

p53MVA Therapy in Patients with Refractory Gastrointestinal Malignancies Elevates p53-Specific CD8⁺ T-cell Responses

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Abstract

Purpose: To conduct a phase I trial of a modified vaccinia Ankara (MVA) vaccine delivering wild-type human p53 (p53MVA) in patients with refractory gastrointestinal cancers.

Experimental Design: Three patients were vaccinated with 1.0×10^8 plaque-forming unit (pfu) p53MVA followed by nine patients at 5.6×10^8 pfu. Toxicity was classified using the NCI Common Toxicity Criteria and clinical responses were assessed by CT scan. Peripheral blood samples were collected pre- and post-immunization for immunophenotyping, monitoring of p53MVA-induced immune response, and examination of PD1 checkpoint inhibition *in vitro*.

Results: p53MVA immunization was well tolerated at both doses, with no adverse events above grade 2. CD4⁺ and CD8⁺ T cells showing enhanced recognition of a p53 overlapping peptide library were detectable after the first immunization, particularly in the CD8⁺ T-cell compartment ($P = 0.03$). However, in most patients, this did not expand further with the second and third immunization. The frequency of PD1⁺ T cells detectable in patients' peripheral blood mononuclear cells (PBMC) was significantly higher than in healthy controls. Furthermore, the frequency of PD1⁺ CD8⁺ T cells showed an inverse correlation with the peak CD8⁺ p53 response ($P = 0.02$) and antibody blockade of PD1 *in vitro* increased the p53 immune responses detected after the second or third immunizations. Induction of strong T-cell and antibody responses to the MVA backbone were also apparent.

Conclusion: p53MVA was well tolerated and induced robust CD8⁺ T-cell responses. Combination of p53MVA with immune checkpoint inhibition could help sustain immune responses and lead to enhanced clinical benefit. *Clin Cancer Res*; 20(17); 1–12. ©2014 AACR.

Introduction

Immune-based therapies have been most extensively studied in cancers such as melanoma, but there is evidence that antitumor immune responses in patients with gastrointestinal cancer correlate with improved prognosis (1, 2). The obstacles to stimulating effective antitumor immunity

are considerable, but there is encouraging evidence that immunotherapy can improve outcomes in patients with cancer (3–5). Furthermore, it is now recognized that radiotherapy and some chemotherapy agents confer therapeutic benefit, at least partly, by stimulating immune responses that directly target cancer cells or induce immunostimulatory side effects (6).

Wild-type p53 protein maintains normal cell division and mutations in this gene are present in the majority of solid tumors (7). p53 gene mutations result in the accumulation of high levels of oncogenic p53 protein within tumor cells. In contrast, the concentration of normal p53 in healthy cells is low, making p53 an attractive target for immunotherapy of a wide range of malignancies. Immune recognition of p53 in tumor cells has been demonstrated both *in vitro* (8, 9) and in mouse models (10, 11). Furthermore, clinical trials targeting p53 by administration of synthetic peptides and dendritic cell (DC)-based vaccines have yielded promising results (12, 13). Most notable are trials using DC infected with a p53 adenoviral vector (Advexin), which showed evidence of clinical benefit when administered to patients with lung cancer (14). However, the p53 vaccines tested to date are restricted to patients with

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Translational Relevance

Mutations in the p53 gene are present in the majority of solid tumors and result in high levels of p53 protein within tumor cells. In contrast, the concentration of wild-type p53 in normal tissue is low. Hence, p53 is an attractive target for immunotherapy of a wide range of malignancies, and data from *in vitro* studies, murine models, and clinical trials support the rationale of targeting this protein. We conducted a first-in-human, phase I trial of a modified vaccinia Ankara (MVA) vector delivering wild-type human p53 (p53MVA) in patients with refractory gastrointestinal cancers. p53MVA was well tolerated and elevated the p53-specific CD8⁺ T-cell response. Furthermore, higher anti-p53 immune responses were detected in patients with lower frequencies of PD1⁺ T cells and enhanced responses were achievable with antibody blockade of PD1 *in vitro*. These initial findings support the continued development of p53MVA, particularly in combination with immune checkpoint inhibition.

certain tissue types, or require individual manufacture for each recipient, and hence are laborious and costly to produce.

We have developed a strategy using the genetically engineered version of the modified vaccinia Ankara (MVA) virus to immunize patients with the wild-type p53 antigen (p53MVA). Using a viral vector to deliver full-length p53 has the potential to generate sustained antigen expression and the presentation of numerous antigenic determinants on different HLA molecules. In preclinical studies, Hupki mice (human p53 knockin) were engineered to substitute the mouse p53 gene with the human form, enabling tolerance, thereby developing an immunologic milieu similar to what the human vaccine will encounter clinically. Hupki mice immunized with p53MVA showed regression of established 4T1 syngeneic breast tumors with murine p53 knock-out and engineered human p53 expression, and generation of systemic antitumor immunity (15). Finally, studies with peripheral blood mononuclear cells (PBMC) collected from patients with cancer with solid tumors showed that specific recall immune responses to p53 could be stimulated *in vitro* with p53MVA (16).

MVA has a demonstrated safety record, being used in numerous clinical trials with only mild side effects. The initial vaccine dose of 1.0×10^8 plaque-forming unit (pfu) was chosen because a previous trial using MVA expressing IL2 and MUC1 reported low toxicity, as well as disease stabilization and cellular immune responses (17). In the MVA-5T4 trials for colorectal cancer, which used doses of 5.0×10^8 pfu, immunologic and clinical responses were achieved in the absence of toxicity (18). Murine studies conducted by us (10) and others (19) have demonstrated that p53-based immunotherapy is most effective when used in combination with anti-CTLA4. Furthermore, comparable

human data were reported in patients with prostate cancer treated with a combination of a PSA-fowlpox vaccine and ipilimumab (Bristol-Myers Squibb; ref. 20). This adds weight to the rationale of combining viral-based vaccines with other immunostimulatory agents. However, because this was a first-in-human trial of p53MVA, a single-agent study was optimal to assess properties of the vaccine construct. Here, we report the findings of this study in regard to safety, clinical response, and immunologic endpoints.

Materials and Methods

p53MVA vaccine formulation

The therapeutic agent tested in this study was a MVA vector expressing full-length wild-type human p53. The p53MVA vaccine product was manufactured at the Center for Biomedicine and Genetics at City of Hope (Duarte, CA) using GMP-grade materials and the final formulation was diluted in phosphate-buffered saline (PBS) and 7.5% lactose. The p53MVA vaccine was previously evaluated in an IND-directed toxicology study in mice. There was no significant toxicity in terms of weight loss, physical examination, activity level, or chemical or hematologic studies (data not shown). p53MVA was vialled at two different concentrations, 1.3×10^8 and 7.0×10^8 pfu/mL, and stored at -80°C . Vaccine doses were thawed at room temperature and administered within 1 hour of thawing. Previous studies showed that the vaccine was stable at room temperature for 4 hours (data not shown). Patients received injections in a volume of 0.8 mL. There were no other therapeutic products involved.

