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Langerhans cells from women with cervical precancerous lesions become functionally responsive against human papillomavirus after activation with stabilized Poly-I:C

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Abstract

Human papillomavirus (HPV)-mediated suppression of Langerhans cell (LC) function can lead to persistent infection and development of cervical intraepithelial neoplasia (CIN). Women with HPV-induced high-grade CIN2/3 have not mounted an effective immune response against HPV, yet it is unknown if LC-mediated T cell activation from such women is functionally impaired against HPV. We investigated the functional activation of *in vitro* generated LC and their ability to induce HPV16-specific T cells from CIN2/3 patients after exposure to HPV16 followed by treatment with stabilized Poly-I:C (s-Poly-I:C). LC from patients exposed to HPV16 demonstrated a lack of costimulatory molecule expression, inflammatory cytokine secretion, and chemokine-directed migration. Conversely, s-Poly-I:C caused significant phenotypic and functional activation of HPV16-exposed LC, which resulted in *de novo* generation of HPV16-specific CD8⁺ T cells. Our results highlight that LC of women with a history of persistent HPV infection can present HPV antigens and are capable of inducing an adaptive T cell immune response when given the proper stimulus, suggesting s-Poly-I:C compounds may be attractive immunomodulators for LC-mediated clearance of persistent HPV infection.

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Disclosures

GMM holds ownership interest in Akela Pharma, Inc. (formerly Nventa). AMS holds ownership interest in Oncovir, Inc.

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Keywords

Langerhans cells; human papillomavirus; HPV16; immune evasion

1. Introduction

Cervical intraepithelial neoplasia (CIN)¹ lesions of the squamous epithelium are premalignant transformations caused by high-risk human papillomavirus (hrHPV) infection [1]. Greater than 15% of women with hrHPV infections cannot initiate an effective immune response against HPV, and among those that do, viral clearance is very slow, leaving these women at higher risk for the development of CIN [2]. A high proportion of low-grade CIN lesions (CIN1) are naturally eliminated by the host's immune system over an extended period of time, however regression is much less common for high-grade lesions (CIN2/3), indicating restricted immune responses in women whose lesions have progressed [3]. Particularly, high-grade CIN2/3 patients have a well-documented risk of progressing to invasive squamous cell carcinoma or adenocarcinoma [4]. These observations highlight the need for therapeutic treatments that can stimulate the immune system to clear HPV infections, especially in patients with consecutive positive hrHPV DNA tests or in patients with low-grade CIN lesions in which the virus is still in a replicative phase. Current clinical management strategies for hrHPV⁺ women with or without low-grade CIN lesions are conservative, especially in young women, and include follow-up with repeated cytologic and HPV co-testing but no definitive treatment [5]. The lack of treatment options for persistent HPV infection leaves room for improvement in the management of cervical abnormalities including increased investigations into immunostimulants that could non-invasively activate an anti-HPV immune response.

The HPV family of viruses establishes persistent infections because it has evolved mechanisms that allow it to evade the human immune system. Langerhans cells (LC) are the resident antigen presenting cells (APC) of the epithelial layer, and are responsible for initiating an adaptive immune response against epithelium invading viruses. We have previously demonstrated that a key mechanism through which HPV evades the immune system is through HPV-mediated suppression of LC immune function [6–8]. Since HPV infections are strictly intraepithelial, HPV should be detected by LC. Studies on LC freshly isolated from human tissues or derived from progenitor cells *in vitro* demonstrate that these specialized APC possess a high capacity for antigen cross-presentation and stimulation of both CD4⁺ and CD8⁺ T cells [9–13]. This contrasts with murine LC, which seem to play a more immunoregulatory role in immune responses to epidermal antigens (reviewed in [14]). Nevertheless, in both cases, in the absence of immunostimulatory, or “danger” signals, LC presenting either self or foreign antigens without costimulation have the potential to induce T cell tolerance [15]. Proper antigenic stimulation of LC normally results in the initiation of activating signaling cascades, up-regulation of co-stimulatory molecules, and the release of

¹Abbreviations: APC, antigen presenting cell; CIN, cervical intraepithelial neoplasia; DC, dendritic cell; dsRNA, double stranded RNA; ELISpot, enzyme linked immunospot; HPRT, hypoxanthine phosphoribosyltransferase 1; HPV, human papillomavirus; hrHPV, high-risk HPV; HPV16, HPV type 16; IFN, interferon; IRG, interferon response gene; LC, Langerhans cell; MLR, mixed lymphocyte reaction; PAMP, pathogen associated molecular pattern; s-Poly-I:C, stabilized polyinosinic-polycytidilic acid; TLR3, toll-like receptor 3; cVLP, chimeric virus-like particle; VLP, virus-like particle

pro-inflammatory cytokines [16]. These activated LC then travel to lymph nodes where they interact with antigen specific naïve T cells and initiate an adaptive T cell response [17]. Effector T cells should then migrate back to the site of infection and destroy infected epithelial cells [18]. In the case of HPV infections, we have demonstrated that LC derived from healthy donors that are exposed to hrHPV capsids do not become functionally mature APC, and are therefore unable to initiate HPV16-specific cytotoxic T cell responses [6, 7], but this can be overcome with toll-like receptor (TLR) agonist stimulation [19, 20].

TLRs are membrane-spanning molecules that recognize pathogen associated molecular patterns (PAMPs). We have shown that treatment of LC from healthy donors with a TLR8 agonist *in vitro* is activating whereas a TLR7 agonist is not [19], suggesting that the specific PAMP engagement on LC has a major effect on the resulting immune response. TLR3 is an intracellular receptor responsible for the detection of viral dsRNA, and TLR3 signaling pathways initiate antiviral and inflammatory responses [21]. In LC, TLR3 is found in endosomes, but has also been detected on the surface of epithelial cells [22, 23]. Both natural and synthetic dsRNAs analogs are capable of providing the necessary warning signals to induce type I IFNs and other cytokines through TLR3 engagement. The synthetic dsRNA, polyinosinic-polycytidylic acid (Poly-I:C), is known as the strongest type I IFN inducer through TLR3 [24], and can induce LC maturation [25]. However, *in vivo* Poly-I:C is rapidly inactivated by blood enzymes making it inadequate for clinical applications [26]. To counter this, positively charged polypeptides have been demonstrated to stabilize Poly-I:C (s-Poly-I:C) thereby promoting their use in clinical settings [27]; poly-arginine stabilized Poly-I:C is known as Poly-ICR and poly-lysine and carboxymethylcellulose stabilized Poly-I:C is known as Poly-ICLC. We recently confirmed that monocyte derived LC express intracellular TLR3 and demonstrated that s-Poly-I:C could functionally activate LC from healthy donors after HPV exposure [20]. However it is unclear whether the LC from women with a history of persistent hrHPV infection, as evidenced by high-grade CIN (CIN2 or CIN3), would respond similarly since their LC and HPV-specific immune responses could be already suppressed. As women with existing persistent HPV infection would be the intended patient population to potentially benefit from antiviral inducing immune modulation, the primary objective of this study was to investigate whether s-Poly-I:C can overcome HPV-induced immune suppression by functionally activating LC exposed to HPV16, and inducing activation of HPV16-specific T cells *in vitro* from women who did not mount an effective immune response against HPV, in this case, women with clinical evidence of HPV-induced high-grade CIN2/3 lesions.

