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Toward the development of multi-epitope p53 cancer vaccines: an *in vitro* assessment of CD8⁺ T-cell responses to HLA class I-restricted wild-type sequence p53 peptides

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Abstract

Wild-type sequence (wt) p53 peptides are attractive candidates for broadly applicable cancer vaccines. Six HLA-A2 or HLA-A24-restricted wt p53 peptides were evaluated for their *ex vivo* immunogenicity and their potential for use in cancer vaccines. Peripheral blood mononuclear cells (PBMC) obtained from HLA-A*0201⁺ and/or HLA-A*2402⁺ normal donors and subjects with squamous cell carcinoma of the head and neck (SCCHN) were analyzed for p53 peptide-specific reactivity in ELISPOT IFN γ assays. CD8⁺ T cells in 7/10 normal donors (HD) and 11/23 subjects with SCCHN responded to at least one of the wt p53 peptides. CD8⁺ T-cell precursors responsive to wt p53 epitopes were detected in the circulation of most subjects with early disease, and an elevated blood Tc1/Tc2 ratio distinguished wt p53 peptide responders from non-responders. The identification of multiple wt p53 peptides able to induce cytolytic T lymphocytes in most subjects with cancer promotes the development of multi-epitope p53 vaccines.

Keywords

p53; CD8⁺ T cells; immune response; head and neck cancer; ELISPOT

Introduction

As development of vaccine-based immunotherapy of cancer progresses, there is a consensus that the use of multiple epitopes targeting either a single tumor antigen or several tumor antigens would enhance the efficacy of cancer vaccines. Wild-type sequence (wt) p53 peptides represent one of a few tumor antigens available for vaccines targeting a wide spectrum of epithelial

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tumors and, consequently, they are attractive candidates for use in broadly applicable immunotherapy of cancer. A loss of p53 function is the most common abnormality in human cancer, with upwards to 80% of most tumors showing defects in p53 due to mutation or alterations in its stability [1]. In many instances, these alterations lead to enhanced presentation of wt p53 epitopes (non-mutated peptide sequences derived from the p53 molecules expressed in tumor cells) by tumor cells for recognition by HLA class I-restricted T cells. Evidence of cellular as well as antibody-mediated anti-p53 immune responses in subjects with cancer is well documented [2-10], and wt p53 peptide-based vaccines have been shown in preclinical murine tumor model studies to be effective in inducing anti-tumor immunity in prevention and therapy settings [11].

To date, a number of wt p53 epitopes defined by HLA-A2 or HLA-A24-restricted, CD8⁺ T cell cells have been identified [2-10]. Some of these wt p53-derived peptides, mainly those defined by HLA-A*0201(HLA-A2)-restricted, CD8⁺ T cells are being clinically evaluated in cancer vaccines [12,13]. In our previous studies, we used *in vitro* stimulation (IVS) of CD8⁺ T cells with wt p53 peptide-pulsed autologous dendritic cells (DC) to induce either HLA-A2-restricted, wt p53₁₄₉₋₁₅₇ and/or wt p53₂₆₄₋₂₇₂ peptide-specific responses. Using single epitopes, wt p53 peptide-specific CD8⁺ T cells were generated in only a third of healthy donors or subjects with cancer tested [14]. Comparable findings have been reported by Azuma et al [15] as well as Nikitina et al [16]. The observed limited responsiveness to HLA class I-restricted wt p53 peptides among HLA class I-compatible healthy donors and subjects with cancer suggests that multiple wt p53 peptides are needed in order to maximize donor responsiveness. If such were the case, then identifying the combination of wt p53 epitopes best able to induce CD8⁺ T cell responses would be necessary for the development and optimization of p53-based cancer vaccines. This requires extensive pre-clinical studies to best define the composition of vaccines for future therapy of cancer subjects expressing a restricting HLA class I allele.

Subjects with cancer, especially those with advanced disease, are known to be immunologically suppressed. Data from our and other laboratories have demonstrated various abnormalities in DC numbers, degree of their maturation, expression levels of antigen processing machinery (APM) from subjects and decreased HLA-DR expression on DC in subjects with cancer compared to healthy donors [17-19]. With respect to T cells, evidence for CD8⁺ effector T-cell apoptosis as well as decreased expression of the ζ chain, which plays an important role as a transmembrane signaling molecule in lymphocytes, was found in T cells isolated from the tumor site or from the peripheral circulation of cancer subjects [20,21]. Recently, we and others have shown that proportions of regulatory T cells (Treg) were significantly elevated at the tumor site and in the peripheral circulation of subjects with cancer [17,22,23]. Such increases in proportions of Treg may be, in part, responsible for down-regulation of antitumor immune responses in cancer subjects. The data suggest that the induction of anti-tumor antigen-specific CTL responses in cancer subjects appears to be regulated by complex interactions between the tumor and the host's immune system.

