Use of Fluorogenic Histocompatibility Leukocyte Antigen-A*0201/HPV 16 E7 Peptide Complexes to Isolate Rare Human Cytotoxic T-Lymphocyte-recognizing Endogenous Human Papillomavirus Antigens

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INTRODUCTION

Cervical cancer (CaCx) is the second most common female malignancy worldwide and remains a clinical problem despite improvements in early detection and therapy. CaCx and preinvasive cervical intraepithelial neoplasia (CIN3) are strongly associated with infection by human papillomavirus (HPV), particularly types 16 and 18. Two nonstructural viral proteins, E6 and E7, are constitutively expressed in cervical tumors and are crucial for the maintenance of the transformed phenotype. These proteins thus provide attractive targets for immunotherapy of CaCx mediated by CD8+ CTLs. However, reliable detection and generation of HPV-specific CTLs in humans has been difficult. Recently, soluble fluorogenic MHC-peptide complexes (tetramers) have greatly increased the sensitivity of antiviral and antitumor CTL detection. To examine the feasibility of this approach for detecting HPV-specific CTLs, we constructed a tetramer consisting of HLA-A*0201 and the best studied HPV peptide epitope, HPV 16 E711–20. Between 2 and 12% of short-term HPV 16 E711–20 CTL lines derived from CaCx patients stained highly with the tetramer. Direct ex vivo staining of peripheral blood mononuclear cells revealed CD8+ tetramer+ high cells at low frequencies in both CIN3 patients (1 of 1,260 to 1 of 19,073) and normal controls (1 of 1,855 to 1 of 42,004). However, short-term in vitro stimulation with the HPV 16 E711–20 peptide expanded CD8+ tetramer+ cells to a greater extent in the peripheral blood mononuclear cells from CIN3 patients. Furthermore, the tetramer provided a powerful tool to isolate polyclonal and clonal peptide-specific CTLs from an established HPV 16 E711–20-specific CTL line. These purified CTLs were able to lyse both peptide-pulsed targets and targets expressing endogenously processed HPV antigens. This tetramer may therefore be useful for selecting rare high-affinity HPV-specific CTLs for the immunotherapy of CaCx.

MATERIALS AND METHODS

Media. RPMI 1640 (Life Technologies Inc., Gaithersburg, MD) was always used with the following additions: 0.02 m HEPES (Sigma-Aldrich Co. Ltd., Irvine, United Kingdom), 2 m sodium-pyruvate (Life Technologies), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies). For the culture of T cells, this medium was supplemented with 10% pooled human AB serum; PE, phycoerythrin; IL-2, interleukin 2; FACS, fluorescence-activated cell sorting; TCR, T-cell receptor; CTL, cytotoxic T lymphocyte.

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6 The abbreviations used are: CaCx, cervical cancer; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; CD, cluster of differentiation; PBMC, peripheral blood mononuclear cell; RAB, RPMI 1640 supplemented with 10% pooled human AB serum; PE, phycoerythrin; IL-2, interleukin 2; FACS, fluorescence-activated cell sorting; TCR, T-cell receptor; CTL, cytotoxic T lymphocyte.
Technologies) and 400 μg/ml G418 (Life Technologies). CaSkis, an HLA-A*0201 cervical carcinoma cell line expressing HPV-16 E6 and E7 proteins (obtained from ATCC) was maintained in RPMI containing 10% FCS (31). MDA-231, an HLA-A*0201 breast epithelial carcinoma cell line (kind gift from Linda Sherman, Scripps Clinic, La Jolla, CA) was maintained in RPMI containing 10% FCS (32).

Patients and Controls. CaCks were patients who were recruited with informed consent from patients presenting for surgical treatment at the University Hospital of Wales, Cardiff (18). All CIN3 patients had histologically confirmed CIN3 and were recruited when they attended colposcopy clinics at the University Hospital of Wales or presented for surgery at Llandough Hospital (22). For tetramer analyses, PBMCs from 10 HLA-A*0201 patients were used, with samples from nine CIN3 patients and one CaCk patient. For controls, nine HLA-A*0201 laboratory volunteers (ages, 26–50 years) were used: four males and five females. None of the females had any history of abnormal cervical cytology, and none of the controls were in high-risk groups for acquisition of sexually transmitted diseases.

