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SHORT COMMUNICATION

# Naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN2/3

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**Abstract** Essentially all squamous cervical cancers and their precursor lesions, high grade cervical intraepithelial neoplasia (CIN2/3), are caused by persistent human papillomavirus (HPV) infection. However, not all CIN2/3 lesions progress to cancer. In a brief, observational study window monitoring subjects with CIN2/3 from protocol entry (biopsy diagnosis) to definitive therapy (cervical conization) at week 15, in a cohort of 50 subjects, we found that 26% of CIN2/3 lesions associated with HPV16, the genotype most commonly associated with disease, underwent complete histologic regression. Nonetheless, HPV16-specific T cell responses measured in peripheral blood obtained at the time of study entry and at the time of conization were marginally detectable directly ex vivo, and did not correlate with lesion regression. This finding suggests that, in the setting of natural infection, immune responses which are involved in elimination of cervical dysplastic epithelium are not represented to any great extent in the systemic circulation.

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**Abbreviations**

| | |
|---|---|
| HPV | Human papillomavirus |
| CIN | Cervical intraepithelial neoplasia |
| SCC | Squamous cervical cancer |
| PBMC | Peripheral blood mononuclear cells |
| ELISPOT | Enzyme-linked immunosorbent spot assay |

## Introduction

Despite the availability of potentially effective screening methods, and, more recently, the introduction of prophylactic vaccines, disease associated with human papillomavirus (HPV) remains common. On a global scale, persistent infection with HPV is the proximate cause of 10% of human malignancies, including squamous cell carcinoma of the cervix (SCCx), vagina, vulva, anus, penis, and oropharynx [1]. A single genotype, HPV16, accounts for over half of all cervical malignancies [2].

High grade cervical intraepithelial neoplasia (CIN2/3), the immediate precursor lesion to invasive cancer, is associated with integration of the HPV genome into the host genome, with subsequent expression of two HPV gene products, E6 and E7, which inactivate p53 and pRb, respectively. Expression of these viral, non-'self' proteins is functionally required to initiate and maintain the transformed phenotype [3, 4]. However, while all cervical squamous cancers arise from untreated CIN2/3 lesions, not all CIN2/3 progress to invasive cancer. We and others have reported that across all HPV types, approximately 35% of CIN2/3 undergo complete regression in a timeframe of 4–6 months [5, 6]. Lesions associated with HPV16 are less likely to

undergo regression than lesions associated with other HPV types [6, 7]. Because it is not possible to distinguish lesions which are likely to regress from those that are not, all CIN2/3 lesions are treated by excision, or, in some cases, ablation.

As CIN2/3 is associated with functionally obligate expression of the E6 and E7 viral proteins, it represents a lesion that could be susceptible to a virus-specific immune response. To date, most translational investigations have focused on the induction of systemic HPV-specific T cell responses, in patient cohorts ranging from late-stage disease to those with early, preinvasive lesions of the genital tract (reviewed in Ref. [8]). However, while the overall approach of eliciting measureable systemic immune recognition of HPV antigens has proven to be effective for vaccines which prevent genital mucosal HPV infection, in contrast, to date, eliciting detectable systemic T cell responses to HPV viral antigens has not been a robust predictor of clinical outcome for immune therapeutic strategies for HPV disease. Overall, the translation of therapeutic vaccines has had more limited success. This may be explained in part because vaccines tested thus far have not been sufficiently immunogenic in humans, and also because an effective cellular immune response must traffic specifically to the site of the lesion, and successfully access it, in order to eliminate established disease.

We report here on a prospective cohort of subjects with HPV16 + CIN2/3 who were followed on a brief, observational protocol for 15 weeks prior to standard therapeutic excision of the lesion site. In this cohort, one in four lesions underwent complete histologic regression in the study window. HPV16 E6 and E7-specific T cell responses measured in the peripheral blood were marginally detectable directly ex vivo, and did not correlate with lesion regression.

