Differential Uptake and Cross-Presentation of Human Papillomavirus Virus-like Particles by Dendritic Cells and Langerhans Cells

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Abstract

A causal link between cervical cancer and high-risk human papillomaviruses has been established. The virus infects basal cells of the mucosa, where Langerhans cells are the resident antigen-presenting cells. Langerhans cells and dendritic cells, which are targeted by vaccination, internalize similar amounts of human papillomavirus virus-like particles (VLPs), albeit through different uptake mechanisms. VLP uptake by dendritic cells results in activation and cross-presentation of MHC class I-restricted peptides with costimulation to T cells. Conversely, VLP uptake by Langerhans cells leads to cross-presentation in the absence of costimulation. Efficient VLP cross-presentation by Langerhans cells with costimulation can be achieved by addition of CD40 ligand. The lack of a protective immune response after viral contact with Langerhans cells may explain why some women fail to mount an immune response against the virus or why the immune responses that do develop may allow the virus to persist. Because VLPs are currently being tested as a vaccine against cervical cancer, our data are very topical and have implications for optimal vaccination strategies against this disease.

Introduction

HPVs are the causative agents associated with the development of cervical cancer and other cancers such as penile, anal, and head and neck cancers (1). Although the majority of virally induced lesions are cleared, the time for clearance ranges from months to years. Some women do not initiate an effective immune response against HPV (2). A HPV vaccine would greatly contribute to women worldwide; however, HPV cannot be produced in vitro, hindering vaccine development. One strategy currently being explored as a vaccine, with promising results, is based on VLPs comprising the HPV major capsid protein L1 (3). cVLPs composed of L1 or L2 fused to desired antigens are capable of inducing a potent cellular immune response against the incorporated antigens (4). This is a result of the cVLPs being endocytosed, processed, and presented by APCs to naïve T cells (5).

Immature DCs and LCs are professional APCs that specialize in the uptake of antigen in the periphery. Immature DCs reside in the dermis, whereas immature LCs are found in the epidermis of the skin and in the epithelial layers of all mucosa (6). Because HPV infects basal cells of the mucosa, LCs are the only APCs that HPV will interact with during its natural life cycle. Immature DCs and LCs lack costimulatory signals necessary for efficient T-cell activation but are very efficient in capturing antigens. Pathways that have been described for the uptake of antigen by APCs are phagocytosis, clathrin-mediated uptake, caveolae-mediated uptake, macroendocytosis, and non-clathrin non-caveolae-mediated uptake (7). Although MHC class I molecules are generally complexed with peptides derived from cytosolic antigens, APCs can also process and present exogenous, endocytosed antigens for class I presentation, a process termed cross-presentation. Cross-presentation by APCs can result in the generation of a CD8+ immune response to extracellular antigens (8). Contact with microbial products, inflammatory cytokines, or CD40-CD40L interactions with CD4+ Th cells have all been shown to activate APCs (9). Costimulatory molecules (i.e., CD80 and CD86), receptors for chemokines (CCR7), and signaling molecules (CD40) appear on the surface of these activated cells, endocytosis is down-regulated, and the APCs migrate toward regional lymph nodes (6). Presentation of the processed antigen and enhanced expression of MHC and costimulatory molecules lead to the activation and proliferation of antigen-specific naïve T cells (10).

We reported previously that human LCs are able to bind and internalize HPV VLPs in a manner quantitatively equivalent to DCs (11). However, in contrast to DCs, LCs do not up-regulate activation markers, do not initiate a HPV16-E7 epitope-specific immune response when chimeric HPV16 L1L2-E7 VLPs are used, and do not migrate out of the epidermis in an in vivo skin explant assay in response to VLP stimulation. This work prompted us to determine whether there is a difference in the HPV VLP uptake mechanisms for DCs and LCs and whether there is a difference in the presentation of HPV VLP-derived peptides by DCs or LCs to T cells. Our data indicate that LCs endocytose HPV VLPs via a non-clathrin, non-caveolae, actin-independent pathway, whereas DCs take up HPV VLPs both by a clathrin-mediated mechanism and via macropinocytosis in an actin-dependent manner. This difference in endocytosis results in LCs processing and presenting similar HPV VLP peptides as DCs on their surface, but in the absence of costimulation. The lack of costimulation by LCs can be overcome by the addition of CD40L. With the addition of CD40L, LCs incubated with HPV VLPs produce robust amounts of the proinflammatory cytokine IL-12 and are capable of stimulating a HPV-specific immune response after incubation with T cells. Taken together, these results elucidate newly identified and essential differences in uptake and presentation of chimeric HPV VLP antigens by two professional APCs, DCs and LCs, that have profound effects on designing optimal vaccination strategies against HPV and cervical cancer.

