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ORIGINAL REPORT

Safety and Immunological Efficacy of a DNA Vaccine Encoding Prostatic Acid Phosphatase in Patients With Stage D0 Prostate Cancer

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
Prostatic acid phosphatase (PAP) is a prostate tumor antigen. We have previously demonstrated that a DNA vaccine encoding PAP can elicit antigen-specific CD8+ T cells in rodents. We report here the results of a phase I/IIa trial conducted with a DNA vaccine encoding human PAP in patients with stage D0 prostate cancer.

Patients and Methods
Twenty-two patients were treated in a dose-escalation trial with 100 μg, 500 μg, or 1,500 μg plasmid DNA, coadministered intradermally with 200 μg granulocyte-macrophage colony-stimulating factor as a vaccine adjuvant, six times at 14-day intervals. All patients were observed for 1 year after treatment.

Results
No significant adverse events were observed. Three (14%) of 22 patients developed PAP-specific IFNγ-secreting CD8+ T-cells immediately after the treatment course, as determined by enzyme-linked immunospot. Nine (41%) of 22 patients developed PAP-specific CD4+ and/or CD8+ T-cell proliferation. Antibody responses to PAP were not detected. Overall, the prostate-specific antigen (PSA) doubling time was observed to increase from a median 6.5 months pretreatment to 8.5 months on-treatment ($P = .033$), and 9.3 months in the 1-year post-treatment period ($P = .054$).

Conclusion
The demonstration that a DNA vaccine encoding PAP is safe, elicits an antigen-specific T-cell response, and may be associated with an increased PSA doubling time suggests that a multi-institutional phase II trial designed to evaluate clinical efficacy is warranted.

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INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy, and the second leading cause of cancer-related death among men, in the United States.[1] Approximately one third of patients will have recurrent disease after definitive surgery or radiation therapy. The first evidence of recurrence is usually an increase in the serum prostate-specific antigen (PSA) blood test, so-called stage D0 disease. In several retrospective analyses, it has been demonstrated that the rate of increase, or PSA doubling time (DT), is prognostic in terms of the time to radiographic evidence of metastases and death.[2-6] At present there is no standard treatment for patients with biochemical recurrence in the absence of radiographically apparent metastases. While androgen deprivation is commonly used, it is not advocated in all circumstances due to potential adverse consequences of long-term treatment. Thus, there is a need to identify therapies that could delay or abrogate the progression of prostate cancer in this early stage.

Active immunotherapies, or antitumor vaccines, are appealing as potential treatments to eradicate micrometastatic disease.[7,8] In principle, a prostate tissue-specific destructive immune response elicited after initial prostatectomy or ablative radiation therapy could eradicate residual microscopic disease. Prostatic acid phosphatase (PAP) is one potential immunotherapy target antigen given that it is a protein whose expression is essentially restricted to normal and malignant prostate tissue.[9] It is also one of only a few known prostate-specific proteins for which there is a rodent homolog, thereby providing preclinical animal models.[10] We

and others have demonstrated in rats that genetic vaccines targeting PAP can result in antigen-specific CD8+ T cells.[11,12] An autologous antigen-presenting cell vaccine loaded ex vivo with a PAP-derivative protein (Provenge, Dendreon Corporation, Seattle, WA) has been demonstrated in a placebo-controlled randomized phase III trial to have possible therapeutic benefit as evidenced by an observed prolonged overall survival in patients with castrate-resistant metastatic prostate cancer.[13] The use of autologous cell-based vaccines is labor-intensive and costly; the development of simpler vaccines targeting this and other relevant antigens could provide a significant advance in the treatment of prostate cancer. Moreover, the best application of immunotherapies may be in the setting of minimal residual disease.[14,15]

We have previously demonstrated that a DNA vaccine encoding PAP was safe and effective in eliciting PAP-specific CD8+ T cells in rats.[12,16] In the current phase I/IIa clinical trial, we hypothesized that patients with stage D0 prostate cancer could be similarly safely immunized with a DNA vaccine encoding PAP, and that immunization would elicit PAP-specific interferon gamma (IFNγ) –secreting CD8+ T cells as evidence of a potentially therapeutic immune response.

PATIENTS AND METHODS

Study Agent and Regulatory Information

pTVG-HP is a plasmid DNA encoding the full-length human PAP cDNA downstream of a eukaryotic promoter.[16] The study protocol was reviewed and approved by all local, sponsor (Human Subjects Review Board of the US Army), and federal (US Food and Drug Administration, National Institutes of Health Recombinant DNA Advisory Committee) entities. All patients gave written informed consent for participation.

