

Combination of p53 Cancer Vaccine with Chemotherapy in Patients with Extensive Stage Small Cell Lung Cancer

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Abstract **Purpose:** The initial goal of this study was to test the immunologic and clinical effects of a new cancer vaccine consisting of dendritic cells (DC) transduced with the full-length wild-type *p53* gene delivered via an adenoviral vector in patients with extensive stage small cell lung cancer. **Experimental Design:** Twenty-nine patients with extensive stage small cell lung cancer were vaccinated repeatedly at 2-week intervals. Most of the patients received three immunizations. *p53*-specific responses were evaluated, and phenotype and function of T cells, DCs, and immature myeloid cells were analyzed and correlated with antigen-specific immune responses. Objective clinical response to vaccination as well as subsequent chemotherapy was evaluated. **Results:** *p53*-specific T cell responses to vaccination were observed in 57.1% of patients. Immunologic responses to vaccination were positively associated with a moderate increase in the titer of antiadenovirus antibodies, and negatively with an accumulation of immature myeloid cells. One patient showed a clinical response to vaccination whereas most of the patients had disease progression. However, we observed a high rate of objective clinical responses to chemotherapy (61.9%) that immediately followed vaccination. Clinical response to subsequent chemotherapy was closely associated with induction of immunologic response to vaccination. **Conclusions:** This study provides clinical support for an emerging paradigm in cancer immunotherapy, wherein optimal use of vaccination might be more effective, not as a separate modality, but in direct combination with chemotherapy.

Small cell lung cancer (SCLC) is an aggressive form of lung cancer, with 5-year survival rates of <10%. Diagnosis of extensive stage (ES) disease comprises approximately two-thirds of new SCLC cases, and the median survival of these patients is only 2 to 4 months if untreated, and survival increases to 6 to 8 months with chemotherapy. This disease is very responsive to first line chemotherapy with response rates of >50% routinely observed. However, these responses almost invariably are short-lived and recurrence in patients with extensive stage disease occurs frequently. After relapse or failure to respond to chemotherapy, patients generally succumb to their disease within a few months (1). Treatment of patients

with relapsed SCLC is especially challenging if patients are platinum-resistant (i.e., disease progression occurs within 3 months of completion of a platinum-containing regimen), where median survival ranges from 3.7 to 4.7 months. For platinum-sensitive patients, median survival ranges from 5.8 to 6.9 months (2).

It is clear that new therapeutic approaches are needed to improve the outcome of this disease. Cancer vaccines may represent one such approach. Although some immunotherapy clinical trials done in recent years have shown encouraging results, most of the trials showed very limited clinical responses (3). The results of these trials have identified major challenges to the successful translation of cancer immunotherapy. One of the most important elements of cancer vaccines is a suitable tumor-associated antigen. The ideal tumor-associated antigen would not only be expressed in a significant proportion of cancer patients, but survival of tumor cells would require the continued presence of the tumor-associated antigen. With this approach, the emergence of antigen-loss variants would not be possible. The tumor suppressor gene, *p53*, has many of the features of an ideal tumor-associated antigen. It plays an essential role as a regulator of cell growth and differentiation. The *p53* gene is mutated in ~90% of SCLC (4, 5). Wild-type *p53* protein has a brief half-life and is therefore present in very low levels in normal cells, whereas mutant *p53* has a significantly prolonged half-life and is present in much greater quantities in tumor cells. This differential level of expression of a protein between normal and malignant cells could provide

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a basis for immunotherapy, and the p53 protein has been suggested as a potential antigenic target to exploit with immunotherapeutic strategies (reviewed in ref. 6). Survival of tumor cells with mutant p53 depends in large degree on the presence of mutant p53. Preclinical studies using mouse models (7, 8) and an *ex vivo* human culture model (9) have shown that the induction of an anti-p53 CTL response selectively killed tumor cells and spared normal cells. Furthermore, anti-p53 T cells have been shown to be present in cancer patients (10–12).

Another critical element of cancer vaccines is that the carrier for the tumor-associated antigen should function to activate the primary immune response and, if necessary, to overcome tolerance to self-proteins. Dendritic cells (DC) are the most potent antigen-presenting cells and are actively used in cancer immunotherapy (reviewed in ref. 13). In recent years, it has become increasingly clear that the success of DC-based immunotherapy depends on the activation status of these cells. Adenovirus provides an effective means of activating DCs. Adenoviral transduction of DC results in up-regulation of cell membrane MHC class II and costimulatory molecules on DC, and an increased production of proinflammatory cytokines such as interleukin-12 (14–16). Adenovirus also provides an excellent tool for gene delivery into DCs, resulting in sustained high levels of transgenic protein production (reviewed in refs. 17, 18).

The goals of this study were to evaluate the immunologic and clinical effects of DC-Ad-p53 vaccine in patients with ES SCLC. The initial results of this trial provide clinical evidence supporting the hypothesis that the combination of immunotherapy with chemotherapy could result in benefit for cancer patients with advanced disease.

Materials and Methods

Clinical protocol. Before enrolling patients, the protocol was reviewed and approved by the Food and Drug Administration (BB-IND 9792), the NIH Office of Biotechnology Activities' Recombinant DNA Advisory Committee (OBA no. 0205-538), the University of South Florida Institutional Review Board, and the University of South Florida Institutional Biosafety Committee. Patients aged 18 or older with a histologic diagnosis of ES SCLC were eligible to participate. Eastern Cooperative Oncology Group performance status of 0 to 2, and adequate organ function (WBC, >3,000/mm³; ANC, >1,500/mm³; platelets, >100,000/mm³; hematocrit, > 25%; bilirubin, <2.0 mg/dL; and creatinine, <2.0 mg/dL) were required. Patients with a preexisting autoimmune disorder, an immunodeficiency condition, a serious ongoing infection, or uncontrolled brain metastases were not eligible.

All patients were treated with conventional cytotoxic chemotherapy prior to receiving the investigational vaccine. Eighteen patients received carboplatin/VP-16, eight received cisplatin/VP-16, and three received cisplatin/CPT-11. Patients who had progressive disease after chemotherapy were eligible if they otherwise met all other inclusion criteria. At least 8 weeks after the last dose of chemotherapy, the patients underwent leukapheresis. Vaccines were produced, and administered by intradermal injection at four separate sites that drain to the bilateral axillary and inguinal lymph node basins. This was repeated on three separate occasions every 2 weeks. Two weeks after the third set of vaccines, the patients were re-staged. Those patients who did not exhibit progressive disease at this point underwent a second leukapheresis procedure, and received three additional sets of vaccines, this time every 4 weeks. Patients who developed progressive disease after the third or sixth vaccination were offered additional cytotoxic chemotherapy.

