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Human papillomavirus 16 virus-like particles use heparan sulfates to bind dendritic cells and colocalize with langerin in Langerhans cells

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Langerhans cells (LC), the immature dendritic cells (DC) that reside in epithelial tissues are among the first immune cells to encounter human papillomavirus (HPV) and are not activated by HPV virus-like particles (VLPs) in contrast to DC. The notion that the differences in response to HPV VLPs between LC and DC are associated with different types of cell binding and intracellular trafficking has been addressed. Inhibition experiments with heparin and sodium chlorate showed that heparan sulfates are necessary for HPV 16 VLPs to bind to DC but not to LC. Electron microscopy analysis demonstrated a colocalization of HPV 16 VLPs and langerin, which is expressed only by LC. This colocalization was observed on the cell surface but also in cytoplasmic vesicles. As anti-langerin antibodies, HPV 16 VLPs were associated with a faster entry kinetics in LC, as reflected by the fact that VLPs were observed near the nuclear membrane of LC within 10 min whereas more than 60 min were needed in DC. However, no difference between LC and DC was observed for the endocytosis pathway. HPV 16 VLPs entered in both DC and LC by a clathrin-dependent-pathway and were then localized in large cytoplasmic vesicles resembling endosomes.

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INTRODUCTION

Cervical cancer, the second most frequent cause of death by cancer amongst females worldwide, is associated with human papillomavirus (HPV) infection and represents one of the best examples of a human cancer preceded by a well characterized preneoplastic period (Richart & Barron, 1967; Richart, 1987). Despite the evidence that specific types of HPV are strongly implicated as causative agents in the aetiology of cervical cancer and its precursors, designated squamous intraepithelial lesions (SILs), HPV alone is not sufficient for cancer development (Chen *et al.*, 1993). The role of the intrinsic immunity in controlling HPV infection and the subsequent development of SILs is shown indirectly by the increased frequency of HPV-associated lesions in patients with depressed cell-mediated immunity (Petry *et al.*, 1994; Calore *et al.*, 1998; Ellerbrock *et al.*, 2000). Initiation and progression of HPV-associated cancer of the uterine cervix have been shown to be related with functional alterations of Langerhans cells (LC) within the cervical epithelium (Giannini *et al.*, 2002). LC are immature dendritic cells (DC) of myeloid origin resident in squamous epithelia, including skin and genital mucosa. The normal function of LC is to survey cell surfaces for pathogens, capture antigens by micropinocytosis or mannose receptor-mediated uptake, process captured proteins into immunogenic peptides, emigrate from tissue to lymph nodes and present peptides in the context of major histocompatibility complex (MHC) molecules to T cells, thereby initiating antigen-specific immune responses. LC are the only cells that constitutively express MHC class II molecules, CD1a (Fithian *et al.*, 1981) and langerin (CD207) (Valladeau *et al.*, 1999) at their cell surface. In addition, LC differ ultrastructurally from other DC through the presence of a unique pentalamellar cytoplasmic organelle, the Birbeck granule.

Because of their role in initiating antiviral immune response, DC and LC represent an ideal target for immune evasion by viruses. Several viruses interfere with DC function, especially measles virus (Grosjean *et al.*, 1997), vaccinia virus (Drillien *et al.*, 2000; Engelmayer *et al.*, 1999) and dengue virus (Tassaneetrithep *et al.*, 2003). The study of the interactions between HPV and DC or LC has been complicated by the fact that native infectious HPV virions cannot be isolated in large enough quantities either *in vitro* or *in vivo*. However, HPV–DC or HPV–LC interactions may be studied by using

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HPV virus-like particles (VLPs) that morphologically mimic native virions (Lenz *et al.*, 2001). HPV VLPs are non-enveloped icosahedral particles, composed of the L1 major capsid protein, which form via self-assembly in the nucleus after high level expression of L1 in eukaryotic cells (Kirnbauer *et al.*, 1993; Hagensee *et al.*, 1993; Rose *et al.*, 1993; Touze *et al.*, 1998). Fausch *et al.* (2002) reported that DC and LC are able to bind and internalize HPV VLPs. However, in contrast to DC, LC incubated with VLPs do not upregulate activation markers, do not initiate immune response and do not migrate *in vivo* out of skin explants (Fausch *et al.*, 2003).

In this study, we showed that HPV 16 VLPs bind similarly to DC and LC but use different types of cell binding and intracellular trafficking. Heparan sulfates are necessary for VLPs to bind to DC but not to LC. Electron microscopy analysis demonstrated a colocalization of HPV 16 VLPs and langerin in LC. However, no difference between LC and DC was observed for the endocytosis pathway.

METHODS

Production and purification of HPV 16 VLPs. HPV 16 VLPs were expressed in Sf9 cells infected with a recombinant baculovirus encoding the HPV 16 L1 protein and incubated at 27 °C for 72 h (Touze *et al.*, 1998; Bousarghin *et al.*, 2002). Cells were harvested by centrifugation, resuspended in PBS containing 0·5 % NP40 and allowed to stand at 4 °C for 30 min. Cell lysates were then centrifuged at 14 000 ***g*** for 15 min at 4 °C. The nuclear fraction was further resuspended in PBS and sonicated. This fraction was then loaded on the top of a preformed CsCl gradient and centrifuged at equilibrium in a Beckman SW28 rotor (24 h, 27 000 r.p.m., 4 °C). L1 positive fractions were pooled in PBS and centrifuged (3 h, 28 000 r.p.m., 4 °C). VLPs were resuspended in 0·15 M NaCl.