A better understanding of the nature and specificity of anti-wt p53 CD8⁺ T cell responses in cancer subjects, as well as their ability to make an immune response, are necessary to better design and develop multi-epitope p53-based vaccines for future immunotherapy of cancer. Therefore, we investigated the *ex vivo* CD8⁺ T cell responsiveness of PBMC obtained from subjects with SCCHN to the HLA-A2-restricted wt p53₁₄₉₋₁₅₇, p53₂₁₇₋₂₂₅, and p53₂₆₄₋₂₇₂ peptides and the HLA-A*2402(HLA-A24)-restricted wt p53₁₂₅₋₁₃₄, p53₁₆₁₋₁₆₉, and p53₂₀₄₋₂₁₂ peptides using enzyme-linked immunospot IFN- γ (ELISPOT IFN- γ) assays. In this study, subjects with squamous cell carcinoma of the head and neck (SCCHN) were the target population. The immunologic response data were evaluated relative to the immune status and disease progression in each subject with SCCHN. Our findings provide insights for further

efforts to develop p53 peptide-based vaccines as well as selection of subjects for future clinical trials employing these vaccines.

Materials and Methods

Subjects

Peripheral blood was obtained from 10 healthy donors and 23 subjects with pathologically and clinically confirmed SCCHN. The study was approved by the Institutional Review Board at Gunma University Hospital. A written informed consent was obtained from each individual. Subject characteristics are summarized in Table 1. Heparinized venous blood (40mL) was obtained from all subjects, and PBMC were isolated by centrifugation over Ficoll-Hypaque gradients (Amersham Biosciences, Uppsala, Sweden), washed, and counted in the presence of a trypan blue dye. All subjects were HLA-A2⁺ and/or HLA-A24⁺, as determined by flow cytometry using HLA-A2-specific antibodies (MA2.1 and BB7.2) and an HLA-A24-specific antibody (anti-HLA-A23, A24 mAb, IgG2b) purchased from American Type Culture Collection (ATCC, Rockville, MD) and One Lambda, Inc. (Canoga Park, CA), respectively.

Cell line and reagents

The T2-A24 cell line used as the peptide-presenting cell in ELISPOT assays was kindly provided by Dr. Kuzushima (Aichi Cancer Center, Nagoya, Japan). It was maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100µg/mL streptomycin, 2 mM L-glutamine and 0.8mg/mL G-418 (all reagents from Invitrogen, Grand Island, NY). GM-CSF and TNF-α were purchased from R&D Systems (Minneapolis, MN). Human recombinant IL-2 and IL-4 were provided by Shionogi & Co., Ltd. (Osaka, Japan) and Ono Pharmaceutical Co., Ltd. (Osaka, Japan), respectively.

Peptides

A total of 6 peptides, 3 HLA-A2-binding peptides (p53₁₄₉₋₁₅₇, p53₂₁₇₋₂₂₅, p53₂₆₄₋₂₇₂) and 3 HLA-A24-binding peptides (p53₁₂₅₋₁₃₄, p53₁₆₁₋₁₆₉, p53₂₀₄₋₂₁₂) were synthesized using standard N-(9-fluorenyl) methoxycarbonyl methodology (Table 2). The peptides were stored as lyophilized preparations. Their amino acid sequences were confirmed by mass spectrometric analysis. As negative control peptides, HLA-A2 binding HIV gag₇₇₋₈₅ (SLYNTVATL) and A24-binding HIV gag₂₈₋₃₇ (KYKLKHIVW) peptides were obtained from Sigma Genosis (Hokkaido, Japan).

Immunohistochemistry for p53 in SCCHN

Immunohistochemical detection of p53 in tumor specimens was performed as previously described [24]. Briefly, formalin-fixed, paraffin-embedded tissue blocks were sectioned (2µm thick) and deparaffinized. The slides were autoclaved in 0.01 M citrate buffer for 15 min (w/v) for antigen retrieval. After washing, sections were incubated with 1% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and 5% (w/v) normal horse serum (IBL, Gunma, Japan) for 30 min and subsequently treated overnight with anti-p53 mAb (DO-7; DAKO Cytomation, Inc., Carpinteria, CA). Next, sections were incubated with biotinylated goat anti-mouse antibodies (DAKO, Copenhagen, Denmark). The avidin-biotin complex technique (Vectastain Elite Kit, Vector Inc, Burlingame, CA) was performed according to the manufacturer's instructions, and 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Wako, Osaka, Japan) was used for color development. Sections were counterstained with hematoxylin. To avoid bias in scoring, evaluation of p53 expression was performed by two independent observers. Staining was scored as either positive (>10% of tumor cell nuclei were stained) or negative.

