

Immunotherapy for Prostate Cancer— Recent Progress in Clinical Trials

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Abstract: Prostate cancer is the most common malignancy affecting men in the United States. Traditional therapy with radical prostatectomy or radiation therapy can be curative for localized disease, but metastatic prostate cancer is currently incurable. The only treatments known to prolong survival in patients with metastatic disease are androgen-deprivation therapy and chemotherapy, both of which have significant side effects. Immunotherapy approaches offer hope in providing new treatments to delay disease progression, ideally with fewer side effects. The results from nearly all early immunotherapy clinical trials for prostate cancer conducted to date have shown minimal toxicity, and many have suggested clinical benefit in terms of delaying disease progression. Several phase III clinical trials are currently under way in patients with metastatic, androgen-independent prostate cancer. The current article reviews recent trials evaluating immune-modulating agents, antigen-specific active immunotherapy, and combination therapies in clinical development for the treatment of prostate cancer.

Prostate cancer is the most common malignancy to affect men in the United States. Traditional therapy with radical prostatectomy or radiation therapy can be curative, but approximately 1/3 of patients will experience disease recurrence, typically heralded by a rise in levels of serum prostate-specific antigen (PSA).^{1,2} Although the minority of patients with this biochemical recurrence progress to overt clinical disease,³ treatment for these patients is currently limited to androgen deprivation, which is associated with significant morbidity. It is difficult to distinguish which patients with biochemical failure will develop clinically significant disease, making the decision to treat patients with biochemical failure even more challenging.⁴ Results from several studies have suggested that the rate of rise of the serum PSA (doubling time) may be useful in this setting to identify patients at greatest risk for disease progression.⁵ Treatment of advanced, metastatic disease includes androgen deprivation and chemotherapy for androgen-independent disease. These agents have many potential side effects.

Keywords

Prostate cancer, immunotherapy, vaccines, antibody, cytokine

Immunotherapies constitute treatments that supply or elicit immune system cells, antibodies, or cytokines for the treatment of cancer. These types of approaches, already in common use for several solid tumor types, offer hope in replacing or complementing the current armamentarium of treatments for advanced prostate cancer, in particular given that these treatments may have significantly fewer side effects. These therapies may be ideal in patients with biochemical recurrence only, due to the low burden of disease, possibly avoiding the adverse effects associated with androgen deprivation. Recent clinical trials have shown that treatments designed to elicit or enhance tumor-specific immune responses, whether by immune-modulating agents or vaccines, are safe, well tolerated, and capable of eliciting immune responses against prostate cancer. In some cases, some clinical benefit has been observed. Many immunotherapy approaches are in various stages of preclinical development. The current review focuses on recent trials evaluating immune modulating agents, antigen-specific active immunotherapy, and combination therapies in clinical development for the treatment of prostate cancer.

Immunomodulating Agents

Granulocyte-Macrophage Colony-Stimulating Factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of a large family of glycoprotein growth factors that act on multiple levels of hematopoietic cell differentiation and development. It has been shown to stimulate the proliferation of granulocytes and macrophages, promote antibody-dependent cell-mediated cytotoxicity of neutrophils, attract and enhance the cytotoxicity of eosinophils, and promote the differentiation and survival of peripheral dendritic cells (DCs).⁶⁻⁸ Given the multiple effects of GM-CSF on several components of the immune system, several groups have investigated it as a vaccine adjuvant to enhance immune responses to targeted antigens. Other groups have evaluated GM-CSF given alone as a pure immunomodulatory agent. For example, the efficacy of GM-CSF in stimulating an antitumor effect in hormone-refractory prostate cancer has recently been investigated by Small and colleagues, who conducted a phase II trial in patients with hormone-refractory prostate cancer.⁹ One cohort of 23 men received 250 $\mu\text{g}/\text{m}^2/\text{day}$ GM-CSF subcutaneously for the first 14 days of a 28-day cycle. An oscillatory effect of PSA coinciding with the administration of GM-CSF was observed, leading to the start of a second cohort to receive continuous GM-CSF. This cohort had 13 patients who received 250 $\mu\text{g}/\text{m}^2/\text{day}$ GM-CSF for 14 days, followed by 250 $\mu\text{g}/\text{m}^2/\text{day}$ 3 days per week until disease progression was observed. PSA levels initially decreased

in 12 of 13 patients, but a decrease of over 50% was seen in only 1 patient at 6 weeks. This patient showed an improved bone scan and maintained a 99% decrease in PSA for more than 14 months.⁹

In a separate trial, Dreicer and associates evaluated the effect of GM-CSF in patients with androgen-dependent or -independent metastatic prostate cancer. They enrolled 16 patients in a phase II study to receive 250 $\mu\text{g}/\text{m}^2/\text{day}$ GM-CSF three times a week for up to 6 months. Three of the 9 androgen-independent and 4 of the 6 androgen-dependent patients completed the trial and did not show progression of their disease after 6 months of GM-CSF therapy.¹⁰

Rini and coauthors further evaluated the efficacy of GM-CSF at 250 $\mu\text{g}/\text{m}^2/\text{day}$ for 14 days of a 28-day cycle in 29 patients who had nonmetastatic disease but increasing PSA despite prior definitive therapy.¹¹ They initially demonstrated a persistent decrease in PSA of at least 50% in 3 patients, and an increase in PSA doubling time in the other 25 evaluable patients, with a mean increase of the cohort's PSA doubling time from 8.4 months pretreatment to 15 months posttreatment. Sixteen patients had a 2-fold increase in their PSA doubling time. Seventeen patients were eventually removed from protocol therapy for progressive disease with increasing PSA, at a mean treatment time of 15.2 months. At the time of publication, 8 patients remained on protocol with treatment duration ranging from 20 to 32 months.¹¹

In these trials, several patients met standard response criteria for PSA response,^{12,13} and many others had evidence of prolonged disease stabilization. Although the exact mechanism of action of GM-CSF as an antitumor agent in these trials is currently unknown, theoretically its use should increase the number or efficacy of tumor-specific T cells. To investigate this, Schwaab and coworkers determined whether PSA-specific T-cell responses were detectable before and after GM-CSF administration. No statistical increase in PSA-specific T-cell response was found. However, a statistically significant correlation between pretreatment PSA and level of PSA-specific CD4-positive T-cell response was found, as well as a positive correlation between pretreatment PSA and the level of PSA-specific CD8-positive T-cell response, suggesting a correlation between disease response and the inherent T-cell response.¹⁴ Furthermore, the difference between the administration of 125 $\mu\text{g}/\text{m}^2/\text{day}$ versus 250 $\mu\text{g}/\text{m}^2/\text{day}$ of GM-CSF was investigated, and a dose-dependent response to GM-CSF administration was found, with 8% of patients in the 125 μg group having a PSA decrease of at least 25%, compared to 7 of 14 patients in the 250 μg group.¹⁴ Further evaluation of the mechanism of action of GM-CSF treatment is clearly needed, and randomized trials designed to evaluate the efficacy of GM-CSF

in delaying disease progression are certainly indicated. Moreover, these results have suggested that GM-CSF could be combined with other tumor therapies. Dreicer and colleagues have recently reported a pilot study in which 22 patients with advanced prostate cancer were treated with GM-CSF in combination with thalidomide (Thalomid, Celgene).¹⁵ All 22 patients initially responded to the therapy at 2 weeks with a decrease in their PSA, and 5 sustained a greater than 50% decline from baseline at 4 weeks.¹⁵

Flt3 Ligand

Flt3 ligand is a hematopoietic growth factor that has been shown to stimulate the growth and differentiation of DCs.^{16,17} Systemic administration of flt3 ligand has been demonstrated in multiple murine tumor models to elicit antitumor responses, presumably by eliciting or augmenting antitumor immune responses.¹⁸⁻²⁰ The use of flt3 ligand in stimulating an immune response against prostate cancer has been investigated in a double-blind phase II trial of 31 patients with hormone-refractory prostate cancer.²¹ In cycle 1, patients were randomized to receive either placebo (n=16) or 25 µg/kg flt3 ligand (n=15) for the first 14 days of a 28-day cycle. For the following cycles, 25 µg/kg flt3 ligand was administered daily for 14 days of a 28-day cycle to all patients. All 31 patients completed three cycles of therapy. Overall, the therapy was well tolerated, with only injection-site reactions occurring more frequently in the experimental group compared to the placebo group during the first cycle. Following six cycles, a 29-fold increase in the number of DCs was observed in the 21 remaining patients. Additionally, though there was no significant change in serum PSA levels, a statistically significant decrease in the PSA velocity was observed (from 0.007/day to 0.002/day), and 11 patients had stable disease with either a decrease or minor increase in PSA (<25%).²¹ These results suggest that flt3 ligand can stimulate DCs *in vivo* and this may result in anti-prostate tumor reactivity. Given the effects of flt3 ligand on antigen-presenting cells, which are similar to GM-CSF, flt3 ligand could be further investigated alone or as a vaccine adjuvant.²²

