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Assessment of CTL Recognizing the HLA-A2.1 Restricted, Wild-type  
Sequence p53 264-272 Epitope

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## Introduction

The focus of this research initiative was to assess the p53-directed anti-tumor cytotoxic T lymphocyte (CTL) present in peripheral circulation of breast cancer patients. Anti-tumor CTL have been shown to be crucial effectors in tumor eradication. CTL recognize short peptides derived from proteins in the cell and are presented on the cell surface in association with class I major histocompatibility complex (MHC) molecules. In humans, these molecules are defined as the human leucocyte antigens (HLA). The consensus that half of all human tumors contain alterations in p53 makes this tumor suppressor gene product an attractive candidate for development of a broadly-applicable cancer vaccine (1-3). Whereas p53 missense mutations may generate or create tumor-specific epitopes, the constraints of antigen presentation limits their vaccine applicability to individuals expressing the class I HLA restriction molecules capable of presenting these epitopes. In contrast, many tumors, including breast carcinoma cell lines, have the potential of enhanced presentation of non-mutated, wild type sequence (wt) p53 epitopes derived from the altered p53 molecules accumulating in the tumor.

Tumor eradication in man and experimental animals has been shown to be dependent on CD4<sup>+</sup> T helper (Th) cell as well as CD8<sup>+</sup> CTL anti-tumor responses. CD4<sup>+</sup> T-cells recognize short peptides associated with class II HLA molecules, i.e, HLA-DR , on the cell surface of antigen presenting cells, such as B-cells, macrophages and dendritic cells. CD4<sup>+</sup> T-cells are considered critical for the induction as well as maintenance of anti-tumor immune responses. Efforts are now being made to identify Th-defined tumor peptides for use in optimizing the efficacies of cancer vaccines.

In addition to promoting CTL responses, Th cells play an important role in generation and production of immunoglobulin gamma (IgG) antibodies. The detection of anti-p53 IgG in ~20-30% of breast cancer patients is indicative, at minimum, of the occurrence in these patients of a Th cell-mediated anti-p53 immune response (4). This conclusion has been confirmed by the detection of anti-p53 proliferative T cell responses using PBMC obtained from p53 sero-positive patients. Whether anti-p53 CD8<sup>+</sup> T cells were involved in these responses is not known. This analysis of anti-p53 cellular

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immune responses of breast cancer patients was undertaken to provide insights into the potential of p53-based vaccines for immunotherapy of breast cancer.

### **Body**

Flow cytometry analysis of lymphoid populations using soluble fluorochrome-conjugated class I HLA /peptide tetrameric complexes (tetramers) are facilitating the identification of anti-tumor peptide T-cells in unstimulated PBMC and *in vitro* stimulated (IVS) cultures of lymphocytes obtained from normal donors and cancer patients (5). In particular, this technology is developing into a useful diagnostic tool for monitoring T-cell mediated anti-tumor responses in cancer patients undergoing immunotherapy in the absence of extensive *in vitro* analyses of T-cell functions.

Five HLA-A2.1-restricted, CTL-defined wt p53 epitopes have been identified. These are p53<sub>65-73</sub>, 149-157, 217-225, 264-272 and 322-330. The wt p53<sub>264-272</sub> epitope has been the most extensively studied. Anti-tumor CTL recognizing the HLA-A2.1-restricted wt p53<sub>264-272</sub> have been generated from peripheral blood mononuclear cells (PBMC) of healthy donors (6-10) and, more recently in this laboratory, from PBMC obtained from HLA-A2<sup>+</sup> oral squamous cell carcinoma (OSCC) patients, as well (11). Autologous dendritic cell (DC) pulsed with peptide are used in these studies for IVS of anti-p53 CTL. Our results indicate that anti-p53<sub>264-272</sub> CTL could be generated from 2/7 healthy donors and 3/7 patients tested. The reactivity of these CTL for the epitope, either pulsed onto HLA-A2<sup>+</sup> target cells or naturally presented by tumors, was detected in standard 4 hour <sup>51</sup>chromium (<sup>51</sup>Cr)-release cytotoxic assays, as well as enzyme linked immune spot (ELISPOT) for interferon gamma (IFN $\gamma$ ) assay. An interesting aspect of this analysis was the correlation between the PBMC responsiveness of patients and the p53 status of their tumors. Patients responsive to this epitope were found to have had tumors which had a low potential to present this epitope: namely, these tumors expressed normal levels of wt p53 or accumulated p53 expressing a missense mutation at codon 273 that has been shown to block processing of the epitope (12).

Our analysis has expanded to include identification of anti-wt p53<sub>264-272</sub> CTL

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present in the peripheral circulation of donors using phycoerythrin (PE)-labeled HLA-A2.1/ wt p53<sub>264-272</sub> tetramers obtained from the National Institutes of Health (NIH) Facility. In the absence of direct evidence of their functionality, the CD3<sup>+</sup>CD8<sup>+</sup> tetramer<sup>+</sup> cells are designated as precursor CTL (pCTL). A four-color flow cytometry protocol, which was designed for detection of rare events, was used to analyze unstimulated PBMC obtained from healthy donors and OSCC patients (n=30 each). HLA-A2.1/ influenza virus matrix (flu) and human immunodeficiency virus (HIV) peptide tetramers, also obtained from the NIH facility, were used in the analysis as positive and negative controls, respectively (13, 14).

Based on the frequencies of anti-wt p53<sub>264-272</sub> pCTL detected using the tetramer in the PBMC of HLA-A2.1<sup>+</sup> donor samples, a frequency of >1/8,000 was determined to indicate a positive level of tetramer<sup>+</sup> cells in PBMC of HLA-A2<sup>+</sup> individuals. Using this tetramer, the average frequencies of anti-wt p53<sub>264-272</sub> pCTL were determined to be ~1/5000 in normal donors and ~1/3500 in oral cancer patients. Although markedly noticeable, the difference in frequencies between patients and normal donors was not significant. All of the patients' PBMC that responded to IVS with the p53<sub>264-272</sub> peptide, however, had frequencies >1/3500. The p53 status of the patients' tumors was then determined and correlated with frequencies of anti-p53 pCTL in their peripheral circulation. The results divided patients into two groups. One group of patients (6 of 23) had tumors with low potential to present the epitope and pCTL frequencies of ~1/1600, while the other patient group had tumors that were most likely able to present the epitope (accumulated mutant p53) and had an average anti-p53<sub>264-272</sub> pCTL frequency in their circulation of ~1/5000, a level comparable to that detected in normal donors (14). Several possible explanations can account for the observed inverse potential between the responsiveness of an OSCC patient to the wt p53<sub>264-272</sub> epitope and the ability of the patient's tumor to process and present this peptide. These include immunoselection, human papilloma virus 16 (HPV 16) early protein 6 (E6) -enhanced degradation of wt p53 molecules, a limited available T-cell receptor (TCR) repertoire, and/or tumor-induced apoptosis of anti-p53 CTL. Although PCR analyses indicate that a significant

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number of oral cancers are HPV 16<sup>+</sup>, there does not appear to be a significant association between wt p53 and HPV (15). Based on several lines of evidence, including the identification of genetic alterations of p53 as an early event in oral cancer, the immunoselection hypothesis is currently favored in our laboratory .

### **Key Research Accomplishments**

#### **1. Identifying variant peptides capable of reversing the non-responsiveness of T lymphocytes to the wt p53<sub>264-272</sub> epitope (15).**

We sought to increase the responsive rate to the wt p53<sub>264-272</sub> peptide of PBMC obtained from normal donors and patients by identifying more immunogenic variants of this peptide. Two such variants were generated by amino acid exchanges at positions 6 (6T) and 7 (7W) of the peptide. These variants were capable of inducing T cells from PBMC of “non-responsive” donors that recognized the parental peptide either pulse onto target cells or naturally presented by tumors. TCR variable beta chain (V $\beta$ ) analysis of two T-cell lines isolated from bulk populations of effectors reactive against the wt p53<sub>264-272</sub> peptide, using either the parental or the 7W variant peptide, indicated that these T cells were expressing identical TCR V $\beta$  13.6 $\tilde{}$  complementary determining region 3 (CDR3) / joining region sequences. This finding confirms the heteroclitic nature of at least one of the variant peptides identified in this study. The use of variant peptides of the wt p53<sub>264-272</sub> epitope represents a promising approach to overcoming the "non-responsiveness" of certain cancer patients to this “self” epitope, thereby enhancing its potential use in tumor vaccines for appropriately selected cancer patients.

#### **2. Defining the specificity of the HLA-A2.1/wt p53<sub>149-157</sub> tetramer:**

In addition to the wt p53<sub>264-272</sub> epitope, the wt p53<sub>149-157</sub> peptide was identified as an HLA-A2.1-restricted, CTL-defined wt p53 epitope. A CD8<sup>+</sup> anti-wt p53<sub>149-157</sub> specific CTL cell line induced by IVS of PBMC obtained from normal donors using autologous

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DC pulsed with the peptide acting as antigen presenting cells (APC) were used to characterize the specificity of a PE-conjugated HLA-A2.1/wt p53<sub>149-157</sub> tetramer. The specificity of the anti-wt p53<sub>149-157</sub> CTL for the p53<sub>149-157</sub> peptide was determined in an ELISPOT IFN $\gamma$  assays using peptide-pulsed T2 target cells, as indicated in Table 1. A control for this assay was the ant-p53<sub>264-272</sub> CTL line. As indicated in bold face in Table 1, both of these CTL lines were specific for the p53 peptide used to induce them. The anti-p53<sub>149-157</sub> CTL cell line was then used in a 3- color flow cytometry analysis to define the specificity of the PE-conjugated HLA-A2.1/p53<sub>149-157</sub> tetramer. The anti-p53<sub>264-272</sub> CTL line was an additional control for these studies, which also involved staining these cell lines with APC-conjugated HLA/A2.1 tetramers containing either the p53<sub>65-73</sub> or p53<sub>217-225</sub> peptide. The p53<sub>149-157</sub> tetramer recognized ~50% of the CD3<sup>+</sup> CD8<sup>+</sup> T cells in the cloned population of anti-p53<sub>149-157</sub> CTL, as shown in Figure 1 Panel A. This tetramer binding was inhibited by anti-CD3 mAb, an indication of tetramer specificity ( ). In contrast, the staining of this cell line with the other three p53 peptide tetramers did not show inhibition of anti-CD3 mAb binding. The specificity of the HLA.A2.1/p53<sub>149-157</sub> tetramer was further confirmed by its lack of binding to anti-p53<sub>264-272</sub> CTL, which did bind the appropriate tetramer, as shown in Figure 1 Panel B. In this analysis, the irrelevant p53<sub>65-73</sub> tetramer showed a relatively high frequency of staining of both CTL cell lines, but this binding occurred in the absence of anti-CD3 mAb inhibition and, therefore, is considered non-specific. As a result of this analysis, a second HLA-A2.1/p53 tetramer is available for determining the frequency of anti-p53 precursor CTL in PBMC, as well as monitor IVS cultures for anti-p53 CTL.

### **3. Defining the frequencies of CD3<sup>+</sup>CD8<sup>+</sup> pCTL recognizing HLA-A2.1-restricted wt p53 epitopes in clinical samples derived from breast cancer patients by flow cytometry analysis.**

The aim was to assess the frequencies of wt p53 pCTL in PBMC and tumor infiltrating lymphocytes (TILs) of breast cancer patients in order to determine whether

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- 1) the frequencies of these anti-p53 pCTL in PBMC of breast cancer patients were comparable to those found in PBMC of oral cancer patients,
- 2) the dichotomy between patients' wt p53 pCTL frequencies and their tumors' p53 status that was observed in oral cancer patients was also observed in breast cancer, and
- 3) an enrichment of these pCTL occurred in TILs.

The tumor microenvironment should be enriched for these T-cells. Recent studies have shown that T-cells in the tumor microenvironment are functionally impaired. Our intention in future studies would be to identify these tetramer<sup>+</sup> T-cells and define their functional activities in order to determine how tumor-specific T-cells are inactivated and gain insights into potential methods to reverse the process.

This retrospective study involved PBMC or TIL samples obtained from breast cancer patients that had been deposited in the UPCI Tumor Bank. The HLA haplotypes of the patients were unknown; ~ 2/3 scored positive when sero-typed for HLA-A2 expression using anti-HLA-A2 mAbs. The anti-p53<sub>264-272</sub> pCTLs frequencies detected in 17 PBMC samples, as determined by 4-color flow cytometry analysis, are shown in Figure 1, Lane 1. They ranged from 1/412 to 1/8469, the latter being the only one below the 1/7850 cutoff and censored from the study. The mean frequency was 1/2350; higher than that the mean frequency of 1/3500 determined for oral cancer patients' PBMCs, of which only ~1/4 had a frequency of anti- p53<sub>264-272</sub> pCTLs  $\geq 1/2400$ (17).

The frequencies of anti-p53<sub>149-157</sub> pCTL in 13/17 patients' PBMC samples were also determined; 11/13 had frequencies above the cutoff and a mean frequency of ~1/1700. (Fig. 2, Lane 2). The mean frequency of these pCTLs in normal donor PBMCs was determined to be ~1/5500, comparable to the frequency of anti-p53<sub>264-272</sub> pCTLs in normal donor PBMCs (data not shown). Among the 13 patients' PBMC analyzed for both wt p53 pCTLs, only 3 showed high frequencies for both. PCR analyses of p53 in these patients' tumor specimens are currently in progress. Therefore, no correlation can

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presently be made between pCTL frequency in the peripheral circulation of the breast cancer patients tested and the p53 status of their tumors.

The frequencies of anti-p53<sub>264-272</sub> pCTLs in 6 TIL samples obtained from breast cancer patients were also determined, 4/6 were above the cutoff with a mean of 1/520. (Fig.2 Lane 3). This high mean frequency in TILs is consistent with anti-tumor effectors being more readily found at tumor sites than the peripheral circulation. For 3 patients, tumor specimens were also available and analyzed for p53. Only one had the potential to present the p53<sub>264-272</sub> peptide; it accumulated mutant p53. The others had a normal level of wt p53 or deletion in p53 exon 6. Based on this limited analysis, our concept of matching the “right vaccine to the right patient” for p53 immunotherapy for oral cancer patients seems most likely to apply to breast cancer patients, as well. This will require, however, establishing a rational basis for this immunotherapy based on defining *ex vivo* the responsiveness of breast cancer patients to wt p53 peptides and identifying which wt p53 peptides their mammary carcinomas are likely to present for T-cell recognition.

#### **4. Identifying the wt p53<sub>110-124</sub> peptide as a naturally presented HLA-DRB1\*0401-restricted CD4<sup>+</sup> Th cell-defined epitope.<sup>1</sup>**

Given the importance of defining Th-defined wt p53 epitopes to optimize immunotherapy, we generated anti-p53 CD4<sup>+</sup> T-cells from PBMC obtained from an HLA-DRB1\*0401 healthy donor using rhp53-pulsed autologous DC. After 3X IVS, the outgrowing T-cells were tested for their proliferative responses against autologous DC pulsed with recombinant human p53 (rhp53) or individually pulsed with one of eight algorithm-predicted HLA-DRB1\*0401-binding wt p53 peptides: wt p53<sub>22-36</sub>, 47-61, 94-108, 106-120, 110-124, 123-137, 127-141 and 192-206. The bulk populations of effectors responded to rhp53

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<sup>1</sup>K.Chikamatsu, A. Albers, J. Stanton<sup>1</sup>, W. W. Kwok, E. Appella, T. L. Whiteside and A. B. DeLeo. p53<sub>110-124</sub>-specific CD4<sup>+</sup> T-helper cells enhance *in vitro* generation/function of anti-tumor CD8<sup>+</sup> T-cells. *Manuscript in preparation*

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as well as the p53<sub>110-124</sub> peptide (RLGFLHSGTAKSVTC). The response to the p53<sub>110-124</sub> peptide was blocked by anti-HLA-DR mAb, L243, but not anti-class I HLA mAb, w6/32. We further confirmed the HLA-DRB1\*0401-restriction of the reactivity of the bulk population of effectors for the wt p53<sub>110-124</sub> peptide in the ELISPOT IFN $\gamma$  assay using peptide-pulsed T2.DR4 target T-cells, a result which also indicated that these effectors were Th1 biased as well. A weaker but noticeable response of these effectors was also detected in this assay system against the overlapping wt p53<sub>106-120</sub> (SYGFRLGFLHSGTA) peptide.

The OSCC cell line, PCI-13, expresses mutant p53 molecules and expresses HLA-DR molecules when treated with IFN $\gamma$  (Fig. 2B). The genotype of PCI-13 was determined to be HLA-DRB1\*0401, -DRB1\*0701. The bulk population of anti-wt p53<sub>110-124</sub> CD4<sup>+</sup> T-cells produced and secreted IFN $\gamma$  in response to cytokine-treated PCI-13 tumor cells. This response was blocked by anti-HLA-DR mAb, L243, but not anti-class I HLA mAb, w6/32, and was consistent with wt p53<sub>110-124</sub> peptide being a naturally presented Th-defined epitope. As this bulk population of CD4<sup>+</sup> T-cells was induced with rhp53, its recognition of PCI-13 cells could have involved a p53-derived epitope(s) other than or in addition to the wt p53<sub>110-124</sub> peptide. Accordingly, we isolated several anti-p53<sub>110-124</sub> peptide-specific T-cell lines from the bulk population by limiting dilution and selected one of the more potent lines, clone #21, for additional characterization. Clone #21 was highly reactive in ELISPOT IFN $\gamma$  assays against p53<sub>110-124</sub> peptide-pulsed T2.DR4 cells, but not against non-pulsed, p53<sub>106-120</sub> or p53<sub>22-36</sub> peptide-pulsed T2.DR4 cells (Fig. 3A). As expected, clone #21 cells also responded in ELISPOT assays to PCI-13 cells pretreated with IFN $\gamma$  alone or in combination with TNF $\alpha$ . This reactivity was blocked by the L234 mAb, but not the w6/32 mAb, confirming wt p53<sub>110-124</sub> peptide represents a naturally presented HLA-DRB1\*0401-restricted, Th-defined wt p53 epitope (Fig. 3B).