Patients and eligibility criteria

Participants were recruited from patients with gastrointestinal cancer attending Medical Oncology clinics at the City of Hope Medical Center (Duarte, CA) between December 2012 and June 2013. The Institutional Review Board (IRB)-approved study was conducted under IND14716 and registered as NCT01191684 at ClinicalTrials.gov. Before treatment, all patients received and signed the informed consent. Patients with unresectable and chemotherapy-resistant primary or recurrent carcinoma of colorectal, gastric, or pancreatic origin were eligible. Patients with colorectal cancer who had failed to respond to 5-fluorouracil (5-FU)-based therapy with oxaliplatin and irinotecan as well as EGFR-directed therapies for KRAS wild-type patients, patients with gastric cancer who failed standard first-line treatment, including Herceptin for Her2⁺ patients, or patients with pancreatic cancer who had failed to respond to at least one chemotherapy regimen were eligible. Only patients with no clinically evident brain metastasis, with an anticipated survival of at least 3 months, and a performance status of 80 to 100 (Karnofsky Performance Status) were admitted to the trial. Evidence of tumoral p53 overexpression by immunohistochemistry or p53 mutational analysis was required. Patients with immunodeficiency (including HIV and organ graft-related), prior radiation to more than 50% of all nodal groups, or those receiving concurrent corticosteroids were excluded. In

Table 2. Healthy controls

	Healthy controls	Age	Sex
Controls for low-dose cohort	1	70	M
	2	61	F
	3	44	F
Controls for high-dose cohort	4	51	51
	5	57	57
	6	58	58
	7	52	52
	8	60	60
	9	60	60

addition, patients with a history of autoimmune disease, severe environmental allergies, myopericarditis, allergy to egg proteins, or a known family history of Li-Fraumeni syndrome were ineligible. Patients could not receive chemotherapy or radiotherapy within the 4 weeks preceding enrollment. The characteristics of all enrolled patients are detailed in Table 1.

Dose escalation

The study included 12 patients treated at escalating doses of 1.0×10^8 followed by 5.6×10^8 pfu p53MVA, following a standard 3+3 design. No inpatient dose escalation was carried out. The first 3 patients received subcutaneous injections of 10^8 pfu p53MVA every 3 weeks for a total of three injections. Dose-limiting toxicity (DLT) was classified using the NCI Common Toxicity Criteria version 4.0. Patients treated in the low-dose group did not experience DLT, hence treatment in the high-dose group was initiated. A second group of 3 patients received three cycles of subcutaneous injections of 5.6×10^8 pfu p53MVA. As none of these patients experienced DLT, an additional 6 patients were then treated at this dose level.

Clinical procedure

Phlebotomy was performed for biochemical, hematologic, and immunologic assays before vaccination and 2 weeks following each immunization according to the schedule shown (Fig. 1). A CT scan and physical examination were carried out before study to establish the extent of disease and was repeated 2 months following the initial injection. Vaccine injections were administered by a study nurse in the subcutaneous tissue of the upper arm over the deltoid muscle on days 0, 21, and 42. All subjects were monitored for 1 hour in the clinic after each immunization for temperature changes and local reactions at the injection site. All subjects were contacted 24 and 48 hours after each immunization to record any vaccine-related complications. Two patients were unable to complete the vaccination schedule due to disease progression, but all 12 enrolled patients provided a pre- and postvaccine blood draw for immunologic analysis.

Table 1. Patients receiving p53MVA

Patient	Age	Sex	Cancer	Rounds of CT/RT	Performance status	p53MVA dose	Highest grade AE	Types of p53MVA related AE	3 month CT scan result	Immunizations + blood draws
1	76	M	Pancreatic	6/2	90	LD	1	ISR, fatigue	PD	3 imm >4 bd
2	64	F	Colon	6/2	90	LD	1	ISR	PD	3 imm >4 bd
3	45	F	Colon	5/1	90	LD	2	Lymphopenia, fatigue, myalgia, arthralgia, ISR, chills, diarrhea	PD	3 imm >4 bd
4	64	F	Colon	9/2	90	HD	2	Headache, fatigue, fever, ISR, nausea, anemia	PD	3 imm 4 bd
5	55	M	Pancreatic	4/2	90	HD	1	ISR, fatigue	PD	3 imm 4 bd
6	48	M	Pancreatic	3/1	80	HD	1	ISR, fever	PD	3 imm 4 bd
7	53	M	Colon	3/0	90	HD	2	ISR, nausea, fatigue, fever	PD	2 imm 3 bd
8	51	F	Colon	6/0	90	HD	1	Hypertension, ISR, fatigue, cough	PD	3 imm 3 bd
9	57	M	Pancreatic	3/1	90	HD	1	ISR	PD	3 imm 4 bd
10	59	M	Colon	3/0	100	HD	1	Chills, bone pain, fever, ISR, fatigue, pain	PD	3 imm 4 bd
11	58	M	Pancreatic	2/0	80	HD	1	ISR	PD	1 imm 2 bd
12	58	F	Colon	9/0	80	HD	2	Fatigue, fever, ISR, pain, headache, malaise	PD	3 imm 4 bd

Abbreviations: AE, adverse event; bd, blood draws; CT/RT, chemotherapy/radiotherapy; imm, immunizations; ISR, injection site reaction; M/F, male/female; LD/HD, low dose/high dose; PD, progressive disease.