Patient Population

Male patients with a histological diagnosis of adenocarcinoma of the prostate and biochemical (serum PSA) recurrence after definitive surgery and/or radiation therapy were eligible, provided there was no evidence of suspected lymph node, bone, or visceral metastatic disease on bone scans or computed tomography scans. Inclusion criteria required that patients have a Karnofsky performance score of ≥ 70, and normal bone marrow, liver, and renal function as defined by a WBC $\geq 3{,}000/\mu L$, hematocrit $\geq 30\%$, platelet count $\geq 100{,}000/\mu L$, total bilirubin ≤ 2.0 mg/dL, and serum creatinine ≤ 1.5 mg/dL or a creatinine clearance ≥ 60 mL/min. Patients were excluded if they had been treated with immunosuppressive therapy, including chemotherapy, corticosteroids, or extensive radiation therapy, within 6 months of study entry, or were on medications with possible anticancer effects.

Study Design

This study was an open-label, single institution, trial using a dose escalation schedule with sequential cohorts receiving increasing doses of pTVG-HP plasmid DNA (level 1 to 100 μg, level 2 to 500 μg, level 3 1,500 μg). A dose-limiting toxicity (DLT) was defined as any adverse event higher than grade 2 during the entire 3-month period of treatment with an attribution of at least possibly related to agent. The maximum tolerated dose (MTD) was defined as the dose level preceding a level at which more than one DLT was observed, or the 1,500 μg dose level in the absence of DLTs. Sixteen total patients were then enrolled at the MTD level for further evaluation. This sample size was chosen to detect an anticipated increase in the immunological response rate from 10% to 40% with 93% power at the one-sided 10% significance level.

Study Procedures

Patients were treated six times at 14-day intervals with pTVG-HP plasmid co-administered with 200 μg granulocyte-macrophage colony-stimulating factor (Leukine, sargramostim). Vaccinations were performed intradermally with a 28-gauge needle on the lateral arm in two to three divided administrations. Patients underwent a leukapheresis procedure within 2 weeks of the first immunization and 2 weeks after the last immunization. Patients also received a tetanus immunization immediately after the baseline leukapheresis. Blood tests were performed monthly and included CBC, creatinine, glucose, bilirubin, AST, alkaline phosphatase, amylase, lactate dehydrogenase, and antinuclear antibodies. All toxicities were graded according to the National Cancer Institute Common Toxicity Criteria grading system, version 3.

Clinical Response Evaluation

PSA values were collected monthly from all individuals, and at a minimum of 3-month intervals for 1 year after the final leukapheresis. PSA values were available from all patients before enrollment. PSA DT were calculated using all serum PSA values available from the same clinical laboratory for the specified period, and using a minimum of four PSA values by the formula log(2)/b, where b denotes the least square estimator of the linear regression model of the log-transformed PSA values on time. For the pretreatment PSA DT, a period of 4 to 6 months was used before treatment, up to and including day 1 of treatment. The on-treatment PSA DT was determined using the PSA values from day 1 to the final leukapheresis. The post-treatment PSA DT was determined using all PSA values from the final leukapheresis until 12 months after treatment, or until the patient began another treatment.

Immunological Response Evaluation

Autologous antigen-presenting cells were prepared by culturing tissue flask-adherent peripheral blood mononuclear cells (PBMC) in the presence of 20 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor and 10 ng/mL recombinant human interleukin-4 for 6 days in X-VIVO medium (Cambrex Corporation, East Rutherford, NJ). CD8+ T cells were sorted from cryopreserved PBMC and cultured with 10^4 autologous dendritic cells in the presence of media only (no antigen), 2 μg/mL PAP protein (Research Diagnostics Inc, Concord, MA), 2 μg/mL PSA protein, 100 ng/mL tetanus toxoid, or 2.5 μg/mL phytohemaglutinin (positive mitogenic control) for 48 hours. Cells were then transferred to enzyme-linked immunospot (ELISPOT) plates previously coated with anti-IFNγ capture antibody, cultured for an additional 48 hours, and spots developed using standard methods. IFNγ spots per well were counted by automated ELISPOT reader. Comparison of experimental wells with control no antigen wells, and with pretreatment experimental wells, was performed using a two-sided t-test, with $P < .05$ used to define a significant response. The Welch approximation was used when group variances were heterogeneous. Assays were conducted in batch fashion for individual subjects, using the same lots of protein antigens for all subjects to control for assay variability.

T-cell proliferation in response to antigen stimulation was determined by a 5-day bromodeoxyuridine (BrdU) incorporation assay, with flow cytometric evaluation of antigen-specific BrdU incorporation in T-cell populations, similar to that previously described.[16] These studies were conducted with fresh PBMC obtained at the time of leukapheresis. To best control for variation over time, the same lots of sera and antigens were used for the entire study. Proliferation index was defined as the % of BrdU+ events for each antigen-stimulated condition compared with the media only control. BrdU+ events were assessed at each time point by gating 0.05% events in the no antigen control group, and applying that gating to all antigen-stimulated conditions.