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## **Reportable Outcomes**

### **Publications:**

T. K. Hoffmann, D. J. Loftus, K. Nakano, M. J. Maeurer, K. Chikamatsu, E. Appella, T. L. Whiteside and A. B. DeLeo. The Ability of Variant Peptides to Reverse the Non-responsiveness of T Lymphocytes to the Wild-Type Sequence p53<sub>264-272</sub> Epitope. *J.Immunol* 2002, 168:1338-1347.

### **Conclusions**

Methodologies have been developed to detect anti-wt p53 CD8<sup>+</sup> and CD4<sup>+</sup> T-cells present in the peripheral circulation and TILs obtained from cancer patients by flow cytometry analysis using soluble HLA/peptide tetramers. Results of tetramer-based studies have shown enhanced frequencies of these anti-p53 T-cells in the tumor environment relative to the peripheral circulation and will permit, in the near future, more detailed flow cytometry analysis of the functional activities of antigen-specific TILs than is presently possible. The results of these analyses may well have prognostic value and provide insights into enhancing the immune responsiveness of cancer patients. In an immediate and translational application, we will be applying tetramer analysis to assess anti-p53 cellular immune responses in breast and ovarian cancer patients undergoing p53-based immunotherapy at the National Cancer Institute under protocols initiated by Dr. Samir Kaheil.

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14. T. K. Hoffmann *et al.* *Cancer Res.* 62: 3521 (2002).
15. T. K. Hoffman *et.al.*, *J. Immunol.* 168:1338 (2002).

**Appendices**

- T. K. Hoffmann *et al.* *Cancer Res.* 62: 3521 (2002).
- T. K. Hoffman *et.al.*, *J. Immunol.* 168:1338 (2002).

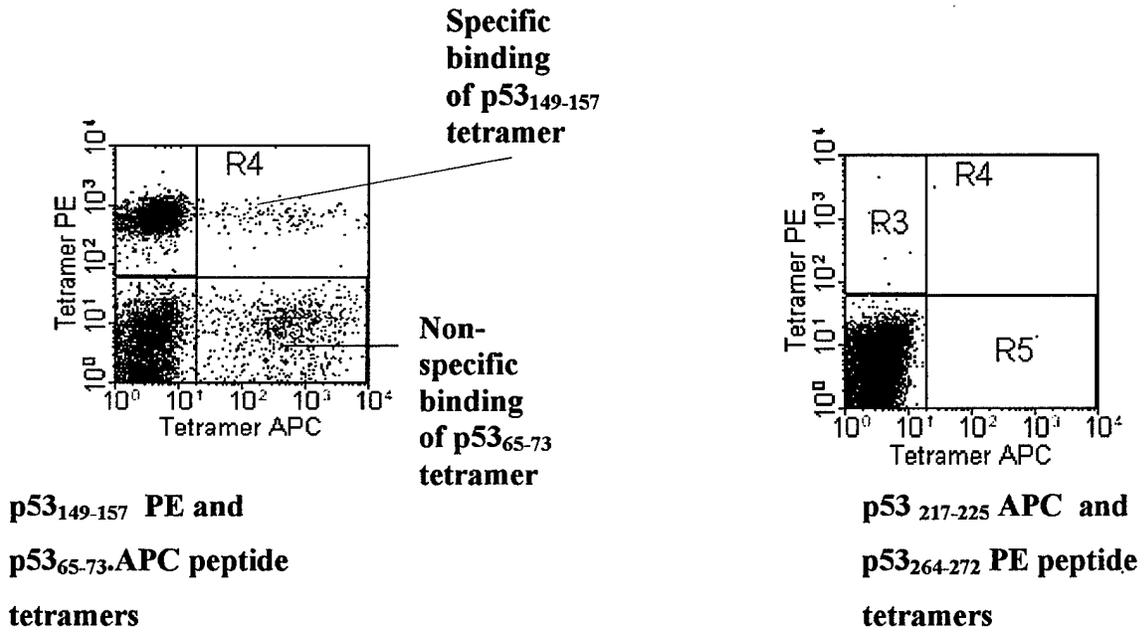
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**Table 1:** Specificities of bulk populations of anti-wt p53 CTL generated by IVS of PBMC obtained from HLA-A2<sup>+</sup> normal donors using autologous dendritic cells pulsed with wt p53 peptides: Analysis using ELISPOT IFN $\gamma$  assays

IVS with peptide	Spots/10 <sup>4</sup> T cells peptide pulsed-T2 target cells		
	None	p53 <sub>149-157</sub>	p53 <sub>264-272</sub>
p53 <sub>149-157</sub>	16.3±1.5	312.3±52.1	13.0±7.9
p53 <sub>264-272</sub>	26.3±3.8	9.0±7.9	630.0±36.4

**Figure 1.** Specificity of binding of p53<sub>149-157</sub> and p53<sub>264-272</sub> peptide/HLA-A2.1 tetrameric complexes against cloned anti-p53 peptide CD8<sup>+</sup> T cell lines by three-color flow cytometry

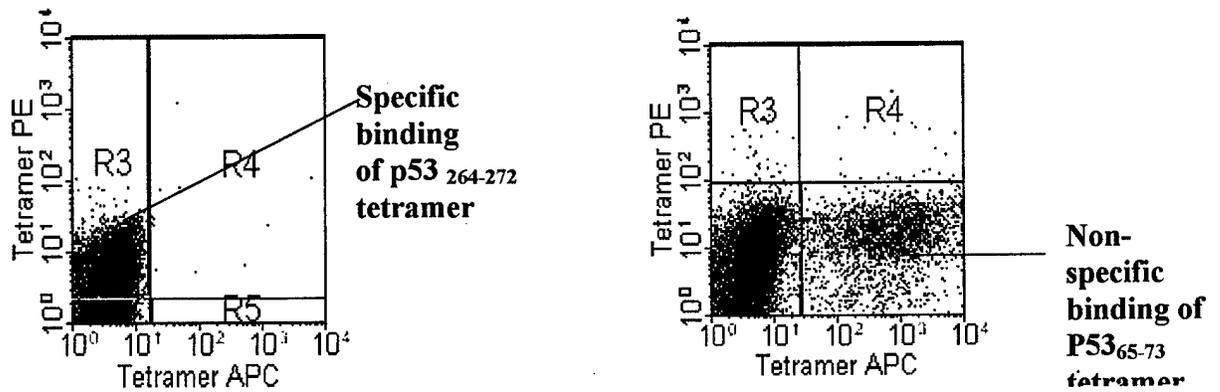
**Panel A:** Analysis of anti-p53<sub>149-157</sub> CD8<sup>+</sup> T cells with HLA-A2.1/p53 peptide tetramers



<u>tetramer</u>	<u>frequencies</u>	<u>anti-CD3 mAb inhibition</u>
65-APC	1/6	none
149 PE	1/2	yes
217-APC	1/1824	none
264-PE	1/839	none

**Figure 1:**

**Panel B:** Analysis of anti-p53<sub>264-272</sub> CD8<sup>+</sup> T cells with HLA-A2.1/p53 peptide tetramers



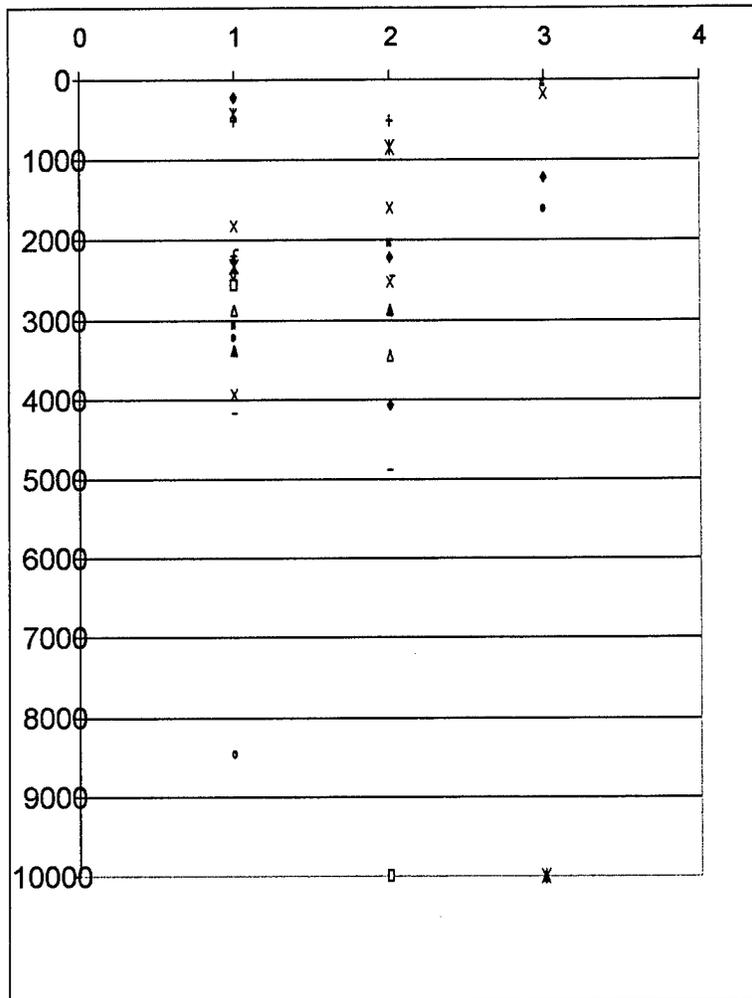
p53<sub>149-157</sub> PE and  
p53<sub>65-73</sub>·APC peptide tetramers

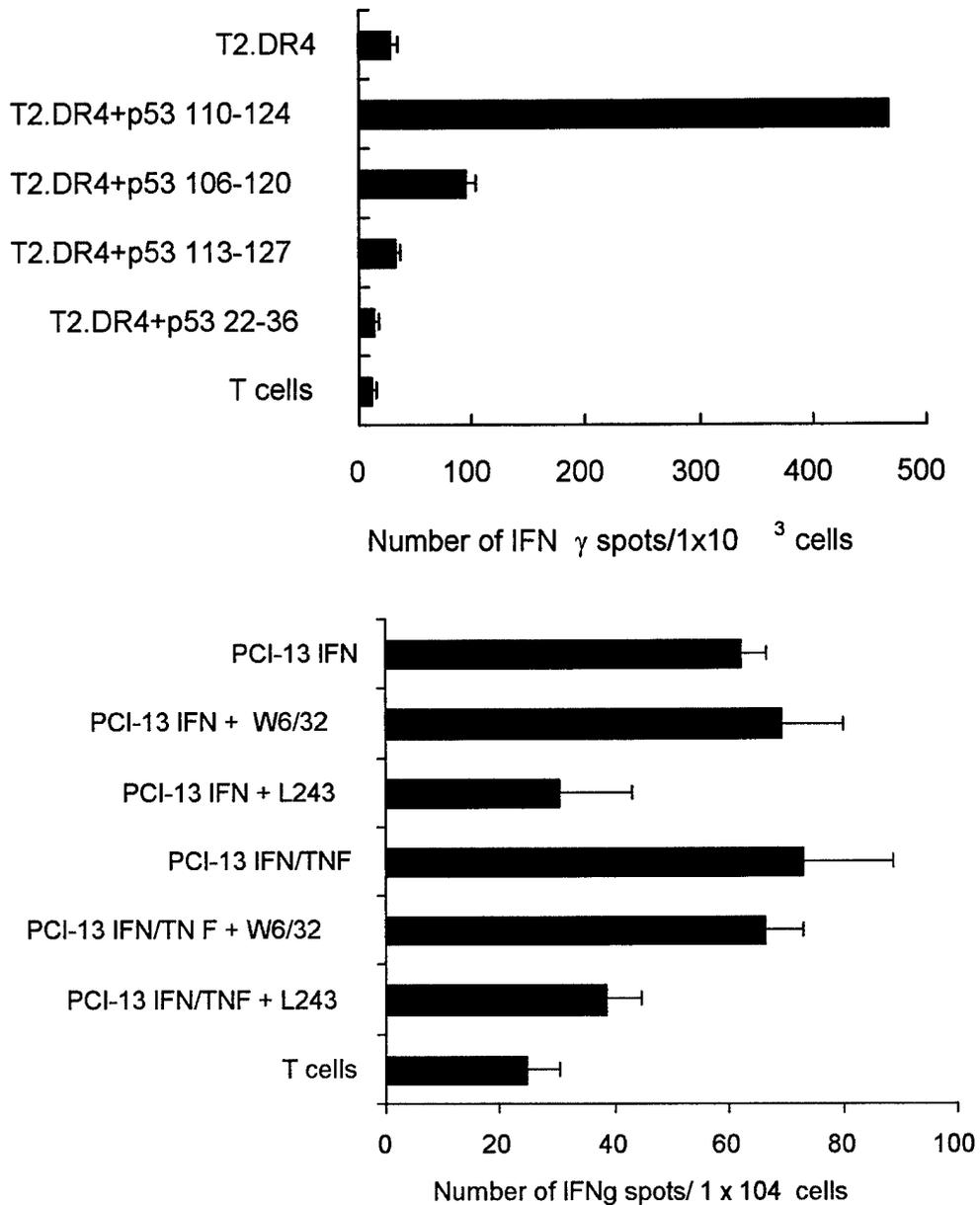
p53<sub>217-225</sub> APC +  
p53<sub>264-272</sub> PE peptide tetramers

<u>Tetramer</u>	<u>frequency</u>	<u>anti-CD3 mAb inhibition</u>
65-APC	1/4	none
149-PE	1/253	none
217-APC	1/3153	none
264-PE	1/1	yes

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**Fig. 2** Frequencies (*fr*) of anti-p53 pCTL detected in PBMC and TIL samples obtained from breast cancer patients. *Lane 1*, anti-p53<sub>264-272</sub> pCTL/ PBMC; *Lane 2*, anti-p53<sub>149-157</sub> pCTL/PBMC; *Lane 3*: anti-p53<sub>264-272</sub> pCTLs / TILs.





**Figure 3.** Specificity analysis of #21 cell line, cloned anti-p53<sub>110-124</sub> CD4<sup>+</sup> T-cells. Clone #21 cells (A) recognize T2.DR4 target cells pulsed with the p53<sub>110-124</sub> peptide, (B) produce IFN $\gamma$  in response to PCI-13 tumor cells pretreated with IFN $\gamma$  alone or in combination with TNF $\alpha$ . Responses were inhibited by mAb to HLA-DR (L243) but not class I HLA molecules (w6/32). A representative example of three sets of analyses is shown. The data are mean values  $\pm$  MSD for triplicate wells.

# Frequencies of Tetramer<sup>+</sup> T Cells Specific for the Wild-Type Sequence p53<sub>264-272</sub> Peptide in the Circulation of Patients with Head and Neck Cancer<sup>1</sup>

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## ABSTRACT

Immunization with wild-type sequence (wt) p53 epitopes represents a novel therapeutic strategy for cancer patients with tumors accumulating mutant p53. To evaluate usefulness of p53-derived peptides as future cancer vaccines, frequencies of wt p53<sub>264-272</sub> peptide-specific CD8<sup>+</sup> T cells were determined in the peripheral circulation of patients with squamous cell carcinoma of the head and neck (SCCHN). T cells of 30 HLA-A2.1<sup>+</sup> patients and 31 HLA-A2.1<sup>+</sup> healthy individuals were evaluated by multicolor flow cytometry analysis using peptide-HLA-A2.1 complexes (tetramers). T cells specific for an influenza matrix peptide (a model recall antigen) or an HIV reverse transcriptase peptide (a model novel antigen) were studied in parallel. Patients with SCCHN had a significantly higher mean frequency of CD8<sup>+</sup> T cells specific for wt p53<sub>264-272</sub> than normal donors ( $P = 0.0041$ ). Surprisingly, the frequency of epitope-specific T cells in the circulation of patients did not correlate with p53 accumulation in the tumor. In patients whose tumors had normal p53 expression or had p53 gene mutations preventing presentation of this epitope, high frequencies of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells were found, of which many were memory T cells. In contrast, patients whose tumors accumulated p53 had low frequencies of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells, which predominantly had a naive phenotype and were unable to proliferate *ex vivo* in response to the epitope, as reported by us previously (T. K. Hoffmann, J. Immunol., 165: 5938-5944, 2000). This seemingly contradictory relationship between the high frequency of epitope-specific T cells and wt p53 expression in the tumor suggests that other factors may contribute to the observed anti-p53 responses. Human papillomavirus-16 E6/E7 expression is common in SCCHN, and E6 is known to promote presentation of wt p53 epitopes. Although human papillomavirus-16 E6/E7 expression was detected in 46% of the tumors, it did not correlate with the frequency of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells or with p53 expression in the tumor. These findings emphasize the complexity of interactions between the tumor and the host immune system, and, thus, have particularly important implications for future p53-based immunization strategies.

## INTRODUCTION

The gene encoding p53 protein is frequently mutated in many human cancers, including SCCHN,<sup>3</sup> which generally results in accumulation (overexpression) of p53 molecules in these tumors (1-4). As most of these mutations involve an alteration of a single amino acid in p53 molecules, the majority of the accumulating mutant protein resembles the wt p53 (4). Therefore, enhanced presentation of wt epitopes derived from p53 accumulating in tumors is possible, and

might lead to their recognition by the immune system and the development of antitumor CTLs (5-9). For this reason, wt p53 epitopes are considered attractive targets for immunotherapy of cancer.

We reported previously on the generation of CTLs recognizing the HLA-A2.1-restricted wt p53<sub>264-272</sub> epitope from PBMCs obtained from SCCHN patients using autologous peptide-pulsed dendritic cells as antigen-presenting cells (9). Surprisingly, we observed that CTLs reactive against this wt p53 epitope could only be generated from T-cell precursors in PBMCs of patients whose tumors either did not accumulate p53 or accumulated mutant p53 molecules that could not present this epitope (9). We hypothesized that *in vivo*, the presence of expandable CTL precursors specific for the wt p53<sub>264-272</sub> epitope led to immunoselection, resulting in the elimination of tumors expressing the epitope and favored the outgrowth of "epitope-loss" tumor cells able to evade the host immune system. On the other hand, it was also possible that HPV infection, known to occur in a substantial proportion of SCCHN, could lead to inactivation of wt p53 and enhanced processing of p53 epitopes (10). The consequence of either phenomenon would be the presence in patients with wt p53 tumors of relatively high frequencies of T cells specific for the wt p53<sub>264-272</sub> epitope. To test these hypotheses, we investigated the frequency of p53<sub>264-272</sub>-specific precursor T cells in the peripheral circulation of 30 HLA-A2.1<sup>+</sup> patients with SCCHN and 31 HLA-A2.1<sup>+</sup> healthy controls, using multimeric peptide-MHC complexes. We also performed PCR analyses of genomic DNA isolated from the patient tumors for p53 and HPV E6/E7.

