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ORIGINAL ARTICLE

High expression of PGE_2 enzymatic pathways in cervical (pre)neoplastic lesions and functional consequences for antigen-presenting cells

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Abstract Although human papillomavirus (HPV) DNA is detected in the majority of squamous intraepithelial lesions (SIL) and carcinoma (SCC) of the uterine cervix, the persistence or progression of cervical lesions suggest that viral antigens are not adequately presented to the immune system. This hypothesis is reinforced by the observation that most SIL show quantitative and functional alterations of Langerhans cells (LC). The aim of this study was to determine whether prostaglandins (PG) may affect LC density in the cervical (pre)neoplastic epithelium. We first demonstrated that the epithelial expression of PGE_2 enzymatic pathways, including cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase 1 (mPGES-1), is higher in SIL and SCC compared to the normal exocervical epithelium and inversely correlated to the density of CD1a-positive LC. By using cell migration assays, we next showed that the motility of immature dendritic cells (DC) and DC partially differentiated in vitro in the presence of PGE_2 are differentially affected by PGE_2. Immature DC had a lower ability to migrate in the presence of PGE_2 compared to DC generated in vitro in the presence of PGE_2. Finally, we showed that PGE_2 induced a cytokine production profile and phenotypical features of tolerogenic DC, suggesting that the altered expression of PGE_2 enzymatic pathways may promote the cervical carcinogenesis by favouring (pre)cancer immunotolerance.

Keywords Prostaglandins · Human papillomavirus · Dendritic cells · Cell migration · Immunotolerance

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Introduction

Chronic infection of keratinocytes in the uterine cervix by human papillomavirus (HPV) is associated with the development of squamous intraepithelial lesions (SIL) and squamous cell carcinoma (SCC) [64]. Despite the evidence that HPV is strongly implicated as the causative agent of cervical cancer and its precursors, HPV infection alone is not sufficient for cancer development. The intrinsic immune status plays a key role in controlling HPV infection and the subsequent development of SIL, as shown indirectly by the increased frequency of HPV-associated lesions in immunodeficient patients such as in AIDS [15, 44].

A substantial majority of cervical SIL and cancers develop within a specific region of the cervix, the transformation zone (TZ), where the glandular endocervical epithelium is transformed progressively into a mature squamous epithelium. This epithelium is composed not only of keratinocytes (the primary target of HPV) but also of a type of immature dendritic cell (DC), the Langerhans cells (LC), which are important for the immunosurveillance of the epithelium. Several groups have reported that the density of

LC is decreased in SIL of the cervix compared with the exocervical epithelium [2, 29, 40]. This quantitative deficiency of LC was also found to be associated with a reduced functional capability [19]. In addition to immunogenic DC, emerging evidence also suggests that DC partially differentiated in a tumour environment may have tolerogenic properties and promote T regulatory cell expansion or proliferation [18].

Among the factors potentially responsible for DC alterations are the prostaglandins (PG) produced in (pre)neoplastic lesions. In response to various stimuli, PG production is initiated by the release of arachidonic acid from membrane phospholipids by A2 phospholipase. Arachidonic acid is then converted into PGH_2 by COX-1 and COX-2 cyclooxygenases. This is followed by the transformation of PGH_2 into different PG by the action of PG-synthases [23]. The most actively investigated molecule is PGE_2 which results from the enzymatic activity of PGE_2 synthases. Many studies have reported the implication of COX-2 in the development or progression of malignant tumors [4]. COX-2 overexpression has been demonstrated in many cancers [58] and is sufficient to induce tumorigenesis in transgenic mice [36]. Inversely, deficiency of COX-2 reduces cancer development in animal models [61]. PGE_2 may promote the carcinogenesis by different mechanisms, such as apoptosis inhibition [52], cell proliferation [58], angiogenesis [49] stimulation and immunosuppression [23]. Accordingly, the increased production of PGE_2 within the tumor environment has been found to be associated with the induction of regulatory T cells [1, 51], T-cell inhibition [6] and PGE_2 was recently qualified as a potential candidate for the induction of tolerogenic DC in tumor environment [63].

In the present work, by using immunohistological techniques, we found a higher epithelial expression of PGE_2 enzymatic pathways (COX-2 and mPGES-1) and a lower density of CD1a-positive LC in cervical epithelial metaplasia (EpM), SIL and SCC compared to the normal exocervical epithelium. In addition, cell migration experiments demonstrated that the motile properties of immature DC and DC generated in the presence of PGE_2 are differentially influenced by PGE_2. Finally, we showed that DC differentiated in the presence of PGE_2 display a tolerogenic phenotype, suggesting that PGE_2 may promote cervical carcinogenesis by decreasing the ability of the immune system to mount a protective response against HPV antigens.

Materials and methods

Cervical biopsy specimens

Formalin-fixed, paraffin-embedded cervical specimens were retrieved from the archives of the Pathology Department at the University Hospital of Liege. These tissues included 12 epithelial metaplasia (EpM), 18 SIL, 15 invasive squamous cell carcinoma (SCC) and 38 paired normal exocervical tissues from the same patients. Seven specimens contained both SIL and SCC components. Except for two biopsies, all tissues were obtained from pre-menopausal women (mean age: 45 years). HPV DNA was not found in the exocervical and metaplastic tissues in contrast to SIL and SCC which were HPV positive. The protocol was approved by the Liege University Hospital Ethics Committee.

Cell culture and biological reagents

SiHa, CaSki, and C4-II are tumorigenic cervical carcinoma-derived keratinocyte cell lines. CaSki and SiHa contain respectively 600 and 1–2 copies of integrated HPV16 DNA [16, 43], whereas the C4-II cell line contains HPV-18 DNA sequences. The CK2 cell line was established by transfection of human cervical keratinocytes with HPV-33 DNA and is not tumorigenic in nude mice [20]. In contrast to these four cell lines, C33-A is a HPV negative cell line derived from a cervical cancer biopsy. All these cell lines were grown and maintained in RPMI (GIBCO, Merelbeke, Belgium) containing 2% fetal calf serum (GIBCO), 1% non-essential amino acid (GIBCO), 1% sodium pyruvate (GIBCO) and 1% penicillin-streptomycine (3,000U/ml, GIBCO).

