Herpes Simplex Virus Down-Regulates Secretory Leukocyte Protease Inhibitor:
A Novel Immune Evasion Mechanism

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Abstract

Secretory leukocyte protease inhibitor (SLPI), an anti-inflammatory mediator of mucosal immunity, inhibits HIV and HSV in cell culture. Epidemiological studies demonstrate that higher concentrations of SLPI in mucosal secretions are associated with a reduced risk of HIV transmission. The current studies were designed to test the hypothesis that HSV triggers a loss in SLPI to evade innate immunity, and that this response may contribute to the increased risk for HIV in the setting of HSV infection. Exposure of human cervical epithelial cells to HSV-1 or HSV-2, but not HIV or VSV, triggered a significant and sustained reduction in SLPI levels. The reduction persisted when cells were infected in the presence of acyclovir, but not following infection with ultraviolet (UV)-inactivated virus, indicating that viral gene expression, but not replication, is required. RT-PCR studies demonstrated that the loss in SLPI is mediated by down-regulation of gene expression. SLPI down-regulation was associated with activation of NF-κB signaling pathways and up-regulation of pro-inflammatory cytokines, consistent with the known inhibitor effects of SLPI on NF-κB pathways. The down-regulation mapped to viral early gene expression as variants impaired in expression of immediate early genes ICP4 or ICP0 failed to down-regulate SLPI or activate NF-κB. Together these results identify a novel role for HSV immediate early gene expression in regulating mucosal immune responses.
Introduction:

Prevention of genital herpes is a global health priority not only because of the morbidity associated with ulcerative disease itself, but also because of the risks for perinatal and sexual transmission as well as the epidemiological link between HSV infection and HIV acquisition and transmission (10, 11). Women and minorities bear a disproportionate burden of disease (54). Approximately 23% of women of child-bearing age in the U.S. are HSV-2 seropositive and the seroprevalence rate among non-Hispanic black women is over 40% (54). In developing countries, 60-80% of the population is infected with HSV-2, the serotype most commonly associated with genital herpes (29, 34). Epidemiological studies consistently demonstrate that HSV-2 infection increases the risk for HIV acquisition and transmission (41). An understanding of the molecular mechanisms underlying this link may facilitate the identification of novel preventative strategies to thwart the overlapping HIV and HSV epidemics.

Cervicovaginal secretions provide intrinsic protection and inhibit HSV infection in vitro by as much as 90% (26, 28). Multiple factors may contribute to this activity including the acidic pH of the healthy female genital tract and antimicrobial proteins such as mucins, defensins, lactoferrin, lysozyme, and secretory leukocyte protease inhibitor (SLPI) (22, 33, 48). To successfully establish infection, HSV must overcome these mucosal defenses. HSV has evolved several strategies to evade the host immune response targeting components of both innate and acquired immunity, including complement proteins, natural killer cells, MHC Class I or Class II molecules and antibody (24). For example, glycoproteins E and C (gE and gC) impair antibody and complement responses. gC inhibits complement activation by binding C3b, whereas gE
binds the IgG Fc domain, blocking Fc-mediated activities, including complement activation and antibody-dependent cellular cytotoxicity. HSV also expresses several viral genes that are associated with resistance to interferons, most notably ICP0 (30).

SLPI is a low molecular weight (11.7 kD) protein found abundantly in mucosal secretions including saliva, breast milk, seminal fluid, and the female genital tract. It has pronounced anti-inflammatory, antibacterial and antifungal activities (8, 13, 23, 45, 47). Importantly, SLPI possesses potent anti-HIV-1 activity at physiological concentrations found in saliva (25, 36, 51), which is presumed to contribute to the endogenous anti-HIV activity of oral secretions. A recent study found that brief exposure of human oral keratinocytes and epithelial cells to HIV-1 stimulated SLPI mRNA and protein production in the absence of direct infection, suggesting that up-regulation of SLPI by the virus may protect the oral cavity against HIV infection (25).

High SLPI concentrations are also found in seminal plasma, but may not provide protection because SLPI may be subject to partial proteolytic cleavage by prostate-specific antigen (39). SLPI binds to the membranes of human macrophages through the phospholipid-binding protein, annexin II, which acts as a cellular cofactor supporting macrophage HIV-1 infection (32). However, SLPI fails to bind cells under basic conditions, suggesting that the alkaline pH of semen may prevent seminal SLPI from binding to HIV target cells (37).