PBMC Isolation. PBMCs were separated from heparinized blood samples (8.5–50 ml) by centrifugation on a Histopaque density gradient (Sigma) and washed three times with RPMI 1640 before use. Patient PBMCs were frozen in aliquots containing 5–10 x 10⁶ cells, stored in liquid nitrogen, and thawed before use for CTL generation (18) and tetramer staining.

Peptides. HPV 16 E7 11–20 (YMLDLQPETT; Ref. (33) was synthesized by Immune Systems Ltd., Paignton, United Kingdom. As controls, two other HLA-A*0201-presented peptides were used; TP (GLLGFGVT), derived from the TAP2 protein (34), synthesized by Immune Systems Ltd.; and CP36 (YLKTIQNSL) from Plasmodium falciparum (35), synthesized by the Peptide and Protein Facility of the University of Wales College of Medicine.

Tetramer Synthesis. MHC class I peptidic complexes were synthesized and tetramerized as described previously (24). Briefly, purified HLA-A2.1 heavy chain and human β2-microglobulin were synthesized using a prokaryotic expression system (PET, R+D Systems, Abingdon, United Kingdom). The heavy chain was modified by deletion of the transmembrane region and cytosolic tail and addition at the COOH terminus of a sequence containing the biotinylation site recognized by the enzyme BirA. Heavy chain and β2-microglobulin (Sigma) were refolded by dilution around peptide 11–20 from the E7 protein of HPV-16 (YMLLDQAPETT). Refolded complexes (45 kDa) were purified by gel filtration and biotinylated using BirA in the presence of biotin (Sigma), ATP (Sigma), and Mg²⁺ (Sigma). Biotinylated complexes were purified by gel filtration and ion exchange, using fast protein liquid chromatography, and then PE-conjugated streptavidin (Sigma) was added at a 1:4 molar ratio to form fluorogenic HPV-16 E7 11–20 tetramer.

In Vitro CTL Induction Using Peptide Stimulation. Thawed PBMCs from patients were stimulated as described previously (18). Briefly, 2 x 10⁷/ml PBMCs in RAB were cultured with peptide at a concentration of 10 μg/ml. On day 4, 1 ml of RAB containing 25 units/ml IL-2 (Chiron UK Ltd, Harefield, Middlesex, United Kingdom) was added. On day 6, 1 ml of medium was aspirated from each well and replaced with 1 ml of fresh medium containing 10 units/ml IL-2. On day 7, fresh or thawed irradiated autologous PBMCs were resuspended at 3 x 10⁶/ml in RAB containing 10 μg/ml peptide and 3 μg/ml β2-microglobulin. Antigen-presenting cells were allowed to adhere for 2 h, and then were washed before the addition of 1–2 x 10⁶/ml effectors. On day 9, 1 ml of RAB containing 25 units/ml IL-2 was added to each well. On day 13, the contents of the wells were split and topped up with medium containing 10 units/ml IL-2. The cells were used in a cytotoxicity assay on day 14.

FACS Staining, Sorting, and Generation of Monoclonal and Polyclonal CTL Lines. Thawed PBMC samples were stained with PE-labeled HPV-E7 11–20 tetramer for 15 min at 37°C before the addition of Tricoulor-anti-CD8 (Caltag, Burlingame, CA) or FITC-anti-CD8 (DAKO AS, Glostrup, Denmark) for 15 min on ice, followed by extensive washes with PBS containing 1% FCS. The cells were fixed in PBS containing 2% paraformaldehyde and 1% FCS before analysis on a FACScan (Becton Dickinson, Mountain View, CA). Small lymphocytes were gated by forward and side scatter profiling, and then up to 1 x 10⁶ cells were collected for analysis of PBMC samples. For some individuals, tetramer staining was performed after 1 week of in vitro culture with the HPV 16 E7 11–20 peptide.

