

Targeting of HPV-16⁺ Epithelial Cancer Cells by TCR Gene Engineered T Cells Directed against E6

Lindsey M. Draper¹, Mei Li M. Kwong¹, Alena Gros¹, Sanja Stevanović¹, Eric Tran¹, Sid Kerkar², Mark Raffeld², Steven A. Rosenberg¹, and Christian S. Hinrichs¹

Abstract

Purpose: The E6 and E7 oncoproteins of HPV-associated epithelial cancers are in principle ideal immunotherapeutic targets, but evidence that T cells specific for these antigens can recognize and kill HPV⁺ tumor cells is limited. We sought to determine whether TCR gene engineered T cells directed against an HPV oncoprotein can successfully target HPV⁺ tumor cells.

Experimental Design: T-cell responses against the HPV-16 oncoproteins were investigated in a patient with an ongoing 22-month disease-free interval after her second resection of distant metastatic anal cancer. T cells genetically engineered to express an oncoprotein-specific TCR from this patient's tumor-infiltrating T cells were tested for specific reactivity against HPV⁺ epithelial tumor cells.

Results: We identified, from an excised metastatic anal cancer tumor, T cells that recognized an HLA-A*02:01-restricted

epitope of HPV-16 E6. The frequency of the dominant T-cell clonotype from these cells was approximately 400-fold greater in the patient's tumor than in her peripheral blood. T cells genetically engineered to express the TCR from this clonotype displayed high avidity for an HLA-A*02:01-restricted epitope of HPV-16, and they showed specific recognition and killing of HPV-16⁺ cervical, and head and neck cancer cell lines.

Conclusions: These findings demonstrate that HPV-16⁺ tumors can be targeted by E6-specific TCR gene engineered T cells, and they provide the foundation for a novel cellular therapy directed against HPV-16⁺ malignancies, including cervical, oropharyngeal, anal, vulvar, vaginal, and penile cancers. *Clin Cancer Res*; 21(19); 4431–9. ©2015 AACR.

Introduction

Adoptive T-cell therapy (ACT) is a promising cancer treatment modality that is showing encouraging results in clinical trials (1). Adoptive transfer of tumor-infiltrating lymphocytes (TIL), T cells isolated from a metastatic tumor and expanded *ex vivo*, can cause complete regression of metastatic melanoma and cervical cancer in some patients (2, 3). TIL therapy might be applied to a broader range of cancers through advanced genomics and immunology (4), but this technology is not widely available. A more "off-the-shelf" approach can be achieved by administering peripheral blood T cells genetically engineered to express a tumor-targeting chimeric antigen receptor (CAR) or T-cell receptor (TCR). This strategy has shown encouraging early results in clinical trials for B-cell malignancies, melanoma, and synovial cell sarcoma (1). Efforts to extend this approach to epithelial cancers have generally targeted antigens that are shared by tumors and healthy tissues, and they have been limited by T-cell-mediated on-target, off-tumor toxicity to important tissues (5–7). This toxicity might be avoided by targeting a tumor antigen that is not expressed by

healthy tissues, but few antigens are both exclusive to malignant cells and expressed commonly by a particular family of epithelial cancers (1, 5).

The human papillomavirus (HPV) E6 and E7 oncoproteins are in principle ideal targets for TCR gene therapy. They are constitutively expressed by and important for the survival of HPV⁺ cancers of the uterine cervix, oropharynx, anus, vulva, vagina, and penis, and they are absent from healthy tissues (1). The viral origin of these antigens suggests that they might be targeted with T cells, the arm of cellular immunity evolved for specific recognition and killing of cells harboring viral proteins. However, despite extensive studies, including clinical trials of vaccines directed against E6 and E7 (8–12), evidence that T cells targeting these antigens can recognize HPV⁺ tumor cells is limited.

Indeed, HPV⁺ tumor cells possess diverse means of immune evasion that may protect them from T-cell targeting (13, 14). HPV⁺ cervical cancer and head and neck cancer cell lines have been reported to elude recognition by oncoprotein-specific T cells through impaired antigen processing and presentation, including low expression of transporter associated with antigen processing (TAP), proteasome subunits, and human leukocyte antigen (HLA) class I proteins (15, 16). Other mechanisms may also undermine antitumor T-cell responses, including tumor production of inhibitory cytokines, attenuated tumor responsiveness to interferons, and limitation of T-cell avidity through molecular mimicry of human proteins by HPV oncoproteins (13, 14). We sought to determine whether these immunosuppressive mechanisms could be overcome by targeting HPV⁺ tumor cells with genetically engineered T cells expressing an avid TCR directed against an HPV oncoprotein.

HPV-16 and HPV-18 are the most common high-risk HPV types to cause human cancers. The E6 and E7 proteins of these HPV

¹Surgery Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland. ²Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corresponding Author: Christian S. Hinrichs, NIH, National Cancer Institute, Clinical Research Center, Room 3-3888, Bethesda, MD 20892. Phone: 301-435-3027; Fax: 301-402-1738; E-mail: Hinrichs@nih.gov

doi: 10.1158/1078-0432.CCR-14-3341

©2015 American Association for Cancer Research.

Translational Relevance

TCR gene therapy, infusion of T cells genetically engineered to express a tumor-specific TCR, is an emerging cancer treatment modality. Its application to epithelial tumors, the most common human cancers, has been limited by injury to healthy tissues that express target antigen. Here, we describe the discovery of a novel TCR for gene therapy that might circumvent toxicity to healthy tissue by targeting HPV-16 E6, a viral tumor antigen that is absent from healthy tissues. We demonstrate that gene engineered T cells directed against HPV-16 E6 can recognize and kill cervical, and head and neck cancer cells and that they are unlikely to possess cross-reactivity against human proteins. This work is the basis for a new personalized cellular therapy for HPV-16⁺ epithelial cancers.

types do not share potential HLA class I-restricted epitopes. Therefore, T-cell therapy directed against the oncoproteins of HPV-16 or HPV-18 must be restricted to either viral type. HPV-16 is responsible for most cervical cancers and nearly all HPV⁺ cancers at other anatomical sites, and is therefore the preferred target (17, 18). Human leukocyte antigen (HLA)-A*02:01 is the most common HLA allele in Caucasian patients, and HLA-A*02:01-restricted naturally processed and presented epitopes of HPV-16 E6 and E7 have been described (19). We therefore sought to identify an HLA-A*02:01-restricted, HPV-16-oncoprotein-reactive TCR with which to assess the targeting of HPV⁺ tumor cells by TCR gene engineered T cells.

Materials and Methods

Patients

The tissues studied were acquired with informed consent from patients enrolled on NIH Clinical Center (Bethesda, MD) Protocol Number 03-C-0277, which was approved by the NIH Clinical Center Institutional Review Board. Leukapheresis was performed by the Surgery Branch Leukapheresis Unit. Peripheral blood mononuclear cells (PBMC) were isolated from leukapheresis samples and cryopreserved by the Surgery Branch TIL Laboratory using standard operating procedures.