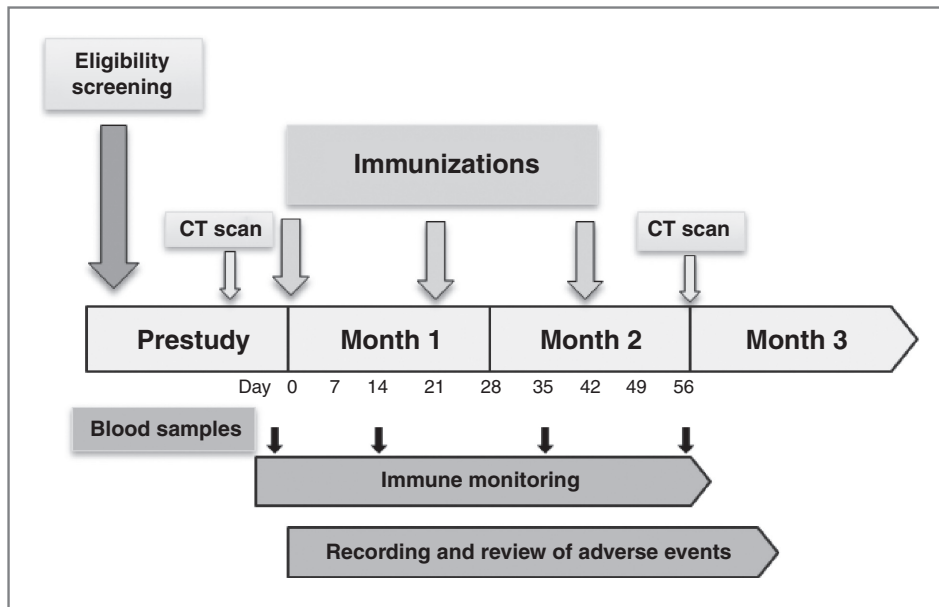


Figure 1. Clinical trial schema: vaccination and phlebotomy schedule for a p53MVA phase I clinical trial.

Cell culture

Peripheral blood samples were collected from patients by venipuncture in acid-citrate-dextrose (ACD) tubes and processed within an hour. PBMCs were purified by density gradient separation using Ficoll-Paque Plus (GE Healthcare). CD3⁺ cells were isolated from PBMCs using human CD3⁻ selection microbeads (Miltenyi Biotec) as per the manufacturer's instructions. All primary cells were cultured in serum-free conditions in X-VIVO 20 medium (Lonza). The CD3⁻ fraction was retained to generate DC as follows: after 2-hour plastic adherence, nonadherent cells were removed and the adherent population cultured in X-VIVO 20 media supplemented with IL4 (50 ng/mL) and GM-CSF (100 ng/mL) for 5 days. Nonadherent cells were washed and infected with Adp53 (a kind gift from Scott Antonia, Moffitt Cancer Center, Tampa, FL) or control adenoviral vector (AdGFP) at an multiplicity of infection (MOI) of 15. After 2 hours, additional media containing polyinosinic-polycytidylic acid (50 µg/mL) was added. After 48 hours, transduced, matured DC were washed and used to set up cocultures with autologous CD3⁺ cells at an effector:stimulator ratio of 10:1. For PD1-blocking experiments, azide-free αPD1 (eBioscience) or IgG1_k isotype control (BD Pharmingen) antibodies were added at 5 µg/mL. *In vitro* stimulation was carried out for 7 days, with 10 µ/mL IL2 added on day 2. Stimulated cells were then washed and reseeded in the presence of test antigens, including VenusMVA, a library of overlapping p53 peptides, or positive (pp65) or negative (BKV-VP1) control peptides at a concentration of 10 µg/mL. Peptides were synthesized in the laboratory as previously described (15). After 24 hours, T cells were analyzed for the expression of the activation marker CD137 by flow cytometry (see below) or IFN γ secretion by ELISPOT assay (see below). Blood draws 3 and 4 from Patient 6 were unevaluable in functional cellular assays due to poor viability.

Flow cytometry

Immunophenotyping. Flow cytometry analysis of cell-surface molecules on PBMCs was conducted using antibodies from Becton Dickinson or eBioscience. Cells were washed and stained with antibody for 30 minutes at room temperature, in the dark, in the presence of 1% FBS. For regulatory T cells (Treg) intranuclear FOXP3 analysis, permeabilization was performed using the eBioscience anti-FOXP3 staining set, according to the manufacturer's instructions. Tregs were identified as CD3⁺CD4⁺CD25⁺CD127^{low/-}FOXP3⁺ and expressed as a percentage of CD4⁺ cells. Myeloid-derived suppressor cells (MDSC) were identified as HLADR⁻LIN1^{low/-}CD33⁺CD11b⁺. T-cell markers CD4, CD8, CD27, CD28, CD57, and PD1 were used to assess T-cell differentiation status. Blood samples from age- and sex-matched healthy donor controls were obtained through the City of Hope Blood Donation Clinic (Table 2).

Measurement of T-cell activation. Cells from each *in vitro* stimulation were costained with anti-CD4, anti-CD8, and anti-CD137-PE or a phycoerythrin (PE)-labeled isotype control. CD137 positivity was assessed relative to the isotype control PE signal. Flow-cytometric analyses were carried out using a FACSCanto (BD Biosciences) or Gallios (Beckman Coulter) flow cytometer. All data were analyzed with FlowJo7.5.6.

MVA neutralization assays

Plasma was obtained by centrifugation of whole blood for 10 minutes, at room temperature for 1,500 rpm. Aliquots were frozen and stored at -80°C until analysis. A modified version of the protocol, described in Cosma and colleagues (21), was used to assess the ability of patient plasma to neutralize MVA. Plasma samples were heat-treated for 30 minutes at 56°C to inactivate complement and then cooled. Serial dilutions of plasma from 1:10 to 1:1,000

were prepared in RPMI-1640 (CellGro) with 10% fetal calf serum (FCS), to which was added 1×10^6 pfu of VenusMVA (previously described; ref. 22). After 90 minutes of incubation at 37°C, the different infection media were added to HeLa cell monolayers and incubated for a further 2 hours. Infection media was then removed and replaced with RPMI, 10% FCS, and the plates were incubated overnight at 37°C, 5% CO₂. Infected cells were trypsinized from plates, washed with PBS, and fixed in 1% paraformaldehyde. The percentage of MVA-infected HeLa cells was evaluated by measuring GFP analogue (Venus) expression by flow cytometry. Intact cells were discriminated from cellular debris according to FSC/SSC (forward scatter/side scatter). The percentage neutralization was calculated in relation to the %Venus⁺ cells after infection with VenusMVA incubated with media alone. Plasma from MVA naïve individuals and purified anti-vaccinia rabbit sera (a kind gift from Dr. Mary Marovich, Henry Jackson Foundation, Bethesda, MD) were included in each assay as negative and positive controls, respectively. Plasma samples from patient 3 were found to be unevaluable by this assay.

IFN γ ELISPOT assays

Whole thawed PBMCs or stimulated effector cells were washed and seeded at 2×10^5 per well into ELISPOT plates coated with IFN γ capture antibody as per the manufacturer's instructions (BD Biosciences). VenusMVA at an MOI of 0.2 was added to triplicate wells. Additional control wells, media alone and PMA + ionomycin or PHA, were included to assess the background stimulation and viability of T cells, respectively. Plates were incubated for 24 hours at 37°C, 5% CO₂, after which cells were removed and the wells were washed with distilled water. IFN γ spots were visualized according to the manufacturer's instructions and counted by computer-assisted video image analysis using an AID ELISPOT reader (Autoimmun Diagnostika GmbH).

