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Immune Response of Plasmacytoid Dendritic Cells Stimulated by Human Papillomavirus (HPV) E6 in an *In Vitro* System

Authors' Contribution:

Study Design A

Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

Literature Search F

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Background: This study aimed to analyze the changes in plasmacytoid dendritic cell (pDC) immunophenotypes when co-cultured with Caski cells and stimulated by human papillomavirus (HPV) E6 *in vitro*, and thus to discuss the immunoregulatory roles of pDCs in the tumorigenesis of cervical cancer.

Material/Methods: The immunophenotypic expression of pDCs was analyzed under stimulation of HPV E6 and co-culturing with Caski cells *in vitro*.

Results: HPV E6 infection caused significantly increased expression of CD40 in HPV16 M and HPV16 H groups MyD88 in HPV16 M, HPV16 H, and HPV18L groups; and TRAF6 in HPV16 M, HPV16 H, and HPV18L groups. pDCs co-cultured with Caski cells showed significantly lower expression of MyD88 and TRAF6 compared with the control.

Conclusions: The expression of MyD88 and TRAF6 might vary in different stages of HPV infection. pDCs might regulate CD40 to participate in the tumorigenesis and progression of cervical cancer, but related mechanisms still need further investigation.

MeSH Keywords: **Dendritic Cells • DNA Probes, HPV • Human Papillomavirus 16 • Human Papillomavirus 18 • Uterine Cervical Neoplasms**

Abbreviations: **pDC** – plasmacytoid dendritic cell; **CIN** – cervical intraepithelial neoplasia

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Background

Cervical cancer is the fourth most common malignant tumors among women, and it is also the fourth leading cause of cancer-related death among women worldwide [1]. The relationship of human papillomavirus (HPV) and the progression of cervical cancer was first reported over 30 years ago. Certain types of HPVs, such as HPV-16, HPV-18, and HPV-31, which are designated “high-risk” or “oncogenic” have been identified as causes of cervical and anal cancers. HPV infects keratinocytes then replicates and assembles exclusively in the nucleus in a differentiation-dependent manner, which first happens in squamous basal layer of the cervical epithelium. The procedure of viral gene expression and replication is tightly regulated by keratinocyte differentiation. The sustained expression of HPV oncoprotein E6/E7 leads to the initiation/transformation and progression of cervical cancer by interacting with and eliminating several key inhibitors of the tumor proteins p53 and pRb in the cell cycle checkpoint, thereby affecting the proliferation and apoptosis of cell, inducing immortalization, chromosomal instability, malignant transformation, and even carcinogenesis [2,3]. Evidence suggests that HPV can evade the surveillance and clearance of host immune system through a variety of mechanisms. Han et al. reported that that HPV E7 proteins could promote the differentiation and maturation of pDCs and activate the TLR and MAPK pathway to induce host innate immune response [4]. Dendritic cells are the most influential antigen-presenting cells, and their subgroup, plasmacytoid dendritic cells (pDCs), play a pivotal role in the natural immune response against HPV, by connecting the innate and adaptive immune responses. Upon stimulation of viral infection, pDCs can secrete high levels of type-I interferon (IFN) that has an antiviral effect but also participates in post-infection immune tolerance, thereby facilitating persistent viral infection. The mechanism of pDCs is yet to be clarified. Whether HPV E6/E7 realize persistent infection and induce cancer development by interfering with pDC functions is also worth exploring. This study aimed to analyze the changes in pDC immunophenotypes when co-cultured with Caski cells and stimulated by HPV E6 *in vitro*, and thus to discuss the immunoregulatory roles of pDCs in the development of cervical cancer.

Material and Methods

Cell culture

The HPV18-positive human cervical cancer cell line Caski was purchased from American Type Culture Collection (ATCC), USA and cultured in RPMI 1640 (GIBCO, Invitrogen) medium supplemented with 10% (vol/vol) fetal bovine serum, 1% penicillin/streptomycin and incubated at 37°C under constant 5% CO₂ supply.

HPV E6 preparation

Sequences of HPV16/18 E6 were obtained from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The *Sac-I* and *Hind-III* restriction sites were added to the 5' and 3' ends, and the gene was synthesized by Shanghai Generay Bioengineering Co., Ltd. The synthesized gene was then cloned to pRSET-A plasmid to construct a prokaryotic expression vector pRSET-A-HPV16 E6, and the recombinant vector was recovered and sequenced for validation. Later, the vector was transformed into an *Escherichia coli* expression strain BL21 (DE3). The HPV E6 fusion protein was isolated, purified, and quantified using bicinchoninic acid (0.5 mg/mL) (Figure 1).