Cell lines

Cell lines were cultured in media consisting of RPMI-1640 (T2, CaSki, 624, 3748 TC, 3569 TC, 2191 TC) or DMEM (SCC90, SCC152, 293 lines) supplemented with 10 percent FBS, L-glutamine 2 mmol/L (Quality Biological), 2-mercaptoethanol 50 μmol/L (GIBCO), penicillin 100 U/mL (Life Technologies), and streptomycin 100 μg/mL (Life Technologies). 3748 TC is an HPV-18⁺ cervical cancer cell line, 3569 TC is a melanoma cell line, and 2191 is a cholangiocarcinoma cell line (all generated at the NCI-Surgery Branch). SCC90 and SCC152 are HLA-A*02:01⁺ HPV-16⁺ squamous cell tumors of the head and neck that were attained from Dr. Susanne Gollin at the University of Pittsburgh. CaSki, T2, and 293 lines were authenticated by and purchased from ATCC and cultured for fewer than 6 months. The other cell lines were authenticated by HPV and mycoplasma testing and by morphology and growth characteristics. The 293-A2 and 293-GFP lines are 293-based lines with stable expression of HLA-A2 and GFP, respectively. 624-E6 and 624-E7 are 624 melanoma lines

with stable expression of E6 and E7, respectively. They were generated by retroviral transduction with LXS_N 16E6 or LXS_N 16E7 (ATCC). The p1322 and p1324 vectors (Addgene) were used for transient expression of E6 or E7 by plasmid transfection. Transfection was accomplished with Lipofectamine 2000 according to the manufacturer instructions (Life Technologies). Epstein-Barr Virus (EBV) LCLs were generated by culturing PBMC with B95-8 cell line supernatant supplemented with 0.5 μg/mL cyclosporine A. HLA typing was performed by the NIH Department of Transfusion Medicine Clinical Laboratory.

Generation of TIL

TILs were grown from 2 mm fragments of fresh tumor in 24-well plates using culture media composed of 50% AIM V (Life Technologies) and 50% RPMI-1640 (Life Technologies) that was supplemented with 10% human serum (Division of Transfusion Medicine, NIH), IL-2 300 IU/mL (Novartis), gentamicin 5 μg/mL, L-glutamine 2 mmol/L (Quality Biological), 2-mercaptoethanol 50 μmol/L (GIBCO), penicillin 100 U/mL (Life Technologies), and streptomycin 100 μg/mL (Life Technologies; ref. 20). TIL cultures were split when confluent and studied when they reached sufficient numbers, typically four wells of confluent cells.

Generation of DCs

DCs were generated from the adherent fraction of PBMC and cultured in media composed of DMEM supplemented with 10% FBS, L-glutamine 2 mmol/L, streptomycin 100 μg/mL, penicillin 100 U/mL and 2-FBS mercaptoethanol 55 μmol/L, recombinant human granulocyte macrophage-colony stimulating factor 1,000 U/mL (Peprotech), and recombinant human IL4 1000 U/mL (Peprotech) for 6 days. Cytokines were replenished every other day.

T-cell transductions

PBMCs were stimulated with soluble anti-CD3 (OKT3) 30 ng/mL and IL2 300 IU/mL. Transduction was performed 2 days after stimulation using retrovirus encoding the E6 TCR. Retrovirus was generated by transient transfection of 293GP cells with MSGV1-E6 TCR plasmid (insert synthesized and subcloned into MSGV1 by Gene Oracle) and a RD114 envelope plasmid. Transductions were performed using retronectin coated plates (Takara Bio) as described previously (21).

Determination of the E6 TCR nucleotide sequence

TILs generated from tumor fragments were cocultured with autologous DCs loaded with the E6 peptide pool for 24 hours. Cells expressing CD137 were positively selected using magnetic beads (Miltenyi Biotec), pooled, and expanded for 2 weeks using a rapid expansion protocol (20). These cells were then cocultured for 24 hours with 293-A2 cells transfected with a vector encoding full-length E6, and the CD137⁺ cells were positively selected with magnetic beads. RNA was isolated from the selected cells using an RNeasy Mini Kit (Qiagen), and TCRα- and β-chains were amplified with using a 5'RACE-PCR SMARTer RACE cDNA Amplification Kit (Clontech) with primers specific for the TCRα (5'-GCC-ACAGCACTGTTGCTCTTGAAGTCC-3') and TCRβ (5'-CAGG-CAGTATCTGGAGTCATTGAG-3') constant regions. 5'RACE products were cloned with a TOPO TA Cloning Kit (Life Technologies), transformed into TOP10 chemically competent E. coli (Life Technologies), and single colonies sequenced (Macrogen) and analyzed using the IMGT/V-Quest online tool.

Immunologic assays

TILs were tested for reactivity against the E6 and E7 antigens using as targets DCs loaded with pools of 15-mer peptides overlapping by 11 amino acids and spanning the full length of the antigen indicated in the figure legend (Miltenyi Biotec). Following overnight coculture, the concentration of IFN γ in the supernatants was quantified by ELISA (R&D Systems). The E6_{29–38} peptide was synthesized by Peptide 2.0, Inc. Reactivity of E6 TCR-transduced cells with target cells was assessed by overnight coculture of 5×10^4 effector cells with 5 to 10×10^4 target cells followed by determination of supernatant concentrations of IFN γ and TNF α by ELISA (R&D Systems). Specific cytotoxicity was assessed by chromium (Cr^{51}) release assay. Target cells were labeled with Cr^{51} for 2 hours at 37°C, washed, and plated at 1×10^4 cells per well in a 96-well plate. Effector cells were added at the ratios indicated and incubated for 4 hours. Supernatants were harvested and dried on luminescence plates, which were counted with a Microbeta luminometer.

Analysis of excised tumor

IHC was performed by the NIH Clinical Center Laboratory of Pathology using standard operating procedures. Tumor expression of HPV oncoproteins was assessed by real-time RT-PCR. RNA was isolated from tumor using an RNeasy Mini Kit (Qiagen). cDNA was generated with qScript cDNA SuperMix (Quanta Biosciences). Gene expression was analyzed using custom gene-specific TaqMan primers and probes. TCR deep sequencing was performed by ImmunoSEQ (Adaptive Biotechnologies).

Flow cytometry

Cells were labeled with APC-H7-conjugated anti-CD3 (BD Biosciences), PE-Cy7-conjugated anti-CD8 (BD Biosciences), or PE-conjugated anti-mouse TCR β (eBioscience). E6_{29–38}/HLA-A*02:01 and E7_{11–19} monomers were provided by the NIH Tetramer Core Facility. Tetramers were assembled and labeled following NIH Tetramer Core Facility protocols. Data were acquired with a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC).

Results

A patient with an ongoing, prolonged disease-free interval after her second resection of metastatic anal cancer harbored tumor-infiltrating lymphocytes reactive against HPV-16 E6

To identify HLA-A*02:01-restricted, HPV-16 oncoprotein-reactive T cells, we studied TIL cultures from the tumor of an HLA-A*02:01⁺ patient with metastatic anal cancer (Patient 3809). This patient had an unusual clinical course marked by a prolonged, ongoing disease-free interval after her second resection of distant metastatic disease (Fig. 1A). Her second metastasectomy was of a portal lymph node tumor (Fig. 1B), which was excised for the generation of TIL to be administered when she developed measurable disease at another site (3). Additional metastatic disease did not materialize, and she remains disease free more than 22 months later.