Materials and Methods

Antibodies and Reagents. Anti-clathrin heavy chain antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). rhu-IL-4 and rhu-GM-CSF were purchased from Intergen (Purchase, NY). Human transforming growth factor β1 was purchased from PeproTech (Rocky Hill, NJ). Cytochalasin D, amiloride, and filipin complex were purchased from Sigma (St. Louis, MO). rhu-CD40L-trimer was obtained from Amgen (Thousand Oaks, CA).
VLPs. HPV16-L1L2 VLP and HPV16-L1L2-E7 cVLP were produced in insect cells and purified by sucrose and cesium chloride ultracentrifugation as described previously (4).

**Donor Material.** Peripheral blood lymphocytes from healthy donors were obtained by leukapheresis. Leukocytes were purified by Ficoll gradient centrifugation (Nycomed) and stored in liquid nitrogen. HPV serology analysis of all donors showed negative results indicating no prior exposure to the virus.

**DC and LC Generation.** DCs and LCs were generated as previously described (11). Briefly, frozen peripheral blood lymphocytes were thawed and washed once with RPMI 1640 containing 10 mM sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD), 10 mM nonessential aa (Life Technologies, Inc.), 100 μg/ml kanamycin (Sigma), and 10% FCS [HyClone, Logan, UT (complete medium)]. For DCs, plastic adherent cells were selected by plating 200 × 10^6 cells in a 175-cm² tissue culture flask for 2 h at 37°C. Nonadherent cells were washed away, and the remaining adherent cells were cultured for 6 days in medium containing 1000 units/ml rhu-GM-CSF and 1200 units/ml rhu-IL-4, of which 50% was replenished every other day. For LCs, adherent cells were cultured for 6 days in medium containing 1000 units/ml rhu-GM-CSF, 1200 units/ml rhu-IL-4, and 10 ng/ml rhu-transforming growth factor β1, of which 50% was replenished every other day.

**VLPL Uptake Experiments.** VLPs were labeled with CFDA (Vybrant CFDA Cell Tracer Kit; Molecular Probes, Eugene, OR) for 4 h at room temperature. After this incubation, the preparation was dialyzed with 4 liters of PBS-0.5% NaCl overnight at 4°C. DCs and LCs were collected and incubated with 10 μg of VLP-CFDA for 90 min at 37°C. In control experiments, 1 μg/ml FITC-dextran or 1 μg/ml FITC-BSA was added to DCs and LCs for 90 min at 37°C. After this incubation, the preparation was dialyzed with 4 liters of PBS-0.5% NaCl overnight at 4°C. FITC-dextran or 1 μg/ml FITC-BSA was added to the culture medium for the remainder of the experiment. The cells were then mixed with autologous CD8+ T cells for 48 h. Supernatants were collected, and the amount of IL-12 was determined using an IL-12 [human IL-12 (p70)] ELISA (Endogen, Woburn, MA). Student’s t test using MS EXCEL assessed statistical significance.

**In Vitro Immunization Assay.** In vitro immunization assays were performed as described previously (11). Briefly, DCs or LCs were loaded with 1 μg/ml CD40L or HPV16 VLP or VLPs and subsequently 1 μg/ml CD40L were incubated in complete medium for 48 h. Supernatants were collected, and the amount of IL-12 was determined using an IL-12 [human IL-12 (p70)] ELISA (Endogen, Woburn, MA). Student’s t test using MS EXCEL assessed statistical significance.

**Results**

Uptake of HPV VLPS by DCs and LCs. As stated above, five main endocytic mechanisms used by APCs have been characterized. Each of these mechanisms has characteristics that can be inhibited by specific inhibitors to identify what pathway a particular pathogen utilizes to gain entry into its target cell (7, 13–15). A list of the inhibitors used in the present study and the mechanism(s) inhibited is shown in Table 1. CFDA-labeled HPV16 VLPs were used to determine the mechanism of VLP uptake used by DCs and LCs. This method was used previously to show that the uptake of HPV VLPS is quantitatively equivalent for DCs and LCs (11). FITC-dextran and FITC-BSA were used as controls for inhibition of uptake by the chemical or molecular inhibitors. FITC-dextran is taken up via receptor-mediated events, and FITC-BSA is taken up via macropinocytosis (16). Cytochalasin D and the anti-clathrin antibody specifically inhibited FITC-dextran uptake by DCs and LCs, whereas cytochalasin D and amiloride inhibited the uptake of FITC-BSA by DCs and LCs. Therefore, at the inhibitor concentrations used, clathrin-mediated uptake, macropinocytosis, and actin-mediated uptake could be effectively inhibited without affecting cell viability (data not shown).