Vaccine production. Mononuclear cells for DC production were obtained after leukapheresis and kept stored in liquid nitrogen. After thawing, cells were placed in X-VIVO-15 medium (Biowhittaker, Walkersville, MD) in tissue culture flasks at a concentration of 1.3 to 1.7 × 10⁶ cells/cm² of available culturing surface. After 2 hours of culture, nonadherent cells were removed and the flasks were recharged with X-VIVO-15 medium supplemented with 5 ng/mL granulocyte macrophage colony-stimulating factor (Amgen, Thousand Oaks, CA) and 5 ng/mL interleukin-4 (R&D Systems, Minneapolis, MN). The flasks were incubated for 48 hours, at which time additional cytokine-supplemented medium was added to the flasks. The flasks were then incubated for an additional 72 hours. At the completion of incubation, the nonadherent and loosely adherent cells were collected and used for 2-hour infection with Ad-p53 at a viral particle to cell ratio of 15,000:1. This optimal dose of adenovirus that would produce the highest level of human p53 expression with the least amount of toxicity to the dendritic cells was determined in preliminary experiments. At the end of the 2-hour incubation, X-VIVO medium was added to a final cell concentration of 10⁶ cells/mL, and cells were incubated in flasks for an additional 46 hours, at which time the cells were harvested, washed, and analyzed. Vaccine release criteria includes, (a) negative Gram's staining, (b) negative *Mycoplasma* test by PCR analysis, (c) maximum endotoxin concentration of 5 EU/mL, and (d) a mature DC phenotype with evidence of intracellular p53 expression by flow cytometry analysis. Mature DC phenotype was defined as lineage (CD3, CD14, CD19, CD20, and CD56)-negative, HLA-DR-positive, and CD86-positive cells. To determine p53-positive DCs, cells were treated with "fix and perm" reagent (Caltag, Burling, CA). Staining with the p53-specific antibody was followed by staining with a phycoerythrin-labeled antimurine immunoglobulin antibody. After washing off excess antibody, surface staining for lineage markers with FITC-conjugated monoclonal antibodies, and for HLA-DR with phycoerythrin-conjugated monoclonal antibodies was done. The final product was analyzed by flow cytometry.

Analysis of IFN- γ -producing cells in ELISPOT assays. Peripheral blood mononuclear cells were collected from patients prior vaccination, 2 to 3 weeks after completion of the third vaccination (postvaccine time point), and then 2 months later. Samples were kept in aliquots in liquid nitrogen. Samples from each patient were thawed and analyzed simultaneously. ALVAC-p53, a recombinant canarypox virus containing full-length wild-type p53 was obtained from Aventis Pasteur (Toronto, Canada). Mononuclear cells were infected with ALVAC-p53 or ALVAC-control (empty vector) for 2 hours in serum-free medium at a multiplicity of infection of four plaque-forming units per cell. After infection, cells were seeded in quadruplicate in complete culture medium supplemented with interleukin-2 (1 × 10⁵ cells/well) in 96-well plates precoated with an anti-IFN- γ antibody and incubated for 36 hours. The number of IFN- γ -producing cells was evaluated using an automated ELISPOT reader (CTL) as described previously (9, 19). The mononuclear cells from HLA-A2-positive patients were incubated in parallel for 36 hours with 10 μ g/mL of either a p53-derived HLA-A2-binding peptide (LLGRNSFEV) or a control PSA-derived peptide (FLTPKKLQCV). The number of IFN- γ -producing cells were evaluated in ELISPOT assays as described previously (19).

Tetramer staining. Tetramer HLA-A-0201/LLGRNSFEV was produced by the National Institute of Allergy and Infectious Diseases MHC tetramer core facility at Yerkes Regional Primate Research Center. Mononuclear cells were stained for 60 minutes at 4°C with an antigen-presenting cell-conjugated anti-CD8 antibody and the phycoerythrin-conjugated tetramer (1:100 dilution). As a control, we used a tetramer containing HLA-A2 peptide from the melanoma antigen gp100 (KTWGQYWQV). The proportion of tetramer-positive cells within the CD8⁺ T cell population was calculated.

Evaluation of humoral immune response. The levels of anti-p53 and antiadenovirus antibodies (IgG and IgM) were evaluated in ELISA using at least four serial dilutions. Internal controls provided by the manufacturers were used to establish a "cutoff" level. Samples

were always assayed in duplicate. The absorbance was read on a spectrophotometer at a wavelength of 450 nm against a reference filter of 620 nm in order to compensate for differences in the material of the microtiter plate. The p53-Autoantibody Elisa^{PLUS} kit (Calbiochem, San Diego, CA) was used to measure circulating antibodies to p53 in human serum samples. Adenovirus IgG/IgM ELISA kits were purchased from IBL Immuno-Biological Laboratories (Hamburg, Germany).

Phenotype and function of T cells and DCs. After thawing, the mononuclear cells were cultured overnight in complete culture medium supplemented with 10% FCS. Cell phenotype was evaluated by multicolor flow cytometry using a FACSCalibur flow cytometer and monoclonal antibodies obtained from Becton Dickinson (Franklin Lakes, NJ).

To evaluate T cell proliferation, mononuclear cells (1×10^5) were cultured in triplicate in U-bottomed 96-well plates in the presence of 0.1 μ g/mL tetanus toxoid or 5 μ g/mL phytohemagglutinin (Sigma, St. Louis, MO). These concentrations were selected after preliminary testing. ³H-thymidine (1 μ Ci) was added on day 3 and cells were harvested 18 hours later. Thymidine incorporation was evaluated using a liquid scintillation counter. The stimulation index was calculated as the ratio between cell proliferation in the presence versus absence of stimuli.

To evaluate DC function, responder T cells were isolated from control donors using T cell enrichment columns (R&D Systems). T cells (10^5 /well) were cultured with mononuclear cells obtained from cancer patients in U-bottomed 96-well plates. T cells with mononuclear cell ratios from 1:1 to 1:8 were used. All experiments were done in triplicate. ³H-thymidine (1 μ Ci) was added on day 3, and cells were harvested 18 hours later. Thymidine incorporation was evaluated using liquid scintillation counter. Each mononuclear cell sample was tested against T cells from at least two different donors and the maximum result was used.

Statistical analysis. We used the SAS (SAS, Inc. v. 9.2., Cary, NC) and GraphPad Prism software for statistical analyses. p53-specific immune responses to immunization were analyzed by descriptive statistics. The Wilcoxon Mann-Whitney test was used to determine a relationship between two groups with continuous variables. Univariate associations between frequency of responders and groups were analyzed by Fisher's exact test considering small sample sizes. No attempt was made to adjust to multiple tests in this exploratory study. Any results which seem to be statistically significant require further confirmation. Overall survival was estimated using the duration from the first vaccine administration to the end of follow-up by the Kaplan-Meier method. Two-sided tests were used for all calculations. The statistical significance for all analyses was determined with $P < 0.05$.

