Research Article

Cancer Immunology Research

Immune Impact Induced by PROSTVAC (PSA-TRICOM), a Therapeutic Vaccine for Prostate Cancer

James L. Gulley[1], Ravi A. Madan[1], Kwong Y. Tsang[1], Caroline Jochems[1], Jennifer L. Marté[1], Benedetto Farsaci[1], Jo A. Tucker[1], James W. Hodge[1], David J. Liewehr[2], Seth M. Steinberg[2], Christopher R. Heery[1], and Jeffrey Schlom[1]

Abstract

PSA-TRICOM (PROSTVAC) is a novel vector-based vaccine designed to generate a robust immune response against prostate-specific antigen (PSA)-expressing tumor cells. The purpose of this report is to present an overview of both published studies and new data in the evaluation of immune responses to the PSA-TRICOM vaccine platform, currently in phase III testing. Of 104 patients tested for T-cell responses, 57% (59/104) demonstrated a ≥2-fold increase in PSA-specific T cells 4 weeks after vaccine (median 5-fold increase) compared with pre-vaccine, and 68% (19/28) of patients tested mounted post-vaccine immune responses to tumor-associated antigens not present in the vaccine (antigen spreading). The PSA-specific immune responses observed 28 days after vaccine (i.e., likely memory cells) are quantitatively similar to the levels of circulating T cells specific for influenza seen in the same patients. Measurements of systemic immune response to PSA may underestimate the true therapeutic immune response (as this does not account for cells that have trafficked to the tumor) and does not include antigen spreading. Furthermore, although the entire PSA gene is the vaccine, only one epitope of PSA is evaluated in the T-cell responses. Because this therapeutic vaccine is directed at generating a cellular/Th1 immune response (T-cell costimulatory molecules and use of a viral vector), it is not surprising that less than 0.6% of patients (2/349) tested have evidence of PSA antibody induction following vaccine. This suggests that post-vaccine PSA kinetics were not affected by PSA antibodies. An ongoing phase III study will evaluate the systemic immune responses and correlation with clinical outcomes. *Cancer Immunol Res; 2(1); 1–9. ©2013 AACR.*

Introduction

PROSTVAC is a vector-based therapeutic cancer vaccine composed of a series of poxviral vectors (vaccinia during the initial priming vaccine and fowlpox for all boosts) engineered to express prostate-specific antigen (PSA) and a triad of human T-cell costimulatory molecules (B7.1, ICAM-1, and LFA-3, designated TRICOM; ref. 1). The vaccine is designed to break immunologic tolerance to PSA and initiate a robust immune response against prostate cancer cells. Early studies of PROSTVAC demonstrated the safety and immunologic activity of this approach in men with advanced prostate cancer (2–6), and a multicenter randomized phase II study showed preliminary evidence of improved overall survival (OS; Fig. 1; ref. 7). On the basis of these findings, an international randomized placebo-controlled phase III study is currently underway (8). This registration endpoint study is designed to confirm an association between the use of PROSTVAC and improved OS in men with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (mCRPC).

Findings of a small-phase II study ($n = 32$) suggested that patients who mounted the highest (≥6-fold) increase in PSA-specific T cells, as measured by ELISPOT assay pre- and post-vaccine, had improved OS compared with patients who did not mount as great an increase in PSA-specific T cells (9). This hypothesis-generating finding is consistent with the putative mechanism suggested by preclinical models: an increase in tumor-specific $CD8^+$ T cells.

Preclinical studies of poxviral vector vaccines containing tumor-associated antigens (TAA) and encoding TRICOM in self-antigen murine models have shown that the vaccine's ability to treat tumors is completely abrogated by the depletion of $CD8^+$ T cells, and partially abrogated by the depletion of $CD4^+$ T cells and natural killer (NK) cells (10). Thus, we have sought to understand the impact of this vaccine primarily by analyzing compiled raw data from multiple trials (some previously published), focusing on the vaccine's ability to generate tumor-specific T cells. This information will directly expand our understanding of the vaccine's potential mechanism of action, and provide data for the first time to suggest that using PSA kinetics following this PSA-based vaccine is not confounded by the generation of a PSA antibody response.

Authors' Affiliations: [1]Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute; and [2]Biostatistics and Data Management Section, National Cancer Institute, NIH, Bethesda, Maryland

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

Corresponding Author: James L. Gulley, Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, NIH, 10 Center Drive, 13N208 MSC-1750, Bethesda, MD 20892. Phone: 301-435-2956; Fax: 301-480-5094; E-mail: gulleyj@mail.nih.gov