2. Materials and Methods

2.1 Patient material

Ten CIN patients were enrolled into this prospective tissue and blood collection study. Eligible patients gave informed consent, were immune competent for leukapheresis collection, were not pregnant, and had a biopsy-confirmed diagnosis of CIN2/3. Written informed consent for blood and tissue sampling was obtained from all individuals under a protocol approved by the Institutional Review Board. Patients were recruited from gynecology clinics at USC-affiliated hospitals. Cervical cells were collected via cervical

swab using the Digene Cervical sampler (Qiagen, Valencia, CA) prior to DNA extraction using the QIAamp MinElute Media Kit. Tissue specimens were obtained prior to cervical excision of high-grade lesions per the standard of gynecologic practice. Pathologic findings were confirmed on surgically excised cervical tissue. HIV status was determined by a HIV-1/HIV-2/HIV-0 multiplex antibody-screening test. HPV genotyping was performed using the INNO-LiPA HPV Genotyping Extra kit (Innogenetics, Seguin, TX). Low-resolution DNA typing for HLA-A2 was performed for all patients using standard endpoint PCR, and was then confirmed on isolated leukocytes by flow cytometry using an anti-HLA-A2 antibody (BD Biosciences, San Jose, CA). For patients testing positive for HLA-A2, high-resolution genotyping was performed by PCR to provide allele level typing at the HLA-A2 locus with the A*02 SSP UniTray Kit (Life Technologies, Carlsbad, CA). Leukapheresis was performed on blood samples to enrich peripheral blood mononuclear cells (PBMC), which were additionally purified immediately after collection using Lymphocyte Separation Media (Corning, Manassas, VA) by density gradient centrifugation, and cryopreserved in liquid nitrogen. Subjects were excluded from leukapheresis if they had a hematocrit <33% or a leukocyte count <4300/ μ L.

2.2 Antibodies, reagents and HPV16 viral particles

HLA-ABC FITC (MHC I), HLA-DP,DQ,DR FITC (MHC II), CD80 FITC, CD83 PE, CD86 FITC, and purified anti-human CCR7, were purchased from BD Biosciences (San Jose, CA). CD40 PE, purified rat IgG2a, goat anti-rat IgG PE, mouse IgG1 FITC, mouse IgG1 PE were purchased from Biolegend (San Diego, CA). Recombinant human (rhu)-CCL21 was purchased from R&D Systems (Minneapolis, MN), Rhu-GM-CSF from Berlex (Seattle, WA), and rhu-TGF β 1 and rhu-IL-4 from Biosource (Carlsbad, CA). Poly-ICR was provided by Nventa Biopharmaceuticals/Akela Pharma (Austin, TX). Poly-ICLC (Hiltonol®) is a clinical grade current good manufacturing practices (cGMP) form of s-Poly I:C that was provided by Oncovir, Inc. (Washington, D.C.) HPV16 L1L2 virus-like particles (VLP) and chimeric HPV16 L1L2-E7 VLP (HPV16 cVLP) were produced in insect cells and purified following published procedures [28], and endotoxin levels were below 0.06 EU as measured using an E-toxate kit (Sigma-Aldrich).

2.3 Langerhans cell generation

LC were derived from human PBMC following published procedures [19, 29]. In brief, monocytes were maintained in complete RPMI media with the addition of 1000 U/mL GM-CSF, 1000 U/mL IL-4, and 10 ng/ml TGF β for 7 days. The phenotype of monocyte derived immature LC was then confirmed by flow cytometry as MHC-I⁺MHC-II⁺CD1a⁺Langerin⁺TLR3⁺CD14⁻, similar to LC derived from healthy donors [20]. LC population was >90% enriched after 7 days in these culture conditions.

2.4 LC activation assay and flow cytometry

LC were treated with HPV16 VLP for 4 h before stimulation with s-Poly-I:C, and activation-associated surface markers were measured by flow cytometry following established procedures [29]. In brief, 10⁶ LC were seeded in a 6-well plate and left untreated, treated with 10 μ g HPV16 VLP, or 10 μ g HPV16 VLP for 4 h followed by 72 h incubation with s-Poly-I:C compounds. Cells were harvested, washed, stained for surface

MHC I, MHC II, CD40, CD80, CD83, CD86, CCR7 or isotype controls, and analyzed on an FC500 flow cytometry using CXP software (Beckman Coulter). Geometric mean fluorescence intensities (MFI) were used to calculate fold change in expression from untreated LC baseline values.

2.5 In Vitro Migration of LC

In vitro chemokine-directed migration towards CCL21 of LC was performed in transwell plates (Costar, Cambridge, MA) following established procedures [30]. In brief, 2×10^5 LC, untreated or treated with HPV16 VLP and/or s-Poly-I:C as described above, were added to the upper chamber wells and incubated for 4 h at 37°C. The cells that migrated to the lower chambers containing CCL21 or media alone after 4 h were counted using a Beckman Coulter Z1 Particle Counter.

2.6 Cytokine and chemokine analysis

Supernatants from different HPV16 VLP and s-Poly-I:C treated LC groups as described above were collected after 72 h and assayed using the Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA) following established procedures [29]. Analytes including IFN α , IL-1 β , IL-6, IL-12p70, IP-10, TNF α , MCP-1, MIP-1 α , MIP-1 β , and RANTES were assayed using a custom MilliPlex MAP Human Cytokine/Chemokine Panel following the manufacturer's instructions (Millipore, Billerica, MA).

