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Evaluation of immunomodulatory compounds for identification of an anti-herpetic innate immune response profile

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Evaluation of immunomodulatory compounds for identification of an anti-herpetic innate immune response profile

by

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Dedication

This dissertation and all of the blood, sweat and tears associated with its composition is dedicated to my grandmother Florence Virva. Her life and death solidified my devotion to spend my life in the aid of others.

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Evaluation of immunomodulatory compounds for identification of an anti-herpetic innate immune response profile

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Herpes simplex virus type 2 (HSV-2) is a prevalent world-wide sexually transmitted infection (STI) that commonly infects the genital mucosa and establishes a life-long latent infection. The virus periodically reactivates producing recurrent shedding episodes that increase the risk of acquiring other STI. There are no FDA-approved HSV-2 vaccines and treatment involves chronic therapy that does not prevent all recurrences; therefore novel anti-herpetic intervention strategies are needed that provide resistance to HSV-2 infection. Because genital HSV-2 infections are more prevalent in women than men, most anti-herpetic strategies focus on vaginal application. One promising intervention involves the use of immunomodulatory compounds to engender an HSV-2 resistant environment by stimulating the natural innate immune defenses of the vaginal mucosa. The compounds consist of evolutionarily conserved pathogen-associated molecular patterns termed toll-like receptor (TLR) agonists that are recognized by vaginally expressed TLR and elicit specific cytokine response profiles. As an initial step in identifying cytokines that are associated with resistance to HSV-2 infection, selected

TLR agonists were evaluated in human cell culture or small animal models. An assay scheme was developed to identify anti-herpetic and non-anti-herpetic compounds and identified, for the first time, fibroblast stimulating ligand-1 as a novel anti-herpetic TLR agonist. Immunological evaluations of the compounds in human vaginal epithelial cells (EC) identified IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β and RANTES as important for establishing an HSV-2 resistant environment. The profile was confirmed and expanded to include IL-12(p40) and IFNβ following evaluations in a mouse model of genital HSV-2 infection. Additionally, the profile was observed in a novel in vitro air-interface vaginal EC model of the human vaginal mucosa. Colonization of the in vitro model with common vaginal commensal bacteria showed a temporally-dependent tempering of the TLR agonist elicited cytokine response and enhancement of agonist induced anti-herpetic activity. The identified anti-herpetic cytokine response profile provides an invaluable resource for the future design of novel immunomodulatory compounds that will aid in reducing HSV-2 transmission world-wide. Additionally, the studies in the novel in vitro model of the human vaginal mucosa showed that commensal bacterial play an important role in the vaginal defenses against pathogenic infection.

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List of Abbreviations

ACV	Acyclovir
BV	Bacterial vaginosis
cDNA	Complementary DNA
cfu	Colony forming unit(s)
CpG	Unmethylated CpG oligonucleotides
CML	Cumulative mean lesion score
CPE	Cytopathic effect(s)
DC	Dendritic cell(s)
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EC	Epithelial cell(s)
EC_{50}	Effective concentration 50%
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
FDA	Food and Drug Administration
FSL-1	Fibroblast stimulating ligand-1
FLAG	Flagellin
gD; gB	Glycoprotein D; Glycoprotein B
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GE	Genome equivalent(s)
GFP	Green fluorescent protein
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte monocyte-colony stimulating factor

GLN	Genital lymph node
H&E	Hematoxylin and Eosin
HIV-1	Human immunodeficiency virus type 1
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
ID_{50}	Infectious dose 50%
IFN	Interferon(s)
IL	Interleukin(s)
IMI	Imiquimod
LD_{50}	Lethal dose 50%
LPS	Lipopolysaccharide
MIP-1α	Macrophage inflammatory protein-1α
MIP-1β	Macrophage inflammatory protein-1β
MOI	Multiplicity of infection
MQAP	Microbicide Quality Assurance Program
MRS	De Man, Rogosa, Sharpe
MyD88	Myeloid differentiation primary response gene 88
NIH	National Institutes of Health
OTC	Over-the-counter(s)
Pam	$Pam_3CysSerLys_4$
PAMP	Pathogen-associated molecular pattern(s)
PAS	Periodic acid Schiff
pfu	Plaque forming unit(s)
PI	Post inoculation
PGN	Peptidoglycan

PIC	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor(s)
R-848	Resiquimod
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
ssRNA	Single-stranded ribonucleic acid
SCV-07	Gamma-d-glutamyl-l-tryptophan
SEM	Standard error of the mean
SRI	Southern Research Institute
STI	Sexually transmitted infection(s)
TE	10mM Tris/1mM ethylenediaminetetraacetic acid
TEER	Transepithelial electrical resistance
TGFβ	Tumor growth factor beta
T_H1	Type 1 T helper cells
TLR	Toll-like receptor(s)
TNF-α	Tumor necrosis factor alpha
TRIF	Toll/interleukin-1 receptor domain-containing adapter inducing interferon-beta
TS	Tryptic soy

CHAPTER 1: NOVEL ANTI-HERPETIC STRATEGIES FOR REDUCING HSV-2 INFECTION OF THE VAGINAL MUCOSA

HERPES SIMPLEX VIRUS TYPE 2

Herpes simplex virus type 2 (HSV-2), a member of the Herpesviridae family, is an enveloped double-stranded DNA (dsDNA) virus (Figure 1) that commonly infects the human genital mucosa [1-4]. Following infection, the virus replicates in the genital epithelial cells (EC) and in symptomatic individuals causes the formation of genital or other nonspecific lesions [4-6]. As the virus penetrates through the dermis, it encounters the sensory nerves and through retrograde transport enters the lumbosacral ganglia [3, 4, 6, 7]. The virus transitions to a latent phase in the ganglion and periodically undergoes reactivation and anterograde transport back to the epithelium near the original infection site due to several factors including stress, physical trauma or changes in the host immune system [3, 4, 6, 8]. As with the primary infection, genital lesions are formed during periodic recurrent episodes in symptomatic individuals [3, 4, 6]. Life-long intermittent clinical and subclinical reactivation and shedding of HSV-2 results in significant transmission that accounts for a high worldwide prevalence [3, 4, 6].

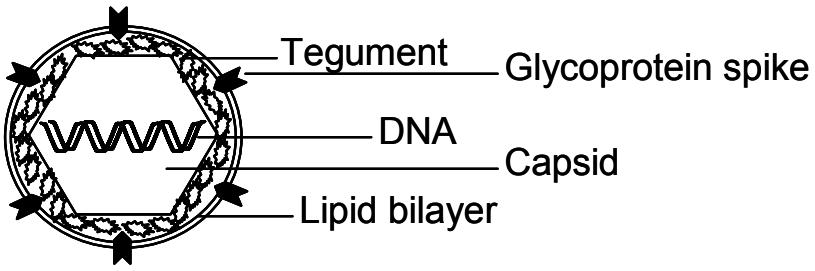


Figure 1. Structure of the HSV-2 virion. The double-stranded liner DNA genome is encased in an icosadeltahedral capsid surrounded by tegument. A lipid bilayer containing glycoprotein spikes surrounds the tegument.

HSV-2 prevalence varies from 16-18% in adult Americans [3, 9] and up to 97% in some African countries [3, 10]. Because women are more susceptible to infection than men [4, 6, 9, 11], HSV-2 is the most common sexually transmitted infection (STI) among the adult female population in the United States [4, 6, 12, 13]. The increased prevalence of HSV-2 infections in women compared to men is likely due to increased mucosal surface area for potential exposures, lack of a keratinized apical-most layer and preexisting pathogenic infections that can disrupt morphological or immunological defenses of the vaginal mucosa [4, 6, 12, 13]. Ashley and Wald summarized data indicating that only 20% of HSV-2 infected individuals exhibited typical lesions while 60% showed atypical lesions and 20% were completely asymptomatic [4, 14]. Asymptomatic viral shedding is important because roughly 70% of all heterosexual HSV-2 transmissions occur during this period [4, 6, 15]. The continued transmission of HSV-2 results in a staggering 500,000 new cases reported each year [3, 4, 6]. Because HSV-2 is the leading cause of genital ulcerative disease worldwide [16-18], preventing transmission is important for reducing the spread of this and other STI.

Recent studies showed that HSV-2 infected individuals are at a higher risk of acquiring other STI including bacterial vaginosis (BV), human immunodeficiency virus type 1 (HIV-1) and *Neisseria gonorrhea* [3, 11, 13, 19]. For HIV-1, the increased risk of acquisition is potentially due to the macro- or micro-lesions formed during symptomatic primary or recurrent genital HSV-2 disease [20, 21]. The lesions act as portals of entry for pathogens by disrupting the integrity of the genital mucosa [11, 20, 21], a critical barrier to pathogenic infection [22, 23; Chapter 6]. Additionally, leukocytes are recruited to the site of genital lesion formation and the increased population of permissive cells offers another potential explanation as to why HSV-2 infection is a significant risk factor for HIV-1 acquisition [24, 25]. Based on the lesion-associated increase in STI risk [3, 11,

13, 19], effective anti-herpetic strategies are needed to reduce the prevalence of genital HSV-2 infections.

Currently, there are no Food and Drug Administration (FDA)-approved HSV-2 vaccines [3, 4, 26]. A potential vaccine containing a truncated HSV-2 glycoprotein D (gD) in a novel lipid adjuvant showed limited success in a phase III clinical trials [27]. The gD subunit vaccine resulted in significant prevention of HSV-2 transmission but only in women that were seronegative for herpes simplex virus type 1 (HSV-1) and HSV-2 prior to vaccination [27]. A confirmatory trial for the gD subunit vaccine is underway [3, 26] and other potential options including live attenuated, DNA or envelope protein subunit vaccines also are under evaluation [26, 28-30]. Due to the high prevalence of existing infections [3, 10] and limited vaccine efficacy [27], therapeutic interventions also are needed to aid in reducing HSV-2 transmission.

While there is no cure for HSV-2 infection, several antiviral compounds are available that can reduce recurrent disease episodes [3, 4, 6, 31]. Current suppressive therapy consists of several nucleoside analog compounds including acyclovir that inhibit HSV-2 replication by blocking viral DNA synthesis [3, 4, 6, 31]. Several studies showed that the antiviral compounds significantly decreased lesion healing time, viral shedding and appearance of new lesions [32-36]. In another study, valacyclovir significantly reduced transmission among HSV-2 discordant couples [33]. Although the studies showed that suppressive therapy is an effective treatment for recurrent genital HSV-2 episodes, the need for chronic application due to lack of durability and failure to prevent all recurrences limits the usefulness of the antiviral compounds [3, 4, 6, 37]. Reducing lesion formation and viral shedding is critical for minimizing the spread of HSV-2; therefore novel anti-herpetic strategies that can reduce transmission by augmenting the innate defenses of the vaginal mucosa are necessary [Chapters 2-6].

VAGINAL DEFENSES AGAINST HSV-2 INFECTION

The EC of the vaginal mucosa are the site of first contact for genital HSV-2 infection [38-41]. The vaginal EC form a non-keratinized stratified squamous epithelium that acts as a physical barrier to potential pathogens [22, 23]. The vaginal mucosa is composed of 3 distinct types of EC known as parabasal, intermediate and superficial cells (Figure 2) [22, 23, 42, 43]. Parabasal cells are rounded with a high nuclear to cytoplasmic ratio and form the generative basolateral layer that connects to the basement membrane [22, 23, 42, 43]. The parabasal cells actively divide into intermediate cells that form the middle layers and the elongated anucleate superficial cells comprise the apical or luminal layer [22, 23, 42, 43]. Tight junction complexes between vaginal EC serve as structural scaffolding and connect the cell layers within the mucosa [22, 23, 44]. Vaginal mucosa and tight junction integrity plays a critical role in defending against STI by restricting passage of pathogens into the cell layers [43, 45]. Vaginal defenses also are aided by the presence of a robust innate immune system and commensal flora that provide resistance to pathogenic infection [11, 13, 46-51].

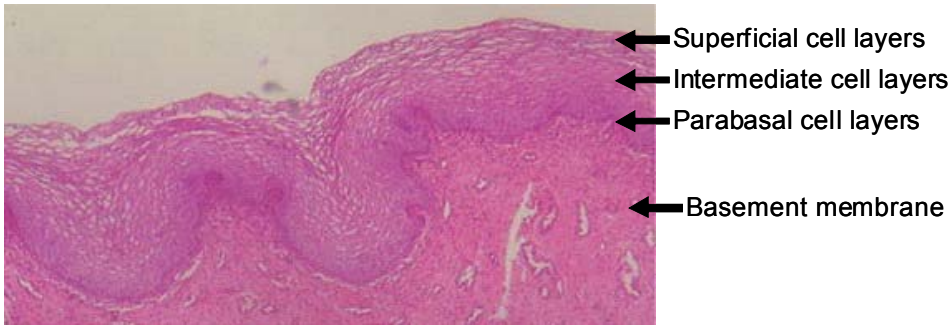


Figure 2. Human vaginal mucosa. The human vaginal mucosa is a stratified squamous epithelium composed of 3 distinct cell layers. The vaginal mucosa also forms an air (lumen)/liquid (vascular system) interface containing tight junction complexes, glycogen in the intermediate and superficial layers and microvilli on the luminal surface. (Image from http://commons.wikimedia.org/wiki/File:Vagina_(mucosa).jpg 10/21/09)

Innate immunity of the vaginal mucosa

The EC of the vaginal mucosa are active participants in the innate immune response against HSV-2 infection [48, 50, 51]. Vaginal EC express pattern recognition receptors (PRR) that recognize evolutionarily conserved pathogen structures termed pathogen-associated molecular patterns (PAMP) [48, 50, 51]. PRR are divided into different families based on receptor structure with toll-like receptors (TLR) being the most extensively studied family [51-53]. The TLR family consists of type I transmembrane receptors that are highly conserved across mammals, insects and even plants [51-55]. TLR are located on the plasma membrane (TLR1-2 and 4-6) or within endolysosomal compartments (TLR3 and 7-9) in vaginal EC (Figure 3) [48, 51-53]. Each TLR as a homo- or heterodimer binds to a specific PAMP or TLR agonist (Table 1) and, based on the ligand, initiates a particular signaling transduction cascade [48, 51-53]. TLR signaling elicits cytokine production and leukocyte recruitment resulting in a highly coordinated innate immune response against genital HSV-2 infection [48, 50, 51].

Table 1: TLR recognize specific PAMP represented by TLR ligands.

	PAMP recognized	**TLR agonist (TLR ligands)**
TLR1/2	Triacyl lipopeptides	$Pam_3CysSerLys_4$ (Pam)
TLR2/6	Diacyl lipopeptides, Lipoteichoic acid	Fibroblast stimulating ligand-1 (FSL-1)
TLR2	Lipoproteins, Peptidoglycan	Peptidoglycan (PGN)
TLR3	dsRNA	Polyinosinic:polycytidylic acid (PIC)
TLR4	Lipopolysaccharide	Lipopolysaccharide (LPS)
TLR5	Flagellin	Flagellin (FLAG)
TLR7/8	ssRNA	Imiquimod (IMI), Resiquimod (R-848)
TLR9	Unmethylated CpG oligonucleotides	Unmethylated CpG oligonucleotides (CpG)

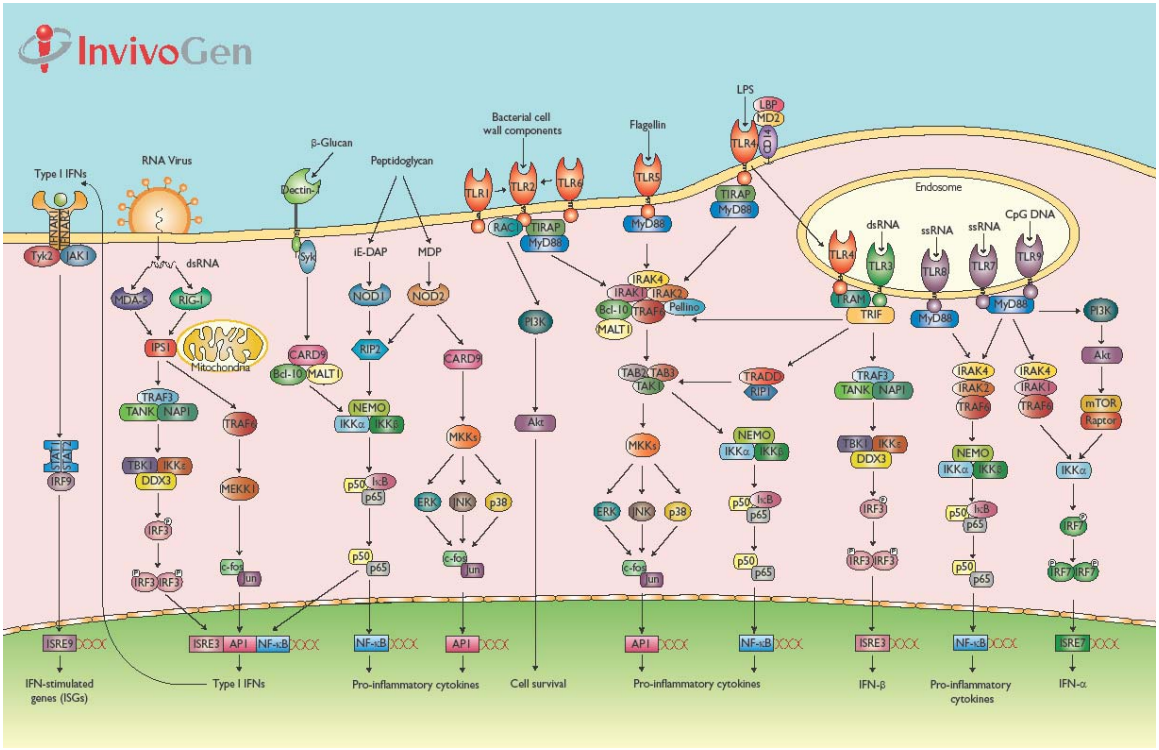


Figure 3. Cellular distribution of PRR and associated signaling pathways. Image reproduced in accordance with copyrights from www.invivogen.com.

HSV-2 recognition via toll-like receptors

Human vaginal EC express TLR2, 3, 5 and 6 at high levels and TLR1 and 4 at moderate levels with a subpopulation expressing low levels of TLR7-9 [50]. HSV-2 recognition at the vaginal EC plasma membrane occurs through TLR2-mediated binding of the glycoprotein spikes within the viral lipid envelope [51, 56, 57]. Studies using TLR2 knockout mice showed an altered neuropathogenesis following HSV-1 infection [58, 59] and reduced cytokine production after HSV-2 infection [60]. TLR1 or 6 often dimerizes with TLR2 [51, 57] indicating HSV-2 recognition could be aided by either of the two receptors. Additionally, viral glycoproteins expressed by respiratory syncytial

virus were shown to activate TLR4 [51, 56, 57, 61] indicating that HSV-2 glycoproteins also could potentially be recognized by TLR4.

HSV-2 infection of vaginal EC exposes the viral dsDNA for recognition via TLR9 present within the cells [51, 56, 57]. Plasmacytoid dendritic cells (DC) express high levels of TLR9 and are capable of recognizing the dsDNA genome of HSV-2 [62, 63]. Sorensen et al. showed that even in TLR2/TLR9 double knockout mice HSV-2 infection elicited an innate immune response [60] indicating a potential for recognition via other TLR. HSV-2 replication or latency associated transcript formation commonly produces single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) intermediates that are recognized by TLR7 or 3; respectively [64-69]. Collectively, HSV-2 recognition by membrane-bound or endolysosomal TLR activates intracellular signaling pathways leading to an anti-herpetic innate immune response [51, 56, 57].

Upon engagement of all TLR except for TLR3 by HSV-2 PAMP, the receptor undergoes a conformational change and initiates signal transduction through myeloid differentiation primary response gene 88 (MyD88) [51, 53, 70, 71]. TLR3 uses the toll/interleukin-1 receptor domain-containing adapter inducing interferon-beta (TRIF) signaling molecule [51, 53, 70, 71]. Recent work with MyD88 knockout mice showed increased susceptibility to genital HSV-2 infection [72] underscoring the importance of TLR mediated signaling for HSV-2 resistance. Activation of the signaling pathways leads to transduction of nuclear factor kappa-light-chain-enhancer of activated B cells, mitogen-activated protein kinases or interferon regulatory factors [51, 53, 70, 71]. Each transcription factor regulates expression of genes coding for different cytokines resulting in activation of TLR-specific innate immune responses [51, 53, 70, 71].

Cytokine-mediated anti-herpetic activity

Cytokines are proteins, peptides or glycoproteins expressed by vaginal EC and other cell types that act as extracellular chemical signaling molecules and are responsible for coordinating the innate immune response against pathogens like HSV-2 [57, 73, 74]. One cytokine subfamily, termed interleukins (IL), activates and stimulates leukocytes and also aids in the production of interferon (IFN) [57, 74]. The importance of IL for mediating the innate immune response to pathogens was highlighted by studies in guinea pigs that showed significant resistance to genital HSV-2 infection following application of recombinant IL-2 [75]. Other studies showed that IL-12 knockout mice are more susceptible to genital HSV-2 infection than wild type mice and intraperitoneal delivery of IL-12 enhanced resistance to genital HSV-2 infection in wild type mice [76-78]. In addition to providing resistance to HSV-2 infection [57, 73-78], IL-2 and IL-12 synergistically induce IFN production [77, 79, 80].

Type I and II IFN are another cytokine subfamily and are responsible for inhibiting viral replication, priming cytokine responses in uninfected cells and activating leukocytes [51, 74, 81, 82]. Both IFNβ and IFNγ exhibit potent activity against genital HSV-2 infection following production via TLR activation or IL stimulation [81, 82]. The importance of IFNβ induced anti-herpetic activity was confirmed in studies that showed significantly enhanced HSV-2 pathogenicity in mice lacking the receptor for IFNα/β [82, 83]. IFNγ, an IFN induced through synergistic IL-12 and IFNβ signaling, provided significant resistance to genital HSV-2 infection in mice by inhibiting viral replication and activating T cells [77, 84, 85].

The highly coordinated innate immune response against genital HSV-2 infection also involves induction of chemokines [57, 81, 86]. As another subfamily of cytokines, chemokines are extracellular signaling molecules that establish a concentration gradient

at the infection site resulting in leukocyte recruitment [51, 57, 81, 86]. Thapa et al. showed that mice lacking the chemokine receptor CCR5 were more susceptible to genital HSV-2 infection due to reduced recruitment of natural killer cells [87]. Granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage-CSF (GM-CSF) also are important for production and maturation of leukocytes [51, 73, 81], but thus far have not been linked with establishment of an HSV-2 resistant environment. In addition to producing chemotactic activity, chemokines like macrophage inflammatory protein (MIP)-1α, MIP-1β and regulated upon activation, normal T-cell expressed, and secreted (RANTES) inactivated HSV-1 by directly binding to viral surface proteins [88]. Collectively, in vitro and in vivo evaluations of the cytokine response profile elicited following TLR activation by HSV-2 PAMP are necessary to identify cytokines important for providing resistance to genital HSV-2 infection.

Phagocyte recruitment in response to HSV-2 infection

Phagocytes, a subset of leukocytes, are recruited to the infection site by chemokines and are critical to the innate immune response against HSV-2 [40, 51, 81]. Milligan et al. showed that neutrophils, a type of phagocyte, play an important role in limiting and clearing HSV-2 vaginal infections in mice [89, 90]. The observed anti-herpetic activity occurred through neutrophil mediated killing of HSV-2 infected cells via phagocytosis, release of reactive oxygen species or nitric oxide following recruitment to the infection site by an IL-8 chemokine gradient [40, 51, 81, 89]. Additionally, neutrophils release anti-herpetic proteins like defensins and secretory leukocyte peptidase inhibitor that actively block pathogen entry into cells, inhibit replication and prevent cell-to-cell spread [40, 81, 91, 92]. Like vaginal EC, neutrophils also express TLR and

following pathogen recognition aid in recruitment of other phagocytes through chemokine induction [51, 93, 94].

Macrophages, another phagocyte, migrate to the infection site in response to an established CCR5 chemokine gradient [81, 95, 96]. Cytokines including IL-12, IFNβ and RANTES are produced by macrophages following recognition of HSV-2 PAMP [81, 97, 98]. Macrophages also are important to the innate immune response by engulfing HSV-2 infected cells through compliment-assisted phagocytosis [81, 99-101]. Following opsonization, macrophages process viral components for presentation to T cells resulting in initiation of an adaptive immune response [51, 81, 102].

Langerhans cells, a specialized type of DC present within the vaginal mucosa and other epithelia, continuously sample the vaginal environment and initiate an innate immune response following pathogen recognition [40, 51, 81, 103, 104]. DC also are recruited to the infection site by established CCR5 chemokine gradients following genital HSV-2 infection [40, 81, 103, 104]. DC express a subset of TLR and are important mediators of the innate immune response through cytokine production [51, 104, 105]. Studies with DC depleted mice showed an increase in susceptibility to HSV-1 infection likely reflecting a lack in natural killer and T cell activation [106]. Like macrophages, DC also serve as a bridge to the adaptive immune response through antigen presentation to T cells [40, 51, 81, 103, 104].

Commensal bacteria of the vaginal mucosa

In addition to the highly coordinated innate immune response elicited by vaginal EC following pathogen recognition [48, 50, 51], vaginal defenses also are supported through commensal bacteria produced antimicrobial activities [107-112]. Many different species of bacteria including *Lactobacillus*, *Corynebacterium*, *Prevotella* and

Peptostreptoccoccus colonize the vaginal mucosa in varying quantities [107, 113-115]. The specific bacterial populations present in the vaginal environment vary among women and even can vary over time within the same woman [107, 113-116]. Overall, lactobacilli are the most commonly isolated bacteria and the population is comprised predominantly of *Lactobacillus crispatus* and *L. jensenii* [114, 115]. Lactobacilli are rod shaped gram-positive facultative anaerobes that can ferment glycogen produced by the vaginal mucosa [22, 109, 117, 118; Chapter 6].

Commensal bacteria, specifically *Lactobacillus spp.*, are a vital component of the vaginal defenses against HSV-2 infection through pH modulation, adherence prevention and antimicrobial protein production [107-112]. *L. crispatus* and *L. jensenii* fermentation of glycogen produces lactic acid [109, 117; Chapter 6] and the resulting acidic pH (3.6-4.5) of the vaginal environment inhibits STI replication [108, 109]. Also, the presence of lactobacilli on the vaginal mucosa prevents attachment of pathogenic organisms like *N. gonorrhoeae* [111, 112, 119]. Spurbeck and Arvidson showed that *N. gonorrhoeae* was unable to effectively bind to vaginal EC colonized with *L. jensenii* [112]. Lactobacilli also aid resistance to pathogenic infection by secretion of antimicrobial peptides and H_2O_2 production [107, 109, 110, 120-123]. Bacteriocins are peptides that exert antimicrobial activity by disrupting membranes likely through pore formation [110, 121, 122] and H_2O_2 production can kill or inhibit the growth of other bacteria lacking peroxidase enzymes [107, 120].

Loss of *Lactobacillus spp.* from the vaginal environment results in the development of a pathogenic state termed BV in which lactobacilli are replaced with gram-negative and other types of bacteria including *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Mobiluncus spp*. and/or *Atopobium vaginea* [124-127]. BV-associated bacteria colonization of the vaginal mucosa results in a loss of the

protective effects afforded by a low pH environment [117, 126, 127]. Several studies showed that the bacteria involved in BV elicited chronic production of inflammation-related markers like IL-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) resulting in an increased susceptibility to STI [128-131]. The BV induced responses also damaged the integrity of the vaginal mucosa [128, 130, 131] offering another potential explanation for the observed higher risk of STI in women with BV [130, 132-135]. Additionally, HSV-2 acquisition was significantly higher in women with BV [11, 13, 46, 47, 49] underscoring the importance of lactobacilli in the vaginal defense against herpetic infection.

NOVEL ANTI-HERPETIC STRATEGIES

While the innate immune response and commensal bacteria of the vaginal mucosa coordinately provide a potent defense against HSV-2 infection [11, 13, 46-51], vaginal defenses can be compromised or evaded resulting in increased HSV-2 acquisition. The loss of commensal bacteria, specifically lactobacilli, and resulting development of BV increases susceptibility to genital HSV-2 infection [11, 13, 46, 47, 49]. Additionally, HSV-2 has evolved several mechanisms to inhibit the innate immune response of the vaginal mucosa [26, 40, 83, 136, 137]; therefore novel anti-herpetic intervention strategies are necessary to augment the vaginal defenses. Currently, microbicides and immunomodulatory compounds are two types of anti-herpetic strategies under evaluation in preclinical and clinical trials [51, 57, 138-141]. The anti-herpetic compounds are divided into two different types based on their mechanisms of producing activity against genital HSV-2 infection [51, 57, 138-141].

Microbicides are compounds designed for topical application to the vaginal or rectal mucosa prior to or just after a sexual encounter to reduce the spread of STI [138,

139, 141]. Activity of microbicides occurs through direct interaction with the pathogen or by maintaining, enhancing or replacing a natural vaginal defense like the acidic environment [138, 139, 141]. Immunomodulatory compounds, consisting of TLR agonists or dipeptides, are designed for application prior to potential transmission events and act by initiating an innate immune response that establishes an environment resistant to pathogenic infection [51, 57, 140]. An advantage of the two anti-herpetic strategies over the use of nucleoside analogs like acyclovir is that the microbicides or immunomodulatory compounds can prevent initial HSV-2 infection of the vaginal EC as opposed to preventing cell-to-cell spread after infection [3, 4, 6, 31, 51, 57, 138-141]. Acyclovir initiates chain termination during replication; therefore HSV-2 must infect vaginal EC before the compound is effective [3, 4, 6, 31]. The anti-herpetic strategies act directly against the HSV-2 virion or "prime" an anti-herpetic innate immune response prior to HSV-2 introduction into the vaginal environment thereby preventing initial infection [51, 57, 138-141]. Identification of the innate immune response profile associated with anti-herpetic activity [Chapters 2-4] is critical for the future design of novel vaginally-applied anti-herpetic intervention strategies.

Another advantage of the vaginally-applied microbicides or immunomodulatory compounds is that the intervention strategies place control of STI prevention in the hands of women [138, 139, 141]. Condoms provide an effective barrier to many STI but in some cultures women lack the ability to control their use during a sexual encounter [138, 139, 141]. Additionally, condoms are a potential concern for STI-discordant couples interested in protection against transmission without preventing conception [138, 139, 141]. Vaginally-applied anti-herpetic strategies allow the option for women to covertly apply the compound and can be formulated to provide resistance to HSV-2 infection without preventing conception [138, 139, 141]. The vaginally-applied anti-herpetic

compound also can be formulated in combination with over-the counter (OTC) feminine products to increase ease of use and compliance [141-144].

Based on the proposed use of OTC products as carriers for vaginally applied anti-herpetic compounds [141-152], quantifying their impact on vaginal EC is an important preliminary safety evaluation [Chapters 2 and 3]. OTC products can contain herbal ingredients that have only undergone limited human testing [147] or compounds like nonoxynol-9 that disrupt the integrity of the vaginal mucosal barrier [148, 149]. Additionally, semen is occasionally introduced into the vaginal environment and consists of a complex mixture of many different proteins including cytokines like tumor growth factor beta (TGFβ) [145, 146]. The alkaline chemical components present in semen also could potentially alter the ability of the vaginal EC to respond to pathogens [141-144]. Evaluating the impact of semen on HSV-2 infections or vaginal EC innate immune responses are critical preliminary safety evaluations [Chapters 2 and 3]. Additionally, quantifying the potential impact of anti-herpetic compounds on vaginal defenses is another important safety measure [Chapters 3 and 6]. Evaluation of repetitive anti-herpetic compound application [Chapter 4] is necessary due to the potential for multiple transmission events in the case of female sex workers [11, 153-155]. Because commensal bacteria are an integral component of vaginal defenses, anti-herpetic compounds also must be evaluated for potential impact on the commensal population and vice versa [Chapter 6].

Microbicides

Microbicides, one type of anti-herpetic compound, are further subdivided into 3 different classes based on their mechanism of action [138, 139, 141, 156]. The membrane disruptors (or surfactants), nonoxynol-9 and SAVVY, act by inhibiting lipid-

lipid interactions thereby destroying lipid membranes and rendering the pathogens inactive [148, 157]. While nonoxynol-9 and SAVVY showed promising activity against HSV-2 in vitro and in small animal models [148, 157-161], clinical trials were stopped due to toxicity [148, 162] or lack of an observed effect [163, 164], respectively.

Microbicides of the entry blocker class inhibit HSV-2 infections by binding to the surface of the virions and preventing receptor mediated entry [165-167]. Unfortunately, clinical trial evaluations for the entry blockers cellulose sulfate and carraguard were stopped due to lack of a protective effect [168, 169]. Also, clinical trials for another entry blocker termed PRO2000 showed an increase in adverse events including mild vaginal bleeding or irritation following application of a 4% PRO2000 gel [170, 171].

Acidiform and BufferGel are vaginal defense enhancers (or vaginal milieu protectors) that consist of a polymeric acidic buffer designed to maintain a low vaginal pH even in the presence of semen [172, 173]. Both pH buffering compounds exhibited promising preclinical activity against HSV-2 leading to ongoing evaluations in clinical trials [173-177]. Overall, the toxicity and lack of activity observed for many tested microbicides [162-164, 168, 169] illustrates a need for alternative strategies to reduce genital HSV-2 infections with improved efficacy and safety.

Immunomodulatory compounds

Immunomodulatory compounds are PAMP designed to elicit specific innate immune responses following PRR activation [51, 57, 140]. Several TLR agonists including fibroblast stimulating ligand-1 (FSL-1) polyinosinic:polycytidylic acid (PIC), imiquimod (IMI), resiquimod (R-848) and unmethylated CpG oligonucleotides (CpG; Table 1) elicited potent activity against HSV-2 infection in vitro and in small animal models [80, 82, 178-185; Chapters 2-4]. Interestingly, pam3CysSerLys4 (Pam),

peptidoglycan (PGN), lipopolysaccharide (LPS) and flagellin (FLAG; Table 1) were found to exhibit no anti-herpetic activity [182, 183, 185] indicating that production of an HSV-2 resistant environment requires induction of specific cytokine response profiles [Chapters 2-4].

Additionally, in vitro and in vivo antimicrobial activity also was observed following application of immunomodulatory dipeptides [186-190]. Oral application of gamma-D-glutamyl-L-tryptophan (SCV-07), an immunomodulatory dipeptide, significantly reduced recurrent genital HSV-2 disease in guinea pigs [191; Chapter 5] supporting evaluation of alternative delivery routes for immunomodulatory compounds. In addition to eliciting anti-herpetic activity through multiple delivery routes [82, 178, 180, 181, 191], several studies showed that immunomodulatory compounds enhanced vaccine efficacy when used as adjuvants [192-194]. Immunomodulatory compounds offer the unique ability to selectively augment the innate immune response to provide potent activity against genital HSV-2 infection through the activation of TLR using evolutionarily conserved PAMP.

SCOPE OF DISSERTATION

The central hypothesis of this research is that an HSV-2 resistance-inducing cytokine response profile can be identified and quantified using selected immunomodulatory compounds with and without anti-herpetic activity in human cell culture or small animal models. To test the hypothesis, anti-herpetic and non-anti-herpetic compounds were identified using the two assay schemes developed in Chapter 2. A preliminary cytokine response profile associated with resistance to HSV-2 infections was quantified in human vaginal EC cultures using the identified compounds. Immunomodulatory compound induced anti-herpetic activity and the associated cytokine

response profile also was evaluated in the mouse model of genital HSV-2 infection. Additionally, the guinea pig model of genital HSV-2 infection was used to evaluate the efficacy of an immunomodulatory compound delivered therapeutically, as well as testing alternate treatment routes. Also of importance, the interactions between the identified immunomodulatory compounds and vaginal defenses were quantified in a novel model of the human vaginal mucosa colonized with commensal bacteria. Collectively, the research detailed in this dissertation furthers understanding of the complex interactions between the vaginal mucosa and pathogenic organisms and aids in the design of more efficacious anti-herpetic intervention strategies to prevent STI.

CHAPTER 2: IDENTIFICATION OF NOVEL ANTI-HERPETIC COMPOUNDS USING TWO DEVELOPED ASSAY SCHEMES

OVERVIEW

HSV-2 is a prevalent (16-97%) often asymptomatic infection that is linked to an increased risk of acquiring other STI. Based on the high prevalence and asymptomatic shedding, a system for rapidly identifying novel anti-herpetic compounds is necessary to reduce the transmission of HSV-2. Several laboratories collaboratively established the Microbicide Quality Assurance Program (MQAP) to develop a validated standard for consistently quantifying the activity of potential anti-herpetic compounds. Using the same cells, virus and blinded compound test panel provided by a quality controlled reference laboratory, the results showed that only the use of different assay schemes produced significantly variable outcomes in quantified compound activity. An assay scheme consisting of compound application 1h prior to viral inoculation was selected as the standard by the MQAP based on the ability of the system to model application of topical intervention strategies. Cross-validation of the standardized assay scheme by the MQAP laboratories showed no significant difference in quantified compound activity. The assay scheme was successfully adapted to identify immunomodulatory compounds by using human vaginal EC and adding the compounds at 24 or 6h prior to viral challenge. FSL-1, a TLR2/6 agonist, showed potent anti-herpetic activity when delivered 24 or 6h prior to HSV-2 inoculation. The adapted assay scheme also was used to evaluate the impact of OTC products on herpetic infection and showed no activity providing support for the use of OTC as potential vehicles for vaginally-applied anti-herpetic compounds. Collectively, several anti-herpetic compounds were rapidly and

consistently identified using developed standardized or adapted assay schemes. The compounds, identified across multiple laboratories, potentially can significantly reduce genital HSV-2 transmission. Part of the research described in this chapter was presented at the Microbicides 2006 meeting and is in preparation by the MQAP for submission as a manuscript.

INTRODUCTION

Seventeen percent of adult Americans are infected with genital HSV-2 [3, 9]. In other regions of the world the prevalence is as high as 97% [3, 10]. HSV-2 establishes a life-long latent infection that only produces typical lesions in 20% of infected individuals while 60% show atypical lesions and 20% are completely asymptomatic [4, 14]. Seventy percent of all heterosexual transmission occurs during asymptomatic viral shedding [4, 6, 15]. Also, recently published data showed that HSV-2 infected individuals are at a higher risk of acquiring other STI like BV, *N. gonorrhea* or HIV-1 [3, 11, 13, 19]. Because of the associated risks, there is a critical need for novel anti-herpetic compounds that can reduce the spread of HSV-2 infections.

Currently, there are no FDA approved vaccines for HSV-2 and only limited success was observed in phase III clinical trials for a potential gD subunit vaccine [27]. Treatment of infected individuals involves chronic suppressive antiviral therapy that only reduces the recurrence of genital lesions but does not prevent all recurrences [3, 4, 6, 37]. Additionally, use of the antiviral compounds for treatment of HSV-2 infected individuals to prevent acquisition of other STI like HIV-1 was shown to be ineffective [195]. Based on the limited and ineffective prophylactic options for reducing infections [27, 195], new types of anti-herpetic compounds derived from natural extracts, preexisting antivirals or PAMP were evaluated for activity against HSV-2 [51, 57, 138-141]. The compounds are

divided into two types of anti-herpetic strategies based on their mechanisms of action [51, 57, 138-141].

One strategy, termed microbicides, involves compounds that are applied vaginally or rectally prior to or just after a sexual encounter and exhibit direct activity against the pathogen through membrane disruption or entry blocking [138, 139, 141]. While, compounds like nonoxynol-9, SAVVY or cellulose sulfate exhibited promising anti-herpetic activity in vitro and in vivo [148, 157-161, 166], the compounds were shown to be ineffective [163, 164, 168] or harmful [148, 162] following evaluations in clinical trials. The recent clinical trial failures of microbicides [148, 162-164, 168] underscores the need for new types of anti-herpetic compounds and improved preclinical efficacy and safety evaluations of microbicides.

Immunomodulatory compounds are another novel anti-herpetic strategy and elicit resistance against pathogenic infection by "priming" host cell innate immune responses prior to exposure [51, 57, 140]. Immunomodulatory compounds include TLR agonists that are recognized by specific cellular PRR and can result in induction of an anti-herpetic innate immune response [80, 82, 178-184]. Some TLR agonists like PIC previously showed promising anti-herpetic activity [184, 185], while others (FLAG) were ineffective [185] or untested for activity against HSV-2 (FSL-1). Testing of microbicides or immunomodulatory compounds using plaque reduction assays or other methods [196-198] are costly and time consuming processes that can produce variable outcomes based on the employed method. The methodological issues necessitated the development of a system that can rapidly and consistently quantify the anti-herpetic potential of the novel compounds.

The MQAP, a National Institute of Health (NIH)-funded collaborative effort among five laboratories, was established to develop a standardized methodology for

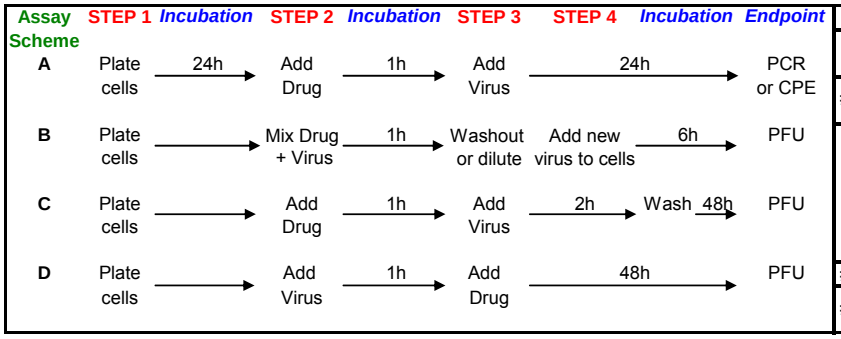
identifying anti-herpetic compounds. Using the same blinded compound panel, cells and virus provided by a quality controlled reference laboratory, the MQAP laboratories evaluated the impact of different cell types, endpoint analyses, statistical analyses or assay schemes (compound and virus application times as well as endpoint analysis times) on quantified compound activity. Based on the results only the use of different assay schemes produced significant variation in quantified compound activity; therefore a standardized assay scheme consisting of compound application 1h prior to viral challenge was selected for further evaluations. The developed standardized assay scheme was cross-validated in several MQAP laboratories. By using human vaginal EC and adding the compounds at 24 or 6h prior to viral challenge, the standardized assay scheme also was successfully adapted to identify anti-herpetic immunomodulatory compounds. We showed, for the first time, that FSL-1 elicited significantly activity against HSV-2 infection in vitro using the adapted assay scheme. Additionally, the adapted assay scheme was employed to evaluate the impact of OTC compounds on herpetic infections as an initial assessment for their use as vaginal delivery vehicles. Collectively, the results indicated that the developed assay schemes provide an invaluable system for rapidly and consistently identifying anti-herpetic compounds necessary to reduce the spread of HSV-2 infections.

