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# T cell ignorance is bliss: T cells are not tolerized by Langerhans cells presenting human papillomavirus antigens in the absence of costimulation

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## ABSTRACT

Human papillomavirus type 16 (HPV16) infections are intra-epithelial, and thus, HPV16 is known to interact with Langerhans cells (LCs), the resident epithelial antigen-presenting cells (APCs). The current paradigm for APC-mediated induction of T cell anergy is through delivery of T cell receptor signals via peptides on MHC molecules (signal 1), but without costimulation (signal 2). We previously demonstrated that LCs exposed to HPV16 *in vitro* present HPV antigens to T cells without costimulation, but it remained uncertain if such T cells would remain ignorant, become anergic, or in the case of CD4<sup>+</sup> T cells, differentiate into Tregs. Here we demonstrate that Tregs were not induced by LCs presenting only signal 1, and through a series of *in vitro* immunizations show that CD8<sup>+</sup> T cells receiving signal 1+2 from LCs weeks after consistently receiving signal 1 are capable of robust effector functions. Importantly, this indicates that T cells are not tolerized but instead remain ignorant to HPV, and are activated given the proper signals.

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## 1. Introduction

Human papillomavirus (HPV) affects millions of individuals worldwide as it is causally linked to the development of cervical, vaginal, anal, and head and neck cancers [1–5]. Of the oncogenic high-risk HPV (hr-HPV) genotypes, HPV type 16 (HPV16) is the most common and accounts for more than 50% of all cervical cancers and 90% of HPV-related head and neck squamous cell carcinomas [6–9]. Various studies have found that HPV capsids, also known as virus-like particles (VLPs), can bind to and stimulate the activation of dendritic cells (DCs) *in vitro* [10–13], providing

evidence that they can induce the maturation of antigen-presenting cells (APCs), which could in turn mediate adaptive immune responses. Despite the ability of HPV capsid proteins to initiate immune responses in human DCs *in vitro*, more than 15% of women that have hr-HPV infections do not initiate effective immune responses against HPV, and among those that do, viral clearance is slow with an average time of 8–16 months [14–18], indicating that HPV is escaping immune detection *in vivo*.

How HPV infection remains undetected by the immune system, and what cells and cellular mechanisms are involved have been central questions to our research. Through multiple prior investigations, we have demonstrated that HPV-mediated manipulation of Langerhans cell (LC) immune function is a key mechanism by which HPV evades immune detection [19–23]. LCs are the resident professional APCs of the mucosal epithelial layer that account for roughly one in twenty cells of the epithelium [24], and are responsible for initiating immune responses against skin invading viruses [25]. However, our group has demonstrated that HPV16 manipulates human LCs in such a manner that internalization of HPV16 VLPs into LCs results in suppressive signaling and defective activation, which differs from human DC responses [26,27]. When stimulated with HPV16 VLPs, the mitogen-activated protein kinase

**Abbreviations:** cVLP, chimeric virus-like particles; DC, dendritic cells; ELISpot, Enzyme Linked Immunosorbant Spot assay; HPV, human papillomavirus; HPV16, HPV type 16; hr-HPV, high-risk HPV; LC, Langerhans cell; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; TLR, Toll-Like Receptor; IFN, interferon; TNF, tumor necrosis factor; Tregs, regulatory T cells; VLP, virus-like particles

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(MAPK) pathway is activated in DCs, yet remains inactivated in LCs. Conversely, HPV16 VLP exposure of LCs initiates a signaling cascade that activates the phosphoinositide 3-kinase (PI3K) pathway, yet inactivates Akt [19]. The phenotypic consequence of this is that LCs exposed to HPV16 VLPs *in vitro* are able to present HPV antigens in the absence of costimulation [28]. The lack of costimulation by LCs may be one reason that T cell immunity is lacking in those with persistent HPV infections (reviewed in [29]).

According to current textbook understanding, the presentation of antigens on major histocompatibility complex (MHC) molecules to T cell receptors (TCR) (providing signal 1) by APCs without the concurrent presentation of costimulatory molecules (providing signal 2) induces T cell anergy or tolerance [30–32]. Alternatively, T cells can remain in an ignorant state with the ability to respond to antigens upon future encounters. Costimulatory molecule recognition by their corresponding receptor on T cells, i.e. CD80 or CD86 by CD28, was proposed by early studies to be essential for the prevention of clonal anergy of CD4<sup>+</sup> T cells either through direct inhibition on the production and function of anergic factors, [33] or indirectly through cell-cycle effects via stimulation of IL-2 [34,35]. There has been significant experimental evidence to support the latter hypothesis involving IL-2 stimulation (reviewed in [36,37]). Similarly, the original demonstration of induced anergy of CD8<sup>+</sup> T cells by APCs lacking costimulatory molecules was made in CD8<sup>+</sup> clones where the phenotype was described as inhibition of IL-2 production and proliferation, though less effect on interferon gamma (IFN- $\gamma$ ) production or cytotoxic activity was observed [38]. Despite the apparent retention of cytotoxic activity in tolerized CD8<sup>+</sup> T cells, the lack of clonal expansion hinders any measurable adaptive immune response.

Naïve CD4<sup>+</sup> T cells play a key role in effective anti-tumor immunity and may differentiate into effector or regulatory subsets depending on the stimulus received from APCs. Beyond anergic CD4<sup>+</sup> T cells, recent studies have shown a significant role for regulatory T cells (Tregs) in the development of HPV-associated malignancies and these cells are found in high frequencies in cervical intraepithelial neoplastic (CIN) lesions [39–42]. Tregs are suppressive T cells that inhibit the proliferation and activation of effector T cells to prevent an autoimmune attack [43]. Naïve CD4<sup>+</sup> T cells can differentiate into regulatory subsets when costimulatory molecules from immature DCs are lacking; however, this has not been investigated for LCs. Tregs may be expanded from a naïve population after exposure to HPV16-presenting LCs, which could be an additional HPV escape mechanism. Hence, the differentiation of CD4<sup>+</sup> T cells into Tregs, Th1, or Th2 cells after incubation with HPV16-exposed LCs was explored in this study.