2.7 In vitro immunization with HPV16 E7 and ELISPOT assay

LC from HLA-A*0201⁺ CIN patients and autologous purified CD8⁺ T cells and were co-cultured *in vitro* over several weeks to induce primary CD8⁺ effector T cell responses against defined HLA-A*0201 HPV16 E7 epitopes following established procedures [30]. Specifically, LC were left untreated or exposed to HPV16 L1L2-E7 cVLP, and were then treated with s-Poly-I:C or left alone. Additional s-Poly-I:C treated LC were exogenously loaded with a pool of HLA-A*0201 binding peptides (E7₁₁₋₁₉, E7₈₂₋₉₀ and E7₈₆₋₉₃) as a positive control. Isolated autologous CD8⁺ T cells were then co-cultured with irradiated LC at a 20:1 ratio for 7 days at 37 °C. Cultures were restimulated with untreated or treated irradiated LC at days 7, 14 and 21. T cells were harvested after 28 days and tested for HPV peptide-specificity via ELISPOT following an established laboratory protocol [30]. Spots were counted using the KS ELISPOT analysis system (Carl Zeiss, Thornwood, NY). HPV16 E7-reactive T cells against E7₁₁₋₁₉, E7₈₂₋₉₀ and E7₈₆₋₉₃ pooled peptides were quantified after subtraction of background spots from medium control wells.

2.8 Innate and adaptive immune response gene expression array

Quantitative mRNA expression analysis of 84 genes involved in the inflammatory and anti-viral immune response was performed with the Human Innate and Adaptive Immune Responses RT² Profiler PCR Array (SA Biosciences, Qiagen, Valencia, CA). LC were treated with 50 μ g/mL Poly-ICLC with or without prior exposure to HPV16 VLP. After 24 h, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). A total of 2 μ g of RNA from each sample were then converted to cDNA using the RT² First Strand Kit (SA Biosciences). PCR array cycling conditions and data collection were performed according to

the manufacturer's protocol with the RT² Real-Time SYBR Green PCR Master Mix on a CFX96 real time thermal cycler (Bio-Rad). PCR data were analyzed using templates downloaded from the manufacturer's website (<http://www.sabiosciences.com/pcrarraydataanalysis.php>). mRNA expression of each gene was normalized using the average expression of hypoxanthine phosphoribosyltransferase 1 (HPRT), one of five housekeeping genes included on every plate that showed the least variability between samples. Gene expression data were analyzed for all genes below a set default lower limit of detection threshold cycle (C_T) cut-off of 35. All samples passed quality control checks for PCR array reproducibility, reverse transcription efficiency, and genomic DNA contamination. Data from the untreated LC (control group) were compared to treatment groups according to the 2^{-CT} method [31]. Correlation between results of RT² Profiler PCR arrays and qPCR is reported to be good [32].

2.9 Statistical analysis

GraphPad Prism 6 was used for all statistical analyses (San Diego, CA). Significance was determined by a non-parametric Kruskal-Wallis statistical test following by a Dunn's multiple comparisons test comparing each treatment group for pooled experiments from individual patient samples. A one-way analysis of variance (ANOVA) was used for normally distributed data, followed by a Tukey's post-test. A two-tailed Non-parametric Mann-Whitney *U* test was performed for representative experiments of technical replicates of single donors performed in triplicate wells. Results with *P*-values <0.05 were considered significant.

3. Results

3.1 Patient population

Ten patients with histologically confirmed high grade CIN2/3 (average age, 34 ± 9.8 yrs) gave consent to participate in this study. All patients were tested for the presence of HLA-A*0201 allowing for the examination of HPV16 specific CD8⁺ T cell responses to HLA-A*0201-binding HPV16 E7 peptides [33]. A subset of CIN2/3 patients (n=3) were seropositive for HIV; allowing examination of an important population due to the higher prevalence, incidence, and persistence of HPV infection in HIV⁺ women [34]. Table 1 lists the baseline characteristics of study participants. Seven of ten CIN patients were diagnosed with CIN3 lesions, the remaining patients were diagnosed with CIN2. Several cases were multi-focal, having more than one area of dysplasia. Similar to what has been described for the distribution of hrHPV types in high-grade lesions [35], HPV16 was found in 50% of the CIN2/3 cases.

3.2 Upregulation of MHC and costimulatory molecules on HPV16-exposed LC from CIN2/3 patients using s-Poly-I:C (Poly-ICR)

LC from CIN2/3 patients were exposed to HPV16 VLP, followed by treatment with s-Poly-I:C (Poly-ICR), and then analyzed for the expression of antigen presentation and co-stimulatory molecules. In their immature state, LC express lower levels of MHC class I and class II molecules, but show little to no expression of CD40, CD80, CD86 costimulatory molecules, the CD83 maturation marker, or chemokine receptor CCR7 (Fig. 1a, untreated).

Upon treatment of LC from CIN2/3 patients with HPV16 alone, the expression of these markers did not change, demonstrating a lack of phenotypic activation. As hypothesized, LC pre-exposed to HPV16 that were then subsequently stimulated with s-Poly-I:C showed a significant increase in the expression of MHC and costimulatory molecules (Fig. 1a). As expected, LC treated with s-Poly-I:C alone also demonstrated phenotypic activation. Quantification of activation-associated markers from all ten CIN2/3 patients indicated that treatment of LCs with s-Poly-I:C after pre-exposure to HPV16 caused a significant upregulation of all surface markers analyzed compared to untreated LC and LC exposed to HPV16 alone (Fig. 1b). No significant differences were found in the activation status of LC derived from the small cohort of patients co-infected with HPV and HIV compared to patients infected with HPV alone (data not shown). These results indicate that HPV16-exposed LC from CIN patients are responsive to s-Poly-I:C stimulation and are capable of proper expression of molecules involved in antigen presentation.