MATERIALS AND METHODS

MQAP Study Group

The following five laboratories were involved in developing a standardized assay scheme for identifying anti-herpetic microbicides: Lab #1 = Pyles group, Lab #2 = Brandt group, Lab #3 = Ptak group, Lab #4 = Isaacs group, Lab #5 = Herold group

(Figure 4). The standardized assay scheme was cross-validated by laboratories #1-3. Southern Research Institute (SRI), a quality controlled reference laboratory, provided the same low passage number of ME-180 cells, clinically isolated HSV (provided to SRI by our laboratory), the blinded panel of compounds used to develop a standardized assay scheme (H1, H2, H3, H4) and the blinded panel of compounds used to validate the standardized assay scheme (H1, H2, H4, H5, H6, H7). The compound code was later revealed to be H1 = Dextran Sulfate, H2 = PRO-2000, H3 = SPL7013 in DMSO, H4 = Acyclovir (ACV), H5 = SPL7013 in H2O, H6 = Anti-HSV-2 plantibody, H7 = Dextran.



Lab	Cells	Assay	Endpoint
#1 Pyles	ME-180	A	PCR
	Vero	A	PCR
#2 Brandt	ME-180	B	PFU
	Vero	B	PFU
#3 Ptak	ME-180	D	PFU
	Vero	D	PFU
	CCD	D	PFU
	ME-180	A	CPE
	Vero	A	CPE
	CCD	A	CPE
#4 Isaacs	ME-180	B	PFU
#5 Herold	ME-180	C	PFU
	CASKI	B	PFU

Figure 4. Assay schemes used by each laboratory for quantifying compound activity to develop a standardized assay scheme. The quality controlled reference laboratory (SRI) supplied all laboratories (Lab), identified by a specific number (#1-5), with the same low passage number of ME-180 cells, blinded panel of 4 compounds and virus. Each assay scheme (Assay), coded to a specific letter (A-D), shows when the compounds and virus were added to the cells and when samples were collected for endpoint analysis. Each laboratory also evaluated different cell types in addition to ME-180 (Cells) and analyzed the results using different endpoint analyses (Endpoint; PCR = real-time PCR for the HSV gB gene, PFU = plaque titration assay, CPE = cytopathic effect scoring).

Cell culture

ME-180, a cervicovaginal carcinoma cell line, was supplied to all laboratories at the same passage number (P2) by SRI (obtained from ATCC #HTB-33) to control for the potential impact of different passage numbers on the observed results. The ME-180 cells were cultured in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 10% heat inactivated FBS (Cellgro), 0.3mg/mL L-Glutamine (Invitrogen, Carlsbad, CA), 1% non-essential amino acids (Sigma, St. Louis, MO), and 0.1mg/mL of Primocin (InvivoGen, San Diego, CA). ME-180 cells were expanded then frozen aliquots were created to provide a stock of cells used for all MQAP experiments. Vero (African green monkey kidney cells; ATCC #CCL-81) were cultured in DMEM (Cellgro) supplemented with 10% heat inactivated FBS and 0.1mg/mL of Primocin. Both ME-180 and Vero cells were subcultured by removal of medium and washing with Dulbecoo's Phosphate Buffered Saline (DPBS) without calcium or magnesium (Cellgro) then detachment with 0.2% trypsin EDTA (Cellgro) for 5min. Cells were enumerated using a hemocytometer (Hausser Scientific, Horsham, PA) and resuspended in supplemented RPMI or DMEM then subcultured into new culture flasks (BD, Franklin Lakes, NJ).

Human immortalized vaginal EC from two different donors (V11I and V19I) [50] at passage numbers ranging from 10-30 were cultured in a 1:1 mixture of VEC-100 (MatTek, Ashland, MA) and keratinocyte serum-free medium (KSFM; Invitrogen) supplemented with 5ng/mL of recombinant Epidermal Growth Factor (Invitrogen), 50μg/mL of Bovine Pituitary Extract (Invitrogen), 44.5μg/mL of $CaCl_2$ (Sigma) and 0.1mg/mL of Primocin. The vaginal EC were subcultured by removal of medium and washing with DPBS then detachment with 0.2% trypsin EDTA for 5min. Cells were suspended in RPMI-1640 with 10% heat inactivated FBS to neutralize residual trypsin activity then centrifuged at 250rcf for 5min. Following resuspension in the 1:1 medium,

cells were enumerated using a hemocytometer then subcultured into new culture flasks (BD). All cell types were maintained in 37^0C, 5% CO_2 humidified incubators and were confirmed mycoplasma-free using the MycoSensor PCR Assay Kit (Stratagene, La Jolla, CA).

Viruses

HSV samples were clinical isolates collected by the University of Texas Medical Branch Clinical Microbiology Department. The viruses were shipped to SRI then propagated and titered before distribution to the MQAP laboratories. Clinically-isolated viruses were used to prevent potential confounding results based on different laboratory adaptations of established HSV-2 strains. HSV-1 UTMB-1 was used for the first study and supplied at a titer of $6.3x10^8$ plaque forming units (pfu)/mL. During the course of developing a standardized assay scheme, the UTMB-1 virus was confirmed to be mistyped as HSV-2 by a follow up PCR using an HSV-1 specific probe described below in the real-time PCR section. SRI supplied a virus confirmed using real-time PCR to be HSV-2 (HSV-2 UTMB-2) at a titer of $2.5x10^4$pfu/mL for use in validating the standardized assay scheme.

Development of a standardized assay scheme

Assay scheme A (Figure 4) was used by our laboratory to quantify the anti-herpetic activity produced by each of the blinded test compounds. ME-180 or Vero cells were seeded into 96-well culture plates (BD) at $5x10^4$cells/well in 100µL of supplemented RPMI (ME-180) or DMEM (Vero) then incubated for 24h to achieve 90-100% confluency. Compounds H1 (40mg/mL), H2 (40mg/mL), H3 (20mg/mL) and H4

(40mg/mL) were diluted to 200μg/mL, 100μg/mL, 20μg/mL, 10μg/mL and 2μg/mL concentrations in supplemented RPMI or DMEM for ME-180 or Vero cells, respectively. Medium was removed from the culture plates and 50μL of each compound at the 5 dilutions were added in triplicate to both cell types then incubated for 1h. HSV-1 UTMB-1 was added to each well at a multiplicity of infection (MOI) of 1 ($5x10^4$pfu/50μL) to achieve a 100μL/well final volume. Additionally, virus or medium only (RPMI or DMEM) was added to triplicate wells and served as virus positive and negative controls, respectively. Culture plates were incubated for 24h to allow time for approximately 2 viral replication cycles prior to collection of the cell layers/supernatants. For cytopathic effect (CPE) scoring, 50μL of each sample was added to confluent Vero cell monolayers in 24 well plates (BD) then the plates were incubated for 24h. After incubation, the plates were scored for CPE as described below in the CPE scoring section. For real-time quantitative PCR, the remaining 50μL of each sample was used for DNA extraction and PCR analysis as described below in the DNA extraction and real-time PCR section. Assay schemes, cell types and endpoint analyses employed by the other laboratories for the development of a standardized assay scheme are summarized in Figure 4.

Validation of the standardized assay scheme

Assay scheme A (Figure 4) was followed by all laboratories for validation of the standardized assay scheme. ME-180 or Vero cells were seeded into 96-well culture plates as described in the development of a standardized assay scheme section. All laboratories used the same 6 dilutions consisting of 200μg/mL, 40μg/mL, 8μg/mL, 1.6μg/mL, 0.32μg/mL and 0.064μg/mL for compounds H1 (40mg/mL), H2 (40mg/mL), H4 (40mg/mL), H5 (20mg/mL) and H7 (40mg/mL). Compound H6 (280μg/mL) was

diluted to 20μg/mL, 4μg/mL, 0.8μg/mL, 0.16μg/mL, 0.032μg/mL and 0.0064μg/mL concentrations. One hour prior to viral inoculation, the 6 dilutions for each compound were added (50μL/well) in triplicate to both cell types after medium removal from the culture plates. Due to the lower titer of HSV-2 UTMB-2, the virus was added at an MOI of 0.1 ($5x10^3$pfu/50μL) to achieve a 100μL/well final volume. Virus positive and negative controls consisted of virus or medium only (RPMI or DMEM) added to triplicate wells. The plates were incubated for 24h prior to collection of the cell layers/supernatants for real-time quantitative PCR. The other laboratories used different cell types in addition to ME-180 cells and different endpoint analyses also were used to quantify compound produced anti-herpetic activity.

CPE Scoring

For CPE scoring, visual observations were performed at 200x magnification on an Axiovert 25 microscope (Zeiss, Thornwood, NY) and used a plus (viral CPE observed) or minus (no viral CPE observed) system to identify infected wells.

DNA extraction and real-time PCR

Each sample (50μL) was lysed for DNA extraction using buffered proteinase K (Qiagen, Valencia, CA) then incubated for 1h at 37^0C. Lysed samples and no template controls (H_2O) were extracted using the Qiagen DNeasy 96 Kit (Qiagen) then samples were eluted into 200μL of 10mM Tris/1mM EDTA (TE; Sigma). For quantification of HSV-2 gB genome equivalents (GE), 12.5μL of Supermix (Bio-Rad, Hercules, CA), 5μM of gBHSV-F (5' – GCT CGC ACC ACG CGA – 3'; IDT, Coralville, IA), 5μM of gBHSV-R (5' – CGC ATC AAG ACC ACC TCC TC – 3'; IDT), 7.5μM of gBHSV2-TP

(5' – FAM – CGG CGA TGC GCC CCA G – BHQ1 – 3'; IDT), 9μL of H_2O and 1μL of sample DNA or no template control were combined per reaction and run on an iCycler outfitted with real time optics (Bio-Rad) with the following protocol: 95^0C for 2min then 45 cycles of 95^0C for 20sec and 60^0C for 2min. Identification of HSV-1 UTMB-1 as an HSV-1 strain was achieved using the same PCR protocol and reaction mix but instead used 15μM of gBHSV1-TP (5' – TXRED – TGG CAA CGC GGC CCA AC – BHQ2 – 3'; IDT) as the probe. For PCR assays, a dilution series of cloned amplimers was utilized for quantification standards and all reactions produced efficiencies between 90% and 110% and correlation coefficients greater than 0.95.

Adaptation of the assay scheme for identifying anti-herpetic immunomodulatory compounds

The standardized assay scheme A was adapted to identify anti-herpetic immunomodulatory compounds by including addition of the test compounds 24 or 6h prior to viral challenge (Figure 5). Additionally, human vaginal EC were utilized for the adapted assay scheme instead of ME-180 cells. Vaginal EC were seeded into 96-well culture plates at $5x10^4$cells/well in 100μL of 1:1 medium then incubated for 24h to achieve 90-100% confluency. At 24, 6 or 1h prior to viral inoculation FSL-1 (1μg, 0.1μg; Invivogen), PIC (100μg; Sigma) and FLAG (10μg, 1μg, 0.1μg; kindly provided by Tonyia Eaves-Pyles [199]) were added (50μL/well) in triplicate following medium removal from the culture plates. HSV-2 was added at an MOI of 0.1 ($5x10^3$pfu/50μL) to achieve a 100μL/well final volume. Virus, medium only (1:1) and 20μg/mL of acyclovir (GlaxoSmithKline, Research Triangle Park, NC) were added to triplicate wells as virus, no virus and positive intervention controls, respectively. After 24h of incubation, cell layer/supernatants were collected for DNA extraction and quantitative PCR analyses.

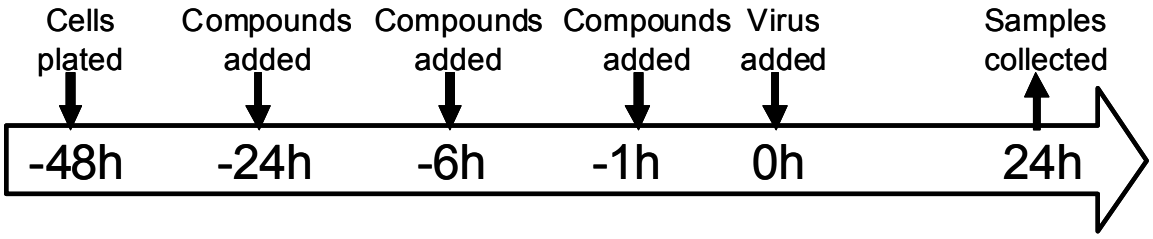


Figure 5. Adapted assay scheme for identifying anti-herpetic immunomodulatory compounds. Cultured cells were plated then incubated for 24h. After incubation, compounds were added to vaginal EC at 24, 6 or 1h prior to HSV-2 challenge (MOI 0.1). Cell layer/supernatants were collected after 24h of incubation for DNA extraction and real-time PCR analyses.

Evaluation of OTC compound anti-herpetic activity using the adapted assay scheme

The adapted assay scheme (Figure 5) was employed to evaluate the impact of selected OTC compounds on herpetic infection in human vaginal EC. Conceptrol Vaginal Contraceptive Gel™ (Johnson & Johnson, Langhorne, PA), Summer's Eve Vaginal Douche™ (C.B. Fleet Company, Lynchburg, VA), KY warming liquid™ (Johnson & Johnson), Replens™ (Lil' Drug Store Products, Cedar Rapids, IA) and Zestra™ (Semprae Laboratories, Saddle Brook, NJ) were added (50µL/well) in triplicate at 24, 6 or 1h prior to inoculation of HSV-2 at an MOI of 0.1 ($5x10^3$pfu/50µL) resulting in a 100µL/well final volume. Virus, no virus and positive intervention controls were added in triplicate. The cultures were incubated for 24h then the cell layer/supernatants were collected for DNA extraction followed by real-time PCR analyses.

Statistical analyses

Calculation of the concentration required to reduce HSV-2 replication by 50% (effective concentration 50% value; EC_{50}) for each compound as quantified using CPE scoring or PCR was achieved using the endpoint estimation method of Reed and Muench [200]. During development of the standardized assay scheme, the mean EC_{50} for the compounds quantified by each laboratory was compared based on assay scheme, cell type, endpoint analysis or statistical analysis to identify significant differences via the Kruskal-Wallis test using the SAS software package v9.1 (Statistical Analysis System, Cary, NC). The same statistical analysis was used during validation of the standardized assay scheme to identify significant differences in cell type, endpoint analysis or statistical analysis. Significant differences in the quantified HSV-2 GE were identified using Student's t-test for the TLR agonists and OTC compound evaluations. The Student's t-test statistical analyses were performed using the Prism software package v4.0 (Graph Pad, San Diego, CA).

RESULTS

A standardized assay scheme for identifying anti-herpetic microbicides was developed through the collaborative MQAP

To develop a standardized assay scheme, each laboratory evaluated the same panel of 4 blinded compounds (H1, H2, H3, H4) using the same virus (HSV-1 UTMB-1) and cells (ME-180), with allowances for other cell types, to identify if differences in assay scheme, cell type, endpoint analysis or statistical analysis significantly contributed to variability in calculated compound EC_{50} across the laboratories. Our laboratory used assay scheme A (Figure 4) to quantify the anti-herpetic activity of the compounds. Initially, analysis of the extracted DNA using real-time PCR with an HSV-2 specific

probe indicated that no HSV-2 was present in any of the tested samples. Results of the CPE scoring endpoint analysis showed the presence of viral CPE in several of the wells that were inoculated with HSV-2. The possibility that the virus had been mistyped by the clinical laboratory was evaluated using the same PCR protocol with an HSV-1 specific probe. The HSV-1 PCR results showed the presence of virus in the viral control wells and in several of the compound test wells. Information on the mistyping of the virus was relayed to SRI for virus type confirmation and for distribution of the correct virus for further studies.

The PCR data showed that in ME-180 cells all compounds were capable of reducing HSV-1 replication by 50% at concentrations of 0.91μg/mL for H1, 2.45μg/mL for H2, 0.07μg/mL for H3 and 0.03μg/mL for H4. Similar compound EC_{50} were observed when calculated from the CPE scoring results indicating that PCR is a comparable endpoint analysis method. Vero cells have long been used as the standard cell line for propagation of certain viruses including HSV and for evaluating the EC_{50} of compounds against the viruses [196-198]. Our laboratory also quantified the anti-herpetic activity of the 4 compounds in Vero cells using PCR and showed that all compounds produced EC_{50} (H1 = 0.98μg/mL, H2 = 7.41μg/mL, H3 = 1.32μg/mL, H4 = 0.04μg/mL) that were comparable to values obtained in ME-180 cells. Again the CPE scoring results observed in Vero cells correlated well with the PCR data.

The results obtained in both cell types and endpoint analyses from our laboratory were submitted to SRI for comparison to data obtained from the other 4 laboratories to identify any significant differences in the compound EC_{50} based on assay scheme, cell type, endpoint analysis or statistical analysis. The use of different assay schemes, timing of drug and virus addition and endpoint collection time, resulted in significantly ($p<0.01$; Kruskal-Wallis test) different mean EC_{50} for the tested compounds among all of the

laboratories (Figure 6). No significant ($p>0.05$; Kruskal-Wallis test) differences were observed when comparing cell types, endpoint analyses or statistical analyses. Interestingly, the assay schemes used by Labs #4 and #5 showed no activity, indicating a failure, for some tested compounds that the other laboratories found to be potent anti-herpetic compounds (Figure 6). Due to the significant variability in quantified compound EC_{50} among the laboratories, assay scheme A was selected as the standard for quantifying compound produced anti-herpetic activity. The assay scheme was selected based on the similar results obtained for the two laboratories that used scheme A (Lab #1 and Lab #3) and the ability of scheme A to model proposed topical anti-herpetic compound application just prior to a potential exposure [138, 139, 141, 201].

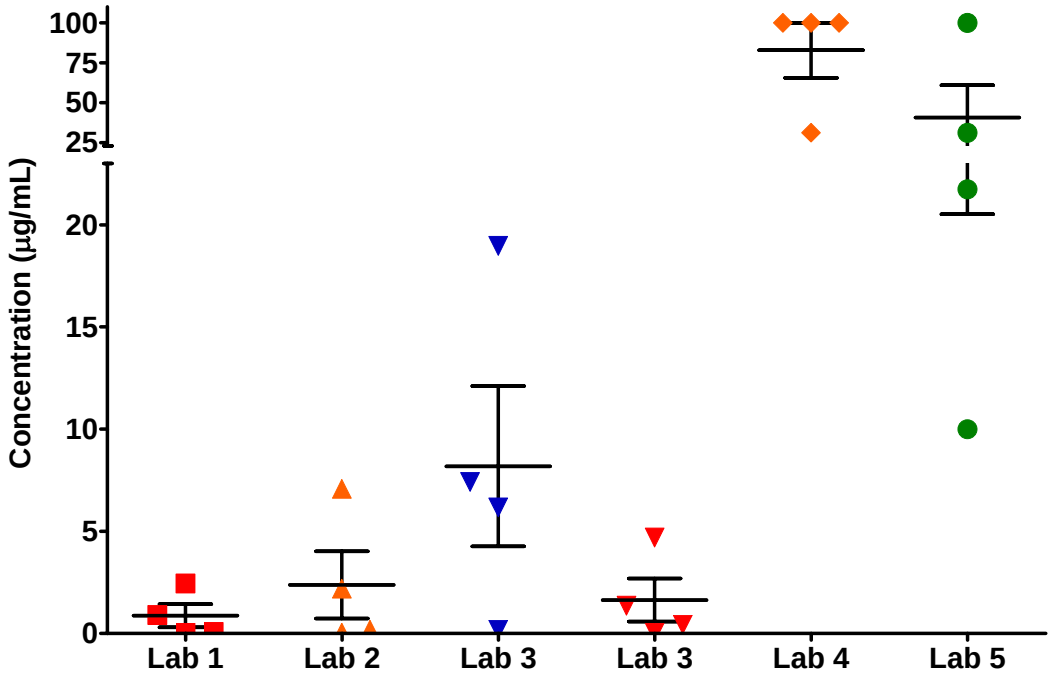


Figure 6. Non standardized assay schemes resulted in significantly variable quantified EC_{50} for the tested compounds. The colors correspond to assay schemes (A = red; B = orange; C = green; D = blue) and the symbols represent the EC_{50} for each of the 4 compounds evaluated by each laboratory (square = Lab 1; triangle = Lab 2; inverted triangle = Lab 3, diamond = Lab 4, circle = Lab 5). Lab 3 quantified the EC_{50} of the compounds in ME-180 cells using two different assay schemes (D and A; Figure 4). The horizontal bars depict the calculated mean EC_{50} for the compounds ± standard error of the mean (SEM). Significant variation ($p<0.01$; Kruskal-Wallis test) was observed comparing the mean EC_{50} of the compounds in ME-180 cells across all laboratories.

Consistent anti-herpetic activity was observed across several laboratories using the developed standardized assay scheme

Assay scheme A (Figure 4) was used as the standard for quantifying anti-herpetic activity of a second blinded compound panel by the MQAP laboratories. HSV-2 UTMB-2 was used for the standardized study because of our earlier identification of the mistyped HSV-1 UTMB-1 virus. The lower titer of HSV-2 UTMB-2 necessitated the use of a lower MOI. The ability of the standardized assay scheme to identify a potential anti-herpetic compound (H6) or a negative control compound (H7) also was evaluated by the MQAP laboratories. Additionally, the impact of different cell types, endpoint analyses or statistical analyses on the quantified EC_{50} of the compounds compared across all laboratories was evaluated using the standardized assay scheme.

For validation of the standardized assay scheme, our laboratory used only PCR for endpoint analysis based on the similar calculated compound EC_{50} for PCR and CPE scoring obtained during development of the standardized assay scheme. The quantified EC_{50} for each compound in ME-180 cells was calculated as 0.22μg/mL for H1, 3.80μg/mL for H2, 0.81μg/mL for H4, 4.79μg/mL for H5, 0.35μg/mL for H6 and >100μg/mL for H7. Comparison of the calculated EC_{50} with the outcomes from the other two laboratories showed that H7 was not able to reduce viral replication by 50% at any tested concentration. The negative control compound (H7), identified by all laboratories, was removed from further data analyses. The potential anti-herpetic compound (H6) was identified as effective using the standardized assay scheme and resulted in a similar EC_{50} as quantified by all laboratories. Comparison of the mean EC_{50} for all tested compounds across the 3 laboratories showed that use of a standardized assay scheme produced significantly ($p>0.05$; Kruskal-Wallis test) consistent results (Figure 7).

Analysis of the PCR results from the Vero cells again showed similar calculated EC_{50} for the compounds tested by our laboratory (H1 = 0.52µg/mL, H2 = 7.41µg/mL, H4 = 0.98µg/mL, H5 = 7.94µg/mL, H6 = 0.89µg/mL, H7 >100µg/mL) compared to the ME-180 results. The mean EC_{50} obtained for the compounds tested in both cell types were compared to the results from the other laboratories and confirmed that the use of different cell types, endpoint analyses or statistical analyses did not produce significant ($p>0.05$; Kruskal-Wallis test) variability. The results showed that by using the standardized assay scheme potent anti-herpetic microbicides can be identified and cross-validated by several laboratories even with allowances for different parameters like cell type.

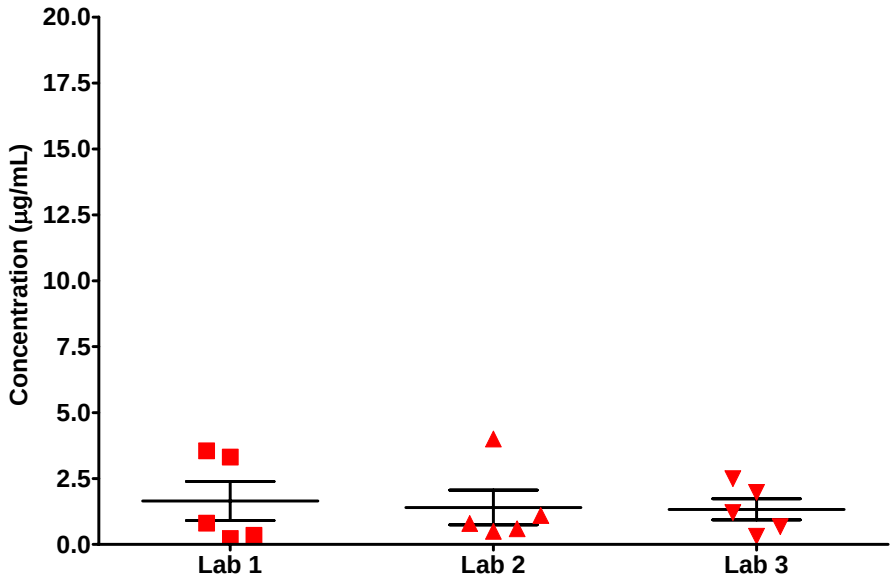


Figure 7. Use of a standardized assay scheme produced similar EC_{50} across all laboratories for the tested compounds. The symbols represent the EC_{50} for the 5 compounds evaluated by each laboratory (square = Lab 1; triangle = Lab 2; inverted triangle = Lab 3). The horizontal bars depict the calculated mean EC_{50} for the compounds ± SEM. No significant variation ($p>0.05$; Kruskal-Wallis test) was observed comparing the mean EC_{50} of the compounds in ME-180 cells across all laboratories.

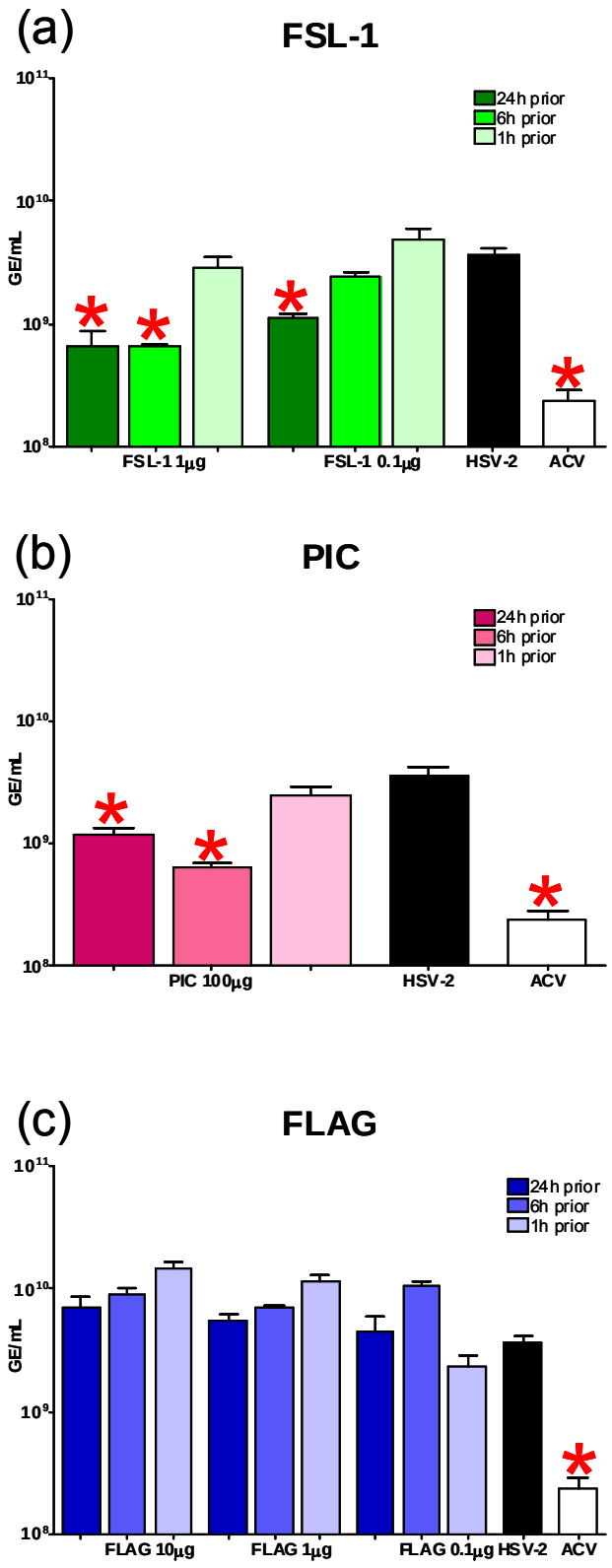


Figure 8. FSL-1 and PIC inhibited HSV-2 replication in human vaginal EC. Different concentrations of FSL-1, PIC or FLAG were applied at selected times prior to viral inoculation (MOI 0.1) of V19I cells. (a) FSL-1 significantly (*, $p<0.05$; Student's t-test) reduced HSV-2 GE when applied at 24 (1µg and 0.1µg) or 6h (1µg) prior to viral challenge. (b) Similar significant (*, $p<0.05$; Student's t-test) results were observed for PIC application at 24 or 6h prior to HSV-2 inoculation. (c) FLAG did not significantly ($p>0.05$; Student's t-test) reduce HSV-2 replication at any of the tested time points or concentrations. A significant (*, $p<0.05$; Student's t-test) reduction was observed for the ACV positive intervention control. Each bar represents the mean ± SEM for 3 replicates.

An anti-herpetic immunomodulatory compound was identified using an adapted assay scheme

The standardized assay scheme was adapted to identify anti-herpetic immunomodulatory compounds through the inclusion of additional compound application times at 24 and 6h prior to viral inoculation (Figure 5). The two time points were selected to allow time for establishment of a potential HSV-2 resistant environment based on previously observed in vitro and in vivo results [181, 184, 185]. Because the proposed application site for anti-herpetic compounds is the vaginal mucosa [138, 139, 141, 201], human vaginal EC were used in the adapted assay scheme rather than the cervicovaginal ME-180 cells. Human vaginal EC express high levels of TLR2, 3, 5 and 6 [50], therefore FSL-1 (TLR2/6) was selected for initial evaluations while PIC (TLR3) and FLAG (TLR5) served as anti-herpetic and non-anti-herpetic controls, respectively [185]. FSL-1 was tested at concentrations of 1μg/mL or 0.1μg/mL based on previous in vitro evaluations [202]. Similar rational was used for selection of the PIC (100μg/mL) test concentration [184, 185] and the series of evaluated FLAG (10, 1, 0.1μg/mL) concentrations [199].

FSL-1 application (1μg/mL) at the 24 or 6h prior time points significantly ($p<0.05$; Student's t-test) inhibited HSV-2 replication compared to virus only controls (Figure 8a). Reductions in HSV-2 GE also were observed following FSL-1 (0.1μg/mL) application at 24 or 6h prior to viral challenge. FSL-1 applied at the 1h prior time point did not produce significant ($p>0.05$; Student's t-test) anti-herpetic activity for either of the two tested concentrations. Similar activity was observed for application of PIC (100μg/mL) at the 24, 6 or 1h prior time points (Figure 8b). FLAG did not significantly ($p>0.05$; Student's t-test) alter HSV-2 replication at any of the tested concentrations or application time points (Figure 8c). Evaluation of FSL-1 in V11I cells produced similar

results (data not shown). Collectively, the adapted assay scheme identified FSL-1 as a novel anti-herpetic immunomodulatory compound and also successfully identified the anti-herpetic and non-anti-herpetic controls confirming the feasibility of using the assay to evaluation other TLR agonists or OTC compounds.

OTC products did not provide resistance to HSV-2 infection in the adapted assay scheme

Because OTC compounds are occasionally applied to the vaginal mucosa and are under consideration as potential vehicles for vaginal application of topical anti-herpetic compounds [149-152], evaluation of their potential impact on HSV-2 replication is necessary. Selected commonly used OTC products were applied to vaginal EC at 24, 6 and 1h prior to viral inoculation. No significant ($p>0.05$; Student's t-test) alteration in HSV-2 GE was observed for any of the tested OTC compounds at any of the delivery time points compared to virus only controls (Figure 9). The lack of observed impact on HSV-2 replication supports the use of OTC products as potential vehicles for delivery of anti-herpetic compounds but additional studies are needed to evaluate the safety of the compounds [Chapter 3].

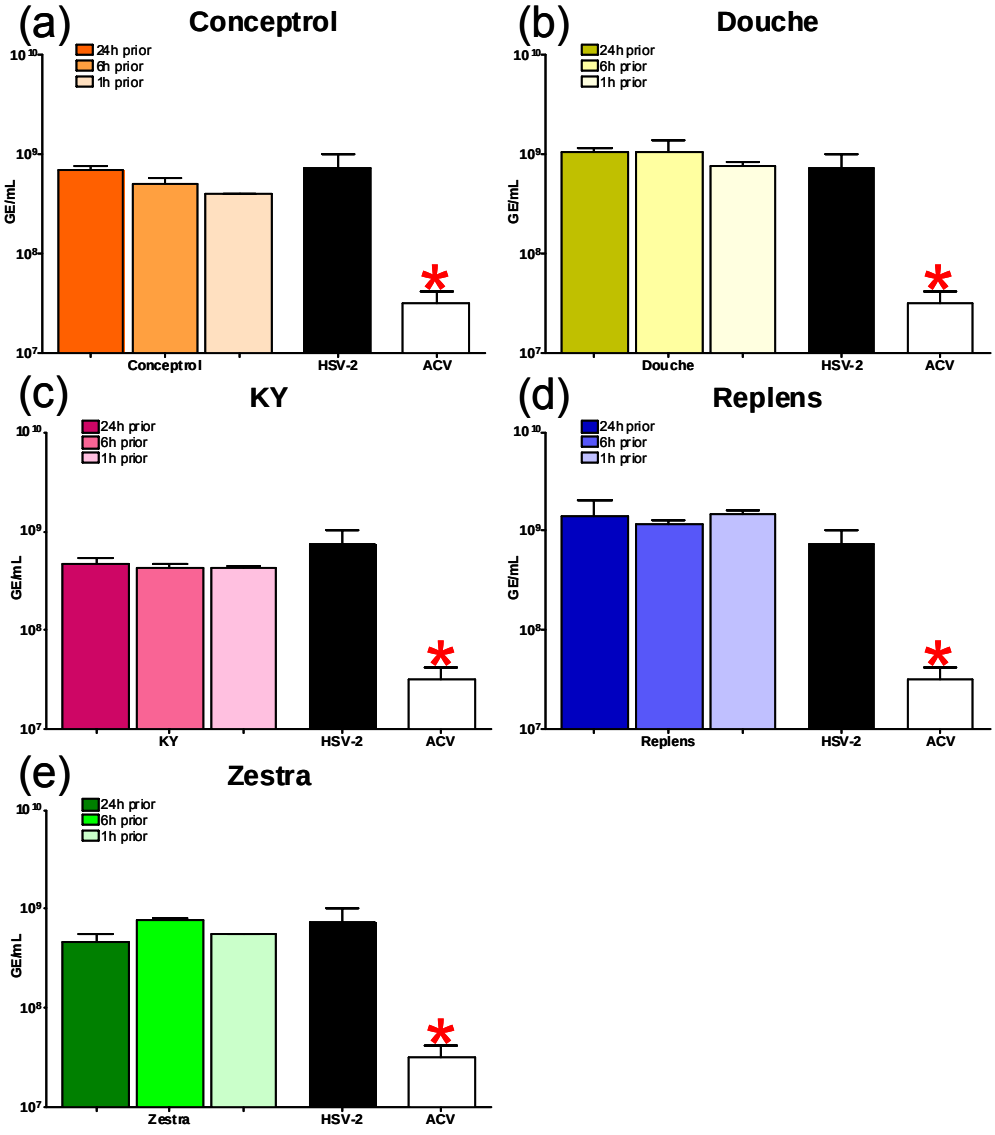


Figure 9. OTC products did not impact HSV-2 replication in human vaginal EC. (a) Conceptrol™, (b) Douche, (c) KY warming liquid™, (d) Replens™ or (e) Zestra™ were applied at selected times prior to viral inoculation (MOI 0.1) of V19I cells. None of the tested OTC compounds significantly ($p>0.05$; Student's t-test) altered HSV-2 GE compared to the virus control at any of the application time points. A significant (*, $p<0.05$; Student's t-test) reduction was observed for the ACV positive intervention control. Each bar represents the mean ± SEM for 3 replicates.

DISCUSSION

Using the collaboratively developed standardized assay scheme we identified several anti-herpetic compounds and our results were validated by the MQAP laboratories. Previous evaluations of anti-herpetic compounds showed highly variable quantified EC_{50} [203-205] underscoring the need for a standardized assay scheme to reliably identify efficacious compounds. The variability in compound activity was further highlighted by identification of tested compounds as effective for some laboratories but not others during the development of a standardized assay scheme (Figure 6). Of the variables evaluated by the MQAP laboratories during development of a standardized assay, the use of different cell types did not significantly impact quantified compound EC_{50} nor did the use of different statistical analyses.

Additionally, our laboratory selected PCR as the endpoint analysis method due to its high throughput capabilities, quantitative outcome measurements and rapid assay times compared to other methods [206, 207]. Plaque assays and yield reduction assays both require days of assay time, multiple dilutions of each sample and handling of infectious materials [196-198]. One potential problem with PCR as an endpoint is that it does not discriminate between live and dead virus but results from the MQAP evaluations and previously published results showed that PCR outcomes correlated well with other methods [207, 208]. Even with the use of different endpoint analyses by the MQAP laboratories, statistical comparisons of the results showed no significant differences in quantified compound EC_{50}.

Only the use of different assay schemes (Figure 4) was identified as a significant source of variability in quantified compound EC_{50} among the laboratories. For anti-herpetic microbicides, the proposed application time is prior to or just after a potential exposure [138, 139, 141, 201]. The time required for HSV-2 to attach and begin entry

into permissive cells in vitro is less than 30min [209, 210]. Because Dextran sulfate (H1), PRO-2000 (H2) and SPL7013 (H3, H5) are entry blocker microbicides [141, 196, 211], varied compound and virus addition timing could produce vastly different quantified compound EC_{50}. The possibility was addressed by standardizing the assay scheme. Assay scheme A (Figure 4) was selected as the standard based on comparable results with another laboratory (Figure 6) and the ability of the assay scheme to model the proposed application of anti-herpetic microbicides [138, 139, 141, 201]. The standardized assay scheme produced consistent compound EC_{50} results across several laboratories indicating the potential for adapting the assay to identify other types of anti-herpetic compounds.

The standardized assay scheme was successfully adapted (Figure 5) by our laboratory to identify anti-herpetic immunomodulatory compounds. The proposed mechanism by which immunomodulatory compounds engender anti-herpetic activity is through elicitation of an innate immune response that can increase resistance to HSV-2 replication [51, 57, 140]. FSL-1 or PIC application elicited significant resistance to HSV-2 infection when applied 24 or 6h prior to viral inoculation in human vaginal EC (Figure 8). Interestingly, no anti-herpetic activity was observed for either compound applied at the 1h time period. The observed temporal dependent activity is consistent with the mechanism of action for immunomodulatory compounds [51, 57, 140]. As described previously [Chapter 1], TLR agonist recognition triggers a signaling cascade resulting in production of cytokines that provide direct antiviral activity and stimulate neighboring cells to elicit innate immune responses [48, 57, 74, 140]. Application of FSL-1 or PIC at 24 or 6h prior to HSV-2 inoculation would allow time for innate immune response priming to occur and offers a potential mechanistic explanation for the observed anti-herpetic activity. Of note, FLAG did not elicit resistance to HSV-2 infection

indicating that anti-herpetic activity likely occurs through elaboration of a specific innate immune response profile that was evaluated in immortalized vaginal EC cultures [Chapter 3].

Agonists were selected for evaluation in the adapted assay scheme based on potential HSV-2 recognition via highly expressed TLR of the human vaginal mucosa. Vaginal EC are initial targets of HSV-2 during genital infection [38-41] and unlike ME-180 cells, a cervicovaginal carcinoma, vaginal EC express high levels of TLR2, 3, 5 and 6 [50]. Additionally, human vaginal EC were selected for use in the adapted assay scheme because the MQAP studies indicated that use of different cell types did not significantly impact quantified compound EC_{50}.

TLR3 recognizes dsRNA produced by viral replication [64, 68, 69]; therefore PIC (TLR3), a synthetic dsRNA, was used as a positive intervention control based on previously exhibited in vitro and in vivo anti-herpetic activity [184, 185]. Conversely, FLAG (TLR5), derived from *Salmonella dublin* flagellin [199], served as a negative control based on previously observed lack of activity against HSV-2 infection [185] and the lack of HSV-2 recognition through TLR5. FSL-1 (TLR2/6) was selected because it is a diacyl lipopeptide [202, 212, 213] that was not previously tested for activity against HSV-2 and previous experiments showed that TLR2 recognizes HSV-2 glycoproteins [51, 56, 57]. We showed that FSL-1 elicited significant anti-herpetic activity in human vaginal EC indicating the potential for HSV-2 recognition through TLR2 dimerization with TLR6.

