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A gynecologic oncology group phase II trial of two p53 peptide vaccine approaches: subcutaneous injection and intravenous pulsed dendritic cells in high recurrence risk ovarian cancer patients

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Abstract

Purpose—Peptide antigens have been administered by different approaches as cancer vaccine therapy, including direct injection or pulsed onto dendritic cells; however, the optimal delivery method is still debatable. In this study, we describe the immune response elicited by two vaccine approaches using the wild-type (wt) p53 vaccine.

Experimental design—Twenty-one HLA-A2.1 patients with stage III, IV, or recurrent ovarian cancer over-expressing the p53 protein with no evidence of disease were treated in two cohorts. Arm A received SC wt p53:264-272 peptide admixed with Montanide and GM-CSF. Arm B received wt p53:264-272 peptide-pulsed dendritic cells IV. Interleukin-2 (IL-2) was administered to both cohorts in alternative cycles.

Results—Nine of 13 patients (69%) in arm A and 5 of 6 patients (83%) in arm B developed an immunologic response as determined by ELISPOT and tetramer assays. The vaccine caused no serious systemic side effects. IL-2 administration resulted in grade 3 and 4 toxicities in both arms and directly induced the expansion of T regulatory cells. The median overall survival was 40.8 and 29.6 months for arm A and B, respectively; the median progression-free survival was 4.2 and 8.7 months, respectively.

Conclusion—We found that using either vaccination approach generates comparable specific immune responses against the p53 peptide with minimal toxicity. Accordingly, our findings suggest that the use of less demanding SC approach may be as effective. Furthermore, the use of low-dose SC IL-2 as an adjuvant might have interfered with the immune response. Therefore, it may not be needed in future trials.

Keywords

p53; IL-2; Ovarian cancer; Cancer vaccine

Introduction

Antigenic peptides have been administered to humans in many vaccine clinical trials; however, the method of vaccine administration has varied. One simple and effective strategy of administration is direct subcutaneous (SC) injection along with adjuvant [1–3]. Another method of administration involves pulsing the peptide on dendritic cells (DC), the most potent antigen-presenting cells, which have been also extensively used to deliver antigens in vaccine trials [4–12].

It has been suggested that the use of ex vivo-generated and peptide-pulsed DC for vaccination might be more effective than direct peptide SC injection since DC are

functionally compromised in cancer patients [13–16]. Accordingly, we designed our clinical trial to test the effectiveness of these two methods of administration. Dendritic cell vaccines can be administered intranodally, intradermally, intralymphatically, and intravenously (IV) [17, 18]. Our preclinical data showed the IV route to be the most effective [19]. Accordingly, based on those findings, we chose to administer DC as IV injections in this trial.

Here, we used the wt p53 peptide 264–272 (p53:264-272), a highly immunogenic HLA-A2-restricted epitope [20], as the antigen. The wt p53:264-272 peptide has been shown to be an effective peptide and has been used for vaccination in many clinical trials [21–23]. In breast cancer clinical trials, vaccination with wt p53:264-272 peptide demonstrated evidence of disease stabilization, minimal toxicity and a correlation between the wt p53:264-272-specific immune responses and the clinical outcome [21, 22].

Since IL-2 has been found to enhance vaccine immune effect [24, 25], we used low-dose IL-2 with both groups. Furthermore, our preclinical data had shown that the combination of granulocyte–macrophage colony stimulating factor (GM-CSF) and IL-2 as local adjuvant has synergy in enhancing peptide vaccines and provides long-term tumor protection [26, 27]. GM-CSF has also been found to enhance vaccine efficacy by increasing the numbers of immature DC (iDC) at vaccine sites [28] and enhancing DC maturation and migration [29]. Accordingly, we administered GM-CSF to patients on the SC group.

We found in this phase II trial that p53:264-272 peptide vaccine in ovarian cancer patients with no evidence of disease, administered either SC along with Montanide and GM-CSF or pulsed onto mature DC (mDC) and infused IV, resulted in comparable specific immune responses with no overall difference in toxicity or clinical outcome.

Materials and methods

Eligibility criteria

Eligible patients were at least 18 years of age with a history of stage III, IV, or recurrent ovarian carcinoma but no evidence of disease (NED) defined as no visible disease by imaging studies at enrollment. The patients had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, with HLA-A2.1 haplotype and tumors over-expressing the p53 protein. The study protocol was approved by the Institutional Review Boards of the National Cancer Institute (NCI) and the National Naval Medical Center (NNMC), Bethesda, MD. Written informed consent was obtained from all patients.

Peptide manufacturing and vaccine preparation

The p53:264-272 peptide was manufactured by Multiple Peptide Systems, San Diego CA, packaged in vials by the NIH Clinical Center Department of Pharmacy, and provided by the NCI Cancer Therapeutics Evaluation Program.

