIVEAMERFGSRNGKTSKKITIADCGQLE
HE4	MPACRLGPLAAALLLSLLLFGFTLVSGTGAEKTGVCPELQADQNCTQECVSDSECADNLKCCSAGC ATFCSLPNDKEGSCPQVNINFPQLGLCRDQCQVDSQCPGQMKCCRNGCGKVSCVTPNF
Lipocalin 2	MPLGLLWLGLALLGALHAQAQDSTSDLIPAPPLSKVPLQQNFQDNQFQGKWYVVGLAGNAILREDK DPQKMYATIYELKEDKSYNVTSVLFRKKKCDYWIRTFVPGCQPGEFTLGNIKSYPGLTSYLVRVVS TNYNQHAMVFFKKVSQNREYFKITLYGRTKELTSELKENFIRFSKSLGLPENHIVFPVPIDQCIDG
Trappin-2	MRASSFLIVVVFLIAGTLVLEAAVTGVPVKGQDTVKGRVPFNGQDPVKGQVSVKGQDKVKAQEPVK GPVSTKPGSCPIILIRCAMLNPPNRCLKDTDCPGIKKCCEGSCGMACFVPQ

Figure 3.4. Several Proteins are Enriched in Proviral CM Fraction.

A) Desalted 3-30 KDa CM fractions were concentrated and resolved by Tricine SDS-PAGE, then visualized by silver stain. Indicated bands were excised for MS analysis. Gel image is one representative of three independent experiments. B) Four proteins

that were identified in both the soluble 3-30 KDa fraction and in excised SDS-PAGE bands are shown. Entire sequence (including signal) for each protein is annotated with ions identified in the initial soluble MS approach highlighted red, while ions identified in SDS-PAGE bands are underlined.

No proteins could be visualized in the media control, or *A. vaginae* alone conditions. The End1 condition did contain low molecular weight proteins that were just discernible by silver stain. In contrast, the End1 cells inoculated with *A. vaginae* produced pronounced protein bands. The pattern observed in this sample mirrored that of the End1 alone condition, but staining intensity suggested upregulation of these proteins in response to bacterial inoculation.

The seven major bands were excised and again subjected to mass spectrometric analysis. Again, over 50 human proteins identities were returned (Appendix C: Table C.2). To narrow these results, we selected proteins of interest based on the following criteria: identity was returned in both mass spectrometry analyses; protein molecular weight is <30 KDa; ions identified are specific for human sequence (not bovine); protein has been previously isolated from human cervicovaginal fluid, and protein has been previously implicated in innate immunity or HIV infection. These requirements allowed us to select four proteins to investigate further [10, 78, 83, 84]. Figure 3.4 panel B shows the sequence of the four proteins of interest: lipocalin 2 (NGAL), cyclophilin A (PPIA), trappin-2 (PI3), and HE4 (WFDC2).

3.3.4 Immunoblot Confirms Upregulation of Selected Proteins in the 3-30 KDa CM Fraction

To confirm the upregulation of these proteins, we performed immunoblots of the 3-30 KDa CM fraction from either mock-inoculated or *A. vaginae*-inoculated End1 cells. Recombinant protein standards were analyzed alongside the fractions in order to quantitate the protein content. Figure 3.5 demonstrates the upregulation of each of these 4 proteins in the *A. vaginae*-inoculated condition compared to a matched mock-inoculated fraction. Based on semi quantitative immunblot estimates, we approximated the following average concentrations of each protein in *A. vaginae*-inoculated End1 3-30 CM fraction at 1X: 2.36 ng/mL for lipocalin 2, 4.89 ng/mL for cyclophilin A, 1.29 ng/mL for trappin-2, and 3.90 ng/mL for HE4.

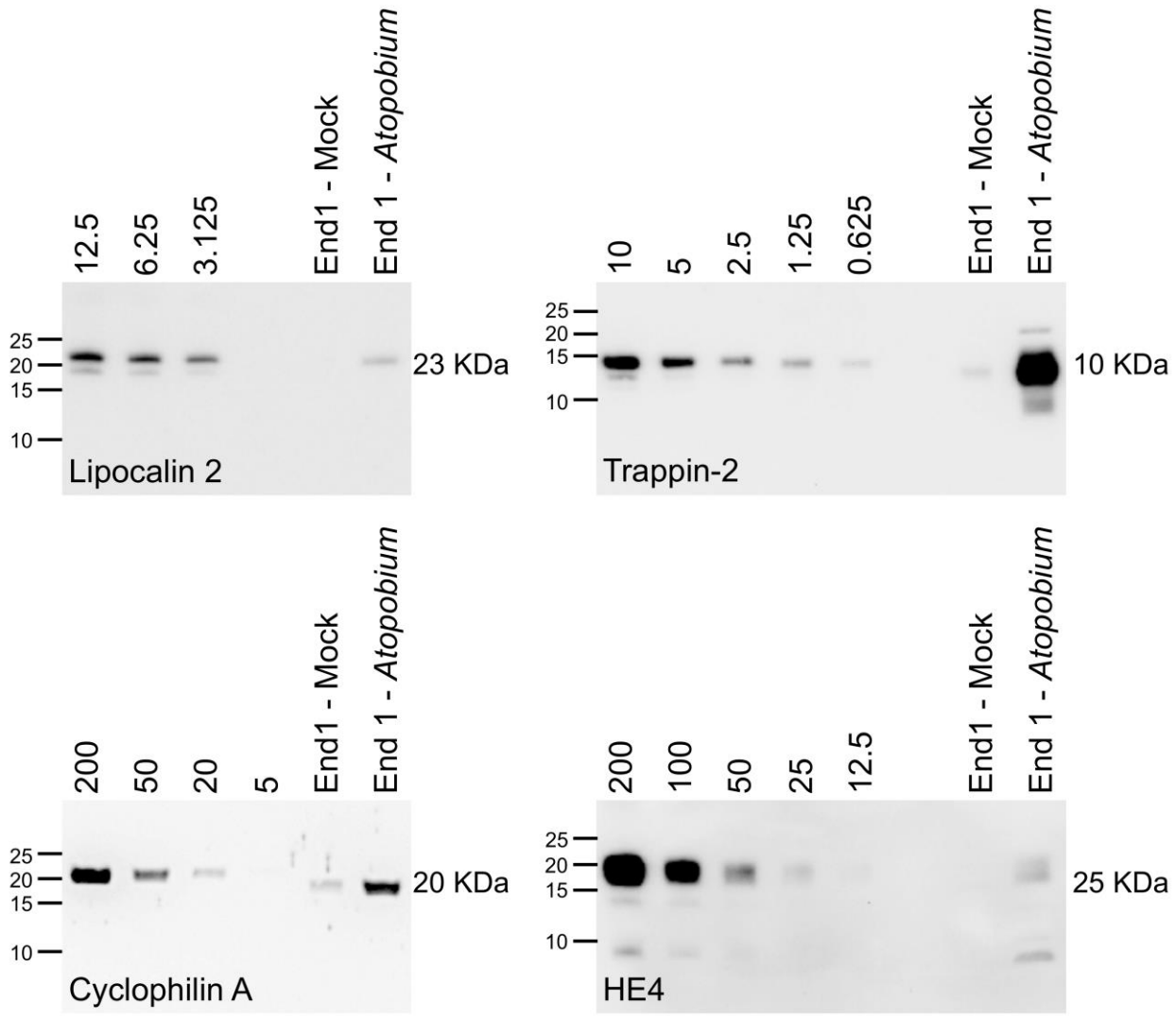


Figure 3.5. Western Blotting Confirms Upregulation of Proteins Identified by MS. The following volumes of 3-30 KDa CM fraction from either mock-inoculated or *Atopobium*-inoculated End1 cells were concentrated, resolved by Tricine SDS-PAGE, and immunoblotted for protein detection: 300 µL for Lipocalin 2; 400 µL for Cyclophilin A; 450 µL for Trappin-2 and 2000 µL for HE4. Recombinant standards (labeled as ng/lane) were resolved alongside CM fractions for quantitative comparison. One representative of three independent experiments for each analyte is shown.

3.3.5 HIV Enhancement is Mediated by the Combined Activity of Upregulated Effectors

Having seen that each of the selected proteins was upregulated by *A. vaginae*-inoculation of End1 cells in the proviral 3-30 KDa CM fraction, we sought to determine whether any one of these proteins might be responsible for the corresponding proviral activity. Recombinant preparations of each of the proteins were added to a TZM-bl infection in the presence of 1X DMEM media background to gauge viral enhancement activity. Surprisingly, none of the four proteins altered HIV infection when applied at concentrations up to 100 ng/mL (data not shown).

We next considered that the proviral activity of the 3-30 KDa CM fraction is likely a combined effect of host proteins, and that the addition of any single element might not recapitulate the viral enhancement observed in the complex CM fraction. To evaluate this possibility, we again performed the TZM-bl assay, this time adding each recombinant protein in addition to the mock-inoculated End1 3-30 KDa CM fraction. Figure 3.6 panel A demonstrates a similar lack of proviral activity observed for any of the four recombinant proteins in the End1 Mock CM condition. As the addition of only four of many upregulated proteins might neglect important proviral determinants, we continued to explore possible synergistic proviral effects of the upregulated epithelial proteins. We next added each of the four recombinant proteins along with the 3-30 KDa CM fraction from *A. vaginae*- inoculated End1 cells, in which many host response proteins are present at increased concentration, to provide a background composition in which proviral activity might be observed. Figure 3.6 panel B demonstrates a significant increase in proviral activity exerted by each of the four proteins of interest when applied

at 100 ng/mL in the *A. vaginae*-inoculated CM composition. Furthermore, a cocktail of the four proteins of interest exhibited significant increases in infection only in the presence of the *A. vaginae*-stimulated CM condition, as shown in Figure 3.6 panel C.