In addition to identifying anti-herpetic TLR agonists the adapted assay scheme (Figure 5) also was used to evaluate the activity of selected OTC compounds. None of the tested OTC products significantly altered HSV-2 replication in human vaginal EC (Figure 9). Quantifying the potential impact of OTC compounds on HSV-2 replication is

an important preliminary step in assessing their utility as potential vehicles for anti-herpetic compounds [149-152]. Conceptrol™ was selected because it contains nonoxynol-9, a compound previously shown to increase susceptibility to STI like HIV-1 [162]. Zestra™ contains herbal ingredients [147] that have not been tested for anti-herpetic activity. KY warming liquid™, Replens™ and vaginal douche were selected as negative controls based on their lack of an active compound or herbal ingredients. Lack of observed impact for any of the tested OTC products lends support to their use as vehicles because effective carriers should not interfere with the activity of anti-herpetic compound [141, 142].

Collectively, two different assay schemes (Figure 4 and 5) were developed for rapidly identifying anti-herpetic microbicides or immunomodulatory compounds using a method that enables cross-validation in several laboratories. Due to the high prevalence of HSV-2 infections [3, 9, 10] and the associated increased risk for STI acquisition [3, 11, 13, 19] there was a need for an assay to identify novel anti-herpetic compounds. Using the adapted assay scheme, FSL-1 was identified as an immunomodulatory compound that provided significant resistance to HSV-2 infection. Identifying anti-herpetic and non-anti-herpetic immunomodulatory compounds using the developed assay scheme was critical for identifying a cytokine response profile-associated with resistance to HSV-2 infection [Chapter 3].

CHAPTER 3: IMMUNOMODULATORY COMPOUNDS ELICITED SPECIFIC CYTOKINE RESPONSE PROFILES IN HUMAN VAGINAL EPITHELIAL CELLS

OVERVIEW

The human vaginal mucosa provides a physical and immunological barrier to many STI including HSV-2. Recognition of PAMP via TLR expressed by the vaginal mucosa produces an innate immune response that provides activity against potential pathogens. Immunomodulatory compounds, a novel anti-herpetic strategy, selectively induce cytokine responses that can produce activity against HSV-2 infection. Initially, the ability of human immortalized vaginal EC to serve as a model of the vaginal mucosa was evaluated using selected TLR agonists (FSL-1, PIC or FLAG). Quantification of induced cytokines profiles confirmed that the immortalized vaginal EC provided a renewable and reproducible model of the human primary cells. The immortalized vaginal EC were used to identify a preliminary cytokine profile associated with resistance to HSV-2 infection using anti-herpetic and non-anti-herpetic TLR agonists. Comparison of induced profiles identified IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β, and RANTES as important anti-herpetic cytokines. Additional cytokine and TLR RT-PCR experiments were performed to evaluate the potential impact of OTC products or semen on immortalized vaginal EC responsiveness to TLR agonists. No significant alterations in cytokine elaboration or TLR profile expression was observed and supported the use of OTC compounds as vehicles for TLR agonists even in the presence of semen. Identification of a preliminary anti-herpetic cytokine response profile is of critical importance for the future design of TLR agonists or vaccine adjuvants to reduce the

spread of genital HSV-2 infections. Part of the research described in this chapter was published in the *American Journal of Reproductive Immunology*.

INTRODUCTION

The vaginal mucosa is the first site of infection for many STI including HSV-2 [38-41]. One vaginal defense against STI is the physical barrier formed by the vaginal EC [22, 23]. Additionally, vaginal EC are active participants in the innate immune response and are considered non-conventional innate immune cells due to their ability to recognize PAMP and express cytokines [48, 50, 51]. Recognition occurs through PRR of which the most studied are a subfamily designated TLR [51-53]. The transmembrane proteins of TLR contain ligand binding leucine rich domains and a signaling toll/interleukin-1 receptor-like domain [51, 53, 70, 71]. Each TLR recognizes a particular PAMP then initiates a specific signaling transduction cascade that results in cytokine production [48, 51-53]. The elicited cytokine profile initiates a highly coordinated innate immune response against genital HSV-2 infection [48, 50, 51].

The ability of vaginal EC to produce activity against HSV-2 infection following exposure to specific PAMP was evaluated as a novel anti-herpetic strategy using TLR agonists [80, 82, 180-185]. Human vaginal EC express high levels of TLR2, 3, 5, and 6, moderate levels of TLR1 and 4 and a subpopulation of the cells express low levels of TLR7-9 [50]. Vaginal application of PIC (TLR3) to human vaginal EC in vitro or small animal models elicited significant resistance to HSV-2 infection [82, 183-185; Chapter 2]. Additionally, FSL-1 (TLR2/6) induced anti-herpetic activity in human vaginal EC [Chapter 2] and in mice [Chapter 4] while Pam (TLR1/2) showed no activity [185]. Similar lack of activity against HSV-2 infection was observed for LPS (TLR4) or FLAG (TLR5) application in vitro and in vivo [182, 183, 185]. Of note, IMI (TLR7/8) or CpG

(TLR9) application resulted in substantial resistance to genital HSV-2 infection in vivo [80, 82, 180, 181] but showed reduced activity in vitro [185]. Collectivity, the observed activity is likely not due to activation a particular TLR but rather the induction of a specific cytokine response profile [48, 50, 51]; therefore anti-herpetic and non-anti-herpetic TLR agonists were evaluated in human vaginal EC to identify cytokines important for providing resistance to HSV-2 infection.

Once identified, an effective delivery vehicle for the anti-herpetic TLR agonists is necessary to facilitate compliant application of the compounds to reduce STI including HSV-2 [149-152]. OTC compounds are a potential vehicle for vaginally-applied anti-herpetic intervention strategies [141-144]; however some products (e.g. Zestra™) can contain herbal ingredients [147] that are not subject to the strict FDA testing requirements as with other compounds. Additionally, nonoxynol-9 is the active ingredient of some OTC products and previous evaluations showed that the compound disrupted the integrity of the vaginal mucosal barrier resulting in an increase in STI acquisition [148, 149]. Also of concern, semen is occasionally introduced into the vaginal environment and consists of different proteins including cytokines like TGFβ and several alkaline chemical components [145, 146] that could alter the vaginal EC response to the TLR agonists. Because OTC products or semen can potentially elicit an innate immune response or alter the responsiveness of vaginal EC to TLR agonists [141-144], preliminary safety evaluations are necessary to aid in the design of novel anti-herpetic intervention strategies.

To address the critical questions, we first evaluated the cytokine profiles induced by agonists specific for highly expressed TLR in primary human vaginal EC. The short life span of the primary vaginal EC limits their usefulness for experimental reproducibility; therefore vaginal EC from different donors were immortalized through

viral transduction [50] and evaluated for their ability to serve as a model of primary cells. Using a panel of TLR agonists (Table 1) [Chapter 1] with and without anti-herpetic activity [80, 82, 180-185], induced cytokine profiles were evaluated in immortalized human vaginal EC to identify cytokines important for resistance to HSV-2 infection. Additionally, potential TLR agonist induced alterations in the vaginal EC TLR expression profile were quantified using TLR RT-PCR as a preliminary safety evaluation. Immortalized vaginal EC also were used to quantify the potential impact of OTC compounds or a semen simulant on cytokine elaboration and TLR expression. The experiments showed that TLR agonists elicited specific cytokine profiles and comparison of the profiles identified cytokines important for producing anti-herpetic activity. Additionally, induction of similar cytokine profiles confirmed the utility of immortalized vaginal EC as a reproducible and renewable model of primary cells.

MATERIALS AND METHODS

Cell culture

Primary vaginal EC from two subjects (V11, V19) were purchased (MatTek) and maintained in VEC-100 medium supplemented with 44.5μg/mL of $CaCl_2$ and 0.1mg/mL of Primocin. To provide a renewable and reproducible source, the primary cells were treated with PA317/LXSN-16E6E7 (ATCC #CRL-2203) conditioned medium then immortalized heterogeneous populations were selected using G418 (50μg/mL; Sigma) as performed previously [50]. The immortalized vaginal EC (V11I, V19I) were maintained in a 1:1 mixture of VEC-100 medium and supplemented KSFM. All cell types were subcultured, maintained and confirmed mycoplasma-free as described previously [Chapter 2].

Evaluation of TLR agonist induced cytokine responses in human vaginal EC

Primary vaginal EC were seeded into 96-well culture plates at $5x10^4$cells/well in 100μL of VEC-100 medium. After 24h of incubation to achieve 90-100% confluency, 10μL of FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or the DPBS vehicle were added in triplicates to the 96 well plates. The plates were incubated for 24h then 100μL of the supernatant was collected and stored at -80^0C until analyzed for cytokine elaboration.

For comparison to the primary cell data, immortalized vaginal EC were seeded as described for the primary vaginal EC. Following 24h of incubation, 10μL of FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL), Pam (10μg/mL), LPS (1μg/mL), IMI (0.1mg/mL), CpG (0.1mg/mL) or the DPBS vehicle were added in triplicates to the 96 well plates. Supernatant samples (100μL) were collected 24h later then cell layers were harvested in 100μL of the lysis buffer RTL (Bio-Rad) with 1% B-mercaptoethanol (Sigma) for RNA extraction. All samples were stored at -80^0C until analyzed for cytokine elaboration or TLR expression.

Cytokine response evaluations of selected OTC compounds and a semen simulant in human immortalized vaginal EC

As described in the evaluation of TLR agonist induced cytokine responses in human vaginal EC section, immortalized vaginal EC were seeded and incubated for 24h. Conceptrol™, KY warming liquid™, Zestra™, a semen simulant (prepared as described by Owen and Katz [146]) or an equivalent volume of DPBS (10μL) were added in triplicates to the 96 well plates. After 24h of incubation, supernatant samples (100μL) were collected for cytokine analyses and stored at -80^0C. Cell layers were then collected

in 100μL of the lysis buffer RTL with 1% B-mercaptoethanol for RNA extraction and stored at -80^{0}C.

Cytometric bead array

For cytokine quantification, 50μL of each supernatant sample was analyzed using the BioPlex Human Group I cytokine kit (Bio-Rad). A series of kit-provided standards diluted with 1:1 medium, to control for any potential medium effects, were utilized for quantification standards. Absolute cytokine quantities (pg/mL) were extrapolated from a standard curve run in parallel.

Quantitative TLR RT-PCR

RNA was extracted from each cell layer sample (100μL) by using the Aurum 96 well RNA extraction kit (Bio-Rad). Samples and no template controls (H_2O) were Dnase treated (Bio-Rad) and eluted into 60μL of the provided elution solution. The iScrpit complementary DNA (cDNA) synthesis kit (Bio-Rad) was used to prepare the cDNA reaction consisting of 3μL of sample RNA or no template controls, 2.5μL of reverse transcriptase, 10μL of 5x iScrpit reaction mix, and 7.5μL of H_2O and performed using the following protocol: 25^{0}C for 5min, 42^{0}C for 30min and 85^{0}C for 5min.

For the TLR PCR reactions, 12.5μL Supermix with SYBR Green (Bio-Rad) was combined with 5μM of TLR primers (Table 2; IDT) [50], 9.5μL H_2O and 1μL of sample cDNA, no template controls or RNA alone controls. Samples were run on an iCycler with the following protocol: 95^{0}C for 1min 30sec then 50 cycles of 95^{0}C for 30sec, 30sec at annealing temperatures of 59^{0}C for TLR1-6 and 8, 63^{0}C for TLR7 or 60.7^{0}C for TLR9

and a 72^0C for 30sec extension followed by 1 cycle of 95^0C for 1min and 75^0C for 1min ending with a melt curve.

Table 2: Human TLR and GAPDH forward and reverse primer sets used for RT-PCR analyses.

	Forward 5'-3'	**Reverse 5'-3'**
TLR1	TGGTATCTCAGGATGGTGTGCC	CACCCAGAAAGAATCGTGCCC
TLR2	GAAAATGATGTGGGCCTGGCT	GCATCCCGCTCACTGTAAGAAA
TLR3	TTCTCGATTTGCAGCATAACAACT	TGGACTCCAAGTTAAGGATGTGG
TLR4	TGGCATGAAACCCAGAGCTTT	AACGGCAGCATTTAGCAAGAAG
TLR5	GACAACGAGGATCATGGGAGAC	CCATCAAAGGAGCAGGAAGGAA
TLR6	TACTTGGATCTGCCCTGGTATCT	TGGAGGTTTCTTTGGAGTTCTTCT
TLR7	CATTTGACAGAAATTCCTGGAGGT	GGGAGATGTCTGGTATGTGGTTA
TLR8	TGAGCAACACCCAGATCAAATACA	TCACAAGGCACGCATGGAAAT
TLR9	GCAATGTCACCAGCCTTTC	GTTCCACTTGAGGTTGAGATG
GAPDH	CAACTACATGGTTTACATGTTC	CTCGCTCCTGGAAGATG

Human glyceraldehydes 3-phosphate dehydrogenase (GAPDH) PCR was performed as a standard for normalization of TLR results and to confirm proper cell counts, cell viability, RNA quality and extraction efficiency as well as cDNA quantity and quality [50]. For GAPDH PCR, 12.5µL of Supermix, 5µM of GAPDH-F (5' – CAA CTA CAT GGT TTA CAT GTT C – 3'; IDT), 5µM of GAPDH-R (5' – CTC GCT CCT GGA AGA TG – 3'; IDT), 7.5µM of GAPDH-TP (5' – TxRed – CAT GGC ACC GTC AAG GCT GAG AAC – BHQ2 – 3'; IDT), 9µL of H_2O and 1µL of sample cDNA, no template controls or RNA alone controls. The GAPDH PCR protocol consisted of 95^0C

for 1min 30sec then 40 cycles of 95^0C for 20sec and 62^0C for 1min. The TLR RT-PCR procedure produced cDNA representing approximately 1000cells/1μL. For PCR assays, a dilution series of cloned amplimers was utilized for quantification standards and all reactions produced efficiencies between 90% and 110% and correlation coefficients greater than 0.95.

Statistical analyses

Significant differences in the cytokine or TLR expression levels following compound application compared to the DPBS vehicle control were analyzed via Student's t-test using the Prism software package v4.0. Primary cell experimental results represent triplicate samples from V11 or V19 cells. The immortalized vaginal EC data is representative of two experiments using triplicate samples.

RESULTS

TLR agonists elicited specific cytokine response profiles in primary vaginal EC

The human vaginal mucosa is the first line of defense against many STI including HSV-2 [48, 50, 51]; therefore understanding the innate immune response elicited by vaginal EC following pathogen exposure is of critical importance for the future design of better models that will be used to develop immunomodulatory intervention strategies. FSL-1 (anti-herpetic bacterial-derived agonist), PIC (anti-herpetic viral-associated agonist) and FLAG (non-anti-herpetic bacterial-derived agonist) were selected for initial cytokine evaluation in human primary vaginal EC based on previously observed activity in the adapted assay scheme (Figure 8) [Chapter 2]. The tested agonists also were selected because of the increased likelihood of observing significant increases in cytokine

elaboration due to the high expression of TLR2, 3, 5 and 6 by human primary vaginal EC [50]. The results from the adapted assay scheme showed that application of FSL-1 or PIC 24h prior to viral challenge produced significant reduction in HSV-2 replication (Figure 8) [Chapter 2]; therefore elicited cytokine profiles were quantified at 24h post-application of the selected agonists. Concentrations of TLR agonists evaluated for cytokine elaboration also were selected based on the previously observed activity (Figure 8) [Chapter 2].

Primary vaginal EC exposed to PIC for 24h elicited a cytokine profile consisting of IL-2, IL-6, IL-8, IL-12(p70), G-CSF, GM-CSF, IFNγ, MIP-1α, MIP-1β and RANTES (Figure 10). Application of FSL-1 resulted in significant ($p<0.01$; Student's t-test) induction of a similar profile except for the lack of IL-2 and IL-12(p70) induction. Interestingly, FLAG application only produced significant ($p<0.01$; Student's t-test) increases in G-CSF and GM-CSF. The TLR agonist induced cytokine profiles also were evaluated using vaginal EC from another donor (V19) to confirm that activity was not donor specific. Similar profiles as observed in V11 cells were elicited by FSL-1 or PIC following a 24h exposure (Figure 10). FLAG application did not produce significant ($p>0.05$; Student's t-test) increases in any of the tested cytokines but showed a trended increase in G-CSF and GM-CSF. The results showed that primary human vaginal EC were capable of selectively responding to pathogen exposure, represented by TLR agonists, and that the elicited cytokine profiles were consistent across two different donors and with previously published cytokine evaluations [50]. Additionally, the cytokines induced by FSL-1 or PIC application (Figure 10) are associated with blocking HSV-2 entry into vaginal EC and directly inhibiting HSV-2 replication as described previously [Chapter 1].

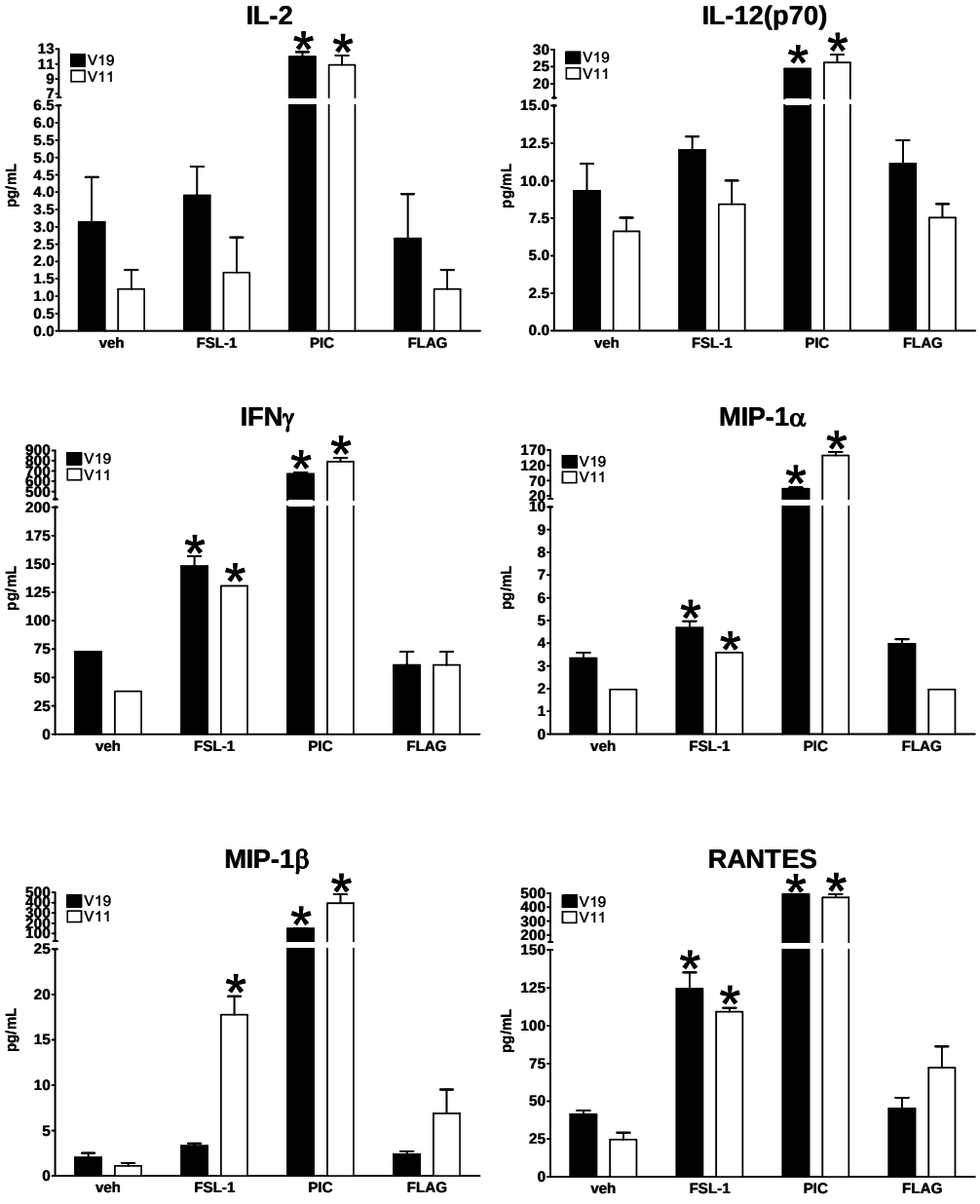


Figure 10. TLR agonists elicited specific cytokine profiles in primary vaginal EC. FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or an equivalent volume of the DPBS vehicle (10μL) were added to V19 or V11 cultures. Supernatant samples (100μL) were collected 24h later for cytokine analyses. *$p<0.01$ (Student's t-test) compared to DPBS vehicle. Data are mean ± SEM of 3 replicates for each cell type.

Immortalized vaginal EC served as a model of primary cells for cytokine evaluation

The limited life span of primary vaginal EC restricts their usability and hampers reproducible experimentation. To provide a renewable model, primary vaginal EC were immortalized by HPV16E6/E7 transduction as performed previously [50]. For comparison purposes, the immortalized vaginal EC were treated with FSL-1, PIC and FLAG at the same concentrations and application times as with the primary vaginal EC experiments (Figure 10). With the addition of IL-12(p70), FSL-1 elicited a significant ($p<0.01$; Student's t-test) increase in the same cytokines as observed for the primary vaginal EC. A trended increase in IL-2 and MIP-1β also was observed 24h post FSL-1 application (Figure 11). The PIC induced cytokine profile in immortalized vaginal EC was indistinguishable from the primary vaginal EC profile and produced a significant ($p<0.01$; Student's t-test) increase in the same cytokines. FLAG application led to a significant ($p<0.01$; Student's t-test) increase in RANTES with a trended increase in G-CSF and GM-CSF. Overall, the results were consistent with previously published results [50] and induced cytokine profiles observed in primary vaginal EC (Figure 10) confirming that the immortalized vaginal EC cells provided a reproducible and renewable model of the primary cells.

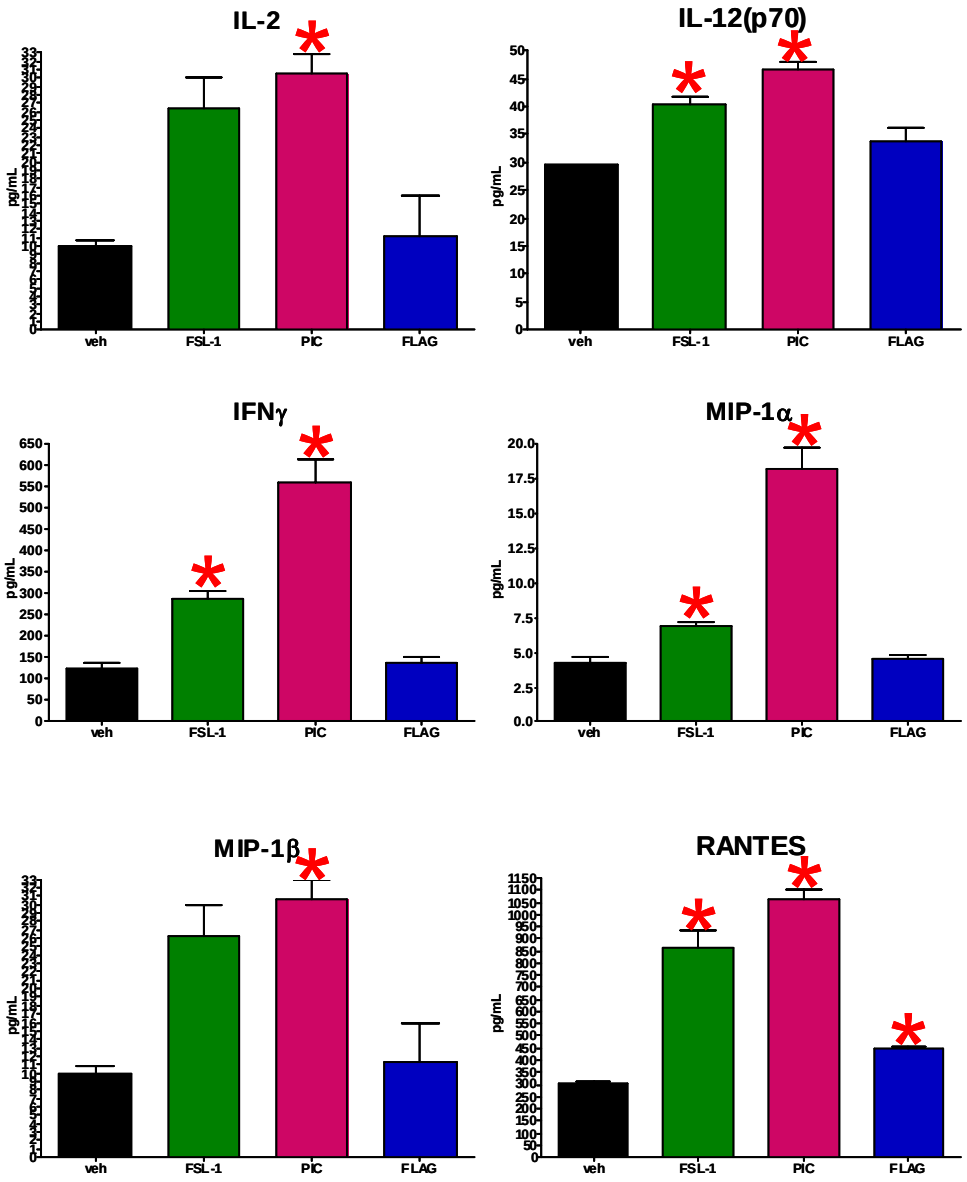


Figure 11. Immortalized vaginal EC elaborated similar cytokine profiles as primary vaginal EC following TLR agonists application. FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or an equivalent volume of the DPBS vehicle (10μL) were added V19I cultures. Supernatant samples (100μL) were collected 24h later for cytokine analyses. *p<0.01 (Student's t-test) compared to DPBS vehicle. Data are the mean ± SEM of 3 replicates from a representative experiment.

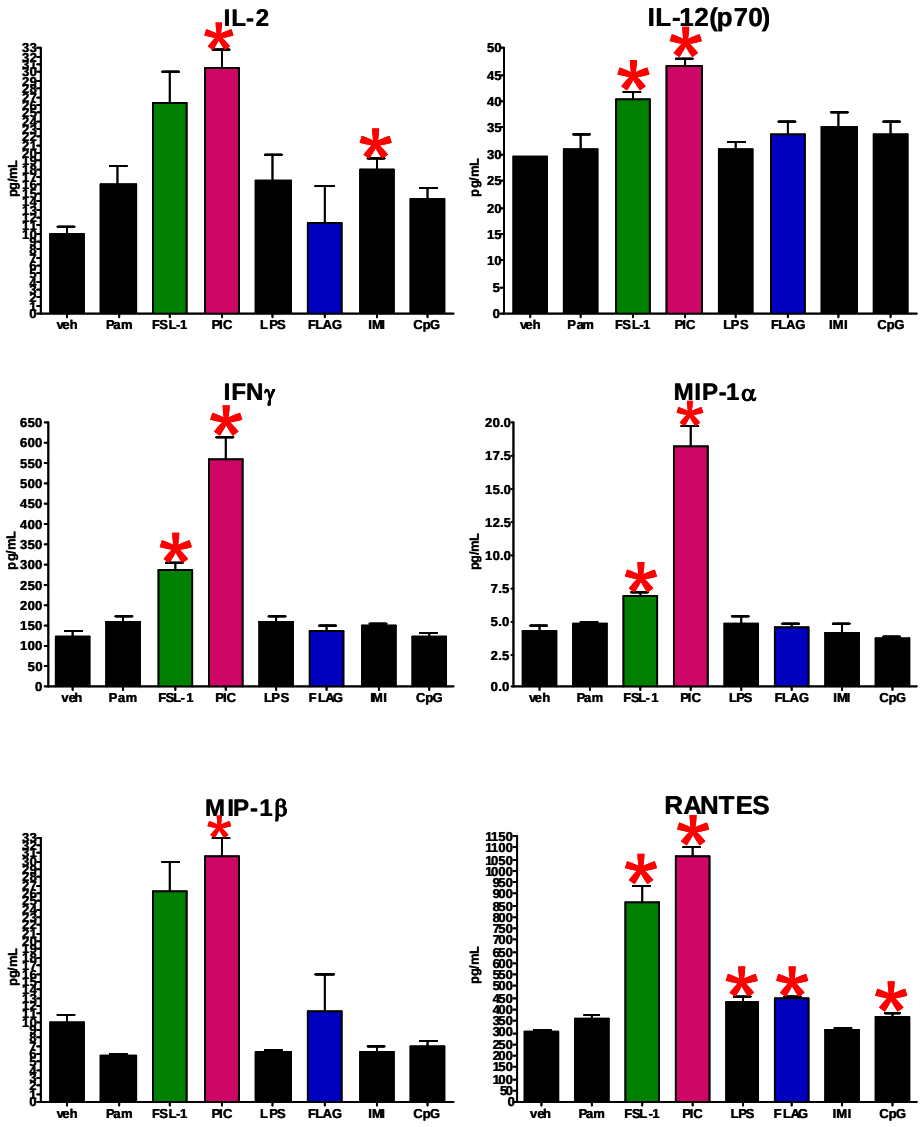


Figure 12. Cytokines important for HSV-2 resistance were identified using anti-herpetic and non-anti-herpetic TLR agonists. Pam (10μg/mL), FSL-1 (0.1μg/mL), PIC (0.1mg/mL), LPS (1μg/mL), FLAG (1μg/mL) IMI (0.1mg/mL), CpG (0.1mg/mL) or an equivalent volume of the DPBS vehicle (10μL) were added to V19I cultures. Supernatant samples (100μL) were collected 24h later for cytokine analyses. *$p<0.01$ (Student's t-test) compared to DPBS vehicle. Data are the mean ± SEM of 3 replicates from a representative experiment.

A preliminary cytokine response profile associated with HSV-2 resistance was identified using anti-herpetic and non-anti-herpetic TLR agonists

The immortalized vaginal EC were used as a renewable model for evaluating TLR agonist induced cytokine response profiles to identify cytokines associated with anti-herpetic activity following TLR agonist stimulation. As described previously [Chapter 1], TLR agonists are recognized by specific TLR and elicit production of cytokines that can potentially engender an HSV-2 resistant environment [51, 57, 140]. Cytokine profiles elaborated following application of selected anti-herpetic (FSL-1, PIC, IMI, CpG) [80, 82, 180-185; Chapter 2] or non-anti-herpetic (Pam, LPS, FLAG) [182, 183, 185] TLR agonists were evaluated as a preliminary step in identifying cytokines important for anti-herpetic activity. Additionally, TLR agonists were selected to provide wide range of bacteria-derived, viral-associated, natural or synthetic agonists covering the TLR expressed by vaginal EC [50]. All agonist were applied 24h prior to collection of the supernatant for cytokine analyses based on the time required to provide resistance against HSV-2 observed previously (Figure 8) [Chapter 2]. The same data set was used to select tested concentrations of FSL-1, PIC or FLAG while previously observed in vitro or in vivo activity was used as the basis for tested concentrations of the other agonists [80, 82, 180-185].

PIC was the most robust inducer of cytokines and significantly ($p<0.01$; Student's t-test) elicited a profile consisting of IL-2, IL-6, IL-8, IL-12(p70), G-CSF, GM-CSF, IFNγ, MIP-1α, MIP-1β and RANTES (Figure 12). FSL-1 application resulted in significant ($p<0.01$; Student's t-test) induction of a similar cytokine profile except IL-2 and MIP-1β only showed a trended increase. Surprisingly, IMI only elicited significant ($p<0.01$; Student's t-test) production of IL-2 while CpG, a potent anti-herpetic TLR agonist in vivo [80, 181], only significantly ($p<0.01$; Student's t-test) increased RANTES

production after a 24h application (Figure 12). The minimal immunomodulatory observed following IMI or CpG application potentially was due to the low expression of TLR7, 8 and 9 by a subset of vaginal EC [50]. The non-anti-herpetic TLR agonists LPS and FLAG significantly ($p<0.01$; Student's t-test) induced only RANTES while Pam showed no activity. Based on the induction of specific cytokines by the anti-herpetic and non-anti-herpetic TLR agonists and previously published evaluations [80, 82, 180-185], a preliminary cytokine response profile associated with HSV-2 resistance would include production of IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β, and RANTES.

TLR agonist application did not substantially alter the TLR expression profile of immortalized vaginal EC

Regulation of TLR expression is important for the selective recognition of PAMP [48, 51-53]; therefore TLR RT-PCR was used to evaluate the potential impact of TLR agonists on the immortalized vaginal EC expression profile. FSL-1, PIC and FLAG were selected for evaluation based on the robust immunomodulatory activity of FSL-1 and PIC observed previously (Figure 11) and the high expression of TLR2, 3, 5 and 6 by vaginal EC [50]. The same concentrations and 24h prior to collection application time were used for correlation of potential TLR alterations to observed cytokine elaboration. Application of FSL-1 or PIC resulted in no significant ($p>0.05$; Student's t-test) alteration in the immortalized vaginal EC TLR expression profile (Figure 13). FLAG application significantly ($p<0.01$; Student's t-test) increased expression of TLR8 and 9 but did not produce a substantial alteration in the overall profile. The observed lack of major TLR expression profile changes, as a preliminary safety evaluation, indicated that TLR agonist activity should be evaluated further in vivo [Chapter 4].

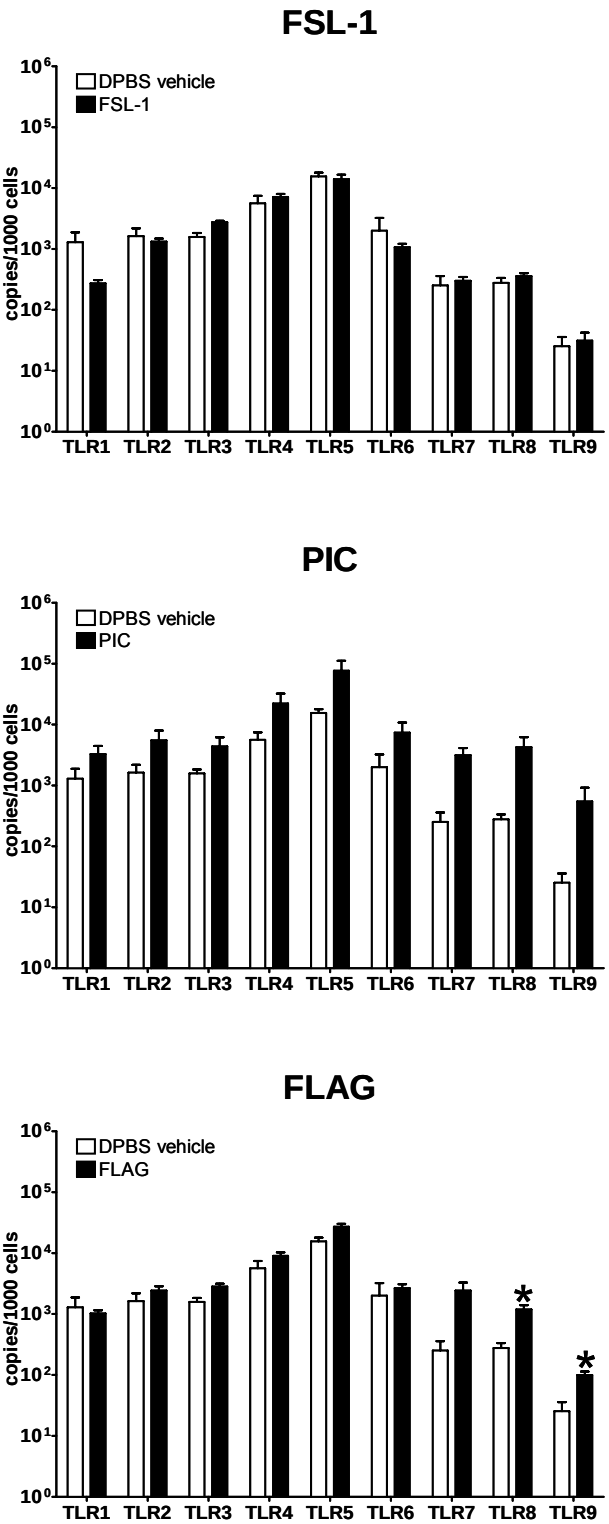


Figure 13. TLR agonists application did not substantially alter the immortalized vaginal EC TLR expression profile. FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or an equivalent volume of the DPBS vehicle (10μL) were added to V19I cultures. After 24h V19I cell layers were collected for quantitative TLR RT-PCR analyses. *$p<0.01$ (Student's t-test) compared to DPBS vehicle. Data are the mean ± SEM of 3 replicates from a representative experiment.

OTC compounds or a semen simulant applied to immortalized vaginal EC did not induce cytokines or alter the TLR expression profile

The use of OTC products as potential vehicles for anti-herpetic compounds [149-152] was supported by the observed promising preliminary safety results (Figure 9) [Chapter 1] but additional evaluations were necessary to identify any immunological impact on immortalized vaginal EC. Also, semen is occasionally introduced into the vaginal environment and contains a mixture of many different alkaline chemical components including cytokines like TGFβ [145, 146] that potentially could alter responsiveness of vaginal EC to TLR agonists. KY warming liquid™, as a representative of the inactive ingredient compounds [Chapter 2], Zestra™ and a semen simulant that represented the alkaline chemical components of semen were applied to vaginal EC for 24h then analyzed for potential cytokine induction. Conceptrol™ was not evaluated due to the lack of quantifiable human GAPDH messenger RNA indicating toxicity (data not shown). Figure 14 shows that no significant ($p>0.05$; Student's t-test) induction of the tested cytokines was observed for KY warming liquid™, Zestra™ or the semen simulant compared to the DPBS vehicle.

The potential impact of OTC products and the semen simulant on immortalized vaginal EC TLR expression profile also was quantified as an additional preliminary safety evaluation. KY warming liquid™, Zestra™ or the semen simulant did not significantly ($p>0.05$; Student's t-test) alter the TLR expression profile after a 24h exposure (Figure 15). The cytokine and TLR RT-PCR data confirmed the feasibility of OTC as vehicles for TLR agonists and showed that the presence of semen should not impact the responsiveness of vaginal EC to TLR agonist immunomodulation.

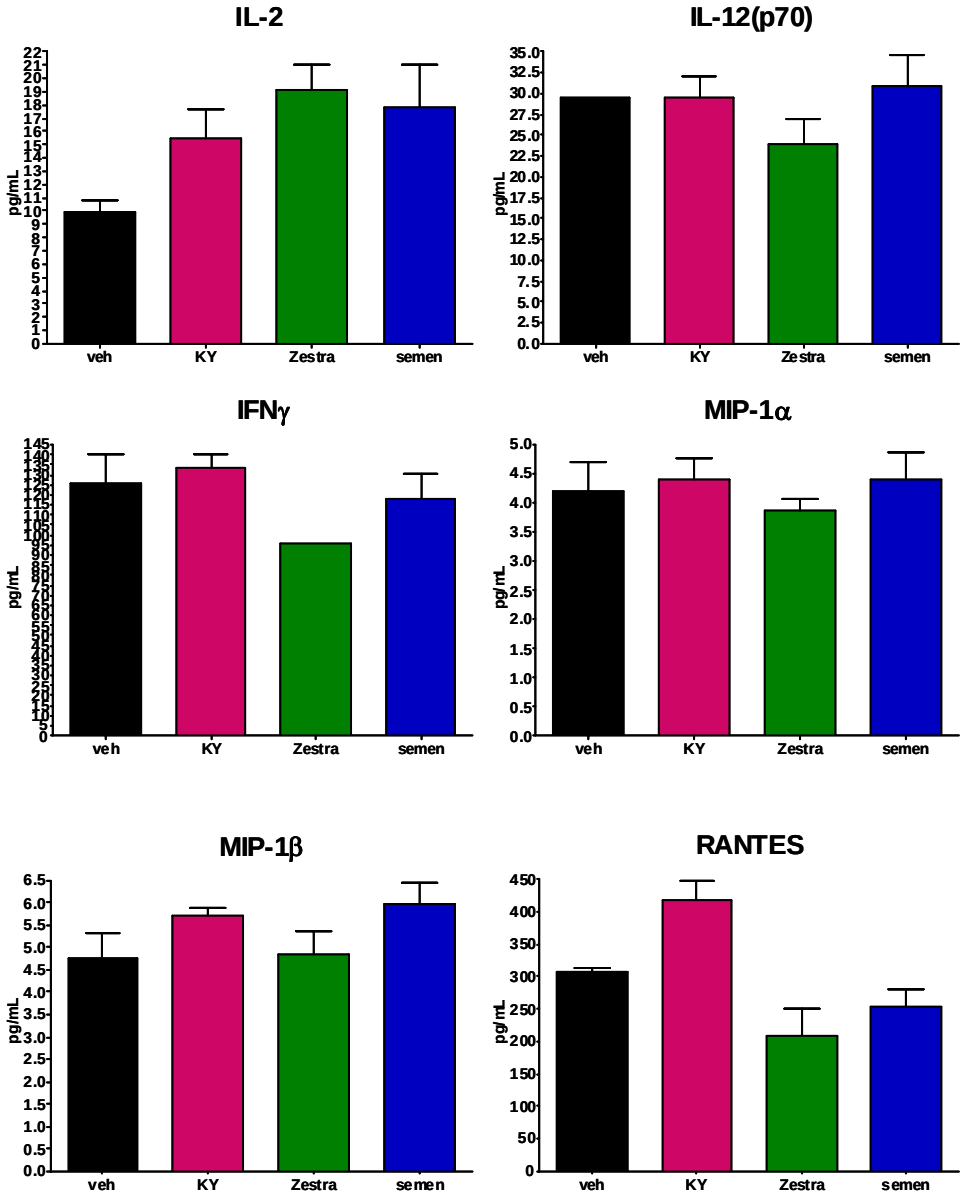


Figure 14. OTC compound or semen simulant application did not induced cytokines in immortalized vaginal EC. KY warming liquid™, Zestra™, a semen simulant or an equivalent volume of the DPBS vehicle (10μL) were added to V19I cultures. Supernatant samples (100μL) were collected 24h later for cytokine analyses. *$p<0.01$ (Student's t-test) compared to DPBS vehicle. Data are the mean ± SEM of 3 replicates from a representative experiment.

