Herpes simplex virus downregulation of secretory leukocyte protease inhibitor enhances human papillomavirus type 16 infection

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Herpes simplex virus (HSV) was originally implicated in the aetiology of cervical cancer, and although high-risk human papillomavirus (HPV) is now the accepted causative agent, the epidemiological link between HSV and HPV-associated cancers persists. The annexin A2 heterotetramer (A2t) has been shown to mediate infectious HPV type 16 (HPV16) uptake by human keratinocytes, and secretory leukocyte protease inhibitor (SLPI), an endogenous A2t ligand, inhibits HPV16 uptake and infection. Interestingly, HSV infection induces a sustained downregulation of SLPI in epithelial cells, which we hypothesized promotes HPV16 infection through A2t. Here, we show that in vitro infection of human keratinocytes with HSV-1 or HSV-2, but not with an HSV-1 ICP4 deletion mutant that does not downregulate SLPI, leads to a >70% reduction of SLPI mRNA and a >60% decrease in secreted SLPI protein. Consequently, we observed a significant increase in the uptake of HPV16 virus-like particles and gene transduction by HPV16 pseudovirions (two- and 2.5-fold, respectively) in HSV-1- and HSV-2-infected human keratinocyte cell cultures compared with uninfected cells, whereas exogenously added SLPI reversed this effect. Using a SiMPull (single-molecule pulldown) assay, we demonstrated that endogenously secreted SLPI interacts with A2t on epithelial cells in an autocrine/paracrine manner. These results suggested that ongoing HSV infection and resultant downregulation of local levels of SLPI may impart a greater susceptibility for keratinocytes to HPV16 infection through the host cell receptor A2t, providing a mechanism that may, in part, provide an explanation for the aetiological link between HSV and HPV-associated cancers.

INTRODUCTION

Until the late 1970s it was believed that the aetiological agent in both cervical and oral cancers was herpes simplex virus (HSV) (Shillitoe & Silverman, 1979; Smith et al., 1979). Despite the well-accepted causal relationship between persistent high-risk human papillomavirus (hrHPV) infection and cervical cancer, and a continuously rising association between hrHPV and oropharyngeal cancers (Bosch et al., 1995; Hammarstedt et al., 2006; Kreimer et al., 2005), a link between HSV and HPV still remains. For example, persistent hrHPV infections are associated with the development of >99% of cervical cancers and ~25% of head and neck squamous cell carcinomas (HNSCCs) (Mehanna et al., 2013), whilst HSV-2 infections have been identified as cofactors in cervical cancer. Specifically, it has been reported that there is a threefold increase in the risk of developing cervical cancer in patients who are seropositive for hrHPV and HSV-2 versus hrHPV alone (Smith et al., 2002). Additionally, individuals with evidence of HPV infection who are seropositive for HSV-1 have a twofold increased risk of developing oral squamous cell cancer compared with HSV-1-seronegative individuals with similar HPV exposure. These observations, amongst others, suggest that HSV and HPV may act synergistically in cancer development (Paba et al., 2008; Starr et al., 2001; Zhao et al., 2012).
Of all the hrHPV genotypes, HPV16 is by far the most common, accounting for ~50% of cervical cancers and an estimated 90% of HPV-related HNSCCs (Chen et al., 2005; Raff et al., 2013; Ragin & Taioli, 2007). It is interesting to note that in the USA, the number of HPV-positive oropharyngeal cancers is expected to rise and is predicted to surpass the annual number of cervical cancers by the year 2020 (Chaturvedi et al., 2011). Given that current vaccination rates against HPV within the USA for young women and men are only 37.6 and 13.6%, respectively (Stokley et al., 2014), it is clear that the identification of common risk factors that can enhance the acquisition rate of hrHPV is important in order to promote prevention programs aimed at high-risk individuals.

HSV-1 and -2, and HPV16 are dsDNA viruses that establish infection within the epithelial cells of mucous membranes through sexual activity. Whilst the viral uptake of HSV into target cells is well defined (Spear, 2004), cellular entry events of HPV16 are still under investigation. The current model for HPV16 entry involves the L1 major capsid protein binding to heparan sulfate proteoglycans (HSPGs) at or near the basement membrane followed by interactions with cyclophilins, furin, α6β1/4 integrins, growth factors, growth factor receptors and tetraspanins (Raff et al., 2013). After these initial cell surface interactions, conformational changes result in increased exposure of the N terminus of the HPV16 L2 minor capsid protein and interaction of the virion with the S100A10 subunit of the annexin A2 heterotetramer (A2t), which facilitates HPV16 internalization (Woodham et al., 2012). HPV16 is then co-internalized with A2t, which mediates intracellular trafficking required for nuclear translocation of viral DNA (Dziduszko & Ozbun, 2013). The interaction between A2t and HPV16 has been effectively inhibited in vitro to reduce HPV16 internalization into both epithelial cells and Langerhans cells by anti-A2t antibodies, the natural A2t ligand secretory leukocyte protease inhibitor (SLPI), and A2t-specific inhibitory molecules (Dziduszko & Ozbun, 2013; Woodham et al., 2012, 2014, 2015).