We recently demonstrated that SLPI also inhibits HSV infection in vitro by binding to epithelial cell surfaces and preventing viral infection, although the precise mechanisms have not yet been elucidated (26). While no epidemiological studies have evaluated the role played by SLPI in protecting against HSV, several studies demonstrate...
a protective role for SLPI in preventing HIV infection. Higher levels of SLPI in vaginal fluid correlated with a reduced rate of perinatal HIV transmission and higher salivary levels in infants were associated with reduced transmission through breast milk (17, 40). The paradigm being tested in the current studies is that HSV modifies expression of SLPI as an escape mechanism, which may facilitate HIV infection.
Materials and Methods:

Cells: The human cervical epithelial cell line, CaSki (CRL-1550), immortalized endocervical cells (Endo, CRL-2615) (18), monkey kidney epithelial cells (Vero) and human osteosarcoma cell lines (U-2 OS) were obtained from the American Type Culture Collection (ATCC). Endocervical cells were propagated in Keratinocyte-Serum Free medium (GIBCO-BRL 17005-042) with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, and additional calcium chloride (final concentration 0.4 mM). U-2 OS cells were propagated in McCoy’s 5a Medium with 10% fetal bovine serum (FBS). FO6, a derivative Vero cell line expressing ICP4, ICP27, and ICP0 under their own promoters (42) and Vero 2.2, a derivative of Vero cell line expressing ICP27 under its own promoter (44) were maintained in DMEM containing 5% FBS. All other cells were grown and maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FBS.

Viral strains: The wild-type strains used in these studies were HSV-1(KOS, HSV-1(F), HSV-1(17+)) and HSV-2(G). The ICP0 deficient virus (7134) and its repair, (7134R), which were derived from HSV-1(KOS), were a gift from P. Schaffer (Harvard Medical School Boston, MA) (5). The viral strains dl1403, an ICP0 deletion virus, FXE, which expresses a form of ICP0 lacking the RING finger, and M1 an ICP0 mutant defective in ubiquitin specific protein USP7 binding M1 and their respective repairs were derived from HSV-1(17+) and were gifts from R. Everett (MRC Virology Unit, Institute of Virology, Scotland, United Kingdom) (3, 4, 16). The HSV-2(333) vhs deletion mutant (333d41) virus was a gift from D. Leib (Washington University School of Medicine, St. Louis, MO) (43). The KOS derivative vBSΔ27 (44) which contains a replacement of the
a27 gene with the *Escherichia coli* lacZ gene was propagated and titered on Vero 2.2 cells. Strain 17 derivative HSV-1(CgalΔ3), which contains the *E. coli* lacZ gene under the HCMV IE promoter inserted into an intergenic site in the US portion of a mutant genome deleted for 3.6 kb of the ICP4 coding region (27); this virus was propagated and titers were determined on FO6 cells. The strain F derivative HSV-1(R7802) (obtained from Bernard Roizman, University of Chicago), which lacks the coding domain of ICP22 (27) was grown and titered on Vero cells as previously described (42).

Vesicular stomatitis virus (VSV) (Indiana) was a gift from P. Palese, Mount Sinai School of Medicine, New York, NY). The laboratory-adapted HIV-1 strain HIV-1_BaL (CCR5-utilizing strain) was grown in PM-1 cells and stored at -180°C after filtration through 0.2 µm filters (Millipore, MA). All herpes viruses were propagated on Vero cells, except for the ICP0 deletion viruses, which were propagated on U2-OS cells. Viruses were titrated on CaSki cells. HSV-2(G) was inactivated by exposure to UV light (at a distance of 10 cm from the light source for 7 minutes).

**Preparation of infected cell lysates, gel electrophoresis and immunoblotting:** Infected cell lysates were prepared 24 hour pi and proteins separated in 8 % polyacrylamide gel and transferred to nitrocellulose membranes for immunoblotting as previously described (9). The following primary antibodies were used to detect viral proteins: (i) 1113, mouse anti-ICP27 monoclonal antibody (Goodwin Institute for Cancer Research, Plantation, FL.); (ii) 1114, mouse anti-ICP4 monoclonal antibody (Goodwin); (iii) 1112, mouse anti-ICP0 monoclonal antibody (Goodwin); (iv) RGST22, rabbit polyclonal antibody directed against the ICP22 protein (2); and mAb to detect B-actin (AC-15, Sigma). Secondary goat anti-rabbit (Bio-Rad) and goat anti-mouse antibodies conjugated with alkaline.
phosphatase were purchased from (Calbiochem). The blots were scanned and analyzed using ImageJ (Bethesda, MD).

