Effect of Human Papillomavirus-16 Infection on CD8+ T-Cell Recognition of a Wild-Type Sequence $p53_{264-272}$ Peptide in Patients with Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Purpose: **Wild-type sequence (wt) p53 peptides are attractive candidates for broadly applicable cancer vaccines, currently considered primarily for patients whose tumors overexpress p53. Circumstances exist, however, where increased p53 degradation may result in appreciable presentation of p53-derived peptides, despite low p53 expression. Squamous cell carcinoma of the head and neck is associated with oncogenic human papillomavirus (HPV) subtypes, which inactivate p53 through proteasomal degradation. The criterion of p53 overexpression would exclude these individuals from wt p53-based immunotherapy.**

Experimental Design: **We tested the correlation of HPV infection with enhanced antigenicity of the p53 protein and postulated that removal of HPV-16$^+$ tumors with enhanced $p53_{264-272}$ peptide presentation might lead to a drop in T cells specific for this peptide *in vivo*. Circulating frequencies of T cells specific for the HLA A*0201:$p53_{264-272}$ complex were measured *ex vivo* using dimeric HLA:peptide complexes in 15 head and neck cancer patients before and 6 months after tumor excision.**

Results: **CD8+ T-cell recognition of HLA A*0201-restricted wt $p53_{264-272}$ peptide presented by HPV-16$^-$ squamous cell carcinoma of the head and neck lines was enhanced by HPV-16 E6 expression, sometimes exceeding that of a naturally transformed, HPV-16$^+$ wt p53 expressing squamous cell carcinoma of the head and neck cell line. In patients with HPV-16$^-$ tumors, the frequency of wt $p53_{264-272}$–specific T cells remained largely unchanged after tumor removal. However, a significant decline in frequency of anti-$p53_{264-272}$ T cells was observed postoperatively in HPV-16$^+$ patients ($P < 0.005$).**

Conclusions: **Recognition of HPV-associated squamous cell carcinoma of the head and neck appears associated with levels of wt p53-specific T cells and inversely with p53 expression. p53 peptides may be useful tumor antigens for squamous cell carcinoma of the head and neck immunotherapy in addition to viral gene products.**

INTRODUCTION

The tumor suppressor protein p53 is an attractive target for a broad spectrum tumor immunotherapy, because elevated levels of p53 protein (referred to as "accumulation" or "overexpression") occur in a high proportion of human carcinomas, including squamous cell carcinoma of the head and neck (1–3). Because most p53 mutations involve a single base missense alteration, the remainder of the accumulated protein is wild type in sequence. This accumulated protein undergoes processing and presentation, leading to the generation of wild-type (wt) p53 epitopes and stimulating cytolytic T-lymphocytes (CTLs; refs. 4–6). Enhancing immune recognition of the tumor through the expansion of antitumor, wt p53-specific CTL may be one immunotherapeutic strategy to consider. A critical question remains, however, as to whether accumulation of p53 protein inside the tumor cells is necessary for wt p53 peptide presentation on the tumor cell surface and whether wt p53-based vaccination should be ruled out in patients whose tumors do not accumulate p53.

Several groups have demonstrated the generation of CTLs recognizing the HLA-A*0201-restricted wt $p53_{264-272}$ epitope from peripheral blood mononuclear cells (PBMCs) obtained from squamous cell carcinoma of the head and neck patients, using autologous peptide-pulsed dendritic cells (DCs) as stimulators. Interestingly, CTLs reactive against this wt p53 epitope could only be generated from PBMCs of patients whose tumors either did not accumulate p53 or accumulated mutant p53 molecules that could not present the $p53_{264-272}$ peptide (7). A related study measured frequencies of wt $p53_{264-272}$–specific

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CTL in the peripheral blood of HLA A*0201$^+$ squamous cell carcinoma of the head and neck patients (8), using multicolor flow cytometry with tetrameric HLA A*0201 molecules (tetramers) complexed with wt $p53_{264-272}$. This analysis also suggested an inverse correlation between accumulation of p53 in the tumor and frequency of anti-wt p53 T cells and that, *in vivo*, the presence of wt-p53–specific CTL could have resulted in immunoselection of "epitope-loss" tumor cells. We show here that the seemingly contradictory relationship between the high frequency of p53 epitope-specific T cells and low/undetectable p53 expression in the tumor could also be attributed to human papillomavirus (HPV)-16/18 infection, which is relatively common (~30%) in squamous cell carcinoma of the head and neck (9, 10). HPV-16/18 infection results in loss of p53 function by targeting p53 for proteasomal degradation (11, 12). In addition to disrupting the tumor suppressor functions of p53, E6-mediated proteasomal targeting has also been shown to enhance processing of wt p53 epitopes in murine tumor models for T-cell recognition and tumor protection (13). We postulated that such enhanced $p53_{264-272}$ peptide presentation might lead to differences in T-cell responses to this peptide *in vivo*. The frequency or avidity of circulating T cells, specific for wt p53 peptides, is impacted by density and avidity of peptide presentation (14) and might be altered by the removal of tumors, in which p53 is undergoing increased degradation and subsequently enhanced peptide presentation. In human tumors, we show that both wt and mutant p53 molecules are sensitive to E6-mediated degradation and that this HPV-induced p53 degradation appears to be correlated with increased T-cell recognition of the tumor cells *in vitro* and *in vivo*. These findings also indicate that T cell-mediated immunotherapy against wt p53 should not be restricted to tumors overexpressing p53, a factor that should be considered in the context of wt p53 based cancer immunotherapy.

