

Andreas E. Albers · Robert L. Ferris · Grace G. Kim
Kazuaki Chikamatsu · Albert B. DeLeo
Theresa L. Whiteside

Immune responses to p53 in patients with cancer: enrichment in tetramer+ p53 peptide-specific T cells and regulatory T cells at tumor sites

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Abstract Objective: A majority of human cancers, including head and neck cancer (HNC), “overexpress” p53. Although T cells specific for wild-type (wt) sequence p53 peptides are detectable in the peripheral blood of patients with HNC, it is unknown whether such T cells accumulate in tumor-involved tissues. Also, the localization of “regulatory” T cells (Treg) to tumor sites in HNC has not been investigated to date. **Methods:** Tumor infiltrating lymphocytes (TIL), tumor-involved or non-involved lymph node lymphocytes (LNL) and peripheral blood mononuclear cells (PBMC) were obtained from 24 HLA-A2.1+ patients with HNC. Using tetramers and four-color flow cytometry, the frequency of Treg and CD3+CD8+ T cells specific for wt p53 epitopes as well as their functional attributes were determined. **Results:** The CD3+CD8+ tetramer+ cell frequency was significantly higher ($P < 0.001$) in TIL than autologous PBMC as was the percentage of CD4+CD25+ T cells ($P < 0.003$). TIL were enriched in FOXP3+, GITR+ and CTLA-4+ Treg. CD8+ TIL had low Z expression and produced little IFN- γ after ex vivo stimulation relative to autologous PBMC or PBMC from NC. **Conclusions:** Anti-wt p53 epitope-specific T cells and Treg preferentially localize to tumor sites in

patients with HNC. However, despite enrichment in tumor peptide-specific T cells, the effector cell population (CD3+CD8+) in TIL or PBMC was unresponsive to activation in the tumor microenvironment enriched in Treg.

Keywords TIL · Tetramers · wt p53 epitopes · CD4+CD25+ T cells · Regulatory T cells (Treg) · Tumor escape

Introduction

Effective antitumor responses in individuals with cancer depend on the presence and function of immune cells that are able to recognize and eliminate tumor cells. Although such tumor antigen-specific immune T cells are known to be present in the peripheral circulation and tissues of patients with cancer, the tumor often evades their effects through various mechanisms referred to as “escape mechanisms” [17, 29]. An obvious consequence of tumor escape from immune surveillance is cancer progression and metastasis. Molecular events that result in a loss of p53 function [11, 12] characterize the majority of human malignancies [5, 20]. By a variety of mechanisms, wild-type (wt) sequence p53 peptides are processed and presented to the host immune cells either by the tumor or by dendritic cells (DC), because wt p53 peptide-specific T cells and, in some instances, p53-specific antibodies are detectable in patients with cancer [1, 7, 25]. We have recently reported that the frequency of tetramer+ CD8+ T cells specific for the HLA-A2.1-restricted wt p53₂₆₄₋₂₇₂ peptide is significantly higher in the peripheral circulation of HLA-A2.1+ patients with head and neck cancer (HNC) than in normal donors [7]. Further, vaccination of patients with ovarian carcinoma with p53-based vaccines leads to an

A. E. Albers · R. L. Ferris · G. G. Kim
K. Chikamatsu · A. B. DeLeo · T. L. Whiteside
University of Pittsburgh Cancer Institute
and Departments of Pathology (ABD, TLW),
Immunology and Otolaryngology (RLF, TLW),
University of Pittsburgh School of Medicine,
Pittsburgh, Pennsylvania 15213 USA

T. L. Whiteside (✉)
Research Pavilion at the Hillman Cancer Center,
University of Pittsburgh Cancer Institute,
5117 Centre Avenue, Suite 1.27, Pittsburgh,
PA, 15213 USA
E-mail: whitesidetl@upmc.edu
Tel.: +1-412-6240096
Fax: +1-412-6240264

increased frequency of tetramer+ wt p53₂₆₄₋₂₇₂ specific T lymphocytes in the peripheral circulation, as recently shown by us [6]. However, the presence of wt p53 peptide-specific T cells in the periphery has no apparent effect on tumor progression, and it has been suggested that these T cells either do not reach the tumor nor are not functional in the tumor microenvironment.

In this manuscript, we investigated these two possibilities by: (a) determining the frequency of wt p53₂₆₄₋₂₇₂ peptide- or wt p53₁₄₉₋₁₅₇ peptide-specific T cells in the circulation and at the tumor site in a cohort of HLA-A2.1+ patients with HNC; and (b) evaluating the extent of accumulation of CD4+CD25+ T cells at the tumor site in the same patients. Recent reports in the literature on suppressor activities of Treg in patients with tumors [2, 16, 31] provide a strong rationale for this experimental approach. The hypothesis tested in this study predicts that the accumulation of CD4+CD25+ T cells, which are enriched in FOXP3+, GITR+ and CTLA-4+ Treg, at the tumor site could negatively impact antitumor functions of tumor-specific CD8+ effector lymphocytes.

Materials and methods

Patients and tumor tissues

Patients with HNC included in this study ($n=24$) were cared for at the Oral Cancer Center, University of Pittsburgh. All patients underwent surgery for the treatment of primary squamous cell carcinoma of the head and neck (SCCHN). Tumor tissues or lymph nodes were collected after the patients signed the IRB-approved informed consent. The tissues were processed and banked at the University of Pittsburgh Cancer Institute (UPCI) Tissue Procurement Facility. Samples of peripheral blood mononuclear cells (PBMC) were obtained from the same patients (#1–#17) and from an additional five patients prior to surgery. All patients were HLA-A2+, as previously determined by serotyping of their PBMC, using monoclonal antibodies (mAbs) BB7.2 and MA2.1 produced by hybridomas obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The age, sex and clinicopathologic characteristics of the patients are listed in Table 1.

