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) icosahedral-shape 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 carcinomas (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 E748–62 epitope in all donor

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

Dominic E. Warrino,1 Walter C. Olson,2 William T. Knapp,2 Meera I. Scarrow,3 Lori J. D’Ambrosio-Brennan,3 Richard S. Guido,3 Robert P. Edwards,4 W. Martin Kast,5 and Walter J. Storkus1,2,6

Departments of 1Immunology and 2Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; the 3Magee-Womens Hospital Department of Obstetrics & Gynecology, Pittsburgh, Pennsylvania; the 4Department of Obstetrics and Gynecology, University of Louisville, Louisville, Kentucky; the 5Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California; and the 6Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

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 in vitro T-cell sensitization assays. Using IFN-γ/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 T helper epitopes (E71–12, E748–62, and E762–75), 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 E71–12 and E762–75 peptides but not to the latter two epitopes evoked Th2-type responses. Notably all responders to the E748–62 and E762–75 peptides expressed the HLA-DR4 or -DR15 alleles, whereas all responders to the E748–62 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.

Received 10/29/03; revised 12/10/03; accepted 12/16/03.

Grant support: This work was supported by an NIH Immunology training grant to D. E. W. and by NIH Grants 2RO1 CA 57840 (W. J. S.) and 1RO1 CA 73497 and PO1 97296 (W. M. K.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Walter J. Storkus, Departments of Surgery and Immunology, 1.32 Hillman Cancer Center-University of Pittsburgh Cancer Institute’s Research Pavilion, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: (412) 623-3240; Fax: (412) 623-7709; E-mail: storkuswj@msx.upmc.edu.

Clinical Cancer Research 3301–3308, May 15, 2004
cohorts, CD4+ T-cell responses against the E71-12 and E746-52 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 circumsporozoite 3126-345 (CS3126-345) Th epitope (17) were synthesized using 9-fluorenlymethoxycarbonyl chemistry by the University of Pittsburgh Cancer Institute’s Peptide Synthesis Facility. Peptides were >96% pure based on high-performance liquid chromatography, with identities validated by mass spectrometric (tandem mass spectrometry) analyses performed by the University of Pittsburgh Cancer Institute’s Protein Sequencing Facility.

Isolation of Peripheral Blood T Cells and Dendritic Cells (DCs). Patient information is provided in Table 2. Briefly, the CIN or invasive cancer status of patients was determined by pathology review. No patient received any adjunctive (chemo-, radio-, or immuno-) therapy within 6 weeks before blood donation. Vaginal hysterectomy patients were clinically treated for various reasons unrelated to CIN or invasive cervical cancer and were considered normal in the context of the current study. Normal donors are asymptomatic women with no history of CIN. Fifty to one hundred ml of heparinized donor blood was obtained with informed consent under an Institutional Review Board-approved protocol and diluted 1:2 in PBS before being centrifuged (550 × g for 25 min at room temperature) on discontinuous Ficoll-Hypaque gradients (Cellgro; Mediatech, Inc., Herndon, VA) per the manufacturer’s instructions. Bouyant peripheral blood mononuclear cells at the gradient interface were recovered and washed three times with PBS (BioWhitaker, Walkersville, MD) to remove residual platelets and Ficoll-Hypaque. The generation of donor DCs from adherent mononuclear cells and the isolation and cryo-preservation of autologous CD4+ T cell from nonadherent cells were performed as described previously (18).

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 × 10^6 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 ELISPsot 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 1 Predicted pan-DR* binding peptides derived from the HPV-16 E7 protein

