Real-Time Immune Monitoring to Guide Plasmid DNA Vaccination Schedule Targeting Prostatic Acid Phosphatase in Patients with Castration-Resistant Prostate Cancer

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Abstract

**Purpose:** We have previously reported that a DNA vaccine encoding prostatic acid phosphatase (PAP) could elicit PAP-specific T cells in patients with early recurrent prostate cancer. In the current pilot trial, we sought to evaluate whether prolonged immunization with regular booster immunizations, or "personalized" schedules of immunization determined using real-time immune monitoring, could elicit persistent, antigen-specific T cells, and whether treatment was associated with changes in PSA doubling time (PSA DT).

**Experimental Design:** Sixteen patients with castration-resistant, nonmetastatic prostate cancer received six immunizations at 2-week intervals and then either quarterly (arm 1) or as determined by multiparameter immune monitoring (arm 2).

**Results:** Patients were on study a median of 16 months; four received 24 vaccinations. Only one event associated with treatment was observed. Six of 16 (38%) remained metastasis-free at 2 years. PAP-specific T cells were elicited in 12 of 16 (75%), predominantly of a Th1 phenotype, which persisted in frequency and phenotype for at least 1 year. IFNγ-secreting T-cell responses measured by ELISPOT were detectable in 5 of 13 individuals at 1 year, and this was not statistically different between study arms. The overall median fold change in PSA DT from pretreatment to posttreatment was 1.6 (range, 0.6–7.0; \(P = 0.036\)).

**Conclusions:** Repetitive immunization with a plasmid DNA vaccine was safe and elicited Th1-biased antigen-specific T cells that persisted over time. Modifications in the immunization schedule based on real-time immune monitoring did not increase the frequency of patients developing effector and memory T-cell responses with this DNA vaccine. *Clin Cancer Res; 20(14); 3692–704. ©2014 AACR.*

Introduction

Despite several new agents recently approved for the treatment of advanced prostate cancer, this remains the most commonly diagnosed malignancy in the United States and the second leading cause of male cancer-related death (1). Approximately one third of patients will have recurrent disease after definitive surgery/radiotherapy, the first evidence of which is usually an increase in the serum PSA blood test. At present, there is no standard treatment for patients with biochemical recurrence in the absence of radiographically apparent metastases, although androgen deprivation is commonly used. In these individuals, ultimately the disease recurs, heralded again by an increase in serum PSA before the tumor becomes radiographically detectable. No therapies have been approved for this castration-resistant "biochemically-relapsed" stage of disease on the basis of clinical benefit, and surveillance is generally recommended until metastases are identified. The baseline PSA, and rate of PSA change (doubling time, PSA DT), were found to be independently predictive of time to metastasis (which occurs with a median time of 30 months; ref. 2) and overall survival (3). Thus, this nonmetastatic castration-resistant prostate cancer (nmCRPC) represents a stage for which there is not a standard therapy, yet one for which prognostic criteria are available. Moreover, the relatively long period of time during which patients generally have no disease-related symptoms provides an opportunity to evaluate novel therapies, including antitumor vaccines, which may exert effects over many months.

*Sipuleucel-T* was approved by the FDA for the treatment of patients with mCRPC based on an improvement in overall survival compared with placebo (4). This landmark event represents the first FDA approval of an antitumor vaccine for the treatment of existing cancer (4, 5). These
Translational Relevance

We previously showed that a DNA vaccine targeting prostatic acid phosphatase (PAP) can elicit antigen-specific T-cell immune responses in patients with early recurrent prostate cancer, and these responses were associated with favorable changes in PSA doubling time (DT). In this pilot clinical trial, we evaluated whether repetitive immunization up to 2 years could elicit durable effector and memory T-cell immune responses, and whether the frequency of these immune responses could be augmented using "personalized" immunization schedules determined by multiparameter real-time immune monitoring, compared with an empirically fixed schedule of immunization. Repetitive immunizations elicited Th1-biased immune responses in 12 of 16 (75%) patients, and these remained Th1-biased even after multiple immunizations. Individualized schedules of vaccination determined by immune monitoring did not appear advantageous as measured by either the development of Th1-biased immune responses or changes in PSA DT.

findings demonstrate that antitumor vaccines can have activity and might be investigated in earlier stages of prostate cancer. Moreover, the specific findings with sipuleucel-T suggest that the target of this vaccine, prostatic acid phosphatase (PAP), is a rational vaccine target.