3.3 HPV16 exposed LC from CIN2/3 patients migrate to CCL21 after Poly-ICR treatment

LC migration to lymph nodes after peripheral activation is required for successful induction of antigen-specific T cells. As an *in vitro* surrogate of LC migration *in vivo*, a transwell chemotaxis assay was performed to assess the capacity of LC to migrate towards CCL21 after exposure to HPV16 VLP followed by treatment with s-Poly-I:C (Poly-ICR). CCL21 is a lymphoid organ chemokine that directs LC towards lymph nodes through expression of the maturation-induced CCR7 receptor on activated LC [17]. Treatment of LC from CIN2/3 patients with s-Poly-I:C after HPV16 exposure resulted in a significant increase in CCR7 expression (Fig. 2a) and a significant increase in migration compared to untreated LC or LC exposed to HPV16 alone (Fig. 2b), indicating that women with a history of persistent hrHPV infection have LC that retain their capacity for chemokine-directed migration. No significant differences were observed between untreated LC and HPV16-exposed LC. Similar CCR7 expression and migration increases were observed in the s-Poly-I:C treated LC, with or without exposure to HPV16.

3.4 HPV16-exposed LC from CIN2/3 patients produce significant amounts of inflammatory cytokines and chemokines in response to Poly-ICR

Analogues of dsRNA such as Poly I:C are well known for inducing a type I interferon response through activation of the transcription factor interferon regulatory factor 3 (IRF3), as well as the NF- κ B and activator protein 1 (AP-1) signaling cascade leading to production of additional inflammatory cytokines and chemokines [21, 24]. Since the induction of anti-viral CD8⁺ T cells requires APC to produce pro-inflammatory cytokines and chemokines, LC from CIN2/3 patients were tested for their ability to secrete a variety of inflammatory cytokines and chemokines after exposure to HPV16 followed by treatment with s-Poly-I:C (Poly-ICR). LC that were pre-exposed to HPV16 prior to treatment with s-Poly-I:C produced a significant amount of cytokines and chemokines compared to untreated or HPV16-exposed LC, most notably TNF α , IFN α , IL-1 β , IL-6, IL-12p70, IFN- γ -inducible protein 10 (IP-10), MCP-1, MIP-1 α , MIP-1 β , and RANTES (Fig. 3). These results demonstrate that s-Poly-I:C treated HPV16-exposed LC of CIN2/3 patients are capable of secreting inflammatory cytokines and chemokines that can activate and attract T cells to the site of the lesion after antigen presentation and T cell priming.

3.5 LC from CIN2/3 patients treated with Poly-ICR induce HPV16-specific CD8⁺ T cell responses

Next, we tested whether LC from CIN patients pre-incubated with HPV16 followed by treatment with Poly-ICR could induce an HPV16-specific CD8⁺ T cell response in HLA-A2⁺ donors. To accomplish this, HPV16 L1L2-E7 cVLP containing an L2-E7 fusion protein encapsidating the E7 protein inside the VLP was used for antigen processing and presentation [36]. Several defined HLA-A*0201 binding E7 peptides [33] were used to detect the HPV16 E7-specific CD8⁺ T cell reactivity. In three out of six HLA-A2-positive CIN patients tested (CIN 01, 05, 07), LC pre-exposed to HPV16 cVLP and subsequently treated with s-Poly-I:C were able to induce significantly more IFN γ secreting HPV16 E7-peptide-specific CD8⁺ T cell responses after s-Poly-I:C treatment compared to LC exposed to HPV16 cVLP alone (Table 2). Three patients (CIN 02, 05, 08) were characterized as low responders (<100 antigen-specific CD8⁺ T cells per million cells) at baseline. While two patients (CIN 02, 08) remained low responders with s-Poly-I:C treatment, one patient (CIN 05) became a high responder (>300 antigen-specific CD8⁺ T cells per million cells). One patient (CIN 01) was a mid-level responders (>100 to <300 antigen-specific CD8⁺ T cells per million cells) at baseline and became a high responder after s-Poly-I:C treatment. The last patient (CIN 10) was a high responder at baseline and remained a high responder. Importantly, all HLA-A2-positive patients (6 out of 6) exhibited at least some HPV16-specific CD8⁺ T cell response after treatment with s-Poly-I:C. T cell responses were observed in both patients with HPV16-positive lesions and HPV16-negative lesions, indicating that HPV16 positivity did not preclude generation of *de novo* immunity to HPV16 E7 antigen. Individual LC surface activation marker data, migration indices, and cytokine responses are summarized for each CIN2/3 patient in Table 2. Collectively, these results demonstrate that LC from CIN2/3 patients exposed to HPV16 and subsequently treated with s-Poly-I:C are able to become functional APC capable of enhancing the number of IFN γ -producing CD8⁺ T cells, and in some cases inducing a *de novo* HPV-specific CD8⁺ T cell response *in vitro* using HPV16 E7 as a model viral antigen. Additionally, as three of the responding CIN2/3 patients were also HIV⁺, these data suggest that s-Poly-I:C can enhance the function of LC against HPV antigens in HPV/HIV co-infected women.

3.6 Poly-ICLC induces LC activation in HPV16-exposed LC

The above results show that poly-arginine stabilized Poly-I:C (Poly-ICR) induces phenotypically and functionally activated LC in the presence of HPV16. Poly-lysine stabilized Poly-I:C (poly-ICLC, Hiltonol), is a dsRNA viral mimic similar to Poly-ICR that has been used as an investigational drug in several past and present clinical trials as a vaccine adjuvant [37–39]. Because of its good safety profile in humans and availability for immediate clinical testing, we wanted to confirm the activity of Poly-ICLC on HPV16-exposed LC from CIN patients, with the expectation that Poly-ICLC would elicit a similar activation phenotype on LC activation as Poly-ICR. Therefore, LC from CIN2/3 patients that were pre-exposed to HPV16 were treated with Poly-ICLC, and activation was evaluated through upregulation of antigen presentation and costimulatory molecules, *in vitro* migration, and secretion of pro-inflammatory cytokines and chemokines. As anticipated, Poly-ICLC was able to activate LC from CIN2/3 patients pre-exposed to HPV16 such that

expression of MHC I, MHC II, CD40, CD80, CD83, and CD86 were significantly increased (Fig. 4a), similar to Poly-ICR (Fig. 1b). Treatment of HPV16-exposed LC from CIN2/3 patients with Poly-ICLC similarly resulted in a significant increase in CCR7 expression (Fig. 4b) and chemotactic migration towards CCL21 (Fig. 4c). Treatment with Poly-ICLC alone induced similar trends in phenotypic markers compared to the HPV16-exposed group treated with Poly-ICLC when the drug was used at lower concentrations, mirroring responses observed with Poly-ICR (supplemental Fig. S1). Additionally, similar to Poly-ICR-induced cytokine induction shown in Fig. 3, treatment of HPV16-exposed LC with Poly-ICLC resulted in significant increases in the amounts of inflammatory cytokines and chemokines analyzed (supplemental Table S1). Collectively these results demonstrate that both Poly-ICLC and Poly-ICR can induce functional activation of HPV16-exposed LC from CIN2/3 patients.

