Regulation and Function of IL-36γ in Genital HSV-2 Infection and Disease Pathogenesis

by

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ABSTRACT

An estimated 267 million women worldwide are HSV-2 seropositive, including roughly 20% of reproductive-aged American women. HSV-2 is a neurotropic virus that establishes a persistent, life-long infection that increases risk for STI acquisition in individuals. The vaginal epithelium represents a critical first line of defense against infection, and during acute infection, underlying immune mechanisms in the epithelium may be critical to protect against disease pathogenesis. The recently identified pro-inflammatory cytokine IL-36γ has been shown to be expressed at mucosal epithelia, including the female reproductive tract (FRT) and may be an important factor in host defense. Although IL-36γ has been shown to be induced in the FRT after exposure to microbial products, the contributions of IL-36γ to host defense mechanisms in response to this clinically relevant STI pathogen are not well understood. This dissertation describes the regulation of IL-36γ in the FRT and explores its contribution to the host response against genital HSV-2 infection.

To test the hypothesis that IL-36γ is a key regulator of mucosal inflammation and immunity in the FRT, hormonal regulation of IL-36γ in the FRT was investigated using estrogen- and progesterone-conditioned mice. From this preliminary study, it was shown that progesterone dampens *IL36G* expression relative to estrogen and may potentially increase susceptibility to infection. Next, the impact of IL-36γ treatment on HSV-2 infection and replication in human 3-D vaginal epithelial cells was explored. In parallel, the impact of intravaginal IL-36γ delivery on HSV-2 disease pathogenesis was evaluated using a lethal murine challenge model. IL-36γ pre-treatment significantly limited HSV-2

replication *in vitro* and *in vivo* and was associated with transient neutrophil infiltration that corresponded with decreased disease severity and increased survival in mice. Last, the requirement for IL-36γ in host defense was investigated utilizing IL-36$\gamma^{-/-}$ mice in a lethal HSV-2 murine challenge model. Following infection, IL-36$\gamma^{-/-}$ mice exhibited significantly impaired neutrophil recruitment, decreased overall survival time, and significantly increased viral neuroinvasion relative to wild type mice. Collectively, these data indicate that IL-36γ is a crucial regulator of HSV-2-induced neutrophil infiltration and appears to function in a previously uncharacterized manner to limit viral neuroinvasion in genital HSV-2 disease pathogenesis.

DEDICATION

I dedicate this body of work to my wife, daughter and supportive family

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There are countless individuals that have influenced me as I reach this stage in my education and career. It all started with Gretel von Bargen, my high school biology teacher. Her passion for teaching and learning was infectious and gave me a clear direction as I started university. At Brigham Young University, my undergraduate research mentor Dr. William McCleary was instrumental in getting me into his lab early on in my studies and teaching me how to work in a lab. Drs. Brian Poole and Don Breakwell let me work for them as a teaching assistant and helped to foster a love of teaching and expanded my understanding in the principles of microbiology, immunology, and virology. I am grateful for these and numerous others that provided meaningful experiences and influence that led me to attend graduate school

I need to recognize, thank, and express my gratitude for my mentor, Dr. Melissa Herbst-Kralovetz. From the first time I talked with her over Skype, I could tell that she cared about me and my own development. I am so grateful that she took a chance on me as her first doctorate student. Melissa leads in the lab by example and I couldn't ask for a more supportive mentor. She has taught me how to think, how to face and overcome challenges, and how to plan for and achieve my goals.

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TABLE OF CONTENTS

Page

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF ABREVIATIONS xii

CHAPTER

1 INTRODUCTION 1

The IL-36 Cytokines: New Kids on the Block 1

HSV-2 and Genital Herpes 11

Overview of Dissertation 16

2 THREE-DIMENSIONAL ROTATING WALL VESSEL-DERIVED CELL CULTURE MODELS FOR STUDYING VIRUS-HOST INTERACTIONS 17

Publication Note 17

Abstract 17

Introduction 18

Virus Infection and Replication in 3-D Bioreactor Models 23

Three-Dimensional Models of Human Neuronal Cells to Study Persistent Viral Infection 26

Three-Dimensional Models of Lymphoid Tissue and Circulating Lympocytes for Long-Term Culture to Study Virus Replication, Latency and Reactivation 28

CHAPTER Page

Three-Dimensional Models for Respiration Viral Infection 30

Three-Dimensional Models for Studying Viral Gastroenteritis 32

Three-Dimensional Models of Liver Tissue for Studying Hepatitis Viral Infections 35

Three-Dimensional Models of Female Reproductive Tract Tissues for Studying Sexually Transmitted Infections and Emerging Viruses 37

Future Directions for RWV Bioreactors 42

Conclusions 44

3 HORMONAL REGULATION OF IL-36γ AND HOST DEFENSE MECHANISMS IN THE FEMALE REPRODUCTIVE TRACT 45

Abstract 45

Introduction 46

Materials and Methods 49

Results 51

Discussion 56

4 IL-36γ INDUCES A TRANSIENT HSV-2 RESISTANT ENVIRONMENT THAT PROTECTS AGAINST GENITAL DISEASE AND PATHOGENESIS 61

Publication Note 61

Abstract 61

CHAPTER Page

Introduction ... 62

Materials and Methods ... 66

Results ... 72

Discussion ... 83

5 IL-36γ IS A KEY REGULATOR OF NEUTROPHIL INFILTRATION IN THE VAGINAL MICROENVIRONMENT AND LIMITS NEUROINVASION IN GENITAL HSV-2 INFECTION ... 89

Publication Note ... 89

Abstract ... 89

Introduction ... 90

Materials and Methods ... 93

Results ... 98

Discussion ... 110

6 FINAL SUMMARY AND IMPLICATIONS OF THE RESEARCH PRESENTED ... 116

REFERENCES ... 127

APPENDIX

A AUTHOR APPROVAL FOR PUBLISHED PAPERS ... 161

B CHAPTER 4 SUPPLEMENTARY MATERIAL ... 163

C CHAPTER 5 SUPPLEMENTARY MATERIAL ... 167

LIST OF TABLES

Table Page

1. Cell Expression Profiles of the IL-36 Cytokines 4

2. Tissue- and Disease- Specific Expression of the IL-36 Cytokines......................... 5

3. Host-Virus Interactions in RWV Bioreactor-Derived 3-D Aggregates................ 25

4. Primers Used in qPCR 51

5. Primers Used in This Study 71

6. Primers Used in qPCR Analysis 95

7. IL-36γ Treatment Delayed Disease Onset and Increased Survival Time in a Dose- and Time-Specific Manner when Challenged with 35×LD50 Dose of HSV-2. 166

LIST OF FIGURES

Figure Page

1. Il-36R Signaling Activates MAPK and NFκB Pathways 2
2. IL-36γ Signaling in Host Defense 9
3. Culturing 3-D Aggregates in the RWV Bioreactor 22
4. Example of Physiological Features of RWV Bioreactor-Derived Human 3-D Vaginal Aggregates 39
5. Effect of Sex Hormones on Mouse FRT Tissue 52
6. Sex Hormones Regulate Expression of Barrier Features in the FRT 53
7. Sex Hormones Regulate Immune Mediator Expression in the FRT 54
8. Hormonal Regulation of the IL-36 Cytokines 56
9. IL-36γ is Induced by HSV-2 and IL-36γ Treatment Limits HSV-2 Infection in 3-D VEC 73
10. IL-36γ Treatment 4h Prior to Infection Significantly Protects Against HSV-2 Disease Incidence, Reduces Disease Severity, and Enhances Survival 77
11. IL-36γ Treatment Induces the Transient Production of Immune Mediators in Murine Lower FRT Tissue and Vaginal Lavages 80
12. IL-36γ Transiently Promotes Polymorphonuclear Leukocyte Infiltration in the Vaginal Microenvironment 83
13. IL-36γ Induces Transient Neutrophil Recruitment to the Vaginal Microenvironment in WT Mice 99

Figure Page

14. Neutrophil Recruitment is Significantly Reduced in IL-36γ$^{-/-}$ Mice After Genital HSV-2 Infection 101

15. Recruitment of Mature Neutrophils to the Vaginal Microenvironment is Impaired in HSV-2 Challenged IL-36γ$^{-/-}$ Mice 103

16. Ccxl1/KC Expression is Reduced in IL-36γ-/- Vaginal Tissue After HSV-2 Challenge 104

17. IL-36γ Protects Against Genital HSV-2 Infection in a Neutrophil Independent Manner. 106

18. Increased Incidence of Hind Limb Paralysis and Productive Infection in the Brainstem in IL-36γ$^{-/-}$ Mice After Genital HSV-2 Infection 107

19. Rapid Systemic Spread of HSV-2 in IL-36γ$^{-/-}$ Mice 109

20. IL-36γ Does Not Limit HSV-2 Replication in a Dose-Dependent Manner and Pre-Treatment with IL-36Ra Does Not Significantly Alter HSV-2 Replication 164

21. IL-36γ Induces Expression of Cytokines, Chemokines and Immune Signaling Molecules in Murine FRT Tissue 4h After Treatment 165

22. Neutrophil Recruitment is Impaired in Vaginal Tissue From IL-36γ$^{-/-}$ Mice After Genital HSV-2 Infection 168

23. Neutrophil depleted mice exhibit disease progression and survival similar to WT mice.. 169

LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
CLR	C-type lectin
CVL	Cervicovaginal lavages
dPI	Days post infection
FRT	Female reproductive tract
h	Hour
HSV-2	Herpes simplex virus 2
ID50	Infectious dose, 50%
IL-	Interleukin-
LD50	Lethal dose, 50%
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PMN	Polymorphonuclear leukocyte
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern-recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
STI	sexually transmitted infection
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor

CHAPTER 1: INTRODUCTION

The IL-36 Cytokines: New Kids on the Block

Characteristics and Mechanisms of Action

At the turn of the 21st century researchers identified a series of novel, uncharacterized genes located on chromosome 2, near the Interleukin-1 (IL-1) locus (Barton et al. 2000, Busfield et al. 2000, Mulero et al. 1999). These genes exhibited homology to several IL-1 family members, and were originally hypothesized to function as important inflammatory mediators (Barton et al. 2000, Busfield et al. 2000, Mulero et al. 1999). Two years after their initial discovery, these cytokines were named IL-1F6, IL-1F8, IL-1F9 and IL-1F5 and designated as members of the IL-1 superfamily (Sims et al. 2001). Over time these new IL-1 family members were shown to exhibit distinct biologic functions and were subsequently renamed and given unique interleukin designations as IL-36alpha (-α), beta (-β), gamma (-γ), and receptor antagonist (-Ra) (Dinarello et al. 2010). The IL-36 cytokines share approximately 20-50% homology to the pro-inflammatory cytokine IL-1β, and also share homology to IL-1Ra (Gresnigt and van de Veerdonk 2013). Additionally, the structure of both IL-36γ and IL-36Ra contain a 12-fold β-trefoil core structure, a common structural feature in many classical IL-1 cytokines (Dunn et al. 2003, Gunther and Sundberg 2014). Similar to other IL-1 cytokines, the IL-36 agonists lack a signal peptide and require cleavage at the N-terminus to be fully activated (Towne et al. 2011). A recent report has elucidated mechanisms of IL-36 family activation and showed that IL-36α is cleaved and activated by neutrophil elastase and

cathepsin G, IL-36β by cathepsin G, and IL-36γ is cleaved and activated by neutrophil elastase and proteinase-3 (Henry et al. 2016).

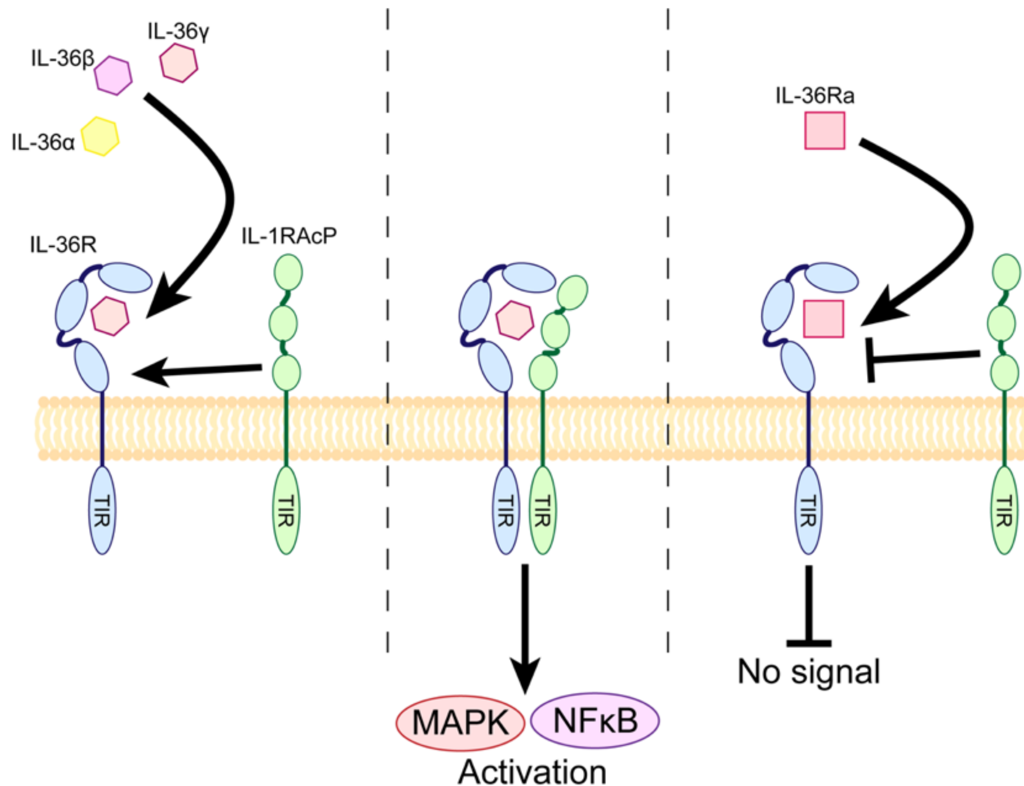

Figure 1. IL-36R signaling activates MAPK and NFκB pathways. Binding of the cleaved IL-36 agonists (α, β, or γ) recruits IL-1RAcP to the IL-36R complex on the plasma membrane surface. IL-36R signaling through Toll/Interleukin-1 receptor (TIR) domains is MyD88 dependent.

Activated IL-36 family members signal through their own unique receptor, IL-36R. Binding of the IL-36 agonists (-α, -β, or -γ) to IL-36R initiates the recruitment of the IL-1 receptor accessory protein (IL-1RAcP) as an essential co-receptor for signaling (**Fig. 1**) (Towne et al. 2004). Both IL-36R and IL-1RAcP contain an intracellular Toll/IL-1 (TIR) domain that will then trigger downstream signaling cascades, including NFκB and MAPK pathways, through the adaptor protein MyD88 (Towne et al. 2004). Activation of NFκB and other transcription factors robustly induce the production of

immune mediators that promote inflammation, direct immune cell recruitment and polarization, and regulate cellular proliferation and gene expression among other processes (Gabay and Towne 2015). In addition to paracrine signaling effects, the IL-36 cytokines can signal in an autocrine manner, thus amplifying IL-36R signaling and downstream immune effects. IL-36Ra functions as an antagonist in the family and will bind to IL-36R to prevent agonist binding to the receptor and the subsequent downstream signaling cascade (Towne et al. 2004, Towne et al. 2011).

Tissue Expression Profiles of the IL-36 Cytokines

Although classical IL-1 family members are generally ubiquitously expressed throughout the human body, the IL-36 cytokines are expressed in a more tissue-restricted manner. The IL-36 family members are expressed in both immune and non-immune cells, with predominant expression in the skin and at mucosal epithelia (Gresnigt and van de Veerdonk 2013). Researchers have also shown that some IL-36 family members are additionally expressed in adipose tissue (Do et al. 2006, van Asseldonk et al. 2010), joints (Frey et al. 2013, Magne et al. 2006), the kidneys (Ichii et al. 2010), and the central nervous system (Berglof et al. 2003, Li et al. 2018). As outlined in **Table 1**, the IL-36 family members are expressed in a wide range of cell types, and in some instances, in a non-overlapping manner, indicating that the IL-36 family members may have distinct cell- and tissue-specific functions.

Table 1.

Cell Expression Profiles of IL-36 Cytokines

IL-36 Family Member	Cells Expressed In	References
IL-36α	Keratinocytes Epithelial Cells Endothelial Cells Chondrocytes Monocytes T/B Lymphocytes	(Blumberg et al. 2007, Boutet et al. 2016, Chustz et al. 2011, Conde et al. 2015, Debets et al. 2001, Smith et al. 2000)
IL-36β	Keratinocytes Epithelial Cells Endothelial Cells Monocytes/Macrophages Dendritic Cells T/B Lymphocytes	(Boutet et al. 2016, Chustz et al. 2011, Johnston et al. 2011, Li et al. 2010, Magne et al. 2006, Smith et al. 2000)
IL-36γ	Keratinocytes Epithelial Cells Monocytes/Macrophages Dendritic Cells Spinal Neurons	(Ahsan et al. 2016, Bachmann et al. 2012, Boutet et al. 2016, Debets et al. 2001, Li et al. 2018, Takahashi et al. 2015, Winkle, Throop, and Herbst-Kralovetz 2016)
IL-36Ra	Keratinocytes Epithelial Cells Monocytes Dendritic Cells	(Blumberg et al. 2007, Boutet et al. 2016, Chustz et al. 2011)
IL-36R	Keratinocytes Epithelial Cells Monocytes/Macrophages Dendritic Cells T Cells Glia	(Ahsan et al. 2016, Berglof et al. 2003, Foster et al. 2014, Johnston et al. 2011, Vigne et al. 2011, Winkle, Throop, and Herbst-Kralovetz 2016)

As described above, the IL-36 cytokines are predominately expressed in the skin and by epithelial cells at mucosal surfaces, including the gastrointestinal tract, respiratory tract, and female reproductive tract (FRT). In the FRT, all IL-36 family members have been detected in uterine tissue in mice, with IL-36γ as one of the most robustly expressed family members (Murrieta-Coxca et al. 2016). Further, Winkle et al. demonstrated that IL-36γ and IL-36R are expressed in human vaginal and cervical tissue and 3-D human vaginal and endocervical epithelial cell models (Winkle, Throop, and Herbst-Kralovetz

2016). Data from studies in the FRT and other tissues indicate that IL-36 family member expression may be differentially regulated in various tissues and in response to disease (**Table 2**), and future studies may elucidate mechanisms controlling expression patterns and responses to different stimuli.

Table 2.

Tissue- and Disease-Specific Expression of the IL-36 Cytokines

Tissue	IL-36 Family Members	Disease/Infection	References
Adipose	IL-36α	Obesity	(Do et al. 2006, van Asseldonk et al. 2010)
Blood	IL-36α IL-36γ	SLE	(Ichii et al. 2010)
Central Nervous System	IL-36γ IL-36Ra	Brain tissue inflammation Chronic inflammation	(Berglof et al. 2003, Li et al. 2018)
Colon	IL-36α IL-36γ	IBD Ulcerative colitis Crohn's disease Colorectal carcinoma	(Nishida et al. 2016, Russell et al. 2016, Weinstein et al. 2017, Weinstein et al. 2019)
Female Reproductive Tract	IL-36α IL-36β IL-36γ IL-36Ra	*L. monocytogenes* Group B *Streptococcus*	(Murrieta-Coxca et al. 2016, Patras et al. 2015, Patras et al. 2013)
Joints	IL-36α IL-36β	Rheumatoid arthritis Psoriatic arthritis	(Frey et al. 2013, Kim et al. 2008, Magne et al. 2006, Wu and Gu 2007)
Kidney	IL-36α	Nephrotic syndrome Tubulointerstitial lesions Glomerulonephritis Streptozocin-induced diabetes	(Ichii et al. 2010)
Lungs	IL-36α IL-36γ IL-36Ra	COPD Asthma *K. pneumoniae* *S. pneumoniae* *L. pneumophilia* Influenza virus	(Aoyagi et al. 2016, Chen et al. 2012, Kovach et al. 2017, Nanjo et al. 2019, Ramadas et al. 2006)
Skin	IL-36α IL-36β IL-36γ IL-36Ra	Psoriasis DITRA HSV-1 *S. aureus*	(Blumberg et al. 2007, Carrier et al. 2011, Mahil et al. 2017, Milora et al. 2017, Ohko et al. 2018)

IL-36 and Chronic Inflammatory Diseases

Given the pro-inflammatory nature of the IL-36 family members and expression in keratinocytes, the IL-36 cytokines were initially investigated in the context of chronic inflammatory diseases, including psoriasis. Blumberg et al. first demonstrated that transgenic mice overexpressing IL-36α in basal keratinocytes exhibit increased skin inflammation and a psoriasis-like phenotype (Blumberg et al. 2007). Subsequent investigations utilizing clinical samples and animal models further demonstrated that IL-36 signaling in psoriasis drives a Th1 and Th17 response and robust leukocyte infiltration in psoriatic lesions (Carrier et al. 2011, Mahil et al. 2017). Further, increased levels of the IL-36 cytokines, including IL-36γ, have been measured in psoriatic lesions, and the IL-36 agonists were shown to upregulate psoriasis-related genes in keratinocytes to drive psoriasis development (Ohko et al. 2018). Deficiencies in IL-36Ra have also been observed in individuals, and is associated with increased inflammatory markers, high fever and generalized pustular rash referred to as deficiency of the IL-36 receptor antagonist (DITRA), highlighting the importance of this antagonist in regulation of IL-36R signaling and the inflammatory response (Cowen and Goldbach-Mansky 2012, Marrakchi et al. 2011). In the lungs, the IL-36 cytokines have been linked to chronic obstructive pulmonary disease (Chen et al. 2012) and asthma (Ramadas et al. 2006), and in the gut, inflammatory bowel disease (Nishida et al. 2016) and ulcerative colitis (Russell et al. 2016), further demonstrating the crucial role the IL-36 cytokines play in the regulation of inflammation at epithelia.

IL-36γ and Cancer

Recent reports have additionally shown that there is increased IL-36γ expression in colorectal carcinoma primary tumors and in cervicovaginal lavages from women with cervical cancer (Weinstein et al. 2019, Laniewski et al. 2018). Because IL-36γ and IL-36R signaling promotes Th1 differentiation, IL-36γ has been hypothesized to exhibit anti-tumor activity. Indeed, researchers recently demonstrated that co-delivery of the chemotherapeutic doxorubicin with an IL-36γ expression plasmid promoted an anti-metastatic effect and drove a Th1 anti-tumor response that was linked to improved overall anti-tumor activity (Chen et al. 2019). Other reports have also indicated that IL-36γ signaling can transform the tumor microenvironment and limit tumor growth by regulating both dendritic cell and T cell polarization and function (Tsurutani et al. 2016, Wang et al. 2015, Weinstein et al. 2017). Though preliminary, these exciting findings indicate a potential function for IL-36γ in the tumor microenvironment to limit metastasis and regulate the anti-tumor response that may be incorporated into novel cancer therapeutics in the future.

Function of IL-36γ in Host-Pathogen Interactions

Over the past few years, researchers have additionally begun investigating the function of IL-36γ in the context of host-pathogen interactions at mucosal epithelia (**Fig. 2**). Notably, in the lungs, IL-36γ signaling has been shown to stimulate type-1 and Th17 cytokine production and promote macrophage polarization to aid in *Streptococcus pneumoniae* and *Klebsiella pneumoniae* clearance and protect against disease

pathogenesis (Kovach et al. 2017). Additionally, in *Mycobacterium tuberculosis* infection of macrophages, IL-36γ stimulated the production of crucial antimicrobial peptides and pro-inflammatory cytokines that limited *M. tuberculosis* replication (Ahsan et al. 2016). Nanjo et al. also demonstrated that IL-36α and IL-36γ may have overlapping roles in host defense against *L. pneumoniae* lung infection, as IL-36R$^{-/-}$ mice, but not IL-36α$^{-/-}$ or IL-36γ$^{-/-}$ mice exhibited increased mortality, delayed bacterial clearance, and decreased cytokine and chemokine production (Nanjo et al. 2019). The pro-inflammatory response induced by IL-36 signaling can also have negative effects that exacerbate disease pathogenesis. IL-36 signaling has been shown to drive neutrophil infiltration to the bronchial epithelium that contributes to disease pathogenesis during influenza virus infection (Aoyagi et al. 2016). IL-36γ-mediated production of prostaglandin E2 in response to *P. aeruginosa* infection also led to decreased bacterial clearance, and increased lung damage and mortality after infection (Aoyagi et al. 2017). The IL-36 cytokines have recently been shown to exhibit important functions in host-pathogen interactions at the skin. Using an epicutaneous skin exposure model, Liu et al. demonstrated that IL-36R/MyD88 signaling in response to *S. aureus* exposure induced robust skin inflammation (Liu et al. 2017). Further, it was shown that IL-36α signaling in T- cells drove the T-cell mediated production of IL-17 and subsequent inflammatory cascade (Liu et al. 2017). Milora et al. additionally demonstrated that in a flank skin HSV-1 infection model, IL-36β$^{-/-}$ mice exhibited increased mortality after HSV-1 infection (Milora et al. 2017). IL-36β$^{-/-}$ mice did not exhibit an altered B- or T-cell response, and future studies are needed to investigate the impact of IL-36β and the other

IL-36 agonists on immune signaling and innate immune cell recruitment in the skin (Milora et al. 2017).

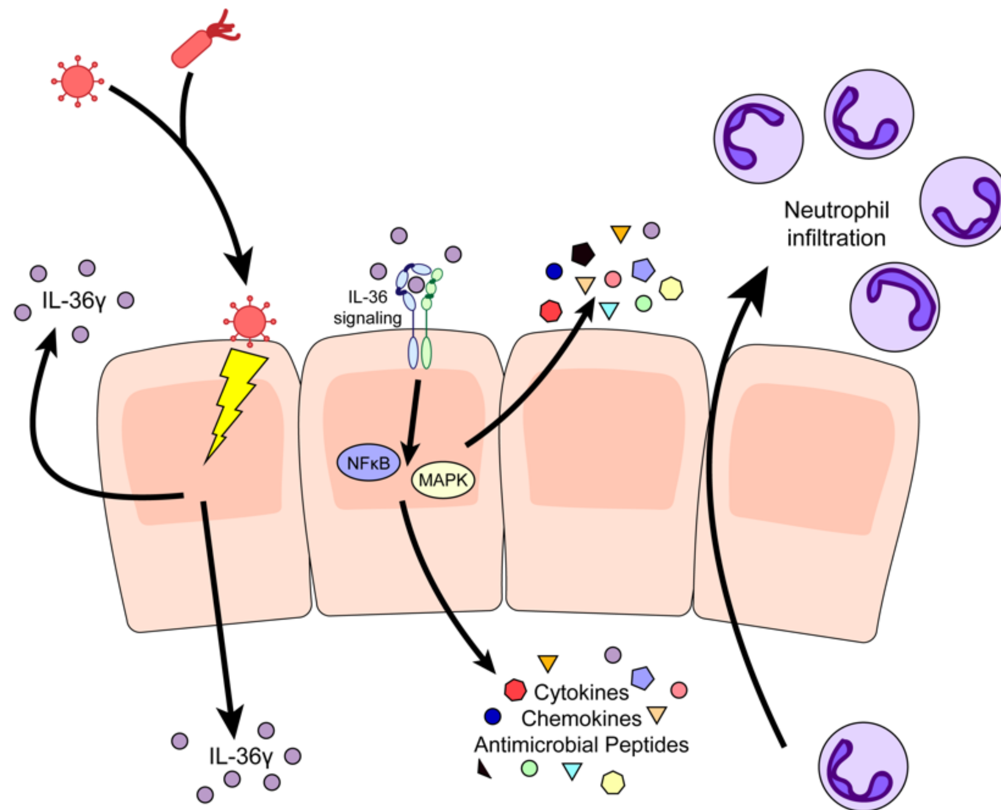

Figure 2. IL-36γ signaling in host defense. IL-36γ is induced in response to bacterial and viral infection at mucosal epithelia. Signaling through IL-36R activates MAPK and NFκB transcription factors to upregulate production of cytokines, chemokines and antimicrobial peptides. IL-36γ signaling additionally promotes neutrophil infiltration to the mucosal epithelia.

In addition to functioning in response to infection, a recent report demonstrated that the IL-36 cytokines can potentially function as vaccine adjuvants to protect individuals against infection. Louis et al. showed that delivery of a plasmid expressing DNA-encoded viral antigen and IL-36β promoted a more effective $CD4^+$ T cell response, whereas an IL-36γ-expressing plasmid combined with the viral antigen drove a more

robust $CD8^+$ T cell response in the context of HIV, Influenza, and Zika virus immunization (Louis et al. 2019). Delivery of the IL-36γ adjuvant further improved the humoral response, leading to enhanced antibody binding without a decrease in antibody avidity. When evaluating the efficacy of the IL-36γ adjuvant *in vivo*, it was shown that delivery of a plasmid encoding IL-36γ and Zika prME antigen protected mice against lethal intraperitoneal challenge and disease in the murine Zika model (Louis et al. 2019). Although this report is the first of its kind and required additional investigation, the study provides exciting insights into the potential prophylactic function the IL-36 cytokines can play in host defense.

While the functions of the IL-36 cytokines are beginning to be well-characterized in host-pathogen interactions in the lungs and skin, in the FRT the IL-36 cytokines remain largely unstudied. Murrieta-Coxca et al. have demonstrated that the IL-36 cytokines are expressed in murine uterine tissue and are induced by *Listeria monocytogenes* infection in the uterus (Murrieta-Coxca et al. 2016). Additionally, Patras et al. found that Group B *Streptococcus* clinical isolates significantly induced IL-36γ in human vaginal, ectocervical, and endocervical epithelial cells (Patras et al. 2015, Patras et al. 2013). Further, utilizing 3-D human vaginal and endocervical epithelial cell models, it was shown that a broad array of microbial products, including the viral mimic poly(I:C), robustly induced IL-36γ expression (Winkle, Throop, and Herbst-Kralovetz 2016). Winkle et al. also demonstrated that treatment of 3-D vaginal and endocervical epithelial cells with recombinant IL-36γ robustly stimulated the production of cytokines, chemokines, and antimicrobial peptides (Winkle, Throop, and Herbst-Kralovetz 2016). Together, these data suggest that IL-36γ may exhibit an important function in host

defense mechanisms in response to bacterial and viral pathogens in the FRT and has led us to develop the hypothesis that IL-36γ is a crucial regulator of mucosal inflammation in the FRT.

HSV-2 and Genital Herpes

Epidemiology and Disease Pathogenesis

The World Health Organization recently published a new report that there are over 1 million new cases of curable sexually transmitted infections every day (Rowley et al. 2019). Genital HSV-2 infection remains one of the most common sexually transmitted infections, affecting an estimated 276 women - 15% of all women worldwide (Looker et al. 2015b). HSV-2 is a neurotropic alphaherpesvirus that establishes a persistent, lifelong infection in individuals that will periodically reactivate. In women, primary infection occurs in the vaginal epithelium, where it replicates and spreads to sensory nerve endings innervating the vaginal epithelium. Once in neurons, the virus will spread, infect, and establish latency in dorsal root ganglia (DRG) in the vertebral column. During reactivation episodes, HSV-2 will exit latency and spread back through sensory neurons to the vaginal epithelium where it will enter a lytic replication cycle that results in shedding at the vaginal mucosa (Johnston and Corey 2016).

Although herpetic lesions are often formed at the vaginal epithelium during reactivation episodes, HSV-2 reactivation and subsequent shedding is frequently subclinical, potentially contributing to increased HSV-2 transmission (Wald et al. 1995, Wald et al. 2000a). Pregnant women can additionally transmit HSV-2 to newborns *in utero* and during delivery causing neonatal herpes, an infection associated with high

morbidity and high mortality as the virus spreads throughout the central nervous system (Corey and Wald 2009, Hass 1935). Neuroinvasion of HSV-2 in adults has been observed clinically in HSV-2 seropositive individuals complaining of acute urinary retention (Caplan, Kleeman, and Berg 1977, Goodell et al. 1983). Individuals with recurrent genital herpes generally average approximately five reactivation episodes during the first two years after acute infection, with less frequent episodes afterwards (Sen and Barton 2007). These ulcerative lesions in turn have been shown to increase risk for STI acquisition, including HIV (Freeman et al. 2006, Patel et al. 2012, Wald and Link 2002).

