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IL-36γ induces a transient HSV-2 resistant environment that protects against genital disease and pathogenesis

Jameson K. Gardner[1,2] and **Melissa M. Herbst-Kralovetz**[1,3,*]

[1]Department of Basic Medical Sciences, College of Medicine-Phoenix, University of Arizona, Phoenix, AZ, USA

[2]Molecular and Cellular Biology Graduate Program, School of Life Sciences, Arizona State University, Tempe, AZ, USA

[3]Department of Obstetrics and Gynecology, College of Medicine-Phoenix, University of Arizona, Phoenix, AZ, USA

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 wellcharacterized 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.

[*]**Correspondence:** Melissa M. Herbst-Kralovetz, mherbst1@email.arizona.edu.

Keywords

vaginal epithelial cells; chemokines; antimicrobial peptides; polymorphonuclear leukocytes; 3-D bioreactor model; HSV-2 mouse model; IL-36 family members; host defense; sexually transmitted infections

1 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 [1]. HSV-2 causes a persistent, lifelong infection that increases risk for STI acquisition, including human immunodeficiency virus [2]. 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 [3–5].

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 [6, 7]. 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 [8–11]. 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) [12]. 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 [12].

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 [12–19]. 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 [20]. 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 [21, 22]. 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 [23]. 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 [14, 18, 19, 24, 25]. 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 [18]. In *Mycobacterium tuberculosis* infections, IL-36γ promotes the production of AMPs that limit bacterial growth

[13, 26]. 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[14]. 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 [12, 27, 28]. 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 [20]. 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 [29]. Recently, our laboratory measured increased levels of IL-36γ in cervicovaginal lavages (CVLs) from cervical cancer patients [30]. 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 [31], and an increase in IL-36 cytokines in women with bacterial vaginosis [32]. 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 [33] 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.

1 Materials and Methods

2.1 3-D VEC culture

Three-dimensional human vaginal epithelial (V19I) cells were cultured as previously described [12, 34, 35]. 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).

2.2 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 [36]. 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 [37]. The University of Arizona (UA) Institutional Biosafety Committee (IBC) approved all safety and handling of HSV-2 in the laboratory.

2.3 *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 [12], 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 [25, 38–41]. 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.

2.4 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 [37]. 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 [37]. 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.

2.5 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.

2.6 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.

2.7 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 1.

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).

2.8 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).

2.9 Statistics

Disease incidence and survival in mouse models were analyzed by log-rank analysis as previously described [37]. 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 twoway 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.

2 Results

3.1 IL-36γ is induced by 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 [12, 25, 27, 28]. 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. 1A). 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. 1B).

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 [33]. 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. 1C). 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 [42–44], the

administration of a single dose of ACV over a 24h period [44], 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 (Supplemental Figure 1). 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.

3.2 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 (Supplemental Table 1). 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 [37, 45, 46]. 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 2A). 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 2B). 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 2C). 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 2D). 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.

3.3 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 proinflammatory cytokines and chemokines 24h after treatment in a human 3-D VEC model [12], 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 (Supplemental Figure 2), 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 [47–51]. We also identified several antimicrobial peptides (AMP) that have previously been demonstrated to limit HSV-2 infection [8–10] 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 3A). 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 (Supplemental Figure 2). 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.

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. 3B). 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 3A, B) and may have contributed to limiting HSV-2 replication and increasing survival.

3.4 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 4). There 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 [52, 53]. 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.

3 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 [12]. 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 [31, 32]. 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[54] 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 [55, 56]. Poly(I:C), a viral dsRNA mimic, has previously been shown to protect against lethal HSV-2 challenge [37], and induce IL-36γ *in vitro* [12, 25, 27, 28], 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 [56].