MATERIALS AND METHODS

Patients. This study was approved by the Institutional Review Board at The Johns Hopkins Medical Institutions, and written informed consent was obtained from each participating individual. Patients were selected for this study from consecutive squamous cell carcinoma of the head and neck patients seen in the Department of Otolaryngology/Head and Neck Surgery at the Johns Hopkins University and enrolled based on the presence of surgically resectable disease, willingness to participate, and HLA A*0201 genotype. All of the patients were recruited into this study over a 12-month period and were followed postoperatively for the indicated times. Archived tissue from all of the patients in this study was reviewed by one of the authors (W. W.), and the diagnosis was reconfirmed histologically. Table 1 shows the demographic and tumor characteristics for the patients studied in this trial. These patients are similar to historical squamous cell carcinoma of the head and neck patients in our practice, with tumors distributed throughout all of the head and neck subsites. In the 15 patients studied, histologically verified squamous cell carcinoma originated in one of the following primary sites: oral cavity (4), oropharynx (4), hypopharynx (1), larynx (4), and undetermined (3).

Cells and Cell Lines. Tumor cell lines were derived from squamous cell carcinoma of the head and neck patients treated at the University of Pittsburgh, as described in Table 1 (15), and were cultured as described (16). Naturally HPV-16–transformed UPCI:SCC090 (referred to as SCC90) cells were isolated, characterized and described recently (17).[6] PBMCs were isolated from squamous cell carcinoma of the head and neck patients and healthy normal controls by centrifugation over Ficoll-Hypaque gradients (Amersham Pharmacia Biotech, Piscataway, NJ). HLA-A*0201 expression was determined first by flow-cytometry using the anti-HLA-A2 mAb BB7.2 (American Type Culture Collection, Manassas, VA), and verified using PCR (15). PBMCs were either used fresh or were cryopreserved at a concentration of 5 to 10×10^6 cells/mL until additional use. The $p53_{264-272}$–specific CTL clone, generated in $p53^{-/-}$ HLA A*0201-K^b transgenic mice (5), was a generous gift from Dr. Linda Sherman (Scripps Research Institute, La Jolla, CA).

Plasmids and Constructs. HPV-16 E6 constructs used were pcDNA3-E6 (generous gift from Dr. T. C. Wu, Johns Hopkins University) or recombinant adenovirus encoding the HPV-16 E6 protein (generous gift of Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA; ref. 18). E6 was expressed by liposomes or adenovirus-mediated transfection in cell lines for 18 hours before experiments assaying for p53 protein or T-cell recognition were carried out.

Immunoblot. SDS-PAGE and immunoblot experiments were carried out as described (19). Equal protein loading was ensured by loading 20 μg of total protein per lane, staining for β-actin, or both. Detection of specific proteins was performed using anti-p53 [DO-7 monoclonal antibody (mAb; Labvision, Fremont, CA) or anti HPV-16/18 E6 (Oncogene, Inc., Boston, MA) mAb DP12 or (Abcam, Inc., Cambridge, MA) mAb C1P5]. Proteasome inhibitors lactacystin (Calbiochem, San Diego, CA) and MG-132 (AG Scientific, San Diego, CA) were applied to tumor cells for 6 hours at 50 μmol/L or 100 μmol/L, respectively, before immunoblot.

Peptides. Peptides were loaded onto A2-immunoglobulin dimeric reagent in several hundred-fold molar excess at 4° for 72 hours. Peptides used were GILGFVFTL, an influenza matrix immunodominant peptide (residues 58 to 66) as a positive control, the HTLV-1 Tax_{11-19} peptide (LLFGYPVYV), or the LLGRNSFEV peptide corresponding to wt $p53_{264-272}$. All of the HLA-A*0201-binding peptides (Macromolecular Resources, Colorado State University, Fort Collins, CO) were provided at >90% purity as determined by high-performance liquid chromatography and confirmed by mass spectrometry (5, 20, 21).

Generation and Maturation of Dendritic Cells. DCs were generated from plastic adherent human PBMCs and phenotyped as described (22). DC were incubated for 4 hours in the presence of the wt $p53_{264-272}$ peptide before the addition of CTL lines.

IFN-γ Enzyme-Linked Immunospot Assays. Enzyme-linked immunospot assay were performed as described (22, 23). Anticlass I (W6/32) and control, anticlass II HLA mAb (L243) were used for blocking HLA:peptide complexes (22). Enzyme-

[6] R. L. Ferris, S. Gollin, and S. A. Khan, unpublished observations.

Table 1 Patient demographic and clinical data

ID#	Sex/Age	Site	T	N	M	F/U (mo)	Status	Xrt*	p53 overexpression	p53 mutation	HPV E6/E7†
1	M/63	OC	1	0	0	26	NED	+	+	H193R	0
2	M/70	L	3	0	0	4	NED	+	0	WT	0
3	M/48	L	1b	0	0	26	NED	+	+	WT	0
4	M/62	Hypo	4	2	1	26	DOD	−	+	R273L	0
5	M/58	OP	3	2	0	24	DOD	+	0	WT	1
6	M/68	OP	1	2	0	29	NED	+	0	WT	0
7	F/58	OP	1	0	0	16	NED	−	+	Y220C	1
8	M/60	OC	1	2	0	23	DOD	+	+	C275Y	0
9	M/54	nd	0	2	0	7	NED	+	0	WT	1
10	M/50	L	3	0	0	16	NED	+	0	Q192stop	1
11	F/65	OC	4	0	0	12	NED	+	nd	nd	nd
12	M/61	nd	0	2	0	19	NED	+	0	WT	1
13	M/62	OC	4	0	0	26	AD	+	+	Y220H, Q317L	0
14	M/56	nd	3	2	0	29	NED		0	nd	nd
15	M/55	OP	0	2	0	24	NED	+	+	WT	0

Abbreviations: OC, oral cavity; OP, oropharynx; HP, hypopharynx; L, larynx; nd, not determined; TNM, tumor-node-metastasis; NED, no evidence of disease; AD, active disease; DOD, dead of disease.

* Received radiation therapy ending 3 months postoperatively.