Statistical analysis

The comparison of experimental values before and after immunization was evaluated using a paired *t* test. A two-group *t* test was used to compare the endpoint values between patients and healthy controls. The correlation between two normally distributed endpoints was evaluated using Pearson correlation analysis. Because all study endpoints consistently showed normality patterns, the use of parametric methods was considered more appropriate than nonparametric tests. *P* values less than 0.05 were considered significant and less than 0.005 as highly significant.

Results

p53MVA vaccination is well tolerated

p53MVA immunization was well tolerated, with no adverse events exceeding grade 2 attributable to the vaccine. Table 1 details the adverse events that were related to p53MVA vaccination. Injection site reaction (ISR) was the

most commonly reported side effect. Other commonly reported adverse events included fatigue, fever, and nausea. Two patients did not complete the course of three immunizations and left the study early due to events not related to the vaccine injection.

p53MVA vaccination stimulates anti-p53 immune responses

p53-specific T-cell responses were initially evaluated in the low-dose patient cohort by quantification of CD137⁺ T cells after stimulation with p53 peptides. The CD137 marker has been validated for measuring both CD8⁺ and CD4⁺ responses (23–25), with the suggestion that this assay may detect a broader repertoire of antigen-specific T cells than measurement of IFN γ (23). Figure 2A shows a representative flow cytometry plot from patient 1. An isotype control for the CD137 antibody was used to set the negative gates. To control for nonspecific stimulation the %CD137⁺ T cells after stimulation with an irrelevant peptide was subtracted from the %CD137⁺ T cells after stimulation with the p53 peptide library (details in Supplementary Table S1). Figure 2B shows the peak response detected after vaccination compared with the prevaccine value in the CD4⁺ and CD8⁺ compartments. The initial dose of 1.0×10^8 pfu was not predicted to be therapeutic but some enhanced p53-immune reactivity was detected in these 3 patients, most notably in the CD8⁺ T cells from patient 1. However, in most cases, the frequency of p53-reactive T cells was comparable with that detected using PBMCs from unvaccinated, healthy controls.

Vaccine-enhanced p53-immune reactivity was higher in the patient cohort receiving the 5.6×10^8 pfu dose of p53MVA. This was most striking in the CD8⁺ T-cell compartment. Figure 3A shows a representative flow cytometry plot from patient 7. As with the low-dose patient cohort, background stimulation was apparent, but even after subtraction of the CD137 signal from an irrelevant peptide, an enhancement of CD8⁺ p53 reactivity was significant after the first vaccination. This was seen in the majority of the high-dose patients, and reached statistical significance (*P* = 0.03). Furthermore, when the highest p53-specific responses detected after vaccination (peak response) were compared with prevaccine levels, significance was even higher (*P* = 0.002) in the CD8⁺ compartment. Despite the p53 peptide library containing both CD4 and CD8 epitopes, the increased p53 reactivity in the CD4⁺ compartment was less apparent in the CD8⁺ population and did not reach statistical significance (Fig. 3B). The expansion of p53-reactive CD8⁺ T cells was greatest after the first vaccination, and subsequent immunizations did not further amplify the response in the majority of patients.

p53MVA vaccination stimulates strong antivector immune responses

A VenusMVA vector was used to assess the humoral and T-cell response to the MVA backbone pre- and postvaccine in the low-dose cohort and the first 3 patients of the high-dose cohort. Figure 4 shows the anti-MVA T-cell response as

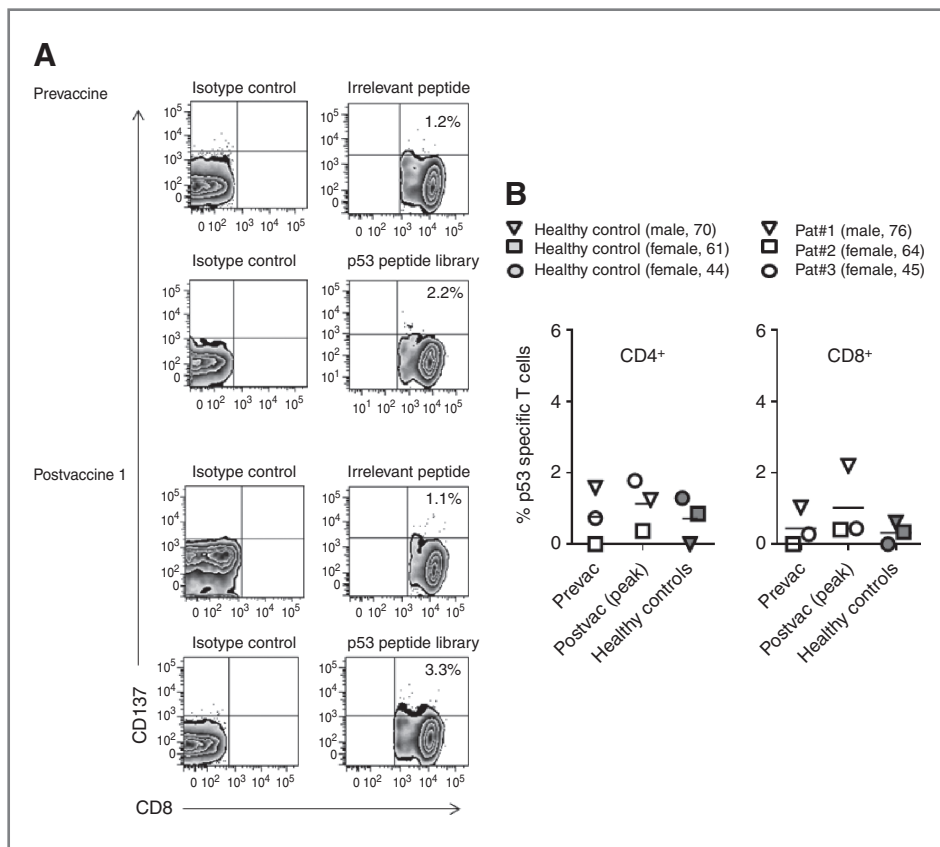


Figure 2. T cells collected from healthy donors and patients with gastrointestinal cancer receiving a subtherapeutic dose of p53MVA show low levels of p53 reactivity, despite *in vitro* stimulation. Patient T cells were cocultured for 5 days with p53 expressing APC (antigen presenting cell) and 10 U/mL IL2. Stimulated cells were then washed and reseeded in fresh media for 2 days. After a final, overnight stimulation with a p53-peptide library, the percentage of CD4⁺ or CD8⁺ T cells expressing the activation marker CD137 was determined. Comparative analysis using PBMCs from three unvaccinated, matched healthy controls was carried out in parallel. A, representative flow cytometry plots of the CD8⁺ population, pre- and postvaccination 1 from patient #1. B, the peak postvaccine p53 response compared with prevaccine for both CD4⁺ and CD8⁺ T cells in patients 1 to 3. To control for nonspecific stimulation %CD137⁺ T cells in response to p53 peptide – %CD137⁺ T cells in response to an irrelevant peptide were plotted. Each patient (open symbols) and the corresponding matched control (closed symbols) is represented by a different symbol.