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 [12]. 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 3A). 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 [48–50, 57–59]. AMP, including Slpi, Defb3, and Ltf, are also key soluble immune mediators that have been shown to inhibit HSV infection [8–10], and are induced by IL-36γ in human VEC [12]. Likewise, we measured elevated levels of *Slpi* following IL-36γ treatment in murine FRT tissue (Fig 3). Interestingly, IL-36γ exposure significantly increased expression of the C-type lectin *Clec4e* 24-fold (Fig 3A). 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 [60, 61]. 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 [14, 62–64]. 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. 3). KC and CCL20 can both function to recruit polymorphonuclear leukocytes (PMN), including neutrophils, that are important for protection against HSV infection in animal models [51, 65–67]. HSV-2 infection does promote the recruitment of PMN in the FRT; however, this is not until approximately 24h after infection [65, 68]. 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 3B, 4). Vaginal smears contained a homogenous infiltration of cells that were consistent with PMN morphology, however, other immune cell populations were not morphologically identified 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 [65], 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 2A, 4). Neutrophils are important producers of proteases, including elastase, which has recently been shown to cleave and activate IL-36γ [69]. 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* [70–72]. 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. 3B). 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 [12]. 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 [29]. 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 both 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.

4 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5 Funding

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- HSV-2 infection induces IL-36γ expression in human vaginal epithelial cells
- HSV-2 replication is limited by IL-36γ treatment *in vitro* and *in vivo*
- IL-36γ induces immune mediators and PMN recruitment in the vaginal epithelium
- Treatment with IL-36γ protects against genital HSV-2 disease and pathogenesis
- IL-36γ may function as a key defense mechanism in the female reproductive tract

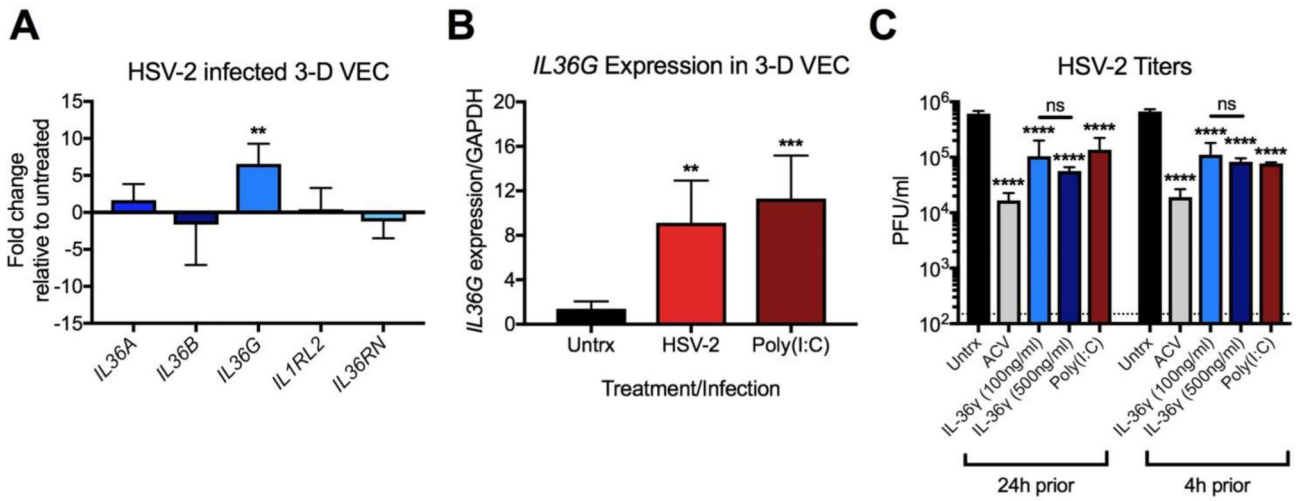

Figure 1. IL-36γ is induced by HSV-2 infection and IL-36γ treatment limits HSV-2 infection in 3-D VEC.

(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). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