The availability of multimeric peptide-MHC complexes, which are generically referred to as tetramers, allows for direct identification and phenotyping of antigen-specific T cells in the peripheral circulation. Tetramers bind to more than one TCR on a specific T cell and, therefore, have a relatively slow dissociation rate (11, 12). However, the specificity of tetramer binding to the TCR has to be carefully controlled, particularly when the frequency of peptide-specific precursor T cells in the peripheral circulation is expected to be very low. Anticipating that discrimination between nonspecific and specific tetramer binding to p53<sub>264-272</sub>-specific T cells might be difficult, we used a novel four-color flow cytometry assay that simultaneously measures tetramer, CD3, CD8, and CD14 binding (13). Furthermore, binding of the p53 tetramer was compared with that of the influenza virus matrix peptide GILGFVFTL (FLU, a model for recall responses) and the HIV reverse transcriptase peptide ILKEPVHGV (HIV, a model for responses to a new antigen).

In this study, the frequency of wt p53<sub>264-272</sub> peptide-specific CD8<sup>+</sup> T cells in the circulation of patients with SCCHN was correlated with the p53 expression in each patient tumor, its HPV status, and the presence of p53 antibodies in the serum. Our results provide significant insights into the *in vivo* interactions that might occur between the developing tumor and immune system in these patients.

## MATERIALS AND METHODS

**PBMCs.** Peripheral blood samples or leukapheresis products were obtained from 30 HLA-A2.1<sup>+</sup> SCCHN patients, 31 HLA-A2.1<sup>+</sup> healthy donors, and 10

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<sup>3</sup> The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; Ab, antibody; CTL, cytotoxic T lymphocyte; HPV, human papillomavirus; HLA, human leukocyte antigen; IL, interleukin; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; ECD, electron capture detection; TCR, T-cell receptor; wt, wild-type sequence; FLU, influenza.

HLA-A2.1<sup>-</sup> healthy donors. PBMCs were isolated by centrifugation over Ficoll-Hypaque gradients (Amersham Pharmacia Biotech, Piscataway, NJ). Leukapheresis products were obtained from the Institute of Transfusion Medicine, Pittsburgh, PA. The study was approved by the Institutional Review Board at the University of Pittsburgh, and written informed consent was obtained from each participating individual. All of the products were tested and found to be negative for HIV-I antigens and antibodies to HIV. PBMCs were phenotyped for expression of HLA-A2 molecules by flow cytometry using the anti-HLA-A2 mAb BB7.2 (American Type Culture Collection, Manassas, VA) and an IgG isotype as a control. The verification of the A2.1 subtype was performed using polymerase chain reaction with sequence-specific primers as described previously (9, 14). PBMCs were either used fresh or were frozen at a concentration of  $50 \times 10^6$  cells/ml in the freezing medium consisting of human AB serum (Pel-Freeze, Brown Deer, WI) plus 10% DMSO (Fisher Scientific, Pittsburgh, PA).

In 30 patients, histologically verified squamous cell carcinomas originated in one of the following sites: the nose ( $n = 1$ ), oral cavity ( $n = 7$ ), oropharynx ( $n = 4$ ), larynx ( $n = 16$ ), and hypopharynx ( $n = 2$ ). Tumors were classified for tumor stage ( $T_1 = 10$ ;  $T_2 = 9$ ;  $T_3 = 3$ ;  $T_4 = 8$ ; and  $T_x = 1$ ), nodal stage ( $N^0 = 22$ ;  $N_1 = 2$ ;  $N_2 = 5$ ; and  $N_3 = 1$ ), and the presence of distant metastases ( $n = 0$  of 30).

**Tetrameric Peptide-MHC Class I Complex (Tetramer) Assay.** Tetramers were obtained through the National Institute of Allergy and Infectious Diseases Tetramer Facility and the NIH AIDS Research and Reference Reagent Program. Stock solutions contained 0.5  $\mu$ g monomer/ml. Peptides provided to the National Institute of Allergy and Infectious Diseases Tetramer Facility were either GILGFVFTL, an influenza matrix immunodominant peptide (residues 58–66), the HIV-1 reverse transcriptase peptide (pol 476–484) ILKEPVHGV, or the HLA-A2.1-binding peptide LLGRNSFEV (15, 16), corresponding to wt p53<sub>264-272</sub> peptide. The specificity of the LLGRNSFEV tetramer was confirmed by staining against the anti-p53-specific CTL line as described previously (9) and by the lack of staining with irrelevant CTLs, as well as HLA-A2-negative PBMCs of healthy donors.

To minimize background staining each tetramer was titrated and used at the lowest concentration that still gave a clearly discernible positive population in a donor vaccinated for influenza (for FLU<sub>58-66</sub> tetramer) as well as in an HIV-infected individual (for pol<sub>476-484</sub>). The final dilution of both preparations during staining, relative to the stock reagent supplied by the NIH, was 1/300. Within a 2-fold range of tetramer concentrations bracketing the concentrations used here, the frequency of tetramer-positive events and competition of CD3 binding (13) were stable, and tetramer fluorescence intensity was within 80% of that obtained at saturating concentrations. At saturating concentrations, CD3 competition decreased, fluorescence intensity of tetramer positive cells increased, and tetramer frequency increased, the latter attributable chiefly to a greater number of tetramer-dim events.

**Antibodies.** The default panel of antibodies used for these studies was CD14-FITC (RMO52; Immunotech, Miami, FL), Tetramer-PE, CD3 ECD (HIT3a; Beckman Coulter, Miami, FL), and CD8-PC5 [SFCl21Thy2D3(T8); Beckman Coulter]. Additionally, antihuman CD45RA FITC (Immunotech) and anti-CD45RO ECD (Beckman Coulter) were used for the characterization of the CD45 phenotype.

**Flow Cytometry Analysis.** Immediately before staining, cells were washed twice with the staining medium, consisting of PBS +0.1% (w/v) BSA +0.1% (w/v) sodium-azide, and resuspended at a concentration of  $5 \times 10^6$ /ml in a volume of 150  $\mu$ l. Tetramer (5  $\mu$ l of 1:10 dilution of stock solution) was added at room temperature for 30 min, followed by a 30 min incubation with antibodies (7.5  $\mu$ l of each) at 4°C. After two additional washes, the cells were resuspended in ~1 ml of 0.5% methanol-free formaldehyde in PBS. At least  $1 \times 10^6$  events were collected using a four-color Coulter Epics XL cytometer set on low or medium flow rate at a maximum of 1000 events/sec. Flow cytometry data were analyzed in real time using Beckman-Coulter System II software. In initial experiments, the region defining tetramer-positive events was determined by evaluating PBMCs stained with the Ab panel but without tetramer. This region was held constant throughout the analysis. Data were saved as FCS 2.0 Listmode files for subsequent reanalysis in System II or WinList (Verity Software House, Topsham, ME).

**Confocal Microscopy.** A wt p53<sub>264-272</sub>-specific CTL line (9) was used as a positive control for the flow cytometric evaluation and additionally to visualize tetramer binding to the specific T cells by confocal microscopy. The

CTL line was incubated with the p53<sub>264-272</sub> peptide containing tetramer in azide-free PBS followed by incubation with FITC-conjugated anti-CD8 Ab. The cells were then fixed with 1% (w/v) paraformaldehyde, placed on a slide, and analyzed by confocal laser scanning microscopy at  $\times 600$  original magnification (Leica TCS NT confocal LSM; Leica Lasertechnik, Heidelberg, Germany). Images were edited using the Adobe PhotoShop software program (Adobe Systems, Mountain View, CA).

**p53 Mutation Analysis, Immunohistochemistry, and Detection of p53 Antibodies.** Tumors of 30 SCCHN patients included in this study were available as paraffin blocks archived at the University of Pittsburgh Medical Center. The histology of each case was reviewed by a pathologist (S. D. F.), and representative tissue sections containing areas of invasive SCCHN were selected for microdissection. Normal-appearing salivary gland tissue or skeletal muscle was microdissected separately to serve as an internal nontumor control. Using 4- $\mu$ m thick recut, unstained histological sections, normal and malignant tissue samples were removed under stereomicroscopic observation. Sufficient material was collected from a single histological section to afford replicate analysis. Samples were treated with proteinase K at a final concentration of 100  $\mu$ g/ml for 2 h and then boiled for 5 min to remove protease activity. PCR used sets of amplification primers flanking exons 5–8 of the p53 gene in four separate PCRs (17). Amplified DNA from microdissected tissues also included splice sites. PCR products were electrophoresed in 4% agarose, and the ethidium bromide-stained bands were excised and then isolated with glassmilk. DNA sequencing used antisense PCR primers for each exon with [<sup>32</sup>P]dATP as the reporter molecule, and sequence analysis was read from overnight exposed autoradiograms of 6% polyacrylamide gels.

For p53 immunohistochemistry, formalin-fixed, paraffin-embedded tumor tissues were sectioned (3–5  $\mu$ m), air-dried overnight at 37°C, deparaffinized, and dehydrated and stained with a mAb against p53, D0-7 (Dako, Carpinteria, CA), which recognizes an epitope in the NH<sub>2</sub> terminus between amino acids 35–45, and reacts with wild-type and most mutant forms of the p53 protein. The avidin-biotin-peroxidase method was used to visualize the p53, according to the instructions supplied by the manufacturer (Dako). The immunostained slides were evaluated by light microscopy for p53 accumulation. The tumor was considered p53-positive when >25% of the tumor cells showed staining intensity of 2+ and higher on the scale of 0–4+. IgG isotype mAb used at the same concentration as the primary mAb served as a negative control.

Ab to p53 in the patient and control sera were detected by an Enzyme ELISA purchased from PharmaCell Immunotech Coulter, Miami, FL, using microtiter plates coated with recombinant human wt p53 protein. Peroxidase-conjugated goat antihuman IgG was used for detection of human anti-p53 Ab by a colorimetric reaction. Staining intensity was compared with a standard curve, and anti-p53 levels  $\geq 1.1$  units/ml were considered to be positive. Assays were performed twice in triplicates and included sera obtained from seropositive as well as seronegative individuals as internal positive/negative controls.

**PCR Analysis for HPV-16.** PCR analysis was performed using amplification primers for HPV-16 E6/E7 (ATGCACCAAAGAGAACTGC and TGCCCATTAACAGGTCTTCC) and  $\beta$ -actin (GCGAGAAGATGACCCAG and GCCTGGATAGCAACGTA) as control, using tumor DNA isolated as described above. DNA aliquots obtained from 25 of 30 specimens were screened in four separate PCR reactions. A solution (50  $\mu$ l) consisting of 25 mM MgCl<sub>2</sub>, 1.5  $\mu$ M of each primer, 1.25U of Taq DNA-polymerase (Promega), 2 mM of deoxynucleotide triphosphate, and double-distilled H<sub>2</sub>O was added to each amplification tube. Amplification was performed with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 2 min. The process was repeated for the total of 40 cycles. In all of the PCR reactions, DNA obtained from HPV-16 E6/E7+ Caski and C33 cell lines were included as positive controls. PCR products (700-bp for HPV-16 E6/E7 and 500-bp for  $\beta$ -actin) were electrophoresed in 4% agarose and the bands visualized in the presence of ethidium bromide. Twelve of 25 tumors tested were positive for HPV-16 E6/E7.

**Statistical Analysis.** Tetramer-positive cells were quantified by flow cytometry and expressed as frequencies (e.g., 1/1000) or reciprocal frequencies (e.g., 1000). We examined raw reciprocal frequency data and log-transformed reciprocal frequency data using normal probability plots (13). For all three tetramers, the log-transformed data were better modeled by the normal distribution. Accordingly, descriptive statistics (means, SDs, and confidence intervals) and statistical analyses (Student's *t* test, two-tailed), were performed on

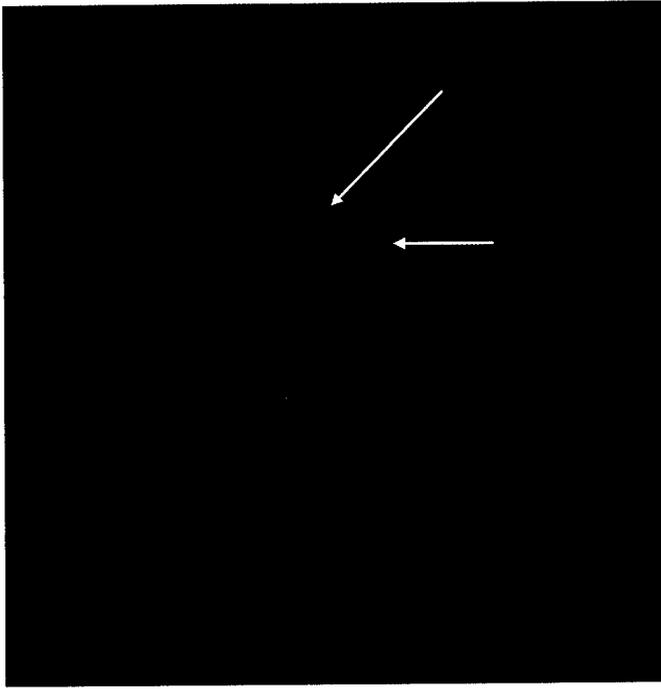


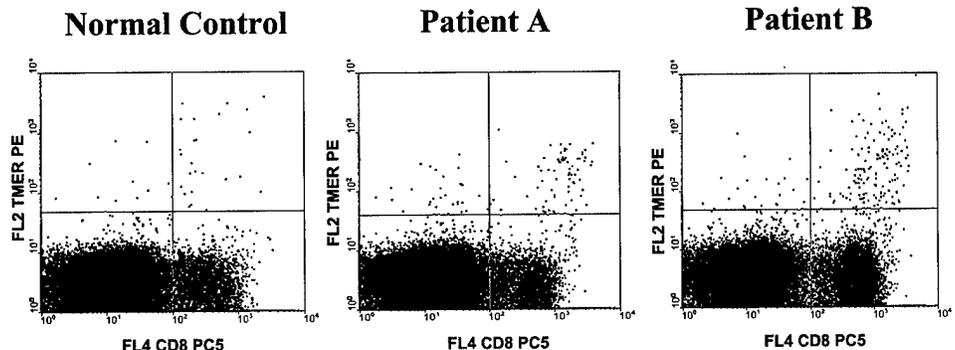
Fig. 1. Confocal microscopy demonstrating internalization of the tetrameric p53<sub>264-272</sub>-MHC-class I complexes by Ag-specific CD8 T cells. A T-cell bulk line specific for the HLA-A2.1-restricted p53 epitope LLGRNSFEV was stained with PE-labeled tetrameric p53<sub>264-272</sub>-MHC-class I complexes at room temperature for 30 min, washed, and incubated with anti-CD8 (FITC) for 30 min on ice. After fixation, the cells were examined in a confocal microscope (midplane image).

log-transformed reciprocal frequencies. For the comparison of multiple parameters, ANOVA (Tukey-Kramer multiple comparison test) was applied. The specificity of tetramer binding to the TCR was measured by competition of CD3 binding (13). Competition of CD3 binding was expressed according to the formula:

$$\% \text{ Competition} = \frac{[(\text{CD3MnI}_{\text{CD3}^+\text{CD8}^+\text{tmer}^-}) - (\text{CD3MnI}_{\text{CD3}^+\text{CD8}^+\text{tmer}^+})]}{(\text{CD3MnI}_{\text{CD3}^+\text{CD8}^+\text{tmer}^-})} \times 100$$

where CD3MnI is the log MFI of CD3 staining of the population designated by the subscript. For tetramer frequencies as well as competition of Ab binding, lower limits of detection were established by estimating the 99<sup>th</sup> percentile (geometric mean plus 2.6 SDs) of responses measured in PBMCs from HLA-A2<sup>-</sup> subjects. Linear regression was performed by the method of least squares. In regression plots the line of best fit and the 95% confidence intervals about the line of best fit are shown. The coefficient of correlation ( $r^2$ ), the slope of the regression line, and the  $P$  associated with the slope are reported as regression summary statistics. Multivariate analysis was performed to determine the relationship between the frequency of p53-specific CTL, accumulation of p53 in the tumor, and the HPV status of the tumor. Statistical analysis

Fig. 2. Dot plots showing the frequency of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells in a normal control (left) and two SCCHN patients (middle and right). Compound gating strategy was used to identify tetramer<sup>+</sup> CD8<sup>+</sup> T cells. PBMC from an HLA-A2.1<sup>+</sup> individuals were stained with p53<sub>264-272</sub> tetramer followed by anti-CD14, anti-CD3, and anti-CD8 mAbs, using four-color flow cytometry. Tetramer-positive cells were evaluated within a Boolean gate, excluding CD14<sup>+</sup> events and including cells within an extended lymphoid light scatter gate and a generous CD3<sup>+</sup> gate. In the example shown, CD3<sup>+</sup> CD8<sup>+</sup> tetramer<sup>+</sup> events were present at a frequency of 1/5757 CD3<sup>+</sup> CD8<sup>+</sup> T cells for the healthy individual, and 1/3063 and 1/1140 for 2 patients with SCCHN.



and statistical graphics were performed using Systat Version 9 (SPSS Inc, Chicago, IL).

## RESULTS

**Binding of the Tetramer to wt p53<sub>264-272</sub>-specific T Cells.** A wt p53<sub>264-272</sub>-specific CTL line established earlier (9) was stained with the FITC-conjugated anti-CD8 (green) and PE-conjugated tetramer (red). Its confocal microscopy mid-plane image is shown in Fig. 1. On binding to the TCR, tetrameric p53<sub>264-272</sub>-MHC class I complexes were internalized in the absence of sodium azide. This p53<sub>264-272</sub>-specific T-cell line served as positive control in subsequent flow cytometry analyses of precursor T cells in the peripheral circulation of human subjects.