Results

Patient characteristics and treatment summary. Between January 2003 and June 2005, 29 patients were treated with the vaccine. All patients had ES SCLC at the time of vaccination (17 patients with newly diagnosed ES disease and 12 with relapsed disease; Table 1). The median age was 63 years (range 39-76). Twenty patients were vaccinated after only one prior chemotherapy regimen (six patients after two regimens, and three patients after three regimens). All patients had received prior platinum therapy (Table 1).

DCs were generated from peripheral blood mononuclear cell precursors, and then infected with an adenoviral construct containing wild-type p53 (ADVEXIN; Ad-p53). A typical example of the cell phenotype after Ad-p53 infection is presented in Fig. 1. Patients were scheduled to receive three doses of vaccine intradermally at 2-week intervals. If patients showed stable disease or better, they were given three or more doses of the vaccine, once per month. The phase I component of the trial had an initial goal to escalate the vaccine dose from

Table 1. Patient characteristics

Characteristics	n (%)
No. of subjects	29 (100)
Gender	
M	13 (45)
F	16 (55)
Age, median (range)	63 (39-76)
Eastern Cooperative Oncology Group performance status	
0-1	28 (98)
2	1 (2)
Clinical stage	
Extensive	17 (59)
Relapsed	12 (41)
No. of chemotherapy regimens before vaccine	
1	20 (69)
2	6 (21)
≥ 3	3 (10)
No. of leukaphereses	
1	18 (62)
≥ 2	11 (38)
No. of vaccines	
< 3	1 (3)
3	20 (69)
> 3	8 (28)

5×10^6 to 5×10^7 p53⁺ DC. However, generation of $> 5 \times 10^6$ p53⁺ DC per dose was difficult to achieve. Therefore, due to feasibility, the doses of p53⁺ DC were not escalated beyond 5×10^6 cells. On average, 7.7×10^7 total DC and 8.6×10^6 p53⁺ DC were generated for each dose (Table 2). The number of injected p53⁺ DC was limited to 5×10^6 even if more cells were generated.

Antigen-specific cellular immune response to the vaccine. p53-specific immune responses were evaluated in IFN- γ ELISPOT assays using a canarypox virus (ALVAC) containing wild-type p53 or control virus for stimulation. Use of ALVAC containing the full-length p53 gene allowed for evaluation of p53-specific responses regardless of the patients' HLA haplotype. Response to vaccination was considered significant if a p53-specific response after immunization (2-3 weeks after third vaccination) was more than 2 SD higher than the p53-specific response before immunization and at least 2 SD higher than the response to control ALVAC. Representative ELISPOT data are shown in Fig. 2A. Immune responses were further evaluated in a subset of 12 HLA-A2-positive patients, using p53-derived or control peptides presented by HLA-A2. Illustrative results are shown in Fig. 2B. In these HLA-A2-positive patients it was also possible to evaluate the antigen-specific CD8⁺ T cells using tetramer staining (data not shown).

Modest but significant p53-specific T cell responses to vaccination were found in 13 out of 25 patients (52%) using ALVAC-p53, and in 7 out of 12 patients (58.3%) using the p53-derived peptide (Fig. 2C and D). Three patients who had a significant response to vaccination measured using the p53-derived peptide were not tested with ALVAC-p53 due to technical reasons. Overall, 16 out of the 28 tested patients (57.1%; termed p53 responders) had statistically significant

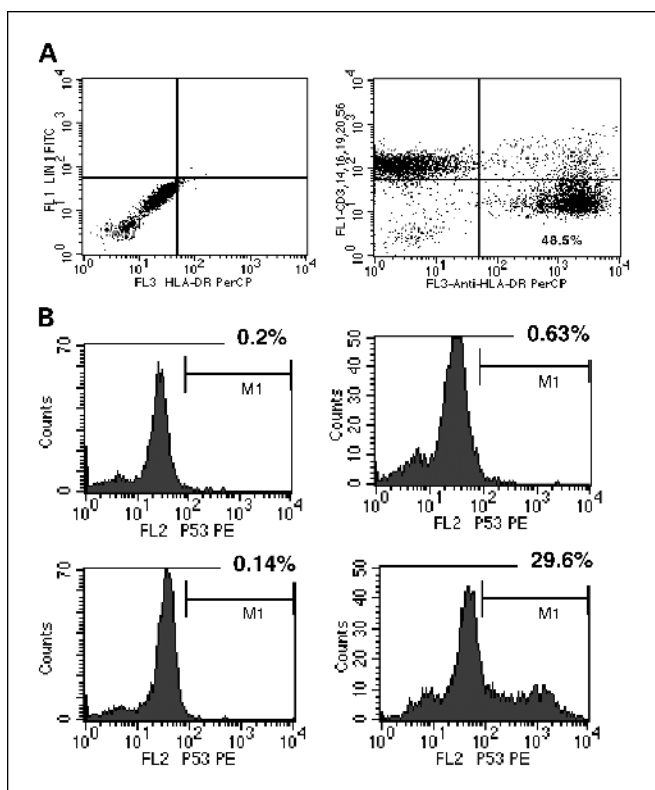


Fig. 1. Characteristics of DCs generated from mononuclear cells. DCs were prepared from frozen samples of mononuclear cells and infected with adenovirus-p53 as described in the text. *A*, on day 7, cells were collected and labeled with a cocktail of FITC-conjugated lineage-specific antibodies and a PerCP-conjugated HLA-DR antibody (*right*) or isotype control IgG (*left*). *B*, surface-stained cells were fixed, permeabilized, and stained with isotype control (*top right*) or anti-p53 antibody (*bottom right*). To illustrate the specificity of the staining, noninfected cells were stained with isotype control IgG (*top left*) or anti-p53 antibody (*bottom left*). Lin⁻HLA-DR⁺ DCs were gated, and staining with p53 was analyzed within this population of DCs.

p53-specific responses to immunization. When both assays were used to test peripheral blood mononuclear cells from the same patient, the response rate to ALVAC-p53 compared with p53-derived peptide was not significantly different ($P > 0.1$), however, a lower frequency was seen using tetramer staining. Only 3 out of 11 tested patients (27.2%) showed a significant increase in tetramer staining. The prevaccination level of p53-specific immunity was similar in patients who responded immunologically to the vaccine to those who did not respond (data not shown).

We analyzed a possible link between the number of DCs injected with each vaccine and p53-specific immune response developed as the result of immunization. On average, 14

patients received the maximum dose (5×10^6 p53⁺ DCs per vaccine), 2 patients received more than 4×10^6 cells, 5 patients received more than 3 but less than 4×10^6 cells, 6 patients received more than 2 but less than 3×10^6 cells, and 2 patients received less than 2×10^6 p53⁺ DCs. No correlation between the number of injected DCs and p53-specific immune response was found.