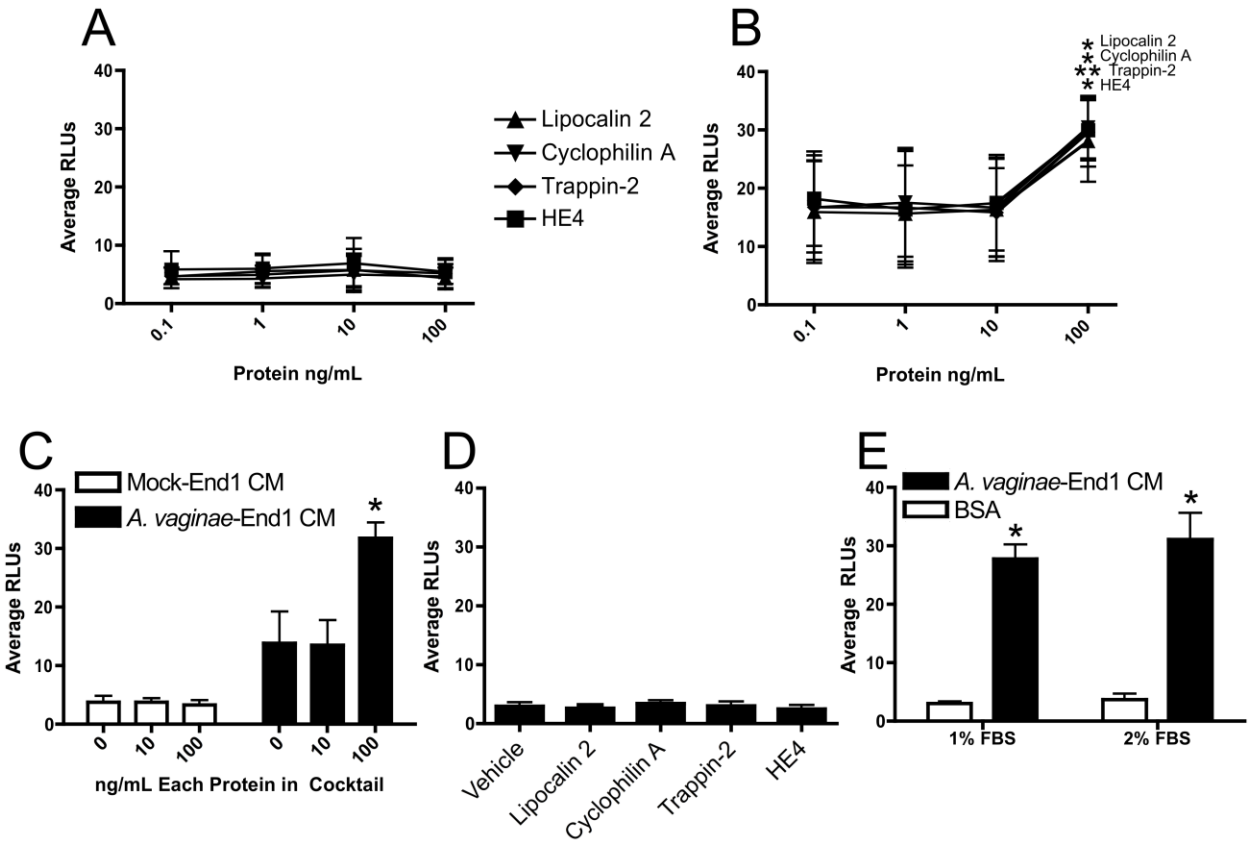


Figure 3.6 Recombinant Proteins Enhance HIV Infection in the Presence of *A. vaginae*-Inoculated End1 CM.

Recombinant proteins lipocalin 2, cyclophilin A, trappin-2 or HE4 were added to the TZM-bl reporter assay at concentrations of 0.1-100 ng/mL, in the presence of A) mock-inoculated End1 3-30 KDa CM fraction, or B) *A. vaginae*-inoculated End1 3-30 KDa CM fraction. CM fractions were added at 4X concentration in all conditions. One or two asterisks indicate a significant ($p<0.05$ and $p<0.01$, respectively) increase in RLUs

compared to control condition containing CM fraction without recombinant protein. n = 3-5. C) All four recombinant proteins were added to the TZM-bl assay as a cocktail, with each protein added at 0, 10 or 100 ng/mL in either mock- or *A. vaginae*-End1 CM at 4X. Asterisk indicates a significant ($p<0.05$) increase in RLUs compared to control condition containing CM fraction without recombinant protein. n = 3-5. D) Individual proteins were assayed at 100 ng/mL in the presence of 10 µg/mL BSA. No recombinant treatments resulted in significant differences from BSA alone. n = 3. E) BSA or *A. vaginae*-inoculated End1 CM were added to a TZM-bl assay at 10 µg/mL in the presence of 1% or 2% FBS. Asterisks indicate a significant ($p<0.01$) difference from control condition without BSA or CM treatment. n = 3-5.

As we had previously observed an increased in total protein content in the *A. vaginae*-inoculated End1 CM fraction, we considered that the proviral activity of this fraction, with or without added recombinant proteins, could be a nonspecific artifact of increased protein concentration in the TZM-bl reporter assay. To confirm that the viral enhancement was not nonspecific, we also assayed each recombinant protein in the presence of an irrelevant protein. Bicinchoninic acid (BCA) protein quantification revealed that when applied at 4X final concentration, the *A. vaginae*-inoculated End1 3-30 KDa fraction contributed 3 µg/mL total protein to the TZM-bl assay. Therefore, we added bovine serum albumin (BSA) at an excess concentration of10 µg/mL to examine potential proviral enhancement by nonspecific effects of the increased protein content. Figure 3.6 panel D shows that the BSA composition failed to facilitate proviral activity of any recombinant protein, confirming that the observed HIV enhancing activity was

specific to the *A. vaginae*-inoculated End1 CM composition. To further evaluate the specificity of the viral enhancement, we also tested the proviral capacity of both BSA and the active CM fraction in increased FBS conditions. Figure 3.6 panel E demonstrates sustained viral enhancement by the *A. vaginae*-inoculated End1 CM fraction in both 1% and 2% FBS, whereas BSA failed to increase infection in either FBS concentration. These data suggested that the proviral activity of the *A. vaginae*-inoculated End1 3-30 KDa CM fraction is specific, and that the complex mixture of upregulated proteins likely functions by synergistic mechanisms to enhance HIV infection.

3.4 Discussion

In this study, we evaluated the immune response initiated by FRT epithelia in response to BV-associated bacteria, and showed that the interaction between host and bacterial cells resulted in the secretion of proviral effectors that enhanced downstream HIV infection. In order to observe this proviral activity, we found it necessary to reduce the FBS concentration of our HIV reporter assay. This served to lower baseline infection considerably, revealing differences in infection that were not readily observable in the presence of additional FBS. This method has been used before to reveal antibacterial effects of antimicrobial peptides [30]. Further, only the End1 cell type exhibited increased proviral activity in CM fractions after inoculation with *A. vaginae*. This observation is in line with our previous characterization, which revealed End1 cells as the most responsive of the three cell types upon inoculation with BV-associated

bacteria, secreting increased concentrations of cytokines and host defense peptides in response to BVAB [40]. Combined with the data reported herein, these results support the notion that heightened epithelial immune response coincides with increased downstream HIV infection.

We initially hypothesized that the lower molecular weight fraction of epithelial CM would contain proviral effectors upon stimulation with *A. vaginae*, based on previous reports of <30 KDa proviral cytokines and innate immune effectors (IL-1 [85] and HD5 [75]). In fact, both the >30 KDa and the 3-30 KDa fractions of *A. vaginae*-inoculated End1 CM exhibited significant proviral activity, however the undesirable proliferative effects of the higher molecular weight fraction made this effect difficult to interpret, and we therefore pursued the 3-30 KDa CM fraction. Yet the absence of cytokines in this fraction was both unexpected, and revealing; though we had expected to find IL-1, IL-8, and other cytokines <30 KDa in this fraction [85, 86], they were surprisingly retained in the >30 KDa fraction. At the same time, the retention of nearly the entire cytokine content in the >30 KDa fraction provides a likely explanation for the infection trends we observed for the >30 KDa CM fraction or combined fractions (Figure 3.2 panel B); for the mock-inoculated condition, the presence of baseline cytokines could be responsible for the dose-dependent increase in RLUs as CM fractions were applied at increasing concentrations. In contrast, the *A. vaginae* condition, which contained significantly increased cytokine concentrations (Figure 3.3), exerted an overall proliferative effect when applied at 2X, perhaps on account of upregulated cytokine content [87] (Figure 3.2 panels B and C). These considerations solidified our focus on the 3-30 KDa CM fraction, whose proviral activity was specific.

Our proteomic analysis of the 3-30 KDa CM fraction from *A. vaginae*-inoculated End1 cells revealed a complex mixture, and from these potential effectors, we chose four peptides to research further. Interestingly, while the secreted epithelial effectors studied here *increased* viral infection, other groups have previously demonstrated *anti-HIV* activity of one of the four peptides evaluated: trappin-2/elafin [88]. Trappin-2 and elafin are post-translational processing variants of the *PI3* gene product, with elafin (5.9 KDa) representing the cleavage product of its precursor, trappin-2 (9.9KDa) [89]. The anti-HIV activity of the *PI3* gene product was demonstrated in a similar TZM-bl system using similar peptide concentrations (0.01 – 10 ng/mL). However, the assay conditions were considerably different, including, amongst other variations, a preincubation of virus with peptide prior to addition to TZM-bl reporter cells. This preincubation was subsequently shown to be essential for observing anti-HIV activity [89]. Further, it was demonstrated that the anti-HIV activity of elafin is more potent than that of its precursor, trappin-2, which we utilized in our study [89]. Our utilization of trappin-2 coincided with the apparent size of the major immunoreactive band detected in our 3-30 KDa CM fraction by immunoblot. While these treatment and cleavage distinctions could account for the observed differences in activity, it would be of interest to determine which of these protein variations represents the major product in cervicovaginal fluid.

In addition to trappin-2, each of the other three proteins was confirmed by immunoblot to be upregulated in the 3-30 KDa *A. vaginae*-End1 CM fraction in comparison to the mock-inoculated condition. Each recombinant protein was then tested in the context of the 3-30 KDa CM fraction from either mock- or *A. vaginae*-inoculated End1 cells. Interestingly, while none of the four individual exhibited proviral

activity in the presence of mock-inoculated End1 CM, *all four* enhanced infection in the presence of the *A. vaginae*-inoculated End1 CM fraction. Further evaluation revealed that an increase in nonspecific proteins did not provide the same stimulatory background as the *A. vaginae*-inoculated End1 CM, indicating that the proviral enhancement of this active fraction was specific. These results suggest that just as antiviral effectors work in concert to inhibit HIV infection [10], so might proviral factors synergize to enhance viral infection. This possible scenario supports further evaluation of additional proteins identified in the 3-30 KDa CM fraction from stimulated FRT epithelia. These proteins are likely mediators of increased viral susceptibility *in vivo*, and could represent molecular targets for combatting heterosexual HIV acquisition in women.

4. THE ANTI-HIV MICROBICIDE CANDIDATE RC-101 INHIBITS PATHOGENIC VAGINAL BACTERIA WITHOUT HARMING ENDOGENOUS FLORA OR MUCOSA

4.1 Introduction

Sexual transmission remains a major mode of female HIV acquisition, and therefore, prophylactic approaches designed to halt the transmission of HIV in the female reproductive tract (FRT) are being actively pursued. [1]. These methods include the development of vaginal microbicides, antimicrobial agents that prevent the transmission of HIV and reduce the user's susceptibility to viral acquisition [1,26]. Retrocyclins are a promising anti-HIV microbicide candidate; as restored innate immune effectors, they exhibit potent antiviral activity, with little adverse toxicity to host mucosa or microflora [90].

It stands to reason that since retrocyclins are derivatives of endogenously encoded primate peptides, their reintroduction to the human FRT would be well-tolerated by both human tissue and healthy bacterial inhabitants that evolved in the presence of host theta-defensins. In agreement with this hypothesis, safety studies in an *ex vivo* human cervical organ model [27] and *in vivo* pigtailed macaque studies showed that application of RC-101 was well-tolerated by vaginal and cervical tissue, inducing no inflammation or adverse side effects upon gynecological examination [90]. At the same time, recovered RC-101 peptide remained bioactive [90] and is stable in the macaque vaginal environment for up to 14 days after application (our unpublished data). Furthermore, RC-101 application did not disrupt endogenous populations of commensal lactobacilli [90]. This is an important consideration, since the tissues and

microbes of the macaque vaginal canal are highly similar to those of the human FRT [91]. These studies suggest that RC-101 will remain safe, stable and active among the complex environment of the human FRT.