**Gating Strategy for Tetramer Analysis.** For the detection of unstimulated precursor T cells specific for the wt p53<sub>264-272</sub> peptide, PBMCs of SCCHN patients and healthy donors were directly stained with the tetramer and analyzed by flow cytometry. To assure the specificity of tetramer binding, we developed previously a gating strategy to eliminate CD14<sup>+</sup> monocytes as well as apoptotic and necrotic cells, all of which could bind tetramer and/or Ab nonspecifically (13). Furthermore, CD3-negative cells were eliminated by compound gating, which finally resulted in discriminative dot plots showing CD8<sup>+</sup> tetramer<sup>+</sup> T cells. Fig. 2 shows the representative dot plots of stained PBMCs obtained from a healthy individual and from 2 patients with SCCHN. The cases shown are representative of relatively low (Fig. 2, left; 1 of 5757), intermediate (Fig. 2, middle; 1 of 3063) and high (Fig. 2, right; 1 of 1140) p53<sub>264-272</sub> tetramer binding frequencies, respectively.

**Definition of the Lower Limit of Detection of Tetramer-positive T Cells.** To establish the lower detection limit for tetramer binding in HLA-A2.1<sup>+</sup> individuals, we stained PBMCs obtained from 10 HLA-A2<sup>-</sup> individuals. Despite the application of the gating strategy described above, low levels of p53<sub>264-272</sub> tetramer<sup>+</sup> CD3<sup>+</sup> CD8<sup>+</sup> T cells were detected in PBMCs of HLA-A2<sup>-</sup> donors (geometric mean = 1/23,397). Because these events were nonspecific by definition, we established a cutoff for the lower detection limit of this assay at the upper 99<sup>th</sup> percentile of tetramer<sup>+</sup> CD8<sup>+</sup> T cells in HLA-A2<sup>-</sup> individuals. This cutoff frequency of 1 of 7,805 was applied to all of the data obtained from testing of the 10 HLA-A2<sup>-</sup> subjects, 30 HLA-A2.1<sup>+</sup> SCCHN patients, and 31 HLA-A2.1<sup>+</sup> healthy controls. As shown in Fig. 3, none of the HLA-A2<sup>-</sup> individuals had frequencies of p53<sub>264-272</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells exceeding the established cutoff. On the other hand, 23 of 30 HLA-A2.1<sup>+</sup> SCCHN patients and 25 of 31 healthy controls had frequencies of p53<sub>264-272</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells above the cutoff point (geometric means = 1/3,533 and 1/5,207, respectively).

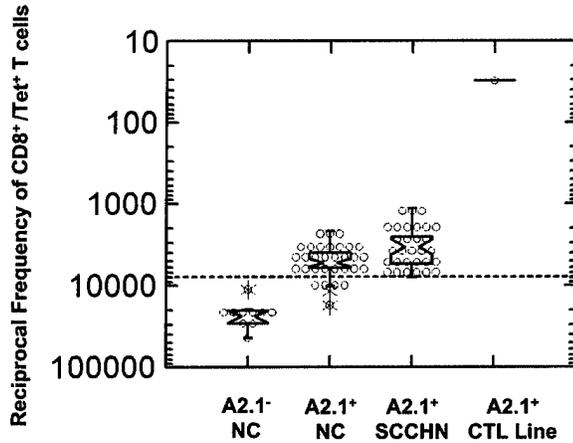


Fig. 3. Reciprocal frequencies of p53<sub>264-272</sub> tetramer<sup>+</sup> CD8<sup>+</sup> CD3<sup>+</sup> T cells.  $\circ$  show individual data points. Superimposed notched box plots display nonparametric descriptive statistics. The waist indicates the group median, the hinges (upper and lower boundaries of the box) indicate interquartile distances. The notches show simultaneous 95% confidence intervals about the median. The whiskers (bars) give the ranges, exclusive of outliers. Outliers ( $>1.5$  times the hingespread from the median) are shown by \* and far outliers (more than three times the hingespread from the median) are shown by double circles. Reciprocal frequencies of p53<sub>264-272</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells in 10 HLA-A2.1<sup>-</sup> and 30 HLA-A2.1<sup>+</sup> healthy donors, 30 HLA-A2.1<sup>+</sup> patients with SCCHN, and the HLA-A2.1<sup>+</sup> p53<sub>264-272</sub>-specific T cell line. The dashed line represents the cutoff for the lower detection limit as determined in HLA-A2.1<sup>-</sup> donors (geometric mean  $+2.6$ ); bars,  $\pm$  SD.

#### Confirmation of the Specificity of Tetramer Binding to TCR.

We have reported previously that tetramer-positive T cells stained dimmer for CD3 than did tetramer-negative T cells in PBMCs obtained from HLA-A2.1<sup>+</sup> subjects. However, this is not the case for the spurious tetramer-positive events seen in PBMCs obtained from HLA-A2<sup>-</sup> subjects (13). We demonstrated that this phenomenon results from a competition between tetramer and anti-CD3 Abs binding to TCR. This competition was subsequently quantified and introduced as a marker for the specificity of tetramer binding to the TCR complex (13). Because there was no detectable competition between tetramer and anti-CD3 Ab binding for CD8<sup>+</sup> T cells in 9 of 10 HLA-A2.1<sup>-</sup> individuals, we were able to define a cutoff based on the 99<sup>th</sup> percentile of CD3 competition in these HLA-A2<sup>-</sup> subjects (3.2%). Competition by anti-CD3 Ab in excess of this cutoff was considered to be significant. The mean of competition for the wt p53<sub>264-272</sub> tetramer was  $10.0 \pm 1.4\%$  (mean  $\pm$  SE) in T cells obtained from patients and  $5.3 \pm 1.5\%$  in normal controls. PBMCs of 23 of 30 SCCHN patients but only 10 of 31 normal controls exceeded both the cutoff for competition as well as the cutoff for frequency (see above), and, thus, were considered to exhibit specific binding of the p53<sub>264-272</sub> tetramer (Fig. 4).

**Comparison of Frequencies of wt p53<sub>264-272</sub>-specific versus HIV- or FLU-specific T Cells.** Next, frequencies of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells were compared with those obtained with the HIV tetramer or the FLU tetramer. These comparisons were performed to evaluate p53-specific responses in the context of those to known novel and recall antigenic peptides. The frequencies of FLU- and HIV-specific T cells are displayed in Fig. 5 as normal distribution curves. The frequencies of wt p53<sub>264-272</sub>-specific T cells are shown as individual circles. For healthy individuals (Fig. 5, left), the majority of p53 frequencies fall within the HIV normal distribution curve. In contrast to PBMCs from the healthy control group, PBMCs from SCCHN patients seem to have a bimodal distribution, with a majority of the frequencies located within the normal HIV distribution curve, and six frequencies shifted to the right (Fig. 5, note the log scale) toward FLU frequencies.

**Frequencies of wt p53<sub>264-272</sub>-specific T Cells in Patients and Controls.** On more careful examination, when the frequencies of wt p53<sub>264-272</sub> peptide-specific CD8<sup>+</sup> T cells in the circulation of patients (see Table 1) were compared with those obtained in normal controls, it appeared that the patients could be divided into two different groups. The first subset consists of patients with wt p53 tumors that do not accumulate p53 or those with tumors unlikely to present the wt p53<sub>264-272</sub> epitope because of the type of mutation they harbor. These patients have significantly higher frequencies ( $P \leq 0.0005$ ) of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells in the peripheral circulation than the patients in the second subset, whose tumors accumulate p53 and, in theory, could have a higher potential for presentation of this epitope (Fig. 6). The SCCHN patients in the second subset, those with tumors accumulating p53, have lower frequencies of CD8<sup>+</sup> T cells specific for this p53 epitope, which do not significantly differ from those obtained for normal controls (Fig. 6). Confirming our initial observations, this result suggests that accumulation of p53 in the tumor does not positively correlate with the frequency of the epitope-specific CD8<sup>+</sup> T cells detectable in the circulation of patients with SCCHN.

**Analysis of wt p53<sub>264-272</sub>-specific Memory versus Naive T Cells.** Expression of CD45 isoforms on the surface of T cells is routinely used to discriminate between naive (CD45RA<sup>+</sup>) and memory (CD45RO<sup>+</sup>) T-cell subsets (18). To determine whether tetramer<sup>+</sup> CD8<sup>+</sup> T cells detected in the circulation of patients with SCCHN belong to the memory or naive T-cell subsets, multicolor flow analysis including anti-CD45 Abs was performed. The gates for CD45RO and CD45RA expression were set on CD8<sup>+</sup> tetramer<sup>-</sup> T cells, as shown in the left panel of Fig. 7. We have shown previously that the majority of CD8<sup>+</sup> tetramer<sup>+</sup> cells for the recall antigen, FLU, were CD45RO<sup>+</sup>/CD45RA<sup>-</sup> memory T cells, whereas those recognizing the novel HIV antigen were predominantly CD45RO<sup>-</sup>/CD45RA<sup>+</sup> naive T cells (13). This analysis was performed on samples obtained from two groups of representative patients: one with relatively high frequencies (average  $\sim 1/2700$ ), the other with low frequencies (average  $\sim 1/5500$ ) of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells as well as from normal donors (see Table 2). Normal donors (Fig. 7, middle panel) and patients with low frequencies of p53<sub>264-272</sub> tetramer<sup>+</sup> T cells had similar percentages of memory ( $\sim 10\%$ ) and naive ( $\sim 70\%$ ) T cells in the peripheral circulation. In contrast, a significantly higher percentage of memory cells (36.5%) was found in patients with high frequencies of wt p53<sub>264-272</sub>-specific T cells (Fig. 7, right panel).

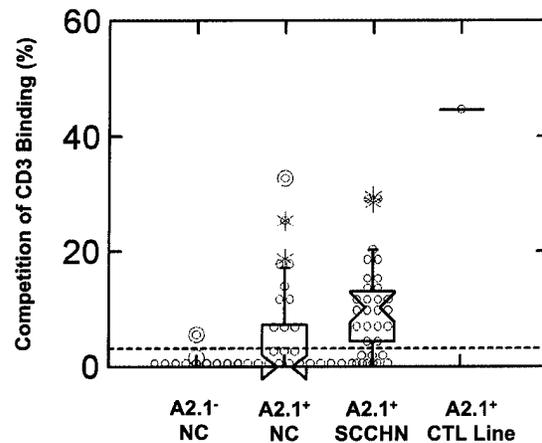


Fig. 4. Competition of anti-CD3 Ab binding by the p53<sub>264-272</sub> tetramer on CD8<sup>+</sup> CD3<sup>+</sup> T cells. Notched box plots show individual data points and nonparametric statistics, as described in Fig. 3. Competition in percentage is shown for p53<sub>264-272</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells obtained from 10 HLA-A2.1<sup>-</sup> and 30 HLA-A2.1<sup>+</sup> healthy donors, 30 HLA-A2.1<sup>+</sup> patients with SCCHN, and the HLA-A2.1<sup>+</sup> p53<sub>264-272</sub>-specific T cell line. The dashed line represents the cutoff for the lower limit of competition as determined in HLA-A2.1<sup>-</sup> normal controls; bars,  $\pm$  SD.

## Normal Controls

## SCCHN Patients

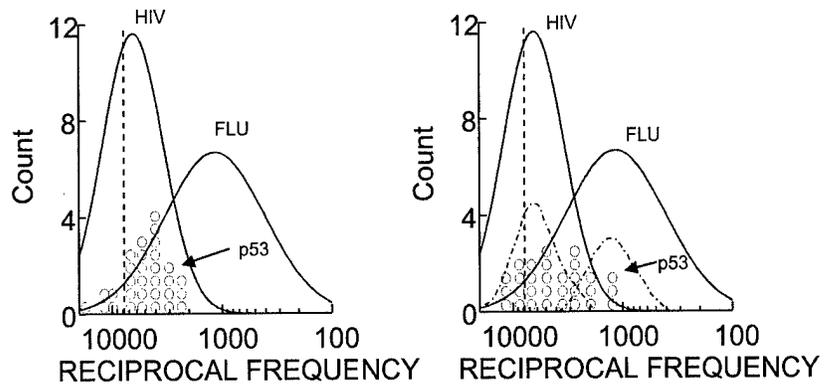


Fig. 5. Reciprocal frequency of p53<sub>264-272</sub> tetramer<sup>+</sup> CD8<sup>+</sup> CD3<sup>+</sup> T cells in the peripheral circulation of patients with SCCHN. ○ show individual data points. Superimposed normal distribution curves were calculated from sample means, and SDs of HIV and FLU tetramer data obtained in the normal control group. The dashed line represents the cutoff for the lower detection limit of tetramer frequency.

**Immunohistochemistry and Genomic PCR Analysis of p53 in Patient Tumors.** Immunohistochemistry of p53 protein and sequencing of genomic PCR products of p53 exons 5–8 in patient tumors were done to evaluate the potential of these tumors to present the wt p53<sub>264-272</sub> epitope, and ultimately relate this information to the frequencies of tetramer<sup>+</sup> CD8<sup>+</sup> T cells detected in the peripheral circulation of these patients (Table 1). Although exceptions have been noted in the literature, most tumor cells lines sensitive to CTL recognizing this epitope have been shown to accumulate p53. Of the 30 primary tumors analyzed, more than half (17 of 30) showed accumulation of p53. The primary tumor of one patient, #28, scored negative, but a lymph node metastasis was positive. From this total of 31 tumors (18 of 31 with p53 accumulation), 28 underwent sequencing of

genomic PCR products of exons 5–8 (3 cases were not available). Of the 16 available tumors (2 of 18 not available) showing p53 accumulation, 13 were found to express missense mutations within p53 exons 5–8. In two tumors (patients #7 and #10), p53 missense mutations were located within or directly next to the wt p53<sub>264-272</sub> peptide sequence. Such a mutation (R273H) was shown previously to prevent presentation of the epitope (19). For subsequent analysis, these tumors were considered as unlikely to be presenting the wt p53<sub>264-272</sub> epitope, and they are designated by brackets for p53 accumulation in Table 1.

A lack of agreement between the mutated p53 exon 5–8 genotype and p53 accumulation was reported for patients with SCCHN (20). In general, the results of our study indicated a good correlation between

Table 1 Summary of wt p53<sub>264-272</sub>-specific T-cell frequencies in patients with SCCHN and the p53 status in their tumors, as well as the presence of p53 autoantibodies<sup>a</sup>

Patient	Tumor site	Tumor-Node-Metastasis status	Frequencies of wt p53 <sub>264-272</sub> -specific T cells among CD8 <sup>+</sup> T cells	Tumor p53 genotype	Tumor p53 protein accumulation	Serum p53 autoantibodies	HPV16 status
1	Larynx	T2N0M0	1/1140	Wild type	–	–	+
2	Oral cavity	T3N2M0	1/1264	Wild type	–	–	+
3	Larynx	T1N0M0	1/1450	Wild type	–	–	+
4	Oral cavity	T1N0M0	1/1798	Wild type	–	–	–
5	Larynx	T2N0M0	1/1992	Wild type	–	–	–
6	Larynx	T2N0M0	1/2099	Wild type	–	–	ND
7	Oral cavity	T2N0M0	1/2566	E8 R273H	+ <sup>b</sup>	–	ND
8	Larynx	T4N2M0	1/2707	E6 213 Stop	–	–	–
9	Oropharynx	T1N2M0	1/2746	Wild type	–	+	+
10	Hypopharynx	T4N0M0	1/3063	E8 E271K	+ <sup>b</sup>	+	–
11	Larynx	T4N0M0	1/3126	Wild type	–	–	+
12	Larynx	T2N0M0	1/3151	E5 V157F	+	–	ND
13	Larynx	T4N0M0	1/3237	n.a.	+	–	–
14	Lip	T1N0M0	1/4527	E7 R248W	+	–	+
15	Nose	TxN0M0	1/4782	E6 Y220C	+	–	–
16	Larynx	T4N1M0	1/4933	Wild type	–	–	+
17	Oral cavity	T1N0M0	1/4958	n.a.	+	–	–
18	Larynx	T4N0M0	1/5025	E 6 deletion	–	–	+
19	Oropharynx	T1N0M0	1/6219	Wild type	+	–	–
20	Larynx	T3N0M0	1/6333	Wild type	+	–	–
21	Larynx	T1N0M0	1/6916	E6 G226R	+	–	–
22	Oral cavity	T2N0M0	1/7097	E6 Y220C	+	–	–
23	Oropharynx	T4N1M0	1/7236	E8 E286K	+	–	–
24	Hypopharynx	T4N2M0	1/7805 <sup>c</sup>	E5 S149C	+	+	+
25	Oral cavity	T2N0M0	<1/7805 <sup>c</sup>	n.a.	–	–	–
26	Larynx	T2N0M0	<1/7805 <sup>c</sup>	E5 H168Y	+	–	–
27	Larynx	T1N0M0	<1/7805 <sup>c</sup>	Wild type	+	–	+
28	Oropharynx	T2	<1/7805 <sup>c</sup>	Wild type	–	–	+
29	Larynx	N3 M0	<1/7805 <sup>c</sup>	E7 G245V	+	–	–
30	Larynx	T3N2M0	<1/7805 <sup>c</sup>	E5 T150R	+	–	+
				E8 C278T	+	–	ND

<sup>a</sup> The table is arranged in descending order according to detected frequencies of p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells; n.a., not available; Breaks are cutpoints as explained in the legend to Figure 8; ND, not done.

<sup>b</sup> The R 273 H mutation has been shown to prevent presentation of p53<sub>264-272</sub> epitope (19). The E271K mutation occurs within this epitope.

<sup>c</sup> These values were below the limit of detection or did not meet criteria of specific tetramer binding because competition with CD3 was lower than the cutoff.