Flow cytometry analysis

To determine the percentage of regulatory T cells (Treg) and the Th1/Th2 as well as Tc1/Tc2 ratios, flow cytometry analysis was performed as previously described [17]. Briefly, isolated PBMC were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (Immunotech, Marseille, France) mAb and phycoerythrin (PE)-conjugated anti-CD4 (Immunotech) mAb in order to detect Treg. Respective IgG isotype-matched controls (Immunotech) were used for negative controls. To identify Treg population, the gate was set to acquire CD4⁺CD25^{high} T cells. The Th1/Th2 and Tc1/Tc2 ratios were determined by intracytoplasmic staining. Isolated PBMC were stimulated with phorbol 12-myristate 13-acetate (PMA, 25ng/mL) and ionomycin (1μg/mL) for 4 h in 2μM monensin (all purchased from Sigma). The cells were harvested, washed twice in PBS, and then incubated with phycoerythrin cyanine 5 (PC5)-conjugated anti-CD4 or PC5-conjugated anti-CD8 mAb (Immunotech). After washing twice, the cells were fixed and permeabilized with 1% (w/v) saponin in PBS (Sigma). Then, the cells were stained with FITC-conjugated anti-INF-γ and PE-conjugated anti-IL-4 mAb (Immunotech) for 30 min at 4°C. As a negative control, cells were stained with IgG isotype-controls. Two-color flow cytometry analyses were performed using Epics XL (Beckman Coulter, Miami, FL), and the data were analyzed using EXPO 32 program (version 1.0, Applied Cytometry Systems, Sheffield, UK). At least 10,000 events for analysis were acquired for each sample.

IVS of PBMC using p53 peptides

Peptide stimulation of PBMC was performed using a modification of the method reported by Tatsumi et al [25]. Briefly, PBMC were incubated for 1h at 37°C in AIM-V medium (Invitrogen), and nonadherent cell were removed by gentle washing with warm medium. AIM-V medium containing 1000u/mL GM-CSF and 10ng/mL IL-4 was added, and cells were incubated for 5 days. TNF-α (10ng/mL) was added to culture medium for 48 h, and matured DCs were harvested and used as antigen-presenting cells (APCs). DC were resuspended at the concentration 10⁶ cells/mL in AIM-V medium, and then irradiated (3000 rad), washed, and resuspended in AIM-V medium containing 10% (v/v) human AB serum (complete medium: CM). CD8⁺ T cells were positively isolated from nonadherent PBMC with immunomagnetic beads (Miltenyi Biotec, Gladbach, Germany). CD8⁺ T cells (2x10⁶) and irradiated DCs (2x10⁵) pulsed with 10μg/mL of a p53 peptide and co-cultured in wells of 24-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in a final volume of 2mL CM/well. On day 5, 20IU/mL IL-2 was added to culture wells. On day 7, responder CD8⁺ T cells were harvested and tested in ELISPOT assays.

ELISPOT IFN-γ assays

ELISPOT assays were performed in wells of 96 well flat-bottomed plates with nitrocellulose membrane inserts (Millipore, Bedford, MA) as previously described [8]. Briefly, the plates were coated overnight at 4°C with anti-human IFN-γ mAb (1-D1K: Mabtech, Nacka Strand, Sweden) in PBS. T2-A24 cells used as APCs were pulsed with a p53 peptide (10μg/mL) and plated in triplicate wells at 1x10⁵ cells/well. The responder cells (1x10⁵ cells/well) were added in AIM-V medium in a final volume of 200μL. Negative controls were responder cells in the presence of T2-A24 pulsed with the HIV gag-derived peptide. CD8⁺ T cells stimulated with 12.5ng/mL PMA and 1μg/mL ionomycin were also used as a positive control. After incubation for 20 h at 37°C, the plates were washed with PBS/0.05% Tween 20, and supplemented with the biotinylated anti-IFN-γ mAb (7-B6-1: Mabtech). After 2 h incubation, plates were washed with PBS/0.05% Tween 20, and developed with the avidin-peroxidase complex (Vectastain Elite kit; Vector) for 1 h. Peroxidase staining was performed with 3-amino-9-ethyl-carbozole (Sigma, St. Louis, MO) for 5 min. The mean number of spots in control wells (the HIV gag peptide) was subtracted from the mean number of spots in experimental wells. A CD8⁺ T cell

response to a given wt p53 peptide was considered to be positive if at least 10 cells per 1×10^5 CD8⁺ T cells secreted IFN- γ , as described by Nagorsen et al [26].