We have similarly been evaluating PAP-targeted vaccines, using plasmid DNA as the means of antigen delivery (6, 7). In a phase I trial in patients with non-castrate, nonmetastatic prostate cancer, we found vaccination 6 times at 2-week intervals was safe and elicited PAP-specific CD4+ and CD8+ T cells with a Th1 phenotype (8). Moreover, PAP-specific T cells could be boosted with subsequent immunization, and the development of durable Th1-biased immune responses (detectable up to 1 year after treatment) appeared to be associated with favorable changes in PSA DT (9). These findings suggested that prolonged schedules of immunization (beyond 6 vaccinations) with the goal of eliciting/maintaining a Th1-biased PAP-specific cellular immune response might be further investigated.

We describe here the results of a randomized, pilot clinical trial (NCT00849121) in patients with nmCRPCs to specifically evaluate different schedules of immunization with a DNA vaccine. The goal of this study was to determine whether ongoing immunization could elicit and maintain Th1-biased memory immune responses over many months, and if immune monitoring conducted in real-time could assist in "personalizing" a schedule of immunization able to maintain tumor antigen-specific effector and memory T cells. Secondary objectives were to determine whether the elicited immune response changed over time with repetitive immunization, and whether immunization was associated with changes in PSA DT. We report that immunization with plasmid DNA encoding PAP elicited Th1-biased PAP-specific T cells in the majority of patients, and responses elicited persisted over many months with ongoing booster immunizations. While a small study, there was no indication that changes to the vaccine schedule by real-time immune monitoring increased the percentage of patients who developed long-term durable immune responses.

Materials and Methods

Study agent and regulatory information

pIVG-HP is a plasmid DNA encoding the full-length human PAP (ACP5 gene) cDNA downstream of a eukaryotic promoter (6). The study protocol was reviewed and approved by all local (University of Wisconsin Human Subjects’ Review Board, Madison, WI) and federal (FDA, NIH Recombinant DNA Advisory Committee) entities. All patients gave written informed consent for participation.

Patient population

Male patients with a histologic diagnosis of prostate adenocarcinoma prostate and PSA recurrence following castration (surgical or ongoing luteinizing hormone-releasing hormone agonist therapy) were eligible, provided there was no evidence of suspected lymph node, bone, or visceral metastatic disease on bone scans or CT scans. All patients had to have been previously treated with an anti-androgen, but with increasing PSA on treatment and persistent increase in PSA after withdrawal. Patients were required to have an Eastern Cooperative Oncology Group performance score of ≤2, and normal bone marrow, liver, and renal function as defined by a WBC ≥3,000/μL, hematocrit ≥30%, platelet count ≥100,000/μL, 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 (chemotherapy, corticosteroids, or extensive radiotherapy) within 6 months of study entry or were on concurrent medications with possible antinecancer effects. Patients were required to have at least 4 serum PSA values, from the same clinical laboratory, over a 3- to 6-month period of time immediately before entry to calculate a pretreatment PSA DT. For eligibility purposes, PSA DT was determined by linear regression, calculated using an online nomogram (http://nomograms.mskcc.org/Prostate/PsaDoublingTime.aspx). Patients were further excluded if they had a history of HIV, hepatitis B, or hepatitis C infection or if they had received a prior antinecancer vaccine.

Study design

This study was an open-label, single-institution, 2-arm pilot trial evaluating different schedules of administration (Fig. 1). Specifically, subjects in arm 1 received immunizations at 2-week intervals for 6 total doses and then every 3 months beginning 2 weeks after the last immunization, for a maximum of 2 years. Subjects in arm 2 received immunizations at 2-week intervals for 6 total doses and then immune monitoring (defined below) was conducted to determine the subsequent schedule; patients without a PAP-specific immune response continued immunizations at 2-week intervals with immune monitoring every month until there...
was evidence of a PAP-specific immune response. Subjects with a detectable immune response decreased the frequency of immunization to a 3-month interval of dosing, with immune monitoring accompanying each immunization. If an immune response was lost with subsequent visits, the schedule was changed to monthly vaccination. If an immune response was again not detectable after 3-monthly vaccinations, the schedule was changed to biweekly. Subjects received a maximum of 24 immunizations or 2 years of treatment, whichever came first. Patients came off-study at the time of detecting metastatic disease (radiographic progression), at any time of undue toxicity, or at the discretion of the patient or treating physician that other therapies for prostate cancer were warranted. Thirty patients were planned for accrual. On the basis of the prior phase I/II study (8), it was anticipated that the immune response rate in arm 1 would be 20% to 40%. The goal was to detect a difference of at least 50% in the immune response rates between the 2 arms. The proposed sample size of 15 patients per arm would have been sufficient to detect a difference of 50% in the immune response rates between arms with at least 80% power at the 2-sided 0.05 significance level.