† HPV-16 E6/E7 copy number was determined by quantitative real-time PCR (as described in Materials and Methods), with a lower threshold defined as at least one copy of E6 and/or E7 DNA per cellular genomic DNA equivalents by β-globin amplification.

linked immunospot analyses used *in vitro* stimulation cultures of PBMCs or enriched populations of $CD8^+$ T-cells (CTL lines) obtained from HLA $A*0201^+$ healthy donors or squamous cell carcinoma of the head and neck patients (22).

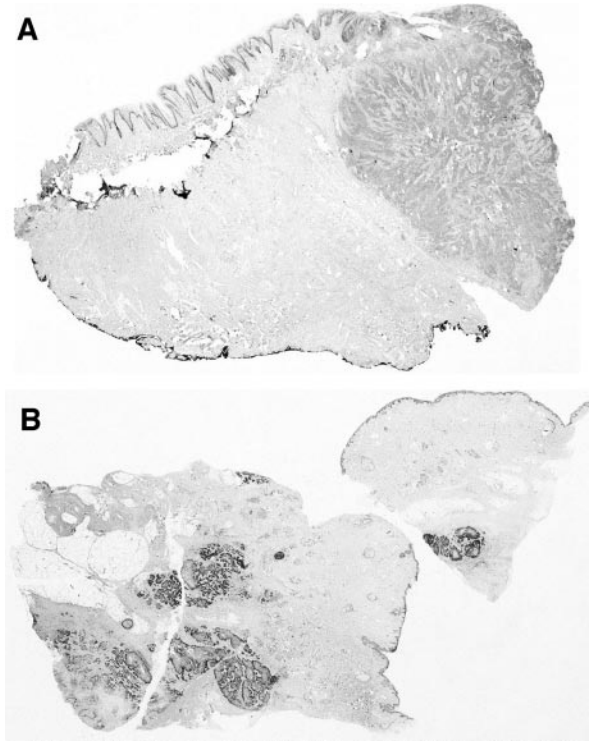

Fig. 1 Immunohistochemical staining for p53 (DO-7 mAb) demonstrated (*A*) undetectable or (*B*) overexpressed p53 molecules, in squamous cell carcinoma of the head and neck patients studied for circulating wt $p53_{264-272}$ T-cell frequencies. Using this technique, patient tumors were scored as listed in Table 1.

Immunohistochemistry of Tumors for p53. For p53 staining, the primary antibody was D0–7, mouse antihuman p53 (Fig. 1) (24). Whereas there was some heterogeneity of accumulation within the p53+ tumors, positive results for p53 "accumulation" was scored if >10% of tumor cells staining positive for p53 was observed by the study pathologist (W. W.).

***In vitro* Stimulation T-Cell Culture.** *In vitro* stimulation cultures for stimulation and propagation of CD8+ T-cell lines specific for wt $p53_{264-272}$ were established and propagated as described (22) using autologous DCs for stimulations from PBMC and peptide-pulsed T2 cells (25) thereafter.

Dimer Analysis of PBMCs of Squamous Cell Carcinoma of the Head and Neck Patients. The following antibodies were used: anti-CD8-FITC, antimouse IgG1-phycoerythrin for detection of HLA-A*0201-IgG1, and anti-CD3-APC (BD PharMingen, San Diego, CA). Additionally, some samples were counterstained with antihuman CD45RO-CyChrome (BD PharMingen) or antihuman CD14 (used for electronic monocyte exclusion gate). The dimeric reagent, HLA A*0201-immunoglobulin, was produced as described previously (26). Because A2-immunoglobulin contained the Fc portion of the murine IgG1 molecule, detection was performed using a phycoerythrin-conjugated antimouse IgG1 2^o Ab (BD PharMingen). Fresh or frozen cells (after thawing) were washed twice with flow cytometry medium (PBS or RPMI 1640 with 2% fetal calf serum and 0.1% NaN_3) and resuspended at a concentration of 5×10^6/mL in a volume of 100 μL. A2-immunoglobulin dimer (3 to 5 μL of stock solution normalized to a working concentration of 500 μg/mL, as determined by previous titrations) was added for 60 minutes at 4°, the cells were washed twice, and a 30-minute incubation with secondary antibody (7.5 to 10 μL of antimouse IgG1-phycoerythrin) followed. After two additional washes, the cells were stained in a volume of 100 μL with 7.5 μL to 10 μL of fluorochrome-conjugated surface markers as described above. Cells were washed again, resuspended in PBS, fixed in 4% (w/v) paraform-

aldehyde, and analyzed on a FACScalibur four-color flow cytometer. Analysis was performed using CellQuest software (Mountain View, CA) or WinMidi (Joe Trotter, Scripps Research Institute, La Jolla, CA, freeware).

Dividing the same batch of dimer reagent into two aliquots and loading each with its peptide at several hundred-fold molar excess ensures that the only difference between the negative control and experimental peptide loaded ($p53_{264-272}$–loaded) dimers was specificity for its cognate T-cell receptor. Counterstaining of dimer+ cells was performed using antibodies to CD3 and CD8 and populations identified through back gating electronically on the flow cytometer. Monocytes were excluded by identifying a CD14+ population and using size criteria on forward scatter/side scatter dot plots (not shown).

Dimer Molecules Efficiently Quantify a $p53_{264-272}$ Specific T-Cell Line by Flow Cytometry. The ability of the $p53_{264-272}$ peptide-loaded A2-immunoglobulin molecule to detect its cognate T-cell receptor by flow cytometry was tested (Fig. 2). A wt $p53_{264-272}$–specific CD8+ population is identified using this reagent, which is not stained with Tax_{11-19} loaded dimer, confirming the specificity of peptide-loaded dimer molecules for detection of wt p53-specific T cells in additional studies.

