

Phase I Dendritic Cell p53 Peptide Vaccine for Head and Neck Cancer

Patrick J. Schuler^{1,7}, Malgorzata Harasymczuk¹, Carmen Visus³, Albert DeLeo³, Sumita Trivedi^{1,4}, Yu Lei¹, Athanassios Argiris⁶, William Gooding², Lisa H. Butterfield⁵, Theresa L. Whiteside^{1,4}, and Robert L. Ferris^{1,4}

Abstract

Background: p53 accumulation in head and neck squamous cell carcinoma (HNSCC) cells creates a targetable tumor antigen. Adjuvant dendritic cell (DC)-based vaccination against p53 was tested in a phase I clinical trial.

Experimental Methods: Monocyte-derived DC from 16 patients were loaded with two modified HLA-class I p53 peptides (Arm 1), additional Th tetanus toxoid peptide (Arm 2), or additional Th wild-type (wt) p53-specific peptide (Arm 3). Vaccine DCs (vDC) were delivered to inguinal lymph nodes at three time points. vDC phenotype, circulating p53-specific T cells, and regulatory T cells (Treg) were serially monitored by flow cytometry and cytokine production by Luminex. vDC properties were compared with those of DC1 generated with an alternative maturation regimen.

Results: No grade II–IV adverse events were observed. Two-year disease-free survival of 88% was favorable. p53-specific T-cell frequencies were increased postvaccination in 11 of 16 patients (69%), with IFN- γ secretion detected in four of 16 patients. Treg frequencies were consistently decreased ($P = 0.006$) relative to prevaccination values. The phenotype and function of DC1 were improved relative to vDC.

Conclusion: Adjuvant p53-specific vaccination of patients with HNSCC was safe and associated with promising clinical outcome, decreased Treg levels, and modest vaccine-specific immunity. HNSCC patients' DC required stronger maturation stimuli to reverse immune suppression and improve vaccine efficacy. *Clin Cancer Res*; 20(9); 2433–44. ©2014 AACR.

Introduction

Despite considerable recent advances in therapy of head and neck squamous cell carcinoma (HNSCC), survival has not improved >50% for decades, largely due to tumor recurrence, high frequency of second primary tumors, development of distant metastases, and resistance of the tumor to chemoradiotherapy (1). Novel therapeutic strategies are needed, and tumor antigen-specific vaccination has been perceived as a potentially effective approach to improve outcome by mobilizing antitumor immunity and

reversing immune escape in patients with HNSCC. Immune suppressive mechanisms described in these patients are extensive and include impaired dendritic cell (DC) maturation (2, 3), inefficient antigen presentation (4), and an increased frequency as well as suppressive activity of regulatory T cells (Treg; ref. 5). As a result of HNSCC-induced immune suppression, antitumor responses of patients are compromised, and a reversal or removal of immune suppression would be expected to restore these responses. Consequently, antitumor vaccines offer an opportunity for normalization of immune responses and of the immune balance responsible for the control of tumor progression.

In all antitumor vaccines, the selection of a target antigen is one of the most critical issues. The requirements for the selection of a tumor-associated antigen (TAA) to be used in the vaccine have been defined (6). Such a TAA should be selectively expressed in the tumor cells but not normal tissues, naturally processed and presented by APC and immunogenic, that is, able to induce TAA-specific effector and Th cells. On the basis of previous studies, p53 meets these criteria: it is the most frequently mutated gene in HNSCC (7) and the mutated protein accumulates in most HNSCC cells (8), allowing for overexpression and processing of wild-type (wt) p53 peptides (8–11). We have previously demonstrated that the presentation of wt p53 peptides on DC induced peptide-specific immune responses *in vitro* in normal donors and in patients with HNSCC

Authors' Affiliations: ¹Cancer Immunology Program; ²Biostatistics Facility, University of Pittsburgh Cancer Institute; Departments of ³Pathology and ⁴Otolaryngology, University of Pittsburgh School of Medicine; ⁵Division of Hematology/Oncology, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; ⁶Department of Medicine, Hematology/Oncology, University of Texas-San Antonio Cancer Center, San Antonio, Texas; and ⁷Department of Otolaryngology, University of Ulm, Germany

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M. Harasymczuk and C. Visus contributed equally to this article.

Corresponding Author: Robert L. Ferris, University of Pittsburgh Cancer Institute, Research Pavilion at the Hillman Cancer Center, 5117 Centre Avenue, Suite 2.26, Pittsburgh, PA 15213-1863. Phone: 412-623-0327; Fax: 412-623-4840; E-mail: ferrisr@upmc.edu

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Translational Relevance

We performed a novel phase Ib clinical trial of adjuvant p53 peptide-loaded dendritic cell (DC) based adjuvant vaccination for patients with head and neck cancer. To the best of our knowledge, this is the largest clinical trial experience of a DC-based cancer vaccine for these patients. With the recent U.S. Food and Drug Administration approval of cellular and antibody-based immunotherapeutic strategies, and the confirmation the TP53 alteration as the major genomic abnormality in head and neck cancer, we believe that our study of adjuvant p53-based vaccination is timely and valuable to warrant further phase II testing. In particular, our observation of consistent declines in regulatory T cells in vaccinated patients, which are a poor prognostic subset of T cells, suggests that clinical benefit would result in larger vaccinated cohorts.

(8, 12–14). Similar *in vivo* studies with wt p53-pulsed DC in animal tumor models have also led to the development of antitumor immunity and inhibited tumor growth (11). Also, DC-based wt p53 peptide vaccines have been used for immunotherapy of human ovarian, breast, lung, and colon cancers (15).

Here, we report the results of a phase Ib trial of autologous monocyte-derived DC loaded with selected wt p53 peptides. Although the study follows the design used for other wt p53-based DC vaccines (15, 16), it includes several novel aspects. First, to determine whether the addition of Th peptides to the vaccine results in the improvements of TAA-specific antitumor immunity, we included the HLA class II p53 helper peptide (p53₁₁₀₋₁₂₄; ref. 17) or tetanus-

derived memory peptide (18) in separate vaccine arms. Second, we used two altered (optimized) HLA-A*0201-binding p53 peptides (p53₁₄₉₋₁₅₇ T150L variant; ref. 9; and p53₂₄₆₋₂₇₂ F270W variant; ref. 19) to enhance the peptide binding to T cell receptors (TCR) or to HLA-A2.1 with higher avidity, respectively. Third, serial immune monitoring of all vaccinated patients allowed for the consideration of TAA-specific immunity generated in response to the vaccine in the context of clinical outcome. Finally, *in vitro* generation of DC from the patients' monocytes in parallel, comparing properties of DC used in the vaccine DC (vDC) with an alternative maturation protocol (DC1; refs. 20, 21), allowed for the identification of improvements in the DC maturation that can boost their adjuvant activity in future trials. The study provides novel insights into optimization of the production and monitoring of adjuvant DC-based vaccines for patients with HNSCC and other p53-overexpressing epithelial malignancies.

