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

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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.

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 and 85% sequence

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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.

2. 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.