Anti-CTLA-4

It is believed that the activation of T lymphocytes requires at least two signals, a specificity signal involving binding of the T-cell receptor to the peptide-major histocompatibility complex (MHC) and a signal from costimulatory molecules, including CD80 or CD86 binding CD28. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is a receptor present on T cells that competes for binding of CD80 or CD86 and provides a negative regulatory role in T-cell activation.²³ Blockade of CTLA-4 binding by

monoclonal antibodies has been shown in several murine models to enhance antitumor immunity, and to enhance T-cell immunity induced by vaccines.²⁴⁻²⁶ A monoclonal antibody specific for blocking human CTLA-4, ipilimumab (MDX-010, Medarex), has been tested in patients with a variety of different cancers and in patients with cancer previously treated with vaccines.²⁷⁻²⁹ Multiple antitumor objective responses have been observed, and multiple autoimmune breakthrough events have also been observed, including dermatitis, enterocolitis, hepatitis, uveitis, and hypophysitis.³⁰ These autoimmune events demonstrate efficacy of this agent in deregulating normal immune tolerance. Trials conducted in patients with hormone-refractory prostate cancer, alone or in combination with GM-CSF, have similarly shown antitumor responses.^{31,32} An ongoing trial using ipilimumab in combination with a GM-CSF-transfected allogeneic cellular vaccine (GVAX, Cell Genesys) has preliminarily shown striking objective disease responses in patients with hormone-refractory prostate cancer.³³ The use of CTLA-4 blockade, either alone or in conjunction with other anticancer agents, is a particularly promising area for further clinical development.

Active Immunotherapies—Vaccines

In contrast to broad amplification of immune responses with systemic immunomodulating agents, active immunotherapy seeks to target the immune response against a specific antigen or group of antigens. Additionally, active immunization should elicit a memory response, potentially creating a longer-lasting antitumor response than that limited by the duration of immunomodulating agent or passive antibody administration. Vaccines targeting several specific protein antigens, including PSA, prostatic acid phosphatase (PAP), and prostate-specific membrane antigen (PSMA), as well as whole-cell vaccines and combination therapies, have been investigated.

Antigen-targeted Vaccines—PSA

PSA is a protease produced by columnar epithelial cells in the prostate. Production of this protease in the prostate occurs following exposure to androgens.^{34,35} Release into the body occurs as a result of disruption of prostate architecture, such as with benign prostatic hypertrophy and prostate cancer.³⁶ The expression of PSA by most prostate cancer cells makes it an obvious candidate as a target for immunotherapy. Preclinical studies have demonstrated that cytotoxic T-cell immune responses specific for PSA can be cultured from peripheral blood cells in some patients with prostate cancer, and that these can lyse prostate cancer cells.³⁷⁻³⁹ These findings have provided a rationale to elicit and/or augment similar responses in

vivo by means of vaccination. A potential disadvantage of therapies targeting PSA, however, is that this confounds the interpretation of serum PSA levels as a marker of tumor response from these therapies.

Protein-based Immunization A series of phase I/II trials conducted by Jenner Biotherapies were among the first trials investigating PSA as a target antigen. In these trials, recombinant PSA protein was delivered in a lipid adjuvant, OncoVax-P, by a variety of routes and with a series of immunomodulatory agents (bacillus Calmette-Guérin [BCG], GM-CSF, interleukin [IL]-2, and cyclophosphamide).⁴⁰ PSA-specific T cells were identified in some patients, but few clinical responses were observed. There has been no further clinical development of this approach.

Peptide-based Vaccines Several MHC class I peptide epitopes derived from the amino acid sequence of PSA have been identified. Most of these preclinical studies focused on peptides restricted to HLA-A2.^{37-39,41} Clinical trials have now been conducted evaluating immunization with some of these peptides directly or with autologous DCs pulsed with these peptides. Perambakam and colleagues have reported a clinical trial in which 28 patients with locally advanced or metastatic prostate cancer were immunized three times with either an HLA-A2–restricted PSA epitope (PSA 146–154) and GM-CSF, or with autologous DCs primed with this peptide.⁴² A delayed-type hypersensitivity response was generated in 50% of the patients, and isolated CD8-positive T cells were capable of lysing PSA-expressing cells.⁴² These results suggest that vaccination with PSA peptides is capable of generating a cytotoxic immune response. Further studies are needed to evaluate clinical responses following vaccination approaches with PSA-derived peptides.

Viral Vector Vaccines Vaccinia virus is a DNA orthopox virus that multiplies in the cell's cytoplasm, resulting in infected cells expressing peptides from virally derived proteins in MHC class I molecules,⁴³ stimulating a vigorous cell-mediated response against the antigenic proteins.^{44,45} Additionally, vaccinia virus has the ability to carry a large genome, making it ideal for gene delivery requiring transduction of multiple genes or genes encoding large proteins.^{46,47} Early studies demonstrated that recombinant vaccinia virus expressing a target antigen can elicit antigen-specific cytolytic T-cell responses.⁴⁸ Similar approaches in primates demonstrated that T-cell responses specific for PSA could be elicited by immunization with recombinant vaccinia viruses expressing PSA (rV-PSA).⁴⁹

The ability to deliver the PSA antigen as a vaccine antigen with vaccinia was first investigated in human

trials by Sanda and colleagues.⁵⁰ In this trial, 6 patients were enrolled with increasing PSA following definitive therapy and were initially treated with limited androgen deprivation. Then, rV-PSA was administered 1 week after stopping androgen-deprivation therapy. PSA and testosterone were checked weekly for 6 weeks and monthly thereafter. Minimal toxicity was reported. Initial tests showed the presence of IgM anti-PSA at baseline in 5 of the 6 patients tested, and IgG anti-PSA antibodies in 2 of the 6. One patient developed anti-PSA IgG antibodies over the course of treatment. Following the removal of antiandrogen therapy, PSA rose within 2 months of testosterone increase in all but 1 patient, in whom PSA did not increase after more than 8 months of testosterone exposure. These results demonstrated the feasibility of using removal of androgen deprivation as a model to study prostate cancer and suggested that rV-PSA could be safely administered.⁵⁰

The ability to deliver rV-PSA multiple times has been investigated in at least two clinical trials.^{51,52} Eder and associates reported a trial in which patients with progressive prostate cancer received increasing doses and multiple vaccinations with rV-PSA.⁵¹ Specifically, patients were given three doses of rV-PSA at 4-week intervals with 2.65×10^6 plaque-forming units (pfu; n=6), 2.65×10^7 pfu (n=6), or 2.65×10^8 pfu (n=11). An additional 10 patients received 2.65×10^8 pfu with 250 $\mu\text{g}/\text{m}^2$ GM-CSF administered at the site of vaccination -1, 0, 1, and 2 days postvaccination. Minimal toxicity was seen, with most patients experiencing injection-site reactions. One out of 33 tested patients developed anti-PSA antibodies by enzyme-linked immunosorbent assay (ELISA) after three vaccinations. Of 7 patients tested with enzyme-linked immunosorbent spot (ELISPOT) for evidence of a PSA-specific T-cell response, an increase of at least 2-fold in PSA-specific T-cell response was observed in 5 patients, with the largest increase following the first administration of the vaccine. PSA levels did not increase more than 80% (defined as stable) for at least 6 months after initial immunization in 14 of 33 patients, with 9 patients stable more than 11 months. Six patients remained on study with stable PSA at the time of publication, all treated at the highest dose, 4 of whom received GM-CSF. Additionally, the patients that remained on study had not shown any evidence of advanced disease. Gulley and coworkers reported a similar dose-escalation trial in which patients with metastatic androgen-independent prostate cancer received rV-PSA three times at 4-week intervals (2.65×10^5 pfu, 2.65×10^6 pfu, 2.65×10^7 pfu, or 2.65×10^8 pfu).⁵² A fifth cohort similarly received 2.65×10^8 pfu with 100 $\mu\text{g}/\text{day}$ GM-CSF administration on -1, 0, 1, and 2 days postvaccination. The vaccine was generally well tolerated, with 1 patient

exhibiting two grade 4 toxicities, and 3 patients exhibiting grade 3 toxicities. The most common toxicity was injection-site reactions, which decreased with each round of vaccination. No objective tumor responses were observed. No antibody response to PSA was observed, however 3 of 5 patients tested by ELISPOT showed a greater than 2-fold increase in PSA-specific T-cell response.⁵² Taken together, these results demonstrated the safety of repeated immunization with rV-PSA, and suggested the efficacy of including GM-CSF as an adjuvant. However, failure of the repeat immunizations to enhance the T-cell response suggested that an immune response generated against the vaccinia vector itself might preclude boosting an immune response to the targeted antigen.⁵¹ Moreover, the disappointing results in patients with metastatic androgen-independent disease, potentially due to the high tumor burden and/or poor immunologic function of these individuals, suggested that patients with earlier stage disease should be preferentially evaluated in future studies with this approach.

