Disease-Stage Variance in Functional CD4\(^+\) T-Cell Responses
Against Novel Pan-Human Leukocyte Antigen-D Region
Presented Human Papillomavirus-16 E7 Epitopes

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ABSTRACT

Given the anticipated clinical importance of helper and regulatory CD4\(^+\) T cells reactive against human papillomavirus-16 E7 in the cervical carcinoma setting, we performed this study to identify novel E7-derived T helper (Th) epitopes and to characterize functional anti-E7 Th responses in normal donors and patients with cervical intraepithelial neoplasia I-III or cervical cancer.

Candidate pan-HLA-DR (D region) binding peptides were identified and synthesized based on results obtained using a predictive computer algorithm, then applied in short-term \textit{in vitro} T-cell sensitization assays. Using IFN-\(\gamma\)/IL-5 (interleukin 5) enzyme-linked immunospot assays as readouts for Th1-type and Th2-type CD4\(^+\) T-cell responses, respectively, we identified three E7-derived Th helper epitopes (E7\(_{1-12}\), E7\(_{48-62}\), and E7\(_{62-75}\)), two of which are novel.

Normal donor CD4\(^+\) T cells failed to react against these E7 peptides, whereas patients with premalignant cervical intraepithelial neoplasia I-III lesions displayed preferential Th1-type responses against all three E7 epitopes. Th1-type responses were still observed to the E7\(_{48-62}\) but not to the latter two epitopes evoked Th2-type responses. Notably all responders to the E7\(_{1-12}\) and E7\(_{48-75}\) peptides expressed the HLA-DR4 or -DR15 alleles, whereas all responders to the E7\(_{48-62}\) peptide failed to express the HLA-DR4 allele.

Our results are consistent with a model in which cervical cancer progression is linked to an undesirable Th1- to Th2-type shift in functional CD4\(^+\) T-cell responses to two novel E7-derived epitopes. These peptides may prove important in vaccines to promote and maintain protective Th1-type antihuman papillomavirus immunity and in the immune monitoring of treated patients harboring HPV-16\(^+\) malignancies.

INTRODUCTION

Human papillomaviruses (HPVs) have been linked to cervical dysplasia and cervical cancer and are associated with 12% of all cancers in women (1). An estimated 30–60% of sexually active men and women are infected with genital HPVs, although most are asymptomatic (2). There are approximately 370,000 cases of cervical cancer diagnosed worldwide each year and a 50% mortality rate has been reported (3, 4).

HPV is a papillomavirus consisting of a nonenveloped (55 nm) isosahedral-shaped virion, with HPV genotypes divided into several risk groups. The low-risk genotypes, such as HPV-6 and HPV-11, are detected in benign genital warts and low-grade squamous intraepithelial lesions but are rarely found in invasive carcinomas (5). HPV DNA of high-risk genotypes (HPV-16, -18, -31, -33, -45, or -56) are detected in nearly all cases of cervical cancer (5, 6). Expression of early viral proteins, such as HPV-16 E6 and E7 are essential in promoting and maintaining the transformation of cells by binding to and inactivating p53 and phosphorylation of the retinoblastoma protein, respectively (7–13).

Why some individuals clear the virus, whereas others do not, remains unknown. The observed increase in frequency of HPV\(^+\) lesions in immunosuppressed individuals, however, suggests that the immune system may play an important protective role (14), with T cell-mediated immunity appearing crucial to the control and eradication of HPV-transformed tumors (15). Although most attention has been traditionally dedicated to the study of anti-HPV CD8\(^+\) CTL responses, it is also clear that CD4\(^+\) T-cell recognition of E6- and/or E7-derived peptides may be critical for optimal prophylactic or therapeutic efficacy against HPV-related malignancies. In the current study, we have identified or confirmed three E7-derived T helper (Th) epitopes and analyzed the magnitude and polarization of specific CD4\(^+\) T-cell responses in the peripheral blood of normal donors and patients with cervical intraepithelial neoplasia (CIN) or cervical carcinoma. We have observed that in contrast to the undisturbed Th1-type responses against the E7\(_{48-62}\) epitope in all donor

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cohorts, CD4+ T-cell responses against the E71-12 and E762-75 epitopes showed a cancer-related bias away from clinically preferred Th1-type and toward a potentially undesirable Th2-type immunity.