In addition to the aforementioned in vitro data, a strong inverse correlation exists between the expression of the innate immune protein SLPI, and the HPV status and degree of metastasis of HNSCC in vivo (Cordes et al., 2011; Hoffmann et al., 2013). Specifically, it was found that higher SLPI levels were correlated with lower HPV expression in vivo. These results parallel the protective role that SLPI has been shown to have in preventing infection of macrophages by human immunodeficiency virus type 1 (HIV-1) (Drannik et al., 2011; Ma et al., 2004). In particular, SLPI inhibits HIV-1 infection of macrophages by binding to A2t and blocking A2t–HIV interactions (Ma et al., 2004). SLPI is a serine protease inhibitor produced by salivary glands and different epithelial cell types (Vogelmeier et al., 1991), and is found in physiological mucosal concentrations of 10–50 μg ml\(^{-1}\) in vivo (Kramps et al., 1984). Of particular interest to us, HSV-1 and HSV-2 have been shown to induce a sustained downregulation of SLPI even during non-replicative HSV life cycles, i.e. during concurrent acyclovir treatment (Fakioglu et al., 2008). This downregulation of SLPI is dependent on HSV immediate-early gene expression and independent of viral replication, making it possible that even abortive or asymptomatic HSV infection could lead to a sustained localized decrease in SLPI in vivo.

Given that (1) the A2t receptor is a critical component in HPV16 uptake and gene transduction, (2) there is evidence that HSV-1 and HSV-2 downregulate SLPI, a protein that prevents HPV gene transduction through A2t, in HPV-transformed cells, and (3) recent reports have shown that levels of SLPI are inversely correlated with positive HPV16 status in HNSCC, we aimed to provide a molecular mechanism that could potentially explain the continued epidemiological link between HSV infection and HPV-related cancers. Specifically, we investigated the hypothesis that HSV infection of HPV-naive human keratinocytes causes a reduction in secreted SLPI that promotes A2t-mediated HPV16 uptake and reporter gene transduction.

**RESULTS**

**HSV-1 and HSV-2 infection decreases SLPI mRNA and secreted protein levels**

It was previously demonstrated that SLPI is a specific target for downregulation by HSV-1 and HSV-2 in HPV16-positive CaSki cells (Fakioglu et al., 2008). Therefore, we hypothesized that SLPI reduction would be similar in the HPV-naive HaCaT human keratinocyte cell line. To examine changes in SLPI mRNA, cells were treated with a mock (PBS), HSV-1 or HSV-2 inoculum at the indicated m.o.i., and RNA was isolated at different time points and analysed via quantitative real-time reverse transcription (qRT)-PCR. There was a significant decrease in SLPI mRNA at both 6 and 24 h post HSV-1 infection compared with that in mock-infected groups, with a >90% reduction at 24 h (Fig. 1a), and similar results were observed at 48 h post HSV-2 infection (Fig. 1b). The levels of SLPI protein secreted by HaCaT cells were measured via ELISA at the same time points with increasing m.o.i. of HSV and significant decreases in SLPI protein levels were observed in all HSV-treated groups. Specifically, there was a 49–61% reduction in secreted SLPI in HaCaT cells exposed to HSV-1 (m.o.i. 1 and 3, respectively) and a 59–66% reduction in cells treated with HSV-2 (m.o.i. 1 and 3, respectively) compared with those of the mock-infected group (Fig. 1c, d).

**Endogenous SLPI colocalizes with A2t from epithelial cell lysates and binds to the A2 subunit**

A2 has been shown to co-immunoprecipitate with SLPI (Ma et al., 2004); however, these results hinged on the
addition of saturating amounts of SLPI to macrophage cell lysates. Additionally, the binding interaction between SLPI and either A2t subunit was not investigated. Therefore, to investigate endogenous SLPI–A2t interactions, we used a newly developed SiMPull (single-molecule pulldown) assay to detect SLPI secreted from epithelial cells in culture bound to A2t from the same cells as individual complexes. Specifically, A2 complexes from whole epithelial cell lysates were captured on SiMPull slides via anti-A2 antibodies and S100A10–SLPI colocalization was assessed. Our results showed that >40% of the detected S100A10 colocalized with SLPI (Fig. 2a). In order to determine which subunit of A2t interacts with SLPI, purified A2t, A2 or S100A10 were bound to an ELISA plate prior to the addition of increasing concentrations of SLPI (Fig. 2b). There was a similar low amount of SLPI detected in the negative control wells compared with those found in S100A10-bound wells. The highest levels of SLPI binding were observed in wells with A2t, which is the relevant conformational cell surface receptor form of the protein. These results indicated that SLPI bound to the A2 subunit of A2t.

