HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2023 March 02.

Published in final edited form as:

J Immunol. 2019 November 15; 203(10): 2655–2664. doi:10.4049/jimmunol.1900280.

IL-36γ is a key regulator of neutrophil infiltration in the vaginal microenvironment and limits neuroinvasion in genital HSV-2 infection

Jameson K. Gardner[*,†], **Alison Swaims-Kohlmeier**[‡], **Melissa M. Herbst-Kralovetz**[*,§]

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

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

[‡]Laboratory Branch, Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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

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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ mice. Additionally, IL-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ 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.

Correspondence: Melissa M. Herbst-Kralovetz, mherbst1@email.arizona.edu, 602-827-2247.

Keywords

HSV-2 mouse model; neuroprotection; sexually transmitted infections; host defense; IL-36 cytokines; female reproductive tract

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 (1). 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 (2–6). The IL-36 cytokines were first characterized as members of the IL-1 superfamily that are expressed by epithelial cells at mucosal sites (7, 8). 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 (9–13). 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 (14–18). 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 (19). 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 (20). We further revealed that IL-36γ transiently induced polymorphonuclear cell recruitment in the FRT that corresponded with decreased vaginal viral titers and increased survival (20).

Neutrophils are one of the predominant immune cells present in the FRT, and function as early responders to pathogens (21, 22). 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 (23). 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 (20). 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 (21). Interestingly, neutrophil elastase has recently been shown to cleave and activate IL-36γ from its pro-form (24, 25), 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 (26–29). Periodic virus reactivation can cause ulcerative disease in individuals, however asymptomatic virus shedding can also occur (30, 31). 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 (32–34). 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 (35). Symptoms of HSV-2 neuroinvasion following i.vag. infection in mice include urinary retention, toxic megacolon, and hind limb paralysis (34–36).

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 (37). Virus titers in HSV-2 stocks and infected samples were determined using standard plaque assay as previously described (3).

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 (3). 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 (20). 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 (20). 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 1.

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 (38). 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 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 (20). Disease incidence and survival were analyzed by log-rank analysis as previously described (3). 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. 1A). This finding is consistent with a recent report from Cora et al. that describes neutrophils as the most abundant cells during diestrus in mice (39). Following treatment with IL-36γ, we measured a significant influx of neutrophils into the vaginal microenvironment at 4 (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. 1C). 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.

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

We next quantified cellular infiltration in CVLs from IL-36$\gamma^{-/-}$ 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. 2). 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. 2B). 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 24 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. 2C, 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. 2E, Supplemental Figure 1). 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 (Supplemental Figure 1). 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.

Interestingly, we observed two distinct populations of CD11b^{+}Ly6G^{+} cells when analyzing the neutrophil population in vaginal lavages (Fig. 3A, 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. 3C). The number of mature neutrophils in WT mice dramatically increased at 24 and 48h ($p < 0.05$) post-infection (Fig. 3D), whereas the number of immature neutrophils remained relatively constant (Fig. 3E). 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. 3C, 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.

Reduced Cxcl1/KC expression in IL-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ and WT mice at 24 and 48h post-infection (Fig. 4). 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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ 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 chemokines assayed. Together, these data suggest that IL-36γ may function to regulate neutrophil recruitment in the FRT in-part through the neutrophil chemoattractant KC.

Decreased survival time in IL-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ mice. We observed that IL-36$\gamma^{-/-}$ mice exhibited more rapid disease symptoms ($p < 0.0001$) compared to WT mice (Fig. 5A). IL-36$\gamma^{-/-}$ mice developed hair loss and erythema around the introitus 2.5 days earlier than controls (Fig. 5B). 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. 5C), 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. 5). Isotype control treated mice exhibited similar disease progression and survival as both WT and neutrophil depleted WT mice (Supplemental Figure 2). 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.

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

Surprisingly, while monitoring disease progression and survival after lethal challenge, we observed an increased incidence of hind limb paralysis in IL-36γ$^{-/-}$ 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. 6A, B). In contrast, 81.5% of IL-36γ$^{-/-}$ 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-36γ$^{-/-}$ mice with productive infections in the brainstem (data not shown). The increased spread of HSV-2 to the CNS in IL-36γ$^{-/-}$ mice indicates a potential neuroprotective function for IL-36γ in the antiviral response.

Delayed clearance of HSV-2 from the vaginal epithelium in IL-36γ$^{-/-}$ 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. 7A). 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 (Supplemental Figure 2). 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. 7E). 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-36$\gamma^{-/-}$ mice relative to wild type mice (Fig 7B–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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ mice relative to wild type mice (Fig 7F–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.

Discussion

Initially, IL-36γ was investigated in the context of a chronic inflammatory disease, such as psoriasis (40). Recently, however, we and others have demonstrated that IL-36γ plays an important role in host defense mechanisms at mucosal sites (19, 20, 41, 42). IL-36γ induces the production of immune mediators and stimulates immune cell recruitment, suggesting a critical role of IL-36γ in immune protection (16, 17, 19, 20). 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 (19, 20). Using flow cytometry, we performed a time course evaluation of IL-36γ-mediated neutrophil recruitment in the cervicovaginal compartment. Similar to our earlier findings (20), 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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ mice (Fig. 2). Consistent with a previous report, we measured a sharp increase in neutrophils in WT mice following i.vag. HSV-2 challenge (23). Surprisingly, there was a notable decrease in neutrophil counts in IL-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ and WT mice at 24 and 48h post-infection. Although there was little change in expression of several chemokines in vaginal tissue between IL-36$\gamma^{-/-}$ and WT