Carboxy-fluorescein diacetate succinimyl ester (CFDA-SE) labelling of HPV 16 VLPs. Purified HPV 16 VLPs were diluted in PBS (pH 8·5) to 0. 5 mg ml^{-1}. CFDA-SE (Molecular Probes) was then added to a final concentration of 100 μM (Drobni *et al.*, 2003; Bergsdorf *et al.*, 2003). Succinimyl ester group reacts with primary amines on the VLPs and forms stable conjugates. Inside cells, acetate on the CFDA-SE is cleaved off by intracellular esterases and yields highly fluorescent particles. After incubation for 2 h at room temperature, reactions were subjected to centrifugation in a sucrose density gradient using 30 and 70 % to separate VLPs from free CFDA-SE. Fractions (1 ml) were collected, L1 positive fractions were pooled in PBS and sedimented by ultracentrifugation. The pellet was resuspended in 0·15 M NaCl.

To characterize the integrity of CFDA-SE-labelled VLP, VLPs were tested by ELISA with the H16.V5 monoclonal antibody (mAb) (Christensen *et al.*, 1996). Plates were coated with the VLP at different dilutions, stored overnight at 4 °C, washed four times with PBS and blocked with 1 % fetal calf serum (FCS). The mAb was diluted at 1 : 5000 in PBS-FCS and allowed to bind to HPV VLP for 1 h at room temperature. After four stringent washes, the bound primary antibody was probed with the relevant streptavidin-labelled secondary antibody and the absorbances on the ELISA plate were read at 450 nm.

Generation of human DC and LC

DC culture. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized leukocyte enriched buffy coats of healthy adult donors by Lymphoprep (Lucron Bioproduct) density-gradient centrifugation applying 400 ***g*** at room temperature. After two washes, PBMCs were plated on tissue culture dishes at a density of 1×10^7 cells ml^{-1} in RPMI 1640 without serum and incubated for 24 h at 37 °C to select adherent cells. Non-adherent cells were washed away with RPMI 1640 and remaining adherent cells were cultured for 6 days in medium containing 800 U granulocyte–macrophage colony-stimulating factor (GM-CSF; Amoytop Biotech) ml^{-1} and 20 U interleukin (IL) 4 (Biosource) ml^{-1}. Routinely, about 70 % of DC displayed a $CD1a^+$ phenotype as assessed by flow cytometry.

LC culture. $CD34^+$ cells (1×10^5), isolated from cord blood mononuclear cells using the MACS Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec), were seeded in T25 flasks (Sarstedt) in 10 ml RPMI 1640 medium supplemented with 10 % FCS, antibiotics and 50 μM mercaptoethanol (all from Gibco-BRL). Cultures were supplemented with previously optimized concentrations of the following human molecules: 20 ng stem cell factor ml^{-1}, 10 U thrombopoietin ml^{-1}, 25 ng Flt3 ligand ml^{-1}, 200 U GM-CSF ml^{-1}, 50 U tumour necrosis factor (TNF)-α, ml^{-1} 100 U IL4 ml^{-1} and 5 ng transforming growth factor (TGF)-β ml^{-1}. All these agents were purchased from PeproTech, except for GM-CSF and IL4, which were obtained from Amoytop Biotech and Biosource, respectively. The cells were cultured at 37 °C in a humidified atmosphere and in the presence of 5 % CO_2. At day 7, the cellular density was adjusted to 2×10^4 per cm^2 and the cells were fed, at days 7 and 14, with GM-CSF, IL4, TNF-α and TGF-β. At day 18, cells were collected from cultures by vigorous pipetting to prepare single-cell populations.

Cellular uptake assay. To detect cell-associated VLPs, DC and LC were collected, respectively, on day 6 and 18 and transferred to new 24-well plates at a density of 2×10^5 cells ml^{-1}. DC or LC were exposed to CFDA-SE-labelled VLPs or VLPs alone (10 μg ml^{-1}) in RPMI 1640 medium for 1 h at 37 °C. Unbound labelled VLPs were removed by extensive washing with PBS. RPMI 1640 medium was then replaced with RPMI 1640 containing 10 % FCS and cells were incubated at 37 °C for 6 h. Flow cytometry measured the fluorescence of DC and LC, resulting from the uptake of labelled VLPs. The relative fluorescent intensity was measured using FACSVantage SE and by Cellquest software (Becton Dickinson). Typically, 20 000 events were collected for each experiment.

Transmission electron microscopy. DC and LC were incubated with VLPs as described above for different lengths of time. Cells were centrifuged at room temperature and prepared for electron microscopy. They were fixed at room temperature in 4 % glutaraldehyde (Laborimpex), post-fixed in 1 % osmium tetroxide (Laborimpex) for 1 h at 4 °C. Cells were then dehydrated in graded (70, 90, 100 %) ethanol solutions (VWR International). Fixed cells were embedded in Epon (Serva) and propylene oxide (Laborimpex), and hardened at 60 °C. Ultrathin sections were stained with uranyl acetate (Fluka) and lead citrate (Leica). These sections were examined using a transmission electron microscope LEO 906E (60 kV) (Zeiss).

Immunogold assay. DC and LC were incubated with HPV 16 VLPs. After two washes with PBS, specimens were incubated for 1 h at 4 °C with anti-langerin antibody (Beckman). Other sections were incubated with an unrelated antibody (anti-CD3 mAb) and with anti-α6 integrin antibody (Sigma). Fixed antibodies were revealed with anti-mouse IgG coupled to 20 nm colloidal gold particles (Tebu-bio). Cells were washed, fixed and treated as above for electron microscopy analysis.