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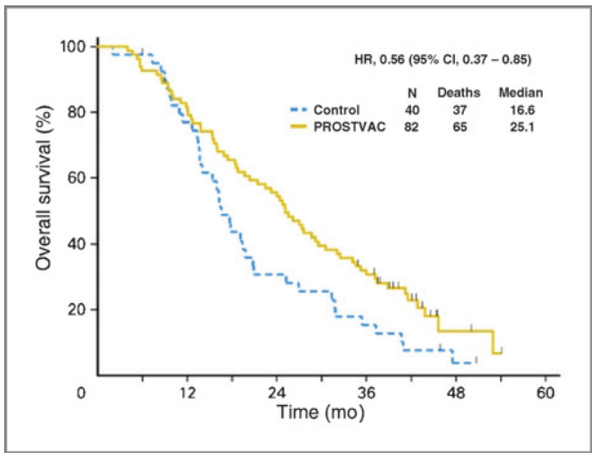

Figure 1. Kaplan–Meier curve of overall survival. Solid gold line, PROSTVAC arm; dashed blue line, control arm; vertical ticks, censoring times. Estimated median overall survival: 25.1 months (PROSTVAC arm) versus 16.6 months (control arm). Used with permission from (7).

Materials and Methods

Collection of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC; apheresis for some patients) were collected before and 4 weeks after vaccinations (around day 28). PBMCs were isolated by Ficoll (Amersham Biosciences) density gradient separations, washed three times, and cryopreserved in liquid nitrogen at a concentration of 1–2 $\times$ 10^7 cells/mL until assayed.

ELISPOT

A modification of the procedure described by Gulley and colleagues (11) was performed, using K562/A*0201 as antigen-presenting cells (APC), as previously reported (12). We used T-cell responses to influenza MP 58 to 66 and HIV peptides as positive and negative controls, respectively, and analyzed T-cell responses to the TAA PSA, mucin 1 (MUC1), prostatic acid phosphatase (PAP), prostate-specific membrane antigen, prostate stem cell antigen, brachyury, AN07, XAGE-1, and PAGE-4. A positive response was defined as a $\geq$2-fold increase in TAA-specific T cells following vaccination. In addition, the same frozen aliquot of PBMCs from a healthy donor was used in all assays with the positive and negative controls to confirm that there was no deviation above 20% of spots from assay to assay. The post-vaccine immune responses are noted as the maximal post-vaccine immune response.

Flow cytometry analysis

Multicolor flow cytometry analysis was performed on cryopreserved PBMCs by staining for 30 minutes at 4°C with CD3-V450, CD8-FITC or APC, HLA-DR-PerCPCy5.5, CD25-PECy7, CD45RA-PerCP-Cy5.5, CD62L-FITC, CD127-V450, CCR7-PE-Cy7, Tim-3-AF700, CD4-APC-Cy7, CTLA-4-FITC, and FOXP3-APC (BD Biosciences). For NK cells, CD3-V450, CD16-APC-Cy7, and CD56-PE-Cy7 were used. For myeloid-derived suppressor cells (MDSC), CD33-PE, CD11b-APC-Cy7, HLA-DR-PerCP-Cy5.5, CD14-V450, and CD15-APC were used. A total of 1 $\times$ 10^5 cells were acquired on an LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star Inc.,). The appropriate isotype controls were used, and dead cells were excluded from the analysis.

Analysis of anti-PSA antibodies

Anti-PSA antibodies in the serum of patients pre- and post-vaccine were assessed by ELISA, as described by Madan and colleagues (13). Patient sera and normal human serum were diluted starting at 1:50. We performed three serial dilutions of 1:5 and a final dilution of 1:6250. We used purified mouse anti-PSA immunoglobulin (IgG1) antibody (Fitzgerald Industries) as positive control for PSA binding. MOPC-21 (IgG1 antibody; Sigma-Aldrich) was used as isotype-matched control.

Cytokine detection

Serum samples pre- and post-vaccine were screened for IFN-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF-α using a multiplex cytokine/chemokine kit (Meso Scale Discovery).

Statistical analysis

The Kaplan–Meier method was used to analyze overall survival and the log-rank test was used to compare strata. Distributions of paired data were tested against zero with the Wilcoxon signed-rank test. Spearman correlation coefficient was used to show the association between two variables.

Results

Induction of PSA-specific T cells (compiled data)

To date, the National Cancer Institute (Bethesda, MD) has conducted seven clinical trials of poxviral vaccines encoding PSA, some of which have yielded data on immune responses. All of these trials were prospectively designed to evaluate immune response, and all have previously been published (Supplementary Fig. S1; refs. 5, 9, 11, 13–17). Of the 104 patients tested, 57% (59/104) had a $\geq$2-fold increase in PSA-specific T cells following vaccine, as measured by IFN-γ ELISPOT assay (Table 1, top). Of these 59 patients, the median number of PSA-specific T cells 28 days post-vaccine was 30/million PBMCs (Table 1, bottom). By comparison, the median baseline level of influenza-specific T cells in those same 59 patients was 33.3/million PBMCs. These levels of influenza-specific T cells are similar to what we have observed in multiple studies.