Dendritic cell cultures

Dendritic cells (DC) were generated by culturing adherent fraction of human peripheral blood mononuclear cells (PBMC) as previously described [27, 47]. Briefly, PBMC were isolated from leukocyte-enriched buffy-coats by centrifugation on Ficoll-Hypaque. After washings at low centrifugation speed to discard a maximum of platelets, PBMC were plated in six-well dishes (Nunclon Multidishes, NUNC, Denmark) at a density of 10×10^6 PBMC per well in 3 ml of medium without FCS. After 18 h at 37°C, non-adherent cells were discarded by two gentle washes and the plastic adherent fraction was cultured with 800 U/ml human recombinant GM-CSF (Amoytop Biotech, Xiamen, China), 40 U/ml IL-4 (ImmunoTools, Friesoythe, Germany) in 3 ml of RPMI 10% FCS-50 µM Mercaptoethanol. Cultures were incubated or not with PGE_2 (Cayman Chemical, Ann Arbor, USA) and harvested with PBS-EDTA 1 mM at day 7. In some experiments (phenotype assessment, mixed lymphocyte reaction assay and ELISA), 1 mg/ml LPS (Sigma-Aldrich, Saint Louis, USA) was added in DC cultures at day 6 to induce the maturation. Phenotype analysis was performed by flow cytometry with the following mouse monoclonal antibodies: NA1/34 (IgG2a, anti-CD1a), HB15e (IgG1, anti-CD83), AB3 (IgG2a, anti-HLA-DR), W6/32 (IgG2a, anti-HLA-ABC), Leu-3a (IgG1, anti-CD4)

from Dako (Glostrup, Denmark), 2331 (FUN-1) (IgG1, anti-CD86), ICAM-1 (IgG1, anti-CD54), Leu-4 (IgG2a, anti-CD3), Leu-M3 (IgG1, anti-CD14) from BD PharMingen (San Diego, CA) and 150503 (IgG2a, anti-CCR7) from R&D systems (Mineapolis, MN). Results were expressed as percentages of positive cells. DC generated for this study constituted a 90% pure cell population based on several criteria including morphology, forward-scatter and side-scatter values observed by FACS analysis and surface phenotype ($CD1a^+$, $HLA\text{-}DR^+$, $HLA\text{-}ABC^+$, $CD4^+$, $CD54^+$, $CD86^{dim}$, $CD3^-$, $CD20^-$ and $CD14^-$).

Immunohistochemistry

Serial sections of cervical biopsy specimens underwent immunohistochemical staining using monoclonal antibodies directed against COX-2 (Cayman Chemical, 1/200), mPGES-1 (Cayman Chemical; 1/400) and CD1a (Novocastra, Newcastle, UK; 1/50). After deparaffination and antigen retrieval with EDTA buffer at 120°C in a pressure boiler for 11 min or in citrate buffer heated for 5 min at 750 watts and at 350 watts in a microwave oven, sections were incubated with the primary antibodies for 1 h at room temperature or overnight at 4°C. After washings, the revelation was performed with the use of appropriate secondary antibodies and the Envision kit (Dako, Glostrup, Denmark) (COX-2) or the peroxydase LSAB®2 system (Dako) (mPGES-1, CD1a), according to the supplier's recommendations. Positive cells were visualized by a 3,3′-diaminobenzidin substrate (Dako) and the sections were counterstained with hematoxylin. The control staining conditions were either the use of a control IgG (for CD1a and COX-2 immunostaining), as a negative control or the neutralization of the mPGES-1 antibody with mPGES-1 blocking peptide (Cayman Chemical) mixed together in a 1:1 ratio.

Immunostaining assessement

The immunolabelled epithelium was evaluated by using a semi-quantitative score of the intensity and extent of the staining according to an arbitrary scale. For staining intensity, 0 represented samples in which the immunoreactivity was undetectable whereas 1, 2 and 3 denoted samples with, respectively, a low, moderate and strong staining. For staining extent, 0, 1, 2 and 3 represented samples in which the immunoreactivity was detectable, respectively, in <5%, 6–25%, 26–75% and >75% of the cells. In order to provide a global score for each case, the results obtained with the two scales were multiplied, yielding a single scale of 0, +1, +2, +3, +4, +6 et +9 [13]. For CD1a immunostaining, the scores (0, 1, 2, 3) were given according to the number of positive cells per high magnification (400×) field (0, 1–5, 5–20 and >20 positive cells).

DC chemotaxis assay

DC migration was evaluated using a chemotaxis microchamber technique (48-well Boyden microchamber; Neuroprobe, Cabin John, MD,USA). The lower wells of the chemotaxis chamber was filled with 27 µl of human fibroblast culture supernatants. This conditioned medium is known to produce chemoattractant molecules for DC [34] and was supplemented or not with PGE_2 (Cayman Chemical, 1 mM). A non conditioned medium was used as control for random migration. Each condition was repeated ten times. After phenotypic characterisation, DC differentiated or not in the presence of PGE_2 were harvested and 55 µl of DC suspension (2×10^6 cells/ml) were applied to the upper wells of the chamber, with a polyvinylpyrollidone-free polycarbonate membrane 8-µM pore filter (Poretics Corp., Livermore, CA) separating the lower wells. These membranes were coated by incubation with 100 µg/ml gelatin in 0.1% acetic acid solution. The chamber was incubated for 5 h at 37°C in a 5% CO2/95% air atmosphere. The cells having migrated to the underside of the filter were fixed and stained with Diff Quick Stain set (Baxter Diagnostics AG, Düdingen, Switzerland). The upper side of the filter was scraped to remove residual non migrating cells. One random field was counted per well using an eyepiece with a calibrated grid to evaluate the number of fully migrated cells.

Cytokine measurement by ELISA

Cytokine production by control DC and DC differentiated in the presence of PGE_2 was quantified by ELISA with the following commercial kits: IL-10 (Biosource, Nivelles, Belgium) and IL-12 p70 (R&D Systems).