**Study procedures**

Each immunization consisted of 100 μg pTVG-HP plasmid DNA, co-administered with 200 μg GM-CSF (Sargramostim, Genzyme), and delivered as an intradermal injection with a 28-gauge needle and syringe. Patients underwent a leukapheresis procedure within 2 weeks before the first immunization and at 1 year on trial. Patients also received a tetanus immunization immediately following the baseline leukapheresis. Blood tests were performed every 1 to 3 months and included complete blood count (CBC), creatinine, aspartate aminotransferase, alkaline phosphatase, amylase, lactate dehydrogenase (LDH), serum PAP, and serum PSA. Anti-nuclear antibody (ANA) testing and urinalysis was performed before study entry, at 12 weeks, and at end of study. Serum testosterone was performed at baseline to confirm that patients were functionally castrate (testosterone levels < 50 ng/mL). All toxicities were graded according to the NCI Common Terminology Criteria Grading System, version 3.

**Immunological monitoring**

For each time point, measures of antigen-specific immune response were performed with fresh (not cryopreserved) peripheral blood mononuclear cells (PBMC). Cryopreserved cells from the pretreatment specimen were also assessed at each time point to evaluate the reproducibility of the methods over time and as a benchmark for each time point evaluated. A PAP-specific immune response elicited after immunization (defined below) detected in at least 2 of
these 3 tests defined an immune response used for altering the immunization schedule for patients treated in arm 2 (Fig. 1).

ELISPOT
Studies were conducted as previously described, using monoclonal capture antibodies specific for IFNγ (Thermo Scientific) or granzyme B (GeneTex Inc.; ref. 9). For these analyses, all antigens and sera were used from the same lots to control for possible variation over time. Plates were then washed, developed with biotinylated detection antibodies for either IFNγ (Thermo Scientific) or granzyme B (GeneTex Inc.), and spots enumerated (9). A response resulting from immunization was defined as a PAP-specific response detectable posttreatment that was both significant (by t-test compared with media-only control), at least 3-fold higher than the pretreatment value, and with a frequency > 1:100,000 PBMCs.

T-cell proliferation
PBMCs were labeled in vitro with PKH26 dye (Sigma) and cultured at 2 × 10^5 cells per well in 96-well, round-bottom microtiter plates (Corning) using the same antigen-stimulating conditions as above. After 7 days of culture at 37°C/5% CO₂, cells were stained (CD4-V450, CD8-FITC, CD45RO-APC, CCR7-PECy7) and enumerated by flow cytometry (LSRII, Becton Dickinson). The precursor frequency of antigen-specific CD4+ and CD8+ lymphocytes was determined among PKH26-labeled CD4+ or CD8+ events (ModFit software, Verity Software House) and subtracting the mean precursor frequency of proliferating cells under media-only conditions. Results are presented as the mean and SD of antigen-specific proliferative precursors per 10⁶ CD4+ or CD8+ T cells using triplicate assessments for each antigen-stimulation condition. Antigen specificity was defined as above using a 2-tailed t-test. A response resulting from immunization was defined as a PAP-specific response detectable posttreatment that was both significant (compared with media-only control), at least 3-fold higher than the pretreatment value, and with a frequency > 1:100,000 CD4+ or CD8+ T cells.

Other immunologic evaluation

Antigen-specific IgG. IgGs specific for PAP, PSA, or tetanus toxoid were evaluated by indirect ELISA, as previously described (10).

Antigen-specific cytokine staining and release. Cryopreserved PBMCs obtained from different time points were thawed and cultured in T-cell medium with 2 µg/mL PAP protein, PSA protein, PHA, or anti-CD3- (BioLegend) and anti-CD28 (BioLegend)-coated latex beads (InNviro) for 72 hours at 37°C/5% CO₂. Culture supernatants were assessed for IFNγ, granzyme B, IL2, IL4, IL6, IL10, IL17, or TGFβ by direct ELISA using standard methods (11). In similar studies, cells were treated after 18 hours in culture with monensin and cultured an additional 4 hours before cell surface staining (CD3-V500, CD4-PE-Cy5.5, CD8-eFluor625), cell permeabilization, and staining for intracellular cytokines (IL2-APC, IL4-PE, IL6-PE-CF594, IL10-APC705, IL17-PacBlue, IFNγ-PerCP-Cy5.5, and TNFα-PE-Cy7) using standard methods (BD cytofix/cytoperm kit). Results are reported as percentage of individual populations expressing specific cytokines as compared with fluorescein labeled isotype controls for each cytokine and subtracting the percentage of populations in media-only conditions.