To determine the evidence of cross-priming and generation of an immune response to TAAs not found in the vaccine (a phenomenon known as antigen cascade or antigen spreading; ref. 18), we tested patients for TAA-specific immune responses to nonvaccine (i.e., non-PSA) antigens in four separate clinical trials (11, 13, 15, 16). Of the 28 patients tested, 68% (19/28) showed evidence of antigen-cascade post-vaccination (Table 2). Eight of the 28 patients had only one additional cascade antigen tested (MUC1 along with PSA), whereas 20 had two to four cascade antigens tested. Of those 20, 16 had a $\geq$2-fold increase in TAA-specific T cells to at least one cascade antigen, and nine of 16 had a positive response to at least two cascade antigens (Supplementary Table S1).

Q7 **Table 1.** PSA-specific T cells induced after vaccination with poxviral vaccines encoding PSA

Disease state	Percentage of patients with PSA$^+$ ELISPOT (≥2-fold increase)	Trial (NCT #; ref)
Localized	72.0% (18/25)	NCT00005916 (11, 15)
bCRPC	62.5% (5/8)	NCT00020254 (14)
bCRPC	25.0% (1/4)	NCT00450463 (17)
mCRPC	48.6% (17/35)	NCT00060528 (9)
mCRPC	11.1% (1/9)	NCT00113984 (13)
mCRPC	73.9% (17/23)	NCT00045227 (16)
Total	56.7% (59/104)	
	Median	**Min.–Max.**
Baseline PSA-specific T cells[a]	5.00	5.00–20.00
Maximum post-vaccine PSA-specific T cells[a]	30.00	10.00–202.51
Fold increase in PSA-specific T cells post-vaccine	5.00	2.00–19.33
Flu-specific T cells[a]	33.33	6.67–343.29

NOTE: Levels of circulating PSA-specific T cells in patients whose PSA-specific T cells increased 2-fold or more following vaccine (57% or 59/104 evaluated patients), and comparison with baseline levels of circulating influenza matrix protein-specific T cells in these same patients. Of 193 post-vaccine ELISPOTs, 60% (115/193) had a 2-fold increase in PSA-specific T cells compared with baseline, with 31 of 59 patients having more than 1 post-vaccine ELISPOT.
Abbreviations: Localized, localized prostate cancer; bCRPC, biochemically progressive (nonmetastatic) castration-resistant prostate cancer.
[a]Spots per million.

Vaccine effects on the number and the function of regulatory T cells (compiled and new data)

An analysis of alterations in the number and the function of regulatory T cells (Tregs) in patients treated with PSA-TRICOM on two phase II studies indicated that the number and the suppressive function of Tregs decreased in some patients post-vaccination (19). In addition, there were trends indicating an association between decreased function of Tregs following vaccination ($P = 0.029$; $r = -0.45$) and improved OS. Tregs expressing CTLA-4 were more suppressive. Those patients who lived longer than predicted as estimated by the Halabi nomogram (20) had an improved effector T cell: CTLA-4$^+$ Treg ratio post-vaccine ($P = 0.029$). Additional immune studies are reported here for the first time. Preliminary data from one study (21) suggested a significant decrease in Tregs relative to CD4$^+$ T cells within the tumor following vaccination. In another of these studies (9), patients showed a trend demonstrating that a decrease in the percentage of Tregs post-vaccine was associated with longer OS (log-rank, $P = 0.058$; Fig. 2A). In addition, an increase in the frequency of the CD4 effector memory T-cell subsets showed a trend toward an association with longer OS (log-rank $P = 0.044$; Fig. 2B). Interestingly, we also found a direct correlation between the change in Treg percentages and the change in the frequency of monocytic myeloid-derived suppressor cells (mMDSC; Spearman $r = 0.82$; Fig. 2C).

Table 2. Evidence of antigen spreading by induction of antitumor T-cell responses to antigens not found in the vaccine

Vaccine	% of positive patients	Reference
rV-PSA+rV-B7.1	75% (6/8)	(11)
rV-PSA+rV-B7.1	62.5% (5/8)	(15)
rV-PSA+rV-B7.1	100% (3/3)	(16)
PSA-TRICOM	55.6% (5/9)	(13)
Total	67.9% (19/28)	

Antibody responses (compiled and new data)

When pre- and post-vaccine sera from 349 patients enrolled on 11 studies (2–7, 9, 11, 14, 16, 22, 23) of PSA-based poxviral vaccines were analyzed for evidence of free PSA antibodies, only two patients showed evidence of PSA antibody response post-vaccine (Table 3). Because PSA is a secreted protein not found on the cell surface, intact tumor cells could not act as a sink to PSA antibodies. However, it is possible that circulating PSA protein could bind free anti-PSA antibody. Knowing that these circulating antigen/antibody complexes (immune complexes) can be measured, we collaborated with Dr. Gabriel Virella of the Department of Microbiology and Immunology at the Medical University of South Carolina (Charleston, SC) to analyze samples from 20 patients treated on one study with PROSTVAC (9) for evidence of PSA-specific circulating immune complex before and after 3 months of vaccine treatment. Patients selected included all those, whose PSA decreased following vaccine. For the first time we show that of these 20 patients, 14 showed no