MATERIALS AND METHODS

Peptide Selection and Synthesis. The HPV-16 E7 protein (GenBank accession no. AAB70738) was scanned using a MHC Class II HLA-DR (D region) peptide binding algorithm (16), with predicted high-affinity HLA-DR binding peptides reported in Table 1. Predicted E7 epitopes and the known malarial circumsporozoite326 peptide-pulsed DC groups varied at most by 5 spots/10^5 CD4+ T cells and analyzed for HPV-16 E7 peptide-reactive specificity in enzyme-linked immunospot (ELISPOT) assays.

HLA-DR Typing. The HLA-DR4+ status of peripheral blood monocytes was confirmed by flow cytometry as described previously (18). Patient HLA-DR genotyping was performed using the Dynal Allset+ SSP DR “low resolution” kit (Dynal Inc., Lake Success, NY), with template DNA extracted from patient lymphocytes expanded for 5 days in the presence of 25 μg/ml phytohemagglutinin (Sigma Chemical Co., St. Louis, MO). DNA extraction was performed using the DNeasy tissue kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol.

Induction of Th Effector Lymphocytes. On day 7 of DC culture, autologous CD4+ T cells were thawed in 10 ml of RPMI 1640 containing 10% fetal bovine serum supplemented with 20 units/ml DNase I (type II, from bovine pancreas; Sigma) to increase harvested cell yield and to reduce clumping. Harvested, nonadherent DCs (2 × 10^5) were co-cultured with 2 × 10^5 thawed, autologous CD4+ T cells in the presence of 10 μM E7-derived synthetic peptides in RPMI 1640 media containing 10% fetal bovine serum. No exogenous cytokines were added to these cultures, to prevent any Th-polarizing effects. Responder CD4+ T cells were then harvested on days 7–10 and analyzed for HPV-16 E7 peptide-specific reactivity in enzyme-linked immunospot (ELISPOT) assays.

IFN-γ and IL-5 ELISPOT Assay for Peptide-Reactive CD4+ T-Cell Responses. To evaluate the frequencies of peripheral blood, CD4+ T cells recognizing peptide epitopes, ELISPOT assays for IFN-γ and IL-5 were performed as described previously (18, 19). Briefly, 10^5 CD4+ T cells and autologous thawed DCs (2 × 10^4 cells) were seeded in ELISPOT wells. Synthetic peptides (stocks at 1 mg/ml PBS) were then added to appropriate wells at a final concentration of 10 μg/ml. Negative control wells contained CD4+ T cells and DCs pulsed with CS326−345 peptide, with DCs alone serving as the antigen-presenting cell control. Data are reported as actual numbers of E7-specific T-cell spots above T-cell background responses to the negative control CS326−345 peptide. Additionally, when sufficient T cells were available, CD4+ T cells co-cultured with nonpeptide pulsed DCs were included as an additional negative control. The nonpeptide pulsed DC and CS326−345 peptide-pulsed DC groups varied at most by 5 spots/10^5 CD4+ T cells, which was statistically insignificant (data not...
Table 2  Patient characteristics and T helper responses to HPV-16 E7 peptides

Patient clinical disease stage (patient identification no. in brackets), age, HLA-DR typing, HPV (geno- and antibody) typing and CD4⁺ T cell responsiveness to HPV-16 E7-derived peptides are indicated. HPV and HLA-DR genotype status were determined by PCR, as outlined in “Materials and Methods,” with data qualitatively reported as + or −. For HPV genotype status, HPV-16-specific (E6 and E6/E7) and pan-HPV (L1 capsid) primers were employed. IFN-γ/IL-5 ELISPOT assays were performed using 10⁵ CD4⁺ T cells, 2 × 10⁶ thawed autologous monocyte-derived DCs, and 10 mg/ml final concentration of specific peptide, as described in “Materials and Methods.” CD4⁺ T cells were subjected to one round of in vitro stimulation using autologous, immature DCs and 10 mg/ml of specific peptide, and then assayed in the ELISPOT assay on day 10–14. Plus (+) signs appearing in the E7 columns indicate statistically significant (P < 0.05) spot numbers in response to E7 peptides versus the malarial circumsporozoite (negative control) peptide background. Minus (−) signs in the E7 columns indicate no significant response to E7 peptides versus the circumsporozoite control peptide. Mean and SD are provided for the age of each cohort of patients or normal donors. The IFN-γ versus IL-5 response data are tabulated for each peptide within each donor cohort.