Collection of PBMC

Peripheral venous blood (20–30 ml) was drawn into heparinized tubes. The samples were hand-carried to the laboratory and immediately centrifuged on Ficoll-Hypaque. PBMC were recovered, washed in AIM-V medium (Invitrogen, Carlsbad, CA, USA) counted in trypan blue and either cryopreserved or immediately used for experiments.

Isolation of tumor infiltrating lymphocytes (TIL)

Tumor infiltrating lymphocytes (TIL) were isolated at the UPCI Tissue Bank according to a standard operating procedure (SOP) as previously described [3]. Briefly, after removal of fat, blood or necrotic areas, primary or metastatic solid human tumor tissue or tumor-involved and non-involved lymph nodes were washed in RPMI-1640 containing 50 µg/ml gentamycin (Invitrogen), cut into 1 mm³ pieces in a petri dish covered with RPMI-1640, washed again with RPMI-1640 and upon transfer to flasks, dissociated using 0.05% collagenase (type IV, Sigma, St. Louis, MO, USA) and 0.02% DNase (type I; Sigma) in RPMI-1640 supplemented with 5% (v/v) fetal calf serum and antibiotics (Invitrogen). Tissues were dissociated for up to 4 h using a magnetic stirrer at 37°C. The digest was then passed through 90 µm and 50 µm nylon mesh to remove clumps, and the filtrate was washed 2–3× in medium followed by centrifugation at 350× *g* for 10 min. To separate tumor cells from lymphocytes, the cell suspension was layered onto a discontinuous Ficoll-Paque gradient of 75% over 100% Ficoll-Paque in medium and centrifuged for 800× *g* for 20 min at RT. Lymphocytes were collected from the interphase between 75% and 100% Ficoll-Paque and washed twice before further use.

Isolation of LNL was performed as previously described [15].

Table 1 Clinicopathologic characteristics of patients with SCCHN who donated PBMC and/or TIL for this study

	<i>n</i>
Age (years)	
Mean range	41–82
Sex	
Male	19
Female	5
Total	24
Tumor site	
Larynx	5
Oral cavity	10
Pharynx	4
Hypopharynx	3
Other	2 (1 skin and 1 unknown)
Tumor stage	
T1	1
T2	5
T3	8
T4	8
Unstaged	2
Nodal status	
N0	6
N1	5
N2	11
unknown	2
Tumor differentiation	
Well	3
Moderate	15
Poor	4
Undifferentiated	2

Cryopreservation of TIL and PBMC

Peripheral blood mononuclear cells, TIL or LNL were cryopreserved at a cell concentration of up to 20×10^6 cells/ml in a freezing medium consisting of 90% (v/v) human AB serum (Nabi, Miami, FL, USA) plus 10% dimethylsulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA, USA). Cells were cooled at a rate of 1°C per min to -80°C (Cryomed, Thermo Forma, Pittsburgh, PA, USA) and stored in liquid N_2 . Prior to use, cells were thawed at 37°C and immediately resuspended in an excess of warm AIM-V medium (Invitrogen). Cells were washed twice in medium, equilibrated in medium for 2–4 h, adjusted to the concentration of 5×10^6 /ml and used for experiments.

Tetrameric peptide-MHC class I complex (tetramer) assay

The streptavidin-APC-labeled tetramers were made at the Tetramer Core Facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA, USA). Tetramer specific for the wt sequence p53 peptides: wt p53₂₆₄₋₂₇₂, wt p53₁₄₉₋₁₅₇ and wt p53₆₅₋₇₃ were used. Four-color flow cytometry-based assays were performed as previously described [9], using FACS (Becton Dickinson, San Jose, CA, USA). As a negative control, an irrelevant HLA-A2-restricted tetramer (e.g., HIV pol peptide IL-KEPVHGV) obtained from Beckman Coulter (Miami, FL, USA) was used. We have previously established a lower limit of detection (LLD) for the assay based on the upper 99th percentile of tetramer+CD8+ T cells in HLA-A2*0201-negative individuals as the frequency of 0.01% or 1/7,600 cells [8]. This LLD was used in evaluating the results of all tetramer experiments.

Antibodies

All the mAbs used for surface staining of lymphocytes were purchased from Becton Dickinson (San Jose, CA, USA) and included mAbs to: CD3, CD4, CD8, CD14, CD25, and the respective isotypes used as negative controls. In addition, anti-IFN- γ mAb was purchased from Beckman Coulter, and anti-CD247 mAb from Santa Cruz, Biotechnology, Santa Cruz, CA, USA. Unlabeled polyclonal anti-FOXP3 and the secondary labeled Ab were purchased from Abcam Ltd., Cambridge, MA, USA. Carboxyfluorescein-conjugated mAb to glucocorticoid-induced TNF receptor (GITR) was purchased from R&D Systems (Minneapolis, MN, USA) and labeled anti-CTLA-4 Ab was from Beckman Coulter. Prior to use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions. Checkerboard titrations were first performed to determine optimal dilutions of primary and secondary Abs used for the indirect staining of FOXP3.