Materials and Methods

Patient cohort

16 HLA-A2.1⁺ patients with advanced HNSCC were enrolled in this study. Six patients were treated with surgery alone and 10 patients had received adjuvant chemoradiotherapy after surgery. At the time of vaccination, all patients were free of disease. The patients' clinical and demographic data are listed in Table 1. All patients were treated at the University of Pittsburgh Cancer Institute (Pittsburgh, PA) and signed written informed consent to participate in this trial, which was approved by the Institutional Review Board of the University of Pittsburgh (UPCI 03-156; IRB# 0507062, NCT00798655), under U.S. Food and Drug Administration IND #12273 (sponsor R.L. Ferris).

Table 1. Clinical and demographic data

ID	Arm	Age	TNM	Location	Status (mo)
1	1	78	T2 N2c	Base of tongue	Alive (30)
2	1	66	T3 N1	Oropharynx	Alive (29)
3	1	55	T3 N2b	Base of tongue (r)	Alive (41)
4	1	27	T2 N1	Base of tongue	Alive (06)
5	1	58	T2 N2b	Lateral tongue	DOD (17)
6	1	55	T4 N2b	Base of tongue (r)	Alive (26)
7	2	68	T4 N2b	Hypopharynx	Alive (27)
8	2	60	T1 N0	Tonsil	Alive (42)
9	2	42	Tx N2a	CUP	Alive (38)
10	2	57	Tx N2a	CUP	Alive (39)
11	3	57	T4 N2c	Pan-pharynx	DOD (25)
12	3	56	Tx N2a	CUP	Alive (26)
13	3	77	T3 N0	Maxilla (r)	DOD (16)
14	3	59	T4 N2b	Base of tongue	Alive (39)
15	3	62	T2 N0	Hypopharynx	Alive (33)
16	3	54	T4 N2b	Larynx	Alive (29)

Abbreviations: CUP, cancer of unknown primary; (r), recurrent disease before vaccination.

Immunohistochemistry of tumors for p53

Staining was performed with p53 monoclonal primary antibody (D011, 1:400 dilution) and secondary antibody "Dual Envision Polymer," using Antigen Retrieval-Citrate pH6 buffer (Dako) and 3,3'-diaminobenzidine chromogen (Dabiosys). p53 protein accumulation was scored as positive when >10% of tumor cells were stained positive as observed by the study pathologist.

Generation of DC

Monocytes were isolated from autologous leukapheresis products by the Elutra System (Caridian BCT). The monocyte-rich fraction #5 was collected and analyzed for cell viability, cell counts, and the cell phenotype by flow cytometry. Following 6-day culture of separated monocytes in Cellgenix DC medium containing granulocyte macrophage colony-stimulating factor (GM-CFS; 1,000 IU/mL) and interleukin (IL)-4 (10 ng/mL, both Cellgenix), immature DC (iDC) were harvested and counted. Aliquots containing 10,000 harvested iDC were replated in T-75 culture flasks in medium containing GM-CFS and IL-4 as above, and matured by the addition of a proinflammatory cytokine cocktail for 48 hours.

DC used for vaccination of patients (vDC) were matured in a cytokine cocktail containing IL-1 β (10 ng/mL), IL-6 (10 ng/mL), TNF- α (10 ng/mL), and prostaglandin E (PGE₂; 1 μ g/mL). The maturation cytokine cocktail for DC1, which were generated in parallel with vDC but were not used for vaccination, contained IL-1 β (10 ng/mL), TNF- α (50 ng/mL), IFN- α (3,000 IU/mL), IFN- γ (1,000 IU/mL), and Poly I:C (20 μ g/mL). Supernatants of both DC preparations were collected for cytokine measurements (IL-10, IL-12, VEGF) by Luminex according to the manufacturer's protocol (SinglePlex Bead Kit, Invitrogen). DC1 was used for *in vitro* studies only. Cytokines were purchased from the following manufacturers: IL-1 β , IL-4, IL-6, and TNF- α (R&D Systems); IFN- α (Schering); IFN- γ (Intermune); GM-CSF (Immunex); and PGE₂ and Poly I:C (Sigma-Aldrich). All DC cultures were tested negative for endotoxin and mycoplasma contamination.

Patient randomization and administration of vaccines

Patients were randomized into three treatment arms as shown in Supplementary Table 1 and Supplementary Fig. S1. The following peptides were used for DC loading: modified HLA-A2 binding wt p53₁₄₉₋₁₅₇ [S(T->L)PPPGTRV], with T150L substitution (9) and wt p53₂₆₄₋₂₇₂ [LLGRNS(F->W)EV], with F270W substitution (22). In Arm 2, the modified, pan-DR-binding Th epitope from tetanus toxoid (AQYIKANSKFIGIGL; refs. 18, 23-25) was used. HLA DR4⁻ patients were randomized to Arm 1 (no helper peptide) or Arm 2 (tetanus helper peptide). All HLA-DR4⁺ patients were assigned to Arm 3 due to DR4⁺ restriction of the Th p53₁₁₀₋₁₂₄ peptide sequence (26).

Fresh or cryopreserved mDCs were loaded with the respective combinations of class I/class II peptides (10 μ M each) for 18 hours. Autologous DCs (100 μ L) were injected into an inguinal lymph node under ultrasound guidance

biweekly for three time points. The vaccination schedule and the injection site were selected on the basis of previously reported successful experience at our institution (15, 27). Lymph nodes in the head and neck region were avoided, as they could have been affected by the proximity of tumor cells.

Collection of peripheral blood mononuclear cells

Blood samples of study patients (40-50 mL) were collected at the following time points: (i) baseline, (ii) one day before the second vaccination, (iii) one week, and (iv) one month after the third vaccination. Blood was drawn into heparinized tubes and centrifuged on Ficoll-Hypaque gradients (GE Healthcare Bioscience). Peripheral blood mononuclear cells (PBMC) were recovered, washed, and used for experiments or cryopreserved in RPMI medium (Invitrogen) containing FBS (40%) and dimethyl sulfoxide (10%).

Antibodies and flow cytometry

The following anti-human monoclonal antibodies (mAb) were used for staining CD3-FITC, CD4-PC5, CD8-PC5, CD14-ECD, CD80-FITC, CD86-PE, DR-ECD, CD83-PC5, CD1a-PC5 (Beckman Coulter), HLA-A2-PE (Biolegend), CCR7-FITC (R&D Systems), CD39-FITC, (eBioscience), CD25-PE (Miltenyi), including their respective isotypes, which served as negative controls for surface as well as intracellular staining. All Abs were pretitrated using PBMC to determine the optimal staining dilution for each. For HLA typing and determination of antigen-processing machinery (APM) components, we used BB7.2 δ -specific mAb SY-4, LMP 2-specific mAb SY-1, TAP1-specific mAb, TAP2-specific mAb NOB-2, tapasin-specific mAb TO-3, which were generated by Dr. Soldano Ferrone (Harvard University, Cambridge, MA) and generously donated for these studies (3).