The absence of T cell immunity during persistent HPV infections may be a direct result from the lack of APC costimulation. However, studies have not yet explored the resultant phenotypes of CD4<sup>+</sup> or CD8<sup>+</sup> T cells after incubation with LCs presenting HPV antigens in the absence of costimulation, which was a focus of the current study. Hence, the fate of CD4<sup>+</sup> and CD8<sup>+</sup> T cells exposed to potentially tolerizing LCs that express HPV antigens without signal 2 was investigated to determine whether the resultant T cells were irreversibly tolerized, ignorant to HPV antigens, or in the case of CD4<sup>+</sup> T cells, became Tregs. Additionally, we determined whether toll-like receptor (TLR) agonist-matured LCs presenting proper signal 1 and signal 2 stimuli could restore CD8<sup>+</sup> T cell cytotoxic activity against HPV16 antigens after long-term exposure to LCs providing only signal 1.

## 2. Materials and methods

### 2.1. Donor material

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors via leukapheresis. PBMCs were subsequently purified over lymphocyte separation medium (Cellgro, Manassas, VA), cryopreserved, and stored in liquid nitrogen [22]. Donor PBMCs were HLA-A and HLA-DR typed. Low-resolution DNA typing for HLA-A2 was performed using standard endpoint PCR, which was confirmed by flow cytometry using an anti-HLA-A2 antibody (BD Biosciences, San Jose, CA). For HLA-A2<sup>+</sup> samples, high-resolution genotyping was performed at the HLA-A2 locus with the A\*02 SSP UniTray Kit (Life Technologies, Carlsbad, CA). HPV serology was negative for all donors. All protocols were approved by the University of Southern California's Institutional Review Board, and informed consent was obtained from each donor.

### 2.2. Antibodies and reagents

The following antibodies were purchased from BD Biosciences: CD4 FITC, CD4 PE, HLA-ABC FITC (MHC I), HLA-DP,DQ,DR FITC (MHC II), CD80 FITC, and CD86 FITC. The following antibodies were purchased from Biolegend (San Diego, CA): CD4 PC5, CD4 PC7, CD45RA FITC, IFN- $\gamma$  PC7, IL-10 PE, IL-4 FITC, and CD25 PE. The following antibody was purchased from eBioscience (San Diego, CA): Foxp3 FITC. Appropriate isotype controls were purchased from either BD Biosciences or Biolegend. Human IFN- $\gamma$  capture and detection antibodies were purchased from Mabtech (Cincinnati, OH). Poly-ICLC (Hiltonol) is a clinical grade current good manufacturing practices (cGMP) poly-lysine stabilized form of Poly polyinosinic-polycytidylic acid (PolyIC) provided by Oncovir, Inc. (Washington, D.C.).

### 2.3. Primary cell culture and LC generation

PBMC monocyte-derived LCs were generated following published procedures [44,45]. Frozen PBMCs were thawed and washed once in complete medium: RPMI 1640 (Life Technologies) containing 10 mM sodium pyruvate (Life Technologies), 10 mM non-essential amino acids (Life Technologies), 100  $\mu$ g/ml kanamycin (Sigma-Aldrich, St. Louis, MO), 50  $\mu$ M  $\beta$ -Mercaptol Ethanol (Life Technologies), and 10% FBS (Omega Scientific, Tarzana, CA). PBMCs were plated in a 175-cm<sup>2</sup> tissue culture flask for 2 h at 37 °C. Nonadherent primary cells were then washed off and remaining adherent monocytes were cultured for 7 days in complete medium containing 1000 U/ml rhGM-CSF (Genzyme, Boston, MA), 1000 U/ml rhIL-4 (Life Technologies), and 10 ng/ml rhTGF- $\beta$ 1 (Life Technologies). Cytokines were replenished on days 3 and 6. LC phenotype in the resultant cells containing the hallmark Birbeck granule structures was confirmed as Langerin<sup>+</sup>CD1a<sup>+</sup>E-Cadherin<sup>+</sup> (data not shown), as previously demonstrated [23].

### 2.4. Chimeric virus like particles

HPV16 L1L2 VLPs and HPV16 L1L2E7 chimeric VLPs (cVLPs) were generated in insect cells and purified by sucrose and cesium chloride ultracentrifugation as previously described [46], and endotoxin levels were measured below 0.06 EU using an E-toxate kit (Sigma-Aldrich). Western blot analysis confirmed the presence of E7, L1, and L2 proteins. Transmission electron microscopy was used to validate intact VLP and cVLP structure.

## 2.5. LC stimulation assay and flow cytometry

LCs were treated with HPV16 VLPs only or treated with HPV16 VLPs for 4 h before stimulation with Poly-ICLC, a TLR3 agonist that has been shown to effectively activate LCs [47,48], and activation-associated surface markers were measured by flow cytometry following established procedures [45]. In brief,  $10^6$  LCs were seeded in a 6-well plate and left untreated, treated with 20  $\mu$ g HPV16 VLPs, or 20  $\mu$ g HPV16 VLPs for 4 h followed by 72 h incubation with Poly-ICLC. Cells were harvested, washed, stained for surface MHC I, MHC II, CD80, CD86, or isotype controls, and analyzed on an FC500 flow cytometer using CXP software (Beckman Coulter, Brea, CA). Geometric mean fluorescence intensities (MFI) were used to calculate fold change in expression from untreated LC baseline values.

## 2.6. CD4<sup>+</sup> *in vitro* immunization (IVI) assay and phenotyping

LCs from HLA-A\*0201<sup>+</sup> donors and autologous CD4<sup>+</sup> T cells were co-cultured *in vitro* over several weeks to evaluate T cell phenotypes following established procedures [49]. Specifically, LCs were either left untreated or treated with HPV16 L1L2E7 cVLPs for 1 h at 37 °C, and then medium was supplemented with 1000 U/ml of rhGM-CSF and LCs were incubated for an additional 4 h. LCs were then cultured with or without Poly-ICLC for 20 h at 37 °C. As a positive control, LCs treated with Poly-ICLC were peptide pulsed with HLA-A2 restricted E7 peptides (E7<sub>1–12</sub>, E7<sub>48–62</sub> and E7<sub>62–75</sub>) [50,51]. All LC groups were subsequently irradiated (30 Gy). Autologous Naïve CD4<sup>+</sup> T cells were isolated using a negative Magnetic Cell Separation Kit (Miltenyi Biotec, Auburn, CA) and  $5 \times 10^5$  cells/well were seeded in a 48-well plate and were co-cultured with  $2.5 \times 10^4$  irradiated LCs. Additional treated and irradiated LCs were added on day 7, 14, and 21 at the same T cell to LC ratio (20:1). Culture medium was supplemented with IL-10 (10 ng/ml) 24 h after each LC addition, and supplemented with IL-2 (50 U/ml) 48 and 96 h after each LC addition. On day 28, CD4<sup>+</sup> T cells were collected from each group and assessed for phenotypic T cell markers via flow cytometry. Single cell suspensions of CD4<sup>+</sup> T cells were washed and subsequently stained for surface marker expression following manufacturer instructions for each antibody. Cells that required permeabilization for intracellular staining were fixed and permeabilized (FoxP3 kit, eBioscience) overnight and stained the following day. Cells were analyzed by flow cytometry as described above.