Uptake inhibition studies. DC and LC were incubated with cell trafficking inhibitors at the following concentrations: 25 μM

chlorpromazine (Sigma), which induces the misassembly of clathrin-coated pits at the plasma membrane by preventing clathrin recycling, 2 μM cytochalasin D (Sigma), which causes the disruption of actin filaments and actin polymerization inhibition, 25 μM nystatin and 5 μg filipin ml^{-1} (Sigma), which inhibit the caveolae pathway. For the blocking of mannose-type receptors, LC were also preincubated with mannan from *Saccharomyces cerevisiae* 5 mg ml^{-1} (Sigma). Labelled VLPs were incubated with DC and LC in the presence of inhibitors for 1 h. Washed cells were then incubated in culture medium for 6 h. Fluorescence of DC and LC, resulting from the uptake of CFDA-SE-labelled VLPs, was measured by flow cytometry as described earlier.

Analysis of VLP–heparan sulfate interactions. Heparan sulfate expression in LC and DC was detected by anti-syndecan-1 and anti-syndecan-4 mAb (Santa Cruz Biotechnology) followed by goat anti-mouse phycoerythrin (PE) antibody and analysed by flow cytometry.

Labelled VLPs were incubated with heparin (heparin leo; Leo Pharma) at 37 °C for 1 h before adsorption to DC and LC. These cells were then washed with PBS and incubated with appropriate medium for 6 h at 37 °C. Internalization efficiency was assessed by detecting cell fluorescence by flow cytometry.

To examine the role of heparan sulfates in cell binding, DC and LC were also incubated with heparinase II (1 U ml^{-1}; Sigma), which cleaves α-*N*-acetyl-D-glucosaminidic linkage, in medium containing 20 mM Tris/HCl, 50 mM NaCl, 4 mM $CaCl_2$, 0·01 % BSA, pH 6·8. After incubation for 1 h at 37 °C and one wash with PBS, DC and LC were incubated with labelled VLPs.

To reduce the sulfation of heparan sulfate on the cell surface, DC and LC were cultured for 48 h in the presence of sodium chlorate (100 μM) as described previously (Bousarghin *et al.*, 2003a). Sodium chlorate acts as a competitive inhibitor for sulfate recognition by ATP sulfurylase, the first enzyme in the sulfate assimilation pathway (Farley *et al.*, 1978). Pretreated cells were incubated with labelled VLPs and fluorescence was measured by flow cytometry.

RESULTS

Binding and uptake of labelled VLPs by DC and LC

To investigate the interactions between viral particles and antigen-presenting cells, we studied the binding of HPV 16 VLPs to DC and LC generated *in vitro*. *In vitro* generated LC exhibited morphological (Birbeck granules) and immunohistochemical features of LC as observed by FACS analysis and surface phenotype [$CD1a^+$, $CD207^+$(langerin), E-cadherin$^+$ and $CCR6^+$] (Fig. 1). The proportion of cells expressing CD207 (langerin) in the entire population was 57 ± 9 % ($n = 8$). DC generated from adherent PBMC in the presence of GM-CSF and IL4 also presented classical phenotypical markers of DC ($CD1a^+$, $HLA\text{-}DR^+$, $CD80^+$) (data not shown). By electron microscopy, HPV 16 VLPs were shown to bind to both DC and LC (Fig. 2a). HPV 16 VLPs internalization by DC and LC was studied by using CFDA-SE/VLPs and flow cytometry. In order to investigate the integrity of VLP after CFDA-SE-labelling, an ELISA with the H16.V5 mAb specific for conformational epitopes (Christensen *et al.*, 1996) was performed. This antibody reacted similarly with VLP alone or with CFDA-SE/VLP, suggesting that the labelling of VLP with CFDA-SE does not induce VLP epitope conformation changes (Fig. 2b). To determine a suitable incubation time to study VLPs cell-internalization, a kinetics curve for CFDA-SE fluorescence