Statistical Analysis

Categoric data were summarized as proportions and percentages. Continuous data were summarized and reported as medians and ranges. The Wilson score method was used to construct the 95% CI for the immunological response rate. PSA DT was estimated for each patient using a linear regression of the log-transformed PSA values on time, assuming an exponential growth model. For analysis purposes, negative PSA DT estimates and high positive PSA DT estimates (> 36 months) were censored at 36 months. The comparisons of PSA DT between periods (pretreatment v on-treatment v follow-up) were performed using a two-sided, nonparametric Wilcoxon signed rank test. Nonparametric Spearman's rank correlation analysis was used to evaluate the association between pTVG-HP

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plasmid DNA dose and changes in PSA DT. A two-sided significance level of $P < .05$ was used for all tests and comparisons.

RESULTS

Patient Population and Course of Study

Twenty-two patients were enrolled in this trial between April 2005 and May 2007 at the University of Wisconsin Paul P. Carbone Comprehensive Cancer Center (Table 1). The median age of participants was 63 years (range, 44 to 74 years). Patients had been off any prior therapy for a median of 38 months (range, 1 to 106 months). Overall, the median PSA DT before treatment for all patients was 6.5 months (range, 2.5 to 30.3 months).

No adverse events higher than grade 2 were observed during the dose escalation portion of the trial, consequently nine patients (three patients per dose level) were accrued. Thirteen additional patients were accrued at the 1,500 μg dose in the extension cohort. One grade 3 serum amylase was observed in one patient at the time of the fifth immunization, resulting in a 1-week dosing delay. Apart from this, there were no dosing delays, and all patients underwent all study immunizations and leukaphereses.

Adverse Events

As demonstrated in Table 2, no significant adverse events were observed. As described, a single grade 3 amylase level was observed in one patient. In the absence of recurrence with two further vaccinations in that individual, this was not believed to be related to treatment. No other laboratory abnormalities were observed. The major events noted were low-grade skin site reactions and constitutional symptoms (fatigue, myalgias, arthralgias, and chills).

Table 1. Demographics for All Patients Enrolled (N = 22)

Parameter	No.	%
Median age, years	63	
Range	44-74	
Race/ethnicity		
White	21	95
African American	1	5
Prior treatment		
Prostatectomy	14	64
Radiation therapy*	16	73
Androgen deprivation	7	32
Chemotherapy	1	5
Gleason score		
≤ 6	9	41
7	7	32
≥ 8	6	27
Baseline PSA, ng/mL		
Median	7.7	
Range	2.1-18.3	
2.0-5.0	5	23
5.0-10.0	13	59
> 10	4	18
Baseline PAP, ng/mL		
Median	1.7	
Range	0.6-3.9	
< 2.0	14	64
≥ 2.0	8	36

Abbreviations: PSA, prostate-specific antigen; PAP, prostatic acid phosphatase.
*Eight of 22 subjects received primary radiation therapy; eight of 22 received radiation therapy after prostatectomy.

Table 2. Adverse Events

Adverse Event	Grade 1 No.	Grade 1 %	Grade 2 No.	Grade 2 %
Injection site reactions				
Redness, induration, pruritis, pain	22	100		
Constitutional symptoms				
Fatigue	9	41	1	5
Nausea	4	18		
Chills	2	9		
Headache	3	14		
Dyspnea	2	9		
Light headed/dizziness	3	14		
Malaise	1	5		
Musculoskeletal				
Pain, back spasm	2	9	2	9
Arthralgias	3	14	1	5
Myalgias	2	9	1	5
GI				
Constipation	1	5		
Diarrhea	3	14		
Cardiovascular				
Tachycardia	1	5		
Edema	1	5		

NOTE. All adverse events by grade that were believed to be at least possibly related to treatment are shown. The numbers represent the No. of patients (of 22) experiencing a particular event at any point during the 3-month treatment period, with the highest grade reported for any single individual. No grade 3 or grade 4 events observed related to treatment.

Immunological Response

The primary immunological end point of the trial was the induction of PAP-specific IFNγ-secreting CD8+ T cells. ELISPOT was used as the primary readout using CD8-purified T cells without in vitro stimulation. Three (13.6%) of 22 patients (95% CI, 4.7% to 33.3%) had evidence of a response to PAP (Fig 1A), one in each treatment dose cohort. Two (9%) of 22 patients had a CD8+ IFNγ-secreting response to tetanus toxoid by the same analysis (Fig 1B). Antigen-specific T-cell proliferation was also evaluated pre- and postimmunization. An example of the methodology used is shown in Figure 2, and a summary of T-cell proliferative responses to PAP and tetanus toxoid is demonstrated in Figure 3. Six (27.3%) of 22 patients (95% CI, 13.1% to 48.2%) developed at least a three-fold increase in PAP-specific CD4+ proliferative T-cells (Fig 3A), and three (13.6%) of 22 patients (95% CI, 4.7% to 33.3%) developed at least a three-fold increase in CD8+ proliferative T-cells (Fig 3B). Tetanus-specific T-cell responses were identified at baseline in several individuals, but increases in tetanus-specific CD4+ and CD8+ cells were detectable in only two of 22 patients and zero of 22 patients, respectively (Fig 3C and 3D). Antibody responses to tetanus were augmented after immunization in 21 of 22 patients, however antibody responses to PAP were not detected (data not shown).