Statistical Analysis

Mann-Whitney's U test was used for statistical analysis of data. *p*-values < 0.05 were considered to be significant. Analyses were performed using Statcel2 (OMS Publishing, Tokorozawa, Japan).

Results

CD8⁺ T-cell responses to wt p53 peptides of PBMC obtained from normal donors and subjects with SCCHN following IVS

All six wt p53 peptides, three HLA-A2-restricted wt p53 peptides (p53₁₄₉₋₁₅₇, p53₂₁₇₋₂₂₅, p53₂₆₄₋₂₇₂) and three HLA-A24-restricted wt p53 peptides (p53₁₂₅₋₁₃₄, p53₁₆₁₋₁₆₉, p53₂₀₄₋₂₁₂), were tested in IVS for the ability to induce wt p53 peptide-specific CD8⁺ T cell responses. PBMC obtained from HLA-A2⁺ or HLA-A24⁺ healthy donors and subjects with SCCHN were used in these experiments. Separate cultures of purified CD8⁺ T cells isolated by positive selection from PBMC were set up with each peptide pulsed onto autologous DCs. Following a 7-day IVS (1x IVS), lymphocytes were evaluated for the peptide-specific CD8⁺ T cell responses in IFN- γ ELISPOT assays. The data in Table 3 indicate that PBMC obtained from all 5 HLA-A2⁺ normal donors responded to at least one of the three HLA-A2-restricted wt p53 peptides tested, with one (HD2-5) showing a response to 2/3 peptides. PBMC from a total of 9 HLA-A2⁺ subjects with SCCHN (4 of which were also HLA-A24⁺) were tested. 7/9 samples, including 2 from HLA-A2⁺/HLA-A24⁺ subjects, were tested against all three HLA-A2-restricted wt p53 peptides. Of these, 3/7 yielded a response to one or more of the peptides, with all three responding to the wt p53₂₆₄₋₂₇₂ peptide. Only the PBMC from this "IVS responder" (SCCHN-3) responded to the other two wt p53 peptides. Consequently, the number of responders among the PBMC samples obtained from the 7 subjects with SCCHN to all three HLA-A2-restricted wt p53 peptides was the same whether a single peptide or multiple peptides were used for stimulation. The PBMC from the other 2 subjects with SCCHN analyzed, both of whom also expressed HLA-A24, failed to respond to any of the peptides tested.

IVS responses to the 3 HLA-A24-restricted wt p53 peptides were studied using PBMC obtained from a total of 17 HLA-A24⁺ subjects with SCCHN, with 7/18 subjects also expressing HLA-A2. 15/17 PBMC samples were tested against all 3 HLA-A24-restricted wt p53 peptides. The IVS responses of the PBMC obtained from 5 HLA-A24⁺ healthy donors to the HLA-A24-restricted wt p53 peptides were quite different compared to those obtained from the 17 subjects with SCCHN. Only 2/5 PBMC obtained from the healthy donors yielded IVS responses, both responding to the wt p53₁₂₅₋₁₃₄. HD24-4 PBMC responded to the wt p53₁₂₅₋₁₃₄ and wt p53₂₀₄₋₂₁₂ peptides, while HD24-5 PBMC responded to the wt p53₁₂₅₋₁₃₄ and wt p53₁₆₁₋₁₆₉ peptides. In contrast, responses to the wt p53₁₂₅₋₁₃₄ were the lowest among the subjects with SCCHN (2/17), while 6/17 and 5/15 responded to the p53₁₆₁₋₁₆₉ and p53₂₀₄₋₂₁₂ peptides, respectively (Tables 4 and 5). Of the 15 PBMC tested against all three peptides, 4/15 yielded responses to more than one peptide, with one sample (SCCHN-20) responding to all three peptides. However, when one takes into account responsiveness to all three HLA-A24-restricted peptides, the frequency of responses from PBMC of healthy donors (2/5 or 40%) was only slightly lower than 8/15 (53%) for subjects with SCCHN.