Dimer Staining of HLA A*0201$^+$ Healthy Volunteer PBMCs Defines Low Background Staining. PBMCs from 5 healthy HLA A*0201$^+$ individuals were stained using the control or wt p53 peptide-loaded dimer technique. Fig. 3 shows a representative experiment with PBMCs obtained from a squamous cell carcinoma of the head and neck patient and a healthy A*0201+ control volunteer. No significant $p53_{264-272}$–specific staining was found in this representative individual above negative control staining using Tax_{11-19}–loaded dimer. For the 5 healthy control subjects, the mean net $p53_{264-272}$–specific T-cell frequency, corrected for irrelevant Tax_{11-19}–specific dimer staining as described above, was <1:10,000 dimer$^+$ T cells.

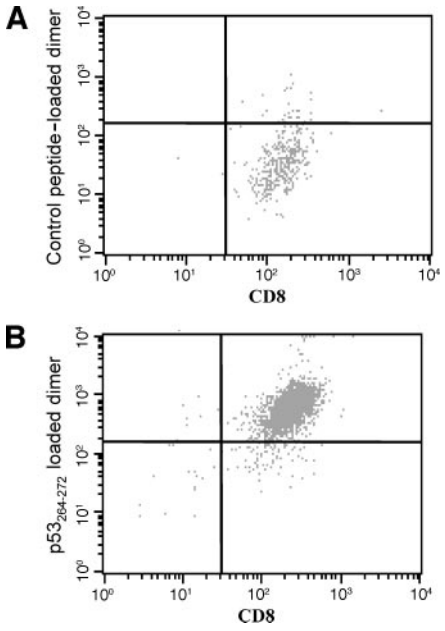

Fig. 2 Specific detection of a T-cell clone specific for $p53_{264-272}$ using peptide-loaded dimers. T cells were stained (see Materials and Methods) and analyzed by flow cytometry, as described in the text. Shift of the CD8+ dimer+ population is seen specifically with staining using wt $p53_{264-272}$ loaded dimer (*B*) and not with control peptide (Tax_{11-19}) loaded dimer staining.

Calculation of $p53_{264-272}$-Specific T-Cell Frequencies. Every staining procedure included an internal negative control for staining the PBMCs of each patient using A2-immunoglobulin loaded with an irrelevant HLA A*0201 binding peptide, the HTLV-1 Tax_{11-19} peptide. This irrelevant peptide-loaded dimer staining was then subtracted from the specific, $p53_{264-272}$–loaded dimer staining of PBMCs from the same patient. Furthermore, normalization to the percentage of CD8+ T cells in the PBMCs was performed after subtraction of irrelevant staining from $p53_{264-272}$–specific staining. Specific $p53_{264-272}$ dimer staining was calculated according to the following equation, to correct for differences in the CD8+ T-cell pool size and for staining of an irrelevant, nonspecific antigen, by the following equation, as a function of %CD8 T cells: $A2{:}p53_{264-272} - A2{:}Tax_{11-19}$ / total CD3+CD8+ cells

Log_{10} reciprocal frequencies were expressed and values determined for data analysis.

Tumor Genotyping for p53 and HPV. Microdissection and DNA extraction were performed as described (27). DNA of sufficient quality for PCR amplification based on β-globin amplification was obtained in 13 of 15 cases with informative dimer staining data. Using the Affymetrix GeneChip p53 Reagent kit, genomic DNA from tumor or cell line samples was PCR amplified for p53 exons 5 to 9. Next, the amplified DNA was fragmented with calf intestine alkaline phosphatase (Roche, Indianapolis, IN). The fragmented DNA was fluorescently end-labeled using the Enzo BioArray Terminal Labeling kit. The samples were then hybridized to the GeneChip p53 array, washed, then visualized using a GeneArray scanner. A reference DNA sample (provided in the Affymetrix kit) was also run to use as a baseline sequence for analysis. Mutated samples were manually sequenced for confirmation of alteration (28).

Quantitative real-time PCR was used to determine the presence of DNA in patient tumors for HPV-16 E6 and E7 genes compared with an endogenous control gene, β-*globin*, using a minimum HPV DNA:cellular genome ratio of ≥0.1:1 (27). No other HPV subtypes were investigated in this study. This lower threshold uses the enhanced sensitivity of quantitative real-time PCR to detect very low copy numbers of DNA, whereas relating this copy number to tumor cellular genomic DNA for a known housekeeping gene, β-*globin*, to give a reliable indication of likely carcinogenic effect of HPV DNA.

Data Analysis. Associations among patient classes defined by HPV status, p53 (wt or mutant) or qualitative immunohistochemistry were examined by Fisher's exact test. Group differences in the number of circulating $p53_{264-272}$–specific dimer T cells were determined with the Wilcoxon test. Changes in $p53_{264-272}$–specific T cells over time and by HPV status were modeled and tested with mixed linear models using the log_{10} of the reciprocal frequency (*i.e.,* 1 specific T cell per 10,000 cells = reciprocal frequency of 10,000, log 10,000 = 4). Interaction between time (0, 3, and 6 months) and HPV status (±) was tested, and specific contrasts were examined. Overall type