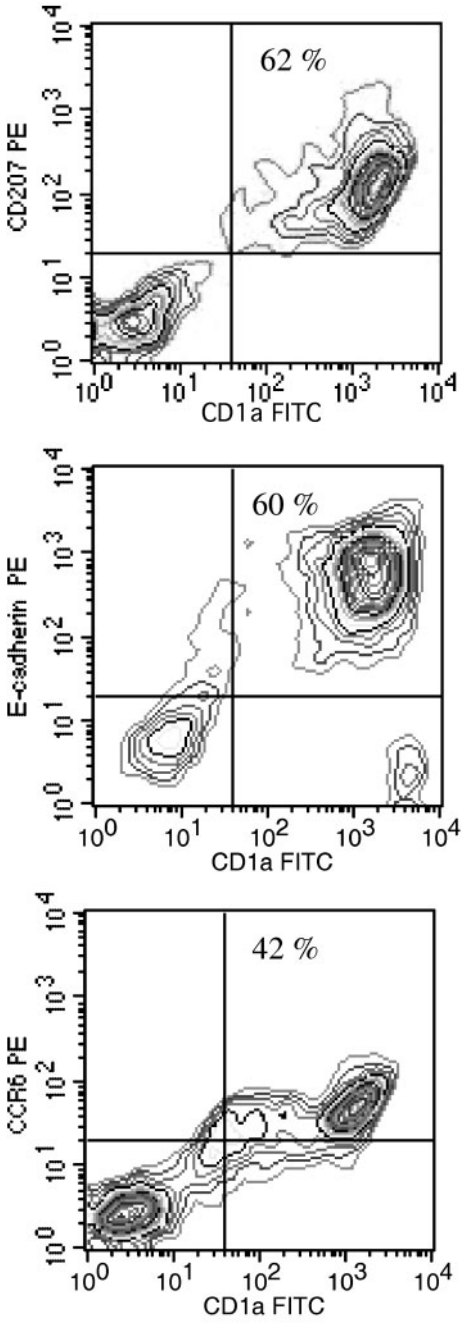

Fig. 1. Phenotypical analysis of LC generated *in vitro*. $CD34^+$ cells cultivated for 18 days in the presence of TNF-α, GM-CSF, IL4 and TGF-β1, after amplification with stem cell factor, thrombopoietin and Flt3 ligand, display the surface phenotype of LC, with a high expression of CD1a, CD207, E-cadherin and CCR6. The values correspond to % of $CD207^+$, E-cadherin and CCR6 cells in the entire population. One representative result is shown out of eight experiments.

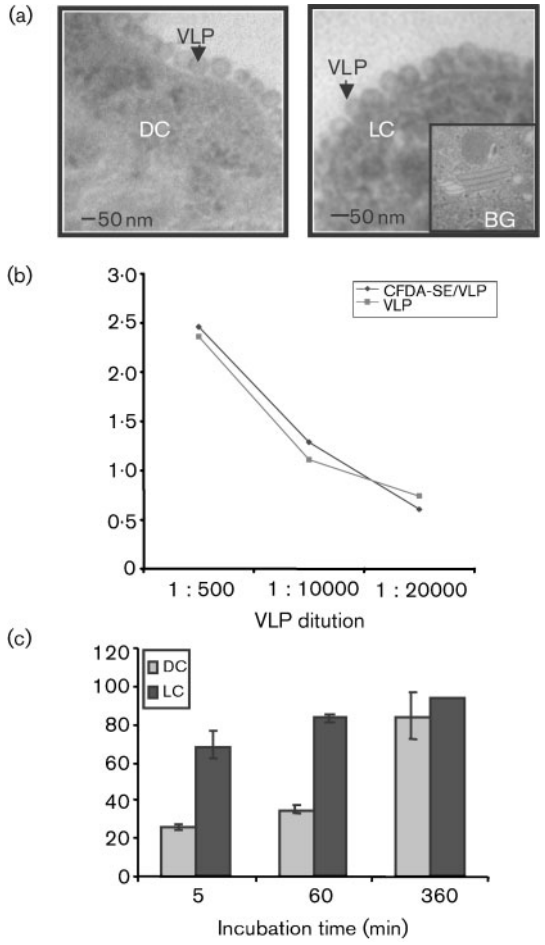

Fig. 2. Binding and uptake of HPV 16 VLPs in DC and LC. (a) HPV 16 VLPs binding in DC and LC at 4 °C (BG, Birbeck granules). (b) ELISA immunodetection of CFDA-SE/VLP with H16.V5 mAb. (c) Entry kinetics of CFDA-SE-labelled HPV 16 VLPs in DC and LC. Cells were incubated with labelled VLPs at 4 °C for 1 h, washed and incubated at 37 °C for different lengths of time. They were then trypsinized and incubated for 6 h at 37 °C. Fluorescence was detected by flow cytometry.

was studied. Mean fluorescence increased with time of incubation and after 6 h, 80 % of cells were fluorescent. To exclude the possibility that the detected fluorescence is caused by non-bound CFDA-SE dye co-migrating with the labelled VLPs, DC and LC were incubated with the fraction of control gradient loaded with CFDA-SE alone. No fluorescence was detected in DC and LC when incubated with this control gradient fraction (data not shown).

To study the cell entry kinetics, DC and LC were first incubated with labelled VLPs at 4 °C, washed and then incubated at 37 °C for 5, 10, 30, 60 min and 6 h (Fig. 2c). After a trypsin treatment to remove uninternalized VLPs and washings, cells were incubated at 37 °C for 6 h. Trypsin treatment reduced binding of VLPs as observed by Evander *et al.* (1997). When cells were incubated for 5 min with labelled VLPs, 69 ± 7 % of LC were fluorescent compared with 27 ± 1 % for DC (Fig. 2c). This difference was also observed after 1 h of incubation (fluorescence in 84 ± 2 % of LC and 35 ± 2 % of DC), suggesting that VLP entry in DC is slower than in LC. With increased incubation times, differences between the two cell populations decreased and at 6 h of incubation 84 ± 12 % of DC and 94 ± 1 % of LC were fluorescent. The faster VLPs entry in LC compared with DC was confirmed by electron microscopy (see Fig. 6). VLPs were observed in LC cytoplasm after only 5 min of incubation (see Fig. 6b). In contrast, a slower kinetic of VLPs entry in DC was observed since VLPs were detected in the cytoplasm after only 20 min of incubation (see Fig. 6e). In LC, VLPs were found near the nuclear membrane after an incubation time of 10 min (see Fig. 6c), whereas more than 1 h was needed for VLPs to reach the nucleus in DC (data not shown).