HSV-1 and HSV-2 infection results in a significant increase in HPV16 internalization and infection, restricted to non-HSV-infected cells

We next sought to determine if in vitro HSV infection increased the susceptibility of epithelial cells to HPV16

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Fig. 1. HSV-1 infection downregulates SLPI at both the mRNA and secreted protein level. (a) HaCaT cells were mock or HSV-1 infected and the expression of SLPI was analysed via qRT-PCR against the 18S reference gene (ΔΔCq) at the indicated time points. Results are presented as mean±SD SLPI gene expression. *P<0.05 and **P<0.01 as determined by a two-tailed, unpaired Student t-test. Data are representative of three independent experiments. (b) HaCaT cells were mock or HSV-2 infected and the expression of SLPI was analysed via qRT-PCR against the 18S reference gene (ΔΔCq) at the indicated time points. Results are presented as mean±SD SLPI gene expression. **P<0.01 as determined by a two-tailed, unpaired Student t-test. Data are representative of three independent experiments. (c) HaCaT cells were mock or HSV-1 infected with the indicated m.o.i. followed by assessment of SLPI concentrations after 24 h in cell supernatants via Quantikine SLPI ELISA. Results are presented as mean±SD concentration from triplicate groups. **P<0.01 as determined by a one-way ANOVA followed by Tukey’s multiple comparison test against the mock-infected group. Data are representative of two independent experiments. (d) HaCaT cells were mock or HSV-2 infected with the indicated m.o.i. followed by assessment of SLPI concentrations after 48 h in cell supernatants via Quantikine SLPI ELISA. Results are presented as mean±SD concentration from triplicate groups. **P<0.01 as determined by a one-way ANOVA followed by Tukey’s multiple comparison test against the mock-infected group. Data are representative of two independent experiments.
SLPI binds to the A2 subunit of A2t. (a) SiMPull chamber slides were coated with an anti-A2 capture antibody and subjected to quantified HeLa cell lysates and washing, followed by detection of S100A10 and SLPI in complex with captured A2. (i) SiMPull colocalization images. (ii) Colocalization events were quantified from the overlap of their respective wavelengths and are presented as mean ± SD percentage S100A10 colocalized with SLPI. (iii) Diagram of assay system. (b) Molar equivalents of A2t, A2 or S100A10 were bound to an ELISA plate and blocked overnight. Indicated amounts of SLPI were added to each well. Binding of SLPI to A2t or individual subunits was detected using an anti-SLPI antibody. Results are presented as mean ± SD A450. Data are representative of three independent experiments.
entry and infection by examining HPV16 virus-like particle (VLP) internalization and pseudovirion (PsV) reporter gene transduction within HSV-infected and non-infected HaCaT cell cultures. The 24 h post HSV-1 and 48 h post HSV-2 exposure time points were chosen for HPV16 addition due to the aforementioned maximum reductions in measured SLPI levels. To examine the specific effects of HSV on HPV16 internalization, mock- or HSV-treated cells were incubated with HPV16 VLPs directly conjugated to a pH-dependent fluorescent rhodamine dye (pHrodo Red) that only fluoresces at late endosomal pH. A twofold increase in HPV16 internalization was observed in HSV-1-infected cultures compared with that in the mock-infected controls (Fig. 3a) and this increase was even greater in HSV-2-treated cultures (2.5-fold increase) (Fig. 3b). Next, gene transduction studies were carried out utilizing HPV16 PsVs containing a GFP reporter plasmid. We observed a twofold increase in the number of HPV16 PsV-transduced HaCaT cells in cultures pre-infected with HSV-1 and a twofold increase in HPV16 PsV-transduced cells in cultures pre-infected with HSV-2 compared with that in mock-treated cultures, which mirrored the results observed using VLPs (Fig. 3d, e). We further examined which cells were HPV-positive in the HSV-infected culture populations and found that the uptake of HPV16 VLPs and reporter gene transduction by HPV16 PsVs was restricted to the non-HSV-infected cells. This indicated that the increases in HPV16 uptake and gene transduction were not due to superinfection by both HSV and HPV in the same cells, but were rather independent events caused by concurrent HSV infection within the same populations (Fig. 3c, f). These data suggested that non-HSV-infected cells within HSV-treated cultures were more likely to internalize HPV and were more susceptible to HPV pseudo-infection compared with non-infected groups.