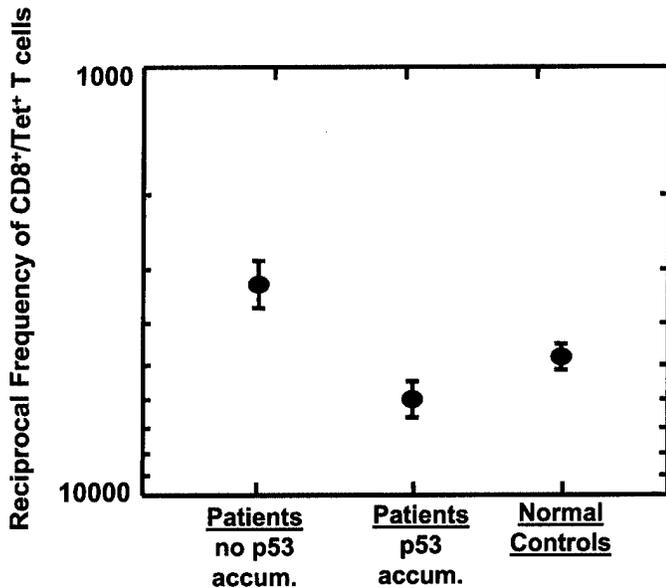


Fig. 6. Reciprocal frequency of p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells in patients with SCCHN and normal controls. The mean frequencies for normal controls and for patients with SCCHN with tumors found to accumulate p53 or not to accumulate p53 (see Table 1) were determined. Paired tumors and PBMC samples of 30 HLA-A2.1<sup>+</sup> patients with SCCHN were evaluated. Tumors showed either normal p53 protein expression, accumulated p53 protein, or had a mutation within or next to the p53<sub>264-272</sub> epitope, most likely preventing presentation of the epitope (18). Such tumors were considered to have normal p53 expression; bars,  $\pm$  SD.

p53 missense mutations and accumulation of the altered protein in tumors (Table 1). In 3 cases (patients #19, #20, and #27) a discrepancy between the p53 genotype and p53 expression was observed (Table 1). All three represented tumors with a wt p53 genotype and with p53 accumulation, which could possibly be because of a mutation outside the exons 5–8 or in genes impacting on the stability of p53, such as mdm2 (21).

**Association between p53 in Tumors and Frequency of wt p53<sub>264-272</sub>-specific T Cells.** Stratification of the frequencies of wt p53<sub>264-272</sub>-specific T cells detected in the circulation of patients with SCCHN identified three groups of patients, those displaying high, intermediate, or low frequencies of these cells (Table 1). The cutoff for the high frequency designation was based on the upper 95<sup>th</sup> percentile ( $>1/2128$ ) frequency of tetramer<sup>+</sup>CD8<sup>+</sup> T cells established in HLA-A2<sup>+</sup> healthy controls. The tumors obtained from the 6 patients with the highest frequencies of these T cells were wt p53 and did not accumulate p53 (Table 1; Fig. 8).

In the second patient group, the intermediate reciprocal frequencies of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells were lower than 1/2128, yet

they exceeded the geometric mean of the patient group as a whole (1/4767). This group of 7 patients was heterogeneous in respect to p53 expression in the tumor: 3 tumors were p53 wt, 2 had p53 mutations and accumulation, and 2 tumors (#7 and #10) expressed missense mutations within or next to the p53<sub>264-272</sub> epitope (positions 273 and 271, respectively) and were probably unable to present this epitope to T cells (Fig. 8).

The third and largest group of the patients ( $n = 17$ ) had the lowest frequencies of wt p53<sub>264-272</sub>-specific T cells. In this group, 14 of 17 primary tumors ( $>80\%$ ) accumulated p53 and had the potential to present this epitope. The frequencies of wt p53<sub>264-272</sub>-specific T cells exceeded the lower limit of detection (1/7805) in 7 patients. The mean frequency for the group was significantly lower than that for the other two groups of patients. As indicated in Table 2, wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells present in low frequencies in the peripheral circulation of these patients predominantly expressed a naive phenotype (CD45RA<sup>+</sup>/CD45RO<sup>-</sup>). On the other hand, in patients with high frequencies of wt p53<sub>264-272</sub> epitope-specific T cells, memory T cells (CD45RA<sup>-</sup>/CD45RO<sup>+</sup>) significantly increased in proportion (Fig. 7). Therefore, it would appear that in the peripheral circulation of patients whose tumors have a low potential for presenting the epitope, the frequency of wt p53<sub>264-272</sub>-specific T cells with a memory phenotype is high. Therefore, it is likely that these T cells had a previous interaction with targets capable of presenting the p53<sub>264-272</sub> epitope.

**HPV-16 Positivity and Frequency of wt p53<sub>264-272</sub>-specific T Cells.** PCR analysis indicated that 12 of 26 (46%) tumors we examined contained HPV-16 E6/E7 DNA (Table 1). Among 12 tumors with p53 mutations, 4 (33%) were E6/E7<sup>+</sup>, whereas 8 of 12 (66%) wt p53 tumors were E6/E7<sup>+</sup>. One tumor (#28) was heterogeneous, with cells expressing either wt or mutated p53, and it was also E6/E7<sup>+</sup>. The p53 genotype was not available for 3 tumors analyzed for HPV. We found that 3 of 5 patients with the highest frequencies of wt p53<sub>264-272</sub>-specific T cells and wt p53 in the tumor were HPV-16<sup>+</sup>. The patient group with the intermediate frequencies (Fig. 8), contained 7 patients of whom 2 could not be tested for HPV and 1 was not genotyped for p53. Of the remaining 4, 2 were wt p53 and HPV<sup>+</sup>, whereas the other 2 had mutated p53 and were HPV negative. The cohort of 17 patients with low CTL frequencies contained 13 informative cases (tumor #28 was excluded from analysis), with 4 of 9 mutated p53 tumors and 2 of 4 wt p53 tumors positive for HPV-16. By fitting loglinear models to the frequencies of each variable in the  $3 \times 2 \times 2$  contingency table, we determined that the frequency of p53<sub>264-272</sub>-specific CTL was significantly correlated with the p53 status of the tumor ( $P = 0.016$ ). In contrast, both the frequency of CTL and p53 status of the tumor were found to be independent of HPV E6/E7 positivity ( $P = 0.9260$  and  $P = 0.2924$ , respectively).

Fig. 7. Representative data (patient #1 in Table 1) for CD45 isoform expression on p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells. Cells were stained with the tetramer followed by CD45RO-FITC, CD8-ECD, and CD45RA-PC5. Monocytes and high side scatter natural killer cells were eliminated from the analysis by using a tight lymphocyte light scatter gate. In normal controls as well as patients with low frequencies of p53<sub>264-272</sub>-specific T cells (not shown), these cells were predominantly CD45RO<sup>-</sup>/CD45RA<sup>+</sup>. In SCCHN patients with high frequencies of p53<sub>264-272</sub>-specific T cells (right panel), a significant number of CD8<sup>+</sup> tetramer<sup>+</sup> cells were CD45RO<sup>+</sup>/CD45RA<sup>-</sup>.

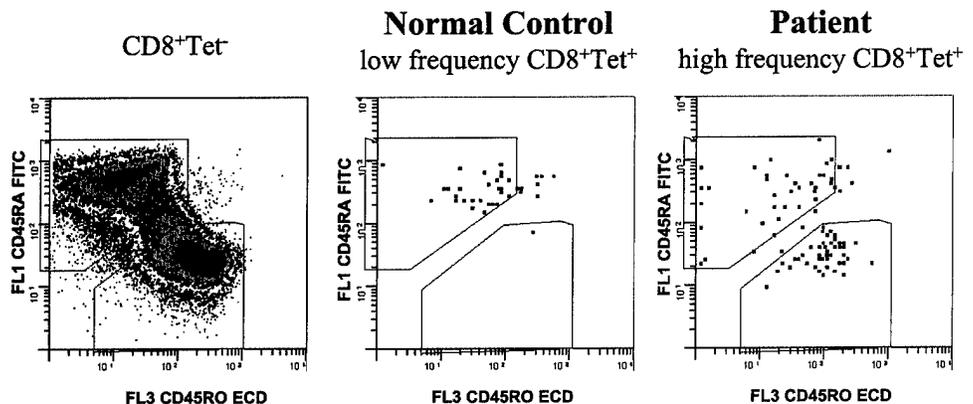


Table 2 Frequencies of wt p53<sub>264-272</sub>-specific memory and naive T cells in healthy controls and patients with SCCHN

Peptide	% of CD8 <sup>+</sup> tetramer <sup>+</sup> cells		Transitional cells
	CD45RA <sup>+</sup> / CD45RO <sup>-</sup> (naive)	CD45RA <sup>-</sup> / CD45RO <sup>+</sup> (memory)	
Healthy controls			
HIV (novel; n = 5)	71.9 ± 9.4	16.6 ± 5.7	11.5 ± 3.9
FLU (recall; n = 5)	11.2 ± 1.3	76.7 ± 2.7	12.1 ± 2.3
wt p53 <sub>264-272</sub> (n = 4)	74.7 ± 8.4	7.5 ± 5.6	17.8 ± 4.3
SCCHN patients			
wt p53 <sub>264-272</sub> (n = 3) <sup>a</sup>	50.3 ± 8.5	36.5 ± 12.8	13.2 ± 4.3
wt p53 <sub>264-272</sub> (n = 3) <sup>b</sup>	72.7 ± 2.5	10.0 ± 6.6	17.3 ± 6.3

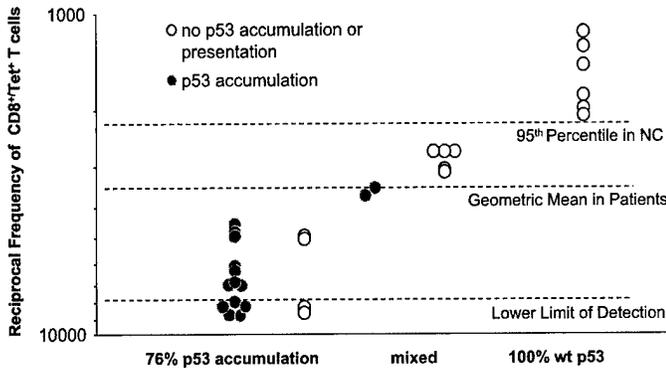
<sup>a</sup> High frequencies of wt p53<sub>264-272</sub>-specific T cells (~1/2700).<sup>b</sup> Low frequencies of wt p53<sub>264-272</sub>-specific T cells (~1/5500).

Fig. 8. The summary of associations between the frequency of p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells in patients with SCCHN and p53 accumulation in the patient tumors. Paired PBMCs and primary tumors from 30 HLA-A2.1<sup>+</sup> patients with SCCHN were evaluated. The bottom dashed line represents the cutoff for the lower detection limit of tetramer frequency, the intermediate line shows the geometric mean of the patient group as a whole, and the top dashed line indicates the upper 95<sup>th</sup> percentile (>1/2128) of tetramer<sup>+</sup>CD8<sup>+</sup> T cells in 30 HLA-A2.1<sup>+</sup> normal controls. According to the T-cell frequency and p53 tumor status, 3 groups of patients were identified. The evaluated tumors either showed normal p53 protein expression, had mutations within or next to the p53<sub>264-272</sub> epitope, shown previously to prevent epitope presentations (18), or displayed p53 protein accumulation.

**Association of p53 Antibodies and Frequency of wt p53<sub>264-272</sub>-specific T Cells.** An analysis of IgG antibodies to p53 in the sera of SCCHN patients by Bourhis *et al.* (22) identified nearly 20% as seropositive. Because the presence of IgG antibodies to p53 implies a T-cell mediated response, it was of interest to determine whether the frequencies of wt p53<sub>264-272</sub>-specific T cells present in the peripheral circulation of our seropositive patients were higher than the mean for all of the SCCHN patients. As indicated in Table 1, 3 of the 30 patients (#9, #10, and #24) scored p53 seropositive. The mean frequency (1/4538) of wt p53<sub>264-272</sub>-specific T cells in these 3 patients was not significantly higher than the geometric mean frequency for all of the SCCHN patients (1/4767). In 2 of these patients the tumor accumulated mutant p53, whereas in the third (#9), it did not. Interestingly, the tumor of this patient contained ~2% of cells positive for p53 (Fig. 9), and it was HPV-16 E6/E7 positive. The presence of p53 autoantibodies in the serum, which is usually associated with p53 accumulation (22), and the relatively high frequency of wt p53<sub>264-272</sub>-specific T cells detected in this patient's circulation (1/2746), suggest that the virus-related enhanced processing of p53 might contribute to effective CTL generation *in vivo*.

## DISCUSSION

SCCHN, which arise at or in close proximity to mucosal surfaces, interact closely with the host immune cells during tumor initiation, promotion, and progression. As a result of this interaction, tumor

cells, which are recognized by immune effector cells, can be eliminated, whereas tumor cells able to evade immune recognition can grow and become resistant to the host immune cells. Tumors can evade immunodetection by a general down-regulation or loss of antigen-presenting molecules, or, more specifically, the loss of immunogenic epitopes (23–25). An outgrowth of epitope-loss tumor variants, which are resistant to immune effector cells, gives the tumor a “competitive edge” for growth in a hostile environment. Another general mechanism of tumor evasion may involve tumor-associated factors, which can cause dysfunction or even death of immune effector cells (26).

It has been generally assumed that p53 accumulation provides an opportunity for presentation to T cells of immunogenic wt p53 epitopes (7, 27) and generation of wt p53 epitope-specific T cells in tumor-bearing hosts. The expected result would be the presence of relatively high frequencies of wt p53 epitope-specific T cells in the circulation of patients with tumors accumulating p53. However, contrary to expectations, the results of our earlier study indicated that CTL could be generated only from PBMCs of the patients whose tumors either did not accumulate p53 or accumulated, but could not present, the p53<sub>264-272</sub> epitope (9). To confirm these unexpected results, we recruited a larger group of HLA-A2.1<sup>+</sup> patients with SCCHN and using tetramer technology, determined frequencies and phenotypic characteristics of T cells specific for the wt p53<sub>264-272</sub> peptide in the peripheral circulation of these patients and a group of healthy controls.

We found the highest frequencies of wt p53<sub>264-272</sub>-specific CD8<sup>+</sup> T cells in a subset of patients with SCCHN whose tumors did not accumulate p53 protein and had a wt p53 genotype. Furthermore, in a subset of patients with tumors accumulating mutant p53, the mean frequency of p53<sub>264-272</sub>-specific T cells did not differ significantly from that in healthy controls. In principle, p53 accumulating in the tumor has an increased opportunity to be presented to immune cells (28). However, it is known that some tumors with mutated p53 are unable to process the wt p53<sub>264-272</sub> epitope. The precedent for blocking of the epitope processing by tumor with a missense mutation at the hotspot codon 273, flanking wt p53<sub>264-272</sub> epitope, has been described by Theobald *et al.* (19). It is possible that additional instances of blocked, altered, or incomplete processing of this as well as other p53 epitopes exist. Another plausible explanation for the observed low



Fig. 9. Immunostaining for p53 on a section of SCCHN in patient #9 (see Table 1). The tumor was HPV-positive. The arrow indicates a tumor cell nucleus stained for p53. Original magnification, ×250. Note that <2% of tumor cells were p53-positive. The features characterizing the immune response of this patient to the tumor are as follows: Antibodies to p53 present in serum. Frequency of the wt p53<sub>264-272</sub> epitope-specific CD8<sup>+</sup> T cells in the peripheral circulation = 1/2746. HPV-16 E6/E7+

frequency of the epitope-specific T cells in patients with mutated p53 could be that recognition of tumors by CTL depends not on p53 accumulation alone but on its turnover and processing by malignant cells, as recently reported by Vierboom *et al.* (29). Thus, it is possible that processing of mutated p53 by the tumor cell proteasome may not lead to optimal presentation of the wt p53<sub>264-272</sub> epitope and effective generation of specific CTL. On the other hand, it has been shown that even when no accumulation of p53 is evident, wt p53 epitope presentation can occur, rendering the tumor susceptible to wt p53-specific CTL (8, 29-31). Thus, accumulation of mutated p53 is not the only criterion associated with the presentation of wt p53 epitope by the tumor and generation of CTL with p53 specificity.

The presence of HPV E6 in tumor cells could also influence p53 processing and CTL generation (29). HPV-16 E6/E7 expression has been reported in a substantial proportion of oral SCCHN (32). Expression of wt p53 in HPV E6-transformed cells is compatible with p53 inactivation, its proteolytic degradation, and enhanced processing, as well as presentation of its epitopes to T cells (29). For this reason, we examined the tumors studied for HPV-16 E6/E7 expression, and sought to correlate it with p53 expression and the frequency of CTL specific for wt p53<sub>264-272</sub> epitope. Multivariate analysis indicated that the frequency of wt p53-specific CTL depended on the p53 status of the tumor and not on its positivity for HPV. Nevertheless, it is interesting to note that in 5 of 7 patients with a relatively high frequency of wt p53-specific CTL, who did not accumulate p53, HPV-16 DNA was detected.

Another explanation for the observed low frequency of wt p53<sub>264-272</sub>-specific T cells in patients with tumors accumulating p53 is that wt p53 epitopes are "self" determinants, and, thus, tolerance to them has to be overcome to induce an immune response. Studies by Theobald *et al.* (33) and Hernández *et al.* (34) demonstrated that tolerance to "self" p53 epitopes in mice is associated with deletion of high avidity T cells and retention of low to intermediate affinity T cells. We have consistently generated comparable anti-wt p53 CTL in humans following *in vitro* sensitization in the presence of epitope-pulsed dendritic cells (8, 9). Others have also reported generation of such CTL (35, 36). Furthermore, the current study shows that precursors of tetramer-positive anti-p53<sub>264-272</sub> T cells exist, albeit with low frequencies, in PBMCs of patients with tumors accumulating p53. Therefore, it is unlikely that CTL precursors of wt p53<sub>264-272</sub>-specific T cells are deleted in cancer patients, as suggested previously. The mechanisms responsible for the failure of these precursor cells to expand *ex vivo* are presently unknown. It is possible that a certain threshold frequency of these precursor cells is needed to overcome anergy to self or immunosuppressive effects of the tumor microenvironment.

This study emphasizes the complexity of tumor-host interactions relevant to anti-wt p53 responses and to the development of wt p53-based vaccines. Our findings suggest that immune precursor cells of the wt p53<sub>264-272</sub> epitope are present in the circulation of HLA-A2<sup>+</sup> patients with SCCHN and that in a subset of these patients, this epitope is immunogenic, results in CTL development, and contributes to shaping immunological memory. In this subset of SCCHN patients, CTL specific for wt p53<sub>264-272</sub> epitope might well have been responsible for elimination of tumors presenting the epitope and the outgrowth of epitope-loss tumor cells able to avoid these effector cells. On the other hand, it is also possible that tumors accumulating mutant p53, which are associated with poor prognosis (37), actively participate in elimination of tumor-specific effector cells, as suggested by studies reported from our laboratories.<sup>4</sup> Tumor-associated apoptosis of such epitope-specific T cells might ac-

count for low frequencies of tetramer-positive CD8<sup>+</sup> T cells in patients with tumors accumulating p53.