## 2.7. CD8<sup>+</sup> IVI and ELISpot assay

LCs and autologous purified CD8<sup>+</sup> T cells were co-cultured *in vitro* over several weeks to induce primary CD8<sup>+</sup> effector T cell responses against defined HLA-A\*0201 HPV16 E7 epitopes following published protocols [49]. Specifically, LCs were treated and irradiated as described above in the CD4<sup>+</sup> IVI assay. Autologous CD8<sup>+</sup> T cells were isolated from cryopreserved PBMCs using a negative Magnetic Cell Separation Kit (MACS by Miltenyi).  $5 \times 10^5$  CD8<sup>+</sup> T cells were then co-cultured with irradiated LCs ( $2.5 \times 10^4$  cells/well). Additional irradiated LCs were added on day 7 as described above. By day 14, non-proliferative, unresponsive CD8<sup>+</sup> T cells were generated by HPV16 cVLP-exposed LCs, while proliferative, activated CD8<sup>+</sup> T cells were generated with exposure to Poly-ICLC-treated and peptide pulsed LCs. On day 14 and 21, LC additions were altered such that non-proliferative CD8<sup>+</sup> T cells were given peptide pulsed LCs and activated CD8<sup>+</sup> T cells were given HPV16 cVLP-exposed LCs. Co-culture medium was supplemented with IL-7 (10 ng/ml) within 24 h of LC additions. Culture medium was supplemented with IL-10 (10 ng/ml) 24 h after each LC addition, and supplemented with IL-2 (50 U/ml) 48 and 96 h

after each LC addition. 96-well ELISpot plates (Millipore Multi-screen HTS IP; Millipore, Temecula, CA) were coated with IFN- $\gamma$  capture antibody (Mabtech mouse anti-human IFN- $\gamma$ , Clone 1-D1K) in PBS overnight at 4 °C and plates were blocked with complete medium. CD8<sup>+</sup> T cells ( $1 \times 10^6$  cells/ml) with HLA-A2 binding E7 peptides or media only (2  $\mu$ g/ml) were seeded on the ELISpot plate in quadruplicates and incubated overnight at 37 °C. After 24 h, plates were washed and incubated with IFN- $\gamma$  detection antibody (Mabtech mouse anti-human IFN- $\gamma$  biotin, Clone 7-B6-1) for 2 h followed by streptavidin-horse radish peroxidase (Sigma-Aldrich) for 1 h at room temperature. Plates were then developed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich) substrate for 5 m, and spots were counted using the KS ELISpot analysis system (Carl Zeiss, Thornwood, NY), and the number of spots in media only containing wells was subtracted from HPV E7 peptide containing wells to determine the number of spots above background.

## 2.8. Cytokine and chemokine analysis

Supernatants were collected from LC cultures on day 3 and CD4<sup>+</sup> IVI cultures on day 14 and subjected to a multiplex enzyme linked immunosorbent assay (ELISA) for cytokine and chemokine analysis. The experiment was performed using a Millipore Milliplex Human Cytokine Kit and the Bio-Plex Suspension Array System (Bio-Rad, Irvine, CA).

## 2.9. Statistical analysis

All statistical analyses were performed on Graphpad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). Statistical significance for ELISpot and cytokine results were assessed with a two-tailed t-test comparing data to negative controls. Significance was defined at  $p < 0.05$  for all experiments.

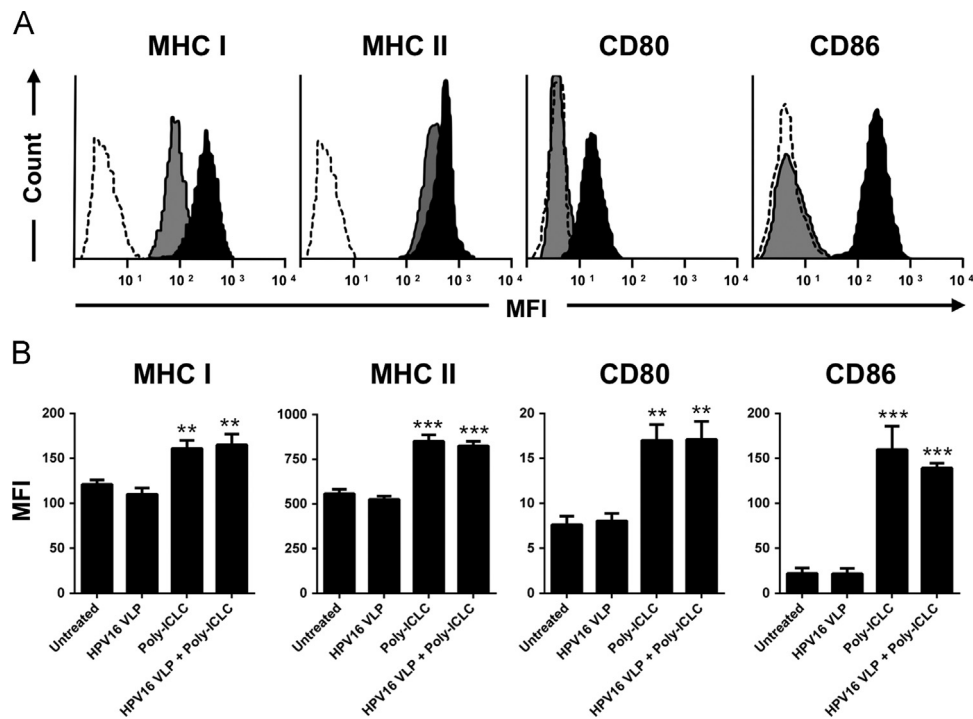
# 3. Results

## 3.1. HPV16-exposed LCs present antigen without costimulation

Immature LCs express lower, albeit substantial, levels of MHC class I and class II molecules than activated LCs, but exhibit little to no expression of CD80 and CD86 costimulatory molecules (Fig. 1). Upon treatment of LCs with HPV16 alone, the expression of these markers did not change, demonstrating a lack of phenotypic activation. Interestingly, we have previously demonstrated that similarly treated DC become activated [22], indicating a unique manipulation of LC by HPV16. More importantly, this shows the ability of LCs to present antigen without costimulation, which is in agreement with our previous results that demonstrated that HPV16-treated LCs could specifically present HPV antigens without signal 2 [28]. Conversely, LCs stimulated with the TLR3 agonist Poly-ICLC both alone and after HPV16 exposure showed a significant increase in the expression of antigen presentation and costimulatory molecules, which are the hallmarks of activated LCs that provide both signal 1 and 2.