Clinical Response

There were no complete PSA responses, and no PSA values that declined by ≥ 50%.[17] However, several patients were observed

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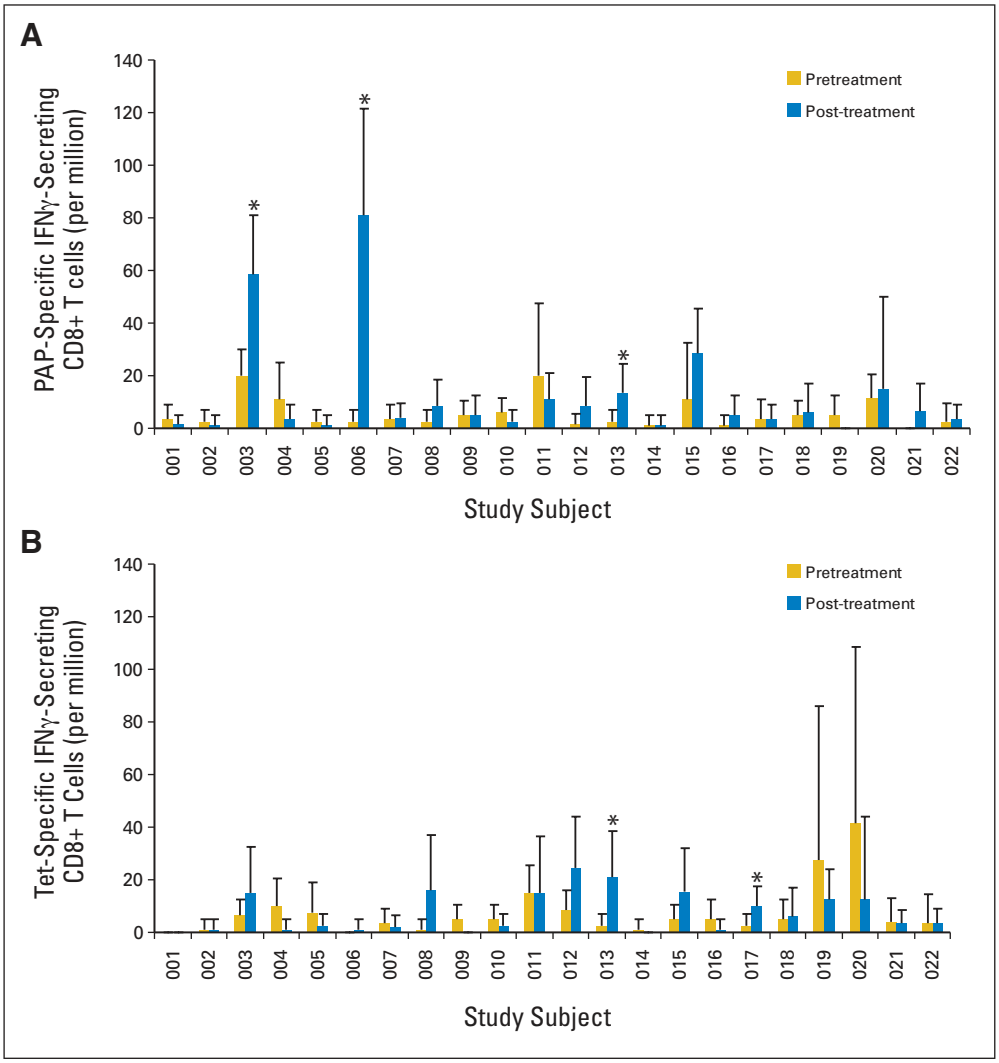

Fig 1. Immunological response. CD8+ interferon gamma (IFNγ) enzyme-linked immunospot (ELISPOT): CD8+ T cells purified from peripheral blood mononuclear cells pretreatment or post-treatment were cocultured with (A) prostatic acid phosphatase (PAP) or (B) tetanus toxoid (Tet) and autologous antigen-presenting cells, and assessed for IFNγ secretion by ELISPOT. (*) Significant responses ($P < .05$, two-sided *t*-test).

to have a decrease in the rate of serum PSA rise after treatment (Fig 4A). PSA DTs were calculated for all patients for a 4-month period of time pretreatment, for the 4-month on-treatment time, and for a 12-month period post-treatment (or until beginning a new therapy; Fig 4B). Seven of 22 patients experienced at least a doubling of the PSA DT. Overall, the median PSA DT pretreatment was 6.5 months (range, 2.5 to 30.3 months), 8.5 months on-treatment (range, 2.6 to 36.0+ months), and 9.3 months post-treatment (range, 2.3 to 36.0+ months). The median increase in PSA DT from the pretreatment to the on-treatment period was 1.3 months (range, −4.4 to 24.0 months; $P = .033$; Fig 4C). The median increase in PSA DT from the pretreatment to the post-treatment follow-up period was 1.0 months ($P = .054$). There was no significant change in PSA DT from the on-treatment to the post-treatment period (median, 0.0 months; $P = .896$). Two patients (2 and 5) were observed to have decreasing PSA values while on treatment, resulting in a negative PSA DT estimate, and an additional patient (14) was observed to have a negative PSA DT estimate in the year post-treatment. There was no significant correlation between pTVG-HP plasmid DNA dose and change in PSA DT from the pre- to the on-treatment ($r_s = -0.29$; $P = .19$) or follow-up ($r_s = -0.23$; $P = .31$) periods.