Overall, these results indicate that PBMC samples obtained from about 1/3 of individuals, whether HLA-A2⁺ and/or HLA-A24⁺ or healthy donor or subject with SCCHN, were responsive to a wt p53 peptide. In general, the wt p53₂₆₄₋₂₇₂ peptide yielded the most responses of the three HLA-A2-restricted wt p53 peptides tested, while among the HLA-A24-restricted

wt p53 peptides, the wtp53₁₆₁₋₁₆₉ and wt p53₂₀₄₋₂₁₂ were more immunogenic than the wt p53₁₂₅₋₁₃₄ peptide.

Correlations of CD8⁺ T-cell responses to wt p53 peptides with clinicopathological factors

We next examined the possibility that the clinicopathological factors, including the p53 accumulation in the tumor, influences their CD8⁺ T-cell responses to wt p53 peptides. Among subjects with an early disease (stages I and II), 5/7 (71%) generated CD8⁺ T-cell responses. In contrast, only 4/13 subjects (31%) with an advanced disease (stages III and IV) responded *in vitro* to wt p53 peptides. This difference in the ability to respond approached significance at $p=0.081$ despite relatively small subject numbers in both cohorts. No significant correlations between CD8⁺ T-cell responses to wt p53 peptides and clinicopathological factors such as age, sex, tumor site, and tumor differentiation were observed. Staining for p53 expression in subjects' tumors showed that 13/23 tumors (57%) accumulated p53 protein (Table 6), confirming our previous report on p53 accumulation in SCCHN [27]. Seven of 13 subjects whose tumors accumulated p53 generated CD8⁺ T-cell responses to wt p53 peptides, as did 5/10 subjects whose tumor showed no p53 protein accumulation. Thus, *ex vivo* immune responses to wt p53 peptides were equally elicited from subjects with tumors that accumulated p53 and those that did not.

Correlations of CD8⁺ T-cell responses to wt p53 peptides with circulating T-cell subsets

In cancer subjects, a loss of balance between type 1 and type 2 T-cell subsets may have negative effects on antitumor immunity. Therefore, we next assessed the T type 1/type 2 balance and the percentages of CD4⁺CD25^{high} (Treg) cells in 18/23 subjects with SCCHN, using multicolor flow cytometry. The T type 1/type 2 balance was determined based on the cytokine profiles expressed in response to *ex vivo* stimulation. As shown in Table 6, the median values for the Th1/Th2 ratio, the Tc1/Tc2 ratio, and the percentage of Treg in these subjects were 5.1 (range 1.4-20.6), 7.0 (range 1.3-14.2), and 3.6 (range 1.2-10.0), respectively. When these subjects were divided into two groups: negative or positive for the ability to generate wt p53 peptide-specific immune responses (Figure 1), only the Tc1/Tc2 ratio in SCCHN subjects who generated CTL was significantly higher compared to that in subjects who were unresponsive. The median Th1/Th2 ratio was also somewhat higher (NSD) in responsive subjects, while the median percentage of Treg tended to be lower (NSD) in the unresponsive group. These results were obtained with small numbers of subjects. They suggest, nevertheless, that the attributes of immune cells in the subjects' circulation, and especially the Tc1/Tc2 ratio, are important for *in vitro* priming of responses to wt p53 peptides.

Discussion

The primary objective of this study was to evaluate six HLA class I-restricted peptides for their ability *in vitro* to prime PBMC obtained from subjects with SCCHN and generate wt p53 peptide-specific CD8⁺ T cell responses. Enhanced expression of naturally occurring, CTL-defined wt p53 epitopes presented by HLA class I molecules on the surface of tumors relative to normal cells has been established [2-9]. Therefore, wt p53 has become a promising candidate for use in broadly applicable, off-the-shelf anti-tumor vaccines. However, we have reported earlier that CTL responses to the wt p53 epitopes, which were selected on the basis of computer-generated algorithms could only be induced in PBMC obtained from some but not all of the HLA-compatible healthy donors or subjects with cancer who were tested [14,27]. Most of these studies utilized one of two wt p53 peptides (i.e., wt p53₂₆₄₋₂₇₂ and/or wt p53₁₄₉₋₁₅₇), previously shown to be immunogenic using transgenic mouse models [28]. In the present study, we simultaneously evaluated *ex vivo* responses of PBMC to wt p53 peptides restricted by HLA-A2 and/or HLA-A24 in cohorts of healthy donors and subjects with SCCHN. This type of approach represents a more rational approach to selection of wt p53 peptides for use in future

multi-epitope cancer vaccines, and also helps identify likely responders to these peptides among subjects who qualify and may benefit from this form of immune therapy.