**Impact of viral exposure on SLPI:** Cells were inoculated with different strains of HSV, VSV, or HIV-1_BaL at the indicated moi (pfu/cell for HSV and VSV and 50 ng p24 for HIV) or mock-infected with PBS in duplicate at 37°C. After a 1-2 h adsorption period, the inoculum was removed and the cells were washed and overlaid with serum free media (DMEM) in the absence or presence of acyclovir (100µg/ml) (American Pharmaceutical Partners, Schaumburg, IL). The cell culture supernatants were collected at various times post-infection (pi). In some experiments, cell lysates were prepared by incubating the cells with lysis buffer for 10 minutes (2% Tris base, 0.3% NaCl, 0.5% NP-40, 0.05% deoxycholate). Protease inhibitors cocktail was added to culture supernatants and lysates prior to storage at -20°C. As a positive control, cells were treated with poly (I:C) (25µg/ml) for (InvivoGen, San Diego, CA)

**SLPI ELISA:** Frozen samples were thawed and diluted 1:10 in serum free media and SLPI levels were detected by Quantikine Human SLPI Immunoassay (R&D Systems, Minneapolis, MN); sensitivity 25 pg/ml. The optical density reading (450 nm) was obtained with multimode detector (Beckman Coulter DTX 880). Standard curves were derived for each individual assay.

**Transcription factor assay:** Nuclear extracts were prepared using the Active Motif's nuclear extract kit (Carlsbad, CA). Nuclear protein was quantified by the Quick Start Bradford Protein Assay (Bio-Rad), and nuclear extracts were stored at -80°C. The samples were analyzed for NF-kB (p65) levels using the Active Motif TransAM NF-κB
Kit. Chemiluminescence was determined by a multimode detector (Beckman Coulter DTX 880).

**Total RNA extraction and real-time quantitative reverse transcriptase PCR (qRT-PCR):** Total RNA was extracted using Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). RNA (200ng) was reverse transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystem, Foster City, CA). RT-PCR amplification was performed in duplicate using an ABI PRISM 7000 detection system and analyzed using sequence detector software. Commercially available probes for human SLPI (Hs00268204_m1), IL-6 (Hs00985639_m1) and the ribosomal large protein subunit (RPLPO) (4333761F) housekeeping gene were obtained from Applied Biosystems. Quantification was normalized against the number of RPLO transcripts in the same RNA extracts. Standards for each transcript gave a linear amplification curve over at least 4 logs of template concentrations.

**Statistical analysis:** GraphPad Prism (version 4; GraphPad Software) was used for statistical analysis. Results were compared using Student’s t-test and differences were considered significant with p values < 0.05.
RESULTS:

HSV-1 and HSV-2 trigger a reduction in SLPI independent of viral replication. Cells were mock-infected or infected with HSV-2(G) or HSV-1(F) in the absence or presence of acyclovir and the SLPI concentration in culture supernatants measured 6, 12 or 24 h pi. Mock-infected CaSki and immortalized End1/E6E7 cells released over 10,000 pg/ml of SLPI into the media within 24 h (Fig 1A, B). Infection of both cell types with HSV-1 or HSV-2 led to a significant and sustained reduction in SLPI, which was detected as early as 6 h pi. (p< 0.01). The reduction in SLPI increased following exposure to higher inoculums (Fig. 1C), but was independent of viral replication as comparable reductions in SLPI levels were observed when cells were infected in the presence of 100µg/mL of acyclovir (Fig. 1A, B). This concentration of acyclovir consistently inhibits viral replication as evidenced by a significant loss in viral yields and a reduction in plaque formation in all of the cell types studied (data not shown). Similar down-modulation of SLPI was observed with primary clinical isolates and following infection of CaCo-2 cells (colonic epithelial cell line) (data not shown).