## 3.2. HPV16-exposed LCs do not secrete pro-inflammatory cytokines

Since anti-viral CD8<sup>+</sup> T cell induction requires APCs to secrete pro-inflammatory cytokines and chemokines, the secretion of various inflammatory cytokines and chemokines by LCs after exposure to HPV16 alone or followed by activation with poly-ICLC was assessed. LCs that were pre-exposed to HPV16 showed no increase in cytokine production (Fig. 2), whereas LC activation by poly-ICLC, whether or not in the presence of HPV16, induced



**Fig. 1.** HPV16-exposed LCs present antigen without costimulation. LC surface marker expression of antigen presentation and costimulatory molecules was evaluated via flow cytometry. LC were left untreated or treated with HPV16 VLP, Poly-IJCLC, or HPV16 VLP prior to Poly-IJCLC. Average mean fluorescence intensity (MFI) of surface marker staining  $\pm$  SD is shown ( $n=4$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to untreated). A. Representative histograms, and B. Each graph is representative of at least four independent experiments.

significant secretion of cytokines and chemokines, which could in turn activate T cells after antigen presentation.

### 3.3. Activated LCs induce activated CD4<sup>+</sup> T cells from naïve CD4<sup>+</sup> T cell populations

The phenotypic characteristics of CD4<sup>+</sup> T cells after exposure to HPV16-presenting LCs with and without costimulation had previously been unknown. Here, the fate of naïve CD4<sup>+</sup> T cells, isolated based on CD4 and CD45RA expression, was determined subsequent to co-culture with either immature (lacking signal 2) or mature LCs (with signal 2) in an IVI assay. Naïve CD4<sup>+</sup> T cells were co-cultured with either medium (no LCs), untreated LCs, peptide pulsed + Poly-IJCLC-treated LCs, HPV16 cVLP-exposed LCs, or HPV16 cVLP + Poly-IJCLC-treated LCs for four consecutive weeks, with additional LCs exposed in the same manner added each week. We then phenotyped the resultant CD4<sup>+</sup> T cells for surface and intracellular markers via flow cytometry. Exposure of naïve CD4<sup>+</sup> T cells to untreated LCs or HPV16 cVLP-exposed LCs resulted in a high frequency of remaining naïve CD4<sup>+</sup> T cells and a low frequency of induced effector CD4<sup>+</sup> T cells compared to those exposed to activated LCs, as demonstrated by the co-expression of CD4 and CD45RA, a marker for naïve T cells (Fig. 3A). Conversely, exposing naïve CD4<sup>+</sup> T cells to activated LCs, including peptide pulsed + Poly-IJCLC-treated LCs and HPV16 cVLP + Poly-IJCLC-treated LCs, caused a complete depletion of the naïve CD4<sup>+</sup> T cell population (Fig. 3A) and a higher frequency of induced effector CD4<sup>+</sup> T cells, as demonstrated by increased expression of the activation marker CD25 (Fig. 3B).

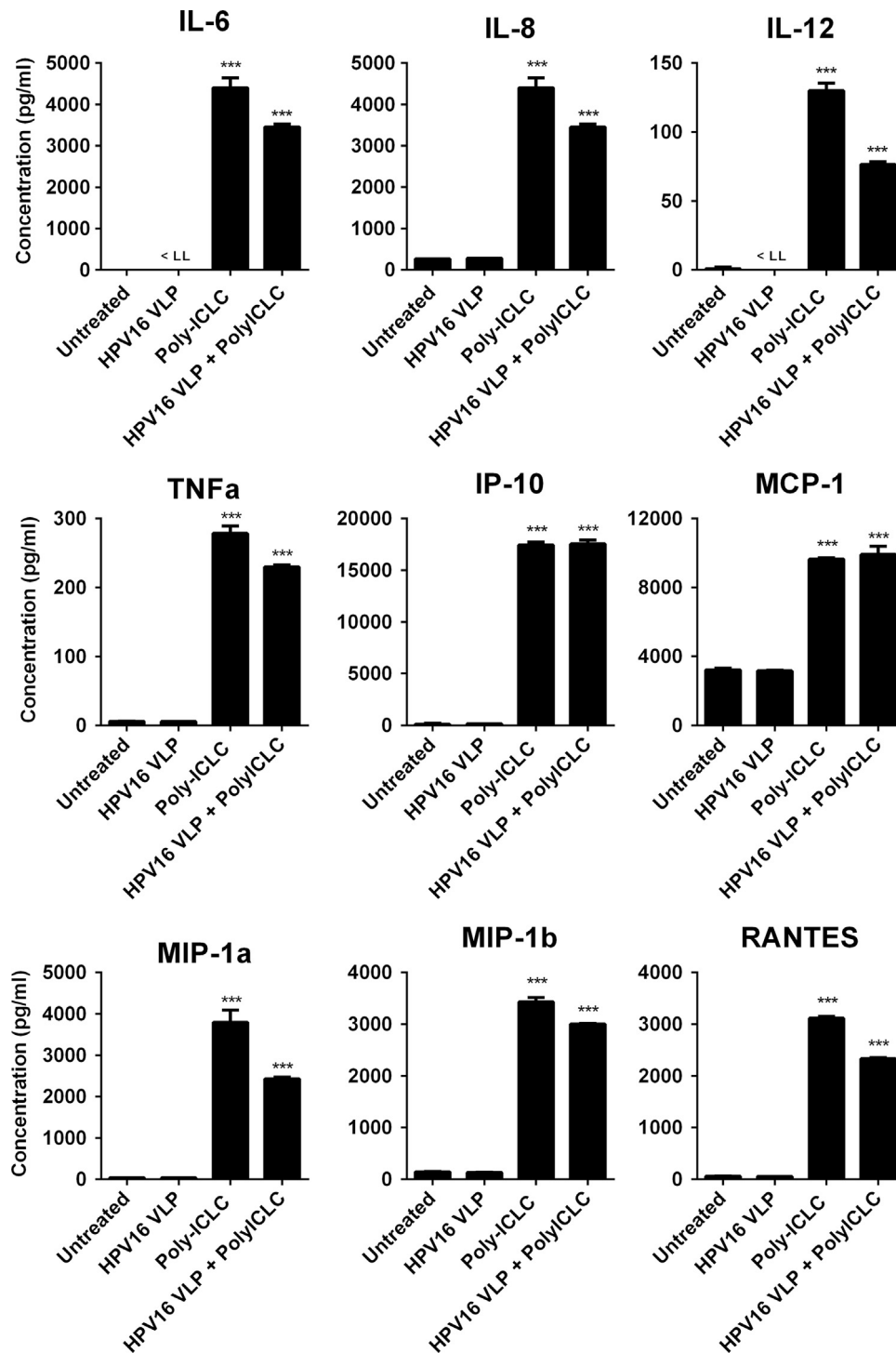
### 3.4. HPV16-exposed LCs do not induce Tregs from a naïve CD4<sup>+</sup> T cell population