HPV 16 VLPs use heparan sulfates to bind to DC

Since the difference observed in entry kinetics between DC and LC could be explained by the use of different types of cell binding, we investigated if heparan sulfates are used by VLPs to enter into DC or LC. We first examined the ability of heparin to block binding and cell entry. Labelled HPV 16 VLPs were preincubated with heparin for 1 h at 37 °C and their capacity to enter into DC or LC was investigated by cell fluorescence (Table 1). In the presence of heparin, fluorescence was inhibited by 76 ± 5 % in DC whereas no inhibition (12 ± 2 %) was observed with LC (Table 1). To determine if this difference was not due to the origin of cells used to induce the differentiation of DC and LC, we also generated DC with $CD34^+$ cells and showed that the entry of VLPs was inhibited in the presence of heparin (51 ± 13 % of inhibition), suggesting that heparan sulfates are receptors for DC but not for LC. We also investigated the heparan sulfates expression by immunostaining with anti-human syndecan-1 and syndecan-4 antibodies. The results showed that DC and LC express similar levels of syndecan-4 whereas syndecan-1 was poorly expressed (Fig. 3).

The role of proteoglycans in the binding of HPV 16 VLPs to DC and LC was also assessed by inhibiting their sulfation with sodium chlorate. For this purpose, LC and DC were grown in the presence of sodium chlorate for 48 h before the addition of labelled VLPs. As shown in Table 1, HPV 16 VLPs entry was reduced by 71 ± 5 % in DC treated with 100 μM sodium chlorate compared with untreated cells. In contrast, only a 13 ± 2 % inhibition was detected in LC. To confirm the role of heparan sulfate in the DC entry of VLPs, DC were incubated with heparinase II, which induced a 51 % infection inhibition in DC whereas LC were not affected (data not shown).

Langerin is colocalized with HPV 16 VLPs

Since VLPs did not enter in LC by heparan sulfates and VLPs entry was faster in LC, we investigated if VLPs use the same

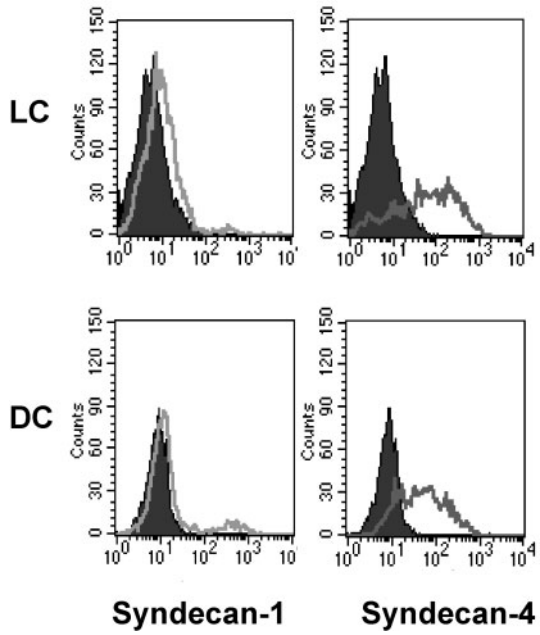

Fig. 3. Heparan sulfate expression in DC and LC. DC and LC were stained with anti-human syndecan-1 and syndecan-4 and analysed by flow cytometry.

method as langerin (CD207), an endocytic receptor in LC, to enter quickly into LC. Electron microscopy analysis showed that after an incubation at 37 °C for 5 min, anti-langerin mAb were present in LC in coated pits (Fig. 4a) or in small vesicles with clathrin morphology (Fig. 4b, c). A proportion of langerin was also localized intracellularly in large multivesicular compartments (Fig. 4d). Electron microscopy also showed that VLPs and langerin are colocalized (Fig. 4e, f, g, h). We observed a colocalization at the cell surface (Fig. 4e), but also in coated pits (Fig. 4f), which lead to the formation of vesicles with clathrin morphology (Fig. 4g). Langerin and VLPs were found inside large cytoplasmic vesicles in LC (Fig. 4h), whereas no langerin was detected in DC. To exclude potential artefacts due to a

Table 1. Effect of heparin and sodium chlorate on labelled HPV 16 VLPs entry in DC generated from either PBMCs or $CD34^+$ cells and LC

Results are expressed as mean percentages of cell fluorescence inhibition (±standard deviation).

Cell type	Cells fluorescence inhibition (%)	
	Heparin (250 IU ml^{-1})	Sodium chlorate (100 μM)
DC (PBMCs)	76±5	71±5
DC ($CD34^+$)	51±13	47±5
LC ($CD34^+$)	12±2	13±2