The line from patient J, stimulated as described (18), was stained with PE-labeled HPV-E7 11–20 tetramer for 15 min at 37°C before the addition of Tricoulor-anti-CD8 (Caltag) for 15 min on ice, followed by extensive washes, and then was sorted using a FACSVantage (Becton Dickinson). Small lymphocytes were gated by forward and side scatter profiling and then sorted according to tetramer/CD8 double staining. Single cells, or 500 cells for the monospecific, polyclonal line D4, were sorted directly into a U-bottomed 96-well plate. Each well had been coated overnight at 4°C with anti-CD3 and anti-CD28, both at 100 ng/ml in PBS, and contained 10⁴ irradiated B cells (LG2) in CTL medium [Isco’s medium (Sigma) with 5% human serum containing 100 units/ml IL-2]. Plates were incubated at 37°C in 5% CO₂ for 7–14 days without any manipulation, and then proliferating blasts were expanded in CTL medium, followed by restimulation using 5 μg/ml phytohemagglutinin with irradiated allogeneic peripheral blood lymphocytes and B cells as feeders (36).

Cytotoxicity Assays. Cytotoxicity was measured in a standard 4-h ³¹Cr-release assay as described previously (18). CIR-A2 target cells were pulsed with 10 μg/ml peptide for 2 h after labeling with ³¹Cr (Na₂³¹CrO₄; Amersham International, Little Chalfont, United Kingdom). Cytotoxicity against HPV 16 and 18 E6 and E7 was measured using a recombinant vaccinia virus, TA-HPV (gift of Cantab Pharmaceuticals, Cambridge, United Kingdom; Ref. 37). TA-HPV has been shown to express HPV antigens by both Western blot (37) and by recognition by HPV-specific CTLs (18, 22). CIR-A2 cells were infected with the vaccinia viruses (multiplicity of infection, 15) for a maximum of 12 h before ³¹Cr labeling. After a 4-h incubation, radioactive counts were obtained by β-plate liquid scintillation counting (Wallac, Turku, Finland).

RESULTS

Characterization of HLA-A*0201/HPV 16 E7 11–20 Tetramer. Previously, we have shown that HPV 16 E7 11–20-specific CTL lines could be generated from four of five CaCk patients but not from control subjects unless dendritic cells were used as antigen-presenting cells (18). We used some of these CTL lines to assess the specificity of an HLA-A*0201 tetramer incorporating the peptide HPV 16 E7 11–20. These CTL lines all demonstrated specific HLA-A*0201-restricted recognition of the HPV 16 E7 11–20 peptide and were able to lyse targets expressing endogenous HPV antigens after infection with recombinant vaccinia virus (Fig. 1, A–E; Ref. 18). Clear populations of CD8⁺ tetramer⁺ cells could be distinguished in all CTL lines tested (Fig. 1, F–J), and this varied between 2 and 12% of the T cells analyzed. No CD8⁺ tetramer⁺ cells could be detected in an HLA-A*0201-restricted CTL line recognizing influenza M1 58–66 peptide (data not shown).

Enumeration of HPV 16 E7 11–20-specific CD8 T Cells in Peripheral Blood of CIN3 Patients and Healthy Controls. Tumors have been used in other viral and tumor systems to directly quantify antigen-specific CD8⁺ T cells in human peripheral blood without in vitro antigen-specific stimulation (27, 29, 38). PBMC samples from 10 HLA-A*0201 patients with cervical neoplasia (9 CIN3 and 1 CaCk) and 9 HLA-A*0201 healthy controls were stained using the HPV 16 E7 11–20 tetramer (Table 1). Previous work has shown that tetramers can be used directly to visualize and clone human T cells constituting at least 0.01% of CD8⁺ T cells (36, 39). Using this criterion, CD8⁺ tetramer⁺ T cells could be detected in PBMCs of both the patient and control groups. However, there was variation in the frequencies of CD8⁺ tetramer⁺ cells detected (Table 1). A range of 0.079–0.005% (1 of 1,260 to 1 of 19,073) was found in patients, with a similar range, 0.054–0.002% (1 of 1,855 to 1 of 42,004) in the normal control group (Table 1). These low frequencies are similar to those found for melanoma-specific CTLs in PBMCs (36, 39, 40) but are an order of magnitude lower than the frequencies detected for systemic antiviral CD8 T cells (24, 29, 41). In some patients and controls, e.g., P6–P10 and C5–C9 in Table 1, the number of CD8⁺ tetramer⁺ cells was close to the limits of detection.