Association between cellular and humoral immune responses to the vaccine. A detectable preimmunization level of anti-p53 antibody was observed in 10 out of 22 (45%) tested patients, and only 3 patients showed a significant increase in the level of anti-p53 antibodies after immunization. Interestingly, all three of these patients had a detectable prevaccine level of the antibody. Four out of 10 patients (40%) with a detectable preimmunization level of anti-p53 antibody developed a positive p53-specific T cell response to vaccination, which was not statistically different from the response rate in patients with no preexisting level of anti-p53 antibody (data not shown).

Antiadenovirus antibodies could potentially play a critical role in limiting the effectiveness of adenovirus-based cancer vaccines. We measured antiadenovirus IgG and IgM antibodies by ELISA using serial dilution of the patients' sera. Most patients had a detectable preimmunization level of antiadenovirus IgG antibody. After three rounds of immunization with adenovirus-p53 DC, the titer of antiadenovirus antibody increased in 16 out of 28 tested patients (57.1%). A moderate increase (>2 and <8-fold) was observed in 14 patients and a substantial (>8-fold) increase in 2 patients. Both patients with the substantial increase in antiadenovirus antibodies did not develop p53-specific cellular responses to immunization. p53-specific cellular immune responses to vaccination were observed in 12 out of 14 patients (85.7%) who had a moderately increased titer of antiadenovirus antibodies, and in only 5 out of 13 patients (38.5%) who had no detectable increase in antibody titer ($P = 0.018$; Fig. 2E). The antiadenovirus antibodies detected in those patients had neutralizing activity with a median neutralizing titer of 1:1,600.

Association of immune response to vaccination with prevaccination levels of T cell function, DC activity, and the presence of immature myeloid cells. The generation of a p53-specific T cell response depends not only on the quality of antigen stimulation but also on the functional activity of T cells and antigen-presenting cells in the host, specifically DCs. In order to determine whether the preexisting condition of the host immune system influenced the outcome of vaccination, mononuclear cells isolated from patients prior to vaccination were stimulated with either tetanus toxoid or phytohemagglutinin, and cell proliferation was measured by uptake of ³H-thymidine. Nine out of 20 (45%) tested patients had a tetanus toxoid response below the lower limit of normal

Table 2. The number of DCs generated for vaccines

	Total number of DCs generated per vaccine	The number of p53 ⁺ DCs generated per vaccine	The number of p53 ⁺ DCs injected per vaccine
Median	2.42×10^7	4.66×10^6	4.78×10^6
Maximum	1.59×10^8	2.7×10^8	5×10^6
Minimum	1.47×10^6	2.4×10^5	2.4×10^5