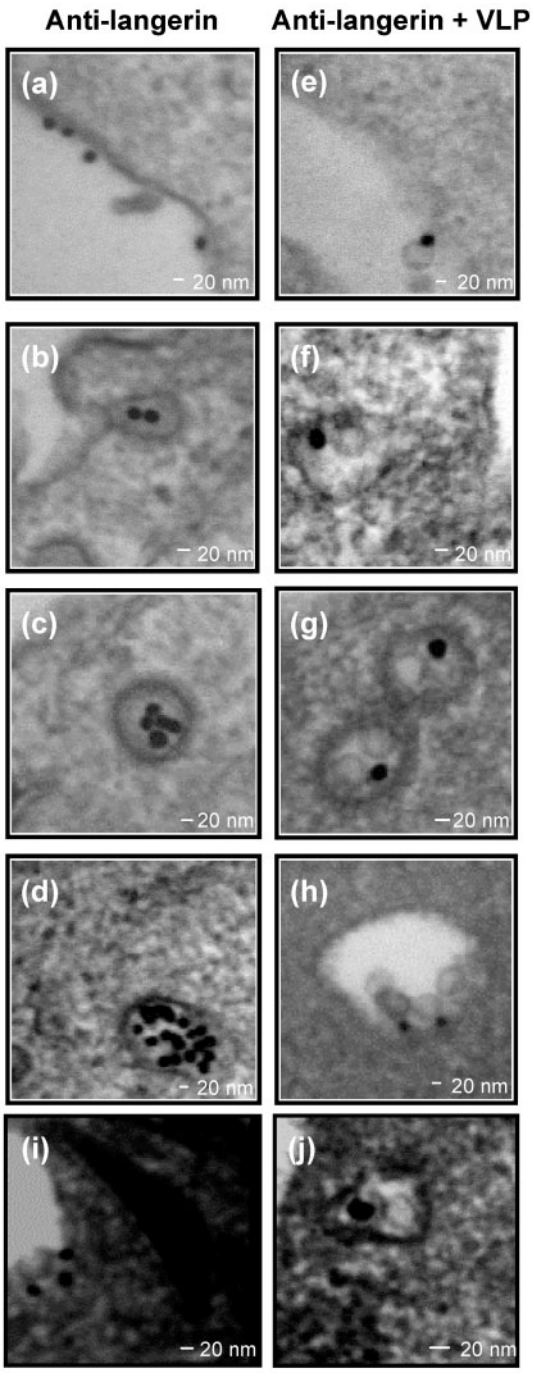

Fig. 4. Colocalization of HPV 16 VLPs and langerin in LC by immunogold electron microscopy. LC were incubated with anti-langerin alone (a–d) or with anti-langerin and HPV 16 VLPs (e–h). Langerin is detected by a secondary antibody associated with gold particles (20 nm), which were found at the cell surface (a, e), in clathrin vesicles (b, c, f, g) and in large vesicles resembling endosome (d, h). Colocalization of VLP (0·2 μg ml^{-1}) and langerin (i, j) in LC.

high level of VLP (10 μg ml^{-1}), we used VLP at a lower concentration (0·2 μg ml^{-1}) and showed, by electron microscopy, that langerin and VLP also colocalize in these conditions (Fig. 4 i, j).

As a negative control, we preincubated LC with an antibody not specific for LC such as anti-CD3 mAb. In the presence of anti-CD3 mAb, no gold particles were detected (data not shown). We have also tested the ability of anti-α6 integrin mAb to interact with the HPV 16 VLPs. The results revealed a low expression of integrin on LC and the absence of colocalization with HPV16 VLPs (data not shown).

To determine the specificity of VLP/LC-binding, cells were preincubated with 5 mg mannan ml^{-1} before adding labelled VLP. In the presence of mannan, a 40 % inhibition of fluorescence was observed for LC (see Fig. 5e).

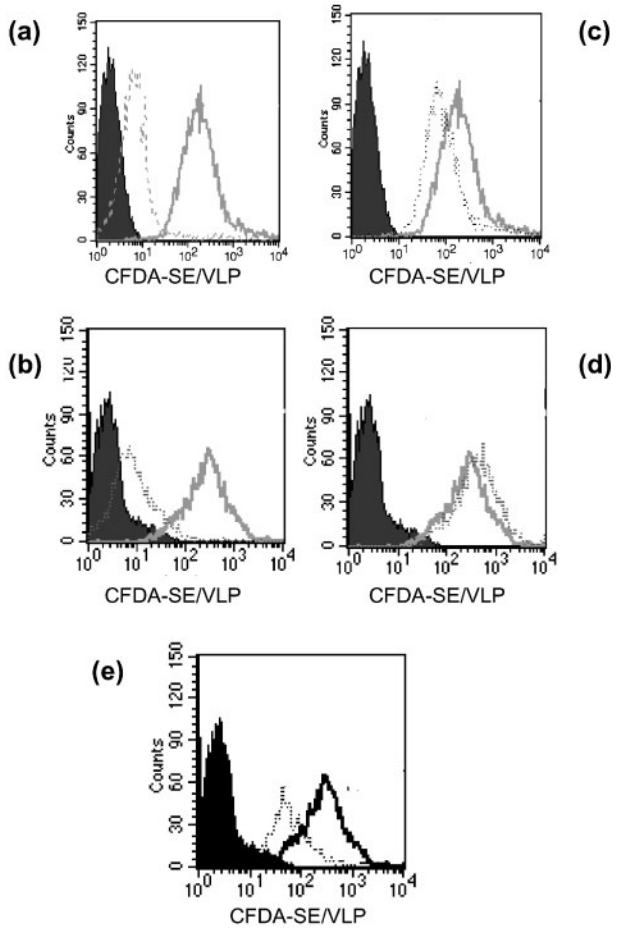

Fig. 5. HPV 16 VLPs endocytosis in LC. Cells were pretreated with chlorpromazine (a), cytochalasin D (b), nystatin (c), filipin (d) and mannan (e). After 1 h of incubation, CFDA-SE-labelled VLPs were added. Cells were then washed with PBS and incubated with culture medium for 6 h before detecting fluorescence by flow cytometry. Solid black line, LC + PBS; solid grey line, LC + CFDA-SE/VLP; dashed line, LC + CFDASE/VLP + drug.