Clinical response evaluation
Staging studies (CT of abdomen/pelvis and bone scintigraphy) were performed every 6 months or as clinically indicated. PSA values (same clinical laboratory) were collected at 1- to 3-month intervals. A minimum of 4 PSA values collected over a 6-month period of time (minimum of 3 months), including the screening value, was used to determine the pretreatment PSA DT, and all values collected from the 6-month period beginning at study week 12 (months 3–9 on study) for the posttreatment PSA DT. PSA DT was calculated as log(2) divided by the slope parameter estimate of the linear regression model of the log-transformed PSA values on time. For analysis purposes, negative PSA DT estimates and high positive PSA DT estimates (>36 months) were censored at 36 months. On-treatment PSA DT and PAP DT were calculated using all available serum PSA or PAP values from the same clinical laboratory from day 1 to the time off-study.

Statistical analysis
Categorical data were summarized as proportions and percentages. Continuous data were summarized and reported as medians and ranges. Profile plots were generated to display changes in PSA values over time. Changes in PSA DT from the pretreatment to the posttreatment period were assessed by calculating fold changes in PSA DT. The SEs of the fold-changes in PSA DT were estimated using Taylor expansion method. Fold changes in PSA DT from the pretreatment to the posttreatment posttreatment period were evaluated using a one-sample t test. Analogously, comparison of PSA DT fold changes between arms was performed using a 2-sample t test. Because the distribution of PSA DT fold changes was skewed, the fold change values were log-transformed before conducting the comparisons. To account for variability in the PSA DT estimates within each subject, these analyses were weighted using the inverse of the SEs of the fold change estimates. Fold change in PSA DT were displayed in graphical format using a waterfall plot. The associations between time to radiographic progression and immune response, PSA DT, or PAP DT were evaluated using Kaplan–Meier analysis and the stratified log-rank test was used to determine differences between groups. The results of these analyses were summarized in terms of HRs. Weighted logistic regression analysis was conducted to evaluate the association between each marker of immune response and fold change in PSA DT. A 2-sided significance level of P < 0.05 was used for all tests and comparisons. Statistical analyses were conducted using SAS software version 9.2 (SAS Institute Inc.).
**Results**

**Patient population and course of study**

Seventeen patients were enrolled in this trial between 2009 and 2012 at the University of Wisconsin Carbone Cancer Center (Madison, WI). As shown in Table 1, the median age of participants was 74 years (range, 47–89 years), the median serum PSA at the time of study entry for all participants was 5.6 ng/mL (range, 2.3–54.4 ng/mL), and the median pretreatment PSA DT for all patients was 3.0 months (range, 1.4–5.5 months). Grade 1 injection site reactions were common, experienced by all subjects. One subject experienced a grade 3 allergic reaction, angioedema, within 30 minutes of his 11th vaccine administration. The angioedema responded to treatment with an oral antihistamine, and the patient was removed from further treatment, but followed until he met a study endpoint. As depicted in the Supplementary Table, no other events greater than grade 2, at least possibly attributed to treatment, were observed, and there were no dosing delays due to adverse events. In addition to the one patient who came off-study for an adverse event, seven patients came off-study for evidence of disease progression, one of these at the final 24-month time point, and 2 patients came off-study for physician discretion due to increasing PSA. Twelve of 16 (75%) evaluable patients, who received at least 6 immunizations, were progression-free at 1 year and 6 of 16 (38%) remained metastasis-free at 2 years. Four patients in arm 2 received 24 immunizations total. Patients were on-study for a median of 16 months. While the original goal was to enroll 30 patients, given the long period of treatment and observation, the trial was closed early due to slow accrual and funding constraints (Supplementary Fig. S1).