A complex interplay of factors, which might determine tumor survival or regression, is best illustrated in patient #9 (Table 1; Fig. 9). A relatively high frequency of the epitope-specific T cells in the circulation, the presence of anti-p53 Abs, accumulation of p53 in a small proportion (~2%) of tumor cells, and tumor positivity for HPV in this patient, suggest that the patient's immune system is actively modulating tumor growth. Delivery of wt p53-based vaccines to patients such as this one could result in a rapid expansion of CTL, which might drive the selection of epitope-loss tumor variants. On the other hand, patients with tumors harboring p53 mutations and a low frequency of wt p53-specific CTL are unlikely to benefit from wt p53-based vaccines, because the expected postvaccine expansion of CTL is unlikely. Strategies that might help to overcome these difficulties include the use of an altered peptide ligand of the p53<sub>264-272</sub> epitope (38), identification of other wt p53 epitopes, which might be more immunogenic than p53<sub>264-272</sub>, as well as MHC-class II restricted p53 epitopes to provide help for generation of antitumor effector cells. We and others are in the process of evaluating several other known HLA-A2.1-restricted wt p53 epitopes in hope of identifying those that may be able to support generation of high-affinity CTL (39). In planning for future p53 vaccines, the use of individualized or personalized vaccines targeting mutant p53 also needs to be revisited, particularly in light of newer evidence that a considerable number of p53 mutations occur within known CTL-defined epitopes in HLA-A2<sup>+</sup> SCCHN patients. However, the efficiency of such vaccines depends on the demonstration that a given mutated p53 epitope can be processed and is immunogenic. Therefore, the selection of an optimal wt p53 peptide for vaccination must await additional studies to define characteristics of other available p53 epitopes.

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# The Ability of Variant Peptides to Reverse the Nonresponsiveness of T Lymphocytes to the Wild-Type Sequence p53<sub>264-272</sub> Epitope<sup>1</sup>

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Recently, we observed that CTL specific for the wild-type (wt) sequence p53<sub>264-272</sub> peptide could only be expanded ex vivo from PBMC of a subset of the HLA-A2.1<sup>+</sup> normal donors or cancer patients tested. Surprisingly, the tumors of the responsive patients expressed normal levels of wt p53 and could be considered unlikely to present this epitope. In contrast, tumors of nonresponsive patients accumulated mutant p53 and were more likely to present this epitope. We sought to increase the responsive rate to the wt p53<sub>264-272</sub> peptide of PBMC obtained from normal donors and patients by identifying more immunogenic variants of this peptide. Two such variants were generated by amino acid exchanges at positions 6 (6T) and 7 (7W) of the peptide. These variants were capable of inducing T cells from PBMC of nonresponsive donors that recognized the parental peptide either pulsed onto target cells or naturally presented by tumors. TCR V $\beta$  analysis of two T cell lines isolated from bulk populations of effectors reactive against the wt p53<sub>264-272</sub> peptide, using either the parental or the 7W variant peptide, indicated that these T cells were expressing identical TCR V $\beta$ 13.6/complementarity-determining region 3/J region sequences. This finding confirms the heteroclitic nature of at least one of the variant peptides identified in this study. The use of variant peptides of the wt p53<sub>264-272</sub> epitope represents a promising approach to overcoming the nonresponsiveness of certain cancer patients to this self epitope, thereby enhancing its potential use in tumor vaccines for appropriately selected cancer patients. *The Journal of Immunology*, 2002, 168: 1338-1347.

Genetic alterations in p53 occur in a wide range of human tumors, including oral squamous cell carcinomas (OSCC)<sup>3</sup> (1). The most common type of genetic alteration in p53 involves a missense mutation that is usually accompanied by accumulation of the altered molecules in the cytosol of tumor cells. Initially, the effort to develop p53-based vaccines focused on these missense mutations, which are tumor specific in nature. However, missense mutations have limited clinical usefulness, because of the requirement that they occur within or create epitopes that could be presented by MHC molecules expressed by the individual patient. On the other hand, the majority of p53 epitopes derived from these altered p53 molecules would be wild type in sequence, representing a new class of tumor-associated self

Ags that are candidates for use in the development of broadly applicable cancer vaccines (1-5).

To date, five MHC class I-restricted, naturally presented human wild-type (wt) sequence p53 epitopes have been identified. They have been shown to be able to induce epitope-specific CTL from PBMC obtained from healthy individuals (1, 6-11). The p53<sub>125-134</sub> epitope is HLA-A24 restricted (11), while the other four, p53<sub>65-73</sub>, p53<sub>149-157</sub>, p53<sub>217-225</sub>, and p53<sub>264-272</sub>, are HLA-A2.1 restricted. Among these, the wt p53<sub>264-272</sub> peptide has been the most intensively investigated (1, 2, 6-8, 12).

The potential of wt p53 epitopes as targets for immunotherapy, however, remains uncertain due to the several critical concerns related to immunological recognition of this truly self tumor Ag. Using HLA-2.1-transgenic wt (p53<sup>+/+</sup>) and p53<sup>null</sup> (p53<sup>-/-</sup>) mice, Sherman and colleagues (13-15) have demonstrated that the CTL repertoire available for wt p53 self epitopes in p53<sup>+/+</sup> mice is limited to intermediate affinity T cells, because the higher affinity CTL are either deleted or tolerated. Apparently, this situation occurs in humans as well, as only CTL with intermediate affinity for the wt p53<sub>264-272</sub> epitope have been generated to date from PBMC obtained from normal donors as well as cancer patients (7, 12). This observation raises the question of whether such CTL are potent enough to be effective in tumor eradication.

Another concern relates to our experience that PBMC obtained from only some HLA-A2.1<sup>+</sup> healthy donors and patients with OSCC were responsive to in vitro stimulation (IVS) with the wt p53<sub>264-272</sub> peptide pulsed onto autologous dendritic cells (DC) (7, 12). Furthermore, CTL reactive against this epitope could only be generated from T cell precursors in PBMC of patients whose tumors were not likely to present this epitope. The analysis of these

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<sup>3</sup>Abbreviations used in this paper: OSCC, oral squamous cell carcinoma; APL, altered peptide ligand; CDR, complementarity-determining region; DC, dendritic cell; IVS, in vitro stimulation; wt, wild type.

tumors indicated no accumulation of p53 or accumulation of mutant p53 with a missense mutation at codon 273, a site known to block processing of the wt p53<sub>264-272</sub> epitope (16). In contrast, PBMC obtained from patients with tumors considered capable of presenting the wt p53<sub>264-272</sub> epitope (i.e., tumors that accumulate mutant p53) were nonresponsive to IVS with wt p53<sub>264-272</sub>-pulsed autologous DC. These findings have led us to conclude that CTL specific for the wt p53<sub>264-272</sub> epitope might play a role in the outgrowth of epitope-loss tumor cells, which are able to escape from the host immune system. This conclusion was further strengthened by the results of a recently completed study in our laboratories that used tetrameric peptide/MHC class I complexes to determine frequencies and characteristics of the p53<sub>264-272</sub>-specific CTL in unstimulated PBMC obtained from 30 OSCC patients and 31 normal donors (data not shown).<sup>4</sup>

Because these observations suggest that it may be possible to accurately predict *ex vivo* the responsiveness of cancer patients to immunotherapy targeting this epitope, we felt that a means of circumventing the nonresponsiveness of individuals needed to be investigated to proceed with the development of wt p53-based vaccines. One solution is to identify a heteroclitic peptide or, in more precise terms, an altered peptide ligand (APL) with enhanced functional activity relative to the parental wt p53<sub>264-272</sub> peptide. By substituting amino acids at various positions of an epitope that contact MHC class I and/or TCR, an array of APL with biological potencies higher than those of the parental epitopes has been identified for various antigenic determinants (17-26). In applying this strategy to the wt p53<sub>264-272</sub> epitope, we anticipated that an APL might induce CTL-mediated responses that cross-react with the parental epitope and that these CTLs also might demonstrate enhanced avidities relative to CTLs induced by the parental peptide. Most importantly, we sought to determine whether an APL would be able to induce anti-wt p53<sub>264-272</sub> CTL from PBMC that were nonresponsive to the parental peptide, particularly the PBMC obtained from patients whose tumors accumulate mutant p53 and are considered to have the potential to present this epitope.

## Materials and Methods

### Cell lines and cell culture

The following HLA-A2<sup>+</sup> OSCC cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA): SCC-4 and SCC-9. The SCC-4 cell line expresses and accumulates p53 expressing a missense mutation at codon 151 but does not present the wt p53<sub>264-272</sub> epitope (6). The SCC-9 cell line expresses an altered p53 molecule with a deletion of codons 274-285. It does not accumulate p53 molecules, yet presents the wt p53<sub>264-272</sub> epitope. In addition, the p53<sup>null</sup> osteosarcoma cell line, SaOS-2, was obtained from ATCC. The cloned p53<sup>+</sup> cell line, SaOS-2Cl3, was derived by transduction of SaOS-2 cells with a p53 cDNA expressing a missense mutation in codon 143 (7). The HLA-A2<sup>+</sup> OSCC cell line PCI-13 has been described previously (27). It expresses a p53 missense mutation in codon 286 (Glu to Lys) and presents the wt p53<sub>264-272</sub> epitope. Tumor cells were cultured in plastic culture flasks (Costar, Cambridge, CA) under standard conditions (37°C, 5% CO<sub>2</sub> in a fully humidified atmosphere) in complete medium consisting of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 50 µg/ml streptomycin, and 50 IU/ml penicillin (all from Life Technologies). The T2 cell line was also obtained from ATCC and maintained in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics. The cultures were routinely tested and found to be free of mycoplasma contamination (Gen-Probe, San Diego, CA).

<sup>4</sup> T. K. Hoffmann, A. Donnenberg, S. Finkelstein, K. Chikamatsu, V. Donnenberg, U. Friebe, E. Appella, A. B. DeLeo, and T. L. Whiteside. Frequencies of tetramer<sup>+</sup> T cells specific for the wild type sequence p53<sub>264-272</sub> peptide in the circulation of patients with head and neck cancer. Submitted for publication.

### Peptides

The CTL-defined, HLA-A2.1-binding peptide, LLGRNSFEV (1), corresponding to wt p53<sub>264-272</sub>, as well as single amino acid exchange variants of this peptide were synthesized by standard *N*-(9-fluorenyl)methoxycarbonyl methodology. Peptides were purified by reversed-phase HPLC, and their amino acid sequence was confirmed by mass spectrometry analysis. All peptides were dissolved in DMSO (Fisher Scientific, Pittsburgh, PA) at 1 mg/ml and diluted with PBS just before use. The 19 variant peptides contain single amino acid exchanges with a bias toward retention of a high degree of similarity to the central region of the parental peptide. The variant peptides are designated 1E, 1F, 1V, 3L, 3F, 3W, 4K, 4L, 5K, 5L, 6G, 6T, 6Y, 7L, 7P, 7Y, 7W, 8A, and 8Y, in which numbers denote the position within the parental sequence and letters refer to exchanged amino acids.

### MHC stabilization assay

T2 cells were incubated overnight at room temperature before use in this assay. Cells were washed and incubated at a cell density of  $2 \times 10^5/0.2$  ml of complete medium with various peptides at final concentrations of  $1 \times 10^{-5}$ – $1 \times 10^{-10}$  M for 3 h at room temperature, followed by a 3-h incubation period at 37°C. After washing with PBS, cells were incubated at 4°C for 30 min with anti-HLA class I mAb, W6/32 (HB95; ATCC), and then with FITC-conjugated goat anti-mouse Ig (Caltag Laboratories, Burlingame, CA) as a secondary Ab. Fluorescence of viable T2 cells was measured at 488 nm in a FACScan flow cytometer (BD Biosciences, San Jose, CA), and the level of MHC class I expression was determined by evaluating the mean fluorescence intensity of stained T2 cells. Cells incubated either at room temperature or 37°C in the absence of peptide served as controls.

### Generation of anti-p53 CTL with peptide-pulsed autologous DC

Peripheral blood or leukapheresis products were obtained from previously studied HLA-A2.1<sup>+</sup> individuals: seven normal donors and six OSCC patients (12). PBMC were isolated by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). The study was approved by the Institutional Review Board at the University of Pittsburgh, and written informed consent was obtained from each individual donating peripheral blood. PBMC were phenotyped for HLA-A2 expression by flow cytometry, using anti-HLA-A2 mAb, BB7.2 (HB82; ATCC), and a mouse IgG isotype as a control. The verification of the A0201 subtype was performed using PCR with sequence-specific primers, as previously described (12).

Human DC were generated from PBMC according to a modification of the method of Sallusto and Lanzavecchia (28), as described by us earlier (7). DC were harvested on day 6, phenotyped by flow cytometry, and then resuspended in AIM-V medium ( $2 \times 10^6$  cells/ml) containing 10 µg/ml peptide and incubated at 37°C for 4 h. The peptide-pulsed DC were then cocultured with autologous PBMC in 24-well tissue culture plates (Costar) in a final volume of 2 ml/well AIM-V medium supplemented with 10% (v/v) human AB serum (Pel-Freez Biologicals, Brown Deer, WI) and 25 ng/ml IL-7 (Genzyme, Cambridge, MA) for the first 72 h and, additionally, with 20 IU/ml IL-2 (Chiron-Cetus, Emeryville, CA) for the remaining time in culture. The lymphocytes were restimulated 1 wk later with peptide-pulsed autologous DC. Irradiated (3000 rad) autologous PBMC were used as APC after the third round of restimulations. Microcultures of CTL lines recognizing the wt p53<sub>264-272</sub> or 7W peptide were isolated from bulk populations of effectors by limiting dilution (1 cell/well/96-well plates), and the lines were maintained in cytokine-supplemented media plus peptide-pulsed APC, as previously described (7). Specificities of generated T cells were determined using one or more of a panel of assays detailed below. The TCR Vβ expression on T cells in bulk CTL populations and cell lines derived from them was done using the IOTest β Mark TCR Vβ Repertoire kit (Beckman Coulter, San Diego, CA).

### ELISPOT assay for IFN-γ

The ELISPOT assay was performed in 96-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA), as previously described by us (29). The capture and detection anti-IFN-γ mAb were purchased from Mabtech (Nacka, Sweden). The spots were counted by computer-assisted image analysis (ELISPOT 4.14.3; Zeiss, Jena, Germany). For Ab-blocking experiments, target cells were preincubated with anti-HLA class I mAb for 30 min. Cryopreserved aliquots of PBMC obtained from a normal donor were thawed and, after stimulation with PMA (1 ng/ml) and ionomycin (1 µM; both from Sigma-Aldrich, St. Louis, MO), were used as a positive control for each assay.

The interassay reproducibility of the assay was acceptable with a coefficient of variation = 15% ( $n = 30$ ).

#### Cytotoxicity assay

The 4-h <sup>51</sup>Cr release assay was performed at various E:T ratios, as previously described (7). Briefly, sensitized targets were labeled with <sup>51</sup>Cr for 45 min at 37°C, washed, and added to wells of 96-well plates ( $1 \times 10^4$  cells/well). Effector T cells were then added to give various E:T ratios. When Ab-blocking experiments were performed, target cells were incubated with anti-HLA class I mAb or the anti-HLA-DR mAb, L243 (HB55; ATCC), for 30 min before adding effector cells. The percentage of specific lysis was calculated according to the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{control cpm}}{\text{maximal cpm} - \text{control cpm}} \times 100$$

#### Flow cytometry analysis using HLA-A2.1/peptide tetrameric complexes (tetramer)

The streptavidin-PE-labeled tetramers used in this study were obtained from the tetramer core facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA). Three-color flow cytometry assays (FACSscan; BD Biosciences) were performed with PerCP anti-CD3, FITC anti-CD8, and PE-tetramer. The specificity of the HLA-A2.1/p53<sub>264-272</sub> tetramer was confirmed by its staining of a CTL line specific for this p53 epitope and by the lack of staining of irrelevant CTL or HLA-A2<sup>-</sup> PBMC of healthy donors, as previously described (30). The additional PE-conjugated HLA-A2.1/tetramer used in this study contained the 7W variant peptide. Generally, 75,000 events per sample were collected progressively after live gating on lymphocytes by forward and side scatter.

#### TCR and CDR3 spectratyping

RNA was extracted from p53<sub>264-272</sub>-specific CTL lines generated using parental or the 7W variant peptide, followed by reverse transcription into cDNA, as previously described (31). Screening for expression of TCR V chains was performed using the primers described by Puisieux et al. (31) for TCR V $\beta$  amplification, followed by a runoff reaction with fluorophore-labeled primers specific for the C region of the TCR $\beta$  (5'-TGTCAC CTCCTTCCATTCCACC) chain. Labeled runoff products were subjected to DNA fragment analysis, as described (32). Finally, amplified products were directly subjected to DNA sequence analysis using ABI 310 sequencer (PerkinElmer, Weiterstadt, Germany).

#### Statistical analysis

A two-tailed Wilcoxon rank sum test was performed to analyze ELISPOT data. An unpaired two-tailed Student's *t* test was used to interpret differences in CTL reactivities against different target cells and in the presence of blocking Ab in cytotoxic assays, and differences between the number of spots obtained from T cells incubated with T2 cells pulsed with relevant p53 peptides vs that obtained using T2 cells pulsed with the irrelevant

gp100 peptide in ELISPOT assays. Differences were considered significant when  $p < 0.05$ .