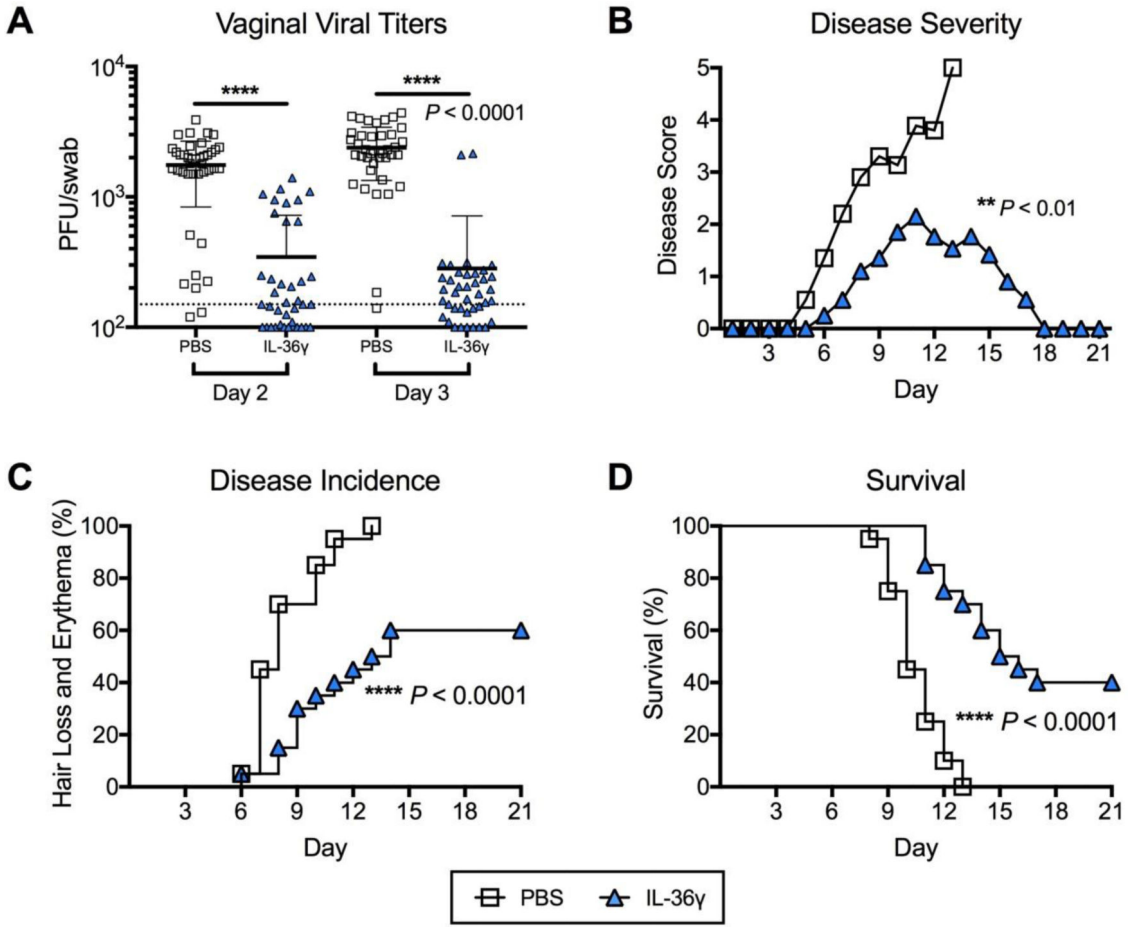

Figure 2. IL-36γ treatment 4h prior to infection significantly protects against HSV-2 disease incidence, reduces disease severity, and enhances survival.

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). **, $P < 0.01$; ****, $P < 0.0001$.