3.7 Poly-ICLC induces innate and adaptive immune response gene expression in HPV16-exposed LC

Poly-I:C is a well-established activator of the endosomal TLR3 and cytosolic MDA-5 pathways, resulting in activation of innate immunity through induction of type I interferons [24, 40]. Moreover, Poly-ICLC has been shown to induce a systemic transcriptional profile similar to live dsRNA virus infection in humans [41]. We thus evaluated the expression of multiple innate and adaptive immune response genes using a PCR gene expression array to confirm the expected effect of Poly-ICLC on genes involved in the anti-viral immune response in LC derived from CIN patients. Of particular interest were genes known to be downstream of TLR3, TRIF, IRF3, and NF κ B signaling, and genes whose proteins were found to be upregulated and secreted upon s-Poly-I:C treatment of HPV16-exposed LC. Untreated LC and HPV16-exposed LC showed similar gene expression profiles, with generally low expression of genes involved in T cell activation. As predicted, genes involved in the interferon signaling pathway (IRF3, IRF7), downstream transcription factors (JAK2, STAT1, TRAF6), and interferon response genes (IRGs) (MX1, IFNA1, IFNB1), were all upregulated in response to Poly-ICLC with and without pre-exposure to HPV16 (Fig. 5). Specifically, IFNA1 and IFNB1 mRNA were significantly upregulated in Poly-ICLC treated LC (IFNA1, 28.6-fold; IFNB1, 61-fold) and in LC exposed to HPV16 followed by Poly-ICLC treatment (IFNA1, 32-fold; IFNB1, 65-fold) compared to untreated LC. Similarly, increased gene expression was observed for IL1B (Poly-ICLC, 155-fold increase; HPV16+Poly-ICLC, 176-fold increase), IL-6 (Poly-ICLC, 1846-fold increase; HPV16+Poly-ICLC, 2062-fold increase), CXCL10/IP10 (Poly-ICLC, 189-fold increase; HPV16+Poly-ICLC, 198-fold increase), and CCL5/RANTES (Poly-ICLC, 135-fold increase; HPV16+Poly-ICLC, 133-fold increase), consistent with the increased secretion of their respective cytokines and chemokines observed in Fig. 3. TLR-associated genes (TLR3, TLR8, DDX58/RIG-I, TICAM1/TRIF) and genes associated with LC maturation and antigen presentation to T cells (HLA-A, CD40, CD80, CD86, ICAM1) were also upregulated with Poly-ICLC treatment in the presence of HPV16 with increases ranging from 3-fold to 24-fold (Fig. 5). Gene expression associated with various T cell subsets (CD8A, CD4, IL4, IL5, IL13, IL17A) was not detected, confirming the high purity of the LC population (data not shown). Taken together, these data verify the activation of the

TLR3 pathway in HPV16- and Poly-ICLC-exposed LC leading to upregulation of genes involved in viral recognition and innate and adaptive immunity.

4. Discussion

FDA-approval of the Cobas® HPV Test for first line cervical cancer screening following the ATHENA (Addressing **THE** Need for **A**dvanced HPV Diagnostics) trial [42–45] has the potential to change clinical management of persistent hrHPV infection as well as detect clinically meaningful hrHPV infections. As HPV DNA testing becomes more widely implemented as an initial screening tool, there will be a larger number of women who test positive for infection with hrHPV genotypes. Many will have normal cytology or low-grade dysplasia for which there is no definitive treatment, creating a clinical need for non-HPV genotype specific treatment options such as s-Poly-I:C that may induce HPV clearance through activation of LC-mediated immune responses.

During hrHPV infection of the basal cells of the epithelial layer, HPV antigens should be processed and presented by LC, the resident professional APC of the parabasal and lower suprabasal layers of squamous epithelium [46]. The lack of an effective immune response suggests that viral antigens are not adequately presented to the immune system. *In situ* immunohistochemistry studies of patient-derived cervical tissue indicate that progressive CIN is associated with a relative increase in LC with an immature phenotype that is negative for expression of adhesion and costimulatory molecules (e.g., ICAM-1, CD86), particularly at the cervical transformation zone. Moreover, these LC exhibit reduced ability to activate T cells, partly due to a decreased expression of TNF α and an increase in the immunosuppressive cytokine IL-10 [47–49]. The immature phenotype of mucosal LC is suggestive of limited immune activation within cervical lesions, although the functional outcome of *in vivo* viral antigen presentation is difficult to predict since maturation may occur during migration to lymphoid organs. While prior investigations have found that HPV16 VLP can stimulate the activation of dendritic cells (DC) demonstrating their natural immunogenicity [50–54], HPV16 VLP internalization into LC results in dysregulated PI3K-Akt signaling and defective cellular activation, which we have determined to be HPV16 L2 capsid protein driven [55, 56]. Immature LC already express little to no costimulatory molecules and do not secrete inflammatory cytokines, thus their lack of activation and maturation by HPV capsids results in APC that present HPV antigens without the necessary signals for optimal T cell activation. Beyond HPV16, we have shown that other HPV genotypes such as high-risk (HPV18, HPV31, and HPV45), low-risk (HPV11), and a cutaneous (HPV5) genotype, also suppress LC activation through a similar mechanism [8]. Unlike murine LC which have in recent studies been shown to suppress T cell responses towards epicutaneous antigens [14], human LC are highly adept at class I-mediated antigen processing, cross-presentation of antigens, and inducing primary CD8⁺ T cell responses [11], having a transcriptional signature similar to murine dermal DC-specific subsets with similar cross-presenting capabilities [9]. Together this suggests that human LC may be antagonistically manipulated by HPV compared to other APC, such as DC, and represents a specific mechanism utilized by HPV to avoid alerting the immune system to its presence.