## Results

### Selection of variant p53<sub>264-272</sub> peptides recognized by anti-p53<sub>264-272</sub> CTL

Because the parental peptide binds efficiently to HLA-A2.1 molecules, all of the APL considered in this study represent single amino acid exchanges at nonanchor residues for the purpose of enhancing the interactions of the variant peptides with the TCR rather than MHC class I molecules. Nineteen variants of the wt p53<sub>264-272</sub> peptide were screened for their recognition by a bulk population of anti-wt p53<sub>264-272</sub>-specific CTL that was maintained in our laboratory (7). T2 cells pulsed with the individual peptides at a fixed concentration of  $1 \times 10^{-6}$  M peptide served as targets for these CTL in a <sup>51</sup>Cr release cytotoxic assay. Significant cytotoxic reactivity against T2 cells pulsed with three of the 19 variant peptides, namely, 6T, 7W, and 7P, was detected (data not shown). Therefore, these three variant peptides were selected for further characterization.

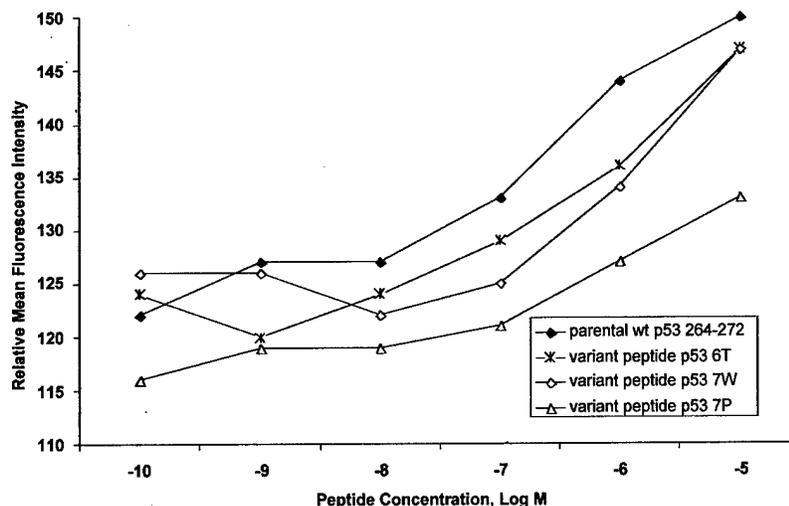
### Variant peptide binding to HLA-A2.1 molecules

Binding of the 6T, 7W, and 7P variant peptides to HLA-A2.1 molecules was compared with that of the parental peptide in an MHC stabilization assay. The relative mean fluorescence intensity of parental and variant peptide-stabilized HLA-A2 molecules on T2 cells is shown in Fig. 1. All the peptides showed stabilization of HLA-A2 molecules in a dose-dependent manner within the concentration range of  $1 \times 10^{-5}$ – $1 \times 10^{-9}$  M. However, in general, the binding affinities of the variant peptides to HLA-A2.1 molecules on T2 cells were slightly lower than that of the parental wt peptide (wt > 6T  $\geq$  7W > 7P).

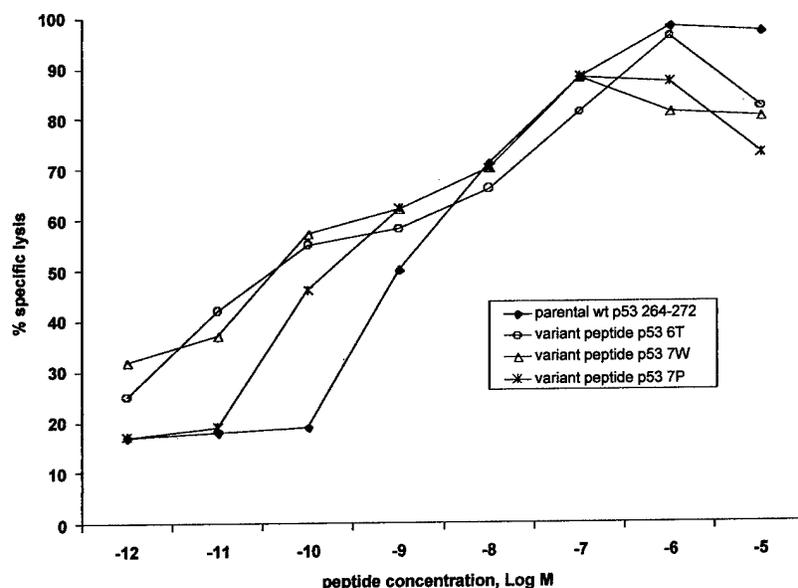
### Affinity of p53<sub>264-272</sub>-specific CTL for variant peptides

The affinity of the bulk population of anti-p53<sub>264-272</sub>-specific CTL for the variant peptides was determined in a 4-h <sup>51</sup>Cr release assay using T2 cells pulsed with these peptides at concentrations ranging from  $1 \times 10^{-5}$  to  $1 \times 10^{-12}$  M as target cells. As shown in Fig. 2, at concentrations  $< 1 \times 10^{-8}$  M, the dose-response curves of the three variant peptides were shifted to the left relative to that of the parental wt peptide. Because the increased responsiveness of the CTL for these variant peptides cannot be attributed to enhanced binding to HLA-A2.1 molecules, these results are consistent with an increased affinity of TCR for the variant peptides.

**FIGURE 1.** Identification of three HLA-A2.1-binding variant peptides of the wt p53<sub>264-272</sub> epitope. T2 cells were incubated with parental p53<sub>264-272</sub> peptide (LLGRNSFEV) or 6T, 7P, or 7W variant peptides at final concentrations of  $1 \times 10^{-5}$ – $1 \times 10^{-10}$  M. The relative mean fluorescence intensities of FITC-conjugated anti-MHC class I mAb (W6/32) are indicative of peptide-stabilized MHC class I molecules on T2 cells.



**FIGURE 2.** Variant peptides are recognized by anti-wt p53<sub>264-272</sub>-specific CTL line. T2 cells were pulsed with different peptide concentrations and tested as targets in a 4-h <sup>51</sup>Cr release assay at the E:T ratio of 10:1.



#### Characterization of wt p53<sub>264-272</sub>-specific CTL generated from PBMC obtained from normal donors using variant peptides

Previously, we reported that CTL reactive against the wt p53<sub>264-272</sub> epitope could be generated from PBMC obtained from only two of the seven HLA-A2.1<sup>+</sup> normal donors tested (12). Analyses involving multiple cryopreserved samples derived from leukopaks obtained from two of the normal donors (a responder and a nonresponder) confirmed the consistency of responses of these donors' PBMC to the parental peptide. In the same experiments in which the seven donors' PBMC were tested for induction using the parental peptide, CTL reactive against this peptide could be generated from five of these seven PBMC using either the 6T or 7W variant peptide. Included in this group were three nonresponsive PBMC (Table I); PBMC obtained from donors 6 and 7 responded to the 7W variant, while PBMC obtained from donor 4 responded to the 6T peptide. None of the seven PBMC tested responded to the 7P variant peptide.

The bulk populations of variant-induced cells generated from PBMC obtained from donors 6 and 7 effectively recognized and lysed T2 cells pulsed with the parental peptide in ELISPOT for IFN- $\gamma$  and cytotoxicity assays. Fig. 3, A and B, shows the results obtained with the effectors generated from PBMC obtained from donor 7. Unpulsed T2 target cells or T2 cells pulsed with an ir-

relevant HLA-A2.1-binding peptide, the melanoma-associated gp100 peptide (33), were not recognized by these CTL in either assay to any noticeable extent. More importantly, these effector cells were also capable of recognizing the naturally presented epitope, as evidenced by their ability to lyse PCI-13 and SCC-9 tumor cells as well as mutant p53-transfected SaOS-2 C13 cell lines in a MHC class I-restricted manner (Fig. 3C). No significant cytotoxicity was noted against HLA-A2<sup>+</sup> tumor cell lines, SCC-4 and SaOS-2, which do not present the epitope.

The CTL generated from PBMC obtained from donor 4 using the 6T peptide yielded effectors with reactivity comparable with that of the 7W variant-induced CTL. The 6T-induced T cells were responsive to wt p53<sub>264-272</sub>-pulsed T2 cells in the ELISPOT for IFN- $\gamma$  assay (Fig. 4A), and cytolytic against the OSCC lines, SCC-9 and PCI-13, as well as SaOS-2C13 (Fig. 4B). This response was blocked by anti-HLA class I mAb but not anti-HLA-DR mAb. No significant reactivity was obtained against the tumor cell line SCC-4. The reactivity of these effectors against SaOS-2 cells in the analysis shown in Fig. 4B was higher than normally detected against this p53<sup>mut</sup> cell line, using bulk populations of anti-p53 effectors (7, 12). However, the reactivity of the 6T-induced effectors against SaOS-2 targets was not significantly blocked by anti-HLA class I mAb and thus could be attributed to nonspecific effectors present in the bulk population. In summary, variant-induced effector T cells had similar reactivities against the parental epitope as those reported previously for the parental peptide-induced effectors from responsive normal donors as well as OSCC patients (7, 12).

Table I. Summary of the anti-p53 CTL responses of PBMC obtained from normal donors following IVS using variant p53<sub>264-272</sub> peptides<sup>a</sup>

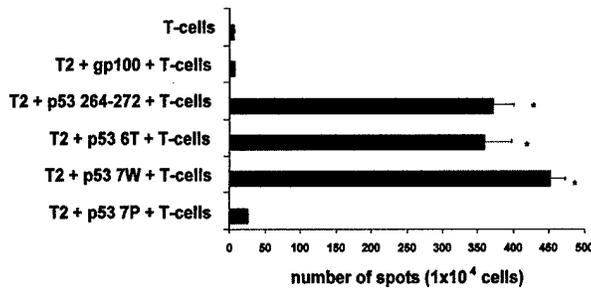
Donor	Anti-p53 CTL Response After IVS With			
	wt p53 <sub>264-272</sub>	Variant 6T	Variant 7P	Variant 7W
1	+	-	-	+
2	+	-	-	+
3	-	-	-	-
4	-	+	-	-
5	-	-	-	-
6	-	-	-	+
7	-	-	-	+

<sup>a</sup> PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; - indicates that no specific reactivity against the wt p53<sub>264-272</sub> peptide was observed, while + indicates that effectors were reactive against variant and parental peptides. Results using the parental wt p53<sub>264-272</sub> were reported in a previous publication from this laboratory (12).

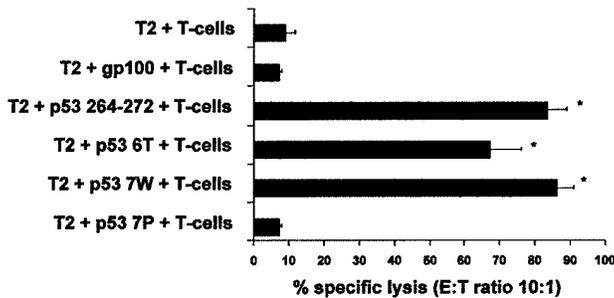
#### Characterization of wt p53<sub>264-272</sub>-specific CTL generated from PBMC of a nonresponsive OSCC patient using a variant peptide

The critical test of the variant peptides was whether their use could induce CTL capable of recognizing the anti-wt p53<sub>264-272</sub> epitope from nonresponsive patients whose tumors were considered capable of presenting this epitope (12). The nonresponsiveness of PBMC obtained from at least one of these donors, patient 3, has been repeatedly confirmed during the past 2 years using blood samples obtained at different times, as well as multiple cryopreserved leukapheresis samples obtained from this patient. As shown in Table II, none of the PBMC from three of these patients responded to the 6T or 7P variant peptides. However, the 7W variant

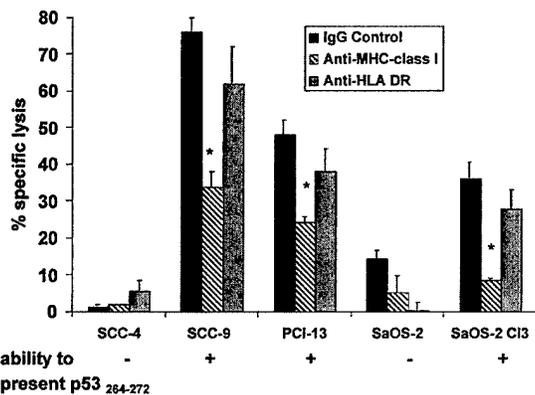
**A. ELISPOT**



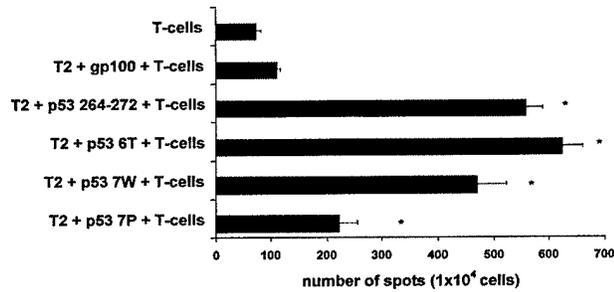
**B. <sup>51</sup>Cr-Release Assay**



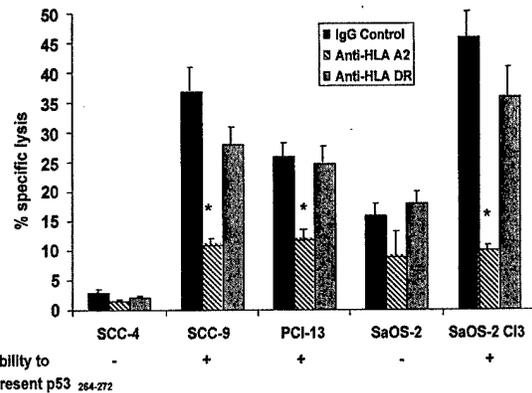
**C. <sup>51</sup>Cr-Release Assay**



**A. ELISPOT**



**B. <sup>51</sup>Cr-Release Assay**



**FIGURE 4.** The 6T variant peptide-induced effectors induced from PBMC obtained from nonresponsive healthy donor recognize the parental wt p53<sub>264-272</sub> epitope pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT assays. Effectors were tested against T2 cells pulsed with either an irrelevant gp100 peptide, the wt p53<sub>264-272</sub> peptide, or a variant peptide at 10 μg/ml. *B*, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. \*, A significant ( $p < 0.05$ ) difference relative to IgG controls.

**FIGURE 3.** The 7W variant peptide-induced effectors induced from PBMC obtained from a nonresponsive healthy donor recognize the parental wt p53<sub>264-272</sub> peptide pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT for IFN-γ assays. Effectors were tested against T2 cells pulsed with an irrelevant gp100 peptide, the wt p53<sub>264-272</sub> peptide, or the variant peptides at 10 μg/ml. *B*, Lysis of T2 cells pulsed with various peptides at an E:T of 1:10. *C*, Lysis of tumor targets naturally presenting the epitope at an E:T ratio of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. \*, A significant ( $p < 0.05$ ) difference relative to IgG controls.

peptide in immunotherapy targeting the wt p53<sub>264-272</sub> epitope in individuals like OSCC patient 3.

*Tetramer-binding and TCR Vβ usage by T cell microcultures reactive against parental and/or variant peptides*

The ability of the variant peptides to induce the generation of CTL specific for wt p53<sub>264-272</sub> from nonresponder PBMC raised the question of the relationship between these CTL and those induced by the parental peptide in responder PBMC. The need to investigate this relationship became evident when the cross-reactive bulk population of CD8<sup>+</sup> T cells induced with the 7W variant peptide from normal donor 7 was stained with the parental or variant tetramer. Whereas only ~2% tetramer<sup>dim</sup> cells were detected with the parental tetramer, a cluster of ~40% tetramer<sup>bright</sup> cells was detected with the 7W tetramer (Fig. 6, *A* and *B*). One possible explanation for this observed difference was that the variant peptide induced a single CD8<sup>+</sup> T cell population that bound the variant tetramer with higher avidity/stability than did the parental tetramer. Another possible explanation was that the variant peptide induced two distinct populations of CD8<sup>+</sup> T cells; one was cross-reactive and bound both tetramers (most likely with different avidities), while the other was specific for the 7W variant and bound the 7W tetramer with high avidity. The two possibilities could be

peptide did induce the ex vivo generation of anti-wt p53<sub>264-272</sub> CTL from PBMC of patient 3, whose autologous tumor, PCI-13, presents this epitope (7, 12). The affinity of these effectors for the parental epitope was comparable with that of a bulk population of CTL induced using the parental epitope, and was in the range of  $1 \times 10^{-9}$  M (Figs. 2 and 5A). Furthermore, the 7W-induced CTL were cytotoxic against a panel of tumor cell lines naturally presenting the wt p53<sub>264-272</sub> epitope, including the autologous PCI-13 cell line, and this reactivity was MHC class I restricted (Fig. 5B). This result clearly illustrates the potential value of the 7W variant

Table II. Summary of the anti-p53 CTL responses of PBMC obtained from nonresponsive OSCC patients following IVS using variant p53<sub>264-272</sub> peptides

Patient	Tumor <sup>a</sup>		Anti-p53 CTL Response After IVS <sup>b</sup>		
	p53 genotype	p53 protein	Variant 6T	Variant 7P	Variant 7W
1	Mutant R248W	+	-	-	-
2	Mutant V 157 F	+	-	-	-
3	Mutant E286K	+	-	-	+

<sup>a</sup> Patients' tumors were analyzed for genetic alterations in p53 exons 5-8, and the identified codon and missense mutations are denoted. The level of p53 expression in tumors was determined by immunohistochemistry, using anti-p53 mAb, and + denotes accumulation of p53 (12).

<sup>b</sup> PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; - indicates that no specific reactivity against the wt p53<sub>264-272</sub> peptide was observed, while + indicates that effectors were reactive against variant and parental peptides.

distinguished based on TCR usage of the T cells involved in recognition of these peptides. To accomplish this, T cell microcultures were established by limiting dilution from bulk CTL populations induced with either parental or variant peptide. Several T cell clones from each type of microculture were expanded for further analysis. Based in part on their rates of proliferation as well as

peptide specificities (Fig. 7), four oligoclonal T cell lines, designated 2, 4, 53, and 68, were selected for TCR analysis by complementarity-determining region (CDR)3 spectratyping.