The results of the analysis of PBMC obtained from the subjects with SCCHN tested for IVS responses indicated that of the three evaluated HLA-A2-restricted wt peptides, the wt p53₂₆₄₋₂₇₂ peptide was the most active, while among the three HLA-A24-restricted peptides tested, the wt p53₁₆₁₋₁₆₉ and p53₂₀₄₋₂₁₂ peptides were comparable to each other and the wt p53₂₆₄₋₂₇₂ peptide in activity. Surprisingly, the responses to the HLA-A24-restricted peptides were more prevalent than those induced by the HLA-A2-restricted wt p53 peptides. This suggests that HLA-A24-restricted peptides may be more immunogenic than the HLA-A2-restricted peptides tested. PBMC from 3 HLA-A2⁺/HLA-A24⁺ subjects with SCCHN (SCCHN-13, -14, and -15) were tested against all six wt p53 peptides, but the results were marginal (0/3 for the HLA-A2 restricted peptides and 1/3 for the HLA-A24-restricted peptides). More extensive studies will be necessary for determining whether there is a distinction in the responsiveness of individuals to the two sets of HLA class I-restricted wt p53 peptides.

To date, most studies have shown that subjects with tumors expressing antigens such as MAGE, NY-ESO or CEA generate tumor antigen-specific CTL at a higher frequency compared to subjects whose tumors do not express these antigens or healthy donors [26,29,30]. Thus, it is reasonable to assume that p53 accumulation in the tumor represents an increased opportunity for more effective presentation of wt p53 epitopes to immune cells and to postulate that it might lead to the development of potent humoral and cellular anti-tumor responses. While this may not always be the case, as indicated by results of Hoffmann et al [27], in the present study, CD8⁺ T-cell responses to multiple wt p53 peptides were detected in peripheral circulation of healthy donors as well as subjects with tumors that accumulated p53 or not. These results highlight the fact that factors other than p53 accumulation in tumor cells are involved in determining responses to wt p53-specific epitopes. In this context, host-mediated factors that might exert significant effects on the subject's ability to generate anti-p53 immune responses and have to be considered. We have reported earlier that anti-wt p53 peptide-specific CTL can be consistently generated from PBMC of normal donors following *in vitro* sensitization with DC (27). This indicates that tolerance against these self peptides is not complete, providing a window of opportunity for CTL expansion.

Immune suppression or deviation of anti-tumor responses is a generalized finding in cancer [31]. Subjects with tumors expressing mutated p53 generally have high-grade malignancies, a poor prognosis and readily detectable immune abnormalities [20,32-34]. In our study, subjects with p53 accumulation in their tumors showed a distinct trend toward immune dysregulation (i.e., had lower type1/type2 responses and increased circulating Treg) compared to subjects with tumors not accumulating p53. On the other hand, the presence in the subjects' circulation of antibodies to p53 indicative of a Th2-biased anti-p53 immune response has been associated with shorter survival and more frequent tumor recurrence [35]. While superficially contradictory, these findings further emphasize the dual potential of mechanisms regulating tumor antigen-specific immune responses that may either promote or retard tumor progression, as recently described [36].

Few studies are available that have examined associations of the subject's immune status and *ex vivo* generation of anti-wt p53 epitope-specific responses. For this reason, we evaluated the immune system components in the SCCHN subjects and correlated the data with their ability to *ex vivo* generate wt p53 peptide-specific CD8⁺ T cells. We specifically examined the balance between type 1 and type 2 T cells and the percentages of Treg in the circulation of each subject. Importantly, we observed that subjects with SCCHN with positive *ex vivo* responses to wt p53 peptides had a significantly elevated Tc1/Tc2 ratio, somewhat higher Th1/Th2 ratio and lower

percentages of circulating Treg compared to non-responsive subjects. Thus, a shift of balance toward Tc1 polarization in the subject's PBMC, which not unexpectedly was most pronounced in subjects with early (stage I and II) disease, helps in eliciting immune responses to wt p53 peptides. As PBMC are readily available for examination, this finding might be useful in the selection of responders who are most likely to benefit from future multi-peptide cancer vaccines incorporating wt p53 epitopes.

Several recent reports have demonstrated that cellular responses to p53 are associated with clinicopathologic parameters, including accumulation of T cells at the tumor site, tumor differentiation and disease stage [37,38]. In this study, we found that subjects with stage I or II disease showed a tendency to respond to wt p53 peptides relative to subjects with advanced disease who were largely in the non-responder category.