To expand the promising profile of RC-101 as a vaginal anti-HIV microbicide, we sought to confirm its compatibility with epithelia, primary tissues, and commensal bacteria of the human FRT. Furthermore, we examined the stability and activity of RC-101 amongst pathogenic BV-associated bacteria, to ensure that this common affliction would not disrupt treatment regimens of RC-101. These studies demonstrate the compatibility of RC-101 with host tissues and microflora, and additionally demonstrate that RC-101 can inhibit BV-associated bacteria, thereby promoting healthy vaginal flora and providing a dual mechanism of HIV prevention.

4.2 Methods

4.2.1 Epithelial and Tissue Cultures

HeLa cells (CCL-2) were purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum. Primary vaginal epithelial cells (VEC-CRY-OV) and full-thickness EpiVaginal tissues (VLC-100-FT) are engineered specimens that were purchased from MatTek Corporation and maintained in provided media according to supplier's instructions. For epithelial experiments, treatments were prepared in maintenance media and applied to confluent monolayers. For tissues, treatments were prepared in 100 µL PBS and applied apically. After incubation, epithelial treatment media or tissue underlay media were collected, clarified, and stored

at -80°C until analysis. Cells were rinsed with PBS before lysing for phosphoprotein analysis.

4.2.2 Bacterial Cultures

The following bacterial cultures were purchased from ATCC: *Lactobacillus crispatus* (33197); *Lactobacillus acidophilus* (4356); *Lactobacillus johnsonii* (11506); *Lactobacillus jensenii* (25258); *Lactobacillus gasseri* (9857); *Lactobacillus vaginalis* (49540); *Gardnerella vaginalis* (49145); *Atopobium vaginae* (BAA-55); *Mobiluncus curtisii* (35241); *Prevotella bivia* (29303). All lactobacilli were grown in de Man, Rogosa and Sharpe (MRS) broth or on MRS agar plates at 37°C, 5% CO_2 atmosphere. *G. vaginalis*, *A. vaginae*, *M. curtisii*, and *P. bivia* maintenance cultures were all grown in tryptic soy broth (TSB) supplemented with 5% defibrinated rabbit blood (Becton, Dickinson and Company), or on agar plates of the same composition. *G. vaginalis* was grown at 37°C, 5% CO_2, while the other three bacteria were grown in anaerobic GasPak chambers (Becton, Dickinson and Company) at 37°C.

4.2.3 Bacterial Inhibition Assays

For experiments, anaerobic BV-associated bacteria (*A. vaginae*, *P. bivia* and *M. curtisii*) were taken directly from snap-frozen vials, and were washed and resuspended in prereduced brain heart infusion (BHI) media. Bacterial suspensions were mixed with preparations of RC-100, RC-101, clindamycin, or vehicle diluted in the same media. 5 µL of the final culture were placed under 3 µL liquid wax on a Terasaki microtiter plate

as previously described [30] and incubated anaerobically for up to 24 h. Extended incubation times such as this are typical for these anaerobic species [92, 93, 94]. Cultures were periodically diluted in prereduced BHI and plated on prereduced 5% blood TSA plates. Plates were incubated anaerobically, and colony forming units (CFUs) were subsequently quantified for each condition.

For lactobacilli, snap-frozen vials of each species were first grown for 2 h in MRS broth at 37°C, 5%CO_2 to allow cultures to recover. Actively growing bacteria were then mixed with RC-101 or vehicle diluted in the same media, and plated in Terasaki wells as done for anaerobes. These cultures were incubated at 37°C, 5%CO_2 for up to 6 h. This duration was chosen based on previous studies demonstrating that RC-101 inhibits susceptible aerobic species in less than three hours [30], and because with longer incubations the density of some cultures began to decline. Culture growth was monitored by diluting and plating on MRS, then incubating at 37°C, 5% CO_2 for CFU determination.

4.2.4 RC-101 Recovery from Pathogenic Bacterial Cultures

For coincubation with RC-101, snap-frozen vials of *G. vaginalis* were grown for 2 h in TSB to achieve log-phase growth, while anaerobic bacteria were taken directly from snap-frozen vials. All bacteria were diluted in maintenance media, and combined with RC-101 diluted in the same preparation for a final culture volume of 100 µL. These cultures were incubated at the appropriate atmosphere for 24 h at 37°C, after which each culture was acid extracted as previously described [90]. Soluble extracts were

neutralized by sequential drying and dilution with water, and neutralized extracts were resolved by Tricine-SDS PAGE, followed by western blotting with an anti-RC-101 antibody. Recovered peptide was run alongside a peptide standard for semi-quantitative comparison.

4.2.5 Bio-plex Analysis of Lysates and Conditioned Media

For phosphoprotein quantification, cells were harvested with Bio-Rad Cell Lysis kit, and equal amounts of total protein were assayed by multiplex phosphoprotein array. For cytokine analysis, conditioned media were clarified and equal volumes were assayed by multiplex cytokine array. Experimental analysis was performed according to manufacturer's instructions. In addition to cytokines appearing in our results, the following cytokines were assayed, but were not produced by our cells or tissues at measurable levels: PDGF-BB, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p70, IL-13, IL-15, IL-16, IL-17, IL-2Rα, IL-18, Eotaxin, FGF-β, G-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, LIF, MCP-3, β-NGF, SCF, SCGF-β, SDF-1α, TFN-β, TRAIL, HGF, IFN-α2.

4.2.6 Statistical Analyses

For bacterial inhibition assays, culture densities were log-transformed and treatments were compared to vehicle at each time point by a two-tailed paired Student's t-test [30]. For RC-101 recovery analysis, densitometric quantification between bacteria condition or media alone was compared by a two-tailed paired Student's t-test. For Bio-

Plex analysis of conditioned media, cytokine concentrations of RC-101 treated cultures were compared to appropriate vehicle by a two-tailed paired Student's t-test.

4.3 Results

4.3.1 Retrocyclin Theta-Defensins are Active Against BV-Associated Bacteria

Retrocyclins RC-100 and RC-101 have been previously shown to inhibit a variety of microbes, including viruses, fungi, and Gram-positive and Gram-negative bacteria [29,30,95]. As potential vaginal microbicides, the ability of retrocyclins to provide simultaneous protection against both HIV and pathogenic bacteria of the FRT is of immediate interest. To determine whether the restoration of retrocyclins to the FRT can inhibit bacterial pathogens, we first incubated RC-100, the endogenously encoded theta-defensin, with BV-associated bacteria *Atopobium vaginae*, *Mobiluncus curtisii* and *Prevotella bivia*. Treated cultures were incubated anaerobically for up to 24 h to determine the effects of the peptide on bacterial growth. Figure 4.1 shows that the peptide RC-100 significantly inhibited two of the three pathogenic anaerobes tested. *M. curtisii* was inhibited 95% by RC-100 after 24 h, while *P. bivia* inhibition was 85%. The third species, *A. vaginae*, was inhibited by RC-100 at 8 h, but the effects of RC-100 decreased by the completion of the 24 h experiment.

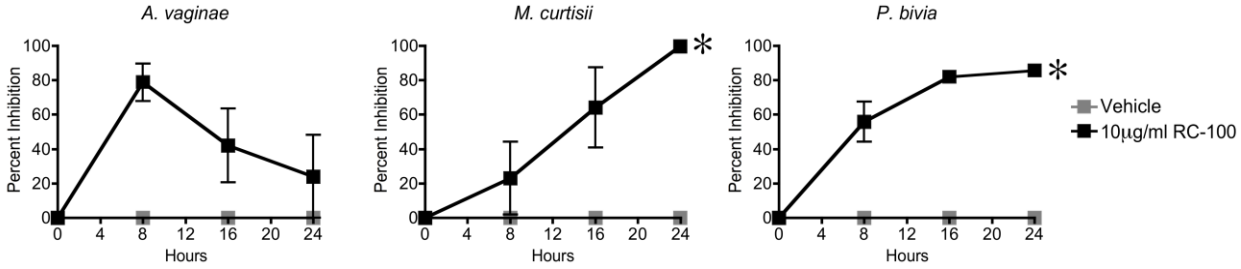


Figure 4.1. RC-100 Inhibits BV-Associated Bacteria.

RC-100 was incubated with BV-associated bacteria *Atopobium vaginae, Mobiluncus curtisii,* or *Prevotella bivia* (5×10^6 CFU/mL) anaerobically and cultures were plated at indicated timepoints to determine culture density. Percent inhibition was calculated relative to vehicle-treated bacteria. All inhibition values less than zero were plotted as zero. Asterisks indicated treatments for which one or more timepoints were significantly ($p<0.05$) different from vehicle. n = 3-4 for each condition.

Having determined that RC-100 inhibited BV-associated bacteria, we next investigated whether the retrocyclin analogue RC-101, which is being actively developed as a topical microbicide, exhibits similar antibacterial activity. The same panel of pathogenic bacteria was incubated with RC-101 at concentrations ranging from 0.5 - 10 μg/mL, or with clindamycin, a standard antibiotic used to treat BV [96], for comparison. Similar to trends observed for RC-100, RC-101 exhibited significant, dose-dependent inhibition of both *M. curtisii* and *P. bivia*, while inhibition of *A. vaginae* was not significant (Figure 4.2). Interestingly, clindamycin was unable to inhibit the pathogen *M. curtisii*, in contrast to RC-101, which exerted >95% inhibition of *M. curtisii* by 24 h. *P. bivia*, on the other hand, was significantly inhibited by RC-101, and also by clindamycin.

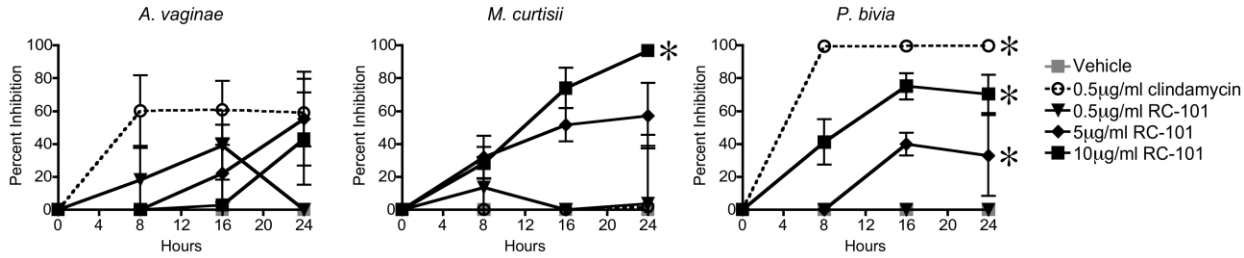


Figure 4.2. BV-Associated Bacteria are Inhibited by RC-101.

RC-101 at 10 µg/mL, 5 µg/mL or 0.5 µg/mL, or clindamycin at 0.5 µg/mL was incubated with BV-associated bacteria anaerobically and cultures were plated at indicated timepoints to determine culture density. Percent inhibition was calculated relative to vehicle-treated bacteria. All inhibition values less than zero were plotted as zero. Asterisks indicate treatments for which one or more timepoints were significantly ($p<0.05$) different from vehicle. n = 3-5 for each condition.

