The Inhibitory Role of Human α-defensin 5 in HPV16 Uptake and Infection

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Abstract

Human papillomavirus (HPV) is the number one sexually transmitted virus worldwide, of which high-risk HPV type 16 (HPV16) is causally associated with 50% of all cervical cancers and 90% of HPV-positive oropharyngeal cancers. Defensins are small peptides of the innate immune system that are expressed by epithelial cells in the genitourinary tract. Human α-defensin 5 (HD5) has been shown to inhibit HPV16 infection. However, the mechanisms proposed by different studies are conflicting. Therefore, we have aimed to delineate the role of defensins in HPV16 uptake, infection, and its mucosal immune responses. Our study shows that HPV16 infection is blocked to near 100% when pretreating HPV16 PsV with HD5, indicating that HD5 does not require direct surface interaction with cellular receptors to block infection. Uptake assays give evidence that HD5 pretreated HPV16 are internalized by the cell, but are unable to reach the nucleus. In fact, a build-up of virus particles within acidic environments of the cell was observed, suggesting viral trafficking was halted at late endosomes or possibly lysosomes. From this data we conclude that HD5 interacts directly with the viral capsid, resulting in a build-up of virus particles in late endosomes and prevention of viral trafficking to the nucleus that completes infection. Throughout the investigation we have continuously refined our hypothesis for the mechanism by which HD5 blocks HPV16 infection. As viral uncoating is required for the HPV16 to escape the late endosomes, we currently hypothesize that HD5 inhibits this viral capsid dissociation by stabilizing the viral capsid.

Samenvatting

Humaan papillomavirus (HPV) is het meest voorkomende seksueel overdraagbare virus wereldwijd, waarvan hoog-risico HPV type 16 (HPV16) geassocieerd is met de ontwikkeling van verschillende maligniteiten, waaronder cervicale carcinomen. Defensinen zijn kleine peptiden van het aangeboren immuunsysteem en worden uitgescheiden door epitheelcellen van het genito-urinaire slijmvlies. Er is een inhiberende rol aangetoond van een specifiek type defensine op HPV16 infectie van keratinocyten, namelijk het humaan α-defensin 5 (HD5). Echter, het mechanisme waarmee deze peptiden HPV16 infectie voorkomen is nog niet geheel duidelijk. Het doel van dit onderzoek was om te bepalen wat de specifieke rol is van HD5 in de opname, infectie en verworven immuunreactie op HPV16. Dit onderzoek laat zien dat infectie met HPV16 bijna volledig wordt geïnhibeerd wanneer het virus voorbehandeld is met HD5, en dat HD5 hierbij geen specifieke interactie aangaat met cellululaire receptoren om infectie te voorkomen. Daarbij geven onze resultaten weer dat HD5-voorbehandelde HPV16 nog wel werd opgenomen door cellen, maar echter de celkern niet kon bereiken. Een opbouw van virus werd geobserveerd in vesikels met een lage pH, namelijk late endosomen of zelfs lysosomen. Hieruit kan worden geconcludeerd dat HD5 de virale ontsnapping uit late endosomen voorkomt, waardoor HPV16 de celkern niet kan bereiken om infectie te laten plaatsvinden. Gedurende dit project hebben wij onze hypothese aangepast naar de verschillende bevindingen die wij hebben gedaan. Om uit late endosomen te kunnen ontsnappen is dissociatie van het HPV16 eiwitmantel noodzakelijk. Om deze reden luidt onze hypothese momenteel dat HD5 het eiwitmantel zodanig stabiliseert dat dissociatie van het virale kapsel in endosomen wordt voorkomen, en als resultaat hiervan HPV16 infectie niet kan plaatsvinden.
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**Introduction**

Human papillomavirus (HPV) is the number one sexually transmitted virus worldwide with over one hundred individual genotypes identified based on sequence diversity (1). Most HPV types are classified as low-risk (lr-HPV) and cause benign, non-carcinogenic epithelial overgrowths on either cutaneous or mucosal sites of the body (2). However, persistent infection with a high-risk HPV genotype (hr-HPV) is causally associated with the development of several human cancers, including cervical cancer and head and neck squamous cell carcinomas (HNSCC) (3).

HPV type 16 (HPV16) is the most common of the hr-HPV genotypes, and together with HPV type 18 (HPV18) accounts for approximately 70% of all cancers of the cervix (4). More specifically, HPV-induced cervical cancers was responsible for 7.5% of all female cancer deaths in 2012 worldwide (5). Next to this, HPV DNA is being detected in an increasingly greater proportion of HNSCC cases, and newly diagnosed HPV-positive HNSCCs are expected to surpass the number of cervical cancer cases in the United States by the year 2020, expanding the global burden and reach of HPV-associated disease (6–8). In addition to this, although prophylactic vaccination against the most prominent hr-HPV types exists, uptake of the vaccination series has been slow. Specifically, it is estimated that 33.6% of women age 10-20 years receive the full vaccination series in developed countries, while only 2.7% of the same demographic of women report being vaccinated in less developed countries (9). Because of the low rate of uptake and given that prophylactic vaccinations do not have a therapeutic effect on infection, the need for novel therapeutic strategies against HPV remains high and extends beyond cervical cancer.

