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## The Effect of Bacterial Vaginosis Associated Bacteria on Epithelial Factors Mediating HIV Transmission

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THE EFFECT OF BACTERIAL VAGINOSIS ASSOCIATED BACTERIA  
ON EPITHELIAL FACTORS MEDIATING HIV TRANSMISSION

by

APRIL H. NGUYEN

A thesis submitted in partial fulfillment of the requirements  
for the Honors in the Major Program in Biomedical Sciences  
in the College of Medicine  
and in the Burnett Honors College  
at the University of Central Florida  
Orlando, Florida

Spring Term 2015

Thesis Chair: Alexander M. Cole, Ph.D.

## ABSTRACT

Bacterial vaginosis (BV), a common female reproductive tract (FRT) condition characterized by an overgrowth of anaerobic species concurrent with the disappearance of commensal Lactobacilli species, is associated with a 60% increased risk of HIV-1 transmission. However, the role of the FRT epithelia in bacterial vaginosis-associated bacteria (BVAB)-augmented HIV-1 transmission is unclear. To evaluate the increased risk of HIV-1 acquisition, we treated FRT epithelia with *Atopobium vaginae*, a prevalent BVAB, to determine the nature of the host response to BVAB exposure. Treatment of endocervical cells with *A. vaginae* resulted in a 1500-fold increase in the expression of the antimicrobial peptide hBD-2, an inflammatory cytokine response, and delocalization of the tight junction protein ZO-1 from cell borders. Conditioned media (CM) from the coculture of FRT epithelia and *A. vaginae* also generated an inflammatory immune response and lowered the transepithelial electrical resistance in polarized endocervical monolayers. Changes in HIV-1 infection were measured in TZM-bl reporter cells, which contain a luciferase gene under the control of an HIV-1 long terminal repeat (LTR) region that is activated by the binding of Tat, an HIV-1 protein that drives viral replication. NF $\kappa$ B is a major host-derived transcription factor that regulates the expression of many genes involved in inflammation and the innate immune response. Interestingly, NF $\kappa$ B has been reported to bind Tat-activated response elements within the LTR of HIV-1, driving viral transcription. TZM-bl cells were treated with CM in the absence of HIV-1, which resulted in increased luciferase production that could be suppressed by the NF $\kappa$ B inhibitor TPCA-1. These data suggest that epithelially derived products from the coculture of FRT cells and *A. vaginae* enhance HIV-1

infection by causing cervical barrier dysfunction and increasing HIV replication efficiency through NF $\kappa$ B.

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## **CHAPTER I: INTRODUCTION**

The human immunodeficiency virus (HIV) continues to be a worldwide epidemic, currently infecting over 34 million people [1]. There is evidence of a gender discrepancy in the infection rate, especially in Sub-Saharan Africa where over 60% of new infections occur heterosexually in women [2]. Therefore, as the predominantly affected group, studying and preventing infection in women can have significant impact on the overall reduction of global HIV infection. As the primary route of infection occurs through mucosal surfaces, mainly through heterosexual transmission, further studies of the interaction between HIV and the female reproductive tract (FRT) epithelia can help identify new ways to reduce HIV infection in women [3].

Heterosexual HIV transmission mainly occurs through the epithelium of the lower FRT, targeting immune cells such as T cells, macrophages, and dendritic cells which are located below the epithelium [4]. The lower FRT consists of the vagina, the ectocervix, and the endocervix. The vagina and ectocervix consist of multiple layers of non-keratinized squamous epithelium, while the endocervix consists of single-layer columnar epithelium. It is currently believed that the endocervix is especially susceptible to HIV infection compared to the vagina and ectocervix due to its single layered nature and the higher concentration of CD4<sup>+</sup> T cells, the main target cell for HIV replication, in the region [5].

Heterosexual HIV transmission to women is typically low, ranging from 1/100 to 1/1000 sexual acts [6, 7]. HIV virions must 1) survive a low pH vaginal environment consisting of

cervicovaginal fluid, which contains a variety of antimicrobial peptides that help fight off invading pathogens; 2) traverse the epithelium of the FRT to target host immune cells that reside below the epithelium; and, 3) be efficiently replicated by host immune cells that travel to regional lymph nodes [4, 8]. The cervico-vaginal fluid is a mixture of fluids from the uterus, cervix, oviducts, and cervical vestibular glands. This fluid contains a variety of antimicrobial peptides, chemokines, and cytokines that together demonstrated shown anti-HIV activity [9]. The upper layers of the epithelium in the lower tract are also regularly sloughed off, which constantly removes pathogens from the tract [10]. The epithelium itself also confers innate immunity as it acts as a physical barrier to transmission of virions to underlying immune cells. Virions can pass through the epithelium by traversing through gaps in a compromised epithelial barrier, by transcytosis through epithelial cells, or paracellular movement between epithelial cells [4].

The barrier function of epithelial cells is conferred by three types of cell-to-cell adhesions that work to seal gaps between epithelial cells: the tight junction, adherens junction, and desmosomes. The tight junction is comprised of multiple proteins: occludins, claudins, and junctional adhesion molecules. Those proteins are anchored to cytosolic proteins and seal the gaps between neighboring epithelial cells. The adherens junction is located below the tight junction and is the main connection that joins cells together by connecting their actin filaments. Similarly, desmosomes connect keratin filaments together. The structure is highly dynamic, quickly responding to a variety of stimuli either to seal intercellular gaps or allow small particles to pass through. Together, the three adhesive components organize the epithelia into a semi-

permeable, polarized barrier that regulates particle diffusion and can hinder HIV translocation [11, 12].

The innate immune system can provide over 99% immunity in HIV infection to healthy women [6, 7]. However, it can be postulated that common gynecological conditions can alter the FRT and its innate defense capabilities. One condition that may contribute is bacterial vaginosis (BV). BV is an idiopathic condition where the Lactobacilli-dominated healthy vaginal microbiota shifts to a microbiota consisting mainly of anaerobic bacteria [13]. Commonly identified bacterial vaginosis associated bacteria (BVAB) include *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus curtisii*, and *Prevotella bivia* [13-15]. The relative scarcity of clinical data has made it difficult to determine the exact polymicrobial composition of the BV microbiota. In addition, many of the species associated with BV are also present in some healthy women [13, 16, 17]. The role that each species plays in the pathogenesis of the condition is unclear, a fact that is further exacerbated by the limitations on current FRT model systems that can replicate the physiological properties of the FRT epithelia in order to more accurately explore host-pathogen interactions.