4.3.2 Commensal Vaginal Lactobacilli are not Inhibited by RC-101

The ability of RC-101 to inhibit BV-associated bacteria is a favorable secondary effect that complements the peptide's anti-HIV activity. However, vaginal microbicides must not exert antibacterial effects on the commensal bacteria that inhabit the FRT and promote reproductive health. Thus, we next examined the effect of RC-101 on the beneficial lactobacilli that comprise healthy vaginal flora. Six strains of lactobacilli that are common to the FRT [97, 98], were subjected to microassay analysis to determine whether they were equally affected by RC-101. Figure 4.3 shows the effect of different concentrations of RC-101 on *Lactobacillus acidophilus, crispatus, gasseri, jensenii, johnsonii and vaginalis* over a 6 h time course of treatment.

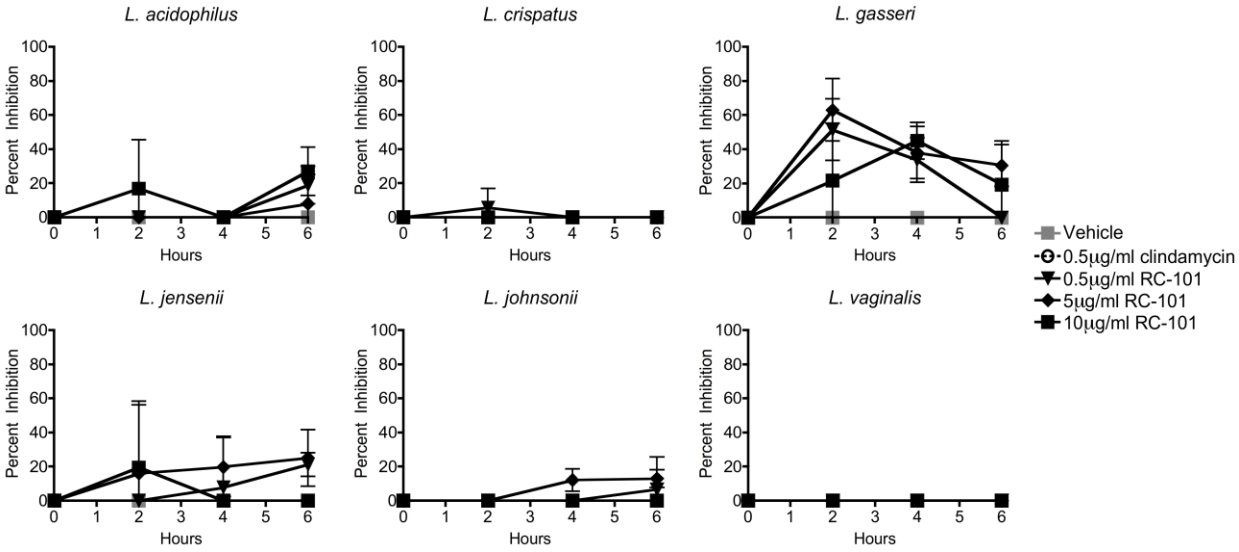


Figure 4.3. RC-101 Does Not Inhibit Commensal Vaginal Lactobacilli.

RC-101 at 10 µg/mL, 5 µg/mL or 0.5 µg/mL was incubated with six different species of vaginal lactobacilli, and cultures were plated at indicated timepoints. Percent inhibition was calculated relative to vehicle-treated bacteria and all inhibition values less than zero were plotted as zero. No treatments resulted in significant ($p<0.05$) inhibition of lactobacilli. n = 3 for each condition.

Unlike the pathogenic bacteria, none of the commensal lactobacilli were significantly inhibited by RC-101 at treatments as high as 10 µg/mL. Overall, the lack of significant antibacterial effects on lactobacilli suggests that RC-101 administered at anti-HIV concentrations would not disrupt the endogenous healthy bacterial flora of the FRT.

4.3.3 RC-101 is Recovered from BV-Associated Bacterial Cultures

While the recovery and bioactivity of RC-101 has been characterized in the presence of commensal microflora, we sought to ensure that BV-associated bacteria would not affect the stability of this peptide microbicide. To do so, we incubated RC-101 in cultures of BV-associated pathogens, and analyzed peptide recovery and electrophoresis after 24 h. Figure 4.4 panel A shows that RC-101 was recovered from all cultures, and that the peptide migrated at the appropriate size. Based on densitometric quantification, there were no significant differences in percent recovery between bacterial cultures and media alone, however there was a trend toward lower recovery from *P. bivia* cultures compared to the other three cultures (Figure 4.4 panel B). We occasionally observed a slower migrating band in this sample extract, which was not included in densitometric quantification. Ongoing studies are investigating whether RC-101 is actively degraded by *P. bivia*, or whether the complexity of this bacterial culture alters peptide recovery or migration.

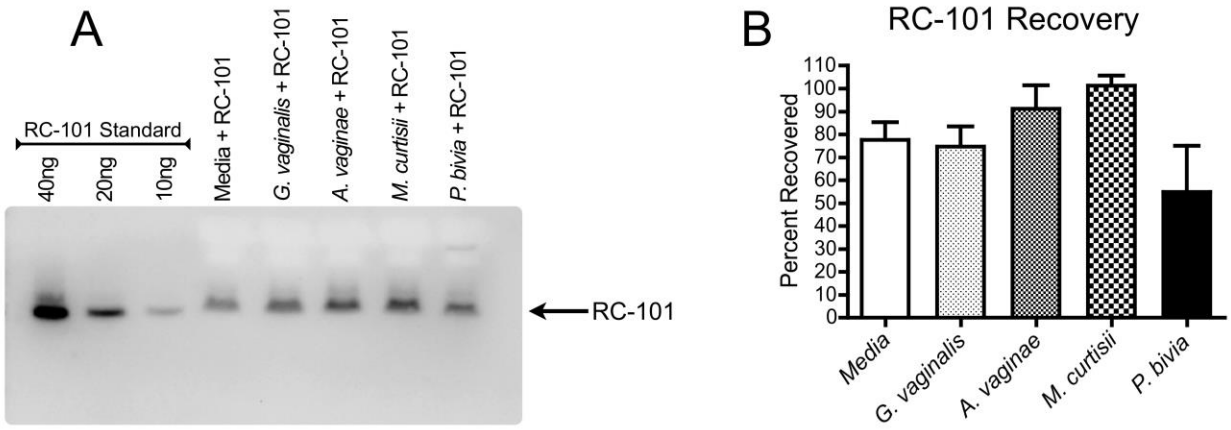


Figure 4.4. RC-101 is Recovered from BV-Associated Bacterial Cultures.

RC-101 (at 5 µg/mL) was incubated with BV-associated bacteria (5×10^6 CFU/mL) anaerobically for 24 h, then culture extracts were immunoblotted for RC-101 recovery determination. A) A representative immunoblot demonstrates the recovery of RC-101 from bacterial cultures or media alone, run alongside a standard of known RC-101 concentrations. B) Densitometry from three independent experiments. RC-101 recovery from bacterial cultures was not significantly different from media alone.

4.3.4 RC-101 is Well-Tolerated by Reproductive Cells and Tissues

In addition to testing the compatibility of RC-101 with commensal vaginal bacteria, we also examined the effects of RC-101 on the epithelial cells that line the FRT to ensure that the peptide would be well-tolerated by host tissues. Recent studies have monitored select cytokine responses to RC-101 in a cervical organ model, but do not provide a comprehensive cytokine and signaling evaluation [27]. To more broadly survey host response to RC-101, HeLa cells were treated with RC-101, and cellular response was gauged by monitoring intracellular signaling pathways. Figure 4.5 shows

the phosphoprotein signaling response of reproductive epithelia to 30 min of RC-101 treatment.

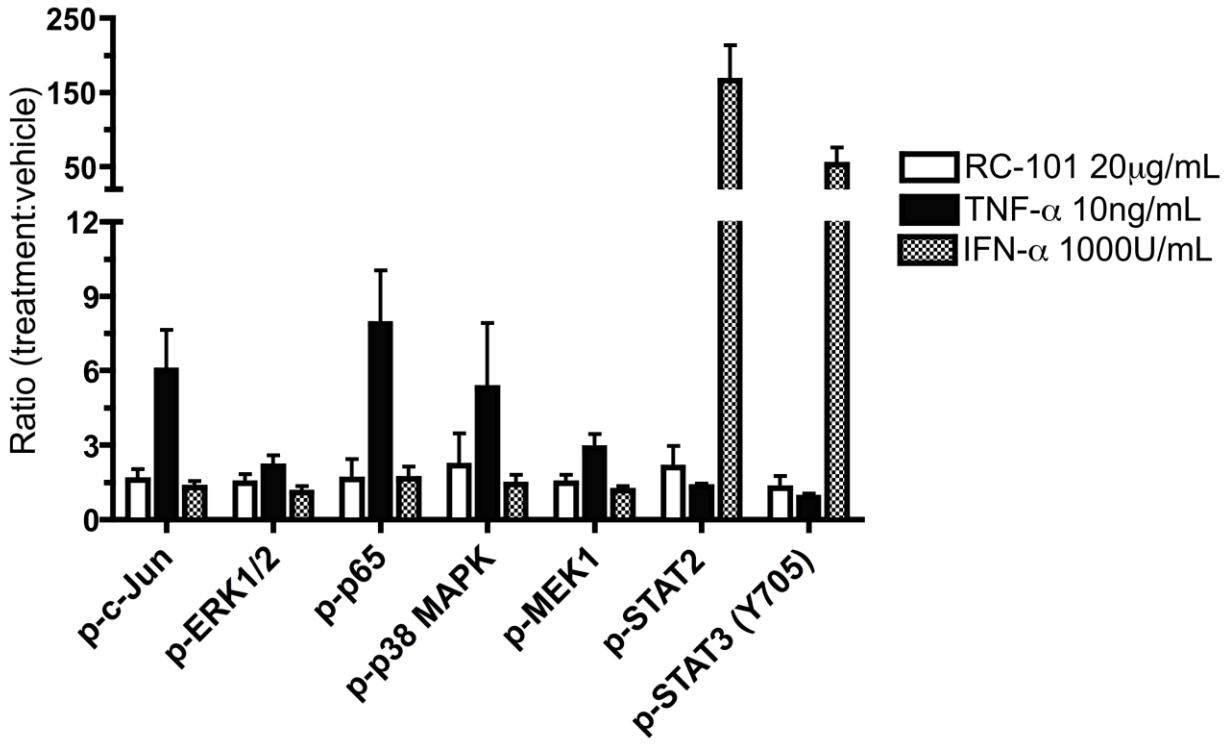


Figure 4.5. Reproductive Epithelial Cells Exhibit Minimal Phosphoprotein Response to RC-101.

HeLa cells were treated with vehicle, 20 μg/mL RC-101, or positive stimuli 10 ng/mL TNF-α or 1000 U/mL IFN-α for 30 min, then lysed for phosphoprotein quantification. Phosphoprotein ratios relative to vehicle-treated cells are shown for seven intracellular signaling proteins. Ratios are averaged from three or more independent experiments, except for quantification of p-MEK1 and p-STAT3 after RC-101 treatment, which are averaged from two independent experiments.