## Methods

### Study subjects and cell samples

This protocol was a prospective, observational cohort study conducted at the Johns Hopkins Medical Institutions. The study protocol was approved by the Johns Hopkins Institutional Review Board, and all subjects enrolled provided written informed consent. Subjects with colposcopically directed, biopsy-confirmed CIN2/3, with visible residual disease after the diagnostic biopsy, underwent surveillance for a period of 15 weeks prior to standard therapeutic resection of the cervical squamocolumnar junction at week 15 (conization or LEEP procedure). Data reported in this analysis include subjects whose lesions were HPV16+ by PCR. All histological slides underwent two independent histologic reviews. For study protocol eligibility, slides were first reviewed by the JHMI institutional gynecologic pathology service as part of standard medical procedures, blinded as to study participation. Subsequently, all cases were re-reviewed by the study pathologist (CLT). Regression was defined as absence of CIN2/3 in the resection specimen at week 15.

Peripheral blood was obtained at study entry ($t_0$), at an interval colposcopic (visual) exam ($t_{wk6–8}$), at the time of definitive excision ($t_{wk15}$), and at the postoperative visit ($t_{wk19}$), and lymphocytes cryopreserved within 3 h of venipuncture.

### Primary IFN-$\gamma$ ELISPOT assays

Unfractionated PBMC were thawed, resuspended at a concentration of $2 \times 10^6$ cells/ml in media, which consisted of IMDM with 10% human AB serum (Invitrogen, Gemini Bio-Products), and plated at 100 µl/well ($2 \times 10^5$ cells/well) in 96-well, nitrocellulose-backed plates (Millipore Corp, Bedford, MA) previously coated with anti-IFN-$\gamma$ monoclonal antibody (I-DIK, 5 µg/ml, Mabtech Technologies, Nacka, Sweden). Cell stimuli used were either $CEF_{32}$, a standardized peptide pool comprised of 32 peptides corresponding to cytomegalovirus, Epstein–Barr virus, and flu [9] as the positive control, medium alone as the negative control, or pools of 15-mer peptides overlapping in sequence by 11 amino acids, spanning the entire length of HPV16 E6 and E7, at a concentration of 2 µg/ml as the epitope-specific tests. Testing was performed using triplicate wells of cells. Plates were incubated at 37° for 20 h, harvested, dried, and read on a Zeiss KS ELISPOT reader. Mean numbers of spot forming cells (SFC) from triplicate wells were calculated and expressed as spots per $1 \times 10^6$ PBMC. Mean spot numbers from wells with PBMCs incubated with medium alone (background) were subtracted from means of PBMCs stimulated with peptides. Values of greater than 25 spots/$1 \times 10^6$ PBMC after background subtraction, and $\geq$2 SD above background were considered a detectable response.

### ELISPOT assay using in vitro sensitized (IVS) PBMC

Positive and negative controls were as described for the direct ELISPOT assay. This protocol differed from the direct assay in the following ways: cells were incubated at 37° in 24-well plates at a density of $2 \times 10^6$ cells/(ml well), with peptides at a concentration of 10 µg/ml. On day 3, cultures were diluted with R10-AB supplemented with 50 IU/ml human interleukin-2, to a final concentration of $1 \times 10^6$ cells/ml. Cultures were incubated for an additional 6 days, with replacement of media every other day before being harvested, washed, and rested overnight. The ELISPOT assay was performed as for the non-expanded

PBMC, testing cells at a concentration of $1 \times 10^5$ cells/well, in 96-well plates. A minimum of 200 SFC/$10^5$ PBMC was considered a detectable response for the assay using the IVS PBMC.

### HPV typing

HPV testing was carried out real-time by the Hopkins Molecular Pathology Core Lab, using the HPV16-specific TaqMan real-time PCR method developed by Gravitt et al. [10].

### Statistical analysis

The primary statistical outcome of the analysis was complete histologic regression, defined as CIN1 or less at 15 weeks. The association of categorical variables with lesion regression was assessed using contingency tables, by estimated odds ratios (OR) and by the $\chi^2$ statistic. Continuously distributed variables in subjects whose lesions regressed versus those whose lesions did not regress were compared using means and *t* tests. All analyses were performed with GraphPad Prism version 5.0 software.