Mouse Model of Genital Herpes

Mouse models have been developed to investigate genital HSV-2 infection and have proved valuable tools to investigate disease pathogenesis and mechanisms of the immune response to acute infection. Inbred C57Bl/6 or BALB/c mice are the most commonly used strains by immunologists, and animals are conditioned with depot-medroxyprogesterone acetate (DMPA) to synchronize cycles and increase susceptibility to infection (Kollias et al. 2015, Parr et al. 1994, Parr and Parr 2003). Upon infection, animals are monitored daily and disease progression is scored throughout the course of infection. As genital disease progresses, animals will lose hair around the introitis and will develop erythema and ulcerative lesions (Gardner and Herbst-Kralovetz 2018). Following intravaginal inoculation, the virus will actively replicate and spread throughout the vaginal epithelium, infecting innervating autonomic ganglia in the pelvis and spread to the DRG (Parr and Parr 2003). Once HSV-2 has infected the DRG, the virus can then spread to the autonomic ganglia of the enteric nervous system (ENS) (Khoury-Hanold et

al. 2016). Infection of the ENS ganglia leads to the destruction of enteric neurons by infiltrating neutrophils that results in a loss of peristalsis, compaction of fecal matter, and death (Khoury-Hanold et al. 2016). Spread of HSV-2 to the DRG additionally provides HSV-2 with access to the spinal cord and central nervous system where the virus may infect the brainstem, causing hind limb paralysis (Parr and Parr 2003, Reinert et al. 2012, Song et al. 2016). Animals are euthanized when they exhibit end-stage disease symptoms, including hind limb paralysis and moribundity, to minimize pain and suffering. Monitoring of disease and survival continues for up to 21 days post-infection (dPI). Over the course of infection vaginal swabs can be collected to monitor viral loads in the vaginal cavity and characterize immune cell infiltration. Upon necropsy, additional tissues can be collected including the genital lymph nodes, colon, spinal cord, dorsal root ganglia, and brainstem for virus titration and flow cytometry analysis or immunohistochemistry.

Immune Response to Genital Herpes

As HSV-2 overcomes the mechanical and chemical barriers to infection at the vaginal epithelium, initial recognition of the virus occurs as virions are sensed by Toll-like receptors (TLRs) on the cellular surface or in endocytic vesicles. As the virus enters the cell and begins to replicate, TLRs and other immune sensing pathways are triggered to lead to the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides. This signaling rapidly recruits innate immune cells including macrophages, neutrophils, and NK cells that can induce apoptosis and phagocytose infected cells and virions, and contribute to the enhanced production of cytokines and chemokines to

protect against infection (Milligan 1999, Milligan, Bourne, and Dudley 2001, Morahan, Morse, and McGeorge 1980, Thapa, Kuziel, and Carr 2007). Dendritic cells recruited to the vaginal epithelium play a vital role in the processing and presentation of viral antigens to lymphocytes in the genital lymph nodes to promote a robust adaptive immune response (Yoneyama et al. 2005). Activated B cells will begin to produce neutralizing antibodies, often targeting surface glycoproteins, that have been shown to reduce viral load and decrease transmission in mouse models (Chu et al. 2008, Sherwood et al. 1996). HSV-2 specific IFNγ-producing T cells will also be recruited to the vaginal microenvironment and are crucial mediators of the cytolytic response (Mueller et al. 2002). Synergy between the type I IFN and IFNγ response in T cells is key for the expansion of anti-HSV-2 CD8+ T cells and in the cross-presentation of HSV-2 antigen to produce a robust adaptive response that can control infection and promote disease resolution in the vaginal epithelium (Le Bon et al. 2006, Le Bon et al. 2003, Sainz and Halford 2002). CD4+ T cells have also been shown to contribute to viral clearance through the production of IFNγ (Johnson, Chu, and Milligan 2008). Ultimately, both the innate and adaptive responses are critically important to promote disease resolution in the vaginal epithelium and rapidly respond during reactivation episodes to limit virus shedding and the duration of episodes.

Drug and Vaccine Development to Combat Genital Herpes

Acyclovir, the current recommended treatment for genital herpes, was first identified in 1974 and clinical trials began three years later (King 1988). A nucleoside analogue, acyclovir functions as it is recognized by viral thymidine kinase and causes

chain termination as it is incorporated in the growing DNA chain during virus replication (Elion et al. 1977). Since the discovery of acyclovir, drugs to treat genital herpes have gone largely unchanged from acyclovir and the acyclovir-analogues that are currently prescribed today (Johnston and Corey 2016). Treatment is administered either episodically as an individual exhibits symptoms of reactivation, or as a chronic suppressive therapy. Although acyclovir is highly specific for HSV-2 and does reduce reactivation episode length and symptoms, treatment with acyclovir does not prevent virus shedding and transmission (Gupta et al. 2004, Johnston et al. 2012). Further, episodic treatment is largely ineffective for individuals experiencing a subclinical reactivation. Daily, suppressive therapeutics are more effective at reducing recurrences, however these regimens require lifelong use and may lead to chronic immunosuppression and drug resistance (Jiang et al. 2016). Additional therapeutics are under development to limit virus reactivation and shedding, however, none of these treatments can eliminate disease due to latent infection in the DRG (Johnston and Corey 2016).

Extensive effort has been spent over the past half-century in the development a prophylactic vaccine to protect against genital HSV-2 infection and the establishment of latency. Glycoprotein subunit vaccines (glycoprotein B2 and glycoprotein D2) have induced the production of neutralizing antibodies in animal models but have been largely ineffective in clinical trials (Belshe et al. 2012). In addition to neutralizing antibodies, an effective local T cell response can improve vaccine efficacy. To promote a robust local memory T cell response, a “prime and pull” vaccination strategy was devised that utilized an initial vaccination to stimulate a robust T-cell response and then the local administration of chemokines to recruit these “primed” T cells to the vaginal cavity to

establish a protective resident population (Shin and Iwasaki 2012). Additional prophylactic vaccines are currently undergoing clinical trials, including one strategy using a replication deficient virus (Dropulic et al. 2017). Ultimately, an understanding of the contribution of underlying immune mediators in the vaginal epithelium to host defense mechanisms can aid in the development of novel therapeutics to treat those with recurrent genital herpes and provide important insights in the continued development of a vaccine to protect individuals against genital HSV-2 infection.

Overview of Dissertation

This body of work evaluates the hypothesis that IL-36γ is a crucial regulator of mucosal inflammation in response to genital HSV-2 infection to protect against disease pathogenesis. To test this hypothesis, a physiologically relevant 3-D human vaginal epithelial cell model and murine lethal genital HSV-2 challenge model were utilized. Hormonal regulation of IL-36γ in the FRT and induction of IL-36γ in response to a clinically relevant STI pathogen were evaluated. Downstream effects of prophylactic IL-36γ treatment on the immune response and the impact on HSV-2 replication and disease pathogenesis were assessed. In addition, the requirement for IL-36γ was investigated in the response to genital HSV-2 infection, providing essential information regarding the function of IL-36γ in host defense in the FRT epithelia.

CHAPTER 2: THREE-DIMENSIONAL ROTATING WALL VESSEL-DERIVED CELL CULTURE MODELS FOR STUDYING VIRUS-HOST INTERACTIONS

Publication Note

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Abstract

The key to better understanding complex virus-host interactions is the utilization of robust three-dimensional (3-D) human cell cultures that effectively recapitulate native tissue architecture and model the microenvironment. A lack of physiologically relevant animal models for many viruses has limited the elucidation of factors that influence viral pathogenesis and of complex host immune mechanisms. Conventional monolayer cell cultures may support viral infection, but are unable to form the tissue structures and complex microenvironments that mimic host physiology and therefore limits their translational utility. The rotating wall vessel (RWV) bioreactor was designed by NASA to model microgravity and was later found to more accurately reproduce features of human tissue *in vivo*. Cells grown in RWV bioreactors develop in a low fluid-shear environment, which enables cells to form complex 3-D tissue-like aggregates. A wide variety of human tissues (from neuronal to vaginal tissue) have been grown in RWV bioreactors and shown to support productive viral infection and physiological meaningful host responses. The *in vivo*-like characteristics and cellular features of the human 3-D

RWV-derived aggregates make them ideal model systems to effectively recapitulate pathophysiology and host responses necessary to conduct rigorous basic science, preclinical and translational studies.

Introduction

In vitro studies of complex virus-host interactions require robust cell culture models that effectively recapitulate *in vivo* properties and characteristics. Researchers have utilized conventional two-dimensional monolayer cell cultures for many decades, increasing understanding in viral life cycles and the host immune response. Cells grown in conventional monolayer cell cultures however, often lack polarization and architectural features of *in vivo* tissues and therefore may improperly represent key virus-host interactions (Abbott 2003, Nickerson, Richter, and Ott 2007, Zhang 2004). Additionally, many newly emerging viruses and difficult-to-propagate viruses (Zika virus, Severe Acute Respiratory Syndrome coronavirus and hepatitis viruses for example) lack sufficient animal models and/or *in vitro* cell culture models to allow for study of these viruses. Therefore, there is a clear need for *in vitro* models that display key cellular components and features that accurately model virus-host interactions. Development of new therapeutic agents and treatments for viral infections requires a more complex understanding of virus-host interactions, as well as, culture systems that model the *in vivo* environment as accurately as possible. Viruses often require distinct cellular architectural features and polarized orientation with receptors for attachment and entry, which may not be present on cells grown in conventional monolayer cell cultures. For example, a recent review article highlighted the important role of tight junctions in viral entry, replication,

dissemination and egress in at least nine different DNA and RNA viruses (Torres-Flores and Arias 2015). Tight junction proteins are also key components of the epithelial barrier function and integrity that protect against viral infection and potentially influence the susceptibility of tissue to viral infection. Mucin production at mucosal epithelial sites additionally influences virus-host interactions at the epithelial barrier, and ultimately impacts viral infection and transmission (Cohen et al. 2013, Zanin et al. 2016, Zanin et al. 2015). While some of these features may be present in conventional monolayer cell cultures, they often lack the polarity and other topographic features that are present *in vivo*. Rotating wall vessel (RWV) bioreactors effectively model many of these features, including tight junctions and unidirectional expression of mucin and key receptor proteins, allowing investigators to more effectively study virus-host interactions as they exist *in vivo* in a reproducible fashion.

Early attempts at three-dimensional (3-D) cell culture utilized explant tissue cultures (Carrel 1912, Strangeways 1924). Collection of explant tissue, however, is limited by donor availability, and such explants have a short life-span in culture (Grivel and Margolis 2009). More recent attempts at 3-D modeling have included scaffold-based systems, scaffold-free systems, transwells, and microfluidics (Haycock 2011, Seyring et al. 2012, van Duinen et al. 2015). NASA initially developed the RWV bioreactor as a way to model the microgravity environment encountered in space and to investigate growth, regulatory, and structural processes (Goodwin et al. 1993, Schwarz, Goodwin, and Wolf 1992). The RWV bioreactor was found to create a modeled microgravity, low fluid-shear environment that provides the necessary oxygenation and nutrients for development and polarization. In this environment, cells were observed to form cellular

structures and features not readily expressed in conventional monolayer cell culture. Since its development, the RWV bioreactor has been utilized for the study of cellular and microbial gene expression in microgravity, cellular differentiation, host-pathogen interactions and tissue engineering (Barrila et al. 2010, Barzegari and Saei 2012, Grimm et al. 2014, Navran 2008).

In the RWV bioreactor, cells are cultured with microcarrier beads or matrices that allow the cells to attach and spontaneously develop 3-D ultrastructures representative of the parental tissue. As the cells grow and develop, cellular aggregates form via bead-to-bead bridges, and cellular aggregates can be sampled at various time points to monitor the progress of development (Cherry and Papoutsakis 1988, Jessup et al. 1994). The low fluid-shear environment prevents the cells from detaching from the microcarriers and protects growing tissue aggregates from damage that can occur from excess agitation during culturing (Cherry and Papoutsakis 1988). The low fluid-shear environment also promotes the co-localization of particles in the fluid, and leads to the formation of the bead-to-bead bridges and cellular aggregates (Schwarz, Goodwin, and Wolf 1992). In addition, this low fluid-shear environment mimics the flow *in vivo* during development and thereby promotes cellular differentiation as cells signal and grow in 3-D. Developing 3-D aggregates are kept in a continuous free-fall state of neutral buoyancy that precludes aggregate sedimentation while maintain the low fluid-shear environment, and allows the cells to grow around the microcarrier and develop complex structures observed *in vivo* (**Fig. 3**) (Cherry and Papoutsakis 1988, Gao, Ayyaswamy, and Ducheyne 1997, Goodwin et al. 2015, Schwarz, Goodwin, and Wolf 1992). In the RWV bioreactor, oxygenation occurs by the diffusion of dissolved gasses, creating a zero headspace environment that

provides the necessary oxygenation for the developing aggregates while maintaining the low fluid-shear environment (Goodwin et al. 2015). Collectively, these growth conditions allow for 3-D tissue aggregates to develop and form organotypic ultrastructures that are not readily present in conventional monolayer cell culture and necessary for advancing development of *in vitro* models that effectively recapitulate *in vivo* tissue. For example, epithelial cells grown in the bioreactor express adhesion proteins, form desmosomes and tight junctions, produce secretory material and mucus, and also form microvilli and microridges(Drummond, Nickerson, and Coyne 2016, Goodwin et al. 1993, Hjelm et al. 2010, McConkey et al. 2016, Radtke et al. 2012, Dill et al. 2012, Zwezdaryk et al. 2012). Notably, cells that are seeded into the bioreactor reflect their phenotype. For example, vaginal epithelial cells form a multi-layered stratified squamous epithelium, whereas endocervical epithelial cells form a single layer as found *in vivo*, therefore reflecting the authentic microanatomical features of the parental tissue (Hjelm et al. 2010, Dill et al. 2012). Cancerous cell lines, on the other hand (e.g. MCF-7 cells) no longer require the extracellular matrix for growth and do not attach and grow on the collagen-coated microcarrier beads, thereby reflecting their cancer phenotype. Fully differentiated aggregates can remain in the bioreactor for infection, or aggregates can be harvested and plated for downstream experiments including infections.

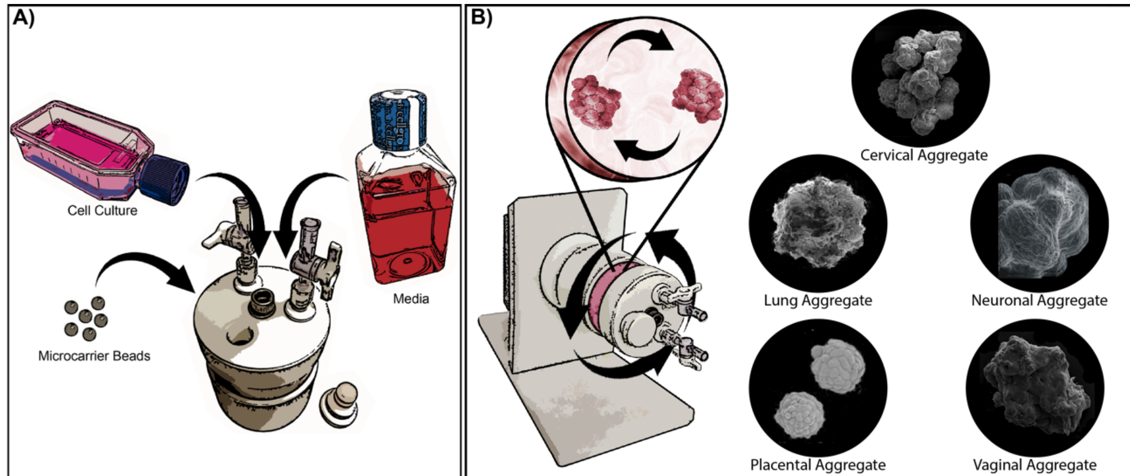

Figure 3. Culturing 3-D aggregates in the RWV bioreactor. A) Cells are grown to confluence in 2-D conventional monolayer cell cultures, then combined with microcarrier beads and appropriate media in the RWV bioreactor. Cells attach to the microcarrier beads in the bioreactor, and culture media can be replaced at any time according to the metabolic needs of the developing aggregates as described in Radtke et al. (Radtke et al. 2012) **B)** The RWV bioreactor is kept in constant rotation at a low speed to create a low fluid-shear simulated microgravity environment that prevents cell detachment and sedimentation. Attached cells grow and form cell-cell junctions creating large aggregates consisting of multiple microcarrier beads. Cells also polarize as they develop and express many characteristics of the parental tissue. Inserts at right show scanning electron micrographs (SEM) depicting representative 3-D aggregates of cells representing cervical, lung, neuronal, placental and vaginal tissues. The cervical tissue SEM is modified from Radtke et al. (Dill et al. 2012) with permission. The lung tissue SEM is taken from NASA/TP-2012-217363, Paramyxovirus infection mimics *in vivo cellular dynamics* in 3-D human broncho-epitlial tissue-like assemblies, and used with permission from NASA (Goodwin et al. 2012). The neuronal tissue SEM is modified from Goodwin et al. (Goodwin et al. 2013) with permission. The placental tissue SEM is modified from McConkey et al. (McConkey et al. 2016) with permission. This work is licensed under CC BY-NC (http://creativecommons.org/licenses/by-nc/4.0/). The vaginal tissue SEM is modified from Hjelm et al. (Hjelm et al. 2010) with permission.

To date, a wide variety of cell lines and tissues have been successfully cultured and reproduced in the RWV bioreactor. The purpose of this review is to highlight those RWV bioreactor studies that focus on modeling virus-host interactions in various tissue settings

(**Table 3**). Additionally, we discuss methodological advancements and future applications of these reproducible and robust RWV bioreactor-derived models for the continued study of virus-host interactions and maximizing their translational utility.

Virus infection and replication in 3-D bioreactor models

Barrier features, including tight junctions, mucus secretion and microvilli, are not often present in cells grown in conventional monolayer cell culture and influence virus-host interactions (Lee and Luk 2010, Torres-Flores and Arias 2015). Tight junctional proteins are receptors for several viruses like hepatitis C virus and adenovirus, and their expression in 3-D cell culture can enhance viral attachment and entry (Torres-Flores and Arias). The presence of tight junction proteins, mucus and other barrier features can also increase resistance to viral infections and more accurately represent *in vivo* virus-host interactions. Our studies, along with those from other researchers, have found that cells grown in the RWV bioreactor are more resistant to infection, requiring a higher virus multiplicity of infection to productively infect 3-D aggregates when compared to conventional monolayer cell culture (Molina-Jimenez et al. 2012, Murakami et al. 2006, Radtke et al. 2012). Likewise, cells grown in the RWV bioreactor show increased resistance to bacterial infection and infected 3-D cell cultures display decreased bacterial replication when compared to infections in conventional monolayer cell culture (David, Sayer, and Sarkar-Tyson 2014, McGowin et al. 2013, Nickerson et al. 2001, Radtke et al. 2012). A study of four veterinary viruses in 3-D RWV bioreactor-derived aggregates further supported these findings by directly comparing replication of the viruses in

conventional monolayer cell cultures vs. 3-D aggregates. Three-dimensional monkey kidney epithelial cell (VERO) aggregates and 3-D bovine kidney epithelial cell (MDBK) aggregates were infected with two DNA viruses (suid herpesvirus 1 and bovine adenovirus) and two RNA viruses (vesicular stomatitis virus and bovine parainfluenza virus). Infections with all four viruses in the 3-D aggregates produced a lower viral titer over the course of infection compared to conventional monolayer cell cultures (Malenovska 2016). However, despite being more resistant to infection, more infectious virions were produced in 3-D aggregates while conventional monolayer cell culture produced more noninfectious virions (Malenovska 2016). These findings allow the speculation that the increased resistance to infection is due to the barrier features that polarized 3-D aggregates exhibit when grown in the RWV bioreactor. The increased infectivity of the virions produced is also noteworthy and suggests again that the 3-D aggregates better mimic the *in vivo* environment. For these and other reasons, we argue herein that the 3-D cellular aggregates grown in the RWV bioreactor provide a more *in vivo*-like simulation of parental tissues and their susceptibility to virus entry, replication and subsequent pathogenesis support their utilization for the study of these processes *in vitro*.

Table 3.

Host-virus interactions in RWV bioreactor-derived 3-D aggregates

Tissue Model	Cell Lines	Virus	Virus Replication	Host Response	Reference
Neuronal	NHNP	VZV	Productive infection	No CPE	(Goodwin et al. 2013)
Tonsil	Primary cells	HIV	Productive infection	Lymphocyte migration tracked	(Margolis et al. 1997)
Lymphoid	P3HR-1	EBV	Suppression of EBV reactivation	ND	(Long, Pierson, and Hughes 1999)
	P3HR-1 Daudi Ramos	EBV	Suppression of EBV reactivation	ND	(Long and Hughes 2001)
	BJAB Raji	EBV	Suppression of EBV reactivation	Microgravity and radiation increased DNA damage in EBV positive cells	(Brinley et al. 2013)
Lung	HBTC BEAS-2B	SARS-CoV	No SARS-CoV replication detected	Vacuolization, mitochondria loss and chromatin alterations	(Suderman et al. 2006)
	HBTC BEAS-2B	RSV	Productive infection	Signs of cellular damage. Mucus produced in 3-D aggregates	(Goodwin et al. 2008)
	HBTC BEAS-2B	RSV & PIV3	Productive infection	Cytokine profile in 3-D aggregates was similar to human airways from RSV and PIV3 infected patients	(Goodwin et al. 2012)
Liver	Huh7	HCV	Productive infection	Expression and localization of TJ proteins enhance HCV infection	(Sainz, TenCate, and Uprichard 2009)
	PLC/PRF/5	HEV	Productive infection	No CPE	(Berto et al. 2013)
Small Intestine	INT-407	HuNoV	Increase in viral RNA copies detected	Vacuolization, shortening of apical microvilli, cell detachment from bead	(Straub et al. 2007)
			No HuNoV replication detected	Clumping and detachment of cells from microcarrier beads	(Herbst-Kralovetz et al. 2013)
			No HuNoV replication detected	No CPE	(Papafragkou et al. 2014, Takanashi et al. 2014)
Colon	Caco-2	HuNoV	Increase in HuNoV RNA copies detected	Shortening of apical microvilli and formation of vacuoles	(Straub et al. 2011)
			No HuNoV replication detected	No CPE	(Papafragkou et al. 2014, Takanashi et al. 2014)
		CVB	Productive infection	Increased expression of proliferation and differentiation genes	(Drummond, Nickerson, and Coyne 2016)
Placenta	TBPC	HCMV	Productive infection	ND	(Swan et al. 2015)
	JEG-3	VSV	No VSV replication detected	Resistance to infection mimics *in vivo* response to infection	(McConkey et al. 2016)
Vagina	V19I	HSV-1 HSV-2	Productive infection	Mucosal epithelial barrier features mimic *in vivo* characteristics, aggregates are more resistant to HSV infection	(Dill et al. 2012, Gardner, Winkle, and Herbst-Kralovetz)

CPE, cytopathic effects; ND, not determined

Three-dimensional models of human neuronal cells to study persistent viral infections

Varicella zoster virus (VZV) is an alphaherpesvirus that belongs to the *Herpesviridae* family that establishes a latent infection in ganglionic neurons following an initial phase of acute infection. The virus will often reactivate in later years, causing zoster, a localized dermatomal rash and also neurologic diseases including meningoencephalitis and myelopathy. Over 90% of the worldwide population is seropositive, and, although a vaccine has been developed and is currently available, there remains a risk of virus reactivation (LaRussa et al. 2000, Virgin, Wherry, and Ahmed 2009). A lack of animal models and limited availability of VZV-free human ganglionic neurons has limited study of the virus. Recently, normal human neuronal progenitor (NHNP) cells have been utilized to study VZV infection and latency. When NHNP cells are cultured in the RWV bioreactor they become partially differentiated, leading to the formation of 3-D aggregates that display features observed in human trigeminal ganglia (Goodwin et al. 2013). These 3-D aggregates express mature neuronal markers such as glial fibrary acidic protein, neuron-specific nuclear protein, β-tubulin III and microtubule associated protein A&B after 180 days in culture (Goodwin et al. 2013). Three-dimensional NHNP aggregates also express additional neuronal markers (nestin and tubulin) at levels similar to those seen in human trigeminal ganglia, however, late stage neuronal development markers CD105, CD90 and CD49f, are expressed at lower levels (Goodwin et al. 2013).

Three-dimensional NHNP aggregates support persistent VZV infections with limited or little lytic replication, and sporadic reactivation at later time points (Goodwin

et al. 2013). NHNP cellular aggregates infected with fluorescently labeled VZV in the bioreactor showed a significant increase of VZV genome copies over an 18-day period, yet aggregates remained viable in culture over a three-month course of infection (Goodwin et al. 2013). The VZV genome was able to stably replicate and infectious virus progeny was detected in the cell culture supernatant intermittently throughout the course of infection (Goodwin et al. 2013). The maintenance of viable 3-D aggregates over a three-month course of infection, while not exactly modeling *in vivo* infections, does allow for the study of persistent VZV infections over an extended period of time. Prolonged studies of VZV infections enable researchers to identify key interactions that may influence virus gene expression, replication and potentially establishment of latency. Prior to the development of the 3-D NHNP aggregates, many other cell lines had been utilized to study VZV infection, including human neuroblastoma (IMR-32), monkey kidney epithelial (VERO), primary human foreskin fibroblasts (HFF), human melanoma (MeWo) and peripheral blood mononuclear cells (PBMC). In these cell lines, however, VZV infections are lytic and preclude prolonged culturing and the study of VZV latency and reactivation cycles (Bourdon-Wouters et al. 1990, Brazeau et al. 2010, Goodwin et al. 2013, Hood et al. 2003, König et al. 2003). It should be noted that a non-lytic VZV infection was achieved with differentiated human neural stem cells (NSC); however, these infections were nonproductive (Pugazhenthi et al. 2011). Despite its limitations, the development of 3-D NHNP aggregates grown in the RWV bioreactor represents a step forward in the study of VZV virus-host interactions. Further development of 3-D aggregates to model virus latency and reactivation can provide new insights into

mechanisms of VZV pathogenesis, and could potentially be utilized in the study of other neurotropic viruses, such as other herpesviruses.

Three-dimensional models of lymphoid tissue and circulating lymphocytes for long-term culture to study virus replication, latency and reactivation

One of the first viruses studied utilizing the RWV bioreactor developed by NASA was Epstein-Barr Virus (EBV) (**Table 3**). EBV is a member of the *Herpesviridae* family and approximately 90% of adults are seropositive for the virus (Cohen 2000). The virus infects epithelial cells and B lymphocytes and establishes latency in resting memory B cells (Babcock et al. 1998). In humans, B cells circulate through the periphery, often in a quasi-gravity state where gene expression and cell metabolism may be different from that observed in conventional monolayer cell culture (Long, Pierson, and Hughes 1999). Simulated microgravity has been shown to alter the gene expression, proliferation and cellular interactions of non-adherent cells compared to when they are cultured in non-rotating, static environments (Bechler et al. 1992, Cogoli and Cogoli-Greuter 1997). As already noted, the RWV bioreactor provides a simulated microgravity environment to reproduce conditions in circulation, and is therefore ideally suited for the *in vitro* study of EBV-infected B cells. P3HR-1, Daudi and Ramos B-cell lines have been cultured in the RWV bioreactor to study factors influencing EBV latency and reactivation (Brinley et al. 2013, Long and Hughes 2001, Long, Pierson, and Hughes 1999). Indirect immunofluorescence assays showed that EBV-positive P3HR-1 and Daudi cells cultured in RWV bioreactors displayed significantly lower expression levels of lytic cycle proteins

compared to cells grown in conventional monolayer cell culture, suggesting that EBV reactivation is suppressed in microgravity environments (Long, Pierson, and Hughes 1999).

Human Immunodeficiency Virus (HIV) is a member of the *Retroviridae* family, that also infects lymphoid tissue and circulating lymphocytes. In addition to the formation of 3-D aggregates from cells grown in conventional monolayer cultures, the RWV bioreactor can be utilized for culturing tissue blocks in the simulated microgravity environment, thereby maintaining their *in vivo* cellular organization and structure while allowing for the delivery of nutrients (Margolis et al. 1997). Margolis et al. inserted blocks of human tonsil tissue into the RWV bioreactor, and cultured these blocks with additional cells from the same tonsil. These cultures were not only viable for up to three weeks, but also contained lymphocytes that migrated throughout the tissue and cell culture media. Infection of these cultures with HIV-1 isolates was productive, with exponential viral replication during the first week of infection (Margolis et al. 1997). *In situ* hybridization for HIV RNA confirmed a productive infection, with 1-3% of the cells in the tissue blocks becoming infected (Margolis et al. 1997). Flow cytometry demonstrated a decrease in $CD4^+$ cells over the course of the infection, confirming the productive infection of $CD4^+$ T cells (Margolis et al. 1997). Additionally, transfer of HIV-positive tissue blocks to RWV bioreactors containing HIV-negative tissue blocks led to the infection of the HIV-negative tissue blocks, and virus was detected in the cell culture supernatant (Margolis et al. 1997). Culturing tissue blocks in the RWV bioreactor thus creates an *in vitro* model system that could provide new insights into HIV infections and virus interactions with lymphoid tissue.

Three-dimensional models for respiratory viral infections

The respiratory epithelium forms a robust barrier to prevent infection. Tight junctions, cilia, and mucus secretion are some of the important features of this barrier. Conventional cell culture techniques are often used for the study of respiratory viruses, but lack many of the important features of the intact human respiratory epithelium like mucus, microvilli and cell-cell junction proteins. Cultures of 3-D respiratory epithelial cell aggregates have therefore been applied to the study of the host immune response to bacterial infections in the lungs, and can be effective models for studying emerging respiratory viruses and difficult-to-propagate viruses that previously lacked a robust model for study (David, Sayer, and Sarkar-Tyson 2014).

Two widely prevalent paramyxoviruses, parainfluenza virus type 3 (PIV3) and respiratory syncytial virus (RSV) cause severe respiratory disease in young children. Both of these viruses have proven to be difficult to propagate in conventional cultures and also lack robust animal models that accurately model viral infection, pathogenesis and the host immune response (Bem, Domachowske, and Rosenberg 2011). Recently, it has been shown that immortalized human broncho-tracheal epithelial (BEAS-2B) cells and primary normal human bronchial epithelial (NHBE) cells grown as 3-D aggregates in the RWV bioreactor are permissive to RSV and PIV3 infection (Table 1). Transmission electron microscopy further demonstrated that the aggregates grow in a multi-layered structure that exhibits tight junctions and microvilli, thus displaying key features of the *in vivo* tissue (Goodwin et al. 2008). These respiratory 3-D aggregates have been cultured for more than 35 days while maintaining functional cell markers and, when challenged with RSV, display cellular damage and active viral infections, including budding virions

(Goodwin et al. 2008). Cytokine profiles from the RSV-infected and PIV3-infected 3-D lung epithelial aggregates demonstrated an induction of pro-inflammatory cytokines, chemokines and other immune factors including IL-1β, IL-8, MIP-1α, RANTES and G-CSF at levels that were similar to those observed in nasal washes from children with RSV and PIV3 infections (Goodwin et al. 2012). In contrast, cytokine profiles from conventional monolayers infected with RSV and PIV3 were not similar to the levels observed clinically. Taken together, these data support the utilization of 3-D aggregates as models of *in vivo* RSV and PIV3 infections with regard to the function of epithelial cells in host response and barrier defense to these viruses (Goodwin et al. 2012).

Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) is an emerging virus first identified in China in 2002 (Zhao et al. 2003). Animal models for SARS-CoV have been developed, although there currently are no FDA-approved vaccines or antivirals available (Gretebeck and Subbarao 2015). Initial experiments with SARS-CoV suggest that 3-D human broncho-tracheal epithelial (BEAS-2B) aggregates provide a more physiologically relevant and species-specific model that may be more permissive to viral infection by SARS-CoV and potentially other emerging human viruses (Table 1). Characterization of the 3-D aggregates has revealed cellular differentiation and increased expression of collagen IV, MUC1 and tight junction protein ZO-1 at levels similar to *in vivo* human tissue (Suderman et al. 2006). Infectious virions were not detected in cell culture supernatants by standard plaque assay when the 3-D aggregates were infected with SARS-CoV; however, immunocytochemistry illustrated cross-reactivity with antibodies to viral spike and nuclear proteins at the cytoplasm and plasma membrane in SARS-CoV infected aggregates (Suderman et al. 2006). Cytoplasmic vacuoles also

increased over the course of the infection, mitochrondria became swollen and decreased in number, and endoplasmic reticula were disrupted, all clear signs of viral infection (Suderman et al. 2006). At 10 days post infection (dPI), cells began shedding off the microcarrier beads, but, again, no budding virions were observed at any time during the course of infection (Suderman et al. 2006). Nevertheless, the positive immunocytochemistry and the visualization of cytoplasmic vacuoles appear to confirm a permissive SARS-CoV infection of the 3-D aggregates, suggesting that these cultures could provide useful information about SARS-CoV infection and pathogenesis, and contribute to the development novel interventions even in the absence of virion formation. These initial studies with 3-D respiratory aggregates are encouraging and could be applied to other newly emerging respiratory viruses such as Middle East Respiratory Syndrome coronavirus (MERS-CoV).

Three-dimensional models for studying viral gastroenteritis

Over 200,000 children under the age of five die from human norovirus (HuNoV) infections, with norovirus infection representing the second leading cause of diarrheal death in this age group (Lopman et al. 2016, Patel et al. 2008, Scallan et al. 2011). An effective cell culture model for modeling HuNoV infections and interactions with intestinal epithelial cells has been elusive, slowing the development of urgently needed interventions for this deadly illness (Riddle and Walker 2016).

Early studies investigated over 27 cell lines and approximately 33 different HuNoV strains for propagation of the virus using *in vitro* monolayers and were

unsuccessful (Duizer et al. 2004). It was hypothesized that 3-D structures or other co-factors may be required for successful cultivation of HuNoV *in vitro.* Human small intestine (INT-407) epithelial cells were initially grown in the RWV bioreactor with collagen coated beads to form 3-D aggregates to study HuNoV (Table 1) (Herbst-Kralovetz et al. 2013, Straub et al. 2011, Straub et al. 2007, Takanashi et al. 2014). INT-407 epithelial aggregates have been shown to differentiate, and subsequently stain positive for tight junction markers occludin, claudin-1, E-cadherin and ZO-1 by immunofluorescence (Straub et al. 2007). The 3-D aggregates possess apical microvilli, though these microvilli are shorter than those on *in vivo* human intestinal epithelial cells (Takanashi et al. 2014). Three-dimensional aggregates developed from an alternate cell line, Caco-2 colon carcinoma, also express tight junction markers ZO-1 and occludin, and apical microvilli on these aggregates were more similar to the human intestinal epithelium *in vivo* (Drummond, Nickerson, and Coyne 2016, Straub et al. 2011, Takanashi et al. 2014). The 3-D Caco-2 aggregates also displayed increased expression of MUC1, MUC13 and MUC17, which are highly expressed mucins in the intestine that could potentially influence virus-host interactions (Drummond, Nickerson, and Coyne 2016). Both 3-D Caco-2 and INT-407 aggregates also expressed histo-blood group antigens (HBGA) H1 and H2, cellular receptors for HuNoVs (Herbst-Kralovetz et al. 2013, Hutson et al. 2003, Takanashi et al. 2014). INT-407 and Caco-2 aggregates were challenged with HuNoV, and harvested for viral RNA titers as measured by RT-PCR. Straub et al. reported successful HuNoV infection and detected viral replication in 3-D INT-407 and 3-D Caco-2 aggregates, however, these results have not been able to be replicated in either 3-D INT-407 or 3-D Caco-2 intestinal epithelial models (Herbst-

Kralovetz et al. 2013, Papafragkou et al. 2014, Takanashi et al. 2014). Additionally, the INT-407 cell line has been reported to be contaminated with HeLa cells, and therefore could explain why these studies of HuNoV cannot be replicated and is not a robust or reproducible model for the study of HuNoV-host interactions (Lavappa 1978, Nelson-Rees, Daniels, and Flandermeyer 1981).

Recent advances in the culture of HuNoV demonstrated the need for additional factors including gut bacteria and/or bile for successful HuNoV replication. Bacteria from the gut have been shown to express HBGA and facilitate HuNoV infection of human B cells (Jones et al. 2014). Additionally, it has been shown that intestinal milieu, including bile, are important factors that enhance HuNoV replication in human intestinal enteroids (Ettayebi et al. 2016).

Although 3-D Caco-2 aggregates alone do not support productive HuNoV infection, they possess *in vivo*-like properties that can aid researchers in understanding the interactions between intestinal epithelial cells and other gastrointestinal viruses like coxsackievirus B (CVB). Currently there is no vaccine for CVB, and CVB infections cause mild gastroenteritis, myocarditis and persistent infections have been linked to type 1 diabetes development (Hober and Sane 2010, Hober and Sauter 2010). Three-dimensional Caco-2 aggregates have supported productive CVB infection, and produce more infectious virions than infections in conventional monolayer cell culture (Table 1) (Drummond, Nickerson, and Coyne 2016). Further study of CVB and other enteric viruses in 3-D colonic epithelial aggregates promises new insights into the factors influencing viral entry and host-virus interactions.

Three-dimensional models of liver tissue for studying hepatitis viral infections

Two hepatotropic viruses, Hepatitis C Virus (HCV) and Hepatitis E Virus (HEV) have proven difficult to propagate *in vitro* and only in recent years have researchers been able to study these viruses in conventional cell culture.

HEV is a member of the *Hepeviridae* family and causes acute liver disease in humans. Transmission of certain HEV genotypes can be zoonotic (from pigs), and affects much of the developing world, especially East and South Asia (179). Human cell culture models for HEV include PLC/PRF/5 hepatocarcinoma cells grown in conventional monolayer cell culture, which have inconsistently supported viral replication (Berto et al. 2013). PLC/PRF/5 have been grown in the RWV bioreactor, where they form aggregates that also support viral replication (Table 1) (Berto et al. 2013, Takahashi et al. 2007, Tanaka et al. 2007). The 3-D aggregates become fully differentiated after a 28-day culture period, and are viable in culture for over five months (Berto et al. 2013). HEV RNA has been detected in supernatants from RWV cultures at the majority of collection points over a 175-day period (Berto et al. 2013). In contrast, PLC/PRF/5 cells grown in conventional monolayer cell cultures contain no HEV RNA in the supernatants (Berto et al. 2013). Scanning electron microscope (SEM) micrographs of RWV bioreactor supernatants demonstrate virions, and subsequent experiments showed that these virions are infective, confirming a productive infection (Berto et al. 2013). Taken together, these data support the use of the RWV bioreactor to study the virus-host interactions that influence HEV infectivity and transmission in a culture system that closely resembles *in vivo* liver tissue.

HCV is a member of the *Flaviviridae* family and has chronically infected over 185 million people worldwide (Mohd Hanafiah et al. 2013). Despite this massive prevalence, the lack of model systems for HCV has limited researchers' ability to study virus-host interactions *in vitro*. Conventional monolayer cell cultures of Huh7 human hepatoma epithelial-like cells are permissive to HCV infection, but have decreased expression of cellular features including occludin and claudin-1, which are known to impact viral uptake (Table 1) (Sainz, TenCate, and Uprichard 2009). Three-dimensional Huh7 aggregates, in contrast, provide a more physiologically relevant system that is highly permissive to HCV infection. Light micrographs have shown that fully differentiated aggregates are multilayered, and RT-PCR of cellular RNA showed increased expression of hepatocyte nuclear factors that regulate hepatocyte differentiation (Sainz, TenCate, and Uprichard 2009). In addition, aggregates stained positive for tight junction proteins (occludin-1 and E-cadherin), cell adhesion proteins and HCV receptor (CD81 and SR-B1) by immunofluorescence (Sainz, TenCate, and Uprichard 2009). This degree of differentiation and polarization of Huh7 cells allows for studies of interactions between HCV and barrier proteins regulating viral entry that was previously impossible in conventional monolayer cell culture. Three-dimensional Huh7 liver aggregates have been productively infected with HCV, and viral RNA was detected in aggregates throughout a two-week period of infection (Sainz, TenCate, and Uprichard 2009). Infection of the Huh7 aggregates was further confirmed through immunofluorescence at days 1, 7 and 14 post infection (Sainz, TenCate, and Uprichard 2009). Cellular differentiation of the 3-D aggregates and the increased expression of tight junction

proteins enables the study of these difficult-to-propagate viruses, and could be utilized in the study of other hepatotropic viruses.

Three-dimensional models of female reproductive tract tissues for studying sexually transmitted infections and emerging viruses

The female reproductive tract (FRT) is divided into the upper tract, consisting of the endocervix, uterus, placenta, fallopian tubes and ovaries, a transitional ectocervix zone, and the lower tract, consisting of the vagina and vulva (Quayle 2002). The FRT is exposed to a myriad of microbes including sexually transmitted pathogens, many of which are able to establish chronic infections that are difficult to clear and can cause long-term reproductive and gynecologic sequelae. However, site-specific differences in the epithelial structure between the upper and lower genital tract necessitate the need to model these different tissues for the study of sexually transmitted infections (STI) and other viruses. Our laboratory was the first to develop and characterize 3-D models of human cervical and vaginal tissue using the RWV bioreactor (Hjelm et al. 2010, Radtke et al. 2012). Human models of the upper FRT, including the endometrium, have also recently been developed and characterized in our laboratory (Laniewski et al. 2017). In addition, human placental models have been successfully created and used to model microbial resistance at this site (LaMarca et al. 2005, McConkey et al. 2016, Swan et al. 2015, Zwezdaryk et al. 2012).

Human vaginal epithelial (V19I) cells cultured in the RWV bioreactor develop *in vivo*-like properties that are not present when cells are grown in conventional monolayer

cell cultures (Hjelm et al. 2010). Scanning and transmission electron microscopy of 3-D vaginal aggregates has demonstrated the presence of tight junctions/desmosomes, microridges, microvilli and mucus secretion, all of which influence viral interactions with host epithelial cells (**Fig. 4**) (Hjelm et al. 2010, Radtke et al. 2012). The 3-D aggregates can be used to test vaginal microbicides and recapitulated toxicity and host cytokine responses similar to human explant tissue thereby demonstrating the translational capability of this model (Hjelm et al. 2010). Three-dimensional cervical and vaginal aggregates exposed to microbial products including poly(I:C) (a viral mimic and toll-like receptor 3 agonist) exhibit induction of acute-phase and pro-inflammatory cytokines and mucus secretion, similar to primary human cells and have been used to model the host immune response to viral infections (Gardner, Winkle, and Herbst-Kralovetz , Radtke and Herbst-Kralovetz 2012, Winkle, Throop, and Herbst-Kralovetz 2016).

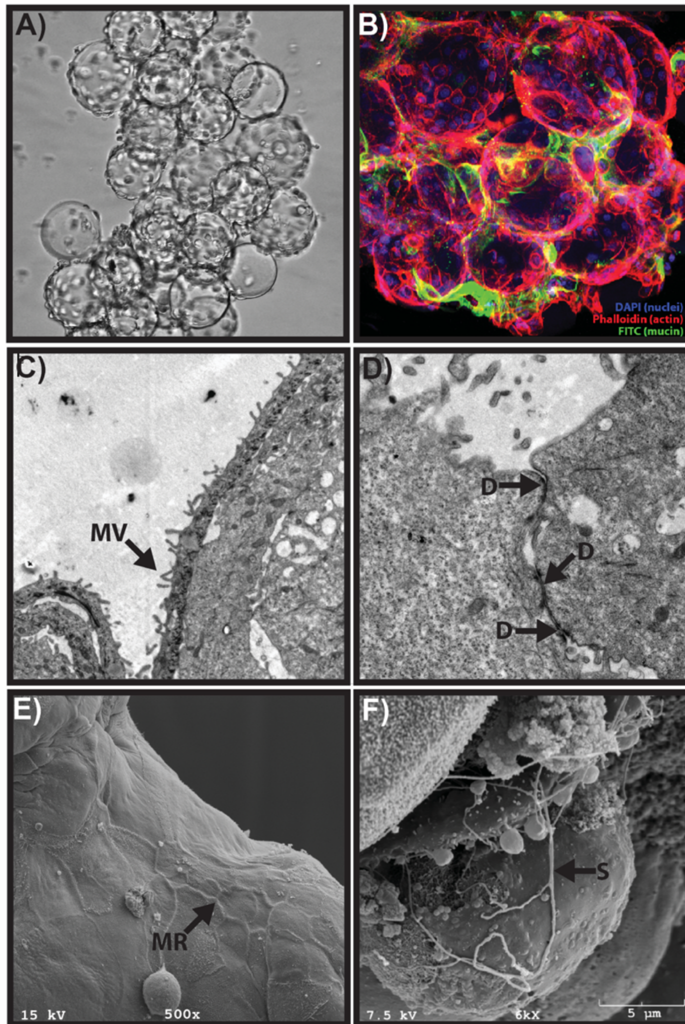

Figure 4. Example of physiological features of RWV bioreactor-derived human 3-D vaginal aggregates. A) Phase micrograph of 3-D vaginal epithelial cell aggregates consisting of multiple microcarrier beads connected by cell-cell junctions at an early stage of development. **B)** Immunofluorescence of 3-D aggregates by laser scanning confocal microscopy demonstrating localized mucus secretion modified from Hjelm et al. (Hjelm et al. 2010) with permission. Nuclei are stained with DAPI (blue), actin filaments are stained with Phalloidin (red) and MUC1 is stained with FITC (green). **C)** Transmission electron microscope (TEM) image of 3-D vaginal aggregates showing microvilli (**MV**) modified from Hjelm et al. (Hjelm et al. 2010) with permission. **D)** TEM micrograph depicting desmosomes (**D**) at cell-cell junctions between vaginal epithelial cells. **E)** Scanning electron microscope (SEM) image showing formation of microridges (**MR**) at cell-cell junctions during late-stage development of 3-D aggregates. **F)** Production of secretory material (**S**) by vaginal 3-D aggregates as shown by SEM.

Herpes simplex virus type 2 (HSV-2) is the leading cause of genital herpes infections and causes persistent, life-long infections that increase risk for STI and human immunodeficiency virus (HIV) acquisition (Garland and Steben 2014). Although antiviral drugs are available, viral reactivation and shedding can occur in many who are asymptomatic and an effective vaccine remains elusive (Wald et al. 2000b). Vaginal and cervical epithelial aggregates are susceptible to FRT pathogens, including HSV-2 as shown by immunofluorescence and standard VERO plaque assay (**Table 3**) (Gardner, Winkle, and Herbst-Kralovetz , Radtke and Herbst-Kralovetz 2012). As mentioned previously, a higher MOI is required to infect 3-D vaginal or cervical aggregates relative to conventional monolayers, most likely due to enhanced epithelial barrier features (Gardner, Winkle, and Herbst-Kralovetz). HSV-2 infections of 3-D vaginal aggregates induce secretion of cytokines, chemokines, mucus and anti-microbial products (AMP), and may provide a more accurate host immune response relative to conventional monolayers (Gardner, Winkle, and Herbst-Kralovetz , Radtke and Herbst-Kralovetz 2012). More recently, our laboratory has reported that exposure of 3-D human vaginal and cervical aggregates to microbial products, including poly(I:C), induces expression of a novel pro-inflammatory cytokine, IL-36γ (Winkle, Throop, and Herbst-Kralovetz 2016). While this cytokine has been identified at other mucosal sites, this was the first report of this cytokine in the FRT and was validated with human tissue (Gabay and Towne 2015, Gresnigt and van de Veerdonk 2013, Winkle, Throop, and Herbst-Kralovetz 2016). We hypothesize it may play a key role in mucosal host defense at this site. Collectively, these physiologically relevant features support the use of 3-D FRT

aggregates from RWV bioreactors to study host immune mechanisms to other viral STI, including HIV and human papillomavirus.

The placenta is an important site for exchange of nutrients and gasses during pregnancy, and virus-host interactions at the placenta have significant health implications for both mother and developing fetus. Human choriocarcinoma trophoblast cells (using the JEG-3 cell line) have been shown to produce 3-D aggregates in the RWV bioreactor when the cells are co-cultured with placental microvascular cells (McConkey et al. 2016). The JEG-3/microvascular aggregates form over a 21-day period and display increased expression of placental differentiation markers including human chorionic gonadotropin beta subunit (βhcg), human placental lactogen (hPL) and syncytian (McConkey et al. 2016). Transcriptome profiles of the 3-D aggregates were significantly different from profiles of cells grown in conventional monolayer, and were more similar to profiles from primary human trophoblasts (McConkey et al. 2016). The 3-D aggregates also form syncytia and brush borders that are additional markers of cellular differentiation and that are not observed in conventional monolayer cell culture (McConkey et al. 2016). The syncytia and brush borders present in the 3-D aggregates provide resistance to vesicular stomatitis virus (VSV) infection, whereas cells in conventional monolayers remain susceptible to VSV infection as shown by RT-PCR (McConkey et al. 2016). These results support the protective nature of placental trophoblasts (both *in vivo* and in 3-D aggregates) against VSV infection. This 3-D placenta model may also be useful for studying the interactions between emerging viruses, including the Zika virus and its severe effects in pregnancy (e.g. irreversible microcephaly) observed in newborn babies from Zika-infected mothers (Johansson et al. 2016).

Future directions for RWV bioreactors

Considering the site-specific microbiome for advancement of RWV-derived tissue models

Recent research has implicated virus-host-microbiome interactions in the processes of viral entry, pathogenesis and the host immune response (Berkhout 2015, Bosch et al. 2013, Lynch 2014, Miura et al. 2013, Pfeiffer and Virgin 2016, Robinson and Pfeiffer 2014). For example, in the FRT commensal microbiota play a key role in maintaining a healthy, homeostatic microenvironment, and disruption of this microbiota increases risk for STI acquisition. Our laboratory has successfully modeled commensal and bacterial vaginosis-associated bacteria from the vaginal microbiome using 3-D RWV-derived vaginal aggregates (Doerflinger, Throop, and Herbst-Kralovetz 2014, Herbst-Kralovetz et al. 2016). Virus-microbiome interactions can also be studied using other mucosal models (e.g. lung and intestinal models), and site-specific microbiota should be included in future tissue modeling of mucosal sites (Doerflinger, Throop, and Herbst-Kralovetz 2014). Use of clinical samples or isolates may be required to recapitulate the microbial milieu at these sites. Culturing of 3-D aggregates with site-specific microbiota in the context of viral infection could provide novel insights into virus-host and virus-microbiome mechanisms.

Advancing RWV bioreactor tissue models with enhanced cellular complexity

Human tissues are complex multi-cellular microenvironments, and interactions between different cell types in a tissue will influence host immune mechanisms. Co-

culturing with lymphocytes and other immune cells increases the complexity of the tissues that can be modeled in the RWV bioreactor and can more faithfully recapitulate the host immune response, however it is challenging to create autologous environments with limited availability and lifespan of primary cell lines and optimization of diverse culture requirements. Application of stem cells and stromal cells to the RWV bioreactor may further enhance tissue complexity of the 3-D aggregates. For example, neural stem cells and neural progenitor cells are able to differentiate and develop into neurons and glia when grown in the RWV bioreactor (Lin et al. 2004, Luo et al. 2015, Ma et al. 2004). Other examples include embryonic stem cells, pluripotent stem cells and mesenchymal stem cells, all which have been successfully propagated in RWV bioreactors and could advance RWV bioreactor-derived models (Cerwinka et al. 2012, Luo et al. 2015, Rungarunlert, Ferreira, and Dinnyes 2016, Teo et al. 2014, Vidyasekar et al. 2016). The increasing utilization of RWV bioreactors for cell lines from a wide variety of tissues holds the promise of enhancing our ability to study emerging viruses that have drastic public health implications. New therapeutic agents and antiviral drugs can also be screened for toxicity and efficacy using 3-D human aggregates to give a more faithful representation of *in vivo* microenvironments and provide superior preclinical data prior to advancing to clinical trials. Although challenging, advancing RWV bioreactor model systems to reproduce these complex *in vivo* cell-cell interactions could improve the translational impact of these robust 3-D models in elucidating key virus-host interactions.

Conclusions

In this review, we have highlighted studies using 3-D RWV-derived models to investigate virus-host interactions (**Table 3**). Three-dimensional RWV bioreactor-derived aggregates express cellular architectural and structural features not readily expressed in conventional monolayer cell culture, including tight junction proteins, mucus and microvilli, that are important for analysis of virus-host interactions. Fully differentiated aggregates can be further utilized in highly reproducible downstream gene expression analyses, high throughput and "omics" analyses, microscopy, toxicology/drug development and gene editing studies. In conclusion, RWV-derived 3-D models can be employed to better understand the key interactions that influence viral pathogenesis and the host immune response to viral infection in a physiologically meaningful context that enhance their translational utility.

CHAPTER 3: HORMONAL REGULATION OF IL-36γ AND HOST DEFENSE MECHANISMS IN THE FEMALE REPRODUCTIVE TRACT

Abstract

The gonadal steroids estrogen and progesterone (commonly referred to as sex hormones) exhibit essential functions in the female reproductive tract (FRT), and levels of these hormones fluctuate through menstrual cycle. Recently, the commonly-used contraceptive Depo Provera (DMPA), a synthetic progestin, has been linked to an increased risk for sexually transmitted infection acquisition among high-risk women. While many factors may contribute to this increased risk for infection, we sought to evaluate the impact of estrogen and progesterone on epithelial barrier feature and immune mediators, including the IL-36 family members. We found that relative to estrogen conditioned mice, DMPA conditioned mice exhibited significantly reduced expression of desmocollin and desmoglein, two key components of desmosomes. We further measured the expression of several immune mediators, including IL-36β and IL-36γ, and found that they were reduced in DMPA conditioned mice relative to estrogen conditioned mice. Additionally, we measured site specific differences in expression of these key barrier features and immune mediators, with elevated expression in the lower FRT relative to the upper FRT. Together, these data indicate that the sex hormones estrogen and progesterone regulate factors influencing host defense in the FRT, and the hormonal regulation of the IL-36 cytokines may ultimately impact susceptibility to infection.

Introduction

Epithelial cells (EC) function as a first line of defense against pathogens at mucosal sites, forming a physical barrier to infection and contributing to the secreted chemical barrier to protect against pathogens. The formation of tight junctions, adherens junctions, and desmosomes that join EC together and form a physical barrier that excludes the transmigration of pathogens across the epithelium. In the lower FRT, vaginal EC form a stratified squamous epithelium, whereas in the upper FRT, endometrial and endocervical EC form a columnar epithelium (Wira, Grant-Tschudy, and Crane-Godreau 2005). ECs additionally contribute to the chemical barrier to infection through the secretion of cytokines, chemokines, and antimicrobial peptides as pattern recognition receptors on the EC plasma member detect pathogen signatures and initiate immune signaling in response to these invaders (Kaushic, Grant, et al. 2000, Kaushic, Zhou, et al. 2000, Robertson, Mayrhofer, and Seamark 1996, Wira, Grant-Tschudy, and Crane-Godreau 2005). These FRT EC barriers are crucial in the maintenance of homeostasis and are regulated in part by the gonadal steroids, estrogen and progesterone, that fluctuate throughout the different stages of the menstrual cycle (Pessina et al. 2006).

Parr and Parr were the first to show that mice treated with Depo Provera, the widely-used synthetic progestin contraceptive, were susceptible to genital HSV-2 infection, although the mechanisms by which DMPA increased susceptibility to infection were not well understood at the time (Parr et al. 1994). The researchers initially hypothesized that the thinned epithelium and increased epithelial permeability in a progesterone-dominant environment may increase susceptibility to infection and facilitate penetration of the virus through the epithelial barrier. This was recently confirmed in a

study that demonstrated that DMPA modifies the physical barrier to infection by decreasing epithelial barrier thickness, increasing permeability, and decreasing barrier integrity that enhance susceptibility to genital HSV-2 infection and virus spread beyond the vaginal epithelium (Quispe Calla et al. 2016). Others have also shown that DMPA treatment thins the vaginal epithelium in non-human primate models (Butler et al. 2013, Butler et al. 2015), however, DMPA-mediated thinning of the FRT epithelium in humans has not consistently been observed and measured (Bahamondes et al. 2000, Bahamondes et al. 2014, Miller et al. 2000). In contrast to DMPA-mediated epithelium thinning, treatment with estrogen to produce an estrogen-dominant environment has been shown to lead to hyperplasia in the vaginal epithelium (Pessina et al. 2006). Estrogen-conditioned mice maintain a robust epithelium that protects against genital HSV-2 infection (Parr et al. 1994, Quispe Calla et al. 2016). In fact, intravaginal bacterial infections often require mice to be conditioned with β-estradiol to thicken the epithelia and provide a more amenable environment for bacterial colonization (Jerse 1999, Jerse et al. 2011, Packiam et al. 2010). Hormonal regulation of FRT tissue varies throughout the menstrual/estrous cycle as levels of estrogen and progesterone fluctuate, and this regulation of the barrier is essential for maintenance of tissue architecture and can potentially impact host defense in the FRT (Pessina et al. 2006).

In addition to the regulation of physical barrier to infection in the vaginal microenvironment, in several reports DMPA has been shown to both dampen and elevate levels of inflammatory mediators in clinical studies that may ultimately influence susceptibility to sexually transmitted infections (STIs). These conflicting reports of up- or down-regulation of immune mediators in women using DMPA may be attributed to

several different factors, including, ethnicity, FRT infections, sexual behavior, and vaginal hygiene practices. Despite these inconsistencies, several recent reports have shown that levels of pro-inflammatory cytokines and chemokines are significantly decreased in cervicovaginal lavages from women using DMPA, and may adversely impact the host response to infection (Govender et al. 2014, Huijbregts et al. 2013, Ngcapu et al. 2015, Smith-McCune et al. 2017). Further, it has been demonstrated that physiologically relevant doses of DMPA inhibits T cell and dendritic cell activation, highlighting additional immunosuppressive effects resulting from DMPA use (Huijbregts, Michel, and Hel 2014). Interestingly, DMPA use has recently been linked to an increased risk for STI acquisition in high-risk women, suggesting that DMPA-mediated dampening of the immune response may contribute to STI susceptibility (Butler et al. 2015, Kleinschmidt et al. 2007, Smith-McCune et al. 2017, Socias et al. 2017). On the other hand, elevated estrogen levels have been linked with enhanced T cell homing (Mo et al. 2005), dendritic cell maturation (Kovats 2012), and the promotion of the type I interferon response (Panchanathan, Liu, and Choubey 2013, Panchanathan et al. 2009, Shen et al. 2010), suggesting an improved immune response in an estrogen-dominant environment. Together, these studies highlight the role of estrogen and progesterone in the FRT in maintaining balance in the immune system and the potential impact that these fluctuations can have during health and disease.

IL-36γ is a pro-inflammatory member of the IL-1 superfamily of cytokines that is expressed at mucosal sites, including the FRT. Murrieta-Coxca et al. showed that the IL-36 cytokines are expressed in the uterus of mice and that cytokine expression fluctuated throughout the estrous cycle, with expression at the lowest level during diestrus, and

peaking during estrus (Murrieta-Coxca et al. 2016). Winkle et al. and Patras et al. have demonstrated that IL-36γ is induced in the FRT after exposure to microbial products and Group B Streptococcus, suggesting a function in host defense mechanisms in the FRT (Patras et al. 2015, Patras et al. 2013, Winkle, Throop, and Herbst-Kralovetz 2016). While the hormonal regulation of several immune mediators in the FRT has been described, regulation of IL-36γ and the other IL-36 family members in the lower FRT by estrogen and progesterone has not previously been investigated and could provide essential insights into the regulation and function of IL-36γ in host defense in the FRT.

In this chapter the impact of the gonadal steroids on epithelial barrier features and immune mediators, including the IL-36 cytokines, was evaluated. Collectively, we demonstrate that in the upper and lower FRT estrogen and progesterone function to regulate expression of the IL-36 cytokines, other immune mediators, and key epithelial barrier features that further suggest that IL-36γ may exhibit a crucial function in host defense mechanisms in the FRT.

Materials and Methods

Ethics Statement

All animals were housed in accordance with Assessment and Accreditation of Laboratory Animal Care (AAALAC) standards, provided unlimited access to food and water, and all procedures and handling for this study were approved by the University of Arizona (UA) Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the Animal Welfare Act.

Hormone preparation and treatment in mice

Water soluble β-estradiol (MilliporeSigma, St. Louis, MO) was resuspended in sterile water for injections (Henry Schein, Melville, NY) at a concentration of 5 mg/ml and filter sterilized. Medroxyprogesterone (DMPA; Upjohn Company, Kalamazoo, MI) was diluted in sterile 0.9% saline to a concentration of 10 mg/ml. Mice were acclimated for one week before being randomly grouped. Female six- to eight-week-old C57Bl/6 mice were conditioned with DMPA (1 mg/mouse) on day -7 and day -1 as previously described (*n* =5). Mice were conditioned with β-estradiol by i.p. injection as previously described (*n* = 5). On day 0, mice were euthanized, and female reproductive tracts were collected. Upper and lower reproductive tracts were separated at the branch point for the uterine horns and stored in DNA/RNA shield until RNA extraction (Zymo Research, Irvine, CA).

RNA extraction and qRT-PCR analysis

RNA was extracted from murine upper and lower FRT tissue using the Zymo Quick-RNA kit following the manufacturer's instructions (Zymo Research). cDNA was synthesized from 1 μg RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and then analyzed by qRT-PCR using iTAQ Universal SYBR Green Supermix (Bio-Rad) with an Applied Biosystems QuantStudio6 Flex Real Time PCR System (Thermo Fisher Scientific Life Technologies, Waltham, MA). Gene expression was normalized to *Gapdh*. Primers sequences are listed in **Table 4**.

Statistics

Statistical analyses were performed by unpaired two-tailed Student *t*-test with Welch's correction or one-way ANOVA with Bonferroni's multiple comparison test using Prism software version 8 (GraphPad, San Diego, CA). A p value of < 0.05 was considered significant.

Table 4.