Addition of excess unlabeled VLPs could inhibit uptake of VLP-CFDA by DCs and LCs, indicating that CFDA labeling does not affect the pathway used by native VLPs (Table 1). HPV VLP-CFDA uptake was inhibited in DCs after treatment with cytochalasin D, the anti-clathrin antibody, and, to a small extent, amiloride. Together, these data indicate that HPV VLPs are taken up by DCs via clathrin-coated pits and macropinocytosis in an actin-dependent manner. In contrast to DCs, the uptake of HPV VLPS by LCs was only inhibited by the macropinocytosis inhibitor amiloride. However, because macropinocytosis is dependent on actin rearrangements, and cytochalasin D was not able to inhibit VLPs-CFDA uptake by LCs, the data indicate that macropinocytosis is not the VLP uptake mechanism responsible in LCs. Based on our results, a clathrin-independent, caveolae-independent, actin-independent mode of uptake is most likely the mechanism of VLP uptake by LCs (Table 1). The presence of FCS in the DC and LC culture medium did not affect HPV VLP uptake because experiments performed in the absence of FCS yielded similar findings (data not shown), indicating that there are no HPV-specific antibodies in FCS.

Reactivated Cytokine Secretion. IL-12 p70 secretion is crucial for the development of Th1-type T-cell responses (17). We previously found that DCs secreted IL-12 p70 after incubation with HPV VLPS, but LCs did not (11). Therefore, we wished to determine whether addition of CD40L, which up-regulates activation markers on the surface of LCs incubated with VLPS (11), would also increase secre-

### Table 1 Endocytic pathways used by DCs and LCs for uptake of HPV VLPS

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mechanism of action</th>
<th>Mechanisms inhibited</th>
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<tr>
<td>VLPs</td>
<td></td>
<td>HPV VLP-CFDA</td>
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<td>(--)</td>
<td></td>
<td>DCs</td>
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<tr>
<td>Unlabeled VLP</td>
<td>Inhibits VLP binding</td>
<td>Labeled VLP uptake</td>
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<tr>
<td>Cytochalasin D</td>
<td>Inhibits actin polymerization</td>
<td>Phagocytosis, macropinocytosis, clathrin-mediated uptake</td>
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<tr>
<td>Amiloride</td>
<td>Inhibits the Na/K transporter, inhibits membrane ruffle formation</td>
<td>Macropinocytosis</td>
</tr>
<tr>
<td>Filipin complex</td>
<td>Sterol-binding complex</td>
<td>Caveolae</td>
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<tr>
<td>Anti-clathrin antibody</td>
<td>Binds clathrin and inhibits clathrin-coated pit formation</td>
<td>Clathrin-mediated uptake</td>
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*Results are representative of three independent experiments at the 90 min time point.

*Represents mean ± SD (%) of uptake of HPV VLP-CFDA as compared with the mean fluorescent intensity of untreated DCs and LCs.
tion of IL-12 p70 by LCs after incubation with VLPs promoting a Th1-type T-cell response. DCs and LCs were incubated with medium alone, CD40L, VLPs, or VLPs followed by CD40L. Levels of IL-12 p70 were determined by cytokine-specific ELISA after 48 h. Untreated DCs and LCs secreted a low level of IL-12 p70 (Fig. 1). DCs incubated with CD40L, VLPs, or VLPs and CD40L secreted large amounts of IL-12 p70. In contrast to DCs, LCs did not secrete IL-12 p70 when incubated with VLPs but did secrete IL-12 p70 when incubated with CD40L or with VLPs followed by CD40L (Fig. 1). These data indicate that the LC’s lack of secretion of proinflammatory mediators after incubation with HPV VLPs can be overcome through treatment with a secondary activation stimulus.

**HPV-E7-specific CTLs Are Generated after LC Reactivation.** It is known that antigen degradation is inefficient in immature APCs, and internalized antigens can remain intact in intracellular compartments for several days (18). It is also known that DCs incubated with cVLPs initiate an E7 epitope-specific immune response, whereas LCs do not (11). Because we found that LCs treated with VLPs and subsequently CD40L up-regulated surface activation markers (11) and increased IL-12 p70 secretion (Fig. 1), we wanted to determine whether treatment with HPV16 L1L2-E7 cVLP and subsequently CD40L would also render the LCs capable of initiating a CD8+ HPV16-E7 epitope-specific immune response. The E7 protein containing cVLP contains a well-characterized human HLA-A*0201-restricted epitope (HPV16-E786–93) recognized by human CD8+ T cells (12).