**HSV-1 ICP4 deletion mutant does not downregulate SLPI or enhance HPV16 infection**

SLPI downregulation in epithelial cells was previously shown to be dependent on immediate-early gene ICP4 expression independent of tegument proteins such as the virus host shutoff (VHS) protein (Fakioglu et al., 2008). Therefore, the effects of the HSV-1 ICP4 deletion mutant [ICP4(−); d120] on SLPI transcription and HPV16 PsV infection in HaCaT cells were examined. At 24 h post HSV-1-ICP4 mutant exposure, cells were either collected and analysed for SLPI gene transcription via qRT-PCR or exposed to HPV16 PsVs. The ICP4 deletion mutant failed to significantly reduce SLPI gene transcription compared with that in mock-infected cells (Fig. 4a), indicating that SLPI downregulation was not part of a general HSV-mediated host shutdown mechanism. Furthermore, there was no increase in the percentage of cells expressing the HPV16 PsV reporter gene compared with that in controls (Fig. 4b). Additionally, there was only a small number of superinfected cells within the group exposed to the ICP4 deletion mutant, which was comparable to what was observed in HSV-1-treated HaCaT cultures (Fig. 4c). Similar results were seen using HPV16 PsVs containing a reporter plasmid with an SV40 promoter (Fig. S1, available in the online Supplementary Material). These data demonstrated that the HSV-1 ICP4 deletion mutant is unable to downregulate SLPI in HaCaT cells and infection with this mutant did not result in increased HPV16 infection.

**Increased uptake and infection of HPV16 VLPs and PsVs in HSV-infected cultures requires the L2 minor capsid protein**

Our previous results indicated that HPV16 entry into cells via A2t is dependent on an interaction between the S100A10 subunit and the L2 minor capsid protein (Woodham et al., 2012). Therefore, to provide further evidence that the increase in HPV16 uptake seen in HSV-infected cell cultures was through the A2t pathway, we utilized HPV16 L1 VLPs consisting of only the L1 major capsid protein. At 48 h post HSV-2 exposure, cell cultures were incubated with either pHrodo Red-labelled HPV16 L1 or HPV16 L1L2 VLPs for 16 h and then analysed via flow cytometry. There was no significant change in the uptake of L1-only VLPs in HSV-positive cultures compared with that in the mock-infected group. However, a significant increase (threefold increase from 9 to 27%) in HPV16 L1L2 VLP uptake in HSV-positive cultures was observed compared with that in controls (Fig. 5a). Additionally, we utilized a PsV mutant that contained L2 substitutions essential for HPV interaction with the S100A10 subunit of the A2t heterotetramer for successful infection through this pathway (Woodham et al., 2012). Briefly, 24 h after HSV-1 or HSV-1 ICP4(−) exposure, HPV16 WT PsVs or mutant HPV16 L1L2(GGDD) PsVs were added. Successful infection was assessed by GFP production 48 h post PsV exposure. There was no significant change in the level of infection by mutant HPV16 PsVs in any of the groups; however, there was a roughly twofold increase in the number of HaCaT cells infected by WT HPV16 PsVs, as seen in previous experiments (Fig. 5b). These results suggested that HSV-mediated susceptibility to HPV16 uptake and infection required the presence of an L2 capable of interacting with the S100A10 subunit, which was necessary for uptake through the A2t-mediated pathway.

**Restoration of SLPI to HSV-1- and HSV-2-infected cultures blocks HSV-mediated increases in HPV16 uptake and gene transduction**