mice, we did measure significantly decreased expression of *Cxcl1*, a robust neutrophil chemoattractant, at both 24 and 48h post-infection in IL-36$\gamma^{-/-}$ 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 (15), 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-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ 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 (43, 44), secrete inflammatory mediators (45, 46), and play a role in viral clearance (23, 47, 48). 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 (49, 50). 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 (51). The dramatic drop in counts of mature neutrophils in IL-36$\gamma^{-/-}$ mice did not impact viral titers at 2 or 4 dPI, but titers at 6 dPI in the IL-36$\gamma^{-/-}$ 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 (23). 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 (52). The specific mechanisms whereby neutrophils participate in HSV-2 clearance from the vaginal epithelium remain unclear, and the production of ROS (46), neutrophil extracellular traps (53), or phagocytosis (43, 44) may potentially contribute to viral clearance (54, 55). 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 (56, 57). 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 (18), and others have shown that IL-36R is expressed in mixed glial cells (58). 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 (59). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are thankful to Dr. Timothy Denning for generously providing a breeder pair of IL-36$\gamma^{-/-}$ mice and Dr. Mrinalini Kala and the Flow Cytometry Core at the University of Arizona College of Medicine Phoenix for assistance with flow cytometry experiments. We thank Dr. Theresa Thomas for critical review of the manuscript.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the U.S. Centers for Disease Control and Prevention or the Department of Health and Human Services.

Funding

This work was supported by the National Institutes of Health NIAID Grant 1R15AI113457–01A1 (to MMH-K).

References

1. Looker KJ, Magaret AS, Turner KM, Vickerman P, Gottlieb SL, and Newman LM. 2015. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. PLoS One 10: e114989. [PubMed: 25608026]
2. Herbst-Kralovetz M, and Pyles R. 2006. Toll-like receptors, innate immunity and HSV pathogenesis. Herpes 13: 37–41. [PubMed: 16895653]
3. Herbst-Kralovetz MM, and Pyles RB. 2006. Quantification of poly(I:C)-mediated protection against genital herpes simplex virus type 2 infection. J Virol 80: 9988–9997. [PubMed: 17005677]
4. Leoni V, Gianni T, Salvioli S, and Campadelli-Fiume G. 2012. Herpes simplex virus glycoproteins gH/gL and gB bind Toll-like receptor 2, and soluble gH/gL is sufficient to activate NF-kappaB. J Virol 86: 6555–6562. [PubMed: 22496225]
5. Lund J, Sato A, Akira S, Medzhitov R, and Iwasaki A. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med 198: 513–520. [PubMed: 12900525]
6. Rasmussen SB, Sorensen LN, Malmgaard L, Ank N, Baines JD, Chen ZJ, and Paludan SR. 2007. Type I interferon production during herpes simplex virus infection is controlled by cell-type-specific viral recognition through Toll-like receptor 9, the mitochondrial antiviral signaling protein pathway, and novel recognition systems. J Virol 81: 13315–13324. [PubMed: 17913820]
7. Gresnigt MS, and van de Veerdonk FL. 2013. Biology of IL-36 cytokines and their role in disease. Semin Immunol 25: 458–465. [PubMed: 24355486]
8. Kumar S, McDonnell PC, Lehr R, Tierney L, Tzimas MN, Griswold DE, Capper EA, Tal-Singer R, Wells GI, Doyle ML, and Young PR. 2000. Identification and initial characterization of four novel members of the interleukin-1 family. J Biol Chem 275: 10308–10314. [PubMed: 10744718]
9. D'Erme AM, Wilsmann-Theis D, Wagenpfeil J, Holzel M, Ferring-Schmitt S, Sternberg S, Wittmann M, Peters B, Bosio A, Bieber T, and Wenzel J. 2015. IL-36gamma (IL-1F9) is a biomarker for psoriasis skin lesions. J Invest Dermatol 135: 1025–1032. [PubMed: 25525775]
10. Foster AM, Baliwag J, Chen CS, Guzman AM, Stoll SW, Gudjonsson JE, Ward NL, and Johnston A. 2014. IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin. J Immunol 192: 6053–6061. [PubMed: 24829417]
11. Johnston A, Xing X, Guzman AM, Riblett M, Loyd CM, Ward NL, Wohn C, Prens EP, Wang F, Maier LE, Kang S, Voorhees JJ, Elder JT, and Gudjonsson JE. 2011. IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. J Immunol 186: 2613–2622. [PubMed: 21242515]
12. Towne JE, and Sims JE. 2012. IL-36 in psoriasis. Curr Opin Pharmacol 12: 486–490. [PubMed: 22398321]
13. Walsh PT, and Fallon PG. 2016. The emergence of the IL-36 cytokine family as novel targets for inflammatory diseases. Ann N Y Acad Sci 1417: 23–34. [PubMed: 27783881]
14. Ahsan F, Moura-Alves P, Guhlich-Bornhof U, Klemm M, Kaufmann SH, and Maertzdorf J. 2016. Role of Interleukin 36gamma in Host Defense Against Tuberculosis. J Infect Dis 214: 464–474. [PubMed: 27389350]
15. Aoyagi T, Newstead MW, Zeng X, Kunkel SL, Kaku M, and Standiford TJ. 2016. IL-36 receptor deletion attenuates lung injury and decreases mortality in murine influenza pneumonia. Mucosal Immunol 10: 1043–1055. [PubMed: 27966554]
16. Kovach MA, Singer B, Martinez-Colon G, Newstead MW, Zeng X, Mancuso P, Moore TA, Kunkel SL, Peters-Golden M, Moore BB, and Standiford TJ. 2017. IL-36gamma is a crucial proximal component of protective type-1-mediated lung mucosal immunity in Gram-positive and -negative bacterial pneumonia. Mucosal Immunol 10: 1320–1334. [PubMed: 28176791]
17. Kovach MA, Singer BH, Newstead MW, Zeng X, Moore TA, White ES, Kunkel SL, Peters-Golden M, and Standiford TJ. 2016. IL-36gamma is secreted in microparticles and exosomes by lung macrophages in response to bacteria and bacterial components. J Leukoc Biol 100: 413–421. [PubMed: 26864267]