<table>
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<th>Status</th>
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<th>HPV genotype</th>
<th>CD4⁺ T-cell response against (IFN-γ/IL-5)</th>
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<td></td>
<td>42.2 ± 6.3</td>
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</table>

* NT, not tested; CIN, cervical intraepithelial neoplasia; ND, normal donors; Vag Hyst, vaginal hysterectomy; HPV, human papillomaviruses; DR, D region; IL, interleukin.

Materials and Methods.

ELISAs.

Transforming Growth Factor β (TGF-β) and IL-10 ELISAs. Supernatants were harvested from ELISPOT plates at the end point of the culture period, pooled for a single

shown). Positive control wells contained T cells plated in the presence of 5 μg/ml phytohemagglutinin (Sigma). All determinations were performed in triplicate, with spots imaged using the Zeiss AutoImager (and statistical comparisons determined using Student’s two-tailed t test analysis). The data are reported as the mean (±SD) number of IFN-γ or IL-5 spots/10⁵ responder CD4⁺ T cells analyzed.
stimulus (i.e., a given peptide, etc.), and then frozen at −20°C until being analyzed by cytokine-specific ELISA. Cytokine capture and detection antibodies and recombinant cytokines for the TGF-β ELISA were purchased from BD-PharMingen (San Diego, CA), whereas the IL-10 ELISA was purchased from Mabtech (Stockholm, Sweden) and used per the manufacturer’s instructions. The lower limit of detection for the TGF-β and IL-10 assays were 50 pg/ml and 12 pg/ml, respectively.

**PCR Analysis.** PCR analysis for HPV-16 E6, HPV-16 E6/E7, and HPV L1 DNA was performed on patient’s loop electrosurgical excision procedure (LEEP) biopsies. Extraction of DNA from the biopsies was performed using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s provided protocol. The following primer sets were used: HPV-16 E6 (forward, ATGCAAAAGGAGAAGTGC; reverse, TTACACGTGGGTTCCTACTC; product size 477 bp with cycles, melting 94°C for 45 s, annealing 59°C for 45 s, extension 72°C for 1 min, for 38 cycles); HPV-16 E6/E7 (forward, ATGCAAAAGGAGAAGTGC; reverse, TGCCCATTAACAGGTCTTCC; product size 735 bp with cycles, melting 94°C for 45 s, annealing 59°C for 45 s, extension 72°C for 1 min, for 40 cycles); and HPV L1 (forward, GCM-CAGGGWCATAAYAATGG; reverse, CGTCMMARRGG-GAWACTGACT; product size 450 bp, with cycles same as E6/E7, M denotes A and C, R denotes A and G, W denotes A and T, and Y denotes C and T). The capsid primers specifically amplify the L1 gene for the majority of (but not all) oncogenic HPV genotypes (20). A patient was defined as HPV-16 if they were qualitatively positive by PCR using either the E6 or E6/E7 primer sets. Hence, we report a given patient’s HPV genotype status as HPV-16+, HPV-16+/HPV-L1+, or HPV-16−/HPV-L1−.

**Statistical Analysis.** Statistical significance of differences was determined between ELISPOT data sets using a Student’s two-tailed *t* test, with statistical significance defined at *P* < 0.05.