measured by IFN γ ELISPOT (Fig. 4A) and the MVA antibody response measured by neutralization assay (Fig. 4B). It was not known which patients had a history of smallpox immunization, but due to the age of all trial participants, it is likely that they had been vaccinated. All tested patients showed low preexisting anti-MVA responses. After vaccination with p53MVA, all patients showed increased T-cell reactivity and neutralizing activity against MVA. The highest induction of anti-MVA T-cell response seen was a 54-fold increase (patient 1). The strongest increase in neutralizing antibodies against MVA observed was a 79.7-fold increase (patient 4). Both the low- and high-dose of p53MVA stimulated comparable levels of anti-MVA T-cell and antibody responses. Unlike T-cell responses against p53, which required *in vitro* expansion to reach measurable levels, MVA responses were detected in unstimulated PBMCs. This suggests that MVA-reactive T cells had greater proliferative capacity and hence reached higher frequencies than p53-specific T cells after vaccination. This is an expected finding because antiviral T-cell responses are less tolerized than those against self-antigens.

MDSC decline transiently after p53MVA in a subset of patients

MDSC were characterized by flow cytometry as LIN⁻/lowHLADR⁻CD11b⁺CD33⁺ gated populations as shown in Supplementary Fig. S1A. Monocytic MDSC and gran-

ulocytic MDSC populations (26) were not differentiated from each other. The frequency of MDSC as a percentage of the live PBMCs did not differ significantly between patients and healthy controls. Interestingly, in five out of nine of the high-dose cohort, the frequency of MDSC in the peripheral blood declined after vaccination; however, in many cases, this was a transient effect. Supplementary Fig. S1C shows a representative example of the MDSC decline in patient 6. However, when analyzed as a group, the difference in MDSC frequency pre- and post-immunization did not reach statistical significance (Supplementary Fig. S1B).

The frequency of PD1⁺ CD8⁺ T cells is greater in patients than healthy controls and shows a correlation with p53 response

The negative T-cell costimulatory molecule PD1 is exploited by tumor cells to evade immune clearance. The percentages of PD1⁺ T cells detected in trial participants PBMCs were significantly higher than in healthy controls (Fig. 5A and B). This was statistically significant in the CD4⁺ T cells ($P = 0.01$) and highly significant in the CD8⁺ compartment revealed a significant correlation between the prevaccine percentage of PD1⁺ T cells and the peak response to p53 peptides after immunization (correlation $r = 0.74$; $P = 0.02$; Fig. 5C, right). To clarify, patients with lower