Importantly, none of seven monitored signal-transducing proteins was phosphorylated >2.1 fold in response to RC-101 compared to vehicle alone. This includes mediators of proinflammatory responses and regulators of cellular proliferation and turnover. The attenuated phosphorylation response to RC-101 is in contrast to control stimuli TNF-α and IFN-α, which elicit robust phosphorylation responses.

The lack of an intracellular phosphoprotein response was corroborated by an equivalent absence of cytokine response. Figure 4.6 shows the cytokine response of primary cultures of vaginal epithelial cells to 24 h of RC-101 exposure.

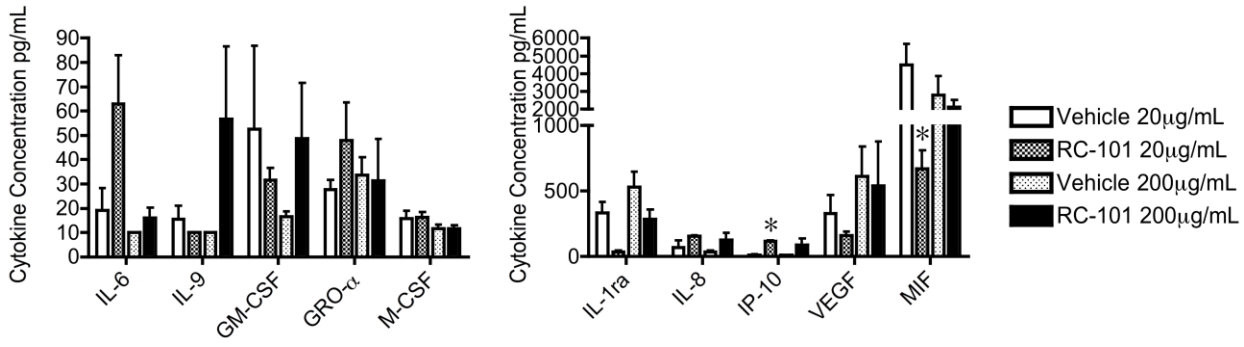


Figure 4.6. RC-101 Does Not Induce Proinflammatory Cytokines In Primary Vaginal Epithelia.

Primary vaginal epithelia were treated with either 20 or 200 μg/mL RC-101, or paired vehicles. After 24 h, conditioned media were collected and analyzed by multiplex cytokine array. The ten cytokines shown are expressed as raw cytokine concentrations and are grouped for graphing purposes. Asterisks indicated significant differences between RC-101 treatment and matched vehicle ($p<0.05$). n = 4-6.

In comparison to matched vehicle control, neither 20 µg/mL nor the excessive 200 µg/mL treatment stimulated significant increases in immune mediators such as IL-6, IL-8, Gro-α, M-CSF or GM-CSF. Of the ten analytes shown, only two (IP-10 and MIF) displayed significant differences from vehicle treatments, and for these two cytokines the significant difference observed at 20 µg/mL did not repeat at the higher dose of 200 µg/mL. These phosphoprotein and cytokine data suggest that RC-101 elicits a very minimal response from reproductive epithelial cells.

To expand this evaluation, we next utilized MatTek *ex vivo* vaginal tissues to examine the effects of RC-101 on intact tissues of the FRT. These full thickness tissues containing stratified epithelia, a basal lamina and submucosal dendritic cells were treated by apical application of RC-101 at the air-liquid interface. After 24 h, the basal media was analyzed for cytokine expression. Figure 4.7 shows the matched ten analytes from our epithelial analysis, with an additional eight characterized cytokines.

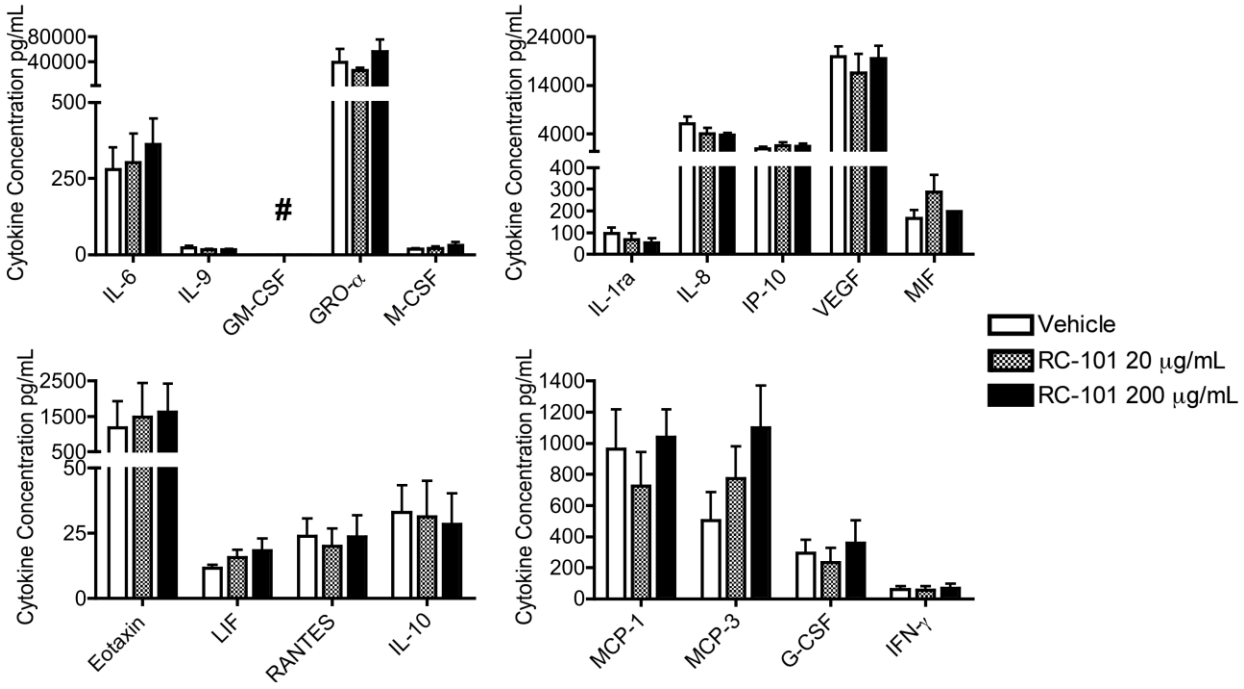


Figure 4.7. RC-101 is Well Tolerated by Organotypic Vaginal Tissue Model. Full-thickness organotypic tissues were treated with apical application of RC-101 at either 20 or 200 μg/mL RC-101 or vehicle. After 24 h, underlay media were collected and analyzed by multiplex cytokine array. In addition to the ten cytokines shown in Figure 6 for epithelia, another eight cytokines are shown here, all expressed as raw cytokine concentrations and grouped for graphing purposes. No RC-101 treatments resulted in significant differences from vehicle. ## = GM-CSF was detected in the maintenance media. n = 3-7.

One cytokine, GM-CSF, could not be accurately quantified, as it was found in tissue maintenance media alone. For the 17 cytokines that were measured, neither the 20 μg nor the 200 μg application per tissue resulted in significant differences from vehicle. This includes the two analytes IP-10 and MIF that showed inconsistent trends

in our epithelial model (Figure 4.6). The absence of any significant cytokine changes in these primary organotypic tissues is in agreement with epithelial trends and suggests that RC-101 is well-tolerated by host tissues of the FRT.

4.4 Discussion

Recent failures of anti-HIV microbicides [27, 99] have prompted more extensive preclinical characterization of candidate prophylactics. In this study, the microbicide candidate RC-101 was evaluated in order to determine its safety for host tissues and microflora prior to clinical trial. In agreement with other recent studies [27,90], we observed a desirable safety profile when RC-101 was applied to human FRT epithelia and tissues. Even at concentrations >40 times its antiviral IC_{50} range, RC-101 did not induce significant changes in cytokine release from primary FRT tissues. This evaluation included chemokines that mediate inflammation and chemotaxis of immune cells, such as IL-8, Gro-α, MCP-1 and MCP-3 [100, 101, 102, 103], and other important immune effectors such as IL-6 [104].

RC-101 did not elicit substantial increases in phosphorylation of the signaling transducers STATs 2 and 3 [105], nor of the proinflammatory mediator, the p65 subunit of NF-κB [106]. The lack of p65 phosphorylation is especially notable, as NF-κB activation is implicated in HIV proviral replication [41], and its unintended activation could counter the anti-HIV activity of applied microbicides. At the same time, the phosphorylation of mitogenic signaling intermediates such as MEK-1, ERK1/2, p38, and c-Jun was essentially unaffected by RC-101 application, indicating that this microbicide

candidate is unlikely to induce unexpected effects on cellular proliferation, turnover or stress response [107].

In addition to exhibiting compatibility with FRT epithelia and tissues, we also observed that RC-101 was well-tolerated by vaginal lactobacilli. Of our panel of six *Lactobacillus spp.*, none was significantly inhibited by RC-101. This validates previous *in vivo* observations of macaque vaginal flora, in which lactobacilli remained unaffected by vaginal film formulations of RC-101 [74]. While lactobacillus growth is not inhibited by RC-101, we observed that BV-associated bacteria were significantly inhibited by retrocyclins. For the two strains that were susceptible, bacterial inhibition occurred at 10 μg/mL, well within expected therapeutic concentrations, and for one species, *M. curtisii*, RC-101 treatment at all concentrations (0.26 - 5.3 μM) exerted significantly greater inhibition compared to clindamycin given at 0.5 μg/mL (1.2 μM), above its reported MIC_{90} of 0.125 μg/mL [92].

Furthermore, RC-101 was recovered from coincubation with these bacterial pathogens, though recovery was decreased when the peptide was coincubated with *P. bivia*. Of note, we occasionally observed a slower migrating immunoreactive band in this sample. As many positively charged antimicrobial peptides exert their antibacterial effect by binding and oligomerizing on bacterial surfaces to permeabilize cells [108], we expect that this band might be either bound or oligomerized RC-101. Interestingly, *P. bivia* is distinct in the panel of bacteria we evaluated, in that it is gram negative. The presence of negatively charged outer cell membrane components in this culture in particular could bind positively charged RC-101, slowing its electrophoretic mobility [109]. While this hypothesis might explain our slightly lower RC-101 recovered from *P.*

bivia culture, overall our results demonstrated good recovery of the peptide from the panel of bacteria, suggesting that RC-101 stability in the FRT would withstand transient fluctuations in microflora.

Though the molecular determinants of susceptibility remain unknown, the specificity of RC-101's antibacterial activity against pathogenic bacteria but not against commensal lactobacilli supports the notion that the dynamic and complex primate vaginal microflora evolved in the presence of similar theta-defensins. Consequently, endogenous lactobacilli are uninhibited by the reintroduction of a theta-defensin analogue, while pathogenic species remain susceptible to this class of antimicrobial host defense peptides.