Western Blotting analysis

Cells were lysed in a buffer containing 50 mM TRIS pH 7,5, 300 mM NaCl, 1 mM EDTA, 1%, Igepal CA-630 (Sigma-Aldrich), 1 mM PMSF (Sigma-Aldrich) and protease inhibitors (Roche, Bale, Switzerland). After quantification (BCA protein assay, Pierce, Rockford, USA), 25 micrograms of proteins were separated by electrophoresis on 4–12% NuPAGE® polyacrylamide gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes. The membranes were subsequently blocked with 5% skim milk during 30 minutes and incubated overnight at 4°C with anti-β-actin (Sigma-Aldrich), anti-mPGES-1 and anti-COX-2 antibodies (Cayman Chemical). The membranes were then washed and incubated with appropriate secondary antibodies. After washings, the protein bands were detected using an enhanced chemiluminescence system (ECL Plus, Amersham Biosciences, Piscataway, USA).

PGE_2 radioimmunoassay

PGE_2 levels were determined in cell culture supernatants according to a previously described radioimmunoassay (RIA) [50] using a polyclonal rabbit antiserum which does not cross react with other prostanoids (TxB_2, 6-keto-PGF1a, PGA_2) or fatty acids (arachidonic, linoleic, oleic). The ^{3}H-labelled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule PGE_2 from Sigma–Aldrich. PGE_2 was measured in triplicate in a 100-µl aliquot of each sample. After 48 h of incubation at 4°C, free antigen was separated from antibodies–antigen complexes by charcoal precipitation and centrifugation (20 min at 1,000*g*, 4°C). The supernatant radioactivity was counted by liquid scintillation. Intra- and interassay coefficients of variation were 6 and 10%, respectively. The recovery of known amounts of PGE_2 from the culture medium was satisfactory (90–97% for the PGE_2 ranging from 2,500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

Mixed lymphocyte reaction assay

The effector population consisted of DC grown in GM-CSF + IL-4 for 7 days. At day 6, 1 mg/ml LPS was added in DC cultures to induce the maturation. Cultures were incubated or not with PGE_2. After centrifugation for 10 min at 200 g, the cells were adjusted to equal number in RPMI 5% human pooled AB serum, with exogenous cytokines (GM-CSF and IL-4) and irradiated with 8,000 rad. Varying numbers of effector cells (625 to 40,000 cells per well) were then added to round-bottomed 96-well Nunclon plates containing 2×10^5 allogeneic PBMC per well (effector:responder ratios from 1:320 to 1:5). The tests were performed in sextuplate. A proliferative response was measured after 6 days of culture by adding 1 µCi of [^{3}H] thymidine to each well. Cells were harvested 18 h later using an automated sample harvester (Packard, Canberra, Tilburg, The Netherlands) and counted using a liquid scintillation counter (Top Count, Packard, Canberra). Thymidine uptake was <700 cpm in irradiated stimulator cells and <150 cpm in responder cells cultures alone.

Statistical analysis

Statistical analysis was performed by using the non-parametric Mann–Withney test (Instat Mac 2.01 software; Graph-Pad Software, San Diego, CA). Differences were considered as statistically significant when $P < 0.05$. The Spearman correlation test was also used to determine whether COX-2 and mPGES-1 labelling scores were significantly correlated.

Results

The density of CD1a-positive LC is inversely correlated in vivo with the expression of COX-2 and mPGES-1

In order to determine the role of PGE_2 in the alterations of LC during cervical carcinogenesis, the density of CD1a-positive intraepithelial cells was correlated with the expression of COX-2 and mPGES-1 in EpM, SIL, SCC and paired normal exocervical tissues from the same patients.

In the normal exocervical epithelium, $CD1a^+$ cells were intermingled with keratinocytes in the (para)basal and intermediate cell layers (Fig. 1a). In contrast, the density of these cells was significantly lower in EpM, SIL and SCC (Fig. 1d, g, j). The CD1a score was higher in normal exocervical epithelium than in EpM, SIL and SCC (Fig. 2a). Figure 1b illustrates the expression profile of COX-2 in the normal exocervix. There was no positive staining in the epithelium nor in the stroma. In contrast, COX-2 was detected in the epithelium of EpM and SIL. However, COX-2 staining was not homogeneous in the entire epithelial layer (Fig. 1e, h). Among the 12 EpM and the 18 SIL tested, respectively, 9 (75%) and 11 (61%) demonstrated an increased COX-2 expression as compared with paired normal exocervical tissues (Fig. 3a, c). Figure 1k illustrates the expression of COX-2 in SCC. Increased COX-2 immunoreactivity was observed in SCC as compared with exocervical epithelium. As in EpM and SIL, the staining was heterogeneous with the presence of COX-2 positive cells adjacent to negative neighbouring cells. Among the 15 SCC tested, 14 (93%) showed an increased COX-2 expression as compared with paired exocervical tissues (Fig. 3e). When the average of all the individual staining scores was considered, COX-2 expression was significantly higher in EpM ($P < 0.01$), SIL ($P < 0.01$) and SCC ($P < 0.001$) than in the exocervix (Fig. 2b).

The staining for mPGES-1 was superimposable to that described for COX-2, except that a very weak immunoreactivity was observed in the (para)basal cell layers of the normal exocervical epithelium (Fig. 1c). As for COX-2, mPGES-1 was more expressed in EpM (Fig. 1f), SIL (Fig. 1i) and SCC (Fig. 1l) than in the normal exocervix. Among the 12 EpM, 18 SIL and 15 SCC tested, respectively, 7 (58%) EpM, 11 (61%) SIL and 7 (46%) SCC demonstrated an increased mPGES-1 expression (Fig. 3b, d, f). When the average of all the individual staining scores was considered, a significantly increased mPGES-1 expression was found in SIL ($P < 0.01$) and in EpM ($P < 0.05$) (Fig. 2c). Moreover, a significant Spearman correlation between COX-2 and mPGES-1 expression was observed in SCC ($P < 0.05$).