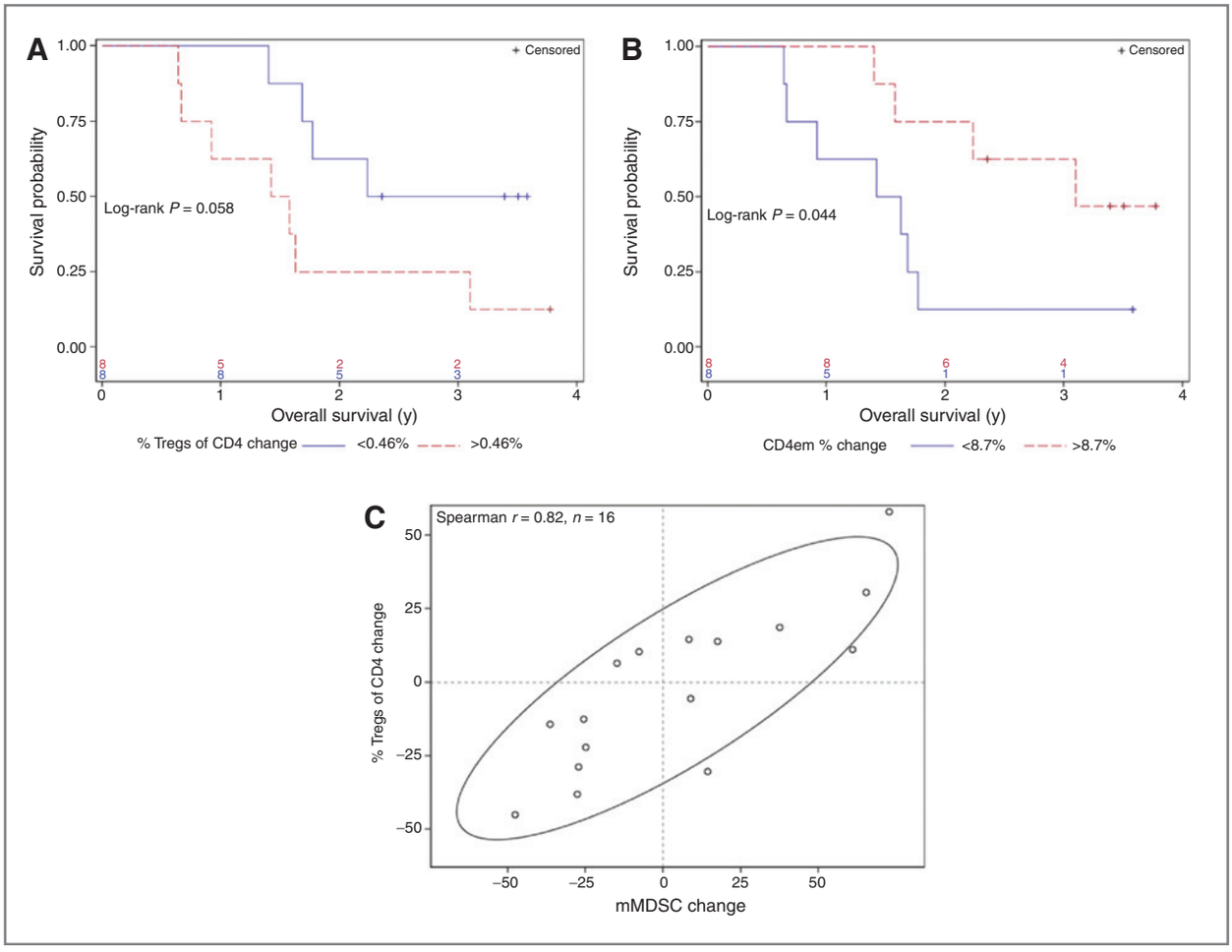

Figure 2. Actuarial analysis and associations of changes in the frequencies of immune cell subsets versus overall survival. We analyzed PBMCs of 16 out of 32 patients in the phase II trial of PROSTVAC pre- versus post-vaccine for immune cell subset phenotypes, as described in Materials and Methods. For actuarial analysis, the data were dichotomized at the median and log-rank test was used to compare strata. Because of the very small number of patient samples analyzed, these data are intended only to be hypothesis generating. A, Kaplan–Meier plot of overall survival versus the change in Treg percentage post- versus pre-vaccination. Blue line, change in Treg percentage below median; red line, change in Treg percentage above median (median change was 0.46%). B, Kaplan–Meier plot of overall survival versus change in frequency of CD4 effector memory ($CD4_{em}$) cells post- versus pre-vaccination. Blue line, decrease below median for $CD4_{em}$ frequency; red line, increase above median for $CD4_{em}$ frequency (median change was 8.7%). C, scatter plot with 75% confidence ellipse showing the % change in Tregs out of all CD4 T cells versus the change in mMDSCs; $r = 0.82$.

evidence of immune complex at any time point, two tested borderline positive (one at baseline only; one following vaccine only), two had new immune complex following vaccination (as well as a 2- to 5-fold increase in PSA), and one had preexisting immune complex that increased following vaccine (along with a proportional increase in PSA).