Staining and flow cytometry for regulatory T cells (Treg)

For flow cytometry, lymphocytes were stained as previously described by us [9, 10]. Briefly, cells were thawed and washed twice in prewarmed AIM-V medium and then washed again with 2 ml PBS + 0.1% (w/v) BSA + 0.1% (w/v) sodium azide (flow buffer). For phenotypic analysis of Treg, 5 μl aliquots of the labeled mAbs to CD3, CD4, CD8 and CD25 were first added to cell pellets (1×10^4 cells). Cells were incubated for 30 min at 4°C in the dark to obtain surface staining. Next, cells were washed twice with the flow buffer and fixed in 2.5% paraformaldehyde (PFA) in PBS for 10 min at room temperature in the dark. After another wash with the same buffer, cells were permeabilized with saponin (0.1% v/v in BSA) and washed with cold saponin solution. Next, Abs to FOXP3, GITR, CTLA-4 or isotype control Abs were added to the cells. Following a 25-min incubation at 4°C in the dark, cells were washed again with 0.1% saponin and then with the flow buffer. In case of FOXP3, the pretitrated secondary Ab was then added for an additional 25-min incubation followed by washing in the flow buffer. Finally, the cells were fixed with PFA in PBS. Staining of cells for intracytoplasmic ζ and IFN- γ expression was performed as described above. All stained samples were immediately analyzed by flow cytometry. Multiparameter flow cytometry for T-cell subsets, ζ or IFN- γ expression in T cell was performed using Coulter Epics XL. The subsequent data analysis was performed with Expo 32 ADC (Beckman Coulter).

Flow cytometry for tetramers in PBMC, TIL and LNL

For tetramer staining, 15 μl aliquots of diluted tetramer stock (1/100) were directly added to cell pellets ($2\text{--}5 \times 10^6$ cells). Cells were incubated for 30 min at room temperature in the dark. Next, 5 μl aliquots of the labeled mAbs were added for surface staining, and cells were incubated for 30 min at 4°C in the dark. Cells were washed twice with 2 ml flow buffer and fixed in 300 μl PBS without Ca^{2+} / Mg^{2+} supplemented with 2% (w/v) paraformaldehyde in PBS and stored at 4°C in the dark. Samples were analyzed within 1 day of staining. Gating strategy consisted of backgating on CD3+CD8+ bright lymphocytes followed by acquisition of tetramer+ cells within this population. All tetramer data were acquired on a BD FACS-Calibur with dual laser capability, using Cell Quest software.

Immunohistochemistry for p53

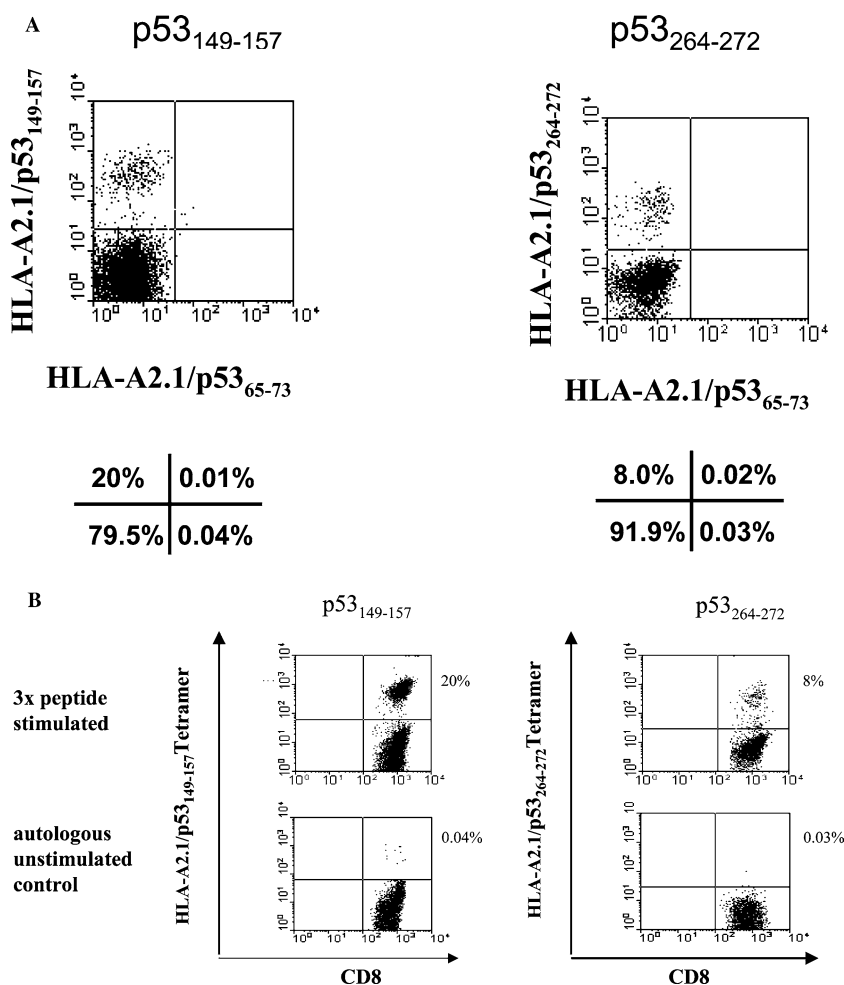
For detection of p53 expression in the tumor, formalin-fixed, paraffin-embedded tumor tissues were sectioned (3–5 μm), air dried overnight at 37°C , deparaffinized, dehydrated, and stained with a mAb against p53, (DO-7, Dako, Carpinteria, CA, USA), which recognizes an epitope in the N-terminus between amino acid 35 and 45

and reacts with the wt and most mutant forms of p53 protein. The avidin–biotin–peroxidase method was used to visualize p53, according to instructions supplied by the manufacturer (Dako). The immunostained slides were evaluated for p53 accumulation by light microscopy. The tumor was considered p53 positive when >25% of the cells showed staining intensity of 1+ or higher on a scale of 0–4+. IgG isotype mAb, used at the same concentration as the primary mAb, served as negative controls.