To overcome the potential difficulties with repetitive immunizations with vaccinia virus, vaccinations with fowlpox virus containing PSA (rF-PSA) have been investigated. In contrast to vaccinia, few patients have pre-vaccination exposure to fowlpox virus, resulting in fewer patients with existing immune responses to the fowlpox vector. Additionally, fowlpox does not infect, replicate, and then reinfect other cells, as does vaccinia, but rather infects and expresses its transgene products for 14–21 days. The inability to reinfect other cells likely leads to a reduced risk of sensitization to the fowlpox vector itself. Kaufman and coauthors have reported a multisite phase II study that evaluated the tolerability and efficacy of prime-boost methods utilizing both rV-PSA and rF-PSA.⁵³ Seventy patients were randomized to receive four vaccines with rF-PSA (group A), three rF-PSA vaccines followed by one rV-PSA (group B), or one rV-PSA followed by three rF-PSA (group C) at 6-week intervals. Subjects received 2.34×10^8 pfu rV-PSA or 1.5×10^9 pfu rF-PSA. Only mild toxicity was observed, with the most common reported adverse events being injection-site reactions and hyperglycemia. The trial was not powered to detect differences in progression rates among the arms, however, after 19 months of follow-up, 45% of patients in all treatment groups were free of PSA progression, and 78% were free of objective disease progression. The median time free of PSA progression was 13.6 months in group A, 9.3 months in group B, and not reached in group C. No antibody responses to PSA were generated in any patients; however, nearly all patients receiving rV-PSA developed antibodies against vaccinia virus. T-cell responses to PSA were evaluated in HLA-A2-expressing individuals by ELISPOT and detected in 46% of 34 patients tested. This study demonstrated the safety of

using vaccinia and fowlpox in a prime-boost approach, and though not powered to detect a difference, suggested that vaccination with rV-PSA followed by rF-PSA may be superior to other methods.⁵³

In addition to GM-CSF, other vaccine adjuvants have been explored in combination with viral vaccines. Specifically, preclinical studies have demonstrated that viral vectors encoding three T cell costimulatory molecules—B7-1, ICAM-1, and ILFA-3 (TRICOM)—along with a target antigen are superior to viral vectors encoding the antigen alone in eliciting antigen-specific T-cell responses.⁵⁴ The safety and feasibility of this approach has recently been demonstrated in patients with prostate cancer by DiPaola and colleagues in a phase I trial evaluating the safety of vaccinia (PROSTVAC-V) and fowlpox (PROSTVAC-F) vectors encoding PSA and TRICOM.⁵⁵ Ten patients with androgen-independent prostate cancer with or without metastases were given 2×10^8 PROSTVAC-V and a booster of 2×10^9 PROSTVAC-F 4 weeks later. No grade 3 or 4 toxicities were reported. The most common adverse reactions were fatigue and injection-site reactions. Four patients demonstrated stable PSA.⁵⁵ At present, two large prospective randomized trials testing this approach with systemic GM-CSF are about to open in the Eastern Cooperative Oncology Group in patients with rising PSA after definitive treatment, or in patients with early androgen-independent disease.

DNA Vaccines DNA vaccines are similar to viral vector vaccines in terms of mechanism of action, and have been demonstrated in animal models to elicit both cellular and humoral immune responses to the targeted antigen.⁵⁶ A potential advantage is that no foreign viral genes need be expressed, reducing the possibility of generating an immune response against the vector. However, DNA vaccines are less efficient than viral vaccines in transfecting host cells, and hence are likely less immunogenic.⁵⁶ A single trial has evaluated the efficacy and safety of a DNA plasmid encoding PSA in which patients with rising PSA after definitive treatment received 100 μ g, 300 μ g, or 900 μ g doses of plasmid DNA administered five times at 4-week intervals.⁵⁷ At each injection, 40 μ g GM-CSF was given for 3 days starting 2 days prior to vaccination and 75 μ g IL-2 was given for 7 days following vaccination. The therapy was well tolerated with adverse events consisting of fever, chills, rhinorrhea, and injection-site reactions such as erythema and swelling. Two patients receiving the highest vaccination dose developed a strong PSA-specific T-cell response, as well as a strong anti-PSA IgG response. The development of a PSA-specific T-cell response correlated with a decrease in the slope of change in PSA.⁵⁷ The low toxicity observed in this study, and the immune response generated, suggest that further studies are warranted.

DC Vaccines The central role of DCs in priming an immune response⁵⁸ has led to a multitude of trials using DCs to directly deliver a target antigen, either as protein, peptide, or nucleic acid.⁵⁹ In vitro studies have shown that DCs derived from prostate cancer patients and transfected with mRNA encoding PSA are potent stimulators of an antigen-specific cytotoxic T-cell response.⁶⁰ These results have led to a small study of 13 patients in which autologous DCs were loaded with PSA-encoding mRNA.⁶¹ The investigators observed a decreased velocity of change in PSA and increased PSA-specific T-cell responses in all patients. Additionally, the PSA-primed DCs showed increased in vitro killing of PSA-expressing cells and transient clearance of circulating prostate cancer cells. Further trials are anticipated.

Antigen-targeted Vaccines—PAP

PAP is another prostate-specific antigen expressed in both normal and malignant prostate cells.⁶² Similar to PSA, it is an androgen-regulated, secreted protein. Given that it is essentially prostate-specific in expression, it is an attractive target for vaccine approaches, particularly as it leaves serum PSA as a potential measure of tumor response. Previous studies have demonstrated that T-cell responses specific for PAP can be identified in patients with prostate cancer,^{63,64} and CD8-positive T-cell epitopes that are able to lyse prostate cancer cells have similarly been identified.^{65,66} These studies, as well as studies in preclinical rat models where there is a homologous prostate-specific PAP antigen,⁶⁷⁻⁶⁹ have formed the basis for clinical investigations targeting this antigen.

Antigen-presenting-cell Vaccines Fong and colleagues demonstrated that rats immunized with a vaccine encoding the human homolog of PAP developed prostatitis.⁶⁸ They subsequently performed a phase I trial in which 21 patients with recurrent and/or metastatic prostate cancer were immunized with autologous DCs loaded with murine PAP protein (mPAP). Patients were divided into three groups to receive the vaccination intravenously, intramuscularly, or intralymphatically at 4-week intervals. No significant toxicity was observed. Six patients developed stable PSA measurements following vaccination and had radiographically stable disease, and 11 of 21 developed a human PAP (hPAP)-specific proliferative T-cell response. All 6 patients with stable PSA developed an hPAP-specific T-cell response, but only 5 of 15 patients with progressive disease had a T-cell response. Four patients developed antibodies against both mPAP and hPAP; however, there was no association between antibody development and clinical outcome.⁷⁰

Studies conducted by Dendreon Corporation have evaluated a similar approach using autologous antigen-

presenting cells prepared by density centrifugation from peripheral blood mononuclear cells, and pulsed ex vivo with a fusion protein containing hPAP and GM-CSF (PA2024). In two phase I/II studies evaluating different schedules of administration in patients with different stages of disease, antigen T-cell proliferation in response to vaccination was observed.^{71,72} In both trials, adverse events were uncommon, with fever and chills being the most common events occurring soon after cell infusion.⁷¹ In terms of clinical responses, 3 patients in one phase I/II trial had a PSA decrease of over 50%, and 3 others had stable PSA.⁷¹ In a follow-on phase II trial conducted at the Mayo Clinic, 1 patient experienced a radiographic complete response with a reduction in PSA to undetectable levels.⁷³ Together, these data provided the foundation for two phase III placebo-controlled randomized clinical trials (D9901 and D9902) designed initially to evaluate as a primary endpoint the time to disease progression in patients with asymptomatic, androgen-independent prostate cancer. In the first study, D9901, 127 patients were randomized to receive three infusions, 2 weeks apart, of PA2024-pulsed autologous antigen-presenting cells (sipuleucel-T; n=82) or placebo (nonpulsed autologous antigen-presenting cells; n=45).⁷⁴ No significant adverse events were observed. A 1.7-week delay in time to progression was observed, but it did not reach statistical significance. However, a statistically significant 4.5-month improvement in median survival, as well as improved survival at 36 months (34% vs 11%) following treatment with sipuleucel-T, was observed. The median survival time of 25.9 months observed in treated patients compared favorably to two other contemporary randomized trials of treatment with mitoxantrone versus docetaxel (Taxotere, Sanofi-Aventis), with median survival times of less than 20 months.^{75,76} Thus, though the D9901 study failed to achieve its primary endpoint, it demonstrated a clinical benefit for patients possibly exceeding that achieved with current therapy and with far less toxicity. Confirmatory studies are underway, and trials comparing this approach to chemotherapy are anticipated.⁷⁴

DNA Vaccines A DNA vaccine encoding PAP has been investigated in animals, demonstrating that PAP-specific CD4- and CD8-positive T-cell responses can be elicited without adverse events.^{69,77} A phase I trial with this approach is currently underway.⁷⁸

Antigen-targeted Vaccines—PSMA

PSMA is a transmembrane glycoprotein found on the surface of many tissues throughout the body.⁷⁹ Due to its presence on the cell surface, generation of an anti-PSMA humoral response with immunization may be effective in decreasing or reversing disease progression, in contrast to

the cytotoxic immune response likely required for immunotherapeutic strategies targeting intracellular or secreted antigens.⁸⁰ Additionally, in contrast to other antigens such as PSA, PSMA expression is increased in higher grade and androgen-independent tumors and is associated with a worse prognosis.⁸¹⁻⁸³ These characteristics make PSMA an attractive, biologically relevant antigen for investigation with targeted immunologic therapy.