Patients were treated with two vaccine preparations: (1) for SC administration, 1,000 µg of wt p53:264-272 peptide was admixed with 100 µg of GM-CSF and emulsified with an equal volume (0.7 mL) of Montanide ISA-51 and (2) for DC peptide-pulsed IV administration, patients underwent apheresis 5–7 days prior to the initial vaccination. Lymphocyte- and monocyte-enriched fractions were separated by counterflow centrifugal elutriation. The monocyte-enriched fraction was cultured in IL-4 (2,000 units (U)/mL) and GM-CSF (2,000 U/mL), or cryopreserved for future doses. On days 4–5 of culture, human CD40 ligand (1 µg/mL) was added to induce DC maturation. The culture was harvested on days 5–6. The p53:264-272 peptide was then added to DC at a concentration of 40–50 µM for 2 h, and the cells were centrifuged and washed. The final preparation of peptide-pulsed dendritic cell

product for infusion was prepared by resuspending 2×10^7 per ml ($\pm 10\%$) of the peptide-pulsed cells in a sterile vial. Prior to release for clinical use, DC were tested using gram staining and blood culture assays as well as for endotoxin.

Protocol design

The study was designed primarily to determine whether endogenous cellular immunity to the HLA-A2 p53 epitope is present in HLA-A2 patients with ovarian cancer and whether vaccination with these peptides can induce or boost the patient's cellular immunity as well as to determine the type and characteristics of the cellular immunity generated. The toxicity of therapy and the correlation of immune response with any objective tumor response were the study's secondary end point.

Patients were assigned to one of two study arms. Patients in arm A received wt p53:264-272 peptide with GM-CSF and Montanide ISA-51 SC. Patients in arm B received 2×10^7 DC pulsed with wt p53:264-272 via slow IV infusion over 5 min. Each arm of the study was intended to try to rule out 20% of patients with immune response and determine whether an immune response rate consistent with as high as 45% could be attained. A Simon two-stage optimal design with $\alpha = 0.20$ and $\beta = 0.10$ was selected in order to not discard potentially active therapy immunologically, even if it meant accepting a therapy that was not as active as desired. Identifying 2 or more of 9 patients with an immune response in the first stage would permit enrollment to a second stage, with a total planned enrollment of 16 patients per arm. Observing 5 or more immune responses on an arm would be considered a successful outcome. Patients were assigned to arm B whenever slots for DC preparation at the NIH Department of Transfusion Medicine were available, and all other patients were assigned to arm A. Thus, although not randomized, the assignment to therapy was determined independently from the preference of the principal investigator, and potential bias in treatment assignments was minimized.

Patients on both arms received the vaccine every 3 weeks for a total of four doses prior to tumor re-evaluation. Patients were observed for 1 h after vaccination to monitor for immediate adverse events. All patients received SC IL-2 at $3-6 \times 10^6$ U/m² starting at vaccine #3 beginning on day 4 for a total of 5 days for 2 consecutive weeks. IL-2 was administered in sets of two consecutive vaccines in an alternating pattern with two vaccines without IL-2 (i.e., IL-2 was given with vaccine 3, 4; 7, 8, etc.). In the absence of disease progression, vaccination was continued for up to 2 years.

Clinical monitoring—Patients gave a targeted history and underwent physical examination, PS evaluation, and laboratory tests prior to each vaccination. Imaging was performed prior to every 2nd vaccination and was reviewed by independent radiologist at National Naval Medical Center. Tumor recurrence was defined as the identification of a new lesion of malignant disease on imaging studies using RECIST criteria or through clinical examination.

HLA testing

All patients were HLA-typed using the molecular testing method.

Assessment of p53 expression and sequencing

Expression of the p53 protein was assessed by immunohistochemistry (IHC) at the NIH Clinical Center. IHC for p53 was performed on sections of formalin-fixed, paraffin-embedded tumor tissues using a microwave antigen retrieval procedure. An automated IHC staining procedure was used according to manufacturer's recommendations (TechMate 1000[®], Ventana, Tucson, AZ). Primary antibodies were DO-7 for p53 (Dako, Santa Barbara,

CA); semiquantitative scores from 0 to 3 were determined for the number of stained nuclei. Sequencing of p53 was done as follows: regions of fixed and paraffin-embedded tumor samples indicated on accompanying H&E stained slides as areas containing >40% tumor were microdissected and subjected to DNA isolation by standard proteinase K/phenol–chloroform extraction techniques. If non-embedded tissue samples were obtained, a ~ 4 mm² piece of tissue was selected at random and subjected to DNA isolation procedures. Exons 5–9 of the p53 gene were amplified from purified genomic DNA by polymerase chain reaction using primers 5F:5′-CCTGAGGTGTAGACGCCAACTCTCT-3′ and 9R:5′-ACGGCATTGTTGAGTGTGTTAGAC-3′. Exons were sequenced using a BigDye terminator cycle sequencing kit (ABI, Foster City, CA) by using primers 5F 6R:5′-GGACTGCTCACCCGGAGGGCCACTGAC-3′, 7F:5′-GGCCTCCCCTGCTTGCCA-3′, 7R:5′-CTCCAGCTCCAGGAGGTG-3′, 8F:5′-ACTGCCTCTTGCTTCT-3′, and 9R:5′-ACGGCATTGTTGAGTGTGTTAGAC-3′. Purified sequencing products were analyzed on an ABI 3100 Genetic Analyzer. The comparison between generated sequences and the p53 reference sequence was conducted using the ABI Sequence Navigator software package.