HPV is a small non-enveloped virus that replicates in stratified squamous epithelial tissue by infecting basal keratinocytes found at the basement membrane (10). Access to these target cells is achieved through micro-lesions caused by physical or chemical disruptions to the tissue that provide direct access to the basement membrane of the epithelium. As the epithelium is re-established, the lower epidermal cells divide and daughter cells migrate to the surface of the epidermis while undergoing differentiation. Upon successful infection and trafficking of its double-stranded DNA to the nucleus of the cell, the viral lifecycle is then dictated by the differentiating mRNA profile of maturing keratinocytes. Newly assembled virus particles are then shed from the upper layers of the dermal tissue to continue and infect a new host or reinfect the original host (11,12).

The HPV genome encodes six early (E) and two late genes (L). The two late genes are the structural proteins L1 major capsid and L2 minor capsid, which comprise the outer protein coat of the virus. The L1 protein forms pentameric capsomeres that interact with one another by carboxy-terminal arms. Together 72 of these pentamers make up the primary icosahedral structure of the virus (13). The L2 minor capsid proteins are positioned in the center of these pentamers with residues of the N-terminus exposed on the outside of the viral capsid while inside the capsid L2 interacts with the viral genome (14,15). Both L1 and L2 play a vital role in HPV16 entry and infection. Studies suggest that L1 mediates initial binding of virions to the cell surface, whereas L2 mediates viral genome escort to the host nucleus by interaction with a network of nuclear import receptors (16,17).

During primary infection, the HPV capsid binds to components of the extra-cellular matrix (ECM). More specifically, L1 is said to engage with heparin sulfate proteoglycans (HSPG), which triggers minor conformational changes in L1 and major conformational changes in L2.
Defensins are small effector peptides (3.4-4kDa) that are part of the human innate immune system. They are comprised of cysteine and arginine residues and have an intricate tertiary structure that is stabilized by three disulfide bonds (27,28). They are extensively studied due to their broad spectrum of primarily antimicrobial activities in the innate immune system. However, they reportedly also have antiviral and antifungal properties (29). Defensins can be classified in three groups; α-, β- and 0-defensins. Humans express only α and β types, which have antiviral activity against both enveloped and non-envelope viruses (30,31). Human α-defensin 5 (HD5) is one of six known human α-defensins which is primarily secreted by Paneth cells in the small intestine and expressed by epithelial cells in the genitourinary tract (32). More specifically, vaginal secretions of healthy patients reportedly contain 0.3-14µM HD5 (30,32). Maximum concentrations of HD5 within the endometrium have been observed during early luteal phase, whereas there is invariant expression of HD5 by the ectocervix throughout the menstrual cycle (33). Previous studies have demonstrated the inhibitory effects of HD5 on HPV16 infection at concentrations below those measured in vivo (30).

Multiple investigators have aimed to determine the exact mechanism by which these peptides block infection. Buck et al provided evidence that defensins block HPV16 infection by preventing endosomal escape, whereas Hubert et al suggested that HPV16 fails to be internalized by keratinocytes when blocked with HD5 (34,35). More recently, Wiens et al has shown that HD5 inhibits furin-mediated cleavage of the HPV16 L2 minor capsid protein via direct binding to the viral capsid, which then prevents the endosomal escape required for infection (36). Because of these opposing outcomes in previous research, the exact mechanism by which HD5 blocks HPV16 infection, whether binding directly to the virus or interacting with cell surface proteins to inhibit infection, has yet to be clarified.

Targeting the A2t receptor prevents HPV16 late endosome escape and trafficking to the nucleus mimicks defensin-mediated blocking as suggested by Buck et al (37). Next to this, defensins have indirectly been linked to A2t through their ability to block plasminogen and tissue plasminogen activator, both ligands of A2t, from binding to endothelial cell surfaces (38). Additionally, expression of secretory leukocyte protease inhibitor (SLPI), an antiviral defensive protein similar to defensins, has been demonstrated to be inversely correlated with
HPV16 infection in HNSCC (6,39). Skeate et al has demonstrated that endogenously secreted SLPI interacts with A2t on the cell surface of keratinocytes (40). With this in mind, the manner in which defensins block HPV16 infection might very well involve surface interaction with A2t at the cell surface.

Next to its role in innate immunity, members of the defensin family have also been reported to have a crucial function in adaptive immune responses (35,41). HPV remains unrecognized by the immune system during initial infection. The life cycle of HPV exclusively takes place within stratified epithelial tissues of the skin and mucosa. These cells are naturally programmed for apoptosis and do not lyse during viral replication. Furthermore, HPV viral replication is largely non-inflammatory as there are no cytokine or chemokine signals produced that would normally activate the local antigen presenting cells (APC) responsible for alerting the adaptive immune system to ongoing infection (42,43). Additionally, as there is no viremia, it is difficult for the immune system outside the epithelium to detect the presence of HPV. This immune evasion property of HPV contributes to the ability of the virus to cause a persistent infection, ultimately leading to malignant tumors in the cervix, esophagus, and anogenital area (44).