Genetic Vaccines DNA vaccines against PSMA have recently been investigated in mice. Vaccination with plasmids encoding human PSMA (hPSMA), but not murine PSMA (mPSMA), stimulated an immune response against denatured mPSMA.⁸⁴ Similar studies are currently underway in human clinical trials. Viral delivery of PSMA, with or without booster immunizations, with a DNA-plasmid-encoding PSMA, has also been investigated in a single phase I/II trial in 26 patients with various stages of prostate cancer.⁸⁵ The authors report the development of delayed-type hypersensitivity responses, but there has been no further development of this approach.

DC Vaccines A phase I trial investigated HLA-A2-restricted peptides derived from PSMA (PSM-P1 or -P2), used to prime autologous DCs, and delivered to patients as a vaccine.⁸⁶ In this trial, 51 patients with hormone-refractory prostate cancer were treated with peptides alone, DCs alone, or DCs pulsed with one of the peptides. Of the 51 patients, 7 partial responders were identified, 2 of whom received either PSM-P1 or PSM-P2 alone and 5 who received DCs pulsed with peptide.⁸⁶ A follow-up investigation of the 7 responders indicated that 4 of the 7 patients continued to have a partial response for more than 220 days.⁸⁷ Several follow-up studies confirmed the safety of this approach, the identification of peptide-specific T cells resulting from vaccination, and the suggestion of clinical responses in some patients.^{88,89}

Antigen-targeted Vaccines—Other Antigens

Carbohydrate Antigens Cancers often result in the production of aberrant short carbohydrate chains that are expressed on the cell surface. One such carbohydrate antigen is globo H (a hexasaccharide), which has increased expression on the surface of both primary and metastatic prostate cancer.⁹⁰ A dose-escalating phase I trial investigating the safety of immunization with globo H fused to keyhole limpet hemocyanin (KLH) was conducted in 18 patients with relapsed prostate cancer, half of whom had radiographic evidence of bone metastases. Patients were assigned to receive subcutaneous vaccinations (3, 10, 30, or 100 μ g) on weeks 1, 2, 3, 7, and 19, along with the saponin immunologic adjuvant (QS21). The vaccine was well tolerated, with minimal toxicity. All immunized

patients developed a strong IgM response to globo H; however, the 30- μ g dose appeared to be optimal. Antibodies generated were capable of reacting with tumor cells, and induced complement-mediated cell lysis in 50% of the patients. All patients showed rising PSA prior to and during the trial. Of 5 patients without bone metastases, 2 developed a small decrease in the slope of PSA change 3 months after receiving the vaccine. These results have supported further investigation of globo H-KLH vaccines in phase II and multivalent trials.⁹¹

Similar results were found in trials targeting clustered α -*N*-acetylgalactosamine-*O*-serine/threonine (Tn(c)) and the Thomsen-Friedenreich (TF) antigens.^{92,93} Tn(c) is often expressed on the surface of epithelial cell tumors, including expression at high levels in prostate cancer.⁹⁰ Immunization with Tn(c) linked with KLH or palmitic acid (PAM) carrier proteins was investigated in 25 patients with biochemically relapsed prostate cancer in a dose-escalation trial. Patients were given vaccinations at weeks 1, 2, 3, 7, 19, and 50 (doses of 3, 7, or 15 μ g Tn(c)-KLH or 100 μ g Tn(c)-PAM). All patients were given 100 μ g QS21 as an adjuvant with the immunization. No significant adverse events were observed. Of those treated with Tn(c)-PAM, 1 patient had a 50% decrease in the log change of PSA; however, in general, antibody responses against Tn(c) were less in the group receiving the PAM conjugate compared to KLH. Of the 15 patients treated with Tn(c)-KLH, 11 patients (44%) had a 25% increase in the log slope of PSA change, 7 patients (28%) had stable disease, and 5 patients (3 receiving the 3 μ g dose and 2 receiving the 7 μ g dose) had a more than 25% decline in the PSA slopes.⁹² Phase II trials targeting multiple carbohydrate antigens have been conducted and continue to be evaluated.⁹⁴

HER2/neu HER2/neu is a proto-oncogene and an epidermal growth factor tyrosine kinase that is expressed in many epithelial-derived cancers.⁹⁵ HER2/neu has been shown to be expressed in prostate cancer, but its prevalence and the significance of its expression remain unclear. Mounting evidence suggests that HER2/neu expression increases as tumors progress towards androgen-independence, suggesting the HER2/neu may play a role in more aggressive tumors.^{96,97} To date, antibody treatments targeting HER2/neu (eg, trastuzumab [Herceptin, Genentech]) have not been shown to be effective as a sole treatment against HER2/neu-expressing prostate tumors.^{98,99} E75 is an HLA-A2-specific peptide derived from HER2/neu, originally identified as the dominant epitope recognized by tumor-infiltrating cytotoxic T lymphocytes in HLA-A2-positive patients with ovarian cancer.¹⁰⁰ Several trials have been conducted immunizing patients with HER2/neu-expressing tumors with this peptide in

adjuvant. An early study using this approach in patients with prostate cancer demonstrated that no significant immune responses were elicited.⁸⁵ In a more recent trial, 17 HLA-A2–positive patients with HER2/neu–expressing prostate cancer and increased risk of recurrence were vaccinated with 100, 500, or 1000 µg E75 monthly for 6 months. The treatment was well tolerated, and peptide-specific immune responses were observed; however, little clinical benefit was demonstrated.¹⁰¹

Prostate Stem Cell Antigen Prostate stem cell antigen (PSCA) is a membrane protein expressed on more than 80% of prostate cancers, with increased levels found in cells with higher Gleason scores and androgen-independent tumors.¹⁰²⁻¹⁰⁴ In a pilot trial, 10 patients with metastatic, androgen-independent prostate cancer were immunized with autologous DCs pulsed with HLA-A2 epitopes derived from PSCA.¹⁰⁵ In this trial, delayed-type hypersensitivity responses were observed in 5 of 10 patients. This response was associated with prolonged survival, as well as a reduced rate of PSA increase, decrease in objective lymph node disease, and improved bone pain.¹⁰⁵ Further studies to investigate the efficacy of this vaccine in patients with a lower tumor burden are anticipated.

Vaccination With Multiple Specific Antigens Vaccines targeting a single antigen have several potential drawbacks, including the absence of potential therapeutic immunity if a tumor does not present, or stops presenting, the target antigen. The lack of response seen in some trials may in part be due to failure of the tumor to express the antigen being targeted. Consequently there is great interest in defining multiple antigens and evaluating immunization strategies targeting multiple antigens.¹⁰⁶ As discussed above, this is currently being investigated in multivalent carbohydrate antigen vaccine trials.⁹⁴ In addition, other groups have investigated DCs vaccines loaded with peptides from multiple antigens. In a pilot trial of patients with prostate cancer immunized with autologous DCs loaded with peptides derived from PSA, PSMA, survivin, prostein, and transient receptor potential p8, there was identification of T cells reactive to PSMA, survivin, and prostein.¹⁰⁷ A similar trial has evaluated autologous DCs pulsed with peptides derived from PSCA, PAP, PMSA, and PSA.¹⁰⁸ Further trials with appropriate clinical endpoints are anticipated.