The results of the current study show that *de novo* generated LC of CIN2/3 patients are not activated by and do not induce an adaptive immune response when exposed to HPV16 alone, which may be one of the first immune escape events that takes place after viral infection. These data appear to be similar to what we have previously demonstrated for LC derived from healthy donors [6, 7]. This observation was seen regardless of whether patients had HPV16-positive or HPV16-negative cervical lesions, suggesting that the LC responses are independent of previous exposure to any particular HPV genotype. Indeed, three of the ten patients tested positive for multiple HPV types. Using HPV16 as the model hrHPV genotype that evades immune detection and E7 as the model tumor antigen, we show that monocyte-derived LC from all CIN patients that failed to initially mature after exposure to HPV16 particles were capable of becoming functionally active APC and were capable of inducing HPV16 E7-specific CD8⁺ T cell responses in the presence of TLR3 stimulation using s-Poly-I:C (either Poly-ICR or Poly-ICLC) *in vitro*. This observation is important because it highlights that, in general, the LC of women with persistent hrHPV infection are not functionally impaired, and are capable of inducing an adaptive anti-viral immune response when a proper “danger” or immunostimulatory signal is applied. We demonstrated that Poly-ICLC induces an interferon and inflammatory gene expression profile in LC derived from CIN2/3 patients similar to that of peripheral blood systemic responses in healthy donors injected subcutaneously with the drug [41], which is highly suggestive of a similar *in vivo* mechanism of action. These data are in line with our previous report demonstrating that monocyte-derived LC derived from healthy donors express TLR3 and are strongly activated by the s-Poly-I:C compounds Poly-ICR and Poly-ICLC [20], which prompted the current investigation of testing s-Poly-I:C on LC derived from CIN patients. Not surprisingly, in a minority of CIN2/3 patients, we were only able to detect very low numbers of HPV16 E7-specific CD8⁺ T cells after LC exposure to E7-containing cVLP and treatment with s-Poly-I:C, despite the fact that the LC from these patients showed functional activation and maturation (Table 2). HIV-negative patients with CIN2/3 have a functional T cell compartment; however, each individual is expected to have a unique T cell repertoire which might be limited in the antigens to which it can respond. Notably, the three HIV-positive patients all mounted a CD8⁺ T cell immune response towards HPV16 E7 HLA-A2-binding epitopes, indicating that this type of immune modulating treatment could have clinical utility for this special population at high-risk for HPV persistence if T cell counts are within the normal range. In all, two HIV⁺ CIN patients and one HIV-negative CIN patient showed that treatment of LC with s-Poly-I:C enhanced the number of IFN γ -producing CD8⁺ T cells against E7 peptides compared to LC exposed to HPV16 cVLP alone. Those patients who remained low responders even after s-Poly-I:C treatment of LC may represent patients whose T cell repertoires are more limited despite having functional LC.

A strength of our study is that we have tested two formulations of s-Poly-I:C (Poly-ICR and Poly ICLC), one of which has been used clinically as a vaccine adjuvant for immunotherapy studies, and therefore can be rapidly translated into a clinical trial for women with persistent hrHPV-positive cervical screening tests. Non-stabilized Poly-I:C is rapidly degraded by nucleases in human serum [26], limiting their applicability to clinical translation. Positively charged polypeptides such as poly-arginine (Poly-ICR) and poly-lysine (Poly-ICLC) have been shown to stabilize Poly-I:C (s-Poly-I:C) increasing the feasibility of using them

clinically [57]. Poly-ICR induced robust responses in LC from all CIN patients, and these results were then confirmed with clinical grade Poly-ICLC. *In vitro*, the mechanism of action of either Poly-I:C or stabilized Poly-I:C (Poly-ICLC) appears to result in similar gene expression and transcriptional profiles after stimulation of PBMC, suggesting that the improved *in vivo* activity of Poly-ICLC may be related to the stabilization effect rather than differences in TLR3 ligation [41]. One might envision topical application of Poly-ICLC to the cervix at intervals that would stimulate local activation of LC and the immune system, analogous to *in situ* vaccination. In this regard, sequential intratumoral Poly-ICLC administration has been shown to result in therapeutic *in situ* autovaccination in the case of a patient with a solid tumor through a mechanism involving release of tumor antigen and activation of DC, followed by T cell priming [37]. An additional potential benefit of Poly-ICLC treatment of persistent HPV infections is reversal of the effects of HPV infection on reduced HLA class I expression by infected epithelial cells, an immune evasion tactic mediated by HPV viral DNA integration and expression of the E5 and E7 viral gene products [58–60]. Poly-ICLC-induced upregulation of HLA class I on infected cells would make them more susceptible to CD8⁺ T cell recognition and killing. Topical Poly-ICLC might also be combined with targeted immunotherapeutic approaches, such as therapeutic vaccination or adoptive cell transfer of genetically engineered CD8⁺ and CD4⁺ T cells with TCR specificity redirected towards either of the viral oncogenes [61, 62]. An increase in local chemokine and interferon production would be expected to enhance the migration of redirected and primed T cells to the site of infection and potentiate their effector functions. A further strength of our study is that we have utilized LC from women who have, for some reason, not responded to their HPV infection, which resulted in viral persistence and development of abnormal cellular cytology. The CIN2/3 patient population is most representative of women in the general population who were unable to clear their hrHPV infection. Therefore, it was important to examine how this high-risk population would respond to s-Poly-I:C, rather than draw on data obtained strictly from healthy donors who are more likely to clear infection, since women with persistent infection are the intended population for this type of immune intervention.

We have shown previously that *de novo* generated LC from healthy donors respond similarly to s-Poly-I:C as the LC from CIN2/3 patients in this study with upregulation of co-stimulatory and maturation markers, as well as increased migration. Healthy donor LC likewise become activated by s-Poly-I:C, leading to HPV16-specific T cell induction [20]. Healthy donor LC also secreted high levels of cytokines and chemokines in response to s-Poly-I:C, similar to CIN2/3 patients in this study. These two studies suggest, therefore, that the primary defect in recognition of HPV is not due to an inherent functional defect in ability of LC to present antigen, which would be anticipated since women with CIN2/3 are not globally immune suppressed. Rather, lack of LC activation upon viral exposure appears to be a shared characteristic between women who clear and do not clear infection, pointing to other causes as being more important for viral persistence, perhaps related to genetics, environmental exposures, or a combination thereof.

Limitations of our study include a small number of patients who were both HPV16-positive and HLA-A*0201-positive, and the source of LC used as proof-of-concept for this study.