The resected tumor was analyzed to determine whether it expressed the HPV oncoproteins and HLA class I molecules. Consistent with the presence of high-risk HPV, it displayed uniform, bright labeling with anti-p16 antibody (Fig. 1C). Real-time reverse-transcription PCR (RT-PCR) with type-specific primers confirmed expression of the HPV-16 E6 and E7 oncoprotein genes (Supplementary Fig. S1). Tumor expression of HLA

class I and infiltration with CD8⁺ cells were evident by IHC, suggesting that HLA class I-restricted antigen presentation might be possible (Fig. 1C). TIL cultures were generated from 23 of 24 tumor fragments and tested for reactivity against HPV-16 E6 and E7 (Fig. 1D). Cultures from nine fragments appeared to show reactivity against E6 but not E7 or gp100. Addition of anti-HLA class I blocking antibody reduced this reactivity in most cultures, but interpretation of the blocking results was limited by a lack of controls for blocking-antibody specificity. However, taken together these findings suggested the presence of CD8⁺ TIL with HPV-16 E6 reactivity.

3809 TIL recognized an HLA-A2-restricted epitope of E6 and an HLA-A2⁺ cell line expressing full-length E6

To test whether TIL from Patient 3809 recognized a naturally processed and presented epitope of E6, we generated from 3809 TIL, a population of T cells enriched for E6 reactivity (E6 TIL) and tested it for recognition of 293-A2 cells (293 line with stable expression of HLA-A*02:01) transfected with a vector encoding full-length E6. Generation of the E6 TIL was accomplished by magnetic bead selection of CD137⁺ cells (22) following overnight incubation of TIL cultures with autologous dendritic cells (DC) loaded with overlapping peptides spanning E6. Isolated CD137⁺ cells from each of the TIL cultures were pooled and expanded using a rapid expansion protocol. These expanded E6 TIL cells showed recognition of 293-A2 cells transfected with a vector encoding full-length E6 (Fig. 2A). The same target cells transfected with a vector encoding GFP were not recognized (Fig. 2A). This result indicated that the E6 TIL recognized a naturally processed and presented epitope of E6, and it suggested that this recognition was HLA-A*02:01 restricted. We postulated that a population of the E6 TIL might be directed against E6_{29–38}, a purported HLA-A*02:01-restricted epitope (19). To assess this possibility, we performed flow-cytometric analysis of T cell binding to HLA-A*02:01/E6_{29–38} tetramers. Thirty-two percent of the cells were CD8⁺ and bound to HLA-A*02:01/E6_{29–38} tetramers (Fig. 2B). Thus, the E6 TIL showed recognition of target cells expressing HLA-A*02:01 and full-length E6 (Fig. 2A), and binding to HLA-A*02:01/E6_{29–38} tetramers (Fig. 2B) suggesting that they might recognize HPV-16⁺ cells through HLA-A*02:01-restricted targeting of E6_{29–36}.

The dominant E6-reactive T-cell clonotype was present at greater frequency in 3809 tumor than peripheral blood

To investigate whether the dominant E6-reactive T-cell clonotype from E6 TIL preferentially localized to tumor *in vivo*, we performed TCR clonotype analysis of 3809 tumor and peripheral blood. To identify the dominant E6-reactive T-cell clonotype from E6 TIL, E6 TILs were cocultured with 293-A2 cells transfected with full-length E6, and CD137⁺ cells were isolated by magnetic bead separation. TCR α -chain and β -nucleotide sequences of isolated cells were amplified by 5' rapid amplification of cDNA ends (5'RACE), and the sequences of the 5'RACE products were determined. A dominant (8/9 bacterial clones sequenced) β -chain clonotype (E6 TCR) was identified and verified as E6 reactive as described in the sections below. The E6 TCR was the eighth most common T-cell clonotype in the tumor with a frequency of 1.6 percent (Fig. 2C). In peripheral blood it was ranked 4,713 with a frequency of 0.004% (Fig. 2C). This greater frequency in the tumor than in the peripheral blood suggested that it preferentially accumulated and/or expanded in the tumor, presumably due to

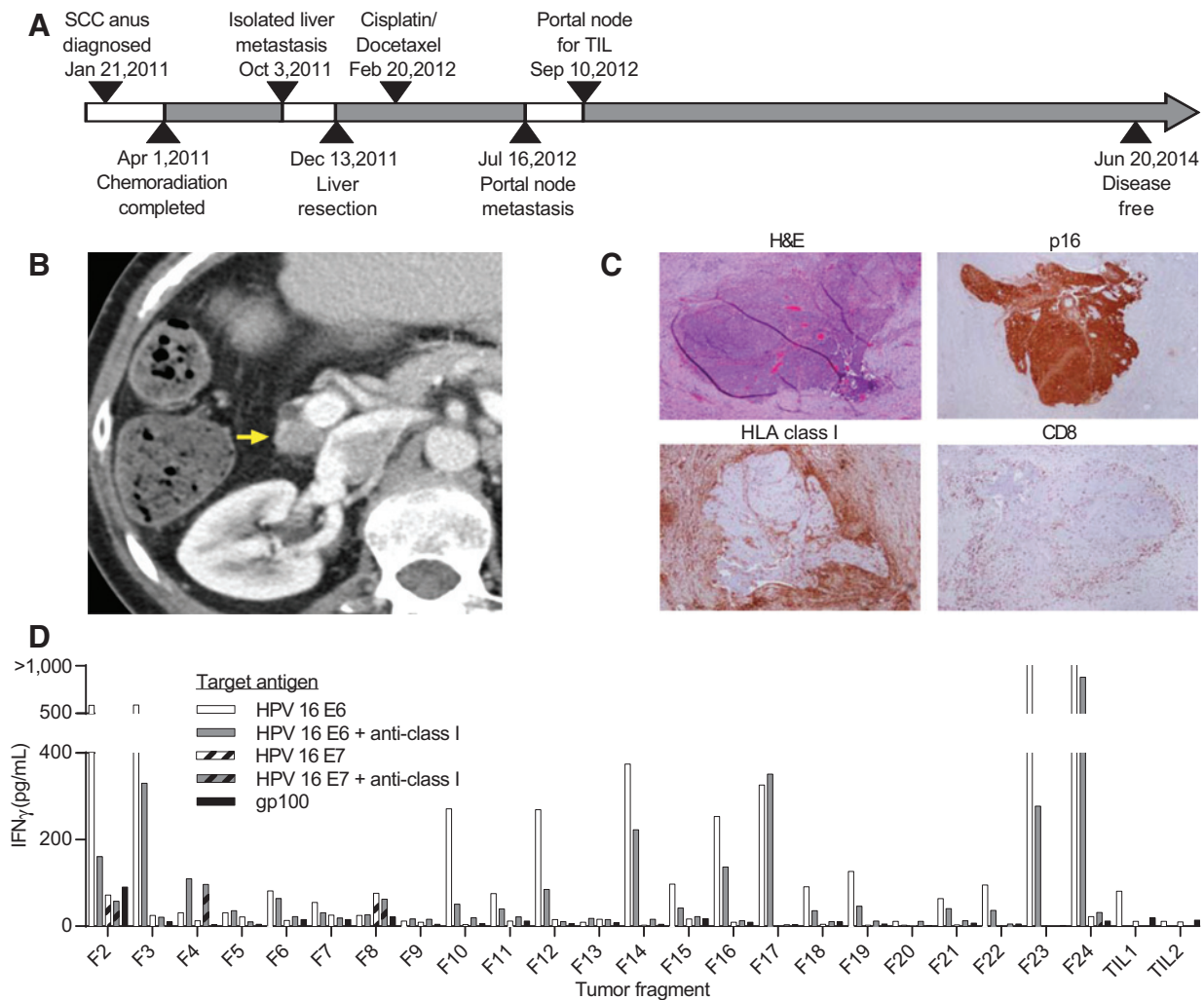


Figure 1. The excised tumor of a patient experiencing a prolonged disease-free interval harbored T cells with HPV-16 E6 reactivity. A, timeline for the clinical course of Patient 3809. Gray segments indicate progressively longer disease-free intervals, including the current, ongoing 22-month period. B, contrast-enhanced CT showing the portal lymph node metastasis (yellow arrow) that was resected and used to generate TIL. C, microscopic examination of the resected tumor including hematoxylin and eosin stain (H&E), as well as p16, HLA class I, and CD8 IHC. D, testing of TIL cultures for HPV-16 E6 and E7 reactivity. The tumor fragment number is indicated on the x axis. Target cells were autologous immature DCs loaded with pools of peptides spanning the antigen indicated in the legend. The W6/32 anti-MHC class I blocking antibody was added as indicated in the legend. TIL1 and TIL2 were TIL cultures generated from melanoma tumors. The concentration of IFN γ in the supernatants following overnight coculture is shown.