**RESULTS**

**Selection and Screening of Candidate HLA-DR-Binding Peptides Derived from the E7 Protein.** Our investigation was designed to identify novel HPV-16 E7-derived peptides capable of being presented in a pan-DR manner to CD4+ T cells and to characterize functional anti-E7 Th responses in patients with HPV-related dysplasia (CIN I-III) or cervical cancer. We subjected the cDNA sequence of the HPV-16 E7 protein to a computer algorithm analysis designed to identify peptides likely to bind a broad range of HLA-DR alleles (i.e., pan-DR binding). Given the relatively high frequency of HLA-DR4 in the general population (~20%) and our past success in identifying HLA-DR4-presented epitopes derived from melanoma and renal cancer antigens (17–19), we initially required that HLA-DR4 be capable of being presented in a pan-DR manner to CD4+ T cells analyzed.

Nine amino acid-long “core” sequences were evaluated and scored for predicted binding to nine distinct HLA-DR4 suballeles, with individual scores summed for the nine sub-alleles (Table 1). The highest aggregate scoring sequence was taken to represent the peptide most likely to bind in a pan-DR4 manner. Peptides E71-12, E71-25, E762-25, E772-86, and E763-77 each contained predicted HLA-DR4-binding peptide nonamers, whereas peptide E748-62 failed to contain a predicted HLA-DR4 binding sequence was selected for analysis because of its otherwise strong-predicted pan-DR binding capacity (Table 1). These six peptides were synthesized and subsequently analyzed for T-cell recognition in vitro.

**CD4+ T Cells Isolated from the Majority of Patients Evaluated Respond to HPV-16 E7-Derived Peptides.** Peripheral blood CD4+ T cells were isolated from 28 patients with CIN I-III or cervical carcinoma, and from five age-matched controls (three normal individuals undergoing vaginal hysterectomy and two normal donors with no history of CIN; see Table 2 for donor characteristics). After a single round of in vitro stimulation with autologous DCs pulsed with a given E7 peptide, the resulting CD4+ T cells were screened for peptide-specific responses in IFN-γ (Th1-type responses) and IL-5 (Th2-type responses) ELISPOT assays. Fig. 1 depicts the Th1-versus Th2-type CD4+ T-cell responses of all donors against the E71-12 and E762-75 peptides, reported as spots/10⁵ CD4+ T cells analyzed. A corresponding analysis of CD4+ T-cell response to the E748-62 peptide is provided in Fig. 2. These data are also summarized in a qualitative format in Table 2 for each individ-

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**Fig. 1.** CD4+ T-cell responses against the HPV-16 E71-12 and E762-75 peptide epitopes display disease stage-dependent Th1 (T helper 1)-/Th2-bias in patients expressing either HLA-DR4 (D region 4) or -DR15. Peripheral blood CD4+ T cells were isolated from patients or normal donors and stimulated for 5–7 days with autologous dendritic cells plus individual HPV-16 E7 peptides, as described in the legend of Table 2. Responder CD4+ T cells were then analyzed for reactivity against autologous dendritic cells pulsed with the indicated HPV-16 E7 peptides in IFN-γ and IL-5 ELISPOT assays. For each peptide analyzed, patient responders have been segregated based on whether they expressed the HLA-DR4 and/or -DR15 class II alleles (left panels) or failed to express these alleles (right panels). Each symbol in a panel represents an individual patient’s data, with patient disease-stage cohorts defined by the type of symbol, as indicated. The number of patients in each cohort is indicated in parentheses for each of the HLA-segregated groups. The IFN-γ and IL-5 ELISPOT data are reported as spot numbers/10⁵ CD4+ T-cells analyzed. HPV, human papillomaviruses; ELISPOT, enzyme-linked immunospot.
Fig. 2  The HPV-16 E7_{48-62} epitope is recognized in a predominantly Th1 (T helper)-biased manner by the CD4^+ T cell isolated from HLA-DR4 (D region 4)-negative patients. Using the experimental protocol outlined in the Fig. 1 legend and text, we analyzed the Th1- versus Th2-type response of patient CD4^+ T cells against the E7_{48-62} peptide presented by autologous dendritic cells in IFN-γ and IL-5 ELISPOT assays. For each peptide analyzed, patient responders have been segregated based on whether they expressed (right panel) or failed to express (left panel) the HLA-DR4 class II allele. Each symbol in a panel represents the data of an individual patient, with patient disease-stage cohorts defined by the type of symbol, as indicated. The number of patients in each cohort is indicated in parentheses for each of the HLA-segregated groups. The IFN-γ and IL-5 ELISPOT data are reported as spot numbers/10^5 CD4^+ T-cells analyzed. HPV, human papillomaviruses; ELISPOT, enzyme-linked immunospot.