DCs and LCs generated from HLA-A*0201-positive donors were loaded with the E786–93 peptide, HPV16-L1L2-E7 cVLP, or HPV16-L1L2-E7 cVLP followed by stimulation with CD40L. DCs and LCs loaded with the E786–93 peptide were previously treated with CD40L to ensure up-regulation of surface costimulatory molecules. The cells were cocultured with autologous CD8+ T cells and restimulated twice with DCs and LCs treated as described in the first stimulation. Seven days after the last restimulation, T cells were collected and tested for a specific response to HPV16-E786–93 by IFN-γ ELISPOT. As expected, HPV16-L1L2-E7 cVLP-loaded DCs, but not LCs, were able to stimulate IFN-γ-secreting T cells that respond to the E786–93 peptide similar to T cells induced by E786–93 peptide-loaded DCs and LC cultures (Fig. 2; Ref. 11). In addition, DCs and LCs incubated with cVLPs and CD40L were also able to elicit IFN-γ-secreting T cells specific for HPV16-E786–93 (Fig. 2). These data demonstrate that LCs incubated with cVLPs and an additional activation stimulus are able to elicit a HPV16-E7 epitope-specific immune response in T cells. The data additionally indicate that similar peptides are processed and presented by the DCs and LCs because both DCs and LCs treated with cVLPs and CD40L were able to stimulate similar CD8+–specific T-cell responses to the HPV16-E786–93 peptide.

**LCs Present Similar Peptides on Their Surface in the Absence of Costimulation.** In the current study, we found that LCs incubated with VLPs did not induce HPV-specific T cells to proliferate without the addition of CD40L. However, we did not know whether peptides are presented on the surface of LCs in the absence of costimulation or whether the VLPs are not processed in the immature LCs. Therefore, we sought to determine whether specific T cells generated from DCs stimulated with HPV-E7 protein containing cVLPs or T cells generated from LCs treated with cVLPs and CD40L could recognize and kill cVLP-loaded DCs or LCs in a cytotoxicity assay.

For this experiment, we generated T cells from DCs or LCs incubated with peptide, cVLPs, or cVLPs and CD40L as described in the previous experiment. These T cells were then assessed for their ability to kill unloaded, peptide-loaded, cVLP-loaded, or cVLP and CD40L–treated DCs and LCs in a standard 4-h51 Cr release assay. T cells generated from DCs treated with peptide, cVLPs, or cVLPs and CD40L were able to kill DCs (Fig. 3A) and LCs (Fig. 3C) treated with peptide, cVLPs, and cVLPs plus CD40L. These data indicate that LCs present HPV VLP peptides on their surface because DC-treated T cells could specifically kill cVLP-loaded DCs (Fig. 3C). Furthermore, the data indicate that similar peptides are presented by DCs and LCs. T cells generated from LCs treated with peptide or cVLPs plus CD40L were able to kill DCs (Fig. 3B) and LCs (Fig. 3D) treated with peptide, cVLPs, or cVLPs and CD40L. T cells generated from LCs treated with cVLPs could not specifically kill any of the targets over unloaded targets (Fig. 3, B and D). These data indicate that cVLP–treated LCs present similar HPV peptides on their surface as DCs, but in the absence of costimulation, rendering the HPV-specific T cells unable to proliferate and become activated. With the addition of CD40L, cVLP–loaded DCs up-regulate costimulatory molecules and provide the signals required for HPV-specific T cells to proliferate and become activated. These T cells are then able to recognize and kill both DC and LC cVLP–loaded targets. When taken together, the data indicate that HPV-specific T cells are likely unresponsive if the T cells interact with LCs that have taken up chimeric HPV VLPs and not encountered a secondary activation stimulus.
Discussion

Although APCs are not important for a productive virus life cycle, the mechanism of virus uptake by different APCs can have a profound effect on the outcome of resulting immune responses. Our data show that although DCs and LCs internalize HPV16 VLPs in a quantitatively equivalent fashion, the two professional APCs differ in the pathways of VLP uptake. DC uptake of HPV16 VLPs depends on the presence of clathrin-coated pits and actin rearrangements. The data also show that, to a small extent, DCs take up HPV16 VLPs by the generally nonspecific mechanism of macropinocytosis. It is not known which of these pathways leads to cross-presentation, but our data clearly show that cross-presentation occurs because DCs incubated with chimeric HPV16 L1L2-E7 VLPs initiate a HPV16-E7-specific CD8+ T-cell response.