Cryopreserved cells were thawed and washed in RPMI medium. For flow cytometry, cells were incubated with mAbs specific for each surface marker in 50 μ L PBS for 30 minutes at room temperature. Flow cytometry was performed using an EPICS XL-MCL flow cytometer equipped with Expo32 software (all Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward and side scatter. At least 1×10^5 events were acquired for analysis and gates were restricted to the CD4⁺ and CD8⁺ T-cell subsets.

The frequency of Treg was measured by multicolor flow cytometry gating on CD4⁺CD25⁺CD39⁺ T cells as previously described (28). The absolute numbers of Treg were calculated using total white cell blood counts determined for each patient. Normal control (NC) values for the frequency and absolute Treg numbers were available in our laboratory having been determined previously.

p53 peptides and measurement of p53-specific CTL

The frequency of CTL specific for wt p53 epitopes was determined by tetramer flow cytometry. Tetramers specific for the wt p53₁₄₉₋₁₅₇ sequences (STPPPGTRV) and wt p53₂₆₄₋₂₇₂ sequences (LLGRNSFEV) were obtained from

the NIH tetramer facility (Atlanta, GA). The specificity of the p53 tetramers was confirmed by staining against a CTL line specific for p53 as well as irrelevant CTL. The gate for tetramer⁺ events was defined by PBMC stained with antibodies (CD3/CD8/CD14) in the absence of tetramer, and the lower limit of detection (LLD) for p53 peptide-specific tetramers was established at the reciprocal frequency of one of 7,805 (0.013%) by staining T cells of ten HLA-A2.1^{neg} donors (13, 29). Approximately 1×10^6 events were acquired, including >50,000 gated CD8⁺ T cells, and positive events were defined as CD3⁺CD8⁺CD14^{neg}tetramer⁺.

ELISPOT assays

Reactivity of PBMC against p53-peptides was tested by IFN- γ ELISPOT assay as previously described (8). Patients' PBMC (1×10^5 /well) were stimulated using T2 cells loaded with wt p53 peptides or tetanus peptide for 18 hours, *in vitro*. All experiments used negative and positive controls to confirm CTL specificity, consisting of T2 cells without peptide or phorbol 12-myristate 13-acetate/ionomycin-treated CTL for maximum spots, respectively. The ELISPOT assay was performed in 96-well plates (Nunc). The capture and detection Abs and 3-amino-9-ethylcarbazole (AEC) substrate reagent were purchased from BD Biosciences (Human IFN- γ ELISPOT pair, AEC substrate reagent set). Spot numbers and sizes were determined with computer-assisted video image analysis (Cellular Technologies). Background, as determined by unstimulated samples, was subtracted in all cases.

APM component expression in DC

APM components TAP1, TAP2, LMP2, and tapasin were determined by flow cytometry (30, 31). Cells were fixed in 2% paraformaldehyde for 10 minutes at room temperature and permeabilized in 100% methanol for >24 hours at -20°C . Cells were then washed and incubated with primary APM component-specific mAb and fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse mAb for 30 minutes at room temperature, respectively. A minimum of 100,000 events were acquired and the cutoff level was set at mean fluorescence intensity (5) based on immunoglobulin G-2a isotype control.

Quantitation of tumor-infiltrating lymphocytes

Hematoxylin and eosin-stained sections of 11 vaccinees' blinded tumor sections were reviewed, and the intratumoral-infiltrating lymphocytes were enumerated in five high power fields ($\times 400$). Tumor cells comprised at least 60% of each field. The extent of tumor-infiltrating lymphocyte (TIL) was determined by the average value of the TIL numbers in the five counted fields. As a relative measure of intratumoral inflammation, lymphocytic tumoral infiltration was considered mild, moderate, or prominent, if the TIL number was ≤ 10 , 11 to 20, ≥ 20 , respectively. Classification of patient clinical outcome and immune responses after vaccination were evaluated for each group to identify patterns associated with extent of TIL present.

Statistical analysis

All averages were calculated as mean. Paired samples were evaluated by Wilcoxon paired signed rank test, and correlations were calculated by the Spearman test using SPSS software (IBM, version 19). The permutation test was used for significance of T-cell reactivity. Comparison of three vaccination arms was performed by Kruskal-Wallis and consecutive Mann-Whitney test. Maturation cocktails were compared for eight different vaccine phenotypes, four APM components, and three cytokines by Friedman test and with adjustment for false discovery for surviving signed rank tests of vDC versus DC1. Kaplan-Meier estimates and the log-rank test were applied for evaluation of disease-free survival (DFS).

Results

Clinical information of vaccinated patients

Demographics of the wt p53 peptide-based DC vaccinated cohort are shown in Table 1. The vaccine was well tolerated by all patients, with skin rash at injection sites and circumscribed hematomas after leukapheresis in two of 16 patients, but no grade II-IV adverse events occurred. No differences were observed between vaccine arms. All patients were followed every 3 months using clinical examination and periodic PET/CT scans for 3 years.

Disease-free survival and tumor immunohistochemistry

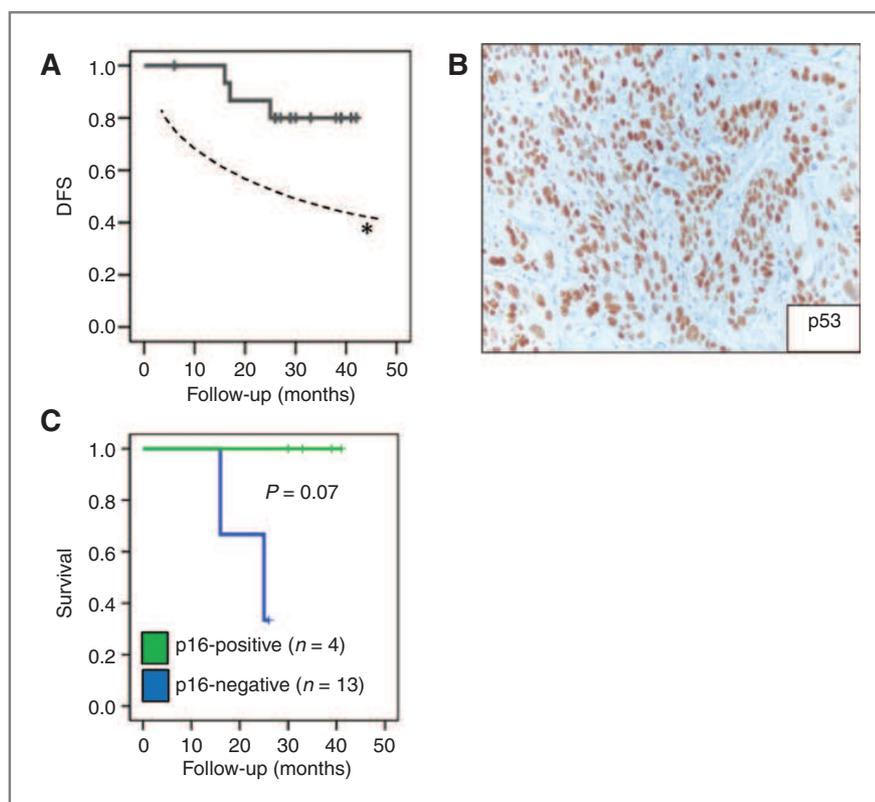
Among the 16 vaccinated patients, three died of disease (DOD) at 16, 17, and 25 months; 13 patients remain alive with no evidence of disease, the median follow-up of 32 months (range 6–42 months). Thus, 2-year DFS was 88% and 3-year DFS was 80% (Fig. 1A). This DFS rate was favorable, as compared with the DFS of a similar, previously untreated stage III/IV clinical trial cohort treated with chemoradiation at our institution, as published previously (70%, 95% confidence interval, 53%–82%; ref. 32)).