Immune monitoring

Peripheral blood mononuclear cells (PBMC) were collected within 1 h prior to therapy and prior to every other vaccine. PBMC were isolated from heparinized venous blood by Ficoll Hypaque centrifugation, washed, and cryopreserved in 2-mL vials, using a CryoMed freezer. Immunologic assays were performed at the Immunologic Monitoring and Cellular Products Laboratory, University of Pittsburgh Cancer Institute, Pittsburgh, PA.

Enzyme-linked immunosorbent spot (ELISPOT) assay

ELISPOT assay was performed as previously described [30]. Responder PBMC obtained from patients at different time points and cryopreserved were thawed, washed with PBS, and plated at a density of 1×10^5 cells per well. Responder cells were stimulated with T2 cells (1×10^4 cells per well), which were pulsed with the relevant peptide (p53:264-272) at the concentration of 10 mg/mL. Negative control wells included responder cells co-incubated with unpulsed T2 or T2 cells pulsed with the CEF peptide pool (a group of 32 peptides with sequences derived from the human cytomegalovirus, Epstein–Barr virus, and influenza virus). Positive control wells included T2 cells pulsed with a recall antigen peptide (influenza matrix 58-66, GIL-GFVFTL). Spots corresponding to IFN- γ secreted by stimulated cells were detected with biotinylated anti-IFN- γ antibody (7-B6-1 mAb, Mabtech, Mariemont, OH) and counted on an automated Zeiss Microimager equipped with KS ELISPOT 4.4 software. The coefficient of variation (CV) for the assay was determined to be 15% ($n = 100$). ELISPOT results were expressed as the “number of spots per 10^5 responder cells” (total PBMC) after subtracting background spots obtained in wells of nonstimulated PBMC. For each subject, PBMC obtained before and after vaccination were pooled and analyzed in the same assay to avoid inter-assay variability. The permutation test was used to determine the significance of differences in the spot counts between experimental and background control values. The percent of CD8⁺ cells in each sample was obtained from flow cytometry analysis of PBMC stained with CD3, CD4, and CD8 antibodies. All ELISPOT results are expressed as numbers of spots per 10^5 CD8⁺ T cells.

Tetrameric peptide-MHC class I complex (tetramer) assay

Tetramers were obtained through the National Institute of Allergy and Infectious Diseases (NIAID) Tetramer Facility and the NIH AIDS Research and Reference Reagent Program. Stock solutions contained 0.5 μ g tetramer/mL. The peptide provided to the NIAID Tetramer Facility was the HLA-A2.1-binding peptide LLGRNSFEV, corresponding to the wt p53:264-272 peptide. An irrelevant HLA-A2 restricted tetramer (HIV pol peptide ILKEPVHGV) purchased from Beckman Coulter (Fullerton, CA) was used as a negative

control. Cells were thawed and washed twice in pre-warmed AIM V medium plated in cell culture flasks and incubated for 45 min at 37°C, 5% CO₂ in a humidified atmosphere to remove monocytes and B cells (cells adherent to plastic). The nonadherent cells were harvested and washed once with 1 mL PBS + 0.1% BSA + 0.1% sodium azide (flow buffer). Then, 10 µL of tetramer solution (1/100 dilution from the stock) was added on the cell pellet. The cells were incubated at room temperature for 30 min in the dark. Five µL of the following undiluted antibodies was then added without prior washing: CD3-APC (BD Pharmingen, San Diego, CA), CD8-FITC (BD Bioscience, San Jose, California), CD45RA-ECD (Beckman Coulter, Fullerton, CA), and CCR7-PC7 (BD Bioscience, San Jose, California). The cells were incubated on ice for 30 min, washed twice with 2 mL flow buffer, and fixed in PBS supplemented with 2% paraformaldehyde (PFA) and stored and protected from light at 4°C. Samples were analyzed within 2 days of staining. The data were acquired on a 5-color FC500 (Beckman Coulter, Fullerton, CA). Data analysis was performed using Expo32 software (Beckman Coulter, Fullerton, CA). FITC-conjugated mouse immunoglobulins were used as isotype control reagents.