Figure 2

The HPV-16 E7_{48-62} epitope is recognized in a predominantly Th1 (T helper)-biased manner by the CD4^+ T cell isolated from HLA-DR4 (D region 4)-negative patients. Using the experimental protocol outlined in the Fig. 1 legend and text, we analyzed the Th1- versus Th2-type response of patient CD4^+ T cells against the E7_{48-62} peptide presented by autologous dendritic cells in IFN-γ and IL-5 ELISPOT assays. For each peptide analyzed, patient responders have been segregated based on whether they expressed (right panel) or failed to express (left panel) the HLA-DR4 class II allele. Each symbol in a panel represents the data of an individual patient, with patient disease-stage cohorts defined by the type of symbol, as indicated. The number of patients in each cohort is indicated in parentheses for each of the HLA-segregated groups. The IFN-γ and IL-5 ELISPOT data are reported as spot numbers/10^5 CD4^+ T-cells analyzed. HPV, human papillomaviruses; ELISPOT, enzyme-linked immunospot.

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Eighteen of the 28 CIN/cancer patients responded significantly to at least one HPV-16 E7 peptide in either the IFN-γ (14 of 28) and/or IL-5 (6 of 27, one patient not tested) ELISPOT assays (Table 2). In the majority (15 of 18) of cases where patients did respond to HPV-16 E7 peptides, they reacted against a single peptide. Reactivity to two E7 peptides was observed in only two patients, and reactivity to all three E7 peptides was noted for a single patient with cervical cancer (patient 35). Overall, six, eight, and eight patients were responsive to the E7_{1-12}, E7_{48-62}, and E7_{62-75} peptides, respectively. Of note, zero of three patients undergoing vaginal hysterectomies and zero of two normal donors were reactive against any of the E7-derived peptides evaluated.

With regard to disease-stage, Type-1 (IFN-γ) responses against any of the three E7 peptides were observed in 5 of 11 CIN I, 3 of 6 CIN II, 3 of 5 CIN III, and 3 of 6 cervical cancer patients. In contrast, Type-2 (IL-5) responses were not seen in CIN I/II patients but were observed in one of five CIN III and five of five patients (27 was not evaluated) cervical cancer patients. Notably, CD4^+ T-cell responses appeared Th1- or Th2-type polarized in the vast majority of responders, with mixed Th1 and Th2 reactivity to E7 peptides evident in only two patients with cervical cancer (patients 12 and 35). We were able to evaluate HPV genotype status by performing a PCR-based analysis of DNA extracted from LEEP biopsies in 16 of the 18 patients responsive to E7-derived peptides. Of these 16 patients, 12 screened as HPV-16^+^, 3 patients (24, 29, and 32) exhibited an HPV-16^+^/HPV-L1^+^ genotype and a single patient (31) was evaluated as HPV-16^+^/HPV-L1^−^.

Of note, zero of three patients undergoing vaginal hysterectomy with CIN III patient 28 representing the sole Th2-type responder (44.4%) evaluable DR4^−^ and/or -DR15 (2 of 6, one patient not tested) ELISPOT responders observed in four CIN I/II patients and Type-2 responses identified in two patients with cervical carcinoma (Fig. 1 and Table 2). Strikingly, all six of these responding patients expressed the HLA-DR4 (four of six) and/or -DR15 (two of six) class II molecules, and were typed as HPV-16^+^ (with the exception of patient 29 who tested as HPV-16^+/HPV-L1^−^) by reverse transcription-PCR.