While it has been reported that CIN and cervical cancer patients have an increased frequency of Tregs in the cervix [41,42], it was not known whether these Tregs were induced from naïve CD4<sup>+</sup> T cells

encountering HPV16-exposed LCs providing only signal 1. As immature LCs could potentially have tolerizing abilities, it was hypothesized that naïve CD4<sup>+</sup> T cells could differentiate into a regulatory phenotype when co-cultured with LCs in the absence of costimulation. Therefore, we assessed the Treg induction through immunophenotyping after naïve CD4<sup>+</sup> T cell exposure to differentially activated LCs. Specifically, CD4<sup>+</sup> T cells were analyzed for CD4 and CD25 cell surface markers, and for the Treg intracellular marker, FoxP3. LCs presenting only signal 1 were expected to generate an increased frequency of Tregs due to the lack of costimulation. Interestingly, we observed that co-culture of naïve CD4<sup>+</sup> cells with either immature or mature LCs did not significantly affect the frequency of Tregs, and their frequency was approximately only 1% of any of the co-culture groups (Fig. 4). Although the literature suggests a significant role for Tregs in HPV-induced malignancies, the lack of costimulation from immature HPV16-exposed LCs did not lead to an induction of Tregs from a naïve CD4<sup>+</sup> T cell population.

### 3.5. CD4<sup>+</sup> T cells produce increased levels of IL-10 when co-cultured with HPV16-exposed LCs

A multiplex-ELISA was performed to investigate whether CD4<sup>+</sup> T cells secrete Th1, Th2, or Treg associated cytokines after exposure to differentially activated LCs. This analysis was performed on day 14 supernatants of each study arm of the IVI. Exposure of CD4<sup>+</sup> T cells to activated LCs (either peptide pulsed + Poly-IJCLC or HPV16 cVLP + Poly-IJCLC treated LCs) induced the secretion of Th1- and Th2-associated cytokines. Most notably, these included the Th1-associated cytokines IFN- $\gamma$  and TNF- $\alpha$ , and the Th2-associated cytokines IL-4 and IL-5 (Fig. 5). There was no difference in the levels of the Treg-promoting cytokine TGF- $\beta$  in any treatment group (data not shown), which is in agreement with the above results with the same low frequency of Tregs in each study arm (Fig. 4). Surprisingly, the inhibitory cytokine IL-10 was elevated in T cells co-cultured with immature LCs lacking costimulation



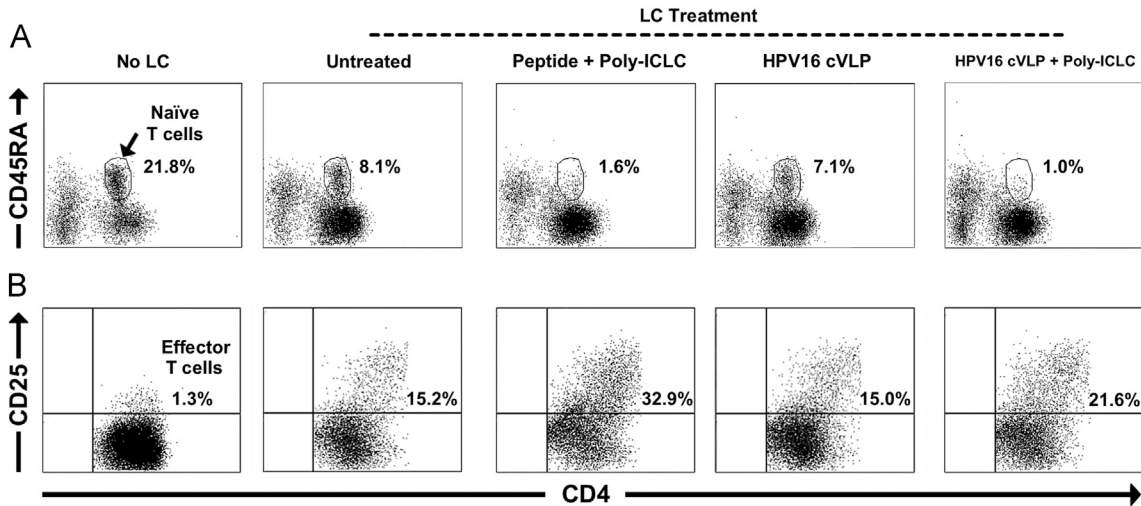
**Fig. 2.** HPV16-exposed LCs do not produce high levels of inflammatory cytokines and chemokines. LCs were left untreated or treated with HPV16 VLP, Poly-I/CLC, or HPV16 VLP prior to Poly-I/CLC. Cell supernatants were analyzed for a panel of 9 cytokines and chemokines using a Bio-Plex Suspension Array System. Data represent the mean  $\pm$  SD analyte concentration ( $n=3$ ;  $***p < 0.001$  compared to untreated). Each graph is representative of at least four independent experiments.

(untreated LCs and HPV16 cVLP-exposed LCs), but was decreased from cells co-cultured with activated LC. These data highlight the importance of activated LCs in an HPV setting to promote Th1-differentiation, which is advantageous for effective viral clearance.