In healthy vaginal conditions, *Lactobacillus* species mainly colonize the epithelium, producing hydrogen peroxide and lactic acid, which help maintain an acidic (pH<4.5) environment [16]. While it is currently unknown which occurs first, BV is characterized by an overgrowth of anaerobes in the vagina concurrent with a depletion of *Lactobacillus* species [13]. This is indicated by a raise in pH, abnormal vaginal discharge, and the presence of bacteria adhering to

epithelial cells, according to the Amsel criteria [18]. The Nugent score is also used to diagnose BV, which is based on the microscopic examination of vaginal smears. The presence and quantity of clue cells, epithelial cells whose edges are covered by small coccobacilli, are used to determine the lack or severity of infection [19]. BV has been associated with a 60% increased transmission rate of HIV among other complications such as preterm delivery, inflammation, and increased risk of contracting other STIs and infections [13, 20, 21]. In some symptomatic cases, symptoms are self-relieving. However, many women seek treatment, most commonly with metronidazole or clindamycin [13, 22]. Current research is exploring the possibility of probiotics as an effective treatment for BV [23].

Currently the link between BV and HIV is not clear though several explanations have been postulated: 1) The inflammatory response to BVAB can damage the mucosa, and cause an increase in chemotaxis and activation of CD4<sup>+</sup> T cells to the site, which may facilitate HIV infection [15, 24]. 2) The increase in pH following the reduction in lactobacilli increases CD4<sup>+</sup> T cell activation and proliferation. 3) When the pH is increased in BV, the negative surface charges on virions remain intact, allowing for higher rates of HIV diffusion through the mucosa for higher accessibility to target cells below [25].

One aspect of BV that remains to be elucidated is the role of the female reproductive tract epithelia in BVAB-augmented HIV transmission and infection. One of the methods of HIV transmission is translocation through gaps in the epithelium, which is held together by cell-cell adhesions [4]. In BV, the endocervix is exposed to pathogenic bacteria, and our lab previously

showed that in End1 cells, an endocervical epithelial cell line [26], BVAB elicit a robust inflammatory response that includes marked increases in expression of  $\beta$ -defensins, inflammatory cytokines and chemokines, as well as other immune modulators such as lipocalin-2, trappin-2, cyclophilin A, and HE4. In End1 cells, the most pronounced response occurred after exposure to *A. vaginae*. Together, these host defense factors exhibited HIV-enhancing effects on TZM-bl target cells [27]. Concurrently, other groups have shown that upregulated cytokine expression may regulate cell-cell junctions via changes in expression and localization of proteins such as occludin, claudin-1/5, ZO-1/2, E-cadherin, and beta-catenin [28]. These proteins are components of tight junctions and serve as markers for epithelial integrity [11].

We confirmed the inflammatory response in FRT cells in response to BVAB, particularly *A. vaginae*, and showed that this exposure also reduces the expression of the tight junction protein, ZO-1, intracellularly. We sought to confirm the barrier disruption by creating an FRT polarized monolayer model using A2EN cells, an endocervical cell line, on Transwell inserts. This model represents an improvement upon the previous *in vitro* model used to explore the host-pathogen interactions, and we were able to observe distinct changes in barrier function after treatment with conditioned media from BVAB-treated FRT epithelia. We also report evidence that this conditioned media may also exhibit proviral activity possibly through NF $\kappa$ B activity.

## CHAPTER II: MATERIALS AND METHODS

### Cell lines

End1 (human endocervical epithelia) and Ect1 (human ectocervical epithelia) cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained with keratinocyte serum-free media (KSFM) supplemented with epidermal growth factor, calcium chloride, and bovine pituitary extract (Life Technologies). A2EN (human endocervical epithelia) cell line was obtained from Dr. Alison J. Quayle (Louisiana State University Health Science Center, New Orleans, LA) and maintained with EpiLife media (Cascade Biologics) supplemented with EpiLife Defined Growth Supplement and calcium chloride. TZM-bl cell line was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.

### Bacterial cultures

*Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Mobiluncus curtisii*, *Atopobium vaginae*, *Prevotella bivia*, and *Gardnerella vaginalis* were obtained from the ATCC. The Lactobacilli strains were cultured in Man, Rogosa and Sharpe (MRS) agar in 37°C 5% CO<sub>2</sub> for 48 hours. The remaining strains were cultured in tryptic soy broth containing 5% defibrinated rabbit blood in 37°C with anaerobic GasPaks for 72 hours. Cultures of each are pre-prepared, aliquoted, and snap frozen for fresh use in experiments.

### Cell culture treatment for End1/Ect1/A2EN experiments

Confluent dishes of cells were treated either supplemented KSFM (mock) or bacteria diluted in supplemented KSFM. Supplemented KSFM and resuspended bacteria were also incubated in cell-free conditions as a control. Bacterial cultures taken directly from snap-frozen aliquots were centrifuged at 13,000 x g for 5 minutes at room temperature and resuspended in appropriate cell growth medium at a final multiplicity of infection (MOI) of 10 bacteria per cell. The bacteria were then placed on confluent cells in 100mm dishes and incubated at 37°C/5% CO<sub>2</sub> for 24 hours. MOI was confirmed through a serial dilution of the starting bacterial culture on the appropriate media to back calculate the number of bacterial colony forming units actually used to treat the cells.

#### Generation and processing of conditioned media

End1 cells were grown to confluence on 100mm tissue-culture treated plates and treated as described above with either a mock (supplemented KSFM) or *A. vaginae*. Conditioned media (CM) from all treatments was collected and clarified at 4000 x g at 4°C for 10 minutes. CM from each treatment was pooled to 15mL, which was then concentrated to 15X to be desalted. For future use in cell treatments, samples were desalted with an Amicon Ultra- 0.5mL Centrifugal Filter with a 3kDa molecular weight cutoff (EMD Millipore). The sample was filtered and washed twice with molecular grade water, resulting in a final salt concentration below 1X. Molecular grade water was used to bring final protein concentration back to 15X, and samples were stored at -20°C until use. Both cell-free KSFM and *A. vaginae* conditions are processed by

the above procedure as well. For convenience, conditioned media samples are labeled as follows:

A) Cell-free KSFM B) Cell-free *A. vaginae* C) End1/KSFM D) End1/*A. vaginae*.

#### Real-time polymerase chain reaction

Confluent End1 cells were treated with same treatment scheme and harvested with TRIzol reagent (Invitrogen). Total RNA was extracted, and contaminating genomic DNA was removed (Ambion's DNA-free kit). 200ng of total RNA was used to create cDNA with iScript cDNA synthesis according to manufacturer instruction (Bio-Rad). Real-time PCR was performed using TaqMan Gene Expression Master Mix with PrimeTime Std qPCR Assay kit (Life Technologies) for desired gene according to the following program: (Cycle 01) 50°C for 2 minutes, (Cycle 02) 95°C for 5 minutes, (Cycle 03) 95°C for 15 seconds followed by 60°C for 1 minute (40X). The housekeeping genes, GAPDH and 18S, were used as a quantitative control to which all samples were normalized to. PCR products were confirmed via 1.5% agarose gel electrophoresis.