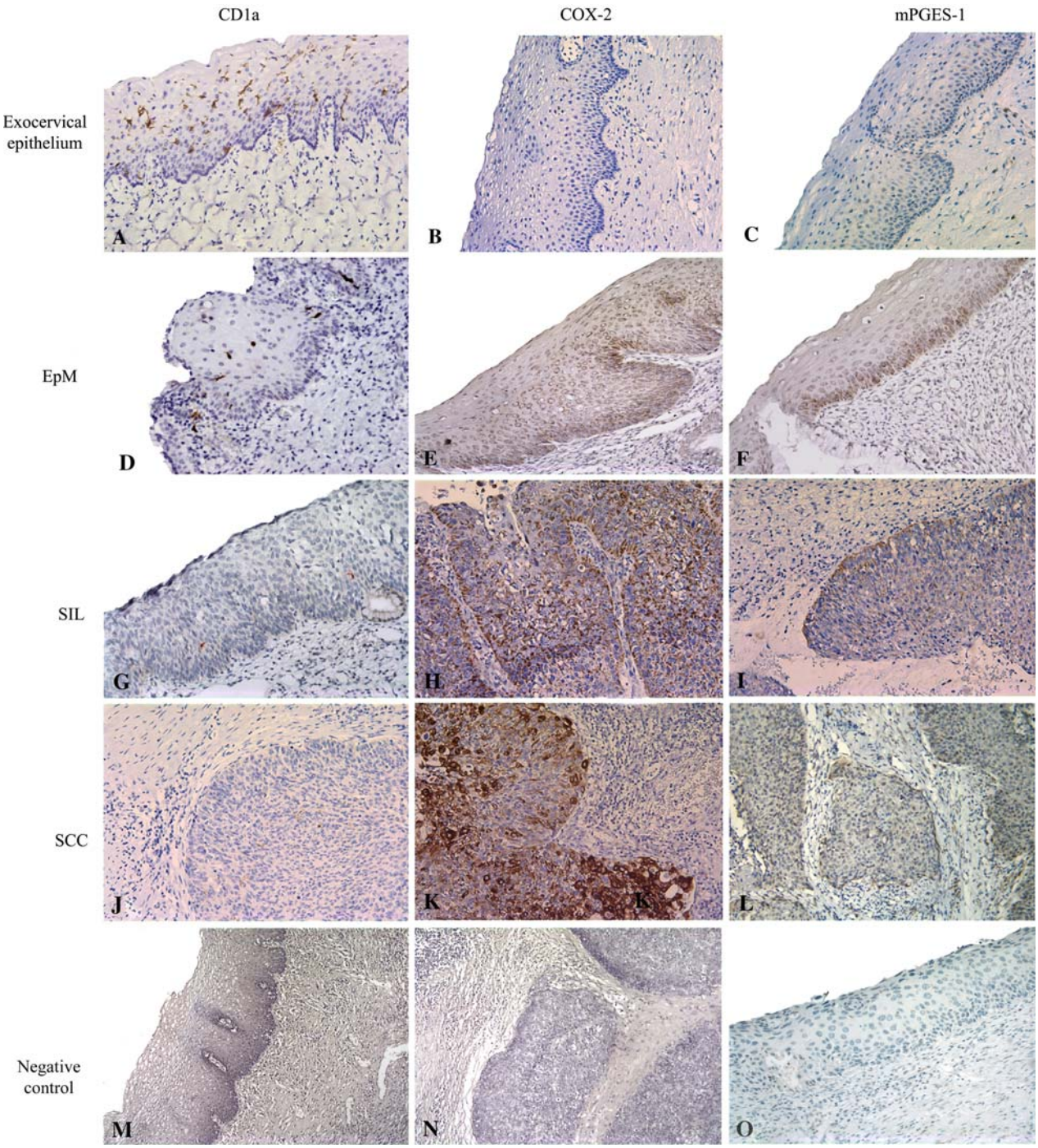


Fig. 1 CD1a, COX-2 and mPGES-1 immunostaining in cervical biopsy specimens. The normal exocervical epithelium shows a high density of $CD1a^+$ cells in basal and supra-basal cell layers (**a**). EpM (**d**), SIL (**g**) and SCC (**j**) demonstrate a low density of $CD1a^+$ cells. The normal squamous epithelium shows a lack of COX-2 (**b**) and mPGES-1 (**c**) immunoreactivity. EpM (**e**, **f**), SIL (**h**, **i**) and SCC (**k**, **l**) demonstrate a high but heterogeneous staining for COX-2 and mPGES-1. The negative control for CD1a, COX-2 and mPGES-1 immunostaining are respectively an exocervical epithelium (**m**), a SCC (**n**) and a SIL (**o**)

Cervical cancer cell lines express COX-2 and mPGES-1 and produce PGE_2 in vitro

In order to confirm the increased expression of COX-2 and mPGES-1 observed in cervical (pre)neoplasic lesions, we investigated the protein levels by western blotting in different cervical cancer cell lines transformed or not by HPV. The different cell lines express COX-2 and mPGES-1 but an increased expression was observed in HPV-positive cell lines (CaSki, C4-II, and

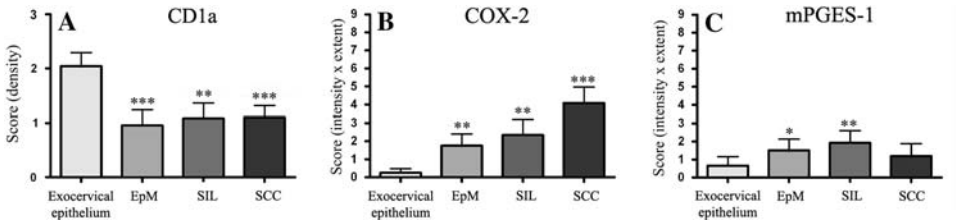


Fig. 2 Semi-quantitative evaluation of CD1a, COX-2 and mPGES-1 expression in normal exocervical epithelium ($n = 38$), EpM ($n = 12$), SIL ($n = 18$) and SCC ($n = 15$). *Asterisks* indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