the presence of cognate antigen. However, other T-cell clonotypes infiltrating the tumor were also more frequent in tumor than peripheral blood, and definitive testing of the E6 TCR for autologous tumor recognition was not possible as a tumor digest or autologous tumor line could not be generated.

T cells gene engineered to express a TCR from E6-reactive TIL (E6 TCR) displayed HLA-A*02:01/E6₂₉₋₃₈ tetramer binding and high functional avidity for E6₂₉₋₃₈

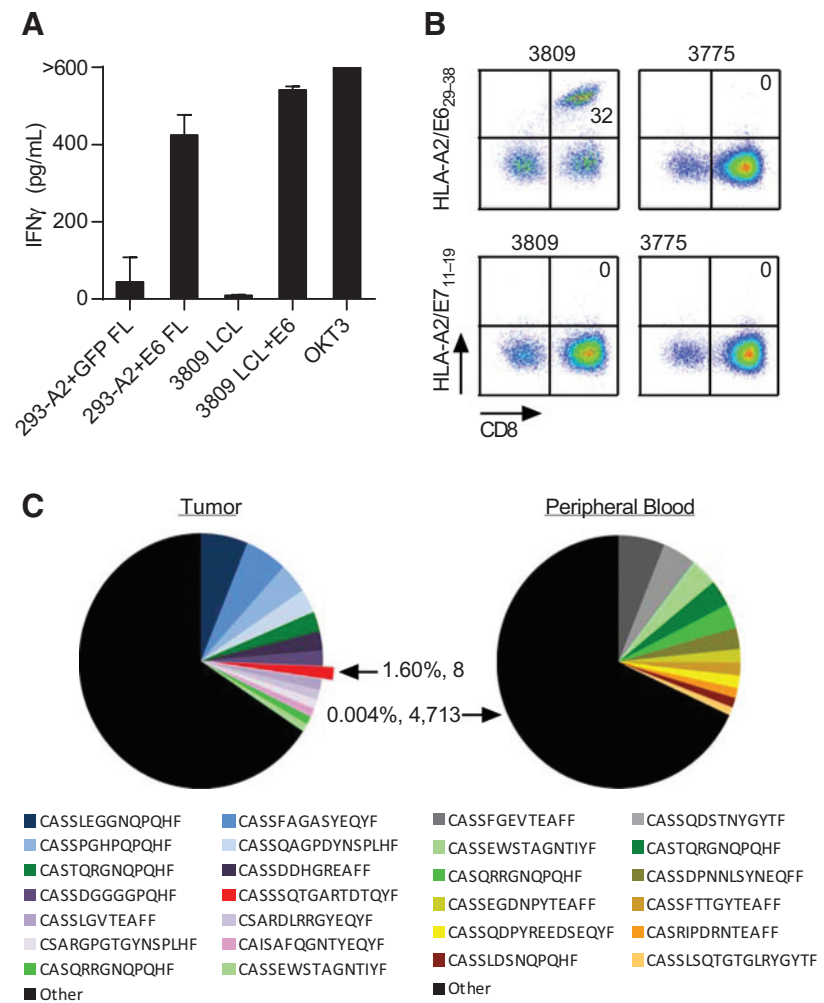
A vector insert was designed encoding the α - and β -chains of the E6 TCR (with the constant regions of each TCR chain exchanged for their murine counterparts) linked by a furin 2A self-cleaving peptide (Fig. 3A). The insert was codon optimized for expression in human cells, synthesized, and cloned into an MSGV1 retroviral expression vector. T cells from peripheral blood mononuclear

cells (PBMC) transduced to express the TCR displayed expression of the murine TCR β -chain constant region and binding to HLA-A*02:01/E6₂₉₋₃₈ tetramers (Fig. 3B). This binding was evident in both the CD8⁺ and CD8⁻ transduced cells indicating avidity sufficient for CD8-independent ligation of cognate peptide-HLA complexes. To measure functional avidity, CD4- or CD8-enriched T cells were transduced to express the E6 TCR, and were cocultured with T2 cells pulsed with titrated concentrations of cognate peptide (Fig. 3C). CD8⁺ T cells displayed greater functional avidity than CD4⁺ T cells, which indicated a contribution to target binding from the CD8 coreceptor. CD8⁺ T cells showed recognition, albeit weak, of T2 cells pulsed with 100 pmol/L E6₂₉₋₃₈ peptide indicating functional avidity similar to TCR gene engineered T cells that have mediated tumor regression in other gene therapy trials (6, 23-26). Although it might be interesting to

Downloaded from <http://aacrjournals.org/clinccancerres/article-pdf/21/19/4434/2026634/4434.pdf> by guest on 09 December 2023

Figure 2.

TIL enriched for E6 reactivity displayed recognition of a naturally processed epitope of E6 and exhibited binding to HLA-A*02:01/E6₂₉₋₃₈ tetramer. A, TILs were enriched for E6 reactivity as described in the Materials and Methods section and tested for recognition of a 293-A2 line transfected with a plasmid encoding full-length E6 (293-A2+E6 FL) or GFP (293-A2+GFP FL). Other targets in this assay were an autologous EBV LCL line with (3809 LCL+E6) or without (3809 LCL) the E6 peptide pool. The OKT3 condition was a nonspecific stimulation with plate-bound anti-CD3 antibody. The quantity of IFN γ produced following overnight coculture with each target is displayed. B, flow-cytometric analysis of E6₂₉₋₃₈ tetramer binding by E6-reactivity-enriched TIL from Patient 3809 or Patient 3775 (negative control). HLA-A2/E7₁₁₋₁₉ was a control tetramer composed of an HLA-A*02:01-restricted epitope of HPV-16 E7. The frequency of events in the right upper quadrant is indicated on each dot plot. The data are graphed on a logarithmic scale. C, the frequency of T-cell clonotypes from the tumor or blood. The β -chain complementary determining regions for clonotypes present at greater than one percent frequency are shown in color and labeled in the legend. The dominant TCR from E6-reactive 3809 TIL is indicated with arrows, and its frequency and clonotypic rank in tumor and peripheral blood is shown in the adjacent text.



compare the avidity of these TCR gene engineered T cells to a panel of naturally occurring T cells targeting the same epitope, we were not able to do so because we have not identified TIL targeting this epitope in other patients.