#### Immunofluorescence

A2EN cells were grown on coverslips coated with poly-L-lysine and treated as described above with either a mock (supplemented EpiLife) or *A. vaginae*. Cells were fixed in 0.4% paraformaldehyde. Cells were then processed with Image-iT Fix-Perm kit (Life Technologies) according to company instructions. Briefly, cells were permeabilized and blocked with the provided buffers. Image iT FX signal enhancer (Life Technologies) was applied, and a monoclonal ZO-1 primary antibody (conjugated to FITC) (Invitrogen, ZO1-1A12) was diluted in the provided block buffer and incubated with the coverslips overnight at 4°C. Nuclear staining was performed with Hoechst 33528 nuclear stain (Life Technologies) followed by slide

mounting with ProLong Diamond Anti-Fade mounting media (Life Technologies). Slides were cured overnight and viewed.

#### A2EN Transwell model

24W Transwells inserts (Corning Incorporated) were coated with Human Placenta Derived Extracellular Matrix (Corning Incorporated) before use. A2EN cells were seeded onto the Transwell insert at 25,000 cells/well and maintained with apical and underlay supplemented EpiLife media containing a final calcium chloride concentration of 0.4mM at 37°C/5%CO<sub>2</sub>. At 10 days past seeding, the transepithelial electrical resistance (TEER) was measured to confirm the initial polarization; and apical media was removed to allow the cells to mature at air-liquid interface. After 3-5 days at air-liquid interface, TEER was measured to confirm complete polarization, and cells were treated at this point. Cells were considered a polarized monolayer when reaching a TEER of approximately 250-300  $\Omega \cdot \text{cm}^2$

#### NF $\kappa$ B Assays

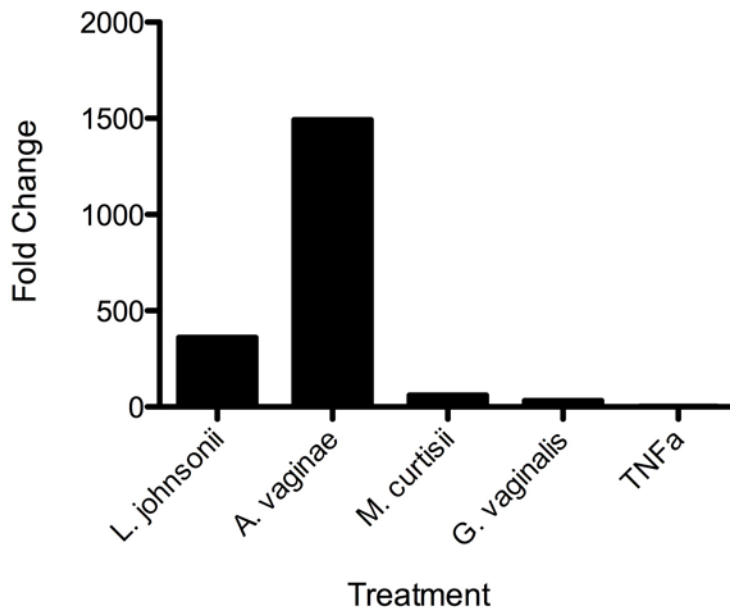
TZM-bl cells were seeded on a 96-well black, clear bottom plate and grown to 60-80% confluence. If an inhibitor, TPCA-1 (Tocris Bioscience), is tested, the inhibitor was applied overnight the previous day to the experiment. On the day of the experiment, CMs were diluted in 2X DMEM/2%FBS to maintain appropriate salt concentration. Treatments were added to the TZM-bl cells. If necessary, HIV was diluted in 1X DMEM/1% FBS and placed on the cells at this point as well. The plate was then allowed to incubate for 24h in 37°C/5%CO<sub>2</sub>. Treatment media was removed from the cells, which were then lysed with Glo Lysis Buffer (Promega) and allowed to complete one freeze-thaw cycle. Upon thaw, BrightGlo Reagent (Promega) was

added, and the luminescence, corresponding to HIV infection, is immediately read using a luminometer.

## CHAPTER III: RESULTS

### BVAB exposure results in an FRT innate immune response

The inflammatory response of the FRT to BVAB was previously proposed to result in increased HIV transmission risk as a result of chemotaxis of immune target cells creating a higher local concentration for potential infection [15]. We initially hypothesized that the BVAB exposure to FRT epithelial cells will also result in a decrease in barrier function that would allow more HIV virions to translocate the epithelia to the immune target cells below. To test the effect of BVAB exposure to FRT epithelia, we first confirmed the inflammatory response of the FRT epithelia using End1 cells, an endocervical cell line. Real-time TaqMan PCR was used to probe for the  $\beta$ -defensin, hBD-2, as a measure of the responsiveness of the cell line to pathogenic exposure. Exposure to *A. vaginae* resulted in a significant 1200-fold increase in hBD-2 expression (Figure 1). These results agree with previous data showing that *A. vaginae* elicits the most robust inflammatory response in this cell line.



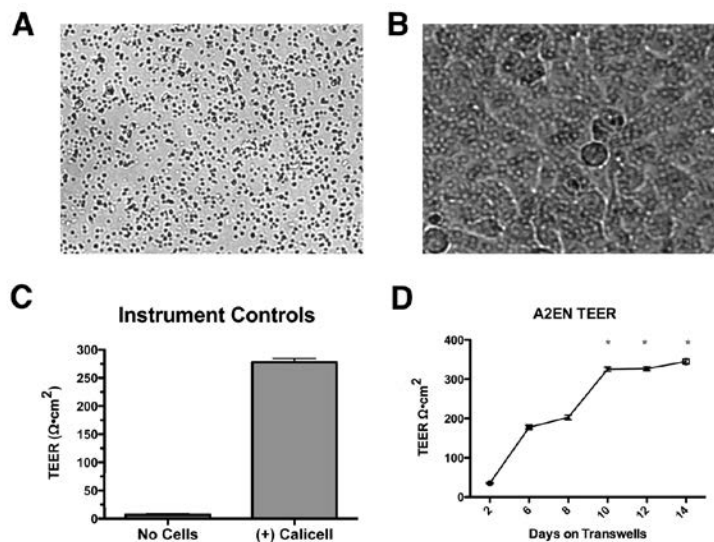
**Figure 1. hBD-2 Expression in End1 Cells.** Confluent End1 cells were treated with either a commensal bacterium (*L. johnsonii*) or BVAB for 24 hours in anaerobic conditions. The cells were lysed and RT-PCR was used to probe for expression of hBD-2 relative to a mock control condition. Bars represent the average and for two experiments.