A substantial level of anti-PSA antibodies should result in a decreased level of circulating PSA, as bound antibodies are pulled from the circulation by reticuloendothelial cells, leading to an artificially reduced PSA level. All of our studies have found a moderately strong association between the values for PSA and PAP, another prostate cancer tumor marker, such that when PSA decreases, so does PAP. For the first time we report here, an analysis of 122 patients treated with PROSTVAC (5, 9, 13, 17, 21, 24), which found a moderately strong association between PSA and PAP both before (Fig. 3A; Spearman $r = 0.76$) and after vaccine (Fig. 3B; Spearman $r = 0.77$). The median of the ratio (log PSA pre/log PAP pre)/(log PSA post/log PAP post) was exactly 1 ($P = 0.78$, Wilcoxon signed-rank test; Fig. 3C). Thus, there was no significant difference in PSA/PAP ratios before versus after treatment.

Vaccine effects on the number of NK cells (new data)

It is possible that a vaccine that induces a Th1 inflammatory response and $CD8^+$ T cells could also alter the quantity or function of NK cells. We performed a small analysis on one study (9) to evaluate whether the vaccine had a significant effect on NK cells. An analysis of 16 paired patient samples available for measurement showed no statistical difference in the number of NK cells pre- versus post-vaccine. An analysis of the subsets indicated no differences in the number of immature ($CD16^{neg}CD56^{bright}$), mature ($CD16^{pos}CD56^{dim}$), and

Table 3. Generation of anti-PSA antibodies following vaccination with PSA-based vaccines

Vaccine	No. of positive patients	No. of patients tested	Data previously published	Reference
rV-PSA	1	33	Yes	(4)
rV-PSA	0	42	Yes	(2)
rV-PSA	1	6	Yes	(3)
rV-PSA/rF-PSA	0	14	Yes	(11)
rV-PSA/rF-PSA	0	15	Yes	(14)
rV-PSA/rF-PSA	0	9	Yes	(16)
rV-PSA/rF-PSA	0	64	Yes	(22)
PSA-TRICOM	0	82	Yes	(7)
PSA-TRICOM	0	10	Yes	(6)
PSA-TRICOM	0	45	Yes	(5, 9)
PSA-TRICOM	0	29	No	(23)
Total	**2**	**349 (0.57%)**		

functional intermediate-stage ($CD16^{pos}CD56^{bright}$) NK cells (data not shown; signed-rank test $P > 0.20$ for all 3).

Th1 versus Th2 cytokines (new data)

An analysis of the pre- and post-vaccine serum levels in 29 patients in the phase II trial of PSA-TRICOM found no consistent alteration in most serum Th1 or Th2 cytokines, including IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF-α (signed-rank test P values: 0.23, 0.84, 0.31, 0.13, 0.0013, 0.78, 0.78, 0.090, 0.89, and 0.14, respectively). Of note, IL-5, a type II cytokine, was significantly decreased following vaccine treatment (Supplementary Fig. S2).

Clinical safety (compiled data)

Generation of a strong immune response could theoretically result in adverse events in both on-target and off-target tissue. The safety of PSA-TRICOM was evaluated in 152 patients treated at the NIH Clinical Center and 82 patients on a randomized phase II study. This primary data safety analysis showed that of the 234 patients, who received 1,341 vaccines, grade 2 or more injection-site reactions were seen following 21.6% of vaccinations (with only 2 grade 3 and no grade 4 injection-site reactions). Other adverse events possibly related to vaccine (largely limited to fatigue and flu-like symptoms) were seen following less than 1.5% (grade 2) and less than 0.5% (grade 3 or 4) of the vaccinations. Furthermore, of the 234 patients who received PSA-TRICOM on study, only two (<1%) had to stop the treatment due to adverse events. All grade 2 or more adverse events believed to be at least possibly related to vaccine are described in Supplementary Table S2.

Discussion

PROSTVAC is known to induce a robust cellular immune response in many patients, with immune responses to PROSTVAC quantitatively similar to the memory responses to influenza. Yet data evaluating immune responses almost certainly underestimate the true therapeutic impact of the vaccine. The ELISPOT assay used to analyze immune response in the trials mentioned above has several inherent limitations. (i) It measures IFN-γ–producing cells circulating in the peripheral blood, but does not take into account those that have trafficked to the site of the tumor. It is possible that the level of tumor-specific T cells measured in the periphery does not reflect the actual number of tumor-specific T cells at the tumor site. Preclinical evidence suggests that TAA-specific $CD8^+$ T cells are enriched at the tumor site. In two studies, the level of TAA-specific T cells detected in the periphery was 2.1% to 2.3% in CEA-transgenic (CEA-Tg) mice following vaccination with CEA/TRICOM vaccine (25, 26). In contrast, in two studies

Table 4. Multiple costimulatory molecules encoded within poxviral vectors dramatically increase avidity of tumor-specific T cells in a murine model

Vaccine	Precursor frequency/10^5 $CD8^+$ T cells	Δ Precursor	Peptide concentration for CTL (nmol/L)	Δ Avidity[a]
rV-CEA	321	1.0X	510	1X
rV-CEA-TRICOM	769	2.4X	5	102X

Abbreviation: CTL: cytotoxic T lymphocytes.