E6 TCR genetically engineered T cells recognized target cells in an HLA-A*02:01-restricted manner

To confirm that recognition of target cells by the E6 TCR was HLA-A*02:01-restricted, we performed HLA-blocking experiments and assessed recognition of cell lines with and without expression of HLA-A*02:01. Recognition of SCC90 and CaSki (HPV-16⁺, HLA-A*02:01⁺ cell lines) cell lines by E6 TCR-transduced cells was blocked by anti-HLA class I but not anti-HLA class II antibodies indicating an HLA class I-restricted interaction (Fig. 4). Transfected 293 cells expressing full-length E6 plasmid were recognized only if they also expressed HLA-A*02:01 (Fig. 4). Thus, the restriction element for the E6 TCR was confirmed to be HLA-A*02:01.

E6 TCR genetically engineered T cells displayed specific targeting of wild-type HLA-A*02:01⁺, HPV-16⁺ oropharyngeal, and cervical cancer cell lines

E6 TCR-transduced cells were tested for specific recognition of a panel of cell lines expressing (or not expressing) HLA-A*02:01 and/or HPV-16 E6. HLA-A*02:01⁺ cell lines loaded with E6₂₉₋₃₈

peptide, transfected with full-length E6 plasmid, transduced with full-length E6-encoding retrovirus, or naturally transformed in humans by HPV infection (Fig. 5A and B), were recognized by E6 TCR-transduced T cells. Recognized tumor cells included CaSki (a cervical carcinoma) and SCC90 and SCC154 (head and neck carcinomas). Negative control lines lacking either the HLA-A*02:01 restriction element or HPV-16 E6 were not recognized. Tumor recognition provoked multiple T-cell effector functions with production of IFN γ and TNF α , as well specific lysis of target cells (Fig. 5A through C). Specific cell lysis did not titrate with the effector-to-target ratio in some target lines, but it was greater than that of the negative controls (untransduced effector cells and 624 target cells). Taken together, these data demonstrated specific targeting of wild-type, unmanipulated HPV-16⁺ cervical, and head and neck tumor cells by E6 TCR gene engineered T cells.

E6 TCR genetically engineered T cells did not cross-react against partially homologous human peptides

The E6 TCR has a lower probability of cross-reactivity against human proteins than TCRs that have been isolated from mice or that have undergone avidity enhancement by complementary region substitutions because it was selected for a lack of auto-reactivity in a human thymus and its complementarity determining regions have not been altered. However, we also tested it for

Downloaded from <http://aacrjournals.org/clinccancerres/article-pdf/21/19/4431/2026634/4431.pdf> by guest on 09 December 2023

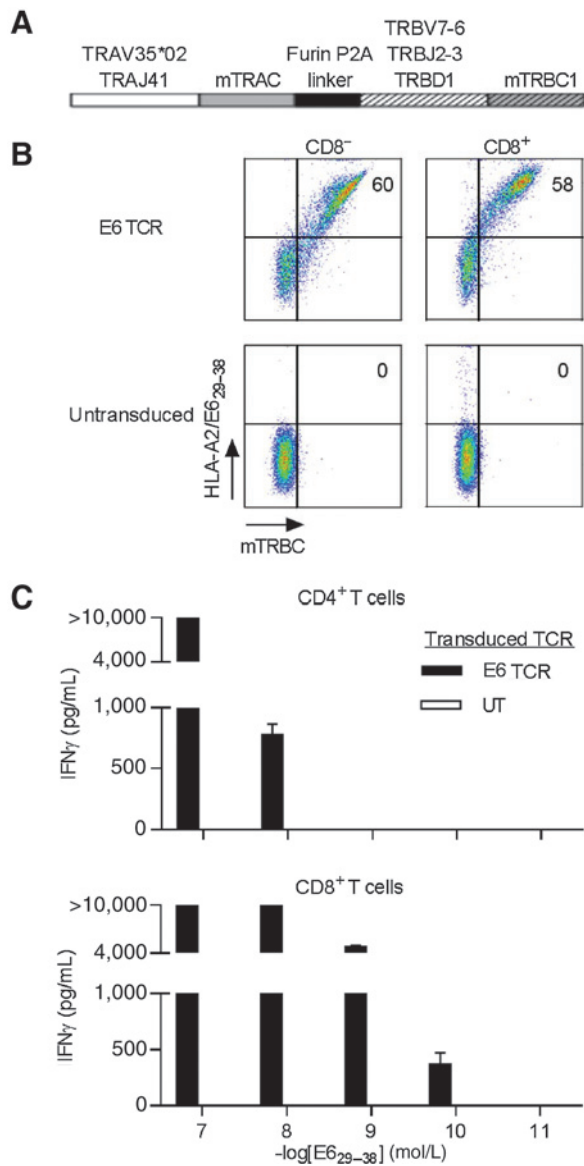


Figure 3. Genetically engineered T cells expressing the E6 TCR displayed high avidity for cognate antigen. A, schematic of the E6 TCR cassette that was cloned into the MSGV1 retroviral vector. Human TCR α - and β -chain constant regions were replaced with their murine equivalents (mTRAC and mTRBC1) and α - (TRAV35*02/TRAJ41) and β - (TRBV7-6/TRBJ2-3/TRBD1) chains were linked with a Furin P2A connector. B, flow-cytometric analysis of CD8⁻ and CD8⁺ fractions of E6 TCR-transduced and untransduced T cells. Mouse TCR β -chain constant region (mTRBC) expression and HLA-A*02:01/E6₂₉₋₃₈ tetramer binding is shown. Dot plots are displayed on a logarithmic scale. C, functional avidity assay testing recognition by T cells transduced to express the E6 TCR (E6 TCR) of T2 cells pulsed with titrated concentrations of E6₂₉₋₃₈ peptide. Control effector cells were not transduced (UT). The concentration of IFN γ in supernatants after overnight coculture is shown. Error bars, SD of duplicate measurements. Data are representative of three separate experiments.

cross-reactivity against human peptides sharing more than five of the 10 amino acids from the E6₂₉₋₃₈ epitope. Sixteen peptides from 15 proteins were identified for testing (Supplementary

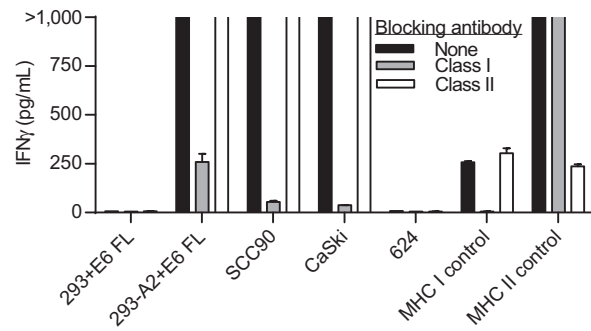


Figure 4. Recognition of cell lines by E6 TCR gene engineered T cells was HLA-A*02:01 restricted. The quantity of IFN γ in supernatants following overnight coculture of E6 TCR transduced T cells with the targets indicated on the x axis is shown. Target cells were 293 or 293-A2 lines transfected with a vector encoding full-length E6 (E6 FL), or the cell lines SCC90, CaSki, or 624. SCC90 and CaSki are HLA-A*02:01⁺ and HPV-16⁺. The 624 line is HLA-A*02:01⁺ and HPV-16⁻. HLA class I (W6/32 clone) or class II (HB145 clone) blocking antibodies were added as indicated in the legend. The HLA class I blocking control consisted of T cells expressing an HLA-A*02:01-restricted MART-1-specific TCR that were cocultured with the 624 cell line. The HLA class II blocking control consisted of T cells expressing an HLA-DR4-restricted E7-specific TCR that were cocultured with dendritic cells loaded with the E7 peptide pool. Error bars, SD of duplicate measurements; data are representative of two experiments.