Tumor samples obtained from the patients who underwent surgery ($n = 10$) before vaccination were examined for expression of p53 in tumor cells. By immunohistochemistry (IHC), eight tumors were p53⁺ by IHC (Fig. 1B) and eight were p53^{neg}. No significant difference in DFS between patients who had p53⁺ versus p53^{neg} tumors was observed. HPV-16 status was available for seven patients, of whom four tumors were HPV-16⁺ using p16 IHC and *in situ* hybridization (32). As expected, HPV status strongly predicted clinical outcome even within this small patient group (Fig. 1C).

Characteristics of vDC

Monocyte-derived matured DC generated for vaccine therapy (vDC) in the patients with HNSCC showed significant upregulation of surface markers HLA-DR, CD86, CD80, and CD83 relative to autologous mononuclear cells or iDC (Fig. 2A). Phenotyping was performed after peptide loading, and there were no differences in expression of surface markers between DC pulsed with class I or class II peptides; therefore, their phenotypic results are grouped

Figure 1. Disease-free survival (DFS) and tumor immunohistochemistry. A, DFS was 88% after 2 years and 80% after 3 years. B, representative IHC of an HNSCC tumor positive for p53 ($\times 10$ magnification). C, DFS for patients ($n = 7$) based on HPV p16 status.



in Fig. 2A. After DC-1 maturation, upregulation of CD80, CD86, and HLA-A2 was stronger, whereas upregulation of CD83 and CCR7 was weaker compared with vDC. Downregulation of CD1a was greater in DC1 than in vDC (all adjusted signed rank $P < 0.01$).

APM components were also measured, and TAP1/2, LMP2, and tapasin were modestly upregulated in vDC as compared with iDC, similar to our previous findings (ref. 30; Fig. 2B). Expression of APM components (TAP1, TAP2, LMP2, and tapasin) was measured in iDC, vDC, and DC1. Although we observed an increased expression of APM components in vDC and DC1 relative to iDC ($P < 0.05$), upregulation of APM components was again significantly stronger in DC1 than in vDC ($P < 0.05$; Fig. 2B). Significant differences were seen in the expression of additional surface maturation markers between DC matured in these two cocktails as shown in Fig. 2C.

Peptide-specific CTL responses to the vaccine

Immune monitoring at baseline and at three postvaccination time points included tetramer analysis for the frequency of wt p53-specific CTL (Fig. 3A). Peptide-specific immune response was considered to be positive when the following criteria were met: (i) the frequency of p53-specific CTL was above LLD of 0.013%, as established previously (13); (ii) the frequency of CTL for both peptide sequences (wt p53₁₄₉₋₁₅₇ and wt p53₂₆₂₋₂₇₄) at any two postvaccina-

tion time points was increased as compared with baseline; and (3) the increase in tetramer-positive events was greater than 0.02%. Applying all three criteria, 69% (11/16) of patients showed a positive tetramer response. Specifically, six of 16 patients (38%) had a positive tetramer response for both peptide sequences ("full response") and five of 16 patients (31%) had a response to one of the two peptides ("partial response") as shown in Fig. 3B. Five patients (31%) were nonresponsive, and although three of five of these patients were in Arm 3, no significant difference in tetramer responses could be attributed to the vaccination arms ($P = 0.46$). Patients in Arm 1 had the most favorable CTL response but without reaching statistical significance. Patients who had tetramer responses were stratified against those with no evident or weak tetramer responses to determine whether wt p53 peptide-specific reactivity predicted DFS. As shown in Fig. 3C, peptide-specific immune responses after vaccination did not differentiate patients based on DFS.

ELISPOT assays

The presence of IFN- γ -producing CTL in patients' PBMC upon stimulation with T2 cell pulsed with the wt p53 peptides was measured in ELISPOT assays. Serial assays were performed at all four time points. Representative data for single tetramer-positive patients (one in each Arm) are shown in Fig. 3D. The data show variably increased