To examine whether the loss in SLPI was specific for HSV, CaSki cells were also infected with VSV or exposed to 50 ng p24 of HIV-1_{BaL}, a concentration that binds to CaSki cells (Mesquita and Herold, work in progress). Previous studies have demonstrated that exposure of human oral epithelial cells or keratinocytes to HIV-1_{BaL} resulted in an increase in SLPI production, although the effects on genital tract epithelial cells were not examined (25). As an additional control, the cells were treated with the TLR-3 agonist, Poly (I:C), which has been shown to augment SLPI production in the genital tract (1). VSV had little or no effect on SLPI production, whereas both HIV (p =
0.047) and Poly I:C (p = 0.005) stimulated a modest increase in SLPI, indicating that the loss in SLPI following HSV infection is specific (Fig. 1D).

**Reduction in SLPI is mediated by transcriptional down-regulation.** HSV could trigger a reduction in SLPI secretion either by blocking its release, triggering its degradation or down-regulating gene expression. First, to address the possibility that the reduction in SLPI reflects a block in secretion, the concentration of SLPI in cell lysates and culture supernatants following HSV or mock infection was compared. HSV triggered a comparable reduction in SLPI in cell lysates and culture supernatants, indicating that HSV does not prevent the protein from being secreted (Fig. 2).

HSV induces rapid destabilization and degradation of host cell mRNA through the activities of the virion host shutoff (vhs) protein (43). To determine whether the reduction in SLPI is mediated by vhs, we took advantage of the HSV-2 vhs deletion mutant, 333d41. A comparable reduction in SLPI was observed following infection with the vhs deletion virus, indicating that the loss of SLPI is independent of vhs activity (Fig. 3).

To determine if the reduction in SLPI was transcriptionally regulated, RNA was harvested from cell lysates 4, 8 and 24 h pi and analyzed for SLPI gene expression by quantitative RT-PCR. Infection with HSV-2(G) resulted in at least a one-log reduction in SLPI gene expression 24 h pi relative to mock-exposed cells. Notably, no significant down-regulation was observed following infection with UV-inactivated virus, suggesting that down-regulation requires viral gene expression (Fig. 4A). Conversely, infection with
HSV-2(G), but not UV-inactivated virus, resulted in rapid and significant up-regulation of the pro-inflammatory cytokine, IL-6, which peaked 8 h pi (Fig. 4B).

Immediate early gene expression is required for down-regulation of SLPI. The observation that SLPI down-regulation requires viral gene expression, but not replication, suggests that one or more of the immediate early genes may play a pivotal role in triggering the loss of SLPI. We initially focused on ICP0 because of its multifunctional roles. ICP0 is a phosphoprotein that transactivates viral and cellular proteins, although no specific promoter sequence has been defined (15). Additionally, ICP0 stimulates the degradation of a number of host proteins, in part because of its E3 ubiquitin ligase activity and its interactions with the cellular ubiquitin-specific protease enzyme, USP7 (4, 50). To examine the impact of ICP0 on SLPI expression, we infected cells with dl1403, FXE, and M1 viruses and their respective repairs. These studies were conducted at a moi of 1 pfu/cell (based on the titer of each virus on CaSki cells) because ICP0 deletion viruses exhibit delayed progression beyond the immediate early gene expression at low moi, which is overcome at higher moi (3, 16). We confirmed that an equivalent number of viral capsids entered the cells, by preparing nuclear extracts and analyzing for VP16 transport to the nuclear pore (data not shown). No reduction in SLPI protein in culture supernatants harvested at 24 h was observed following infection with any of ICP0 deletion viruses indicating that ICP0 and both the FXE and USP7 encoding domains contribute to the SLPI down-modulation (Fig. 5A). The phenotype was restored, at least partially, following infection with the respective repair viruses (Fig. 5). Similar results were obtained by RT-PCR using RNA isolated from the dl1403 and FXE viruses, their
respective repairs, and the parental virus, HSV-1(17+) (Fig. 5B). Notably, the down-regulation of gene expression was delayed relative to results obtained with HSV-2(G) (Fig. 4A).