Taken together, our data and those of others suggest that in subjects with cancer, T-cell responses to wt p53 peptides is negatively impacted by: (a) the “self” nature of these antigens, and (b) the tumor itself, which regulates the Tc1/Tc2 balance and Treg accumulation [39]. Like most human tumors, SCCHN have evolved sophisticated means of escape from the host immune system either via down-regulation of key molecules necessary for the immune response to proceed, by interference with functions and survival of immune cells, as well as “epitope-loss [31]. An inadequate performance of anti-cancer vaccines to date [40] suggests that to overcome tolerance and tumor escape, it will be necessary to employ much more robust vaccination strategies possibly combining several different therapeutic modalities. In targeting wt p53, the choice of more than one immunogenic peptide, the selection of subjects with early disease, and the ability to shift the balance toward type 1 cytokine polarization become of primary importance, if this clinical strategy is to be successful. Given the reality that anti-wt p53 epitope-based vaccines are already in clinical trials [12,13], this report providing data a useful for vaccine improvement is especially timely.

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Figure 1. Correlations of CD8⁺ T-cell responses to wt p53 peptides with circulating T-cell subsets
Positive (+) and negative (-) responsiveness to HLA class I-restricted wt p53 peptides of CD8⁺ T cells present in PBMC obtained from HLA-A2⁺ and/or HLA-A24⁺ subjects with SCCHN following 1x IVS are plotted against (A) Th1/Th2 ratio, (B) Tc1/Tc2 ratio and (c) % Treg of subject's PBMC. As indicated in panel (A) positive wt p53 peptide-specific CD8⁺ T cell responses were only significant relative to Tc1/Tc2 ratios of subject's PBMC.

Table 1
Characteristics of the HLA-A2 and/or A24-positive patients with SCCHN tested in this study

patient	age/sex	HLA typing A2/A24	tumor site	stage	histology	disease status at time of evaluation ^a
SCCHN-1	53M	+/-	Oral cavity	III	SCC (mod)	Local Rec.
SCCHN-2	72M	+/-	Hypopharynx	IV	SCC (poor)	NED
SCCHN-3	65M	+/-	Hypopharynx	III	SCC	Local Dis.+LN mets.
SCCHN-4	36F	+/-	Nasopharynx	IV	SCC (poor)	Local Dis.+LN mets.
SCCHN-5	68M	+/-	Larynx	I	SCC (mod)	Local Dis.
SCCHN-6	56M	++	Hypopharynx	IV	SCC (mod)	Distant mets. (lung)
SCCHN-7	75M	++	Larynx	IV	SCC (mod)	Local Dis.+LN mets.
SCCHN-8	62M	++	Hypopharynx	IV	SCC (poor)	Local Dis.+LN mets.
SCCHN-9	50F	++	Hypopharynx	III	SCC (well)	Local Dis.
SCCHN-10	77M	++	Oral cavity	IV	SCC (well)	Local Dis.
SCCHN-11	84M	++	Larynx	IV	SCC (mod)	Local Dis.+ LN mets.
SCCHN-12	80M	++	Hypopharynx	IV	SCC (mod)	Local Dis.+ LN mets.
SCCHN-13	82M	++	Oropharynx	IV	SCC (mod)	Local Dis.
SCCHN-14	75M	-/+	Hypopharynx	IV	SCC (mod)	Local Dis.+ LN mets.
SCCHN-15	68M	-/+	Hypopharynx	IV	SCC (poor)	Local Dis.+ LN mets.
SCCHN-16	54F	-/+	Oral cavity	III	SCC (mod)	Local Dis.
SCCHN-17	63M	-/+	Larynx	II	SCC (well)	Local Dis.
SCCHN-18	65M	-/+	Hypopharynx	II	SCC (well)	Local Dis.+ LN mets.
SCCHN-19	60M	-/+	Oral cavity	II	SCC (well)	Local Dis.
SCCHN-20	56M	-/+	Larynx	II	SCC (well)	Local Dis.
SCCHN-21	77M	-/+	Hypopharynx	III	SCC (mod)	Local Dis.+ LN mets.
SCCHN-22	66M	-/+	Larynx	II	SCC (mod)	Local Dis.
SCCHN-23	64M	-/+	Hypopharynx	I	SCC (mod)	Local Dis.