Nine of 22 patients went on to receive other treatments during the 1 year of follow-up. Of these, two patients developed seminal vesicle recurrences and received radiation therapy. One patient developed lymph node metastases and was started on androgen deprivation. None of the other patients had known radiographic evidence of disease recurrence after 1 year of follow-up.

DISCUSSION

Rising serum PSA after definitive surgery or radiation therapy for prostate cancer typically signifies persistent disease that ultimately will become radiographically detectable. While androgen deprivation is commonly used in this situation, the optimal timing for initiating treatment remains unknown, and no therapies have proven benefit. We report here the results of a phase I/IIa trial evaluating the safety and immunological efficacy of a DNA vaccine encoding PAP in patients with stage D0 prostate cancer. This treatment was not associated with significant adverse events; as anticipated, patients experienced local injection site reactions, and a few experienced low-grade constitutional symptoms such as fever, chills, myalgias, and arthralgias lasting

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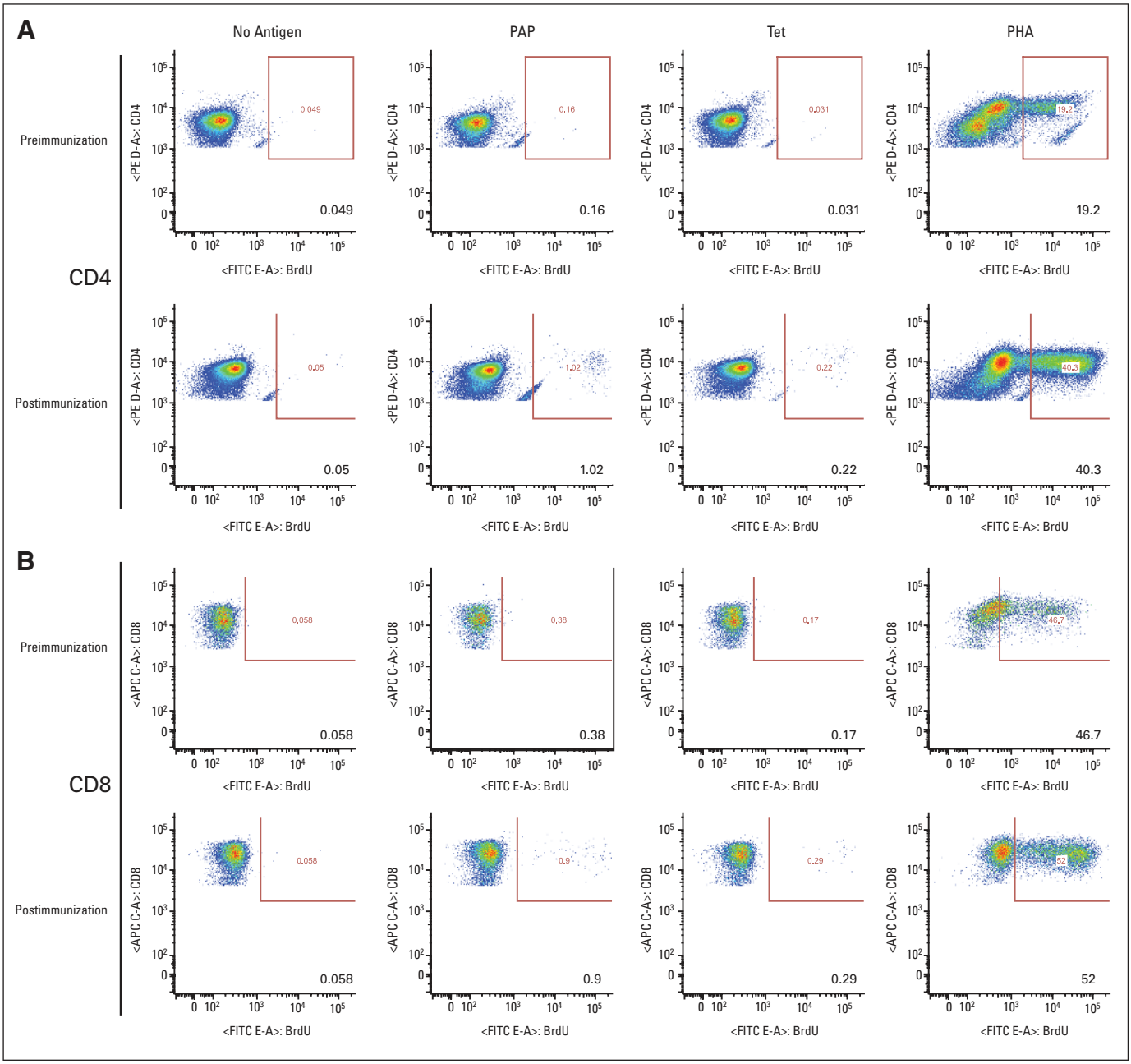