18. Milora KA, Uppalapati SR, Sanmiguel JC, Zou W, and Jensen LE. 2017. Interleukin-36beta provides protection against HSV-1 infection, but does not modulate initiation of adaptive immune responses. Sci Rep 7: 5799. [PubMed: 28724920]

19. Winkle SM, Throop AL, and Herbst-Kralovetz MM. 2016. IL-36gamma Augments Host Defense and Immune Responses in Human Female Reproductive Tract Epithelial Cells. Front Microbiol 7: 955. [PubMed: 27379082]

20. Gardner JK, and Herbst-Kralovetz MM. 2018. IL-36gamma induces a transient HSV-2 resistant environment that protects against genital disease and pathogenesis. Cytokine 111: 63–71. [PubMed: 30118914]

21. Amjadi F, Salehi E, Mehdizadeh M, and Aflatoonian R. 2014. Role of the innate immunity in female reproductive tract. Adv Biomed Res 3: 1. [PubMed: 24592358]

22. Lee SK, Kim CJ, Kim DJ, and Kang JH. 2015. Immune cells in the female reproductive tract. Immune Netw 15: 16–26. [PubMed: 25713505]

23. Milligan GN 1999. Neutrophils Aid in Protection of the Vaginal Mucosae of Immune Mice against Challenge with Herpes Simplex Virus Type 2. Journal of Virology 73: 6380–6386. [PubMed: 10400730]

24. Clancy DM, Sullivan GP, Moran HBT, Henry CM, Reeves EP, McElvaney NG, Lavelle EC, and Martin SJ. 2018. Extracellular Neutrophil Proteases Are Efficient Regulators of IL-1, IL-33, and IL-36 Cytokine Activity but Poor Effectors of Microbial Killing. Cell Rep 22: 2937–2950. [PubMed: 29539422]

25. Henry CM, Sullivan GP, Clancy DM, Afonina IS, Kulms D, and Martin SJ. 2016. Neutrophil-Derived Proteases Escalate Inflammation through Activation of IL-36 Family Cytokines. Cell Rep 14: 708–722. [PubMed: 26776523]

26. Fleck M, Podlech J, Weise K, Muntefering H, and Falke D. 1993. Pathogenesis of HSV-1/2 induced vaginitis/vulvitis of the mouse: dependence of lesions on genetic properties of the virus and analysis of pathohistology. Arch Virol 129: 35–51. [PubMed: 8470958]

27. McDermott MR, Brais PL, P. L. GC, Evelegh MJ, and Goldsmith CH. 1987. Expression of immunity to intravaginal herpes simplex virus type 2 infection in the genital tract and associated lymph nodes. Arch Virol 93: 51–68. [PubMed: 3813891]

28. McDermott MR, Smiley JR, Leslie P, Brais J, Rudzroga HE, and Bienenstock J. 1984. Immunity in the female genital tract after intravaginal vaccination of mice with an attenuated strain of herpes simplex virus type 2. J Virol 51: 747–753. [PubMed: 6088797]

29. Parr MB, Kepple L, McDermott MR, Drew MD, Bozzola JJ, and Parr EL. 1994. A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2. Lab Invest 70: 369–380. [PubMed: 8145530]

30. Johnston C, and Corey L. 2016. Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. Clin Microbiol Rev 29: 149–161. [PubMed: 26561565]

31. Wald A, Zeh J, Selke S, Ashley RL, and Corey L. 1995. Virologic characteristics of subclinical and symptomatic genital herpes infections. N Engl J Med 333: 770–775. [PubMed: 7643884]

32. Caplan LR, Kleeman FJ, and Berg S. 1977. Urinary retention probably secondary to herpes genitalis. N Engl J Med 297: 920–921. [PubMed: 198656]

33. Goodell SE, Quinn TC, Mkrtichian E, Schuffler MD, Holmes KK, and Corey L. 1983. Herpes simplex virus proctitis in homosexual men. Clinical, sigmoidoscopic, and histopathological features. N Engl J Med 308: 868–871. [PubMed: 6300674]

34. Khoury-Hanold W, Yordy B, Kong P, Kong Y, Ge W, Szigeti-Buck K, Ralevski A, Horvath TL, and Iwasaki A. 2016. Viral Spread to Enteric Neurons Links Genital HSV-1 Infection to Toxic Megacolon and Lethality. Cell Host Microbe 19: 788–799. [PubMed: 27281569]

35. Parr MB, and Parr EL. 2003. Intravaginal administration of herpes simplex virus type 2 to mice leads to infection of several neural and extraneural sites. J Neurovirol 9: 594–602. [PubMed: 14602572]

36. Reinert LS, Harder L, Holm CK, Iversen MB, Horan KA, Dagnaes-Hansen F, Ulhoi BP, Holm TH, Mogensen TH, Owens T, Nyengaard JR, Thomsen AR, and Paludan SR. 2012. TLR3 deficiency

renders astrocytes permissive to herpes simplex virus infection and facilitates establishment of CNS infection in mice. J Clin Invest 122: 1368–1376. [PubMed: 22426207]