We have previously reported that SLPI inhibits HPV16 infectious uptake through A2t (Woodham et al., 2012) and here our data demonstrate that HPV16 gene transduction was increased in HSV-infected cultures, potentially through HSV-mediated reduction in SLPI. To examine if SLPI downregulation was one of the mechanisms responsible for this increase in HPV16 uptake and infection in HSV-treated cultures, increasing amounts of SLPI were
Fig. 3. HSV infection results in increased HPV16 internalization and percentage of cells with reporter gene transduction restricted to non-HSV-infected cells. (a) HaCaT cells were mock or HSV-1 infected for 2 h. Inoculum was removed, media replaced and cells incubated for 24 h. pHrodo Red-labelled HPV16 L1L2 VLPs were then added for 12 h. VLP internalization was assessed by flow cytometry and results are displayed as mean ± SD percentage positive cells of triplicate wells normalized to the mock-infected groups. H16.E70 was used as a positive control for blocking internalization. Data are representative of two independent experiments. (b) HaCaT cells were mock or HSV-2 infected as described above and incubated for 48 h. pHrodo Red-labelled HPV16 L1L2 VLPs were then added to the cells for 12 h. VLP internalization was assessed by flow cytometry and results are displayed as mean ± SD mean fluorescence intensity (MFI) of triplicate wells. Results are a representative example of two independent experiments. (c) Flow plot data showing HPV16 VLP uptake (x-axis) and HSV-2 infection (y-axis). Results are a representative example of two independent experiments. (d) HaCaT cells were infected with HSV-1 as described above. HPV16 PsVs containing a GFP reporter plasmid were then added and GFP expression was analysed 48 h post-infection by flow cytometry. Results are presented as mean ± SD infection of triplicate wells normalized to mock-infected groups. Data are representative of three independent experiments. (e) HaCaT cells were infected with HSV-2 as described above and incubated for 48 h. HPV16 WT PsVs containing a GFP reporter plasmid were then added to the cells and GFP expression was analysed 48 h post-infection by flow cytometry. Results are presented as mean ± SD mean fluorescence intensity (MFI) of triplicate wells. Data are representative of three independent experiments. (f) Flow plot data showing percentage of cells with HPV16 PsV gene transduction (y-axis) and detectable HSV-2 infection (x-axis). Results are a representative example of two independent experiments. **P < 0.01 as determined by a one-way ANOVA followed by Tukey’s multiple comparison test against the mock-treated group.
added to both mock- and HSV-2-infected cells, and internalization of pHrodo Red-labelled HPV16 L1L2 VLPs and HPV16 pseudo-infection were assessed. HPV16 L1L2 VLP uptake was significantly decreased upon the addition of SLPI in a dose-dependent manner (Fig. 6a). Additionally, pre-treatment of cells with SLPI resulted in a 75% reduction of HPV16 PsV reporter gene transduction within the HSV-1-treated cultures (Fig. 6b). Taken together, these data suggested that HSV infection increased neighbouring keratinocyte susceptibility to HPV16 gene transduction by reducing local concentrations of SLPI.

DISCUSSION

hrHPV infection is a widely accepted necessary cause of cervical cancer, as is HPV’s contributing role in anogenital cancers, such as vaginal, vulvar, anal and penile cancer, in addition to HNSCC. The viral oncogenes E6 and E7 disrupt the cell cycle, inhibit apoptosis, and contribute to genetic instability and immune evasion, ultimately leading to cellular transformation (Moody & Laimins, 2010). Despite the causal relationship, the epidemiological link between HSV seropositivity and increased risk of HPV-associated cancer development suggests that a potential association between the two viruses exists, although the molecular mechanism(s) for this association have remained uncertain. HSV has not been shown to have direct cellular transforming abilities. Thus, any biological effect of co-infection by HSV and HPV that enhances the development of epithelial cell transformation is expected to be indirect, meaning that HSV does not necessarily need to exert an effect on the cells that ultimately become transformed, but may instead change the environment in such a way as to predispose tissue to either infection by HPV or catalyse HPV-induced carcinogenesis. Here, we extended our work that showed that SLPI is an effective inhibitor of HPV16 uptake and gene transduction through the A2t pathway in both epithelial and Langerhans cells (Woodham et al., 2012, 2014). Specifically, the data presented herein suggest that modification to the cellular microenvironment through downregulation of SLPI by HSV infection results in an increase in HPV16 uptake and infection through the A2t pathway.

Early experiments that examined the interaction between SLPI and A2t relied on the addition of exogenous SLPI to macrophage cell lysates prior to detection of A2–SLPI

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**Fig. 4.** HSV-1 ICP4 deletion mutant is unable to downregulate SLPI and does not enhance HPV16 infection. (a) HaCaT cells were mock infected or infected with an HSV-1 ICP4 deletion mutant [ICP4(–)] and incubated for 24 h. Expression of SLPI was analysed via qRT-PCR against the 18S reference gene (ΔΔCt). Results are presented as mean ± SD SLPI gene expression. NS, Not significant. (b) HaCaT cells were mock infected or infected with HSV-1 or an HSV-1 ICP4 deletion mutant [ICP4(–)] for 2 h. Inoculum was removed, media replaced and cells incubated for 24 h. HPV16 WT PSVs containing a GFP reporter plasmid were then added to the cells, and GFP expression was analysed 48 h post-infection along with HSV infection by flow cytometry. Results are presented as mean ± SD percentage of positive cells of triplicate wells. ***P<0.001 as determined by a one-way ANOVA followed by Tukey’s multiple comparison test against the mock-infected group; NS, not significant. (c) Flow plot data showing percentage of cells with HPV16 PsV gene transduction (x-axis) and with detectable HSV-1 infection (y-axis). Results are a representative example of two independent experiments.
co-immunoprecipitation (Ma et al., 2004). As these systems do not reflect natural conditions, we utilized the novel and sensitive SiMPull technique to show that endogenous A2t–SLPI interactions exist at the cell surface of epithelial cells. In these experiments, A2 was captured on SiMPull slides as the bait, and S100A10 and SLPI colocalization was thus interpreted as A2t–SLPI colocalization as all three of these proteins were together as one molecular complex. Through ELISA, we further demonstrated that the interaction between SLPI and A2t was through the A2 subunit as minimal binding was observed between SLPI and purified S100A10. Interestingly, our previous findings revealed that HPV16 L2 interacts with S100A10 (Woodham et al., 2012), which may indicate that SLPI inhibits HPV16 interaction with A2t through steric hindrance or through conformational changes in A2t that prevent S100A10–L2 interactions.