Fig 2. Antigen-specific T-cell proliferation. Peripheral blood mononuclear cells from patient 6 obtained preimmunization or postimmunization were cultured in the presence of 2 μg/mL prostatic acid phosphatase (PAP), 250 ng/mL tetanus toxoid (Tet), 2.5 μg/mL phytohemaglutinin (PHA), or media only (no antigen) for 96 hours. Ten μmol/L bromodeoxyuridine (BrdU) was added for the last 8 hours of culture before flow cytometric analysis. (A) CD4+ or (B) CD8+ T cells costaining for BrdU. The *x*-axis in each graph shows BrdU staining and the numbers represent the percentage of BrdU+ events among CD4+ or CD8+ cells. FITC, fluorescein isothiocyanate.

up to 24 hours. Several patients developed T-cell immune responses specific for PAP, and CD8+ T-cell responses in particular, after six immunizations. In addition, several patients were observed to have an increase in PSA DT after treatment.

The evaluation of T-cell immune responses to target self antigens after vaccine clinical trials presents several challenges. Antigen-specific T cells can be evaluated by their peptide target specificity (eg, tetramer staining), proliferative capacity, cytokine secretion, cytolytic activity, and membrane markers of activation. At present the best measure of antigen-specific T cells is not known, and the optimal time to evaluate immune responses is not known. In most analyses reported from previous antitumor vaccine trials in vitro stimulation has been used to augment the number of antigen-specific cells from peripheral blood samples to detectable ranges. ELISPOT has become a preferred methodology for clinical trials, given its sensitivity and reproducibility even with cryopreserved cells.[18,19] In our current analysis, we wished to evaluate only antigen-specific Th1-biased CD8+ T-cell responses, and used IFNγ ELISPOT without in vitro stimulation to provide a more direct quantitative assessment after immunization. Thus, while we

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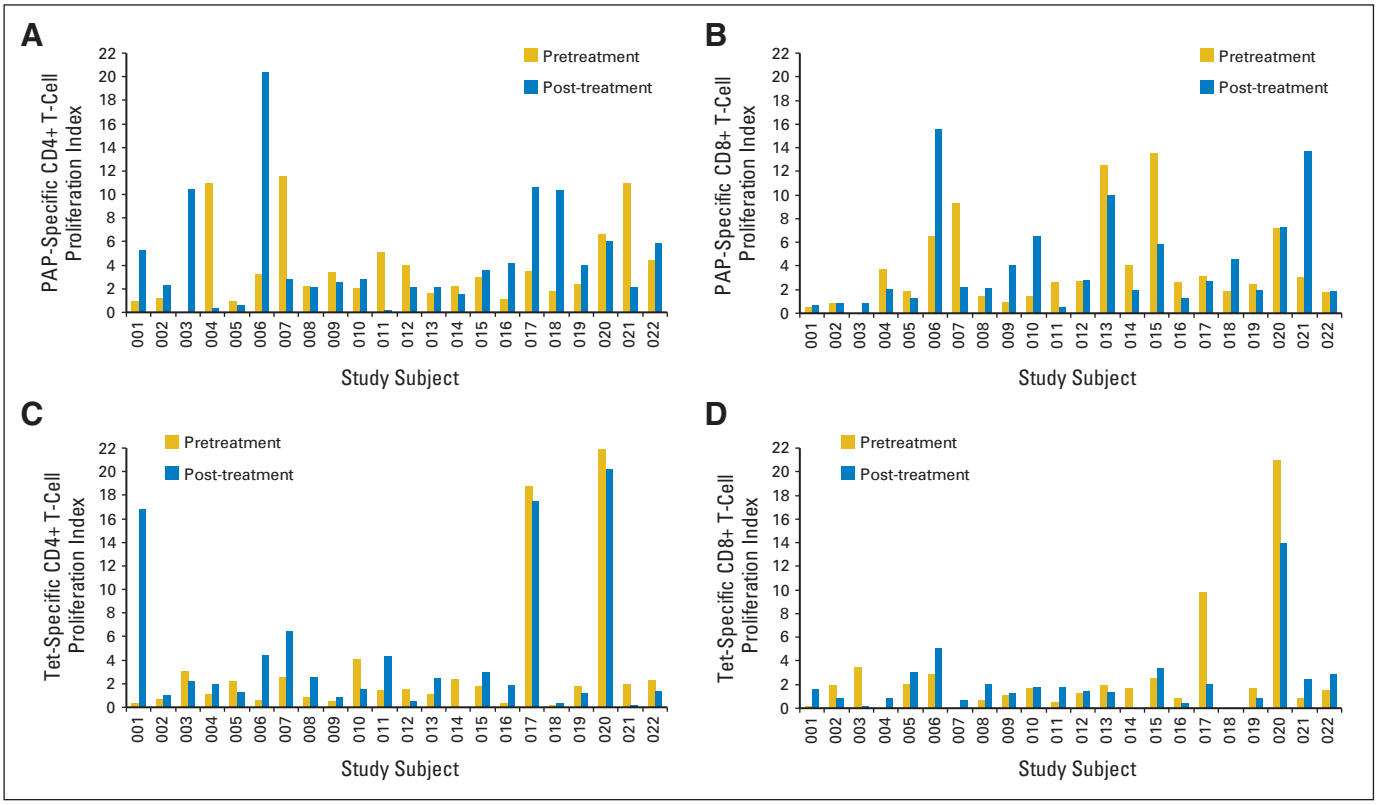