Table S1). A test for reactivity of E6 TCR-transduced T cells against T2 cells loaded with these peptides revealed no recognition of the partially homologous peptides (Fig. 6). These findings suggest that the E6 TCR is unlikely to possess cross-reactivity against human proteins.

Discussion

HPV-16 E6 and E7 are constitutively expressed by HPV⁺ malignancies and absent from healthy tissues; they are therefore attractive targets for T-cell-based immunotherapy. However, HPV⁺ tumor cells can evade T cells targeting the HPV oncoproteins (13, 14, 27–29), and it is not known if oncoprotein-specific T cells can recognize HPV⁺ cancer cells. The CaSki cervical cancer cell line has been reported to escape recognition of E6₂₉₋₃₈-specific T cells through low expression of LMP2, LMP7, TAP1, and TAP2 and the proteasome activator PA28 (15). Similarly, in a separate study, the inability of E7-specific T cells to engage the SCC90 head and neck cancer cell line was attributed to nearly absent levels of LMP2, TAP1, and tapasin in the SCC90 cells (16). These reports illustrate how reduced antigen processing can permit HPV⁺ tumor cells to evade oncoprotein-specific T cells. Our results show that this evasion can be overcome by targeting HPV⁺ tumor cells with TCR gene engineered T cells bearing an avid oncoprotein-specific TCR.

Our initial efforts to raise HPV-16 E6- or E7-reactive TCRs through *in vitro* stimulation of human PBMC and by vaccination of HLA-A*02:01 transgenic mice generated receptors with low functional avidity, weak or absent tetramer binding, and no recognition of HPV⁺ tumor lines (Supplementary Figs. S2 and S3). These results were unexpected given the presumed immunogenicity of these viral proteins. However, to our knowledge, there are no published reports that describe high-avidity T cells targeting E6 or E7 that can specifically recognize HPV⁺ tumor

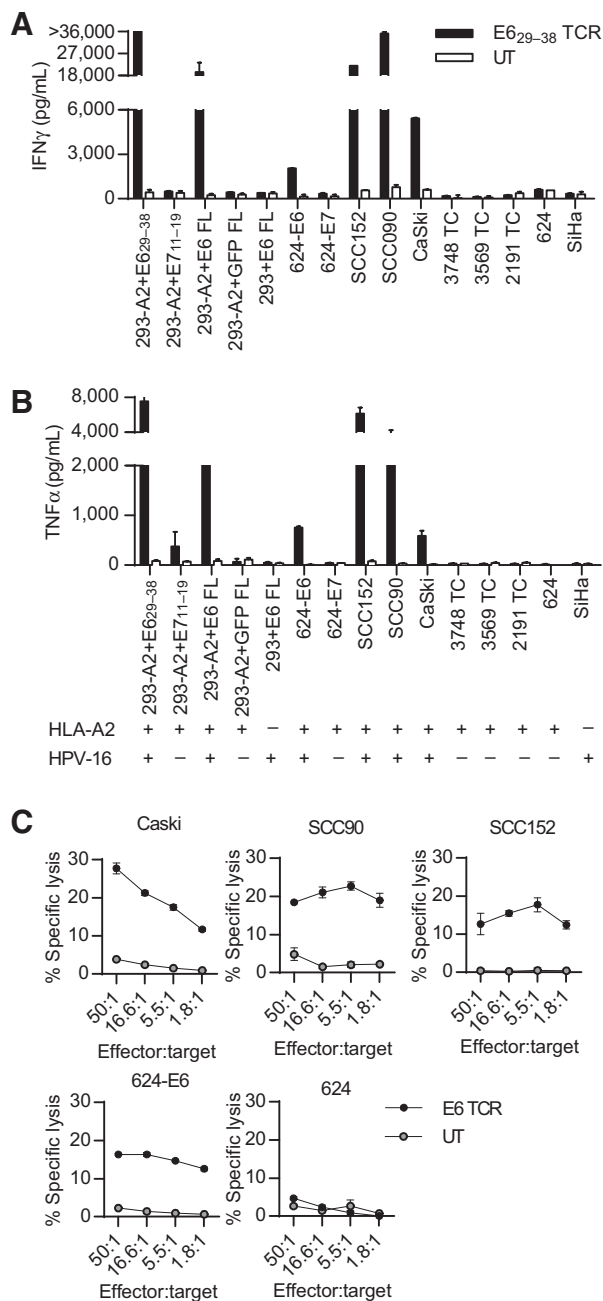


Figure 5. E6 TCR gene engineered T cells specifically recognized and killed HLA-A2⁺ HPV-16⁺ tumor cells. A, IFN γ and (B) TNF α production by E6 TCR gene engineered T cells cocultured with a panel of targets possessing or lacking the HPV-16 E6 antigen and/or HLA-A*02:01 restriction element. Target expression of HLA-A*02:01 and HPV-16 E6 is indicated below the graph in B. Error bars, SD of duplicate measurements; data are representative of two independent experiments. In some experiments, recognition of SCC90 and SCC152 was weaker. C, specific cytolysis of tumor cells by E6 TCR gene engineered (E6 TCR) or untransduced (UT) T cells as measured by Cr⁵¹ release assay. The target cell line is indicated above each graph. Error bars, SEM of triplicate measurements.

lines. That high avidity T cells against E6 and E7 may be uncommon is also intimated by clinical observations. Despite sustained oncoprotein expression in the basal epithelial layer, HPV-16-

infected patients routinely fail to clear the virus for months or even years (30). Similarly, in the face of constitutive oncoprotein expression by tumor cells, therapeutic cancer vaccines targeting E6 and E7, although promising in premalignant HPV⁺ vulvar intraepithelial neoplasia (31, 32), have been unsuccessful in mediating regression of invasive cancers (8-12).

Multiple factors likely contribute to the apparently weak T-cell response against E6 and E7, but one may be a lack of highly avid T-cell precursors reactive against these target antigens. In vaccine studies, the frequency of HPV-reactive T cells has been studied, but the functional avidity of these cells has not been reported (8-12). Similarly, TILs targeting HPV oncoproteins have been isolated from HPV⁺ tumors, but the avidity of these T cells was not determined (33-35). Our group has reported cervical cancer regression in patients following administration of TIL cultures selected for HPV oncoprotein reactivity, but the avidity of the HPV-specific T cells was not studied, and whether tumor destruction was mediated by HPV-specific T cells or "bystander" T cells in these cultures is unknown (3). The avidity of the T cells targeting E6 or E7 may be an overlooked but important factor in the success of immunotherapies directed against the HPV oncoproteins.