After confirming the responsiveness of our FRT cell line, we wanted to determine whether the BVAB exposure resulted in changes in the innate barrier function by analyzing changes in ZO-1 expression, a tight junction protein, using Western blot. Results with this cell line, however, were inconclusive as the commercial antibody failed to identify a distinct band for ZO-1.

#### *A2EN cells polarize in a Transwell model*

End1 cells were not suitable for analysis of tight junctions via Western blot. We attempted to produce a polarized End1 monolayer on Transwell inserts and determine barrier function through transepithelial electrical resistance (TEER) measurements. According to company specifications,

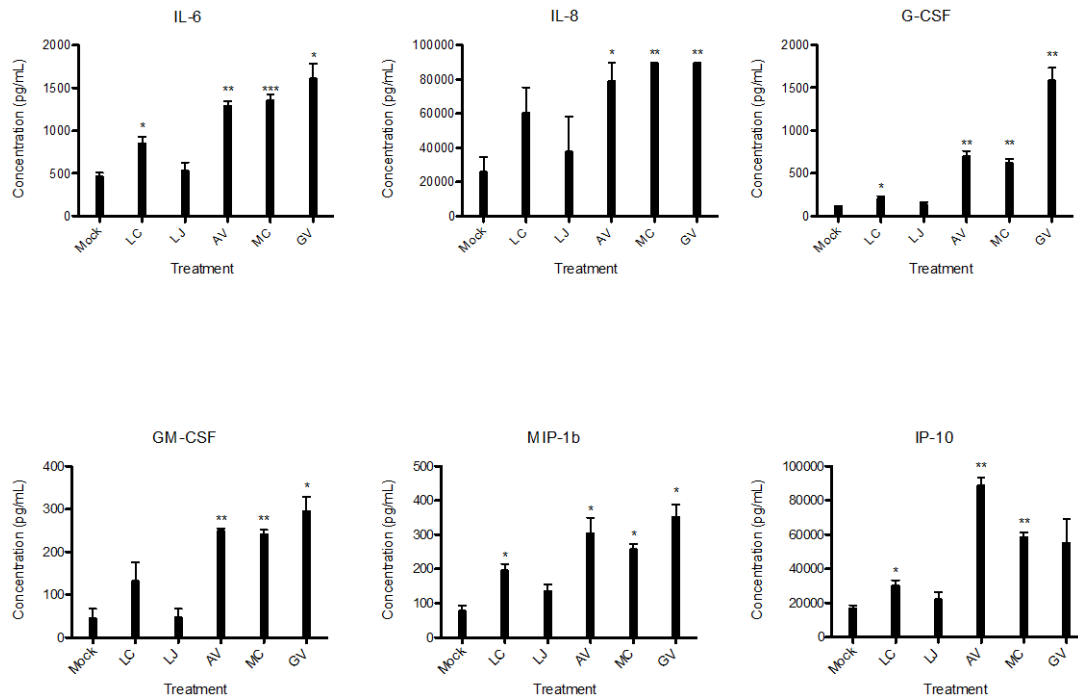
a completely polarized monolayer has TEER values ranging between 200-300  $\Omega\cdot\text{cm}^2$  based on positive control tested at various media temperatures (Figure 2C). The End1 and Ect1 cell lines were unable to produce polarized monolayers and did not produce TEER values above the positive control for a polarization. As a result, we generated a new *in vitro* FRT model that was more physiologically relevant, containing intact barrier function, mucin production, and an air-liquid interface characteristic of the lower FRT. This approach developed polarized monolayers with complete barrier function using the endocervical A2EN cell line (Figure 2B). At 10 days post-seeding, TEER values plateaued between 300-380  $\Omega\cdot\text{cm}^2$  and were considered mature with intact barrier function (Figure 2D).



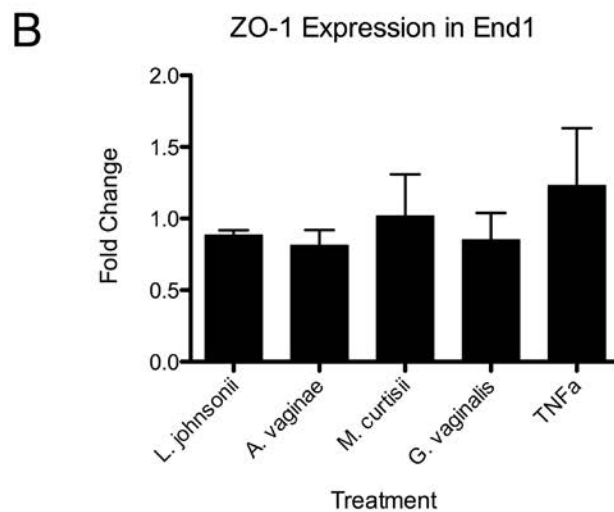
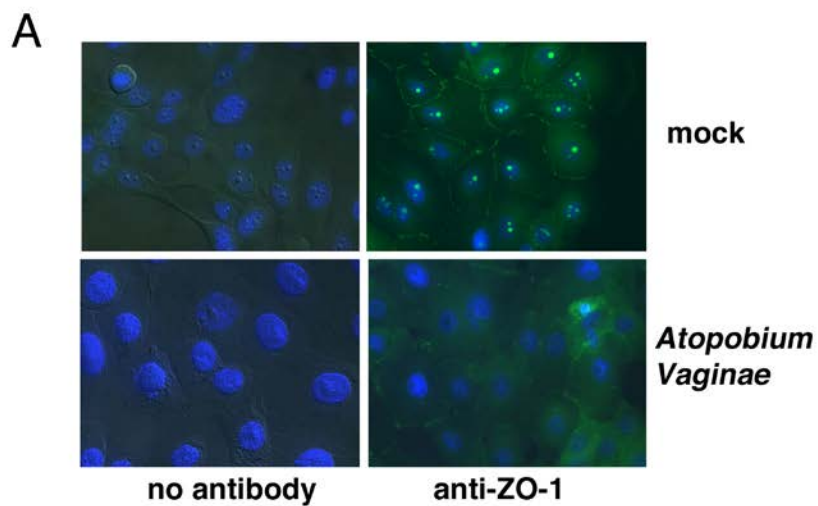
**Figure 2. A2EN Cells Produce Polarized Monolayer** A) Transwell insert with no cells seeded on membrane B) Transwell insert with confluent A2EN monolayer on membrane C) Calibration results of the EVOM<sup>2</sup> volttohmmeter using an ECM-coated Transwell insert and a company-provided positive control Calicell. D) A2EN cells were seeded onto 24 well Transwell inserts, and TEER was measured over a period of 14 days. N=7-12 for each data point. (\*p<0.0001 versus Calicell)

### A2EN submerged cells respond to BVAB and CM exposure

A2EN cells were cultured on coverslips and treated with commensal Lactobacilli and BVAB to determine 1) their immune response to BVAB, and 2) the presence of tight junctions via Bioplex analysis of cytokine levels and measurement of TEER changes. A2EN cells produced an inflammatory response to the presence of BVAB as shown by 3 fold increases in IL-6 and IL-8 concentration, 7-15 fold increases G-CSF concentration, 2 fold increases in GM-CSF concentration, 4 fold increases in MIP-1b and IP-10 concentration after treatment compared to the mock-treated control (Figure 3).