**Immunological evaluation—immune monitoring to determine timing of immunization**

The primary immunologic goal of this study was to determine whether real-time immune monitoring could be used to define a personalized schedule of immunization, able to elicit and maintain a PAP-specific cellular immune response. Patients were randomly assigned to 1 of 2 arms, with immunizations delivered on an empiric fixed schedule (arm 1) or as determined by immune monitoring (arm 2; Fig. 1). All subjects received a tetanus booster immunization before beginning the immunization series, providing a test of an individual’s immune competence (although tetanus responses are predominantly humoral (IgG) and CD4-biased; ref. 8). Responses to PSA, a nontarget prostate-specific protein, were concurrently evaluated as a negative control. Patients had PBMCs collected by leukapheresis at baseline and at 1 year for multiple analyses and by peripheral blood draw at immune monitoring time points. An example of these immune analyses, performed over time for 2 particular individuals, is shown in Fig. 2 (and Supplementary Fig. S2A). As demonstrated, PAP antigen-specific T-cell proliferation (Fig. 2A), IFNγ release (Fig. 2B), and granzyme B release (Fig. 2C) could be identified following immunization that persisted over the period of treatment. In multiple patients, antigen-specific proliferating cells were predominantly of an effector (CCR7−/CD45RO+/-) and effector memory (CCR7−/CD45RO+) phenotype (illustrated in Fig. 2D). At each time point, cryopreserved pretreatment cells were evaluated to assess reproducibility (Supplementary Fig. S2C). A summary of all real-time immune monitoring assessments, for the 16 evaluable subjects who received at least 6 immunizations, is shown in Fig. 2E. As demonstrated, PAP-specific cellular responses were augmented and detected in most individuals (12 of 16, Table 1. Demographics for all patients enrolled

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<th>n (Mean ± SD)</th>
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<th>Arm 2 (n = 9)</th>
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<td>67.7 (60–76)</td>
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<td>3</td>
<td>5</td>
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<td>Baseline serum PSA, ng/mL</td>
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<td>4.20 (2.22–5.28)</td>
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</table>
by one or more methods and were persistent over time, up to 2 years in 5 of 6 (83%) individuals who remained on study. Responses to PSA were infrequent, as expected. Granzyme B–secreting responses to PAP were elicited in the majority (11 of 16, 69%) of individuals and persisted over time, and this type of response was essentially not detected to tetanus. IFNγ-secreting responses determined by ELISPOT were detectable for at least 1 year in 5 individuals (2 patients in arm 1 and 3 patients in arm 2). Using purified CD4+ or CD8+ T cells, granzyme B ELISPOT responses were found to consist of both CD4+ and CD8+ T cells, whereas PAP-specific IFNγ ELISPOT responses were predominantly CD8-mediated (data not shown). While immune responses were elicited to PAP in all patients in
the immune monitoring arm (arm 2) by one or more methods, responses by 2 or more methods at any one time point were not detectable in 3 of 8 (38%) individuals (subjects 3, 6, and 8), despite 2 of these individuals (#6 and #8) receiving 24 immunizations at 2-week intervals. Two subjects (#4 and #17) were found to have an immune response after immunization that was subsequently lost and had their immunization schedule changed to biweekly as per protocol.

**Immunologic evaluation—changes in immune response over time**

We next questioned whether the character or magnitude of the immune response changed over time, particularly in patients receiving multiple biweekly immunizations. As suggested in Fig. 2E, PAP-specific immune responses elicited were frequently detected at later time points, and these tended to be of a Th1/cytolytic (IFNγ- and granzyme B-secreting) phenotype. To further address this, the cytokine profile of PAP-specific T cells was assessed by intracellular cytokine analysis for concurrent antigen-specific expression of IFNγ, TNFα, IL2, IL4, IL6, IL10, and/or IL17. As shown in Fig. 3A and B, CD4+ and CD8+ T-cell responses to PAP tended to be Th1-biased (expressing IFNγ, TNFα, and/or IL2). IL4 and IL6 expression was also observed in some patients. These responses persisted beyond 12 weeks, and the type of immune response (Th1 vs. Th2 vs. Th17) did not appreciably change over time. Notably, patients #4, 6, 8, and 17 who received the greatest number of immunizations (n = 24) did not develop increased IL4- or IL10-biased responses with multiple immunizations. Th1-biased responses to tetanus and PSA were only occasionally detected (Supplementary Fig. S3). Similar results were obtained by ELISA using culture supernatants assessed for cytokines following antigen stimulation (Supplementary Fig. S4). While antibody responses to tetanus toxoid were common (and amplified following booster immunization), IgG responses to PAP were not detected in any individual, even after 24 immunizations, further consistent with a Th1-biased immune response (Fig. 3C) and as we had previously observed (8). CD4+ and CD8+ cells expressing more than one Th1 cytokine (IFNγ, TNFα, and/or IL2) were detected (Fig. 3D) and in several patients (patients 9, 10, 13, 15, 17) were found to persist for at least 1 year. An example of this analysis demonstrated that multifunctional Th1-biased cells remained present 1 year after beginning immunization (patient #9, Fig. 3E and F).