HSV has been shown to downregulate SLPI in vitro through expression of ICP0 and ICP4 genes whilst
independent of the presence of the VHS tegument protein, which is responsible for the majority of host mRNA degradation (Fakioglu et al., 2008). We were intrigued by this observation as SLPI is a natural ligand for A2t, an HPV16 entry receptor identified by our group. As HPV16-transformed CaSki cells were used in the previous study we posited that HPV gene expression may also effect normal cellular functions. Therefore, we verified that downregulation of mRNA and secreted SLPI protein occurs in HSV-infected HaCaT keratinocytes, which are HPV-negative and similar to untransformed epithelial cells. Our data showed a significant (>60%) reduction of SLPI mRNA at just 6 h post-infection with HSV-1. However, measurable differences of secreted SLPI protein levels were found at 24 h for HSV-1 and 48 h for HSV-2, which is when we examined the effects of a reduced SLPI environment on HPV16 VLP uptake and successful PsV infection. Whilst we observed a decrease in the level of SLPI when only a small percentage of cells had detectable surface HSV proteins (25–30% based on flow cytometry surface staining for glycoprotein D), whilst simultaneously controlling the HSV lytic cycle/HaCaT cell viability with acycloguanosine (Fig. S2), this phenomenon may be the result of extracellular signalling events instigated by HSV-infected cells to neighbouring uninfected cells. This phenomenon has been previously demonstrated in HaCaT cell migration assays used to study wound healing where it was found that HSV-infected HaCaT cells are able to enhance the migratory capacity of non-HSV-infected cells through an unidentified paracrine signalling mechanism(s) (Abaitua et al., 2013).

We have previously provided evidence that HNSCC patients with lower SLPI levels are more likely to have HPV-positive cancers, demonstrating a correlation between reduced SLPI and increased HPV infection in vivo (Cordes et al., 2011; Hoffmann et al., 2013). With this observation in mind, we utilized the HSV-mediated reduced SLPI environment to examine the effect of HPV16 uptake and infection in vitro. In initial experiments we found that HSV-1-infected cells showed similar increases in uptake of HPV16 VLPs labelled with either a pH-dependent rhodamine dye or pH-independent carboxy-fluorescein diacetate (CFDA) succinimidyl ester dye (data not shown); however, as it is not possible to assess whether CFDA-labelled particle are trafficked to late endosomes we chose the pHrodo Red system. HSV-1 and HSV-2 infection both showed similar increased levels of VLP uptake and percentages of cells with HPV PsV gene transduction. To our surprise we found that <1% of cells were co-infected, an important observation because it suggests that the non-HSV-infected cells within an SLPI-reduced environment are more susceptible to subsequent HPV16 infection. Furthermore, we identified that enhanced

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### Fig. 6. Addition of SLPI to HSV-1- and HSV-2-depleted cultures blocks increase in HPV16 gene transduction.

(a) HaCaT cells were mock or HSV-2 infected and incubated for 48 h. Increasing concentrations of recombinant human SLPI (rhSLPI) were added for 1 h at 4 °C followed by pHrodo Red-labelled HPV16 L1L2 VLPs for 12 h to measure uptake. Uptake was assessed by flow cytometry and results are presented as mean ± SD MFI of triplicate wells and normalized to the mock-infected groups. Results are representative of two independent experiments. *P < 0.05 and **P < 0.01 as determined by utilizing a two-tailed, unpaired Student’s t-test. (b) HaCaT cells were mock or HSV-1 infected for 24 h and then treated with increasing concentrations of SLPI for 1 h at 4 °C, followed by exposure to HPV16 PsVs. After 24 h, the cells were washed and media replaced. Gene transduction was assessed via flow cytometry 48 h after initial HPV16 PsV exposure. Results are presented as mean ± SD gene transduction, with data normalized to the mock-infected groups. ***P < 0.001 as determined by a one-way ANOVA followed by Tukey’s multiple comparison test against the mock-infected group. Data are representative of two independent experiments.
HPV16 uptake and infection are dependent on the presence of an L2 capsid protein that is capable of interacting with the S100A10 subunit of A2t, demonstrating that increased HPV uptake is not due to a global effect on endocytosis by HSV infection, but rather specific to the identified A2t pathway (Dziduszko & Ozbun, 2013; Woodham et al., 2012). The inability of the HSV ICP4 deletion mutant to downregulate SLPI or increase the number of HPV16-positive cells, coupled with the observation that exogenously added SLPI reverses the HSV-enhanced HPV uptake and infection, suggests that HSV-mediated reduction of SLPI may partially explain the increased susceptibility to HPV infections. Whilst the amount of SLPI added back is within the physiological ranges found in vitro (Kramps et al., 1984), the amount needed to block HPV infection exceeded the change in protein concentration measured by ELISA. We posit that a higher concentration of recombinant SLPI was needed because of missing important post-translational modifications that would normally occur when produced in eukaryotic cells, reducing its affinity for naturally occurring ligands. Additionally, it is possible that localized SLPI concentrations at the surface of the cells may be much higher than the unbound SLPI sampled in the culture supernatant as secreted SLPI can immediately bind to cell surface receptors in an autocrine/paracrine manner upon release into the extracellular environment.