These data suggest that RC-101 would be an ideal anti-HIV microbicide. In addition to being well-tolerated by human vaginal epithelia and tissues, by restoring a lost host defense mechanism, RC-101 provides not only potent antiviral activity, but also specific antibacterial activity that stabilizes the mucosal microflora. These desirable attributes make RC-101 a promising candidate for vaginal anti-HIV microbicide development.

5. GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE CONSIDERATIONS

Globally, the HIV pandemic is fuelled by sexual transmission, with overall sexual acquisition rates estimated to be 1-2 for every 1000 coital acts [110]. This combined estimate can be stratified by geographic location, with low-income countries exhibiting a 15-fold higher transmission rate per coital act than high-income countries [111]. In sub-Saharan Africa, where 68% percent of HIV-infected individuals reside, this increased transmission rate, compounded by cultural gender inequity, has given rise to a disproportionate burden of infection on the female population [24].

There are 1.4 times as many HIV-positive women in sub-Saharan Africa than HIV-positive men [1]. This skewed demographic likely perpetuates the viral pandemic, with the potential for mother-to-child transmission estimated at 12-40% in the absence of antiviral intervention [112]. Thus, the mechanisms and risk factors for male-to-female heterosexual transmission of HIV are critical targets for curbing viral dissemination. Toward this aim, a better understanding of mucosal integrity and immunity in the FRT is essential for halting heterosexual HIV transmission. In this dissertation, we explored the complex environment of the human FRT, taking into account host immunity, bacterial interactions, and topical anti-HIV prophylactic approaches, in an effort to combat HIV infection in women.

In Chapter 2, we described interactions between FRT epithelia and bacteria in order to elucidate immune interactions that affect HIV infection. We demonstrated distinct immune response profiles from different types of FRT epithelia, and further, revealed that specific FRT bacteria stimulate a more robust immune response than

others. We were surprised to find that *L. vaginalis* induced a heightened immune response, reminiscent of a BVAB-induced response, in contrast to the general consensus that lactobacilli are well-tolerated by host cells. Interestingly, this species is distinct from the other 5 lactobacillus species evaluated herein; based on taxonomic and phylogenetic analyses, *L. vaginalis* has been assigned to the *L. reuteri* group of lactobacilli, whereas the other species evaluated in coculture (*L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. johnsonii*) are assigned to the *L. delbrueckii* group [113]. Of interest is whether the metabolic and genetic determinants that separate this species from other FRT lactobacilli might contribute to the enhanced stimulatory activity it exerts on host epithelia.

Future investigations may benefit from exploring the stimulatory activity of FRT lactobacilli from diverse phylogenetic groups on host epithelia, an effort that relies on detailed characterization of the complexity of FRT microbiota. While prior descriptions of FRT bacterial inhabitants lacked species-level identification of microbial inhabitants [45, 46, 58], advanced sequencing approaches have provided a better characterization of vaginal microflora [50]. This approach will facilitate a better understanding of the diverse species, both commensal and pathogenic, that comprise the vaginal microbiome, and permit further characterization of host-bacterial interactions in the FRT.

In Chapter 3, we expanded our analysis of bacterial-epithelial interactions to investigate the mechanism by which the BVAB *A. vaginae* enhances downstream HIV infection. We demonstrated a significant increase in HIV infection in the presence of secreted effectors from epithelial-bacterial coculture, and identified several host proteins that were upregulated in the stimulatory CM treatment. Though we were unable to

recapitulate proviral activity by the addition of select individual proteins, we hypothesize that the synergistic activity of many upregulated effectors may contribute to the enhanced infection induced by the complex mixture of increased proteins. In future analyses, it would be of interest to characterize the *in vivo* composition of cervicovaginal fluid from women with or without BV, to determine if the epithelial upregulation described herein extends to the physiological setting of BV. While this analysis has been conducted for a small set of proteins [43], proteomic methodology including 2D-PAGE and isobaric tags for relative and absolute quantitation (iTRAQ) could provide useful insight into the complete cervicovaginal proteome [114]. These approaches could delineate important proteins of interest, and define biomarkers and critical mediators of BV-associated sequelae.

Such biomarkers of FRT pathogenesis could also be useful for the development of prophylactic topical microbicides engineered for FRT application. As discussed in Chapter 4, it is essential to define the effects of a microbicide candidate on both host and bacterial components of the FRT. In line with this requirement, retrocyclins represent promising candidate microbicides; in addition to being well-tolerated by host tissues, their selective antimicrobial activity inhibits pathogen BVAB, while leaving commensal lactobacilli unaffected. As peptide microbicides, retrocyclins such as RC-101 also have the potential to benefit from new a technique being explored by the vaginal microbicide field: the recombinant expression of antimicrobial proteins by transgenic lactobacilli.

As the benefits of commensal flora have been elucidated, the expression of anti-HIV peptides by probiotic lactobacilli has been eagerly explored. The transformation of

lactobacilli has been improved, and it has been shown that these bacteria can achieve superior folding of recombinant antiviral proteins compared to mammalian expression systems [115]. Initial *in vivo* studies have demonstrated successful colonization of the human vaginal canal when lactobacilli were administered in repeated doses [116], and most recently, recombinant lactobacilli expressing the antiviral protein cyanovirin-N were administered vaginally to macaques, which successfully prevented vaginal infection by SHIV up to 63% [117]. This rate of inhibition is likely a combined effect of the endogenous protective factors contributed by the lactobacilli in addition to the antiviral protein they were engineered to recombinantly express. Thus, in addition to accomplishing sustained delivery of antiviral compounds, the intravaginal application of transgenic lactobacilli has the dual advantage of also bolstering the endogenous protective barrier of the female reproductive tract. Peptide microbicides like RC-101 represent ideal candidates for pioneering this novel FRT microbicide delivery system.

Such multifaceted prophylactic and therapeutic approaches to FRT health are only now possible, with an expanded understanding of the dynamic interactions that determine mucosal integrity. The interplay between host tissues and the complex microbiome of the FRT is a critical determinant of the inherent innate immunity of the FRT. It is hoped that the studies conducted herein will provide a better understanding of the innate immune interactions in the FRT, and lead to new strategies for maintaining reproductive health and preventing heterosexual HIV acquisition via the FRT.

APPENDIX A: IRB APPROVAL LETTER FOR HUMAN SUBJECT

University of Central Florida Institutional Review Board
Office of Research & Commercialization
12201 Research Parkway, Suite 501
Orlando, Florida 32826-3246
Telephone: 407-823-2901 or 407-882-2276
www.research.ucf.edu/compliance/irb.html

Approval of Human Research

From: **UCF Institutional Review Board #1**
FWA00000351, IRB00001138

To: **Alexander M. Cole, Ph.D.** and Co-PI: **Amy L. Cole**

Date: **July 30, 2012**

Dear Researcher:

On 7/30/2012 the IRB approved the following human participant research until 7/29/2013 inclusive:

Type of Review:	Submission Response for IRB Continuing Review Application Form
Project Title:	Blood collection for PBMC isolationGrant: "Retrocyclins: circular defensins active against HIV-1" NIH Grant# R01 AI052017
Investigator:	Alexander M Cole, Ph.D.
IRB Number:	BIO-10-07092
Funding Agency:	National Institutes of Health
Grant Title:	
Research ID:	1039324

The Continuing Review Application must be submitted 30days prior to the expiration date for studies that were previously expedited, and 60 days prior to the expiration date for research that was previously reviewed at a convened meeting. Do not make changes to the study (i.e., protocol, methodology, consent form, personnel, site, etc.) before obtaining IRB approval. A Modification Form **cannot** be used to extend the approval period of a study. All forms may be completed and submitted online at https://iris.research.ucf.edu .

If continuing review approval is not granted before the expiration date of 7/29/2013, approval of this research expires on that date. When you have completed your research, please submit a Study Closure request in iRIS so that IRB records will be accurate.

Use of the approved, stamped consent document(s) is required. The new form supersedes all previous versions, which are now invalid for further use. Only approved investigators (or other approved key study personnel) may solicit consent for research participation. Participants or their representatives must receive a copy of the consent form(s).

In the conduct of this research, you are responsible to follow the requirements of the Investigator Manual.

On behalf of Sophia Dziegielewski, Ph.D., L.C.S.W., UCF IRB Chair, this letter is signed by:

Signature applied by Patria Davis on 07/30/2012 04:09:17 PM EDT

IRB Coordinator

APPENDIX B: CHAPTER 1 SUPPLEMENT

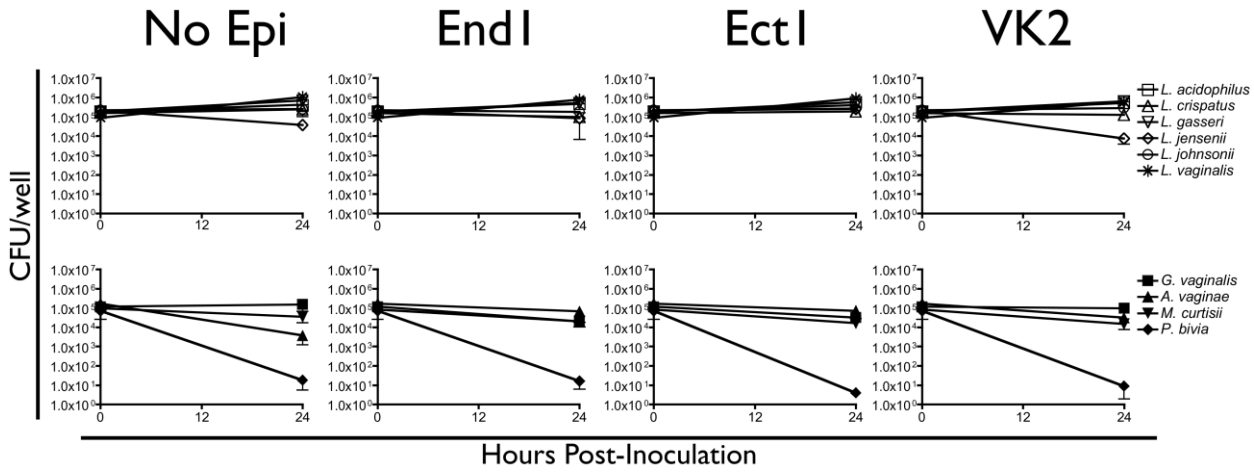


Figure B.1. Bacterial Growth in Coculture is Minimal.

Confluent monolayers of epithelia or no epithelia control wells were inoculated with indicated bacteria as previously described. In addition to calculating starting inocula, we also monitored bacterial density at the experiment endpoint (24 hr) by resuspending the coculture and plating serial dilutions on appropriate bacterial growth media. Bacterial density is represented as back-calculated CFU, and is averaged from three independent experiments.