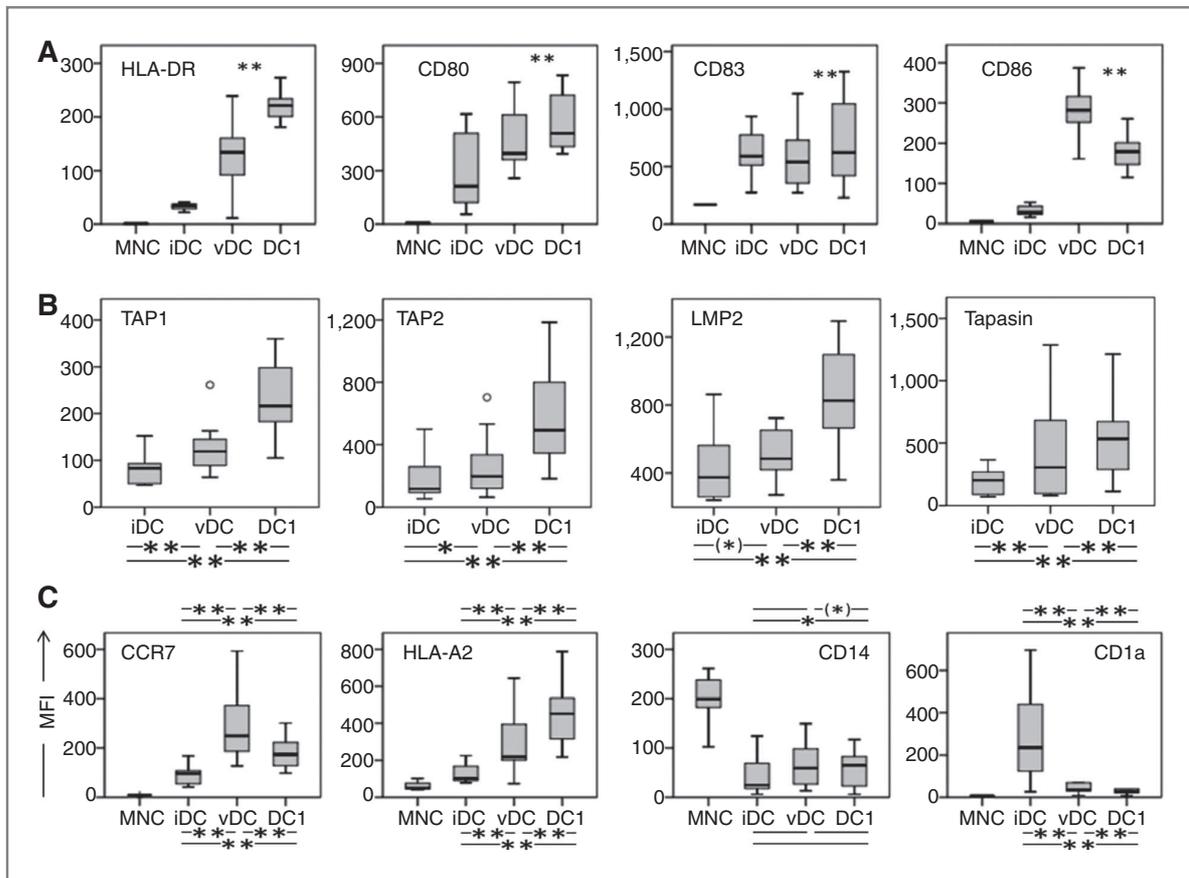


Figure 2. Phenotypic and functional characteristics of vDC. A, monocyte-derived iDC were loaded with wt p53 peptides and matured in a conventional cytokine cocktail before vaccination or in an alternative cytokine cocktail generating DC1. Mature DC for vaccination (vDC) and DC-1 were phenotyped for the maturation markers. Box plots for all patients' vDC show quartiles for 25, 50, 75 as boxes, and values for 0% and 100% as whiskers. B, intracellular expression of APM components TAP1, TAP2, LMP2, and tapasin was determined in iDC and vDC of all study patients ($n = 16$). Intracellular expression of APM components was determined in vDC and DC1 of all study patients ($n = 16$). *, $P < 0.05$; **, $P < 0.01$. C, iDCs were generated from monocytes (MNC) obtained from study patients with HNSCC. iDCs were matured with two different maturation cocktails (vDC and DC1). MNC, iDC, vDC, and DC1 were phenotyped for additional maturation markers. Wilcoxon paired signed rank test; **, $P < 0.005$; *, $P < 0.05$; \ominus , $P < 0.1$.

numbers of spots at the postvaccination time point versus background values for four selected patients who were tetramer responders. These were the only patients among 16 tested with positive ELISPOT results. Interestingly, positive IFN- γ responses to the wt p53 peptides as well as to the tetanus peptide were seen, suggesting epitope spreading and/or nonpeptide-specific activation of immune cells in these four patients.

Regulatory T cells levels after vaccination

In this study, Treg were evaluated by flow cytometry and defined as CD4⁺CD25⁺CD39⁺ T cells, as shown in Fig. 4A. We have previously reported that these Treg cells were highly FoxP3⁺ and mediated suppression of autologous CD4⁺ T-cell proliferation after *in vitro* SEB stimulation (33). The frequency of CD4⁺CD25⁺CD39⁺ Treg was determined at baseline and after administration of the third vaccine (days 35–56). As expected, the frequency of Treg was increased at

baseline in patients with HNSCC ($8.1 \pm 3.5\%$) in comparison with normal controls ($NC, 3.4 \pm 2.1\%$; $n = 40$; $P < 0.001$). Notably, after vaccination, the frequency of Treg (Fig. 4B) as well as the absolute number of Treg (Fig. 4C) were significantly decreased ($P < 0.006$ and $P < 0.005$, respectively). The Treg frequency was decreased in 12 of 15 patients evaluated and the absolute number was decreased in 15 of 16 patients. After vaccination, the mean absolute number of Treg was lower than that present in the circulation of NC (28 ± 6 per μL ; $n = 20$; $P < 0.01$). In some patients, a decrease in the Treg frequency after vaccination was accentuated by an increase in total CD4⁺ T-cell frequencies. In the patient cohort as a whole, the frequency of Treg in the peripheral blood had a strong inverse correlation with the absolute number of CD4⁺ T cells ($P < 0.01$; Fig. 4D).

To determine whether the overall decrease in Treg number is due to vaccination-independent nonspecific contribution of TIL, we analyzed 11 available prevaccine tumor