LCs internalize HPV16 VLPs by a mode of uptake that is clathrin independent, caveola independent, and actin independent. Other viral particles have been shown to be taken up via non-clathrin, non-caveola-mediated uptake in mammalian cells, and actin is not required for endocytosis (19). No inhibitors have been described to inhibit this mode of uptake, but this uptake pathway does result in the presentation of HPV16-E7 peptides similar to DCs presented peptides. In contrast to DCs, however, LCs do not provide the necessary costimulatory signals required for the generation of an immune response from CD8+ T cells. Furthermore, with the addition of CD40L, LCs incubated with VLPs increase surface costimulatory molecule expression (11) and IL-12 p70 secretion. Both of these events, coupled with the fact that HPV VLP peptides are presented on the LC surface, resulted in the generation of HPV-specific CTLs. The CTL assay is more sensitive than the ELISPOT assay because fewer HPV peptide/MHC complexes on the LC surface would be adequate to render the LCs as a suitable target for HPV-specific CTLs. With the addition of CD40L, there may have been an increase in peptide/MHC complexes on the LC surface. This increase in peptide/ MHC density may account for the ability of the cVLP-treated LCs to initiate an epitope-specific response.

Our results clearly show that DCs and LCs use distinct HPV16 VLP uptake mechanisms. The outcome of these uptake mechanisms results in DCs promoting T-cell activation, whereas LCs do not. This finding may due to either an essential difference in antigen cross-presentation by these two cells or a similar presentation, but a difference in the activation of these two APCs in response to HPV VLPs. The latter possibility resembles the response DCs display to immune complexes as opposed to apoptotic cells. DCs endocytose immune complexes, become activated, and cross-prime T cells in the absence of additional stimuli (20). Apoptotic cell fragments enter DCs without triggering activation, but the immature DCs process and present antigen found in the apoptotic cells (21). With the addition of an activation stimulus, such as CD40L, DCs loaded with apoptotic cellular antigens are able to stimulate antigen-specific T cells (22). Therefore, the immune response generated to an antigen greatly depends on both the form of the antigen and the type of APC presenting the antigen. Of note is that in the present study, in vitro-cultured, monocyte-derived LCs were used that display characteristics of in vivo-derived LCs (i.e., Birbeck granules and E-cadherin expression). Differences in behavior between in vitro-cultured and in vivo-derived cells may still be present. Therefore, our results will have to be validated in an in vivo human skin organ culture system.

Various pathogens have learned to exploit uptake pathways as a means to gain entry to cells (7). This finding raises the possibility that some pathogens have developed means to avoid immune detection by avoiding certain endocytic pathways. HPV may have evolved to escape immune surveillance by the interaction with LCs, the resident APCs at the sites of primary infection. During a natural HPV infection, LCs will encounter HPV, and, because there is gradual turnover of LCs in the epidermis (23), over time LCs harboring HPV may migrate out of the epidermis toward T-cell areas of lymph nodes. When a secondary activation stimulus is encountered by the LCs before they interact with naïve HPV-specific T cells, the LCs may become immunostimulatory, thus having the ability to stimulate naïve HPV-specific T cells for the generation of an immune response. When an immunostimulatory signal is not encountered, the immature LCs will present HPV peptides on their surface in the absence of costimulation. When HPV-specific T cells are then encountered, the T cells will not react, reducing the potency of an HPV-specific immune response.

Fig. 3. HPV-E7-specific CTLs are generated after LC treatment with CD40L. CD8+ T cells were collected from in vitro immunizations with DCs and LCs that were either unloaded or loaded with HPV16-E7 peptide (TLGIVCPI), HPV16-L1L2-E7 cVLPs, or HPV16-L1L2-E7 cVLPs and subsequently CD40L. The T cells were washed and assessed for their ability to kill DCs and LCs that were either unloaded, loaded with HPV16-E7 peptide (TLGIVCPI), HPV16-L1L2-E7 cVLPs, or cVLPs and subsequently CD40L in a standard 4-h 51 Cr release assay at an E:T ratio of 100:1. T cells stimulated with the treatments are shown on the X axis, and the specific lysis for each target is shown as the bars on each graph. Results are represented as the mean ± SE of six measurements.
response. Thus, these new viral uptake data define a possible immune escape mechanism used by HPV to evade host immunity and prolong infection, which may explain why it takes some women infected with HPV months to years to clear the virus. Because HPV VLPs are being considered as a possible vaccine candidate against cervical cancer, our data suggest that the most optimal vaccine delivery method should not target LCs but should target other professional APCs, such as DCs, unless a secondary activation stimulus is also administered.

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References

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