## Results

### Complete regression of HPV16 + CIN2/3 in a 15-week observational window

In this cohort, 13/50 (26%) of CIN2/3 lesions underwent complete histologic regression in the study window, and the remainder 37/50 (74%), had persistent CIN2/3 at the time of conization. No subjects had occult, unsuspected invasive disease discovered at the time of resection. The average age of this cohort was 27.3 years (median 26, range 19–48). Subjects who were younger than age 25 were slightly more likely to regress compared to subjects older than age 25 (OR 2.64, 95% CI 0.935–7.46, $P = 0.0618$), but this difference was not statistically significant. Co-infection with HPV types in addition to HPV16 conferred a slightly increased likelihood of regression (OR 1.41, 95% CI 0.393–5.05, $P = 0.744$) that was not statistically significant. Subset analysis of the diagnostic biopsies obtained at $t_0$ did not identify a significant difference in rates of lesion regression in subjects with a biopsy that contained only CIN2; 2/13 (15.4%) of subjects whose lesions had regressed at the time of excision at study week 15 had a CIN2 diagnosis at $t_0$, and 8/37 (21.6%) of subjects who had persistent disease at week 15 had CIN2 at study entry (H.R. 0.6591, 95% CI 0.1206–3.601). Patient characteristics are reported in Table 1.

**Table 1** Clinical characteristics of study subjects: regressors versus non-regressors

| | Regressors ($n = 13$) | Non-regressors ($n = 37$) | *P* |
|---|---|---|---|
| Age ≤25 years | 9/13 (69.2%) | 14/37 (37.8%) | 0.0618 |
| Co-infection with other HPV types | 6/13 (46.2%) | 14/37 (37.8%) | 0.7443 |

### Systemic immune responses to E6 and E7

IFN-$\gamma$ ELISPOT assays were performed on cryopreserved PBMC, using a direct assay after a 20-h stimulation with antigens, as well as on subject-matched specimens that had undergone a cycle of in vitro sensitization. Data are reported for weeks 0 and 15 (study entry and conization, respectively) (Table 2). Using the direct assay, immune responses to HPV16 E6 and E7 measured in samples obtained at study entry ($t_0$) did not correspond to lesion regression in the study window (Fig. 1a). All subjects generated measureable IFN-$\gamma$ responses to the positive control $CEF_{32}$ peptide pool, which were similar in magnitude to what others have reported [9]. The median response to the pool of E6 overlapping peptides in regressors was 0 [range (0–5)] SFC/$10^6$ PBMC, and 0 [range (0–2.5)] SFC/$10^6$ PBMC in non-regressors. Median responses to E7 at study entry were similar; 0 [range (0–3.3)] SFC/$10^6$ PBMC in regressors, and 0 [range (0–10)] SFC/$10^6$ PBMC in non-regressors.

To test for HPV-specific T cell responses present at a frequency below the detection limit of the primary ELISPOT assay, a parallel set of ELISPOT assays was performed on peripheral blood lymphocytes from the same timepoints in the same subjects, after a 9-day in vitro sensitization (IVS) with either E6 or E7 overlapping peptides.

**Table 2** Systemic IFN-$\gamma$ immune responses to HPV16 E6 and E7 at study entry ($t_0$)

| | Regressors | Non-regressors | *P* |
|---|---|---|---|
| Response to E6 | | | |
| Direct ELISPOT median SFC/$10^6$ PBMC (range) | 0 (0–5) | 0 (0–10) | 0.9890 |
| IVS ELISPOT median SFC/$10^6$ PBMC (range) | 570 (150–8,520) | 593 (33–5,640) | 0.4195 |
| Response to E7 | | | |
| Direct ELISPOT median SFC/$10^6$ PBMC (range) | 0 (0–3.3) | 0 (0–10) | 0.3201 |
| IVS ELISPOT median SFC/$10^6$ PBMC (range) | 440 (7–2,170) | 230 (3–4,170) | 0.2934 |