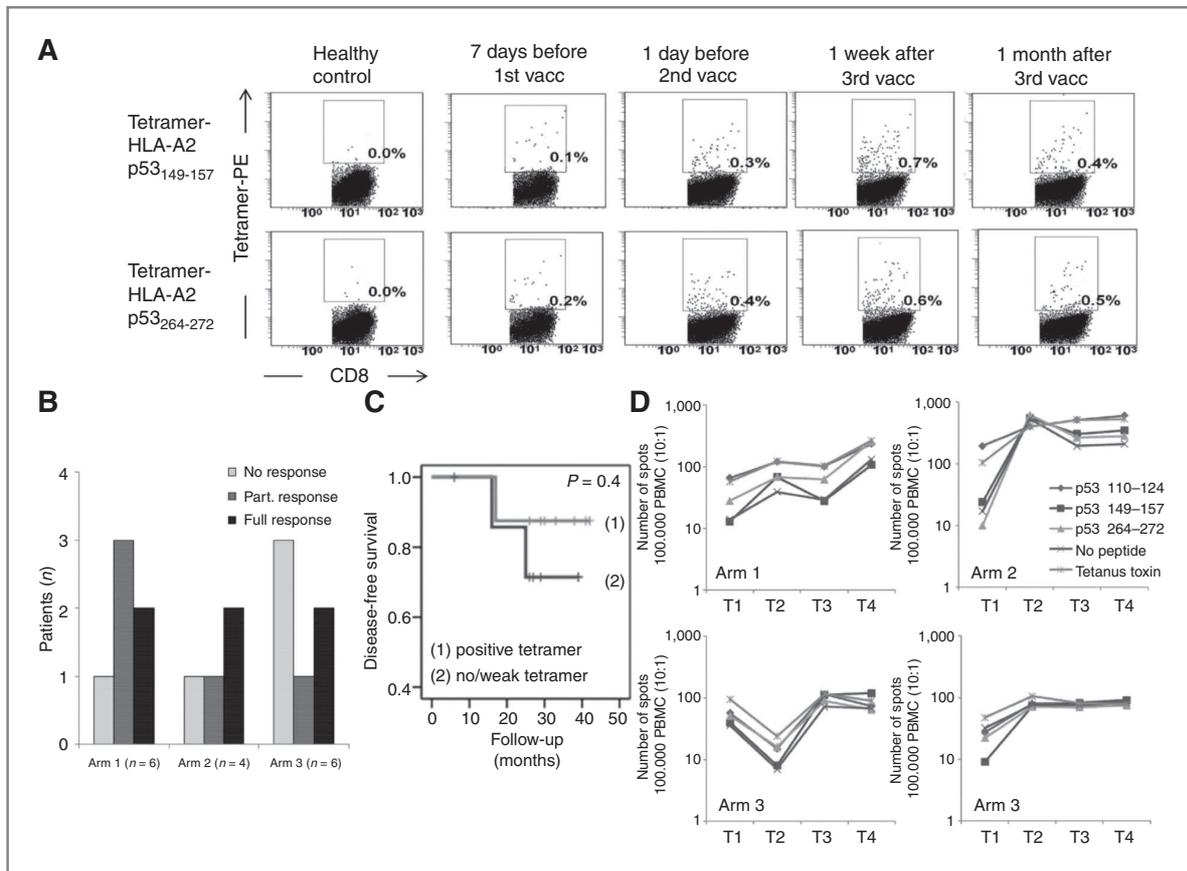


Figure 3. Immune response to the vaccine. **A**, the frequency of CTL specific for wt p53₁₄₉₋₁₅₇ or wt p53₂₆₄₋₂₇₂ sequences in patients' peripheral blood was determined by tetramer-flow cytometry at baseline and at three follow-up time points, as indicated. Dot plots are from one representative patient with HNSCC and one normal control. Boxes indicate tetramer-positive CD8⁺ T cells. **B**, numbers of patients in each treatment arm with positive, partial, or no tetramer response. Six of 16 patients (38%) showed a positive immune response; five of 16 patients (31%) showed a partial response with an elevated CTL frequency for only one peptide; and five of 16 patients (31%) showed no peptide-specific immune response to both wt p53 peptides. Patients in treatment arm 1 had the most favorable immune response, without reaching significance ($P > 0.2$). **C**, DFS by the positivity of tetramer responses; patients are classified as "positive tetramer response" or "no/weak tetramer response." Overall DFS was similar for each tetramer response group (log-rank, $P = 0.754$). **D**, representative IFN- γ ELISPOT data at four different time points: T1, baseline; T2, 1 day before second vaccine; T3, 1 week after third vaccine; and T4, 1 month after third vaccine. CTL reactive against wt p53 peptides, tetanus toxoid, or no peptide were measured in PBMC stimulated with peptide-loaded T2 cells. Longer term follow-up data are shown for four patients with a positive immune response.

specimens histologically. The surgical resection preceded vaccination by 2 to 23 months, and these tumor specimens showed varied extent of TIL levels. Four tumors showed prominent (>20 TIL/HPF), two tumors showed moderate (10–20 TIL/HPF), and four tumors showed mild TIL content (<10 TIL/HPF) within the cancer cell islands. Of the latter four patients with minimal TIL content, two had died (of three total deaths in the trial cohort). No correlation was found between time of resection and clinical or immunologic parameters.

Functional studies with HNSCC patients' DC generated by an alternative protocol

The functional characteristics of DC matured by different maturation regimens were also determined. To test this, iDC generated from cryopreserved CD14⁺ monocytes of 13 of

16 patients were matured by an alternative cytokine protocol. Thus, iDCs were matured for 48 hours in the presence of the DC1 cocktail of cytokines (21), containing IFNs and polyI:C, as described in Materials and Methods. In parallel, using the same iDC, maturation was performed with the cytokine cocktail utilized for maturation of the vDC administered to patients. The peptide-pulsed vDC and DC1 were also tested for cytokine secretion (IL-10, IL-12, and VEGF) by Luminex. As shown in Fig. 5A, vDC produced more IL-10, IL-12, and VEGF than iDC; however, the ratios of IL-10/IL-12 were not different in vDC loaded with combinations of peptides and used in Arms 1 to 3 (Fig. 5B). As shown in Fig. 5A, DC1 produced more IL-12 and less IL-10 than did vDC, and only vDC produced VEGF. The data suggest that vDCs have an immunosuppressive functional profile relative to DC1 which mediate stronger adjuvant effects.

Schuler et al.

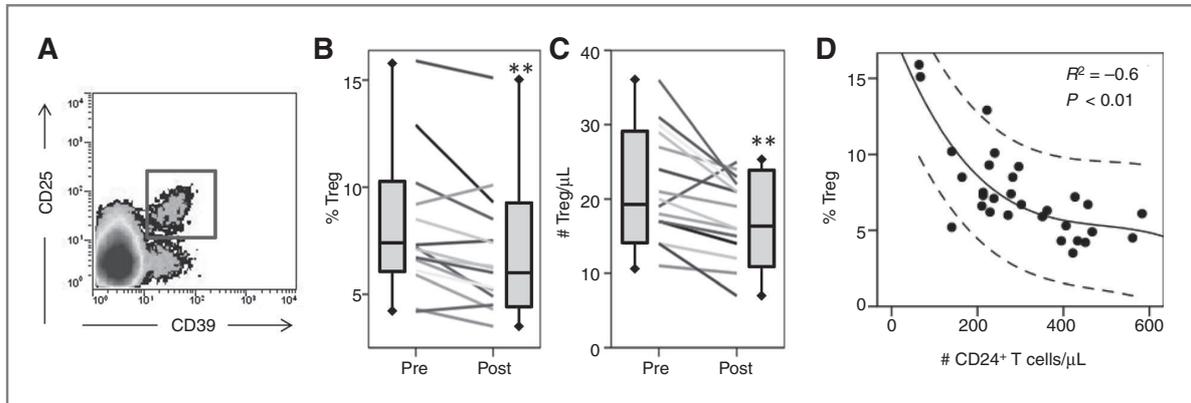


Figure 4. Treg in the peripheral blood and phenotype of DCs. A, Treg were defined as CD4⁺CD39⁺CD25⁺ cells by flow cytometry, which are regularly identified as a defined population. B, percentages of CD4⁺CD39⁺CD25⁺ Treg in the peripheral blood of patients (*n* = 16) before and after vaccination. Wilcoxon paired signed rank test; **, *P* = 0.006. C, absolute numbers of CD4⁺CD39⁺CD25⁺ Treg in the peripheral blood of patients (*n* = 16) before and after vaccination. Wilcoxon paired signed rank test; **, *P* = 0.005. D, the frequency of Treg in the peripheral circulation inversely correlates to the absolute number of CD4⁺ T cells (*R*² = -0.6, *P* < 0.01). Top and bottom lines denote 95% confidence interval.