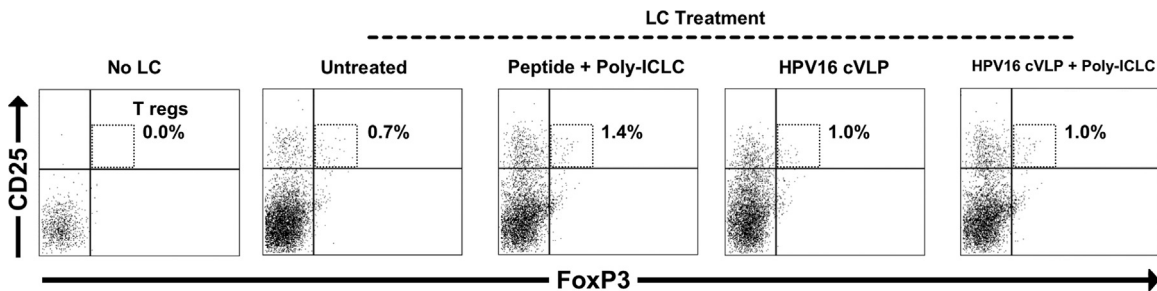
### 3.6. $CD8^+$ T cells are not tolerated by HPV16-exposed LCs in the absence of costimulation

HPV16-exposed LCs are not phenotypically or functionally activated as shown above (Figs. 1 and 2), and as such we have previously

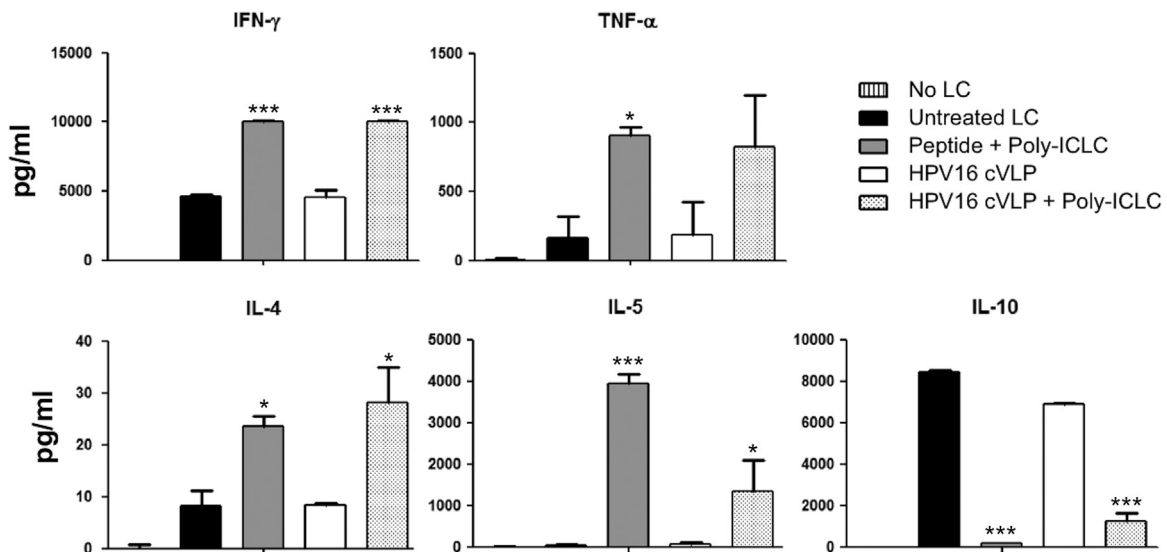
demonstrated that these LCs do not induce HPV16-specific  $CD8^+$  T cell responses despite HPV antigen presentation [23,28,44]. However, it was uncertain if these LCs induced T cell anergy or if the  $CD8^+$  T cells remained ignorant, which was a focus of the current study. Here, we incubated naïve  $CD8^+$  T cells with either HPV16-exposed LCs providing only signal 1, or activated LCs providing both signal 1 and 2. Our results demonstrate that unresponsive (non-proliferative and lacking  $IFN-\gamma$  secretion)  $CD8^+$  T cells were generated by co-culturing naïve  $CD8^+$  T cells for 14 days with HPV16-exposed LCs that lack costimulatory molecule expression (Fig. 6A), although it



**Fig. 3.** Activated LCs induce activated CD4<sup>+</sup> T cells from naïve CD4<sup>+</sup> T cell populations. (A) CD4<sup>+</sup> T cells were analyzed via flow cytometry for CD4 and CD45RA surface markers representing the naïve CD4<sup>+</sup> T cell population subsequent to CD4<sup>+</sup> IVI. Naïve CD4<sup>+</sup> T cells were co-cultured with differentially treated LC as indicated by the following treatments for 4 weeks: medium, untreated LC, peptide pulsed and Poly-ICLC activated LC, HPV16 cVLP-exposed LC, or HPV16 cVLP-exposed and Poly-ICLC-activated LC. The frequency of CD4<sup>+</sup>CD45RA<sup>+</sup> T cells are indicated. (B) Frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells are indicated that represent the percentage of activated CD4<sup>+</sup> T cells. All experiments were repeated three times in different donors and representative data are shown.



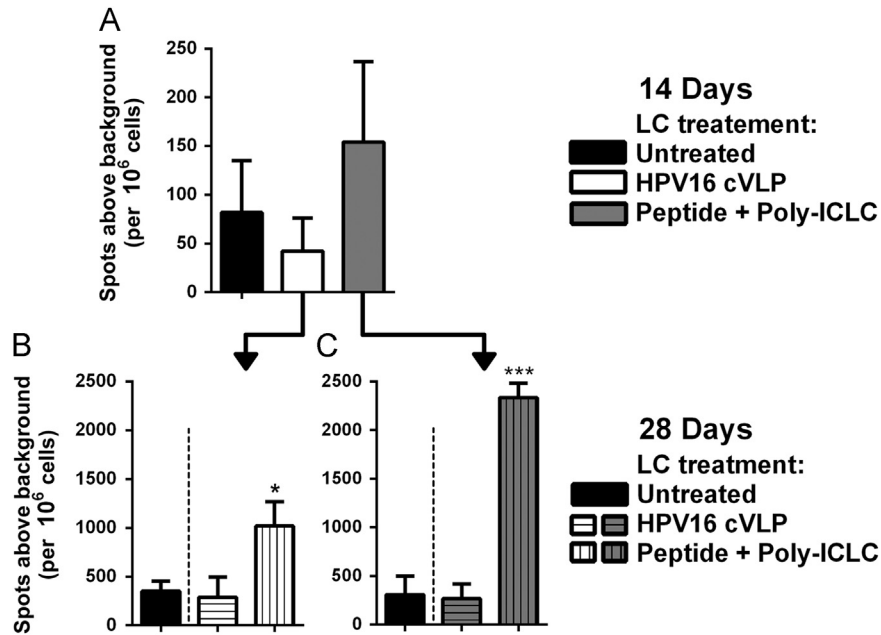
**Fig. 4.** HPV16-exposed LCs do not induce Tregs from a naïve CD4<sup>+</sup> T cell population. CD4<sup>+</sup> T cell populations were analyzed for the presence of Tregs. The percentage of CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (Tregs) are shown (upper right quadrant). All experiments were repeated for three different donors and representative data are shown.