Similarly, seven of eight patients with CD4^+^ T-cells reactive against the HPV-16 E7_{62-75} peptide were either HLA-DR4^−^ or -DR15^−^ (Table 2), with Type-1 responses observed in three patients with CIN II/III and Type-2 responses identified in five patients with cervical carcinoma (Fig. 1). Cancer patient 27, who expresses neither HLA-DR4^−^ nor HLA-DR15^−^, was responsive to the HPV-16 E7_{62-75} epitope in the IFN-γ ELISPOT assay (but was not tested for IL-5 response because of an insufficient number of T cells). All responders tested positive for expression of HPV-16, with the exception of CIN II patient 24, who was typed as HPV-16^+^/HPV-L1^−^.

This patient’s (24) plasma also failed to contain detectable levels of anti-HPV-16 E7 IgG antibodies by ELISA (data not shown).

Peptide E7_{48-62} Appears to Be Primarily Recognized by Patients Who Do Not Express HLA-DR4. Eight of 28 patients evaluated with CIN I–III or cervical carcinoma displayed statistically significant CD4^+^ T-cell responses to the E7_{48-62} peptide in either the IFN-γ or IL-5 ELISPOT assays, as summarized in Table 2. In marked contrast to Th responses against the E7_{1-12} and E7_{62-75} peptides, none of the responders to peptide E7_{48-62} were HLA-DR4^+^ (Fig. 2). Hence, of 18 (44.4%) evaluable DR4^−^ women exhibited CD4^+^ T cells reactive against this peptide. Seven of these eight responders displayed Th1-type biased reactivity to the E7_{48-62} peptide, with CIN III patient 28 representing the sole Th2-type responder to this epitope. Interestingly, two HLA-DR15^+^ cancer patients displayed strong Th1-type immunity to the E7_{48-62} peptide, and in the case of patient 35, this response occurred in concert with Th2-biased responses against the E7_{1-12} and E7_{62-75} peptides (Table 2). LEEP biopsies were available for seven of eight responder patients. PCR analyses determined the genotype status of five patients as HPV-16^+^, one patient (32) as HPV-16^+^/HPV-L1^+^ and one patient (31) as HPV-16^+^/HPV-L1^−^ (Table 2). On the basis of analysis of their plasma, these latter two patients displayed detectable levels of anti-HPV-16 E7 IgG antibodies by ELISA (data not shown).

**DISCUSSION**

In the current report, we have identified two novel HPV-16 E7-derived Th epitopes contained within the E7_{1-12} and E7_{62-74}
peptides. Each epitope is seemingly restricted by at least HLA-DR4 and -DR15 and is recognized by CD4+ T cells isolated from CIN and cervical cancer patients but not normal donors. We also synthesized (and evaluated CD4+ T cell responsiveness) the HLA-DR15-presented E7\textsubscript{48–62} epitope defined previously by Van der Burg et al. (23), based on our algorithm analysis result suggesting that this peptide was likely to be pan-DR presented. Our study revealed that the E7\textsubscript{48–62} peptide is recognized by CD4+ T cells isolated from a high frequency (i.e., approximately 44%) of HLA-DR4+ CIN or cervical cancer patients evaluated in this study, including but not restricted to HLA-DR15+ patients. Theoretically, this data argues for the pan-class II presentation of this epitope on multiple HLA-DR, -DP, or -DQ molecules (other than HLA-DR4). Alternatively, this peptide can be presented by HLA-DR4 and is either an immunodominant antagonist or it preferentially promotes Th responses other than Th1- or Th2-type (i.e., T regulatory, etc.). These two possibilities are unlikely because this peptide is predicted to bind to HLA-DR4 very poorly, if at all. Furthermore, in extended studies, we analyzed the supernatants harvested from ELISPOT wells by ELISA to determine whether E7 peptides elicited TGF-β and IL-10 production (an index of T regulatory function; Ref. 18) from any of these donors. We observed E7 peptide-specific TGF-β production from CD4+ T cells isolated from only two patients [CIN II (patient 1), 102 pg/ml; cancer patient 39, 54 pg/ml] that exceeded the 50 pg/ml lower limit of detection for this ELISA. Detectable IL-10 production (i.e., >12 pg/ml, the lower limit for the IL-10 ELISA) was observed only for cancer patient 39 (25 pg/ml). Interestingly, this patient was also one of the two patients producing TGF-β in response to E7-derived peptides. We were unable to evaluate the other TGF-β producer (patient 1) for IL-10 production because of insufficiency in T-cell numbers required for the assay. In aggregate, the available data support the pan-class II- (but non-HLA-DR4-) presented nature of the E7\textsubscript{48–62} peptide.