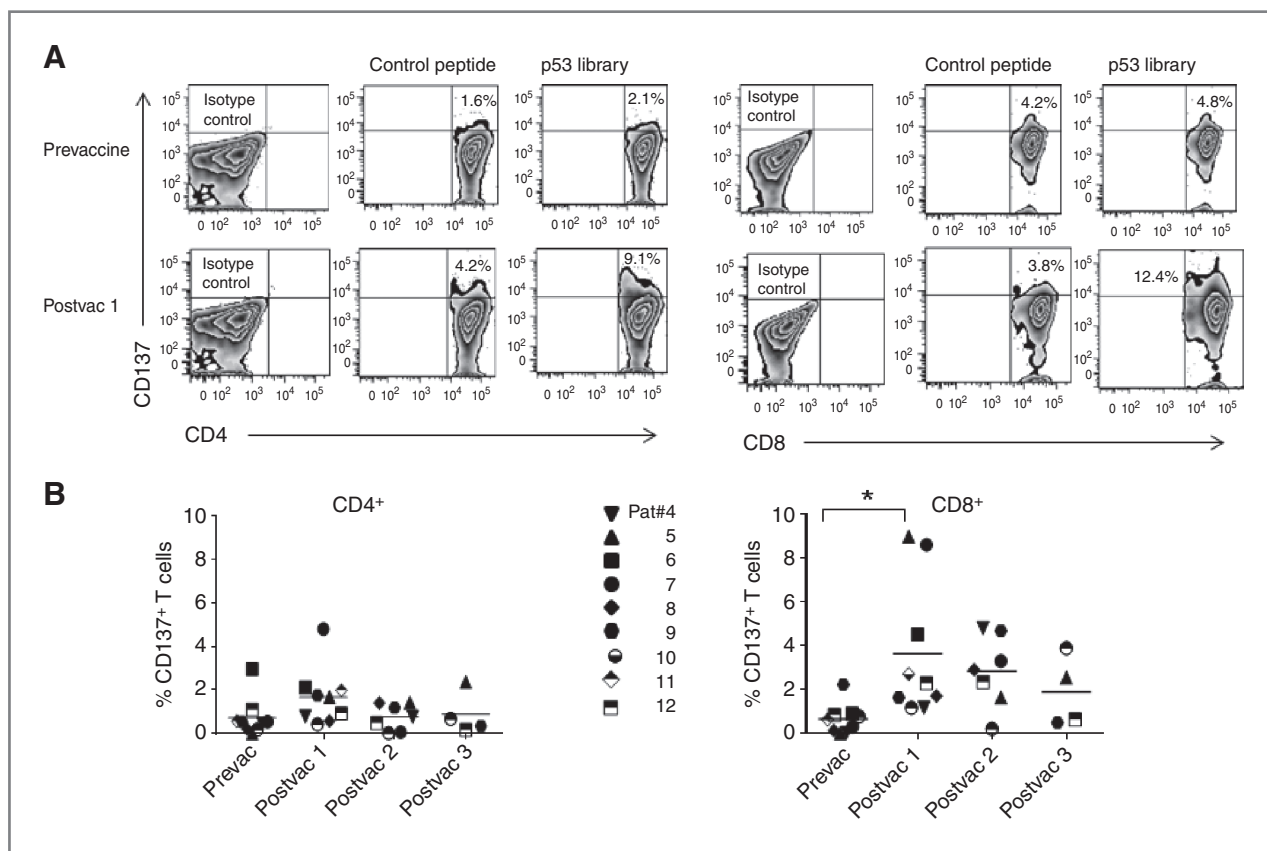


Figure 3. Vaccination with high-dose p53MVA transiently increases the frequency of p53-reactive T cells in the peripheral blood of patients with gastrointestinal cancer. Blood samples were collected prevaccination and 2 weeks after each p53MVA immunization. Purified CD3⁺ cells were expanded *in vitro* for 7 days before an overnight stimulation with a p53 peptide library or control peptides. The T-cell activation marker, CD137, was used as a measure of T-cell responsiveness and quantified by flow cytometry. A, representative flow cytometry plots from patient #7. B, the percentage of CD137⁺ T cells in response to p53 peptide – %CD137⁺ T cells in response to an irrelevant peptide in the CD4⁺ or CD8⁺ populations were plotted. Patients 4 to 12 are each represented by a different symbol. The increase in percentage of p53-responsive CD8⁺ T cells postvaccine 1 compared with prevaccine values was statistically significant ($P = 0.03$) as denoted by the asterisk.

frequencies of PD1⁺ CD8⁺ T cells prevaccine showed greater induction of CD8⁺ p53-reactivity after immunization. However, this is a small dataset, which requires confirmation in a larger patient cohort.

***In vitro* PD1 blockade can increase T-cell responses to p53 peptides**

Because p53 reactivity showed a relationship with frequency of PD1⁺ T cells, PD1 blockade was investigated. Because the p53 reactivity detected after the second or third vaccinations were lower than those detected after the first vaccination, we examined whether these responses could be enhanced by PD1 antibody blockade. A single round of *in vitro* expansions were carried with 6 patient samples as previously described but with the addition of anti-PD1 antibody or an isotype control at the start of the culture period. Figure 5D shows the IFN γ responses of the expanded cells as measured by IFN γ ELISPOT assay. PD1 blockade modestly increased the T-cell response to the p53 library, reaching statistical significance ($P = 0.026$). These results support future

clinical studies in which PD1 antibody blockade is combined with the p53MVA vaccine.

The frequency of early-, intermediate-, and late-differentiated T cells or Tregs does not significantly change after p53MVA immunization

Tregs were defined as the percentage of CD3⁺CD25⁺CD127^{low}/-FOXP3⁺ gated populations within the CD4⁺ T-cell compartment. The frequency of Tregs in patients' PBMCs did not differ significantly from those detected in a set of healthy control PBMC samples. A slight downward trend in the percentage of Tregs within the CD4⁺ T-cell population was apparent after immunization, but this did not reach statistical significance (Supplementary Data).

Malignant disease is thought to cause chronic antigenic stimulation and drive T-cell progression. This results in an expansion of late-differentiated, senescent lymphocytes that can impair antitumor immunity. When the differentiation stage of patients T cells were evaluated, some showed a higher frequency of terminal effector T cells (CD28⁺CD27⁺) and senescent T cells (CD8⁺CD27⁻CD28⁻CD57⁺PD1⁺) than

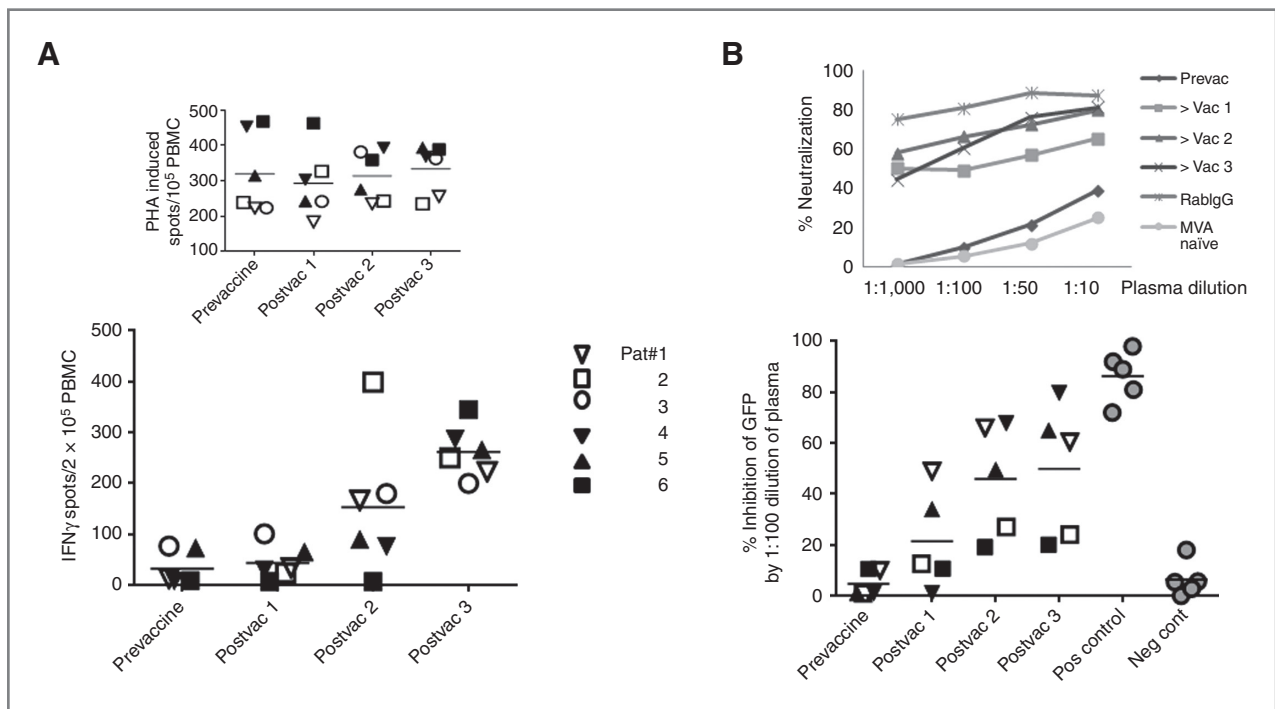


Figure 4. p53MVA immunization induces anti-MVA neutralizing antibody and T-cell responses in patients with advanced gastrointestinal cancer. A, the T-cell response from 3 low-dose patients (open symbols) and 3 high-dose patients (closed symbols) against VenusMVA. PBMCs tested IFN γ ELISPOT assays for reactivity to media alone, VenusMVA or PHA. Values plotted represent the mean number of spots from PBMCs incubated with VenusMVA—the mean number of spots from PBMCs seeded in media alone (background). The number of IFN γ spots from PHA stimulated PBMCs are shown separately above. B, the percentage neutralization of the Venus signal by plasma collected from the same patients. This was assessed by incubating the VenusMVA virus with patient or control plasma before infection of a permissive cell line. Venus expression was determined by flow cytometry. Data are shown for only 5 patients (Patient #3 plasma samples were unevaluable in this assay). Plasma from MVA-naïve individuals and purified IgG from vaccinia vaccinated rabbits were included as negative and positive controls, respectively. Percentage neutralization obtained at 1:100 dilution is shown, with a representative dilution curve for Patient #1 shown above.

healthy controls, but this did not reach statistical significance (Supplementary Fig. S1). In addition, the frequency of early-, intermediate-, or late-stage effector T cells did not change significantly after p53MVA immunization.