Protein isolation and quantification

Briefly, samples were collected from the broken bacterial solution after centrifugation, washing, resuspension, and centrifuged at 10 000 g for 20 minutes. Then we prepared and equilibrated the column and loaded the sample. Finally, we wash the column to collect the outflow peak and the elution peak. Fusion protein concentration was determined by bicinchoninic acid (BCA) method (Figure 2).

In vitro expression of related proteins in HPV16 E6 – stimulated pDCs

pDCs were acquired from healthy human peripheral blood (Zhejiang Blood Center, Zhejiang, China) by magnetic bead sorting. Samples were divided into groups: 1) control group (not treated); 2) HPV16 L group (treated with HPV16 E6 at a low dose, 10 ng/mL); 3) HPV16 M group (treated with HPV16 E6 at a medium dose, 100 ng/mL); and 4) HPV16 H group (treated with HPV16 E6 treatment at a high dose, 1000 ng/mL). All groups were treated with HPV16 E6 for 48 hours. The cells for HPV18 E6 treatment were grouped in the same way. Flow cytometry was used to analyze protein phenotype changes in the expression of IRAK4, TRAF6, IRAK1, MyD88, CD83, CD123, CD40, CD80, CD289, and IRF7.

Co-culture of pDCs with Caski cells

Briefly, the 6-well Transwell inserts (pore size, 0.4 μm; Corning) were used. 1×10⁵ Caski cells, suspended in 1 mL of RPMI 1640, were placed in the upper compartment, and 1×10⁵ pDC cells suspended in 1 mL of RPMI 1640 were added to the lower compartment of Cell Culture Insert. After 48-hour incubation at 37°C in 5% CO₂, cells in the lower compartment were harvested.

Statistical analysis

Statistical analysis was conducted using SPSS 16.0 software. Intergroup comparison was made using Student's *t*-test and

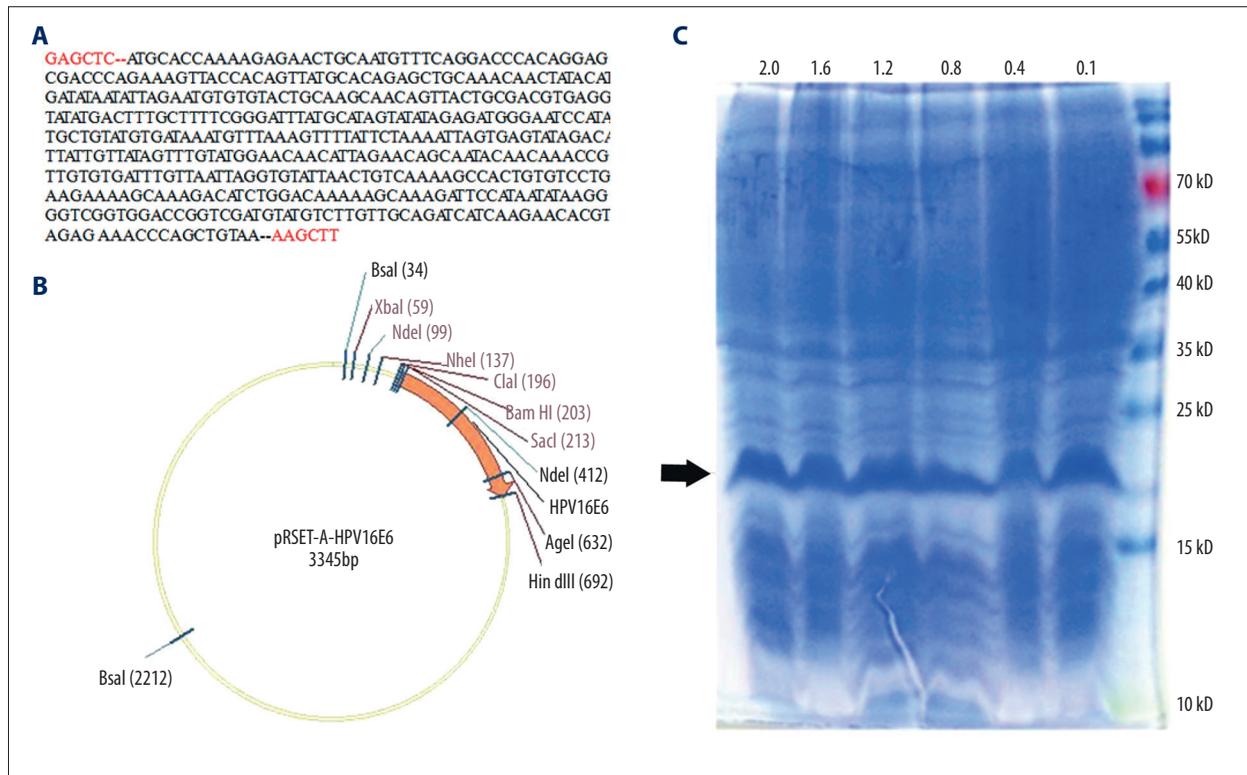


Figure 1. (A–C) HPV E6 preparation.