Because deletion of ICP0 impacts the expression of other IE genes, we next examined a panel of IE mutant viruses. CaSki cells were mock-infected or infected with each virus at a moi ~1 pfu/cell (based on titer on complementing F-06 cells) and 24 h pi, the concentration of SLPI in culture supernatants was determined by ELISA. Whole cell lysates of infected cells were prepared in parallel and viral gene expression evaluated by preparing Western blots and probing for each of the IE proteins and β-actin as a control. No down-modulation of SLPI was observed following infection with any of the IE mutant variants, whereas each of the parental viruses triggered a significant loss in SLPI (Fig. 6A). Analysis of the Western blots suggests that down-modulation of SLPI requires expression of both full-length ICP0 (with intact ring domain) and ICP4. This follows from the observation that the ICP4 deletion virus, CgalΔ3 expresses each of the other IE genes including ICP0, whereas the FXE variant expresses all of the other IE genes including ICP4, but a mutated form of ICP0. Thus, neither protein alone is sufficient to trigger SLPI down regulation (Fig. 6B).

**Down-modulation of SLPI is associated with NF-κB activation** Prior studies have demonstrated that SLPI inhibits LPS-induced NF-κB activation in monocyctic cells by blocking the degradation of IκBα and by competitively binding to the NF-κB consensus binding domain (46). This suggests that the viral induced down-regulation of SLPI might be linked to NF-κB activation. Thus, we would predict that infection with HSV viruses that down-regulate SLPI would be associated with NF-κB activation and conversely, the
IE mutants viruses, that fail to down-regulate SLPI would be impaired in NF-κB activation. To explore this, we compared NF-κB activation in CaSki cells following infection with the IE viral deletion variant viruses 8-h pi. HIV-1_{Bal} was also included for comparison. Activated Jurkat cells served as a positive control in the assay. Each of the parental HSV viruses triggered significant activation of NF-κB, whereas little or no NF-κB activation was observed following exposure of the cells to viral variants lacking ICP0, ICP4, ICP27 and ICP22 or to HIV-1_{Bal} (Fig. 7).
Discussion

These studies demonstrate that HSV-1 and HSV-2 down-regulate SLPI, which may serve as a novel immune evasion strategy. Importantly, down-modulation does not require viral replication, as evidenced by the findings that down-modulation occurs within 6 hours following infection and persists in the presence of acyclovir. However, down-modulation does require expression of both ICP4 and wild-type ICP0, as evidenced by results obtained with IE viral variants and UV-inactivated virus.

The observation that both of these IE proteins are required to trigger the down-modulation of SLPI is consistent with their known functional interactions. Both IE proteins play critical roles as transcriptional activators of viral gene expression and the ability of ICP0 to transactivate promoters is increased synergistically in the presence of ICP4 (35). Their vital role in regulating HSV infection and reactivation from latency is highlighted by a recent study, which found that LAT encodes for several microRNA precursors (miRNAs) and that one of these miRNAs, miR-H2-3p, is transcribed in an antisense orientation to ICP0 and inhibits its expression. Notably, a second miRNA (which derives from a transcript distinct from LAT) inhibits expression of ICP4 (49).

ICP0 has previously been shown to play a major role in immune evasion by overcoming the antiviral interferon (IFN) response. The ability of ICP0 to transactivate cellular proteins may contribute to its capacity to overcome the IFN-induced block to viral transcription (38). ICP0 blocks interferon regulatory factor 3 (IRF-3) and IRF-7 mediated activation of IFN-stimulated genes through the activities of the RING finger domain (31). Additionally, ICP0 stimulates the degradation of a number of host proteins,
in part because of its E3 ubiquitin ligase activity as well as its interactions with the cellular ubiquitin-specific protease enzyme, USP7 (4, 50). In the current studies, we found that the primary mechanism by which HSV reduces SLPI is down-regulation of mRNA, although viral induced degradation of SLPI protein may also contribute. Further studies are required to determine whether ICP0 and ICP4 directly or indirectly triggers the down-regulation and the precise mechanism underlying this response. Notably, the loss of SLPI down-modulation was associated with reduced ability to activate NF-κB at early times pi (8 hours).