IFN-γ and IL-5 ELISPOT assays were used to monitor the functional polarization of patient CD4+ T-cell responses against HPV-16 E7-derived peptides. We observed that a high number of CIN I-III patients (11 of 22) displayed Th1-type immunity to one or more E7 peptides but that only 1 of 22 CIN I-III patients displayed detectable Th2-type responses to these same peptides. In marked contrast, five of six patients with cervical cancer (i.e., patient 27 could not be evaluated for IL-5) displayed Th2-type responses to at least one E7-derived Th epitope, with two of five exhibiting mixed Th1-/Th2-type immune responses. These data suggest that Th2-type dominant CD4+ T-cell responses against HPV-16 E7 epitopes may correlate with advanced disease status in these individuals, a finding consistent with results reported for tumor antigen-specific Th responses in patients with advanced-stage cancers of alternate histologies (18, 24–28).

It should be stressed that polarized Th2-type CD4+ T-cell responses were specific for the HPV-16 E7\textsubscript{1–12} and E7\textsubscript{62–75} peptides tested and do not reflect the general tendency of these donors with advanced-stage malignancy to respond in a generic Th2-biased fashion. Indeed, mitogen (phytohemagglutinin) control spot frequencies obtained for both the IFN-γ and IL-5 ELISPOT assays using CD4+ T cells were indistinct among patients with cancer, CIN patients, and (normal) vaginal hysterectomy patients. In addition, cancer patients exhibited strong Th1-type immunity to the pan-DR-presented EBV Th epitope Epstein-Barr virus nuclear antigen-2\textsubscript{300–290} (Ref. 29; data not shown). Perhaps most salient, cervical cancer patient 35 displayed coordinate Th2-type responses to the E7\textsubscript{1–12} and E7\textsubscript{62–75} peptides and Th1-type reactivity against the E7\textsubscript{48–62} epitope.

The mechanism(s) by which Th2-type skewing may occur in anti-E7 CD4+ T-cell responses of cervical carcinoma patients remains unknown but could include the following: (a) chronic antigenic restimulation (i.e., repetitive reinfection, Refs. 30–34) in situ that may delete Th1-type responders (18, 35); (b) dominant locoregional DC2-type antigen presenting cell function in the advanced cancer setting that favors Th2-type immunity (36); and/or (c) a generalized Th2-polarizing cytokine microenvironment at the tumor site and within the tumor draining lymph nodes of affected patients (37). The maintenance of Th1-type immunity against the pan-DR-presented EBV Th epitope (and against the E7\textsubscript{48–62} peptide in patient 35) argues against dominant DC2-mediated functional, or global Th2-type cytokine-mediated, skewing of peripheral blood CD4+ T-cell responses in cervical cancer patients. However, these important issues will require intense prospective evaluation to determine their unequivocal roles in shaping the HPV-specific immune response in high-risk and progressor patients.

Our analysis of donor CD4+ T cells using ELISAs specific for IL-10 and TGF-β revealed weak evidence for anti-E7 Treg-type activity in only 2 of 28 patients (CIN II patient 1 and cancer patient 39). This suggests, at least in the peripheral circulation of patients, that the detection of Treg-type CD4+ T cells reactive against HPV-16 E7-derived epitopes is not a common event. Such responses if they exist in patients may be more dominant in cervical mucosal sites or the secondary lymphoid organs that drain these tissues (38).