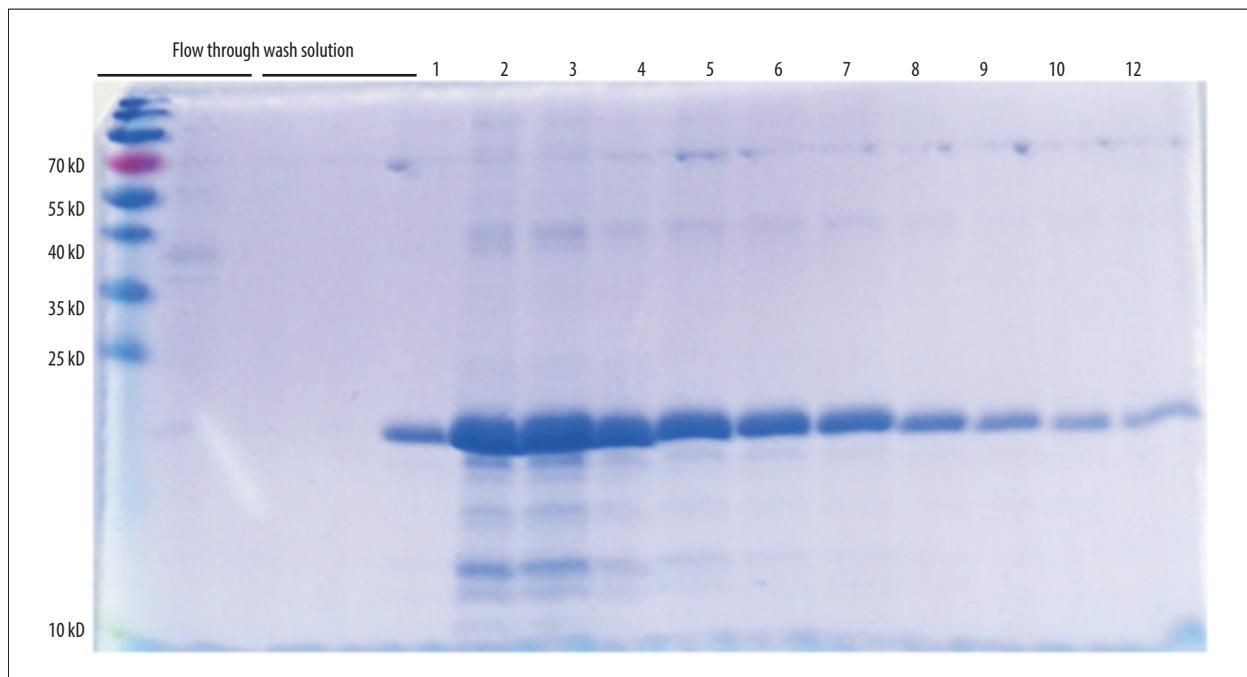


Figure 2. Protein isolation and quantification.