37. Pyles RB, Higgins D, Chalk C, Zalar A, Eiden J, Brown C, Van Nest G, and Stanberry LR. 2002. Use of immunostimulatory sequence-containing oligonucleotides as topical therapy for genital herpes simplex virus type 2 infection. J Virol 76: 11387–11396. [PubMed: 12388699]
38. Jiang J, and Kelly KA. 2012. Isolation of lymphocytes from mouse genital tract mucosa. J Vis Exp: e4391. [PubMed: 22972306]
39. Cora MC, Kooistra L, and Travlos G. 2015. Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. Toxicol Pathol 43: 776–793. [PubMed: 25739587]
40. Ding L, Wang X, Hong X, Lu L, and Liu D. 2018. IL-36 cytokines in autoimmunity and inflammatory disease. Oncotarget 9: 2895–2901. [PubMed: 29416822]
41. Bassoy EY, Towne JE, and Gabay C. 2018. Regulation and function of interleukin-36 cytokines. Immunol Rev 281: 169–178. [PubMed: 29247994]
42. Gabay C, and Towne JE. 2015. Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions. J Leukoc Biol 97: 645–652. [PubMed: 25673295]
43. Boddingius J, Dijkman H, Hendriksen E, Schift R, and Stolz E. 1987. HSV-2 replication sites, monocyte and lymphocytic cell infection and virion phagocytosis by neutrophils, in vesicular lesions on penile skin. Electronoptical studies of a biopsy. J Cutan Pathol 14: 165–175. [PubMed: 3038975]
44. Van Strijp JA, Van Kessel KP, van der Tol ME, Fluit AC, Snippe H, and Verhoef J. 1989. Phagocytosis of herpes simplex virus by human granulocytes and monocytes. Arch Virol 104: 287–298. [PubMed: 2539796]
45. Galani IE, and Andreakos E. 2015. Neutrophils in viral infections: Current concepts and caveats. J Leukoc Biol 98: 557–564. [PubMed: 26160849]
46. Gonzalez-Dosal R, Horan KA, Rahbek SH, Ichijo H, Chen ZJ, Mieyal JJ, Hartmann R, and Paludan SR. 2011. HSV infection induces production of ROS, which potentiate signaling from pattern recognition receptors: role for S-glutathionylation of TRAF3 and 6. PLoS Pathog 7: e1002250. [PubMed: 21949653]
47. Thomas J, Gangappa S, Kanangat S, and Rouse BT. 1997. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. J Immunol 158: 1383–1391. [PubMed: 9013983]
48. Tumpey TM, Chen SH, Oakes JE, and Lausch RN. 1996. Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. J Virol 70: 898–904. [PubMed: 8551629]
49. Deniset JF, Surewaard BG, Lee WY, and Kubes P. 2017. Splenic Ly6G(high) mature and Ly6G(int) immature neutrophils contribute to eradication of S. pneumoniae. J Exp Med 214: 1333–1350. [PubMed: 28424248]
50. Wright HL, Makki FA, Moots RJ, and Edwards SW. 2017. Low-density granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. J Leukoc Biol 101: 599–611. [PubMed: 27601627]
51. Drifte G, Dunn-Siegrist I, Tissieres P, and Pugin J. 2013. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. Crit Care Med 41: 820–832. [PubMed: 23348516]
52. Milligan GN, Bourne N, and Dudley KL. 2001. Role of polymorphonuclear leukocytes in resolution of HSV-2 infection of the mouse vagina. J Reprod Immunol 49: 49–65. [PubMed: 11137112]
53. Agraz-Cibrian JM, Giraldo DM, Mary FM, and Urcuqui-Inchima S. 2017. Understanding the molecular mechanisms of NETs and their role in antiviral innate immunity. Virus Res 228: 124–133. [PubMed: 27923601]
54. Camp JV, and Jonsson CB. 2017. A Role for Neutrophils in Viral Respiratory Disease. Front Immunol 8: 550. [PubMed: 28553293]
55. Drescher B, and Bai F. 2013. Neutrophil in viral infections, friend or foe? Virus Res 171: 1–7. [PubMed: 23178588]

56. Rosato PC, and Leib DA. 2015. Neuronal Interferon Signaling Is Required for Protection against Herpes Simplex Virus Replication and Pathogenesis. PLoS Pathog 11: e1005028. [PubMed: 26153886]

57. Song R, Koyuncu OO, Greco TM, Diner BA, Cristea IM, and Enquist LW. 2016. Two Modes of the Axonal Interferon Response Limit Alphaherpesvirus Neuroinvasion. MBio 7: e02145–02115. [PubMed: 26838720]

58. Berglof E, Andre R, Renshaw BR, Allan SM, Lawrence CB, Rothwell NJ, and Pinteaux E. 2003. IL-1Rrp2 expression and IL-1F9 (IL-1H1) actions in brain cells. J Neuroimmunol 139: 36–43. [PubMed: 12799018]

59. Li Q, Liu S, Li L, Ji X, Wang M, and Zhou J. 2018. Spinal IL-36gamma/IL-36R participates in the maintenance of chronic inflammatory pain through astroglial JNK pathway. Glia 67: 438–451. [PubMed: 30578562]

Key Points

- IL-36γ is crucial for neutrophil recruitment in response to genital HSV-2 infection
- Genital HSV-2 disease pathogenesis progresses more rapidly in IL-36$\gamma^{-/-}$ mice
- IL-36$\gamma^{-/-}$ mice exhibit significantly increased HSV-2 neuroinvasion and virus spread