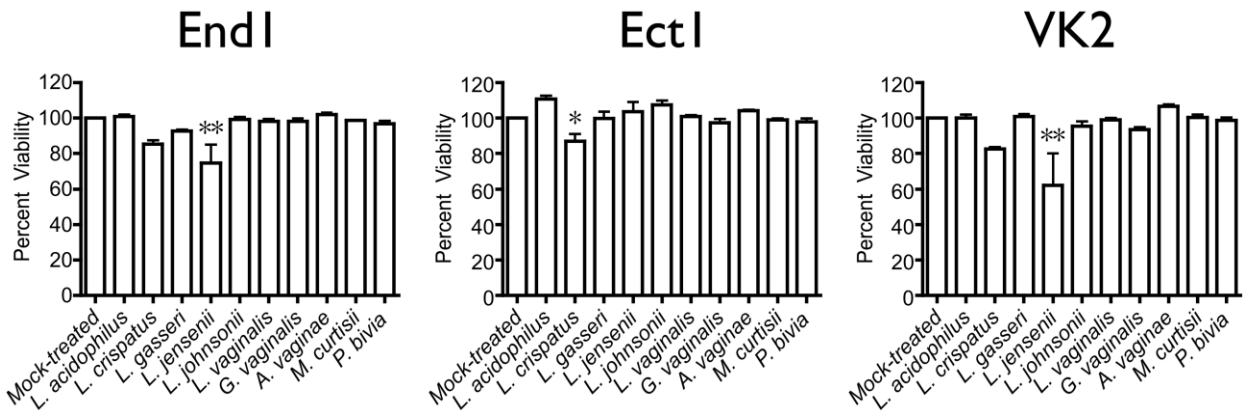


Figure B.2. Stimulatory BVAB do not Affect Epithelial Viability in Coculture. Confluent monolayers of epithelia or no epithelia control wells were inoculated with indicated bacteria as described in Methods. At the coculture endpoint (24 hr) epithelial viability was assessed by CytoTox Glo system. Control wells without epithelia were subtracted from matched coculture conditions to account for background bacterial fluorescence. Percent viability is shown relative to mock-inoculated controls, and is averaged from three independent experiments. One or two asterisks indicate significant ($p<0.05$ and $p<0.01$, respectively) differences in viability compared to mock-inoculated controls.

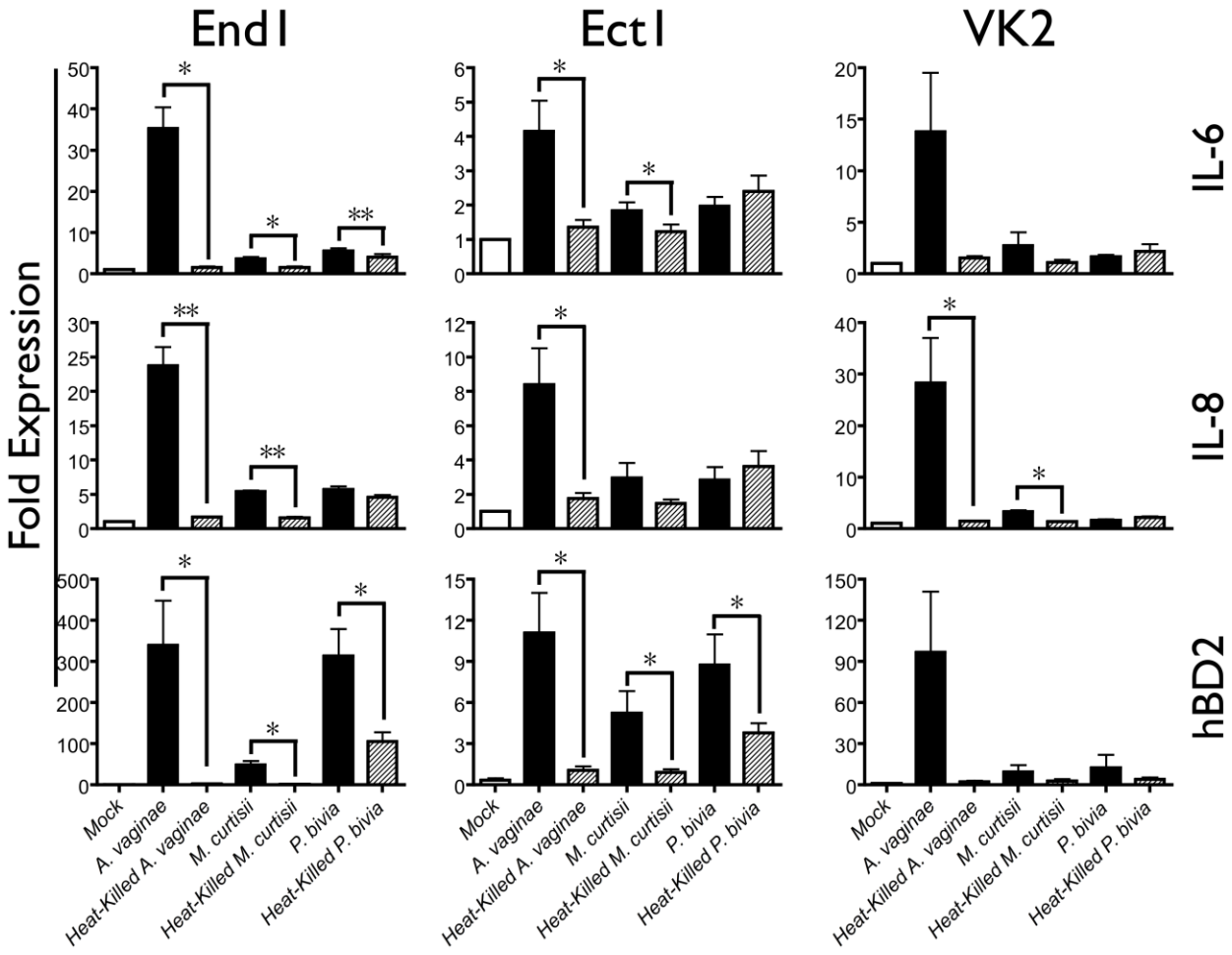


Figure B.3. Heat-Killing of Bacterial Inocula Attenuates Epithelial Response. Confluent monolayers of epithelia were inoculated with the BVAB *A. vaginae*, *M. curtisii* and *P. bivia* alongside heat-killed controls for each species. Heat-killing was achieved by incubating bacterial inocula at 65°C for 30 min, then cooling to 37°C prior to inoculation of epithelia, and was verified by plating. After 24 hr, epithelial response was measured by (A) IL-6 protein secretion (by ELISA), (B) IL-8 protein secretion (by ELISA), and (C) hBD2 transcript expression (by RTqPCR). All data are normalized to mock-inoculated controls and are averaged from three independent experiments. One

or two asterisks indicate significant ($p<0.05$ and $p<0.01$, respectively) decrease in heat-killed condition compared to live bacterial inoculum.

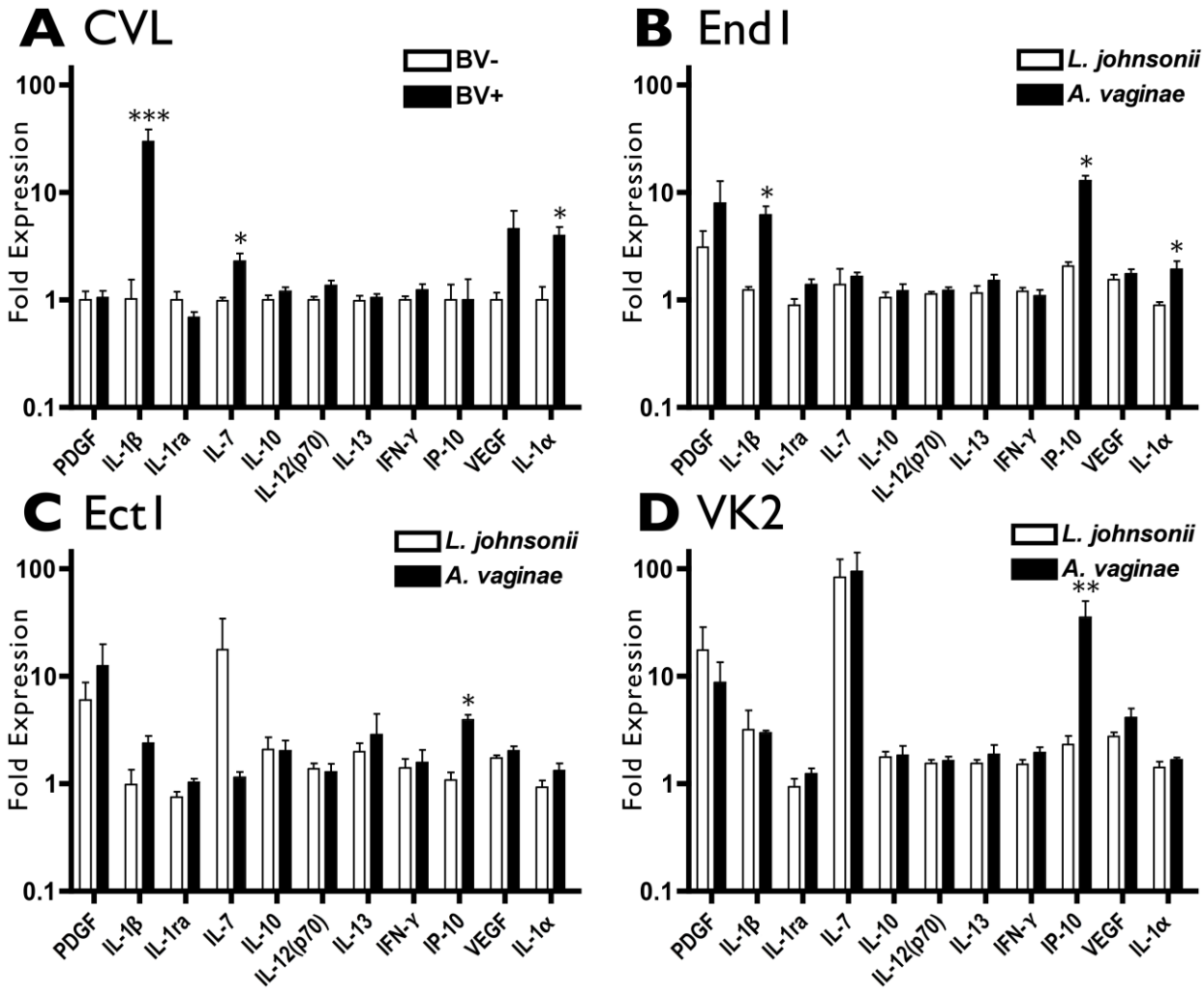


Figure B.4. Supporting Bio-plex Cytokine Panel.

A) Analytes evaluated but not included in Figure 2 are provided for cervicovaginal lavage samples from BV-negative or BV-positive women, where fold expression for each cytokine was calculated relative to the average value of the BV-negative samples, and one ($p<0.05$), two ($p<0.01$), or three ($p<0.001$) asterisks indicate a significant increase for the BV-positive samples over the BV-negative samples. Of note, average values of IL-7 were 3.7 pg/mL for BV-negative group, and 8.5 pg/mL for BV-positive group. Averages for IL-1α were 616.6 pg/mL for BV-negative group, and 2455.1 pg/mL for BV-positive group. Averages for IL-1β were 165.5 pg/mL for BV-negative group, and

4924.4 pg/mL for BV-positive group. Also shown are cytokines for B) End1, C) Ect1, and D) VK2 in response to *L. johnsonii* and *A. vaginae* where one ($p<0.05$) or two ($p<0.01$) asterisks indicate a significant increase in cytokine concentration for the *A. vaginae*-inoculated conditions over the *L. johnsonii*-inoculated conditions. Refer to Figure 1 for average concentrations of each analyte in these conditions.