Non-antigen-targeted Vaccines

Whole-cell Vaccines Some of the earliest studies in animal models of tumor immunology investigated the ability to immunize rodents with syngeneic tumor cells to confer protection from subsequent tumor challenge. More recently, vaccination studies conducted in rats with

allogeneic whole-cell irradiated prostate cancer cells have demonstrated protection against challenge from lethal doses of prostate cancer, suggesting crossreactivity with shared antigens using allogeneic cell lines.¹⁰⁹ This has provided the rationale for similar trials in patients with prostate cancer. In a study reported by Eaton and colleagues, 60 patients with hormone-refractory prostate cancer were divided into four groups and vaccinated with irradiated, allogeneic prostate cancer cell lines (Pr1-4, Onyvac) at 2-week intervals for four vaccinations, followed then by monthly immunizations.¹¹⁰ *Mycobacterium vaccae* was given with each vaccination as an adjuvant. Whereas the initial vaccine and 2-week boosters contained one of the four prostate cancer cell lines, the monthly doses contained three of the four allogeneic lines. A different combination of strains was given to each of the four experimental groups. Little toxicity was observed. No patients showed a sustained PSA during the course of treatment. Several patients exhibited transient decreases in PSA, not necessarily associated with the vaccine treatment itself, but 3 had transient decreases in their PSA without other concurrent therapy. T-cell proliferation was seen following in vitro stimulation with any of the four prostate cancer strain lines, suggesting the immunologic response generated by the vaccination was potent against antigens shared among cell lines.¹¹⁰ In a similar trial, 28 patients with hormone-refractory nonmetastatic prostate cancer were immunized intradermally with a vaccine composed of three irradiated allogeneic cell lines, OnyCap23, LnCaP, and P4E6.¹¹¹ Immunizations were given with BCG as adjuvant at 2-week intervals for the first three vaccines and then monthly. The most common adverse events were injection-site reactions. PSA velocity decreased in 42% of patients, with an increase in the mean PSA doubling time from 26 to 58 weeks. The change in PSA kinetics appeared to be associated with a Th1-type production of cytokines following vaccination.¹¹¹ Based on these results, further evaluation of whole-cell vaccines are anticipated.

GM-CSF–secreting Tumor Cell Vaccines GM-CSF has been shown to augment the immune response against whole-cell vaccines when used in combination. In pre-clinical studies, irradiated B16 melanoma cells (incapable of eliciting a protective immune response alone) were transfected with viruses encoding a variety of cytokines. Cells transfected to express GM-CSF increased the inflammatory response generated at the vaccination site significantly compared to nontransfected cells, resulted in protection from subsequent tumor challenge, and had an antitumor response in tumor-bearing animals.¹¹² Similar results have been demonstrated in other preclinical models. The first application of this approach to patients with prostate cancer was reported by Simons and colleagues.¹¹³

They reported a phase I trial in which 11 patients with metastatic prostate cancer had autologous prostate cancer cells harvested, cultured, transfected *ex vivo* with replication-deficient retrovirus encoding GM-CSF, irradiated, and delivered intradermally as a vaccine.¹¹³ Of the 11 patients entered in the trial, only 8 were able to be treated due to the difficulty obtaining sufficient amounts of tumor tissue for transduction. Three patients developed antibodies recognizing prostate-tumor antigens. Adverse events observed included injection-site reactions, fevers, chills, and malaise. The approach, however, was not felt to be feasible due to the difficulty of culturing autologous cells *ex vivo*, thus precluding possible evaluation in patients with less advanced disease.¹¹³ These investigators have more recently reported the results of a phase I/II trial in which 21 patients with nonmetastatic prostate cancer with rising serum PSA after definitive therapy were treated intradermally weekly for 8 weeks with allogeneic cells (LNCaP and PC3 prostate cancer cell lines) transfected to express GM-CSF and irradiated.¹¹⁴ The authors report that 16 of 21 patients demonstrated a decrease in PSA velocity after vaccination, with 1 patient demonstrating a PSA response of 7 months' duration. Antibody responses elicited to cell line–derived antigens were also detected.¹¹⁴ A similar open-label phase II trial conducted in patients with androgen-independent prostate cancer has suggested that patients treated with GM-CSF gene-transduced allogeneic prostate cancer cell lines (GVAX) may have prolonged overall survival.^{115,116} Given these encouraging findings, two prospectively randomized phase III trials are currently underway to evaluate this approach, both with the primary endpoint of overall survival. The first trial (VITAL-1) is enrolling patients with asymptomatic, castrate-resistant prostate cancer and is comparing treatment with GVAX to docetaxel plus prednisone. The second trial (VITAL-2) is enrolling patients with symptomatic, castrate-resistant prostate cancer and is comparing treatment with GVAX plus docetaxel to docetaxel plus prednisone.

Protein- and mRNA-loaded DC Vaccines DCs have also been used to deliver antigens from allogeneic prostate cancer cell lines. Pandha and colleagues have described a pilot clinical trial of 11 patients with hormone-refractory prostate cancer treated with autologous DCs pulsed with lysates from allogeneic prostate cancer cell lines.¹¹⁷ This study demonstrated safety and evidence of an immune response elicited with vaccination. Further studies are expected. Similarly, it has been demonstrated in preclinical studies that mRNA from autologous tumors could be used to transfect DCs and elicit tumor-specific cytolytic T-cell responses.¹¹⁸ This general approach has been tested in a pilot trial in which 19 patients with androgen-inde-

pendent prostate cancer were treated with four vaccines consisting of 2×10^7 autologous DCs transfected with mRNA from three human prostate cancer cell lines.¹¹⁹ Immunizations were given weekly for at least 4 weeks. Ten patients received the vaccine directly into lymph nodes and others received the vaccine intradermally. The treatment was well tolerated and no events greater than grade 1 were observed. Of 19 patients tested, 10 showed an increased T-cell response following *in vitro* stimulation with prostate cancer cells. This response declined over the course of several weeks but was stimulated with boosting. No improvements were noted in bone metastases as seen by bone scan; however, 13 of 19 patients showed a decrease in the log slope of PSA change postvaccination, with 11 patients meeting the criteria for stable PSA levels. These encouraging results suggest that vaccination with mRNA transfected DCs is safe and, given the change in PSA velocity, warrants further investigation.¹¹⁹

Combination Treatments

The absence of significant adverse events with most of the agents described above has suggested that immunotherapies could readily be coupled with other antitumor agents. Many of these were discussed above, including combinations with GM-CSF and anti-CTLA-4. Other combinations are highlighted below.

Immunotherapy and Bevacizumab

Bevacizumab (Avastin, Genentech) is a monoclonal antibody targeting vascular endothelial growth factor, a proangiogenic factor that also inhibits the function of antigen-presenting cells.¹²⁰ As described above, infusion of autologous antigen-presenting cells loaded with PAP-GM-CSF fusion protein (sipuleucel-T) has been shown to induce an immune response.⁷¹⁻⁷⁴ To investigate whether bevacizumab could be combined with sipuleucel-T to augment immune responses, 22 patients with nonmetastatic, recurrent prostate cancer were treated with sipuleucel-T at weeks 0, 2, and 4 with bevacizumab 10 mg/kg intravenously given every 2 weeks until disease progression. Six grade 3 events were reported resulting in the withdrawal of 4 patients from the trial. Nine patients were evaluated for evidence of antigen-specific T-cell proliferation and found to have an increased response postvaccination. The greatest increase was observed in the patient with the largest PSA response. One patient had a partial PSA response that was durable for 88 weeks. Three had a decrease of at least 25%, and 9 had minor decreases in serum PSA. Six patients had at least a 200% increase in PSA doubling time, and 4 had an increase between 100% and 200%.¹²¹ The toxicities observed were greater than what has been seen with other trials using sipuleucel-T.

In addition, the treatment response to bevacizumab alone is not yet known in this population. These points notwithstanding, the use of bevacizumab will likely be tested in future trials with this vaccine and others.

Vaccines and Radiotherapy

Although vaccines may address systemic disease, their efficacy may be limited in the face of a large local tumor burden. Conversely, intense local treatment, such as radiation therapy, is beneficial in addressing local tumor burden, but has no efficacy for systemic disease. The combination of these two therapies was investigated in 30 patients with proven prostate adenocarcinoma who were eligible for external-beam radiation therapy (EBRT).¹²² Patients were randomized to receive either at least 70 Gy EBRT with rV-PSA and costimulatory molecule B7 (n=19) or radiation therapy only (n=11). Subsequent vaccinations were performed monthly with rF-PSA, for a total of eight vaccines. Subjects received 100 µg GM-CSF subcutaneously on days 1–4 of a 28-day cycle, and each vaccination was performed on day 2. A dose of 4 MIU/m² IL-2 was then administered on days 8–12. Therapy was well tolerated, and 19 of 21 patients received all eight vaccinations. Seventeen of 19 patients developed IFN-α-secreting T cells upon stimulation with PSA postvaccination; however, a decreased number of cells was detected immediately following radiation therapy. Thirteen of the 19 vaccinated patients had a greater than 3-fold increase in the T-cell stimulatory response. No increase in T-cell response was noted in the radiation-only treatment group. T-cell responses were also detected to other prostate-associated antigens, including PSMA, PAP, PSCA, and mucin 1, implying in vivo killing and presentation of other tumor antigens.¹²² These results suggest that combining radiation therapy and a vaccine is safe and generates an immune response specific for not only the antigen being targeted, but also against antigens presented by the tumor itself. These encouraging results warrant further testing in a larger randomized trial.