Peripheral cytotoxic T lymphocyte responses against HPV16 E7 epitopes can be detected in about half of women who have cleared HPV16 infection [63] but are detected very rarely in HPV16⁺ CIN3 patients [64]. Moreover, in some cases, immune tolerance against HPV antigens may develop in women with long term viral persistence in the setting of chronic antigen exposure [65]. The limited number of HPV16-positive and HLA-A*0201-positive patients in our study did not allow us to examine whether HPV16⁺ patients have CD8⁺ T cells peripherally tolerized to E7 with statistical confidence. However, our study differs from these previous observational studies in that the CD8⁺ T cell responses we detect are generated *de novo* through an extended *in vitro* immunization assay as opposed to measuring memory T cell responses after short term stimulation of T cells with peptides. Hence, the E7-specific CD8⁺ T cells reported herein reflects a biological process that is capable of occurring in patients when properly activated HPV-exposed LC and T cells interact optimally, in this case, after activation of LC with s-Poly-I:C. An important finding of this study was the observation that having an HPV16-positive lesion did not preclude peripheral blood T cells from reacting to HPV16 viral antigens when presented appropriately by activated LC. We can therefore infer that, at least in some cases, HPV16-specific T cells in patients with HPV16⁺ lesions remain poised for activation and are not irreversibly tolerized or otherwise rendered unresponsive. The LC used in our *in vitro* assays were blood-derived rather than isolated from the cervical mucosa of patients, the latter being impractical to obtain for functional studies in sufficient numbers and in an inactivated state from standard clinical patient punch biopsies. Therefore, our study cannot confirm whether mucosally-derived tissue-resident LC from CIN patients will respond identically to the *de novo* generated LC from these same individuals. Additionally, our culture system does not replicate the complex interactions that take place *in vivo* between epithelial cells, LC, and intraepithelial lymphocytes along with cytokine cross-talk. Despite this, previous LC studies suggest that freshly isolated epidermal LC and LC derived from peripheral blood monocytes have similar phenotypes, and importantly, respond similarly to Poly-I:C [25, 66]. Additionally, human endocervical, ectocervical, and vaginal epithelial cells treated with Poly-I:C *in vitro* exhibit a dose-dependent increase in the secretion of the chemokine IL-8/CXCL8, indicating that cells at the cervical transformation zone, which is highly susceptible to hrHPV infection, would be responsive to TLR3 agonists [67]. Therefore, we expect cervical epithelium LC to respond to s-Poly-I:C in a similar manner, though this would have to be confirmed through biomarker analysis in a well-designed clinical trial.

LCs make up approximately one in twenty nucleated cells in the epidermis, and are able to form an expansive cellular network through extended dendrites to detect invading antigens [68]. However, observational studies have shown that LC numbers are reduced an average of 2-fold in HPV positive CIN1 lesions compared to normal cervical mucosal tissue due to downregulation of epithelial-expressed E-cadherin, indicating other effects on LC dysfunction associated with HPV infection [69–71]. While these observations may suggest that TLR3 agonists might have reduced efficacy in inducing viral clearance of HPV infected tissue if only LC are targeted, it is worth noting that a study of the TLR transcriptome in the HPV-positive cervical cancer microenvironment revealed an increase in TLR3 expression in pre-malignant and malignant cervical tissue compared to other TLRs [72]. Therefore, even if the number of LC in HPV-infected tissues is reduced, s-Poly-I:C molecules may still have

potent immunomodulatory activity at the cervix through epithelial-expressed TLR3. Additionally, it is not known how many LC require activation in order to initiate the cascade of T cell activation in the draining lymph nodes. *In vivo*, s-Poly-I:C has the potential to act not only on hematopoietic cells but also non-hematopoietic cells, which are the main source of anti-viral type I IFNs. Interestingly, higher TLR3 expression detected from cervical cytobrush samples from HPV-positive patients was correlated to HPV16 clearance, demonstrating a link between TLR3 and HPV clearance *in vivo* [73, 74]. The association between TLR3 expression and HPV clearance is somewhat surprising considering HPV is a DNA virus with no dsRNA genome intermediates and is therefore unlikely to signal through TLR3 directly. However, it highlights the important role of TLR3 signaling pathway in anti-viral immunity even in the absence of direct virus engagement of these intracellular PAMP receptors.

In conclusion, we demonstrate that *de novo* generated LC from high-grade CIN2/3 patients respond strongly to poly-peptide stabilized forms of poly-I:C. While both stabilized compounds demonstrate the capability of initiating potent LC-mediated immune responses, Poly-ICLC is currently being used in clinical trials including studies in healthy donors and in cancer patients to stimulate immunity against solid tumors, and has demonstrated a good clinical safety profile [38, 41, 75–77]. For this reason and with the supporting evidence provided in this study, we suggest that Poly-ICLC should be further explored as a topical immunomodulator to induce anti-HPV immunity and promote viral clearance *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Langerhans cells (LC) from women with CIN2/3 are not activated by HPV16
- S-Poly-I:C-activated LC from CIN2/3 patients are functionally active APCs
- s-Poly-I:C-activated LC from CIN2/3 patients induce HPV16-specific CD8⁺ T cells
- s-Poly-I:C may be clinically useful for treating persistent HPV infection in CIN patients

Fig. 1. MHC and costimulatory molecule upregulation on LC from CIN2/3 patients with Poly-ICR treatment

Immature LC from CIN patients were left untreated or exposed to HPV16 VLP.

Subsequently, cells were treated with s-Poly-I:C (10 µg/mL Poly-ICR) for 48h. Controls were left untreated, exposed to HPV16 VLP alone, or treated with s-Poly-I:C alone. **(A)** Representative flow cytometry data from CIN 02 patient LC untreated or exposed to HPV16 VLP or exposed to HPV16 VLP followed by s-Poly I:C. Expression of indicated surface marker is shown in black histograms. Isotype control staining is shown as gray histogram. **(B)** Fold increases in LC surface marker expression from CIN2/3 patients ($N=10$ individual patients) based on geometric mean fluorescence intensity (MFI). Each data point represents

an individual patient. Horizontal lines indicate group mean $\pm$ 95% confidence interval.
** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Kruskal-Wallis statistical test following by Dunn's multiple comparisons test).