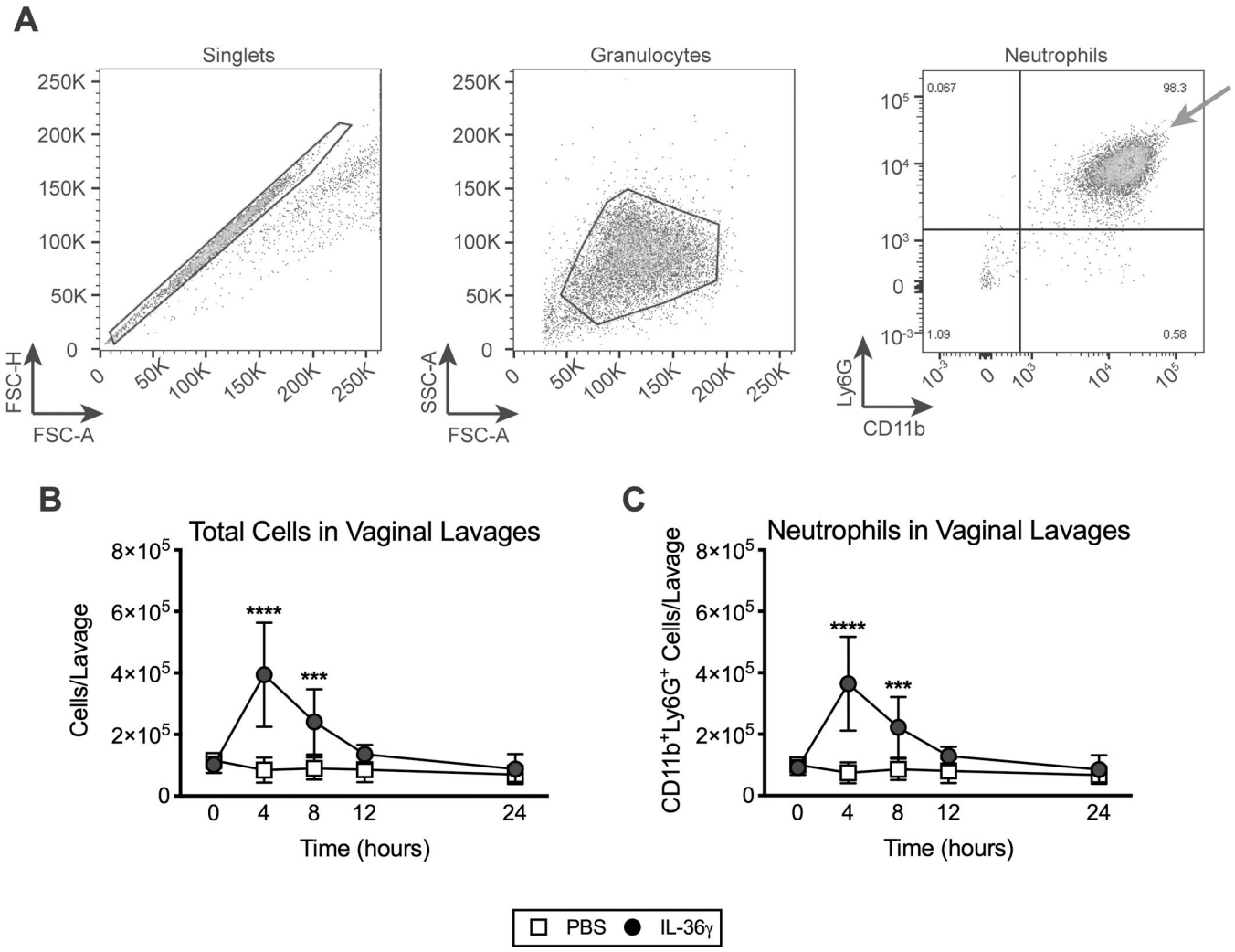

Figure 1. IL-36γ induces transient neutrophil recruitment to the vaginal microenvironment in WT mice.