Understanding how common pathogens can increase the risk of acquiring oncogenic viruses is essential to cancer prevention strategies. Our findings highlight the importance of viral co-infections, given that both HSV serotypes and HPV16 are highly prevalent sexually transmitted infections that share similar modes of transmission, replicate in stratified epithelia and have been epidemiologically linked to cervical cancer since the 1970s. In the case of HSV, ~80–90% of all active infections are thought to be linked and do not lead to ulcerative lesions or localized inflammation (Schillinger et al., 2008). Moreover, the paradigm of HSV-1 infections occurring primarily in the oro-facial region has begun to shift, where over half of newly diagnosed genital HSV infections are attributed to HSV-1. This becomes clinically relevant when taking into account our data showing HSV-1 is able to downregulate SLPI more rapidly than HSV-2 and therefore the agenital regions infected with HSV-1 may be particularly susceptible for hrHPV infection. Given that we examined one specific mechanism that could enhance HPV16 infectious uptake, our data warrant a further investigation into the other cellular changes induced by HSV infection that may lead to an environment that promotes HPV infection. For example, significant increases in the levels of syndecan-1 and syndecan-2 produced by HSV-1-infected HeLa cells have been reported (Bacska et al., 2011). As syndecan-1 is the most abundant HSPG expressed on the surface of keratinocytes, it is logical to hypothesize that epithelium infected by HSV has localized increases in the levels of syndecan-1 and syndecan-2, potentially resulting in faster HSGP-mediated binding and subsequent conformational changes of the HPV16 capsid that expose the N terminus of L2 allowing for furin cleavage (Alexopoulou et al., 2007; Raff et al., 2013; Shafti-Keramat et al., 2003). Even though it has been shown that syndecan-1 knockout mice are susceptible to HPV infection, it remains clear that HSPGs play a vital role in initial targeting of virions to the basement membrane, coating of virions with growth factors and other events preceding viral entry (Huang & Lambert, 2012; Surviladze et al., 2012). In summary, our findings demonstrate that molecular changes caused by HSV to the cellular microenvironment promote HPV acquisition in vitro, providing the first evidence for a mechanism that explains the ongoing epidemiological link between HSV infection and HPV-associated cancers.

METHODS

Cell cultures, antibodies and recombinant proteins. HaCaT cells (Cell Lines Service) are in vitro spontaneously transformed human keratinocytes derived from normal skin (Boukamp et al., 1988), and were maintained in keratinocyte serum-free media (KSFM; Life Technologies) with manufacturer-provided growth supplement at 37 °C with 5% CO2. HeLa cells (ATCC) are human epithelial cells derived from cervical cancer and were maintained in complete medium [Iscove’s modified Dulbecco’s medium (IMDM), 10% FBS, 1 × PenStrep, 1 × β-mercaptoethanol] (Lonzka) at 37 °C with 5% CO2. Monkey kidney epithelial cells (Vero) were obtained from the ATCC for propagation of HSV-1(F) and HSV-2(G), and were maintained in complete medium (IMDM, 10% FBS, 1 × PenStrep) at 37 °C with 5% CO2. The Vero-E5 cell line (a gift from Neal DeLuca, University of Pittsburgh, Pittsburgh, PA, USA) was used for propagation of the HSV(d120) ICP4 deletion mutant, and was maintained in complete medium (IMDM, 10% FBS, 1 × PenStrep) at 37 °C with 5% CO2.