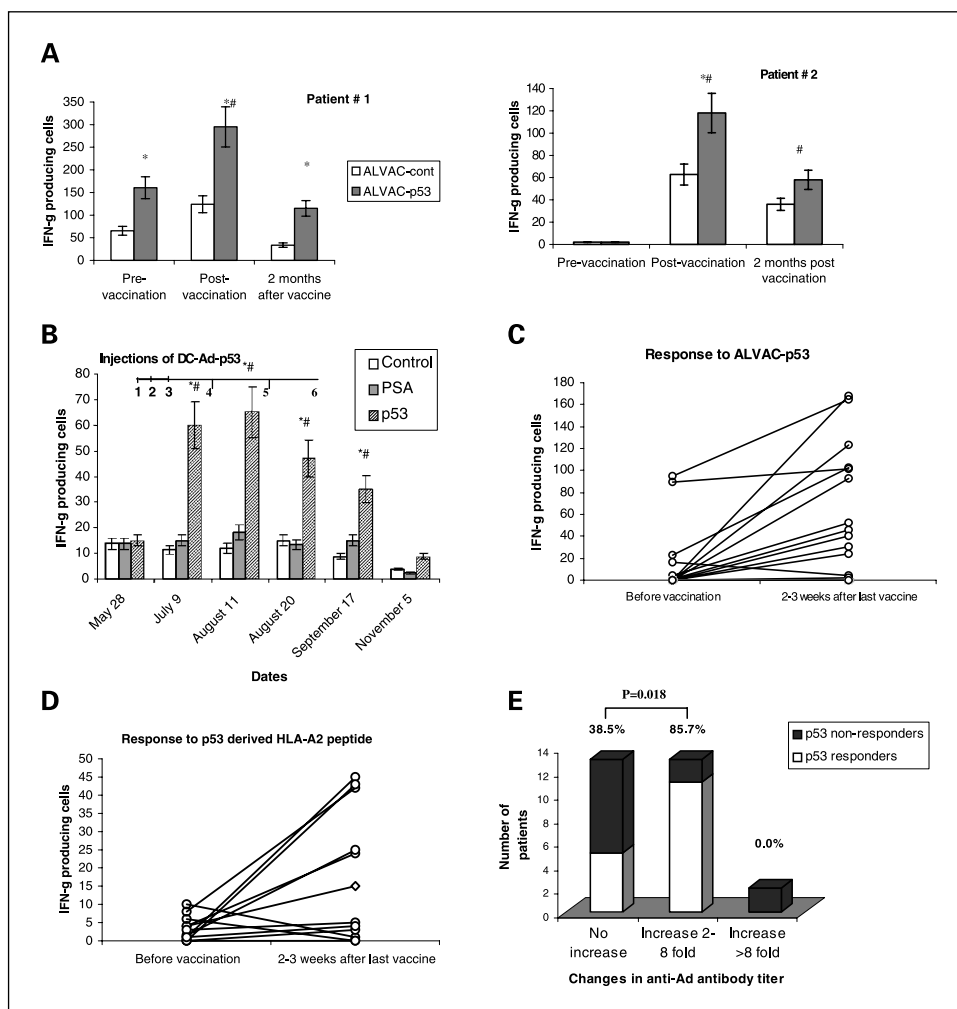


Fig. 2. p53-specific immune response to immunization. **A**, two HLA-A2-negative patients were vaccinated with DC-adenovirus-p53 (three vaccines at 2-week intervals). Blood was collected before immunization, 3 weeks after last vaccine and 2 months later. Cells were stimulated with ALVAC-p53 as described in Materials and Methods. The ALVAC “empty” vector was used as the control (ALVAC-cont). The number of IFN- γ -producing cells was evaluated in quadruplicate in ELISPOT, and normalized per 2×10^5 mononuclear cells added. *Columns*, mean; *bars*, \pm SD; *, $P < 0.05$ between cells incubated with ALVAC-p53 and ALVAC-cont; #, $P < 0.05$ between prevaccine and postvaccine samples. **B**, HLA-A2-positive patients with ES SCLC were vaccinated with DC-adenovirus-p53. Blood was collected before immunization and at different points after immunization. The number of IFN- γ -producing cells per 2×10^5 mononuclear cells were evaluated in quadruplicate in ELISPOT. Cells were stimulated with HLA-A2-matched p53-derived peptide (LLGRNSFEV), PSA-derived irrelevant peptide (FLTPKLLQCV) or left in medium alone (control). *Columns*, mean; *bars*, \pm SD; *, $P < 0.05$ between cells incubated with p53 and PSA peptide; #, $P < 0.05$ between prevaccine and postvaccine samples. **C** and **D**, the results of IFN- γ ELISPOT assays from all tested patients. The background levels of nonspecific IFN- γ production (ALVAC-control or irrelevant peptide) were subtracted. The number of spots per 1×10^5 cells are shown. All measurements were done in quadruplicate. The mean for each sample is shown. Not all HLA-A2-positive patients were tested both with ALVAC-p53 and p53-derived peptide. **E**, the titer of antiadenovirus IgG was calculated using a serial dilution assay. Patients were divided into three groups: patients who had no increase in antibody titer after vaccination (11 patients), patients who had a moderate increase in antibody titer (from 2- to 8-fold, 10 patients), and patients who had a large increase in the titer after vaccination (>8-fold, 2 patients). The proportion of patients who showed a p53-specific cellular immune response was calculated in each group. P value (two-tailed) was calculated using Fisher’s exact test.

samples (stimulation index, <15), and 9 out of 19 (47.3%) tested patients had a phytohemagglutinin response below the control range (stimulation index, <25). However, patients with a decreased T cell response to tetanus toxoid or phytohemagglutinin developed p53-specific immunity to vaccination at a rate similar to the patients with normal T cell responses ($P > 0.1$; data not shown).

Recent studies have suggested that CD4⁺CD25⁺ regulatory T cells (T_{reg}) might play an important role in the down-regulation of antitumor immune responses (reviewed in ref. 20). As an initial evaluation of the T_{reg} population, CD25^{high} cells were enumerated within the total population of CD3⁺CD4⁺ T cells. No differences in the proportion of these cells were found between the groups of healthy subjects and patients with SCLC

prior to vaccination or 2 to 3 weeks after completion of vaccination (Fig. 3A). Furthermore, no statistically significant link was found between the presence of these cells in the patients’ blood before or after vaccination, and p53-specific T cell responses to vaccination (Fig. 3B).

The possible association between the number and functional activity of DCs prior to vaccination, and antigen-specific responses to vaccination was also investigated. Although no statistically significant decrease in the median proportion of DCs (Lin⁻HLA-DR⁺) nor their mature CD83⁺ subset was found in patients with SCLC as compared with normal subjects, a substantial number of individual patients had decreased levels of DCs (below minimum values of the control group; Fig. 3C). Patients with a reduced level of the total DC population did