Langerhans cells (LC) are the main antigen presenting cells (APC) of the epithelium and are responsible for initiating immune responses to invasive pathogens. Upon recognition of viral or bacterial pathogens, LC undergo phenotypic and functional changes including the activation of signaling cascades, the up-regulation of co-stimulatory molecules, and the release of pro-inflammatory cytokines (45,46). Activated LC then travel to lymph nodes via chemokine-directed migration where they interact with naïve T cells to initiate an adaptive T cell response (47). Effector T cells then migrate back to the site of infection and destroy infected cells. Failure to develop this cell-mediated immune response allows persistent HPV infection, resulting in lesion progression from low to high-grade cervical intraepithelial neoplasia (CIN) and even invasive carcinoma (43). HPV16 evades immune recognition by manipulating LC, leading to a non-activated LC phenotype and failure to induce a specific T-cell response necessary for eliciting an adaptive immune response (48–50). Woodham et al found this mechanism to be carried out by viral capsid interaction with surface bound A2t on LC (51).

A subset of human defensins have been shown to independently induce activation and maturation of LC (35,41). However, α-defensins are yet to be examined for this property in context of HPV16 infection.
Hypotheses

Our primary aim was to determine the mechanism by which defensins block HPV16 infection of epithelial cells. We initially hypothesized that HD5-mediated inhibition of HPV16 infection required interaction with the annexin A2/S100A10 heterotetramer (A2t). Secondly, we aimed to investigate the effect of defensins on the mucosal immune response to HPV16. Specifically, we hypothesized that defensins inhibit and reverse the negative cascade of events occurring in Langerhans cells (LC) upon HPV16 virion interaction with A2t.
Materials and Methods

Cell Cultures, Antibodies and Recombinant Proteins
HaCaT cells are an in vitro spontaneously immortalized keratinocyte cell line from normal human skin (Cell Lines Service, Eppelheim, Germany). Cells were maintained in Keratinocyte Serum Free Media (KSFM) (Life Technologies, Carlsbad, CA) supplemented with the included defined growth factors, calcium chloride (at a final concentration of 8mM) and 1X gentamicin (50 µg/mL final w/v). A second line of HaCaTs were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, 10% FBS, 1X gentamicin). HeLa cells are human epithelial cells derived from cervical cancer, and were maintained in complete Iscove’s Modified Dulbecco’s Medium (IMDM, 10% FBS, 1X 2-mercaptoethanol, 1X gentamicin). Two genetically modified HeLa cell lines were generated by a student in the lab (J.R. Taylor) by knocking out either annexin A2 or S100A10 via CRISPR/Cas9 gene editing technology. These HeLa clones were also maintained in complete IMDM. Human PBMC from healthy donors were obtained by leukapheresis. LC were generated from human PBMC as previously described (48). PBMC were maintained in complete RPMI-1640, supplemented with 10% FBS, 1X nonessential amino acids, 1X sodium pyruvate, 1X 2-mercaptoethanol and 1X gentamicin, with the addition of 1000 U/ml (180 ng/ml) GM-CSF, 1000 U/ml (200 ng/ml) IL-4, and 10 ng/ml TGF-β during a seven-day culturing schedule. All studies were approved by the University of Southern California’s Institutional Review Board and informed consent was obtained from all donors.

Antibodies used in this study were H16.V5 mouse anti-HPV16 L1, mouse anti-annexin A2 (BD Biosciences, San Jose, CA), mouse anti-S100A10 (BD Biosciences), rabbit anti-β-actin (Cell Signaling, Danvers, MA), Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and IRDye 800 goat anti-mouse IgG (LI-COR Biotechnology, Lincoln, NE). HLA-ABC-FITC, HLA-DP DQ DR-FITC, CD1a-Cy5, CD40-PE, CD80-FITC, CD83-PE and CD86-Cy5 were all purchased from BD Biosciences. Recombinant human α-defensin 5 (HD5, a gift from the Selsted Lab, University of Southern California) was maintained at 4°C in H2O at a concentration of 5.16mg/ml.