HPV 16 VLPs endocytosis in DC and LC

Since VLP could be internalized by different pathways (Selinka *et al.*, 2002; Bousarghin *et al.*, 2003b; Day *et al.*, 2003; Fausch *et al.*, 2003), we examined the role of clathrin-coated-pit-mediated endocytosis in the entry of HPV 16 VLPs in LC and DC. These cells were preincubated with chlorpromazine, which inhibits clathrin-mediated endocytosis. A 70 % inhibition in HPV 16 VLPs endocytosis was observed in both DC and LC populations after chlorpromazine treatment (Fig. 5a). We also tested the effect of cytochalasin D, a potent inhibitor of actin-dependent cellular processes. In the presence of this drug, VLP fluorescence was shown to decrease (Fig. 5b). LC and DC were also treated with nystatin and filipin, which inhibit caveolae-mediated endocytosis and no inhibition was observed with these drugs (Fig. 5c, d). To visually analyse the uptake of HPV VLPs into DC and LC, transmission electron microscopy analysis was also performed. In the presence of HPV 16 VLPs, DC and LC plasma membranes were rich in coated pits where VLPs accumulated (Fig. 6a, d). These coated pits budded off to yield coated vesicles with clathrin morphology confirming that HPV enter by endocytosis through clathrin-coated pits in both DC (Fig. 6e) and LC (Fig. 6b). VLPs were contained in cytoplasmic coated vesicles (Fig. 6b), which enlarged with time by fusion of different coated vesicles (Fig. 6e). The HPV 16 VLPs remained clustered in large vesicles resembling endosome (Fig. 6c, f). At this step, most vesicles were smooth rather than coated. In LC, vesicles containing VLPs were located near the nucleus after an incubation of 10 min (Fig. 6c), whereas more time was needed for DC (data not shown).

When using VLP at low concentration (0·2 μg ml^{-1}), they were also observed in coated pits and cytoplasmic vesicles of LC with a clathrin morphology (Fig. 6g, h).

DISCUSSION

HPV 16 VLP-based vaccine formulations have been shown to induce strong humoral and cell-mediated immunity and to confer protection against HPV 16-related SILs (Koutsky *et al.*, 2002; Harro *et al.*, 2001; Evans *et al.*, 2001). These data suggest that vaccine antigens are efficiently presented to the immune system and may be correlated to the ability of HPV VLPs to activate DC (Rudolf *et al.*, 2001; Fausch *et al.*, 2002). In contrast, Giannini *et al.* (2002) demonstrated that SILs are deficient in presenting antigens to T cells suggesting that the function of LC is incapacitated in the HPV-transformed epithelium. In agreement with these observations, Fausch *et al.* (2002) demonstrated that LC in the presence of HPV 16 VLPs are unable to activate T cells.

The purpose of the present report was to investigate further the interactions between HPV 16 VLPs and DC or LC. We showed that these antigen-presenting cells differentially interact with HPV 16 VLPs. Although DC and LC are able to bind and internalize HPV 16 VLPs, difference in

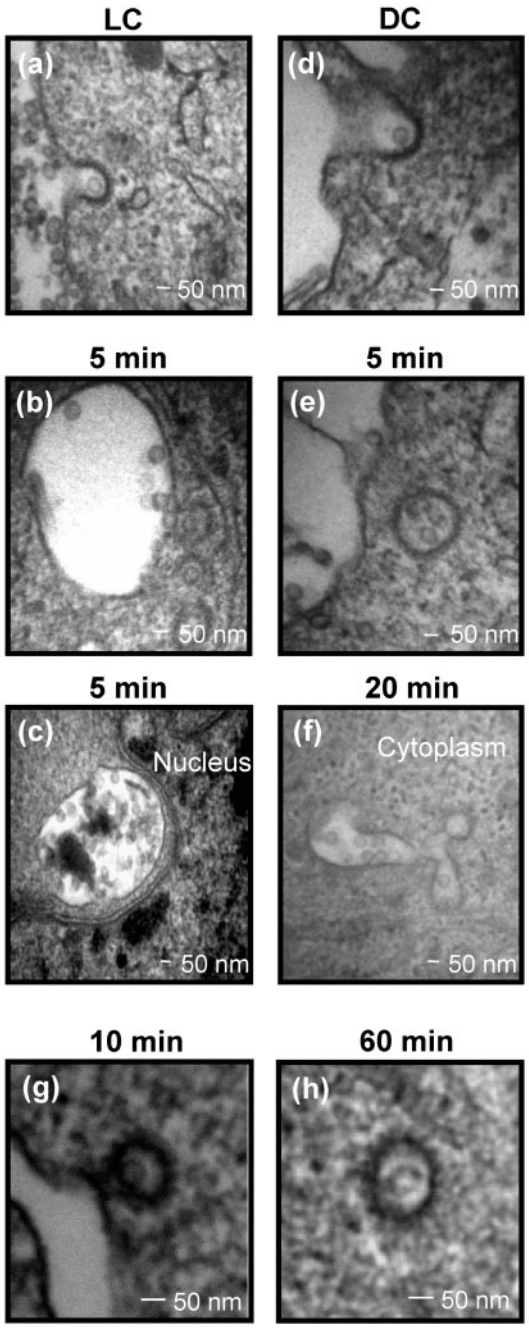

Fig. 6. Clathrin-dependent pathway of HPV 16 VLPs into LC (a–c) and into DC (d–f): binding and coated pits formation (a, d); clathrin vesicle formation (e); clathrin vesicle fusion (b, f); VLPs near nuclear membrane (c); demonstration of HPV 16 VLPs (0·2 μg ml^{-1}) in coated vesicles (g) and clathrin vesicles (h) of LC.