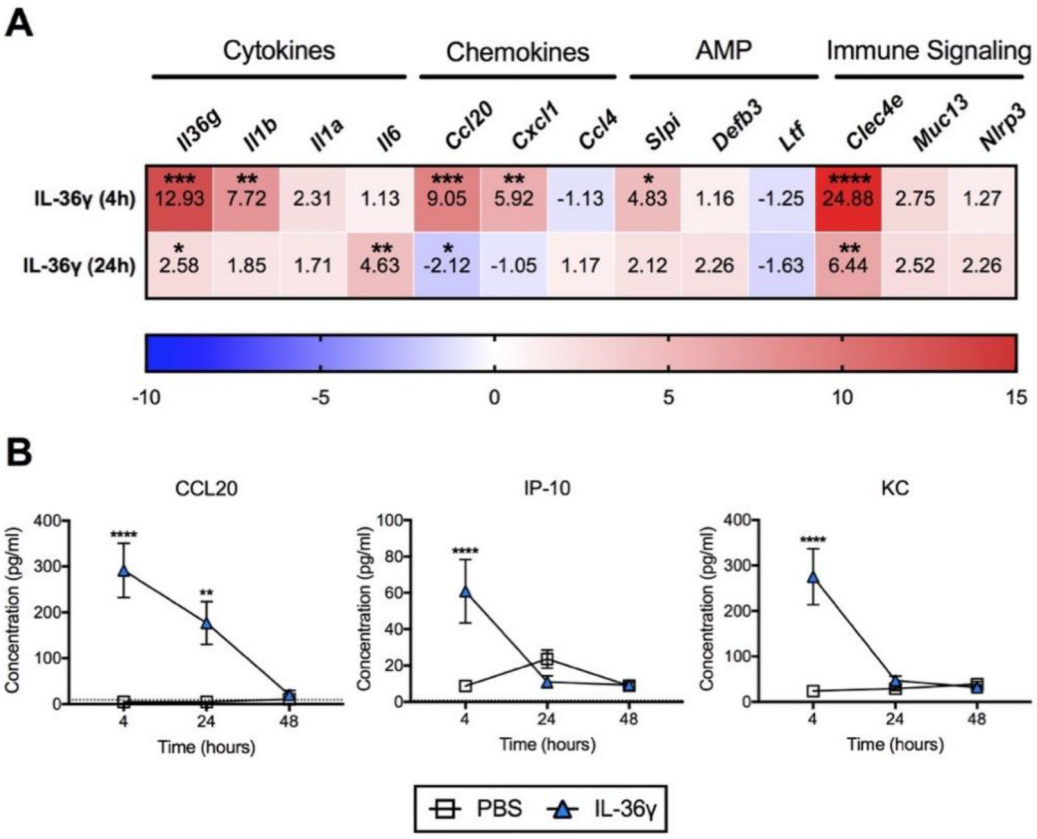

Figure 3. IL-36γ treatment induces the transient production of immune mediators in murine lower FRT tissue and vaginal lavages.

(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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

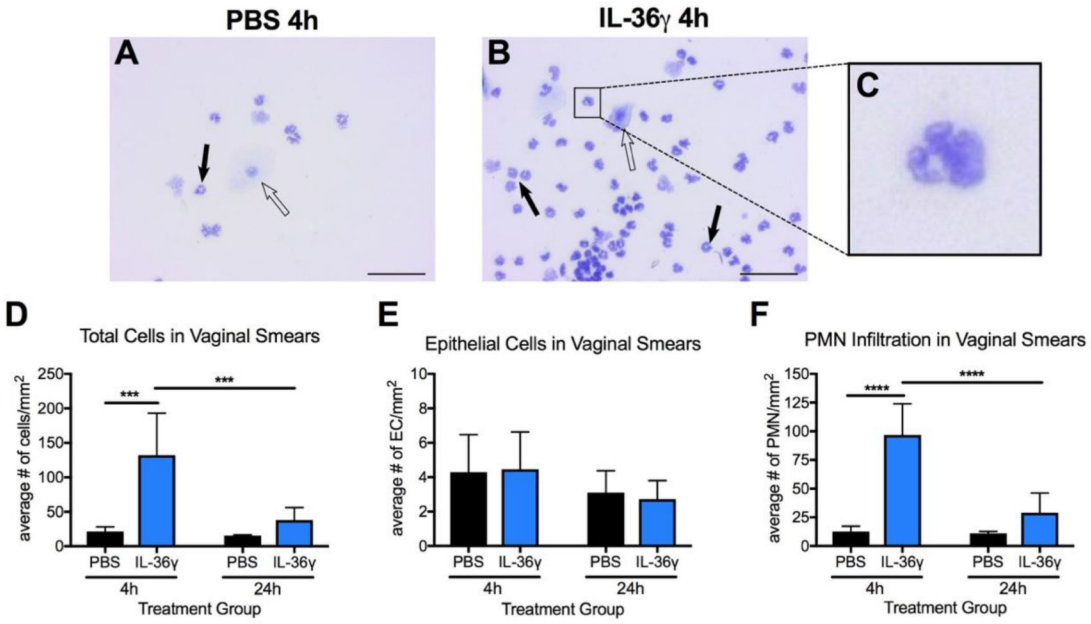

Figure 4. IL-36γ transiently promotes polymorphonuclear leukocyte infiltration in the vaginal microenvironment.

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. ***, $P < 0.001$; ****, $P < 0.0001$.

TABLE 1.

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