<table>
<thead>
<tr>
<th>Peptide position</th>
<th>Peptide sequence</th>
<th>Cumulative DR4 score</th>
<th>Other high-scoring HLA-DR alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>E71-12</td>
<td>MGNDTTLHHEYD</td>
<td>6.5</td>
<td>DR3</td>
</tr>
<tr>
<td>E711-23</td>
<td>YMLDQPETWRLLYCY</td>
<td>10.5</td>
<td>DR3, DR15</td>
</tr>
<tr>
<td>E746-52</td>
<td>DRAHYNIVFTCCKCD</td>
<td>0</td>
<td>DR3, DR7, DR8, DR11, DR13, DR15, DRB5</td>
</tr>
<tr>
<td>E746-52</td>
<td>DSTLRCQVSTHVD</td>
<td>14.3</td>
<td>DR3, DR8, DR13</td>
</tr>
<tr>
<td>E772-86</td>
<td>THVDIRTELEDLZGM</td>
<td>16.4</td>
<td>DR3, DR7, DR11, DR13, DR15</td>
</tr>
<tr>
<td>E783-97</td>
<td>LGMTLGIVCIPICSDK</td>
<td>19.9</td>
<td>DR1, DR3, DR7, DR8, DR11, DR13, DR15, DRB5</td>
</tr>
</tbody>
</table>

a DR, D region; HPV, human papillomaviruses.
b DRβ3*0101 and DRβ4*0101 were not analyzed.
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 10^5 CD4+ T cells, 2 × 10^5 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>
<thead>
<tr>
<th>Status</th>
<th>Age</th>
<th>HLA-DRβ1 (3, 4, 5)</th>
<th>HPV genotype</th>
<th>CD4+ T-cell response against (IFN-γ/IL-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND [01]</td>
<td>28</td>
<td>11, 13</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td>ND [03]</td>
<td>30</td>
<td>03, 12</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td>Vag Hyst [10]</td>
<td>53</td>
<td>04, 11 or 13</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>Vag Hyst [14]</td>
<td>46</td>
<td>07, 13</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>Vag Hyst [21]</td>
<td>31</td>
<td>03, 15</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td></td>
<td>37.6 ± 11.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN I [9]</td>
<td>19</td>
<td>NT</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN I [13]</td>
<td>28</td>
<td>04, 07</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN I [15]</td>
<td>55</td>
<td>NT</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN I [19]</td>
<td>44</td>
<td>01, 15</td>
<td>+</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN I [20]</td>
<td>50</td>
<td>04, 16</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>CIN I [22]</td>
<td>47</td>
<td>07, 08</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>CIN I [28]</td>
<td>81</td>
<td>07, 11</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN I [29]</td>
<td>26</td>
<td>03, 04</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN I [32]</td>
<td>52</td>
<td>11, 15</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN I [36]</td>
<td>65</td>
<td>04, 15</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN II/III [40]</td>
<td>25</td>
<td>03, 14</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td></td>
<td>44.7 ± 19.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN II [1]</td>
<td>20</td>
<td>NT</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN II [2]</td>
<td>20</td>
<td>03, 13</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN II [7]</td>
<td>22</td>
<td>03, 04</td>
<td>3, 4</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN II [25]</td>
<td>34</td>
<td>03, 13</td>
<td>3</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN II [31]</td>
<td>44</td>
<td>03, 11</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN II [33]</td>
<td>39</td>
<td>01, 13</td>
<td>−</td>
<td>−/−</td>
</tr>
<tr>
<td></td>
<td>29.8 ± 10.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN III [6]</td>
<td>46</td>
<td>03, 07</td>
<td>3, 4</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN III [8]</td>
<td>28</td>
<td>14, 15</td>
<td>3, 5</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN III [18]</td>
<td>30</td>
<td>04, 11</td>
<td>3, 4</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN III [24]</td>
<td>34</td>
<td>04, 09</td>
<td>4</td>
<td>−/−</td>
</tr>
<tr>
<td>CIN III [37]</td>
<td>47</td>
<td>07, 14</td>
<td>3, 4</td>
<td>−/−</td>
</tr>
<tr>
<td></td>
<td>39.0 ± 12.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer [12]</td>
<td>34</td>
<td>07, 15</td>
<td>4, 5</td>
<td>−/−</td>
</tr>
<tr>
<td>Cancer [23]</td>
<td>37</td>
<td>03, 15</td>
<td>3, 5</td>
<td>−/−</td>
</tr>
<tr>
<td>Cancer [27]</td>
<td>40</td>
<td>01, 14</td>
<td>NT</td>
<td>−/−</td>
</tr>
<tr>
<td>Cancer [35]</td>
<td>44</td>
<td>07, 15</td>
<td>5</td>
<td>−/−</td>
</tr>
<tr>
<td>Cancer [38]</td>
<td>50</td>
<td>07, 15</td>
<td>5</td>
<td>−/−</td>
</tr>
<tr>
<td>Cancer [39]</td>
<td>48</td>
<td>01, 15</td>
<td>5</td>
<td>−/−</td>
</tr>
<tr>
<td></td>
<td>42.2 ± 6.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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 Autolmager (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/10^6 responder CD4+ T cells analyzed.