**Clinical evaluation**

As described above, patients enrolled had no radiographic evidence of metastatic disease and remained on study until progression or 2 years, whichever came first. Serum PSA values were collected in a protocol-specified fashion to determine PSA DT and changes in PSA DT from pre- to posttreatment. While PSA DT is a known prognostic marker for men with this stage of disease (2, 3), changes in PSA DT have not been validated as a measure associated with clinical benefit. Hence, our use of changes in PSA DT must be viewed as exploratory. Serum PSA values, and serum PAP values, with respect to time points of vaccination are shown for all subjects in Fig. 4. Overall, PSA values increased over time, although some patients exhibited stable PSA over many months (e.g., subjects 5, 8, 9, and 10). Of note, several subjects demonstrated stable serum PAP levels over many months (e.g., subjects 2, 8, 10, 15, 17) in some cases despite increasing serum PSA (e.g., subjects 2, 15, 17). Serum PAP values were not available pretreatment. Seven of 17 (41%) patients experienced at least a 2-fold increase in PSA DT (subjects 2, 5, 6, 8, 9, 10, and 12). Overall, the median fold change in PSA DT from pretreatment to posttreatment was 1.6 (range, 0.6–7.0; P = 0.036). Stratified by study arm, the median fold change in PSA DT was 1.21 (range, 1.13–5.55; P = 0.027) for arm 1 and 1.72 (range, 0.57–7.02; P = 0.293) for arm 2. There was no statistically significant difference in the PSA DT fold change detected between arms 1 and 2 (P = 0.764).

**Association of immune response with exploratory clinical parameters**

Ultimately we wished to determine whether the development of a biologic response from vaccination was associated with favorable changes in serum PSA kinetics or time to disease progression. Immune responses to PAP, identified by the different measures of immune response in Fig. 2, are depicted in Fig. 5A with respect to changes observed in PSA DT. As shown, all measures of immune response tended to be more common in patients who experienced an increase in PSA DT. However, except for CD4 T-cell proliferation (OR, 2.3; P = 0.049), these associations were not statistically significant. A trend toward increased time to disease progression was observed in patients who developed more than one measure of immune response for PAP; however, this was not statistically significant (HR = 2.7; P = 0.153; Fig. 5B). Similarly, and as expected on the basis of prior studies demonstrating PSA DT to be a predictor for time to radiographic progression in this population (3), a trend toward increased time to progression was observed in patients who had higher on-study PSA DT (HR = 3.4; P = 0.088; Fig. 5C). Curiously, patients who had a higher on-study doubling time in serum PAP had a significantly prolonged time to progression (HR = 15.7; P = 0.001; Fig. 5D). While these findings must be viewed as exploratory, given the small number of patients, they suggest that changes in serum PAP kinetics might be evaluated as a future biomarker for response to DNA immunization targeting PAP.

**Discussion**

A common theme emerging from the evaluation of antitumor vaccines and T-cell checkpoint inhibitors is that it can be difficult to recognize immediate clinical benefit, as radiographic progression can occur before regression or stable disease is observed (12, 13). This has led to conclusions that longer term clinical endpoints are necessary for evaluating these therapies (4, 14).
Figure 3. Immune responses to PAP tend to be Th1-biased and persist over time. Intracellular cytokine staining was performed with PBMC samples collected pretreatment, after 12 weeks, and at 1 year (3 patients did not have 1-year samples). The frequency of CD8\(^+\) (A) or CD4\(^+\) (B) T cells expressing IFN\(\gamma\), TNF\(\alpha\), IL2, IL4, IL6, IL10, or IL17 was assessed. Asterisks show significant changes from pretreatment (paired t test). C, sera at these same time points were assessed for IgG responses to PAP, PSA, or tetanus toxoid (TET). Antibody titers are shown. D, intracellular cytokine staining permitted the concurrent evaluation of cells expressing more than one Th1 cytokine. Shown is an example (subject 9) of CD8\(^+\) gated T cells expressing IFN\(\gamma\) and/or TNF\(\alpha\) in response to antigen stimulation at the time points indicated. E, single cytokine CD4\(^+\) and CD8\(^+\) intracellular cytokine analysis at these same time points (pretreatment, 12 weeks, and 1 year) for subject 9. F, the fraction of PAP-specific CD4\(^+\) T cells (left plots) or CD8\(^+\) T cells (right plots) expressing one or more Th1 (IFN\(\gamma\), TNF\(\alpha\), and/or IL2) cytokines. The pie charts demonstrate the frequency of individual cell populations, and numbers below the pie charts indicate the percentage of PAP-specific Th1\(^+\) events among total CD8\(^+\) or CD4\(^+\) T cells. The size of the plots is proportional to the percentage of PAP-specific CD4\(^+\) or CD8\(^+\) T cells over time (*, not to scale).
Results from animal models would further suggest that immune-based treatments may have their greatest role early in the course of disease, before the development of tumor-mediated immunosuppression or immunovasion mechanisms (15). For these reasons, we sought to evaluate the immunologic efficacy of a DNA vaccine and evaluate different schedules of vaccination in patients with early castration-resistant disease, a stage of disease with a long natural history and without standard treatment options (2). This population enabled us to evaluate the development and durability of immune responses elicited over many months. In our experience, this is also a rarer population, as fewer patients elect to begin androgen deprivation therapy in the absence of metastatic disease. As a result of this, our trial closed early due to slow accrual. Nonetheless, we report here that prolonged DNA vaccination, up to 24 times and/or 2 years, was without significant toxicity in this population and could elicit and amplify Th1-biased antigen-specific immune responses that persisted with repetitive immunization.