Coordinate analysis of CD4+ T-cell responsiveness to HPV-16 E7-derived peptides and HPV-16 genotype status of LEEP biopsies indicated that in six of seven cases for the E7\textsubscript{1–12} peptide, five of seven (evaluable) cases for the E7\textsubscript{48–62} peptide and seven of eight cases for the E7\textsubscript{62–75} peptide, the responding patients were HPV-16+. A total of three patients (patient 24 CIN III, patient 29 CIN I, and patient 32 CIN II) that reacted against E7 peptides were genotyped as HPV-16/HPV-L1+ indicating current infection by alternate HPV types, and a single CIN II patient (31) who reacted against the E7\textsubscript{48–62} peptide was genotyped as HPV-16/HPV-L1+. We would hypothesize that each of these latter four patients had at one time been infected with HPV-16 but had cleared the virus, potentially via specific T cell-dependent immunity. This latter possibility is consistent with the detection of (T-dependent) IgG, anti-E7 antibody levels in the plasma of patients 31 and 32 by ELISA (data not shown, patient 29 could not be evaluated). After clearing HPV-16, a subsequent reinfection with alternate HPV genotypes could have resulted in HPV-L1+ status for the CIN lesion in three of these four patients. We currently cannot provide a convincing rationale for the results obtained in patient 31, unless the biopsy tissue provided for analysis failed to contain a sufficient quantity of the CIN II lesion within the LEEP biopsy, resulting in a positive control signal (for β-actin) but a failure to detect the
HPV-16 or HPV-L1 genes. The lack of an L1-specific signal could also occur if the patient’s CIN lesion were infected by a high-risk HPV type that is not amplified by our selected PCR primers. Finally, the data derived from CIN III patient 24 who exhibited Th1-type CD4+ T cells reactive against the E7\textsubscript{62–75} peptide but who failed to display positive HPV-16 PCR or serology (data not shown) results remains an enigma.

One surprisingly finding in this study was that five of six (83%) cancer patients evaluated were tissue typed as HLA-DR15\textsuperscript{+}, although this allele is typically observed only in the general population at a frequency of 17–29%, depending on donor ethnicity (39). Of interest in this regard, recent reports argue that HLA-DR15 may be associated with increased susceptibility to cervical cancer (40, 41). We will continue to monitor whether the HLA-DR15 allele is expressed by a disproportionately high frequency of cervical cancer patients in our prospective studies.

Overall, we believe that the E7\textsubscript{1–12} and E7\textsubscript{62–75} peptides represent good candidate epitopes for implementation in vaccines for (at least) HLA-DR4\textsuperscript{+} or -DR15\textsuperscript{+} individuals to prevent or treat cervical cancer. On the basis of the high frequency of responders against the E7\textsubscript{1–62} epitope in HLA-DR4-negative patients, the inclusion of this peptide in a polyepitope vaccine (including the E7\textsubscript{1–12} and E7\textsubscript{62–75} epitopes), would complement and broaden patient coverage. This would allow for the majority of patients (i.e., >70%) with HPV-16 CIN or cervical carcinomas to be treated by such a modality. Furthermore, given an expanding number of cancer histologies reported to be HPV-positive, HPV-16 E7 peptide-based vaccines may prove clinically applicable to an even larger patient population. When provided with an adjuvant capable of preferentially promoting Th1-type immunity, E7 peptide-based vaccines would be anticipated to stimulate IFN-\gamma secreting, anti-E7 CD4+ effector T cells. These Type-1 Th cells would, in turn, be expected to enhance the frequencies and functionality of anti-HPV-16 reactive CD4+ and CD8+ T cells in situ and to recruit these T cells into HPV-16+ tissues (via a delayed-type hypersensitivity mechanism), yielding enhanced clinical efficacy. These peptides will also likely prove useful in the longitudinal immunomonitoring of functionally evolving CD4+ T-cell responses in patients with, or at high-risk to develop, cervical carcinoma or other HPV-related malignancies.

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**REFERENCES**


40. Gostout BS, Poland GA, Calhoun ES, et al. TAP1, TAP2, and HLA-DR2 alleles are predictors of cervical cancer risk. Gynecol Oncol 2003;88:326–32.

Disease-Stage Variance in Functional CD4+ T-Cell Responses Against Novel Pan-Human Leukocyte Antigen-D Region Presented Human Papillomavirus-16 E7 Epitopes


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