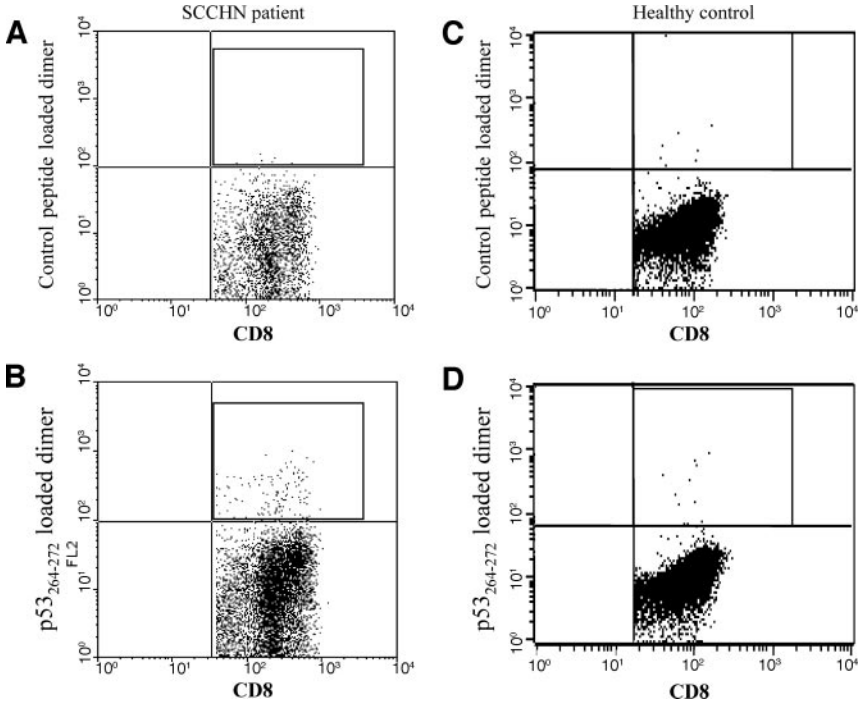

Fig. 3 Representative dimer staining experiment from a squamous cell carcinoma of the head and neck patient (*A* and *B*) and from a healthy control volunteer (*C* and *D*), showing specific detection of wt p53 (264 to 272) –specific T cells ($CD8^+$ wt $p53_{264-272}$ $dimer^+$ population) over background staining in the squamous cell carcinoma of the head and neck patient, but not the healthy control PBMC. *A,* control peptide (Tax_{11-19}) loaded $dimer^+$ staining (see Materials and Methods) and (*B*) wt $p53_{264-272}$–specific dimer staining of $CD8^+$ T cells from an A*0201+ squamous cell carcinoma of the head and neck patient (backgated on CD3+ population). Inverse frequency of $p53_{264-272}$–specific pCTL from this time point is calculated as 1/1,489 (see formula in Materials and Methods). Representative healthy HLA A*0201+ control PBMC staining for (*C*) control peptide (Tax_{11-19}) -loaded $dimer^+$ events and (*D*) wt $p53_{264-272}$–specific dimer staining of $CD8^+$ T cells from a healthy donor (backgated on CD3+ population). Frequency of $p53_{264-272}$–specific pCTL from this time point is subtracted from nonspecific staining by control peptide loaded dimer.

I error rate was controlled by the multivariate *t* distribution simulation method (29). All of the tests were two sided.

RESULTS

HPV-16 E6 Mediates Rapid Proteasome-Dependent Degradation of wt and Mutant p53 Molecules in Squamous Cell Carcinoma of the Head and Neck Cells. We sought to determine whether HPV-16 E6 interacts exclusively with wt p53 or can also target mutant p53 for degradation. Squamous cell carcinoma of the head and neck cells were transfected to express HPV-16 E6 using transient transfection (data not shown) or an Ad-E6 construct. Cell lysates were probed for E6 (data not shown) and p53 before and after expression of HPV-16 E6 by Western blot demonstrating that mutant p53 molecules are sensitive to HPV-16 E6-mediated interaction and degradation (Fig. 4*A*). This phenotype was similar to that seen in immunoblot experiments using naturally HPV-16–transformed SCC90 cells, which were derived from an oropharyngeal tumor, and express wt p53 (data not shown). These cells do not accumulate p53 unless specific proteasome inhibitors are added (Fig. 4*B*).

Table 2 Characteristics of tumor cell lines used in this study

Cell line	Mutation	p53 accumulation	HPV-16	HLA A*0201	Ability to present wt $p53_{264-272}$
PCI-13	E286K	+	−	+	++
SCC90	WT	−	+	+	++

Anti-$p53_{264-272}$ T Cells Recognize HPV-16 E6-Expressing Squamous Cell Carcinoma of the Head and Neck Cells That Do Not Accumulate p53. Anti-wt $p53_{264-272}$ T-cell recognition of $HPV\text{-}16^-$ tumor cells (expressing mutant or wt p53), before and after HPV-16 E6 transfection, was tested in IFN-γ enzyme-linked immunospot assays (Fig. 5). Enhanced recognition of these cells by anti-wt p53 T cells correlated with the presence of E6 and the proteasomal targeting and consequent degradation of p53, as determined by immunoblotting. We also tested recognition of the wt $p53_{264-272}$ epitope on the naturally HPV-16–transformed SCC90 cell line (see Fig. 5). Increased T-cell recognition of the tumor cells correlated with HPV-16 E6-mediated enhancement of p53 degradation in these cells. Transgenic E6 expression in $HPV\text{-}16^-$ cells was found to be 10- to 50-fold higher than endogenous E6 expression in SCC90 cells by quantitative real-time PCR (data not shown). IFN-γ release by wt p53-specific T cells was reduced to background when target cells were treated with a mAb blocking class I but not class II HLA (data not shown) confirming CD8+ T-cell reactivity. Each experiment included squamous cell carcinoma of the head and neck cell targets infected with blank Ad-vector (negative control) or T2 cell targets with or without