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

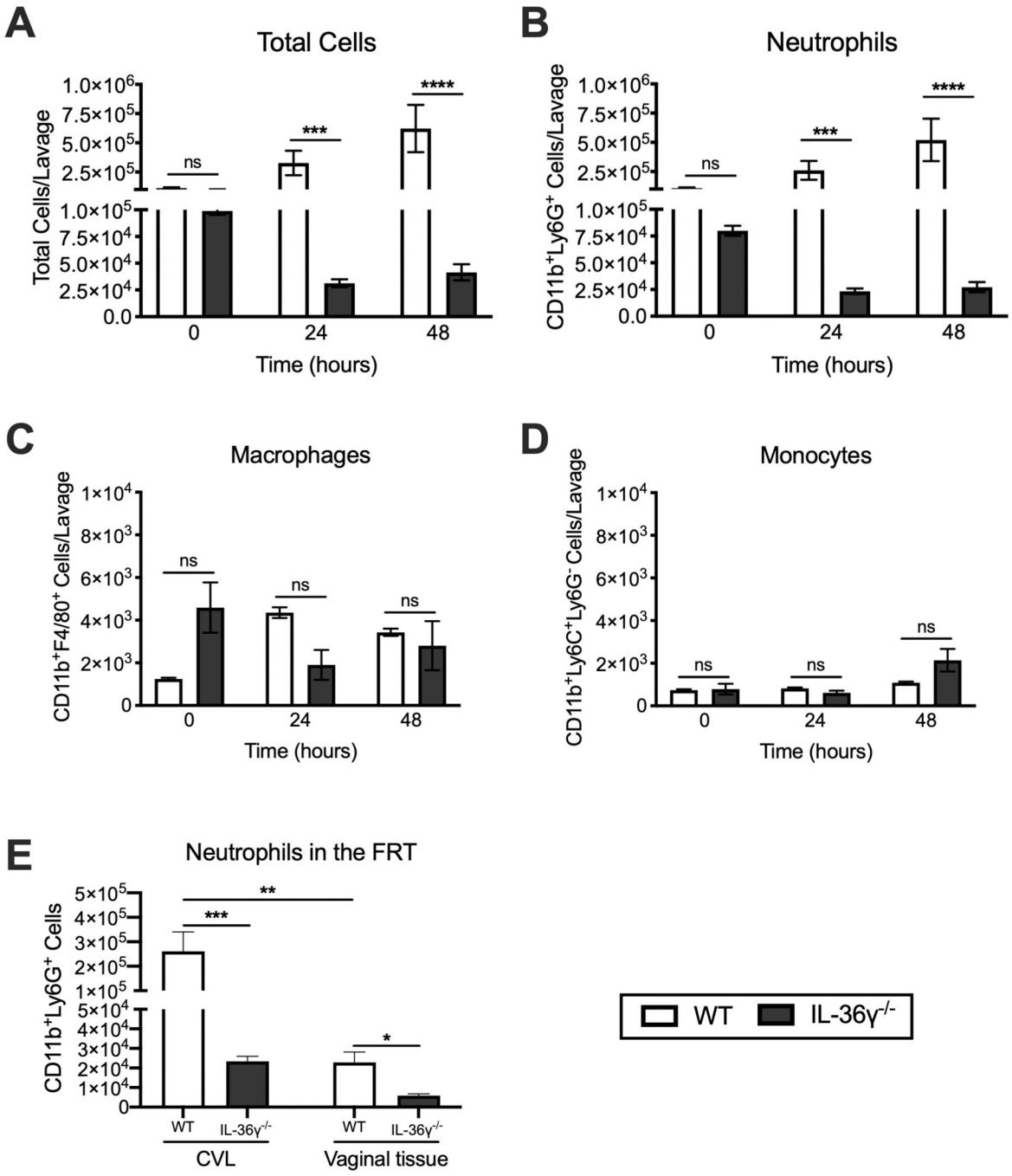

Figure 2. Neutrophil recruitment is significantly reduced in IL-36$\gamma^{-/-}$ mice after genital HSV-2 infection.

Flow cytometry analysis of immune cells in vaginal lavages. Female six- to eight-week-old DMPA conditioned WT and IL-36$\gamma^{-/-}$ 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-36$\gamma^{-/-}$ mice prior to infection, and at 24 and 48h post-infection. (**E**) Comparison of

neutrophil counts in cervicovaginal lavages (CVLs) and vaginal tissue in IL-36$\gamma^{-/-}$ 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.

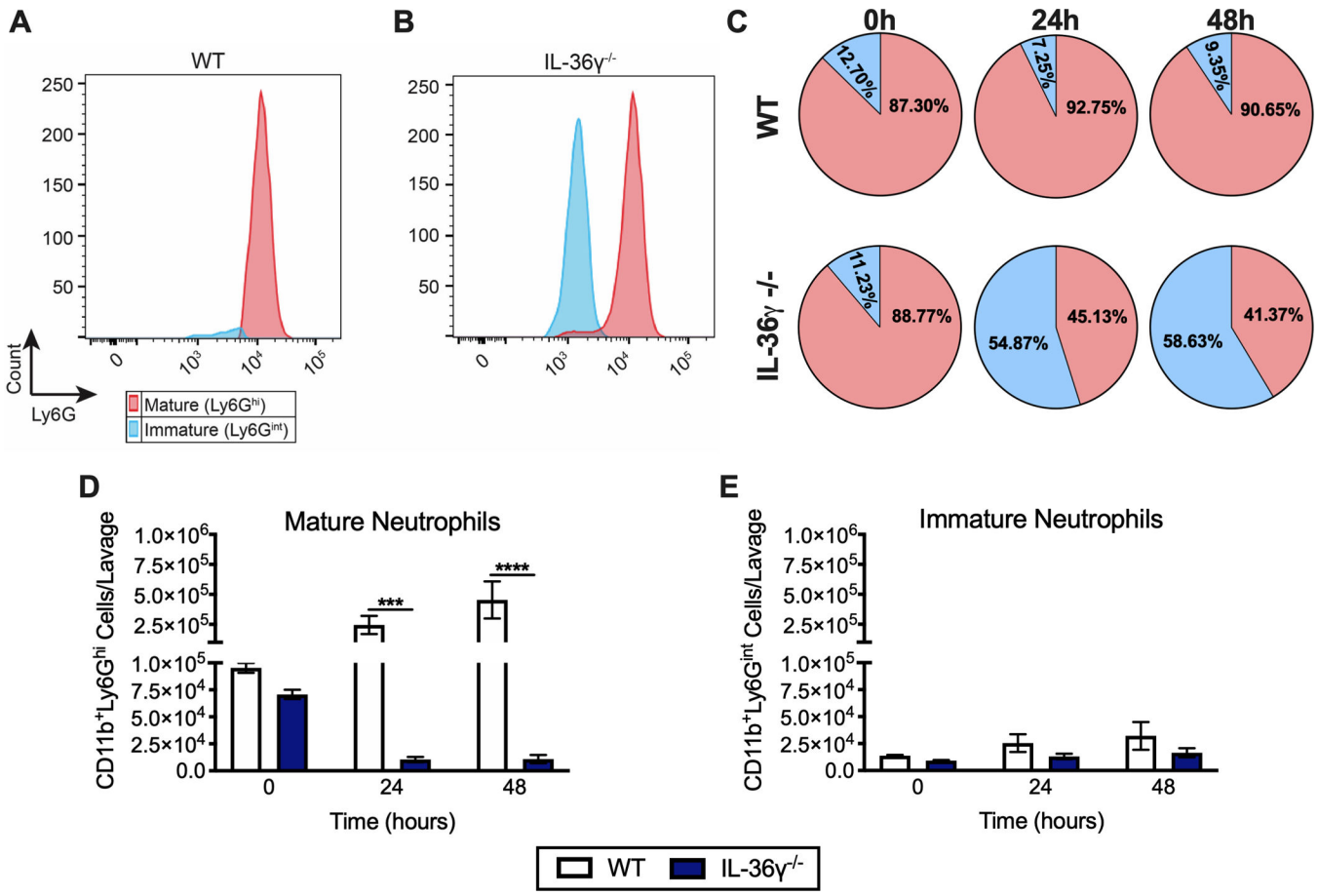

Figure 3. Recruitment of mature neutrophils to the vaginal microenvironment is impaired in HSV-2 challenged IL-36γ$^{-/-}$ mice.

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 histogram plots of 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. ***, $p < 0.001$; ****, $p < 0.0001$.