Vaccines and Chemotherapy Docetaxel was recently approved for the treatment of androgen-independent metastatic prostate cancer based on the results from two large randomized trials demonstrating a survival benefit in this population.^{75,76} Given these findings, and studies which suggest that taxanes themselves may be beneficial in generating tumor-associated immune responses,^{123,124} a pilot trial has recently been reported investigating a combination of these approaches.¹²⁵ Twenty-eight patients with progressive, metastatic, androgen-independent prostate cancer were treated with either poxvirus vaccines encoding PSA or vaccines plus docetaxel. Similar to other trials, the initial vaccine was rV-PSA followed by booster vac-

cines with rF-PSA given 2 weeks apart for the first month and monthly thereafter. A dose of 100 µg of GM-CSF was given for 4 days following vaccination. Patients treated with docetaxel plus vaccine were given docetaxel with dexamethasone one day prior to vaccination beginning in the second month of therapy. Following progression in patients treated with vaccination only, patients were given docetaxel. No significant toxicity was observed. T-cell proliferative studies demonstrated similar immune response rates in both study groups. A decline in serum PSA was observed in 3 of 14 patients treated with vaccine alone, none over 50%. In the vaccine plus docetaxel group, 6 of 14 patients had a decline in PSA levels, with 3 patients decreasing more than 75%. Patients treated with the combination had a median time to progression of 3.1 months, whereas those treated with vaccination alone progressed with a median time of 1.8 months. Patients who failed vaccination alone and received docetaxel following failure had a median time to progression of 6.1 months.¹²⁵ At this point it is unclear whether there is an advantage in combining these treatments, but certainly there was no identifiable disadvantage in delaying chemotherapy in these patients. As described above, other vaccines are being investigated in combination with chemotherapy, and many studies are ongoing to evaluate the effects of different chemotherapy agents and the timing with respect to immunization schedules. It is likely that this will remain an active area of clinical trials research.

Conclusion

Prostate cancer is a significant disease affecting millions of men in the United States. Metastatic prostate cancer is an incurable illness and is the major cause of death from prostate cancer. The only treatments known to potentially prolong survival in patients with metastatic disease are androgen deprivation and chemotherapy. Both of these treatments have significant side effects. The results from nearly all early clinical trials using immunotherapy approaches for the treatment of prostate cancer have shown minimal toxicity, and many have suggested clinical benefit in terms of delaying disease progression. Several phase III clinical trials are currently under way in patients with metastatic, androgen-independent prostate cancer. Several other trials have suggested that immunotherapy approaches can be safely, and perhaps beneficially, integrated with other therapies. These treatments offer challenges to the design of clinical trials where traditional endpoints such as reduction in tumor volume and PSA declines by a certain percentage in patients with large-volume disease may be less relevant. The results of

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ongoing large trials, and the results of trials combining therapies with treatments designed to decrease immunosuppressive responses, are eagerly awaited. Taken together, these findings offer hope to patients with prostate cancer that new therapies with favorable benefit-to-risk ratios are on the horizon.

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References

- Khan MA, Han M, Partin AW, et al. Long-term cancer control of radical prostatectomy in men younger than 50 years of age: update 2003. *Urology*. 2003;62:86-91; discussion 91-82.
- Djavan B, Moul JW, Zlotta A, et al. PSA progression following radical prostatectomy and radiation therapy: new standards in the new Millennium. *Eur Urol*. 2003;43:12-27.
- Ward JE, Blute ML, Slezak J, et al. The long-term clinical impact of biochemical recurrence of prostate cancer 5 or more years after radical prostatectomy. *J Urol*. 2003;170:1872-1876.
- Polascik TJ, Oesterling JE, Partin AW. Prostate specific antigen: a decade of discovery: what we have learned and where we are going. *J Urol*. 1999;162:293-306.
- Slovin SF, Wilton AS, Heller G, et al. Time to detectable metastatic disease in patients with rising prostate-specific antigen values following surgery or radiation therapy. *Clin Cancer Res*. 2005;11:8669-8673.
- Weisbart RH, Golde DW, Clark SC, et al. Human granulocyte-macrophage colony-stimulating factor is a neutrophil activator. *Nature*. 1985;314:361-363.
- Weller PF. Cytokine regulation of eosinophil function. *Clin Immunol Immunopathol*. 1992;62:S55-59.
- Markowicz S and Engleman EG. Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro. *J Clin Invest*. 1990;85:955-961.
- Small EJ, Reese DM, Um B, et al. Therapy of advanced prostate cancer with granulocyte macrophage colony-stimulating factor. *Clin Cancer Res*. 1999;5:1738-1744.
- Dreicer R, See WA, Klein EA. Phase II trial of GM-CSF in advanced prostate cancer. *Invest New Drugs*. 2001;19:261-265.
- Rini BI, Weinberg V, Bok R, et al. Prostate-specific antigen kinetics as a measure of the biologic effect of granulocyte-macrophage colony-stimulating factor in patients with serologic progression of prostate cancer. *J Clin Oncol*. 2003;21:99-105.
- Scher HI, Eisenberger M, D'Amico AV, et al. Eligibility and outcomes reporting guidelines for clinical trials for patients in the state of a rising prostate-specific antigen: recommendations from the Prostate-Specific Antigen Working Group. *J Clin Oncol*. 2004;22:537-556.
- Bubley GJ, Carducci M, Dahut W, et al. Eligibility and Response Guidelines for Phase II Clinical Trials in Androgen-Independent Prostate Cancer: Recommendations From the Prostate-Specific Antigen Working Group. *J Clin Oncol*. 1999;17:3461-3467.
- Schwaab T, Tretter CP, Gibson JJ, et al. Tumor-related immunity in prostate cancer patients treated with human recombinant granulocyte monocyte-colony stimulating factor (GM-CSF). *Prostate*. 2006;66:667-674.
- Dreicer R, Klein EA, Elson P, et al. Phase II trial of GM-CSF + thalidomide in patients with androgen-independent metastatic prostate cancer. *Urol Oncol*. 2005;23:82-86.
- Maraskovsky E, Brasel K, Teepe M, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med*. 1996;184:1953-1962.
- Maraskovsky E, Daro E, Roux E, et al. In vivo generation of human dendritic cell subsets by Flt3 ligand. *Blood*. 2000;96:878-884.
- Chen K, Braun S, Lyman S, et al. Antitumor activity and immunotherapeutic properties of Flt3-ligand in a murine breast cancer model. *Cancer Res*. 1997;57:3511-3516.
- Ciavarrà RP, Somers KD, Brown RR, et al. Flt3-ligand induces transient tumor regression in an ectopic treatment model of major histocompatibility complex-negative prostate cancer. *Cancer Res*. 2000;60:2081-2084.
- Esche C, Subbotin VM, Maliszewski C, et al. FLT3 ligand administration inhibits tumor growth in murine melanoma and lymphoma. *Cancer Res*. 1998;58:380-383.
- Higano CS, Vogelzang NJ, Sosman JA, et al. Safety and biological activity of repeated doses of recombinant human Flt3 ligand in patients with bone scan-negative hormone-refractory prostate cancer. *Clin Cancer Res*. 2004;10:1219-1225.
- McNeel DG, Knutson KL, Schiffman K, et al. Pilot study of an HLA-A2 peptide vaccine using flt3 ligand as a systemic vaccine adjuvant. *J Clin Immunol*. 2003;23:62-72.
- Krummel MF and Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med*. 1995;182:459-465.
- Leach DR, Krummel MF and Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*. 1996;271:1734-1736.
- Kwon ED, Hurwitz AA, Foster BA, et al. Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc Natl Acad Sci U S A*. 1997;94:8099-8103.
- Hurwitz AA, Foster BA, Kwon ED, et al. Combination immunotherapy of primary prostate cancer in a transgenic mouse model using CTLA-4 blockade. *Cancer Res*. 2000;60:2444-2448.
- Hodi FS, Mihm MC, Soiffer RJ, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci U S A*. 2003;100:4712-4717.
- Korman AJ, Peggs KS, Allison JP. Checkpoint blockade in cancer immunotherapy. *Adv Immunol*. 2006;90:297-339.
- Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*. 2003;100:8372-8377.
- Blansfield JA, Beck KE, Tran K, et al. Cytotoxic T-lymphocyte-associated antigen-4 blockade can induce autoimmune hypophysitis in patients with metastatic melanoma and renal cancer. *J Immunother*. 2005;28:593-598.
- Davis TA, Tchekmedyian S, Korman A, et al. MDX-010 (human anti-CTLA4): A phase I trial in hormone refractory prostate carcinoma (HRPC). *Proc Am Soc Clin Oncol*. 2002;21:19a (abstr 74).
- Fong L, Kavanaugh B, Rini BI, et al. A phase I trial of combination immunotherapy with CTLA-4 blockade and GM-CSF in hormone-refractory prostate cancer. *Proc Am Soc Clin Oncol*. 2006;24:102s. Abstract 2508.
- Gerritsen W, Van Den Eertwegh AJ, De Gruijl T, et al. A dose-escalation trial of GM-CSF-gene transduced allogeneic prostate cancer cellular immunotherapy in combination with a fully human anti-CTLA antibody (MDX-010, ipilimumab) in patients with metastatic hormone-refractory prostate cancer (mHRPC). *Proc Am Soc Clin Oncol*. 2006;24:100s. Abstract 2500.
- Young CY, Montgomery BT, Andrews PE, et al. Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. *Cancer Res*. 1991;51:3748-3752.
- Henttu P, Liao SS, Vihko P. Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. *Endocrinology*. 1992;130:766-772.
- Stamey TA, Yang N, Hay AR, et al. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N Engl J Med*. 1987;317:909-916.
- Correale P, Walmsley K, Nieroda C, et al. In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. *J Natl Cancer Inst*. 1997;89:293-300.
- Alexander RB, Brady F, Leffell MS, et al. Specific T cell recognition of peptides derived from prostate-specific antigen in patients with prostate cancer. *Urology*. 1998;51:150-157.
- Xue BH, Zhang Y, Sosman JA, et al. Induction of human cytotoxic T lymphocytes specific for prostate-specific antigen. *Prostate*. 1997;30:73-78.
- Meidenbauer N, Harris DT, Spitzer LE, et al. Generation of PSA-reactive effector cells after vaccination with a PSA-based vaccine in patients with prostate cancer. *Prostate*. 2000;43:88-100.
- Correale P, Walmsley K, Zaremba S, et al. Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligopeptide. *J Immunol*. 1998;161:3186-3194.
- Perambakam S, Hallmeyer S, Reddy S, et al. Induction of specific T cell immunity in patients with prostate cancer by vaccination with PSA146-154 peptide. *Cancer Immunol Immunother*. 2006;55:1033-1042.