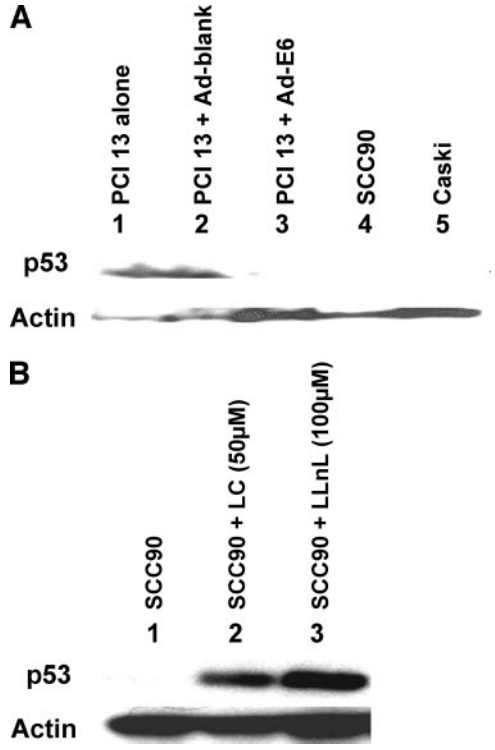

Fig. 4 Destabilization of wt and mutant p53 by HPV-16 E6 expression, determined by immunoblot (DO-7 mAb). *A,* Western blot for p53 of HPV-16$^-$ PCI-13 cell lysates, before (*lane 1*) or 18 hours after transfection with Ad-E6 (*lane 2*). Expression of p53 and HPV-16 E6 in PCI-13 cells is compared after control viral vector transfection (*lane 3*) or compared with HPV-16+ TC-1 cells (*lane 4*). *B,* naturally HPV-16-transformed SCC90 cells untreated (*lane 1*) treated with proteasome inhibitors (lactacystin, *lane 2* and MG-132, *lane 3*) to recover p53 degradation intermediates. Equal protein loading was confirmed by equalizing input protein material or blotting for actin.

preincubation with synthetic wt $p53_{264-272}$ peptide (data not shown) to confirm CTL specificity. These results indicate that E6–induced degradation of p53 leads to enhanced HLA class I presentation of p53-derived peptides, regardless of whether or not the tumor cells accumulate p53.

Because of the reported inverse relationship between p53 accumulation and anti-wt p53 T-cell frequency (8), we studied the correlation between HPV-16 E6 DNA and p53 status in squamous cell carcinoma of the head and neck tumors, isolated from 15 squamous cell carcinoma of the head and neck patients. Accumulation of p53 was detectable by immunohistochemistry analysis (Table 2) and scored as positive in 50% (7 of 14 subjects), consistent with previous reports (1, 30, 31). Patient tumors were also analyzed for genetic alterations in p53. Alterations in p53 gene exons 5 to 9 were determined, and this mainly represented missense mutations as described previously (ref. 1; see Table 2).

HPV-E6 and E7 DNA, determined by quantitative real-time PCR (27), was determined to be at least 1 copy per cellular genome (range, 1 to 140 HPV-16 E6 copies per cell, mean = 5), using β-globin as control (data not shown). No correlation between HPV DNA and p53 overexpression could be determined (Fisher's exact test, $P = 0.1026$) using a binary function for HPV positivity.

$p53_{264-272}$–Specific T Cells Decline After Removal of HPV-16$^+$ Tumors. After specificity of the HLA:peptide dimer for this CD8+ T cell was confirmed, as shown in Fig. 1, dimer staining was performed on PBMCs isolated from a pilot cohort of 15 HLA A*0201+ squamous cell carcinoma of the head and neck patients (representative patient staining is shown in Fig. 2). We correlated the presence and frequency of precursor cytolytic cells with clinicopathologic features, HPV status, p53 (wt or mutant), and levels of p53 expression in the resected tumors. The preoperative mean reciprocal frequency of wt $p53_{264-272}$–specific T cells for all 15 of the patients was determined to be 3,197 ± 663. As shown in Fig. 3, the composite pCTL frequency at 3 months and 6 months postoperatively for all of the patients combined did not change significantly after tumor removal (reciprocal frequencies 3,619 ± 705 at 3 months and 3,379 ± 632 at 6 months). The aggregate dimer$^+$ T-cell frequencies during the 6-month follow up analysis are shown in Fig. 3*A* for all of the squamous cell carcinoma of the head and neck patients in this study. When the HPV-16 status of patient tumors was considered, there was no change in $p53_{264-272}$–specific pCTL levels during 6 months of follow up after surgical tumor removal in HPV$^-$ patients (Fig. 3*B*). However, the frequency of $p53_{264-272}$–specific T cells in HPV-16$^+$ patients declined significantly at 3 months ($P = 0.0327$) and at 6 months postoperatively ($P = 0.0032$) when compared with the presurgery baseline at t = 0 (Fig. 3*C*).

DISCUSSION

Significant recent attention has focused on the association of oncogenic HPV subtypes (types 16 and 18) with squamous cell carcinoma of the head and neck, particularly in the oropharynx (9, 32, 33). Immunotherapeutic strategies for HPV-associated carcinomas have primarily focused on virally derived antigens such as the oncogenic proteins, E6 and E7. Our studies suggest that wt p53 peptides may be attractive tumor-associated antigens for use in immunotherapy of HPV-associated tumors, in combination with virally encoded tumor antigens, and that *a priori* lack of p53 overexpression should not exclude a population of cancer patients from wt p53-based immunotherapy.

Our *in vitro* model system demonstrated that enhanced recognition of these HPV-16$^-$ squamous cell carcinoma of the head and neck cells by anti-wt p53 T cells correlated with the presence of E6 and the proteasomal targeting and consequent degradation of p53, as determined by immunoblotting. The recognition of wt $p53_{264-272}$ on the naturally HPV-16–transformed SCC90 cell line confirms this finding, that undetectable levels of p53 may be associated with sufficient levels of wt p53 peptide presentation for T-cell recognition (see Fig. 2*B*). In naturally HPV-16–infected cells (such as SCC90 cells) the expression of HPV-16 E7, shown to cause down-regulation of HLA class I molecules, may explain the lower recognition of these cells, compared with HPV-16$^-$ squamous cell carcinoma of the head and neck cells transfected with E6 alone (Fig. 2). Alternatively, higher E6 expression in E6–transfected cells *versus* endogenous E6 expression in SCC90 cells by quantitative real-time PCR (data not shown) may also play a role in this difference.