Fig 3. Immunological response: antigen-specific T-cell proliferation. Assessment of antigen-specific T-cell proliferation was conducted as in Figure 2. Shown are the proliferation indices (% bromodeoxyuridine [BrdU] + events under antigen-stimulation condition/% BrdU+ events for media only) pre- and post-treatment for (A, B) prostatic acid phosphatase (PAP) and (C, D) tetanus (Tet). (A, C) CD4+ T-cell proliferation and (B, D) CD8+ T-cell proliferation.

detected responses, these were of low level. Given the multiple manipulations to detect these cells, our method likely underestimated the true number of antigen-specific IFNγ-secreting CD8+ T cells. In fact, we detected few responses to tetanus toxoid, our intended positive control antigen. T-cell proliferation following coculture with antigen was also used to detect antigen-specific T cells, and by this method we detected several additional patients with immune responses to PAP, some present at baseline, and others clearly elicited or augmented after immunization. This method, while not strictly quantitative, further suggested that the DNA vaccine had biologic activity in eliciting a PAP-specific T-cell response. Given the small number of patients treated, it is difficult to determine whether an association exists between the development of an immune response and clinical response. A priori, we did not detect a significant association between either ELISPOT response or T-cell proliferative response and increase in PSA DT. We are, however, currently conducting further immunological analyses at multiple time points to determine whether other markers of T-cell immune response, pre-existing immune responses to PAP,[20] or the development of late immune responses, are associated with changes in PSA DT.

The current treatment schedule, with six immunizations delivered at 2-week intervals, was designed based on previous rodent studies.[12] In an MHC diverse human population, however, the optimal number and schedule of immunizations remains unknown for most vaccines. While not unexpected, we did not detect immune responses in all individuals. It is possible that some patients were entirely tolerant to the PAP antigen, however it is also possible that ongoing immunization could be useful to augment a T-cell response to this autologous antigen. Other immunomodulatory strategies or adjuvants could also potentially be combined with DNA vaccines in the future to further augment T-cell responses. The observation that PSA DT increased in some individuals during the period on treatment, but decreased in the post-treatment period, further suggests that in some individuals there might be a benefit to continued vaccination. At present, however, it should be highlighted that the clinical significance of changes in PSA DT from any treatment remains unknown and awaits the results of prospective randomized trials to determine whether changes are associated with changes in radiographic disease progression. Future clinical trials with pTVG-HP will evaluate other schedules of vaccination continuing beyond six initial immunizations, or with periodic booster immunizations, and with frequent immune monitoring. In any case, the demonstration of a PAP-specific CD8+ T-cell response being elicited serves as evidence that a relatively simple method of repetitive DNA vaccine administration can elicit CD8+ T cell responses to autologous antigens in the absence of heterologous immunization strategies or manipulated cell products. The safety, feasibility, and immunological efficacy of this approach suggest that other antigens could be evaluated by means of DNA vaccines.[21,22]

In summary, our results demonstrate that antigen-specific T cells were elicited after vaccination of patients with biochemical recurrence of prostate cancer using a DNA vaccine encoding PAP. No significant adverse events were observed, and several patients had an increased PSA DT after immunization. These findings demonstrate biologic