43. Moss B, Smith GL, Gerin JL, et al. Live recombinant vaccinia virus protects chimpanzees against hepatitis B. *Nature*. 1984;311:67-69.
44. Hodge JW, McLaughlin JP, Kantor JA, et al. Diversified prime and boost protocols using recombinant vaccinia virus and recombinant non-replicating avian pox virus to enhance T-cell immunity and antitumor responses. *Vaccine*. 1997;15:759-768.
45. Bernards R, Destree A, McKenzie S, et al. Effective tumor immunotherapy directed against an oncogene-encoded product using a vaccinia virus vector. *Proc Natl Acad Sci U S A*. 1987;84:6854-6858.
46. Moss B. Vaccinia virus: a tool for research and vaccine development. *Science*. 1991;252:1662-1667.
47. Mackett M, Smith GL, Moss B. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. *J Virol*. 1984;49:857-864.
48. Bennink JR, Yewdell JW, Smith GL, et al. Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. *Nature*. 1984;311:578-579.
49. Hodge JW, Schlom J, Donohue SJ, et al. A recombinant vaccinia virus expressing human prostate-specific antigen (PSA): safety and immunogenicity in a non-human primate. *Int J Cancer*. 1995;63:231-237.
50. Sanda MG, Smith DC, Charles LG, et al. Recombinant vaccinia-PSA (PROSTVAC) can induce a prostate-specific immune response in androgen-modulated human prostate cancer. *Urology*. 1999;53:260-266.
51. Eder JP, Kantoff PW, Roper K, et al. A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer. *Clin Cancer Res*. 2000;6:1632-1638.
52. Gulley J, Chen AP, Dahut W, et al. Phase I study of a vaccine using recombinant vaccinia virus expressing PSA (rV-PSA) in patients with metastatic androgen-independent prostate cancer. *Prostate*. 2002;53:109-117.
53. Kaufman HL, Wang W, Manola J, et al. Phase II randomized study of vaccine treatment of advanced prostate cancer (E7897): a trial of the Eastern Cooperative Oncology Group. *J Clin Oncol*. 2004;22:2122-2132.
54. Hodge JW, Sabzevari H, Yafal AG, et al. A triad of costimulatory molecules synergize to amplify T-cell activation. *Cancer Res*. 1999;59:5800-5807.
55. Dipaola R, Plante M, Kaufman H, et al. A Phase I Trial of Pox PSA vaccines (PROSTVAC(R)-VF) with B7-1, ICAM-1, and LFA-3 co-stimulatory molecules (TRICOMtrade mark) in Patients with Prostate Cancer. *J Transl Med*. 2006;4:1.
56. Liu MA. DNA vaccines: a review. *J Intern Med*. 2003;253:402-410.
57. Pavlenko M, Roos AK, Lundqvist A, et al. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. *Br J Cancer*. 2004;91:688-694.
58. Steinman R. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. 1991;9:271-296.
59. Gilboa E, Nair SK, Lyerly HK. Immunotherapy of cancer with dendritic-cell-based vaccines. *Cancer Immunol Immunother*. 1998;46:82-87.
60. Heiser A, Dahm P, Yancey DR, et al. Human dendritic cells transfected with RNA encoding prostate-specific antigen stimulate prostate-specific CTL responses in vitro. *J Immunol*. 2000;164:5508-5514.
61. Heiser A, Coleman D, Dannull J, et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. *J Clin Invest*. 2002;109:409-417.
62. Cunha AC, Weigle B, Kiessling A, et al. Tissue-specificity of prostate specific antigens: comparative analysis of transcript levels in prostate and non-prostatic tissues. *Cancer Lett*. 2006;236:229-238.
63. McNeel DG, Nguyen LD, Ellis WJ, et al. Naturally occurring prostate cancer antigen-specific T cell responses of a Th1 phenotype can be detected in patients with prostate cancer. *Prostate*. 2001;47:222-229.
64. McNeel DG, Nguyen LD, Disis ML. Identification of T helper epitopes from prostatic acid phosphatase. *Cancer Res*. 2001;61:5161-5167.
65. Peshwa MV, Shi JD, Ruegg C, et al. Induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes in vitro using antigen-presenting cells pulsed with prostatic acid phosphatase peptide. *Prostate*. 1998;36:129-138.
66. Machlenkin A, Paz A, Bar Haim E, et al. Human CTL epitopes prostatic acid phosphatase-3 and six-transmembrane epithelial antigen of prostate-3 as candidates for prostate cancer immunotherapy. *Cancer Res*. 2005;65:6435-6442.
67. Roiko K, Janne OA and Vihko P. Primary structure of rat secretory acid phosphatase and comparison to other acid phosphatases. *Gene*. 1990;89:223-229.
68. Fong L, Ruegg CL, Brockstedt D, et al. Induction of tissue-specific autoimmune prostatitis with prostatic acid phosphatase immunization; implications for immunotherapy of prostate cancer. *J Immunol*. 1997;159:3113-3117.
69. Johnson LE, Frye TP, Arnot AR, et al. Safety and immunological efficacy of a prostate cancer plasmid DNA vaccine encoding prostatic acid phosphatase (PAP). *Vaccine*. 2006;24:293-303.
70. Fong L, Brockstedt D, Benike C, et al. Dendritic cell-based xenotantigen vaccination for prostate cancer immunotherapy. *J Immunol*. 2001;167:7150-7156.
71. Small EJ, Fratesi P, Reese DM, et al. Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. *J Clin Oncol*. 2000;18:3894-3903.
72. Burch PA, Breen JK, Buckner JC, et al. Priming tissue-specific cellular immunity in a phase I trial of autologous dendritic cells for prostate cancer. *Clin Cancer Res*. 2000;6:2175-2182.
73. Burch PA, Croghan GA, Gastineau DA, et al. Immunotherapy (APC8015, Provenge) targeting prostatic acid phosphatase can induce durable remission of metastatic androgen-independent prostate cancer: a Phase 2 trial. *Prostate*. 2004;60:197-204.
74. Small EJ, Schellhammer PF, Higano CS, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. *J Clin Oncol*. 2006;24:3089-3094.
75. Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med*. 2004;351:1502-1512.
76. Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med*. 2004;351:1513-1520.
77. Johnson LE, Frye TP, Chinnasamy N, et al. Plasmid DNA vaccine encoding prostatic acid phosphatase (PAP) is effective in eliciting autologous antigen-specific CD8+ T cells. *Canc Immunol Immunother*. 2007;(in press).
78. Zlotocha S, Staab MJ, Horvath D, et al. A phase I study of a DNA vaccine targeting prostatic Acid phosphatase in patients with stage D0 prostate cancer. *Clin Genitourin Cancer*. 2005;4:215-218.
79. Kinoshita Y, Kuratsukuri K, Landas S, et al. Expression of prostate-specific membrane antigen in normal and malignant human tissues. *World J Surg*. 2006;30:628-636.
80. Horoszewicz JS, Kawinski E, Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res*. 1987;7:927-935.
81. Bostwick DG, Pacelli A, Blute M, et al. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. *Cancer*. 1998;82:2256-2261.
82. Murphy GP, Kenny GM, Ragde H, et al. Measurement of serum prostate-specific membrane antigen, a new prognostic marker for prostate cancer. *Urology*. 1998;51:89-97.
83. Wright GL, Jr., Grob BM, Haley C, et al. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology*. 1996;48:326-334.
84. Gregor PD, Wolchok JD, Turaga V, et al. Induction of autoantibodies to syngeneic prostate-specific membrane antigen by xenogeneic vaccination. *Int J Cancer*. 2005;116:415-421.
85. Mincheff M, Tchakarov S, Zoubak S, et al. Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: a phase I/II clinical trial. *Eur Urol*. 2000;38:208-217.
86. Murphy G, Tjoa B, Ragde H, et al. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *Prostate*. 1996;29:371-380.
87. Tjoa BA, Erickson SJ, Bowes VA, et al. Follow-up evaluation of prostate cancer patients infused with autologous dendritic cells pulsed with PSMA peptides. *Prostate*. 1997;32:272-278.
88. Tjoa BA, Simmons SJ, Bowes VA, et al. Evaluation of phase I/II clinical trials in prostate cancer with dendritic cells and PSMA peptides. *Prostate*. 1998;36:39-44.
89. Tjoa BA, Simmons SJ, Elgamil A, et al. Follow-up evaluation of a phase II prostate cancer vaccine trial. *Prostate*. 1999;40:125-129.
90. Zhang S, Zhang HS, Reuter VE, et al. Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. *Clin Cancer Res*. 1998;4:295-302.
91. Slovin SF, Ragupathi G, Adluri S, et al. Carbohydrate vaccines in cancer: immunogenicity of a fully synthetic globo H hexasaccharide conjugate in man. *Proc Natl Acad Sci U S A*. 1999;96:5710-5715.