Regulatory T cells (Tregs)

Cryopreserved PBMC were thawed, washed, and incubated in AIM V medium overnight. Staining of T-cell subsets was performed as previously described [31] using pretitrated labeled antibodies for surface and/or intracytoplasmic proteins (i.e., FOXP3, TGF- β , and IL-10). All antibodies were from Beckman Coulter except anti-FOXP3 (clone PCH101), which was purchased from eBioscience (San Diego, CA). Briefly, cells were stained without stimulation or first stimulated with 1 ng/mL PMA + 1 µM ionomycin for 1 h. Monensin (0.7 µL/mL) was then added for the next 3 h of incubation. The cells were washed and stained with labeled antibodies to surface proteins for 30 min at room temperature. To permeabilize the cells, saponin (0.1% in PBS) was added for 15 min prior to staining for IL-10, and TGF- β or FOXP3 was added for 30 min at room temperature. Beads were added to selected wells to obtain absolute cell numbers. The cells were washed three times, fixed with 2% paraformaldehyde in medium, and examined by flow cytometry. Data were acquired in an FC500 using Expo32 software and are presented as percentages of positive cells. The following antibody panels were used based on our previous reports [31, 32]: (1) nTregs: CD3⁺CD4⁺CD25^{high}; (2) nTregs: CD3⁺CD4⁺CD25^{high}FOXP3⁺; (3) nTregs: CD3⁺CD4⁺CD25^{high}CTLA4⁺; (4) Tr1: CD3⁺CD4⁺IL-10⁺CD132⁺; and (5) Tr1: CD3⁺CD4⁺TGF- β ⁺CD132⁺.

Statistical analysis

A positive immune response was defined as the change from baseline observed at a minimum of two time points, with an increase of twofold or greater in either ELISPOT or tetramer assays. Furthermore, the following 17 immune endpoints were examined to determine whether the vaccine was associated with changes in the immune profile (from cycle 1 to cycle 5), using a two-tailed Wilcoxon signed rank test: % CD8⁺ T cells, absolute number of CD8⁺ T cells, % CD8⁺CD45RA⁺CCR7⁺, % CD8⁺CD45RA⁺CCR7⁺, % CD8⁺CD45RA⁻CCR7⁺, % CD8⁺CD45RA⁻CCR7⁻, % CD8⁺tetramer⁺CD45RA⁻CCR7⁺, % CD8⁺tetramer⁺CD45RA⁻CCR7⁻, % CD8⁺tetramer⁺CD45RA⁺CCR7⁻, % CD4⁺CD25⁺, % CD4⁺CD25^{high}, % CD4⁺FOXP3⁺CD25^{high}, % CD4⁺GITR⁺CD25^{high}, % unstimulated CD132/IL-10⁺, % stimulated CD132/IL-10⁺, and % CD4⁺CD132/TGF- β ⁺. In view of the large number of evaluations made, only changes with *P* values <0.005 were interpreted as statistically significant. Wilcoxon test of the equality of changes between treatment arms was also conducted to directly compare the immunologic changes in T-cell repertoire. Progression-free survival (PFS) and overall survival (OS), considered only to be exploratory outcomes, were calculated from the on-study date until the date of progression, death, or last known follow-up, as appropriate. The

probability of OS or PFS as a function of time was calculated using the Kaplan–Meier method. A two-tailed log-rank test was used to determine the statistical significance of the difference between two Kaplan–Meier curves. The significance of the difference between the two arms in the change in CA125 levels from on-study to off-study was determined by an exact two-tailed Wilcoxon rank sum test. Patients who received less than two vaccines were excluded from the statistical analysis.

Results

Patient profile

Twenty-one patients with a history of stage III, IV, or recurrent ovarian cancer with no evidence of disease (NED) were enrolled in this study. Fourteen of 21 patients received direct SC injection of the peptide along with Montanide and GM-CSF (arm A), and 7 patients received IV peptide-pulsed mDC injections (arm B). Accrual on both arms was stopped when it became apparent that the statistical criterion for declaring a successful result was met in each arm prior to reaching the intended accrual goal for that arm. Patient #13A voluntarily withdrew from the study after receiving one vaccine and was excluded from the evaluation. Characteristics of treated patients are summarized in Table 1. The average age was 57 years in arm A and 54 in arm B. Six of 15 patients sequenced for the *p53* gene had a point mutation in exon 5–9. All patients had grade 3 adenocarcinoma except 2 patients in arm A and 3 patients in arm B who had grade 2 adenocarcinoma. Patients were heavily pretreated. Five patients in arm A and two in arm B had elevated CA125, and 16 patients had a PS of “0” (Table 1).