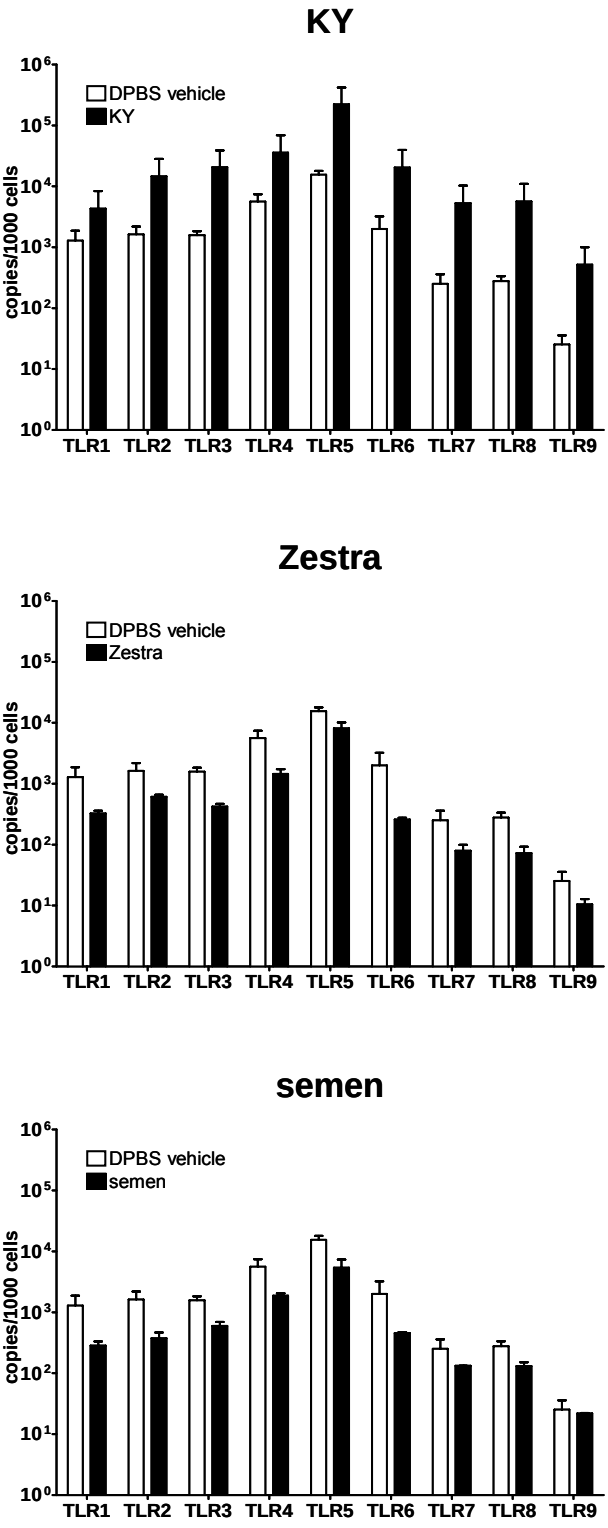


Figure 15. Application of OTC compounds or a semen simulant showed no impact on the TLR expression profile of vaginal EC. KY warming liquid™, Zestra™, a semen simulant or an equivalent volume of the DPBS vehicle (10μL) were added to V19I cultures. After 24h V19I cell layers were collected for quantitative TLR RT-PCR analyses. Data are the mean ± SEM of 3 replicates from a representative experiment.

DISCUSSION

We showed that human immortalized vaginal EC responded to TLR agonist application through induction of cytokine response profiles similar to the profiles observed in primary vaginal EC. The limited nature of primary vaginal EC often provides too few cells to address biological questions or reproduce observed results; therefore the primary cells were immortalized through viral transduction to provide a renewable source of vaginal EC [50]. Exposure to PIC for 24h produced the same cytokine response profiles in immortalized or primary vaginal EC and similar results were observed for FSL-1 or FLAG application (Figures 10 and 11). TLR agonist evaluation in other donor vaginal EC confirmed that the results were not donor specific. Collectively, the cytokine data illustrated that immortalized vaginal EC will provide a renewable and reproducible model of primary vaginal EC. The renewable nature of the immortalized vaginal EC also is critical for enabling cross-validation of the observed cytokine results similar to the adapted assay scheme [Chapter 2].

Additionally, the immortalized vaginal EC allowed for evaluation of anti-herpetic and non-anti-herpetic TLR agonists and resulted in identification of a preliminary anti-herpetic cytokine profile consisting of IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β, and RANTES. The observed TLR agonists induced cytokine profiles also were consistent with previously published results [50, 185]. Application of the anti-herpetic TLR agonists, FSL-1 or PIC [184, 185; Chapter 2], 24h prior to cytokine analyses both induced production of IL-2, IL-6, IL-8, IL-12(p70), G-CSF, GM-CSF, IFNγ, MIP-1α, MIP-1β and RANTES (Figure 12). Interestingly, the other anti-herpetic TLR agonists, IMI and CpG [80, 82, 180, 181], exhibited only minimal immunomodulatory activity following application and resulted in induction of IL-2 and RANTES. The minimal cytokine activity offers a potential explanation for the reduced anti-herpetic activity

observed for IMI and CpG in vitro [185]. The non-anti-herpetic agonists, Pam, LPS and FLAG [182, 183, 185], only elicited G-CSF and RANTES. The observation of RANTES production by the non-anti-herpetic TLR agonists indicates that resistance to HSV-2 infection likely is engendered through highly coordinated activation of several anti-herpetic associated cytokines.

Comparison of the cytokine profiles elicited by anti-herpetic and non-anti-herpetic TLR agonists identified IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β, and RANTES as cytokines associated with induction of resistance to HSV-2 infection. In vivo experiments showed that IL-2 delivered alone or as a vaccine adjuvant increased resistance to genital HSV-2 infection [75, 214]. Additionally, IL-2 and IL-12 stimulate and activate leukocytes and induce type I (IFNα, IFNβ) and type II (IFNγ) IFN production [75, 77, 79, 80, 215, 216]. The production of IFN by IL-2 and IL-12 skews the immune system towards a T_H1-baised response that is important for controlling early replication of HSV-2 and for effective clearance of the infection [84, 85, 217, 218]. HSV-2 replication is directly inhibited by IFN-inducible proteins [219] and is aided by the activity of MIP-1α, MIP-1β and RANTES [88]. The chemokines also recruit additional leukocytes to the infection site [88, 220, 221] that are in turn activated by the activity of IL-2 and IL-12 [75, 77, 79, 80, 215, 216]. Identification of a preliminary anti-herpetic cytokine response profile provides a basis for the future design of novel TLR agonist interventions to reduce the spread of HSV-2. Additional evaluations in small animal models [Chapter 4] and refined in vitro models [Chapter 6] also were performed to confirm and expand the identified profile.

Additional safety evaluations in immortalized vaginal EC showed that OTC products or a semen simulant did not significantly induced cytokine production or alter the vaginal EC TLR expression profile. In addition the anti-herpetic assays performed

previously [Chapter 2], immunological evaluations of OTC compounds were necessary because of their proposed use as potential vehicles for anti-herpetic compounds [149-152]. OTC products also are occasionally applied to the vaginal environment prior to or during sexual intercourse [222-224] and can potentially interfere with TLR agonist induced anti-herpetic activity. OTC compound application showed no significant induction of cytokines (Figure 14) or alteration in the immortalized vaginal EC TLR expression profile (Figure 15). Semen contains a unique mixture of proteins, including cytokines and alkaline chemical components [145, 146], that could potentially impact the responsiveness of the vaginal mucosa to TLR agonists induced anti-herpetic activity. Even after 24h of exposure to a semen simulant immortalized vaginal EC showed no significant increase in cytokines or TLR expression (Figures 14 and 15). The immunological evaluations, combined with the previous anti-herpetic assays [Chapter 2], further supported the feasibility of OTC products as vehicles for anti-herpetic TLR agonists. Also, the presence of semen in the vaginal environment should not impact the activity of the TLR agonists.

We have preliminarily identified IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β, and RANTES as cytokines important for inducing resistance to HSV-2 infection through the use of anti-herpetic and non-anti-herpetic TLR agonists in human vaginal EC. The identified anti-herpetic cytokine profile was associated with a highly coordinated innate immune response to HSV-2 infection [75, 88, 214, 219]. Additionally, immunological evaluations were facilitated by the generation of immortalized vaginal EC [50]. TLR agonist application resulted in similar cytokine profiles in both cell types and confirmed that immortalized vaginal EC provided a reproducible and renewable model of the primary cells [50]. The immortalized vaginal EC also allowed for evaluation of potential immunological alterations induced by OTC products or semen. The observed lack of

cytokine or TLR expression alterations indicated that anti-herpetic TLR agonists should be efficacious even when occasionally exposed to OTC compounds or semen. The preliminary safety evaluations coupled with identification of an anti-herpetic cytokine profile is of key importance for the development of TLR agonist intervention or vaccine adjuvant strategies to reduce HSV-2 infections. Additionally, the preliminary anti-herpetic cytokine profile was evaluated further in a mouse model of genital HSV-2 infection using FSL-1 [Chapter 4] and in a refined in vitro model of the human vaginal mucosa using selected TLR agonists [Chapter 6].

CHAPTER 4: FSL-1, A BACTERIAL-DERIVED TLR2/6 AGONIST, ENHANCED RESISTANCE TO GENITAL HSV-2 INFECTION

OVERVIEW

HSV-2 is a leading cause of genital ulceration that can predispose individuals to an increased risk of acquiring other STI. There are no approved HSV-2 vaccines and current suppressive therapies require daily compound administration that does not prevent all recurrences. A promising experimental strategy is the use of TLR agonists to induce an innate immune response that provides resistance to HSV-2 infection. Previous studies showed that anti-herpetic activity varied based on origin of the agonists and activation of different TLR indicating that activity likely occurs through elaboration of a specific innate immune response. To test the hypothesis, we evaluated the ability of FSL-1 (TLR2/6) to increase resistance to experimental genital HSV-2 infection. Additionally, cytokines induced by FSL-1 were kinetically quantified to identify cytokines important for anti-herpetic activity and establish a preliminary safety profile. The results showed that vaginally-applied FSL-1 created an environment resistant to a 25-fold higher HSV-2 challenge dose. Mechanistically, vaginal FSL-1 application led to transient elaboration of cytokines linked to anti-herpetic innate immune responses. No gross local or peripheral immunotoxicity was observed even after multiple dosing. The results showed, for the first time, that the bacterial-derived TLR2/6 agonist FSL-1 induced significant resistance to HSV-2 infection when applied in mice. Additionally, the identified anti-herpetic cytokine profile provides an invaluable resource for the future design of novel compounds to reduce genital HSV-2 infection and improves understanding of the

complex innate immune response to potential pathogens elicited by the vaginal mucosa. Part of the research described in this chapter is in press in *Virology Journal*.

INTRODUCTION

Genital HSV-2 is one of the most wide spread STI and prevalence varies from 16-97% based on age, culture, ethnicity, geographic location, sex and other factors [3, 9, 10]. Following genital infection, HSV-2 establishes a life-long latency in lumbosacral ganglia and periodically can reactivate resulting in the formation of genital lesions [3, 4, 6, 7]. Controlling the spread of HSV-2 is important because the genital lesions can serve as portals of entry for other STI including HIV-1 [3, 11, 13, 19]. Also important to transmission cycles, HSV-2 is frequently shed from the genital tract in the absence of symptoms [4, 14]. Recent studies showed that HSV-2 is associated with coinfections and can exacerbate the pathologies produced by STI [11, 13, 19, 225]. So prevention of transmission is a laudable goal, however, there are no FDA-approved vaccines for HSV-2 [3, 4, 26]. Further, treatment of existing infections involves daily antiviral therapy that only suppresses but does not completely prevent all recurrences [3, 4, 6, 37]. New approaches are needed that can prevent or reduce the likelihood of transmission.

One promising experimental strategy is the use of evolutionarily conserved pathogen structures termed PAMP that are recognized by TLR [48, 50, 51]. As described previously [Chapter 1], each PAMP is recognized by a particular TLR that activates specific signaling molecules like MyD88 or TRIF resulting in production of cytokines that can engender an environment resistant to pathogenic infection [51, 53, 70, 71]. Previous work showed that viral-associated TLR agonists vaginally-applied prior to challenge increased resistance to HSV-2 infection in mice [80, 82, 181-185]. Application of PIC, a synthetic TLR3 agonist, 24h prior to viral inoculation significantly increased

resistance to genital HSV-2 infection and mice that were infected showed significantly delayed disease signs and increased survival times [184]. Similar anti-herpetic activity was observed following application of IMI or CpG, synthetic TLR7/8 or 9 agonists respectively [80, 82, 181-183]. Interestingly, murine experiments using bacterial-derived TLR agonists including PGN (TLR2), LPS (TLR4) or FLAG (TLR5) showed no significant activity [182, 183]. In a recent study, FimH derived from *Escherichia coli* elicited significant resistance to genital HSV-2 infection through TLR4 activation and IFNβ production [226]. The varying activity observed following TLR agonist application indicates that establishment of an HSV-2 resistant environment likely occurs through elaboration of a specific innate immune response.

To test the hypothesis that HSV-2 resistance could be engendered through elaboration of a specific cytokine response, we selected a synthetic diacyl lipopeptide derived from *Mycoplasma salivarium*, FSL-1, that is recognized by TLR2/6 heterodimers [202, 212, 213]. TLR2 and 6 are highly expressed by human [50] and murine [227] vaginal and lower genital tract EC. Studies also showed that HSV-2 is recognized through TLR2-mediated binding of the glycoprotein spikes within the viral lipid envelope [51, 56, 57]. Additionally, previous data [Chapter 2] illustrated that, like PIC, FSL-1 induced significant resistance to HSV-2 infection in human vaginal EC. The anti-herpetic activity correlated with induction of IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β and RANTES similar to the profiles observed for other anti-herpetic TLR agonists [50, 185; Chapter 3]. Based on the previously observed cytokine-associated anti-herpetic activity of FSL-1 in human vaginal EC [Chapters 2 and 3], we hypothesized that the FSL-1 elicited cytokine pattern would engender resistance to HSV-2 infection in an experimental mouse model.

FSL-1 was tested at several doses and application times in the mouse model to evaluate potential induced resistance to genital HSV-2 infection. Cytokines induced by FSL-1 application were quantified for comparison to other TLR agonists to identify an innate immune response profile associated with anti-herpetic activity. Kinetic cytokine profiles elicited by a single or by multiple doses of FSL-1 were quantified and weights of relevant organs were measured to establish a preliminary safety profile of the induced innate immune response for the rational design of future anti-herpetic compounds. Collectively, the results indicated that FSL-1 induced significant resistance to experimental genital HSV-2 infection through elicitation of a specific cytokine response profile.

MATERIALS AND METHODS

Virus preparation

HSV-2 strain 186 (obtained from Dr. Lawrence Stanberry [228]) was added to a Vero cell monolayer in a T-150 cell culture flask (BD). The infected Vero cells were incubated at 37^0C, 5% CO_2 for 48h to allow for several cycles of viral replication. The culture flask was rapped lightly to dislodge infected cells then the supernatant containing the cells was collected into a 50mL oak ridge centrifuge tube (Fisher). Virus was released from the cells using 2 freeze (-80^0C) then thaw (37^0C) cycles. Clarified virus was aliquoted (1mL) and stored at -80^0C. Plaque titration assay was used to quantify the titer of the virus.

HSV-2 plaque titration assay

Vero cells were seeded into 24-well culture plates (BD) at $1x10^6$cells/well in 1mL of supplemented DMEM then incubated for 24h to achieve 90-100% confluency. One of the HSV-2 strain 186 aliquots was thawed and 100μL was added to 900μL of Vero medium in a 1.7mL centrifuge tube (BD) to make a 10^{-1} dilution. Additional serial dilutions (10^{-2}-10^{-6}) were created then the medium was removed from the 24-well culture plate and 100μL of each dilution was added to the wells in triplicate. The plate was incubated for 1h and during incubation the plate was rocked every 15min. After 1h, 1mL of Vero medium supplemented with 0.3% human immune globulin (Talecris Biotherapeutics, Research Triangle Park, NC) was added to each well. The medium was removed from the plates after 48h of incubation then the plates were stained with 0.8% crystal violet (Sigma) for 5min, rinsed and air dried. Plaque counts were recorded through visual observation on a dissecting microscope (Unico, Dayton, NJ) and used to calculate the average pfu/mL for the virus.

Mouse model of genital HSV-2 infection

Female Swiss-Webster mice (Harlan Sprague Dawley, Indianapolis, IN) weighing 20-25g were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved quarters and provided with unlimited access to food and water. All procedures were performed humanely and approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Following a 7d acclimation to the facility, mice were hormonally-conditioned by subcutaneous delivery of 150 mg/kg of Depo-Provera (UpJohn, Kalamazoo, MI) to the scruff of the neck 7d prior to viral inoculation as described [181]. Hormonal conditioning was used to increase the susceptibility of the mice to genital HSV-2 infection [181, 229].

FSL-1 was diluted in DPBS to achieve a 0.1μg/μL solution and delivered vaginally using a positive displacement pipet (Rainin, Oakland, CA) at 2 or 6μg/mouse prior to or following viral challenge as specified for each experiment. Doses were selected based on previously observed anti-herpetic activity of FSL-1 in human vaginal EC [Chapter 2] and volumetric constraints of the murine vagina. DPBS vehicle treated mice were followed in parallel to serve as controls for all experiments. Mice were swabbed with a wet then dry calcium alginate swab (Fisher) just prior to instilling 25μL of HSV-2 strain 186 (10^4pfu/mouse) vaginally using a positive displacement pipet. The challenge dose, a 100 times greater than the dose required to cause lethal outcomes in 50% of the mice (LD_{50}) [181, 184], was selected to ensure that enough mice in the DPBS vehicle treatment group exhibited disease signs to allow for statistical comparisons.

Mice were assessed daily (d1-14) for the appearance of disease signs and were considered infection positive following the development of hair loss and erythema around the vagina (Figure 16a) indicating viral replication in the vaginal mucosa [181, 184, 230]. As viral replication progressed to the lumbosacral ganglia [229], chronic wetness due to loss of bladder control (Figure 16b) and hind limb paralysis occurred followed by fatal encephalitis [181, 184, 230]. Moribund mice or those with debilitating hind limb paralysis were euthanized to prevent suffering of the animal.

Mice were confirmed as infection positive using CPE scoring of vaginal swabs collected 2d post inoculation (PI) [181, 184, 230]. Vaginal swabs were placed in 1mL of Vero medium then 100μL from each swab tube was added to 24-well culture plates containing Vero cells ($1x10^6$cells/well) in 1mL DMEM at 24h post-seeding. The plates were incubated at 37^0C, 5% CO_2 for 48h then scored for CPE as described previously [Chapter 2].

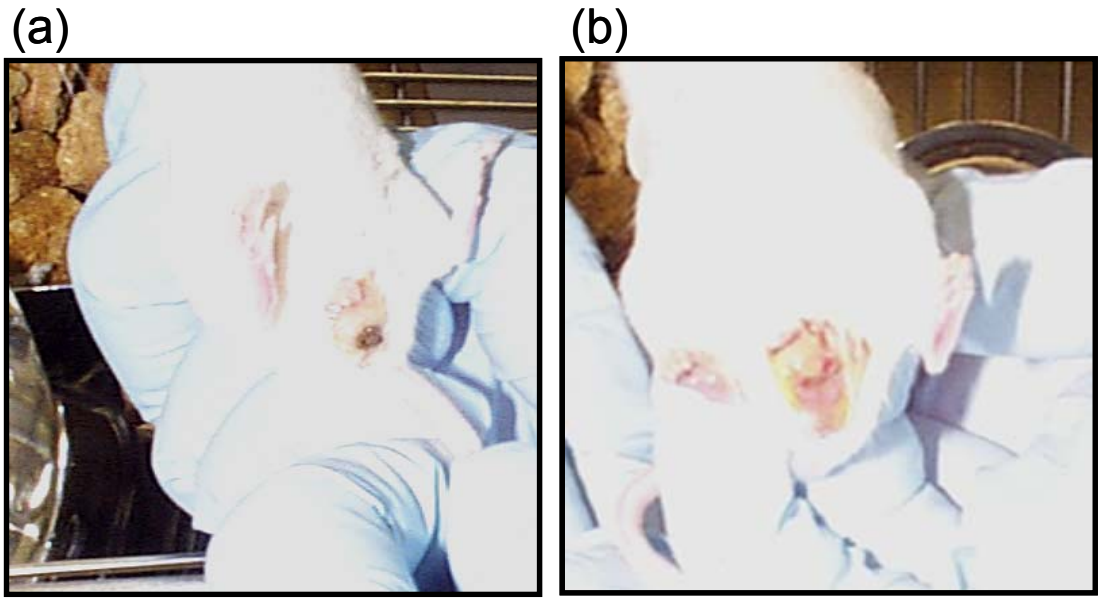


Figure 16. Appearance of disease signs in a mouse infected with HSV-2 genitally. (a) Hair loss and erythema are observed around the vagina at 4 days post infection. (b) The severity of hair loss and erythema increased and chronic wetness due to loss of bladder control is observed as the infection progresses. Hind limb paralysis also is observed during the later stages of infection in some mice prior to fatal encephalitis.

Evaluation of FSL-1 induced anti-herpetic activity using the ID_{50} shift paradigm

FSL-1 induced resistance to HSV-2 infection was quantified using the infectious dose 50% (ID_{50}) shift paradigm [184]. FSL-1 doses of 2 or 6μg/mouse were delivered at indicated times then groups of mice were challenged with viral inoculums of 10^2, 10^3 or 10^4pfu/mouse (n = 10). DPBS treated groups were employed to control for the potential impact of the vehicle on HSV-2 infection and were challenged with viral inoculums of 10^1, 10^2 or 10^3pfu/mouse (n = 10). Mice were followed daily for survival and infection was confirmed using CPE scoring as described previously [Chapter 2].

Cytokine quantification

Vaginal lavages were collected at 0 and 24h after application of FSL-1 (6μg) or an equivalent volume of the DPBS vehicle (60μL) or at 6 and 48h after treatments from a second group of mice (n = 5 mice/treatment for each group). Lavages were collected by instilling 25μL of DPBS five times then pooling into a 1.7mL centrifuge tube with an average recovery of approximately 100μL followed by storage at -80^{0}C until cytokine analyses [184]. Lavage collections were scheduled to prevent an artificial "washout" of vaginal cytokines by only sampling an animal once within a 24h period. For repetitive dosing evaluations, vaginal lavages were collected from FSL-1 (6μg) or DPBS vehicle (60μL) treated mice at 6h (n = 5 each; group 1) or 24h (n = 5 each; group 2) after a once daily application for 5 consecutive days. Vaginal cytokines in the recovered lavages were quantified using the BioPlex Mouse Group I cytokine kit (Bio-Rad) as described previously for the cytometric bead array [Chapter 3]. Vaginal IFNα and IFNβ were quantified using a mouse specific ELISA kit (PBL, New Brunswick, NJ). A series of kit-provided standards diluted with DPBS, to control for any potential vehicle effects, were utilized for quantification standards. Absolute cytokine quantities (pg/mL) were extrapolated from a standard curve run in parallel.

Organ weight analyses

Mice (n = 5/treatment at each collection time) received FSL-1 (6μg) or DPBS vehicle (60μL) vaginally as a single dose or as 5 consecutive daily doses. Treated mice were euthanized 2 or 5d after the last dose then the genital lymph nodes (GLN), liver and spleen were collected from each mouse and weighed using an Accu-413 balance (Fisher). Organ weights were compared to the total body weight to obtain percent body weight values for each organ.

Statistical analyses

For quantifying effective FSL-1 doses and delivery times, the average time to symptoms and survival times were analyzed using ANOVA with Dunnett's test to identify significant differences among the treatment and control groups. The endpoint estimation method of Reed and Muench [200] was used to calculate the ID_{50} and LD_{50} of each FSL-1 or DPBS vehicle treatment group for the ID_{50} shift paradigm experiment. Fisher's exact test was used to identify significant differences in the treatment groups compared to the DPBS vehicle group. Significant differences in FSL-1 induced cytokines or organ weights were identified by comparison to the DPBS vehicle groups using Student's t-test or one-way ANOVA, respectively. All data were analyzed using the Prism software package v4.0.

RESULTS

FSL-1 impacted genital HSV-2 infections in mice

FSL-1 doses were applied vaginally at selected time points relative to viral challenge to identify doses and delivery times that could induce significant resistance to HSV-2 infection. Initially, a single application of FSL-1 (2μg) delivered at 24 or 6h (n = 10 mice/group) prior to HSV-2 inoculation (10^4pfu) was evaluated based on efficacy results from experimentation with other TLR agonists [182, 184] and previously observed anti-herpetic activity in vitro [Chapter 2]. FSL-1, delivered at either time, significantly ($p<0.05$; Student's t-test) delayed the appearance of disease signs (disease incidence) and increased survival times compared to DPBS vehicle treated controls (Table 3).

An additional dosing regimen of FSL-1 was evaluated by employing a potential topical anti-herpetic compound delivery schedule similar to the time course for the

standardized assay scheme [Chapter 2]. FSL-1 (2μg/dose) was applied vaginally 1h prior and 1h after HSV-2 inoculation (10^4pfu) to a separate group of mice (n = 10). Interestingly, the treatment did not impact HSV-2 disease incidence nor alter survival times compared to DPBS vehicle treatment and was significantly ($p<0.05$; Student's t-test) less effective than FSL-1 (2μg) applied at 24 or 6h prior to viral challenge (Table 3). In an additional study, 2 other groups of mice received 3 repetitive FSL-1 doses (2μg/dose) at 6, 5 and 4h prior to HSV-2 inoculation or a single 6μg dose 6h prior to viral challenge. The single FSL-1 (6μg) dose produced significantly ($p<0.05$; Student's t-test) improved outcomes compared to DPBS vehicle application or FSL-1 (2μg) applied at 1h prior and 1h after viral inoculation (Table 3) indicating a time dependence for FSL-1-mediated resistance to HSV-2 similar to observed in vitro activity [Chapter 2].

Table 3. Vaginal application of FSL-1 significantly delayed HSV-2 disease development and increased survival times.

Treatment groups[a]	Time to symptoms[b] (days)	Survival time[c] (days)	Survival[d] (%)
FSL-1 2μg 24h prior	8.0 (6.4-9.7)[e,f]	9.4 (7.8-11.0)[e,f]	1/10 (10)
FSL-1 2μg 6h prior	9.0 (8.1-9.9)[e,f]	10.1 (8.9-11.3)[e,f]	0/10 (0)
FSL-1 2μg 1h prior, 1h after	6.0 (5.5-6.4)	7.3 (6.8-7.7)	0/10 (0)
FSL-1 2μg 6h, 5h, 4h prior	6.4 (5.9-7.0)	9.1 (7.0-11.2)	1/9 (11)
FSL-1 6μg 6h prior	8.5 (6.7-9.9)[e,f]	10.0 (8.4-11.6)[e,f]	4/10 (40)
DPBS vehicle control	5.8 (5.0-6.4)	7.2 (6.9-7.6)	0/8 (0)

[a] Intravaginal application of FSL-1 or DPBS vehicle control at selected times prior to or after vaginal inoculation of HSV-2 (10^4pfu).

[b] Mean (95% confidence interval) for day that each mice first showed disease signs within 14d PI

[c] Mean (95% confidence interval) for day that each mice succumbed to disease within 14d PI.

[d] Number of mice that did not succumb to disease within 14d PI/total number of animals in the group (percent survival).

[e] $p<0.05$ compared to DPBS vehicle control (ANOVA, Dunnett's Test).

[f] $p<0.05$ compared to FSL-1 2μg 1h prior, 1h after (ANOVA, Dunnett's Test).

Vaginal FSL-1 application increased resistance to HSV-2 infection by 25-fold

Having identified 2 effective in vivo treatment times (Table 3) that correlated well with in vitro times (24 and 6h prior to inoculation), the ID_{50} shift paradigm [184] was employed to quantify the resistance to HSV-2 challenge afforded by FSL-1. Single doses of 2μg or 6μg per mouse were evaluated at the 2 time points to identify the most effective FSL-1 regimen (n = 30 mice per treatment). Treated animals then were distributed randomly into subgroups (n = 10) for subsequent challenge with escalating viral doses (10^2, 10^3, or 10^4pfu) (Table 4). FSL-1 (2 or 6μg) delivered at 24 or 6h prior to viral inoculation resulted in similar outcomes as observed in Table 3 when genitally challenged with 10^4pfu of HSV-2 confirming the previously observed FSL-1 in vivo activity. Of the tested doses and times, the lower dose of FSL-1 (2μg) applied 24h prior to HSV-2 inoculation produced a significant shift in the ID_{50} (760pfu) resulting in a 25-fold increase in resistance to viral infection compared to DPBS vehicle treated control animals (ID_{50} = 31pfu). For the animals that were infected, the treatment also resulted in a 10-fold increase in survival (LD_{50} = 760pfu). FSL-1 (2μg) delivered 6h prior to HSV-2 challenge increased the ID_{50} (260pfu) and LD_{50} (660pfu) by 10-fold compared to DPBS vehicle control (Table 4). Application of FSL-1 (6μg) at 24 or 6h prior to viral inoculation produced similar increases in the ID_{50} and LD_{50} as FSL-1 (2μg) delivered 6h prior to HSV-2 challenge. The cytokine response elicited by FSL-1 application in vivo was quantified to confirm that observed anti-herpetic activity occurred through induction of specific cytokines as seen previously in vitro [Chapters 2 and 3].

Table 4. FSL-1 application significantly protected against genital HSV-2 challenge in mice.

HSV-2 dose	FSL-1 2μg 24h prior[a]		FSL-1 2μg 6h prior		DPBS vehicle control	
(pfu)	%Infected[b]	%Survival[c]	%Infected	%Survival	%Infected	%Survival
$1x10^1$	ND[d]	ND	ND	ND	30	70
$1x10^2$	30	70	10[e]	90	70	50
$1x10^3$	50[e]	50[e]	100	40	100	0
$1x10^4$	90	10	100	0	ND	ND
ID_{50}[f]	760 (80-7000)		260 (10-6000)		31 (10-80)	
LD_{50}[g]	760 (80-7000)		660 (300-1000)		68 (7-700)	

[a] Intravaginal application of FSL-1 or DPBS vehicle control at indicated times prior to vaginal HSV-2 inoculation (n = 10 mice/treatment at each HSV-2 dose).

[b] Percentage of mice with infectious virus in d2 vaginal swabs.

[c] Percentage of mice that survived HSV-2 vaginal challenge up to d14 PI.

[d] ND, not determined.

[e] $p<0.05$ compared to DPBS vehicle control (Fisher's exact test).

[f] Dose of the virus required to infect 50% of the mice based on %Infected data; ID_{50} (95% confidence interval).

[g] Dose of the virus required to cause lethal outcomes in 50% of the mice based on %Survival data; LD_{50} (95% confidence interval).

Table 5. Vaginal application of FSL-1 in mice induced a specific cytokine profile.

	6h post FSL-1 application (pg/lavage ± SEM)	
Cytokine[a]	**FSL-1**	**DPBS vehicle**
IL-1α	1083.2±143.5[b]	527.0±65.7
IL-1β	1157.0±320.2[c]	169.2±46.3
IL-2	34.8±5.2[b]	BDL(3.9)[d]
IL-6	418.5±94.1[b]	35.4±8.5
IL-12(p40)	104.2±10.5[b]	44.9±3.8
IL-12(p70)	64.0±19.1[c]	17.1±3.1
G-CSF	5157.0±1095.0[c]	2208.0±433.1
GM-CSF	198.6±44.6[b]	22.6±8.7
IFNα	30.3±4.4[b]	0.7±0.6
IFNβ	445.3±93.2[b]	13.8±3.5
IFNγ	76.8±18.8[c]	BDL(43.1)
MIP-1α	2770.7±484.0[b]	440.0±127.0
MIP-1β	4140.0±570.5[c]	2232.0±218.5
TNF-α	1109.6±212.7[b]	232.6±71.6

[a] The indicated cytokines were found to be induced by FSL-1 6h post-application by BioPlex or ELISA as indicated in the methods. Data are presented as mean ± SEM of three replicates from two independent experiments.

[b] $p<0.01$ compared to DPBS vehicle (Student's t-test).

[c] $p<0.05$ compared to DPBS vehicle (Student's t-test).

[d] BDL; Below Detection Limit (for statistical comparisons with these cytokines the value for the lowest standard of each cytokine was utilized).

FSL-1 elicited a cytokine response profile associated with resistance to genital HSV-2 infection

Previous in vitro and in vivo work showed that secretion of specific cytokines correlated with resistance to HSV-2 infection [75, 77, 80, 82, 85, 88, 183-185; Chapters 2 and 3]. To compare established cytokine profiles to the one produced by FSL-1, vaginal lavage samples from mice treated with FSL-1 or equal volumes of the DPBS vehicle were collected at 0, 6, 24 and 48h post-application. Individual lavages then were analyzed to quantify specific cytokines that were significantly increased by FSL-1 treatment compared to DPBS vehicle. The FSL-1 profile, evident by 6h, included significant ($p<0.05$; Student's t-test) induction of IL-1α, IL-1β, IL-2, IL-6, IL-12(p40), IL-12(p70), G-CSF, GM-CSF, IFNα, IFNβ, IFNγ, MIP-1α, MIP-1β and TNF-α (Table 5). Cytokine induction profiles produced by a single 2μg or 6μg dose were indistinguishable (data not shown). Lavage samples collected at later time points showed that the induced cytokines had returned to levels similar to DPBS vehicle controls within 24h indicating a lack of chronic stimulation (Figure 17). Overall, the observed cytokine profile was consistent with the profiles elaborated by other TLR agonists that significantly reduced genital HSV-2 infection in mice [80, 82, 183, 184].

Multiple doses of FSL-1 applied vaginally did not induce chronic inflammation or organ enlargement

Effective anti-herpetic compounds should only transiently induce cytokines even after multiple dosing cycles to prevent disruption of the vaginal mucosa thereby increasing susceptibility to STI [184, 231]. To determine if FSL-1 caused chronic cytokine inducation, vaginal lavages were collected at 6 and 24h following daily 6μg FSL-1 applications delivered over five consecutive days. The 6μg dose was selected because it was the highest tested dose that elicited resistance to genital HSV-2 infection.

Cytokine elaboration was similar to the single dose results (Figure 17) following the first dose and showed significant ($p<0.05$; Student's t-test) induction of the indicated cytokines 6h after FSL-1 application (Table 5) with a return to DPBS vehicle levels by 24h. The cycle of induction and return to DPBS vehicle levels was observed after each daily dose of FSL-1 (Figure 18). Importantly, no additive effect of repeated FSL-1 dosing was observed; cytokine levels 24h after the last dose were indistinguishable from DPBS vehicle treated mice (120h) (Figure 18). As an additional preliminary evaluation of potential chronic inflammation, the weights of GLN, liver and spleen ($n = 5$ mice/group) were determined at indicated time points after delivery of a single or five consecutive daily 6μg doses of FSL-1. GLN, liver and spleen weights collected 2d after 5 consecutive daily FSL-1 doses (6μg) were statistically ($p>0.05$; Student's t-test) indistinguishable from DPBS vehicle treated mice (Figure 19). Similar statistically ($p>0.05$; Student's t-test) indistinguishable results were observed for organs collected 2 or 5d after a single FSL-1 dose (data not shown). Together, the data indicate that repetitive 6μg doses of FSL-1 did not elicit chronic cytokine elaboration or cause organ enlargement, as a marker of potential systemic inflammation, unlike the outcomes observed following GpC application in mice [80].

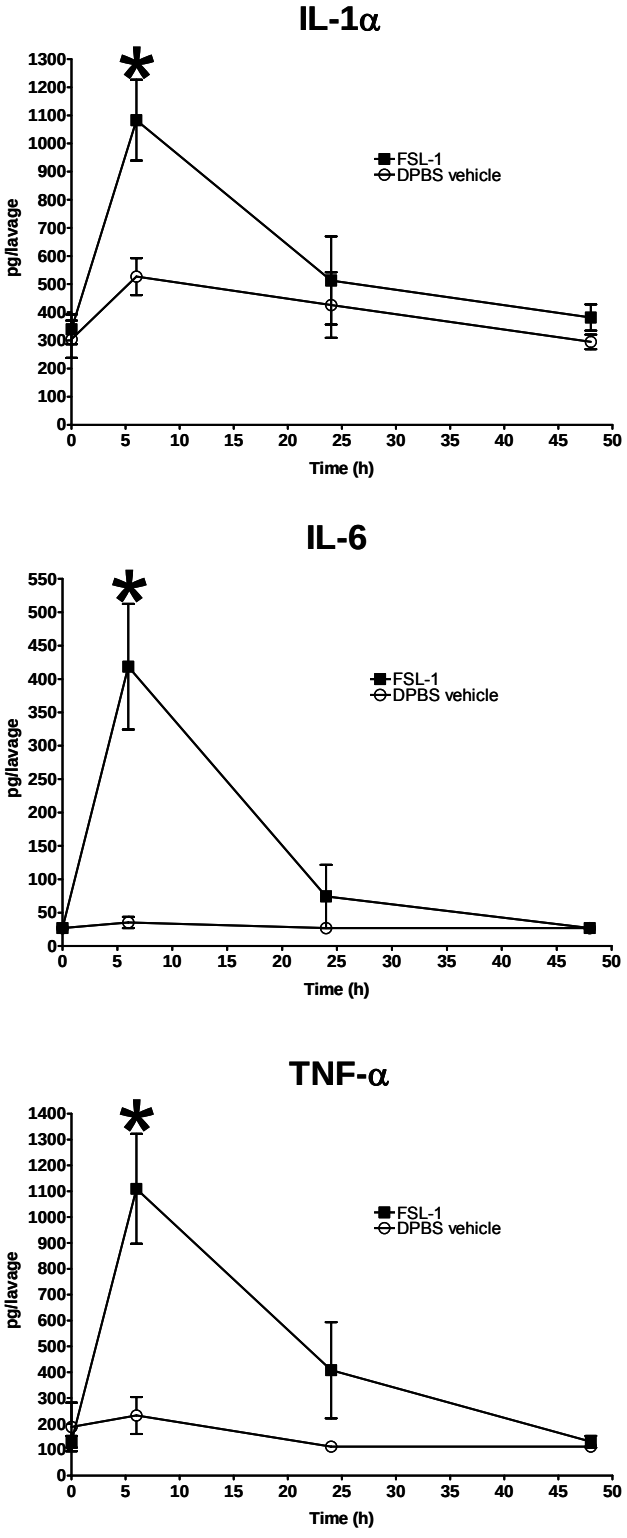


Figure 17. FSL-1 application transiently induced production of inlammation-related markers. Selected cytokines were quantified kinetically from murine vaginal lavages (n = 5/treatment) collected 0, 6, 24 and 48h after FSL-1 (6μg; closed square) or DPBS vehicle (60μL; open circle) application. The inflammation-related markers IL-1α, IL-6 and TNF-α were significantly increased (*, $p<0.05$ Student's t-test) 6h after FSL-1 application compared to DPBS vehicle. By 24h, induced cytokines had returned to DPBS vehicle levels indicating a lack of chronic induction. Each collection time point is presented as mean ± SEM of five replicates from two independent experiments.

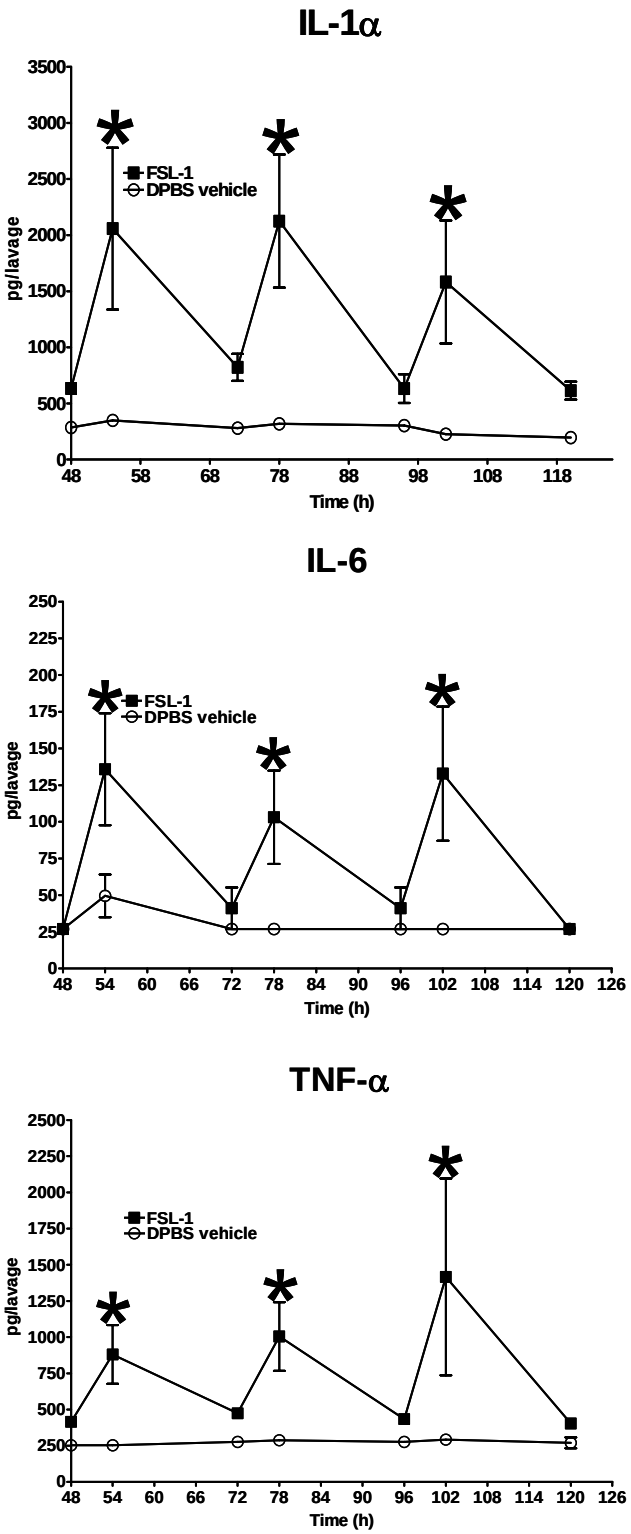


Figure 18. FSL-1 delivered once daily for 5 consecutive days did not induce chronic cytokine production. As a marker of an inflammatory state, IL-1α, IL-6 and TNF-α were kinetically quantified in murine lavages of repetitive daily dosed animals (n = 5/treatment). Each cytokine was significantly induced (*, $p<0.05$; Student's t-test) 6h after FSL-1 (6μg; closed square) application compared to DPBS vehicle (60μL; open circle). Notably, by 24h after each FSL-1 dose, induced cytokines had returned to DPBS vehicle levels and were not significantly ($p>0.05$; Student's t-test) increased 24h after the final FSL-1 application. Each collection time point is presented as mean ± SEM of five replicates from two independent experiments.