Activation of TIL and PBMC

Lymphocytes ($1\text{--}2 \times 10^6$ ml) were incubated in the presence of anti-CD3 mAb (OKT3, UPCI Pharmacy) at the final concentration of 5 $\mu\text{g}/\text{ml}$ in AIM V medium supplemented with 10% (v/v) human serum (Nabi, Miami, FL, USA) in 12-well plates (2–4 ml/well) for 24 h at 37°C. Monensin (2 μM ; Sigma) was added to each well for the last 4 h of culture. The cells were harvested, stained for surface markers using relevant mAbs washed, permeabilized, as described previously [21], and stained for intracellular expression IFN- γ . Flow cytometry was performed as described above.

Fig. 1 Specificity of tetramer binding to T cells expressing TCR for wt p53 epitopes. In **a**, T-cell lines were generated using wt p53 peptides presented on DC obtained from PBMC of HLA-A2+ patients with SCCHN. These T-cell lines were simultaneously stained with two different p53 tetramers and were shown to react only with the tetramers specific for the wt p53 peptides used for priming. In **b**, the generated wt p53 peptide-specific CTL lines were stained with the relevant and irrelevant (e.g., wt p53₆₅₋₇₃) tetramers. Only the relevant wt p53 peptide tetramers bound to CTL lines generated in cultures with wt p53₁₄₉₋₁₅₇ or wt p53₂₆₄₋₂₇₂ peptides. An irrelevant tetramer (wt p53₆₅₋₇₃) did not bind to these CTL. The percentages of positive cells are indicated below or next to every dot plot



Statistical analysis

Differences between TIL and PBMC obtained from the same patients were evaluated using paired Student's *t* test. The *P* values < 0.05 were considered significant.

Results

Specificity of tetramer binding to CD8+ T cells

Specificity of tetramer binding was determined by: (a) positive staining of peptide-specific vs. no staining of irrelevant T-cell lines, (b) double staining of a T-cell line with two differently labeled tetramers (a tetramer mix) to show that tetramer+ T cells are single-stained and not double-stained, and (c) a lack of staining of CD8+ T cells from HLA-A2 negative patients and healthy donors [7]. In addition, all p53-specific tetramers were preiterated using T-cell lines or T-cell clones specific for wt p53 peptides to determine optimal staining dilutions and distinguish positive from negative signals [9].

Binding of p53-specific tetramers to wt p53 epitope-specific T-cell lines was tested using T-cell lines generated by in vitro sensitization (IVS) of PBMC, obtained

from HLA-A2.1+ patients with SCCHN with wt p53 epitopes pulsed on DC as previously described [7]. Figure 1a shows that these T-cell lines contained CD8+ T cells which were reactive with the relevant tetramers. The generated T-cell lines were then used to test the specificity of tetramers. As shown in Fig. 1b, a T-cell line generated by IVS in response to wt p53₁₄₉₋₁₅₇ epitope only bound the relevant tetramer and not the irrelevant wt p53₆₅₋₇₃ peptide-specific tetramer. Similarly, a T-cell line generated by IVS in response to wt p53₂₆₄₋₂₇₂ epitope only bound the relevant tetramer and not the irrelevant wt p53₆₅₋₇₃ tetramer. In these preliminary experiments, the available tetramer reagents were demonstrated to be sufficiently specific for testing of T lymphocytes obtained from patients with HNC.

Frequency of tetramer+ T cells in TIL versus PBMC

Using the validated tetramers, we determined the frequencies of CD3+CD8+ T cells specific for wt p53₂₆₄₋₂₇₂ peptide or wt p53₁₄₉₋₁₅₇ peptide in patients' PBMC as

well as TIL by multicolor flow cytometry (Table 2). It is apparent that these frequencies are significantly higher in TIL than autologous PBMC in every case (Table 2). The PBMC frequency of T cells recognizing wt p53₂₆₄₋₂₇₂ epitope is lower than that of T cells specific for wt p53₁₄₉₋₁₅₇ epitope. In fact, taking the frequency value of 1/7,600 as a LLD for the wt p53 tetramer-based assays, as previously determined [8], only 6/10 (~50%) patients tested had detectable wt p53₂₆₄₋₂₇₂ tetramer+CD8+ T cells in the peripheral circulation. In contrast, in 9/10 patients frequency values for circulating T cells specific for wt p53₁₄₉₋₁₅₇ epitope were high (i.e., lower than 1/7,600). The data in Table 2 indicate that the frequency values for wt p53-peptide-specific CD8+ cells do not appear to be related to p53 overexpression in the tumor, as determined by immunohistochemistry. Overall, the frequency values for TIL with specificity for these epitopes are high, ranging from 1/87 to 1/2,175, an indication that the epitope-specific T lymphocytes accumulate at the tumor site. Consistent with the frequency values for PBMC, more CD8+ T cells specific for wt p53₁₄₉₋₁₅₇ epitope than for wt p53₂₆₄₋₂₇₂ epitope