**Fig. 5.** CD4<sup>+</sup> T cells produce increased levels of IL-10 when co-cultured with HPV16-exposed LCs. Cytokine profiles of CD4<sup>+</sup> T cells co-cultured with differentially treated LC are presented. The different treatment groups are shown for each cytokine presented. The experiment was repeated for three different donors and representative data are shown as the mean  $\pm$  SD (\* $p$  < 0.05 and \*\*\* $p$  < 0.001 compared to untreated).

was not certain if these co-cultured T cells would respond to stimuli thereafter. This was in contrast to naïve CD8<sup>+</sup> T cells co-cultured with activated LCs (peptide pulsed and Poly-ICLC treated), which exhibited a robust HPV16-specific response in the same time period.

A vital question that remained was if T cells co-cultured with the HPV16-exposed LCs (displaying signal 1 only) were tolerized to HPV16 antigens. To investigate this, the day 14 unresponsive CD8<sup>+</sup> T cells were co-cultured with peptide pulsed Poly-ICLC-activated



**Fig. 6.** CD8<sup>+</sup>T cells are not tolerized by HPV16-exposed LCs in the absence of costimulation. 14- and 28-day ELISpot results are presented. (A) The number of E7 specific IFN $\gamma$  producing CD8<sup>+</sup> T cells per million T cells induced by stimulation of autologous CD8<sup>+</sup> T cells with untreated, HPV16 cVLP-exposed, or E7 peptide+Poly-ICLC treated LC in HLA-A\*0201 positive patients after 14 days. Spots from no E7 peptide wells have been subtracted to display the number of peptide-specific T cells induced in each treatment group above background levels. (B) T cells co-cultured with HPV16 cVLP-exposed LC for 14 days were either cultured with HPV16 cVLP exposed LC or E7 peptide+Poly-ICLC treated LC for an additional 14 days. (C) T cells co-cultured with E7 peptide+Poly-ICLC treated LC for 14 days were either cultured with HPV16 cVLP exposed LC or E7 peptide+Poly-ICLC treated LC for an additional 14 days. Data are shown as the mean  $\pm$  SE of six separate experiments (\* $p < 0.05$  and \*\*\* $p < 0.001$  compared to cVLP).

LCs or were alternatively exposed to the same HPV16-exposed LCs that generated the unresponsive cells (Fig. 6B). We hypothesized that if anergy occurred, then the T cells would be tolerant to subsequent antigen delivery. Interestingly however, HPV-specific T cell induction was increased in previously unresponsive T cells co-cultured with HPV16-exposed LCs. This data suggests that these T cells were not tolerized by LCs providing only signal 1, but were rather ignorant to viral antigens. Lastly, it was unknown whether HPV16-exposed LCs could functionally suppress active HPV-specific CD8<sup>+</sup> T cells. Therefore, we generated a population of HPV16 E7-specific CD8<sup>+</sup> T cells through co-culture with peptide pulsed Poly-ICLC-activated LCs. These responsive HPV16-specific CD8<sup>+</sup> T cells were subsequently co-cultured with HPV16-exposed LCs lacking costimulation, and their response to the E7 epitope was assessed. Our results indicate that HPV-specific CD8<sup>+</sup> T cells continue to show immune responses against E7, but lack proliferative ability without signal 2, as the number of total spots increased to only a fraction of those with continual signal 1 and 2 stimulation (Fig. 6C). These results suggest that immature LCs providing only signal 1 slow down or halt the adaptive immune response such that no further clonal expansion occurs.

#### 4. Discussion

The phenotype of immature LCs derived from monocytes *in vitro* has been defined as high expression of MHC class I, class II, and CD1a with low expression of the co-stimulatory molecules and maturation markers CD40, CD80, CD83, CD86, and CCR7 [52]. Similar low levels of CD80 and CD86 were shown for immature LCs isolated from skin *in vivo* [53], which was comparable to what was observed herein. Upon proper pathogenic stimulation, LCs undergo dramatic changes including the activation of signaling cascades, the release of pro-inflammatory cytokines, and importantly, the up-regulation of co-stimulatory molecules [54–56]. *In vivo*, activated LCs then migrate to lymph nodes, processing viral

antigens en route, where they interact with naïve T cell populations by presenting antigens (signal 1) with costimulation (signal 2) to initiate an adaptive T cell response [54,57]. Additionally, it has been shown that LCs can spontaneously migrate [58,59], and thus immature LCs undergoing spontaneous migration may present antigens without signal 2, which may lead to T cell anergy or the induction of Tregs. Activated effector CD8<sup>+</sup> T cells migrate back to the site of infection and destroy infected cells. However, with regard to HPV infections, LCs from healthy donors exposed to high-risk HPV strains do not become functionally mature APCs, but can still spontaneously migrate [60]. Therefore, the focus of this study was to investigate T cell subsets induced by these immature LCs, as the phenotypes and functionality of T cells resulting from co-culture with HPV16-exposed LCs *in vitro* had yet to be explored.