**Figure 3. A2EN Submerged Monolayer Produces Inflammatory Response to BVAB** A2EN cells are grown submerged on coverslips which are then treated with either *L. crispatus* (LC), *L. jensenii* (LJ), *A. vaginae* (AV), *M. curtisii* (MC), or *G. vaginalis* (GV) at an MOI of 10 for 24 hours. Cytokine quantification is completed by Bioplex analysis over three repeated experiments. (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 versus mock-treated control)



**Figure 4. Exposure to BVAB Causes Dysregulation of Cell-Cell Junctions in A2EN Cells** A) A2EN cells were grown on ECM-coated coverslips and treated with either a mock infection control or *A. vaginae* for 24 hours. Cells were then probed for the presence of ZO-1 (green) and counterstained with Hoechst 33528 nuclear stain (blue). B) End1 cells were treated with either a mock infection control or *A. vaginae* and lysed. Changes in ZO-1 expression were analyzed via RT-PCR.

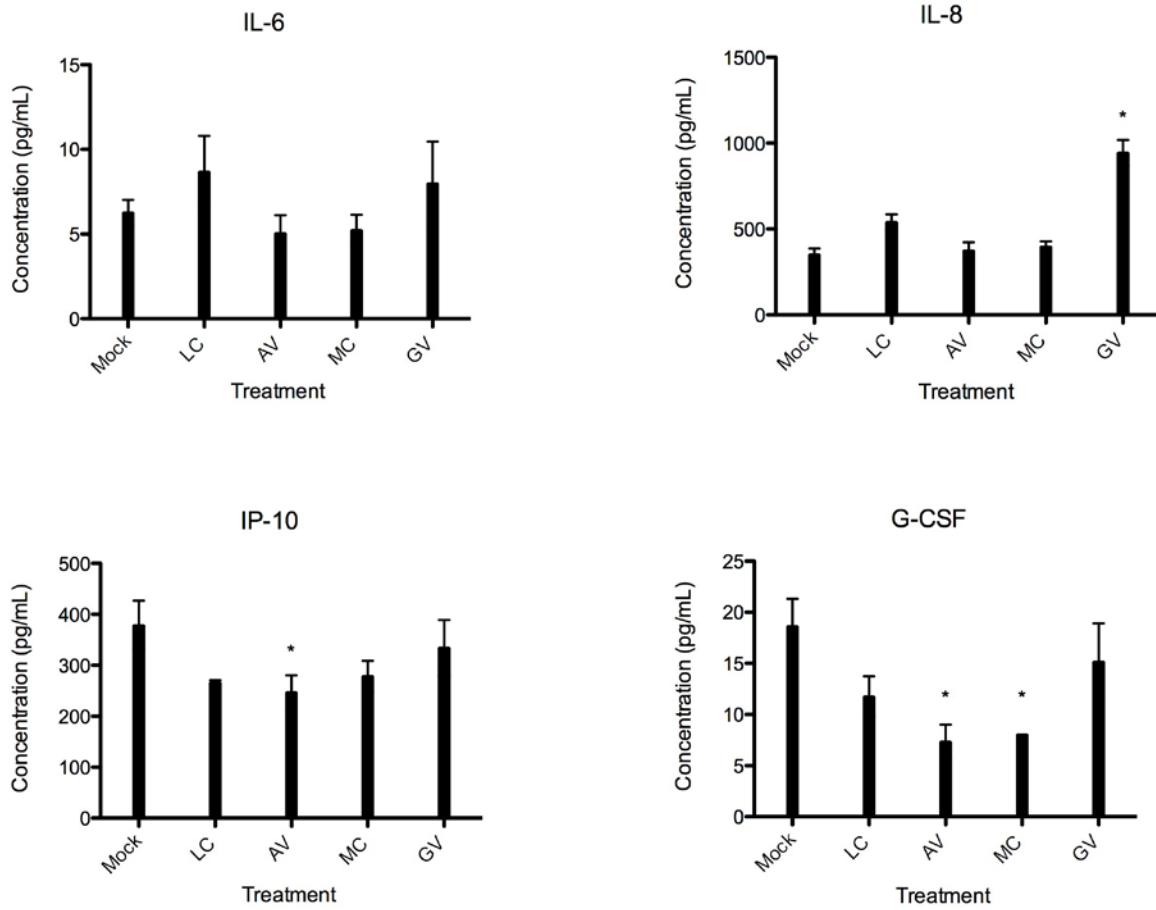
To determine whether this response also corresponded with a decrease in barrier function, we used immunofluorescence to view ZO-1 expression to visualize any changes in tight junctions between individual cells (Figure 4A). ZO-1 expression was identified at the borders between untreated A2EN cells, suggesting that these cells produce tight junctions and are capable of polarizing as ZO-1 is a major component of barrier formation [29]. Previous real-time PCR analysis in a parallel endocervical cell line, End1, indicate that after BVAB exposure, there is no significant change in ZO-1 mRNA expression (Figure 4B). These results are supported in the A2EN cell line. Treatment of A2EN cells with *A. vaginae* results in diffuse ZO-1 presence throughout the cell as opposed to ZO-1 concentration at the cell-cell border. ZO-1 expression did not decrease; instead the ZO-1 protein was delocalized after BVAB exposure, diffusing outward from the cell-cell border.