Pseudovirions and Virus Like Particles
HPV16 pseudovirions (PsV) with an incorporated green fluorescent protein (GFP) reporter plasmid were generated by co-transfection of 293TT cells with plasmids encoding HPV16 L1 & L2, and GFP under a CMV promoter (pCI-neo-GFP) (52). The infectious titer, or multiplicity of infection (MOI), was calculated by treating 293TT cells with serial dilutions of purified PsV stock followed by infection analysis via flow cytometry. HPV16 L1 and HPV16 L1L2 virus like particles (VLP) were generated using a recombinant baculovirus expression system as previously described (53). Presence of L1 and L2 was confirmed via Western Blot, and ELISA analysis with a neutralizing antibody confirmed intact particles. HPV16 L1L2 VLP were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA, Thermo Fisher Scientific, Waltham, MA) or pHrodo Green STP ester, or pHrodo Red succinimidyl ester (Thermo Fisher Scientific) following manufacturer’s instructions. Western blot confirmed presence of VLP in collected fractions, which were quantified against BSA standards via Olymms Blue staining using Odyssey Imaging software.
Infection assay with HD5-pretreated HPV16 PsV
Infection and HD5 blocking conditions were optimized to the following method. HaCaT cells were seeded on a 24-well plate (3x10^4 cells/well in 500µl KSFM) and incubated overnight at 37°C. Prior to exposure, pseudovirions carrying the GFP reporter plasmid were incubated with HD5 (10µl/ml) for 1 hour at 37°C. After this hour, cells were washed with PBS and exposed to HD5-pretreated PsV or PsV alone at an MOI of 400. Cells were incubated at 37°C for 72 hours, after which they were analyzed for infection via flow cytometry on a Cytomics FC500 flow cytometer using CXP software (Beckman Coulter). Infection was measured by detecting the percent GFP positive cell. GFP reporter plasmids fluoresce green once present in the host cell nucleus, and thus a signal indicates successful infection with the pseudovirion. Neutralization of PsV with H16.V5 mouse anti-L1 prior to exposure to cells served as a negative control. This procedure was also optimized for HeLa cells, where fewer cells were seeded (2x10^4 cells/well of a 24-well plate in 500µl complete IMDM) and PsV were used at an MOI of 50. Cells were collected after 48 hours and analyzed for infection via flow cytometry.

Infection assay in A2- and S100A10 knockout cells with HD5-pretreated HPV16 PSV
Following optimization, we examined whether HD5 requires specific cell surface interactions to inhibit infection, such as interaction with the A2t heterotetramer. HeLa A2 knockout cells and HeLa S100A10 knockout cells were seeded in separate 24-well plates (2x10^4 cells/well in 500µl complete IMDM). Using optimal conditions outlined above, PsV were pretreated with HD5 1 hour prior to exposure. In this hour, cells were also pretreated with HD5 (10µg/ml) to test for direct surface interaction. Cells were analyzed via flow cytometry 48 hours post PsV exposure.

Internalization assay with CFDA- and pHrodo labeled HPV16 L1L2 VLP
HaCaT cells were seeded in a 24-well plate (3x10^4 cells/well in 500µl KSFM) and incubated overnight at 37°C. Prior to exposure, CFDA or pHrodo labeled HPV16 L1L2 VLP were pretreated with HD5 (10µg/ml) for 1 hour at 37°C. After this hour, cells were washed with PBS and either left untreated, or exposed to HD5-pretreated VLP or VLP only (both 5µg/million cells) and incubated at 37°C. Cells were harvested and analyzed via flow cytometry at indicated time points post VLP exposure.

LC activation assay
Langerhans cells (LC) were generated as outlined above, harvested, and counted with an automatic cell counter (Countess, Invitrogen). LC were then seeded in a 12-well plate (2x10^5 cells/well in 1ml complete RPMI 1640) and incubated overnight at 37°C. HPV16 L1L2 PsV were pretreated with HD5 (10µg/ml) for 1 hour at 37°C. LC were either left untreated or exposed to HD5-pretreated PsV or PsV only (both at 50µg PsV per 1x10^6 LC) for 48 hours, after which cells were harvested and stained for activation- and presentation markers using appropriate fluorescently labeled antibodies against MHC-I, MHC-II, CD1a, CD40, CD80, CD83 and CD86. Stained cells were analyzed via flow cytometry.

Statistical Analysis
All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Unless stated otherwise, statistical significance was determined by one-way ANOVA, followed by Tukey’s multiple comparisons test. P-values ≤0.05 were considered statistically significant.
Results

Pretreating HPV16 PsV with HD5 results in near complete blocking of infection in all models tested. In both HeLa and HaCaT cells, pretreatment of HPV16 PsV with 10µg/ml HD5 for 1 hour at 37°C resulted in near to 100% blocking of infection. For HaCaT cells infection readout required a longer incubation time compared to HeLa cells (72 vs. 48 hours, respectively) and a higher MOI (400 vs. 50, respectively), which may be due to variable expression of infection cofactors involved, or doubling time differences between the cell lines. The optimal MOI for each cell type was chosen to give 50% infection. Experiments using either HaCaT cells cultured in KSFM or HaCaT cells maintained in DMEM showed similar results. Three biological repeats were carried out for each cell type. Neutralization of HPV16 PsV with H16.V5 mouse anti-L1 for 1 hour at 37°C served as a negative control for experiments and yielded <1% infection.