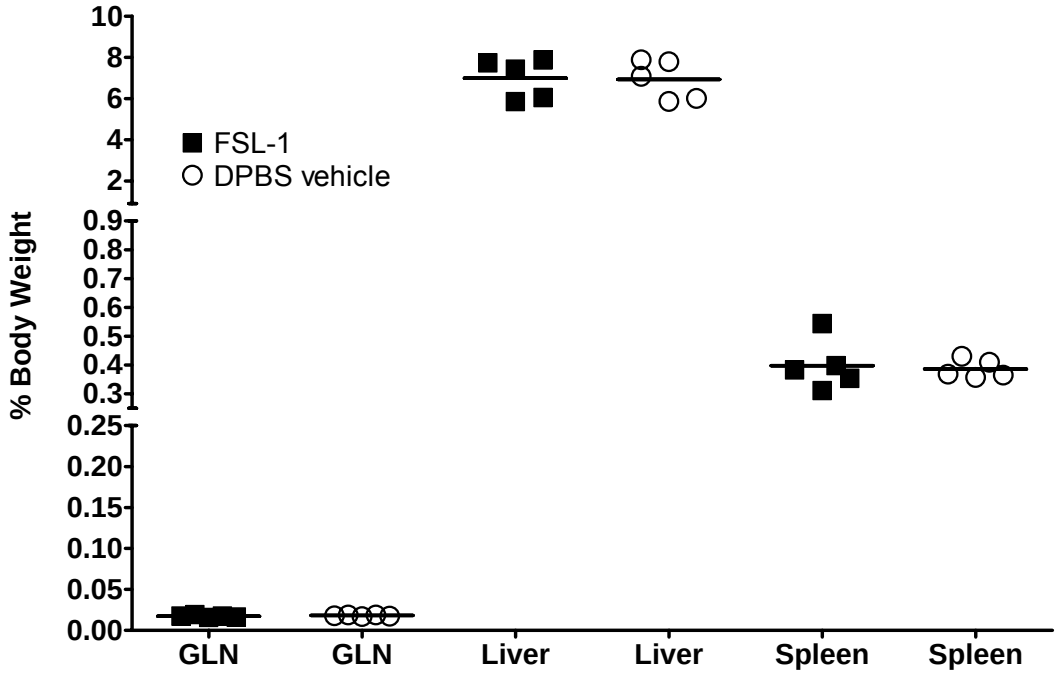


Figure 19. Single or multiple consecutive doses of FSL-1 did not induce organ enlargement. As an additional measure of peripheral immunotoxicity, immune cell migration was evaluated by gross observation of associated lymphoid tissues following FSL-1 application. GLN, liver and spleen weights for organs collected 2 or 5d after delivery of FSL-1 (6μg; closed square) or DPBS vehicle (60μL; open circle) in a single or 5 multiple once daily doses. By organ, the mean % of total body weight for each group is demarcated by the horizontal line with each point representing one mouse (n = 5/group). Weights of organs collected 2d after the last of 5 consecutive daily FSL-1 doses were not significantly ($p>0.05$; Student's t-test) different than organs collected from mice treated with an equivalent volume of the DPBS vehicle. Groups were compared for statistical significance by one-way ANOVA.

DISCUSSION

Murine studies illustrated, for the first time, that a synthetic bacterially-derived TLR2/6 agonist, FSL-1, significantly increased resistance to genital HSV-2 infection. The observed increase in resistance has potential implications for HSV-2 transmission prevention by increasing the threshold of infection above the amount of virus shed during a sexual encounter [232, 233]. FSL-1 (2μg) application at 24h prior to viral inoculation engendered the greatest resistance to experimental HSV-2 challenge of the tested doses (25 fold increase over the DPBS vehicle ID_{50}) (Table 4). Based on recent epidemiological studies, even an anti-herpetic strategy that did not completely protect against but induced significant resistance to infection could substantially reduce HSV-2 transmission [234]. The murine data also confirmed the previously observed results in human vaginal EC showing that FSL-1 elicited significant anti-herpetic activity when applied 24 or 6h but not 1h prior to HSV-2 inoculation [Chapter 2].

The FSL-1 induced increase in resistance to genital HSV-2 infection in mice is consistent with activity observed for other anti-herpetic TLR agonists. Application of PIC, IMI or CpG prior to viral challenge elicited significant resistance to genital HSV-2 infection while PGN, LPS or FLAG showed no activity [80, 82, 181-185]. The HSV-2 genome is composed of dsDNA that is recognized by TLR9 [56, 57, 62]. Also, viral replication potentially produces dsRNA or ssRNA intermediates that are recognized by TLR3 or TLR7/8, respectively [64-69]. There are no published reports showing HSV-2 recognition through TLR5 and recent data indicated that PGN recognition occurs through NOD1/2 or TLR2 [51, 235, 236]. Interpretation of the results would suggest that induction of anti-herpetic activity is specific to viral-associated agonists, but recent work by Ashkar et al. showed that FimH, a TLR4 agonist derived for *E. coli*, provided significant resistant to genital HSV-2 infection while LPS from *E. coli* was ineffective

[226]. Additionally, we showed that FSL-1, a bacterial-derived diacyl lipopeptide [202, 212, 213], significantly increased resistance to HSV-2 infection in the mouse model. The varying anti-herpetic activity observed following activation of different TLR pathways indicates that effective agonists likely induce a specific innate immune response profile that produces an HSV-2 resistant environment.

FSL-1 elicited a cytokine response profile consistent with other anti-herpetic TLR agonists. Vaginal application of CpG in mice resulted in induction of IL-12, IL-18, IFNγ and RANTES [80, 82, 183]. The cytokine profile elicited by PIC or IMI vaginal treatment was similar to CpG and FSL-1 and included secretion of IL-1α, IL-1β, IL-6, IL-12, IFNβ, IFNγ, MIP-1α and RANTES [82, 183, 184]. Interestingly, vaginal delivery of PGN or FLAG in mice did not result in production of IFNα or IFNβ [183]. Combined with the observed cytokine profile elicited by FSL-1 6h post-application (Table 5) and other in vitro or in vivo experiments showing TLR agonists mediated activity against HSV-2 in wild type or knockout animals [75, 77, 80, 82, 84, 85, 88, 183-185, 214, 220; Chapters 2 and 3], a preliminary anti-herpetic cytokine profile would include IL-2, IL-12(p40), IL-12(p70), IFNβ, IFNγ, MIP-1α, MIP-1β and RANTES.

The identified cytokines are important for establishing an HSV-2 resistant environment through viral entry and replication inhibition and leukocyte recruitment and activation. Gill et al. and others showed that IFNβ production correlated with protection of mice against experimental genital HSV-2 infection [82, 183]. Type I interferons (IFNα, IFNβ) act by directly inhibiting HSV-2 replication and skew the immune system towards a T_H1-baised response [84, 85, 219]. A T_H1-associated immune response is important for controlling early replication of HSV-2 and for effective clearance of the infection [84, 85, 217, 218]. Production of IL-2 and IL-12 also aids the innate immune response against HSV-2 through stimulation and activation of leukocytes and induction

of type II interferon (IFNγ) [75, 77, 79, 80, 215, 216]. Resistance to HSV-2 is enhanced by the direct anti-herpetic activity of MIP-1α, MIP-1β and RANTES [88]. The chemokines also are important for the recruitment of leukocytes that aid in the regulation of the anti-herpetic immune response [88, 220, 221].

Importantly, the TLR agonist induced innate immune response against HSV-2 infection must provide adequate resistance without producing chronic inflammation that can potentially increase acquisition of STI due to disruptions in the vaginal mucosa. Kinetic vaginal cytokine induction and organ weights following FSL-1 application were evaluated and showed no chronic or systemic outcomes. Chronic inflammation could result in disruption of the vaginal mucosal barrier leading to increased susceptibility to STI [149, 231] so careful analyses of prolonged inflammation is necessary. Our initial studies indicated that even repeated dosing with FSL-1 only elicited a transient cytokine response but did not cause chronic inflammation of the vaginal tract (Figure 18). Also, the treatment regimen resulted in no enlargement of the GLN, spleen or liver (Figure 19). PIC application in mice produced similar results but specific inflammation-related markers remained elevated until 48h post-application [184]. Further, Ashkar et al. showed that delivery of CpG resulted in significant enlargement of the spleen in mice due to a large influx of immune cells [182]. The observed systemic immunotoxicity following CpG application highlights the need for a TLR agonist that not only provides resistance to genital HSV-2 infection but does not negatively impact the vaginal mucosa or associated lymphoid tissues [149, 231, 237]. Comparison of the preliminary FSL-1 safety profile to other TLR agonists confirmed that the mouse model is useful for assessing the preliminary safety profile of immunomodulatory compounds prior to evaluation in expensive and lengthy clinical trials.

In summary, we showed that the TLR2/6 agonist FSL-1 is capable of significantly increasing resistance to HSV-2 infection in mice through induction of a specific cytokine response profile. The observed FSL-1 induced cytokine response profile and resulting anti-herpetic activity is consistent with previous in vitro outcomes [Chapters 2 and 3], and is of critical importance for the future design of TLR agonist interventions. Additionally, the innate responses to FSL-1 from the genital tract provided rationale for investigation into the impact of secondary reproductive tract infections by genital mycoplasmas or other potential coinfecting pathogens on HSV-2 transmission. Indeed, HSV-2 is associated with increased susceptibility to other important bacterial and viral STI including HIV-1 [3, 11, 13, 19]. Continued investigation into the innate response to specific immunomodulatory compounds like FSL-1 will provide a greater understanding of how pathogens are recognized in the polymicrobial environment of the vagina, as in the context of commensal microorganisms [Chapter 6], and how these responses can be exploited to develop novel strategies for STI prevention or HSV-2 treatment [Chapter 5].

CHAPTER 5: THE IMMUNOMODULATORY DIPEPTIDE, SCV-07, ELICITED THERAPEUTIC ACTIVITY AGAINST RECURRENT GENITAL HSV-2

OVERVIEW

HSV-2 infection produces a recurrent disease state characterized by genital lesion formation that is associated with increased susceptibility to other STI. There are no current treatments that can cure this life-long herpetic infection; therefore novel compounds that reduce the formation of recurrent genital lesions are needed to control the spread of STI. To address the need for novel compounds, the therapeutic potential of SCV-07 (gamma-d-glutamyl-l-tryptophan), a synthetic dipeptide with potent immunomodulatory and antimicrobial activity, was evaluated in the guinea pig model of recurrent genital HSV-2 infection. Initial experiments showed that when delivered orally, but not subcutaneously, SCV-07 significantly reduced recurrent lesion incidence and severity. Oral dose ranging studies indicated that, of the tested amounts, 5μg/kg was the most effective when delivered after an overnight fast. Interestingly, fasting induced a significant increase in recurrent lesions in vehicle treated guinea pigs relative to non-fasted animals potentially due to stress. Despite the fasting-associated increase, SCV-07 significantly reduced lesion formation in treated animals but showed no durable reduction in lesion formation following cessation of treatment. In fact, the SCV-07 treatment regimen produced statistically indistinguishable outcomes compared to topical acyclovir application. The observed therapeutic activity illustrated that SCV-07 may provide an easily administered alternative or supplemental treatment option for recurrent genital HSV-2 disease. Additionally, the efficacy of SCV-07 application in guinea pigs supported evaluation of other immunomodulatory compounds for potential therapeutic

anti-herpetic activity. Part of the research described in this chapter was published in the *Journal of Antimicrobial Agents and Chemotherapy*.

INTRODUCTION

Currently, 16-18% of adult Americans are infected with HSV-2 [3, 9] with a higher prevalence observed in other areas of the world [3, 10]. Several studies concluded that HSV-2 infected individuals are at a higher risk of acquiring other STI [3, 11, 13, 19]. The increased risk of STI acquisition is potentially due to lesions formed during symptomatic primary or recurrent genital HSV-2 disease [20, 21]. The lesions disrupt the integrity of the vaginal mucosa creating portals of entry for pathogens [11, 20, 21]. Additionally, physiological or psychological stress can increase the frequency of recurrent episodes [8]; therefore reducing genital lesion formation is critical to reducing susceptibility to other STI.

Potential HSV-2 vaccines have thus far only resulted in limited success within specific seronegative individuals [27], and current therapeutic treatment involves chronic administration of oral antiviral compounds [3, 4, 6, 31]. While administration of daily suppressive therapy significantly reduced the frequency of genital lesion formation it did not completely prevent all recurrences [3, 4, 6, 37]. Because of the lesion-associated increase in STI acquisition [11, 20, 21], new therapeutic agents are needed that alone or in combination with existing treatments can reduce recurrent genital HSV-2 lesions.

A promising therapy for treatment of HSV-2 infected individuals is the use of immunomodulatory compounds. TLR agonists previously elicited potent anti-herpetic activity following application in vitro and in vivo through the induction of specific innate immune response profiles [75, 77, 80, 82, 84, 85, 88, 183-185, 214, 220; Chapters 2-4]. Dipeptides also exhibited promising antimicrobial activity following activation of cellular

immune responses [186-189]. Application of muramyl dipeptides in vitro and in vivo resulted in significant antiviral activity against HIV-1 and murine cytomegalovirus, respectively [187, 189].

In other work, a bacterial-derived dipeptide, SCV-07 (gamma-d-glutamyl-l-tryptophan), significantly increased the efficacy of antituberculosis therapy, stimulated thymic and splenic cell proliferation and improved macrophage function [190]. SCV-07 application in mice also resulted in the elaboration of cytokines, specifically IL-2 and IFNγ [190]. Several studies showed that cell-mediated production of IL-2 and IFNγ is important for reducing the formation of recurrent genital lesions [215, 216]. Based on the previously observed immunomodulatory responses [190], SCV-07 was evaluated for activity against established experimental genital HSV-2 infection.

Guinea pigs infected genitally with HSV-2 exhibit primary and recurrent disease states that closely mimic human HSV-2 infections [238-240]; therefore the animals provide an invaluable model for evaluating therapeutic intervention strategies. The guinea pig animal model was used to evaluate the therapeutic potential of SCV-07 administered orally or subcutaneously. Appearance of recurrent genital lesions in HSV-2 infected guinea pigs was scored during treatment to identify SCV-07 induced reductions in lesion severity and frequency. Orally delivered SCV-07 significantly reduced the incidence and severity of recurrent disease in the animal model even during stress-induced reactivation events caused by overnight fasting. The collective findings supported the continued development and evaluation of immunomodulatory compounds as alternative anti-herpetic therapies for control of HSV-2 infections.

MATERIALS AND METHODS

Guinea pig model of recurrent HSV-2 disease

Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) weighing 250–300g were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved quarters and provided with unlimited access to food and water. All procedures were performed humanely and approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Animals were acclimated to the facility for at least 7d prior to the start of any experimentation.

HSV-2 strain MS (ATCC VR-540) was propagated and titered as described previously for HSV-2 stain 186 [Chapter 4]. Guinea pigs were swabbed with a wet then dry calcium alginate swab just prior to instilling 100μL of HSV-2 strain MS (10^5pfu/animal) vaginally using a positive displacement pipet. The same viral inoculum was used for all experiments to provide infection of nearly every animal without causing lethal outcomes [181]. To confirm infection, vaginal swab samples were collected 3d PI and assayed for infectious virus using CPE scoring as described previously [Chapter 2].

As described previously [238, 239], guinea pigs exhibit primary and recurrent genital lesions following HSV-2 infection. The severity of primary genital skin disease was assessed daily from d0–14 PI and scored using a 1–4 lesion scale [238]. For the scoring system, modified from Stanberry et al. [238], 0 was normal skin, 1 was 1-5 discrete lesions, 2 was >6 discrete lesions, 3 was confluent lesions and 4 was necrotic lesions. Animals without primary lesions (score <1) or virus in d3 PI vaginal swabs were counted as uninfected and removed from further study. Following primary disease resolution, animals were scored for the appearance of recurrent genital lesions (Figure 20) using a system of 0 for no disease, 0.5 for a discrete erythematous area and 1 for a

discrete lesion [238]. The scores were used to calculate incidence (lesion appearance) and average severity (cumulative mean lesion score; CML) for each group [238, 240].

Before treatment initiation in all experiments, animals were distributed such that each group contained statistically similar average recurrent disease incidence and severity. The average guinea pig weight was 600g by treatment time, and the average weight was used for preparing SCV-07 (SciClone Pharmaceuticals, Foster City, CA) doses (100μL/animal).

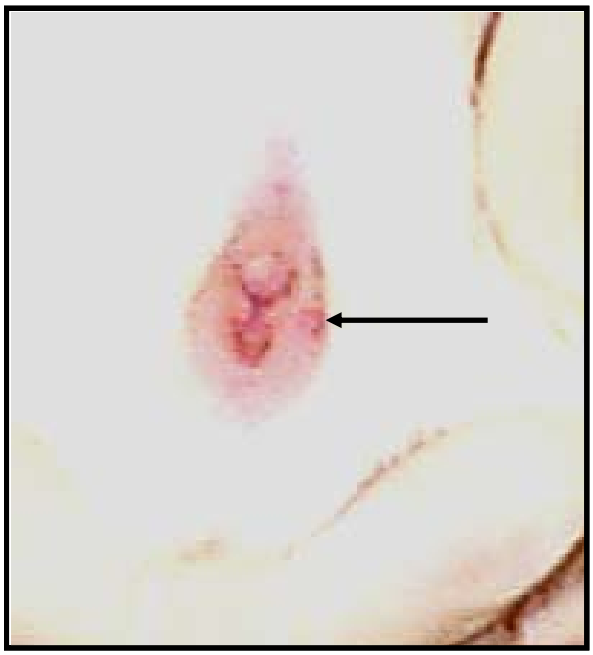


Figure 20. Appearance of a discrete recurrent genital lesion in a latently infected guinea pig. The black arrow indicates the presence of a vesicular lesion on the vagina of an HSV-2 infected animal.

Evaluation of SCV-07 therapeutic potential

Following confirmation of infection and basal recurrent disease readings, 33 animals were assigned to 3 groups. The first group (n = 11) received 100μg/kg SCV-07 orally in a sterile 2% sucrose solution (Fisher) selected to increase palatability. The second group (n = 11) received 100μg/kg SCV-07 in DPBS injected subcutaneously.

The control group (n = 11) was untreated. Recurrences were scored from d22–42 PI and each treatment was administered for 5 days (d29–33 PI).

Impact of fasting on SCV-07 efficacy

Fifty-four newly infected animals that showed primary infection and recurrent lesions were distributed into one of 3 groups as described above in the evaluation of SCV-07 therapeutic potential section. Two groups (n = 18/group) received 100μg/kg SCV-07 orally in 2% sucrose. To establish if bioabsorption could be enhanced by an empty stomach, one of the 2 SCV-07-treated groups was fasted by removing food but not water for ≤12h before each treatment. Animals in the final group (n = 18) were fasted overnight but received oral 2% sucrose vehicle only. Groups were treated for 5 consecutive days.

Evaluation of lower doses of SCV-07 and in combination with topical acyclovir

To establish if SCV-07 doses lower than 100μg/kg provided therapeutic activity, 86 animals experiencing recurrent genital lesions were allocated into one of 6 groups. Each of 3 groups (n = 14/group) received one of 3 selected oral daily doses of SCV-07 using a 2-fold increasing dose scheme consisting of 5, 10 or 20μg/kg in 2% sucrose. The dosing scheme was designed to test similar doses to those utilized previously in mice [190]. As a control, another group (n = 14) was treated daily with oral 2% sucrose vehicle only.

To investigate potential synergistic or antagonistic therapeutic activity with a common anti-herpetic treatment, SCV-07 was administered orally at 10μg/kg SCV-07 (n = 15) in combination with twice daily vaginal application of 5% acyclovir cream (Glaxo-

SmithKline). As a comparator group (n = 15), animals received twice-daily vaginal acyclovir only, based on previously observed recurrence reduction [181]. All groups were fasted each night (≤12h) before 5 consecutive days of treatment.

Statistical analyses

Differences in the mean lesion scores among the groups during the primary disease state were compared using one-way ANOVA. Student's t-test was used to identify differences in recurrent genital lesion incidence or severity for each treatment group compared to the control group. All data were analyzed using the Prism software version 4.0.

RESULTS

Oral but not subcutaneous SCV-07 reduced experimental recurrent genital HSV-2 disease

In previous experiments, mice that received oral or subcutaneous SCV-07 showed significant induction of IL-2 and IFNγ [190] and both cytokines were associated with reduction in recurrent genital lesion formation [215, 216]. Thus, an initial study was conducted to evaluate if SCV-07 administered by either route provided therapeutic activity against experimental recurrent genital HSV-2 disease in guinea pigs. Following viral inoculation, scoring of the primary disease state (d0-14 PI) showed that all animals exhibited statistically ($p>0.05$; one-way ANOVA) similar mean lesion scores (Figure 21). Additionally, each animal was scored to establish a recurrence profile before treatment in order to distribute the animals into groups with similar average disease. As shown in Table 6, there were no significant ($p>0.05$; Student's t-test) differences between the groups during the week before initiation of therapy (d22–28 PI).

For SCV-07 treatments, an initial dose of 100μg/kg was selected based on an extrapolation from previously performed dosing studies [241]. During the week starting with the 5 days of treatment (d29–33 PI), animals that received 100μg/kg SCV-07 orally exhibited a reduction in lesion incidence from 55% (pre-therapeutic week) to only 18% (2/11 animals). In animals with lesions, severity was reduced from 1.27 CML to 0.45 CML. Compared with untreated animals, orally delivered SCV-07 reduced the incidence (5/11 vs. 2/11 in the treated group) and average lesion severity (0.80 CML vs. 0.45 CML). Additional studies with orally delivered SCV-07 were warranted by the observed data while SCV-07 delivered subcutaneously did not produce significant ($p>0.05$; Student's t-test) disease reduction (Table 6). The lack of observed activity was potentially due to differences in activity in the guinea pig or bioavailability between the two administration routes [241].

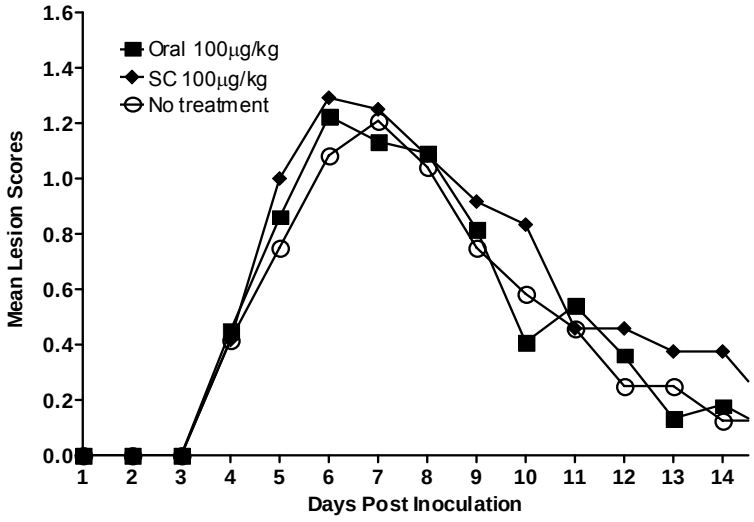


Figure 21. guinea pigs experienced a similar primary disease state following genital HSV-2 inoculation. No significant ($p>0.05$; one-way ANOVA) differences in the mean lesion scores among each group were observed during the primary disease state (d0-14).

Table 6. Orally delivered SCV-07 reduced the recurrence of genital HSV-2 lesions.

	Pre-therapeutic week		Therapeutic week	
Group	**Incidence[a]**	**CML±SEM[b]**	**Incidence**	**CML±SEM**
Oral SCV-07 100μg/kg	6/11 (55%)	1.27 ± 0.45	2/11 (18%)	0.45 ± 0.31
Subcutaneous SCV-07 100μg/kg	7/11 (64%)	1.00 ± 0.30	5/11 (45%)	1.00 ± 0.47
Untreated	5/11 (45%)	0.90 ± 0.35	5/11 (45%)	0.80 ± 0.33

[a] Number of guinea pigs with recurrent lesions/total number of guinea pigs (% with lesions).

[b] Measurement of the mean recurrent disease severity by group ± SEM.

Overnight fasting improved the therapeutic activity of orally delivered SCV-07

Recently, orally delivered SCV-07 exhibited increased bioavailability when delivered following overnight fasting [241]. Based on the fasting data and the observed reduced recurrent disease following oral treatment, it was hypothesized that an empty gastrointestinal tract may enhance SCV-07 efficacy in guinea pigs. Groups were again established with similar primary and recurrent disease prior to therapy initiation. During oral 100μg/kg SCV-07 treatment, the fasted and non-fasted groups showed significant ($p<0.01$ and $p<0.05$; Student's t-test, respectively) reduction in lesion incidence and average severity compared with the fasted vehicle treated group (Table 7). As hypothesized, fasted animals that received oral SCV-07 showed greater reductions in incidence and severity than non-fasted SCV-07 treated animals. In fact, fasting-associated stress during the treatment period increased lesion incidence (14/18) and average severity (1.22 CML) compared with the pre-therapeutic week (5/18 and 0.6

CML) for the vehicle treated group. Despite fasting-associated increases, significant ($p<0.01$; Student's t-test) reductions in lesion incidence and severity were generated by orally-delivered SCV-07 in fasted animals (only 2/18 and 0.22 CML), supporting testing of additional doses.

Table 7. Overnight fasting prior to treatment increased the efficacy of orally-delivered SCV-07.

	Therapeutic week	
Group	**Incidence[a]**	**CML±SEM[b]**
Oral SCV-07 100μg/kg	7/18 (39%)[c]	0.50 ± 0.17[c]
Oral SCV-07 100μg/kg, fasted	2/18 (11%)[d,e]	0.22 ± 0.15[d,f]
Vehicle treated, fasted	14/18 (78%)	1.22 ± 0.25

[a] Number of guinea pigs with recurrent lesions/total number of guinea pigs (% with lesions).

[b] Measurement of the mean recurrent disease severity by group ± SEM.

[c] $p<0.05$ compared to fasted vehicle treated group (Student's t-test).

[d] $p<0.01$ compared to fasted vehicle treated group (Student's t-test).

[e] $p=0.06$ compared to oral SCV-07 100μg/kg group (Student's t-test).

[f] $p=0.22$ compared to oral SCV-07 100μg/kg group (Student's t-test).

Lower doses of orally delivered SCV-07 exhibited enhanced therapeutic activity

Administration of SCV-07 in a murine tuberculosis model [190] indicated that doses lower than the initially tested 100μg/kg dose also may be effective. A series of 2-fold lower concentrations of SCV-07 were tested in overnight fasted guinea pigs to

evaluate if therapeutic activity was increased relative to the 100μg/kg dose. Groups with similar primary and recurrent disease were again generated using pre-therapy scoring. Therapeutic intervention with orally delivered 5μg/kg SCV-07 resulted in a significant ($p<0.05$; Student's t-test) reduction in the incidence (2/14) and average severity (0.14 CML) of recurrent lesions compared with fasted animals that received vehicle only (10/14 and 2.07 CML). Significant ($p<0.05$; Student's t-test) reduction in recurrent lesions also was observed for the 10μg/kg dose but 20μg/kg SCV-07 produced a non-significant ($p>0.05$; Student's t-test) reduction following treatment (Table 8). Disease reduction observed following 5μg/kg SCV-07 treatment was significantly ($p<0.05$; Student's t-test) more effective than the 20μg/kg dose (8/14 and 0.93 CML). As observed previously, the fasted vehicle treated group experienced stress-induced increases in recurrent disease average severity (2.07 CML) compared with the week prior to (1.21 CML) and after treatment (0.50 CML). Recurrence scoring of the post-treatment period illustrated that any therapeutic benefit of SCV-07 had limited durability (Table 8).

SCV-07 did not impact acyclovir therapy

Although current anti-herpetic therapies are capable of significantly reducing recurrent genital disease [3, 4, 6, 31] they do not prevent all recurrences. To evaluate the possibility of synergism or antagonism with such treatments, a low dose of SCV-07 (10μg/kg) delivered once daily was tested in combination with twice-daily vaginally applied acyclovir. All animals were fasted during treatment that again elevated recurrent disease. The results indicated that animals receiving topical acyclovir and oral SCV-07 (10μg/kg) showed no significant ($p>0.05$; Student's t-test) difference in recurrent disease compared with acyclovir alone (Table 8). Both treatment groups experienced

significantly ($p<0.05$; Student's t-test) reduced recurrent disease relative to the fasted vehicle treated group (Table 8).

Table 8. Lower doses of SCV-07 elicited therapeutic activity and were not antagonistic to acyclovir therapy.

	Therapeutic week		Post-therapeutic week	
Group	**Incidence[a]**	**CML±SEM[b]**	**Incidence**	**CML±SEM**
Oral SCV-07 5μg/kg, fasted	2/14 (14%)[c,d]	0.14 ± 0.10[c,d]	5/14 (36%)	0.57 ± 0.23
Oral SCV-07 10μg/kg, fasted	4/14 (29%)[d]	0.71 ± 0.44	4/14 (29%)	0.50 ± 0.25
Oral SCV-07 20μg/kg, fasted	8/14 (57%)	0.93 ± 0.29	4/14 (29%)	0.50 ± 0.25
Vehicle treated, fasted	10/14 (71%)	2.07 ± 0.60	4/14 (29%)	0.50 ± 0.25
Acyclovir and oral SCV-07 10μg/kg, fasted	5/15 (33%)[d,e]	0.67 ± 0.30[d,e]	6/15 (40%)	0.80 ± 0.28
Acyclovir	3/15 (20%)[d]	0.33 ± 0.21[d]	4/15 (27%)	0.40 ± 0.19

[a] Number of guinea pigs with recurrent lesions/total number of guinea pigs (% with lesions).

[b] Measurement of the mean recurrent disease severity by group ± SEM.

[c] $p<0.05$ compared to fasted oral SCV-07 20μg/kg group (Student's t-test).

[d] $p<0.05$ compared to fasted vehicle treated group (Student's t-test).

[e] $p>0.05$ compared to acyclovir group (Student's t-test).

DISCUSSION

Using the guinea pig model, we have established the potential of an orally delivered immunomodulatory dipeptide, SCV-07, as a novel strategy for reducing the

incidence and severity of recurrent genital HSV-2 lesions. Reducing genital lesion formation in HSV-2 infected individuals is important because erosions in the vaginal mucosa allow pathogens to bypass the natural vaginal EC barrier [22, 23] resulting in increased STI susceptibility [11, 20, 21]. Oral delivery of 5μg/kg SCV-07 after an overnight fast resulted in the most effective reduction in recurrent genital lesions (Table 8). A potential explanation for the observed reduction was that SCV-07 directly inhibited viral replication. Application of 100μg/kg of SCV-07 to Vero cells shortly before or after HSV-2 inoculation showed no direct impact on viral replication [191]. Based on the in vitro results [191] and immunomodulatory evaluations in mice [190], it is likely that SCV-07 administration elicited an innate immune response that accounts for the observed reduction in genital lesions.

Previous studies with other immunomodulatory dipeptides showed that the compounds were capable of producing antimicrobial activity against a wide range of pathogens through elicitation of immune responses [186-189]. The mechanisms of action for the dipeptides included induction of cytokines, T-cells, natural killer cells, macrophages and other leukocytes [186-189]. As an initial step in identifying the mechanism of immunomodulation for SCV-07, Simbirtsev et al. showed that in mice significant levels of IL-2 and IFNγ were induced with a concurrent decrease in IL-4, indicating stimulation of a T-helper cell shift to a T_H1-biased immune response [190]. IFNγ directly inhibits HSV-2 replication and IL-2 is important for activation of NK and cytotoxic T cells that clear HSV-2 infected cells [215, 216]. The immunomodulatory activity of the 2 cytokines provides a potential mechanism of action for the observed SCV-07 reduction in genital lesions but the lack of suitable immunological reagents limited similar evaluations in the guinea pigs.

The immunostimulatory capacity of dipeptides can vary greatly depending on the route of administration as observed for oral, subcutaneous, intranasal or intrarectal delivery of muramyl dipeptides derivatives [242, 243]. Application of SCV-07 in mice, rats and humans indicated that SCV-07 activity was dependent upon the route and dosage [241]. Altered bioavailability could explain the lack of therapeutic activity observed for subcutaneous delivered SCV-07. Additionally, dose dependent activity of SCV-07 is consistent with results from a murine tuberculosis model that showed lower doses of SCV-07 elicited increased immunotherapeutic activity compared to higher doses [190]. Of the tested doses, fasted guinea pigs orally delivered 5μg/kg SCV-07 exhibited significantly less recurrent lesions with a reduced severity compared to vehicle treated animals or even 20μg/kg SCV-07 delivered orally (Table 8). While lower doses of the compound could potentially further improve therapeutic activity, during treatment the 5μg/kg dose resulted in only 2/14 animals with one lesion each of the lowest score. Alternatively, SCV-07 efficacy could be improved through combination with antiviral compounds.

SCV-07 induced activity was not antagonistic with topical anti-herpetic therapies. Orally delivered SCV-07 in combination with topically-applied acyclovir cream produced a reduction in recurrent genital lesion incidence and average severity that was statistically indistinguishable from acyclovir treatment only (Table 8). Although treatment outcome, observed in separate experiments, is limited and does not show direct drug interactions, it is an important first step because any alternative strategy should ideally be synergistic with existing therapies but should not antagonize standard antiviral approaches. Importantly, all SCV-07 doses were well tolerated and the animals did not show any adverse reactions or behavior changes. However, this does not preclude other

types of toxicity that should be evaluated carefully like the potential for systemic immunotoxicity that was studied previously [Chapter 4].

An interesting outcome associated with overnight fasting was observed during SCV-07 therapeutic evaluations. Reported for the first time, increased lesion incidence and severity due to fasting-induced stress was observed in the guinea pigs. Similar stress-associated recurrences also are observed in human HSV-2 infections [8]. The ability to induce a stress response provides an important enhancement to the guinea pig model for future studies. Also, the fasting-associated increase in HSV-2 disease enhanced our ability to evaluate the therapeutic potential of SCV-07. Results from the fasting studies indicated that despite the substantial increases in HSV-2 lesion incidence and severity, oral SCV-07 still produced significant reductions in recurrent HSV-2 disease relative to the vehicle treated controls (Tables 7 and 8).

Collectively, evaluation of SCV-07 in the guinea pig model showed that the immunomodulatory dipeptide was capable of significantly reducing experimental recurrent genital HSV-2 disease and should be evaluated further as an anti-herpetic therapy. Based on observed SCV-07 immunomodulatory activity in mice [190], reduction in recurrent genital lesion formation in guinea pigs following SCV-07 application likely occurred through activation of a specific cytokine response. Interestingly, muramyl dipeptides enhanced the activity of an HIV-1 subunit vaccine through the induction of specific immune responses in vitro [189]. The observed in vitro activity indicates that other immunomodulatory dipeptides like SCV-07 potentially could be used as vaccine adjuvants. Additionally, in vivo efficacy of SCV-07 supports further evaluation of other immunomodulatory compounds for therapeutic activity against HSV-2 to reduce the spread of STI.

CHAPTER 6: COMMENSAL BACTERIA COLONIZATION MODULATED THE INNATE IMMUNE RESPONES OF AIR-INTERFACE VAGINAL EC MULTILAYER CULTURES

OVERVIEW

Human vaginal microbiome alterations like BV are associated with increased susceptibility to HSV-2 possibly due to changes in innate defense responses related to the loss of commensal bacteria colonization. Current cell culture models of the human vaginal mucosa provide limited options for evaluating the impact of commensal bacteria on vaginal EC engendered innate immune responses to pathogens. To address the shortcomings and quantify the impact of commensal bacteria on the vaginal defenses against HSV-2 infection, we developed a renewable model of the human vaginal mucosa capable of supporting commensal bacteria colonization using immortalized vaginal EC. The developed model included several hallmarks of the human vaginal mucosa including multiple cell layers, an air/liquid interface, tight junction complexes, glycogen and microvilli. Application of FSL-1 or PIC to the vaginal EC multilayer cultures elicited cytokine responses similar to the profiles observed in primary vaginal EC cultures. Additionally, the induced cytokines, specifically IL-8, elicited significant neutrophil transepithelial migration in the model, an important component of the innate defenses against HSV-2 infection. Based on the presence of glycogen in the multilayer cultures, we hypothesized that lactobacilli could colonize the cultures due to the ability of the bacteria to metabolize glycogen. *Lactobacillus crispatus* and *L. jensenii*, commonly isolated vaginal commensals, were selected to evaluate multilayer culture colonization in addition to the impact of the bacteria on vaginal EC and the ability of the cells to respond to TLR agonist stimulation or HSV-2 infection. The results showed that lactobacilli

associated intimately with the vaginal EC and localized strictly to the apical surface. Neither *Lactobacillus spp.* caused cytokine induction but *Staphylococcus epidermidis*, a common skin commensal, was inflammatory. Relative to uncolonized cultures, *L. jensenii* and *L. crispatus* colonized vaginal EC elicited similar but substantially reduced production of specific cytokines following TLR agonist application. Similar cytokine responses were observed in cultures colonized with *L. rhamnosus*, an uncommon vaginal lactobacilli, or *L. casei*, a non-vaginal lactobacilli, indicating the immunological outcomes were specific to commensal lactobacilli. Additionally, *L. jensenii* colonization enhanced TLR agonist induced anti-herpetic activity in the multilayer cultures. The results provided valuable insights into the potential contribution of the commensal bacteria, specifically lactobacilli, to the vaginal defenses against pathogens and offered a potential explanation for the pathologies observed in women with BV. Also, the developed air-interface vaginal EC multilayer cultures provided a renewable and reproducible model for evaluating anti-herpetic intervention strategies that could potentially complement existing models of HSV-2 infection. Part of the research described in this chapter is in preparation for submission to *Cellular Microbiology*.

INTRODUCTION

The human vaginal mucosa is composed of a non-keratinized stratified squamous epithelium that forms a physical barrier to pathogens [22, 23]. The vaginal epithelium has a distinctive polarized architecture consisting of a liquid-interface at the basolateral surface and an air-interface at the apical surface [22, 23, 42, 43]. The large parabasal vaginal EC are in contact with the highly vascularized lamina propria while the elongated often anucleate superficial vaginal EC are in contact with the vaginal lumen [22, 23, 42, 43]. The vaginal mucosa contains many tight junction complexes that serve as structural

scaffolding [22, 23, 44]. The tight junction complexes also play a critical role in defending against STI by selectively permitting transepithelial migration of leukocytes while restricting passage of pathogens into the cell layers [43, 45]. Additionally, vaginal defenses are aided by the presence of commensal bacteria that colonize the apical surface of the vaginal mucosa [107-112].

Many different species of commensal bacteria including *Lactobacillus*, *Corynebacterium*, *Prevotela* and *Peptostreptoccoccus* colonize the vaginal mucosa [107, 113-115]. The commensal organisms are intimately associated with vaginal EC and play crucial but poorly defined roles in vaginal health [244-246]. In healthy women, the most prevalent commensal lactobacilli are *L. crispatus* and *L. jensenii* [114, 115]. Interestingly, *L. iners* is another vaginal commensal but is associated with recovery states following cessation of antimicrobial therapies and is implicated as a potential contributor to the loss of a lactobacilli-dominated vaginal environment [116]. In general, lactobacilli commensals are important for maintenance of a healthy vaginal environment through the production of antimicrobial agents and lactic acid via glycogen metabolism that lowers vaginal pH [22, 108, 109, 117]. In fact, vaginal EC contain many intercellular glycogen vacuoles [22, 118] supporting the concept of a symbiotic relationship. Lactobacilli also provide a physical barrier to adherence of pathogenic organisms [111, 112]. Clinically, the loss of vaginal lactobacilli due to antibiotic therapy, douching, sexual activity, pathologies or other unknown factors is associated with increased susceptibility to subsequent infection [46, 47, 130, 135].

BV represents a transition from predominately *Lactobacillus spp.* to other pathogenic bacteria including *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Mobiluncus spp*. and/or *Atopobium vaginea* [124-127]. Several studies showed that in women with BV there was a significantly increased production of

inflammation-related markers like IL-1β, IL-6, IL-8 and TNF-α compared to women with normal commensal flora [128-131]. The inflammatory response can produce physical disruptions of the vaginal mucosa and potentially alter the innate immune response of vaginal EC to pathogen insults [128, 130, 131]. Additionally, BV is an important risk factor for many clinical disorders and was linked to increased acquisition of HIV and other STI including HSV-2 [130, 132-135]. Because the human vaginal epithelium is the first site of contact for HSV-2 [38-41], understanding the importance of commensal bacteria colonization to the vaginal EC innate immune response is critical for the future design of novel anti-herpetic intervention strategies.