Table 2 Reciprocal frequency values for tetramer+CD3+CD8+ T cells in tumor infiltrating lymphocytes (TIL) and autologous peripheral blood mononuclear cells (PBMC) in patients with SCCHN relative to p53 expression in the tumor

Patient no.	CD3+CD8+ tetramer ₂₆₄₋₂₇₂ ⁺		CD3+CD8+ tetramer ₁₄₉₋₁₅₇ ⁺		p53 accumulation in the tumor ^a
	PBMC	TIL ^b	PBMC	TIL ^b	
#1	5,270	1,207	3,022	668	+
#2	10,000	1,503	10,000	776	+
#3	4,460	2,175	2,863	87	NA
#4	10,000	2,015	5,637	NA	-
#6	8,963	240	3,211	456	+
#7	8,129	1,660	2,521	1,355	NA
#8	2,626	1,881	3,368	351	+
#9	6,369	817	2,687	620	+
#10	1,822	1,056	2,923	1,426	-
#11	NA	1,077	NA	2,569	NA
#12	NA	2,839	NA	1,349	NA
#13	NA	5,105	NA	2,440	NA
Mean	6,405	1,774	4,026	1,100	
±SD	3,075	1,198	2,424	819	

Paired TIL and PBMC were obtained from patients as described in Materials and methods

NA not available

^aDetermined by immunocytochemistry, using the DO-7 Ab

^bThe differences in the frequency of tetramer+ cells in PBMC versus TIL are significant at $P < 0.001$ for wt p53₂₆₄₋₂₇₂ tetramer and for wt p53₁₄₉₋₁₅₇ tetramer

Fig. 2 The log frequencies of CD3+CD8+ tetramer+ cells in PBMC and TIL of an HLA-A2+ patient with SCCHN (#1). The frequencies of wt p53₁₄₉₋₁₅₇ tetramer+ T cells as well as p53₂₆₄₋₂₇₂ tetramer+ T cells are significantly higher in TIL than in PBMC ($P < 0.001$) for both

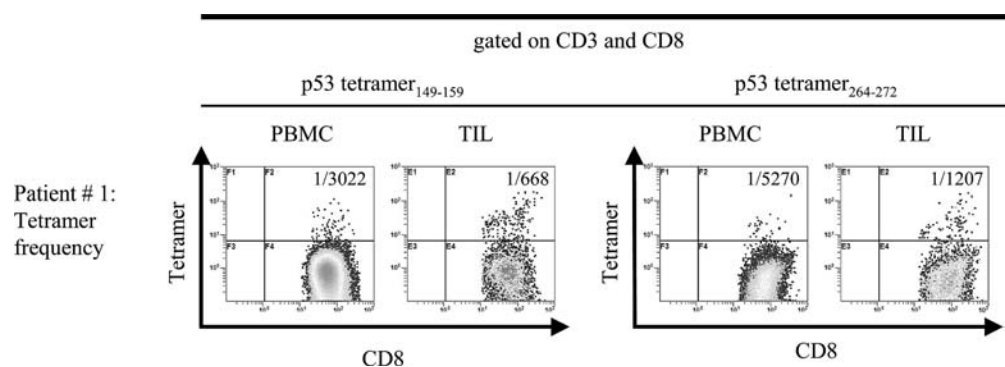


Table 3 Reciprocal frequency values for tetramer+CD3+CD8+ T cells and percentages of CD4+CD25+ T cells in tumor-non-involved lymph nodes relative to autologous peripheral blood lymphocytes (PBMC) and tumor-infiltrating lymphocytes (TIL) in patients with SCCHN

Patient no.	CD3+CD8+						CD4+CD25+		
	wt p53 ₂₆₄₋₂₇₂ tetramer+			wt p53 ₁₄₉₋₁₅₇ tetramer+			Percentage of positive		
	PBMC	TIL	Tumor-non-involved LN	PBMC	TIL	Tumor-non-involved LN	PBMC	TIL	Tumor-non-involved LN
#1	5,270	1,207	5,021	3,022	668	6,042	9	65	9
#12	NA	2,839	8,686	NA	1,349	7,166	NA	48	11
#13	NA	5,105	10,035	NA	2,440	6,429	NA	34	12
Mean		3,050	7,914		1,486	6,546		49	11
±SD		1,958	2,595		894	571		16	1.5

The frequency of wt p53 peptide-specific tetramer+CD8+ T cells and the percentages of CD4+CD25+ T cells were determined by flow cytometry, as described in Materials and methods

are present at the tumor site. Figure 2 presents representative flow cytometry data, illustrating a clear-cut enrichment in wt p53 epitope-specific CD8+ T cells among TIL as compared to autologous PBMC.