Two of the cell lines analyzed, 53 and 68, were derived from the bulk population of 7W-induced CTL that was described above and shown to exhibit differential staining with the parental and 7W

### A. ELISPOT

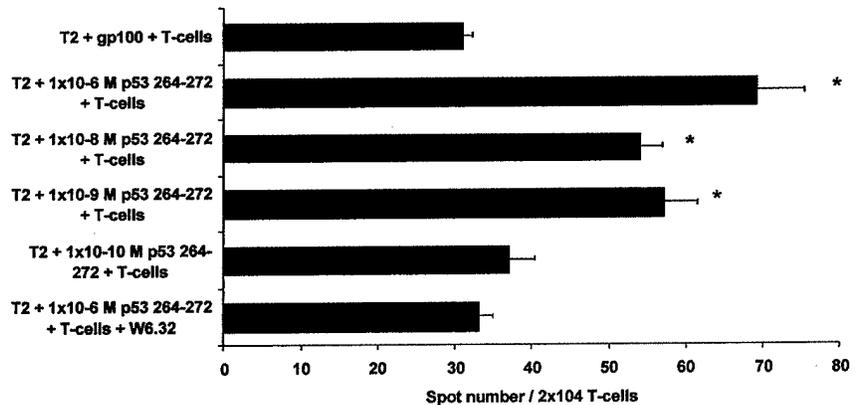
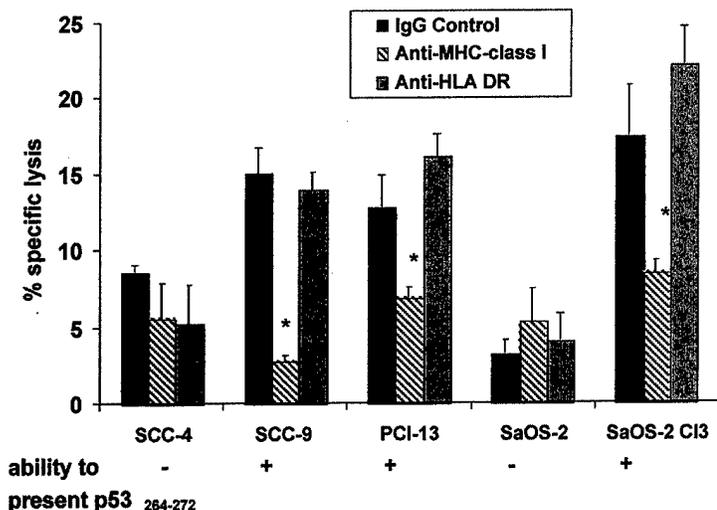
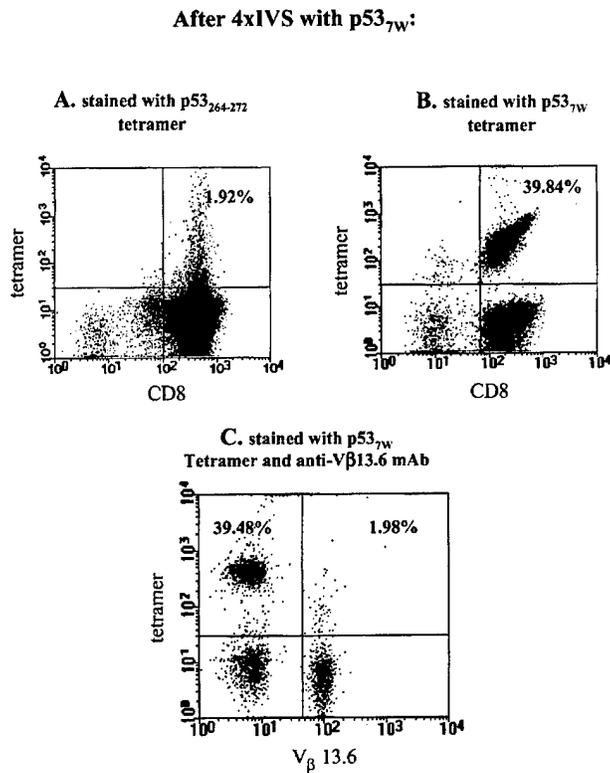


FIGURE 5. The 7W variant peptide-induced effectors obtained from a nonresponsive OSCC patient recognize the parental wt p53<sub>264-272</sub> peptide pulsed onto target cells or naturally presented by tumors, including PCI-13, the autologous tumor cell line. A, Affinity of effectors for the parental wt p53<sub>264-272</sub> peptide as determined in ELISPOT assay. T2 cells were incubated with parental peptide at concentrations of 1 × 10<sup>-6</sup>–1 × 10<sup>-10</sup> M. T2 cells pulsed with an irrelevant gp100 peptide served as a control. B, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. \*, A significant (p < 0.05) difference relative to IgG controls.

### B. <sup>51</sup>Cr-Release Assay





**FIGURE 6.** CD8<sup>+</sup> cells induced from PBMC of a nonresponsive normal donor using the 7W variant peptide that recognizes the parental peptide express TCR Vβ13.6. Three-color flow cytometry analysis of CD8<sup>+</sup> cells stained with HLA-A2.1 tetramers containing either the parental peptide (A), 7W variant peptide (B), or the 7W tetramer and anti-Vβ13.6 mAb (C). The numbers in the *upper right quadrants* indicate the percentage of tetramer<sup>+</sup> cells. The analyses shown in A and B involved 75,000 events, while in C 10,000 events were analyzed.

tetramers (Fig. 6). Although tetramer analysis of the bulk population suggested that the vast majority of CTL were 7W specific, one of the several T cell clones isolated was cross-reactive. The 53 cell line, which was specific for the 7W variant, was found to express Vβ9. The 68 cell line, which recognized the variant as well as the parental peptide, was found to express Vβ13.6 with completely different CDR3 and J regions from those expressed by line 53 (Table III). Consistent with these findings was the result of a combined tetramer/Vβ expression flow analysis of the bulk population from which the 53 and 68 cell lines were derived. We observed that the ~40% cells that stained as a distinct cluster with the 7W tetramer were Vβ13.6<sup>-</sup>, whereas the ~2% cells that stained weakly with this tetramer were Vβ13.6<sup>+</sup> (Fig. 6C).

The other two cell lines analyzed, 2 and 4, were derived from bulk populations of effectors induced with the parental peptide from PBMC obtained from a patient (patient 2 in Ref. 12) and a normal donor (donor 2, Table I), respectively. The 2 cell line was found to express Vβ13.6, with identical motifs for the CDR3 and J regions as the 68 cell line, which was induced from a different individual using the 7W peptide (Table III). The observation that cross-reactive T cells induced by the variant peptide from PBMC obtained from one donor express the identical TCR/CDR3/J region as that expressed by T cells induced with the parental peptide from another donor illustrates the heteroclitic nature of the 7W variant peptide.

The 4 cell line was shown to express Vβ1 with a CDR3 and J region sequence distinct from those of any of the other cell lines analyzed (Table III). Interestingly, Vβ analysis of the bulk popu-

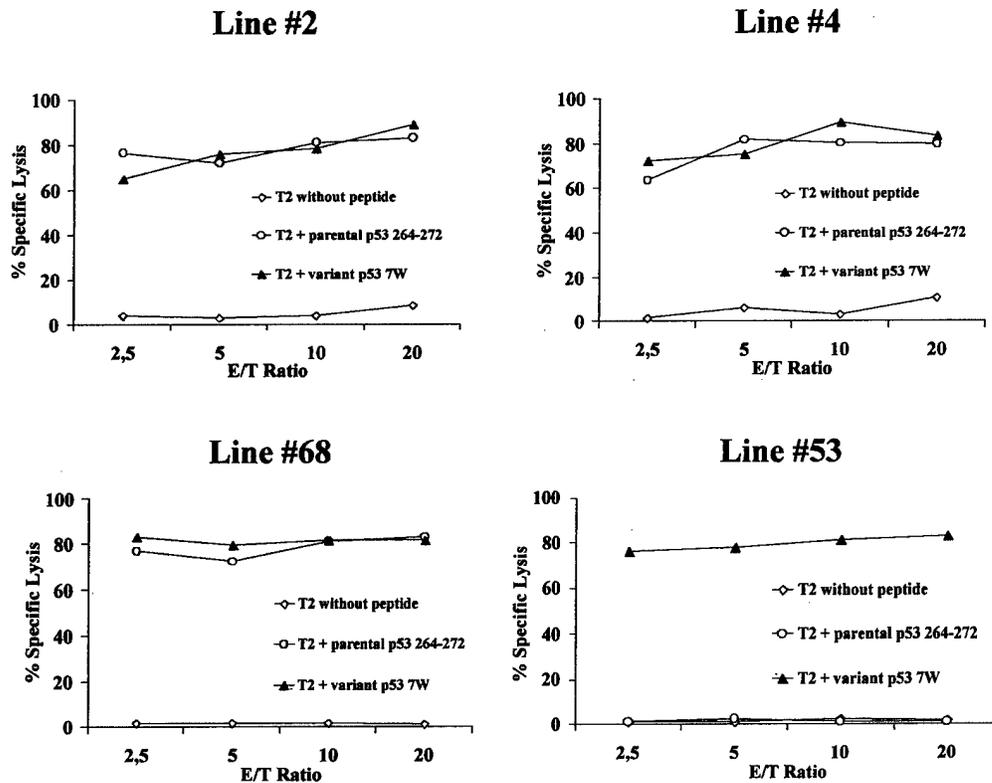
lation of effectors from which the 4 cell line was isolated detected mainly Vβ1 and Vβ13 CD8<sup>+</sup> cells. Another bulk population of parental peptide-induced CTL, which was obtained from a different normal donor (7) and used to identify 7W and 6T peptides as potential APLs (Table II), was found to consist of >90% Vβ1<sup>+</sup> cells (data not shown). These results, summarized in Table IV, are strongly suggestive of a relatively limited TCR Vβ usage being involved in recognition by CTL of the HLA-A2.1-restricted, wt p53<sub>264-272</sub> epitope, regardless of whether these cells are induced by the parental or variant peptide.

## Discussion

Most studies of APL of tumor Ags involve amino acid exchanges at anchor residue positions of the peptide to enhance its binding to class I MHC molecules. The recent modification of the HLA-A2.1-restricted wt p53<sub>149-157</sub> peptide at anchor position 2 to improve its binding to the restriction element and immunogenicity is one example of this approach (26). Particularly relevant to this study are variants designed to enhance TCR/peptide interactions rather than increase MHC binding, such as the HLA-A2.1-restricted, melanoma-associated MART1/Melan A<sub>27-35</sub> and carcinoembryonic Ag, CAPI, peptides, which involve amino acid exchanges in residues other than anchor positions (18-21). While the binding affinities of these variant and parental peptides to HLA-A2.1 molecules are comparable, amino acid exchanges of these peptides at nonanchor positions yielded variant peptides that were more immunogenic than the parental peptides.

Since the parental wt p53<sub>264-272</sub> peptide has a reasonable affinity for HLA-A2.1 molecules ( $>1 \times 10^{-9}$  M), the 19 p53<sub>264-272</sub> variants designed for this study had unmodified anchor positions. Among the amino acid exchanges tested, those at position 6 (6T) and position 7 (7W) appeared to be promising. Since both variants have lower affinities than the parental peptide for HLA-A2.1 molecules, their ability to increase the frequency of anti-p53<sub>264-272</sub> CTL responses generated from nonresponsive PBMC does not appear to be due to their enhanced binding to HLA-A2.1 molecules. Instead, their increased immunogenicity might be due to the replacement or counterbalancing of residues causing adverse TCR-peptide interactions. Such a replacement could result in an improved interaction of the peptide/MHC complex with TCR and a subsequent expansion of T cells capable of recognizing the parental epitope (23, 24). Two lines of evidence support this conclusion. First, using the parental tetramer to determine the frequency of tetramer<sup>+</sup> precursor T cells in unstimulated PBMC obtained from normal donors and patients with cancer, we found that most of the nonresponsive individuals had markedly lower frequencies of these cells in their peripheral circulation than did the responders (data not shown).<sup>4</sup> Second, the parental and variant peptides were found to engage and expand T cells expressing the same TCR in PBMC obtained from responsive and nonresponsive donors (see Tables III and IV). These findings support the concept that increased stability of interaction with the TCR is the basis for the enhanced functional activity of the 7W variant peptide.

Although the use of variant peptides did reverse the nonresponsiveness in IVS of PBMC obtained from some donors, their use did not yield high-affinity CTL. The persistence of low-affinity CTL against self tumor peptides, such as wt p53 epitopes, which is considered a true consequence of tolerance (15), might be due to a limited TCR repertoire being available for recognition of these epitopes. Our analyses detected the predominant use of only two TCR Vβ families, Vβ1 and Vβ13.6, being involved in CTL recognition of the wt p53<sub>264-272</sub> epitope in four different donors. Furthermore, in two different donors, identical usage by the parental



**FIGURE 7.** Specificity of T cell lines analyzed for TCR V $\beta$  usage. Lines 2 and 4 were derived from bulk populations of effectors induced from an OSCC patient and a normal control, respectively, using the parental peptide. Lines 53 and 68 were derived from a bulk population of effectors induced from a nonresponsive healthy donor with the 7W variant peptide. The 53 cell line is specific for the 7W variant, while the 68 cell line is cross-reactive.

and variant peptide was detected. In contrast, an analysis of responses in HLA-A2.1<sup>+</sup> patients to repeated immunizations with an anchor position-variant peptide of the melanoma-associated gp100<sub>209-217</sub> epitope demonstrated that the appearance of higher-affinity T cells was associated with an expansion of the TCR repertoire rather than an increased oligoclonal response (33). In the future, additional data on TCR usage of cross-reactive and variant-specific CTL could allow for extensive molecular modeling of the interactions within the trimeric complexes and, perhaps, the design of APL with more enhancing properties than those of the 6T and 7W variants. These variants might engage more diverse populations of T cells that are capable of cross-recognition of the parental epitope with, perhaps, higher avidity. However, the apparent outgrowth of epitope-loss tumors in OSCC patients responsive to this epitope suggests that even intermediate-affinity CTL recognizing wt p53<sub>264-272</sub> might be effective in tumor eradication (12).

To fully estimate the potential of p53-based vaccines in immunotherapy of cancer, it is becoming increasingly apparent that an array of T cell-defined wt p53 epitopes needs to be analyzed, and strategies for optimal induction of T cells recognizing these epitopes need to be further evaluated. In this regard, the use of genetically modified DC expressing intact wt p53 appears to enhance the generation and increase the frequency of antitumor effectors from PBMC of normal donors and cancer patients (34). The p53-based immunotherapy also might be critically dependent on targeting the right epitopes and matching a patient's ability to respond *ex vivo* to wt p53 epitopes with the potential of his/her tumor to present these epitopes for immune recognition. Again, of course, it is necessary to be aware that a patient's *ex vivo* responsiveness to these epitopes does not guarantee a successful *in vivo* response to immunization with them. In this study, HLA-A2.1<sup>+</sup> patient 3 with OSCC, for whom the tumor cell line and tumor-specific CTL are available in the laboratory, has been

**Table III.** Amino acid sequences of monoclonal TCR transcripts expressed in four parental and/or variant p53<sub>264-272</sub>-specific CTL lines<sup>a</sup>

Line	IVS	Specificity	V $\beta$ Family	Sequences		
				V $\beta$	CDR3 region	J region
2	wt	wt + 7W	V $\beta$ 13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3
4	wt	wt + 7W	V $\beta$ 1	LELGDSALYFCA	SSEGGL	ETQYFGPGTRLT/BJ2-5
53	7W	7W	V $\beta$ 9	LGDSAVYFCA	SSAGTNT	YEQYFGPGTRLT/BJ2-7
68	7W	wt + 7W	V $\beta$ 13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3

<sup>a</sup> Lines 2 and 4 were stimulated with the parental wt p53<sub>264-272</sub> peptide (wt) and were reactive against the parental and 7W variant peptides. Lines 53 and 68 were stimulated with the 7W variant peptide. Line 53 was reactive against the 7W variant peptide only, while line 68 was reactive against the parental and 7W variant peptides. Single peaks in individual TCR variable chain families, suggesting clonality, were analyzed by direct sequencing of the PCR products.

Table IV. Summary of evidence of limited TCR V $\beta$  usage for CTL recognition of the wt p53<sub>264-272</sub> epitope<sup>a</sup>

PBMC Donors <sup>b</sup>	Induced with p53 <sub>264-272</sub> Peptide	Bulk T Cell Population V $\beta$ Usage	Derived T Cell Lines <sup>c</sup>	
			V $\beta$ usage	p53 peptide specificity
Normal donor 2 (R)	wt	V $\beta$ 1, 13.6	4 V $\beta$ 1	wt/7W
Normal donor 4 <sup>d</sup> (R)	wt	V $\beta$ 1	ND	
Normal donor 7 (NR)	7W	V $\beta$ 9, 13.6	53 V $\beta$ 9	7W only
OSCC patient 2 <sup>f</sup> (R)	wt	V $\beta$ 13.6	68 V $\beta$ 13.6 <sup>e</sup>	wt/7W
			2 V $\beta$ 13.6 <sup>e</sup>	wt/7W

<sup>a</sup> See Table III for the details on TCR V $\beta$  usage.

<sup>b</sup> Normal donors and patients identified in Tables I and II; R, responsive to IVS of PBMC to the wt p53<sub>264-272</sub> peptide; NR, nonresponsiveness.

<sup>c</sup> T cell lines derived by limiting dilution.

<sup>d</sup> Normal donor used as source of PBMC for induction of a bulk population of CTL specific for wt p53<sub>264-272</sub>. Generation and characterization of this cell line was detailed in Ref. 7.

<sup>e</sup> These T cell lines express identical V $\beta$ , CDR3, and J region sequences (see Table III).

<sup>f</sup> The OSCC patient 2 identified in Table I in Ref. 12.

of particular interest. The tumor cell line established from this patient and designated PCI-13 accumulates p53 molecules expressing a missense mutation at codon 286 and naturally presents the p53<sub>264-272</sub> epitope, albeit following pretreatment with IFN- $\gamma$  (7, 12). The ability to generate anti-p53<sub>264-272</sub> CTL with the 7W variant from this patient's PBMC, which were nonresponsive to the parental peptide, provides a basis for the potential use of the 7W variant peptide in immunotherapy of this patient and, perhaps, other nonresponsive OSCC patients with tumors expressing similar characteristics. Concurrently, it needs to be determined whether the trends observed in OSCC patients regarding their responsiveness to wt p53 epitopes and the potential of their tumors to present these epitopes are also apparent in patients with other types of cancers.

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