The development of promising microbicides has not translated to success in clinical trials [162-164, 168, 169]. The clinical failures likely were precipitated by unintended alterations of the vaginal bacterial ecology that abrogate the protective effects produced by commensals. We theorized that commensal bacteria may also modulate the response of vaginal EC to pathogens offering a potential explanation for the chronic cytokine production observed in women with BV. Arguably, development of a human culture model for evaluating the impact of common vaginal commensals is necessary to define the role of the bacteria in vaginal defenses and allow for better predictions of clinical outcomes for promising anti-herpetic intervention strategies like TLR agonists.

Currently, only the rabbit vaginal irritation model is FDA approved for evaluating the safety of vaginally applied compounds [247]. Unfortunately, the sensitivity of the rabbit model is poor [248] and the rabbit vaginal mucosa is composed of columnar epithelial cells not a stratified squamous epithelium as in humans [249]. Other animal models are available [43, 250, 251] but are cost prohibitive [43, 250] or differ physiologically from the human vaginal mucosa [252]. Human vaginal explant cultures were evaluated to address the concerns with animal models but showed high intra-subject

variability and were limited to available donors [253]. Also, studies using bacteria in the explant or other models, thus far, only evaluated the impact of dead bacteria or short-term (<8h) colonization [112, 123]. Because of the limitations with current models, a novel model of the human vaginal mucosa capable of supporting long-term (>72h) colonization of commensal bacteria is necessary for evaluating the safety and efficacy of potential anti-herpetic compounds.

We developed a novel renewable air-interface vaginal EC multilayer culture using immortalized vaginal EC [50] to provide an in vitro model of the human vaginal mucosa. The model was used to evaluate the impact of commensal bacteria on vaginal EC responsiveness to previously observed TLR agonist induced cytokine response profiles and associated resistance to HSV-2 infection [Chapters 2 and 3]. The developed vaginal EC cultures were evaluated morphologically and histologically for comparison to the human vaginal mucosa and showed the presence of several hallmarks including multiple cell layers, an air/liquid interface, glycogen and tight junction complexes. Additional immunological evaluations were performed to compare TLR agonist induced cytokine responses to previously observed profiles in primary vaginal EC [50; Chapter 3] and to quantify potential neutrophil transepithelial migration. The results showed that the vaginal EC multilayer cultures selectively responded to TLR agonist stimulation through elicitation of cytokine response profiles associated with resistance to HSV-2 infection. The induced profiles also elicited significant recruitment of neutrophils in the model, an important contributor to vaginal defenses.

Additionally, we evaluated the ability of the air-interface vaginal EC multilayer cultures to support stable colonization of several commensal bacteria species as further validation of the in vitro human vaginal mucosa model. The cultures supported long-term (>72h) apical colonization by common commensal bacteria including *L. crispatus*

and *L. jensenii*. The cultures also were used to evaluate the immunological impact of commensals on vaginal EC and their response to TLR agonist stimulation or HSV-2 infection. We showed that colonized vaginal EC multilayers selectively responded to specific commensal bacteria and that colonization of the multilayers selectively tempered cytokine response profiles elicited by TLR agonist application. Lactobacilli colonization also enhanced TLR agonist induced resistance to HSV-2 infection. Collectively, the results provide valuable insights into the role that commensal bacteria play in aiding the immune response of the vaginal mucosa against pathogenic infection. Also, the air-interface vaginal EC multilayer cultures provide a renewable and reproducible model of the human vaginal mucosa for evaluating potential anti-herpetic compounds across several laboratories.

MATERIALS AND METHODS

Air-interface vaginal EC multilayer culture model

To develop a novel in vitro model of the human vaginal mucosa, human immortalized vaginal EC from two different donors (V11I and V19I) [50] at passage numbers ranging from 10-30 were cultured as described previously [Chapter 2]. V11I and V19I were selected for initial validation of the model because they were the most abundantly available of the 3 donor cell types. The cultured cells were added to 24 or 96 well cell culture insert systems (BD) and maintained using KSFM supplemented as described in Chapter 2 except no Primocin was added to the medium. The 96 well multilayer cultures were generated by addition of vaginal EC ($1x10^5$cells/well) in 75μL of KSFM to the top chambers of a 96 well 1.0μm insert system in an angled bottom plate containing 260μL of KSFM in the bottom chambers. For the generation of 24 well

multilayer cultures used to quantify the impact of commensals on HSV-2 infection, vaginal EC ($1x10^6$cells/well) in 250μL of KSFM were added to the top chambers of 24 well 0.4μm cell culture inserts containing 500μL of KSFM in the bottom chambers. Both 24 and 96 well cultures were incubated for 48h before the medium from the top chamber was removed to form an air-interface. The cultures were refed with KSFM every other day starting 48h after addition of the vaginal EC to the cultures. As an indirect measurement of multilayer formation, an electronic volt and ohm meter with the STX-2 (24 well) or STX-100 (96 well) probe (World Precision Instruments, Sarasota, FL) was used to measure transepithelial electrical resistance (TEER). For TEER measurements, 500μL (24 well) or 75μL (96 well) of DPBS, a low resistance solution, was added to the apical chamber then removed after recording the value. TEER measurements of selected d0 cultures were performed to confirm that the TEER values had significantly increased compared to initial values (d-9). Prior to the start of experiments (d0), medium was replaced in the bottom chamber. Figure 22 is a schematic summarizing the timeline for creation of the multilayer cultures.

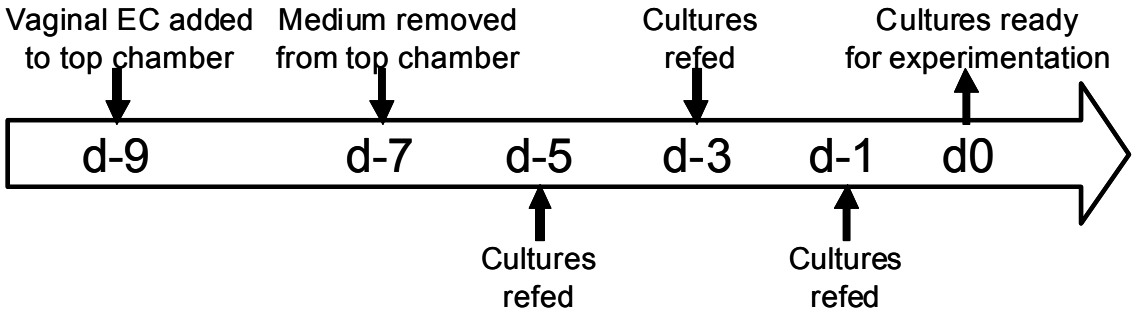


Figure 22. Timeline for generation of the air-interface vaginal EC multilayer cultures. Vaginal EC were added to the top chamber and the cultures were incubated for 48h prior to medium removal from the top chamber. The cultures were refed with KSFM every other day starting 48h after addition of the vaginal EC to the cultures. After creation of the air-interface, the cultures were incubated for an additional 7d prior to the start of any experiments.

The inverted vaginal EC multilayer cultures for the neutrophil transepithelial migration assays were generated as described for the regular multilayer cultures with the following exceptions: The 24 well cell culture inserts (3.0μm pore size; BD) were inverted and vaginal EC ($1x10^6$cells/well) in 150μL of KSFM were added to the topside of the inverted insert filter. The inserts were incubated for 24h then righted into a 24 well plate (BD) containing 500μL of KSFM and 500μL of KSFM was added to the top chambers. After an additional day of incubation the medium from the bottom chamber was removed to form an air-interface. Medium in each top chamber was replaced every other day starting 48h after addition of the vaginal EC to the cultures and TEER was used to monitor multilayer formation. Figure 23 is a schematic summarizing the timeline for creation of the inverted vaginal EC multilayer cultures.

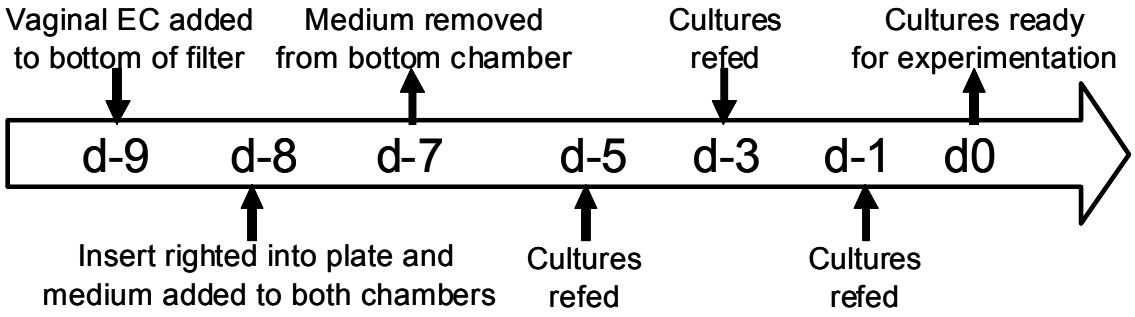


Figure 23. Timeline for generation of the inverted air-interface vaginal EC multilayer cultures. Vaginal EC were added to the topside of the inverted insert filters then incubated for 24h. The inserts were righted into a plate and medium was added to both chambers. After 24h of incubation the medium was removed from the bottom chamber. The cultures were refed with KSFM every other day starting 48h after addition of the vaginal EC to the cultures. After creation of the air-interface, the cultures were incubated for an additional 7d prior to the start of any experiments.

Bacterial culture, identification and lactic acid production

For colonization of the multilayers, commensal bacteria were selected to represent common vaginal lactobacilli (*L. crispatus*, *L. jensenii*), uncommon vaginal lactobacilli (*L. rhamnosus*), non-vaginal lactobacilli (*L. casei*) or non-vaginal non-*Lactobacillus spp.* (*S. epidermidis*). All clinically isolated commensal bacteria were collected under UTMB IRB approval from clinically obtained vaginal swabs. The clinical commensal bacteria were isolated through serial passaging of individual colonies using agar plates containing medium specific for growth of the particular commensals. Bacteria were cultured in De Man, Rogosa, Sharpe (MRS) broth (BD) for *L. crispatus* (ATCC 33820), *L. jensenii* (ATCC 25258), *L. jensenii* (clinically isolated), *L. rhamnosus* (clinically isolated) and *L. casei* (isolated from DanActive®). *S. epidermidis* (ATCC 35984) and *S. epidermidis*

(clinically isolated) were cultured in Tryptic Soy (TS) broth (BD). Cultures were harvested after overnight growth then stored at -80^{0}C in bacterial specific medium with 15% glycerol (Fisher). *L. iners* (CCUG 28746) was cultured on chocolate agar plates (PML, Wilsonville, OR) in Bio-Bags (BD) then stored at -80^{0}C in Litmus Milk (BD).

To confirm the identity of each bacterial strain, 10μL samples of each bacteria were added to 40μL of sterile H_2O (Invitrogen) in a 96 well plate then incubated at 95^{0}C for 10min using an iCycler. From the heat lysed bacterial samples, 5μL was added to 12.5μL of Supermix from Bio-Rad, 0.5μM of 27-F (5' – AGA GTT TGA TCA TGG CTC AG – 3'; IDT), 0.5μM of 518-R (5' – ATT ACC GCG GCT GCT GG – 3'; IDT) and 5.5μL of H_2O based on the 16S rRNA PCR assay described by Gillan et al. using primers specific for the domain *Bacteria* [254]. The reaction mixes were incubated at 95^{0}C for 5min then subjected to 50 cycles of denaturation at 95^{0}C for 30sec, annealing at 49^{0}C for 30sec, and primer extension at 72^{0}C for 30sec. The PCR products were purified using the Montage PCR Centrifugal Filter Device system (Millipore, Billerica, MA). From the purified PCR products, 10μL of each bacterial sample was added to 1μL of 27-F primer and 1μL of H_2O then the samples were submitted the UTMB Protein Chemistry Core for DNA sequencing. The samples were sequenced using a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and data were analyzed using the included software. The DNA sequences coding for the 16S rRNA, termed ribotype, for each sample were compared to known sequences using the basic local alignment search tool to confirm the identity of each bacterial strain.

For identification of lactic acid production, supernatant samples (1mL) from 18h cultures of each bacterial species were centrifuged at 10,000g for 10min at 4^{0}C. Using the D/L-lactic acid assay kit (R-Biopharm, Marshall, MI), 100μL from each of the

centrifuged samples was assayed to identify production of the D or L isomer of lactic acid.

Bacterial colonization of the multilayer cultures and viability quantification

Vaginal EC multilayer cultures (d0) were used for all bacterial colonization experiments. Frozen bacterial aliquots were thawed into 5mL of MRS broth for *L. crispatus*, *L. jensenii*, *L. rhamnosus* and *L. casei* or TS broth for *S. epidermidis* and cultured for 18h in a sealed 15mL conical tube (BD). After 18h, the amount of viable bacteria present, represented as colony forming units (cfu)/mL, was quantified using MRS or TS agar plates (BD) or by the liquid-based viability quantification assay. Bacterial samples were serially diluted then plated (100μL) on duplicate agar plates for each dilution. The agar plates were incubated for 24h then the number of colonies present on each plate was enumerated and used to calculate the cfu/mL of bacteria for each sample. For the liquid-based viability assay, MRS or TS broth (90μL) was added to all wells of a 96 well culture plate and 10μL of each bacteria sample was added to the first column (Figure 24). Serial dilutions into the remaining 11 columns were performed using a multichannel pipet (Rainin). The plates were incubated for 24h then visually inspected using an Axiovert 25 microscope at 200X magnification to identify the last well that contained bacteria. The approximate amount of bacteria present in each tube after 18h of growth was calculated based on the serial dilutions (Figure 24). The viability assay system also was used for quantifying bacterial loads in the vaginal EC multilayer cultures. For quantification of *L. iners*, the bacteria were cultured for 48h on chocolate agar plates in Bio-Bags. After 48h, the bacteria were collected from the plate, and the amount of viable bacteria present was quantified using serial dilutions on chocolate agar

plates. All bacterial species were pelleted and washed extensively before dilution in KSFM prior to culture colonization.

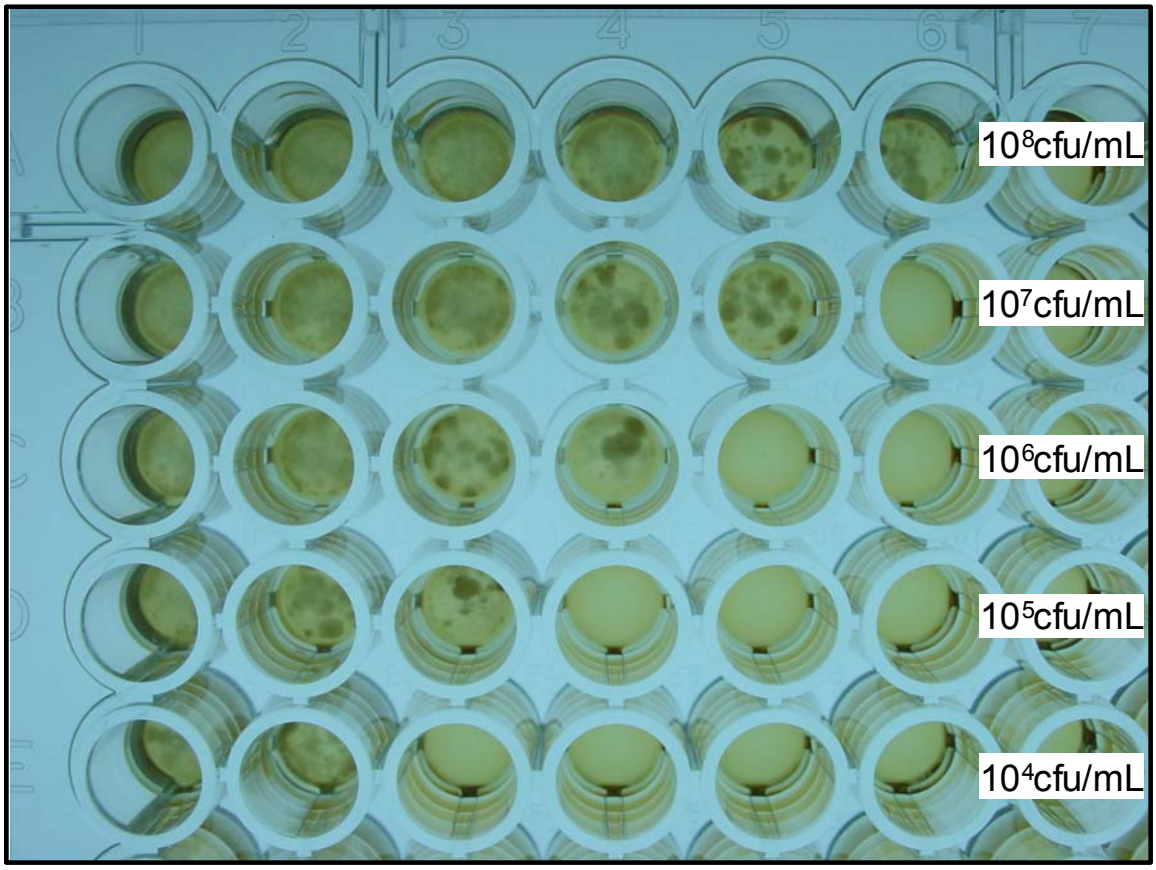


Figure 24. High-throughput liquid-based commensal bacteria vaibility quantification assay. Bacteria samples (10μL) were added to the first well of a 96 well plate containing MRS or TS broth then serially diluted and incubated for 1d. The image is an example of the liquid-based viability assay after 1d of incubation showing a 10-fold serial dilution series plated in each row. The last well with bacteria present was identified and used to calculate the cfu/mL based on the serial dilutions.

To quantify long-term bacterial viability in the multilayer cultures and bacterial impact on vaginal EC health, 10^3cfu of selected bacteria (10μL) or KSFM (10μL) was

added to the apical air-interface of d0 multilayers. The cultures were incubated for 72h and apically (top chamber) supplemented at 48h with 10μL of KSFM. At 24, 48 and 72h 50μL KSFM was added to the top chamber of triplicate wells for each time point then the colonized multilayers were scrapped and collected for analyses. Of the 50μL collected for each sample, 20μL was used for duplicate viability analyses. The remaining 30μL was analyzed via MTT assay using 1mg/mL Thiazolyl Blue Tetrazolium Bromide to evaluate cell health (Sigma). MTT samples were incubated for 3h and optical density measurements (560nm) were collected on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). Potential bacterial produced pH changes were quantified by removal of culture medium and replacement in selected cultures with saline (Fisher) or fresh medium at 24 or 6h prior to the addition of 10^3cfu of selected bacteria (10μL) to the top chamber. Changes in apical cell surface pH were measured in triplicate wells for each condition using pH 4.0-7.0 indicator strip material (EMD, Gibbstown, NJ) at 6, 18 or 24h post-addition of the bacteria.

Histological staining and immunofluorescent labeling

Vaginal EC multilayer cultures (d0) were fixed with Z-fix (Anatech, Battle Creek, MI) for 24h at 4^0C. The filters were excised, paraffin embedded and 5μm sections were cut using a Microm HM 310 microtome (Fisher) then placed on slides. The sections were Hematoxylin and Eosin (H&E) or periodic acid Schiff (PAS) stained to visualize the cell layers or glycogen, respectively. For confocal observation of multilayer formation, the cultures were Z-fixed and washed 3X with tris-buffered saline (Fisher) then the filters were excised and placed on slides. Cells were labeled using Vectashield with DAPI (Vector Laboratories, Burlingame, CA) then coverslipped and visualized on a LSM 510 UV Meta Laser Scanning Confocal Microscope (Zeiss).

To observe bacterial colonization of the apical cell surface, 10^3cfu of selected bacteria were added to d0 green fluorescent protein (GFP) expressing V19I cultures. After 24h of colonization the fixed culture inserts were labeled with a Gram positive bacteria marker monoclonal antibody (clone 3801; Santa Cruz Biotechnology, Santa Cruz, CA) and a goat anti-mouse secondary conjugated to AF555 (Invitrogen). Filters were removed, mounted using Vectashield with DAPI and visualized by confocal microscopy.

Transmission, immunogold occludin-labeled and scanning electron microscopy

To identify tight junction complexes or visualize commensal bacteria colonization of the multilayers, d0 cultures at 24h post-addition of medium or 10^3cfu of selected bacteria were Z-fixed and used for electron microscopy (EM) experiments. Cultures were rinsed with cacodylate buffer, postfixed in 1% OsO_4 in 0.1M cacodylate buffer for non immunogold EM samples, rinsed with water, stained en bloc with 2% aqueous uranyl acetate and dehydrated in ethanol. Filters were excised and embedded into Poly-Bed 812 resin (Polysciences Inc., Warrington, PA) or LR White (Electron Microscopy Sciences, Hatfield, PA) for immunogold EM samples. Ultrathin sections were cut on a Sorvall MT-6000 ultramicrotome (RMC, Tucson, AZ) then labeled with rabbit anti-human occludin (clone H-279; Santa Cruz Biotechnology, Santa Cruz, CA) and a 15nm gold-labeled goat anti-rabbit IgG (Amersham, England) for immunogold EM samples. Sections were counterstained with aqueous uranyl acetate and lead citrate then examined on a Philips CM100 TEM at 60kV (Philips, Netherlands).

For scanning EM, the cultures were fixed as above then dehydrated in ethanol and processed through hexamethyldisalazane followed by air drying. The filters were excised, mounted onto metal stubs and sputter-coated with iridium in an Emitech K575x

Sputter Coater (Emitech, Houston, TX) at 20mA for 20sec. The filters were examined in a Hitachi S4700 SEM (Hitachi High Technologies, Schaumburg, IL) at 2kV.

Cytokine quantification assays

To quantify TLR agonist induced cytokine response profiles, selected commensal bacteria (10μL) at 10^3cfu/well or KSFM (10μL) were added to d0 vaginal EC multilayers. The cultures were incubated for 24h prior to the addition of FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or an equivalent volume of the KSFM vehicle (10μL) to the apical surface. After 6h, 100μL of KSFM was added to apical surface, to simulate cervicovaginal lavaging, then collected and stored at -80^0C. Supernatants (50μL) were quantified using the BioPlex Human Group I cytokine kit as described [Chapter 3].

To evaluate potential temporal alterations of TLR agonist induced cytokine response profiles in colonized cultures, selected commensal bacteria (10μL) at 10^3cfu/well or KSFM (10μL) were added to d0 vaginal EC multilayers. The cultures were incubated for 24 or 48h prior to the addition of PIC (0.1mg/mL) or an equivalent volume of the KSFM vehicle (10μL) to the apical surface. The colonized cultures were exposed to agonist stimulation for 6 or 24h then 100μL of KSFM was added to apical surface and collected for cytokine analyses. Figure 25 is a schematic summarizing the timeline for bacterial addition, TLR agonist addition and sample collection as described in the cytokine quantification assays.

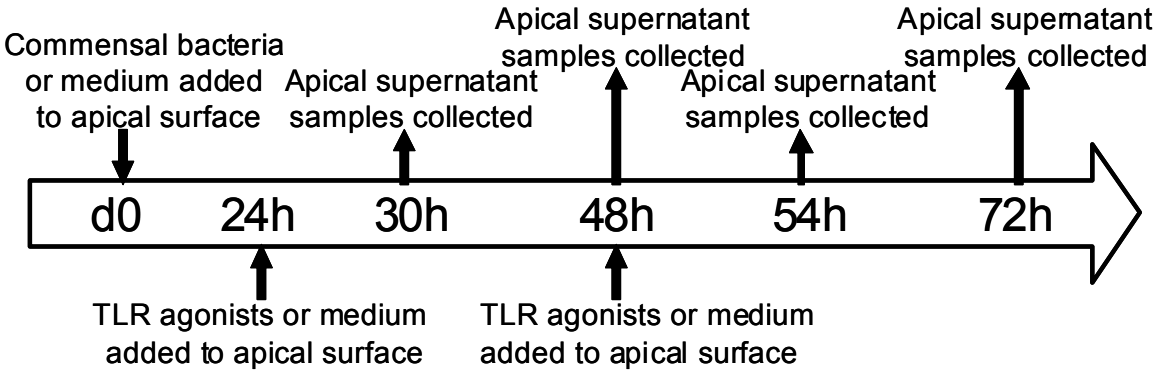


Figure 25. Timeline for bacterial addition, TLR agonist application and sample collection for the cytokine quantification assays. Commensal bacteria or medium were added to d0 multilayer cultures. TLR agonist or medium were added 24 or 48h post-addition of the commensals. Apical supernatant samples were collected at 30, 48, 54 or 72h post addition of the commensals.

Neutrophil isolation, preparation and transepithelial migration

To evaluate the ability of the multilayer cultures to model leukocyte transepithelial migration, neutrophils were collected from fresh anti-coagulant treated human venous blood obtained from healthy volunteers (UTMB IRB approved protocol). Neutrophils were isolated from the donated blood using Mono-Poly Resolving Medium (MP Biomedicals, Solon, OH) gradient centrifugation then enumerated using a hemocytometer.

FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or an equivalent volume of the KSFM vehicle (10μL) were added to the apical surface of inverted 24 well vaginal EC multilayer cultures (d0) then incubated for 24h. Neutrophils ($1x10^6$neutrophils/well) were added to the basolateral surface (top chamber) in 250μL of KSFM. The cultures were incubated for 4h then apical wash (100μL) and basolateral supernatant (100μL)

samples were collected from each well for neutrophil counting. The neutrophil samples were processed using the Cell Titer-Glo assay (Promega, Madison, WI) and analyzed with a TR 717 Microplate Luminometer (Applied Biosystems). The number of neutrophils present in each apical wash sample was divided by the total number of neutrophils added to calculate percent migration.

Quantification of commensal bacteria impact on TLR agonist induced activity against HSV-2 infection

Vaginal EC multilayer cultures (d0) were incubated for 48h with selected bacteria (10μL) at 10^3cfu or KSFM (10μL). After 48h, FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or an equivalent volume of the KSFM vehicle (10μL) were added at 48h to the apical surface and the cultures were incubated for 24h. HSV-2 186 (100pfu/well) in 20μL of KSFM was added to the apical surface and the cultures were incubated for 24h. The cell layers from each culture were collected in 500μL of KSFM then the amount of HSV-2 present was quantified as described for the plaque titration assay [Chapter 4]. Figure 26 is a schematic summarizing the timeline for TLR agonist addition, HSV-2 addition and sample collection as described in the commensal bacterial impact on HSV-2 infection assay.

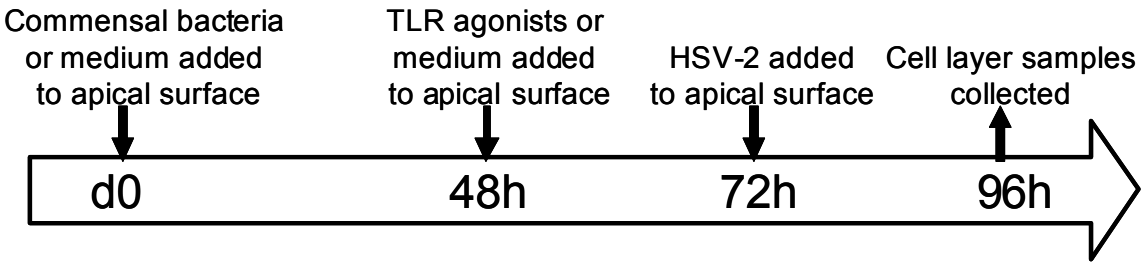


Figure 26. Timeline for TLR agonist application, HSV-2 inoculation and sample collection for the commensal impact on HSV-2 infection assay. Commensal bacteria or medium were added to d0 multilayer cultures. TLR agonist or medium were added 48h post-addition of the commensals. The cultures were inoculated with HSV-2 at 72h post-addition of the commensals. Cell layer samples were collected at 96h post addition of the commensals.

Statistical analyses

All data were analyzed for significance differences compared to the KSFM vehicle control using the Student's t-test in the Prism software package v4.0. Each study was repeated at least once with triplicate samples to confirm results. The TEER values present are from 96 well multilayer cultures and are presented as the mean of 3 culture inserts ± SEM for each time point.

RESULTS

Human immortalized vaginal EC formed multilayers consistent with stratified squamous epithelium

The human vaginal mucosa is a stratified squamous epithelium that contains glycogen and tight junction complexes [22, 23, 42, 43, 118]. To develop a vaginal EC culture model that contained several hallmarks of the human vaginal mucosa,

immortalized vaginal EC were seeded into culture inserts. After 48h, the growth medium was removed from the apical chamber forming an air/liquid interface, a hallmark of the human vaginal mucosa. TEER monitoring of 96 well vaginal EC cultures established that a steady state value was achieved after at least 9d post-seeding. Specifically, TEER values significantly ($p<0.001$; Student's t-test) increased after 9d (mean = 228.9 ± 6.4 Ωcm^2) relative to TEER values at seeding (mean = 41.4 ± 0.3 Ωcm^2) and were maintained for at least 14d. To address histological characteristics, established multilayers (d0; Figure 22) on the filter support (Figure 27) were fixed and then carefully excised from the inserts for paraffin embedding. H&E stained cross-sections showed that vaginal EC formed multiple cell layers with nucleated cells at the basolateral surface and elongated, often anucleate cells at the apical surface (Figure 28a). The observed characteristics were consistent with the formation of distinct types of vaginal EC based on their location within the vaginal mucosa [22, 23, 42, 43]. PAS staining of vaginal EC multilayer culture cross-sections showed the presence of glycogen within the cell layers (Figure 28b). Again, the observation of glycogen within the cellular multilayers is consistent with the presence of glycogen within the human vaginal mucosa [22, 118].

To further characterize the vaginal EC multilayers, transmission EM of d0 cultures was performed and multiple cell layers (>7) were observed (Figure 29) supporting the confocal and histological data. Large nucleated cells were observed at the basolateral surface in contact with the filter membrane and elongated anucleate vaginal EC were observed at the apical surface. The EM images also indicated the presence of tight junction complexes between the plasma membranes of cells (Figure 30a). Immunogold-labeling for occludin, a protein involved in the tight junction complex [44], confirmed the identity of the structures (Figure 30b). Secondary antibody alone did not label the tight junction complexes in subsequent sections (Figure 30b, insert). Vaginal

EC from an additional donor and multilayer cultures in 24 or 96 well formats also were evaluated with similar outcomes confirming that the results were not donor or format specific, respectively (data not shown). Collectively, the results indicated that immortalized vaginal EC reproducibly formed multilayer cultures that developed tight junction complexes, contained glycogen and closely modeled the human vaginal mucosa. The presence of glycogen within the multilayers supported the possibility of commensal bacteria colonization of the cultures based on the ability of lactobacilli to metabolize glycogen. Immunological evaluations also were necessary prior to multilayer colonization to confirm that the vaginal EC selectively responded to TLR agonist stimulation by eliciting cytokine responses similar to previously observed primary vaginal EC profiles [50; Chapter 3].

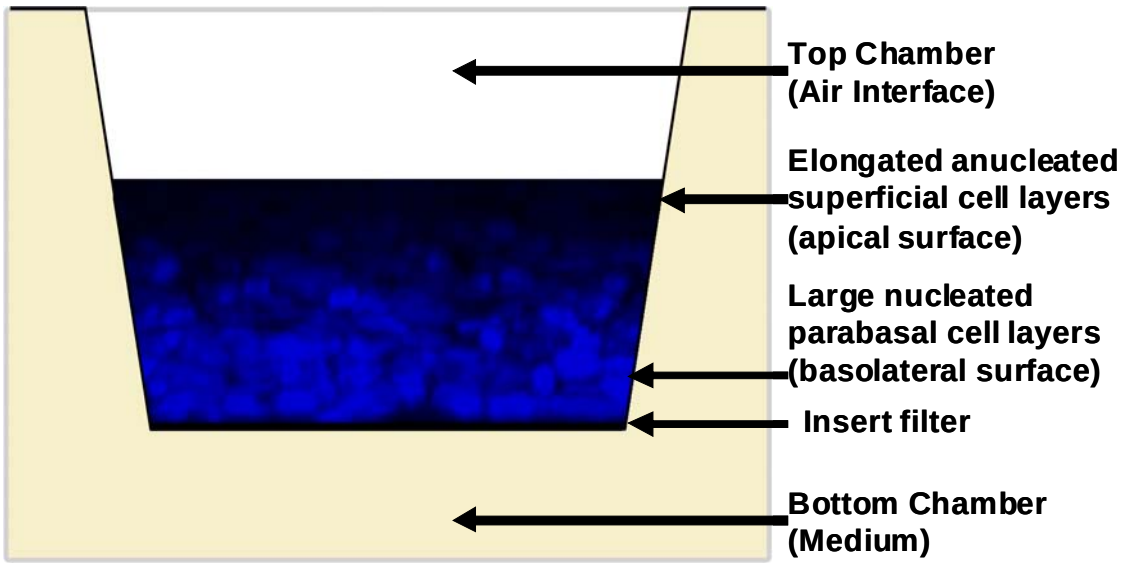


Figure 27. Human vaginal EC formed air-interface multilayer cultures. Confocal Z-axis projection (630X magnification) of a DAPI-labeled (blue nuclei) d0 V19I vaginal EC culture showing multiple (>7) cell layers.

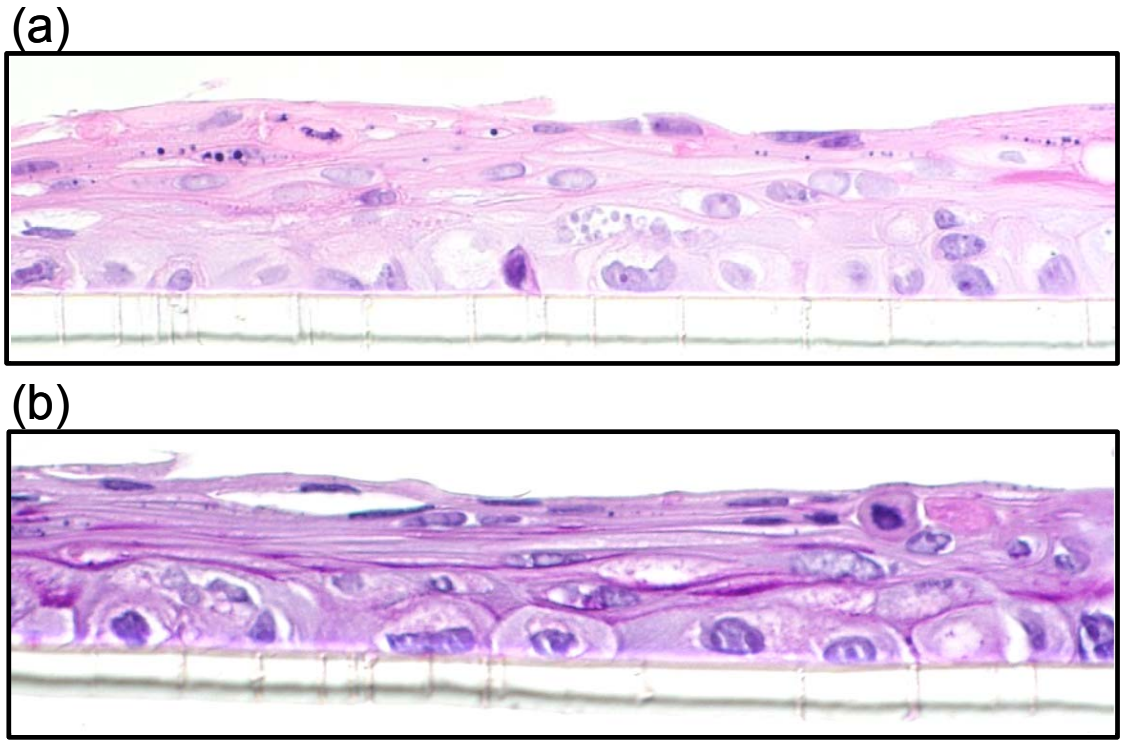


Figure 28. Human immortalized vaginal EC cultures formed multiple glycogen containing layers. (a) H&E stained 5μm cross-section (400X magnification) of a d0 V19I vaginal EC culture showing formation of multiple cell layers (7-10) with large nucleated cells present at the basolateral surface and elongated anucleate superficial cells at the apical surface. (b) PAS stained 5μm cross-section (400X magnification) of a d0 V19I vaginal EC culture illustrating the presence of glycogen (dark purple) within the cell layers.

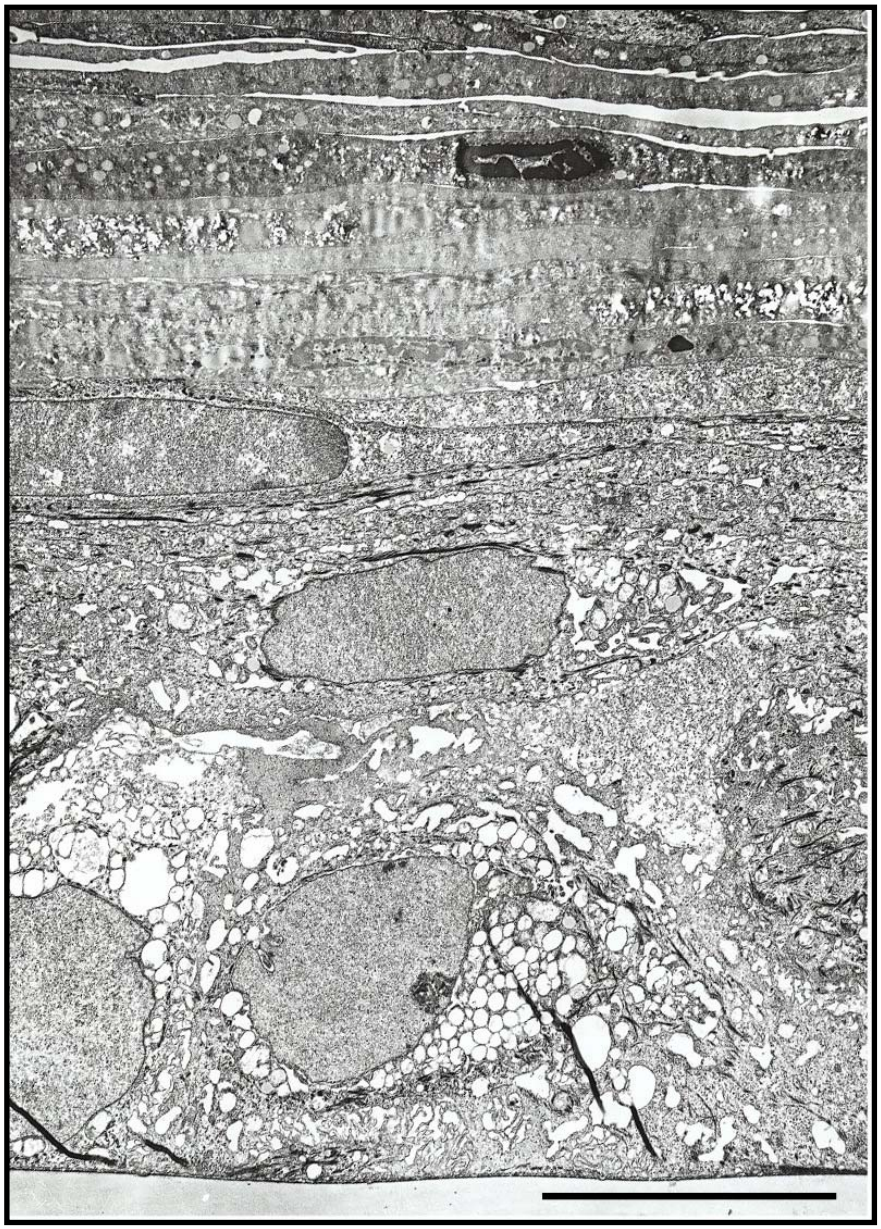


Figure 29. Human immortalized vaginal EC formed multilayer air-interface cultures. Transmission EM image of a 1μm cross-sectioned d0 V19I vaginal EC multilayer culture showing the formation of multiple cell layers (>7) with large nucleated cells present at the basolateral surface and elongated anucleate superficial cells at the apical surface (bar = 10μm). The image is representative of >25 fields of view and the mean multilayer thickness was 48μm for d0 cultures.

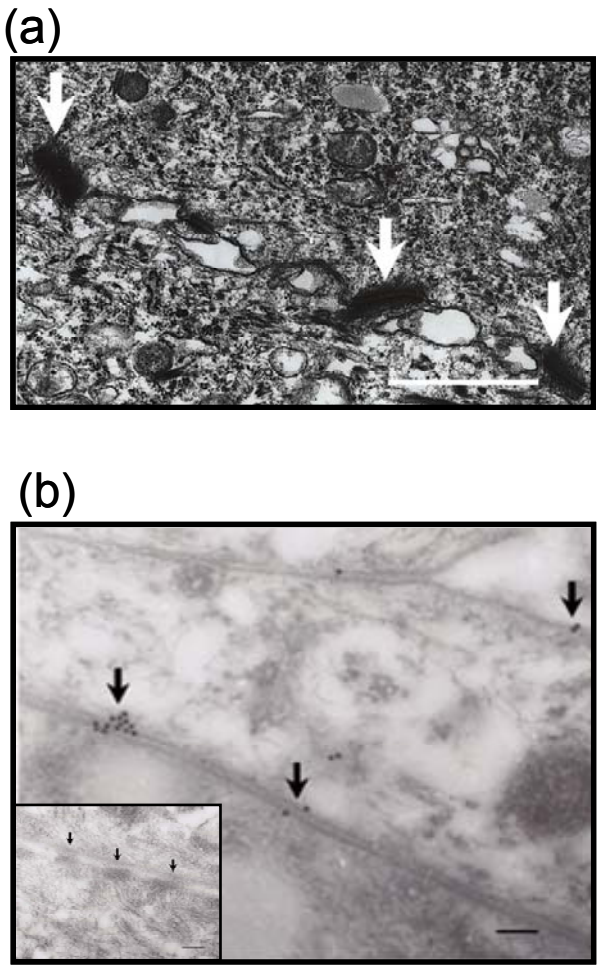


Figure 30. Vaginal EC formed multilayers cultures with tight junction complexes between the plasam membranes of the cells. (a) Transmission EM image of a 1μm cross-sectioned d0 V19I vaginal EC multilayer culture illustrating the presence of tight junction complexes (white arrows) connecting the plasma membranes of two cells (bar = 1μm). (b) Immunogold transmission electron micrograph of a 1μm cross-sectioned d0 V19I vaginal EC multilayer culture showing tight junction complexes (black arrows) containing gold-labeled occludin (black dots) in the plasma membranes of adjacent cells (bar = 100nm). (b, insert) A secondary antibody control image showed no labeling of the tight junction complexes (black arrows) indicating that the gold-labeled antibody specifically bound to occludin (bar = 100nm). Images are representative of >25 fields of view.