[a]Defined as natural log of the peptide concentration that results in 50% maximal target lysis. Similar results were seen with tetramer dissociation. Adapted from ref. (26).

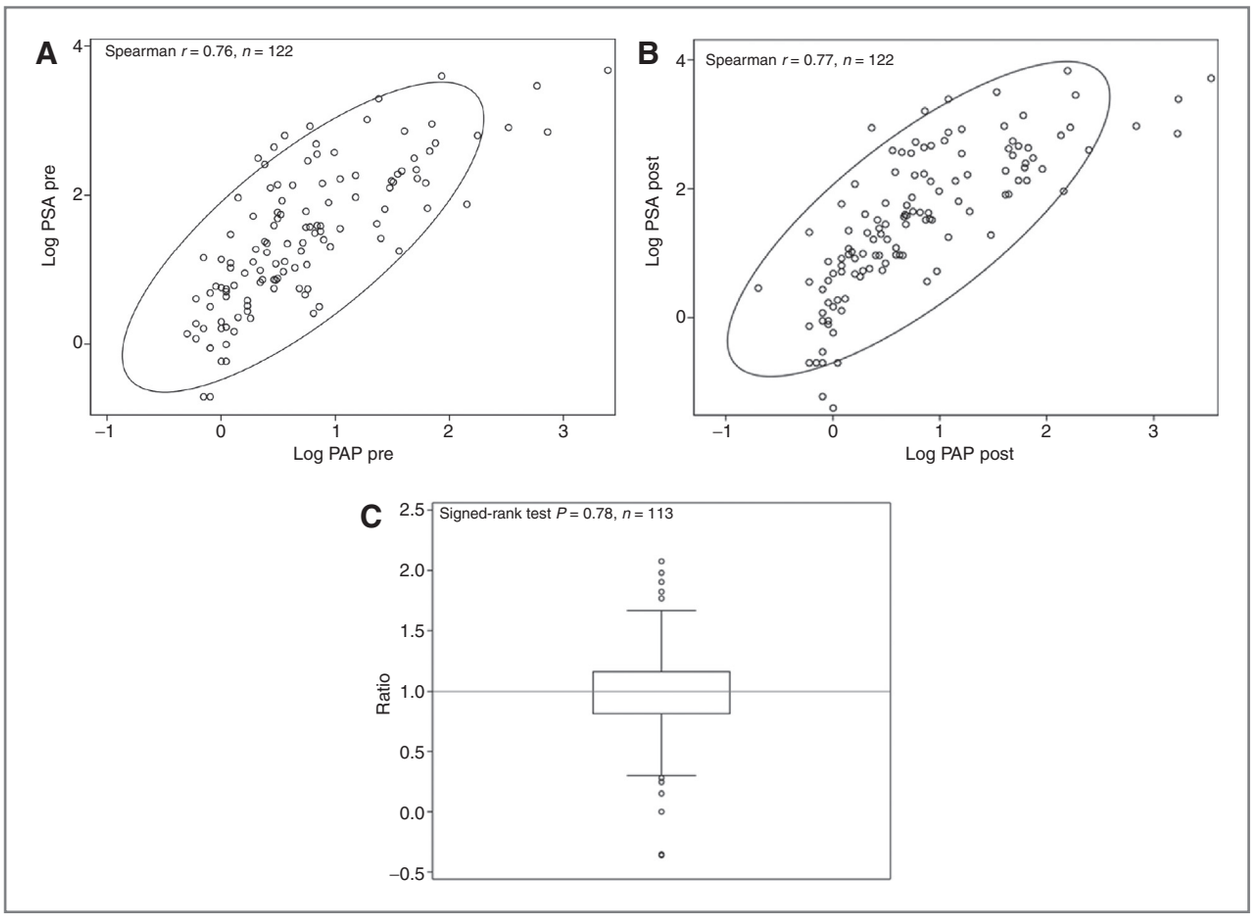

Figure 3. Unchanged correlation of PSA to PAP in 122 patients treated with PROSTVAC. A, pre-vaccine scatter plot with 90% confidence ellipse. B, post-vaccine scatter plot with 90% confidence ellipse. C, box- and whisker plot of the ratio (log PSA pre/log PAP pre)/(log PSA post/log PAP post), $n = 113$. There were nine missing observations because of division by zero (five cases in the pre-ratio and four cases in the post-ratio). If PSA antibodies were pulling PSA out of circulation, the amount of PSA per unit of PAP would decrease.