^aRec, recurrence; NED, no evidence of disease; LN mets, lymph node metastasis; mets, metastasis

Table 2
HLA-A2 and -A24 restricted wt-p53 derived peptides used in this study

restriction element	amino acid position	amino acid sequence
A2	149-157	STPPPGTRV
	217-225	VVPYEPPEV
	264-272	LLGRNSFEV
A24	125-134	TYSPALNKMV
	161-169	AIYKQSQHM
	204-212	EYLDNRNTF

Table 3
Healthy donor CD8+ T-cell responses to wt p53-derived peptides

HLA-A2 restricted wt p53 peptides		CD8+ T-cell response to the peptide (number of IFN- γ spots/ 10^5 CD8+ T-cell)		
Healthy donor #		149-157	217-225	264-272
HD2-1		3	3	14
HD2-2		0	2	13
HD2-3		0	25	0
HD2-4		27	3	0
HD2-5		29	4	26
HD2-6		ND	ND	4
Total responses		2/5	1/5	3/6
HLA-A24 restricted wt p53 peptides		CD8+ T-cell response to the peptide (number of IFN- γ spots/ 10^5 CD8+ T-cell)		
Healthy donor #		125-134	161-169	204-212
HD24-1		0	2	0
HD24-2		0	0	0
HD24-3		1	1	3
HD24-4		12	3	12
HD24-5		13	17	1
Total responses		2/5	1/5	1/5

CD8+ T-cell responses to wt-p53 derived peptides in HLA-A2-positive patients with SCCHN

Table 4

patient #	CD8+ T-cell response to the peptide (number of IFN- γ spots/10 ⁵ CD8+ T-cell)		
	149-157	217-225	264-272
SCCHN-1	7	5	0
SCCHN-2	5	2	24
SCCHN-3	26	16	21
SCCHN-4	0	0	0
SCCHN-5	0	0	26
SCCHN-10	0	ND	2
SCCHN-11	1	3	1
SCCHN-12	ND	ND	0
SCCHN-13	6	7	0
Total responses	1/8	1/7	3/9

CD8+ T-cell responses to wt-p53 derived peptides in HLA-A24-positive patients with SCCHN

Table 5

patient	CD8+ T-cell response to the peptide (number of IFN- γ spots/ 10^5 CD8+ T-cell)		
	125-134	161-169	204-212
SCCHN-6	0	33	36
SCCHN-7	20	3	32
SCCHN-8	3	3	1
SCCHN-9	0	0	7
SCCHN-10	0	4	0
SCCHN-12	3	0	27
SCCHN-13	0	ND	ND
SCCHN-14	2	ND	ND
SCCHN-15	0	4	4
SCCHN-16	6	8	5
SCCHN-17	0	0	0
SCCHN-18	0	33	0
SCCHN-19	1	0	0
SCCHN-20	23	20	19
SCCHN-21	3	21	0
SCCHN-22	2	28	0
SCCHN-23	6	30	25
Total response	2/17	6/17	5/15

Table 6
Summary of CD8+ T-cell responses, percentages of T-cell subsets, and the p53 status in their tumors in patients with SCCHN

patient	CD8+ T-cell response against wt p53- derived peptides	Th1/Th2 ratio	Tc1/Tc2 ratio	%Treg (CD4+CD25 ^{high})	tumor p53 accumulation
SCCHN-1	+	2.3	12.1	10.0	+
SCCHN-2	+	ND	ND	ND	-
SCCHN-3	+	5.1	7.9	2.9	-
SCCHN-4	-	1.4	1.3	2.5	+
SCCHN-5	+	9.7	9.4	1.2	+
SCCHN-6	+	7.4	14.2	3.9	-
SCCHN-7	+	ND	ND	ND	-
SCCHN-8	-	ND	ND	ND	-
SCCHN-9	-	ND	ND	ND	-
SCCHN-10	-	6.3	7.8	2.5	-
SCCHN-11	-	3.1	3.9	3.2	+
SCCHN-12	+	7.4	5.1	2.9	+
SCCHN-13	-	5.1	5.6	5.0	+
SCCHN-14	-	4.6	5.7	5.7	-
SCCHN-15	-	5.8	5.8	4.3	+
SCCHN-16	-	4.7	4.9	3.1	-
SCCHN-17	-	2.4	6.9	5.4	+
SCCHN-18	+	20.6	8.8	6.1	+
SCCHN-19	-	5.4	7.1	4.2	+
SCCHN-20	+	5.1	4.8	3.9	+
SCCHN-21	+	2.3	9.4	1.6	+
SCCHN-22	+	ND	ND	ND	+
SCCHN-23	+	15.6	12.2	1.6	-
Total response	12/23				