In the present study, we examined CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets induced by HPV16-exposed LCs providing only signal 1, and determined whether unresponsive CD8<sup>+</sup> T cells encountering these LC could regain cytotoxic functions after proper signal 1 and 2 stimulation. It is tacit that effective CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses play a crucial role in viral clearance and anti-tumor immunity against HPV-induced malignancies. Importantly, our lab has previously demonstrated that HPV16 evades the host immune response by not activating LCs despite their presentation of HPV antigens resulting in the absence of effector CD8<sup>+</sup> T cells against HPV antigens [23,26,44,61]. This process includes the dysregulation of the PI3K-Akt signaling pathway and defective cellular activation [23,26]. Accordingly, it is reasonable to hypothesize that antigen presentation is also disrupted in these cells. However, HPV16 E7-specific T cells can recognize and kill LCs exposed to HPV16 cVLPs [28], indicating that LCs are able to effectively cross-present HPV-derived peptides after cVLP internalization.

We have previously demonstrated that treatment with a TLR8 agonist can activate LCs, whereas a TLR7 agonist does not [44], suggesting that the specific TLR molecule engaged on LCs has an effect on the resulting immune response. More recently, we demonstrated that TLR3 is expressed by monocyte-derived LCs,

and that Poly-ICLC, a TLR3 agonist, is a potent activator of LC [52,62]. While it was shown that some TLR agonists work synergistically to increase cytokine production by APC, no increase was observed on the expression of costimulatory molecules [63]. Moreover, overstimulation can lead to impaired APC function, which decreases their ability to induce adaptive immune responses [64,65], and thus Poly-ICLC was chosen as the single potent LC activating agent herein.

It has previously been concluded that the lack of responsive cytotoxic CD8<sup>+</sup> T cells against HPV16 is linked to the progression of CIN lesions and persistence of HPV infection [66]. Here, we demonstrate that co-culturing naïve CD8<sup>+</sup> T cells with HPV16-exposed immature LCs results in non-proliferative, unresponsive CD8<sup>+</sup> T cells, whereas co-culture with HPV peptide pulsed Poly-ICLC-activated LCs results in a robust cytotoxic T cell response. In a crossover design, we demonstrate that LCs providing proper signal 1 and 2 elicit functional effector properties against HPV antigens in the unresponsive T cells, and this data suggests that CD8<sup>+</sup> T cells are ignorant rather than tolerant to HPV antigens after consistently receiving signal 1 without signal 2. Furthermore, we demonstrated that HPV16-exposed LCs lacking signal 2 essentially stopped stimulating the previously activated T cells. Together, these results indicate the importance of signal 2 in inducing functional activity in evidently ignorant CD8<sup>+</sup> T cells, and indicate a different fate from the current paradigm of irreversible anergy caused by antigen presentation in the absence of costimulation. These results also imply that the development of new therapies to induce activation of HPV-exposed LCs may lead to potent CD8<sup>+</sup> T cell responses *in vivo* even after T cells have encountered HPV-16 exposed LC providing only signal 1.

Beyond CD8<sup>+</sup> T cells, it was suspected that regulatory T cell subsets may be induced by these immature HPV16-exposed LCs from naïve CD4<sup>+</sup> T cells based on the high frequency of Tregs seen in the cervix of women with HPV-induced lesions [41,42,67,68]. The results of the current study demonstrate that Poly-ICLC-activated and HPV16 exposed LCs induced effector CD4<sup>+</sup> T cells from a naïve CD4<sup>+</sup> population, whereas HPV16-only-exposed LCs were immature and induced few effector CD4<sup>+</sup> T cells and likewise a higher proportion of the naïve CD4<sup>+</sup> population remained in these cultures. Contrary to our hypothesis, these HPV16-exposed immature LCs did not induce Tregs. Furthermore, there were no significant differences in Tregs induced among any of the study groups. Therefore, the source of the large quantity of Tregs found in cervical cancer biopsies remains unknown, but suggests that Tregs may be recruited into the tumor microenvironment rather than being induced by HPV itself. Alternatively, suppressive cytokines such as TGF- $\beta$  and IL-10 may dictate the regulatory fate of naïve CD4<sup>+</sup> T cells in the cervix, which suggests that Tregs may be induced by local stromal cells and not by immature LC.

While our results show that HPV16-exposed immature LCs did not directly lead to Treg induction *in vitro*, these LCs did promote a suppressive environment by mediating the secretion of the suppressive cytokine IL-10 by co-cultured T cells, which can suppress the differentiation of naïve T cells to effector cells. Our cytokine analysis also showed that there was reduced secretion of Th1 and Th2 cytokines (IL-4, IL-5, TNF- $\alpha$ , and IFN- $\gamma$ ) by T cells co-cultured with the HPV16-exposed immature LCs. The IL-10 secretion by T cells co-cultured with HPV16-exposed LC was similar to what was observed when T cells were co-cultured with untreated LCs that are expected to present self-antigens without costimulation. These results may reflect a shift in the current LC paradigm. Once thought that their primary function was to take up antigens penetrating the epidermal layer and convey them to draining lymph nodes where they display them in an immunogenic manner to T cells, recent mouse studies suggest that LCs have naturally immunosuppressive functions that can dampen T cell responses

[69–74]. Despite this proposed suppressive role, human LCs have been shown to be highly adept at class I-mediated antigen processing, cross-presentation, and inducing primary CD8<sup>+</sup> T cell responses [75], and have a transcriptional signature similar to murine dermal DC-specific subsets with similar cross-presenting capabilities [76]. In light of the results herein, this may suggest that immature human LCs residing in the epidermis presenting self-antigens dampen cell-mediated immune responses, and this natural suppressive ability is hijacked by HPV as it enters LCs without activating them. Conversely, if these LCs later become activated through proper stimulation, they are capable of inducing robust Th1+ and CD8+ T cell responses.

Overall, our data indicate the importance of therapeutic adjuvants like Poly-ICLC in restoring activity of LCs to educate CD4<sup>+</sup> and CD8<sup>+</sup> T cells for an effective cell-mediated anti-HPV immune response. Our results further indicate that the absence of costimulation by immature LCs does not contribute to Treg induction in the presence of HPV antigens, but does lead to the reduction of essential Th1-associated cytokine secretion. Moreover, the induced secretion of IL-10 in the unresponsive setting potentially prohibited the induction of effector functions while maintaining quiescence in the naïve CD4<sup>+</sup> T cell population. In conclusion, activated LCs presenting antigens with costimulation as signals 1 and 2, such as those stimulated with TLR agonists like Poly-ICLC, are necessary to induce Th1 and Th2 effector functions and can force ignorant CD8<sup>+</sup> T cells into generating a robust anti-HPV immune response.

## Conflict of interest

The authors declare no conflicts of interest.

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