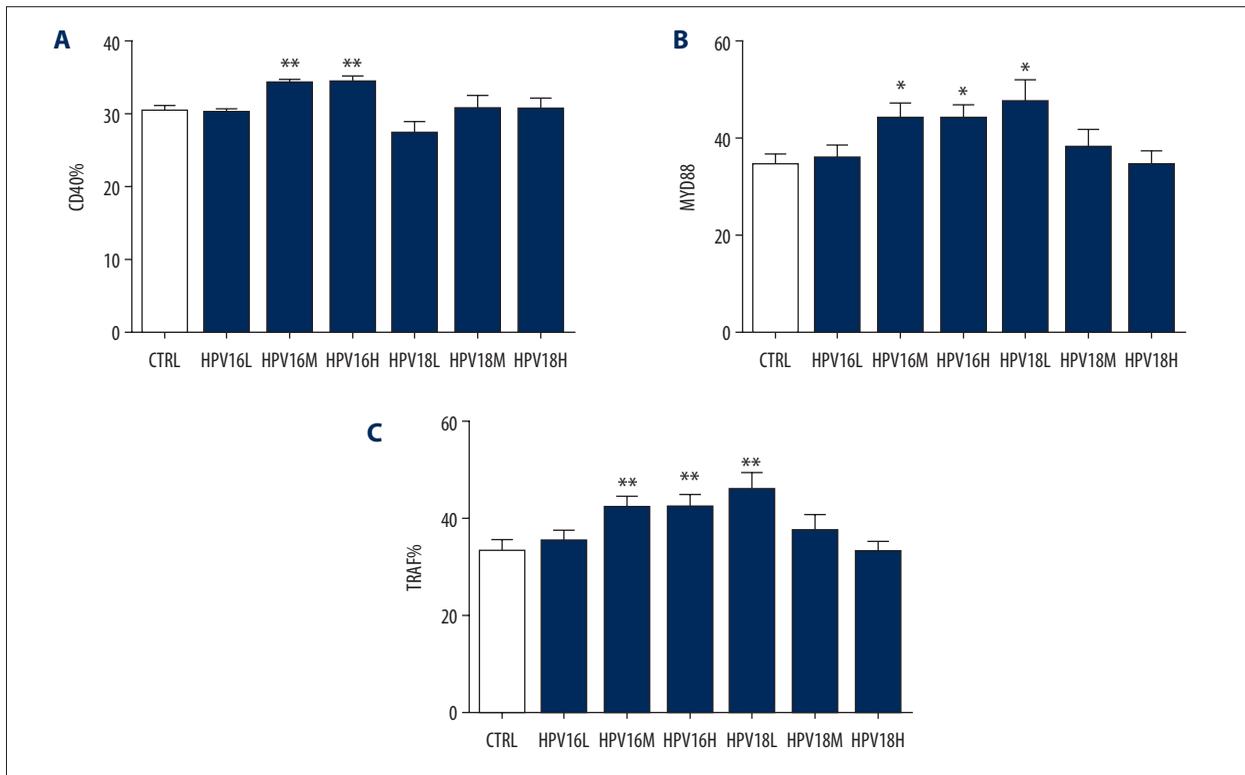


Figure 3. (A–C) Immunophenotypic changes in pDCs under HPV E6 stimulation.

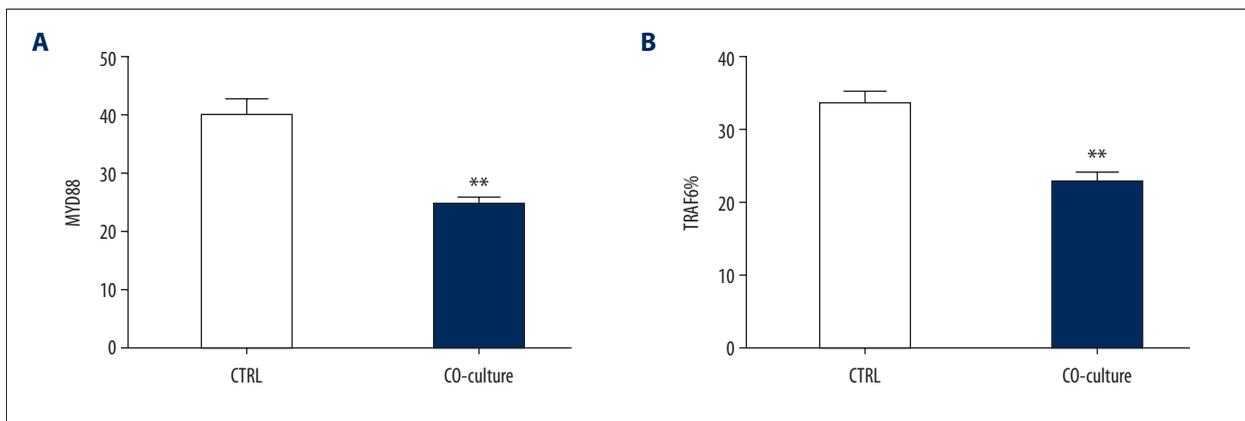


Figure 4. (A, B) Immunophenotypic changes in pDCs co-cultured with Caski cells.

nonparametric test. A *P* value <0.05 was defined as statistically significant.

Results

Immunophenotypic changes in pDCs under HPV E6 stimulation

pDCs sorted from healthy human peripheral blood were stimulated by HPV E6 proteins at 3 different concentrations. The expression of CD40 was significantly upregulated in HPV16 M

and HPV16 H groups. The expression of MyD88 was significantly elevated in the HPV16 M, HPV16 H, and HPV18 L groups. The expression of TRAF6 was significantly raised in HPV16 M, HPV16 H, and HPV18 L groups. No significant difference was identified in the other groups (Figure 3).