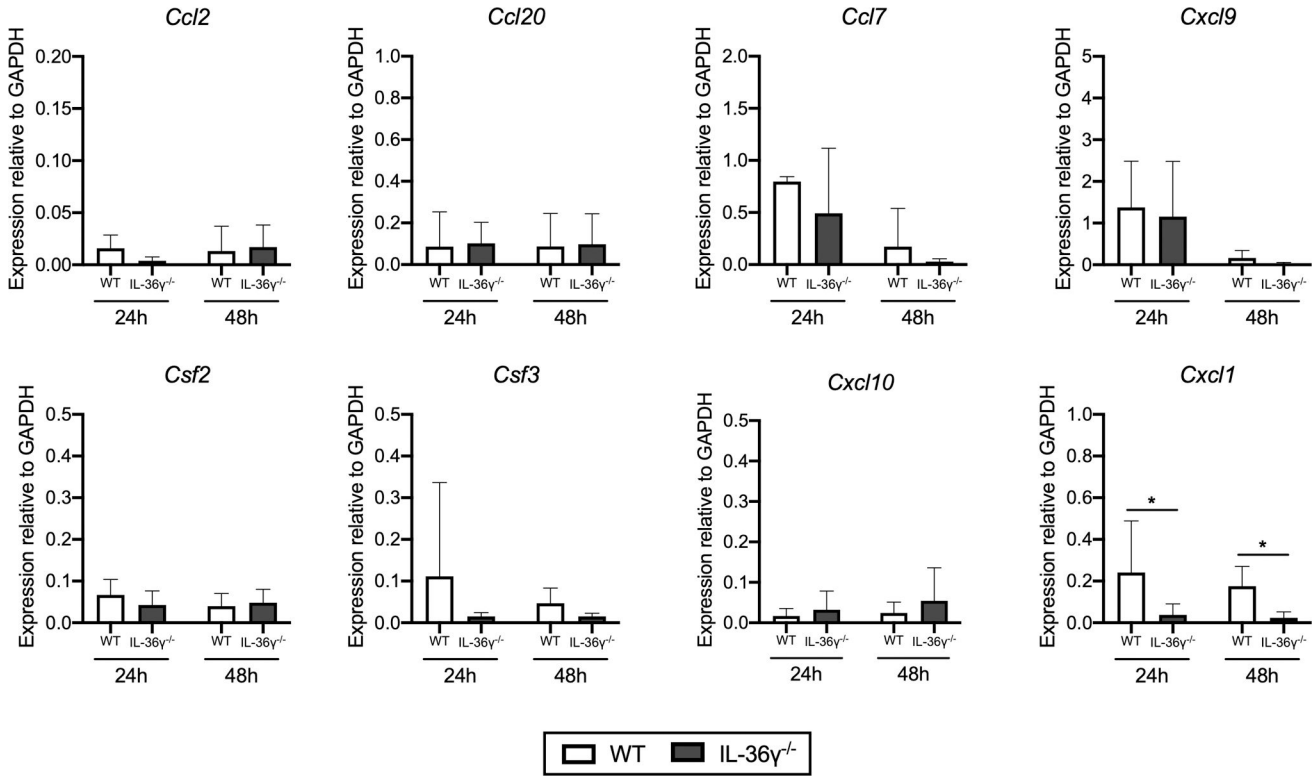

Figure 4. Cxcl1/KC expression is reduced in IL-36γ$^{-/-}$ vaginal tissue after HSV-2 challenge.

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. *, $p < 0.05$.

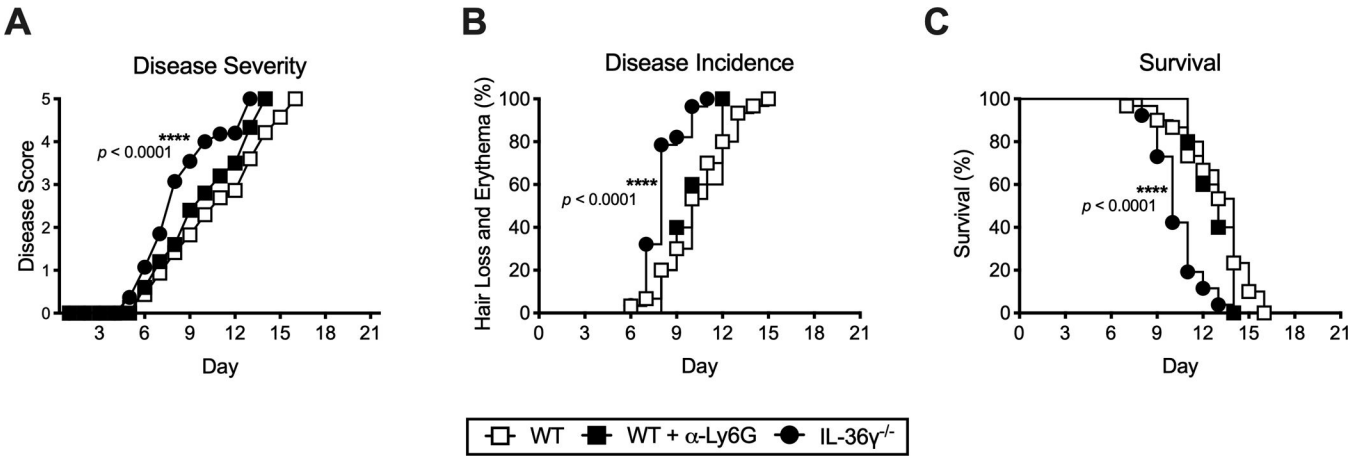

Figure 5. IL-36γ protects against genital HSV-2 infection in a neutrophil independent manner.

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**). ****, $p < 0.0001$.