The following antibodies were used in this study: goat anti-SLPI (R&D Systems), H16.E70 mouse anti-HPV16 L1 (a gift from Neil Christensen, Penn State, Philadelphia, PA, USA), donkey anti-goat HRP (Promega), rabbit anti-HSV-1 (Dako), rabbit anti-A2 (H-50) (Santa Cruz Biotechnology), biotin-conjugated donkey anti-rabbit IgG (Abcam), mouse anti-A2 light chain (BD Biosciences), and Alexa Fluor 488 goat anti-rabbit IgG and phycoerythrin/Cy5.5-conjugated goat anti-rabbit IgG (Life Technologies). Recombinant human SLPI was purchased from R&D Systems. Recombinant human A2t, A2 and S100A10 were a generous gift from Dr Ralf Langen (University of Southern California, Los Angeles, CA, USA).

Virus, PsVs and VLPs. WT HSV-1(F) HSV-2(G) stock virus (a gift from Bernard Roizman, University of Chicago, Chicago, IL, USA) was propagated in Vero cells and titrated utilizing HaCaT cells following published procedures (Blaho et al., 2005). HSV-1(d120), the ICP4 deletion mutant, was propagated and titrated in the Vero-E5 rescue cell line following published procedures (Marconi et al., 1996). HPV16 PsVs containing the GFP reporter plasmid were produced by co-transfection of 293TT cells with plasmids encoding HPV16 L1, L2, pCNeoGFP or pfwB-GFP reporter plasmid following published procedures (Buck & Thompson, 2007). To produce PsVs with a mutated L2 (aa 108–126) region (Kawana et al., 2001), site-directed mutagenesis was performed using overlapping mutated primers on the bicistronic packaging HPV16 PsV plasmid p16shE1 as template (Buck & Thompson, 2007). Forward primer 5′-AGCGACCCCAGC-
**VLP internalization assays.** HPV16 L1L2 VLPs were labelled with pRhodo Red succinimidyl ester (Life Technologies) as per the manufacturer’s instructions and quantified via Coomassie blue staining following SDS-PAGE against BSA standards. HaCaT cells were seeded in KFSM (3 × 10⁵ cells per well in 12 well plates) and incubated overnight at 37 °C. Cells were then inoculated with either HSV-1 or HSV-2 at an m.o.i. which yielded 25–30 % infection or mock infected at 2 h at 37 °C. Cells were washed with PBS, then KFSM containing 0.8 mM acycloguanosine (Sigma-Aldrich) was added and cells were incubated for 24 h for HSV-1 or 48 h for HSV-2 at 37 °C. pRhodo Red-labelled VLPs were then added (2.5 μg per 10⁵ cells) and cells were incubated for 12 h at 37 °C. The cells were next collected with trypsin/EDTA, washed, stained for HSV-1 or HSV-2 surface antigens, and analysed via flow cytometry to measure the percentage of pRhodo Red-labelled HPV16 VLP-positive cells and the percentage of cells with detectable HSV surface antigens. For control experiments, VLPs were pre-incubated with the H16.E70 neutralizing antibody prior to uptake experiments, and minimal signals were observed (<3 %). For SLPI blocking experiments, mock- and HSV-infected groups were treated with PBS or indicated concentrations of SLPI for 45 min at 4 °C prior to addition of labelled VLPs.

**HPV16 PsV gene transduction assays.** HaCaT cells were seeded in KSF M (3 × 10⁵ cells per well in 24-well plates) and incubated overnight at 37 °C. Cells were then inoculated with HSV-1, the HSV(d120) ICP4 deletion mutant or HSV-2 at an m.o.i. which yielded 25–30 % infection or mock infected for 2 h at 37 °C. Post-infection, cells were washed with PBS, and fresh KSF M containing 0.8 mM acycloguanosine was applied. Cells were then incubated for 24 h for HSV-1 or 48 h for HSV-2 infection at 37 °C. At the indicated time points HPV16 PsVs containing a pCIneoGFP vector at m.o.i. 200 were added (as defined by infection of 293TT cells) overnight at 37 °C. The m.o.i. was chosen to achieve 10–15 % GFP-positive cells within the mock-infected group. Following incubation, cells were washed with PBS to remove unbound PsVs and fresh KSF M containing 0.8 mM acycloguanosine was added. Gene transduction of target cells was assessed as the percentage of GFP-positive cells 48 h post HPV16 PsV addition via flow cytometry. For SLPI blocking experiments, either PBS or recombinant human SLPI was added at the indicated concentrations and incubated at 4 °C for 45 min before the addition of PsVs. Treatment groups were normalized to the mock group and the fold changes in gene transduction were assessed. In control experiments, PSVs were pre-incubated with the H16.E70 neutralizing antibody prior to infection of HaCaT cells, resulting in <2 % gene transduction.

**Statistical analysis.** All statistical analyses were performed using Prism 6 (GraphPad).
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