Safety and toxicity

Side effects in both arms were comparable. No acute allergic reaction occurred in either of the treatment arms. The proportion of serious toxicities per patient and per vaccine was not significantly different ($P = 0.27$ and $P = 0.12$, respectively). In arm A, patients received a total of 143 vaccines; all experienced grade 1 or grade 2 toxicities. The most common toxicities were erythema or induration at the sites of the vaccine and IL-2 injections, occurring in 77 and 100% of patients, respectively. Other common side effects included fatigue and elevated liver enzymes (Table 2). Eighty-five percent of the side effects occurred during IL-2 cycles. Grade 3 or 4 toxicities occurred in 11 patients (14% of vaccines) and included fatigue, elevated liver enzymes, and arthralgia. All grade 3 toxicities occurred during the IL-2 cycles and required IL-2 dose reduction. One patient (#2A) who experienced grade 3 cardiac arrhythmia after receiving IL-2 with the third vaccine was removed from the study. Patient #10A developed grade 4 hepatic toxicity after the fourth vaccine and continued vaccination without IL-2.

In arm B, patients received a total of 68 vaccines. Eighty-five percent experienced grade 1 or 2 toxicities in 44% of vaccines, all of which occurred during IL-2 cycles (Table 2). Local inflammation occurred in 100% of IL-2 cycles. Other side effects included fatigue and increases in liver enzymes. Grade 3 toxicities occurred in 5 patients (23% of vaccines) and included fatigue, lymphopenia, and increased liver enzymes. All occurred during the IL-2 cycles and required dose reduction of IL-2.

IL-2 dosing

IL-2 toxicities on both arms were similar. The first 14 patients received IL-2 at the starting dose of 6×10^6 U/m² (8 patients on arm A and 6 on arm B); 11 of 14 patients required dose reduction due to grade 3 toxicities (Table 3). Accordingly, the protocol was amended to decrease the starting dose of IL-2 to 3×10^6 U/m² in the remaining patients. All 11 patients who received 6×10^6 U/m² required at least one dose reduction of 50%; 3 patients required

a second dose reduction of 50%. Of the 5 patients who received a starting dose of 3×10^6 U/m², 2 required a dose reduction of 50% and 1 required a second dose reduction of 50% (Table 3).

Immunologic data

Immune testing was performed in 19 patients (13 in arm A and 6 in arm B) using both ELISPOT and tetramer assays. All patients demonstrated a baseline endogenous immune response to the wt p53:264-272 by either ELISPOT or tetramer assay except for 2 patients (#11A and 5B). There were no significant differences in immune response generated in either arm ($P = 1.00$). Nine patients (69%) in arm A and 5 patients (83%) in arm B had positive immune responses either by ELISPOT or by tetramer assays (Table 3). Interestingly, the positive ELISPOT responses were associated with tetramer responses in all patients except one; on the other hand, 4 patients with positive tetramer responses had negative ELISPOT responses. In some patients, there was a wt p53:264-272-specific CD8⁺tetramer⁺ T cells increase without a corresponding increase in IFN- γ ELISPOT reactive producing CD8⁺ T cells. Supplemental Figures 1a-b and 2a-b show examples of two responding patients, one on each arm (Patients# 10A and 6B). Furthermore, changes in lymphocyte subsets were tested in all 19 patients during the first five vaccinations. There were no changes observed in T-cell subsets, including both central and naïve memory T cells comparing pre- and post-vaccine time points in either arms or in the whole cohort of treated patients (Table 4). Significant increase in the percentage of activated CD4⁺ T cells (CD4⁺CD25⁺) ($P = 0.001$) and Tregs: CD4⁺CD25^{high} ($P = 0.0008$) as well as CD4⁺CD25^{high}FOXP3 ($P = 0.0056$) cells were observed comparing pre- and post-vaccine time points in the total treated cohort. However, Wilcoxon test of the equality of changes showed no difference between treatment arms with respect to these changes.

IL-2 effect on T-cell profile

IL-2 was administered in combination with alternate sets of two consecutive vaccines starting with the 3rd vaccine as described in the Materials and Methods section. Here, we found that patients on either arm who received two or more cycles of IL-2 (eight vaccines or more) had a substantial increase in the frequency of activated T cells (CD4⁺CD25⁺), and Tregs (CD4⁺CD25^{high}) and (CD4⁺CD25^{high}FOXP3⁺), with every exposure to IL-2. As shown in Fig. 1, this increase in Tregs closely corresponded with the IL-2 administration and that was independent of the dose of IL-2.

Clinical outcome

Clinical responses were evaluated in 20 patients. In arm A, only 1 patient (#10A) completed the treatment for the full 2 years, receiving 31 vaccines, and was followed for 44 months without disease recurrence. Patient #2A was removed from the study because of grade 3 cardiac toxicity after the third cycle due to IL-2; however, this patient had no evidence of disease at the end of the 2-year follow-up. The remaining 11 patients had disease recurrence at various times after receiving vaccination (Table 3). In arm B, 2 patients (#6B and #7B) electively terminated therapy; however, both had no evidence of disease after receiving 14 and 8 vaccines, respectively. The other patients had disease recurrence after receiving 2–6 vaccines (#3B and #2B) or 10–16 vaccines (#1B, #4B and #5B) (Table 3).