Figure 1: HD5-pretreatment of PsV results in significant blocking of infection (A) %GFP positive cells 72 and 48 hours after exposure to HPV16 PsV in HaCaT and HeLa cells, respectively. HD5-pretreatment of PsV results in near complete blocking of infection. (****, p≤0.0001). (B) Flow plot showing the population shift in PsV-only treated cells (grey filled), whereas no population shift observed in cells exposed to HD5-pretreated PsV (black). A comparable population shift is observed in both HaCaT and HeLa cell lines.
HD5 does not interact with A2t at the cell surface to block HPV16 infection. Wild type-, A2 knockout- and S100A10 knockout HeLa cells were exposed to HPV16 PsV at an MOI of 50 and analyzed for GFP expression 48 hours post exposure. Between the three clones there is a clear difference in percentage GFP positive cells when comparing cells incubated with PsV only. HPV16 infection decreases by 95.3 and 60.1% in the A2- and S100A10 knockout cells, respectively, as compared to wild type HeLa cells (Figure 2a and b) (p≤0.0001). This data indicates that both annexin A2 and S100A10 play an important role in HPV16 infection, and are both necessary components for optimal HPV16 infection. For the purposes of our research question, however, HD5-pretreatment of cells did not lead to any significant blocking of infection in wild type and A2 knockout HeLa cells. In the S100A10 knockout HeLa cells, HD5-pretreatment of cells did lead to a slightly decreased percentage of infected cells. However, HD5-pretreatment of PsV lead to near complete blocking of infection in all three cell lines. Three biological repeats were carried out for each cell line with similar results. Cell lysates from clones were used to verify knockout of target protein via western blot prior to experiments.

**Figure 2: HD5-pretreatment of HPV16 causes significant blocking of infection in both A2- and S100A10 knockout HeLa cell lines.** (A) Direct HD5 cell surface binding does not inhibit downstream HPV16 infection in wild type HeLa cells, as shown by %GFP positive cells after pretreatment of cells with HD5 opposed to HD5-pretreatment of PsV. There is a 50% population shift in GFP positive cells in the PsV only treatment group (grey filled) as well as in cells pretreated with HD5 prior to PsV exposure (grey line). Exposing cells to HD5-pretreated PsV did not cause a population shift in GFP fluorescence (black line) and %GFP positive cells dropped by almost 100% (****, p≤0.0001). (B) Only in the S100A10 knockout cells does HD5-pretreatment of cells result in partial blocking of infection by 25.4%. Pretreating PsV with HD5 prior to exposure to cells results in near to 100% blocking of infection in both A2 and S100A10 knockout cell lines (****, p≤0.001, ***. p≤0.001, *, p≤0.05). (C) Western blot confirming the absence or presence of both the annexin A2 and S100A10 sub-units of A2t. As S100A10 requires A2 to be stabilized, absence of A2 leads to a very low amount of S100A10 in cells.
HD5 does not inhibit HPV16 L1L2 VLP internalization. Internalization was investigated using CFDA labeled HPV16 L1L2 VLPs. Upon entry into the cell, acetate groups of CFDA are cleaved by internal esterases, causing CFDA to fluoresce a bright green. With flow cytometry we measured the fluorescence of cells exposed to HPV16 L1L2 VLP only or HD5-pretreated VLP after 4, 8, 12, 24, 32 and 48 hours. CFDA fluorescence was measured in triplicate at each time point for all treatment groups. After 12 hours, the MFI in cells treated with VLP only reached a plateau level of intensity (MFI of 16.1, which was normalized to 1 in Figure 3a). This in contrary to the HD5-pretreated VLP treatment group, where MFI kept increasing over time, peaking at 32 hours with an MFI of 61.7, which is a five-fold increase in intensity compared to the VLP only treatment group (p≤0.0001, unpaired t-test with Welch’s correction). During these assays we specifically tested for viability of cells, to ensure changes in cell viability do not compromise interpretation of our results. Staining cells with propidium idiodide gave us an indication of the viability, which remained above an acceptable level for all treatment groups (>75%). Untreated cells and neutralization of HPV16 PsV with H16.V5 mouse anti-L1 for 1 hour at 37°C served as negative controls for experiments. Background fluorescence from these cells was subtracted from all the MFI values measured of each treatment sample. Two biological repeats were conducted for this assay.
Figure 3: HD5-pretreated PsVs are internalized by HaCaT cells. (A) Viability of HaCaT cells dropped by 9.8% when exposed to HD5 only. However, exposing cells to HD5-pretreated VLP and VLP only also resulted in a slight viability drop (-6.6 and -4.6%, respectively) (**, p≤0.01). (B) MFI of CFDA fluorescence was measured at various time points post VLP exposure to cells. The CFDA signal in VLP only treated cells reaches a plateau after 12 hours, whereas the signal in cells exposed to HD5-pretreated VLP increases five-fold after 32 hours (****, p≤0.0001, unpaired t-test with Welch’s correction). (C) Population histograms of CFDA fluorescence at each individual time point. In both treatment groups there is no distinct or single positive population visible. Yet, over time, an entire population shift occurs to the right. This shift is greater in cells exposed to HD5-pretreated VLP (black) as compared to VLP only (grey filled).
Pretreating HPV16 L1L2 VLP with HD5 causes build-up of virus particles in late endosomes. We also carried out time course experiments using pHrodo labeled HPV16 L1L2 VLP. pHrodo is a pH-dependent rhodamine fluorophore that begins to fluoresce in acidic environments. This property of pHrodo makes it a very useful tool to track internalization of virus particles into early and late endosomes as these are highly acidic.