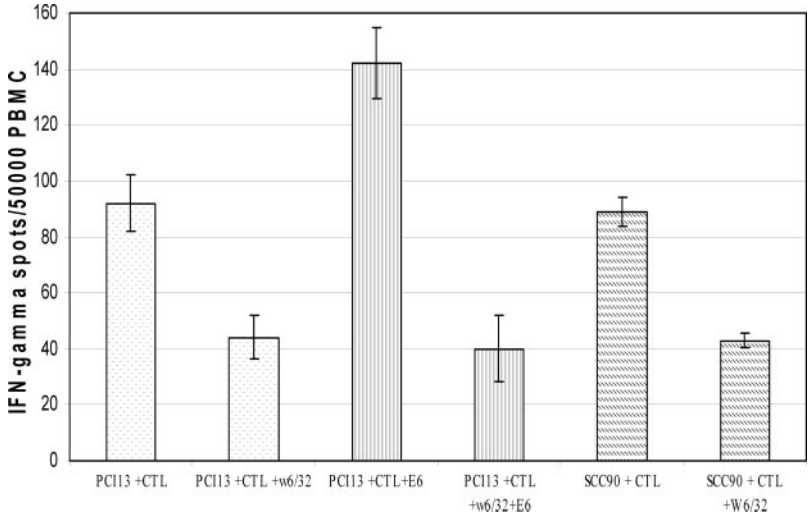

Fig. 5 Rapid degradation of p53 leads to increased wt p53 peptide presentation. Enzyme-linked immunospot assay (mean; *bars,* ± SD) shows increased anti-$p53_{264-272}$ T-cell recognition of IFN-γ–treated HPV-16$^-$ PCI-13 cells before (□) and after (▨) expression with HPV-16 E6 protein. Anti-$p53_{264-272}$ T-cell recognition is shown of IFN-γ–treated, naturally HPV-16$^+$–transformed SCC90 cells (▤). HLA class I-restricted T-cell recognition is confirmed by pretreatment with an anti-HLA A, B, C mAb, w6/32.

In a pilot cohort of patients with squamous cell carcinoma of the head and neck, significant changes in the frequency of circulating $p53_{264-272}$–specific T cells after surgery occurred only in patients with HPV-16$^+$ tumors. Our interpretation of the observed decline in the frequency of wt $p53_{264-272}$ T cells 6 months after tumor removal in HPV$^+$ squamous cell carcinoma of the head and neck patients is based on *in vitro* and *in vivo* findings, presented by us and others, that rapid turnover of p53 leads to enhanced wt p53 peptide presentation. Because HPV-16 E6 protein has been shown to cause polyubiquitination and proteasomal degradation of p53, this initial step in the antigen processing pathway could lead to higher levels of $p53_{264-272}$ peptides presented on the surfaces of tumor cells. Thus, removal of such tumors might be expected to result in a decrease over several months of $p53_{264-272}$–reactive T cells, an effect seen within 3 to 6 months in our analysis. This intriguing result can only be viewed as preliminary due to the small number of patients included in this study. A larger validation study must be performed to determine the reproducibility of these findings.

Several possible explanations might explain this finding. One might hypothesize that patients with HPV-16–associated squamous cell carcinoma of the head and neck possess circulating T cells with varied avidities, compared with those of HPV-16$^-$ squamous cell carcinoma of the head and neck patients. Lower affinity T-cell receptor-expressing T cells might be maintained in patients whose tumors express high levels of the wt p53:HLA A*0201 peptide complex, and these could decline in the absence of cells, with high levels of wt p53 peptide presentation, due to rapidly turning over p53 at steady-state (34). Thus, the decline in $p53_{264-272}$–specific T cells that we observed, after removal of HPV-16$^+$ tumors, may represent the loss of a lower-avidity circulating T-cell population, in the absence of higher levels of wt p53 peptide presentation (35). Another possible explanation may be that direct *versus* cross-priming could explain the differences seen after removal of HPV+ *versus* HPV− tumors. HPV-infected tumors (rapidly degrading p53) would be expected to directly prime wt p53-specific T cells, whereas HPV-negative tumors (containing accumulated p53) can transfer p53 to DCs for cross-priming of these T cells. Removal of HPV+ tumors then removes this antigen source, whereas DCs may present antigen for prolonged periods of time, perhaps continuing to prime CTL (36). Also, because DC are known to prime the most active and long-lived T cells phenotypically, potentially preventing terminal differentiation leading to premature apoptosis in cancer patients (37), this is one potential explanation for differential fate of T cells generated after the removal of HPV+ *versus* HPV− tumors. We are currently pursuing this issue mechanistically, in future experiments analyzing PBMCs from such squamous cell carcinoma of the head and neck patient populations.

An interesting finding was that patients whose tumors contained detectable HPV-16 DNA (range, 1 to 140 HPV-16 E6 copies per cell; mean, 5) did not always contain wt p53. Consistent with a number of studies, our data suggest that HPV-16 infection and mutant p53 may coexist in squamous cell carcinoma of the head and neck tumors. As we showed, mutant p53 is also susceptible to HPV-16 E6-mediated degradation, perhaps leading to presentation of wt p53 peptides.

We note that a previous report (8) showed an inverse relationship between p53 overexpression and wt $p53_{264-272}$ T-cell frequencies but was unable to correlate this with evidence of HPV infection. However, patient PBMCs in the latter study were not measured before and after tumor removal, perhaps obscuring such a finding. Fig. 6 shows that although some variations were detectable over the 6-month postoperative period of this study in squamous cell carcinoma of the head and neck patients with HPV-16$^-$ tumors, these changes were not significant, unless the interaction of variables of time and HPV-16 infection are considered. In this study, tumors of 2 patients accumulated p53 despite having wild-type sequences in these exons. This could reflect mdm-2 mutations, other dysregulation of p53 turnover, or mutations in intron/exon junctions, which were not analyzed in this study (3). Tumor from 1 patient, no. 10, contained a mutated p53 allele, as well as HPV-16 E6 DNA, but no detectable p53 by immunohistochemistry, indicat-