Primers used in qPCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	TCATGACCACAGTCCATGCCA	CCCTGTTGCTGTAGCCAAATT
IL36G	CCCAGTCACTGTTGCTGTTA	CAGTCTTGGCACGGTAGAAA
Cxcl1	CAATGAGCTGCGCTGTCAGTG	CTTGGGGACACCTTTTAGCATC
Dsc1	GATGGTCAAAGGAGGCCACA	ACACCTTTTCACCAAGCCGA
Dsg1	TCCCGCAAGAGCTACTTTGTT	AAACTTTCAAACCTAGGCATCTTC
Gapdh	AAATTCAACGGCACAGTCAAG	TGGTGGTGAAGACACCAGTAG
Il1a	CTCTAGAGCACCATGCTACAGAC	TGGAATCCAGGGGAAACACTG
Il1b	AGCAACGACAAAATACCTGTG	TCTTCTTTGGGTATTGCTTGG
Il36a	ACAAAGGATGGGGAGCAGCCTGT	CCAGGGAAGGCTGCAGACTCAAAT
Il36b	ACAAAAAGCCTTTCTGTTCTATCAT	CCATGTTGGATTTACTTCTCAGACT
Il36g	ATGGACACCCTACTTTGCTG	TGTCCGGGTGTGGTAAAACA
Il36rn	GGGCACTATGCTTCCGAATG	CTTTGATTCCTTGGCCCCGA
Il36r	AAACACCTAGCAAAAGCCCAG	AGACTGCCCGATTTTCCTATG
Slpi	AAGTCCTGCGGCCTTTTACCT	GGCATTGTGGCTTCTCAAGCT
Tnf	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT

Results

Sex Hormones Regulate Expression of Epithelial Barrier Features and Immune Mediators

Because mice are often treated with either DMPA or β-estradiol prior to intravaginal viral or bacterial infection, we first sought to determine the impact of these sex hormones on basal expression of key epithelial barrier features and immune

mediators. Wild type C57Bl/6 mice were hormonally conditioned with either DMPA or β-estradiol and mice were sacrificed and FRT tissue was collected. Interestingly, the FRT in β-estradiol conditioned mice appeared to be larger and more swollen than the FRT from DMPA conditioned mice (**Fig. 5**). The uterine horns were separated from the lower FRT and expression of barrier features and immune mediators was analyzed by qPCR in both upper and lower FRT tissue. We found that basal expression of desmocollin (*Dsc1*) and desmoglein (*Dsg1*), two cadherins present in desmosomes, was elevated in the lower FRT relative to the upper FRT, regardless of hormone treatment (**Fig. 6A, B**). In the lower FRT, expression of these *Dsc1* was increased 89-fold in β-estradiol conditioned mice relative to DMPA conditioned mice. Similarly, *Dsg1* expression was upregulated in the lower FRT in β-estradiol conditioned mice 430-fold relative to DMPA conditioned mice (**Fig. 6B**). Interestingly, in the upper FRT, we measured small differences in *Dsc1* (-1.61-fold change) and *Dsg1* (1.54-fold change) expression in β-estradiol conditioned mice relative to DMPA conditioned mice.

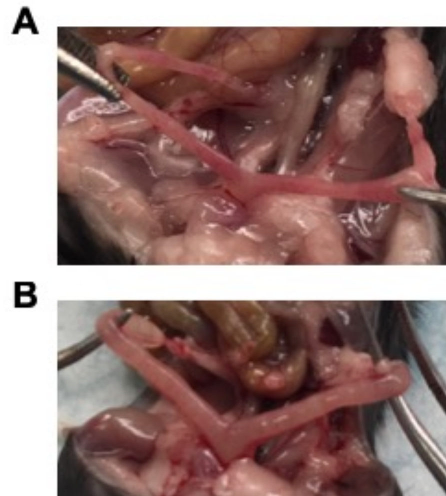

Figure 5. Effect of sex hormones on mouse FRT tissue. Representative images of FRT from DMPA (**A**) and β-estradiol (**B**) conditioned mice.

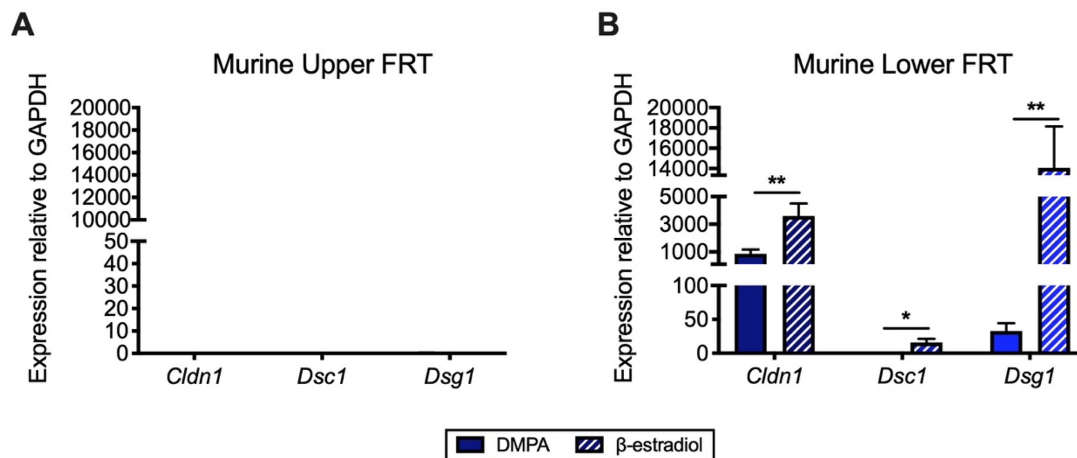

Figure 6. Sex hormones regulate expression of barrier feature in the FRT. Six- to eight-week-old mice (n=5/group) were conditioned with DMPA or β-estradiol. Expression of barrier features in upper (A) and lower (B) FRT was assessed by qPCR. Expression was evaluated relative to GAPDH and data represent two independent animal experiments. Data depict mean expression ± SD and statistical significance was determined by unpaired two-tailed Student *t*-test. *, $p < 0.05$; **, $p < 0.01$.

After evaluating features of the epithelial barrier, we then sought to assess expression of immune mediators in the FRT in estrogen-dominant and progesterone-dominant microenvironments. Similar to our findings in barrier features, we found that basal expression of several immune mediators was elevated in the lower FRT relative to the upper FRT (**Fig. 7**). In the lower FRT, the immune mediators *Il1a*, *Tnf*, *Cxcl1*, and *Slpi* all exhibited elevated expression in β-estradiol conditioned mice (13-fold, 9-fold, 8-fold, and 5-fold, respectively) relative to DMPA conditioned mice. We likewise measured increased expression of *Il1a* (23-fold), *Tnf* (340-fold), *Cxcl1* (42-fold), and *Slpi* (49-fold) in the upper FRT in β-estradiol treated mice relative to DMPA treated mice, albeit at lower expression levels than what we measured in the lower FRT. Together, these data demonstrate site-specific differences in basal expression of barrier feature and

immune mediators between lower and upper murine FRT tissue, and show that estrogen may promote epithelial barrier integrity and elevated expression of immune mediators, whereas progesterone appears to dampen barrier feature and immune mediator expression in the lower FRT.

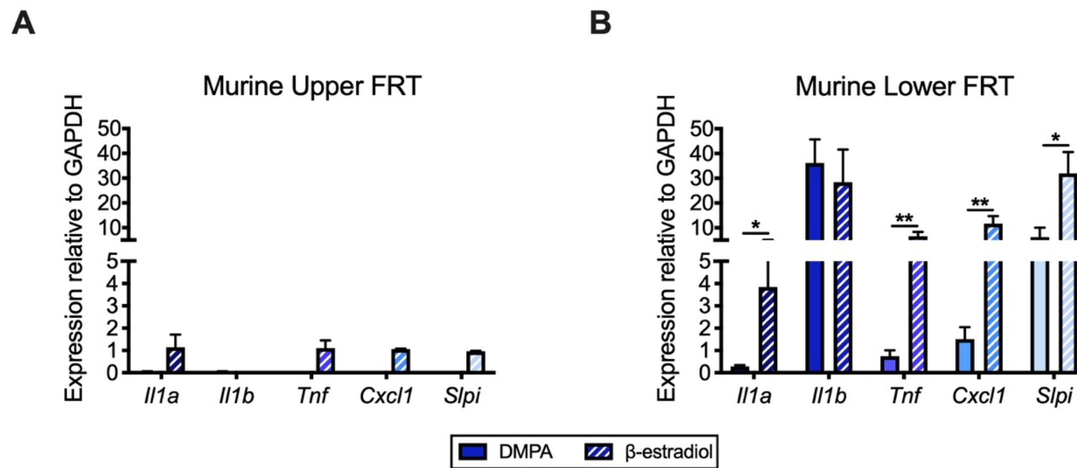

Figure 7. Sex hormones regulate immune mediator expression in the FRT. Six- to eight-week-old mice (n=5/group) were conditioned with DMPA or β-estradiol. Expression of immune mediators in upper (A) and lower (B) FRT was assessed by qPCR. Expression was evaluated relative to GAPDH and data represent two independent animal experiments. Data depict mean expression ± SD and statistical significance was determined by unpaired two-tailed Student *t*-test. *, $p < 0.05$; **, $p < 0.01$.

Hormonal Regulation of the IL-36 Family in the Murine FRT

Because expression of the IL-36 cytokines is more tissue restricted than other immune mediators, we next set out to evaluate the extent to which the sex hormones estrogen and progesterone regulate expression of the IL-36 cytokines in the murine FRT. All IL-36 cytokines were detected by qPCR in the upper and lower FRT, albeit at lower expression levels in the upper FRT relative to the lower FRT (**Fig. 8**). We further found that β-estradiol elevated expression of all IL-36 family members in the lower FRT

relative to expression in DMPA conditioned mice. *Il36b* and *Il36g* expression in lower FRT tissue were significantly increased 17-fold ($p < 0.001$) and 26-fold ($p < 0.0001$), respectively, in β-estradiol conditioned mice relative to DMPA conditioned mice. *Il36a*, *Il36rn*, and *Il1rl2* expression in β-estradiol treated mice was elevated 20-fold, 331-fold, and 8-fold relative to DMPA treated mice, respectively, although these increases were not statistically significant. Interestingly, we observed that IL-36 cytokine expression is less influenced by the sex hormones in the upper FRT relative to the lower FRT. In the upper FRT we measured decreased expression of *Il36a* (-1.8-fold), *Il36rn* (-4.2-fold), and *Il1rl2* (-3.5-fold), and increased expression of *Il36b* (2.3-fold) and *Il36g* (15.6-fold) in β-estradiol treated mice relative to DMPA treated mice. Surprisingly, IL-36γ was the IL-36 family member that exhibited the largest change in expression between the two hormone treatments. Collectively, these data demonstrate that IL-36 cytokine expression is hormonally regulated in the FRT, and this regulation may influence susceptibility to infection and ultimately impact host defense mechanisms.

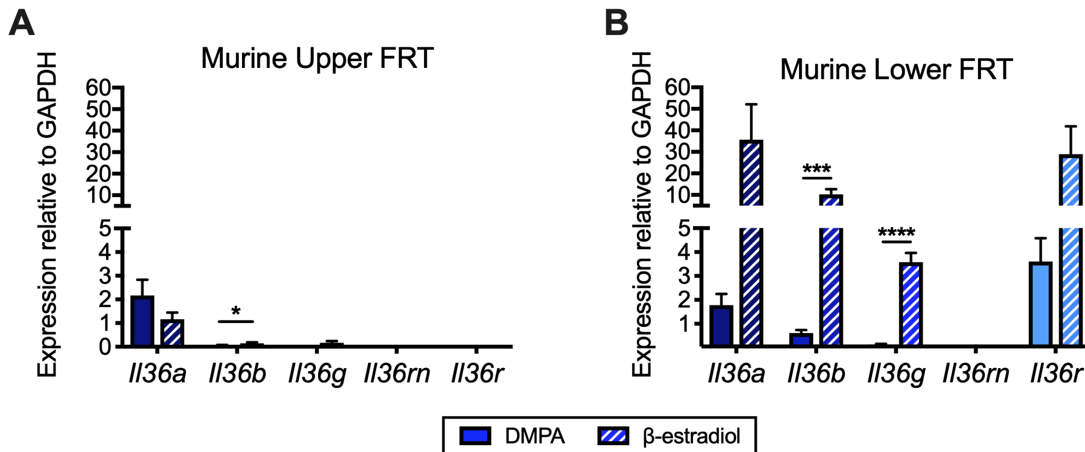

Figure 8. Hormonal regulation of the IL-36 cytokines. Female six- to eight-week-old C57Bl/6 mice were conditioned with either β-estradiol or DMPA. Levels of the IL-36 family members were evaluated in the upper and lower FRT. Expression was calculated relative to Gapdh and data depict mean ± SD. Results are representative of two independent animal studies. Statistical significance was evaluated by one-way ANOVA with Bonferroni's multiple comparisons test. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

Discussion

Several reports have recently linked hormonal contraceptives to increased STI acquisition, directing researchers to investigate the role of hormones in regulating immunity in the FRT. In fact, high-risk women (e.g. sex workers) using DMPA as a contraceptive have been shown to exhibit an increased risk for HSV-2 infection (Grabowski et al. 2015, Socias et al. 2017). DMPA use has also been linked to HIV acquisition, and in 2016 the World Health Organization initiated a large randomized trial to better characterize relationships between DMPA use, STI acquisition, and host defense in the FRT (Hofmeyr et al. 2017, Polis et al. 2016). In this chapter, we demonstrate that the IL-36 cytokines and other factors influencing host defense are hormonally regulated in response to estrogen and progesterone in the upper and lower FRT and may ultimately impact the host response to pathogens and susceptibility to infection.

Desmocollin and desmoglein are two essential cadherins that functionally make up desmosomes, important cell junction molecules that adhere cells together and physically exclude pathogens (Delva, Tucker, and Kowalczyk 2009, Kowalczyk and Green 2013). Quispe Calla et al. demonstrated that DMPA treatment in mice significantly reduced expression of *DSC1* and *DSG1* and decreased epithelium thickness that corresponded with increased genital mucosal permeability in mice and in human ectocervical biopsies (Quispe Calla et al. 2016). Similarly, we show significantly reduced expression of *DSC1* and *DSG1* in DMPA conditioned mice relative to mice conditioned with β-estradiol, indicating the potential role that these sex hormones may exhibit in regulating key barriers to infection. In fact, DMPA conditioning is required for intravaginal viral infection, due in part to the thinning of the vaginal epithelium (Kaushic et al. 2003, Kaushic, Zhou, et al. 2000, Parr et al. 1994, Parr and Parr 2003, Quispe Calla et al. 2016). Quispe Calla et al. and others have demonstrated that mice treated with β-estradiol or in the estrous stage are protected against intravaginal HSV-2 infection, and exhibit decrease epithelium permeability and increased thickness (Kaushic et al. 2003, Kaushic, Zhou, et al. 2000, Quispe Calla et al. 2016). Collectively, our findings support the conclusion that the sex hormones estrogen and progesterone function in an important manner to regulate epithelial barrier integrity and the physical barrier to infection.

In addition to the regulation of the physical barrier to infection, current evidence suggests that the sex hormones estrogen and progesterone additionally function to regulate the chemical barrier to infection and production immune mediators that are crucial components of the host response to pathogens in the FRT mucosa (Wira et al. 2010, Wira, Rodriguez-Garcia, and Patel 2015). In humans, DMPA and other progestins

have inconsistently been shown to suppress levels of immune mediators in the FRT (Deese et al. 2015, Francis et al. 2016, Govender et al. 2014, Huijbregts et al. 2013, Ngcapu et al. 2015, Smith-McCune et al. 2017), and this may be due to a wide-array of factors, including ethnicity (Murphy and Mitchell 2016, Nguyen et al. 2004, Ryckman et al. 2008), vaginal health(Campos et al. 2012, Hemalatha et al. 2012, Mitchell et al. 2008), and vaginal hygiene practices (Alcaide et al. 2017, Birse et al. 2017) among others. In the murine model, we are able to exclude many of these potentially confounding variables to better understand the impact of estrogen and progesterone on the immune mechanisms, and particularly how the sex hormones influence the basal expression of several immune mediators that exhibit crucial functions in mucosal immunity. Our data demonstrate that DMPA treatment results in decreased expression of several immune mediators, including *Il1a*, *Tnfa*, *Cxcl1*, and *Slpi* in naïve FRT tissue. We observed higher expression levels of these immune mediators in lower FRT tissue relative to upper FRT tissue, demonstrating that basal expression of these pro-inflammatory immune mediators is site-specific in the FRT. This can in part be attributed to the fact that the lower FRT faces a barrage of pathogens that require a robust immune response, whereas the upper FRT generally does not encounter FRT pathogens in healthy states (Baker, Chase, and Herbst-Kralovetz 2018). When mice were treated with estrogen, we observed a significant increase in expression of *I1a* (13.2-fold, $p < 0.05$), *Tnf* (8.8-fold, $p < 0.01$), *Cxcl1* (7.7-fold, $p < 0.01$), and *Slpi* (5.1-fold, $p < 0.05$) in the lower FRT relative to levels when mice were treated with DMPA. Altogether, these data indicate that in the murine model, DMPA suppresses expression of several key inflammatory mediators relative to β-estradiol

treatment, suggesting that DMPA use may contribute to increased susceptibility to genital HSV-2 infection through the dampening of the immune response.

Given that we measured decreased expression levels of several immune mediators in DMPA-conditioned mice relative to β-estradiol treated mice, we sought to evaluate hormonal regulation of the IL-36 family, cytokines that are robustly expressed at mucosal sites. Recently, a proteomics study of Kenyan women indicated that elevated IL-36γ levels were associated with DMPA use and various vaginal hygiene practices, including vaginal washing and drying (Birse et al. 2017). Interestingly, 70% of participants using DMPA were HSV-2 seropositive, and 26% of the women had bacterial vaginosis (Birse et al. 2017), and these FRT infections and vaginal hygiene practices may have led to the elevated IL-36γ levels that were detected in the study. In the murine model we can eliminate many of these potentially confounding factors and evaluate hormonal regulation of the IL-36 cytokines in naïve mice. We detected significantly decreased expression of *Il36b* (16.6-fold decrease, $p < 0.001$) and *Il36g* (26.2-fold decrease, $p < 0.001$) in the lower FRT of DMPA treated mice relative to β-estradiol treated mice, indicating that expression of the IL-36 cytokines may in-part be hormonally regulated. All family members were expressed in murine FRT tissue, and we additionally measured decreased expression of *Il36a* (20.1-fold decrease), *Il36rn* (331-fold decrease), and *Il36r* (8-fold decrease) in the lower FRT of DMPA treated mice relative to β-estradiol treated mice, though these decreases were not statistically significant. Similar to our findings with other immune mediators, we measured site-specific differences in expression of the IL-36 family members, with increased expression in the lower FRT relative to the upper FRT. We have previously shown that IL-36γ is induced in response to an array of

microbial products (Winkle, Throop, and Herbst-Kralovetz 2016), and suppression of IL-36γ by DMPA treatment may result in a suppression of IL-36γ and IL-36R signaling, resulting in an insufficient immune response and increased susceptibility to infection. We have demonstrated that expression of the IL-36 cytokines is regulated in-part through the sex hormones estrogen and progesterone, and this regulation may ultimately impact the host response to pathogens in the FRT and susceptibility to STIs.

Collectively, in this chapter we demonstrate that the sex hormones estrogen and progesterone function to regulate the epithelial barrier to infection, as well as the chemical barrier to infection in mice. We show that the synthetic progestin, DMPA, reduces expression of the key epithelial barrier features *Dsc1* and *Dsg1*, suggesting a weakening of the epithelial barrier. We additionally demonstrate that DMPA treatment results in decreased expression of several pro-inflammatory immune mediators, including the IL-36 cytokines, and IL-36γ specifically. Together, we hypothesize that the dampened expression of IL-36γ, coupled with the reduced expression of other immune mediators and weakened epithelial barrier may contribute to the increased susceptibility to FRT infection after DMPA use.

CHAPTER 4: IL-36γ INDUCES A TRANSIENT HSV-2 RESISTANT ENVIRONMENT THAT PROTECTS AGAINST GENITAL DISEASE AND PATHOGENESIS

Publication Note

The research reported in this chapter was previously published in an altered format in *Cytokine.* Jameson K. Gardner and Melissa M. Herbst-Kralovetz. IL-36γ induces a transient HSV-2 resistant environment that protects against genital disease and pathogenesis. *Cytokine*. **111**:63-71. doi: 10.1016/j.cyto.2018.07.034.

Abstract

Herpes simplex virus 2 (HSV-2) causes a persistent, lifelong infection that increases risk for sexually transmitted infection acquisition. Both the lack of a vaccine and the need for chronic suppressive therapies to control infection presents the need to further understand immune mechanisms in response to acute HSV-2 infection. The IL-36 cytokines are recently identified members of the IL-1 family and function as inflammatory mediators at epithelial sites. Here, we first used a well-characterized three-dimensional (3-D) human vaginal epithelial cell (VEC) model to understand the role of IL-36γ in the context of HSV-2 infection. In 3-D VEC, IL-36γ is induced by HSV-2 infection, and pretreatment with exogenous IL-36γ significantly reduced HSV-2 replication. To assess the impact of IL-36γ treatment on HSV-2 disease pathogenesis, we employed a lethal genital infection model. We showed that IL-36γ treatment in mice prior

to lethal intravaginal challenge significantly limited vaginal viral replication, delayed disease onset, decreased disease severity, and significantly increased survival. We demonstrated that IL-36γ treatment transiently induced pro-inflammatory cytokines, chemokines, and antimicrobial peptides in murine lower female reproductive tract (FRT) tissue and vaginal lavages. Induction of the chemokines CCL20 and KC in IL-36γ treated mice also corresponded with increased polymorphonuclear (PMN) leukocyte infiltration observed in vaginal smears. Altogether, these studies demonstrate that IL-36γ drives the transient production of immune mediators and promotes PMN recruitment in the vaginal microenvironment that increases resistance to HSV-2 infection and disease. Our data indicate that IL-36γ may participate as a key player in host defense mechanisms against invading pathogens in the FRT.

Introduction

Genital herpes simplex virus 2 (HSV-2) infections remain one of the most common sexually transmitted infections (STI), affecting over 250 million women worldwide (Looker et al. 2015a). HSV-2 causes a persistent, lifelong infection that increases risk for STI acquisition, including human immunodeficiency virus (Freeman et al. 2006). Despite the availability of therapeutic interventions to limit HSV-2 disease, the virus can be transmitted through asymptomatic shedding and even during chronic suppressive therapy (Johnston and Corey 2016, Johnston et al. 2012, Wald et al. 2000a).

The vaginal epithelium is a first-line of defense against acute genital HSV-2 infection, forming a physical barrier to infection. Initiation of innate immune signaling by

vaginal epithelial cells is crucial for the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides (AMP) to control acute HSV-2 infection (Johnson, Koelle, and Wald 2011, Shin and Iwasaki 2013). These soluble immune mediators produced in the vaginal epithelium are vital components of the barrier to infection, and can protect against HSV-2 infection by blocking binding and entry (Hazrati et al. 2006, John et al. 2005, Shust et al. 2010, Yarbrough, Winkle, and Herbst-Kralovetz 2015). Despite our current understanding of host defense mechanisms, there still exists a need to understand underlying innate immune mechanisms in the vaginal epithelium that can inhibit virus replication during acute HSV-2 infection, and ultimately limit the spread and establishment of latency and recurrent HSV-2 disease. We have previously shown that interleukin (IL)-36γ, a novel pro-inflammatory cytokine, is expressed in the lower FRT and is induced in a Toll-like receptor (TLR)-mediated manner in response to microbial products, including the viral dsRNA mimic, poly(I:C) (Winkle, Throop, and Herbst-Kralovetz 2016). Additionally, we demonstrated that IL-36γ signals in an autocrine manner, creating a self-sustaining loop that amplifies IL-36γ and cytokine, chemokine, and AMP production in the FRT (Winkle, Throop, and Herbst-Kralovetz 2016).

Several studies have recently shown that the IL-36 cytokines, including IL-36α, -β, and -γ, are key inflammatory mediators in host defense against bacteria, fungi, and viruses at various epithelial sites (Ahsan et al. 2016, Aoyagi et al. 2016, Braegelmann et al. 2018, Gabay and Towne 2015, Huynh et al. 2016, Kovach et al. 2017, Kovach et al. 2016, Winkle, Throop, and Herbst-Kralovetz 2016). These family members share between 15-85% sequence similarity at the amino acid level, with IL-36α and IL-36γ

being the most similar among the cytokines (Milora et al. 2017). Despite similarity among the three agonists, the differential expression patterns of the IL-36 family members suggest that these cytokines may have cell- and/or tissue-specific functions (Bassoy, Towne, and Gabay 2018, Gresnigt and van de Veerdonk 2013). IL-36α, - β, and -γ are all expressed in nonhematopoietic cells, including keratinocytes and mucosal epithelial cells, but are induced in response to different inflammatory stimuli (Walsh and Fallon 2016). For example, IL-36α and IL-36γ have been shown to be robustly induced in bronchial epithelial cells in response to microbial products, bacterial infection, and viral infection, suggesting that the IL-36 cytokines may play an important role in epithelial host defense (Aoyagi et al. 2016, Bochkov et al. 2010, Chustz et al. 2011, Kovach et al. 2017, Kovach et al. 2016). Indeed, IL-36γ -/- mice exhibited delayed clearance of *Streptococcus pneumoniae* and *Klebsiella pneumoniae* in lung infections, decreased Th1 and Th17 cytokine levels, and increased mortality (Kovach et al. 2017). In *Mycobacterium tuberculosis* infections, IL-36γ promotes the production of AMPs that limit bacterial growth (Ahsan et al. 2018, Ahsan et al. 2016). In another study, it was found that IL-36R signaling and IL-36α promoted the production of immune mediators and increased influx of neutrophils and monocytes in response to influenza virus infection in the lungs, indicating that the IL-36 cytokines may have distinct functions in response to specific inflammatory stimuli(Aoyagi et al. 2016). It has been well documented that the viral RNA mimic poly(I:C) induces IL-36γ, further demonstrating that IL-36γ may play a role in host antiviral defense mechanisms (Lian et al. 2012, Rana et al. 2015, Winkle, Throop, and Herbst-Kralovetz 2016). However, IL-36β, but not IL-

36α or IL-36γ, has been shown to protect against HSV-1 disease in keratinocytes and a flank skin infection model (Milora et al. 2017). This finding indicates that the IL-36 cytokines may have site-specific functions in host defense. In the upper female reproductive tract it has been shown that *Listeria monocytogenes* infection robustly induced the IL-36 cytokines in the uteri of pregnant mice, however, the impact of the IL-36 cytokines on infection and pregnancy is still unclear (Murrieta-Coxca et al. 2016). Recently, our laboratory measured increased levels of IL-36γ in cervicovaginal lavages (CVLs) from cervical cancer patients (Laniewski et al. 2018). Additionally, clinical proteomic studies have also measured an increase in the relative abundance of IL-36γ in CVLs from HSV-2 seropositive Depo-Provera users (Birse et al. 2017), and an increase in IL-36 cytokines in women with bacterial vaginosis (Borgdorff et al. 2016). Together, these studies suggest that IL-36γ may play an important role in host defense mechanisms in the FRT.

We aimed to better understand IL-36γ in the context of HSV-2, a clinically relevant viral STI. In this study, we utilized an innovative three-dimensional (3-D) human vaginal epithelial cell (VEC) model (Gardner and Herbst-Kralovetz 2016) and a lethal genital infection model to identify the extent to which IL-36γ impacts HSV-2 disease. We also investigated the level to which IL-36γ treatment modulated production of immune mediators and recruitment of immune cells in the vaginal microenvironment as potential mechanisms by which IL-36γ limits genital HSV-2 disease. Collectively, our data suggests that IL-36γ may participate as a key regulator of mucosal inflammation and host defense in the FRT.

Materials and Methods

3-D VEC culture

Three-dimensional human vaginal epithelial (V19I) cells were cultured as previously described (Hjelm et al. 2010, Radtke and Herbst-Kralovetz 2012, Winkle, Throop, and Herbst-Kralovetz 2016). The V19I cell line was validated by short tandem repeat (STR) profiling and shown to be free of contamination from other cell lines. Briefly, V19I cells were combined with collagen-coated dextran microcarrier beads in a 1:1 mixture of supplemented keratinocyte serum free medium (KSFM) and EpiLife medium (Life Technologies, Grand Island, NY). Cell and bead mixtures were transferred to a slow turning lateral vessel bioreactor (Synthecon, Houston, TX) and incubated over a 28-day period at 37°C. Fully-differentiated aggregates were quantified and cell viability was measured by trypan blue exclusion using a Countess machine (Life Technologies). For all *in vitro* experiments, 3-D aggregates were transferred into 24-well plates (1×10^5–5×10^5 cells/ml).

HSV-2 propagation and plaque assay

HSV-2 186 was generously provided by Dr. Richard Pyles (UTMB, Galveston, TX), and used for all studies. Stocks were prepared from infected Vero cell monolayers and frozen at -80°C. Vero cell monolayers (ATCC CCL-81) were grown in Dulbecco's modified Eagle's medium (DMEM; Corning, Manassas, VA) as previously described (Pyles et al. 2002). All HSV-2 infections *in vitro* were performed at a multiplicity of infection (MOI) of 0.1 (1×10^4–5×10^4 PFU/ml depending on 3-D cell density in individual experiments). Virus titers were quantified by standard plaque assay using Vero

cell monolayers as previously described (Herbst-Kralovetz and Pyles 2006a). The University of Arizona (UA) Institutional Biosafety Committee (IBC) approved all safety and handling of HSV-2 in the laboratory.

In vitro cytokine and TLR agonist treatment

Three-dimensional aggregates were treated with poly(I:C) (Invivogen, San Diego, CA) at 100 µg/ml, recombinant human IL-36γ (Peprotech, Rocky Hill, NJ) at 100 or 500 ng/ml as previously described (Winkle, Throop, and Herbst-Kralovetz 2016), or recombinant IL-36Ra (BioLegend, San Diego, CA) at 100 ng/ml. These concentrations are consistent with prior reports in the literature studying IL-36γ in the lung, skin, and intestines (Chustz et al. 2011, Harusato et al. 2017, Jiang et al. 2017, Scheibe et al. 2017, Zhang et al. 2017). Aggregates were treated with acyclovir (ACV; GlaxoSmithKline, Research Triangle Park, NC) at 20 µg/ml as a positive control, or left untreated as a negative control.

Genital HSV-2 mouse model

Female six- to eight-week-old C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were housed in accordance with American Association for Laboratory Animal Care (AALAC) standards, provided unlimited access to food and water, and all procedures and handling for this study were approved by the UA Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the Animal Welfare Act to minimize pain and suffering. Animals were acclimated for 7 days before being grouped (*n*=5-10, groupings described

in figure legends) prior to treatment and HSV-2 infection. All mice were pretreated with medroxyprogesterone acetate (1 mg/mouse; Up John Company, Kalamazoo, MI) at day -7 and day -1 as previously described (Herbst-Kralovetz and Pyles 2006a). Mice were i.vag. treated by instilling recombinant murine IL-36γ (100 ng, 250 ng, or 500 ng; BioLegend) or PBS (Corning, Manassas, VA) in 10 µl total volume. Mice were i.vag. challenged with HSV-2 186 (1×10^3 or 1×10^4 PFU) in 10 µl total volume in DMEM media as previously described (Herbst-Kralovetz and Pyles 2006a). Survival and disease incidence in mice were measured over a 21-day period. The vaginal mucosa was visually inspected daily for hair loss, erythema, and ulceration by the trained investigators in the study. Disease severity was scored daily according to the following scale: no pathology (0), mild vulvar erythema (1), moderate vulvar erythema (2), severe vulvar erythema and hair loss (3), perineal ulceration (4), extension of perineal ulceration to surrounding tissue and/or hind limb paralysis (5). Moribund mice and those scoring a 5 were euthanized to minimize pain and suffering. Mice scoring a 4 that were euthanized were scored a 5 the following day. Disease onset/incidence was defined by erythema and hair loss (a score of 3). Vaginal swabs were collected at 2 and 3 days post inoculation (d.p.i.) using sterile urethro-genital calcium alginate tipped swabs (Puritan; Guilford, ME) in 1 mL DMEM stored at -80°C. Vaginal viral replication was measured in vaginal swabs by standard plaque assay as described.

IL-36γ treatment in mice

Mice were pretreated with medroxyprogesterone acetate as described above and then treated by i.vag. instillation with recombinant murine IL-36γ (250 ng or 500 ng;

BioLegend) in 10 µl total volume. Control mock-treated mice were administered 10 µl PBS. Vaginal lavages were collected from mice by i.vag. instilling 125 µl of sterile PBS and lavaging the vaginal cavity using an oral feeding tube (Fisher Scientific, Waltham, MA). Lavage fluid was stored at -80°C until cytometric bead array analysis. Female reproductive tract tissue was collected at 4h and 24h after treatment and stored in DNA/RNA Shield (Zymo Research, Irvine, CA) at -20°C until RNA extraction and qRT-PCR analysis.