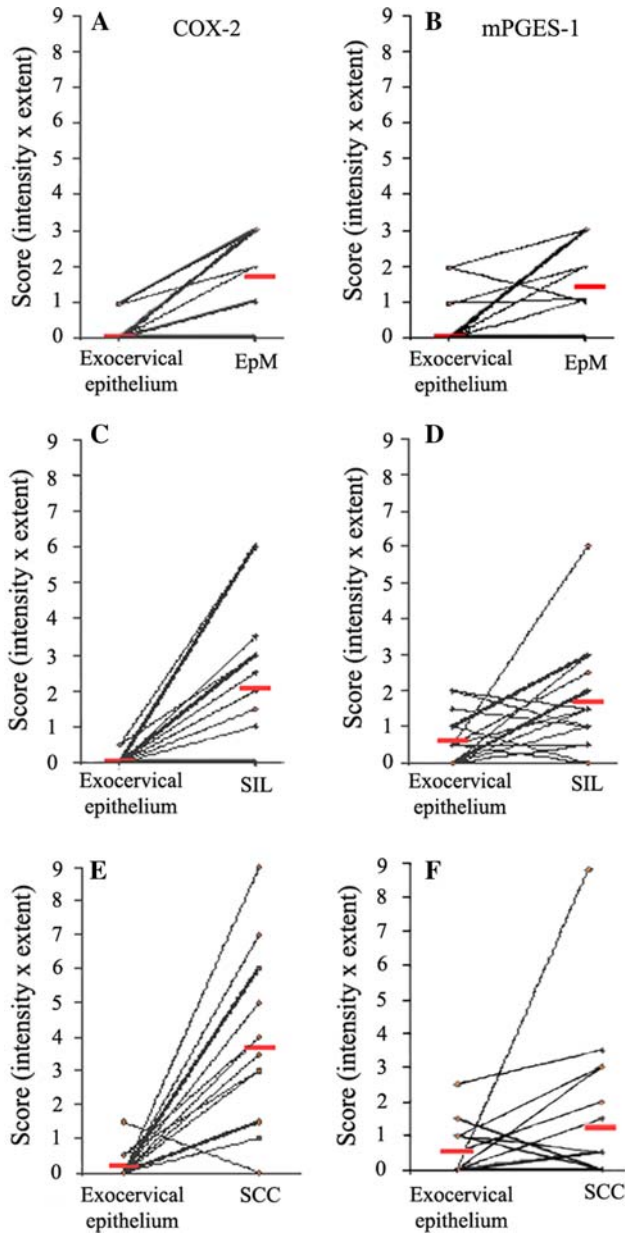


Fig. 3 Comparison of COX-2 (**a**, **b**, **e**) and mPGES-1 (**b**, **d**, **f**) immunostaining in paired biopsy specimens (exocervix vs. EpM, SIL or SCC) of individual patients. The *horizontal bars* indicate the mean score obtained for each diagnostic category. The *bold lines* correspond to biopsies from several patients

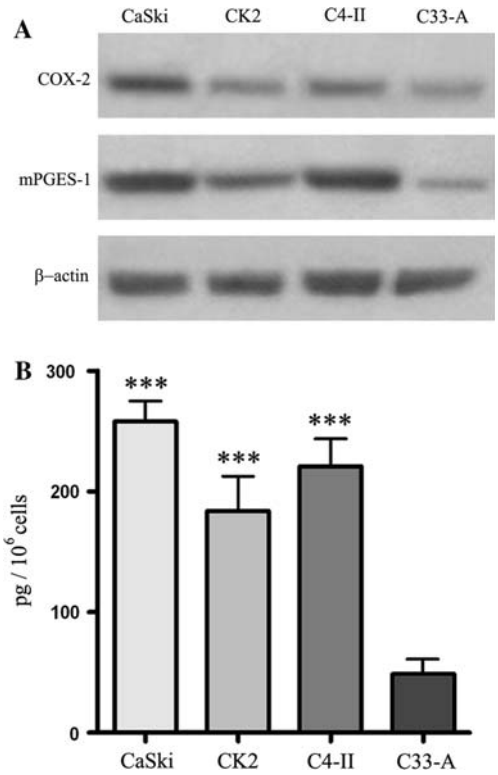


Fig. 4 In vitro activity of the enzymatic pathways responsible for the PGE_2 production in cervical cancer cell lines. **a** Western blot demonstrates higher levels of COX-2 and mPGES-1 proteins in HPV-positive cell lines (CaSki, CK2, C4-II) compared with an uninfected cervical cancer cell line (C33-A). **b** Cells were grown in culture medium for 72 h. The supernatants were then collected to determine by radioimmunoassay the levels of PGE_2 production. Results are representative of three independent experiments performed in duplicates. The means ± standard deviation is shown. *Asterisks* indicate statistically significant differences compared with C33-A cells (***$P < 0.001$)

CK2) compared to HPV-negative cells (C33-A) (Fig. 4a). Moreover, by RIA, we measured the levels of PGE_2 production by the different cell lines. Consistent with the results on COX-2 and mPGES-1 protein levels, HPV-positive cells produced more PGE_2 than the C33-A cell line (Fig. 4b).

Immature DC have a lower ability to migrate in the presence of PGE_2 compared to DC generated in vitro in the presence of PGE_2

In order to evaluate the functional effects of PGE_2 on the ability of antigen-presenting cells to migrate in or from a (pre)neoplastic epithelium, we used a Boyden Chamber assay to study the migration of immature DC or DC generated with PGE_2 in the presence of conditioned media of human fibroblasts (HFM) supplemented or not with PGE_2. A significant increased migration of DC was observed in the presence of HFM compared to a non conditioned medium used as control ($P < 0.001$) and this increase was significantly more marked for DC generated in the presence of different concentrations of PGE_2 (10^{-7} M, 10^{-8} M and 10^{-9} M) compared to immature DC. When conditioned HFM was supplemented with PGE_2, there was a significant decrease in DC migration compared to the results obtained with the HFM ($P < 0.001$) and the migration of immature DC was significantly lower than DC generated with PGE_2 (10^{-7} M, 10^{-8} M) ($P < 0.05$) and similar to that observed with the non conditioned medium (Fig. 5).