Frequency of tetramer+ T cells in tumor-non-involved LN

In three patients with SCCHN, tumor-involved LN were available for analysis in addition to PBMC and TIL. The data in Table 3 show that no enrichment in wt p53 epitope-specific CD8+ T cells was evident in these LN. In fact, the frequency values for p53 tetramer+ T cells in the tumor-non-involved LN were comparable to those

Table 4 Percentages of CD4+CD25+ T cells present in paired PBMC and TIL obtained from patients with SCCHN

Patient no.	PBMC	TIL
	Percentage of CD4+CD25+	
#1	9	65
#2	16	5
#3	10	12
#4	16	27
#5	NA	19
#6	5	20
#7	8	25
#8	NA	15
#9	4	26
#10	11	47
#11	NA	26
#12	NA	48
#13	NA	34
#14	14	42
#15	15	51
#16	NA	5
#17	NA	44
Mean	11	30 ^a
±SD	4.4	17.2

NA not available

^aThe difference in the percentage of CD4+CD25+ Treg between PBMC and TIL is significant at $P < 0.003$

obtained with autologous PBMC (Table 2). The percentage of CD4+CD25+ in the tumor-non-involved LN was also similar to that in PBMC.

Percentages of CD4+CD25+ T lymphocytes in TIL versus PBMC

The subset of CD4+CD25+ cells, which might contain Treg, was of particular interest [2]. We first determined that the percentages of CD4+CD25+ T cells were significantly higher in TIL ($P < 0.003$) than in autologous PBMC (Table 4). In some patients, e.g., #1 or #10, this difference was impressively greater. Figure 3 illustrates enrichment of these cells in TIL vs. PBMC in three representative patients with SCCHN. The data also demonstrate that although TIL were enriched in CD4+CD25+ T cells and tetramer+CD8+ T cells relative to PBMC, there was no correlation between the frequency of these cell subsets at the tumor site.

Treg among CD4+CD25+ T lymphocytes

The subset of CD4+CD25+ T cells could include activated helper T cells as well as Tregs. Tregs are thought to be responsible for downregulation of immune responses to autoantigens [24]. As wt p53 epitopes can be considered “self” as well as tumor-specific peptides, we looked for the presence of Treg in the peripheral circulation and among TIL of patients with SCCHN by multicolor flow cytometry, using antibodies to FOXP3, GITR and CTLA-4 [2, 14, 19, 27]. These markers have been used to distinguish activated CD4+ T cells from Treg [2, 27]. The data in Table 5 indicate that CD4+CD25+ TIL were highly enriched in FOXP3+, CTLA-4 as well as GITR+ T cells relative to normal or patients' PBMC. Among TIL nearly all CD4+CD25+ T cells expressed FOXP3 surface marker and had intracytoplasmic GITR and CTLA-4+. In contrast, only a proportion of CD4+CD25+ T cells in the peripheral