#### *Polarized A2EN cells respond to CM but not BVAB exposure*

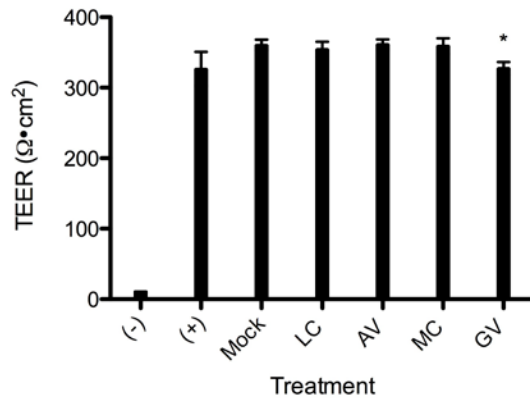
In addition to ZO-1 staining, we used the polarized A2EN cells to determine whether TEER changes as a result of BVAB exposure. Our polarized A2EN cells were treated with live BVAB and commensal bacteria. Surprisingly, the A2EN cells treated with BVAB exhibited a lower inflammatory immune response when growing at the air-liquid interface on Transwell inserts compared to submerged on coverslips (Figure 5). Baseline cytokine values for BVAB-exposed cells were lower compared to baseline measurements from submerged A2EN cells. Compared to the mock-treated control, cytokine concentrations were only significantly higher (2.6 fold increase) in *G. vaginalis* treatments when measuring IL-8. This result supports the decreased

immune response presented by the polarized A2EN cells as the IL-8 concentration for *G. vaginalis* is 95-fold lower than the IL-8 measurements for *G. vaginalis* treatment in submerged A2EN cells. BVAB treatments for *A. vaginae* resulted in significantly lower concentrations for IP-10 and G-CSF. Other treatments produced no significant changes in cytokine concentration compared to the mock-treated control. Overall, these results suggest that the BVAB had less impact on the polarized A2EN cells compared to submerged A2EN cells.

We then measured the corresponding TEER values after 48 hours of treatment with commensal and BV-associated bacteria (Figure 6). Even after 48h, there were no significant changes in TEER values for any treatment except *G. vaginalis* compared to the mock-treated control. *G. vaginalis* treatments did not significantly decrease TEER in polarized A2EN cells compared to the Calicell positive control. Combined with the lowered inflammatory response, we concluded that live bacteria did not appreciably impact the polarized A2EN cells.

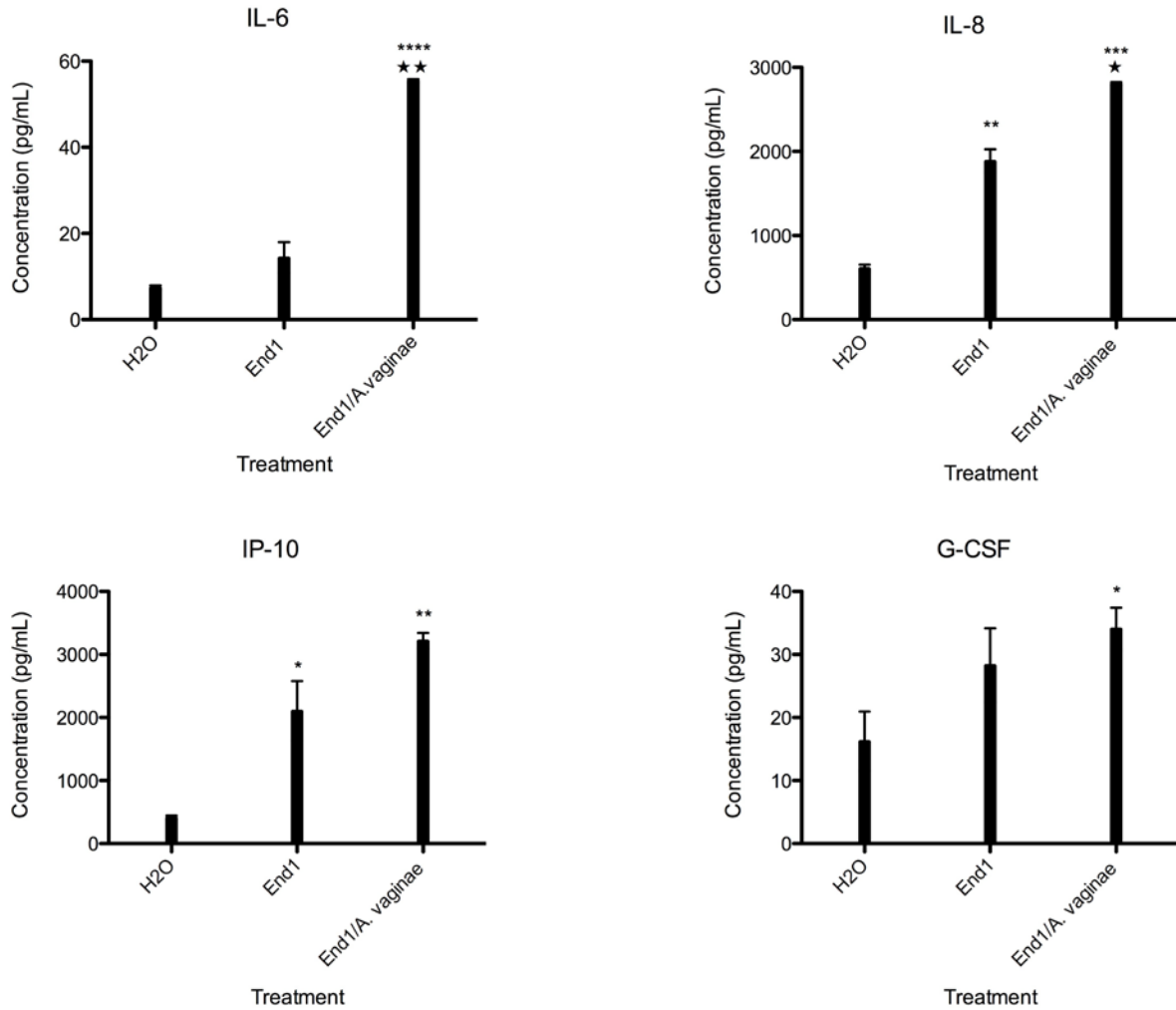


**Figure 5. Polarized A2EN Cells Produce Decreased Inflammatory Response to BVAB** A2EN cells were grown on Transwell inserts at the air-liquid interface. Upon maturation, cells were treated with either *L. crispatus* (LC), *A. vaginae* (AV), *M. curtisii* (MC), or *G. vaginalis* (GV) at an MOI of 10. Cytokine quantification was completed by Bioplex analysis over three repeated samples. (\* $p < 0.05$  versus mock-treated control)



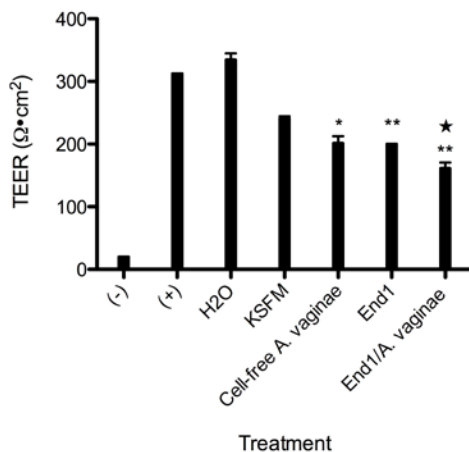
**Figure 6. Polarized A2EN Cells' TEER Values Are Not Impacted by BVAB Exposure** A2EN cells were grown at air-liquid interface on Transwell inserts. Upon maturation, cells were treated with either *L. crispatus* (LC), *A. vaginae* (AV), *M. curtisii* (MC), or *G. vaginalis* (GV) at an MOI of 10 for 48 hours at which point the TEER was measured. Above shows the average of three repeated samples per condition relative to the negative and positive controls.