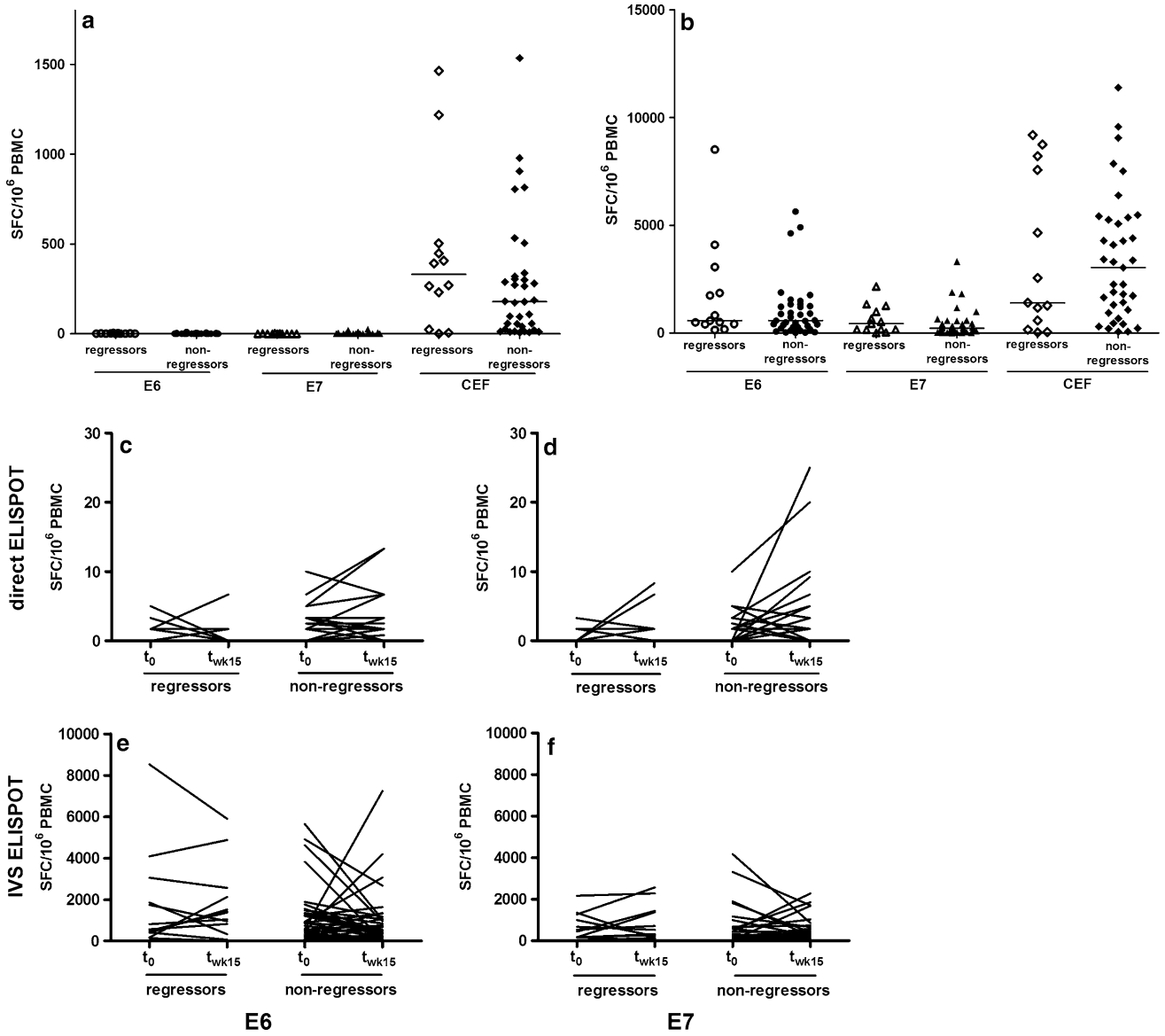


**Fig. 1** **a** Direct IFN-$\gamma$ ELISPOT assays for response to HPV16 E6 and E7, in peripheral blood samples obtained at study entry ($t_0$) do not predict lesion regression in a 15-week observational window. **b** IFN-$\gamma$ ELISPOT assays performed on subject-matched PBMC after a cycle of in vitro sensitization identify T cell responses to HPV16 E6 and E7, but do not predict lesion regression. Immune responses to HPV16 E6 (**c**) and E7 (**d**) measured at study entry ($t_0$) and at the time of conization ($t_{wk15}$), using the direct ELISPOT assay, are weak; responses detected to E6 (**e**) and E7 (**f**) using pre-sensitized PBMC from the same timepoints are of greater magnitude and more frequently identified in subjects whose lesions regressed than in subjects whose lesions failed to regress in the study window, but do not predict regression

Using this method, detectable responses to both E6 and E7 were of greater magnitude, and more frequently identified than in subject-matched samples using the direct ELISPOT method. However, using pre-sensitized cells, a detectable response to E6 at study entry ($t_0$) was not predictive of regression in the study window [R.R. 2.389, 95% CI (0.885, 6.423), $P = 0.1480$], and neither was a detectable response to E7 [R.R. 1.306, 95% CI (0.2449, 6.96), $P = 1.00$] (Fig. 1b). Responses to the positive control peptide pool ($CEF_{32}$) using this method were an order of magnitude greater than those identified in subject-matched specimens using the direct assay.

To assess the possibility that relevant systemic responses to HPV antigens might be transient, responses were determined in specimens obtained at week 15, at the time of cervical conization. The direct assay identified one subject with a detectable response to E7 at week 15; this subject's lesion had undergone complete regression. The IVS ELISPOT assay identified responses to HPV16 E6 at one or both timepoints in 9/50 (18%) of subjects, and to HPV16