TCR gene therapy, through transgenic expression of a well-defined TCR, permits relatively precise control over the avidity of the tumor-targeting T cells. In addition, *ex vivo* T-cell expansion before administration enables treatment with a high number of tumor-specific precursors. Lymphocyte-depleting conditioning regimens given before T-cell infusion reduce negative regulatory elements, increase the availability of homeostatic cytokines, and activate innate immunity thereby enhancing the anti-tumor activity of the infused T cells (1). Finally, TCR gene engineering permits selection or induction of T-cell subsets with augmented capacity to induce tumor regression (21, 36, 37). Hence, TCR gene therapy can be a potent means of targeting a tumor antigen. However, treatments directed against shared tumor/self antigens such as CEA, MART1, and gp100, although they induced tumor regression in some patients, caused severe autoimmune toxicities that prevented their clinical development (5). Identification of high avidity TCRs targeting *bona fide* tumor-specific antigens has been perhaps the limiting factor in the development of effective TCR gene therapy for epithelial cancers (5). Here, we report the discovery of a TCR that enables targeting of a constitutively expressed, tumor-specific antigen. This TCR may permit the realization of the full potential of TCR gene therapy for the treatment of a family of otherwise incurable and difficult to palliate epithelial tumors. One limitation of the present study is that it does not test the *in vivo* antitumor activity of E6 TCR gene engineered T cells in an animal model. A clinical trial of E6 TCR gene therapy is presently active (NCT02280811), and will provide needed insight into the clinical value of this approach.

Cross-reactivity of TCRs against antigens expressed by healthy tissues has been a major obstacle to the development of TCR gene therapy for solid tumors (5). Targeting of antigens with known expression in vital tissues such as eyes, ears, skin, and colon has resulted in dose-limiting toxicities related to tissue injury at those sites. Unrecognized cross-reactivity of TCRs against epitopes other than the intended target has caused fatal toxicity in clinical trials of two different receptors (38-40). In both cases, the TCRs had been affinity-enhanced by complementary determining region amino acid substitutions,

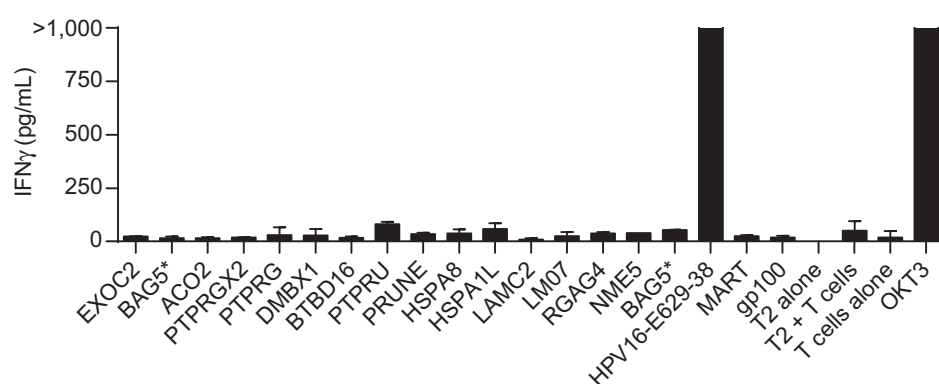


Figure 6.

E6 TCR gene engineered T cells did not display cross-reactivity against human peptides. E6 TCR-transduced T cells were cocultured with T2 cells pulsed with human peptides identified as sharing six (or five plus a conservative substitution) or more amino acids with the E6_{29–38} epitope (Supplementary Table S1). The concentration of IFN γ in the supernatants following overnight coculture is shown. The MART (MART_{127–35}) and gp100 (gp100_{154–162}) peptides are HLA-A*02:01 restricted. The OKT3 condition was stimulation with plate-bound OKT3 antibody. Error bars, SEM of duplicate measurements. Asterisks, peptides derived from the same protein.

which introduced new, unintended reactivities. The E6 TCR is unlikely to possess cross-reactivity against human proteins because it targets an antigen that is expressed only by viruses, it was isolated from a human (i.e., underwent thymic selection, which would be expected to limit autoreactivity), and its complementarity determining regions are unaltered. In addition, our screen for reactivity against peptides with partial homology to E6_{29–38} revealed no recognition of these peptides (Fig. 6). Hence, the E6 TCR appears to be a relatively safe TCR for testing in human subjects.

The patient from whom the E6 TCR was isolated experienced an unusual clinical course characterized by increasing disease-free intervals between surgical tumor resections and a prolonged (and ongoing) disease-free interval following her second metastasectomy. This type of prolonged disease-free survival or cure is known to occur following surgical resection of metastatic cancer despite the presumed presence of widespread microscopic disease, but the mechanism of the phenomenon is poorly understood. It has been speculated that an antitumor T-cell response may eliminate or control micrometastatic tumors preventing the emergence of clinically evident cancer in these patients. However, an immunologic "smoking gun," a T-cell targeting a tumor-specific antigen and capable of tumor recognition, has not been recovered in reported cases. This patient was found to harbor in her resected tumor a population of T cells with high avidity for the HPV-16 E6 tumor antigen. The frequency of this HPV-16 E6 T-cell clonotype was approximately 400-fold greater in the tumor than in the peripheral blood indicating its preferential localization to the tumor presumably due to its targeting of a tumor antigen. Also supporting this notion, T cells genetically engineered to express the TCR from this clonotype displayed specific recognition of a panel of HPV-16⁺ tumors from cervical and head and neck cancers. These findings suggest that the E6 TCR possessed reactivity against the patient's tumor. Whether it played a role in the patient's clinical course is unknown. A clinical trial to test whether

it can be used to the benefit of other patients is now active (NCT02280811).

Disclosure of Potential Conflicts of Interest

C.S. Hinrichs reports receiving commercial research support from Kite Pharma. C.S. Hinrichs and S.A. Rosenberg are listed as co-inventors on a provisional patent application, which is owned by the NIH and licensed to Kite Pharma, on the HPV-16 E6-targeted TCR described in this manuscript. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: L.M. Draper, M.L.M. Kwong, A. Gros, S.A. Rosenberg, C.S. Hinrichs, S. Stevanović

Development of methodology: L.M. Draper, M.L.M. Kwong, A. Gros, C.S. Hinrichs

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.M. Draper, M.L.M. Kwong, A. Gros, S. Kerker, M. Raffeld, C.S. Hinrichs

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.M. Draper, M.L.M. Kwong, S. Kerker, S.A. Rosenberg, C.S. Hinrichs

Writing, review, and/or revision of the manuscript: L.M. Draper, E. Tran, M. Raffeld, S.A. Rosenberg, C.S. Hinrichs, S. Stevanović

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.L.M. Kwong, E. Tran, S. Kerker, S.A. Rosenberg, C.S. Hinrichs, S. Stevanović

Study supervision: S.A. Rosenberg, C.S. Hinrichs

Other (performed one of the experiments shown): A. Gros

Grant Support

This work was supported by the Intramural Research Program of the NIH. 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.

Received December 31, 2014; revised May 21, 2015; accepted May 22, 2015; published online October 1, 2015.