There are several potential consequences of down-regulating SLPI. First, we previously demonstrated that recombinant SLPI interacts with human epithelial cells to inhibit HSV infection in vitro (26). Down-regulation could overcome the anti-viral activity of SLPI in mucosal secretions, thus providing a mechanism for immune evasion. A clinical study is currently ongoing to examine the concentration of SLPI in genital tract secretions in women during an acute HSV outbreak. Second, down-modulation of SLPI could promote the activation of NF-κB by releasing a negative regulator, thus further facilitating HSV infection. The ability of HSV to activate NF-κB has been well documented (2, 20) and interference with NF-κB activation, for example following expression of a dominant-negative IκBα, resulted in a reduction in virus yield. This has been attributed, in part to the role NF-κB may play in preventing viral-induced apoptosis (20). Thus it has been proposed that persistent NF-κB activation, rather than being a host response to virus infection, may play a positive role in promoting efficient virus replication.
Additionally, the HSV-induced down-regulation of SLPI could also contribute to epidemiological observations of an increased risk for HIV acquisition among HSV-2 seropositive individuals (19). SLPI inhibits HIV infection of macrophages and higher concentrations of SLPI in mucosal fluids are associated with a reduced risk of infection (17, 40). Thus, the reduction in SLPI in the setting of HSV, even in the presence of acyclovir, could promote HIV acquisition. The down-regulation of SLPI could also contribute to the increase in HIV viral loads in the genital tract observed during periods of HSV reactivation in co-infected individuals (21). We found that exposure of the chronically HIV-infected monocytic cell line, U1, to wild-type HSV, but not the ICP4 deletion virus, resulted in enhanced HIV replication with a significant increase in p24 production (Mesquita and Herold, work in progress). These results could be explained by NF-κB-mediated activation of the HIV long-terminal repeat (LTR). Whether SLPI down-regulation contributes to this response remains to be determined. It is interesting to speculate whether the down-modulation of SLPI or other changes in the mucosal environment triggered by reactivating HSV, even in the absence of viral replication, played a role in the failure of oral acyclovir suppression to reduce the risk of HIV infection in the recently completed clinical trials among high risk HSV-infected, HIV-negative individuals (6,7,52).

The observation that HSV down-modulates SLPI is unusual as most other microbes induce SLPI and other antimicrobial peptides. VSV induced no change in SLPI and, consistent with prior studies, we found that HIV exposure resulted in a modest increase in SLPI levels. Notably, the response by the cervical cells was less than that previously reported for oral epithelial cells. In the previous study, the authors found that
exposure of oral epithelial cells to HIV resulted in a three-fold increase in SLPI production and speculated that this response may contribute to the poor oral transmission of HIV (25). Possibly, the more modest increase in SLPI following exposure of cervical epithelial cells to HIV-1 contributes to the greater risk for genital compared to oral mucosal transmission.

Increases in SLPI have also been observed in response to other pathogens. For example, Mycobacterium tuberculosis (M. tuberculosis) has also been shown to increase SLPI production by macrophages. Exposure of murine peritoneal macrophages to M. tuberculosis or aerosolized infection of mice with M. tuberculosis triggers an increase in SLPI. Notably, macrophages from TLR2-/- mice are incapable of inducing this response, suggesting a role for TLR2 dependent pathways in triggering the SLPI response (12).

Similar to results obtained here for HSV, several other pathogens appear to have evolved strategies to escape the antimicrobial effects of SLPI. Helicobacter pylori triggers a loss in SLPI in cell cultures and antral biopsies of H. pylori-positive subjects show reduced SLPI expression (53). The H. pylori-induced decrease in SLPI could not be explained by a transcriptional down-modulation and appeared to be regulated post-translationally. Additionally, cysteine proteases of Trichomonas vaginalis have been demonstrated to degrade SLPI, which has been suggested to contribute to the increased risk of HIV in the setting of trichomonas infection (14).

In conclusion, these studies describe a novel mechanism by which HSV may interfere with innate mucosal immunity and identify yet another role for HSV in immune evasion. Defining the precise mechanisms by which ICP4 and ICPO trigger down-regulation of this mediator of mucosal host defense may promote the development of
strategies to prevent this mucosal immune response, which could foster prevention of both HSV and HIV.
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Figure Legends:

**Figure 1. HSV triggers reduction in SLPI.** The concentration of SLPI in culture media was determined by ELISA following mock infection (PBS) or infection with HSV-2(G) or HSV-1(F) (moi 5 pfu/cell) in the absence of presence of acyclovir; (A) CaSki and (B) End1/E6E7 cells. (C) To determine if the effects were dependent on the inoculum, CaSki cells were infected with 0.5, 1 or 5 pfu/cell HSV-2(G). Results are presented as concentration of SLPI (mean ± SD pg/ml) obtained from at least 3 independent experiments conducted in duplicate. (D) To examine the specificity of the response, CaSki cells were infected with HSV-2(G), VSV (Indiana) (5 pfu/cell) or HIV-1 BaL (50 ng p24) or treated with the TLR3 agonist, Poly(I:C) (25 µg/ml)). Results are presented as the percentage change in SLPI concentration relative to PBS-treated cells and are the mean ± SD from 2 independent experiments in duplicate. The differences between treated and mock-exposed cells were compared by unpaired t-tests; p<0.05 was considered to be significant.