E7 in 5/50 (10%). A higher percentage of regressors compared to non-regressors had detectable responses to E6 [3/13 (23.1%) of regressors, compared to 6/37 (16.2%) of non-regressors ($P = 0.6693$)], but this difference was not statistically significant. Responses to E7 at one or both timepoints were also identified in more regressors than non-regressors [2/13 (15.4%) of regressors, compared to 3/37 (8.1%) of non-regressors ($P = 0.09$)] but this difference was not statistically significant either (Fig. 1c–f; Table 2). ELISPOT assays performed on peripheral blood samples obtained at the interval visit ($t_{wk6–8}$) did not identify any immune responses that were predictive of lesion regression (data not shown).

## Discussion

We report here that one in four HPV16 + CIN2/3 lesions undergo complete histologic regression within a relatively brief prospective, observational study window of 15 weeks. Immune responses to viral proteins which are required for cellular transformation, measured in the peripheral blood at the time of study entry, did not predict lesion regression. We found detectable responses to HPV antigens in peripheral blood specimens only after a cycle of ex vivo presensitization. IFN-$\gamma$ responses to E6 were detectable in a higher percentage of study subjects than to E7; however, neither correlated with lesion regression. This finding suggests that the IVS assay identified cells with the potential to recognize HPV antigens, and not necessarily an ongoing active response at the time of sample acquisition.

These observations suggest that in the setting of natural infection, HPV antigens which are required for disease initiation and persistence are not presented systemically in a robust manner, that other antigens expressed by transformed cells may be responsible for lesion rejection, or that clinically relevant immune responses are not reflected in the peripheral blood. From a practical standpoint, these findings also suggest that in the specific setting of HPV16-associated disease, IFN-$\gamma$ ELIspot assays on peripheral blood lymphocytes are not reliable measures of either prevalent or prior cervical HPV infection. Although the measurement of systemic immune responses to vaccine antigens administered parenterally is a reasonable proxy measure of vaccine efficacy, clinical trials designed to test immune therapeutic strategies for HPV-associated disease should include monitoring of the cervical mucosa as well.

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