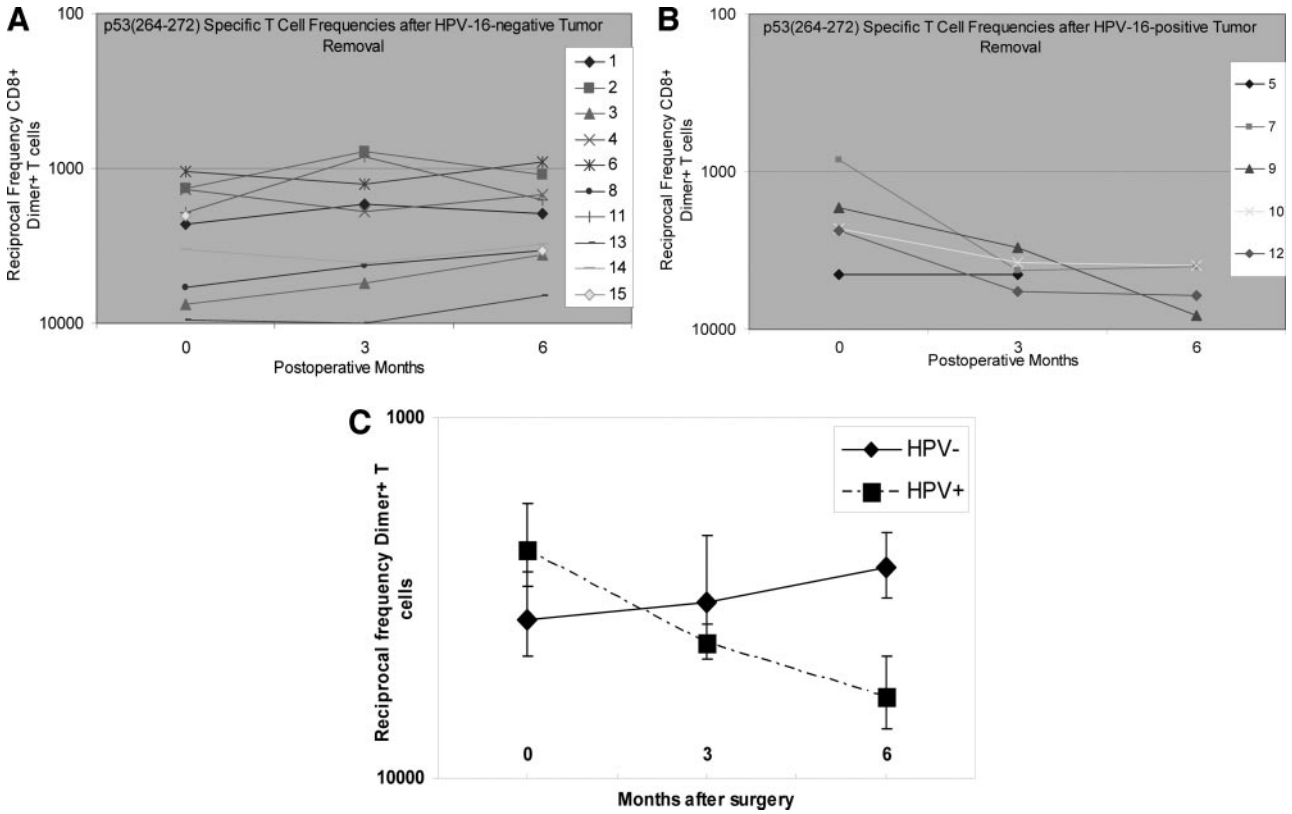

Fig. 6 Anti-$p53_{264-272}$ T-cell frequencies during 6 months after tumor removal in HPV-16^+ or HPV-16^- squamous cell carcinoma of the head and neck patients. All of the plotted values shown take into account background staining, using control peptide (Tax_{11-19}) -loaded dimer. See Material and Methods for staining procedure and frequency calculations. All of the subjects were NED clinically during this period of the study. *A,* dimer staining for wt $p53_{264-272}$–specific T cells in HPV-16-negative squamous cell carcinoma of the head and neck patients. Preoperative frequencies (time 0) were determined while all of the subjects were tumor-bearing, and follow-up measurements were taken every 3 months after tumor removal (time = 3 and 6 months, respectively). *B,* dimer staining for wt $p53_{264-272}$–specific T cells in HPV-16-positive squamous cell carcinoma of the head and neck patients. $CD8^+$ $dimer^+$ T-cell frequencies of squamous cell carcinoma of the head and neck patients in this study at the indicated time points. *C,* evolution of mean $p53_{264-272}$ T-cell frequencies of HPV-16–positive *versus* HPV-16–negative squamous cell carcinoma of the head and neck subjects over 6 months of follow-up analysis. As described above, pCTLs were determined preoperatively and every 3 months after tumors were removed in all of the patients, then retrospective correlation was performed between $p53_{264-272}$ dimer staining and tumor factors (listed in Table 1). The interaction between HPV-16–positive tumor removal and time (at 3 and 6 months) was statistically significant ($P < 0.005$).

ing that in some situations, E6 may even target mutated p53 for rapid turnover, a finding confirmed *in vitro* in this report.

It is important to note that all of the subjects were tumor-free clinically (based on repeated examinations and radiographic studies) during the initial 6-month period of the study, when PBMCs were tested for $p53_{264-272}$–specific T-cell frequencies. Follow up in our study (mean 13 months) was not long enough to gather meaningful outcome data to evaluate the use of pCTL using dimer staining as a biomarker for response to therapy, nor was this study designed for this purpose. Application of the findings presented here to wt p53–based immunotherapy for squamous cell carcinoma of the head and neck would suggest that a reservoir of wt p53-specific pCTL is available for vaccine-induced expansion, if nonresponsiveness can be reversed (22, 23, 38). Thus, low steady-state levels of p53 in tumors should not exclude these patients from wt p53-based vaccination, and wt p53 peptides may represent clinically useful targets for cancer immunotherapy in HPV-associated tumors.

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