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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+ 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 $CD8^+$ 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.

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 histocompatability 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 $CD4^+$ 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 $CD8^+$ T cells by APCs lacking costimulatory molecules was made in $CD8^+$ clones where the phenotype was described as inhibition of IL-2 production and proliferation, though less effect on interferon gamma (IFN-γ) production or cytotoxic activity was observed [38]. Despite the apparent retention of cytotoxic activity in tolerized $CD8^+$ T cells, the lack of clonal expansion hinders any measurable adaptive immune response.

Naïve $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ or $CD8^+$ 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 $CD4^+$ and $CD8^+$ 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+ 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 $CD8^+$ 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 $HLA\text{-}A2^+$ 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-γ 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-γ 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 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO), 50 μM β-Mercaptol Ethanol (Life Technologies), and 10% FBS (Omega Scientific, Tarzana, CA). PBMCs were plated in a 175-cm^2 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-β1 (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 $Langerin^+CD1a^+E\text{-}Cadherin^+$ (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.