Vaginal smears

Mice were conditioned with medroxyprogesterone acetate and treated with recombinant murine IL-36γ (250 ng; BioLegend) or mock-treated with PBS as described above. Four hours and 24h after treatment mice were swabbed with a PBS soaked urethro-genital calcium alginate tipped swab (Puritan). Swabs were smeared on Fisherbrand Selectfrost microscope slides (Fisher Scientific) and allowed to air dry. Slides were stained with modified Wright stain (Volu-Sol, Salt Lake City, UT) and imaged on a Zeiss AxioImager M2 (Zeiss, Oberkochen, Germany) at 20× and 40× magnification to identify cell populations. Cell counting was performed by selecting five unique fields on a smear at 20× magnification. The number of epithelial cells, PMN, and total cells in each field were enumerated for each mouse in the treatment groups and the average count/mm^2 was calculated. The field of view at 20× magnification has an area of 1 mm^2.

RNA extraction and qRT-PCR analysis

RNA was extracted from 3-D V19I aggregates and mouse FRT tissue using the Zymo Quick-RNA kit following the manufacturer's instructions (Zymo Research). Mouse FRT tissue was stored in DNA/RNA shield prior to RNA extraction. cDNA was synthesized from 1 μg RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and then analyzed by qRT-PCR using iTAQ Universal SYBR Green Supermix (Bio-Rad). qRT-PCR was performed with an Applied Biosystems QuantStudio6 Flex Real Time PCR System (Life Technologies). Gene expression was normalized to GAPDH and fold change was calculated relative to controls. Primers used for all qRT-PCR analyses are listed in **Table 5**.

Initial gene expression screens were performed using cDNA synthesized using a RT2 First-Strand kit (Qiagen, Valencia, CA) from 500 ng pooled RNA from murine FRT tissue treated with IL-36γ (500 ng) or PBS collected 4h and 24h after exposure and analyzed using Mouse Antiviral response and Mouse Toll-like receptor signaling RT2 arrays (Qiagen). qRT-PCR was performed as described above. Fold change was calculated by delta delta Ct analysis using RT2 Profiler PCR Array Data Analysis software (v3.5) and then log2 transformed (Qiagen).

Table 5.

Primers used in this study

Gene	Forward (5′-3′)	Reverse (5′-3′)
Human *GAPDH*	TCATGACCACAGTCCATGCCA	CCCTGTTGCTGTAGCCAAATT
Human *IL36A*	CCAGACGCTCATAGCAGTCC	AGATGGGGTTCCCTCTGTCTT
Human *IL36B*	ACCAAGGAGAGAGGCATAACTAAT	AGTGAACTCAGTCGCATAATGATC
Human *IL36G*	CCCAGTCACTGTTGCTGTTA	CAGTCTTGGCACGGTAGAAA
Human *IL1RL2*	GCTGGAGTGTCCACAGCATA	GCGATAAGCCCTCCTATCAA
Human *IL36RN*	ACTCGGCATTGAAGGTGCTTT	GGGACCACGCTGATCTCTT
Murine *Ccl20*	CGACTGTTGCCTCTCGTACA	CACCCAGTTCTGCTTTGGAT
Murine *Ccl4*	AAACCTAACCCCGAGCAACA	CCATTGGTGCTGAGAACCCT
Murine *Clec4e*	CTGTAAGTTCTGCCCGGAAA	GGATGCTTCAAAAACTCCCA
Murine *Cxcl1*	CAATGAGCTGCGCTGTCAGTG	CTTGGGGACACCTTTTAGCATC
Murine *Defb3*	GTCAGATTGGCAGTTGTGGA	GCTAGGGAGCACTTGTTTGC
Murine *Il1a*	CTCTAGAGCACCATGCTACAGAC	TGGAATCCAGGGGAAACACTG
Murine *Il1b*	AGCAACGACAAAATACCTGTG	TCTTCTTTGGGTATTGCTTGG
Murine *Il36g*	ATGGACACCCTACTTTGCTG	TGTCCGGGTGTGGTAAAACA
Murine *Il6*	AGATAACAAGAAAGACAAAGCCAGAGTC	GCATTGGAAATTGGGGTAGGAAG
Murine *Gapdh*	AAATTCAACGGCACAGTCAAG	TGGTGGTGAAGACACCAGTAG
Murine *Ltf*	AAACAAGCATCGGGATTCCAG	ACAATGCAGTCTTCCGTGGTG
Murine *Muc13*	TGCGTGATGCTACAAAGGAC	TGTCCTGGCATTTACTGCTG
Murine *Nlrp3*	TGCTCTTCACTGCTATCAAGCCCT	ACAAGCCTTTGCTCCAGACCCTAT
Murine *Slpi*	AAGTCCTGCGGCCTTTTACCT	GGCATTGTGGCTTCTCAAGCT

Cytometric bead array analysis of CVLs

Cytokine levels in murine vaginal lavages were measured by cytometric bead array analysis using a Mouse High Sensitivity T Cell Magnetic Bead Panel (containing IL-1α, IL-1β, IL-6, TNFα, CCL20, and CXCL1/keratinocyte chemoattractant (KC)) and a Mouse Cytokine/Chemokine Magnetic Bead Panel (containing IL-1β, IL-6, TNFα, IP-10, and KC) following the manufacturer's protocol (Millipore Sigma, Billerica, MA). Assays were performed using a Bio-Plex 200 system with Bio-Plex 5.0 Manager software (Bio-Rad).

Statistics

Disease incidence and survival in mouse models were analyzed by log-rank analysis as previously described (Herbst-Kralovetz and Pyles 2006a). Disease severity and resolution were analyzed by Area Under Curve (AUC) analysis with an unpaired two-tailed Student *t*-test with Welch's correction. Unpaired two-tailed Student *t*-test with Welch's correction, one-way ANOVA with Bonferroni's multiple comparisons test, and two-way ANOVA with Bonferroni's multiple comparisons test were performed for comparisons as indicated using Prism software version 7 (GraphPad, San Diego, CA). A P value of <0.05 was considered significant.

Results

IL-36γ is induced by poly(I:C) and HSV-2, and treatment with IL-36γ limits HSV-2 infection in human 3-D VEC

Microbial products, including the viral mimic poly(I:C), have been shown to induce expression of *IL36G* mRNA and protein (Chustz et al. 2011, Lian et al. 2012, Rana et al. 2015, Winkle, Throop, and Herbst-Kralovetz 2016). To determine if HSV-2 infection induces mRNA expression of IL-36 family members, 3-D human VEC were challenged with HSV-2 186 (MOI of 0.1) or treated with poly(I:C) (100 µg/ml). Aggregates were collected after 4h, and expression of IL-36α, IL-36β, IL-36γ, IL-36R (*IL1RL2*), and IL-36Ra (*IL36RN*) mRNA transcripts was measured by qRT-PCR. HSV-2 infection significantly ($P < 0.01$) induced expression of *IL36G* 6.5-fold relative to

untreated controls (**Fig. 9A**). Expression of other IL-36 family member gene transcripts were not significantly altered after HSV-2 infection, suggesting that IL-36γ may play an important role in antiviral host defense in the FRT. The induction of *IL36G* after HSV-2 infection was similar to poly(I:C) treatment, a viral RNA mimic, and known inducer of IL-36γ (**Fig. 9B**).

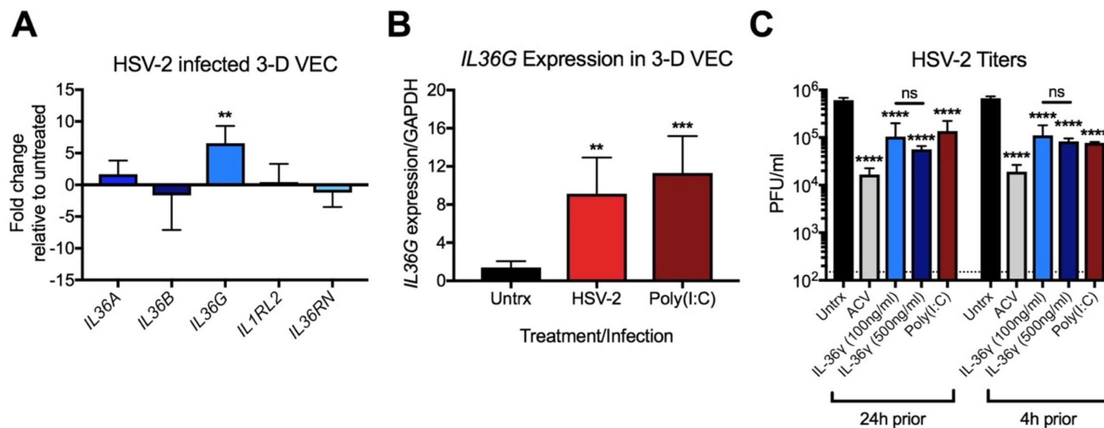

Figure 9. IL-36γ is induced by HSV-2 infection and IL-36γ treatment limits HSV-2 infection in 3-D VEC. (A) Three-dimensional human VEC were infected with HSV-2 186 (MOI 0.1) for 4h. IL-36 family member mRNA transcript levels were measured by qRT-PCR, normalized to *GAPDH* and expressed as fold-change relative to untreated controls. (B) Three-dimensional human VEC were infected with HSV-2 186 (MOI 0.1) or treated with poly(I:C) (100 µg/ml), a known inducer of *IL36G*, for 4h. *IL36G* mRNA transcript levels were measured by qRT-PCR and normalized to *GAPDH*. (C) HSV-2 replication was measured in 3-D VEC treated with recombinant IL-36γ (100 ng/ml or 500 ng/ml), Poly(I:C) (100 µg/ml), or left untreated 24h prior or 4h prior to infection with HSV-2 186 (MOI 0.1). Aggregates were treated with acyclovir (20 µg/ml) 2h prior to infection as a positive control. Viral titers were measured after 24h as PFU/ml by standard plaque assay. Dashed line indicates limit of detection for assay. Data represent mean ± SD from biological replicates from three independent experiments. Statistical significance was determined by one-way ANOVA with Bonferroni's multiple comparisons test (A, B) and two-way ANOVA with Bonferroni's multiple comparisons test (C). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

As a potential host defense mechanism in response to HSV-2 infection, we sought to determine the level to which IL-36γ impacted HSV-2 replication in a well-characterized 3-D human VEC model (Gardner and Herbst-Kralovetz 2016). Three-dimensional VEC aggregates were treated with IL-36γ (100 ng/ml or 500 ng/ml) or poly(I:C) (100 μg/ml) 24h prior or 4h prior to HSV-2 challenge. Aggregates were treated with acyclovir (ACV; 20 μg/ml) 2h prior to infection as a positive control to limit HSV-2 replication, or left untreated as a negative control. Twenty-four hours post inoculation, viral titers were measured by standard plaque assay. Treatment with IL-36γ (100 ng/ml) both 24h and 4h prior to infection resulted in a significant ($P < 0.05$) reduction in viral titers compared to untreated controls (**Fig. 9C**). Exposure to a higher dose of IL-36γ (500 ng/ml) 24h or 4h prior to infection reduced viral titers similar to treatment with IL-36γ at 100 ng/ml. Poly(I:C) treatment also significantly ($P < 0.01$) reduced viral titers similar to IL-36γ treatments at both time points. The relatively short half-life of ACV (Hodge and Perkins 1989, Schiffer et al. 2013, Weinberg et al. 1992), the administration of a single dose of ACV over a 24h period (Weinberg et al. 1992), and the high infectious dose ($1 \times 10^4 – 5 \times 10^4$ PFU) used in this study may explain the incomplete blockade of HSV-2 replication beyond the measured ~50-fold reduction. IL-36 signaling through IL-36R was blocked by treating 3-D human VEC with recombinant IL-36 receptor antagonist (IL-36Ra), and inhibition of IL-36γ signaling was confirmed by qRT-PCR (data not shown). Pretreatment with IL-36Ra (100 ng/ml) prior to HSV-2 challenge did not significantly increase viral titers relative to controls, suggesting that other mechanisms (e.g. NFκB activation, Type I IFN) may compensate to control HSV-2 infection in human VEC

(**Appendix A**). To extend these findings, we sought to understand the role of IL-36γ in the context of HSV-2 disease pathogenesis in a complex multicellular microenvironment using a well-established animal model.

Treatment with IL-36γ limits vaginal viral replication, delays disease onset, and decreases disease severity to protect against lethal HSV-2 challenge

To determine the extent to which IL-36γ exposure limits HSV-2 disease pathogenesis, female C57Bl/6 mice were treated with IL-36γ prior to lethal HSV-2 challenge. In initial studies to examine the impact of IL-36γ dose on HSV-2 disease, medroxyprogesterone acetate conditioned mice were i.vag. treated with recombinant murine IL-36γ (100 ng, 250 ng, or 500 ng) or mock treated with PBS (n = 5-10/group) 4h prior to lethal HSV-2 challenge with a rigorous challenge dose of 1×10^4 PFU (35X LD_{50}). We found that mice treated with IL-36γ (250 ng) 4h prior to infection had a significant ($P < 0.0001$) delay in disease onset, and survived significantly ($P < 0.0001$) longer relative to PBS controls (**Appendix A**). Similar to mice treated with the 250 ng dose, mice treated with IL-36γ (500 ng) exhibited a significant ($P < 0.0001$) delay in symptoms, and increased survival time compared to controls. However, there was no difference in the time to symptoms or survival time between mice that were treated with IL-36γ (100 ng) and controls. Further, when mice were treated with IL-36γ (250ng) 24h prior to lethal challenge, there was no difference in disease onset or survival time relative to controls. These initial studies demonstrated that both the dose of IL-36γ and timing of delivery are important in limiting HSV-2 disease.

We then sought to determine the impact of IL-36γ treatment on HSV-2 disease pathogenesis using a lower challenge dose (LD_{100}) previously reported (Herbst-Kralovetz and Pyles 2006a, Rose, McGowin, and Pyles 2009, Tsunobuchi et al. 2000). Medroxyprogesterone acetate conditioned mice were i.vag. treated with IL-36γ (250 ng) or mock-treated with PBS (n = 10/group) 4h prior to challenge with 1×10^3 PFU HSV-2 186 (LD_{100}). Vaginal viral titers were measured in vaginal swabs collected 2 and 3 days post-inoculation (d.p.i.). Vaginal viral titers were significantly ($P < 0.0001$) reduced in mice treated with IL-36γ compared to PBS controls on both day 2 and 3 p.i., and several IL-36γ treated mice had no detectable titers at either 2 or 3 d.p.i. (**Fig. 10A**). Survival and disease incidence was measured over a 21-day period and the disease severity was recorded daily on a 1-5 scale to monitor disease progression. IL-36γ treated mice had significantly ($P < 0.01$) less severe disease relative to PBS controls, and 3/20 (15%) IL-36γ treated mice exhibited no disease symptoms over the 21-day period (**Fig. 10B**). Of the 17 IL-36γ treated mice that developed disease, five mice (29.4%) exhibited less severe pathology that did not progress in severity beyond a score of 2/5. Treatment with IL-36γ significantly ($P < 0.0001$) delayed the onset of disease, and reduced the number of mice presenting both hair loss and erythema at the introitus and surrounding tissue to just 12/20 (60%), compared to 20/20 (100%) of mice in the PBS group (**Fig. 10C**). In the IL-36γ treated mice that did develop erythema and hair loss, onset was delayed by two days compared to controls. Moreover, IL-36γ treatment significantly ($P < 0.0001$) protected from lethal challenge, as 8/20 (40%) of IL-36γ treated mice survived, whereas 20/20 (100%) of PBS treated mice succumbed to disease (**Fig. 10D**). IL-36γ treated mice that

died from lethal infection exhibited a three-day delay in death. These data demonstrate that IL-36γ treatment limits viral replication, decreases disease severity, and increases survival, suggesting that IL-36γ enhances protection against a lethal HSV-2 challenge in the vaginal epithelium.

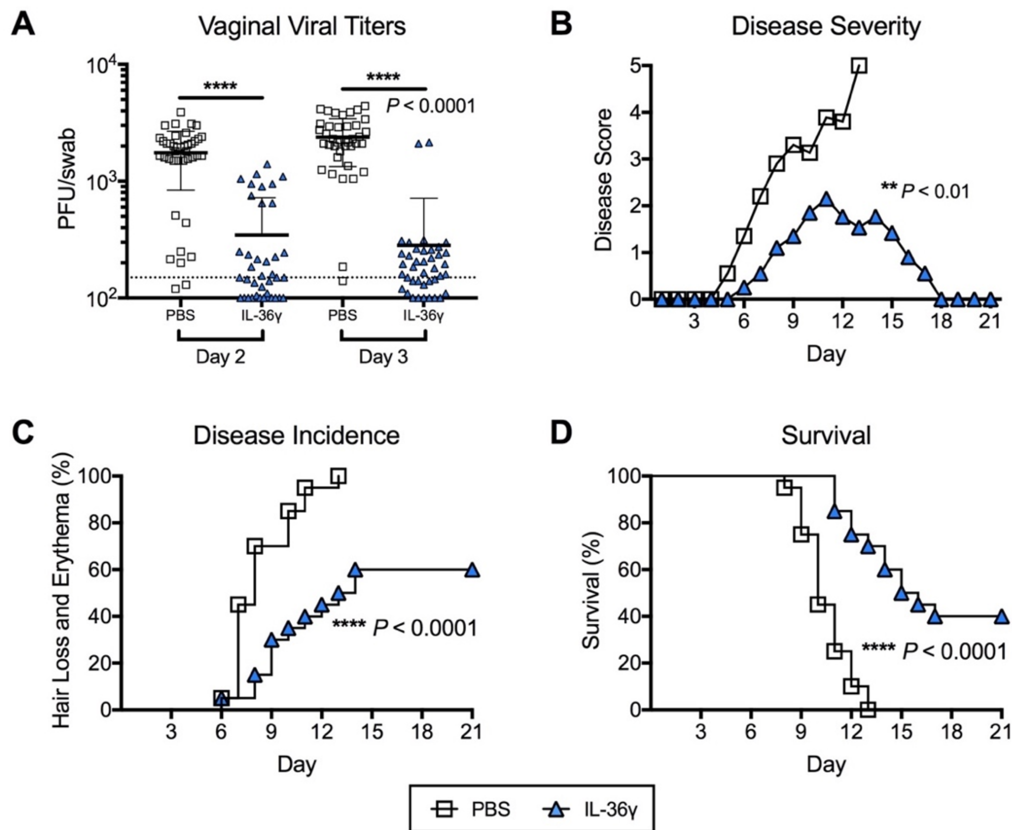

Figure 10. IL-36γ treatment 4h prior to infection significantly protects against HSV-2 disease incidence, reduces disease severity, and enhances survival. Female six- to eight-week-old C57Bl/6 mice were treated with murine recombinant IL-36γ (250 ng) 4h prior to infection (*n*=10), or mock-treated with PBS (*n* = 10). Mice were then intravaginally challenged with a lethal dose of HSV-2 186 (10^3 PFU). (A) Vaginal swabs were collected at days 2 and 3 post-inoculation and HSV-2 replication was measured in duplicate by standard plaque assay. Each symbol represents an individual mouse, and mean ± SD is depicted. Dashed line represents minimum detectable level for assay. Several IL-36γ-treated mice had undetectable titers and are depicted on the graph as half of the minimum detectable level. Disease severity (B) was measured daily and scored on a 0-5 scale. Once a mouse scored 5 and died it was no longer included in

scoring. Incidence of disease was measured by the presence of both erythema and hair loss (C), and survival (D) was recorded over a 21-day period. Data is representative of two independent animal studies. Statistical analyses were performed by one-way ANOVA with Bonferroni's multiple comparisons test (A), Area Under Curve (AUC) analysis with an unpaired two-tailed Student *t*-test with Welch's correction (B), and log-rank analysis (C, D). **, $P < 0.01$; ****, $P < 0.0001$.

IL-36γ drives the transient production of immune mediators in murine lower FRT tissue and vaginal lavages

Previously, we have demonstrated that IL-36γ induces expression and secretion of pro-inflammatory cytokines and chemokines 24h after treatment in a human 3-D VEC model (Winkle, Throop, and Herbst-Kralovetz 2016), and this may be a mechanism whereby IL-36γ treatment can limit HSV-2 replication and disease. In an initial screen, we employed gene expression arrays to assay over 130 unique genes involved in the antiviral response and Toll-like receptor signaling to identify key immunoregulatory genes that could be modulated by IL-36γ exposure in murine FRT tissue. We collected FRT tissue from mice treated with IL-36γ (500 ng) or mock-treated with PBS 4h and 24h after exposure, and pooled RNA from mice in the treatment groups for cDNA synthesis and analysis. From this initial screen (**Appendix A**), we identified several cytokines, chemokines and immune signaling molecules that were upregulated in the murine FRT 4h after treatment with IL-36γ to further investigate and validate. Many of these genes have been shown to be important in the immune response to HSV-2 (Chan et al. 2011, LeBlanc et al. 1999, Milora et al. 2014, Sergerie, Rivest, and Boivin 2007, Thapa and Carr 2008). We also identified several antimicrobial peptides (AMP) that have previously

been demonstrated to limit HSV-2 infection (Hazrati et al. 2006, John et al. 2005, Shust et al. 2010) that were not included in the arrays for subsequent analysis.

Murine lower FRT tissue was collected 4h and 24h after treatment (*n* = 5/time point) with IL-36γ (250 ng) and qRT-PCR was performed. We measured a transient induction of the IL-1 family members *Il1b* and *Il36g* 4h after treatment with IL-36γ, with levels increased 7.7-fold and 12.9-fold, respectively (**Fig. 11A**). Levels of *Il1b* and *Il36g* decreased by 24h treatment, and were induced 1.8-fold and 2.5-fold, respectively. Expression of the cytokine IL-6, in contrast, was significantly ($P < 0.01$) increased 4.6-fold 24h after IL-36γ treatment. Levels of the chemokines *Ccl20* and *Cxcl1* (KC) were transiently increased 4h after treatment 9-fold and 5.9-fold respectively, and levels decreased by 24h after treatment. Additional chemokines were initially screened in the gene expression arrays, including *Ccl2, Ccl3, Ccl4, Ccl5, Csf2, Csf3,* and expression of these targets was not significantly altered by IL-36γ treatment (**Appendix A**). In addition to cytokines and chemokines, we measured levels of the AMP defensin beta 3 (*Defb3*), Lactotransferrin (*Ltf*), and secretory leukocyte peptidase inhibitor (*Slpi*). *Slpi* was transiently induced, with levels at 4h after treatment significantly ($P < 0.05$) increased 4.8-fold relative to PBS controls. No significant changes in expression of *Defb* or *Ltf* was observed. Interestingly, the C-type lectin, *Clec4e*, was robustly induced 24.8-fold 4h after IL-36γ exposure, and at 24h after treatment *Clec4e* expression was increased 6.4-fold relative to controls. We observed no significant changes in expression of the mucin *Muc13* and the inflammasome component *Nlrp3* at either 4h or 24h after IL-36γ exposure. Altogether, these data demonstrate that IL-36γ induces the transient expression of immune

mediators at 4h in the murine FRT that may contribute to resistance to genital HSV-2 disease.

Figure 11. IL-36γ treatment induces the transient production of immune mediators in murine lower FRT tissue and vaginal lavages. (A) Female reproductive tract tissue was removed from C57Bl/6 mice 4h or 24h after i.vag. IL-36γ treatment (250 ng) and the lower FRT was isolated for RNA extraction (*n* = 5/time point). Expression of genes was measured by qRT-PCR and was normalized relative to *Gapdh*. Data is representative of two independent animal experiments and represent mean fold change ± SD relative to PBS treated controls. **(B)** Female C57Bl/6 mice were treated with murine recombinant IL-36γ (250 ng) or PBS. Vaginal lavages (*n* = 5 mice per time point) were collected at 4, 24 and 48h after exposure. Levels of the chemokines; CCL20, IP-10, and KC were quantified by cytometric bead array analysis. Minimum detectable concentration is indicated by dashed line. Data shown are representative of two independent animal experiments and are presented as the mean ± SD. Statistical analyses were performed by two-way ANOVA with Bonferroni's multiple comparisons test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

To understand the kinetics of cytokine and chemokine secretion following IL-36γ treatment, murine vaginal lavages were evaluated by cytometric bead array analysis to measure protein levels of soluble immune mediators. Vaginal lavages were collected from mice treated i.vag. with recombinant murine IL-36γ (250 ng) or PBS in the absence of HSV-2 challenge. Levels of cytokines (IL-1α, IL-1β, IL-6, TNFα) and chemokines (CCL20, KC, IP-10) shown to be important in limiting HSV-2 infection were evaluated in lavages at 4, 24, and 48h after treatment (n = 5/time point) using a high sensitivity murine cytokine/chemokine panel. IL-36γ significantly ($P < 0.05$) increased CCL20 levels 62-fold compared to PBS controls 4h after treatment (**Fig. 11B**). IP-10 levels were increased 4h after IL-36γ treatment, but at a lower fold induction (5-fold). Likewise, IL-36γ treatment significantly ($P < 0.01$) increased KC levels 11-fold compared to PBS controls at 4h. By 24h after treatment, levels of chemokines were lower when compared to 4h, and returned to baseline levels by 48h. The cytokines IL-1α, IL-1β, IL-6, and TNFα were not significantly induced relative to PBS controls at all time points (data not shown). Overall, these results demonstrate a significant transient increase in chemokine secretion following IL-36γ treatment in murine vaginal lavages that correlated with gene expression data (**Fig. 11A, B**) and may have contributed to limiting HSV-2 replication and increasing survival.

IL-36γ promotes the transient recruitment of neutrophils in the local vaginal microenvironment

The high levels of chemokines measured in vaginal lavages and murine FRT tissue following IL-36γ treatment led us to investigate the extent to which IL-36γ treatment impacted immune cell infiltration in the lower FRT. Medroxyprogesterone acetate-conditioned female C57Bl/6 mice were treated i.vag. with recombinant murine IL-36γ (250 ng) or PBS. Vaginal swabs were collected 4h and 24h following treatment (n = 5/treatment), and smeared on slides and allowed to air dry. Slides were then stained with modified Wright stain and imaged to characterize immune cell infiltration. Treatment with IL-36γ significantly ($P < 0.01$) enhanced infiltration of polymorphonuclear leukocytes (PMNs) (shown by black arrows) in vaginal smears 7.7-fold relative to PBS controls at 4h post-treatment (**Fig. 12**). This was a homogenous infiltration of cells that consistently exhibited PMN morphology. The increase in PMNs corresponded with the elevated levels of the chemokines CCL20 and KC after IL-36γ treatment that recruit PMN, as well as the induction of IP-10, a chemokine produced by PMN. PMN infiltration decreased by 24h post-treatment, and was comparable to PBS controls. Epithelial cells were present in vaginal swabs from both treatment groups, albeit at lower levels relative to PMNs. This could be due to the progesterone treatment all mice received, which has been demonstrated to thin the vaginal epithelium, and mice remain in diestrus for at least one month (Kaushic et al. 2003, Quispe Calla et al. 2016). Together, the enhanced recruitment of PMNs following IL-36γ treatment could be a potential mechanism whereby IL-36γ treatment protects against genital HSV-2 disease.

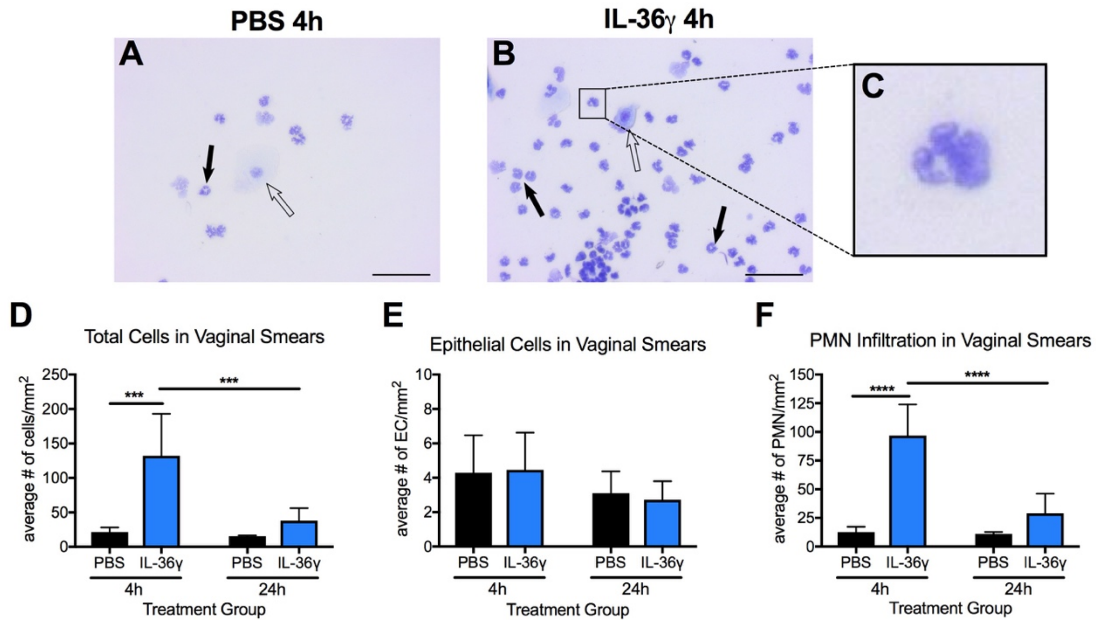

Figure 12. IL-36γ transiently promotes polymorphonuclear leukocyte infiltration in the vaginal microenvironment. Female C57Bl/6 mice were treated with murine recombinant IL-36γ (250 ng) or PBS and vaginal swabs were collected 4h (A, B, C) and 24h (data not shown) after treatment (*n* = 5 mice/treatment). Vaginal smears were prepared on slides and allowed to air dry. Slides were stained with modified Wright stain and imaged at 20× and 40× magnification. Black arrows indicate polymorphonuclear leukocytes (PMN) and white arrows indicate epithelial cells. Scale bar is 50 µm. Cells in fields at 20× were enumerated and graphed as average number of total cells/mm^2 (D), average number of epithelial cells/mm^2 (E), and average number of PMN/mm^2 (F). Statistical significance was determined by two-way ANOVA with Bonferroni's multiple comparisons test. ***, $P < 0.001$; ****, $P < 0.0001$.

Discussion

We have previously demonstrated that IL-36γ is expressed in FRT tissue, and promotes the secretion of cytokines, chemokines and AMP in response to microbial products (Winkle, Throop, and Herbst-Kralovetz 2016). In addition, recent proteomic studies evaluating human CVLs have found an increased relative abundance of IL-36γ in women with bacterial vaginosis and HSV-2 seropositive women using the contraceptive

Depo-Provera (Birse et al. 2017, Borgdorff et al. 2016). In this report, we investigated the role of IL-36γ in the vaginal microenvironment and in protecting against a prototypical viral STI, HSV-2. Herein we demonstrated that IL-36γ treatment can limit HSV-2 replication, decrease disease severity, and protect against a lethal HSV-2 challenge.

Early in infections, microbial products trigger TLRs in the vaginal epithelium(Herbst-Kralovetz et al. 2008) and stimulate the innate immune response. In HSV-2 infections, envelope glycoproteins will be sensed by TLR2, whereas the nucleic acid sensing TLR3 and TLR9 will be triggered by viral nucleic acid products during HSV replication (Herbst-Kralovetz and Pyles 2006b, Ma and He 2014). Poly(I:C), a viral dsRNA mimic, has previously been shown to protect against lethal HSV-2 challenge (Herbst-Kralovetz and Pyles 2006a), and induce IL-36γ *in vitro* (Chustz et al. 2011, Lian et al. 2012, Rana et al. 2015, Winkle, Throop, and Herbst-Kralovetz 2016), suggesting that IL-36γ could participate in antiviral host defense. Herein, we demonstrated that HSV-2 infection robustly induced *IL36G* expression, but did not significantly alter expression of other IL-36 family members. This finding suggests that IL-36γ, specifically, may play a key role in antiviral host defense in the vaginal epithelium. Induction of IL-36γ in response to HSV-2 may be TLR-mediated, as TLR2, TLR3, and TLR9 are all stimulated during HSV infection (Ma and He 2014).