APPENDIX C: CHAPTER 2 SUPPLEMENT

Table C.1. Proteins Identified by MS Analysis of Soluble 3-30 KDa CM Fraction

Sample	Gene Symbol	Description	MW [Da]	Score
3-30 KDa	BASP1	Isoform 1 of Brain acid soluble protein 1	22680	225
3-30 KDa	TPI1; TPI1P1	Triosephosphate isomerase isoform 2	31857	204
3-30 KDa	WFDC2	Isoform 1 of WAP four-disulfide core domain protein 2	13953	196
3-30 KDa	ACTB	Actin, cytoplasmic 1	42052	126
3-30 KDa	IGF2; INS; INS-IGF2	Insulin	12315	122
3-30 KDa	SOD1	Superoxide dismutase [Cu-Zn]	16154	111
3-30 KDa	TPM2	Isoform 2 of Tropomyosin beta chain	33827	102
3-30 KDa	HBB;HBBM13	Putative uncharacterized protein	24369	100
3-30 KDa	KRT17	Keratin, type I cytoskeletal 17	48361	100
3-30 KDa	MT1G	Isoform 1 of Metallothionein-1G	7277	89
3-30 KDa	VGF	Neurosecretory protein VGF	67215	86
3-30 KDa	YBX1	Nuclease-sensitive element-binding protein 1	35863	84
3-30 KDa	LCN2	Isoform 1 of Neutrophil gelatinase-associated lipocalin	22745	84
3-30 KDa	RPS27A	Ubiquitin-40S ribosomal protein S27a	18296	83
3-30 KDa	PPIA	Peptidyl-prolyl cis-trans isomerase A	18229	82
3-30 KDa	PFN1	Profilin-1	15216	79
3-30 KDa	TXN	Thioredoxin	12815	77
3-30 KDa	PKM2	Isoform M1 of Pyruvate kinase isozymes M1/M2	58538	77
3-30 KDa	RPS28	40S ribosomal protein S28	7863	74
3-30 KDa	TPM1	cDNA FLJ16459 fis, clone BRCAN2002473, moderately similar to Tropomyosin, fibroblast isoform 3	37487	73
3-30 KDa	TPM2	Isoform 3 of Tropomyosin beta chain	28666	71
3-30 KDa	SDC4	Syndecan-4	21628	68
3-30 KDa	KRT5	Protein	23267	67
3-30 KDa	KRT5	cDNA FLJ54681, highly similar to Keratin, type II cytoskeletal 5	59842	67
3-30 KDa	ENO2	Gamma-enolase	47581	66
3-30 KDa	SCGB	Uncharacterized protein	21486	67
3-30 KDa	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	25151	65
3-30 KDa	MARCKS	Myristoylated alanine-rich C-kinase substrate	31767	65
3-30 KDa	SUMO4	Small ubiquitin-related modifier 4	10315	64
3-30 KDa	LOC728875; SUMO2	Small ubiquitin-related modifier 2	10889	64
3-30 KDa	CFL1	Cofilin-1	18719	64
3-30 KDa	CANX	Calnexin	67982	62
3-30 KDa	SFN	Isoform 1 of 14-3-3 protein sigma	27871	62
3-30 KDa	VCP	Transitional endoplasmic reticulum ATPase	89950	61
3-30 KDa	UBQLN	Ubiquilin-like protein [SGN]	17933	60
3-30 KDa	HMGA1	Isoform HMG-I of High mobility group protein HMG-I/HMG-Y	51230	59
3-30 KDa	PEBP1	Phosphatidylethanolamine-binding protein 1	21158	56
3-30 KDa	HSPA5	78 kDa glucose-regulated protein	72402	56
3-30 KDa	GAL	Galanin peptides	13294	54
3-30 KDa	PPIA	cDNA FLJ75625, highly similar to Homo sapiens peptidylprolyl isomerase A (cyclophilin A)	11582	52
3-30 KDa	LRRFIP1	Isoform 2 of Leucine-rich repeat flightless-interacting protein 1	94879	51
3-30 KDa	DARET	Protein DJ-1	20850	49
3-30 KDa	TPM3	33 kDa protein	33410	48
3-30 KDa	CALM3; CALM2; CALM1	Calmodulin	16827	47
3-30 KDa	ACTBL2	Beta-actin-like protein 2	42318	47
3-30 KDa	KRT79	Keratin, type II cytoskeletal 79	58885	46
3-30 KDa	KRT2	Keratin, type II cytoskeletal 2 epidermal	66110	46
3-30 KDa	PSR1	Phosphoglycerate kinase 1	44985	45
3-30 KDa	PTMS	Parathymosin	11523	45
3-30 KDa	HNRNPK	Isoform 1 of Heterogeneous nuclear ribonucleoprotein K	51230	45
3-30 KDa	SCG2	Secretogranin-2	70897	44
3-30 KDa	AVP	Vasopressin-neurophysin 2-copeptin	77525	43
3-30 KDa	OXT	Oxytocin-neurophysin 1	13719	42
3-30 KDa	DSC3	Isoform 3A of Desmocollin-3	101218	42
3-30 KDa	EIF4A	Eukaryotic translation initiation factor 4A	46167	42
3-30 KDa	ZNF510	Zinc finger protein 510	73710	41
3-30 KDa	PI3	Elafin	12719	41
3-30 KDa	EIF3J	Eukaryotic translation initiation factor 3 subunit J	29159	41
3-30 KDa	TUBA4A	Tubulin alpha-4A chain	50634	39
3-30 KDa	PTMA	Prothymosin alpha	15849	39
3-30 KDa	MIR1244-3; MIM1244-2; MIM1244-1	Prothymosin alpha	11884	39
3-30 KDa	TRIM29	Isoform Alpha of Tripartite motif-containing protein 29	64478	38
3-30 KDa	THOS10	Thymosin beta-10	5023	38
3-30 KDa	KCNG2	Potassium voltage-gated channel subfamily G member 2	52176	38
3-30 KDa	SNRPA; RPSAP15; SNRPA2; RPSA	Laminin receptor-like protein LAMRL6	33889	38
3-30 KDa	ACO2	Aconitase (Fragment)	65934	38

Shown are protein identities obtained by analyzing the 3-30 KDa CM fraction by mass spectrometry, and referencing against a human database. For each entry, the gene symbol(s), description, and molecular weight are given, followed by the ion score, which was used to order the protein identities.

Table C.2. Proteins Identified by MS Analysis of Excised Tricine SDS-PAGE Bands

Sample	Gene Symbol	Description	MW [Da]	Score
Band 1	MSN	MSN protein	58803	5
Band 2	MSN	MSN protein	28862	5
Band 3	UBC	Ubiquitin	16831	56
Band 3	KRT2	Keratin, type II cytoskeletal 2 epidermal	65395	53
Band 3	KRT9	Keratin, type I cytoskeletal 9	62827	49
Band 3	KRT6C	Keratin, type II cytoskeletal 6C	58432	33
Band 3	PI3	Elafin	12261	8
Band 6	KRT1	Keratin 1	66826	119
Band 6	KRT1	Keratin 1	66813	118
Band 6	KRT2	Keratin, type II cytoskeletal 2 epidermal	65395	93
Band 6	KRT9	Keratin, type I cytoskeletal 9	62827	72
Band 6	KRT6B	Keratin, type II cytoskeletal 6B	60830	31
Band 6	PPIA	Peptidyl-prolyl cis-trans isomerase	18862	28
Band 6	KRT14	Keratin, type I cytoskeletal 14	51529	28
Band 6	SOD1	Superoxide dismutase [Cu-Zn]	15926	27
Band 6	CFL1	Cofilin-1	18491	13
Band 6	KEAP1	Kelch-like ECH-associated protein 1	69621	5
Band 6	MGST1	Isoform m18 of 1,8-dihydro-8-oxoguanine triphosphatase	17940	3
Band 7	KRT1	Keratin 1	66826	154
Band 7	KRT1	Keratin 1	66813	154
Band 7	KRT2	Keratin, type II cytoskeletal 2 epidermal	65395	148
Band 7	KRT10	Keratin, type I cytoskeletal 10	58792	132
Band 7	KRT9	Keratin, type I cytoskeletal 9	62827	124
Band 7	KRT14	Keratin, type I cytoskeletal 14	51529	72
Band 7	KRT5	Keratin, type II cytoskeletal 5	62340	68
Band 7	KRT6C	Keratin, type II cytoskeletal 6C	58432	49
Band 7	PEBP1	Phosphatidylethanolamine-binding protein 1	21044	44
Band 7	JUP	Junction plakoglobin	83369	25
Band 7	PARK7	Protein DJ-1	19878	22
Band 7	CFL1	Cofilin 1	18801	13
Band 7	HRNR	Hornerin	282199	12
Band 7	WFDC2	Isoform 2 of WAP four-disulfide core domain protein 2	8115	10
Band 7	MGST1	Isoform m18 of 1,8-dihydro-8-oxoguanine triphosphatase	17940	9
Band 7	LCN2	Neutrophil gelatinase-associated lipocalin	22574	9
Band 7	SCAF4	Uncharacterized protein	123568	9
Band 7	DCD	Dermcidin	11277	6
Band 7	HPC2	Epididymal secretory protein E1	13870	6
Band 7	GGCT	Gamma-glutamylcyclotransferase	20994	5
Band 7	SERPINB12	SERPINB12 protein	20905	5
Band 7	NXLC1	cDNA, FLJ95121, highly similar to Homo sapiens nucleolar and coiled-body phosphoprotein 1	73561	4
Band 7	ANXA2	Annexin A2	7876	4
Band 7	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	468532	4
Band 7	UBA52	Ubiquitin-60S ribosomal protein L40	14715	4
Band 7	NRS	Uncharacterized protein	14517	4
Band 7	WDR72	WD40 domain-containing protein 2	17665	4
Band 7	TGM1	Protein-glutamine gamma-glutamyltransferase K	83685	4
Band 7	PITPNB	cDNA, FLJ93543, highly similar to Homo sapiens phosphatidylinositol transfer protein, beta	51446	4
Band 7	ABHD14B	Abhydrolase domain-containing protein 14B	19784	3
Band 7	KEAP1	Kelch-like ECH-associated protein 1	69621	3
Band 7	PRSS1	Protease serine 1	9161	3
Band 7	TPI1	Triosephosphate isomerase	17947	3
Band 7	LYZ	Lysozyme C	11481	3
Band 7	S100A8	Protein S100-A8	13829	3
Band 7	PRDX1	cDNA, FLJ92164, highly similar to Homo sapiens peroxiredoxin 1	22186	3
Band 7	STMN1	Stathmin	9858	3
Band 7	CSTA	Cystatin-A	7887	3
Band 7	TCTP	cDNA FLJ57828, highly similar to Translin protein	92713	3

Shown are protein identities obtained by analyzing the seven bands excised from a PAGE separation of 3-30 KDa CM fraction. Each band was analyzed by mass spectrometry, and referenced against a human database. For each band, the identified proteins are listed with their the gene symbol(s), description, and molecular weight, followed by the ion score, which was used to order the protein identities. Gene symbols in italics were manually curated.

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