There was no significant difference between the two arms in the median OS (40.8 months vs. 29.6 months, $P = 0.26$), nor in the median PFS (4.2 months vs. 8.7 months, $P = 0.81$) (Fig. 2). The PFS and the OS for the whole combined group of 20 patients were 5.5 and 40.4 months, respectively. There was no statistical difference between the two arms with respect to CA125 response to treatment ($P = 0.94$) (Supplemental Figure 3).

Discussion

In this pilot phase II clinical trial, the efficacy of immunization with HLA-A2 p53:264-272 peptide was evaluated using two vaccine approaches: Arm A, a SC injection of a 1,000 μ g of the peptide based on previous clinical trials [3,33], administered with Montanide and GM-CSF and Arm B, a systemic IV delivery of autologous mDC pulsed with the peptide. Twenty-one patients with ovarian carcinoma were treated on the two arms; 14 patients were enrolled in arm A and 7 in arm B. Although the trial was not intended to directly compare between the two arms, in the post hoc analysis, we found no difference in the immune responses generated between the two arms. The two vaccination strategies generated specific immune responses to the p53:264-272 peptide in similar proportions of patients: 69% in arm A and 83% in arm B. Furthermore, changes in T-cell subsets profile including T memory cells, central and peripheral, and T regulatory cells among the two methods of vaccination were comparable. We also found that vaccination with either method is safe and comparable with mild vaccine-relevant adverse events (grade 1 and 2). Patients experienced grade 3 or 4 toxicities only during the IL-2 administration.

In a recent study, Slingluff et al. [7] reported a greater frequency of immune responses in advanced melanoma patients vaccinated with a mixture of peptides (gp100 and tyrosinase) administered SC and intradermally with Montanide and GM-CSF, than in patients vaccinated with the peptides pulsed on DC administered IV and SC. The lower response on the DC arm in the Slingluff trial (11–13%) compared to the result obtained on this current trial (83%) can be attributed to: (1) the use of immature DC where iDC have been shown to be less effective than mDC and can induce tolerance [17, 34–37]; (2) the use of lower dose of DC (1.0×10^7) versus 3×10^7 cells used in this current trial; and (3) the use of frozen peptide loaded DC versus freshly prepared DC. Furthermore, the immune responses we generated against wt p53:264-272 were comparable to those seen in other clinical trials [22].

Considering the results of Slingluff et al. and this trial, it may be unnecessary to use a method of delivery as cumbersome and expensive as ex vivo DC preparations; the less expensive and more streamlined SC administration of peptides may suffice in generating adequate immune responses. Accordingly further, larger-scale studies comparing these and other methods of vaccination are required.

In this trial, we chose the IV route for the administration of DC based on data by Takahashi et al. [19] showing that IV DC administration is the most effective route in inducing immune responses in murine models. Other methods of DC administration have been tested including direct intranodal injection [38, 39]. Bedrosian et al. [40] have shown that intranodal administration of mature DC results in superior T-cell sensitization over intradermal or IV DC administration, however, a 50% failure in injection rate [41] may make this method a less desirable approach for vaccine delivery.

We found that vaccination with a wt p53 peptide was safe. The vaccine-relevant adverse events were mild (grade 1 and 2) and comparable between patients receiving vaccines by the two administration routes. Grade 3 or 4 toxicities occurred only during the IL-2 cycles and were accordingly considered to be a result of IL-2 administration. Although the same IL-2 regimen used in this trial has been well tolerated in other clinical trials [42–44], here, we had to decrease the initial 6×10^6 U/m² dose to 3×10^6 U/m² after treating the first 14 patients because of grade 3 toxicity in 11 patients. This group of patients was less tolerant to the subcutaneous IL-2 administration, possibly because patients enrolled in this trial were healthier (NED) and had a more reactive immune system in response to IL-2 as opposed to those treated for advanced disease in other trials.

IL-2 administration has been reported to lead to the expansion of Tregs in vitro and in vivo [45, 46]. Most of those studies involved treatment of patients with advanced disease, making it difficult to distinguish the effects of IL-2 versus tumor progression on systemic changes in the frequency of Tregs. In our trial, IL-2 was used, for the first time, in an off/on treatment schedule in NED patients. It appears that the observed increases in Tregs are tightly linked to IL-2 administration, since IL-2 induced substantial fluctuations in the frequency of Tregs in most patients, correlating to the time of administration and not disease status. Interestingly, we also observed significant increases in the percentage of activated CD4⁺ (i.e., CD4⁺CD25⁺) T cells in the course of this trial, again emphasizing the possibility that IL-2 caused activation and expansion of CD4⁺ T cells, including CD4⁺CD25^{high}FOXP3⁺ Tregs. Because we found that the IL-2 administration led to significant toxicity and induced expansion of Tregs that might have contributed to limited clinical efficacy of the vaccine, we question the need of using IL-2 in future cancer vaccination trials.