Discussion

In this report, we describe the first-in-human clinical trial of p53 delivered by an MVA vector in patients with advanced, refractory gastrointestinal malignancies. Clinical and immunologic responses were assessed, but the primary endpoint was safety. p53MVA was well tolerated in all 12 patients, causing only low-grade side effects (Table 1). The p53MVA tolerability findings presented here are in line with previous reports of MVA clinical studies (17, 27, 28). No clinical responses were detectable at the postvaccine CT scan in any of the 12 patients by RECIST criteria; however, immunologic responses were transiently robust. Stimulating objective clinical responses is challenging in patients with high morbidity due to advanced disease and high levels of pretreatment. Because chemotherapy-naïve patients can respond better to immunotherapy (20), p53MVA therapy in less heavily pretreated patients is an attractive approach.

The role of MDSC and Tregs in tumor immune evasion and cancer progression is well accepted (29, 30). Human

MDSC are a heterogeneous population that, unlike murine MDSC, are still being characterized, but the currently accepted definition for human MDSC is CD33⁺CD11b⁺HLA-DR^{low/-}. Within this population, the CD14⁺CD15^{low/-} and CD14⁻CD15⁺ subsets are considered equivalent to the murine monocytic and granulocytic populations, respectively (26). Tregs also exhibit some heterogeneity, with two main subtypes being recognized, natural Tregs generated during thymic development and inducible Tregs that arise in the periphery after interaction with tolerogenic stimuli. Tregs are crucial in maintaining peripheral tolerance to self-antigens, and hence dampen effector T-cell responses against many tumor antigens (31). These suppressive cell types pose an obstacle to effective antitumor immunity, with both Tregs (32) and MDSC (33) being shown to affect vaccine-induced immune responses. Hence, we thought it valuable to assess the frequency of these suppressive cells in the participants both pre- and postvaccine. In 4 of 9 of the high-dose patients, the frequency of MDSC increased slightly during the vaccination, concurrent with disease progression. However, in five of nine of the high-dose cohort, the frequency of MDSC fell transiently after the first immunization (Supplementary Fig. S1). Tregs also showed a slight downward trend after immunization, but did not reach statistical significance