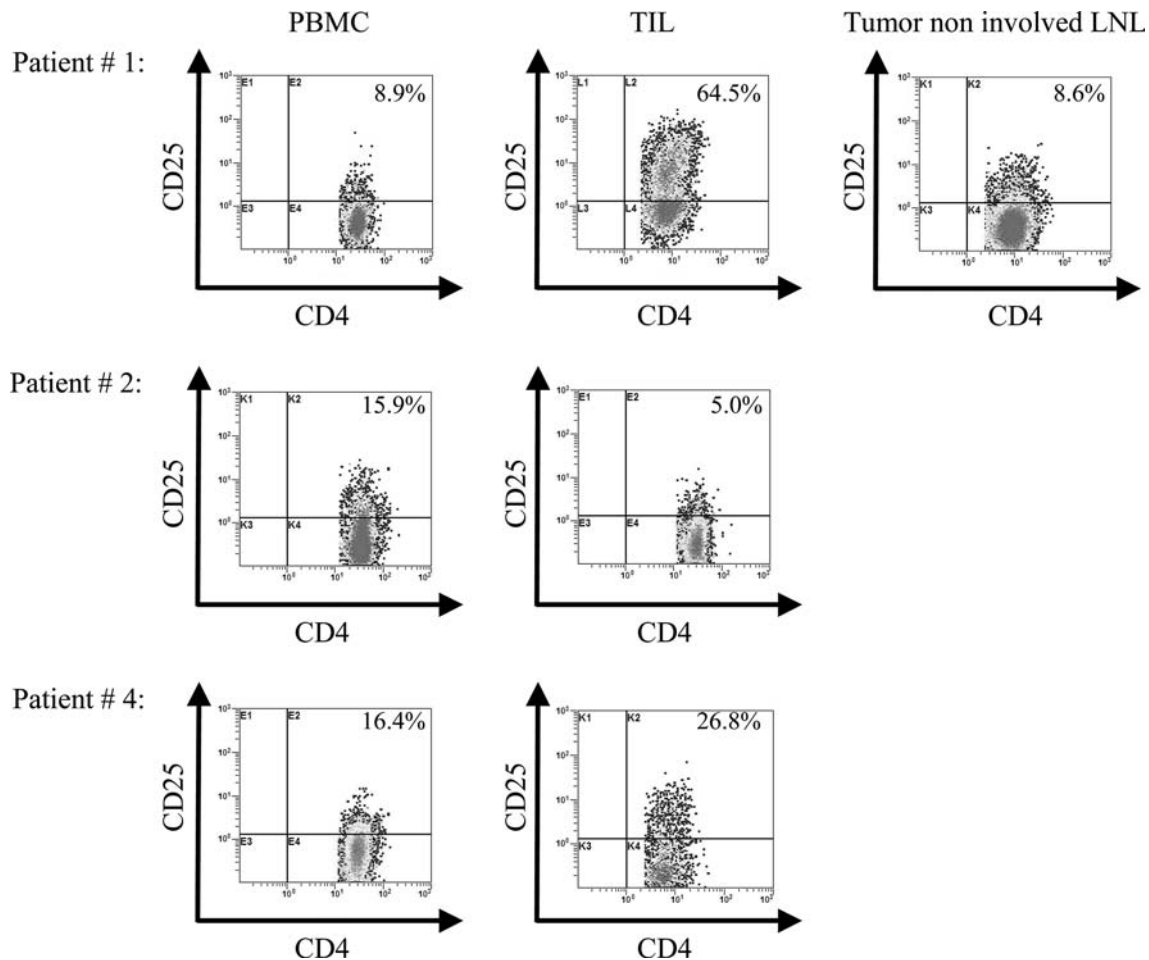


Fig. 3 Increased percentages of CD4+CD25+ T cells in PBMC, TIL and one tumor non-involved LN in representative patients with SCCHN. The gate was set on CD3+CD4+ cells. Patient #1 shows the highest increase in CD4+CD25+ T cells in TIL among the patients studied. The tumor-non-involved LN has the same percentage of CD4+CD25+ T cells as PBMC. Patient #2 is the

circulation of patients or normal donors contained Treg, as defined by these markers, while the majority were non-Treg CD4+CD25+ (i.e., activated) T cells.

Functional properties of TIL in SCCHN

Since TIL were shown to be enriched in CD4+CD25+FOXP3+ Treg relative to PBMC, we expected that TIL would be unresponsive to activating stimuli, and that tetramer+ tumor-specific CD8+ T cells at the tumor site would be functionally compromised. To test this hypothesis, TIL obtained from two patients (#16 and #17) with SCCHN and PBMC from patient #16 were tested for expression of TCR ζ chain in the CD3+CD8+ T population. As shown in Table 6, both CD8+ and CD4+ TIL showed very low expression of the ζ chain, whether based on the percent values or MSFE units measurements. In contrast, CD8+ and

CD4+ T cells obtained from PBMC of normal donors had uniformly high ζ chain expression (Table 6). These results suggest that the CD3+CD8+ population of effector cells among TIL is unable to signal via TCR- ζ , which is downregulated in these cells.

In addition, after 24 h in vitro activation of TIL with anti-CD3 Ab, IFN- γ expression was induced in only a small proportion of CD3+CD8+ TIL. Activation of PBMC obtained from five patients with SCCHN was also accompanied by IFN- γ expression in only a small percent of CD3+CD8+ T cells tested under the same conditions. In contrast, a significantly higher percentage (mean 12% \pm 7; $P < 0.05$) of CD3+CD8+ T cells obtained from PBMC of normal donors expressed IFN- γ when activated with anti-CD3 mAb under the same experimental conditions. Overall, the data are consistent with decreased responsiveness of TIL and PBMC, and especially the CD8+ T-cell subset, in patients with SCCHN to the ex vivo activation, as previously reported [21, 28] (Fig. 4).

Discussion

The presence and frequency of wt p53 epitope-specific T lymphocytes was previously determined in the peripheral

Table 5 Percentages of CD4+CD25+ and Treg populations in the peripheral blood and TIL of patients with SCCHN

	CD4+CD25+	CD4+CD25+GITR+	CD4+CD25+CTLA4+	CD4+CD25+FOXP3+
Percentage (mean \pm SD)				
Pt ($n=5$)	7.2 \pm 1.4	1.3 \pm 0.6	1.5 \pm 0.5	3.1 \pm 2.0
TIL #18	13.0	12.0	12.0	13.0
TIL #19	7.6	5.3	5.6	7.0
NC ($n=4$)	10.0 \pm 3.0	1.8 \pm 1.0	2.0 \pm 0.9	3.7 \pm 0.9

Percentages of positive cells in peripheral blood mononuclear cells (PBMC) and TIL (not matched) obtained from patients with SCCHN and in PBMC of normal controls (NC) were determined

by multicolor flow cytometry. Surface staining for CD4+CD25+ T cells and intracytoplasmic staining for GITR, CTLA-4 and FOXP3 were performed as described in Materials and methods.

Table 6 Expression of the TCR- ζ chain in SCCHN-derived T cells

	CD8+Z+	CD4+Z+
TIL #16	1.3 (8,936)	1.9 (14,540)
PBL #16	4.6 (45,205)	4.7 (46,470)
TIL #17	1.8 (13,566)	2.6 (21,743)
N PBL 1	92 (83,688)	96 (85,373)
N PBL 2	98 (87,900)	98 (76,951)
N PBL 3	97 (85,373)	95 (90,429)

The data are percentages of positive cells determined by flow cytometry as described in Materials and methods. The data in parentheses are the MESF units of fluorescence determined in each specimen by using the calibration beads. The cells were tested without any exogenous stimulation. Three PBL obtained from normal donors were tested in the same assays as TIL or PBL obtained from patients with SCCHN

circulation of HLA-A2+ patients with SCCHN [8] and other malignancies [4]. However, it is not known whether these T cells are also present at tumor sites. We report here for the first time that TIL obtained from patients with HNC are significantly enriched ($P < 0.003$) in wt p53 epitope-specific T cells, as compared to autologous PBMC. The frequency of wt p53₁₄₉₋₁₅₇ epitope-specific T cells was higher than that of wt p53₂₆₄₋₂₇₂ epitope-specific lymphocytes, possibly because the p53₁₄₉₋₁₅₇ epitope is more immunogenic than other wt p53 epitopes, it is also one of the most frequently