We collected cells exposed to pHrodo labeled HPV16 L1L2 VLP only and HD5-pretreated pHrodo labeled VLP at six different time points, in triplicate. Up to 24 hours, the MFI of collected cell populations increased in both treatment groups. At 24 hours, the fluorescence in cells treated with VLP only reached its maximum fluorescent intensity, after which it plateaued over the next time points up to 48 hours. MFI in cells treated with HD5-pretreated PsV, on the other hand, kept increasing at a similar linear trend compared to the CFDA-data in Figure 3. This continuous increase in pHrodo fluorescence indicates either a further drop in pH in the environment that increases the pHrodo signal, or an increase in the amount of pHrodo fluorophore that is within cells. Either way, the virus particles are not likely escaping the acidic environment and thus this data indicates build-up of virus particles in late endosomes. Untreated cells and neutralization of HPV16 PsV with H16.V5 mouse anti-L1 for 1 hour at 37°C served as negative controls for experiments. Background fluorescence from these cells was subtracted from all the MFI values measured of each treatment sample. Three biological repeats were carried out with similar results.
Figure 4: HD5-pretreated HPV16 L1L2 VLPs build up in late endosomes. (A) 24 hours after exposing cells to pHrodo labeled VLP, the MFI of VLP only treated cells (grey) reaches a plateau, whereas fluorescence in cells treated with HD5-pretreated VLP (black) continues to increase linearly, with a two-fold increase in MFI at 48 hours respective to VLP only (***, p=0.0009, unpaired t-test with Welch’s correction). (B) Histograms showing population shift in pHrodo fluorescence at each time point investigated, as compared to untreated cell population (grey line). The pHrodo fluorescence in cells exposed to VLP only (grey filled) and HD5-pretreated VLP (black line) increases in a similar fashion up to 24 hours. At 32 and 48 hours, a clear positive population peak is observed in both treatment groups, with a slightly greater shift in the HD5-pretreated VLP treatment group.
**HD5-pretreatment of HPV16 PsV does not result in an activated LC phenotype.** To investigate the effect of HD5 on the adaptive immune response to HPV16, we carried out an LC activation assay. Previously has been shown that viral capsid interaction with LC results in an immature LC phenotype, and that a subset of defensins are able to activate LC on their own (41,51). Therefore, we tested whether HD5-pretreatment of HPV16 PsV would prevent negative signaling induced by the viral capsid, or if LC treatment with HD5 alone would result in an activated phenotype. We found that pretreating HPV16 PsVs with HD5 did not prevent the immature LC phenotype. No statistical significant difference was observed between the two treatments groups in all presentation- and activation markers, with the exception of a reduced CD86 expression in LC receiving HD5-pretreated PsV (p≤0.05 when compared to the untreated LC). Despite increasing the amount of HD5 in pretreatment of PsV, the LC phenotype remained unchanged. Exposing LC to HD5 also did not lead to an activated phenotype (data not shown). All treatment groups were compared to untreated cells (negative control) and LC incubated with Poly-IC (positive control, data not shown). Three biological repeats were carried out with similar results.
Figure 5: HD5-pretreatment of HPV16 PsV does not result in an activated LC phenotype. Surface marker expression was assessed via FACS in LC left untreated (grey) and LC exposed to PsV only or HD5-pretreated PsV (black). (A) Treatment of LC with either PsV only or HD5-pretreated PsV did not lead to a significant shift in the population in most activation and presentation markers. Expression of MHCII caused a population shift to the left in both treatment groups, however no apparent differences are observed between the two treatment groups. (B) Bar graphs representing fold change indicate that there is no statistical significant difference between treatment groups in all markers tested, except for CD86, in which HD5-pretreated PsV lead to a reduced expression as compared to treatment with PsV only. However, expression of CD86 in the HD5-pretreated PsV group is similar to untreated LC (p=0.38, unpaired t-test with Welch’s correction). Thus this data implies that LC are not activated by HD5-pretreated HPV16 PsV (*, p≤0.05).
Discussion and Conclusion

The risk of acquiring one or more anogenital HPV types is greater than 80%, and it is estimated that two-thirds of women worldwide will be infected with HPV within two years after becoming sexually active (1). Furthermore, recent studies suggest that nearly 50% of men between the ages of 18-59 years in both the United States and Denmark have an ongoing HPV infection, and that 25% of this population harbors at least one high-risk genotype (54,55). As the lifetime risk for HPV infection is high and prophylactic vaccine uptake remains low, there is a significant need to develop new therapeutic and preventative strategies to ultimately combat the HPV epidemic. Defensins are small effector peptides of the innate immune system that have been shown to inhibit HPV16 infection of epithelial cells (56). Currently it is not completely understood which defensive processes are involved in causing certain patients to be more susceptible to HPV infection or the development of HPV-induced cancers. Through future work it may show that patients who have diminished ability to naturally produce defensins may be at a higher risk of developing an hr-HPV driven cancer, given an innate defensive mechanism against the virus is abnormally reduced or absent. Outlining the mechanism by which defensins block HPV infection can thus provide us with a potential approach to prevent infectious uptake of HPV, and ultimately the development of HPV-induced anogenital- and oropharyngeal cancers.