We investigated the role of IL-36γ in the FRT and innate immune mechanisms that can contribute to the inflammatory response during acute HSV-2 infections that may influence virus replication and, ultimately, disease pathogenesis. Data from both our *in vitro* and *in vivo* models suggest that IL-36γ may limit HSV-2 replication and disease

through multiple innate immune mechanisms. We have previously demonstrated that exposure to IL-36γ for 24h promotes the production of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNFα in VEC (Winkle, Throop, and Herbst-Kralovetz 2016). In IL-36γ treated murine FRT tissue we also observed an increase in expression of *Il1a* and *Il1b* however, this was a more transient induction with levels the highest after exposure for 4h (**Fig. 11A**). IL-1α and IL-1β are important pro-inflammatory cytokines that have been shown to regulate HSV replication and disease and may be contributing to the antiviral environment we observed after IL-36γ treatment (Carr and Campbell 1999, Chucair-Elliott et al. 2014, LeBlanc et al. 1999, Lucinda et al. 2017, Milora et al. 2014, Sergerie, Rivest, and Boivin 2007). AMP, including Slpi, Defb3, and Ltf, are also key soluble immune mediators that have been shown to inhibit HSV infection (Hazrati et al. 2006, John et al. 2005, Shust et al. 2010), and are induced by IL-36γ in human VEC (Winkle, Throop, and Herbst-Kralovetz 2016). Likewise, we measured elevated levels of *Slpi* following IL-36γ treatment in murine FRT tissue (**Fig. 11**). Interestingly, IL-36γ exposure significantly increased expression of the C-type lectin *Clec4e* 24-fold (**Fig. 11A**). While C-type lectin receptors (CLR) are mostly known for their role in antifungal immunity, there is evidence of CLRs protecting against viral infection, including HSV-1 (Hoving, Wilson, and Brown 2014, Zelenay et al. 2012). Future studies are required to determine if Clec4e can contribute in host defense against genital HSV-2 infection. Collectively, increased production of these immune mediators may contribute to the HSV-resistant environment that is induced by IL-36γ treatment to limit viral replication and enhance survival.

In the complex multicellular vaginal microenvironment, innate immune cells can also contribute to enhanced resistance to genital HSV-2 infection, control viral replication and clear infections. Signaling by IL-36γ and IL-36R has previously been shown to increase chemokine levels and promote leukocyte recruitment in both the skin and lungs (Ainscough et al. 2017, Aoyagi et al. 2016, Foster et al. 2014, Ramadas et al. 2011). Indeed, we observed that IL-36γ exposure elicited significantly increased levels of the chemokines CCL20 and KC measured in both FRT tissue and vaginal lavages 4h following treatment (**Fig. 11**). KC and CCL20 can both function to recruit polymorphonuclear leukocytes (PMN), including neutrophils, that are important for protection against HSV infection in animal models (Milligan 1999, Thapa and Carr 2008, Thomas et al. 1997, Tumpey et al. 1996). HSV-2 infection does promote the recruitment of PMN in the FRT; however, this is not until approximately 24h after infection (Iversen et al. 2016, Milligan 1999). We observed an increase in the recruitment of PMN in vaginal smears collected from mice 4h after treatment with IL-36γ that corresponded with increased levels CCL20 and KC measured in vaginal lavages (**Fig. 11B, 12**). There was a homogenous infiltration of cells that consistently exhibited PMN morphology. Other immune cell populations besides PMNs were not morphologically identified in vaginal smears from either IL-36γ treated or control animals. Further, we did not measure a significant induction in chemokines that recruit monocytes/macrophages (Ccl2, Ccl3, Ccl5), basophils (Ccl2), eosinophils (Ccl5), T-cells (Ccl3, Ccl4, Ccl5, Cxcl9, Cxcl11), NK cells (Ccl4,), and other immune cell populations. This suggests that these cells are not being recruited to the vaginal microenvironment at 4h or 24h after IL-36γ treatment, which was further supported by the vaginal smears. However, the presence and

contribution of other immune cell subsets cannot be ruled out and require additional analyses. PMN have been shown to be important in suppressing viral replication (Milligan 1999), and our data suggest that the increase of PMN in the vaginal epithelium may contribute to the decreased levels of vaginal viral replication we measured in IL-36γ treated mice (**Fig. 10A, 12**). Neutrophils are important producers of proteases, including elastase, which has recently been shown to cleave and activate IL-36γ (Henry et al. 2016). Therefore, IL-36γ-mediated neutrophil recruitment could increase levels of elastase, and enhance cleavage and activity of IL-36γ. Another important function of PMN is the production of soluble immune mediators, including AMP and other chemokines that could contribute to the HSV-resistant environment induced following IL-36γ treatment. An important chemokine produced by PMN is IP-10, a T-cell chemoattractant that contributes to controlling HSV replication *in vivo* (Molesworth-Kenyon et al. 2012, Wuest et al. 2006, Wuest and Carr 2008). We measured elevated levels of IP-10 (5-fold) in vaginal lavages 4h after IL-36γ treatment, that corresponded with increased CCL20 and KC levels at the same time point (**Fig. 11B**). The robust induction of CCL20 and KC that we measured could be key in recruiting PMN early to combat genital HSV-2 infection in the FRT by decreasing disease severity, promoting disease resolution, and enhancing survival following a lethal viral challenge.

Previously, we demonstrated that microbial products stimulate IL-36γ in human models of the FRT and that IL-36γ could regulate host defense mechanisms at this site (Winkle, Throop, and Herbst-Kralovetz 2016). We extend these findings here in the context of a highly relevant STI pathogen, HSV-2. Consistent with a recent clinical

report, we found that HSV-2 robustly induces IL-36γ (Birse et al. 2017). Furthermore, we found that pretreatment with IL-36γ significantly decreased vaginal viral replication *in vitro*. This translated to significantly limiting vaginal viral replication, decreasing disease severity and incidence, and increasing survival in a lethal HSV-2 mouse model. In summary, our data suggests that IL-36γ exposure induces a HSV-2 resistant environment through the transient induction of cytokines and chemokines that can inhibit viral replication and promote PMN recruitment in the vaginal microenvironment to protect against genital HSV-2 disease. The animal model dependence on the dose and timing of IL-36γ delivery facilitates the pursuit of future studies to investigate IL-36-mediated protection in the FRT, and the translational impact of these findings. Our data indicate that IL-36γ functions as a key regulator of innate immune signaling in genital HSV-2 infection and may play a role in host defense against other STI pathogens in the FRT

CHAPTER 5: IL-36γ IS A KEY REGULATOR OF NEUTROPHIL INFILTRATION IN THE VAGINAL MICROENVIRONMENT AND LIMITS NEUROINVASION IN GENITAL HSV-2 INFECTION

Publication Note

The research reported in this chapter is published in an altered format in *The Journal of Immunology.* Jameson K. Gardner, Alison Swaims-Kohlmeier, and Melissa M. Herbst-Kralovetz. IL-36γ is a key regulator of neutrophil infiltration in the vaginal microenvironment and limits neuroinvasion in genital HSV-2 infection. *J Immunol*. ji1900280; doi: 10.4049/jimmunol.1900280.

Abstract

Herpes simplex virus 2 is a neurotropic virus that causes a persistent, life-long infection that increases risk for other sexually transmitted infections. The vaginal epithelium is the first line of defense against HSV-2 and coordinates the immune response through the secretion of immune mediators, including the pro-inflammatory cytokine IL-36γ. Previously, we showed that IL-36γ treatment promoted transient polymorphonuclear cell infiltration to the vaginal cavity and protected against lethal HSV-2 challenge. In this report, we reveal that IL-36γ specifically induces transient neutrophil infiltration, but does not impact monocyte and macrophage recruitment. Utilizing IL-36γ$^{-/-}$ mice in a lethal HSV-2 challenge model, we show that neutrophils counts are significantly reduced at 1- and 2-days post infection and that KC-mediated mature neutrophil recruitment is impaired in IL-36γ$^{-/-}$ mice. Additionally, IL-36γ$^{-/-}$ mice develop genital disease more

rapidly, have significantly reduced survival time, and exhibit an increased incidence of hind limb paralysis that is linked to productive HSV-2 infection in the brainstem. IL-36γ$^{-/-}$ mice also exhibit a significant delay in clearance of the virus from the vaginal epithelium and a more rapid spread of HSV-2 to the spinal cord, bladder, and colon. We further show that the decreased survival time and increased virus spread observed in IL-36γ$^{-/-}$ mice is not neutrophil-dependent, suggesting that IL-36γ may function to limit HSV-2 spread in the nervous system. Ultimately, we demonstrate that IL-36γ is a key regulator of neutrophil recruitment in the vaginal microenvironment and may function to limit HSV-2 neuroinvasion.

Introduction

Herpes simplex virus-2 (HSV-2) is a sexually transmitted virus that affects an estimated 11% of the global population, with an increased incidence in women (Looker et al. 2015b). The vaginal epithelium is the first line of defense against HSV-2 infection, and the innate immune response triggered by vaginal epithelial cells is crucial in controlling viral replication and spread during the initial stages of infection (Herbst-Kralovetz and Pyles 2006b, Herbst-Kralovetz and Pyles 2006a, Leoni et al. 2012, Lund et al. 2003, Rasmussen et al. 2007). The IL-36 cytokines were first characterized as members of the IL-1 superfamily that are expressed by epithelial cells at mucosal sites (Gresnigt and van de Veerdonk 2013, Kumar et al. 2000). Subsequent study of the IL-36 cytokines, including the agonists IL-36α, -β, and -γ, identified these cytokines as key drivers of chronic inflammatory diseases, including psoriasis (D'Erme et al. 2015, Foster et al. 2014, Johnston et al. 2011, Towne and Sims 2012, Walsh and Fallon 2016).

However, the function of the IL-36 cytokines in host-pathogen interactions is not as well understood. Recently, the IL-36 cytokines have been shown to promote neutrophil recruitment and play a role in both bacterial and viral infections in the lungs (Ahsan et al. 2016, Aoyagi et al. 2016, Kovach et al. 2017, Kovach et al. 2016, Milora et al. 2017). We have demonstrated that IL-36γ is expressed in the female reproductive tract (FRT) and is induced in response to an array of microbial products, suggesting a role in host defense mechanisms (Winkle, Throop, and Herbst-Kralovetz 2016). Indeed, we found that HSV-2 stimulated *IL36G* expression, and that treatment with IL-36γ induced an antiviral state that limited viral replication in a human 3-D cell culture model and protected against disease pathogenesis after lethal challenge in mice (Gardner and Herbst-Kralovetz 2018). We further revealed that IL-36γ transiently induced polymorphonuclear cell recruitment in the FRT that corresponded with decreased vaginal viral titers and increased survival (Gardner and Herbst-Kralovetz 2018).

Neutrophils are one of the predominant immune cells present in the FRT, and function as early responders to pathogens (Amjadi et al. 2014, Lee et al. 2015). Genital HSV-2 infection robustly recruits neutrophils to the vaginal cavity by 24h post-infection, and these neutrophils aid in virus clearance from the vaginal mucosa (Milligan 1999). We have shown that neutrophil infiltration to the vaginal cavity corresponded with decreased HSV-2 production and protection against disease pathogenesis after intravaginal (i.vag.) challenge (Gardner and Herbst-Kralovetz 2018). In addition to aiding in HSV-2 clearance, neutrophils can function to provide broad immune protection against pathogens at the FRT barrier through production of antimicrobial peptides, cytokines,

chemokines, and reactive oxygen species (Amjadi et al. 2014). Interestingly, neutrophil elastase has recently been shown to cleave and activate IL-36γ from its pro-form (Clancy et al. 2018, Henry et al. 2016), suggesting an important link between IL-36γ and neutrophils in host defense mechanisms at mucosal sites.

When genital HSV-2 infection spreads beyond the vaginal epithelium, the virus infiltrates autonomic ganglion of the pelvis and the dorsal root ganglia, where it establishes latency (Fleck et al. 1993, McDermott et al. 1987, McDermott et al. 1984, Parr et al. 1994). Periodic virus reactivation can cause ulcerative disease in individuals, however asymptomatic virus shedding can also occur (Johnston and Corey 2016, Wald et al. 1995). While herpetic lesions are the most well-known symptom of genital HSV-2 disease, HSV-2 is a neurotropic virus that can spread through the nervous system. Spread of HSV-2 into the pelvic ganglion can cause damage to the enteric nervous system that leads to urinary retention and constipation in some individuals (Caplan, Kleeman, and Berg 1977, Goodell et al. 1983, Khoury-Hanold et al. 2016). Parr and Parr also demonstrated that the spread of HSV-2 to the dorsal root ganglia provides a pathway for the virus to spread to the spinal cord and brain (Parr and Parr 2003). Symptoms of HSV-2 neuroinvasion following i.vag. infection in mice include urinary retention, toxic megacolon, and hind limb paralysis (Khoury-Hanold et al. 2016, Parr and Parr 2003, Reinert et al. 2012).

In this study, we aimed to determine the requirement for IL-36γ in protection against genital HSV-2 disease pathogenesis. We evaluated IL-36γ-mediated immune cell

recruitment to the vaginal microenvironment after i.vag. HSV-2 infection, and additionally investigated HSV-2 spread and neuroinvasion to better understand the function of IL-36γ in host defense mechanisms locally in the FRT and systemically after viral infection. Collectively, we demonstrate that IL-36γ plays a key role in recruiting mature neutrophils to the vaginal microenvironment after HSV-2 infection and that IL-36γ may function in a manner that protects against neuroinvasion and HSV-2 disease pathogenesis.

Materials and Methods

HSV-2 propagation and plaque assay

HSV-2 186 was used in all studies and generously provided by Dr. Richard Pyles (UTMB, Galveston, TX). Stocks were prepared from infected Vero cell culture monolayers and frozen at -80°C. Vero cells were grown in DMEM (Corning, Manassas, VA) as described previously (Pyles et al. 2002). Virus titers in HSV-2 stocks and infected samples were determined using standard plaque assay as previously described (Herbst-Kralovetz and Pyles 2006a).

Animals and genital HSV-2 infection model

C57Bl/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-36$\gamma^{-/-}$ mice were kindly provided by Dr. Tim Denning (Georgia State University, Atlanta, GA). All animals were housed in accordance with the American Association for Laboratory Animal Care (AALAC) standards and provided unlimited access to food and water. All procedures and handling were approved by the University of Arizona

Institutional Animal Care and Use Committee and performed in accordance with the Animal Welfare Act to minimize pain and suffering. Mice were treated with depot medroxyprogesterone acetate (DMPA; Greenstone, Peapack, NJ) at day -7 and day -1 to synchronize cycles and increase mucosal permeability and susceptibility to infection as previously described (Herbst-Kralovetz and Pyles 2006a). For IL-36γ treatment, mice were treated with recombinant murine IL-36γ (250 ng, BioLegend, San Diego, CA) or PBS in 10 µl total volume by i.vag. instillation. At time of challenge in HSV-2 infection experiments, 10^3 PFU HSV-2 186 in 10 µl DMEM was i.vag. instilled. Mice were visually inspected daily for disease progression and survival over a 16-day period. Disease severity was scored on a scale from 0-5 as previously described (Gardner and Herbst-Kralovetz 2018). Briefly, no pathology (0), mild vulvar erythema (1), moderate vulvar erythema (2), hair loss & erythema (3), perineal ulceration (4), extension of perineal ulceration to surrounding tissue and/or hind-limb paralysis (5). Moribund mice, or those scoring a 5 were euthanized. Vaginal swabs were collected at days 2, 4 and 6 post-infection using a DMEM soaked urethro-genital calcium alginate swab (Puritan, Guilford, ME) and stored in 1mL DMEM at -80°C. Cervicovaginal lavages (CVL) were collected from mice by i.vag. instilling ~125 µl sterile PBS and lavaging the vaginal cavity using an oral feeding tube (Fisher Scientific, Hampton, NH) as previously described (Gardner and Herbst-Kralovetz 2018). The bladder, distal colon, lumbar spinal cord, and brainstem were collected from mice during necropsy, weighed, and stored in DMEM at -80°C. For titration, tissues were homogenized, and virus titers quantified by standard plaque assay.

RNA extraction and qPCR analysis

Mouse vaginal tissue was collected at 24 and 48h post-HSV-2 infection and stored in DNA/RNA Shield (Zymo Research, Irvine, CA) prior to RNA extraction using the Zymo Quick-RNA plus kit following the manufacturer's instructions (Zymo Research). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) from 1µg RNA and then analyzed by qPCR using iTAQ Universal SYBR Green Supermix (Bio-Rad) with an Applied Biosystems QuantStudio6 Flex Real Time PCR System (Life Technologies, Grand Island, NY). Gene expression was normalized relative to GAPDH, and primer sequences are listed in **Table 6**.

Table 6.

Primers used in qPCR analysis

Gene	Forward (5'-3')	Reverse (5'-3')
Murine *Ccl2*	GATGATCCCAATGAGTAGGC	TCTTGAGCTTGGTGACAAAA
Murine *Ccl20*	CGACTGTTGCCTCTCGTACA	CACCCAGTTCTGCTTTGGAT
Murine *Ccl7*	CCTGGGAAGCTGTTATCTTCAA	TGGAGTTGGGGTTTTCATGTC
Murine *Cxcl9*	GGAACCCTAGTGATAAGGAATGCA	TGAGGTCTTTGAGGGATTTGTAGTG
Murine *Csf2*	CCAGCTCTGAATCCAGCTTCTC	TCTCTCGTTTGTCTTCCGCTGT
Murine *Csf3*	TGGCAGCAGATGGAAAACCTAG	AGGTACGAAATGGCCAGGACA
Murine *Cxcl1*	CAATGAGCTGCGCTGTCAGTG	CTTGGGGACACCTTTTAGCATC
Murine *Cxcl10*	CCTGCCCACGTGTTGAGAT	TGATGGTCTTAGATTCCGGATTC
Murine *Gapdh*	AAATTCAACGGCACAGTCAAG	TGGTGGTGAAGACACCAGTAG

Neutrophil Depletion

WT C57Bl6/J and IL-36$\gamma^{-/-}$ mice were injected i.p. with I*nVivo*MAb anti-mouse Ly6G (α-Ly6G) antibody or *InVivo*MAb rat IgG2a isotype control (Bio X Cell, West Lebanon, NH) to deplete neutrophils using 200 µg of antibody in 200 µl at Day -1 prior to infection and then every other day through the duration of the challenge study. CVLs were analyzed by flow cytometry to confirm the efficiency of neutrophil depletion.

Flow Cytometry

All lavages were passed through 40 µm filters (Corning BD Falcon), and cell counting and viability were performed by Trypan blue exclusion. Murine vaginal tissue was processed into single cell suspensions for flow cytometry analysis using a modified protocol as described by Jiang and Kelly (Jiang and Kelly 2012). Briefly, vaginal tissue was cut into fine pieces (< 1 mm) with surgical scissors and transferred to a 1.5 ml microfuge tube with 1 ml of digestion media: RPMI media (Corning), 10% heat inactivate fetal bovine serum (Corning), 1% penicillin/streptomycin (Corning), 10mM HEPES (Fisherbrand, Hampton, NH), 0.5 mg/ml Collagenase type VIII (Sigma Aldrich, St. Louis, MO). Tissue was incubated at 37°C with shaking for 1h. After 1h, tissue was strained through a 40 µm filter (Corning) and washed with fresh RPMI. Remaining tissue issue underwent a second incubation with fresh digestion media at 37°C with shaking for 1h. After second incubation, tissue was lightly dissociated between sterile glass slides and filtered through 40 µm filters. Cell counting and viability was then evaluated by Trypan blue exclusion. Non-specific binding in samples was blocked by staining with TruStain FcX antibody (anti-mouse CD16/32; BioLegend), and cells were then stained with a

cocktail of antibodies: CD11b Alexa Fluor 647 (clone M1/70), F4/80 PE (clone BM8), Ly6C PerCp (clone HK1.4), Ly6G Alexa Fluor 488 (clone 1A8), and GR-1 FITC (clone RB6-8C5; BioLegend). After staining, cells were fixed in 1% paraformaldehyde and data was acquired on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Unstained, single stained, and "Fluorescence minus one" controls were used to determine positive and negative staining. Data were analyzed with FlowJo software (Treestar, Ashland, OR). Dead cells and debris were excluded by forward and side scatter, and singlet events were selected based on forward scatter area, height and width parameters.

Statistics

Disease severity, scoring, and systemic virus spread were evaluated by Area Under Curve (AUC) analysis with an unpaired two-tailed Student *t*-test with Welch's correction as previously described (Gardner and Herbst-Kralovetz 2018). Disease incidence and survival were analyzed by log-rank analysis as previously described (Herbst-Kralovetz and Pyles 2006a). Unpaired two-tailed Student *t*-test with Welch's correction and ordinary one-way and two-way ANOVAs with Bonferroni's multiple comparison test were performed for comparisons as indicated. A p value of < 0.05 was considered significant. All statistical analyses were performed using Prism software version 8 (GraphPad, San Diego, CA).

Results

IL-36γ induces transient neutrophil recruitment to the vagina

We previously demonstrated that i.vag. IL-36γ treatment induced the recruitment of polymorphonuclear cells to the FRT barrier. Therefore, we sought to further understand the role of IL-36γ and immune cell recruitment in the vaginal microenvironment. $CD11b^{+}Ly6G^{+}$ neutrophils were the predominant cells in CVLs from DMPA-conditioned naïve C57Bl/6 mice, representing approximately 88-94% of all cells in lavages (**Fig. 13A**). This finding is consistent with a recent report from Cora et al. that describes neutrophils as the most abundant cells during diestrus in mice (Cora, Kooistra, and Travlos 2015). Following treatment with IL-36γ, we measured a significant influx of neutrophils into the vaginal microenvironment at 4h (4.9-fold, $p < 0.0001$) and 8h (2.6-fold, $p < 0.001$) post-treatment. Neutrophil infiltration was transient, and neutrophil counts returned to baseline levels by 12 and 24h post-treatment (**Fig. 13C**). We identified macrophages ($CD11b^{+}F4/80^{+}$) and monocytes ($CD11b^{+}Ly6C^{+}Ly6G^{-}$) in CVLs at all time points, but these cells were present at low levels and were not significantly altered by IL-36γ treatment at any time point (data not shown). These results further demonstrate that IL-36γ robustly recruits neutrophils to the vaginal epithelium that can potentially contribute to host defense mechanisms.

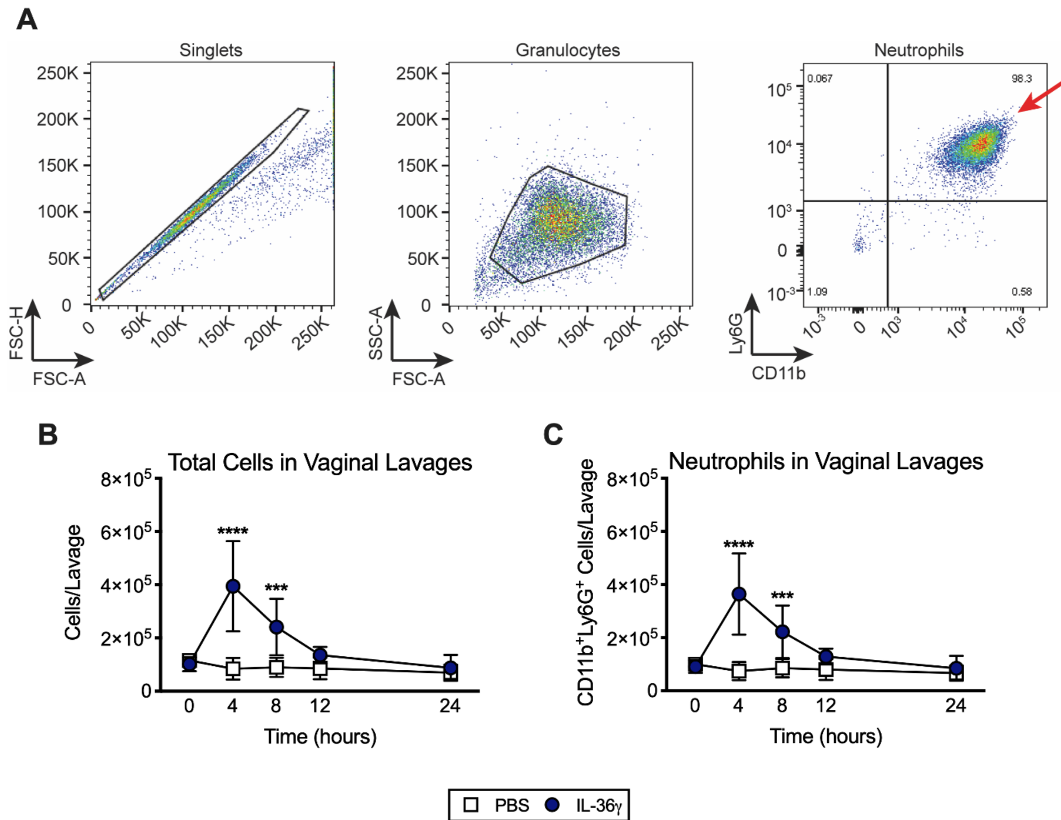

Figure 13. IL-36γ induces transient neutrophil recruitment to the vaginal microenvironment in WT mice. Flow cytometry analysis of neutrophils in vaginal lavages from IL-36γ treated mice. Female six- to eight-week-old C57Bl/6 mice were conditioned with DMPA and i.vag. treated with recombinant murine IL-36γ or PBS (*n*=5 mice/timepoint). Representative staining illustrating gating strategy for CVL immune cell characterization (**A**). Red arrow indicates neutrophils ($CD11b^+Ly6G^+$). Cell counts and positive staining were used to determine total cell (**B**) and $CD11b^+Ly6G^+$ neutrophil (**C**) counts. Data depict mean cell count ± SD and are representative of two independent animal studies. Statistical significance was determined by two-way ANOVA with Bonferroni's multiple comparison test. ***, $p < 0.001$; ****, $p < 0.0001$.

Mature neutrophil recruitment is impaired in IL-36γ$^{-/-}$ mice

We next quantified cellular infiltration in CVLs from IL-36γ$^{-/-}$ and wild-type (WT) mice before lethal HSV-2 challenge, and then at 24 and 48h post-infection to determine if IL-36γ is necessary for cell recruitment in response to viral infection. We

found that there was no difference in the total number of cells or neutrophils in the vaginal microenvironment between naïve IL-36$\gamma^{-/-}$ and WT mice prior to HSV-2 challenge (**Fig. 14**). Following HSV-2 challenge in WT mice, the number of neutrophils steadily increased in vaginal lavages at 24 (2.4-fold) and 48h (4.8-fold, $p < 0.0001$) post-infection (**Fig. 14B**). In contrast, lavages from IL-36$\gamma^{-/-}$ mice exhibited significantly decreased numbers of CD11b^{+}Ly6G^{+} neutrophils at 24 (-3.4-fold, $p < 0.0001$) and 48h (-2.9-fold, $p < 0.0001$) after HSV-2 challenge. Neutrophil counts in IL-36$\gamma^{-/-}$ mice were decreased -11.15-fold ($p < 0.05$) and -19.15-fold ($p < 0.0001$) at 24h and 48h post-infection, respectively, compared to WT mice. Macrophages (CD11b^{+}F4/80^{+}) and monocytes (CD11b^{+}Ly6C^{+}Ly6G^{-}) were present at low levels in lavages and were not significantly altered in either IL-36$\gamma^{-/-}$ or WT mice at all time points evaluated (**Fig. 14C, D**). Similar to our findings in CVLs, we measured significantly lower neutrophil counts in IL-36$\gamma^{-/-}$ mouse vaginal tissue at 24h post-HSV-2 infection compared to WT mice (**Fig. 14E, Appendix C**). We additionally detected macrophages and monocytes in vaginal tissue, and numbers of these cells were not significantly altered in IL-36$\gamma^{-/-}$ mice relative to WT mice (**Appendix C**). Collectively, these results reveal an impaired recruitment of neutrophils to the vaginal microenvironment in IL-36$\gamma^{-/-}$ mice after HSV-2 challenge that suggests a crucial function for IL-36γ in regulating neutrophil recruitment after viral infection.

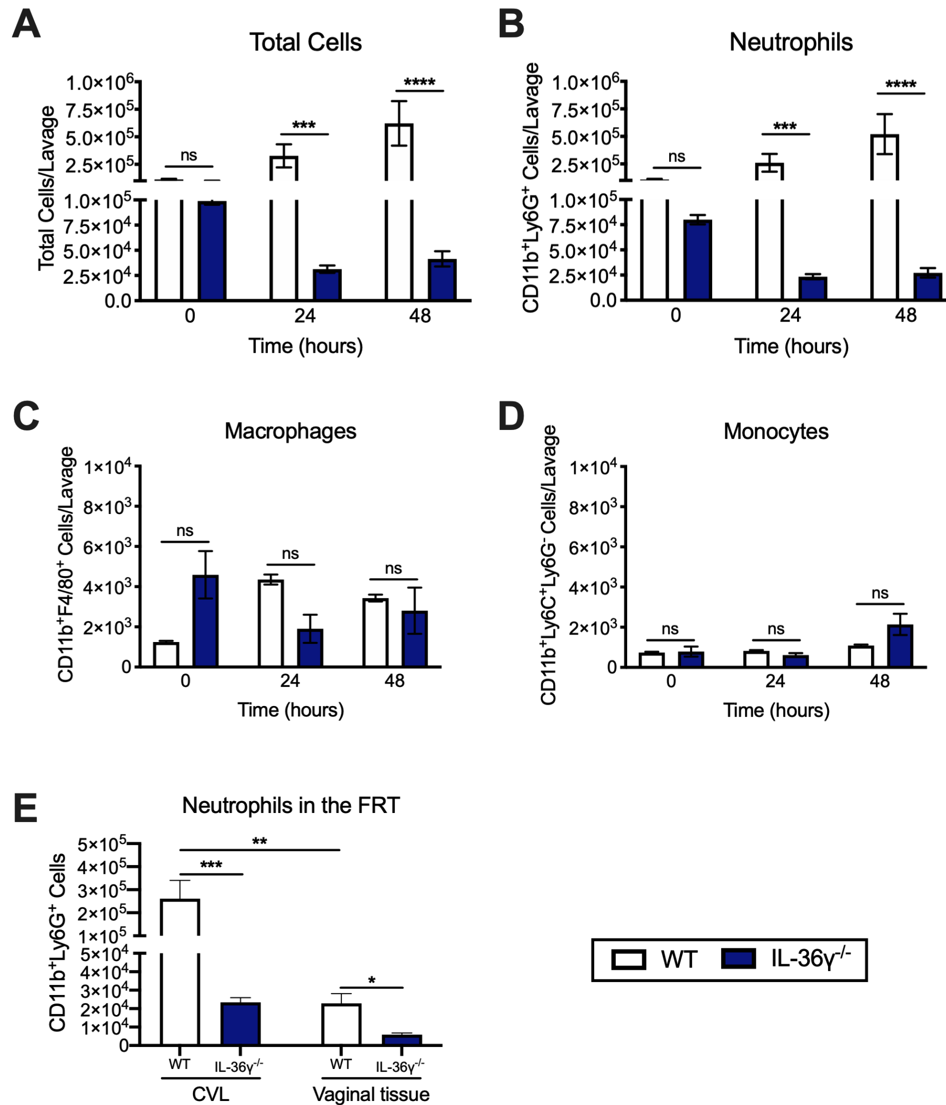

Figure 14. Neutrophil recruitment is significantly reduced in IL-36γ$^{-/-}$ mice after genital HSV-2 infection. Flow cytometry analysis of immune cells in vaginal lavages. Female six- to eight-week-old DMPA conditioned WT and IL-36γ$^{-/-}$ mice (*n*=5 mice/timepoint) were i.vag. challenged with 10^3 PFU HSV-2 186. Lavage cell counts and results from flow cytometry analysis were used to determine numbers of total cells (**A**), neutrophils (CD11b$^+$Ly6G$^+$) (**B**), macrophages (CD11b$^+$F4/80$^+$) (**C**), and monocytes (CD11b$^+$Ly6C$^+$Ly6G$^-$) (**D**) in WT and IL-36γ$^{-/-}$ mice prior to infection, and at 24 and 48h post-infection. (**E**) Comparison of neutrophil counts in cervicovaginal lavages (CVL) and vaginal tissue in IL-36γ$^{-/-}$ and WT mice at 24h post-infection. Data depict mean ± SD and represent results from two independent animal studies. Statistical significance was determined by two-way ANOVA with Bonferroni's multiple comparisons test. ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

Interestingly, we observed two distinct populations of $CD11b^+Ly6G^+$ cells when analyzing the neutrophil population in vaginal lavages (**Fig. 15A, B**). Prior to HSV-2 challenge, mature ($CD11b^+Ly6G^{hi}$) neutrophils were the predominant population in vaginal lavages from both WT and IL-36$\gamma^{-/-}$ mice, with a small pool of immature ($CD11b^+Ly6G^{int}$) neutrophils present. After virus challenge, wild type mice maintained the predominant population of $Ly6G^{hi}$ stained cells, and a small pool of $Ly6G^{int}$ cells (**Fig. 15C**). The number of mature neutrophils in WT mice dramatically increased at 24 and 48h ($p < 0.05$) post-infection (**Fig. 15D**), whereas the number of immature neutrophils remained relatively constant (**Fig. 15E**). Unexpectedly, following infection in IL-36$\gamma^{-/-}$ mice, we recorded relatively similar proportions of $Ly6G^{int}$ and $Ly6G^{hi}$ stained cells. The number of mature neutrophils in IL-36$\gamma^{-/-}$ mice significantly ($p < 0.0001$) decreased at 24 and 48h post-infection while counts of immature neutrophils did not significantly change, shifting the ratio of mature to immature neutrophils in the vaginal microenvironment. This is shown in **Fig. 15C**, illustrating a shift in the neutrophil population in vaginal lavages from IL-36$\gamma^{-/-}$ mice at both 24 and 48h after HSV-2 infection. Collectively, these results demonstrate an important role for IL-36γ in mediating mature neutrophil recruitment in response to genital HSV-2 infection.