analyzing tumor-infiltrating T cells, CEA-Tg mice demonstrated 11% to 22% of the infiltrating $CD8^+$ T cells specific for the TAA following vaccination with CEA/TRICOM vaccine (27, 28). Taken together, these data suggest that TAA-specific T cells may be enriched 5- to 10-fold at the site of the tumor. Moreover, much higher levels of tumor-specific T cells at the tumor than in the peripheral blood have previously been reported in patients with cancer (29). A neoadjuvant study in prostate cancer that will analyze intratumoral immune responses following vaccination with PSA-TRICOM is slated to open shortly. (ii) In each of the clinical studies of PROSTVAC, immune responses were assayed in PBMCs obtained 28 days after the preceding vaccination. Resultant immune responses likely reflected residual circulating memory cells, since 28 days is well beyond the anticipated peak of an effector cell response to vaccine. (iii) ELISPOT uses only one-nanomer PSA peptide in HLA-$A2^+$ patients, whereas patients may mount immune responses to other PSA epitopes expressed by the viral-vector vaccine, which expresses the entire gene (i.e., 244 amino acids). (iv) Unlike the multiple rounds of *in vitro* stimulation reported in some studies, the ELISPOT assays reported here used a single overnight incubation of APCs pulsed with antigen and patient samples. (v) The assays used whole PBMC populations, not pure $CD8^+$ populations. It is estimated that $CD8^+$ populations represent only 20% of total PBMCs; thus, a pure $CD8^+$ population used in the ELISPOT assay should give 5-fold higher results. (vi) Not all patients were HLA-0201. Therefore, the level of binding of the PSA nanomer in these patients could not be determined, which may have resulted in an underestimation of the proportion of patients responding to the vaccine. (vii) Our laboratory has a very conservative definition of what constitutes an ELISPOT, as evidenced by the data for influenza matrix protein-specific T cells (Table 1B).

An additional limitation of the ELISPOT assay is that it does not address two fundamental aspects of an immune response: its avidity and breadth of response to a variety of TAAs. T-cell avidity can be defined by the concentration of antigen required to elicit a response, meaning that higher avidity T cells can be activated to kill tumor cells with much lower concentrations of antigen (30). This is important because only high-avidity T cells can efficiently lyse target cells (31). In preclinical studies, vaccines containing TRICOM produced a 2.4-fold increase in

vaccine-specific T cells compared with vaccines without costimulatory molecules. More importantly, however, there was a more than 100-fold increase in the avidity of T cells produced by TRICOM-containing vaccines compared with vaccines without costimulatory molecules (Table 4; ref. 26).

The most intriguing explanation for the ELISPOT assay's possible underestimation of a clinically relevant antitumor immune response may be the difficulty of identifying the specific tumor rejection antigen(s) present in any given patient with prostate cancer. However, any initial tumor-specific immune response that leads to immune-mediated tumor killing can also lead to cross-priming of other tumor-specific antigens to the immune system in a process called antigen spreading or antigen cascade (32), as demonstrated in multiple preclinical studies (18, 33, 34). One study of intratumoral CEA-TRICOM vaccine demonstrated not only increased numbers of CEA-specific T cells within the tumor compared with the spleen, but also T cells specific to other antigens expressed by the tumor, such as wild-type p53 and an endogenous retroviral epitope of gp70 (18). Moreover, the magnitude of $CD8^+$ T-cell immune responses to gp70 was far greater than responses induced to CEA. In fact, the predominant T-cell population infiltrating the regressing CEA^+ tumor after vaccine was specific for gp70. An expanding, cascading immune response may continue over time, eventually broadening into an immune response potentially more clinically relevant than the initial immune response to the vaccine. Clinical trials of PROSTVAC have reported a T-cell antigen-cascade in 68% of the patients tested (Table 2). Furthermore, immune responses to cascade antigens are often more robust than immune response to the PSA expressed by the vaccine (11, 13, 15). One study with an earlier version of PROSTVAC revealed treatment-associated autoantibody responses in 15 of 33 (45.5%) patients treated with the combination of vaccine and radiation versus one of eight (12.5%) patients treated with radiation alone (35). Others have reported improved clinical outcomes for patients who demonstrated a broadened immune response (36–38).

Multiple studies have demonstrated that the number and/or function of Tregs are increased in patients with prostate cancer compared with healthy volunteers (39–41). PSA-TRICOM may be capable of reversing the immunosuppressive capacity of Tregs by shifting the effector T cell:Treg ratio, or possibly by decreasing the functional capacity of Tregs. Prospective analyses of the correlative trends seen in this analysis, along with other immune endpoints, are planned in the ongoing phase III study.