**Figure 2. HSV triggers reduction in intracellular and secreted SLPI.** CaSki cells were mock- infected or infected with HSV-2(G) and culture media and cell lysates harvested at 6, 12, 24 hours pi and analyzed for SLPI by ELISA. Results are means ± SD from 2 independent experiments conducted in duplicate. As indicated by the asterisk, p < 0.05 was considered to be significant.
**Figure 3.** *HSV retains ability to reduce SLPI following infection with vhs deletion virus.*

CaSki cells were mock-infected or infected with HSV-2(G) or the vhs-deficient virus (moi 5 pfu/cell) and culture media harvested at the indicated times pi and analyzed for SLPI concentration by ELISA. Results are presented as means ± SD obtained from 2 independent experiments; asterisks indicate p<0.05 relative to mock-exposed cells.

**Figure 4.** *SLPI gene expression is down-regulated following infection with HSV-2(G), but not UV-inactivated virus.* CaSki cells were infected with either HSV-2(G) or UV-inactivated virus and the expression of SLPI (A) or IL-6 (B) analyzed by qRT-PCR. Results are presented as the log change in gene expression relative to the expression of the housekeeping gene, RPLPO, and are means of duplicate wells obtained from a representative experiment. Similar results were obtained in at least 2 independent experiments; asterisks indicate p<0.01 relative to mock-exposed cells.

**Figure 5.** *Down-regulation of SLPI requires expression of wild-type ICP0.* CaSki cells were mock-treated or infected with the indicated viruses (moi 1 pfu/cell based on viral titer on CaSki cells). Culture supernatants (A) were collected 24 h pi and analyzed for SLPI protein levels by ELISA. In parallel studies, cell lysates were harvested 4, 8 and 24 h pi and SLPI gene expression determined by qRT-PCR. Results are presented as the percentage change in SLPI concentration relative to PBS-treated cells (A) and as the log change in SLPI gene expression relative to the expression of RPLPO (B) and are means ± SD obtained from duplicate wells and are representative of at least two independent
experiments; asterisks indicate a significant change in SLPI protein (P<0.01) and gene expression (p<0.05).

**Figure 6.** *Neither ICP0 nor ICP4 alone is sufficient to down-regulate SLPI.* CaSki cells were mock-treated or infected with the indicated viruses (moi 1 pfu/cell) and 24 h pi, the culture media was collected and analyzed by ELISA for SLPI protein levels (A). Total cell proteins were separated by SDS-PAGE, and the levels of indicated IE genes were detected by Western blotting (B). The quantification was determined using ImageJ software. The numerical values represent the intensity density of bands relative to β-actin.

**Figure 7.** *IE gene expression is required for activation of NF-κB.* CaSki cells were exposed to the indicated viruses and 8-h pi, nuclear extracts were prepared and analyzed for NF-κB p65. Activated Jurkat cells are included as a positive control. Results are presented as percentage increase in nuclear p65 relative to mock-treated cells and are means of two independent experiments. The asterisks indicate significant increase in nuclear p65 (p<0.05).
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Fig. 1A
Fig. 1C
Fig. 1D
Fig. 2
Fig. 3
Fig. 4A

Log change in SLPI gene expression

time pi (hours)

[Graph with bars for HSV-2(G) and HSV-2(G)-uv at 4, 8, and 24 time points, indicating significant differences at 24 hours. Asterisks denote statistical significance.]
Fig. 4B

 HSV-2(G)
 HSV-2(G)-uv

 Log change in IL-6 gene expression vs. time pi (hours)
SLPI (% control of mock treated cells)

Fig. 5A
Fig. 5B
SLPI (% control of mock treated cells)

Fig. 6A
Fig. 6B
Fig. 7