DC differentiated in the presence of PGE_2 display a tolerogenic phenotype

To evaluate the effects of PGE_2 on DC, adherent PBMC were cultured with GM-CSF and IL-4 in the presence or absence of PGE_2. After 6 days, DC were induced to mature with LPS which, in contrast to TNFα and other cytokines, is able to induce a DC signaling pathway involving Toll-like receptor 4 [17] and to stimulate the production of IL-12p70 [33] which is important to demonstrate the tolerogenic properties of PGE_2. PGE_2 inhibited DC maturation and despite similar morphology, DC generated in the presence of PGE_2 showed a lower expression of CD83, as well as other molecules involved in antigen presentation (CD1a, HLA-ABC, HLA-DR, CD86), compared with control DC (Fig. 6). A similar decreased expression of these cell-surface proteins was observed when DC were differentiated in the presence of HPV-transformed cell line supernatants (data not shown). In addition, by using a mixed lymphocyte reaction assay, we showed that PGE_2 reduces the ability of DC to stimulate T lymphocyte proliferation suggesting that DC differentiated in the presence of PGE_2 have a lower capacity to present antigens to T lymphocytes (Fig. 7). Consistent with their reduced immunostimulatory capacity, DC generated in the presence of PGE_2 produced higher levels of IL-10 and a lower amount of IL-12 compared to control DC (Fig. 8).

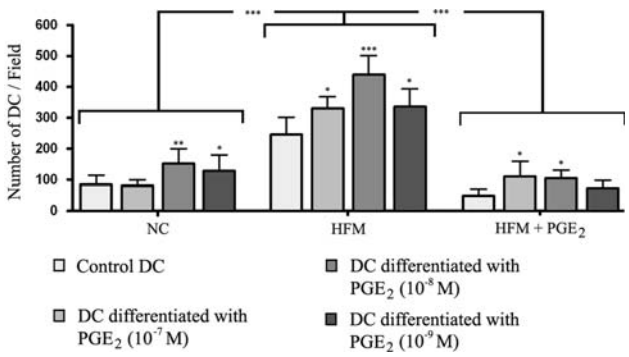


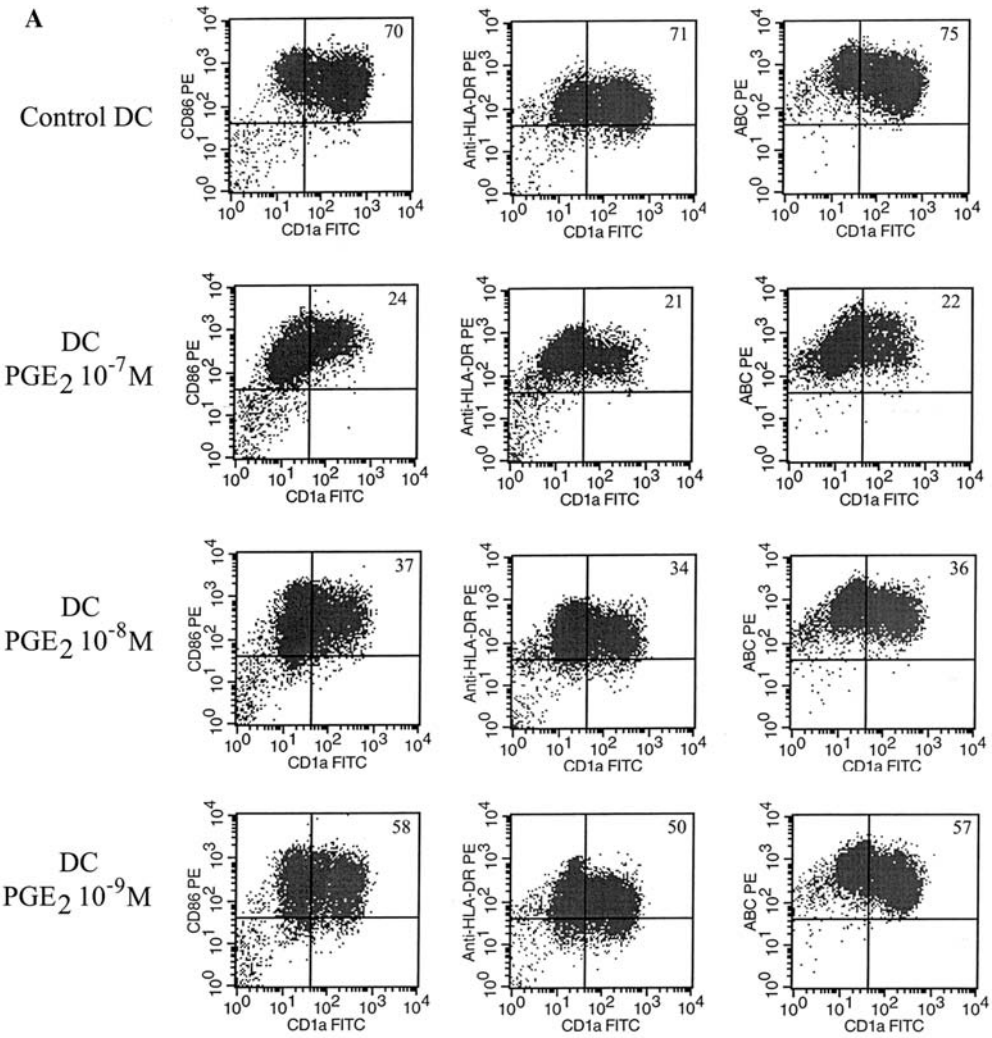
Fig. 5 Influence of PGE_2 on DC migration in a Boyden Chamber assay. *NC* non-conditioned medium; *HFM* conditioned medium of human fibroblasts (positive control); HFM + PGE_2: conditioned medium of human fibroblasts supplemented with PGE_2 (1 mM). Control DC generated in the absence of PGE_2 and DC differentiated in the presence of PGE_2 were tested. Results are the means ± standard deviation of ten wells from three different experiments. *Asterisks* indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Discussion

The chronic infection of keratinocytes of the uterine cervix by oncogenic types of HPV is necessary but not sufficient for the development of cervical cancer. Host cofactors may also contribute to the cervical carcinogenesis. The intratumoral production of immunosuppressive molecules may be an important mechanism by which cancer cells escape from the immunosurveillance. We have previously reported, as other investigators, that cervical (pre)neoplastic lesions are preferentially associated with the presence of a T Helper 2 (TH2) or immunosuppressive cytokine expression pattern in the cervix and in the peripheral blood of patients with (pre)neoplastic lesions [2, 8, 27, 28, 59]. Moreover, the Langerhans/dendritic cells (LC/DC) were shown to display quantitative and qualitative alterations [19].