To enhance detection of rare tetramer⁺ cells, PBMCs from patients
and controls were stimulated with the HPV 16 E711–20 peptide for 1 week in vitro before staining with tetramer (40). This peptide stimulation increased the numbers of CD8"tetramer" cells in six of nine of the patient PBMC samples tested, with approximately 4-fold increases seen for four of the patients. (Table 1 and Fig. 2). In contrast, no expansions of similar magnitude were seen for the eight control PBMC samples (Table 1). These results are consistent with previous reports that document a failure to generate HPV 16 E711–20-specific CTL in normal controls after short-term in vitro peptide restimulation (17, 18).

### Table 1

<table>
<thead>
<tr>
<th>Subject</th>
<th>Histological grade/stage</th>
<th>Sex</th>
<th>Age (years)</th>
<th>CD8&quot;tetramer&quot; (%)</th>
<th>Reciprocal frequency</th>
<th>CD8&quot;tetramer&quot; (%)</th>
<th>Reciprocal frequency</th>
<th>Increase&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>3.825</td>
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<td>42.004</td>
<td>0.003</td>
<td>32784</td>
<td>1.28</td>
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</table>

<sup>a</sup>Half of the thawed PBMCs were stimulated with HPV 16 E711–20 peptide for 1 week before analysis.

<sup>b</sup>Change calculated from [tetramer" cells (%) after peptide stimulation/tetramer" cells (%) before peptide stimulation]. Increases >1, bold.

<sup>c</sup>ND, not determined due to insufficient sample; NK, age not known.
HPV 16 E711–20-specific CTL Isolated by Tetramer Lyse Targets Expressing Endogenous HPV Antigens. A potential pitfall for the use of synthetic peptides to restimulate CTLs in vitro is the selection of low-affinity CTLs that are peptide specific but unable to recognize endogenously processed antigens (42, 43). Previously, we demonstrated that HPV 16 E711–20-specific CTL lines were able to recognize targets expressing endogenous HPV antigens albeit at lower levels of killing than that seen for peptide-pulsed targets (Fig. 1, A–E; Ref. 18). To further investigate the fine specificity of HPV 16 E711–20 CTLs, we used the HPV 16 E711–20 tetramer to isolate purified populations of CD8+ CTLs (Fig. 3A) from the JJ CTL line (Fig. 1, D and E). The resulting polyclonal CTL line D4, and clone C6 stained with HPV 16 E711–20 tetramer at high levels (Fig. 3, B and C) and were phenotypically stable (92 and 99% TCR Vβ6, respectively) after prolonged tissue culture (data not shown).

Both D4 and C6 were tested for HLA-A*0201-restricted cytotoxic recognition of peptide-pulsed targets and targets expressing endogenous HPV antigens; either after infection with recombinant vaccinia virus containing full-length HPV 16 E6 and E7 antigens (TA-HPV) or as a consequence of HPV 16 transformation (CaSki cells). Both D4 and C6 were able to recognize peptide-pulsed target cells more efficiently than the original JJ CTL line (Figs. 1D, 3A, and 3E). Furthermore, both D4 and C6 were able to recognize HLA-A*0201+ target cells infected with TA-HPV vaccinia but not cells infected with control vaccinia (Fig. 3, D and E). However, lysis of TA-HPV-infected targets was lower than HPV 16 E711–20-pulsed target cells (Fig. 3, D and E). Both D4 and C6 populations were also able to lyse HLA-A*0201+, HPV 16+ CaSki cervical epithelial cell targets but not HLA-A*0201+, HPV 16+ breast epithelial target cells (MDA-231). Pretreatment of CaSki and MDA-231 targets with IFN-γ prior to use in CTL assays (44) did not increase the level of CTL lysis despite increases in HLA class I expression (data not shown). Furthermore, there was no CTL recognition of HPV 16-negative target cells that were HLA matched with CaSki for one or more HLA class I alleles (data not shown).

These results confirm that both clonal and polyclonal populations of HPV 16 E711–20-specific CTLs are able to recognize endogenously processed HPV 16 E7 antigens.