For this project we aimed to investigate the effects of human α-defensin 5 (HD5) on HPV16 infection in both the innate and adaptive immunity. The Langerhans cell (LC) activation assessment was our initial assay investigating the effect of HD5 on the adaptive immune response to HPV16 infection. Our results show that pretreating virus with HD5 did not alter the non-immunogenic LC response to HPV16 (Figure 5). This outcome halted this arm of our project aim and we have focused primarily on the specific mechanism by which HD5 blocks HPV16 infection of epithelial cells.

It initially was hypothesized that HD5 would function through surface interaction with the annexin A2/S100A10 heterotetramer (A2t) to block HPV16 infection. To investigate this, we carried out an infection assay on HeLa A2- and S100A10 knockout cell lines with the optimal HD5 blocking conditions as determined before. Our data showed that HD5 does not require interaction with A2t or either of its sub-units (annexin A2 or S100A10) at the cell surface to block HPV16 infection (Figure 2). In fact, our data suggest that defensins do not require any direct surface interaction with epithelial cells to block HPV16 infection. Instead, significant blocking of infection is achieved when the virus is pretreated with HD5 prior to their addition to cells, indicating that direct binding to the virus capsid occurs (Figure 1).

HD5 has been reported to be present in vaginal lavage at concentrations ranging from 0.3-14µM (30,32). To first optimize the inhibitory effects of HD5, we performed initial blocking experiments with various concentrations of defensins that stayed below or within the reported range. Optimal blocking conditions were found within this range, specifically at a concentration of 10µg/ml. This concentration was used in all following experiments to keep HD5-pretreatment of PsV and VLP consistent throughout the investigation. Interestingly, we observed a strong serum effect on the ability of defensins to block infection (data not shown). As this peptide interacts with protein, usage of serum containing media was avoided when pretreating PsV or VLP with HD5. However, as HeLa cells require serum to grow and divide efficiently in cell culture, serum-containing media could not be completely avoided during the various assays as it would have compromised cell proliferation and viability. To work around
this situation, we primarily used HaCaT cells cultured in KSFM (keratinocyte serum free media) for all experiments that followed. A slight drop in the viability of HaCaT cells was observed when cells were exposed to HD5 only. However, there was no significant difference in viability between cells exposed to VLP only and HD5-pretreated VLP (Figure 3A).

The internalization time course assays with CFDA and pHrodo labeled HPV16 VLP gave us an indication as to which stage defensins were blocking infection. The presence of CFDA fluorescence in both the HD5-pretreated VLP and VLP only treatment groups verify that HPV16 does indeed enter the cell when exposed to HD5, despite downstream infection being blocked. However, as shown in Figure 3B, over time there is a five-fold increase in the CFDA fluorescence signal in the HD5-pretreated VLP cells compared to cells exposed to VLP only. As CFDA is mostly used to tag cells, and is not widely used in viral trafficking, it is difficult to fully explain what causes this increase in signal in the HD5-pretreated VLP treatment group. The histograms in Figure 3C show a greater population shift in cells exposed to HD5-pretreated VLP after 24 hours, meaning either more cells have internalized the CFDA or cells are fluorescing brighter when exposed to HD5-pretreated VLP. This indicates that HD5 is acting on one of the processes of HPV16 infection post viral internalization but prior to nuclear infection.

To indicate at which stage virus infection is blocked by HD5, pHrodo labeled VLP were used to track the virus upon entry. The time course experiment using pHrodo dye gives an indication where the virus is traveling in the host cell. Normally, the virus will enter the cell by endocytosis and travel through early and late endosomes, which become highly acidic. pHrodo dye fluoresces in acidic environments, where the degree of fluorescence correlates to the degree of acidity. As the virus is transported from early towards late endosomes the pH continues to drop, triggering eventual viral uncoating (57). These late endosomes normally mediate their internal material towards highly acidic lysosomes for degradation. HPV16, however, has found a way to divert its genome from this degradation process by escaping the late endosome prior to lysosomal fusion (58). As the virus leaves the acidic environment in late endosomes, the pHrodo dye will lose its fluorescence.

Our results show that pHrodo signal in cells treated with VLP only reached a plateau between 12 and 24 hours, as shown in Figure 4A, which is comparable to the plateau observed in CFDA signal in Figure 3B. This plateauing of signal most likely follows the regular process of cycling of virus particles; as the cells remained in the pool of virus media until they were collected for analysis, VLP are continuously being taken up, travelling through endosomes followed by endosomal escape and trafficking to the trans-Golgi network. Internalization of virus particles has been reported to be asynchronous and can take up to 12 hours. However, it must also be noted that under optimal cellular conditions infection readouts have been measured as early as 4 hours post exposure to HPV16 PsV (59,60). This reported large range in uptake and infection supports our virus cycling theory, and also the plateau observed in both the pHrodo and CFDA signal.