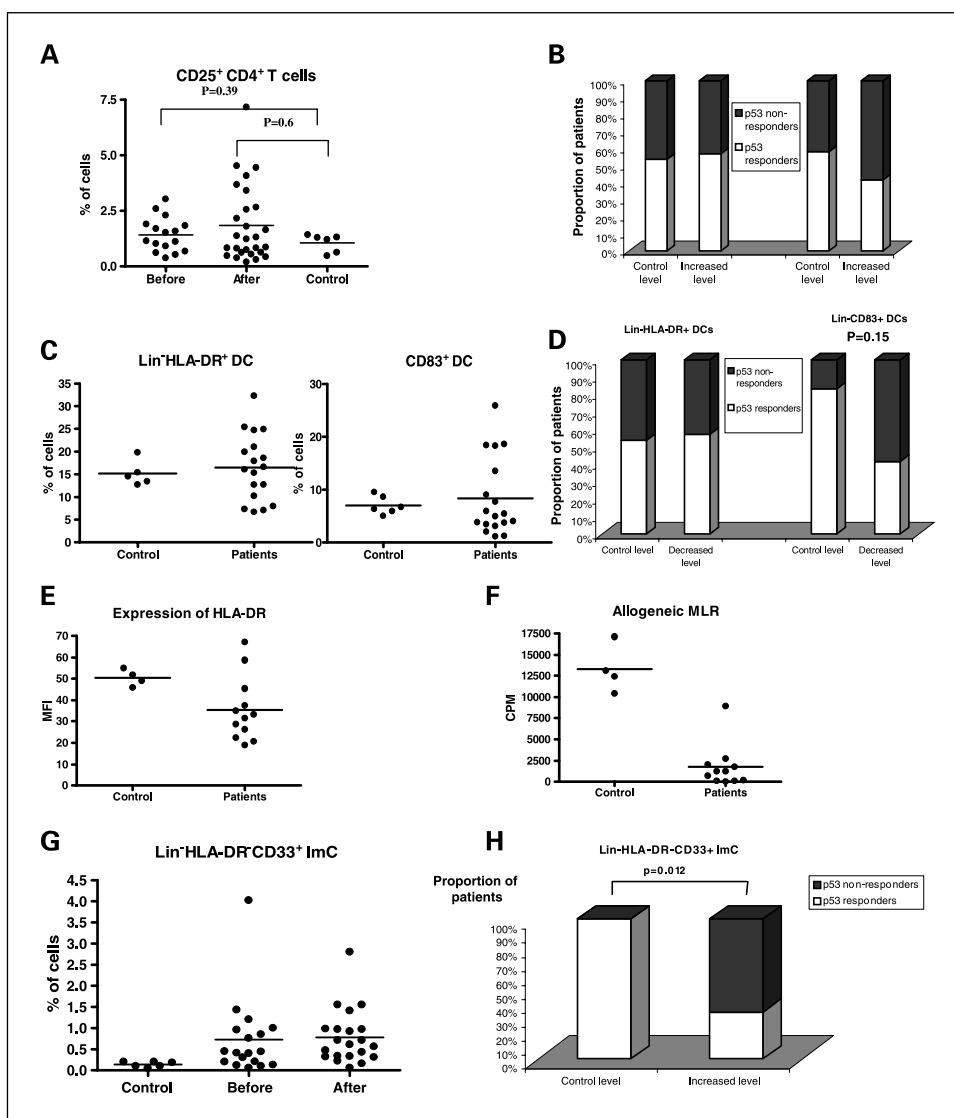


Fig. 3. An association of p53-specific responses with T cell function, DC function, and the presence of immature myeloid cells prior to vaccination. *A*, mononuclear cells collected prior to and 2 to 3 weeks after vaccination were stained with a phycoerythrin-conjugated anti-CD3 antibody, an antigen-presenting cell – conjugated anti-CD4 antibody and a FITC-conjugated anti-CD25 antibody, and analyzed by flow cytometry. The proportion of CD25^{high} cells within the population of CD3⁺ CD4⁺ T cells was calculated. *Bar*, mean of the values in the groups ($P > 0.2$ in Mann-Whitney test). *B*, patients were divided into two groups: with control and increased levels of CD4⁺ CD25⁺ T cells (above maximal control values). The proportion of patients with positive p53-specific responses was calculated within each group. No statistical differences were found between the groups ($P > 0.3$ in Fisher's exact test). *C*, mononuclear cells were isolated from control donors and patients with SCLC prior to vaccination. Cells were stained with a cocktail of antibodies and analyzed using multicolor flow cytometry as described in Materials and Methods. The proportions of DC (Lin⁻ HLA-DR⁺) and mature DCs (Lin⁻ CD83⁺) were evaluated. Two-tailed P values were calculated using the Mann-Whitney test. *D*, percentage of patients with a positive p53-specific response to vaccination (p53-responders) or negative response (p53 nonresponders) was calculated within the groups of patients with control and decreased levels of DCs prior to vaccination. *E*, mean fluorescence intensity (MFI) of HLA-DR in Lin⁻ cells. *F*, mononuclear cells were used as stimulators of allogeneic control T cells as described in Materials and Methods. Results of 1:1 ratio (mononuclear/T cells) are shown. Each experiment was done in triplicate. Two-tailed P values were calculated using the Mann-Whitney test. *G*, mononuclear cells were isolated from control donors and patients with SCLC prior to vaccination. Cells were stained with a cocktail of antibodies and analyzed using multicolor flow cytometry. The proportion of immature myeloid cells (Lin⁻ HLA-DR⁻ CD33⁺) was evaluated. *H*, percentage of p53-responders and nonresponders was calculated within the groups of patients who had control and elevated levels of immature myeloid cells prior to vaccine administration. Two-tailed P value in Fisher's exact test is shown.

not exhibit a significant decrease in the ability to develop a p53-specific cellular immune response to vaccination compared with those who had a normal level of these cells (Fig. 3D). In contrast, there was a trend toward a lower rate of p53-specific responses in patients with reduced levels of CD83⁺ mature DCs. Five out of six patients with control levels of CD83⁺ DCs prior to vaccination developed p53-specific immune response to vaccination, whereas only 5 out of 12 patients with reduced level of these cells responded to the vaccine ($P = 0.15$; Fig. 3D).

Expression of HLA-DR on SCLC patients' DCs was significantly lower than in healthy subjects (Fig. 3E). Furthermore, a substantial decrease in the function of DC was seen in allogeneic mixed leukocyte reactions (Fig. 3F). Analysis of p53-specific immunologic response to vaccination in this case was not relevant because very few patients showed control levels of these variables.

Because immature myeloid cells have been implicated in the immunosuppressive state of tumor-bearing hosts (21, 22)

the patients with SCLC were examined for the presence of these cells. Patients with SCLC had elevated levels of Lin⁻HLA-DR⁻CD33⁺ immature myeloid cells prior to vaccination ($P = 0.01$). After vaccination, their presence increased even further ($P = 0.002$; Fig. 3G). All six patients with a normal level of immature myeloid cells prior to vaccination developed p53-specific immune responses to vaccination (100%), compared with only 4 out of 12 patients who had an elevated level of immature myeloid cells (25.0%; $P = 0.012$; Fig. 3H). Thus, it seems that patients with an increased presence of immature myeloid cells were less likely to develop responses to vaccination compared with the patients with a normal level of immature myeloid cells.

Clinical response to vaccination and its association with an antigen-specific immune response. Adverse events associated with the administration of the vaccine were infrequent and mostly mild. Only two patients experienced grade 2 adverse events (one for fatigue and one for arthralgia) with vaccine administration, and vaccinations were never withheld due to the presence of any adverse events. The occurrence of adverse events was independent of the number of vaccines previously received.

Out of 29 patients treated with the vaccine, 1 patient achieved a partial response, 7 had stable disease, and 21 patients developed progressive disease. The patient with a partial response received four cycles of cisplatin and etoposide concurrent with thoracic radiation therapy immediately after the initial diagnosis. She subsequently progressed 2 months after her last dose of cisplatin, with the appearance of several positron emission tomography–positive enlarged retroperitoneal lymph nodes. She received three vaccines at that time, and was re-staged 2 weeks later. Overall Response Evaluation Criteria in Solid Tumors measurements revealed a 60% decrease in the size of all of her measurable lesions (Fig. 4A). This patient, and five out of seven patients with stable disease after vaccination, eventually progressed 2 to 6 months later. Twenty-three of the 27 patients with progressive disease after vaccine administration were treated with additional chemotherapy (four patients declined). At this time, clinical follow-up was completed for 21 patients. Of these, 13 were platinum-resistant (progressed within 90 days of receiving a platinum-containing regimen). Fourteen patients received paclitaxel, three received carboplatin/CPT-11, two received CDDP/CPT-11, one received carboplatin/VP-16, and one received epirubicin. Previously published objective response rate to second-line chemotherapy in patients with platinum-resistant ES SCLC is 2% to 5%, and for studies where >50% patients had resistant disease (as in our patient population; 6% to 16%; ref. 23). However, we observed objective clinical responses (partial response + complete response) in 61.9% of the 21 patients treated with second-line chemotherapy (Table 3). Only four patients (19%) progressed after second-line chemotherapy. Thirteen of the platinum-resistant patients treated with the vaccine received various chemotherapeutic regimens when they progressed after receiving the vaccine, the response rate was 61.5% (Table 3). The median survival of these platinum-resistant patients ($n = 13$) from the time of the first vaccine administration was 9.3 months with a lower 95% confidence interval of 7.1 months. The median overall survival of all 29 patients was 11.8 months from the time of the first vaccine administration, with a lower 95% confidence interval

of 7.9 months (Fig. 4B). Eleven of the 29 patients (38.1%) were alive 1 year after the administration of the first vaccine.

Twenty-eight treated patients are thus far evaluable for immune responses. One patient was removed from the immune response analysis due to the loss of a blood specimen. We evaluated the connection between the development of p53-specific T cell responses to immunization and clinical response to second-line chemotherapy. As was described above, immune response was measured 2 to 3 weeks after the end of vaccination just before the start of second-line chemotherapy. Nine out of 12 patients (75%) who had a positive immunologic response to immunization developed a complete response or partial response to second-line chemotherapy compared with 3 out of 10 patients (30.0%) who had no detectable immunologic response ($P = 0.08$; Fig. 4C). Patients with positive immunologic response to vaccination showed a slight trend toward improved overall survival (median 12.1 months) compared with patients who did not respond immunologically to vaccination (median survival, 9.6 months).

The administration of second line chemotherapy started in most patients 3 to 4 weeks after the end of vaccination. To follow-up the status of the specific immune response in these patients, we evaluated p53-specific immune responses 2 months after the last vaccination. In most patients, there was a significant decrease in the p53-specific immune responses (Fig. 4D and E). This decrease was not associated with significant chemotherapy-induced lymphopenia (Fig. 4F).