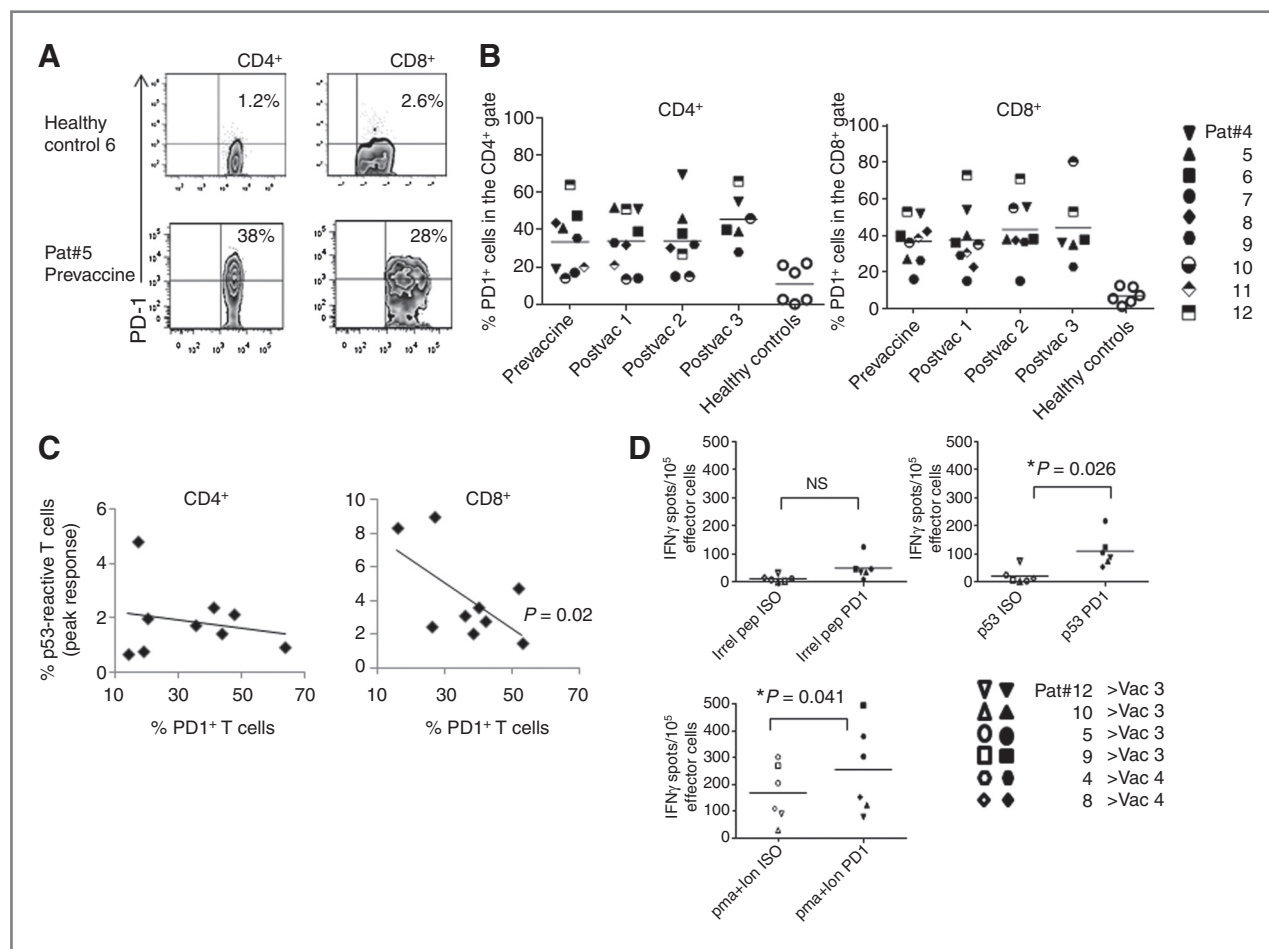


Figure 5. Patients with advanced gastrointestinal cancer show higher frequency of PD1⁺ T cells than healthy controls both pre- and postimmunization with high-dose p53MVA. Peripheral blood T cells collected pre- and postimmunization were assessed by flow cytometry for the expression of the programmed cell death receptor PD1. A, representative plots from a patient and healthy control. Quadrant gates were set according to isotype control staining. B, the percentage of PD1⁺ T cells in the CD4⁺ and CD8⁺ populations at different times in the immunization schedule. Different patients are represented by different symbols. C, an inverse relationship between the frequency of PD1⁺ T cells preimmunization and the peak p53-reactive T cells in the CD4⁺ and CD8⁺ populations. Correlation in the CD8⁺ population reached statistical significance ($P = 0.02$). D, the reactivity of effector cells after *in vitro* expansion in the presence of α -PD1 (closed symbols) or isotype control (open symbols). Stimulated cells were tested in IFN γ ELISPOT assays against control peptide, p53 library, VenusMVA, and PMA + ionomycin. Data shown are from lymphocytes obtained after two or three vaccinations.

(Supplementary Fig. S2, bottom). In addition, the small sample size and the fact that both Treg and MDSC frequency were in the range for healthy donors, makes it difficult to attribute disease relatedness to these findings.

Detection of p53-specific T cells required an *in vitro* restimulation to expand them to detectable levels, a finding reported by other groups developing p53-targeted therapies (13, 34). To ensure that this is not a purely *in vitro* effect (*in vitro* immunization), healthy donor controls were included for comparison. The levels of p53-reactive T cells were generally lower than the responses seen in patients from the low-dose cohort (Fig. 2B). Vaccine-induced responses were of greater magnitude in the high-dose cohort, particularly with regard to CD8⁺ responses. Of note, 7 of 9 patients in the high-dose cohort showed increased p53-reactive CD4⁺ T cells after vaccination, the median fold increase being 3.5 (Fig. 3B, left). All the high-dose patients

showed increased p53-reactive CD8⁺ T cells after immunization, with a median fold increase of 5 (Fig. 3B, right). Our *in vitro* stimulation method using endogenously expressed p53 protein may have favored the expansion of CD8⁺ T cells in our cultures before analysis. However, it has been demonstrated that endogenously processed antigens can enter the MHCII pathway and prime CD4⁺ responses (35). The lower CD4⁺ T-cell response to the p53 peptide library could have clinical implications, because CD4⁺ T cells play a critical role in sustaining the antitumor action of CTL (36). Hence, providing a greater helper signal by combining the vaccine with IL2 or immunologic adjuvants could further expand the CD8⁺ T-cell response. It is possible that Tregs could be expanded during the *in vitro* stimulation and we will address this in future studies.

The enhancement of p53 response did not show continued expansion with successive immunizations in the

majority of patients. However, in the small number of high-dose patients who provided an evaluable fourth blood draw, 2 out of 4 still showed a higher frequency of p53-reactive CD8⁺ T cells than before immunization. It is possible that primed p53-reactive T cells may have moved out of the periphery into tissues, and hence not been accessible in peripheral blood draws. It would be interesting to examine the tumor-infiltrating lymphocyte (TIL) population to test this hypothesis.

Immunodominant viral epitopes present in vector backbones may hamper the priming of responses against transgene encoded epitopes (27). Despite the notable immunogenicity of the MVA backbone (Fig. 4), anti-p53 T-cell responses were detectable after immunization with p53MVA. However, expansion of responses against immunodominant viral antigens could be minimized by a prime boost strategy, delivering alternate doses of p53 with different viral vectors (37).

Chronic antigen stimulation causes upregulation of the "exhaustion marker" PD1 on T cells, including TILs (38, 39), and has been associated with disease progression (40) and poor prognosis (41). The primary ligand, PDL1, has been detected on tumor cells, hence the PD1-PDL1 pathway can hamper antitumor immune responses (42). In addition, blood DC express elevated levels of PDL1 in patients with cancer (43). This could reduce the effectiveness of viral-based vaccines, which require uptake and expression within DC. Inhibition of the PD1-PDL1 pathway has been shown to enhance antitumor responses both *in vitro* (44) and murine models (45). Furthermore, antibodies targeting PD1 or PDL1 exhibited impressive clinical activity in patients with a variety of solid tumors, including lung cancers. Both antibodies were able to induce tumor regression or stable disease, with objective response rates of 18% to 27% for α -PD1 and 6% to 17% for α -PDL1 being reported. (3, 46, 47).

We have conducted *in vivo* studies using p53MVA combined with anti-PD1 antibody in an orthotopic murine model of pancreatic cancer and observed impressive tumor rejection (data not shown). Correspondingly, significantly higher frequencies of PD1⁺ T cells were detected in our trial participants compared with healthy donors (Fig. 5A and B). Furthermore, lower frequencies of prevaccine PD1⁺ CD8⁺ correlated with higher CD8⁺ p53-reactive T cells ($P = 0.02$). In light of these observations and recent clinical reports, we hypothesized that PD1 blockade would enhance the vaccine induced, anti-p53 T-cell responses. *In vitro* expansions conducted in the presence of α PD1 showed that significant enhancement of vaccine-induced responses by PD1 antibody was indeed achievable (Fig. 5D). Amplification of antigen-specific responses by PD1 antibodies *in vitro* have been previously reported (44, 48), but to our knowledge,

this is the first report of enhanced anti-p53 T-cell responses due to PD1 blockade. The combination of viral-based vaccines and chemotherapy has also shown promise in patients with gastrointestinal cancer (18, 49), which raises another possible combination for p53MVA vaccine therapy.

In conclusion, it is likely that the high levels of immunosuppression in this patient group were a barrier to achieving objective clinical responses from p53MVA immunization. However, the tolerability profile and the ability to elevate the p53-specific CD8⁺ T-cell response, support the continued development of p53MVA. Furthermore, the combination of p53MVA with additional agents, such as antibody-based immune checkpoint inhibitors, warrants investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (acquired and managed patients, provided facilities, etc.): N.R. Hardwick, M. Carroll, D. Lim, L. Leong, P. Chu, J. Kim, J. Chao, D.J. Diamond, V. Chung

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.R. Hardwick, D. Qian, L. Leong, P. Chu, J. Kim, J.D.I. Ellenhorn, D.J. Diamond, V. Chung

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p53MVA Therapy in Patients with Refractory Gastrointestinal Malignancies Elevates p53-Specific CD8⁺ T-cell Responses

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