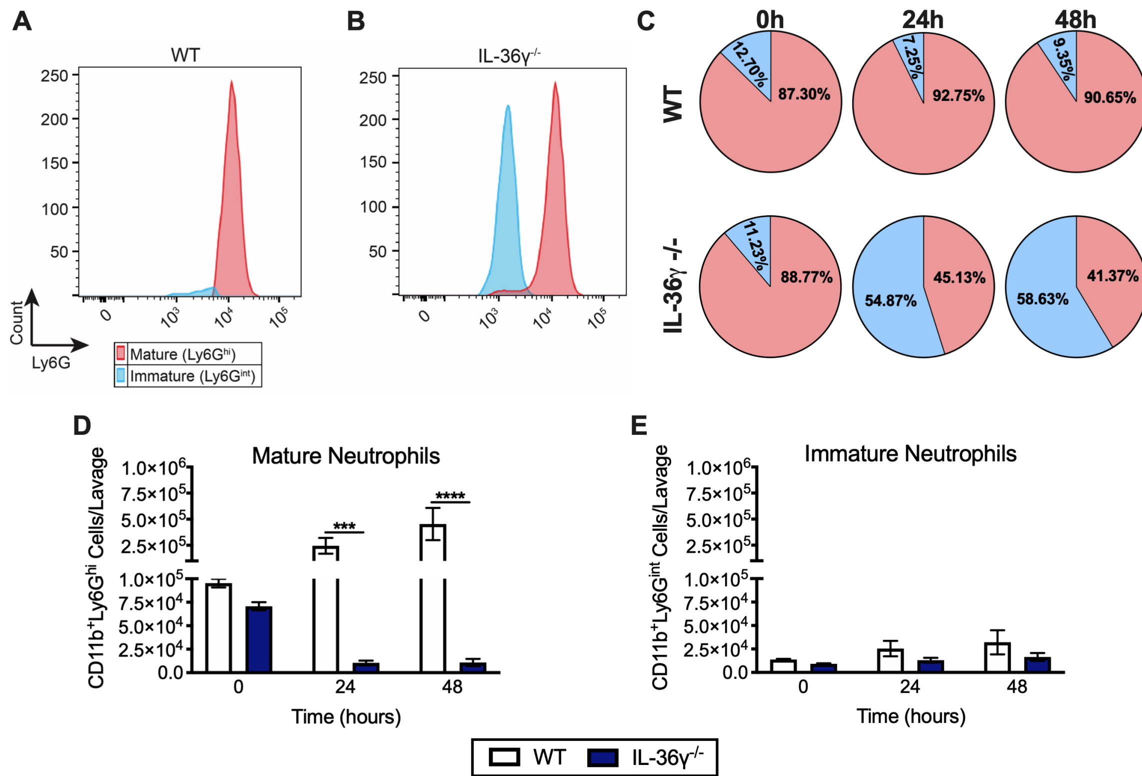

Figure 15. Recruitment of mature neutrophils to the vaginal microenvironment is impaired in HSV-2 challenged IL-36γ$^{-/-}$ mice. Analysis of neutrophils in vaginal lavages from HSV-2 challenged WT and IL-36γ$^{-/-}$ mice. Female six- to eight-week-old WT and IL-36γ$^{-/-}$ DMPA conditioned mice were i.vag. challenged with 10^3 PFU HSV-2 186 (n=5 mice/timepoint). Representative histograms plot Ly6G staining intensity and relative proportions of neutrophils from WT (**A**) and IL-36γ$^{-/-}$ mice at 24h post-infection (**B**), and the ratio of mature to immature neutrophils in lavages are represented in pie charts (**C**). Gating on Ly6G staining intensity was used to determine counts of CD11b$^+$Ly6G^{hi} mature neutrophils (**D**), CD11b$^+$Ly6G^{int} immature neutrophils (**E**). Data indicate mean ± SD and represent results from two independent animal studies. Statistical significance was determined by two-way ANOVA with Bonferroni's multiple comparison test. ***, $p < 0.001$; ****, $p < 0.0001$.

Reduced Cxcl1/KC expression in IL-36γ$^{-/-}$ vaginal tissue

In an effort to understand mechanisms that may be contributing to IL-36γ-mediated regulation of neutrophil recruitment and the potential impact on other immune cells, we evaluated expression of several chemokines in vaginal tissue from IL-36γ$^{-/-}$ and

WT mice at 24 and 48h post-infection (**Fig. 16**). These chemokines include those with chemotactic activity for macrophages, monocytes, neutrophils, NK cells, and T cells, among others. From this panel of chemokines, we found that IL-36γ$^{-/-}$ mouse vaginal tissue exhibited a significant reduction in *Cxcl1* expression relative to WT mice at both 24 (-6.4-fold, $p < 0.05$) and 48h (-5.9-fold, $p < 0.05$) post-infection. We further detected a -7.3-fold reduction in *Csf3* expression in IL-36γ$^{-/-}$ vaginal tissue relative to WT at 24h post-infection, although this was not statistically significant. We did not measure any significant changes in expression of any of the other chemokine assayed. Together, these data suggest that IL-36γ may function to regulate neutrophil recruitment in the FRT in-part through the neutrophil chemoattractant KC.

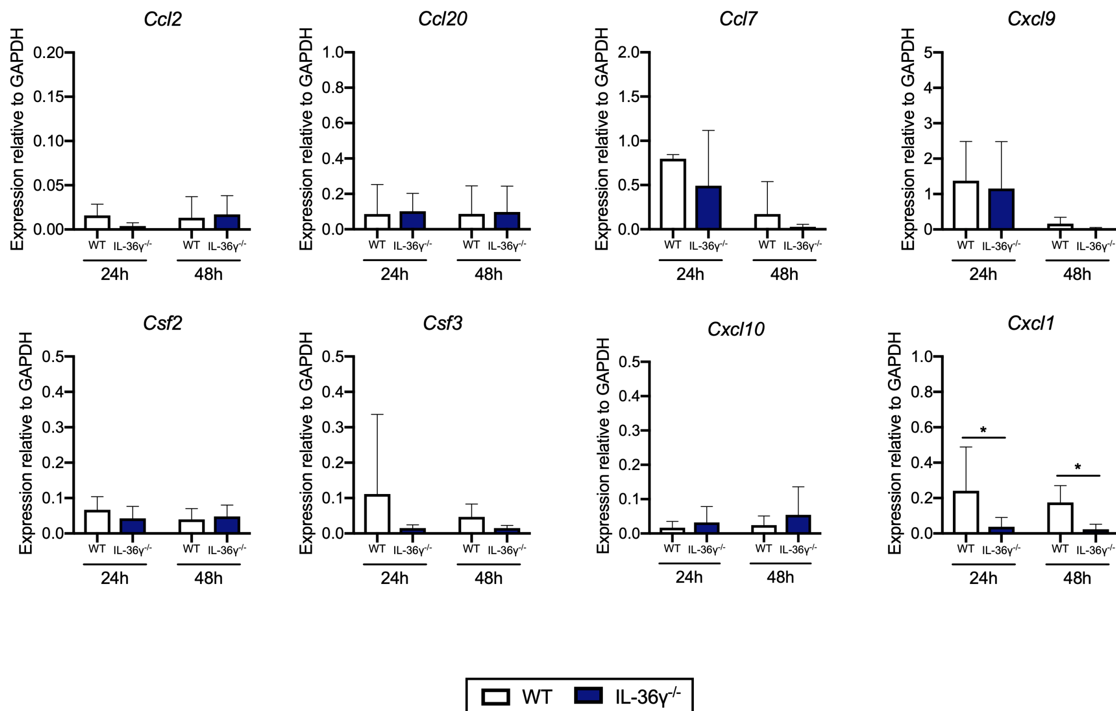

Figure 16. Cxcl1/KC expression is reduced in IL-36γ$^{-/-}$ vaginal tissue after HSV-2 challenge. qPCR analysis of murine vaginal tissue after intravaginal HSV-2 challenge. Female six- to eight-week-old IL-36γ$^{-/-}$ and WT mice were conditioned with DMPA and i.vag. challenged with 10^3

PFU (LD_{100}) HSV-2 186. Vaginal tissue was collected at 24 and 48h post-infection (n=5 mice/timepoint), and expression of chemokines assayed by qPCR. Gene expression was normalized to GAPDH and depict mean ± SD. Data reflect results from two independent animal studies. Statistical significance was determined by two-way ANOVA with Bonferroni's multiple comparisons test. *, $p < 0.05$.

Decreased survival time in IL-36γ$^{-/-}$ mice after genital HSV-2 infection

To assess the requirement for IL-36γ in the host response to genital HSV-2 infection, we evaluated disease progression and survival after lethal i.vag. HSV-2 challenge in WT and IL-36γ$^{-/-}$ mice. We observed that IL-36γ$^{-/-}$ mice exhibited more rapid disease symptoms ($p < 0.0001$) compared to WT mice (**Fig. 17A**). IL-36γ$^{-/-}$ mice developed hair loss and erythema around the introitus 2.5 days earlier than controls (**Fig. 17B**). Corresponding with the more rapid onset of disease symptoms, IL-36γ$^{-/-}$ mice exhibited significantly ($p < 0.0001$) reduced survival time, succumbing to disease 2.6 days sooner than WT mice (**Fig. 17C**), indicating that IL-36γ signaling is involved in the host response to genital HSV-2 infection.

We next sought to determine if IL-36γ-mediated protection against genital HSV-2 infection was neutrophil dependent. Using an anti-mouse Ly6G antibody to deplete neutrophils in mice, we evaluated disease progression and survival after lethal intravaginal challenge. We found that there was no significant difference in disease progression, the incidence of hair loss and erythema, or in survival time between WT mice and neutrophil depleted WT mice (**Fig. 17**). Isotype control treated mice exhibited similar disease progression and survival as both WT and neutrophil depleted WT mice

(**Appendix C**). These data suggest that although IL-36γ regulates neutrophil recruitment in response to HSV-2 infection, IL-36γ-mediated protection against genital HSV-2 infection and disease pathogenesis is not neutrophil dependent.

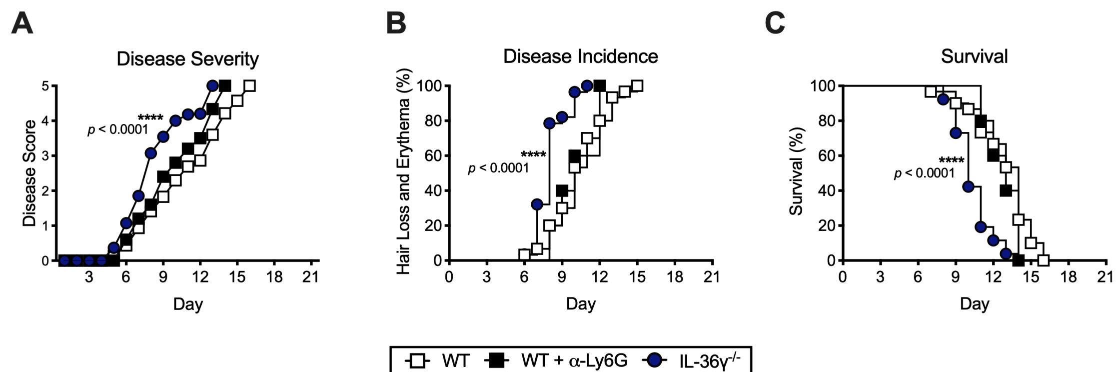

Figure 17. IL-36γ protects against genital HSV-2 infection in a neutrophil independent manner. Lethal i.vag. HSV-2 challenge in WT mice (*n*=30), neutrophil depleted WT mice (α-Ly6G, *n*=5), and IL-36$\gamma^{-/-}$ mice (*n*=27). Female six- to eight-week-old mice were conditioned with DMPA and i.vag. challenged with 10^3 PFU (LD_{100}) HSV-2 186. Disease severity (**A**), incidence (**B**), and survival (**C**) were monitored over a 21-day period. Data are representative of one (Neutrophil depleted WT mice) or three independent animal studies (WT and IL-36$\gamma^{-/-}$ mice). Statistical significance was determined by AUC analysis with two-tailed Student *t*-test with Welch's correction (**A**), and log-rank analysis (**B, C**). ****, $p <$ 0.0001.

Increased incidence of hind limb paralysis in IL-36$\gamma^{-/-}$ mice

Surprisingly, while monitoring disease progression and survival after lethal challenge, we observed an increased incidence of hind limb paralysis in IL-36$\gamma^{-/-}$ mice, an indication of HSV-2 spread to the central nervous system (CNS). We found that 16.67% of WT mice developed hind limb paralysis over the course of infection, and that the onset of hind limb paralysis was linked to productive HSV-2 infection in the brainstem, as

demonstrated by plaque assay (**Fig. 18A, B**). In contrast, 81.5% of IL-36$\gamma^{-/-}$ mice ($p <$ 0.0001) developed hind limb paralysis after lethal challenge, and all mice exhibiting hind limb paralysis had detectable viral titers in the brainstem. There was no significant difference in the viral load in WT and IL-36$\gamma^{-/-}$ mice with productive infections in the brainstem (data not shown). The increased spread of HSV-2 to the CNS in IL-36$\gamma^{-/-}$ mice indicates a potential neuroprotective function for IL-36γ in the antiviral response.

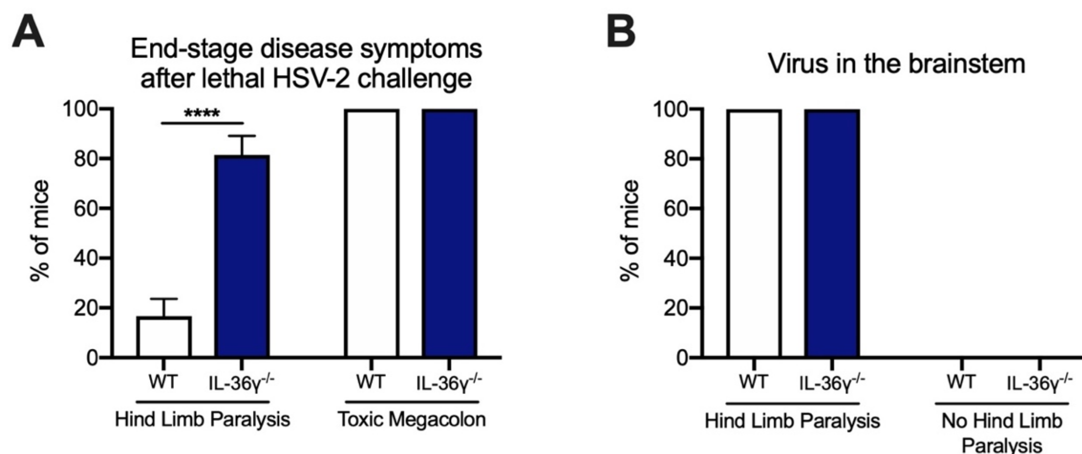

Figure 18. Increased incidence of hind limb paralysis and productive infection in the brainstem in IL-36$\gamma^{-/-}$ mice after genital HSV-2 infection. Incidence of hind limb paralysis and toxic megacolon in HSV-2 challenged mice. Female six- to eight-week-old DMPA conditioned mice were i.vag. challenged with 10^3 PFU HSV-2 186 (*n*=10 mice/strain). Animals were monitored daily for disease progression and survival. (**A**) Incidence of hind limb paralysis was recorded, and toxic megacolon was assessed upon necropsy. Data represent three independent animal studies (**B**) Productive viral infection in the brainstem was analyzed by standard plaque assay. Tissue was collected at time of death. Data represent the detection of viral titers in the brainstem of mice with or without hind limb paralysis and are representative of two independent animal studies. Statistical significance was determined by two-tailed Student t-test with Welch's correction. ****, $p < 0.0001$.

Delayed clearance of HSV-2 from the vaginal epithelium in IL-36$\gamma^{-/-}$ mice and increased systemic spread

We then sought to determine the kinetics of the local HSV-2 infection and systemic spread in IL-36γ$^{-/-}$ mice compared to WT mice to better understand the decreased survival time and increased incidence of neurologic disease in IL-36γ$^{-/-}$ mice. To measure viral titers and clearance of the virus in the vaginal epithelium, vaginal viral titers were quantified at 2, 4 and 6 days after lethal HSV-2 challenge (10^3 PFU; LD_{100}). Viral titers in IL-36γ$^{-/-}$ mice were comparable to WT at both 2- and 4-days post-infection (dPI; **Fig. 19A**). However, at 6 dPI, IL-36γ$^{-/-}$ mice still exhibited detectable titers with viral loads comparable to 2 and 4 dPI, whereas WT mice had mostly cleared the virus. Vaginal viral titers in neutrophil depleted WT mice were similar to WT and IL-36γ$^{-/-}$ mice at 2 and 4 dPI and similar to IL-36γ$^{-/-}$ mice, exhibited delayed clearance at 6 dPI (**Appendix C**). Trend analyses indicated that IL-36γ$^{-/-}$ mice exhibited a significant ($p < 0.05$) delay in HSV-2 clearance from the vaginal epithelium relative to WT mice (**Fig. 19E**). To evaluate systemic spread of HSV-2 after i.vag. infection, viral titers were quantified in the lumbar spinal cord, distal colon, and bladder of IL-36γ$^{-/-}$ and WT mice at 2, 4, and 6 dPI. We detected significantly higher viral titers at 4 and 6 dPI in the spinal cord ($p < 0.0001$, $p < 0.01$, respectively), distal colon ($p < 0.01$, $p < 0.05$, respectively), and bladder ($p < 0.01$, $p < 0.05$, respectively) in IL-36γ$^{-/-}$ mice relative to wild type mice (**Fig. 19B-D**). These differences in titers were most profound at 4 dPI, where there was a 2.3-log increase in titers in the lumbar spinal cord, a 1-log increase in titers in the distal colon, and a 3.3-log increase in HSV-2 titers in the bladder of IL-36γ$^{-/-}$ mice relative to WT mice. Trend analyses indicated that HSV-2 spread more quickly to the lumbar spinal cord ($p < 0.05$), distal colon ($p < 0.05$) and bladder ($p < 0.01$) in IL-36γ$^{-/-}$ mice relative to

wild type mice (**Fig. 19F-H**). Altogether, these data suggest that IL-36γ may function to limit replication and/or systemic spread of HSV-2 through neurons and the nervous system to protect against disease pathogenesis.

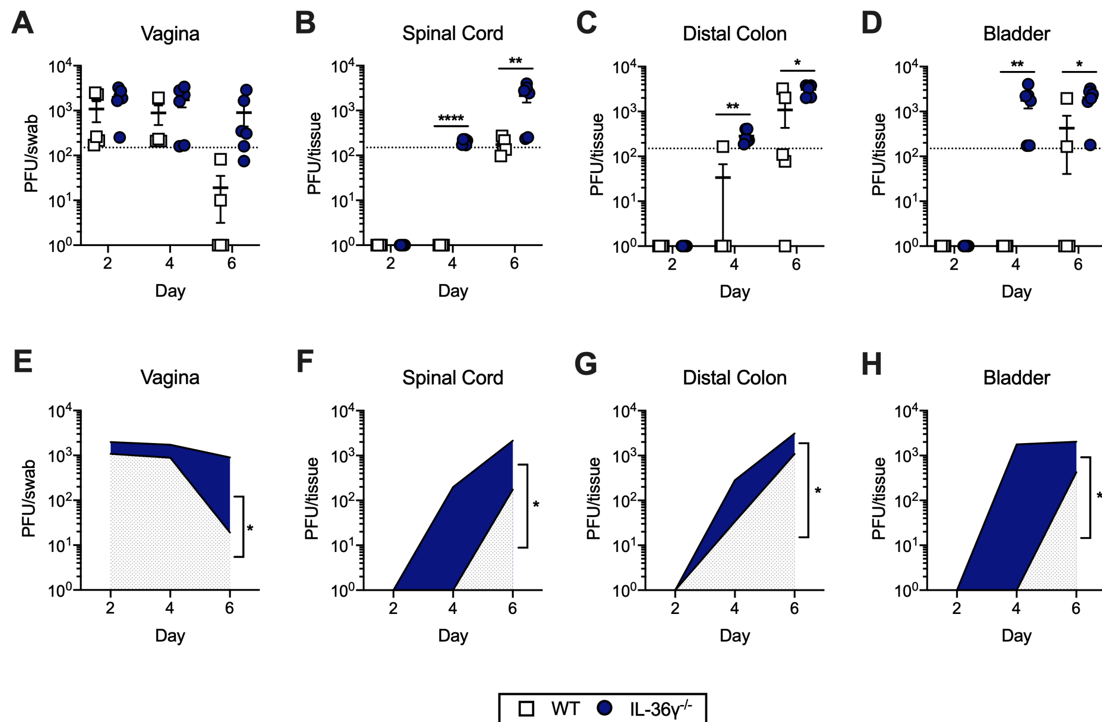

Figure 19. Rapid systemic spread of HSV-2 in IL-36$\gamma^{-/-}$ mice. Analysis of viral titers (**A-D**) and kinetics of virus spread (**E-H**) in WT and IL-36$\gamma^{-/-}$ mice after lethal HSV-2 challenge. DMPA conditioned WT and IL-36$\gamma^{-/-}$ mice were i.vag. challenged with 10^3 PFU HSV-2 186 and then sacrificed at 2, 4, and 6 days post-infection (*n*=5-6 mice/timepoint). Viral titers were assessed in vaginal swabs (**A, E**), spinal cord (**B, F**) distal colon (**C, G**), and bladder (**D, H**) by standard plaque assay. Dashed line indicates minimum detectable level (150 PFU). Data depict mean ± SD and represent two (WT mice) or three (IL-36$\gamma^{-/-}$ mice) independent experiments. Statistical significance was determined by unpaired two-tailed Student *t*-test with Welch's correction (**A-D**) and by AUC analysis followed by unpaired two-tailed Student t-test with Welch's correction (**E-H**). *, $p < 0.05$; **, $p < 0.01$; **** $p < 0.0001$.

Discussion

Initially, IL-36γ was investigated in the context of a chronic inflammatory disease, such as psoriasis (Ding et al. 2018). Recently, however, we and others have demonstrated that IL-36γ plays an important role in host defense mechanisms at mucosal sites (Bassoy, Towne, and Gabay 2018, Gabay and Towne 2015, Gardner and Herbst-Kralovetz 2018, Winkle, Throop, and Herbst-Kralovetz 2016). IL-36γ induces the production of immune mediators and stimulates immune cell recruitment, suggesting a critical role of IL-36γ in immune protection (Gardner and Herbst-Kralovetz 2018, Kovach et al. 2017, Kovach et al. 2016, Winkle, Throop, and Herbst-Kralovetz 2016). In this report, we demonstrate that IL-36γ is an essential component of the innate immune response to genital HSV-2 infection, influencing both neutrophil recruitment and the spread of HSV-2 through the nervous system.

Previously, we showed that IL-36γ is expressed by vaginal epithelial cells, and that treatment with recombinant IL-36γ induced transient polymorphonuclear cell recruitment in addition to the production of cytokines and chemokines, including the neutrophil chemoattractant KC (Gardner and Herbst-Kralovetz 2018, Winkle, Throop, and Herbst-Kralovetz 2016). Using flow cytometry, we performed a time course evaluation of IL-36γ-mediated neutrophil recruitment in the cervicovaginal compartment. Similar to our earlier findings (Gardner and Herbst-Kralovetz 2018), we measured a significant increase in levels of $CD11b^+Ly6G^+$ neutrophils in vaginal lavages at 4 ($p < 0.0001$) and 8h ($p < 0.001$) post-treatment with recombinant IL-36γ (Fig. 2C). Comparing

neutrophil infiltration in WT and IL-36γ$^{-/-}$ mice, we measured no differences in neutrophil counts between the different strains prior to infection, suggesting that the normal influx of neutrophils under homeostatic conditions in the FRT is not dependent on IL-36γ. However, upon exposure to HSV-2 we observed that the number of neutrophils in the vaginal microenvironment differed dramatically between WT and IL-36γ$^{-/-}$ mice (Fig. 2). Consistent with a previous report, we measured a sharp increase in neutrophils in WT mice following i.vag. HSV-2 challenge (Milligan 1999). Surprisingly, there was a notable decrease in neutrophil counts in IL-36γ$^{-/-}$ mice after lethal challenge and these neutrophil levels remained suppressed through 48h post-infection. This was also reflected in vaginal tissue, where we measured a significant decrease in neutrophil counts in IL-36γ$^{-/-}$ mice relative to WT mice. We did not measure any changes in counts of macrophages or monocytes at 24 or 48h post-infection in IL-36γ$^{-/-}$ mice relative WT mice, indicating that IL-36γ signaling may specifically function to regulate neutrophil recruitment in the FRT. To determine potential mechanisms of IL-36γ-mediated regulation of neutrophil recruitment, we evaluated expression of several chemokines in vaginal tissue from IL-36γ$^{-/-}$ and WT mice at 24 and 48h post-infection. Although there was little change in expression of several chemokines in vaginal tissue between IL-36γ$^{-/-}$ and WT mice, we did measure significantly decreased expression of *Cxcl1*, a robust neutrophil chemoattractant, at both 24 and 48h post-infection in IL-36γ$^{-/-}$ mice (Fig. 4). Similarly, Aoyagi et al. reported decreased KC production coupled with reduced neutrophil infiltration to the lungs in IL-36R$^{-/-}$ mice after influenza virus infection (Aoyagi et al.

2016), suggesting that in the FRT IL-36 signaling may drive neutrophil recruitment in part through KC.

By distinguishing mature and immature neutrophil populations, we unexpectedly found that the mature neutrophil population was depleted in the IL-36γ$^{-/-}$ mice through 48h after HSV-2 infection. The drop in mature neutrophil levels created a shift in the vaginal epithelium, whereby immature neutrophils became the dominant neutrophil population, despite no changes in the overall counts of immature neutrophils. We did measure a 7-fold decrease in *Csf3*, an important factor for neutrophil maturation, in IL-36γ$^{-/-}$ vaginal tissue at 24h post-HSV-2 challenge, although this change was not significant. IL-36γ signaling may impact neutrophil maturation factors, and future studies are needed to better understand the impact of IL-36γ on neutrophil mobilization and maturation. While specific anti-HSV-2 functions of neutrophils are not well defined, neutrophils have been shown to phagocytose virions (Boddingius et al. 1987, Van Strijp et al. 1989), secrete inflammatory mediators (Galani and Andreakos 2015, Gonzalez-Dosal et al. 2011), and play a role in viral clearance (Milligan 1999, Thomas et al. 1997, Tumpey et al. 1996). Although immature neutrophils have many of the functions of mature neutrophils, immature neutrophils have been reported to exhibit a reduction in mobility and trafficking behavior (Deniset et al. 2017, Wright et al. 2017). Additionally, immature neutrophils are less efficient in the production of reactive oxygen species (ROS) that function as an important component of the host response to infection (Drifte et al. 2013). The dramatic drop in counts of mature neutrophils in IL-36γ$^{-/-}$ mice did not impact viral titers at 2 or 4 dPI, but titers at 6 dPI in the IL-36γ$^{-/-}$ mice were similar to 2

and 4 dPI, whereas WT mice had almost completely cleared the virus. The decreased neutrophil counts and subsequent delayed clearance of the virus in IL-36$\gamma^{-/-}$ mice is consistent with our findings using neutrophil depleted mice and from a previous report that demonstrated that neutrophil depleted mice exhibit delayed clearance of HSV-2 from the vaginal epithelium (Milligan 1999). Milligan et al. further demonstrated that although neutrophils function in viral clearance from the vaginal epithelium, they appear to have a limited role in preventing virus spread to the dorsal root ganglia after i.vag. inoculation (Milligan, Bourne, and Dudley 2001). The specific mechanisms whereby neutrophils participate in HSV-2 clearance from the vaginal epithelium remain unclear, and the production of ROS (Gonzalez-Dosal et al. 2011), neutrophil extracellular traps (Agraz-Cibrian et al. 2017), or phagocytosis (Boddingius et al. 1987, Van Strijp et al. 1989) may potentially contribute to viral clearance (Camp and Jonsson 2017, Drescher and Bai 2013). Although neutrophils function in virus clearance from the vaginal epithelium, we found that neutrophils were not essential for protection against genital HSV-2 infection, as neutrophil depleted mice exhibited disease progression and an overall survival time that was comparable to WT mice. Neutrophils may exhibit a function in protection against genital disease pathogenesis, but protection afforded by neutrophils appears to be limited. Collectively, our data indicate that IL-36γ-mediated neutrophil recruitment plays a role in HSV-2 clearance from the vaginal epithelium but is not essential for protection against disease pathogenesis or in limiting virus replication during acute infection.

Interestingly, we observed a significant ($p < 0.0001$) increase in the incidence of hind limb paralysis in IL-36$\gamma^{-/-}$ mice after lethal i.vag. challenge compared to WT mice.