DISCUSSION

This study demonstrates for the first time the use of soluble MHC-peptide complexes or tetramers to study HPV-specific CTLs in patients with cervical neoplasia. A tetramer was constructed consisting of HLA-A*0201 and HPV 16 E711–20. This tetramer combined the most frequently occurring HLA allele among Caucasians (33) together with the best-studied CTL peptide epitope (15, 17, 18, 45, 46) from the HPV type most frequently associated with CaCx (3). Furthermore, the HPV 16 E711–20 peptide epitope has been incorporated into peptide vaccines and has been the subject of several clinical trials (47). The HPV tetramer clearly identified 2–12% CD8+ tetramer+ T cells in short-term CTL lines with known HPV 16 E711–20 specificity. These CD8+ tetramer+ T cells could be purified and were shown to specifically recognize both HPV 16 E711–20 peptide-pulsed, HLA-A*0201+ target cells and HLA-A*0201+ target cells expressing endogenous HPV 16 E7 antigens. Previous studies using the HPV 16 E711–20 peptide have suggested that this epitope is processed endogenously, based on the recognition of either HPV-16-transformed CaSki target cells (17, 46) or vaccinia-HPV-infected target cells (18). However, recognition of CaSki cells may be due to allogeneic recognition of non-HLA-A*0201 molecules (15), and vaccinia-infected targets may express HPV antigens at higher levels than seen in cervical tumor cells. In this study we have used both types of target cell to show stringently that HPV 16 E711–20 peptide-specific CTLs (D4 and C6) can recognize endogenous HPV antigens in an HLA-A*0201-restricted fashion. In peptide dose-response experiments, half-maximal lysis was achieved at ~10 pm for both CTL. This is similar to the values obtained with melanoma-specific CTLs capable of recognizing endogenously processed tumor antigens (48).

Tetramer-directed cell sorting offers a precise method to generate both monospecific polyclonal lines (D4) and more importantly, clones such as C6. Both have been propagated for up to 10 months, maintaining phenotype (CD8, TCR Vβ6) and specificity, thus suggesting that these CTLs could easily be propagated for adoptive immunotherapy. By contrast, the use of conventional limiting dilution methods to isolate CTLs from peptide-specific CTL lines is highly inefficient, with only 1% of CTL clones being able to recognize endogenous HPV antigens (49). Another drawback to the limiting-dilution cloning approach is the possibility that so-called “CTL clones” may actually be derived from mixed populations of CD8+ and CD4+ T cells, which may not be stable over time. Thus, the use of the HPV 16 E711–20 tetramer has efficiently isolated purified populations of stable, high-affinity HPV-specific CTLs not obtainable by conventional methods.

Both D4 and C6 CTLs were obtained from CD8+tetramer+ populations from the JJ CTL line (Fig. 3A). This CTL line had a heterogeneous pattern of tetramer staining (Fig. 1D) compared with other HPV 16 E711–20-specific CTL lines (Fig. 1, F, H, and J). This might reflect the presence of both tetramer+ or tetramer+ CTL subpopulations within the polyclonal CTL line as has been described recently for melanoma-specific CTLs (50). However, further in vitro culturing of the parental JJ CTL line in the absence of antigen resulted in a more homogeneous population of predominantly CD8+tetramer+ T cells. This might reflect the presence of nonsynchronously activated CTL.

7 S. Youde, unpublished observations.
8 S. Youde and P. R. Dunbar, unpublished observations.
9 P. R. Dunbar, unpublished observations.
subpopulations (with differing levels of TCR expression) rather than distinct tetramer\textsuperscript{hi} or tetramer\textsuperscript{lo} populations. Alternatively, tetramer\textsuperscript{hi} T cells may have a longer life span in vitro than tetramer\textsuperscript{lo} T cells.