One of the primary objectives of our study was to determine whether the frequency of antigen-specific effector and memory T cells could be augmented by real-time immune monitoring, using this information to adjust the schedule of immunization. We were particularly interested in the development of IFNγ-secreting T-cell responses, given results from a previous phase I trial (9). While the study population was small, we did not observe a higher frequency of IFNγ ELISPOT responses in the immune monitoring arm (3 of 8) relative to the fixed schedule arm (2 of 8; \( P = 0.59 \)) at 1 year. Moreover, some immune responses were not detectable after the initial series but became detectable at later time points. The observation that some immune responses developed over time regardless of the immunization schedule, and IFNγ-secreting T-cell responses in particular were not augmented in more individuals treated in the variable arm, suggested that using real-time immune monitoring to personalize the vaccination schedule was not necessary and was not particularly informative for managing individual schedules.

Plasmid DNA has been used as an antigen delivery vehicle both for antitumor immunization, in which the goal is to elicit a cytolytic cellular response, and for the treatment of autoimmune disease, in which the goal is to elicit a tolerant response (16, 17). Given this, we were particularly keen to determine whether multiple repetitive immunizations could skew an immune response toward a Th2 or tolerant phenotype. This was not observed, including in 4 individuals who received 24 immunizations. Notably, antibody responses, common after treatment with another vaccine (sipuleucel-T) targeting the same PAP antigen (4), were not elicited following this DNA
immunization (Fig. 3C). At present, it remains unknown whether the type of immune response elicited is related to the particular antigen targeted, potentially due to preexisting inflammatory immune responses that are amplified with immunization. Alternatively, the Th1 bias that we observed could be due to the route of immunization and the adjuvant used. We used an intradermal route of immunization and GM-CSF as an adjuvant, a route and adjuvant previously investigated in other models for the specific development of Th1-biased immune responses (18–21). GM-CSF, however, has pleiotropic effects, and its efficacy as an adjuvant has been questioned in the context of peptide-based vaccines (22). While preclinical models support its use with DNA vaccines as an adjuvant (23), the role of GM-CSF, and whether it promotes or inhibits the development of Th1 immunity in humans will be a goal of future trials. In addition, it remains unknown whether the frequency or magnitude of the immune responses we observed could be further augmented by the use of different delivery methods, such as electroporation or particle bombardment being investigated by others (24–27).