Approximately 81% of IL-36$\gamma^{-/-}$ mice developed hind limb paralysis and exhibited detectable titers in the brainstem, whereas only 16% of WT mice developed hind limb paralysis and infection in the brainstem after lethal challenge. Additionally, we measured a more rapid spread of HSV-2 to the spinal cord ($p < 0.05$), and then to peripheral tissues including the distal colon ($p < 0.05$) and bladder ($p < 0.01$) that corresponded with the decreased survival time in IL-36$\gamma^{-/-}$ mice. Further, IL-36$\gamma^{-/-}$ mice exhibited significantly elevated HSV-2 titers in the spinal cord, bladder and colon at 4 and 6 dPI. As described earlier, there were no significant differences in vaginal titers at 2 or 4 dPI in WT mice, neutrophil depleted WT mice, and IL-36$\gamma^{-/-}$ mice, indicating that the increased spread of HSV-2 and neuroinvasion may not be due to increased and/or uncontrolled replication of the virus in the vaginal epithelium as a result of the decreased neutrophil counts in IL-36$\gamma^{-/-}$ mice. Rather, these data suggest that IL-36γ may function to limit viral infection, replication and/or spread in neurons and the nervous system. Related neuroprotective functions have also been shown for interferon signaling in neurons that protect against HSV replication and pathogenesis (Rosato and Leib 2015, Song et al. 2016). Milora et al. suggested that the IL-36 cytokines may have a neurologic role in immune protection against HSV-1 in a flank skin model (Milora et al. 2017), and others have shown that IL-36R is expressed in mixed glial cells (Berglof et al. 2003). A recent report has further shown that IL-36γ was upregulated in spinal neurons and astrocytes in a chronic inflammatory model, indicating an inflammatory function for IL-36γ in the central nervous system that may function to protect against viral infection and spread (Li et al. 2018). Together, our findings suggest a dual role for IL-36γ in neutrophil recruitment in the FRT and in protection against HSV-2 neuroinvasion after lethal genital challenge.

In this study, we reveal that IL-36γ is a crucial innate immune mediator in the FRT that ultimately influences genital HSV-2 disease pathogenesis. Our findings demonstrate that IL-36γ is essential for recruiting neutrophils to the vaginal microenvironment following HSV-2 challenge and may function generally as a regulator of neutrophil recruitment in the FRT. Our data additionally indicate that IL-36γ-mediated protection against genital HSV-2 infection is not neutrophil dependent and suggests that IL-36γ may function in neurons to protect against HSV-2 neuroinvasion. However, future studies are necessary to determine the mechanisms of IL-36γ-mediated neuroprotection. Ultimately, our findings show that IL-36γ is a key component of the antiviral response to genital HSV-2 infection and may function broadly in host defense mechanisms in the FRT and against other sexually transmitted pathogens.

CHAPTER 6: FINAL SUMMARY AND FUTURE DIRECTIONS

An estimated 267 million women worldwide are HSV-2 seropositive, and the WHO recently indicated that over one million curable STIs are transmitted on a daily basis (Looker et al. 2015b, Rowley et al. 2019). Genital HSV-2 infection results in a persistent, life-long infection in individuals that increases risk for additional STI acquisition (Freeman et al. 2006, Patel et al. 2012, Wald and Link 2002). While modern medicine has greatly improved treatment and prevention in the fight against STIs, including genital herpes, there still exists a significant need to understand underlying immune mechanisms in the vaginal epithelium that can ultimately impact disease pathogenesis. This dissertation details studies investigating the regulation and function of the novel pro-inflammatory cytokine IL-36γ in host defense against genital HSV-2 infection in the FRT. These studies addressed the hypothesis that IL-36γ functions as a key regulator of mucosal inflammation in the FRT and protects against genital HSV-2 infection. It was demonstrated that the sex hormones estrogen and progesterone function in part to regulate expression of IL-36γ in the FRT in a mouse model (**Chapter 3**). Exposure to IL-36γ prior to infection was shown to protect against genital HSV-2 infection and disease pathogenesis by promoting transient neutrophil infiltration and production of immune mediators (**Chapter 4, 5**). Additionally, IL-36γ was shown to be a critical regulator of neutrophil recruitment and potentially exhibit a previously uncharacterized function in limiting viral neuroinvasion after intravaginal infection. Together, these studies have provided new insights into the mechanisms whereby IL-36γ

functions in the FRT and has provided new hypotheses that can further define the role of IL-36γ in host defense against other bacterial and viral pathogens.

In **Chapter 2** of this dissertation, the use of human 3-D epithelial cell culture models for virus-host interactions was discussed. These models, including the FRT models developed in the Herbst-Kralovetz lab (Vaginal EC, (Hjelm et al. 2010); Endocervical EC, (Radtke, Quayle, and Herbst-Kralovetz 2012); and Endometrial EC, (Laniewski et al. 2017)) faithfully recapitulate features of host tissue that can be essential for virus infection (Gardner and Herbst-Kralovetz 2016, Radtke and Herbst-Kralovetz 2012). In this dissertation, *in vitro* data is presented utilizing the human 3-D vaginal EC model. These 3-D aggregates robustly produce pro-inflammatory immune mediators in response to pathogens, similar to primary human cells (Gardner and Herbst-Kralovetz 2016, Winkle, Throop, and Herbst-Kralovetz 2016). Altogether, these model systems, coupled with animal models, provide a powerful and unique tool to investigate the host response to infection in a physiologically relevant manner, and can provide important insights into the function of IL-36γ in the FRT.

Because the FRT is regulated by the sex hormones estrogen and progesterone, the impact of these hormones on the IL-36 cytokines and host defense in the FRT was investigated (**Chapter 3**). Consistent with a report from Quispe Calla et al., DMPA treatment in mice decreased expression of barrier features and pro-inflammatory cytokines, including IL-36γ and other IL-36 family members, relative to mice that were treated with estrogen. Further, it was shown that these immune mediators and barrier features were expressed at higher levels in the lower FRT relative to the upper FRT, and this may be due to the differences in structure of the epithelium (stratified squamous vs.

columnar), and the fact that the lower FRT is exposed to pathogens on a regular basis whereas the upper FRT is more protected from potential pathogens. While results from animal models, including mice, do not always translate to humans, there is evidence that the sex hormones and hormonal contraceptives can impact host immunity in the human FRT (Govender et al. 2014, Huijbregts et al. 2013, Huijbregts, Michel, and Hel 2014, Ngcapu et al. 2015, Smith-McCune et al. 2017). As a cytokine that is robustly expressed in the FRT, the hormonal regulation of IL-36γ in the vaginal microenvironment could have an important impact on susceptibility to infection and the host response. Several clinical studies have investigated the link between DMPA use and levels of pro-inflammatory cytokines (Govender et al. 2014, Huijbregts, Michel, and Hel 2014, Ngcapu et al. 2015, Smith-McCune et al. 2017), but few have measured or reported on IL-36γ (Birse et al. 2017). Evaluation of IL-36γ levels throughout the menstrual cycle and in women using hormonal and non-hormonal contraceptives is of great importance in understanding the extent to which hormones can regulate IL-36γ in the FRT. Additionally, investigating the link between the hormonal regulation of IL-36γ and susceptibility to infection is an important next step, and these studies can be performed in the human 3-D vaginal EC model, mouse models, and in clinical studies. Because of the widespread use of hormonal contraceptives and recent links to increased STI acquisition in high-risk women (Grabowski et al. 2015, Socias et al. 2017), an enhanced understanding of hormonal regulation of IL-36γ and the immune response will greatly aid in the development of safe contraceptives.

In **Chapter 4**, IL-36γ treatment was shown to limit HSV-2 replication 3-D human vaginal EC, and IL-36γ pre-treatment protected mice against a lethal intravaginal HSV-2 challenge. Interestingly, treatment of 3-D vaginal EC aggregates with IL-36γ at either 24 or 4h prior to infection led to significantly reduced viral titers after 24h. The exact mechanism whereby IL-36γ treatment limits HSV-2 replication in the human 3-D VEC model has not been elucidated, although exposure to IL-36γ has been shown to robustly induce pro-inflammatory immune mediators (Winkle, Throop, and Herbst-Kralovetz 2016). IL-36γ and IL-36 signaling may limit HSV-2 replication directly or indirectly by inhibiting binding of the virus to target cells, suppressing viral mRNA expression, or by limiting the release of virus progeny to neighboring cells or the cell culture supernatant. Additional studies in human 3-D vaginal EC can elucidate specific mechanisms whereby IL-36γ treatment limits virus replication *in vitro*. Viruses have deployed various immune evasion strategies over time to promote infection of and replication in target cells, including the HSV protein VP22 that is capable of inhibiting IL-1β production and secretion (Jensen 2017, Maruzuru et al. 2018), however, at this time there is currently no suggestion of or evidence for a similar HSV protein that either inhibits IL-36γ production or signaling.

Interestingly, in the mouse model for genital HSV-2 infection, treatment with IL-36γ 4h, but not 24h, prior to infection protected mice against lethal challenge and resulted in reduced viral titers. The protection that was observed with the 4h pre-treatment corresponded with the robust production of immune mediators and the influx of

polymorphonuclear cells to the vaginal microenvironment. In contrast to the 3-D *in vitro* model, where EC are the only cell type present, protection against HSV-2 challenge from IL-36γ treatment in the mouse model appears to be dependent, at least partly, through the recruitment PMN corresponding to the time of challenge. Other reports have indicated that IL-36γ is a crucial factor to promote PMN infiltration at mucosal sites (Aoyagi et al. 2016, Kovach et al. 2017), and this study demonstrates, for the first time, that IL-36γ promotes PMN infiltration in the FRT.

Many immune mediators, including other IL-1 family members, can regulate and direct the host response to pathogens in the vaginal microenvironment, therefore, the requirement for IL-36γ in response to genital HSV-2 infection was evaluated in **Chapter 5**. In characterizing the IL-36γ-mediated immune cell infiltration in CVLs, a robust and transient infiltration of $CD11b^{+}Ly6G^{+}$ neutrophils to the vaginal microenvironment was measured after intravaginal IL-36γ treatment and IL-36$\gamma^{-/-}$ mice exhibited an impaired recruitment of neutrophils to the FRT tissue and CVLs after HSV-2 challenge. Together, these data indicate that IL-36γ not only promotes neutrophil recruitment but is crucial for HSV-2-induced neutrophil recruitment. Neutrophils have been shown to function in viral clearance from the vaginal epithelium (Milligan 1999, Milligan, Bourne, and Dudley 2001), and *in vitro* studies with primary neutrophils can investigate mechanisms whereby neutrophils contribute to clearance of HSV-2 from the vaginal epithelium, as well as, the impact of IL-36γ exposure on neutrophil maturation and activity.

In addition to impaired neutrophil recruitment, IL-36γ$^{-/-}$ mice exhibit a more rapid systemic spread of the virus to peripheral sites and significantly increased HSV-2 neuroinvasion that corresponded with decreased overall survival time. Neutrophil depletion studies have shown that neutrophils do not participate in controlling virus replication during acute infection or limit spread of the virus to the DRG, and these reports, coupled with our data, suggest that IL-36γ may function in a previously uncharacterized manner in the nervous system to protect against viral infection, replication, and/or spread in neurons. The mechanisms of IL-36γ-mediated neuroprotection and sources of neuronal IL-36γ that may be critical to protect against HSV-2 are currently unknown. IL-36γ has recently been shown to be upregulated in a model of CNS chronic inflammatory pain (Li et al. 2018) and may potentially drive inflammatory signaling in the CNS that could impact the host response to viral infection. Primary neurons collected from WT and IL-36γ$^{-/-}$ mice cultured in tri-chamber systems could provide important insights into the function of IL-36γ in virus-neuron interactions and indicate the potential requirement for additional cells (e.g. astrocytes) to limit HSV-2 infection and spread in neurons. TLR3 has been shown to protect against viral neuroinvasion by promoting the type I interferon response in astrocytes (Reinert et al. 2012), and IL-36γ may similarly promote an antiviral response in astrocytes to protect against HSV-2 neuroinvasion. Performing additional studies to define the function of IL-36γ in the nervous system can further delineate the mechanisms of IL-36γ-mediated neuroprotection and may be relevant in the context of other neurotropic viruses (e.g. Lyssaviruses, Poliovirus, and other Herpesviruses) in addition to inflammatory diseases

in the CNS. Research into the function of IL-36γ in the context of Zika virus infection may be of particular interest to many, as this virus spreads to the brain tissue in developing fetuses causing severe birth defects that include microcephaly (Brasil et al. 2016, Rasmussen et al. 2016). IL-36γ may exhibit a crucial function in pregnant women to limit virus spread throughout the FRT epithelium, neurons, and ultimately, to the developing fetus. In fact, a recent study reported that a Zika DNA vaccine containing an IL-36γ plasmid adjuvant exhibited enhanced survival and protection against lethal Zika virus challenge in a mouse model (Louis et al. 2019), indicating that IL-36γ may be a critical component of the host response to protect against Zika virus infection.

The studies in this dissertation have highlighted the function of IL-36γ in acute genital HSV-2 infection but did not investigate the role of IL-36γ in HSV-2 latency and reactivation. Because genital HSV-2 infection is lethal in mice, the guinea pig model is required to evaluate HSV-2 latency and reactivation *in vivo* (Kollias et al. 2015). HSV-2 reactivation in guinea pigs mimics virus reactivation and shedding in humans in terms of frequency and duration of reactivation episodes (Bernstein et al. 1991). Using the guinea pig model, the impact of IL-36γ on the establishment of latency in the DRG could be investigated. Pre-treatment of mice with IL-36γ prior to infection did protect 40% of mice against lethal challenge and leads to the hypothesis that intravaginal IL-36γ treatment could protect against virus spread and the establishment of latency in the DRG. The guinea pig model could also be utilized to evaluate the extent to which IL-36γ treatment could limit the duration of reactivation and shedding episodes. IL-36γ-mediated

recruitment of neutrophils and production of pro-inflammatory mediators could aid in clearing the virus from the vaginal epithelium and decrease the overall time of reactivation episodes. Due to the persist latent nature of genital HSV-2 infections, an understanding of the function of IL-36γ in the establishment of latency and virus reactivation is an important next step to further define the function of IL-36γ in host-pathogen interactions.

For several decades researchers have worked towards the development of a vaccine to protect against genital herpes. To date, although several candidates have been successful in pre-clinical trials, they exhibited limited therapeutic effects and none have successfully completed clinical trials and received FDA approval (Whitley and Baines 2018). Interestingly, as previously mentioned, a recent report by Louis et al. demonstrated that an IL-36γ encoded plasmid functioned as an effective adjuvant to increase the protection afforded by a Zika virus DNA vaccine (Louis et al. 2019). The IL-36γ adjuvant increased the frequency of antigen specific $CD4^+$ and $CD8^+$ T cells to help protect against a lethal Zika virus challenge. T cells function in a key manner in the cytolytic response to HSV-2 infection, and exhibit critical functions in controlling virus shedding (Mueller et al. 2002, Skoberne et al. 2013). The prime-pull strategy for a HSV-2 vaccine devised by Shin and Iwasaki relies on the initial priming of effector T cells followed by their recruitment to the FRT to establish an HSV-2-specific resident memory population (Shin and Iwasaki 2012). The use of an IL-36γ encoded plasmid adjuvant in concert with the prime-pull vaccination may further promote an enhanced T cell response that could function as a protective resident population. Ultimately, the utilization of an

IL-36γ-based adjuvant could be an important and effective strategy in the development of a vaccine to protect against genital HSV-2 infection and/or reactivation.

In addition to its function in genital HSV-2 infection and disease pathogenesis, IL-36γ may exhibit key functions in response to other obstetric and gynecologic diseases. Laniewski et al. recently demonstrated that women with newly diagnosed invasive cervical carcinoma exhibited elevated levels of IL-36γ (Laniewski et al. 2018). IL-36γ has been shown to also be elevated in colorectal cancer (Weinstein et al. 2019). Two recent reports have demonstrated that therapeutic delivery of IL-36γ alone, or alongside chemotherapeutics, promoted a robust anti-tumor response that decreased the overall tumor burden in mice (Chen et al. 2019, Wang et al. 2015). Larger clinical studies can investigate the link between IL-36γ levels and cervical cancer, and additionally investigate potential associations between IL-36γ and other gynecologic cancers, including ovarian and endometrial cancer.

As mentioned earlier, the WHO recently reported that over one million curable STIs are transmitted daily, and IL-36γ may contribute to the host response to other bacterial and viral STIs. Winkle et al. demonstrated that microbial products induce IL-36γ in human 3-D vaginal EC (Winkle, Throop, and Herbst-Kralovetz 2016), and Patras et al. also demonstrated that invasive Group B *Streptococcus* strains induce IL-36γ *in vitro* (Patras et al. 2015, Patras et al. 2013). IL-36γ may particularly be relevant in the context of STI that are neutrophilic, including gonorrhea. During infection, *N.*

gonorrhoeae causes robust neutrophil infiltration, and the bacteria are resistant to neutrophil killing (Johnson and Criss 2011). Extensive neutrophil infiltration can lead to damage in the tissue, and IL-36γ-mediated neutrophil recruitment may ultimately contribute to disease pathogenesis rather than disease resolution (Johnson and Criss 2011). For example, during influenza virus infection of the lungs, IL-36-mediated neutrophil recruitment was shown to exacerbate disease and IL-36R$^{-/-}$ exhibited signs of reduced lung damage and increased survival after lethal challenge (Aoyagi et al. 2016). Studies investigating the requirement for IL-36γ and IL-36γ-mediated neutrophil recruitment in *N. gonorrhoeae* infection could provide important insights into the mechanisms contributing to disease pathogenesis and the function of IL-36γ in a vaginal bacterial infection. In addition to gonorrhea, IL-36γ may play a role in other STIs, including chlamydia, syphilis, human papilloma virus infection, and bacterial vaginosis, the most common bacterial infection worldwide (Allsworth and Peipert 2011). Investigation into the function of IL-36γ in the context of other STIs and gynecologic diseases will increase our understanding of mechanisms regulating and contributing to the immune response in the FRT and be valuable in the development of novel therapeutics to prevent and treat these widely prevalent diseases.

In summary, this dissertation provides the first report on the function of IL-36γ in the FRT in response to a viral infection. These results provide essential information on the regulation of IL-36γ and other IL-36 family members in the FRT by the sex hormones estrogen and progesterone that can potentially impact susceptibility to infection. The role

of IL-36γ in protecting against viral infection in the FRT is defined, and these data show that IL-36γ is a critical regulator of neutrophil recruitment in response to HSV-2 challenge. Furthermore, these studies provide novel insights into the potential role of IL-36γ in the CNS to protect against viral neuroinvasion and support the further study of IL-36γ signaling and host defense in neurons. Together, these data provide compelling evidence that IL-36γ is a key mediator of mucosal inflammation in the FRT and may be an important factor in the host response to other obstetric and gynecologic sequelae.

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APPENDIX A

PERMISSION TO USE PUBLISHED CO-AUTHORED ARTICLES

All authors on previously published and submitted co-authored manuscripts have provided permission to include the articles in this document. References for co-authored publications are provided below.

Jameson K. Gardner and Melissa M. Herbst-Kralovetz. 2016. Three-dimensional rotating wall vessel-derived cell culture models for studying virus-host interactions. *Viruses*. 8(11). pii:E304.

Jameson K. Gardner and Melissa M. Herbst-Kralovetz. IL-36γ induces a transient HSV-2 resistant environment that protects against genital disease and pathogenesis. *Cytokine*. 111:63-71. doi: 10.1016/j.cyto.2018.07.034.

Jameson K. Gardner, Alison Swaims-Kohlmeier, and Melissa M. Herbst-Kralovetz. IL-36γ is a key regulator of neutrophil infiltration in the vaginal microenvironment and limits neuroinvasion to protect against genital HSV-2 disease pathogenesis. *J. Immunol.* ji1900280; doi: 10.4049/jimmunol.1900280.

APPENDIX B

CHAPTER 4 SUPPLEMENTARY MATERIAL

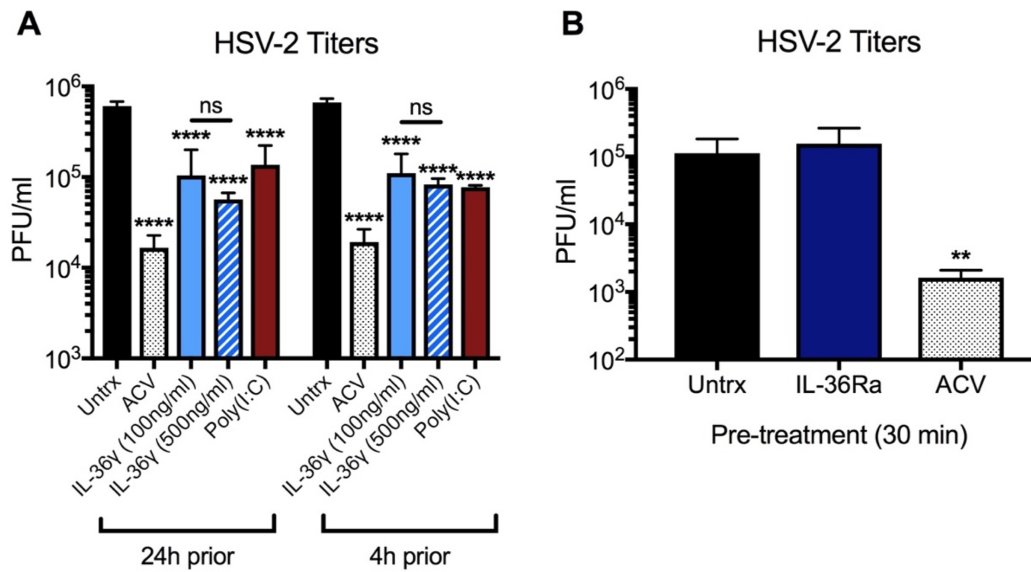

Figure 20. IL-36γ does not limit HSV-2 replication in a dose-dependent manner and pre-treatment with IL-36Ra does not significantly alter HSV-2 replication. HSV-2 replication was measured in 3-D VEC treated with recombinant IL-36γ (100 ng/ml or 500 ng/ml), Poly(I:C) (100 µg/ml), or left untreated 24h prior or 4h prior to infection with HSV-2 186 (MOI 0.1). Aggregates were treated with acyclovir (20 µg/ml) 2h prior to infection as a positive control. Viral titers were measured after 24h as PFU/ml by standard plaque assay. (B) Human 3-D VEC were pre-treated with IL-36Ra (100 ng/ml), ACV (20 µg/ml) or PBS 30 min prior to challenge with HSV-2 186 (MOI 0.1). Aggregates were collected after infection for 24h and viral replication was measured as PFU/ml by standard plaque assay. Data represent mean ± SD from two independent experiments. Statistical significance was determined by one-way ANOVA with multiple comparisons. ****, $P < 0.0001$.

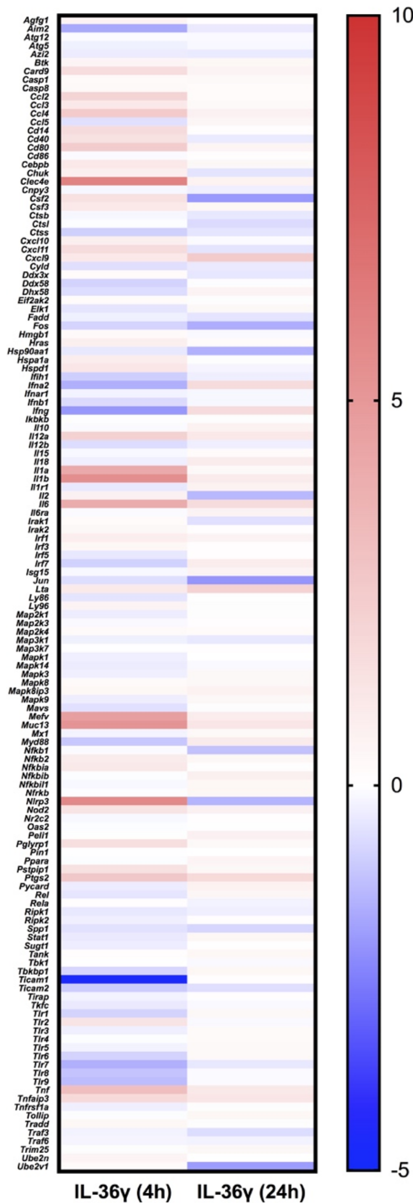

Figure 21. IL-36γ induces expression of cytokines, chemokines and immune signaling molecules in murine FRT tissue 4h after treatment. Female 6- to 8-week-old C57Bl/6 mice were conditioned with medroxyprogesterone as described and then i.vag. treated with recombinant murine IL-36γ (500 ng) or mock-treated with PBS in 10 µl. FRT tissue was collected 4h and 24h after treatment (*n* = 5 mice/time point) for RNA extraction. Gene expression was measured by qRT-PCR analysis using Mouse Antiviral response and Mouse Toll-like receptor signaling RT2 profiler arrays (Qiagen). Data was normalized to endogenous controls and fold change was calculated by delta delta Ct analysis. Data is $\log_2$ transformed.

Table 7.

IL-36γ treatment delayed disease onset and increased survival time in a dose- and time-specific manner when challenged with 35× LD_{50} dose of HSV-2 186

Pre-treatment [a]	Time to symptoms [b] (days)	Survival time [c] (days)
PBS	5.8 ± 1.1	8.1 ± 0.9
IL-36γ (100 ng) 4h	5.2 ± 0.4	8.4 ± 0.9
IL-36γ (250 ng) 24h	5.5 ± 0.5	8.1 ± 0.9
IL-36γ (250 ng) 4h	7.8 ± 1.7 ****	10.6 ± 1.9 ****
IL-36γ (500 ng) 4h	7.6 ± 0.9 ***	9.1 ± 1.9

[a] Intravaginal treatment of IL-36γ was delivered 24h or 4h prior to viral challenge with 1×10^4 PFU HSV-2 186 (35× LD_{50}).

[b] Mean for mice with disease signs (erythema and hair loss). Log rank analysis was used to compare the average time to disease in treatment groups and PBS controls. ***, $P < 0.001$; ****, $P < 0.0001$.

[c] Mean survival time within 15 days. Log rank analysis was used to compare survival between the treatment groups and PBS controls. ****, $P < 0.0001$.

APPENDIX C

CHAPTER 5 SUPPLEMENTARY MATERIAL

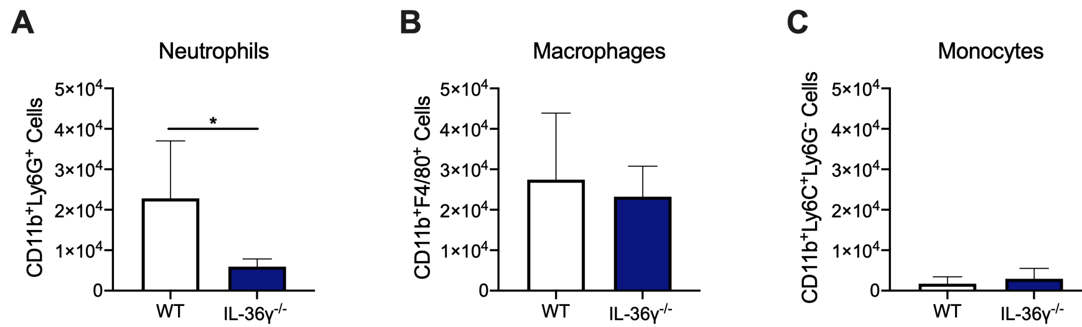

Figure 22. Neutrophil recruitment is impaired in vaginal tissue from IL-36γ$^{-/-}$ mice after genital HSV-2 infection. Flow cytometry analysis of immune cells in vaginal tissue after HSV-2 challenge. Female six- to eight-week old DMPA conditioned WT (n=7) and IL-36γ$^{-/-}$ (n=5) mice were i.vag. challenged with 10^3 PFU HSV-2 186. Vaginal tissue was collected 24h post-infection and analyzed by flow cytometry. Total cell counts from dissociated tissue and results from flow cytometry analysis were used to calculate number of neutrophils (CD11b$^+$Ly6G$^+$) (**A**), macrophages (CD11b$^+$F4/80$^+$) (**B**), and monocytes (CD11b$^+$Ly6C$^+$Ly6G$^-$) (**C**) in vaginal tissue. Data depict mean ± SD and represent results from two independent animal studies. Statistical significance was determined by unpaired two-tailed Student *t*-test with Welch's correction. *, $p < 0.05$.

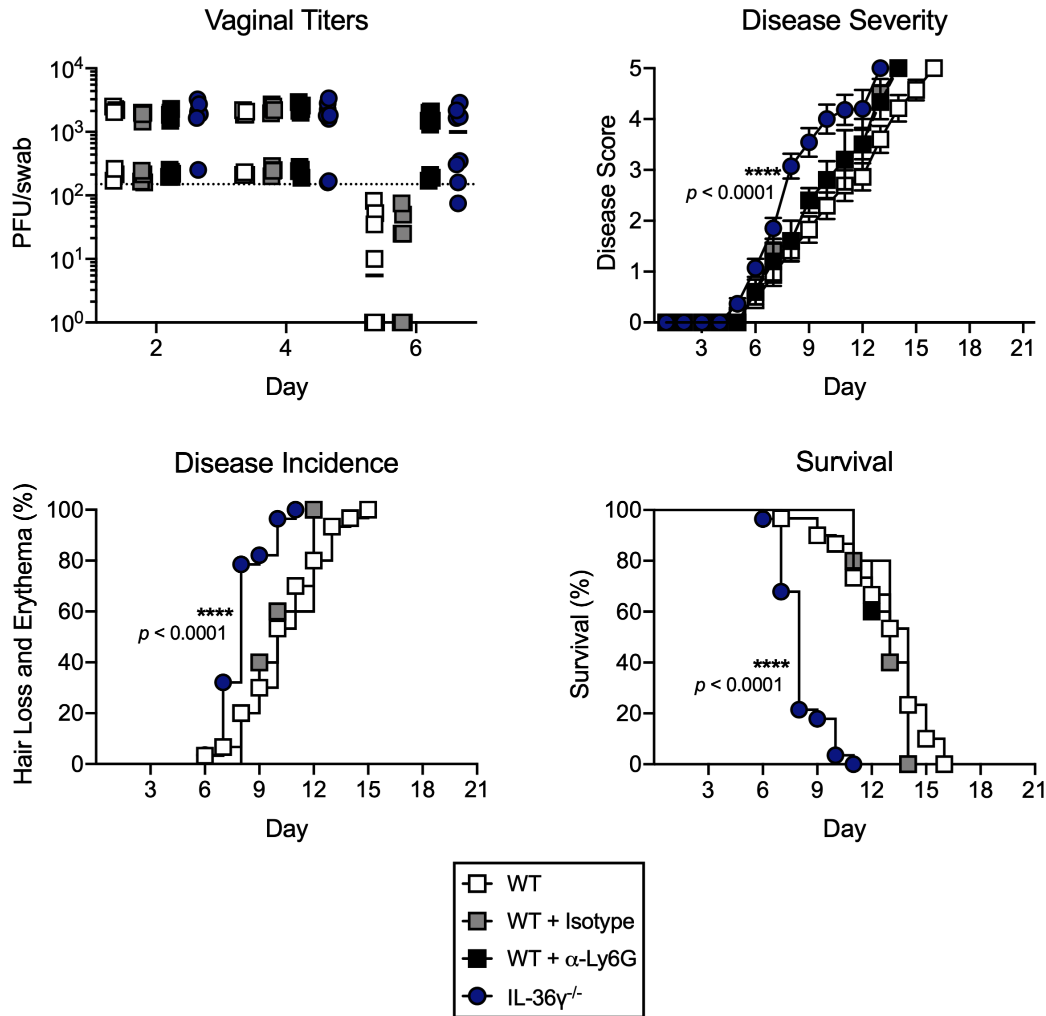

Figure 23. Neutrophil depleted mice exhibit disease progression and survival similar to WT mice. Female six- to eight-week-old DMPA conditioned mice were treated with anti-mouse Ly6G (α-Ly6G, n=5) or isotype control (n=5) antibodies one day prior to infection and then every other day through the remainder of the study. Mice were challenged with 10^3 PFU HSV-2 186. (**A**) Vaginal swabs were collected at 2, 4, and 6 dPI to measure vaginal viral titers. Disease severity (**B**), incidence (**C**), and survival (**D**) were monitored over a 21-day period. Data are presented in comparison with results from WT (n=30) and IL-36$\gamma^{-/-}$ mice (n=27). Data are representative of one (Neutrophil depleted mice and controls) and three (WT and IL-36$\gamma^{-/-}$ mice) independent animal experiments. Statistical analysis was performed by two-way ANOVA with Bonferroni's multiple comparisons (**A**), AUC analysis followed by two-tailed Student *t*-test with Welch's correction (**B**), and log-rank analysis (**C, D**).