Fig. 2. CCR7 expression and migrate to CCL21 in HPV16-exposed LC treated with Poly-ICR LC were exposed to HPV16 prior to s-Poly-I:C (10 μ g/mL Poly-ICR) treatment as described. **(A)** CCR7 expression was analyzed by flow cytometry. Data represent mean fold increase in CCR7 expression ($\pm$ 95% confidence interval) relative to untreated LC based on MFI from CIN2/3 patients ($N=10$ individual patients). **(B)** *In vitro* migration assay. LC were analyzed for chemotaxis to CCL21 or medium alone through a transwell insert. Shown is the mean number of LC migrating to CCL21 (black bars) compared to spontaneous migration (white bars) ($\pm$ SEM) of ten CIN2/3 patients relative to untreated LC. *** $p<0.001$, **** $p<0.0001$ compared to untreated LC (Kruskal-Wallis statistical test, Dunn's multiple comparisons post-test).

Fig. 3. Production of inflammatory cytokines and chemokines by CIN2/3 patient HPV16-exposed LC treated with Poly-ICR

LC from CIN patients ($N=10$) were left untreated or exposed to HPV16 prior to treatment with s-Poly-I:C (10 μ g/mL Poly-ICR). Cell supernatants were analyzed for a panel of 10 cytokines and chemokines using a Bio-Plex Suspension Array System. Data represent the mean ($\pm$ SEM) analyte concentration from 10 individual patients. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$ compared to untreated LC (one-way ANOVA, Tukey's post-test).

Fig. 4. HPV16-exposed LC from CIN2/3 patients treated with Poly-ICLC become phenotypically and functionally active

(A) Poly-ICLC induces upregulation of MHC and costimulatory molecules on LC. LC from a CIN patient were left untreated or exposed to HPV16 prior to treatment with s-Poly-I:C (50 μ g/mL Poly-ICLC), then analyzed by flow cytometry. Control cells were left untreated or exposed to HPV16 alone. Data is representative of one patient (CIN 06) out of three patients analyzed. Data shown is the mean fold increase in surface marker expression ($\pm$ SD) of triplicate replicate values relative to untreated LC based on MFI. (B) Poly-ICLC induces HPV16-exposed LC to express CCR7 as measured via flow cytometry. Data shown for one representative patient out of three is shown as the mean fold increase in CCR7 expression ($\pm$ SD) of triplicate replicate values relative to untreated LC based on MFI. (C) Poly-ICLC induces HPV16-exposed LC to migrate to chemokine CCL21 *in vitro*. LC from a CIN patient were exposed to HPV16 prior to Poly-ICLC. LC were analyzed for migration to medium or medium supplemented with CCL21. Shown is the mean ($\pm$ SD) number of LC migrating to CCL21 compared to spontaneous migration of a representative CIN patient

performed in triplicate wells. *** $p < 0.001$ compared to both untreated LC and HPV16-exposed LC (Student's t test).

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Fig. 5. Poly-ICLC induces gene expression associated with interferon response and innate and adaptive immunity in LC from CIN2/3 patients exposed to HPV

LC were left untreated or exposed to HPV16 prior to treatment with Poly-ICLC (50 µg/mL) for 24 h. Total RNA was isolated and gene expression was analyzed using a PCR gene array profiler for 84 human innate and adaptive immune response genes. Shown is a heat map of expression of genes involved in the interferon response, cytokines and chemokines, antigen presentation, and PAMP receptors and associated adapter molecules that were modulated in unexposed LC or HPV16-exposed LC treated with Poly-ICLC from one representative CIN

patient. Untreated LC served as the control group for gene expression analysis after normalization to the housekeeping gene HPRT1.

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Table 1

Baseline characteristics of CIN patients included in this study.

Subject	Age (yr)	Staging of cervical lesion(s)	Type of neoplasia	HPV type(s)	HLA-A2 genotype	HIV status
CIN 01	35	CIN3, CIN1	Multifocal	16, 31	A*0201	Neg
CIN 02	59	CIN3	Multifocal	58	A*0201	Neg
CIN 03	30	CIN2	Multifocal	58	Negative	Neg
CIN 04	27	CIN2, CIN1	Multifocal	16	Negative	Neg
CIN 05	24	CIN3	Unifocal	52	A*0206	Pos
CIN 06	27	CIN3	Multifocal	16	Negative	Neg
CIN 07	33	CIN2, CIN1	Multifocal	18	A*0201	Pos
CIN 08	39	CIN3	Unifocal	16	A*0201, A*0206	Neg
CIN 09	35	CIN3	Multifocal	16, 82	Negative	Neg
CIN 10	35	CIN3, CIN2	Multifocal	31, 52, 53	A*0201	Pos

Table 2

Summary of analysis of LC function and induction of HPV16 specific CD8⁺ T cell responses in CIN patients.

Subject	LC activation marker increase ^a	LC Migration Index ^b	LC cytokine secretion ^c	HPV16 E7 epitope specific HLA-A*0201 IFN γ producing T cells ^d	
				HPV16 cVLP	HPV16 cVLP + s-Poly-I:C
CIN 01	+	4.5	+	145	358
CIN 02	+	6.0	+	56	12
CIN 03	+	44.5	+	ND ^e	ND
CIN 04	+	11.8	+	ND	ND
CIN 05	+	32.6	+	2	1091
CIN 06	+	14.8	+	ND	ND
CIN 07	+	12.0	+	0	875
CIN 08	+	15.0	+	36	32
CIN 09	+	13.1	+	ND	ND
CIN 10	+	49.7	+	1574	378

^aPositive response defined by at least 2-fold increase in MFI in LC activation markers analyzed.

^bMigration index, average number of CCL21 chemokine directed migrating cells divided by spontaneous number of migrating cells. Shown is migration index of LC in HPV16 + s-Poly-I:C group. Migration index of untreated LC was always 2.0.

^cPositive cytokine response defined by at least 10-fold increase the secretion of pro-inflammatory cytokines and chemokines (TNF α , IFN α , IL-1 β , IL-6, IL-12p70, IP-10, MCP-1, MIP-1 α , MIP-1 β , RANTES) in HPV16 VLP + s-Poly-I:C group compared to untreated LC group.

^dNumber of E7 peptide pool specific IFN γ producing CD8⁺ T cells per million PBMC induced by stimulation of autologous CD8⁺ T cells with HPV16 cVLP-exposed LC alone or HPV16 cVLP + s-Poly-I:C treated LC in HLA-A*0201 positive patients. Average number of spots from untreated LC wells has been subtracted to display number of peptide-specific T cells induced in each treatment group.

^eND, not determined due to non HLA-A*0201 status.