The pHrodo signal in cells exposed to HD5-pretreated VLP, however, continues to increase linearly over the indicated time points, suggesting that there is a build-up of pHrodo labeled particles in acidic environments; most likely the late endosomes, and possibly lysosomes as well. This build-up of virus particles implies that the regular process of cycling is disrupted at a certain stage in the viral infection pathway prior to endosomal escape.
Our results are in accordance with Buck et al, who gave evidence through microscopy that the virus is unable to escape the late endosomes and thus post-endosomal trafficking towards the nucleus cannot take place (34). In addition to this, Wiens et al has shown that HD5 inhibits furin cleavage of the L2 N-terminus at the cell surface in vitro, which is necessary for downstream endosomal escape (36). Interestingly, however, a recent study has shown that in foreskin- and cervical derived tissues the L2 N-terminus is cleaved during native viral assembly and maturation in differentiating cells, indicating that initiation of HPV16 infection may be independent of furin in vivo (61). Therefore, the proposed mechanism of HD5 inhibition of furin cleavage at the cell surface may not be the true mechanism occurring in vivo and thus alternative processes ought to be investigated.

Viral uncoating, in addition to cleavage of the L2 N-terminus, is necessary for the HPV16 genome to escape the endosome. Viral capsid dissociation is triggered by acidification of endocytic vesicles (62). The L2 minor protein, which remains in complex with the viral DNA after uncoating, contains a hydrophobic region and is believed to insert into the endosome, crossing the membrane to engage with a cellular retromer complex within the cytoplasm (38). This retromer complex diverts L2 and the viral genome away from the endosome and continues trafficking to the trans-Golgi network (57). Given our data suggesting endosomal buildup of virus when pretreated with HD5, we hypothesize that HD5 acts to block this viral uncoating step resulting in the inability of the virus to escape from late endosomes.

The L1 major protein is the primary structural protein responsible for forming the viral capsid of HPV16. L1 disulfide bonds stabilize the capsid through covalent linkage of highly conserved cysteine residues (63,64). As HD5 contains 6 cysteine residues it may act as a stabilizing factor for the viral capsid, and even in the presence of acidic environments, the added stabilization from HD5 association may prevent normal capsid disassembly. This is not a surprising mechanism of action for HD5, as Nguyen et al show that human α-defensins block adenovirus uncoating by stabilizing the viral capsid (65). Furthermore, HD5 has also been reported to stabilize the viral structure of JC Polyomavirus in a post-entry step, ultimately disrupting viral trafficking (66).

With this in mind, failure of viral uncoating may very well explain the five-fold increase in CFDA signal in Figure 3, and two-fold increase in pHrodo fluorescence as shown in Figure 4. Normally, late endosomes fuse with lysosomes and contents are degraded. In regular virus infection, dissociated L1 particles are degraded in this manner. A virus particle that has failed to uncoat, however, is a relatively large particle that may not be degraded immediately. This prolonged time within lysosomes and a continuous influx of virus particles may explain the linear increase in fluorescence signal over time.

In summary, we originally hypothesized that HD5 interacts with the annexin A2/S100A10 heterotetramer (A2t) at the cell surface. After giving evidence that HD5 directly acts on the virus and does not require any cell surface interaction, we continued the investigation by looking specifically which step of the HPV16 infectious cycle HD5 acts upon. Throughout our experiments we have continuously refined our hypothesis as we narrowed down which part of the infectious cycle is blocked by HD5. We have shown that HD5-pretreated HPV16 VLP are internalized by keratinocytes and that viral build-up occurs in acidic endosomes and lysosomes.
Currently, assays are being carried out to further investigate our hypothesis that HD5 inhibits viral uncoating within the late endosomes. Confocal microscopy will be used to visualize viral uncoating using the fluorescently labeled 33L1-7 antibody. This antibody solely recognizes the inner epitope of the L1 major protein, and thus can be used to indicate viral capsid dissociation (67). Additionally, co-immunoprecipitation studies are being carried out to quantify the relative amount of CD63 that is pulled down with L1 post uptake, with and without HD5-pretreatment of HPV16. Tetraspanin CD63 has been shown to associate with the L1 major protein and is involved in post uncoating viral processes (67). This assay will therefore give a direct measure of post viral uncoating protein interactions that occur during the course of infection.

Collectively, this research provides a mechanism by which HD5 inhibits HPV16 infection. While HD5 has a very potent inhibitory effect on HPV16 infection in vitro, there are limited studies available investigating the effects in vivo. Future perspectives of this project therefore also include investigating the effects of HD5 on HPV16 infection in vivo, and to what extent diminished HD5 expression in the genitourinary tract may lead to an increased susceptibility of acquiring an HPV infection. The need for novel preventative and therapeutic strategies remains high, especially for populations with low vaccine coverage who will continue to develop HPV-related cancers. Therefore, knowledge from these studies could be used to provide us with a better understanding of HPV16 infection, possibly identify at-risk populations that may be more susceptible to infection and ultimately work towards combating the HPV epidemic.

*After the completion of this thesis an article was released in the American Society for Microbiology’s journal mBio on 24/1/2017 confirming the mechanism proposed through the findings during this research project (68).*
References