Previous data from our laboratory revealed that conditioned media from a coculture of *A.vaginae* and End1 cells (End1/*A.vaginae* CM) had a proviral effect as compared to live bacteria alone. We then proceeded to test whether these conditioned media caused any immune response in polarized A2EN cells (Figure 7). After treatment with conditioned media produced from the End1/*A.vaginae* CM, the polarized A2EN cells produced an increased inflammatory immune response compared to treatment with live bacteria alone. End1/*A.vaginae* CM produced significantly higher concentrations of IL-6, IL-8, IP-10, and G-CSF compared to the vehicle control. Conditioned media from mock-treated End1 cells (End1 CM) without BVAB was used as a more stringent control. Treatment with End1/*A.vaginae* CM resulted in significantly higher concentrations of IL-6 and IL-8 compared to the End1 CM.



**Figure 7. Conditioned Media from Coculture of End1 Cells and *A. vaginae* Induce Inflammatory Response in Polarized A2EN Cells** A2EN cells were grown at air-liquid interface on Transwell inserts and treated with either water or conditioned media from End1 cells alone (End1) or a coculture of End1 cells with *A. vaginae* (End1/*A.vaginae*) for 48 hours. Cytokine quantification was accomplished via Bioplex testing, over three repeated samples. (\* $p < 0.05$  versus water, \*\* $p < 0.01$  versus water, \*\*\* $p < 0.001$  versus water, \*\*\*\* $p < 0.0001$  versus water, □ $p < 0.05$  versus End1, □□ $p < 0.01$  versus End1)

We then determined whether this increased immune response affected the barrier function of the polarized A2EN cells by measuring their TEER values after treatment with conditioned media from End1 cells and End1 cells cocultured with *A. vaginae* compared to additional controls: vehicle, desalted KSFM, and conditioned media from cell-free *A. vaginae* incubated in KSFM (Figure 8). Compared to the vehicle control, all TEER measurements were significantly decreased for the other controls and treatment. The cell-free *A. vaginae* conditioned media and End1 CM controls were also significantly lower than the desalted KSFM controls, however the End1/*A.vaginae* CM treatment resulted in significantly decreased TEER compared to every control. This increased immune response followed by decreased TEER measurements in the polarized A2EN cells after treatment with the End1/*A.vaginae* CM suggests that epithelially derived products from the coculture of End1 cells and *A. vaginae* are the effectors rather than the live bacteria themselves.



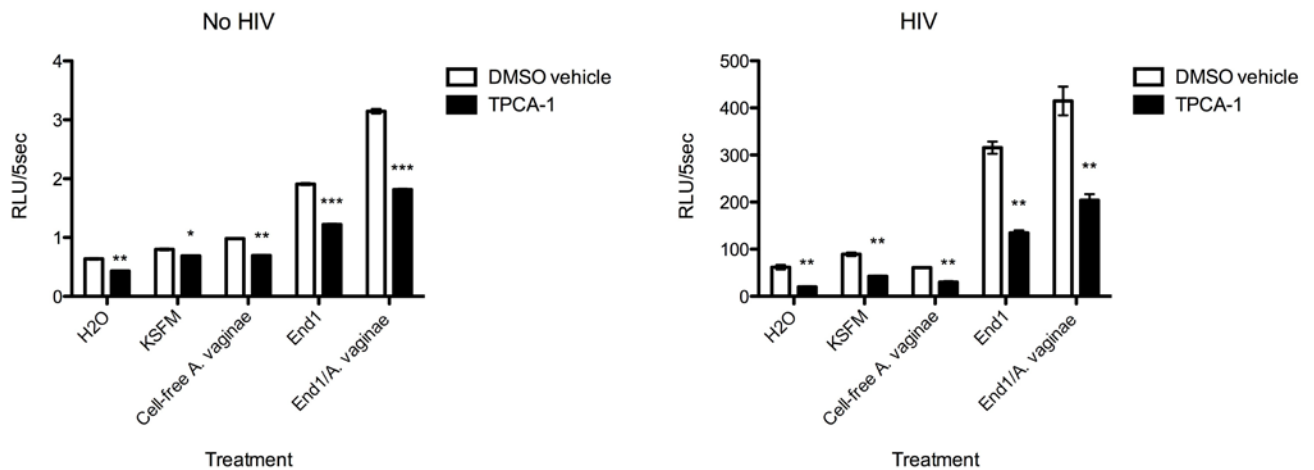
**Figure 8. Conditioned Media from Coculture of End1 Cells and *A. vaginae* Reduce TEER in Polarized A2EN Cells** A2EN cells were grown at air-liquid interface on Transwell inserts. Upon maturation, cells were treated with

conditioned media for 48 hours at which point the TEER was measured. Above shows the average of three repeated samples per condition relative to the negative and positive controls. (\* $p < 0.05$  versus KSFM, \*\* $p < 0.01$  versus KSFM,  $\square p < 0.05$  versus cell-free *A.vaginae* and End1 conditioned media controls)

### *CM shows HIV-enhancing activity potentially through NF $\kappa$ B activation*

We reasoned that the End1/*A.vaginae* CM contained the effector molecules that induce barrier dysfunction for a potential HIV-enhancing effect, and we wanted to explore the pathway by which these effectors function. In TZM-bl cells, luciferase production is under the control of the HIV-1 long terminal repeat (LTR). Activation of the HIV-1 LTR can occur through binding of the HIV-1 tat protein to the Tat-responsive region (TAR) after tat is produced from the integrated virus. Tat then increases transcription of HIV genes and activates NF $\kappa$ B signaling pathways [30, 31]. NF $\kappa$ B is a transcription factor whose activation and subsequent signaling pathway that has been associated with increased HIV transcription and replication through binding its recognition sequence on the HIV-1 long terminal repeat [32]. Through these processes, HIV infection and replication in TZM-bl cells can be determined as a function of luciferase production as measured by production of luminescence. End1/*A.vaginae* CM containing only the 3-30kDa fraction was previously shown to increase HIV infection of TZM-bl cells, indicated by increased luminescence values after the treatment [27]. To determine the pathway by which these CMs act provirally, we tested the End1/*A.vaginae* CM alone on TZM-bl cells to determine whether effectors in the CM could activate the HIV-1 LTR to produce luciferase on its own. Even without the presence of HIV-1 tat, the End1/*A. vaginae* CM increased the tat-induced luciferase production in TZM-bl cells by 3-fold compared to the control. As the proviral activity did not require HIV-1 tat, we tested to determine whether luciferase was produced from NF $\kappa$ B activation

of the HIV-1 LTR. This was tested by treating TZM-bl cells with End1/*A.vaginae* in the presence of the NF $\kappa$ B inhibitor, TPCA-1. Presence of the inhibitor resulted in significantly decreased luminescence in all samples both with and without the presence of HIV to drive luciferase production.