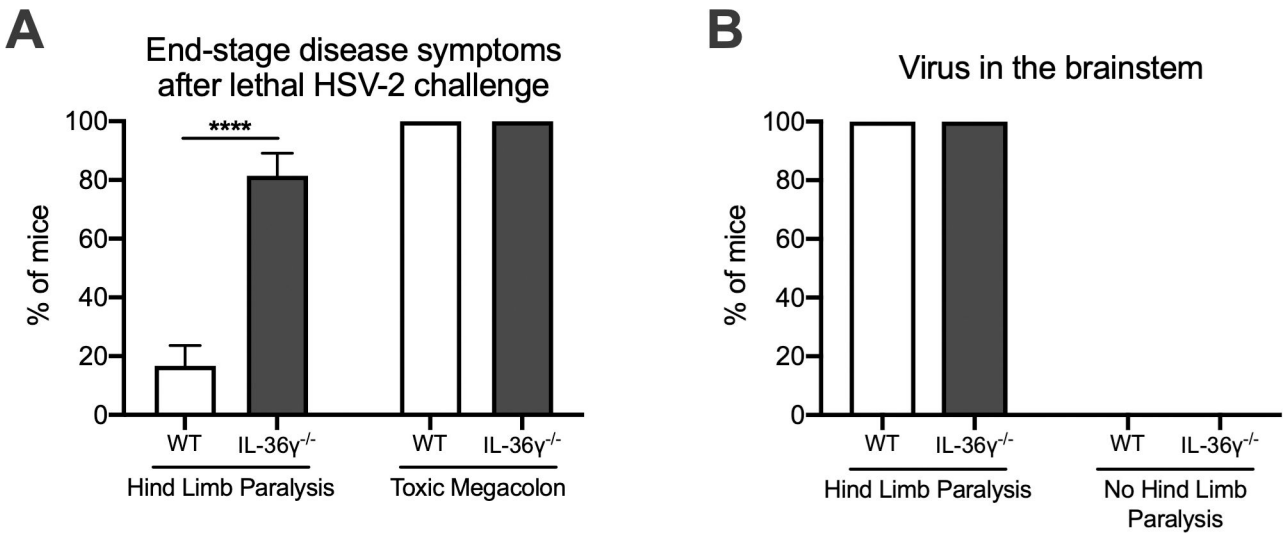

Figure 6. Increased incidence of hind limb paralysis and productive infection in the brainstem in IL-36γ$^{-/-}$ mice after genital HSV-2 infection.

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. ****, $p < 0.0001$.

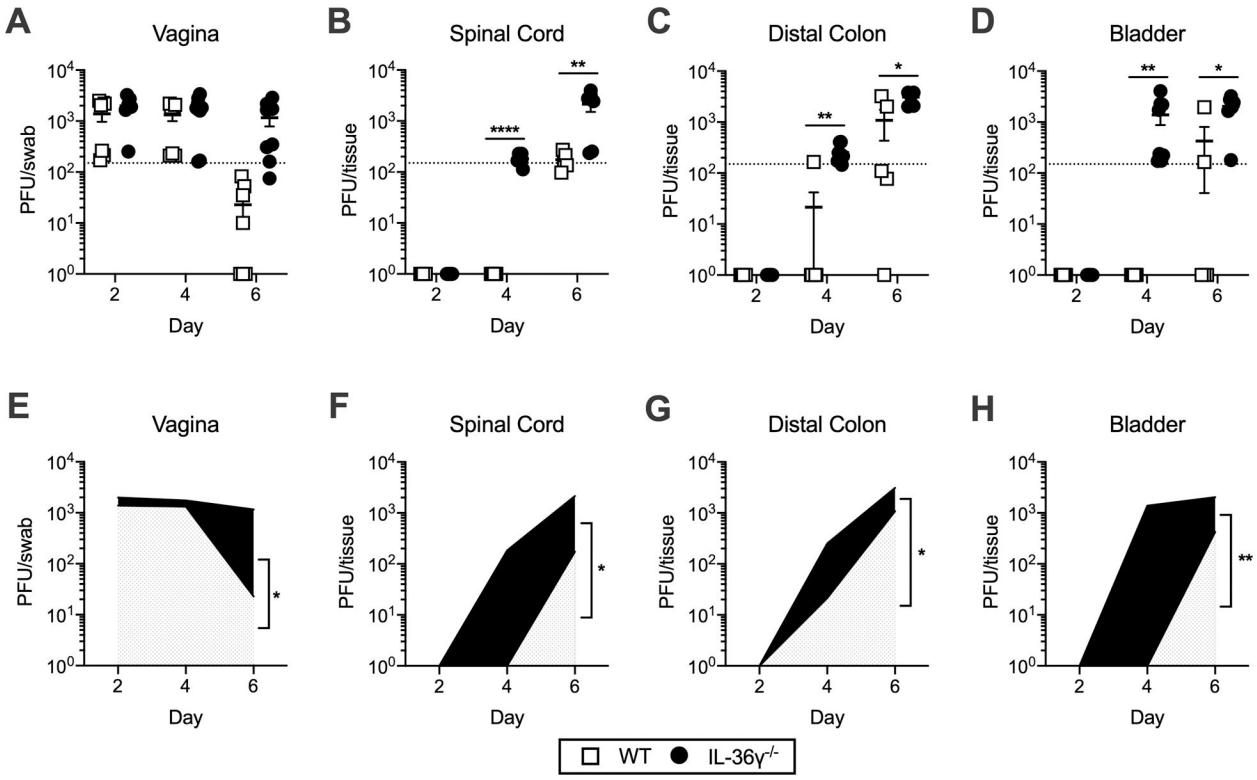

Figure 7. Rapid systemic spread of HSV-2 in IL-36γ$^{-/-}$ mice.

Analysis of viral titers (**A-D**) and kinetics of virus spread (**E-H**) in WT and IL-36γ$^{-/-}$ mice after lethal HSV-2 challenge. DMPA conditioned WT and IL-36γ$^{-/-}$ 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-36γ$^{-/-}$ 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**). *, $p < 0.05$; **, $p < 0.01$; **** $p < 0.0001$.

Table 1.

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