Immunophenotypic changes in pDCs co-cultured with Caski cells

Since HPV16 is the most oncogenic genotype among all the HPVs, we conducted HPV16 positive Caski cells to co-cultured with pDCs, which sorted from healthy human peripheral blood.

Flow cytometry identified significantly lower expression of MyD88 and TRAF6 ($P < 0.01$), whereas the expression of other proteins, such as IRAK4, CD83, CD123, CD40, CD80, CD289, IRF7, and IRAK4, was not significantly different from that of control (Figure 4).

Discussion

Cervical cancer is common in China. As a definite cause of cervical cancer, persistent HPV infection helps in the initiation of normal cervix to cervical intraepithelial neoplasia (CIN), CIN I, CIN II, CIN III, and even invasive cervical carcinoma. Also, E6/E7 are key genes inducing malignant transformation and maintaining malignant phenotypes of cervical cells [5]. Normally, about 50–90% of cervical HPV infection could be cleared by the immune system within 2 years. However, previous studies showed that high-risk HPV E6/E7 could interfere with the initiation of natural antiviral immunity and the activation of acquired immunity, thereby impairing the immune function of host cells and facilitating malignant transformation by maintaining persistent viral infection [6]. Therefore, host immune response is decisive for HPV clearance and patient prognosis.

DCs are the most powerful antigen-presenting cells and the only cells that can induce initial T-cell response. As a special subgroup of DCs, pDCs are pivotal in the innate immune system. When stimulated by viruses, pDCs can release high levels of anti-viral IFN, but they also participate in post-infection immune tolerance, thereby facilitating persistent infection of the virus. As the cells can both trigger immune response and induce immune tolerance, they play critical roles in viral infections.

Many former reports have shown that the expression of CD123, BDCA2, CD2AP, and CD56 increases with the severity of disease from normal tissue to low-grade lesion, high-grade lesion, and cervical cancer tissue ($P < 0.05$). This study simulated the immune response of pDC under HPV infection and identified significantly upregulated levels of CD40 in HPV16 M and HPV16 H groups; MyD88 in HPV16 M, HPV16 H, and HPV18 L groups; and TRAF6 in HPV16 M, HPV16 H, and HPV18 L groups. In addition, after co-culturing with HPV18-positive Caski cells, pDCs showed decreased immunophenotypic expression of MyD88 and TRAF6 ($P < 0.01$). CD40 is a 48-kDa transmembrane

glycoprotein surface receptor. It is key to the activation and maturation of DCs and the initiation of T cells. HPV could inhibit CD40 signal transduction, thereby impairing CD40-mediated DC immune response [7]. CD40 was reported to be upregulated in HPV16/18 E6-positive cervical cancer and was also associated with the expression of vascular endothelial growth factor and the microvessel density. Therefore, CD40 is believed to be related to the neovascularization of tumors [8]. MyD88 participates in TLR signaling transmission as a common transduction protein, and it has a procarcinogenic effect on the skin, liver, spleen, and colon cancers. In this study, MyD88 and TRAF6 showed increased expression under HPV stimulation, but were downregulated when co-cultured with Caski cells. The reason might be that TLR4-MyD88-TRAF6-TAK1 could enhance the NF- κ B signaling pathway, and HPV16 E6 could trigger host cell immune response and promote tumor development via the NF- κ B pathway [9,10]. In addition, Vandermark et al. [11] reported that E7 protein inhibited NF- κ B activation and facilitated malignant transformation of cells. The HPV16 E6 prepared in this study might have promoted NF- κ B activation by upregulating MyD88 and TRAF6, whereas Caski cells might have inhibited NF- κ B signaling via the E7 protein. Hence, the activation of NF- κ B pathway and the expression of MyD88 and TRAF6 proteins were affected by HPV-positive cells at different infection stages [12].

Conclusions

Many studies have reported the association of pDCs with tumor infiltration and immune tolerance [13,14]. In this study, it was also speculated that in the early phase of HPV infection, pDCs might have exerted an anti-HPV function for a short period; however, when HPV could not be cleared and epithelial cells showed malignant transformation, pDCs might have entered immune anergy and even developed immune tolerance under persistent HPV stimulation. pDCs participate in the immune response during cervical cancer development and progression, but the mechanism still needs further investigation.

Conflict interest

None.

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