92. Slovin SF, Ragupathi G, Musselli C, et al. Fully synthetic carbohydrate-based vaccines in biochemically relapsed prostate cancer: clinical trial results with alpha-N-acetylgalactosamine-O-serine/threonine conjugate vaccine. *J Clin Oncol*. 2003;21:4292-4298.
93. Slovin SF, Ragupathi G, Musselli C, et al. Thomsen-Friedenreich (TF) antigen as a target for prostate cancer vaccine: clinical trial results with TF cluster (c)-KLH plus QS21 conjugate vaccine in patients with biochemically relapsed prostate cancer. *Cancer Immunol Immunother*. 2005;54:694-702.
94. Slovin SF, Ragupathi G, Fernandez C, et al. A bivalent conjugate vaccine in the treatment of biochemically relapsed prostate cancer: a study of glycosylated MUC-2-KLH and Globo H-KLH conjugate vaccines given with the new semi-synthetic saponin immunological adjuvant GPI-0100 OR QS-21. *Vaccine*. 2005;23:3114-3122.
95. Meric F, Hung MC, Hortobagyi GN, et al. HER2/neu in the management of invasive breast cancer. *J Am Coll Surg*. 2002;194:488-501.
96. Signoretti S, Montironi R, Manola J, et al. HER2-neu expression and progression toward androgen independence in human prostate cancer. *J Natl Cancer Inst*. 2000;92:1918-1925.
97. Craft N, Shostak Y, Carey M, et al. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER2/neu tyrosine kinase. *Nat Med*. 1999;5:280-285.
98. Morris MJ, Reuter VE, Kelly WK, et al. HER2 profiling and targeting in prostate carcinoma. *Cancer*. 2002;94:980-986.
99. Ziada A, Barqawi A, Glode LM, et al. The use of trastuzumab in the treatment of hormone refractory prostate cancer; phase II trial. *Prostate*. 2004;60:332-337.
100. Fisk B, Blevins TL, Wharton JT, et al. Identification of an immunodominant peptide of HER2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med*. 1995;181:2109-2117.
101. Hueman MT, Dehqanzada ZA, Novak TE, et al. Phase I clinical trial of a HER2/neu peptide (E75) vaccine for the prevention of prostate-specific antigen recurrence in high-risk prostate cancer patients. *Clin Cancer Res*. 2005;11:7470-7479.
102. Gu Z, Thomas G, Yamashiro J, et al. Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene*. 2000;19:1288-1296.
103. Reiter RE, Gu Z, Watabe T, et al. Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc Natl Acad Sci U S A*. 1998;95:1735-1740.
104. Han KR, Seligson DB, Liu X, et al. Prostate stem cell antigen expression is associated with gleason score, seminal vesicle invasion and capsular invasion in prostate cancer. *J Urol*. 2004;171:1117-1121.
105. Thomas-Kaskel AK, Zeiser R, Jochim R, et al. Vaccination of advanced prostate cancer patients with PSCA and PSA peptide-loaded dendritic cells induces DTH responses that correlate with superior overall survival. *Int J Cancer*. 2006;119:2428-2434.
106. Dunphy EJ, Johnson LE, Olson BM, et al. New approaches to identification of antigenic candidates for future prostate cancer immunotherapy. *Update Canc Ther*. 2006;22:273-284.
107. Fuessel S, Meyer A, Schmitz M, et al. Vaccination of hormone-refractory prostate cancer patients with peptide cocktail-loaded dendritic cells: results of a phase I clinical trial. *Prostate*. 2006;66:811-821.
108. Waeckerle-Men Y, Uetz-von Allmen E, Fopp M, et al. Dendritic cell-based multi-epitope immunotherapy of hormone-refractory prostate carcinoma. *Cancer Immunol Immunother*. 2006;55:1524-1533.
109. Hrouda D, Todryk SM, Perry MJ, et al. Allogeneic whole-tumour cell vaccination in the rat model of prostate cancer. *BJU Int*. 2000;86:742-748.
110. Eaton JD, Perry MJ, Nicholson S, et al. Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate cancer. *BJU Int*. 2002;89:19-26.
111. Michael A, Ball G, Quatan N, et al. Delayed disease progression after allogeneic cell vaccination in hormone-resistant prostate cancer and correlation with immunologic variables. *Clin Cancer Res*. 2005;11:4469-4478.
112. Dranoff G, Jaffee E, Lazenby A, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A*. 1993;90:3539-3543.
113. Simons JW, Mikhak B, Chang JF, et al. Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. *Cancer Res*. 1999;59:5160-5168.
114. Simons JW, Carducci MA, Mikhak B, et al. Phase I/II trial of an allogeneic cellular immunotherapy in hormone-naive prostate cancer. *Clin Cancer Res*. 2006;12:3394-3401.
115. Small EJ, Higano C, Smith D, et al. A phase 2 study of an allogeneic GM-CSF gene-transduced prostate cancer cell line vaccine in patients with metastatic hormone-refractory prostate cancer (HRPC). *Proc Am Soc Clin Oncol*, Prostate Cancer Symposium. 2005;# 280.
116. [No_authors_listed]. Cell Genesys reports long-term survival data in Phase II trial of GVAX. *Expert Rev Anticancer Ther*. 2002;2:245-246.
117. Pandha HS, John RJ, Hutchinson J, et al. Dendritic cell immunotherapy for urological cancers using cryopreserved allogeneic tumour lysate-pulsed cells: a phase I/II study. *BJU Int*. 2004;94:412-418.
118. Heiser A, Maurice MA, Yancey DR, et al. Induction of polyclonal prostate cancer-specific CTL using dendritic cells transfected with amplified tumor RNA. *J Immunol*. 2001;166:2953-2960.
119. Mu LJ, Kyte JA, Kvalheim G, et al. Immunotherapy with allotumour mRNA-transfected dendritic cells in androgen-resistant prostate cancer patients. *Br J Cancer*. 2005;93:749-756.
120. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med*. 1996;2:1096-1103.
121. Rini BI, Weinberg V, Fong L, et al. Combination immunotherapy with prostatic acid phosphatase pulsed antigen-presenting cells (provenge) plus bevacizumab in patients with serologic progression of prostate cancer after definitive local therapy. *Cancer*. 2006;107:67-74.
122. Gulley JL, Arlen PM, Bastian A, et al. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. *Clin Cancer Res*. 2005;11:3353-3362.
123. Chan OT, Yang LX. The immunological effects of taxanes. *Cancer Immunol Immunother*. 2000;49:181-185.
124. Mason K, Staab A, Hunter N, et al. Enhancement of tumor radioresponse by docetaxel: Involvement of immune system. *Int J Oncol*. 2001;18:599-606.
125. Arlen PM, Gulley JL, Parker C, et al. A randomized phase II study of concurrent docetaxel plus vaccine versus vaccine alone in metastatic androgen-independent prostate cancer. *Clin Cancer Res*. 2006;12:1260-1269.