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
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, ATGCACCAAAGAGAAGCTGC; reverse, TTACAGCTGCGGTTCCTCCTAC; 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, ATGCACCAAAGAGAAGCTGC; 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 capsid (forward, GCMCAGGGWCATAAYAATGG; reverse, CGTCCMARRGGAWACTGACT; 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 the DR4 allele capable of being presented in a pan-DR4 manner to CD4⁺ T cells.

Nine amino acid-long “core” sequences were evaluated and scored for predicted binding to nine distinct HLA-DR4 sub-alleles, 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, E7₁–₁₁–₂₅, E7₂–₂₅, E7₇₂–₂₅, and E7₄₈–₆₂ each contained predicted HLA-DR4-binding peptide nonamers, whereas peptide E7₄₈–₆₂ that 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 E7₂–₇₅ 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 individual patient's data.
null
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 responsive-
ness) the HLA-DR15-presented E748–62 epitope defined previ-
ously 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 E748–62 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,
-DR15, 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 Th1
cellular responses other than Th1- or Th2-type (i.e., Th 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 har-
vested from ELISpot wells by ELISA to determine whether E7
peptides elicited TGF-β and IL-10 production (an index of Th2
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 produc-
tion (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 produc-
tion 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 E748–62 pep-
tide.

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-dominated CD4+ T-cell responses
against HPV-16 E7 epitopes may correlate with advanced dis-
ease 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 (18–28).

It should be stressed that polarized Th2-type CD4+ T-cell
responses were specific for the HPV-16 E71–12 and E762–75
peptides 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) con-
trol spot frequencies obtained for both the IFN-γ and IL-5
ELISpot assays using CD4+ T cells were indiscriminant
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 A30–390 (Ref. 29;
data not shown). Perhaps most salient, cervical cancer patient 35
displayed coordinate Th2-type responses to the E71–12 and
E762–75 peptides and Th1-type reactivity against the E748–62
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
antigen 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 E748–62 peptide in patient 35) argues against dom-
ant DC2-mediated functional, or global Th2-type cytokine-
mimicked, skewing of peripheral blood CD4+ T-cell responses
in cervical cancer patients. However, these important issues
will require intense prospective evaluation to determine their un-
evocational 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
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 E71–12
peptide, five of seven (evaluable) cases for the E748–62 peptide
and seven of eight cases for the E762–75 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+ indi-
cating current infection by alternate HPV types, and a single
CIN II patient (31) who reacted against the E748–62 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 quan-
tity 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_{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+, 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_{1-12} and E7_{62-75} peptides represent good candidate epitopes for implementation in vaccines for (at least) HLA-DR4+ or -DR15+ individuals to prevent or treat cervical cancer. On the basis of the high frequency of responders against the E7_{1-12} epitope in HLA-DR4-negative patients, the inclusion of this peptide in a polyepitope vaccine (including the E7_{1-12} and E7_{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-γ 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.

ACKNOWLEDGMENTS

We thank Drs. Albert DeLeo, Theresa Whiteside, Russell Salter, Saleem Khan, Pawel Kalinski, and Amy Wesa for constructive comments provided in review of this manuscript.

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


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/10/3301

Cited articles
This article cites 37 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/10/3301.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/10/10/3301.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.