References

- Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev* 2014;257:56–71.
- Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et al. Durable complete responses in heavily pretreated patients with

- metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* 2011;17:4550–7.
3. Stevanović S, Draper LM, Langhan MM, Campbell TE, Kwong ML, Wunderlich JR, et al. Complete regression of metastatic cervical cancer after treatment with human papillomavirus-targeted tumor-infiltrating T cells. *J Clin Oncol* 2015;33:1543–50.
 4. Tran E, Turcotte S, Gros A, Robbins PF, Lu Y-C, Dudley ME, et al. Cancer immunotherapy based on mutation-specific CD4⁺T cells in a patient with epithelial cancer. *Science* 2014;344:641–5.
 5. Hinrichs CS, Restifo NP. Reassessing target antigens for adoptive T-cell therapy. *Nat Biotechnol* 2013;31:999–1008.
 6. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan D-AN, Feldman SA, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther* 2010;19:620–6.
 7. Lamers CHJ, Sleijfer S, Vulto AG, Kruit WHJ, Kliffen M, Debets R, et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 2006;24:e20–2.
 8. Maciag PC, Radulovic S, Rothman J. The first clinical use of a live-attenuated *Listeria monocytogenes* vaccine: a Phase I safety study of LmLLO-E7 in patients with advanced carcinoma of the cervix. *Vaccine* 2009;27:3975–83.
 9. Borysiewicz LK, Fiander A, Nimako M, Man S, Wilkinson GW, Westmoreland D, et al. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *Lancet* 1996;347:1523–7.
 10. Steller MA, Gorski KJ, Murakami M, Daniel RW, Shah KV, Celis E, et al. Cell-mediated immunological responses in cervical and vaginal cancer patients immunized with a lipidated epitope of human papillomavirus type 16 E7. *Clin Cancer Res* 1998;4:2103–9.
 11. Ferrara A, Nonn M, Sehr P, Schreckenberger C, Pawlita M, Dürst M, et al. Dendritic cell-based tumor vaccine for cervical cancer II: results of a clinical pilot study in 15 individual patients. *J Cancer Res Clin Oncol* 2003;129:521–30.
 12. Van Poelgeest MIE, Welters MJP, van Esch EMC, Stynenbosch LFM, Kerpershoek G, van Persijn van Meerten EL, et al. HPV16 synthetic long peptide (HPV16-SLP) vaccination therapy of patients with advanced or recurrent HPV16-induced gynecological carcinoma, a phase II trial. *J Transl Med* 2013;11:88.
 13. Tindle RW. Immune evasion in human papillomavirus-associated cervical cancer. *Nat Rev Cancer* 2002;2:59–64.
 14. Bhat P, Mattarollo SR, Gosmann C, Frazer IH, Leggatt GR. Regulation of immune responses to HPV infection and during HPV-directed immunotherapy. *Immunol Rev* 2011;239:85–98.
 15. Evans M, Borysiewicz LK, Evans AS, Rowe M, Jones M, Gileadi U, et al. Antigen processing defects in cervical carcinomas limit the presentation of a CTL epitope from human papillomavirus 16 E6. *J Immunol* 2001;167:5420–8.
 16. Albers A, Abe K, Hunt J, Wang J, Lopez-Albaitero A, Schaefer C, et al. Antitumor activity of human papillomavirus type 16 E7-specific T cells against virally infected squamous cell carcinoma of the head and neck. *Cancer Res* 2005;65:11146–55.
 17. Wheeler CM, Hunt WC, Joste NE, Key CR, Quint WGV, Castle PE. Human papillomavirus genotype distributions: implications for vaccination and cancer screening in the United States. *J Natl Cancer Inst* 2009;101:475–87.
 18. De Vuyst H, Clifford GM, Nascimento MC, Madeleine MM, Franceschi S. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. *Int J Cancer* 2009;124:1626–36.
 19. Riemer AB, Keskin DB, Zhang G, Handley M, Anderson KS, Brusic V, et al. A conserved E7-derived cytotoxic T lymphocyte epitope expressed on Human Papillomavirus 16-transformed HLA-A2⁺ epithelial cancers. *J Biol Chem* 2010;285:29608–22.
 20. Dudley ME, Wunderlich JR, Shelton TE, Even J, Rosenberg SA. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. *J Immunother* 2003;26:332–42.
 21. Hinrichs CS, Borman ZA, Gattinoni L, Yu Z, Burns WR, Huang J, et al. Human effector CD8⁺ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. *Blood* 2011;117:808–14.
 22. Wolf M, Kuball J, Ho WY, Nguyen H, Manley TJ, Bleakley M, et al. Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8⁺ T cells responding to antigen without requiring knowledge of epitope specificities. *Blood* 2007;110:201–10.
 23. Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z, et al. Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. *J Immunol* 2008;180:6116–31.
 24. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 2011;29:917–24.
 25. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 2009;114:535–46.
 26. Parkhurst MR, Joo J, Riley JP, Yu Z, Li Y, Robbins PF, et al. Characterization of genetically modified T-cell receptors that recognize the CEA:691-699 peptide in the context of HLA-A2.1 on human colorectal cancer cells. *Clin Cancer Res Off J Am Assoc Cancer Res* 2009;15:169–80.
 27. Georgopoulos NT, Proffitt JL, Blair GE. Transcriptional regulation of the major histocompatibility complex (MHC) class I heavy chain, TAP1 and LMP2 genes by the human papillomavirus (HPV) type 6b, 16 and 18 E7 oncoproteins. *Oncogene* 2000;19:4930–5.
 28. Heller C, Weisser T, Mueller-Schickert A, Rufer E, Hoh A, Leonhardt RM, et al. Identification of key amino acid residues that determine the ability of high risk HPV16-E7 to dysregulate major histocompatibility complex class I expression. *J Biol Chem* 2011;286:10983–97.
 29. Dunn LA, Evander M, Tindle RW, Bulloch AL, de Kluyver RL, Fernando GJ, et al. Presentation of the HPV16E7 protein by skin grafts is insufficient to allow graft rejection in an E7-primed animal. *Virology* 1997;235:94–103.
 30. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. *Nat Rev Cancer* 2010;10:550–60.
 31. Kenter GG, Welters MJP, Valentijn ARPM, Lowik MJG, Berends-van der Meer DMA, Vloon APG, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* 2009;361:1838–47.
 32. Welters MJP, Kenter GG, de Vos van Steenwijk PJ, Löwik MJG, Berends-van der Meer DMA, Essahsah F, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. *Proc Natl Acad Sci U S A* 2010;107:11895–9.
 33. Hinrichs CS, Stevanović S, Draper L, Somerville R, Wunderlich J, Restifo NP, et al. HPV-targeted tumor-infiltrating lymphocytes for cervical cancer. *J Clin Oncol [Internet]* 2014 [cited 2014 Jul 20];32:58. Available from: <http://meetinglibrary.asco.org/content/129263-144>
 34. De Vos van Steenwijk PJ, Heusinkveld M, Ramwadhoebe TH, Löwik MJ, van der Hulst JM, Goedemans R, et al. An unexpectedly large polyclonal repertoire of HPV-specific T cells is poised for action in patients with cervical cancer. *Cancer Res* 2010;70:2707–17.
 35. Piersma SJ, Welters MJP, van der Hulst JM, Kloth JN, Kwappenberg KMC, Trimbos BJ, et al. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. *Int J Cancer* 2008;122:486–94.
 36. Hinrichs CS, Borman ZA, Cassard L, Gattinoni L, Spolski R, Yu Z, et al. Adoptively transferred effector cells derived from naive rather than central memory CD8⁺ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci* 2009;106:17469–74.
 37. Hinrichs CS, Kaiser A, Paulos CM, Cassard L, Sanchez-Perez L, Heemskerck B, et al. Type 17 CD8⁺ T cells display enhanced antitumor immunity. *Blood* 2009;114:596–9.
 38. Linette GP, Stadtmauer EA, Maus MV, Rapoport AP, Levine BL, Emery L, et al. Cardiovascular toxicity and titin cross-reactivity of affinity enhanced T cells in myeloma and melanoma. *Blood* 2013;122:863–71.
 39. Cameron BJ, Gerry AB, Dukes J, Harper JV, Kannan V, Bianchi FC, et al. Identification of a titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. *Sci Transl Med* 2013;5:197ra103.
 40. Morgan RA, Chinnsamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* 2013;36:133–51.