mutated in cancer [5, 20]. These results demonstrate preferential localization to the tumor site or tumor-involved LN of wt p53 epitope-specific CD3+CD8+ effector T lymphocytes. In contrast, tumor-non-involved LN had a lower frequency of these epitope-specific CD3+CD8+ T cells, which was comparable to that found in PBMC. The observed localization of tetramer+ tumor-specific effector cells to the tumor or tumor-involved LN implies that these cells are mobilized to accumulate in situ and to exercise antitumor functions. However, numerous previous reports have indicated that T lymphocytes at tumor sites, including SCCHN, are dysfunctional and thus fail to perform expected antitumor functions [17, 21, 29, 30]. In the case of patients with SCCHN, the presence and frequency of wt p53 epitope-specific effector T cells among TIL did not correlate with tumor stage. This implies that the frequency of tetramer+CD8+ effector cells in situ has no effect on tumor progression. It appears that the tumor is successful in escaping from these tumor-specific effector T cells. Indeed, in two patients who had sufficient numbers of TIL available to test in vitro responsiveness of TIL to anti-CD3 mAb, we found that CD3+CD8+ T cells were poorly responsive as measured by IFN- γ expression. While this conclusion is based on a limited data set, our other studies support the concept of tumor escape associated with and perhaps caused by a diminished functional potential of TIL [21].

Although various mechanisms have been proposed to account for tumor escape, recent literature reports emphasize the presence at relatively high proportions of Treg in patients with cancer [2, 16, 31]. Treg were previously phenotypically defined as CD4+CD25^{high} cells, although newer markers are currently available that appear to be more specific, including FOXP3 or GITR and CTLA-4 [2, 19, 22]. The importance of these markers is that activated CD4+CD25+ T cells can now be distinguished from true Treg. These cells have been shown to play an important role in maintaining peripheral tolerance in murine models by controlling potentially harmful autoreactive T cells [24]. Also, these cells, acting as suppressors of functions of both CD4+CD25⁻ and CD8+ T cells, upon cell contact, have been described to be present in the peripheral circulation [23], LN [18] as well as tumors [2, 26, 32] of patients with various malignancies. It has been suggested that the presence or accumulation of Treg, which are

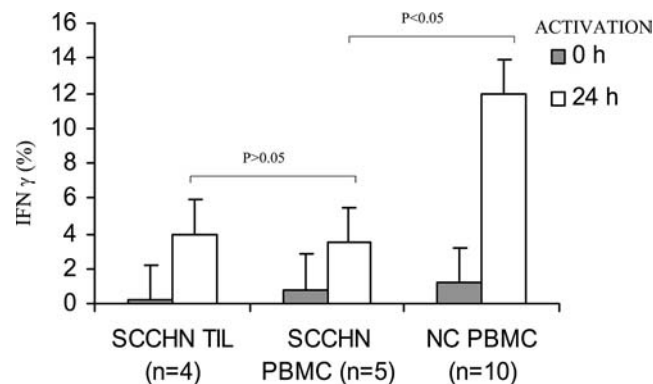


Fig. 4 Low percentages of IFN- γ expressing CD3+CD8+ T cells in TIL or PBMC of patients with SCCHN compared to PBMC of normal donors. The cells were tested for IFN- γ expression by flow cytometry following 24 h activation with anti-CD3 Ab as described in Materials and methods. The gate was set on CD3+CD8+ T cells. The data are means \pm SD

able to produce IL-10 and inhibit T-cell responses in vivo may be responsible in part, for immune suppression was evident in patients with cancer [16, 31]. It was therefore, not surprising to observe that the percentage of CD4+CD25+ T cells was significantly, and in some cases dramatically, increased in TIL as compared to autologous PBMC, in patients with SCCHN. Further, phenotyping for Treg markers, FOXP3, GITR and CTLA-4, confirmed their enrichment in TIL. We suspect that accumulations of Treg in the tumor could be in part responsible for the observed dysfunction of the infiltrating effector cells, although we cannot rule out the possibility that the tumor-derived factors contribute as well [19]. While we did not recover sufficient numbers of T cells for classical mixing experiments for testing suppressor functions of Treg in TIL [13], we were able to show by flow cytometry that expression of the ζ chain, a signaling molecule associated with the T-cell receptor (TCR) was substantially decreased in representative CD3+CD8+ TIL isolated from SCCHN. Further, as indicated above, this TIL population was poorly responsive to anti-CD3 Ab as measured by intracellular IFN- γ expression following activation.

In conclusion, our results show that the wt p53 peptide-specific effector T cells are not only present in the circulation but also preferentially localize to tumor sites in patients with SCCHN. However, the simultaneous presence in the tumor and tumor-involved LN of an excess of Treg probably contributes in part, to poor responsiveness of these tumor-specific CD8+ T lymphocytes in situ. It is likely that the tumor or tumor-derived factors also play a role in inducing dysfunction of effector T cells in situ [19, 25, 26]. Other data from our laboratory confirm depressed functionality or spontaneous apoptosis of CD8+ T lymphocytes in patients with malignancies, including SCCHN [10, 21, 28]. These phenomena appear to be related to effects directly or indirectly mediated by the tumor, and they contribute to tumor escape from the host immune system. In addition to various other mechanisms known to facilitate tumor escape [reviewed in 29], Treg are likely to mediate suppressive effects directed at self-reactive T cells [24]. This immunosuppressive mechanism may be particularly relevant to wt p53 epitope-specific T lymphocytes, which recognize "self" peptides and, thus, are likely to be tolerated, especially at the sites of their accumulation in tumor tissues.

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