As PG are involved both in the function of LC/DC [66] and in the TH1/TH2 decision [32, 56], we analyzed the expression of enzymes responsible for the production of PGE_2 (COX-2 and mPGES-1) in normal and (pre)neoplastic cervical tissues.

We showed that the density of $CD1a^+$ intraepithelial cells and the expression of PGE_2 enzymatic pathways are inversely correlated during the "metaplasia-dysplasia-cancer" sequence with, respectively, a diminished number of LC and an increased expression of COX-2 and mPGES-1 in EpM, SIL and SCC compared to the normal exocervix. These data are in agreement with those of Sales et al. [45, 46], who demonstrated, by using RT-PCR and Western Blot techniques, an increased expression of COX-2 in cervical SCC. However, COX-2 and mPGES-1 expression was heterogeneous, as reflected by a variable immunoreactivity of adjacent cells in a same lesion. This may be related to a different cell response to pro-inflammatory molecules,



B

	Control DC	DC PGE2 10^{-7} M	DC PGE2 10^{-8} M	DC PGE2 10^{-9} M
$CD11c^+$	99 % ± 0.8 %	95 % ± 4.4 %	98 % ± 1.4 %	99 % ± 0.7 %
$CD1a^+$	64 % ± 23 %	26 % ± 2.7 % **	35 % ± 8.7 %	59 % ± 20.3 %
$CD1a^+/CD83^+$	45 % ± 18 %	20 % ± 5 % *	21 % ± 1.3 % **	36 % ± 15.8 %
$CD1a^+/CD86^+$	57 % ± 14 %	23 % ± 1.1 % ***	29 % ± 14 % *	52 % ± 10.7 %
$CD1a^+/HLA\text{-}ABC^+$	60 % ± 20.1 %	25 % ± 2.8 % *	33 % ± 9.7 %	49 % ± 11.8 %
$CD1a^+/HLA\text{-}DR^+$	63 % ± 24.6 %	25 % ± 3.1 % *	32 % ± 9.6 %	65 % ± 9.9 %
$CD1a^+/CCR7^+$	39 % ± 29.9 %	17 % ± 10.2 %	19 % ± 8.8 %	35 % ± 27.1 %

Fig. 6 Phenotype analysis of DC differentiated in the presence or not of different concentrations of PGE_2. **a** Analysis of cell surface expression of CD1a, CD86, HLA-DR, HLA-ABC by different DC populations using flow cytometry. One of five experiments performed for each condition is shown. **b** Mean percentages of positive cells ± standard deviation obtained in the different experiments. The expression of surface markers CD11c, CD1a, CD83, CD86, HLA-ABC, HLA-DR and CCR7 were investigated. *Asterisks* indicate statistically significant differences compared with control DC (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

such as TNFα which is involved in COX-2 [11, 12] or mPGES-1 expression [67] and which has been detected in cervical EpM and (pre)neoplastic lesions [19] or in the presence of chronic acid exposure [54]. It is also possible that the expression profile of viral oncogenes in individual cells influences PGE_2 activity. Recently, Mann and coworkers have reported that EGF receptor (EGFR) signaling can induce the Snail transcriptional factor which binds conserved E-box elements in the prostaglandin dehydrogenase promoter to repress transcription [38]. Consequently,

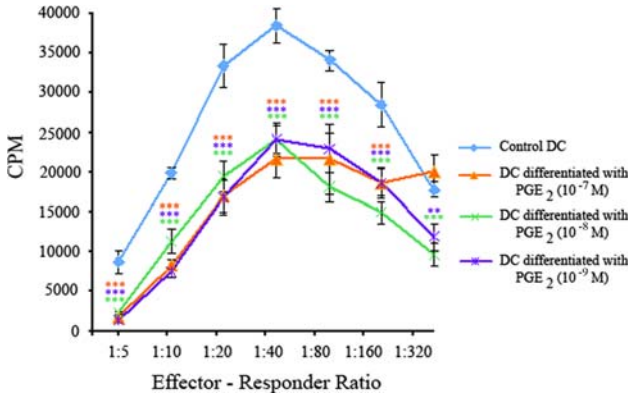


Fig. 7 DC differentiated in the presence of PGE_2 attenuate allogeneic PBMC proliferation in Mixed Leucocyte Reaction. PBMC were cultured with DC generated in the presence or not of different concentrations of PGE_2 for 6 days. Proliferation of PBMC was determined by assessing the uptake of [3H]thymidine for the last 18 h of the culture. For each condition, five independent experiments were performed in sexplicates. Data are presented as means ± standard deviation of these five experiments. *Asterisks* indicate statistically significant differences compared with control DC (**$P < 0.01$, ***$P < 0.001$)

the oncoprotein E5 which has been shown to stabilize EGFR [5, 9] could indirectly influence the concentration of PGE_2 by inhibiting the PGE_2 catabolism pathway. Moreover, HPV16 E6 and E7 oncoproteins have been shown to stimulate COX-2 transcription by inducing a corepressor/coactivator exchange [57].