**Figure 9. TPCA-1 Treatment Results in Decreased HIV-1 Infection in TZM-bl Cells in Presence of CM** TZM-bl cells were treated with either TPCA-1, an NF $\kappa$ B inhibitor, or DMSO vehicle 24 hours prior to the experiment. Subsequently, cells were treated with conditioned media in absence or presence of HIV, as well as with and without TPCA-1 for 24 hours. Luminescence values (RLU/5sec for each well) were measured, and bars represent mean $\pm$ SEM, n=3. (\*p<0.05, \*\*p<0.01 \*\*\*p<0.001 compared to vehicle-control for each condition)

## CHAPTER IV: DISCUSSION

HIV transmission in the FRT is an unlikely event due in part to the layers of innate immunity present in the FRT [6, 7]. However, some gynecological conditions, such as BV, are associated with increased risk of HIV transmission in the host, possibly through mechanisms that affect the innate immunity [4, 20]. Previous studies have shown that exposure of the FRT cells *in vitro* to BVAB result in an inflammatory response [15], with which our data agrees. While it has been suggested that the increased HIV transmission risk is due in part to chemoattractants secreted in the inflammatory response [15], our studies explored the role of the epithelial barrier in this relationship.

We demonstrated an inflammatory response in our endocervical cell lines, End1 and A2EN, after exposure to live BVAB. However, only the A2EN cell line was capable of producing tight junctions in order to measure if the immune response also resulted in changes in barrier function. This is in agreement with previous literature showing that End1 cells do not polarize [33]. Immunofluorescence probing for ZO-1 in A2EN cells on coverslips revealed a change in ZO-1 localization, implying a dismantling of tight junctions, after exposure to BVAB. However, in our polarized A2EN cells, live bacterial treatments elicited a lower cytokine response than in the submerged A2EN cells and little significant impact on barrier function as measured by TEER. This could be a result of the more physiologically accurate representation that this approach provides. Polarized A2EN cells have additional defenses produced at air-liquid interface, such as mucus production [34], which may have inhibited the bacteria from eliciting effects from the epithelial cells.

TEER values decreased when the polarized A2EN cells were treated with conditioned media from the coculture of *A. vaginae* with End1 cells. We determined that epithelially derived products in the conditioned media were the effectors that disrupted the epithelial barrier as a result of an increased cytokine response and decreased barrier function compared to live bacterial treatment of polarized A2EN cells. This supports previous data from our laboratory suggesting that low-weight molecules in BVAB conditioned media acted to enhance viral infection [27].

In addition to analysis of barrier dysfunction, we also assayed our CMs for other mechanisms that promote HIV-enhancing activity. Previous data concludes that the 3-30kDa fraction from End1/*A. vaginae* CMs have HIV-enhancing activity from data showing increased luciferase production when TZM-bl cells were treated with End1/*A. vaginae* CM in the presence of HIV, relative to the control [27]. Our data using whole, unfractionated CM support these results as the End1/*A. vaginae* CM condition shows the highest luminescence values when used to treat TZM-bl cells with HIV present. Unexpectedly, the conditioned media generated from only End1 cells mock-treated with KSFM (End1 CM) also resulted in an increase in HIV infection of TZM-bl cells. As the lower FRT environment is typically antimicrobial and antiviral, we expected this condition to inhibit HIV infection in the TZM-bl cells. However, all conditioned media were produced from supplemented KSFM and then concentrated to 15X. The concentrated supplemented proteins, BPE and EGF, may have played a role that affected interaction between the TZM-bl cells and the HIV virions, thus producing the increased luciferase expression.

We also demonstrate that, even without the presence of HIV, End1/*A. vaginae* CMs are able to induce production of tat-driven luciferase in TZM-bl cells, suggesting that their proviral activity may involve other activators of the HIV-LTR. After treatment with TPCA-1, an NFκB inhibitor [35], we observed that in every condition, there is significantly lower luminescence and therefore luciferase expression in the TZM-bl cells. Decreased luciferase production in the presence of TPCA-1 suggests that NFκB can act independently of HIV-1 tat to activate the HIV-1 LTR. The CMs may activate an NFκB pathway to produce its HIV-enhancing effects by activating HIV-1 LTR. As NFκB is known to drive HIV transcription and replication [32], the HIV-enhancing activity of effectors in End1/*A. vaginae* CMs may be due to its ability to both increase HIV replication efficacy and allow more virions to reach target cells through barrier dysfunction.

We have yet to form a conclusion on whether the decreased TEER in the polarized A2EN cells translates into increased HIV translocation through the epithelial layer. Also, in previous studies, the 3-30kDa CM fractions were created to find the specific effector molecules that induce this HIV-enhancing activity in the presence of HIV [27]. It has not been tested yet whether those HIV-enhancing effectors in TZM-bl cells are also the same effectors that reduce TEER in the polarized A2EN cells. We have not explored whether there are additional proteins that have HIV-enhancing activity present in our whole, unfractionated CM as compared to the previously assayed 3-30kDa fraction. In addition, it has not yet been determined whether End1/*A. vaginae* CM increases viral replication in actual HIV targets cells, such as monocytes, T cells, or dendritic cells. While this is more physiologically relevant, the actual HIV target cells typically

require high serum conditions to survive, proliferate, and propagate HIV; however 10-20% serum masks the activity of fractionated CM. This technical hurdle is the subject of current investigation in our laboratory.

The presented studies, as well as the future proposed studies, will contribute to both the HIV and women's health fields, as the role of the FRT epithelial barrier has been understudied in the context of BV and HIV acquisition. Part of the reason for a paucity of data in the literature may be due to heretofore physiologically limited *in vitro* models for studying the interaction between pathogens and the FRT epithelia. Our new model provides physiologically accurate barrier properties and immune responses that may help provide a novel way to study these interactions. Understanding how BVAB interacts with the FRT could lead to new ways to defend against BV and sexually transmitted infections. A more comprehensive risk analysis of BV for HIV infection also allows clinicians to better gauge the importance of treatment and HIV prevention measures for BV-positive women. This study will also provide new insight into the mechanism by which HIV is transmitted through the mucosa, which could one day be used to develop new methods to prevent or reduce the incidence of HIV transmission.

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