In Fig. 5C, a significant negative correlation between IL-10 and IL-12 levels in DC1 is shown as well as a positive correlation between VEGF and IL-10 in vDC, further illustrating the cytokine profile associated with vDC.

Discussion

Therapeutic vaccination strategies in HNSCC have been limited to the use of virus-modified tumor cells (i) or heat shock protein HSP65 (ii), and both these approaches have reported considerable toxicity and low vaccine efficacy. The present clinical vaccination trial is, to the best of our

knowledge, the largest tumor-peptide-specific DC-based vaccination trial in HNSCC reported to date, and it is the first to incorporate a Th peptide derived from the same TAA, p53, while using modified/optimized HLA class I binding peptides to generate CTL. It has been previously shown that the induction of tumor-specific CTL required cross-presentation of the relevant helper peptides via class II molecules on mDC (17, 22). Activated CD4⁺ Th cells upregulate expression of surface CD40 and produce Th1 cytokines (IFN-γ, IL-2), which have positive effects on DC maturation as well as CTL function (34). The class II wt p53 epitope

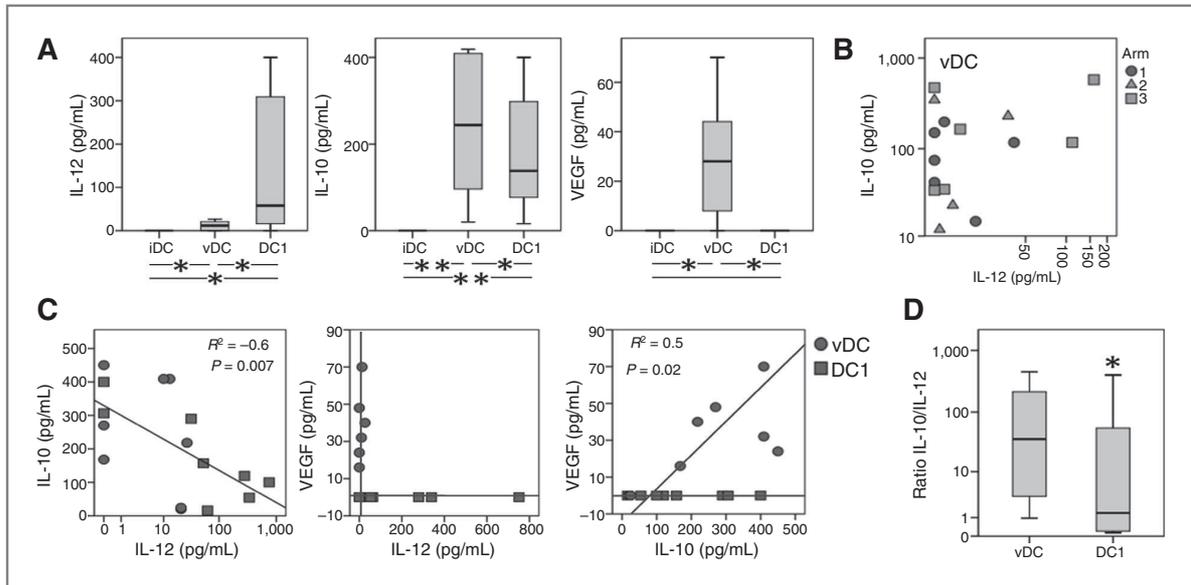


Figure 5. Functional characteristics of cytokine secretion by vDC and DC1. A, production of cytokines IL-10 and IL-12 was measured by Luminex in CD40L-stimulated vDC (B) and DC1. C, production of IL-10 and IL-12 was inversely correlated in all DC (*R*² = -0.6, *P* = 0.007) and production of IL-10 and VEGF was positively correlated in vDC (D), *R*² = 0.5; *P* = 0.02. Wilcoxon paired signed rank test; *, *P* < 0.05; **, *P* < 0.01.

(p53₁₁₀₋₁₂₄), we incorporated in the vaccine, was previously identified as a potent stimulator of p53 class I presentation on DC (17). This phase I DC-based vaccination trial targeting p53 tested the hypothesis that transfer of wt p53 peptide-loaded DC to patients with HNSCC could be therapeutically beneficial and that the vaccine-induced benefits could be related to its ability to induce antitumor immune responses.

Clinical outcome of this vaccination therapy following previous surgery or chemoradiation suggested that 2-year DFS of the patients may have been increased, as compared with historical controls comprising a cohort of similarly staged, unvaccinated patients. DFS is approximately 50% to 70% for advanced HNSCC (stage III and IV) patients after conventional treatment (35–37). In single-arm phase II adjuvant trials, experimental therapy is generally considered worthy of future study, if 2-year DFS is increased from 50% to 70% (38). In the present phase I trial, 2-year PFS was 88%, providing a rationale for a future confirmatory phase II vaccine trial in HNSCC. Interestingly, DFS did not correlate with the presence or expression levels of p53 in the tumor cells or with positive tetramer staining of wt p53 peptide-specific CD8⁺ T cells in the patients' peripheral blood after vaccination, suggesting that reduction of the poor prognostic influence of Treg may provide a global beneficial impact and serve as a biomarker for correlation with clinical outcome in a prospective phase II trial. Indeed, a strong and functional lymphocytic response is beneficial for patients' clinical response. In this phase I trial, two of the three patients who died of disease had only mild TIL within the specimen, suggesting the significance of TIL in antitumor immunity. However, the time lapse between surgery and vaccination in this trial expanded from a few months to a couple of years, making the interpretation of the impact of the baseline TIL on patients' survival of interest for further trials involving larger patient numbers, thus permitting statistical analyses.

The observed limited and weak postvaccination anti-wt p53 peptide-specific immunologic responses in patients with advanced HNSCC could be explained in several different ways. Foremost among them is the well-known immunosuppressive influence of the tumor microenvironment in patients with HNSCC (39). HNSCC is characterized by a potent tumor-induced immune suppression mediated by Treg, Myeloid Derived Suppressor Cell (MDSC), tumor-derived factors, and exosomes. Tumor-induced suppression involves all components of the host immune system, including DC, Th cells, CTL, natural killer, and B cells, and is especially evident in advanced disease. It is, therefore, likely that the vaccine consisting of matured DC loaded with immunogenic cytotoxic and helper wt p53 peptides was unable to overcome extensive immune suppression present in patients with HNSCC and induce strong antitumor immune responses.

Nevertheless, the vaccine was apparently able to induce changes in immunoregulatory mechanisms. One consistent finding in nearly all vaccinated patients was a significant reduction in the frequency and absolute number of circu-

lating Treg after vaccination. This decline in Treg was not restricted to the protocol arms using a class II-restricted peptide in the vaccine but occurred in nearly all vaccinated patients. In addition, the overall decrease in Treg frequency and absolute number in almost every patient in this cohort seemed to be a specific result from vaccination and unrelated to the intratumoral inflammation status, given the fact that the examined tumor specimens displayed a wide range of TIL levels, from as few as 6 TIL per high power field to more than 50 TIL per high power field.