Although this study was not designed to test for differences in clinical outcome between the two arms, the results showed no difference in terms of PFS or OS. Subsequent therapies can also impact OS as well, limiting interpretation of this outcome. Further, the changes from baseline to off-study CA125 did not differ between the two arms. The patient cohort in this study was small, precluding a meaningful statistical analysis of the association between immunologic and clinical data.

Testing different vaccine strategies through small comparative trials incorporating carefully selected immunologic end points has been advocated as an approach that could help in selecting a successful vaccination strategy among many different available [47]. This small phase II clinical trial addressed the use of two different methods of peptide vaccine delivery, an IV administration of peptide-pulsed mDC or a direct SC administration of the peptide. Our finding that both strategies produced comparable immune responses to the vaccine with minimal toxicity supports the use of the less demanding and more cost-effective SC method in future clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

IL-2 effects on T regulatory cells (Tregs). The percentage of activated T-cell *subsets* after each IL-2 cycle is shown in *different colors*: CD4⁺CD25⁺ cells in *red*; CD4⁺CD25^{high} in *green*; and CD4⁺CD25^{high} FOXP3⁺ in *blue*, in patients who received two or more cycles of IL-2 (eight vaccines or more). The vaccine cycles in which IL-2 was given are indicated by *arrows* on the *X* axis (3–4–7–8...etc.). *IL-2 dose reduction

Fig. 2. Kaplan–Meier progression-free survival curve (**a**) and overall survival curve (**b**) for the treated population on both arms

Table 1

Characteristics of the study population

Pt	Age	P53 mutation	PS	Stage	Grade	Previous therapy	Positive energy tests ^a	CA125 ^b
1A	50	273 Arg-His	0	IV	2	S/C/C/C	4	7
2A	68	WT	0	IV	3	S/C/C	2	8
3A	71	WT	1	IIIC	3	S/C/C/H/C	0	280
4A	39	WT	0	IIIC	3	S/C/C	3	36
5A	67	WT	0	IIC	2	S/C/C/C/S/C	1	447
6A	57	WT	0	IIIC	3	S/C	2	14
7A	57	ND	0	IIIC	3	S/C	2	40
8A	67	WT	0	IIIC	3	S/C/S/C/C	3	9
9A	54	214 His-Arg	0	IIIC	3	S/C/T/C/R/C	ND	16
10A	47	ND	0	IIIC	3	S/C/C/C	ND	10
11A	53	ND	0	IIIC	3	S/C/C	ND	8
12A	56	ND	0	IIIC	3	S/C/C/C	ND	6
13A	47	ND	1	IIC	2/3	S/C/S/R/C/C	ND	5
14A	60	ND	0	IIIC	3	S/C/C/C	ND	41
1B	62	214 His-Arg	0	IIIC	3	S/C	3	5
2B	67	157 Val-Phe	1	IIIC	3	S/C/C/C/C/C	2	196
3B	50	WT	1	IIIC	3	S/C/S/C/S	3	12
4B	49	248 Arg-Trp	0	IIIC	2	S/C/S/T/C/S/C	1	35
5B	56	220 Tyr-Cys	0	IIIC	2/3	S/C/C	2	15
6B	55	WT	0	IIIC	2	S/C/H/C	2	6
7B	39	WT	1	IIIC	2	S/C/C/R	3	9

Pt patient, *PS* performance status, *wt* wild type, *ND* not done, *S* surgery, *C* chemotherapy, *H* hormonal therapy, *T* bone marrow transplant, *R* radiation therapy

^aPositive energy tests were defined as positive skin tests to PPD, tetanus, mumps, and candida

^bCA125 levels on enrollment shown in U/mL

Table 2

Vaccine-related toxicities

Arm	Toxicities grade	Local reaction		Fatigue		Hepatic toxicity	
		Patients (%)	Vaccines (%)	Patients (%)	Vaccines (%)	Patients (%)	Vaccines (%)
A. Common toxicities in Arms A and B							
A	I, II	100	77	85	27	78	23
	III			38	3	38	5
B	I, II	100	20	85	35	85	14
	III			57	7	14	3
Toxicity	Arm A		Arm B				
	% Vaccines	% Patients	% Vaccines	% Patients			
B. Grade III/IV vaccine related toxicities							
LFTs—ALT	4.20	41.7	1.47	14.3			
LFTs—AST	4.20	41.7	2.94	14.3			
Lymphopenia	1.40	8.3	7.35	42.9			
Fatigue	3.50	33.3	7.35	42.9			
Arthralgia	1.40	16.7	0	0			
Anemia	0.70	8.3	0	0			
Fever	0.70	8.3	1.47	14.3			
Depression	0.70	8.3	0	0			
Anxiety	0.70	8.3	0	0			
Hyperglycemia	0.70	8.3	0	0			
Pelvic pain	0.70	8.3	0	0			
Constipation	0.70	8.3	0	0			
Diarrhea	0.70	8.3	0	0			
Vomiting	0.70	8.3	0	0			
Hypocalcemia	0	0	1.47	14.3			
Memory loss	0	0	1.47	14.3			
Rigors/chills	0	0	1.47	14.3			