Figure 5. Biomarker associations with changes in PSA DT and time to disease progression. Shown are the fold changes in PSA DT from the pretreatment period to the posttreatment period as a waterfall plot (A). Red columns indicate patients treated in arm 1 and blue columns for patients in arm 2. The detection of an immune response by one of the measures described is indicated with an asterisk. The time to radiographic disease progression was evaluated using Kaplan–Meier analysis with respect to the development of more than one measure of immune response to PAP (B), PSA DT (using all on-study serum PSA values to determine an on-study PSA DT, C), or PAP DT (using all on-study serum PAP values to determine an on-study PAP DT, D).
We had previously observed in a phase I trial that 6 immunizations with plasmid DNA encoding PAP was sufficient to elicit a PAP-specific cellular immune response in some individuals with prostate cancer, but not others, and that immune responses tended to develop over time with repetitive immunization (8). Consequently, an additional goal of the current trial was to determine whether ongoing immunization was necessary to elicit responses in some individuals who did not develop an immune response after 6 immunizations only. Notably, in 2 patients who did not have an immune response at the end of the initial 12-week immunization series, continuous immunization at 2-week intervals did not lead to higher magnitude immune responses. Potential reasons for the inability to effectively immunize these individuals remain unknown and were not addressed in our study. This could be due to differences in these individuals even before beginning treatment, perhaps suggesting that some individuals are completely tolerant to this antigen. It is also conceivable that plasmid DNA immunization is ineffective in some individuals irrespective of the target antigen. It is further conceivable that immune responses were elicited but at levels undetectable in the peripheral blood. While no baseline parameters (e.g., Gleason score) or prior therapy appeared to be associated with the development of immune response (data not shown), the identification of differences leading some patients to develop measurable immune responses, and others not, will be an important area for future investigation.

In our study, PSA protein was included as a prostate-specific negative control for the immunologic assessments. Curiously, we did identify Th1-biased responses elicited to this protein, in one patient detectable at multiple time points after immunization (Fig. 2, subject 17). We have previously reported that Th1-biased T-cell responses to PSA can occur in patients with prostate cancer (11). However, the finding that these were not detectable at baseline but augmented after immunization suggests that antigen spread might have occurred in at least these individuals. Antigen spread to nontarget tumor-associated proteins has been observed following different antitumor vaccine treatments and in some circumstances has been associated with favorable outcomes from vaccination (28). Using antibody responses to prostate-derived proteins, we have previously identified antibody responses to nontargeted proteins elicited following DNA immunization (29). The identification of T-cell antigen spread to other prostate-associated proteins and whether this is associated with better clinical outcome will be a direction for future studies.

Ultimately the goal of our study was to determine whether the development of PAP-specific long-term immunity was associated with improved clinical outcome. However, our desire to evaluate vaccination in the setting of minimal residual disease with a long natural history necessarily confounds the analysis of clinical benefit; this population of patients did not have radiographically apparent disease that could be monitored—the only measure of tumor response was by changes in serum tumor markers. Hence an exploratory objective of our trial was to evaluate biomarkers that might be associated with prolonged time to disease progression. While PSA DT is a known prognostic factor in this stage of disease (2), and changes in PSA DT have been associated with prolonged time to disease progression in non-castrate patients with prostate cancer (30), this has not been prospectively validated in clinical trials and awaits validation in trials currently underway. Notwithstanding, those patients who demonstrated an increase in PSA DT tended to have multiple measures of PAP-specific immunity that were detectable over multiple time points. This is consistent with our previous findings in patients with non-castrate PSA-recurrent prostate cancer (9). We also observed that the 4 patients who experienced the greatest change in PSA DT (patients 5, 9, 10, 12; 2 of whom were on the immune monitoring arm) were effectively treated with the same fixed quarterly-booster schedule. This observation, with the demonstration of Th1-biased immune responses being elicited using this schedule, has led us to select this schedule for further evaluation in a larger, randomized phase II clinical trial (NCT01341652).

In the current trial, we also evaluated the time to disease progression by standard radiographic criteria. The development of immune responses to PAP by more than one measure and a longer on-trial PSA DT were associated with an increased time to radiographic progression; however, these associations were not statistically significant. Intriguingly, a longer on-trial PAP doubling time was significantly associated with a prolonged time to disease progression, potentially due to immunologic targeting of tumor cells expressing PAP. As there was no evidence of PAP antibody responses by immunization, it is unlikely that serum levels were affected by antibody-mediated clearance of PAP. Consequently, it is possible that serum PAP kinetics might serve as a simple biomarker for an effective antitumor cytolytic response following PAP-targeted immunization. At present, it is unknown whether changes in serum PAP might generally be evaluated as a prognostic biomarker or whether changes observed in this trial are specifically related to targeting the PAP antigen. It will be of interest to determine whether serum PAP kinetics are affected by other antiproliferation approaches such as Prostvac-VF (targeting PSA) or sipuleucel-T (targeting PAP). The evaluation of serum PAP and immune biomarkers demonstrating treatment effect will be further explored in randomized phase II trials.

Disclosure of Potential Conflicts of Interest
D.G. McNeel is an employee of, reports receiving a commercial research grant from, has ownership interest in, and is a consultant/advisory board member for Madison Vaccines Inc. No potential conflicts of interest were disclosed by the other authors.

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