Vaginal EC multilayer cultures elicited specific cytokine response profiles following TLR agonist application

To evaluate innate immune responses of vaginal EC multilayers to TLR agonist stimulation, cultures were treated apically with a panel of TLR agonists previously tested

in human vaginal EC cultures in vitro [50; Chapters 2 and 3]. Specifically, FSL-1, PIC and FLAG were tested because they are recognized by the most abundantly expressed TLR in human vaginal EC [50]. Similar to the previously observed results for vaginal EC in vitro (Figure 11) [Chapter 3], PIC application resulted in significant ($p<0.05$; Student's t-test) induction of IL-6, IL-8, GM-CSF, MIP-1β, RANTES and TNF-α at 6h post-application compared to parallel cultures treated with vehicle only (Table 9). FSL-1 elicited a distinct cytokine profile with significant ($p<0.05$; Student's t-test) increases observed in IL-6, IL-8, IL-12(p70), G-CSF, GM-CSF, MIP-1β, RANTES and TNF-α relative to vehicle alone (Table 9). Addition of FLAG led to significant ($p<0.05$; Student's t-test) production of IL-6, IL-8, G-CSF, GM-CSF, MIP-1β and TNF-α (Table 9). Of the tested agonists, PIC application resulted in the most robust induction of cytokines followed by FSL-1. Similar cytokine profiles were observed following application of TLR agonists in V19I vaginal EC multilayer cultures (data not shown). Additional temporal evaluations showed that multilayer cultures stimulated with PIC for 24h resulted in significant induction of the same cytokine profile as the 6h time point (Table 9). PIC also induced IL-2, IL-12(p70), G-CSF, IFNγ and MIP-1α consistent with the previously observed cytokine response profile following 24h of PIC stimulation in vitro (Figure 11) [Chapter 3]. The agonist specific cytokine profiles were consistent with previously observed results in primary vaginal EC [50; Chapter 3] and correlated with induction of an anti-herpetic cytokine response following PIC or FSL-1 application [Chapters 2 and 3].

Table 9. TLR agonists induced specific cytokine response profiles in vaginal EC multilayer cultures.

	TLR agonists[a]			
Cytokines[b]	**vehicle**	**FSL-1**	**PIC**	**FLAG**
IL-6	11 ± 1	155 ± 17[c]	1057 ± 63[e]	137 ± 7[e]
IL-8	96 ± 11	24867 ± 8769[d]	24863 ± 8773[d]	19258 ± 2566[c]
IL-12(p70)	40 ± 4	57 ± 4[d]	42 ± 3	42 ± 4
G-CSF	58 ± 16	524 ± 29[e]	46 ± 4	1002 ± 88[e]
GM-CSF	9 ± 1	23 ± 4[c]	25 ± 2[e]	39 ± 7[c]
MIP-1β	5 ± 2	138 ± 3[e]	41 ± 1[e]	28 ± 7[c]
RANTES	55 ± 21	302 ± 2[e]	313 ± 11[e]	78 ± 10
TNF-α	22 ± 4	263 ± 14[e]	460 ± 3[e]	434 ± 33[e]

[a] FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or an equivalent volume of the KSFM vehicle (10μL) were added to the apical surface of V11I 24h multilayer cultures in the 96 well insert format. Apical samples (100μL) were collected 6h later for cytokine analyses.

[b] Data are the mean ± SEM (pg/mL) of 3 replicates from a representative experiment of 4 independent experiments.

[c] $p<0.01$ compared to KSFM vehicle (Student's t-test).

[d] $p<0.05$ compared to KSFM vehicle (Student's t-test).

[e] $p<0.001$ compared to KSFM vehicle (Student's t-test).

TLR agonists induced neutrophil recruitment in vaginal EC multilayer cultures

The ability of the vaginal EC multilayer cultures to model phagocyte transepithelial migration was evaluated using neutrophils (Figure 31), due to the importance of phagocytes for providing anti-herpetic activity following elaboration of a specific cytokine profile by TLR [48, 50, 51]. Neutrophils were selected for initial evaluations due to ease of availability and the significant induction of IL-8 by the tested TLR agonists (Table 9). A 24h exposure to PIC elicited significant ($p<0.05$; Student's t-test) transepithelial migration within 4h after neutrophil addition compared to parallel cultures treated with vehicle only (Figure 32). FSL-1 and FLAG application also resulted in similar significant ($p<0.05$; Student's t-test) recruitment of neutrophils to the apical surface of vaginal EC multilayer cultures. Manual counting of the migrated neutrophils using a hemocytometer confirmed the Cell Titer-Glo counting outcomes (data not shown). The results showed that TLR agonist induced significant production of IL-8 (Table 9) correlated with significant neutrophil transepithelial migration (Figure 32). Additionally, the data illustrated the feasibility of the vaginal EC multilayer cultures for evaluating transepithelial migration of neutrophils and potentially other leukocytes important for providing resistance to genital HSV-2 infection.

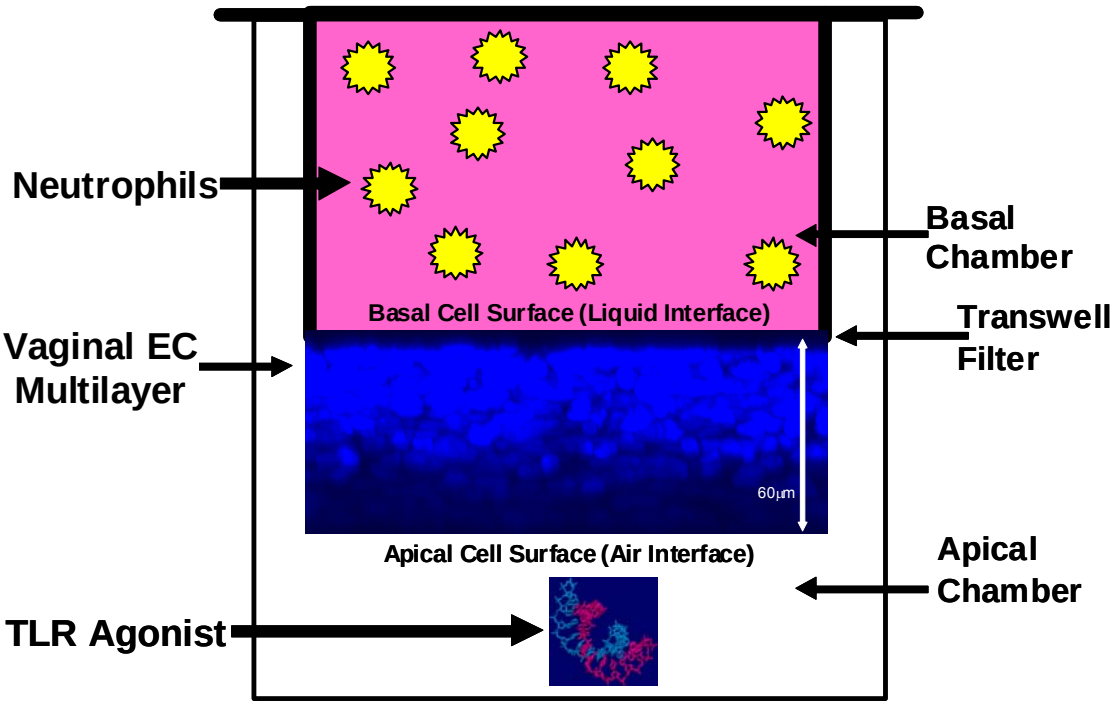


Figure 31. Quantification of neutrophil transepithelial migration using inverted vaginal EC multilayer cultures. Image is a diagram showing the use of the inverted vaginal EC multilayer cultures to model neutrophil migration. TLR agonist were added to the apical cell surface of d0 multilayer cultures and incubated for 24h. After incubation, freshly isolated neutrophils were added to the basal (top) chamber to place the neutrophils in contact with the basolateral cell surface to simulate neutrophils arriving from the vascular system. The cultures were incubated for 4h to allow time for potential TLR agonist induced neutrophil transepithelial migration to occur. After 4h, apical cell surface and basal supernatant samples were collected to quantify induced neutrophil recruitment to the apical cell surface.

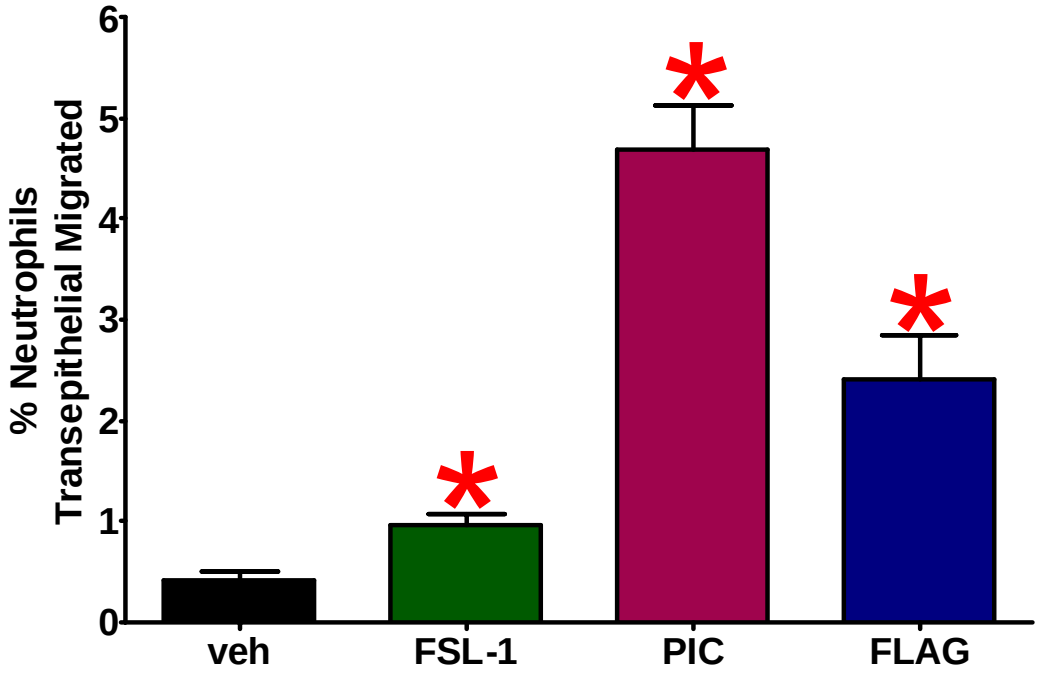


Figure 32. TLR agonists induced neutrophil transepithelial migration in inverted vaginal EC multilayer cultures. FSL-1 (0.1µg/mL), PIC (0.1mg/mL), FLAG (1µg/mL) or an equivalent volume of the KSFM vehicle (10µL) were added to the apical surface of d0 inverted V19I vaginal EC multilayers in the 24 well inserts. After 24h, neutrophils ($1x10^6$neutrophils/culture) were added to the basolateral surface (top chamber) in 250µL of KSFM. Apical wash samples (100µL) were collected after a 4h incubation for neutrophil counting using the Cell Titer-Glo assay. A significant (*; $p<0.05$; Student's t-test) increase in neutrophil transepithelial migration compared to the vehicle control was observed for all tested agonists. Results are the mean of 3 replicates ± SEM.

Commensal bacteria viably colonized the apical surface of vaginal EC multilayer cultures

Having established that the basic morphological and immunological characteristics of air-interface vaginal EC multilayer cultures were similar to the human vaginal mucosa, we next evaluated the ability of the model to support stable commensal bacteria colonization to further validate the model. Commensal bacteria, specifically lactobacilli, are an important component of the vaginal defenses through production of an acidic environment, secretion of antimicrobial proteins and prevention of pathogen adherence [107-112]. Because *L. crispatus* and *L. jensenii* are the most commonly isolated vaginal commensals, they were selected for initial evaluations in vaginal EC multilayer cultures [114, 115]. Additionally, a clinically isolated *L. jensenii* was used to identify potential differences in colonization and immunological impact on vaginal EC between the ATCC strain and lactobacilli present within the human vaginal environment. *L. rhamnosus* and *L. casei* served as served as uncommon and non-vaginal *Lactobacillus spp.* controls [255-257] to identify if observed colonization or immunological outcomes were specific to vaginal lactobacilli. The identity of each selected bacteria was confirmed and the ability of the bacteria to produce lactic acid was evaluated prior to the start of any experimentation (Table 10).

As an initial qualitative evaluation of colonization, bacterial adherence and the potential for penetration into the vaginal EC multilayers was analyzed via confocal and EM. Scanning EM analyses showed the presence of rod-shaped *L. jensenii* tightly associated with vaginal EC on the apical surface of each multilayer examined (Figure 33). In examined fields the bacteria were in contact with microvilli-like structures, another hallmark of the human vaginal mucosa, on the apical vaginal EC and were distributed relatively evenly across the surface. Based on the scanning EM results, the

average length of *L. jensenii* in contact with vaginal EC, calculated by measuring bacteria from 33 fields, was 1.45μm ± 0.08μm. Transmission EM of bacteria colonized cultures further illustrated the superficial contact between the vaginal EC multilayers and *L. jensenii* exclusively on the apical surface (Figure 34). In every case the transmission EM images showed no penetration of *L. jensenii* into deeper layers and no intracellular bacteria were observed in the cultures. To confirm that the colonizing bacteria remained localized only to the apical surface, *L. jensenii* was added to GFP-expressing vaginal EC multilayer cultures then incubated for 24h prior to immunofluorescent labeling for confocal microscopy analyses. Optical sectioning through the culture confirmed that the bacteria did not penetrate into the multilayers as seen in multiple compiled Z-stacks (Figure 35). Individual slices showed that most of the bacteria were on the extreme apical surface with some pooling in the valleys that formed in each culture (Figure 35).

Table 10. Species identification and lactic acid production of bacteria selected for multilayer vaginal EC culture colonization.

Bacteria[a]	Source[b]	Growth[c]	Lactic acid[d]	Ribotype[e]
L. crispatus	ATCC 33820	MRS broth	D/L	AF257097.1
L. jensenii	ATCC 25258	MRS broth	D	AF429513.1
L. jensenii	Clinical (UTMB)	MRS broth	D	AB289172.1
L. rhamnosus	Clinical (UTMB)	MRS broth	L	FJ409226.1
L. casei	DanActive®	MRS broth	L	GQ141813.1
L. iners	CCUG 28746	Chocolate agar	ND[f]	Y16329.1
S. epidermidis	ATCC 35984	TS broth	L	AY688051.1
S. epidermidis	Clinical (UTMB)	TS broth	L	FJ957856.1

[a] Bacteria selected for multilayer colonization. Common vaginal lactobacilli (*L. crispatus*, *L. jensenii*), uncommon vaginal lactobacilli (*L. rhamnosus*), non-vaginal lactobacilli (*L. casei*), lactobacilli associated with an abnormal vaginal environment (*L. iners*) or non-vaginal non-*Lactobacillus* species (*S. epidermidis*)

[b] Source of bacteria selected for evaluation. Clinical isolates were obtained under UTMB IRB approval.

[c] Growth medium used to specifically culture the selected bacteria.

[d] Production of D- or L-lactic acid isomers through fermentation by each bacteria species.

[e] GenBank® accession number corresponding the partial DNA sequence of the 16S rRNA isolated from each bacteria using the PCR protocol and primers described by Gillan et al. [254].

[f] ND; Not determined.

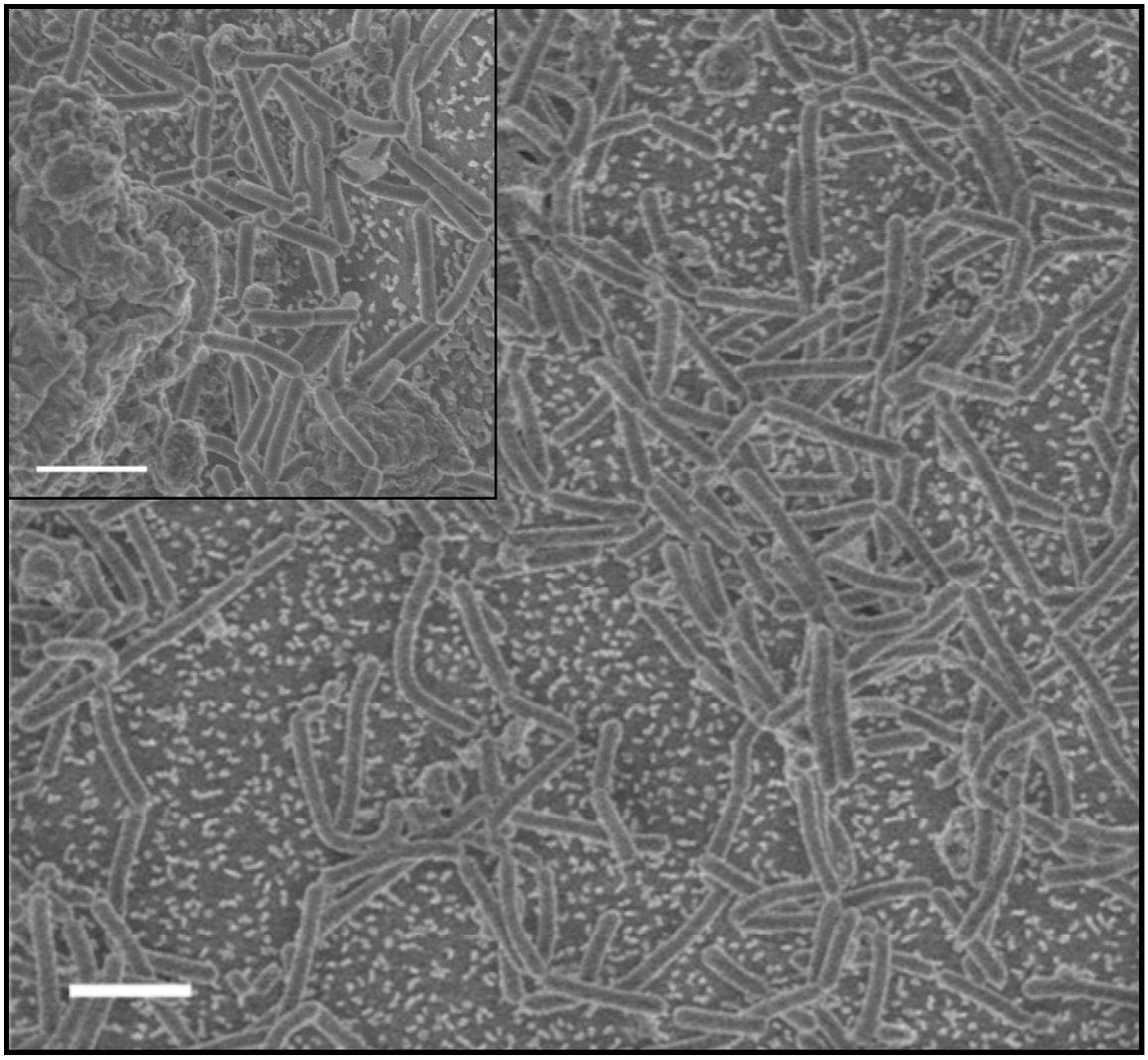


Figure 33. ***L. jensenii*** **(ATCC) colonized the apical surface of air-interface vaginal EC multilayer cultures.** Scanning EM image of a d1 V19I vaginal EC multilayer culture apical surface showing *L. jensenii* colonization at 24h post-addition (bar = 2μm). The images illustrate the rod shaped morphology of *L. jensenii* and show several *L. jensenii* that were in the process of actively dividing prior to fixation of the cultures for EM processing. Bacteria were observed in approximately 25% of the images and the selected image is a representative of >25 fields of view containing bacteria. Additionally, microvilli were present on the apical cell surface.

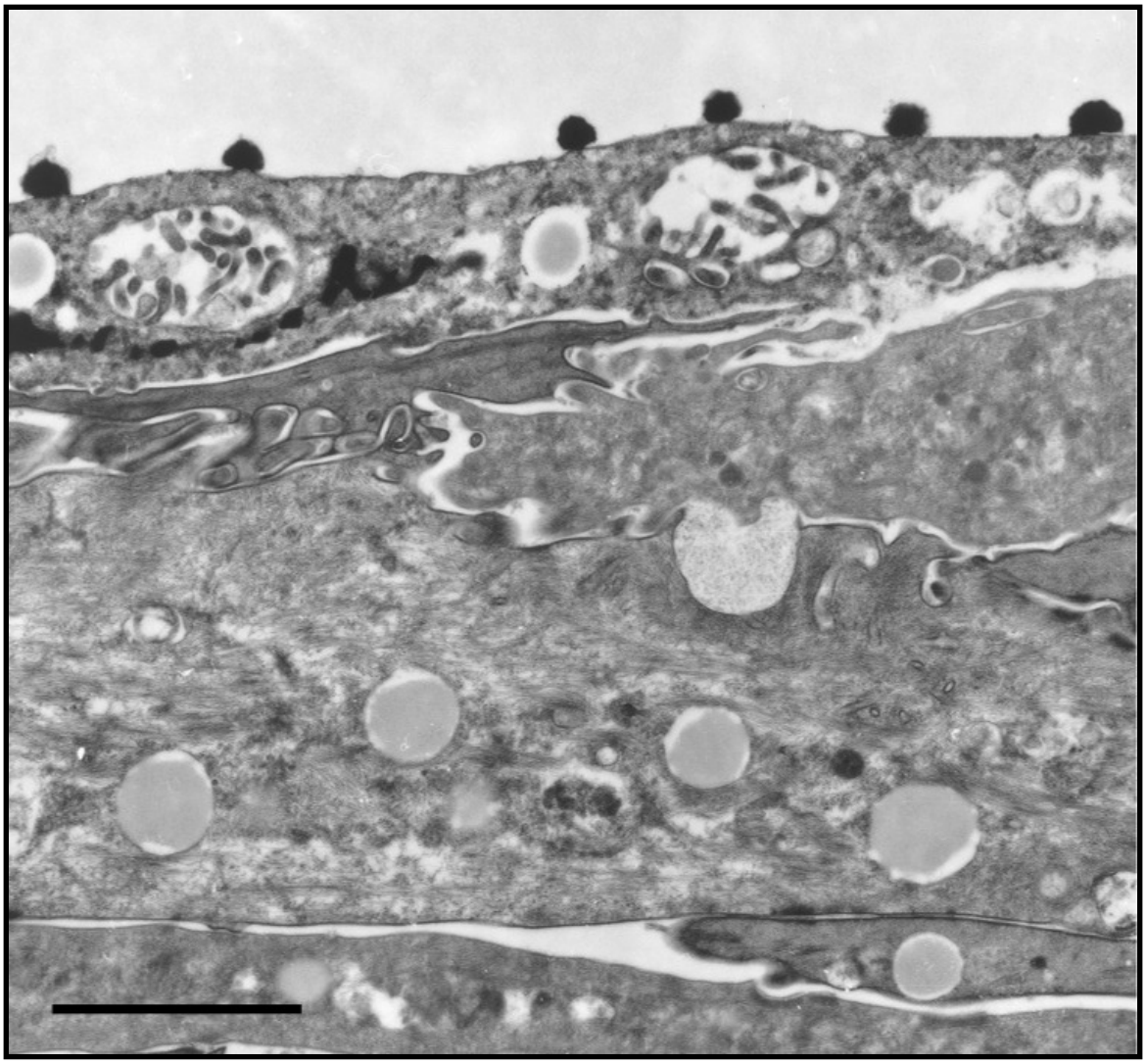


Figure 34. *L. jensenii* (ATCC) colonization was restricted to the apical surface of air-interface vaginal EC multilayer cultures. Transmission EM image of a 1μm cross-sectioned d1 V19I vaginal EC multilayer culture showing *L. jensenii* colonization at 24h post-addition (bar = 2μm). The *L. jensenii* (shown in cross-section as the black circles) were only found on the apical surface of the multilayer cultures. Bacteria were observed in approximately 25% of the images and the selected image is a representative of >25 fields of view containing bacteria.

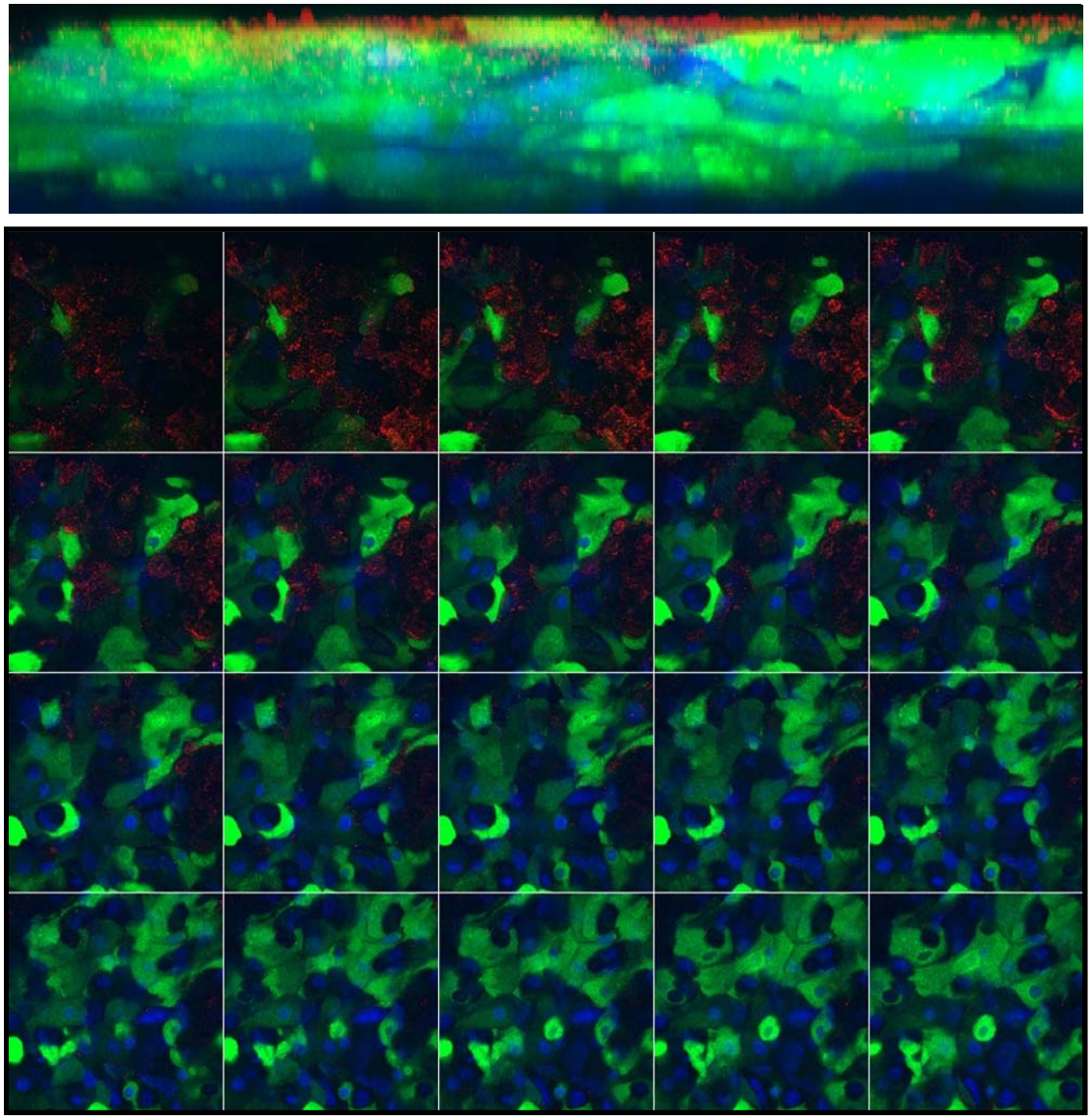


Figure 35. *L. jensenii* (ATCC) only colonized the apical surface of air-interface vaginal EC multilayer cultures without penetrating into the deeper cell layers. Confocal Z-axis projection (630X magnification) of a d1 V19I GFP expressing vaginal EC multilayer culture with DAPI stained nuclei (blue) and antibody labeled *L. jensenii* (red), at 24h post-colonization, showing superficial colonization of the apical cell surface. Serial optical confocal slices through the z-plane moving from the apical surface showed that no red labeling is observed in the deeper cell layers (>slice #11).

The calculated surface area of the vaginal EC multilayer cultures was 80mm^2 representing ~0.1% of the human vaginal mucosal surface [23]. Based on the surface area calculation, we estimated that each culture should stabilize with ~10^5 bacteria according to reported levels of vaginal lactobacilli colonization [114, 115]. To obtain log phase bacteria for addition to the multilayer cultures, the commensals were thawed from frozen aliquots and incubated overnight as described in the bacterial culture section of the methods. The amount of bacteria present in each overnight culture was quantified by adapting the standard serial dilution quantification method with agar plates to a liquid-based, 96-well plate format (Figure 24). The validity of the liquid-based viability assay was confirmed by comparing the two counting methods for both *L. crispatus* and *L. jensenii* cultures. The results showed no significant ($p>0.05$; Student's t-test) difference between the methods for both *L. crispatus* and *L. jensenii* quantification (Figure 36).

The ability of the air-interface vaginal EC multilayers cultures to support commensal bacteria growth and establishment of stable colonization was evaluated using the liquid-based viability assay. The apical surface of the multilayer cultures was seeded with 10^3cfu of selected commensal bacteria from overnight cultures to prevent outstripping of available nutrients and to evaluate potential bacterial growth prior to stabilization at the predicted ~10^5 bacteria per culture. *L. crispatus* (ATCC), *L. jensenii* (ATCC), *L. jensenii* (clinical), *L. rhamnosus* (clinical), *L. casei* (DanActive®) or *L. iners* (CCUG) were added to the multilayer cultures and triplicate cultures from each commensal species were collected 24, 48 and 72h later to quantify viability.

For *L. crispatus*, *L. jensenii* (ATCC), *L. rhamnosus* and *L. casei* the amount of viable bacteria in the cultures significantly ($p<0.05$; Student's t-test) increased over the first 24h to ~10^5cfu then remained relatively stable for at least another 48h (Figure 37). Interestingly, the amount of *L. jensenii* (clinical) in the cultures significantly ($p<0.05$;

Student's t-test) increased by 24h but did not achieve the same amount of colonization as the other commensals. By 48h, the clinical *L. jensenii* strain reached ~10^5 viable bacteria per culture similar to the other commensals and the number of viable bacteria was stable for at least another 24h. The results indicated that the doubling time of the clinical *L. jensenii* was different from the other lactobacilli potentially representing an increased time required to establish stable colonization in the vaginal environment. *L. iners* did not viably colonize the multilayers indicating an inadequate survival niche. The results confirmed the hypothesis that the presence of glycogen in the multilayer cultures could potentially support lactobacilli colonization. Additionally, the data illustrated that the air-interface vaginal EC multilayer cultures were capable of sustaining long-term (>72h) colonization of several commensal bacteria providing additional support for the ability of the cultures to model the vaginal mucosa.

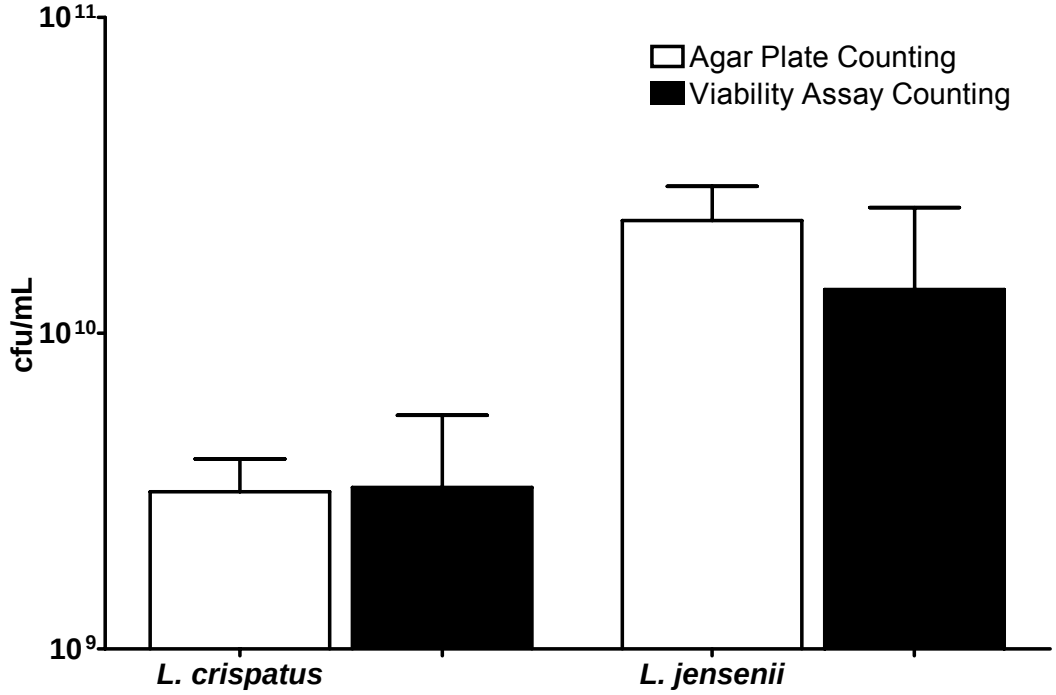


Figure 36. Commensal bacteria were successfully quantified using colony counting on agar plates or a liquid-based vaibility assay. *L. crispatus* (ATCC) or *L. jensenii* (ATCC) were cultured overnight (18h) then the amount of bacteria present was quantified using agar plate counting or the liquid-based viability assay described in the methods section. No significant ($p>0.05$; Student's t-test) difference was observed between the two counting methods for *L. crispatus* or *L. jensenii* quantification. Data are the mean ± SEM of 2 *L. crispatus* or *L. jensenii* cultures for each counting method from 2 independent experiments.

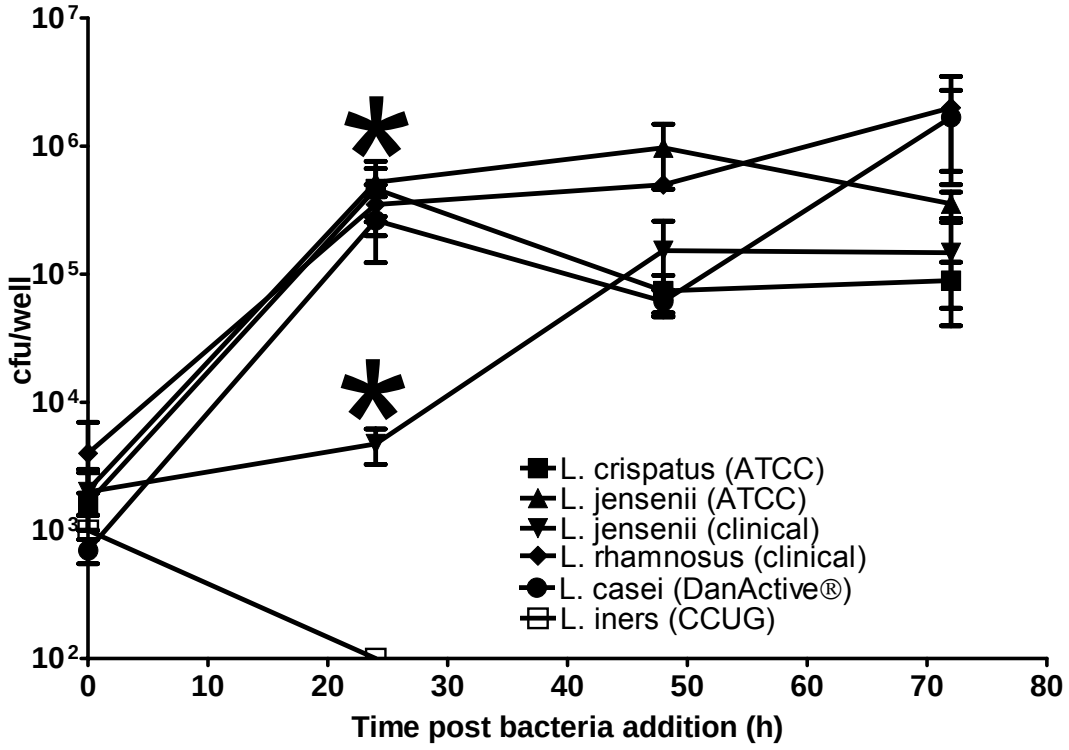


Figure 37. Vaginal EC multilayer cultures supported long-term viability of several commensal bacteria species. *L. crispatus* (ATCC), *L. jensenii* (ATCC), *L. jensenii* (clinical), *L. rhamnosus* (clinical), *L. casei* (DanActive®) or *L. iners* (CCUG) were added at 10^3cfu/well in 10µL of KSFM to d0 vaginal EC multilayer cultures. *L. iners* viability rapidly decreased and no viable bacteria were observed after 24h. *L. crispatus* (ATCC), *L. jensenii* (ATCC), *L. jensenii* (clinical), *L. rhamnosus* (clinical) and *L. casei* (DanActive®) viability significantly (*, $p<0.05$, Student's t-test) increased after 24h of culture and were maintained for at least 72h. Each time point for the selected bacterial species is represented by the mean ± SEM for triplicate wells of each cell type (V11I, V19I) from 2 independent experiments.

The potential physiological impact of commensal colonization on air-interface vaginal EC multilayer cultures was evaluated by measuring cell health, tight junction integrity, and pH changes. *L. crispatus* (ATCC) colonization of d0 V19I multilayers did not significantly ($p>0.05$; Student's t-test) impact the health of the cultures over a 72h time period (0.2 ± 0.03 optical density by MTT assay) compared to vehicle only cultures (0.2 ± 0.02). Evaluation of multilayer integrity using TEER measurements showed no significant ($p>0.05$; Student's t-test) difference between *L. crispatus* (ATCC) cultures ($235.3 \pm 2.2\ \Omega cm^2$) and controls at 72h ($235.6 \pm 4.7\ \Omega cm^2$). Similar outcomes were established for cultures colonized with *L. jensenii* (ATCC). Interestingly, neither *L. crispatus* (ATCC) nor *L. jensenii* (ATCC) altered the pH of the apical surface of colonized vaginal EC multilayers relative to uncolonized cultures (data not shown).

Vaginal EC multilayer cultures elicited cytokine responses to selected commensal bacteria

To evaluate the response of the vaginal EC multilayer cultures to commensal bacteria colonization, we quantified cytokine production in uncolonized and colonized cultures. Because of the commensal bacteria contribution to vaginal defenses [107-112], specifically *Lactobacillus spp.*, vaginal EC must exhibit tolerance towards the commensals while maintaining the capacity to respond to pathogens. Initial evaluations were performed to identify potential cytokine induction in response to the presence of *L. crispatus* (ATCC), *L. jensenii* (ATCC) or *S. epidermidis* colonization. *S. epidermidis* was selected as a non-vaginal commensal control for because the bacteria is isolated occasionally from the human vagina [258] but is more commonly found as a commensal of the skin. Cytokine analyses showed that *L. crispatus* or *L. jensenii* colonization of vaginal EC multilayer cultures did not significantly ($p>0.05$; Student's t-test) elicit

cytokine production compared to that of uncolonized vaginal EC multilayers (Table 11). Interestingly, colonization of the cultures with *S. epidermidis* resulted in a significant ($p<0.05$; Student's t-test) increase in the inflammation-related markers IL-1β, IL-8 and TNF-α (Table 11). Similar results were observed for a clinical isolate of *S. epidermidis* while *L. jensenii* (clinical), *L. rhamnosus* (clinical) or *L casei* (DanActive®) colonization showed no impact (data not shown). The ability of the vaginal EC to selectively respond to bacterial colonization provides a potential explanation for the chronic cytokine production observed in women with BV [128-131].

Table 11. Vaginal EC multilayer cultures selectively responded to commensal bacteria.

	Cytokines[b]			
Bacteria[a]	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
Uncolonized	12 ± 3	11 ± 1	96 ± 11	22 ± 4
L. crispatus (ATCC)	7 ± 1	16 ± 3	83 ± 6	24 ± 3
L. jensenii (ATCC)	10 ± 1	15 ± 2	166 ± 24	17 ± 2
S. epidermidis (ATCC)	43 ±9[c]	31 ± 13	1058 ± 107[c]	130 ± 47[c]

[a] *L. crispatus* (ATCC), *L. jensenii* (ATCC) or *S. epidermidis* (ATCC) at 10^3cfu/well or an equivalent volume of the KSFM vehicle (10μL) were added to the apical surface of V11I d0 multilayer cultures. Apical samples (100μL) were collected 6h later for cytokine analyses.

[b] Data are the mean ± SEM (pg/mL) of 3 replicates from a representative experiment of 4 independent experiments.

[c] $p<0.05$ compared to uncolonized cultures (Student's t-test).

Commensal bacteria colonization altered multilayer vaginal EC cytokine responses to TLR agonist stimulation

The impact of commensal bacteria on the responsiveness of air-interface vaginal EC multilayer cultures to TLR agonist stimulation was evaluated to identify potential immunological contributions of the commensals to the vaginal defenses. Loss of *Lactobacillus spp.* from the vaginal environment and development of BV was linked to chronic IL-1β, IL-6, IL-8 and TNF-α production [128-131]. The observed cytokine response [128-131] is likely due to the presence of BV-associated bacteria; however lactobacilli colonization also potentially modulates the vaginal EC innate immune response to prevent hyper-responsiveness to pathogenic infection. To evaluate the hypothesis, initial experiments were performed at 24h post-colonization with *L. crispatus* or *L. jensenii* (ATCC) because both bacterial species reached a bacterial load of ~10^5cfu by 24h (Figure 37). Based on the ratio of the multilayer culture surface area compared to the vaginal mucosa, a bacterial load of ~10^5cfu in the multilayer cultures is comparable to reported levels of vaginal lactobacilli colonization in women [114, 115]. Also, the multilayer cultures were stimulated with FSL-1, PIC or FLAG, as molecular models of bacterial or viral pathogens, for 6h prior to cytokine analyses to evaluate the impact of commensals on the TLR agonist induced response.