A. The percentage of patients on each arm who had a side effect and the percentage of vaccines where such side effect occurred

B. The percentage of patients on each arm who had grade III/IV vaccine related specific toxicity and the percentage of vaccines where such toxicity occurred

Table 3

Clinical and immunologic outcomes

Pt	Vaccines received	# IL-2 dose reduction ^a	Off-study response	Off-study reason	PFS	OS	Immune response
1A	4	0	RD	R	2.5	75	+
2A	3	0	NED	T	32 ⁺	32 ⁺	+
3A	8	1	RD	R	5.5	41.5	+
4A	5	1	RD	R	3	40.5	–
5A	6	1	RD	R	4	21	+
6A	4	1	RD	R	3	66	+
7A	4	1	RD	R	3	3 ⁺	–
8A	19	2	RD	R	14	14 ⁺	+
9A	30	2	RD	R	22	22 ⁺	+
10A	31	0	NED	C	44 ⁺	44 ⁺	+
11A	19	1	RD	R	15	15 ⁺	+
12A	4	0	RD	R	2.5	22	–
14A	6	1	RD	R	4	19	–
1B	16	1	RD	R	12	15 ⁺	+
2B	6	0	RD	R	4.5	22	+
3B	2	0	RD	R	1	7.5	ND
4B	12	1	RD	R	9	33.5	+
5B	10	2	RD	R	8.5	8.5 ⁺	+
6B	14	2	NED	D	13	42	+
7B	8	1	NED	D	24	60.5	–

Pt patient, RD recurrent disease, NED no evidence of disease, R recurrence, T toxicity, C completion, D withdrawal, ND not done, + positive immune response, – negative immune response, PFS progression-free survival in months, OS overall survival in months. Both PFS and OS were calculated from the on-study date until progression, death or last known follow-up marked as (†)

^aNumber of 50% IL-2 dose reduction

Table 4

Changes in the immunologic end points from baseline that occurred during the first five cycles of vaccination

Immunologic end point	In vitro assay	<i>P</i> value for change cycle 1–5 <i>All patients</i>	<i>P</i> value for change cycle 1–5 <i>Arm A</i>	<i>P</i> value for change cycle 1–5 <i>Arm B</i>
% CD8 ⁺ T cells	Flow cytometry (whole blood)	0.24	0.23	0.81
Abs. # of CD8 ⁺ T cells		1.00	0.73	0.81
% CD8 ⁺ CD45RA ⁺ CCR7 ⁺	Flow cytometry (PBMC)	0.70	0.58	1.0
% CD8 ⁺ CD45RA ⁺ CCR7 ⁺		0.24	0.09	0.81
% CD8 ⁺ CD45RA ⁺ CCR7 ⁺		0.43	0.23	1.0
% CD8 ⁺ CD45RA ⁺ CCR7 ⁺		0.69	0.97	0.62
% CD8 ⁺ tetramer ⁺ CD45RA ⁺ CCR7 ⁺	Tetramer ⁺ surface markers by flow cytometry (PBMC)	0.46	0.22	1.0
% CD8 ⁺ tetramer ⁺ CD45RA ⁺ CCR7 ⁺		0.84	0.69	1.0
% CD8 ⁺ tetramer ⁺ CD45RA ⁺ CCR7 ⁺		0.20	0.44	0.50
% CD8 ⁺ tetramer ⁺ CD45RA ⁺ CCR7 ⁺		0.52	1.0	0.50
% CD4 ⁺ CD25 ⁺	Activated CD4 ⁺ T cells and Treg analysis by flow cytometry (PBMC)	0.0010 [*]	0.0058 [*]	0.1250
% CD4 ⁺ CD25 ^{high}		0.0008 [*]	0.0087 [*]	0.0625
% CD4 + FOXP3 ⁺ CD25 ^{high}		0.0056 [*]	0.0537	0.31
% CD4 + GITR ⁺ CD25 ^{high}		0.011	0.14	0.06
% CD132/IL-10 ⁺ unstimulated	Tr1 analysis by flow cytometry (PBMC)	0.90	0.21	0.08
% CD132/IL-10 ⁺ stimulated		0.13	0.14	0.81
% CD4 ⁺ CD132/TGF- β ⁺		0.39	0.70	0.37

PBMC peripheral blood mononuclear cells, *Abs#* absolute number* Statistically significant at $P < 0.005$