As Tregs are known to suppress effective antitumor immune responses and impair patient prognosis (40, 41), their normalization postvaccine could be expected to restore or upregulate antitumor immunity. However, despite consistently seen decline in the Treg frequency and absolute numbers after vaccination, anti-wt p53-specific responses measured by tetramer analysis or ELISPOT assays were few and weak. The role of Treg in human cancer is still controversial, and our finding implying that Treg normalization by the vaccine is not sufficient for restoration of antitumor immunity further highlights the uncertain role these cells seem to play in cancer progression. In contrast with our study, many DC-based vaccines have been reported to increase the frequency of circulating Treg relative to that before vaccination. This discrepancy could be in part explained by the fact that reliable surface markers are controversial for human Treg. By using CD39, an ectonucleotidase, in addition to CD25 as surface markers for Treg (28), we can reliably distinguish activated CD4⁺CD39^{neg} conventional T cells from CD4⁺CD39⁺CD25⁺Treg and thus increase the specificity of Treg phenotyping. It is perhaps important to note that a decrease in CD39⁺Treg implies that the vaccine also had an impact on the adenosine pathway, which is known to be involved in downregulation of immune cell functions. We acknowledge that additional immunosuppressive subsets exist in patients with HNSCC, including MDSC, and with a small number of available residual specimens, we were unable to detect a consistent vaccine effect on MDSC frequency in PBMC (data not shown). Further vaccine trials will clearly need to incorporate studies and disruption of these cells to permit full efficacy of vaccination, in addition to Treg reduction.

One of the key objectives of DC-based vaccination is to restore suppressed antitumor immunity by providing effective antigen presentation and strong adjuvant activity (30, 31). In patients with HNSCC, DCs are not fully competent for T-cell priming, likely due to their incomplete maturation (19, 21). Thus, DC generation and maturation for vaccine production is a critical step. It is clear that vDC used for therapy in this trial were not functionally optimal despite a normal surface expression of costimulatory and HLA molecules as evaluated by flow cytometry. Importantly, vDC produced little IL-12 and an excess of immunoinhibitory factors, IL-10 and VEGF; they did not upregulate expression of crucial APM components upon maturation in the presence of the conventional cytokine cocktail. The *ex vivo* comparison of vDC with DC1 matured in parallel from iDC of the same patients with HNSCC by an alternative

cytokine cocktail convincingly demonstrates that superior DC can be generated from monocytes derived from the blood of patients with HNSCC using alternative maturation cocktails. Furthermore, it has been determined that phenotypic and functional characteristics of vDC generated from patients with cancer differ from those of DC generated from normal donors (16). Because the vaccination outcome might depend on the quality of injected vDC, it is necessary to ensure that vDCs are mature and functionally effective.

Given the fact that DC generated from patients with HNSCC preferentially produced IL-10 and had only mild adjuvant effects *in vivo*, we considered a possibility that vDCs were not optimally matured. To test this hypothesis, iDC generated from cryopreserved CD14⁺ monocytes of 13 of 16 patients were matured by an alternative cytokine protocol. Thus, iDCs were matured for 48 hours in the presence of the DC1 cocktail of cytokines (21), containing IFNs and polyI:C, as described in Materials and Methods. In parallel, using the same iDC, maturation was performed with the cytokine cocktail utilized for maturation of the vDC administered to patients (Figs. 2 and 5). Our studies suggest that DC generated for clinical applications should be critically monitored not only for surface maturation markers, but also especially for intracellular APM component expression and for their cytokine production profile. For example, APM component TAP1 might serve as a good measure of DC maturation as shown by our recently published data (30).

In addition to vDC quality, the potential of any vaccine to induce antitumor immune responses depends on the selected target TAA. The selection of wt p53 as a vaccination target is based on recent data indicating that p53 mutation is the dominant genetic event in HNSCC (>60% mutated) and that p53 frequently accumulates in tumor cells, making the wt p53 peptides the most ubiquitous TAAs (42). The self-antigen, p53, consists of many low- to moderate-affinity wt peptide sequences, some of which have been shown to be immunogenic (14, 15, 17, 31). Vaccination with the modified (HLA-A2 binding) wt p53 peptides plus Th peptides is designed to stimulate both CD8⁺ and CD4⁺T cells and thus increase the immunogenic effects of the vaccine. Also, the delivery of the combination of wt p53 cytotoxic and helper peptides on matured autologous DC is expected to improve antigen presentation to T cells in the context of strong adjuvant effects needed for improving the vaccine efficacy. However, because the wt p53 peptides are expressed at low levels in normal tissues, vaccines targeting p53 peptides may have to overcome a high level of immunologic tolerance to self (43). Therefore, the three vaccine administrations used in the present study might be insufficient for induction of a

detectable and persistent response. Other p53-peptide-based vaccination studies have applied up to ten courses of DC vaccination to induce peptide-specific immune responses (44). In light of the absence of serious side effects in this study, the number of vaccination courses could be increased, enhancing the likelihood of a positive immune response against wt p53-peptides.

It should be emphasized that the patients with HNSCC enrolled in this trial were treated with either surgery or chemoradiation before receiving the vaccine. To date, the optimal sequence, timing, or combination of various therapeutic cancer regimens is still unclear. Chemotherapeutic drugs, for example, cisplatin, can increase susceptibility of tumor cells to CTL-mediated killing. On the other hand, chemotherapy reduces the T-cell pool available for DC-based vaccination and expands the frequency of chemoresistant Treg. Therefore, it may be an advantage to begin tumor-specific vaccination before chemoradiation therapies. Alternatively, chemotherapeutic regimens or targeted therapies which expand immune responses, such as taxanes or cetuximab (Erbix; ref. 32), might be preferentially selected for combinatorial immunotherapy.

Disclosure of Potential Conflicts of Interest

C. Visus is an employee of Activartis Biotech GmbH. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R.L. Ferris, A.B. DeLeo

Development of methodology: R.L. Ferris, M. Harasymczuk, C. Visus, A.B. DeLeo, T.L. Whiteside

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.L. Ferris, M. Harasymczuk, A.B. DeLeo, S. Trivedi, A. Argiris, L.H. Butterfield, T.L. Whiteside

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.L. Ferris, P.J. Schuler, A.B. DeLeo, Y. Lei, W.E. Gooding, L.H. Butterfield, T.L. Whiteside

Writing, review, and/or revision of the manuscript: R.L. Ferris, P.J. Schuler, M. Harasymczuk, S. Trivedi, A. Argiris, W.E. Gooding, L.H. Butterfield, T.L. Whiteside

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.L. Ferris

Study supervision: R.L. Ferris

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Schuler et al.

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