entry kinetics was observed between both cell types with a faster entry of HPV 16 VLPs into LC compared with DC. As a correlation between entry kinetics and cell receptors has been previously shown with other viruses (Narayan *et al.*, 2003), we looked for potential different types of HPV 16 VLPs binding to DC and LC. The most described epithelial receptors for HPV are heparan sulfates and integrin (Evander *et al.*, 1997; Giroglou *et al.*, 2001; Joyce *et al.*, 1999). Our inhibition experiments with heparin or sodium chlorate confirm that DC use heparan sulfates to bind HPV 16 VLPs in contrast to LC in agreement with the data of Yan *et al.* (2004) showing that HPV 6b L1 VLPs uptake by DC is inhibited in the presence of heparin. Other pathogens such as *Chlamydia trachomatis* also use heparan sulfates to enter DC (Matyszak *et al.*, 2002). Mummert *et al.* (2002) suggested that heparan sulfates moiety detected on DC may also play an immunoregulatory role. In fact, heparan sulfates and their degradation products have been reported to deliver costimulatory signals to T cells and maturation signals to DC (Wrenshall *et al.*, 1991; Kodaira & Platt, 2000; Johnson *et al.*, 2002). This may explain why HPV VLPs initiate DC (but not LC) maturation and activation.

HPV 6b genotype has been shown to interact with epithelial cells by α6 integrin (Evander *et al.*, 1997). However, this receptor is poorly expressed in DC and LC and probably not implicated in the differences of HPV 16 VLPs uptake between both cell types. Yan *et al.* (2004) also reported that α6 integrin is not responsible for the initiation of binding of HPV 6b L1 VLPs by LC.

LC express several receptors that facilitate antigen recognition and uptake, including Fc receptors, DEC205 multilectin and langerin. Langerin is exclusively expressed by LC (Valladeau *et al.*, 1999) and is localized not only on the cell surface, but also intracellularly in close association with Birbeck granules. This molecule functions as an endocytic receptor (Valladeau *et al.*, 1999, 2000). Upon binding to the cell surface, anti-langerin antibodies are rapidly internalized and delivered to Birbeck granules (Valladeau *et al.*, 1999, 2000). Electron microscopy analysis showed that HPV 16 VLPs colocalize with anti-langerin antibodies. Indeed they were detected together in coated pits and in large cytoplasmic vesicles. These data are in agreement with recent studies showing that the processing pathway of HPV 6b L1 VLPs in LC is associated with $langerin^{+}$ compartments (Yan *et al.*, 2004) and that other viruses, such as HIV, also use langerin and lectin or mannose to enter into LC and DC, respectively (Turville *et al.*, 2002). Our electron microscopy analysis showed a colocalization of HPV 16 VLPs and langerin in large cytoplasmic vesicles resembling to endosome, which may be related to the observation that langerin is associated with the CD1a recycling compartment (McDermott *et al.*, 2002). HPV 6 VLPs were also shown to be colocalized with CD1a+ endosome (Yan *et al.*, 2004), but the loading mechanism was not determined. When mannan, which was shown to be able to inhibit HIV entry in LC, was used a 40 % inhibition of VLP entry was observed suggesting that other proteins are also involved in the VLP binding to LC. It will be interesting to determine the direct effects of langerin in VLP binding by further experiments and if other lectins are also involved in the uptake of VLPs by LC.

As the cell surface binding of HPV 16 VLPs differed in DC and LC, we wondered if their internalization was also different as suggested by Fausch *et al.* (2003) and Yan *et al.* (2004). Fausch *et al.* (2003) showed that DC use a clathrin-mediated endocytosis whereas LC use a different pathway, which is not associated with clathrin or caveolae. Yan *et al.* (2004) show that HPV 6 VLP enter in LC by a caveolae-dependent pathway. Entry pathways can be inhibited at different stages by specific inhibitors, such as chlorpromazine, cytochalasin D, nystatin and filipin. When using these drugs, HPV 16 VLP entry was only inhibited in the presence of chlorpromazine and cytochalasin D whereas no inhibition was observed with the caveolae inhibitors, nystatin and filipin. These results suggest that VLP enter in DC and LC by a clathrin-mediated pathway and are in agreement with the direct demonstration of HPV 16 VLPs in coated pits and cytoplasmic vesicles with a clathrin morphology in LC and DC preparations processed for electron microscopy. The differences between these studies might be related to the different methods used to analyse HPV VLPs internalization although we cannot exclude different endocytosis pathways according to the origin of cells used to generate LC. In agreement with this hypothesis, the LC used in this study poorly expressed syndecan-1 in contrast to the LC generated by Yan *et al.* (2004).

The fact that HPV 16 VLPs use different types of cell binding to DC and LC might have important functional consequences. It has been shown that the maturation of DC and LC depends not only on receptors involved in the uptake of pathogens but also on receptors directly acting on DC activation (Niedergang *et al.*, 2004). Moreover, a cooperation between these two types of receptors has been shown to be important for the diversity of DC or LC responses and to determine the outcome of the immune response (Gantner *et al.*, 2003). As receptors implicated in DC maturation activate nuclear factor kappa B and mitogen-activated protein kinase pathways (Janeway & Medzhitov, 2002), it will be interesting to characterize the cytoplasmic factors implicated in DC or LC maturation in the presence of VLPs and to determine if heparan sulfates are able to interact with these factors.

Future research addressing the recognition of HPV VLP by DC and LC will provide insight on how these cell surface receptors regulate antigen-presenting functions and may contribute to the development of novel strategies that specifically prevent or treat HPV infections and associated (pre)neoplasic lesions.

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