Discussion

The goal of this study was to develop a vaccine that would improve the clinical outcome of ES SCLC. We and others have established in preclinical experiments that p53 could be a good target for cancer immunotherapy (7–9, 14, 24–29). In several clinical trials using p53 peptides or a viral vector containing the wild-type p53 gene, generation of a specific immune response in some patients has been reported. However, no objective clinical responses have been observed (11, 30, 31). We hypothesized that the use of the most potent antigen-presenting cells, DCs, transduced with wild-type p53 might induce a more potent p53-specific immune response that would translate into a clinical response. Adenovirus is not only an excellent tool for gene delivery into DCs (reviewed in refs. 17, 18) but also induces activation of these cells manifested by up-regulation of MHC class II and costimulatory molecules on DC, production of interleukin-12 and other proinflammatory cytokines as well as enhancing functional potency (14–16, 32, 33). Thus, adenovirus offers a unique ability to combine Ag delivery and DC activation and might provide additional benefits for DC-based cancer immunotherapy.

Vaccination resulted in the development of p53-specific T cell responses in just over half of the treated patients (57.1%). These data indicate that despite our efforts, from an immunologic standpoint, the vaccine was effective in only half of the patients. There are several factors that could contribute to a suboptimal immune response: defective T cell function, T cell suppression induced by regulatory T cells, the inability of host DCs to maintain antigen-specific immune responses, the presence of immune suppressive populations of DCs or macrophages, suppression of antigen-specific immune responses

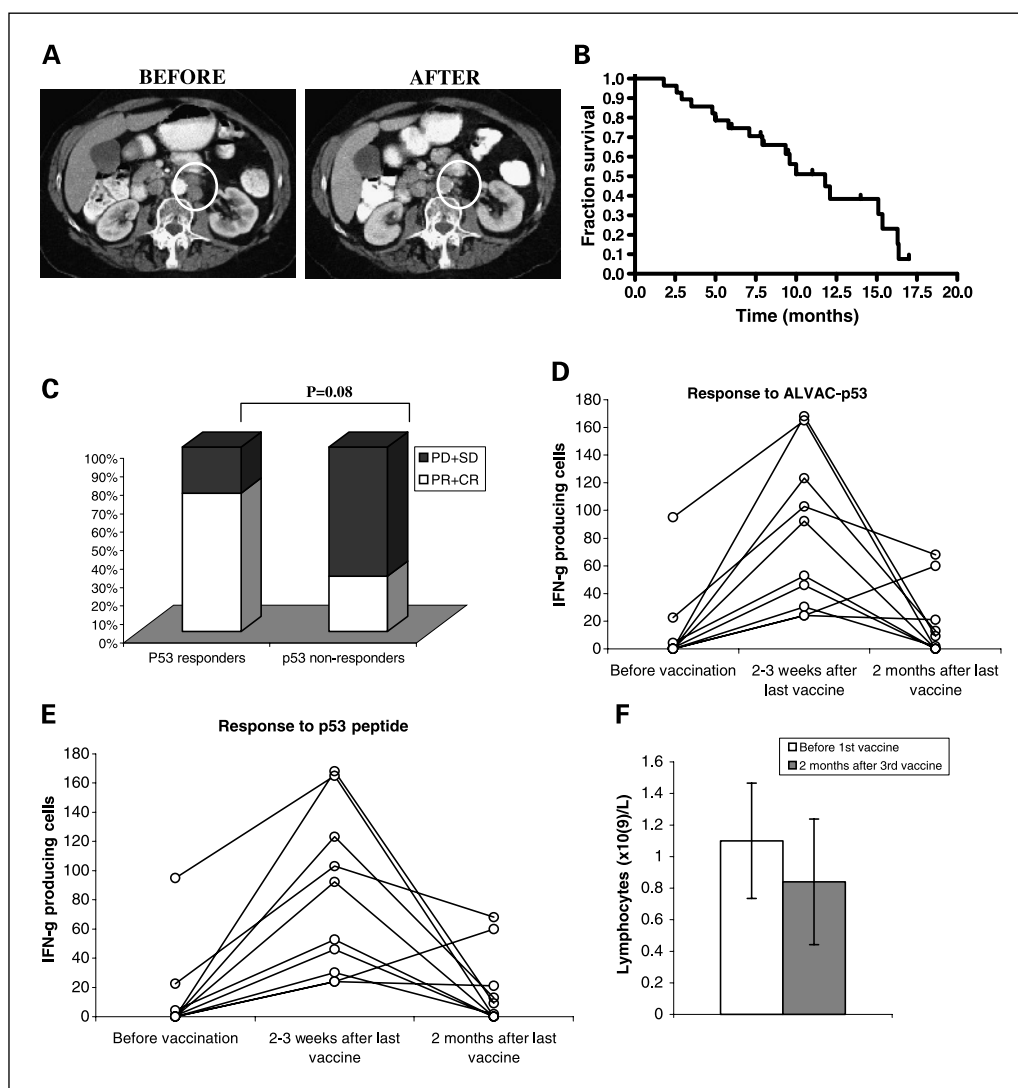


Fig. 4. Association between immunologic and clinical response to vaccination. *A*, clinical response to vaccine. A patient with progressive disease in retroperitoneal lymph nodes (new, and positive on positron emission tomography scan) 2 months after cisplatin/etoposide therapy was treated with three vaccines at the time of progression. A partial response was observed 6 weeks after the first vaccine administration. *Left*, an abdominal CTscan done 1 week prior to the first vaccine shows two enlarged retroperitoneal lymph nodes (circled, each 2 cm in diameter). *Right*, 2 weeks after the third vaccine, the CTscan showed a >60% reduction in the size of both lesions. *B*, overall survival (in months) of all 29 patients treated with the vaccine from the time of the first vaccine injection. *C*, 18 patients who progressed after vaccination and subsequently received chemotherapy were divided into two groups according to their immunologic response to the vaccine. PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response (all according to Response Evaluation Criteria in Solid Tumors). *P* values were calculated using Fisher's exact test. *D* and *E*, the results of IFN- γ ELISPOT assay from patients who developed p53 immune response to vaccination. The background level of nonspecific IFN- γ production (ALVAC-control or irrelevant peptide) was subtracted. The number of spots per 1×10^5 cells are shown. All measurements were done in quadruplicate. The mean for each sample is shown. Not all HLA-A2-positive patients were tested both with ALVAC-p53 and p53-derived peptide. *F*, lymphocyte counts ($\times 10^9/L$) in patients who were treated with second-line chemotherapy. *Columns*, mean; *bars*, \pm SD.

by immature myeloid cells, the induction of antiadenoviral immune responses, etc. In one study, it was not possible to evaluate all possible mechanisms. Our analysis indicated that inhibition of overall T cell reactivity and accumulation of T_{reg} were not associated with failure to develop p53-specific immune responses. Our data do not necessarily indicate a lack of involvement of T_{reg} in SCLC. T_{reg} may migrate from peripheral blood to the site of the tumors. In addition, because all patients were treated with platinum-based chemotherapy 8 weeks before the analysis, it is possible that chemotherapy could have eliminated some of these cells as was previously reported for cyclophosphamide (34). Investigation is currently under way to address this issue.