The results discussed above established the specificity of the tetramer for detecting effector CTLs from in vitro restimulated PBMCs. A goal of this study was to develop tetramer reagents that allow direct quantitation of HPV 16-specific CTLs from ex vivo blood or tissue biopsy samples, as has been demonstrated for melanoma (28, 29, 51) and viral antigens (29, 38, 52). This would be particularly beneficial for rapid monitoring of HPV-specific CD8\textsuperscript{+} T-cell frequencies either in epidemiological studies in developing countries (53) or for vaccine trials (21). Analysis of ex vivo PBMC samples revealed that staining of CD8\textsuperscript{+} T cells with the HPV 16 E7\textsubscript{11–20} tetramer was rare (1 of 1,260 to 1 of 42,000) in both CIN3 patients and controls. On the basis of epidemiological data, HPV would be detected in 65% (54) and 5% (55) of CIN3 patients and controls respectively. Although only small numbers of patients and controls were studied, there was no correlation between the numbers of tetramer\textsuperscript{+} cells detected and HPV-associated disease. It was not possible to examine the HPV 16 E7\textsubscript{11–20} cells for phenotypic markers associated with T-cell memory because of the low frequency of cells detected and the limited PBMC samples available. However, the enhanced detection of tetramer\textsuperscript{+} cells after peptide stimulation of patient samples suggests that these were more readily able to proliferate in vitro. This observation is consistent with previous findings that HPV 16 E7\textsubscript{11–20}-specific CTLs can be generated in patients, but not controls, by standard restimulation protocols (17, 18).

The staining patterns of ex vivo PBMCs were not as clear as for established HPV 16 E7\textsubscript{11–20} specific CTL lines (Fig. 1). This is particularly problematic where the numbers of CD8\textsuperscript{+} tetramer\textsuperscript{+} cells are close to the limit of detection by FACS (Table 1). However, it is not surprising that such low frequencies were seen in PBMCs in the absence of in vitro restimulation (Table 1) because similar results have been observed in melanoma (28). It may be that for HPV-specific CTLs, as for melanoma, PBMCs will not be the best compartment for study. Higher frequencies of tumor-specific CTLs may be detected among tumor-infiltrating lymphocytes (18) or in lymph nodes that have been infiltrated with tumor (28).

The frequencies detected for HPV 16 E7\textsubscript{11–20} CD8\textsuperscript{+} cells (1 of 1,260 to 1 of 42,000) are an order of magnitude lower than found for other viral antigens and tumor antigens. For example, memory CTLs recognizing influenza A M1\textsubscript{58–66} can be found in 1 of 500 small lymphocytes (29), whereas for EBV EBNA3C\textsubscript{325–333}, up to 1 of 25 T cells can be detected using tetramer (41). However, it should be noted that both of these viruses generate strong systemic CTL responses, whereas HPV may have evolved mechanisms to avoid immune recognition (56). Furthermore, the immunodominant peptide epitopes of influenza A and EBV were used to construct tetramers. It is not clear whether HPV 16 E7\textsubscript{11–20}, which was defined using the reverse immunogenetic approach (33), is immunodominant. Even for immunodominant viral epitopes, at least 10-fold variation has been observed in the frequency of CD8 T cells detected by tetramer (29, 41). The variation in the frequency of CD8\textsuperscript{+} HPV 16 E7\textsubscript{11–20} tetramer\textsuperscript{+} T cells among patient PBMCs in this study might explain the contrasting
results obtained for HPV 16 E7 11–20-specific CTL detection (15, 17, 18). However, the current study is limited by the use of tetramers incorporating a single peptide epitope from HPV, and additional HPV CTL epitopes need to be defined. In the future, multiple tetramers comprising different CTL epitopes may allow more precise assessment of the role of CTL in cervical disease, including the possibility that HPV’s can induce immunological tolerance (56). Nevertheless, the HPV tetramer we have characterized may be beneficial for monitoring patients who have been immunized with the HPV 16 E7 11–20 peptide as part of ongoing clinical trials (47).

Recently, tetramer-driven sorting has been used to derive melanoma-specific CTLs suitable for use in adoptive immunotherapy (36, 40, 50). Regardless of the natural role of HPV-specific CTLs, our current studies demonstrate that it is possible to use a HPV tetramer to select high-affinity HPV-specific CTLs, which have potential application in the immunotherapy of CaCx. However, the frequent down-regulation of HLA class I molecules in cervical tumors (57) and the low levels of HPV E6 and E7 expression (58) may confound attempts at CTL-based immunotherapy. The use of the HPV 16 E7 11–20 tetramer to derive large numbers of stable HPV-specific CTL clones will now allow detailed investigation of the factors influencing CTL recognition of HPV-transformed tumor cells.

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Use of Fluorogenic Histocompatibility Leukocyte Antigen-A*0201/HPV 16 E7 Peptide Complexes to Isolate Rare Human Cytotoxic T-Lymphocyte-recognizing Endogenous Human Papillomavirus Antigens

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