The global increased expression of COX-2 and mPGES-1 in (pre)neoplastic lesions was confirmed by Western blot experiments on cervical cancer cell lines and suggests a higher activity of the enzymatic pathways responsible for the PGE_2 production. Consistent with these data, high levels of PGE_2 were detected in culture supernatants by RIA. In order to determine the significance of these observations in terms of DC/LC trafficking, we studied the impact of PGE_2 on cell migration in a Boyden chamber assay. We demonstrated that, when PGE_2 was added during the Boyden assay, the migratory capacity of immature DC was significantly decreased. These data suggest that circulating DC precursors could have a lower ability to infiltrate the cervical preneoplastic epithelium because of the presence of PGE_2 which could counteract the effect of stimulating chemokines, such as MIP3α [25], expressed in the epithelium. Interestingly, previous reports have already described that PGE_2 added to human plasma fibronectin or bovine bronchial epithelial cell-conditioned medium has an inhibitory effect on fetal lung fibroblasts [35] and is able to inhibit transendothelial migration of both human T lymphocytes [42] and neutrophils [3].

Moreover, since the LC/DC pool can also be renewed from precursors present in the epithelium [41] or from monocytes which are specifically recruited to the skin and which proliferate locally before differentiating into LC [22], we also analyzed the migratory ability of DC differentiated in the presence of PGE_2. We demonstrated that the migration of DC generated in the presence of PGE_2 is increased compared to immature DC when a conditioned medium of fibroblasts, which contains, among other molecules, SDF-1 (CXCL12) or MIP3β (CCL19) [10, 39] is used as chemoattractant. This observation is in agreement with previous studies showing that PGE_2 enhances the capacity of DC to migrate towards lymph node-derived chemokines such as SDF-1 or MIP3β [30, 31]. Indeed, PGE_2 contributes, alone or in combination with proinflammatory cytokines or CD40L, to induce the upregulation of CCR7, which is the receptor of MIP3β [37, 48]. Interestingly, we observed an increased expression of CCR7 on DC differentiated in the presence of PGE_2 compared to control DC (data not shown), suggesting that LC/DC derived from a local precursor pool could have a higher capacity to leave the dysplastic epithelium and to migrate towards the lymph nodes. Moreover, in experiments where DC maturation was induced, the only marker which was not significantly downregulated by PGE_2 was CCR7, suggesting that the ability of LC/DC to escape out of the epithelium is retained even after in situ maturation.

In addition to cell mobility experiments, we studied the impact of PGE_2 on the functionality of DC. We demonstrated that DC generated in the presence of PGE_2 displayed a tolerogenic phenotype with a lower expression of

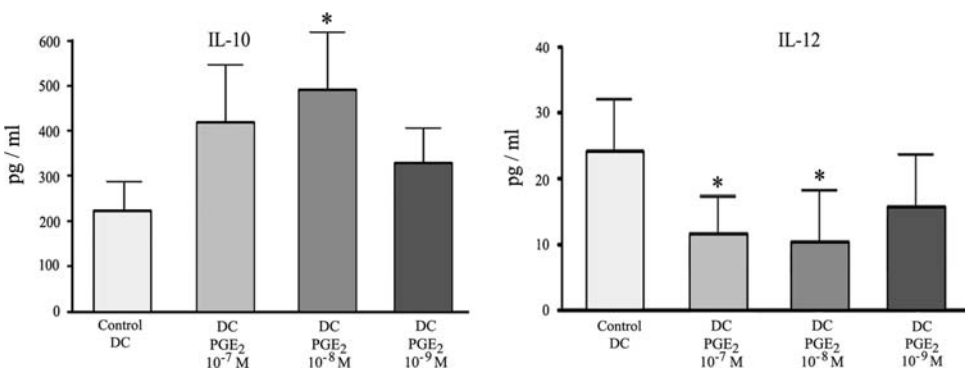


Fig. 8 Secretion levels of cytokines (IL-10 and IL-12p70) in culture supernatants of DC, generated in the presence or not of PGE_2, measured by ELISA after 24 h of incubation with LPS. Data are presented as means ± standard deviation of four independent experiments performed in duplicate. *Asterisks* indicate statistically significant differences (*$P < 0.05$)

accessory molecules and a IL-10/IL-12 ratio modified after maturation with LPS. DC with a IL-10^{high}IL-12^{low} phenotype are reported to promote T regulatory cells [7]. Indeed, it is well known that tumor environmental factors, such as IL-10, TGF-β and PG, can suppress DC differentiation and function, resulting in partially differentiated DC [65]. These tumor-converted DC are able to promote T regulatory cell expansion or proliferation [18]. PGE_2 has also been shown to induce the DC expression of indoleamine 2,3-dioxygenase [63] which catalyzes the depletion of the essential aminoacid tryptophan. This depletion enhances the production of immunoregulatory kynurenine metabolites which inhibit T-cell proliferation [60]. Moreover, an increased intracellular production of cyclic AMP in the presence of PGE_2 down-regulates expression of TH1 cytokines, such as interleukin-2 [56], which is in agreement with the TH2 immunodeviation previously described during the cervical carcinogenesis [2].

In addition to the importance of the intra-tumoral migration of immune cells in the progression of neoplastic lesions, cancer cell motility also plays a crucial role during the invasion and metastatic stages and previous studies showed that PGE_2 can stimulate cancer cells motility [53, 55, 62]. One mechanism may involve the down-regulation of E-cadherin by PGE_2 [14] and the development of an epithelial to mesenchymal transition process which has been associated to increased cell migration [21]. Interestingly, E-cadherin expression has also been shown to be necessary for the homophilic and heterotypic interactions between dendritic cells and keratinocytes in squamous epithelial tissues [24, 26].

In conclusion, we demonstrated a high expression of PGE_2 enzymatic pathways in cervical (pre)neoplastic lesions. The data concerning the influence of PGE_2 on DC migration could partially explain the decreased density of DC observed in HPV-induced cervical lesions. Indeed, circulating DC could have a lower ability to infiltrate the (pre)neoplastic epithelium producing PGE_2 and the DC precursors present in the lesion, i.e. in contact with PGE_2, could have a higher capacity to leave the dysplastic epithelium and to migrate towards the lymph nodes. Moreover, the induction of a tolerogenic phenotype by DC could also partially explain the functional alterations of antigen presenting cells during the cervical carcinogenesis and have important consequences for the progression of HPV-associated preneoplastic lesions.

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