Our data showed a trend in the association between a decreased presence of mature $CD83^+$ DCs in the peripheral blood of patients prior to vaccination and a lack of immune response to the vaccine. These data may point out an important role of host DCs in the development of antigen-specific immune responses. Nearly all patients enrolled in the trial had significantly reduced levels of DC function, suggesting that improvement of DC function prior to vaccination might be necessary to achieve improved efficacy of the vaccine.

Patients with ES SCLC had increased levels of immature myeloid cells, the cells implicated in tumor-associated antigen-specific immune suppression (21, 22, 35). Importantly, 100% of patients with normal prevaccine levels of immature myeloid

cells developed p53-specific responses to vaccination, compared with only 25% of patients with elevated prevaccine levels of immature myeloid cells ($P = 0.012$). Those differences show that an increase in the number of immature myeloid cells might negatively affect antigen-specific responses to vaccine. After vaccination, the presence of immature myeloid cells increased even further, with only two patients having a normal level of these cells (both developed positive responses to vaccination). The increase in immature myeloid cells may have been caused by the fact that most of the patients had progressive disease by the time of evaluation, thus, removal of immature myeloid cells could potentially be beneficial in enhancing the effect of cancer vaccines.

The induction of antiadenovirus antibody response is considered one of the major limiting factors for the use of this vector in gene therapy. However, our results suggested that a moderate increase in the titer of antiadenovirus antibody was associated with p53-specific responses to vaccination. Because both adenovirus- and p53-derived antigens are presented on the same DC, evaluation of antiadenovirus immunity may serve as a correlate for functional activity of DC. These data are consistent with the results obtained in animal models that showed a limited antiadenovirus response after immunization of mice with DCs transduced with different adenoviral constructs which did not affect antigen-specific CTL activity (14, 36).

Despite the induction of p53 antigen-specific immunity in more than half of the patients, an objective clinical response was observed in only one patient after vaccine treatment. Although this objective response indicates that the vaccine could have direct antitumor effects, the frequency of such responses was unsatisfactory. Importantly, it was similar to the response described in previous clinical trials (3). However, unexpected results were observed after treatment of patients with second-line chemotherapy. Most of the vaccinated patients had objective clinical responses (complete response or partial response) to subsequent chemotherapy. Given the very low historic response rate in this group of patients; these data suggest an improvement in the clinical outcome of the disease caused by vaccination. Patients with ES SCLC have a poor prognosis (reviewed in ref. 16), with treated patients having a median survival of 7 to 11 months. Although first line chemotherapy produces tumor responses 40% to 70% of the time, the response rate to second-line chemotherapy is much

lower. Patients who develop progressive disease within 90 days after receiving a platinum compound (platinum resistant) have a particularly poor prognosis, with response rates reported with single agent chemotherapy of 3.7% to 6.4% with corresponding median survivals of 3 to 6.9 months (37–39). One trial where patients were treated with a combination of paclitaxel, cisplatin, and ifosfamide showed a response rate of 70%. Although the median survival of the platinum-resistant patients was not reported separately, the median survival of all patients treated (20 with platinum resistant and 13 with platinum sensitive disease) was 7 months (40).

It is important to point out that the dramatic difference in historic rate of response to second-line chemotherapy of platinum-resistant patients reported previously (<8%) and the one observed in our study (>60%) could be partly caused by the bias introduced during selection of patients. Further studies are required to rigorously evaluate this treatment regimen in the setting of a controlled clinical trial. Importantly, clinical response to vaccination was associated with immunologic response. Only 30% of patients who did not develop a p53-specific response to vaccination responded clinically to second-line chemotherapy, whereas 75% of p53 cellular immune responders had objective clinical responses to chemotherapy after vaccination ($P = 0.08$). The median overall survival of all patients from the time of the first vaccine administration was 11.8 months with a lower limit of the 95% confidence interval of 7.8 months. Furthermore, 38% of patients were alive 1 year after the first vaccine injection. The median survival of the patients treated with second-line chemotherapy in most phase II clinical trials has been ~6 months with <20% of patients living beyond 1 year (39). These data support the hypothesis that vaccination may be beneficial for patients with SCLC receiving chemotherapy. These data are consistent with recent unexpected observations made in cancer patients immunized with the carcinogen activator cytochrome P450 1B1 (CYP1B1). Most of the 17 vaccinated patients progressed, and all but 1 of 11 patients who did not develop immunity to CYP1B1 progressed and did not respond to salvage therapy. Five patients who developed immunity to CYP1B1 required salvage therapy for progressive metastatic disease and showed marked response to their next treatment regimen, most of which lasted >1 year (41). In another recent phase I study, similar results were obtained in patients with glioma (42).

Table 3. Response to second-line chemotherapy in vaccinated patients

All patients who received chemotherapy after vaccine ($n = 21$)		Platinum-resistant patients who received chemotherapy after vaccine ($n = 13$)	
Response	n (%)	Response	n (%)
CR	3 (14.3)	CR	1 (8)
PR	10 (47.6)	PR	7 (54)
SD	4 (19.05)	SD	3 (23)
PD	4 (19.05)	PD	2 (15)
CR + PR	13 (61.9)	CR + PR	8 (61.5)

Abbreviations: PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response (all according to Response Evaluation Criteria in Solid Tumors).

Chemotherapy may blunt the antigen-specific T cell response because p53 immunity was significantly reduced after starting chemotherapy. However, apparently this time frame was sufficient to produce a positive clinical result. It seems that induction of anti-p53 cellular immunity synergizes with subsequent chemotherapy to provide potent systemic antitumor activity. There are a number of potential mechanisms that could explain the observed effect. Chemotherapy might down-regulate the effect of tumor-produced immunosuppressive factors that prevent CTLs from killing tumor cells, it can up-regulate p53 in tumor cells, which could make them more susceptible to recognition by CTLs, it may activate CTLs by up-regulating the level of

expression of perforin or granzymes, or the proapoptotic effect of granzymes and chemotherapy might synergize at a molecular level. We are currently investigating these possibilities.

Preclinical data accumulated in recent years suggest that the combination of cancer immunotherapy and chemotherapy might provide significant benefit (43). Here, we provide direct clinical evidence in support of this emerging paradigm in cancer immunotherapy. Cancer vaccines may be most effective not as a single modality but rather in close combination with other methods of treatment, specifically, chemotherapy. This concept may have broad implications for the further development of new vaccine strategies.

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