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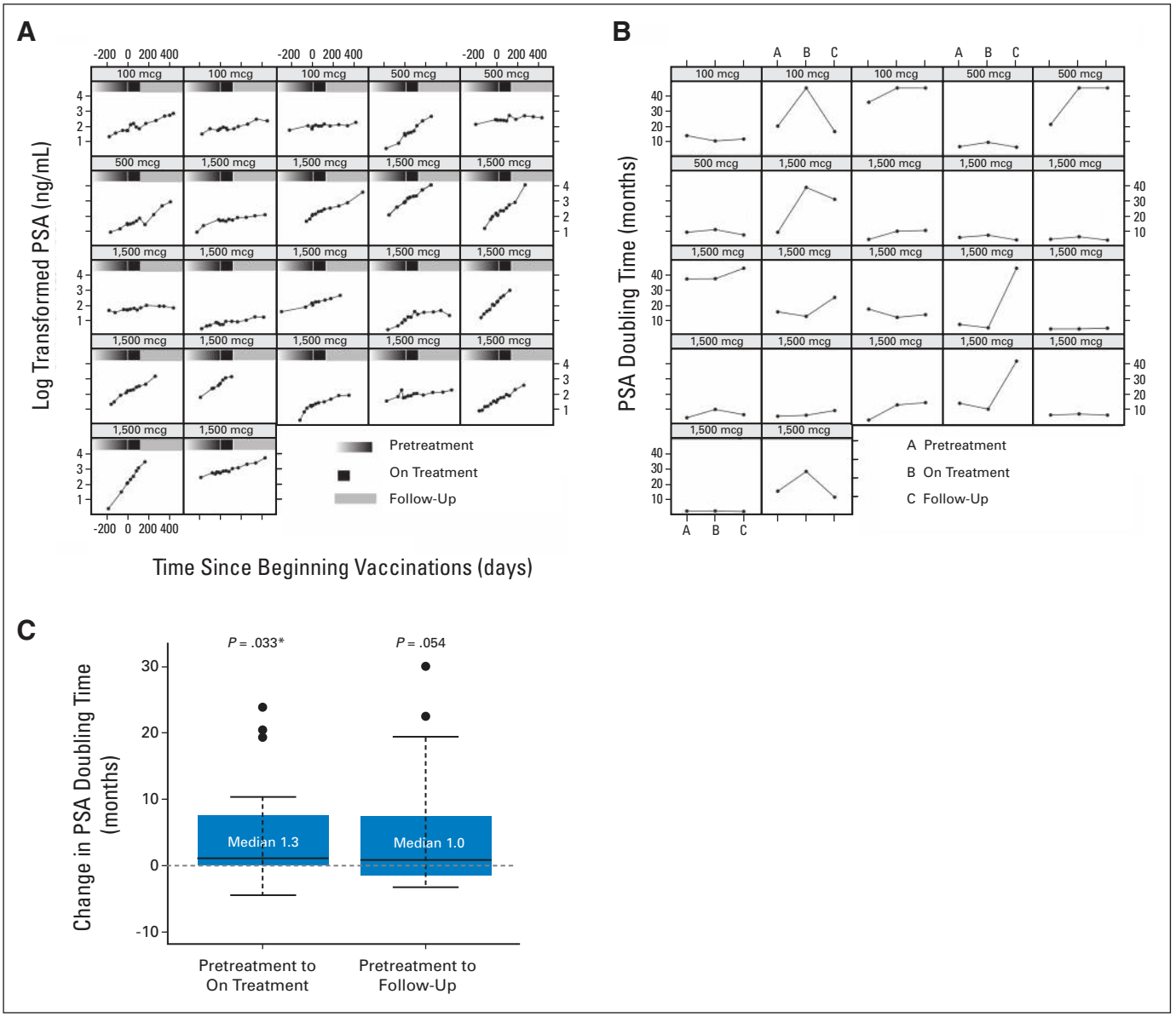

Fig 4. Prostate-specific antigen (PSA) responses. (A) Log-transformed serum PSA values from all patients obtained over time with respect to beginning the vaccination series. (B) PSA doubling time (DT) values were calculated for all patients pretreatment, on-treatment, and post-treatment. Negative and high positive (> 36 months) PSA DT estimates were censored at 36 months. (C) Boxplots for distributions of changes in PSA DT from the pretreatment to the on-treatment period and from the pretreatment to the post-treatment follow-up period are shown. The horizontal bold line shows the median while the box shows the 25th and the 75th percentiles of the changes in PSA DT values.

activity, suggest safety, and suggest possible effects on tumor growth rates. These observations suggest that further investigation in a randomized phase II clinical trial, where clinical benefit in terms of evaluating time to disease progression, is warranted.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Glossary Terms

IFN-γ (interferon gamma): Cytokine that is produced by activated T cells and natural killer cells, its primary action is the activation of macrophages.

ELISpot: Enzyme-linked immunospot that is exquisitely sensitive to assay minute amounts of mediators that are produced by cells. Typically, cells are deposited on a membrane coated with an antibody specific for a given protein. The protein of interest is captured directly around the secreting cell and is detected with an antibody specific for a different epitope. Coupled with colorimetry, the cells are visualized by specialized plate readers. Thus, the molecule is assayed before it is diluted in the supernatant, captured by receptors of adjacent cells, or degraded.

Active immunotherapy: Induction of an immune response in the host, typically to a particular antigen or set of antigens. This is commonly by means of a vaccine, and is in contrast to a "passive immunotherapy" in which cells, antibodies, or cytokines of the immune systems are passively infused into the host.

PAP (prostatic acid phosphatase): A secretory protein produced by cells of the prostate gland.

Antigen: A substance that promotes, or is the target of, an immune response.

PSA doubling time: The calculated time for the serum PSA of a patient to double in value.

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