Commensal bacteria colonization selectively reduced the response of the vaginal EC multilayer cultures to TLR agonist stimulation. Specifically, FSL-1 and PIC induction of IL-6, IL-8 and TNF-α was reduced in *L. crispatus* colonized cultures compared to agonist application in uncolonized cultures (Table 12). Cultures colonized with *L. crispatus* only showed a reduction in the IL-6 and IL-8 response following FLAG application. *L. jensenii* (ATCC) colonized vaginal EC multilayers stimulated with any of the 3 tested TLR agonists showed a reduced induction of IL-6 (Table 12). Unlike the *L.*

crispatus colonized multilayer cultures, *L. jensenii* only significantly ($p<0.05$; Student's t-test) reduced FSL-1 but not PIC induction of TNF-α. The results confirmed the hypothesized impact of commensal bacteria and the enhanced tempering observed for *L. crispatus* colonization provides a potential explanation for why *L. crispatus* is the most commonly isolated vaginal commensal bacteria. Also, the tested agonists did not significantly ($p>0.05$; Student's t-test) increase elaboration of IL-1β confirming that the vaginal EC selectively responded to TLR agonist stimulation. The results indicated that commensal bacteria, specifically lactobacilli, colonization of the multilayer cultures selectively tempered the vaginal EC innate immune response to potential bacterial or viral pathogens represented by TLR agonists.

Table 12. Commensal bacteria colonization of vaginal EC multilayer cultures altered responsiveness to TLR agonist stimulation.

	Cytokines[b]			
Bacteria and TLR agonists[a]	**IL-1β**	**IL-6**	**IL-8**	**TNF-α**
FSL-1	1.0 ± 0.1	14.2 ± 1.6	254.1 ± 95.4	14.5 ± 0.8
L. crispatus (ATCC) + FSL-1	1.3 ± 0.1	5.5 ± 2.4[c]	147.6 ± 55.4	9.2 ± 4.8
L. jensenii (ATCC) + FSL-1	1.9 ± 0.1	5.2 ± 0.7[c]	295.9 ± 95.4	4.0 ± 0.2[c]
PIC	1.5 ± 0.1	97.1 ± 5.7	258.4 ± 91.1	25.4 ± 0.2
L. crispatus (ATCC) + PIC	1.8 ± 0.1	36.4 ± 12.4[d]	150.1 ± 53.0	19.6 ± 5.4
L. jensenii (ATCC) + PIC	2.6 ± 0.4	68.4 ± 17.3	300.8 ± 106.1	24.5 ± 1.9
FLAG	0.8 ± 0.2	12.6 ± 0.6	200.1 ± 26.6	24.0 ± 1.8
L. crispatus (ATCC) + FLAG	0.9 ± 0.1	10.0 ± 0.8	148.6 ± 9.5	30.6 ± 5.5
L. jensenii (ATCC) + FLAG	1.2 ± 0.1	9.0 ± 0.5[e]	205.0 ± 21.3	29.5 ± 1.5

[a] *L. crispatus* (ATCC) or *L. jensenii* (ATCC) at 10^3cfu/well or an equivalent volume of the KSFM vehicle (10μL) were added to the apical surface of V11I d0 multilayer cultures. After 24h, FSL-1 (0.1μg/mL), PIC (0.1mg/mL), FLAG (1μg/mL) or vehicle (10μL) were added to the apical surface and samples (100μL) were collected for cytokine analyses 6h later.

[b] Data are the mean fold change (agonist/vehicle or agonist + bacteria/bacteria) ± SEM of 3 replicates from a representative experiment of 4 independent experiments.

[c] $p<0.05$ compared to FSL-1 (Student's t-test).

[d] $p<0.05$ compared to PIC (Student's t-test).

[e] $p<0.05$ compared to FLAG (Student's t-test).

Commensal bacteria tempering of vaginal EC responsiveness was dependent on colonization time and quantity

Based on the differences in multilayer colonization at 24 and 48h post-bacterial addition for the clinical *L. jensenii* strain (Figure 37), we evaluated the impact of different commensal bacteria quantities on the vaginal EC response to TLR agonist stimulation. PIC was selected for evaluation because it was the most robust inducer of a cytokine response (Table 9). Additionally, a 24h agonist stimulation time point was selected to evaluate the impact of commensal bacteria colonization on cytokines produced at a later phase. *L. jensenii* (clinical) also was evaluated in the multilayer cultures to compare the tempering ability of a *Lactobacillus* strain isolated from the vaginal environment the ATCC *L. crispatus* and *L. jensenii* strains.

Again, PIC application induced significant ($p<0.05$; Student's t-test) elicitation of IL-6, IL-8 and TNF-α but not IL-1β at all tested colonization and stimulation time points confirming the selective responsiveness of the vaginal EC. Evaluation of the PIC induced cytokine response profile after 6h of stimulation showed reduced IL-6 and TNF-α expression in multilayer cultures colonized with *L. crispatus* for 24h (Table 13). The *L. crispatus* colonized cultures also showed a significant ($p<0.05$, Student's t-test) reduction in IL-8 induction after 24h of exposure to PIC. The results indicated that *L. crispatus* colonization was more effective at reducing cytokines elaborated following the acute cytokine response. Interestingly, *L. crispatus* significantly ($p<0.05$, Student's t-test) reduced induction of the same cytokines after 48h of colonization and 6h of PIC stimulation potentially indicating that established commensal populations are more effective at tempering the agonist induce cytokine response. A similar ratio of cytokine reduction was observed in multilayer cultures colonized with *L. crispatus* for 24 or 48h following a 24h PIC exposure.

The ability of *L. jensenii* (clinical) to temper the vaginal EC response to TLR agonist stimulation was compared to the results observed for *L. crispatus* or *L. jensenii* (ATCC) colonization. Interestingly, *L. jensenii* (clinical) colonization of the multilayer cultures even at reduced bacterial quantities (24h of colonization; Figure 37) produced a tempering of the PIC induced cytokine response similar to *L. crispatus* colonized cultures (Table 13). The tempering response was even more pronounced once *L. jensenii* (clinical) colonization stabilized at ~10^5cfu after 48h of incubation in the multilayer cultures. The data indicated that the clinical *L. jensenii* strain is potentially an improved model of the commensal bacteria present in the vaginal environment based on the significant tempering response observed at lower bacterial colonization amounts. *L. jensenii* (ATCC) only significantly ($p<0.05$, Student's t-test) reduced vaginal EC multilayer expression of IL-6, IL-8 and TNF-α after 48h of colonization and 24h of exposure to PIC (Table 13). The observed differences between the ATCC *L. crispatus* and *L. jensenii* compared to the clinical *L. jensenii* were potentially due to laboratory adaptations in the ATCC bacteria that attenuated the tempering response. Collectively, the results confirmed that lactobacilli are capable of temporally and selectively tempering the cytokine response to TLR agonists and provided insights into the immunological contribution of the commensals to the vaginal defenses.

Table 13. Commensal bacteria alteration of vaginal EC responsiveness to TLR agonist stimulation was temporally dependent.

	Bacteria and TLR agonists[a]	Cytokines			
		IL-1β	**IL-6**	**IL-8**	**TNF-α**
24h / 6h	PIC	1.3 ± 0.1	11.1 ± 2.5	3.0 ± 0.2	25.8 ± 6.1
	L. crispatus (ATCC) + PIC	1.2 ± 0.2	8.2 ± 1.1	3.2 ± 0.1	14.7 ± 0.4
	L. jensenii (ATCC) + PIC	0.9 ± 0.1	36.2 ± 6.1	5.5 ± 0.5	34.4 ± 3.6
	L. jensenii (clinical) + PIC	1.1 ± 0.1	8.9 ± 2.3	2.3 ± 0.1	22.3 ± 2.4
24h / 24h	PIC	1.2 ± 0.1	30.9 ± 2.3	26.1 ± 8.8	36.5 ± 6.9
	L. crispatus (ATCC) + PIC	1.2 ± 0.1	16.8 ± 4.2[b]	6.2 ± 1.1[b]	7.4 ± 2.4[b]
	L. jensenii (ATCC) + PIC	1.0 ± 0.1	31.8 ± 8.9	16.7 ± 4.7	37.8 ± 9.6
	L. jensenii (clinical) + PIC	0.9 ± 0.2	2.3 ± 0.4[b]	0.9 ± 0.5[b]	1.1 ± 0.4[b]
48h / 6h	PIC	1.1 ± 0.1	13.8 ± 1.5	2.8 ± 0.2	18.2 ± 1.7
	L. crispatus (ATCC) + PIC	1.2 ± 0.3	3.9 ± 1.5[b]	1.3 ± 0.1[b]	6.2 ± 3.0[b]
	L. jensenii (ATCC) + PIC	0.8 ± 0.1	13.7 ± 2.0	4.1 ± 0.3	15.2 ± 3.0
	L. jensenii (clinical) + PIC	0.8 ± 0.3	1.2 ± 0.5[b]	2.4 ± 1.6	0.9 ± 0.1[b]
48h / 24h	PIC	0.8 ± 0.1	3.7 ± 0.7	2.4 ± 0.2	4.1 ± 0.7
	L. crispatus (ATCC) + PIC	0.7 ± 0.1	1.4 ± 0.3[b]	1.2 ± 0.2[b]	1.6 ± 0.6[b]
	L. jensenii (ATCC) + PIC	0.5 ± 0.1	0.8 ± 0.1[b]	0.3 ± 0.1[b]	0.8 ± 0.1[b]
	L. jensenii (clinical) + PIC	0.4 ± 0.1	0.3 ± 0.1[b]	0.2 ± 0.1[b]	0.9 ± 0.1[b]

[a] *L. crispatus* (ATCC), *L. jensenii* (ATCC) or *L. jensenii* (clinical) at 10^3cfu/well or vehicle (10μL) were added to the apical surface of V11I d0 multilayer cultures. After 24 or 48h, PIC (0.1mg/mL) was added apically and samples (100μL) were collected for cytokine analyses 6 or 24h later. Data are the mean fold change ± SEM of 3 replicates.

[b] $p<0.05$ compared to PIC (Student's t-test).

TLR agonist induced anti-herpetic activity was augmented by commensal bacteria colonization of multilayer vaginal EC cultures

Based on the previous observed activity in vitro [Chapters 2 and 3] and the induction of HSV-2 resistance-associated cytokines (Table 9), the ability of TLR agonists to elicit anti-herpetic activity was evaluated in air-interface vaginal EC multilayer cultures. Also, the impact of commensal bacteria on the TLR agonist induced anti-herpetic activity was evaluated based on importance of commensals to response against HSV-2 infection [107-112]. The same TLR agonists (FSL-1, PIC, FLAG) were tested for comparisons to previously observed anti-herpetic in vitro data (Figure 8) [Chapter 2].

Similar to the results from the adapted assay scheme (Figure 8) [Chapter 2], PIC elicited significant ($p<0.05$; Student's t-test) reduction in HSV-2 replication (Figure 38). FSL-1; however only showed a trended reduction while no activity was observed for FLAG application. Colonization of the vaginal EC multilayer cultures with *L. jensenii* did not significantly ($p>0.05$; Student's t-test) alter HSV-2 replication. Interestingly, application of PIC ($p<0.001$; Student's t-test) or FSL-1 ($p<0.05$; Student's t-test) to *L. jensenii* colonized multilayers elicited a significant reduction in HSV-2 replication compared to vehicle treated uncolonized cultures (Figure 38). A non-significant ($p>0.05$; Student's t-test) reduction in viral titers was even observed for colonized cultures exposed to FLAG. The results also were confirmed in V19I multilayer cultures (data not shown). The anti-herpetic activity data illustrated that the air-interface multilayer cultures were capable of modeling TLR agonist interventions strategies for genital HSV-2 infection. Additionally, the data showed that activity against HSV-2 infection in vaginal EC multilayer cultures induced by previously identified anti-herpetic TLR agonists [Chapter 2] through elaboration of a specific cytokine profile [Chapters 3 and 4] was enhanced by the presence of commensal bacteria (Figure 38).

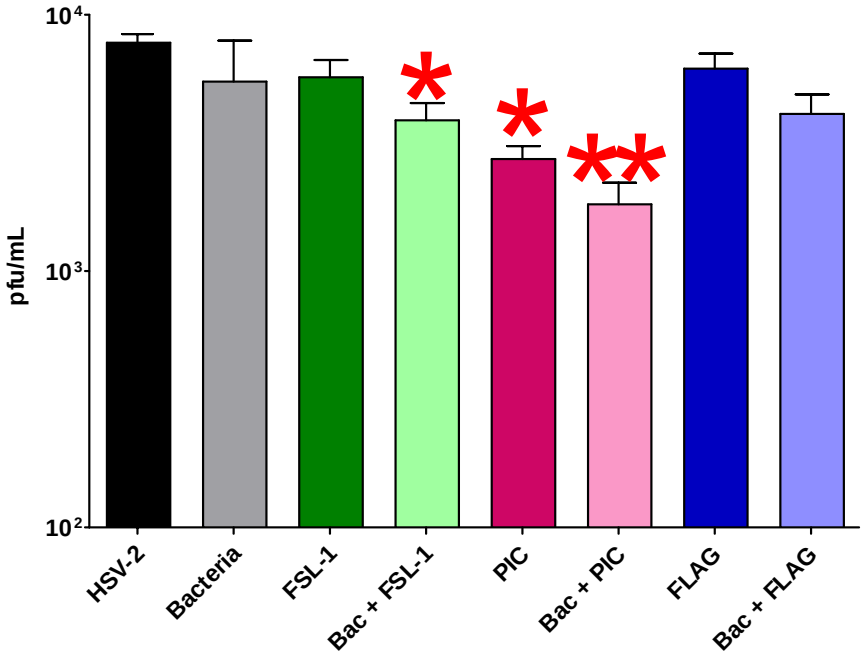


Figure 38. *L. jensenii* (ATCC) colonization of vaginal EC multilayer cultures enhanced TLR agonist induced anti-herpetic activity. *L. jensenii* (ATCC) at 10^3cfu/well or an equivalent volume of the KSFM vehicle (10µL) were added to d0 V11I vaginal EC multilayer cultures. FSL-1 (0.1µg/mL), PIC (0.1mg/mL), FLAG (1µg/mL) or vehicle (10µL) were added 48h later to the apical surface. After 24h, HSV-2 186 (100pfu/well) in 20µL of KSFM was added to the apical surface. The cultures were incubated for 24h prior to sample collection and plaque titration. No significant ($p>0.05$; Student's t-test) reduction in HSV-2 replication was observed for cultures with *L. jensenii* only. PIC alone significantly (*, $p<0.05$; Student's t-test) reduced the viral titer and FSL-1 showed a trended reduction while FLAG exhibited no impact. In *L. jensenii* colonized cultures, a significant reduction in HSV-2 replication was observed for PIC (**, $p<0.001$; Student's t-test) and FSL-1 (*, $p<0.05$; Student's t-test). Results are the mean of triplicate samples ± SEM for a representative experiment from 2 independent experiments with 2 cell types (V11I, V19I).

DISCUSSION

We have produced, for the first time, a air-interface immortalized human vaginal EC multilayer culture containing an air/liquid interface, glycogen, tight junction complexes and microvilli that was capable of supporting long-term (>72h) viable commensal bacteria colonization. The human vaginal mucosa consists of an air/liquid-interface (vaginal lumen/lamina propria) stratified squamous epithelium that contains glycogen and tight junction complexes within the cell layers [22, 23, 42-44]. Using previously immortalized vaginal EC from different donors [50], multilayer cultures were generated and evaluated for tight junction complex and glycogen formation using several different morphological and histological analyses (Figures 28-30). The multilayer cultures showed striking similarities to the human vaginal mucosa including the presence of several hallmark structures [22, 23, 42-44]. Additionally, similar morphological and histological results were observed for vaginal EC multilayer cultures generated using different donor cells and culture formats. The reproducibility confirmed that the vaginal EC could be used by many different laboratories for evaluating the activity and safety of potential anti-herpetic compounds as described previously [Chapter 2]. Additionally, development of the model in a 96 well format enables high-throughput analyses through the use of automation. Due to the renewable nature of the immortalized vaginal EC and the high-throughput capabilities of the model, potential anti-herpetic compounds could be evaluated and the results cross-validated across several laboratories.

Immunological evaluations of the air-interface multilayer cultures showed that the vaginal EC elicited specific cytokine response profiles following TLR agonist application that were consistent with previously observed results for human primary vaginal EC [50; Chapter 3]. Previous in vitro studies showed that FSL-1 or PIC induced anti-herpetic activity that correlated with production of IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β and

RANTES by human immortalized vaginal EC [Chapters 2 and 3]. Similar cytokine response profiles were induced by application of FSL-1 or PIC to the vaginal EC multilayer cultures further validating the ability of the in vitro cultures to model the human vaginal mucosa.

An important aspect of the multilayer cultures was their ability to model phagocyte transepithelial migration. Phagocytes are an important component of the innate immune response to pathogens through phagocytosis, cytokine production and activation of leukocytes like T cells following recruitment to the infection site by chemokine gradients [40, 51, 81]. Based on the significant induction of IL-8, a potent neutrophil chemokine [40, 51, 81], by the tested TLR agonist we evaluated neutrophil transepithelial migration in the multilayer cultures and the results showed that all agonist elicited significant recruitment (Figure 32). The results also indicated that the multilayer cultures provided a model for evaluating recruitment of other leukocytes like DC that are associated with critical activity against HSV-2 infections [40, 51, 81, 103, 104].

As further validation of the model, we evaluated the ability of the air-interface vaginal EC multilayer cultures to support long-term (>72h) stable commensal bacteria colonization. Current animal or ex vivo models are costly, differ from human vaginal physiology and/or lack colonized commensal bacteria [43, 248, 250, 252, 253]. Also, studies involving commensal bacteria in human vaginal mucosa models were limited and did not address the potential impact of bacterial colonization on vaginal EC [112, 123]. Scanning EM of commensal colonized vaginal EC multilayer cultures showed actively dividing *L. jensenii* in intimate contact with the microvilli, another hallmark of the human vaginal mucosa, present on the apical surface of the superficial cells (Figure 33). Additional analyses showed that bacterial colonization was restricted to the apical surface and no penetration of *L. jensenii* was observed in the deeper cell layers (Figures 34 and

35). Using a developed high-throughput liquid-based viability quantification assay (Figure 24), colonization of the vaginal EC multilayer cultures with several species of *Lactobacillus* was observed for at least 72h (Figure 37) except for *L. iners*, a vaginal commensal potentially associated with a transitional environment [116]. The long-term (>72h) stable colonization of the multilayers with several commensal bacteria species further supported the ability of the in vitro cultures to model the human vaginal mucosa. Additionally, the air-interface vaginal EC multilayer cultures provided a model for evaluating to immunological impact of commensal bacteria on the human vaginal mucosa or vaginally-applied anti-herpetic intervention strategies.

Air-interface vaginal EC multilayer cultures were not physiologically impacted by commensal bacteria colonization. Because commensal bacteria, specifically lactobacilli, are present in the vaginal environment and are important for the vaginal defense against pathogens [107-112], it is of critical importance to understand the dynamic interplay between the commensal bacteria and the vaginal mucosa. Colonization of the vaginal EC multilayer cultures with *L. crispatus* or *L. jensenii* did not significantly alter the integrity of the tight junction complexes or the viability of the vaginal EC. Interestingly, no alteration in the pH of the apical multilayer surface was observed after 6, 18 or 24h of colonization with *L. crispatus* or *L. jensenii*. The inability of the lactobacilli to produce a significant pH change was likely due to reduced numbers of bacteria compared to in vivo quantities [114, 115] and the buffering capacity of residual medium in the cultures. Both bacteria shifted the pH from 6.0 to less than 4.0 after 18h of culture in MRS broth and the bacteria were capable of producing lactic acid (Table 10). While both *Lactobacillus spp.* did not significantly alter vaginal EC physiological characteristics, additional evaluations were necessary to identify potential immunological alterations.

The vaginal EC of the multilayer cultures were capable of selectively recognizing and responding to particular commensal bacteria through cytokine elicitation. Previous studies in women with BV linked chronic cytokine production with disruption of the vaginal mucosa and increased susceptibility to STI like HSV-2 [130, 132-135]. *S. epidermidis* colonization of multilayer cultures significantly induced production of IL-1β, IL-8 and TNF-α (Table 11) similar to the cytokine response profiles that were observed for women with BV [128-131]. The observed innate immune response following *S. epidermidis*, a skin commensal, colonization of the multilayer cultures offers a potential explanation for why the bacteria is not commonly isolated from the vaginal environment [258]. The results also indicated that the observed chronic cytokine production in women with BV [128-131] is potentially due to the recognition of and response to BV-associated bacteria by the vaginal mucosa. Interestingly, *L. crispatus* or *L. jensenii*, common vaginal lactobacilli, colonization did not significantly induce a cytokine response in any of the tested cytokines and similar results were observed for clinical isolates of *L. jensenii*, *L. rhamnosus* or *L. casei* from DanActive®. The results indicated that vaginal EC were tolerized to the presence of *Lactobacillus spp.* but retained the capacity to selectively respond to potential pathogens.

Commensal bacteria colonization of the multilayer cultures tempered the response of vaginal EC to TLR agonist stimulation. Loss of *Lactobacillus spp.* from the vaginal environment is associated with the development of BV and chronic production of cytokines [128-131]. The observed cytokine response [128-131] is likely due to the presence of BV-associated bacteria, but it also is possible that lactobacilli colonization modulates the response of the vaginal EC even in the presence of potential pathogens. TLR agonist, as molecular models of bacterial or viral infections, elicited production of IL-6, IL-8 and TNF-α following application to the multilayer cultures. Colonization of

the multilayers with *L. crispatus* or *L. jensenii* (ATCC) showed a reduction in the agonist induced cytokine response compared to uncolonized cultures (Table 12). Interestingly, *L. crispatus* colonization resulted in enhanced tempering compared to *L. jensenii* (ATCC) colonized cultures providing a potential rational for why *L. crispatus* is the most commonly isolated vaginal commensal bacteria. The results showed that the air-interface vaginal EC multilayer innate immune response to potential pathogens was selectively tempered by commensal bacteria colonization.

Vaginal EC multilayer cultures colonized with a clinical isolate of *L. jensenii* showed a significantly reduced cytokine response following TLR agonist stimulation. Interestingly, even with a reduced amount of bacteria present in the cultures (Figure 37) the *L. jensenii* (clinical) tempering activity was as effective as cultures colonized with ~10^5cfu of *L. crispatus* (Table 13). The observed commensal bacteria tempering of the TLR agonist induced cytokine response was more pronounced with increased agonist stimulation or bacterial colonization times. The data illustrated that lactobacilli cultures with low-level sustaining division were more effective at reducing the observed cytokine response then cultures containing commensal bacteria that were actively doubling. Also, the differences in tempering responses between the clinical and ATCC strains of *Lactobacillus spp.* indicated that the clinical stain is potentially a better model of commensal bacteria present in the vaginal environment. Tempering of multilayer inflammation-related marker production by the commensals following TLR agonist stimulation indicated that the bacteria could potentially alter vaginal EC responsiveness to HSV-2 infection or agonist induced anti-herpetic activity.

TLR agonist induced anti-herpetic activity was enhanced by commensal bacteria colonization of air-interface vaginal EC multilayer cultures. Commensal bacteria, specifically *Lactobacillus spp.*, are an important component of the vaginal defense

against STI [107-112]. Colonization of multilayer cultures with *L. jensenii* alone did not alter HSV-2 replication but addition of FSL-1 or PIC to the colonized cultures significantly reduced viral titers (Figure 38). Application of the TLR agonists to uncolonized cultures showed similar activity against HSV-2 infection as observed previously in vitro and in vivo [Chapters 2 and 4]. Additionally, the results are consistent with previous studies that showed loss of lactobacilli colonization was associated with increased susceptibility to HSV-2 infection [11, 13, 46, 47, 49] and provided insights into the potential role of the commensals in the innate immune response of the vaginal mucosa to pathogens.

Collectively, the developed novel air-interface vaginal EC multilayers recapitulated several hallmarks of the human vaginal mucosa and elicited cytokine response profiles similar to primary vaginal EC following TLR agonist stimulation. As further validation of the in vitro human vaginal mucosa model, we showed that the cultures were capable of supporting long-term (>72h) stable colonization of several commonly isolated vaginal commensal bacteria. Additionally, we showed that the vaginal EC multilayer innate immune response to TLR agonist stimulation was selectively tempered by lactobacilli colonization. The results illustrated that lactobacilli play a critical role in maintaining vaginal health and aiding the innate immune response of the vaginal mucosa against pathogenic infection. Based on the ability of the multilayer cultures to support colonization of several commensal bacteria including *S. epidermidis*, a skin commensal, the model could potentially support colonization of BV-associated bacteria enabling evaluations of BV induced pathologies. The developed air-interface vaginal EC multilayer cultures also provide a renewable and reproducible model of the human vaginal mucosa for evaluating and cross-validating anti-herpetic intervention strategies across several laboratories.

CHAPTER 7: IN VITRO AND IN VIVO EVALUATIONS OF SELECTED IMMUNOMODULATORY COMPOUNDS IDENTIFIED AN ANTI-HERPETIC CYTOKINE RESPONSE PROFILE

SUMMARY AND FUTURE DIRECTIONS

HSV-2 primarily infects the genital mucosa and following establishment of latency the virus can periodically reactivate resulting in viral shedding with or without symptoms [3-6]. Up to 70% of heterosexual HSV-2 transmission occurs during asymptomatic shedding episodes [4, 6, 15] and collectively HSV-2 shedding causes 500,000 new infections each year [3, 4, 6]. Worldwide prevalence of HSV-2 varies from 16-97% based on age, culture, ethnicity, geographic location, sex or other factors [3, 9, 10] and of those infected women bear a greater burden of the disease than men [6, 9]. Additionally, recent studies showed that HSV-2 infection is a significant risk factor for acquisition of other STI [3, 11, 13, 19]. Currently, there are no FDA approved HSV-2 vaccines and treatment of infected individuals involves chronic suppressive therapy that does not prevent all recurrences [3, 4, 6, 37]. Considering the prevalence of HSV-2 and the limited treatment options, we undertook a multifaceted approach to identify an HSV-2 resistance-inducing cytokine profile using selected immunomodulatory compounds with and without anti-herpetic activity in human cell culture or small animal models.

In studies presented in Chapter 2, we developed a standardized assay scheme for identifying anti-herpetic microbicides through a NIH-funded collaboration with four other laboratories. Initial evaluations showed that the timing of compound and virus addition and endpoint collection time, termed assay scheme, was the only significant source of variability in quantified compound EC_{50} (Figure 6). The assay scheme developed by our laboratory was selected as the standard for all further MQAP

evaluations based on reproducible quantification of compound EC_{50} and modeling of the proposed microbicide application scheme [138, 139, 141, 201]. Additionally, the misidentification of effective microbicides as non-effective by some of the MQAP laboratories supported the development of a standardized assay scheme for identifying anti-herpetic compounds. Using the developed standardized assay scheme several promising microbicides were identified and cross-validated in the MQAP laboratories (Figure 7). Based on the results of the MQAP studies, the developed standardized assay scheme provides an invaluable resource for identifying novel anti-herpetic microbicides. Through the use of different PCR primers and probes the assay scheme easily can be employed to evaluate the efficacy of microbicides against other STI. The scheme also can be adapted to identify other types of anti-herpetic compounds.

The assay scheme was successfully adapted to identify anti-herpetic immunomodulatory compounds through the use of human vaginal EC and additional compound application times (Figure 5) [Chapter 2]. Due to the altered TLR expression profile of ME-180 cells compared to the vaginal mucosa [50], human vaginal EC were used as an improved model. Additionally, previous experiments illustrated that application of TLR agonists 24 or 6h prior to viral inoculation allowed time for the establishment of a potential HSV-2 resistant environment [181, 184, 185]. The adapted assay showed, for the first time, that FSL-1 significantly inhibited HSV-2 infection and confirmed previously observed PIC activity [184, 185]. The assay also showed lack of activity for FLAG or OTC vaginal products (Figures 8 and 9). Based on the results of the MQAP studies, use of the adapted assay by other laboratories to evaluate TLR agonists or OTC compounds should produce statistically similar results but the outcomes need to be confirmed through another collaborative project. Identification of other anti-herpetic and

non-anti-herpetic TLR agonists is of critical importance for identifying a cytokine response profiles associated with resistance to HSV-2 infection.

Evaluation of the identified anti-herpetic and non-anti-herpetic TLR agonists in human vaginal EC showed that a preliminary anti-herpetic cytokine response profile consisted of IL-2, IL-12(p70), IFNγ, MIP-1α, MIP-1β, and RANTES [Chapter 3]. The induced cytokine profile is associated with the establishment of a T_H1-baised immune response, recruitment of leukocytes and direct inhibition of HSV-2 proteins [51, 57, 74, 77, 79-82, 86, 88]. Initial studies confirmed that the immortalized vagina EC served as a model of the human vaginal mucosa for evaluating TLR agonist induced immunomodulatory activity (Figures 10 and 11). Comparison of cytokine response profiles induced by anti-herpetic (FSL-1, PIC, IMI, CpG) and non-anti-herpetic (Pam, LPS, FLAG) TLR agonists identified selected cytokines of the ones tested that were associated with resistance to HSV-2 infection (Figures 8 and 12). Of note, OTC product or semen simulant application did not induce any of the tested cytokines or alter the vaginal EC TLR expression profile (Figures 14 and 15). Future studies should include evaluation of TLR agonist activity when co-delivered with OTC products to assess the feasibility of their use as carrier vehicles for anti-herpetic compound application in vivo. Additionally, the preliminary anti-herpetic cytokine profile needs to be expanded and refined by evaluating other TLR agonists identified using the adapted assay scheme and by confirming activity using small animal models or refined in vitro models.

FSL-1 applied vaginally in mice significantly increased resistance to experimental genital HSV-2 infection confirming observed in vitro FSL-1 activity [Chapter 4]. Quantification of the protection afforded by FSL-1 application showed a 25-fold increase in resistance to HSV-2 infection and a 10-fold increase in survival of infected mice (Tables 3 and 4). Additionally, resistance to genital HSV-2 infection elicited by FSL-1

application correlated with induction of a cytokine profile consisting of IL-2, IL-12(p40), IL-12(p70), IFNβ, IFNγ, MIP-1α, MIP-1β and RANTES (Table 5). The observed profile was consistent with the previously identified anti-herpetic cytokine profile (Figures 8 and 12) and the profile elicited by other anti-herpetic TLR agonists in vivo [80, 82, 181-185]. Additional studies using knockout mice lacking different combinations of anti-herpetic associated cytokines are warranted to enhance understanding of the interplay between the cytokines and how their coordinated activity produces an HSV-2 resistant environment. Also, preliminary safety evaluations showed that single or multiple consecutive FSL-1 doses did not result in chronic cytokine induction or gross enlargement of selected organs (Figures 17-19). Based on the preliminary safety results, future FSL-1 evaluations should include combinations with OTC compounds or other anti-herpetic compounds to test potential application vehicles for future clinical trials or synergistic preventative approaches, respectively. Evaluation of potential immunomodulatory compound induced therapeutic activity also is necessary due to the high prevalence of HSV-2 infected individuals [3, 9, 10].

SCV-07, an immunomodulatory dipeptide previously shown to elicit IL-2 and IFNγ [190], significantly reduced the incidence and severity of recurrent genital HSV-2 lesions in the guinea pig model [Chapter 5]. Reduction in genital lesions is important because several studies linked HSV-2 infection with increased susceptibility to other STI [3, 11, 13, 19]. SCV-07 applied orally after an overnight fast significantly reduced recurrent genital lesions formation in HSV-2 infected guinea pigs (Tables 7 and 8) confirming the potential use of immunomodulatory compounds as therapeutic interventions. Additionally, SCV-07 combination treatment with acyclovir was not antagonistic (Table 8) supporting the potential augmentation of existing therapies using

immunomodulatory compounds. Future studies should include evaluation of the SCV-07 induced immune response for comparison to the identified anti-herpetic cytokine profile.

In addition to using small animal models, we developed a novel in vitro human vaginal mucosa model for refined evaluations of immunomodulatory compounds [Chapter 6]. Morphological, histological and microscopic analyses confirmed the formation of multiple layers, tight junction complexes, glycogen and microvilli (Figures 28-30, 33) similar to the human vaginal mucosa [22, 23, 42, 43, 118]. Evaluation of TLR agonist induced cytokine response profiles in the multilayer cultures showed similar profiles (Table 9) as observed previously in primary vaginal EC [50; Chapter 3]. TLR agonist induced immunomodulatory activity also significantly induced neutrophil transepithelial migration in the multilayer cultures (Figure 32) supporting use of the model for evaluating recruitment of other phagocytes important for resistance to HSV-2 infection [40, 51, 81]. In addition to the robust and highly coordinate innate immune response [48, 50, 51], vaginal defenses also are aided by the antimicrobial activity of commensal bacteria colonizing the vaginal mucosa [107-112].

To address the contribution of commensal bacteria to vaginal EC elicited innate immune responses, the multilayer cultures were successfully colonized with several different species of commensal bacteria [Chapter 6]. *Lactobacillus spp*., specifically *L. crispatus* and *L. jensenii*, are important for the maintenance of a healthy vaginal environment through pH modulation, adherence prevention and antimicrobial protein production [107-112]. Initial evaluations used lactobacilli based on their ability to metabolize glycogen, produced by the multilayer cultures (Figure 28), and their importance to vaginal defenses [107-112]. The results showed that the bacteria were capable of stably colonizing the apical surface of vaginal EC multilayer cultures (Figures 33-35, 37). Immunological evaluations showed that the vaginal EC selectively elicited

cytokine production in response to *S. epidermidis* colonization but not *Lactobacillus spp.* including the non-vaginal commensal *L. casei* (Table 11). The cytokine results indicated that tolerization of the vaginal EC to vaginal lactobacilli potentially also extends to non-vaginal lactobacilli consistent with published reports concerning the probiotic usage of *L. rhamnosus* or *L. casei* [259, 260]. Recent studies showed significantly increased production of inflammation-related markers like IL-1β, IL-6, IL-8 and TNF-α in women with BV compared to women colonized with lactobacilli [128-131] providing additional support for the importance of lactobacilli for maintaining a healthy vaginal environment.

Lactobacilli tempered the TLR agonist induced cytokine response in colonized multilayer vaginal EC cultures [Chapter 6]. The *Lactobacillus spp*. mediated cytokine reductions were observed for several TLR agonists and dependent upon colonization or stimulation times (Tables 12 and 13). Of the tested commensal bacteria, *L. crispatus* induced significant reductions in the cytokine responses at three out of the four tested time points. A clinical isolate of *L. jensenii* was more effective at reducing cytokine induction than a laboratory adapted *L. jensenii* indicating that clinical isolates potentially provide a better model of vaginal commensal bacteria. The observed varying reductions in the induced cytokine responses also offers a potential explanation for why *L. crispatus* is the most commonly isolated vaginal commensal bacteria followed by *L. jensenii* [114, 115]. Understanding the impact of commensal bacteria on anti-herpetic compound induced immunomodulatory activity is critically important for future clinical trials involving women with varying vaginal bacterial ecologies.

L. jensenii colonization of air-interface vaginal EC multilayer cultures enhanced the anti-herpetic activity of TLR agonists compared to uncolonized cultures [Chapter 6]. *L. jensenii* colonization of vaginal EC multilayer cultures enhanced FSL-1 and PIC induced anti-herpetic activity and even produced a reduction in HSV-2 replication in

cultures treated with the non-anti-herpetic TLR agonist, FLAG (Figure 38). Tempering of inflammation-related markers and enhancement of resistance to HSV-2 infection following TLR agonist stimulation offers potential insights into why loss of lactobacilli colonization is associated with increased susceptibility to STI [130, 132-135]. Additionally, the synergistic effect of *L. jensenii* on the vaginal EC induced anti-herpetic innate immune response following TLR stimulation further underscores the importance of continued evaluations using other commensal bacteria like *L. crispatus* or different colonization or stimulation time periods. The observed anti-herpetic enhancement is potential due to production of D-lactic acid by *L. jensenii*, cytokine tempering or some as yet unidentified factor. Confocal analyses of HSV-2 cell-cell spread in infected vaginal EC multilayers colonized with commensal bacteria and/or treated with TLR agonists or vehicle also could provide insights into the observed replication reduction. Additionally, the in vitro commensal bacteria colonized vaginal EC multilayer cultures provide a renewable and reproducible model for evaluating novel anti-herpetic intervention strategies that could potentially complement existing models of HSV-2 infection.

Continued evaluation and development of vaginally-applied anti-herpetic compounds is of vital importance for placing STI prevention control in the hands of women [138, 139, 141]. Based on the inability of women to dictate condom usage during a sexual encounter in some cultures or due to associated social stigmas [138, 139, 141], vaginally-applied anti-herpetic strategies that allow the option for women to covertly apply the compounds are a critical component in reducing the spread of STI. Additionally, the compounds can be formulated in combination with OTC feminine products to increase ease of use and compliance [141-144]. The success observed with SCV-07, an immunomodulatory dipeptide, against recurrent genital HSV-2 disease following oral delivery [Chapter 5] supports the use of alternate application routes for the

anti-herpetic compounds. Recently, several studies focused on evaluating the activity of rectally-applied compounds against STI potentially introduced through receptive anal intercourse [261-265]. Adaptation of vaginally-applied anti-herpetic compounds for rectal application would provide an additional resource for reducing STI but further evaluations of compound activity in a model of the rectal mucosa are necessary.

Anti-herpetic intervention strategies also are important because they provide an advantage over current antiviral compounds based on their novel mechanisms of action against HSV-2 infections. Nucleoside analogs like acyclovir inhibit HSV-2 replication thereby reducing cell-to-cell spread of the virus but not initial infection of the vaginal EC [3, 4, 6, 31]. Anti-herpetic intervention strategies like immunomodulatory compounds act by eliciting a vaginal EC induced innate immune response that establishes resistance to HSV-2 infection prior to introduction of the virus into the vaginal environment [51, 57, 138-141]. The "primed" innate immune response is capable of reducing initial HSV-2 infection of the vaginal EC [51, 57, 138-141; Chapters 2-4, 6]; therefore combinations with nucleoside analogs potentially could reduce initial infection and replication in infected cells. Support for combination intervention strategies was provided by the studies in Chapter 5 that showed SCV-07 activity was not antagonistic with acyclovir treatments (Table 8). Based on the observed promising in vivo results, additional evaluations of combination anti-herpetic interventions are warranted to further compliment current strategies to reduce HSV-2 transmission.

Collectively, the in vitro and in vivo studies addressed the hypothesis that cytokines important for establishing an environment resistant to HSV-2 infection could be identified using selected anti-herpetic and non-anti-herpetic immunomodulatory compounds. Induction of IL-2, IL-12(p40), IL-12(p70), IFNβ, IFNγ, MIP-1α, MIP-1β and RANTES was associated with significant resistance to genital HSV-2 infection.

Identification of cytokines associated with anti-herpetic activity is important for the future design of immunomodulatory compounds that selectively elicit production of only the HSV-2 resistance-associated cytokines. The identified anti-herpetic cytokine response profile also aids optimization of immunostimulatory compounds for use as vaccine adjuvants. Additional possible uses of the optimized compounds include combinations with existing vaccines, like the gD adjuvant vaccine [27], or current anti-herpetic therapies to improve efficacy. Development and use of improved anti-herpetic immunomodulatory compounds is critical for preventing genital HSV-2 infections and reducing the spread of other STI. Overall, studies detailed in this dissertation expanded understanding of the complex role that human vaginal EC play in coordinating innate immune responses to potential pathogens while maintaining tolerance towards beneficial commensal bacteria that augment the immune response. Additionally, the developed assay schemes and models described in this dissertation easily can be adapted to identify innate immune responses important for resistance to other STI and aid in the design of novel intervention strategies to collectively reduce the spread of the global STI epidemic.

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VITA

William A. Rose II was born on December 27, 1978 in Nederland, Texas. William is the son of Wayne E. Rose and Susan A. Rose. While at graduate school, William received several honors. In 2007, William was selected for a platform presentation of his research entitled "Quantitative Evaluation of the Mechanism of Immunomodulation Afforded by TLR Application in Utilizing Polarized Multilayer Human Vaginal Epithelial Cultures" at the 17th International Society for Sexually Transmitted Disease Research meeting Seattle, Washington in 2007. William also was awarded a travel grant by the ISSTDR to present his work at the meeting. Additionally, in 2007 William received the Christina Fleischmann Award for his outstanding academic performance, exceptional skill in conducting laboratory research and excellence in teaching. In 2008, William was awarded the UTMB Retirees Scholarship Award for his performance as an outstanding graduate student.

While at the University of Texas Medical Branch, William had the opportunity to serve as a research mentor for Alywn Rapose, a fellow in the Department of Infectious Disease. The collaborative research entitled "An Ex-Vivo Model of Human Vaginal Epithelium Colonized by the Commensal *Lactobacillus jensenii*" was selected for a platform presentation at the 15th International Union against Sexually Transmitted Infections (IUSTI) Asia Pacific Congress in Dubai, UAE. William also has served as a judge for both the University of Texas Medical Branch Department of Pathology Annual Research Trainee Day and the Summer Undergraduate Research Program poster presentations.

William has accepted a post-doctoral position in the Department of Microbiology and Immunology at Cornell University in Ithaca, New York.

Education

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Publications

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