A poxviral vector vaccine encoding multiple T-cell costimulatory molecules might be expected to produce an immune response skewed toward Th1, with little or no antibody production. Indeed, some preclinical data support this expectation. An initial safety study of recombinant vaccinia-expressing human PSA, performed in rhesus monkeys, found only transient production of immunoglobulin M antibodies to human PSA and no IgG or IgA antibodies (42). Studies seeking evidence of induction of anti-PSA antibody responses in 349 patients treated with poxviral vaccines encoding PSA found only two patients with increased anti-PSA antibodies following vaccination (Table 3) and little direct evidence of circulating PSA/antibody immune complexes. Furthermore, indirect evidence from 122 patients suggests no change in the ratio of PSA to PAP after vaccination. A substantial increase in the post-vaccine ratio would be consistent with an artificial lowering of PSA (more than PAP) due to induction of a PSA antibody. The almost identical correlation coefficients indicate that the vaccine does not alter the ratio of PSA to PAP, suggesting that it is reasonable to use PSA as a marker to analyze the kinetics of a clinical response to PROSTVAC vaccine. Indeed, this lends further credence to a prior analysis and hypothesis on tumor growth rates following vaccine (Supplementary Fig. S3; ref. 43, 44).

T-cell response (mostly $CD8^+$) appears predominant, with no evidence of B-cell response. It is interesting to note that IL-5, a cytokine involved in a type II immune response, decreases following vaccine. Although this finding would need to be confirmed in subsequent studies, this is consistent with a bias for a cytotoxic T-lymphocyte response over a B-cell response. This T-cell–predominant response is exactly what one would predict to be beneficial, based on preclinical depletion studies. Furthermore, the absence of evidence of antibody/immune complexes to PSA allows for the use of PSA levels to assess disease kinetics in vaccine-treated patients. The ability to generate apparently clinically significant immune response is associated with only a minority of the patients having transient mild to moderate adverse events from the vaccines. This desirable side-effect profile allows for potential combinations with a variety of different agents and facilitates patient acceptability in earlier stages of the disease.

The ELISPOT assays used to evaluate patients' immune responses in the trials reported here, and used by many other investigators to evaluate other vaccines, should be considered first-generation biomarkers. The same ELISPOT assay was used in the trials reported here for consistency and comparison of results from one trial to another. Most ELISPOT assays to date, however, have used 9-mer peptides, which represent only one $CD8^+$ epitope, and only the immune responses of patients with one HLA allele (HLA-A2) can be evaluated. Recent studies have begun to use numerous 15-mer peptides, which span the entire region of the tumor antigen being evaluated. This approach will identify both CD4 and CD8 responses and will not be restricted to evaluating a patient with a particular allele. The use of 15-mer peptides, however, is costly and the concentration of each individual peptide in the assay is limited. In addition to ELISPOT assays, fluorescence-activated cell sorting (FACS)-based assays are being used to evaluate intracellular cytokines and activation markers in response to antigens, as are FACS-based assays using 10 or more color lasers to evaluate numerous immune cell subsets pre- and post-therapy. This approach is being used to determine whether a given patient has the potential to benefit from a particular vaccine therapy, and to determine early in the vaccine regimen whether specific immune cells are being activated. It is unclear at this time, however, whether any immune phenomenon will be a true surrogate for patient benefit. This is due to the complexity of the immune system and the heterogeneous nature of a given patient population in terms of prior and/or current therapy, age, etc. At this time, any one or combination of the above assays can at best define trends in correlation with patient

responses. As more clinical studies demonstrate clinical efficacy, better immune correlations with clinical efficacy may become apparent.

These preliminary immune data from more than 100 patients treated with PSA-TRICOM suggest clear evidence of immune responses to PSA in the majority of patients post-vaccination. Although no surrogates for clinical efficacy have been identified, further detailed analyses of immune endpoints and correlation with clinical endpoints are prospectively designed into the ongoing 1,200-patient randomized, controlled clinical trial of PSA-TRICOM (8).

Disclosure of Potential Conflicts of Interest

J. Schlom has commercial research grant from Cooperative Research and Development Agreement with BN ImmunoTherapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J.L. Gulley, J. Hodge, S.M. Steinberg, C.R. Heery, J. Schlom
Development of methodology: K.Y. Tsang, B. Farsaci, J. Hodge, C.R. Heery
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Gulley, R.A. Madan, C. Jochems, J.L. Marte, J. Hodge, C.R. Heery
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Gulley, R.A. Madan, K.Y. Tsang, C. Jochems, J.L. Marte, B. Farsaci, J.A. Tucker, J. Hodge, D.J. Liewehr, S.M. Steinberg, J. Schlom
Writing, review, and/or revision of the manuscript: J.L. Gulley, R.A. Madan, C. Jochems, J.L. Marte, J.A. Tucker, J. Hodge, D.J. Liewehr, S.M. Steinberg, C.R. Heery, J. Schlom
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Gulley, J.L. Marte, J. Hodge
Study supervision: J.L. Gulley

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