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Human prostate cancer vaccines – proposed mechanisms of action and future combinatorial treatment strategies

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Abstract

Prostate cancer (PCa) is responsible for the deaths of more than 33,000 American men annually. Once PCa has become metastatic there is no curative treatment. Oncologists worldwide are becoming increasingly convinced that alternative therapies to chemotherapy and radical prostatectomy need to be explored. Cancer vaccines (CaVacs) that promote the cancer patient's own immune system to develop a tumor-specific cytotoxic T lymphocyte-mediated immune attack have been investigated in clinical trials with modest yet encouraging results. Here we look at the rationale behind different prostate CaVacs and propose key immune events that are likely to contribute to the efficacy of each vaccine. Finally, we prognosticate upon what improvements may be required to generate more effective CaVacs in the future with full consideration of the mechanisms of action.

Introduction

Prostate cancer (PCa) is the second leading cause of cancer related deaths among men in the United States ¹. Primary therapy involves radiotherapy, radical prostatectomy, cryosurgery or hormonal therapy. Despite these aforementioned therapies, more than one third of patients will experience biochemical recurrence and will ultimately progress to an advanced stage, defined as castrate-resistant prostate cancer (CRPC), for which there is no effective cure. Biochemical recurrence is defined as an increase in serum levels of prostate-specific antigen (PSA) which is usually followed by a slow progression to detectable metastasis (median: 7 years) ². Since it is the metastatic form of PCa that results in the vast majority of patient deaths, it is necessary that any treatment modality be systemic. The current standard treatment for patients with CRPC is the taxane, docetaxel. However, chemotherapeutic drugs only modestly enhance patient survival whilst having a wide range of undesirable side effects ³. There is a dire need for alternative therapies and in the last decade the possibility of using vaccines against PCa has gained momentum to the extent that many clinical trials have been performed to test safety and effectiveness of various cancer vaccine (CaVac)-related approaches. For the most part, these PCa vaccines have been shown to be safe and well tolerated ⁴. Indeed, the results from a randomized phase III trial led to the first therapeutic CaVac, sipuleucel-T (PROVENGE®), to be approved by the United States Food and Drug Administration (FDA). Sipuleucel-T is now available for patients with

asymptomatic or minimally symptomatic metastatic CRPC⁵. In this review we focus on therapeutic PCa vaccines designed to promote adaptive CTL-mediated responses. The roles of passive immunotherapy in PCa as well as vaccines that primarily promote antibody-mediated responses (e.g. TroVax) have been reviewed elsewhere^{6,7}. The primary focus of this review is on the mechanism(s) of action of each PCa vaccine that has reached clinical trials and how awareness of potential mechanisms may be exploited when considering future combinatorial treatments. Only a brief synopsis of the clinical outcomes is provided for most of the current PCa vaccines since most have been recently reviewed in detail elsewhere^{4,8}.

Prostate cancer vaccine principles

There are many reasons why PCa is a good candidate for attack by the adaptive immune system. Firstly, PCa cells are generally slow growing, thus accommodating for the time it takes for an effective immune response to develop⁹. Secondly, the prostate expresses many tissue-specific proteins that can serve as immune targets (detailed below). Thirdly, it has been suggested that the prostate gland is immunologically ignored and therefore heralds the potential for a CaVac-mediated activation of the patient's own prostate-specific T lymphocytes that have not been centrally deleted¹⁰.

Most PCa vaccines, and CaVacs in general, are focused on generating tumor-specific cytotoxic lymphocytes (CTLs). The tumor-specificity is usually determined by a tumor-associated antigen (TAA), used as the key immunogen in the vaccine. Such TAAs may be prostate-specific, mutated, aberrantly expressed or anachronistically expressed. Thus targeting these TAAs should result in death of the tumor cells expressing them and very little damage to healthy tissue outside of the prostate gland. In PCa, many potential TAAs have been identified and can be categorized as either predominantly prostate-specific or, more generally, tumor-associated. These TAAs include prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), prostatic acid phosphatase (PAP), and many others, which have recently been reviewed¹¹.

Dendritic cells (DCs) are key mediators of the immune system and are usually the principle intermediates for CaVacs¹². Alternatively, DCs can be the vaccine vehicle itself¹³. Cross-presentation of tumor antigens is necessary for the generation of antitumor CTL responses and it has been shown *in vivo* that DCs are vital for this process¹⁴. It has long been recognized that DCs need to endocytose an immunogen/TAA in particulate form, or endogenously express the immunogen/TAA in order to favor CTL stimulation over B lymphocyte activation. It is imperative that the DC also simultaneously receive an activation/maturation signal, often generically referred to as a "danger signal"^{15,16}. Danger signals are molecular structures derived from exogenous foreign pathogens or endogenous "stressed" or dying cells that are capable of stimulating DCs to initiate adaptive immune responses. Examples of danger signals include granulocyte-macrophage colony-stimulating factor (GM-CSF), viral or bacterial components, and endogenous proteins, such as high-mobility group box 1 protein (HMGB1), expressed by the host's dying tumor cells¹⁷⁻²⁰.

Viral-based prostate cancer vaccines

One of the potential benefits of viral-based CaVacs is their immunogenicity. Certain viral-based CaVacs possess inherent “danger signals” capable of promoting DC maturation and are particularly potent with adenoviral-based vaccines as an example ¹⁹. In addition, recombinant attenuated viruses, the most common form of viral-based CaVacs, are very efficient at transducing cells *in vivo*, resulting in substantial endogenous expression of encoded cargo, such as TAAs. Finally, viral-based CaVacs have the potential to be manufactured as “off-the-shelf” products (e.g. ProstVac VF) requiring no individualization, as opposed to some other CaVacs, such as the host cell-based sipuleucel-T (see below).

ProstVac VF

ProstVac VF, or PSA-TRICOM, is an optimized heterologous vaccine protocol designed by the National Cancer Institute, which involves a prime and multiple boosts with attenuated vaccinia virus and fowlpox virus respectively ²¹. Both of these recombinant viruses are vectors for DNA encoding human PSA and co-stimulatory proteins CD54, CD58 and CD80. Preclinical studies established that the triad of co-stimulatory proteins, as compared to none, one or two, co-encoded with a TAA, was more effective at stimulating TAA-specific CTL activity and antitumor responses ²². Phase II clinical trials subsequently revealed that ProstVac VF significantly extended patients’ lives over control patients by approximately 9 months and that this benefit was particularly evident in patients with less advanced or less aggressive disease ^{23,24}. It has been suggested by Gulley et al that cancer vaccines for PCa, and for cancers in general, are more likely to benefit patients with less advanced disease ²⁵.

Understanding mechanisms of action of CaVacs is vital when considering future combinatorial approaches. It was shown in one of the ProstVac phase II clinical trials mentioned above that patients with greater PSA-specific T lymphocyte responses trended towards enhanced survival, therefore one can surmise that the vaccine was achieving its desired immunological effect ²⁴. For ProstVac VF, as is the case for most PCa vaccines used in clinical trials to date, the primary mechanism of action can only be deduced from analysis of both clinical and preclinical data. It is quite possible that ProstVac VF for the most part, actually stimulates an antitumor response in an environment of impaired DC function. The vaccinia virus is known to have multiple strategies for evading immune attack that involve impairment of host immune responses ^{26,27}. There is a large amount of conflicting data as to whether vaccinia viruses impair DC maturation ^{28,29} but what appears to be less controversial is that CD4+ T lymphocyte involvement is limited. The vaccinia virus has been shown to inhibit MHC class II presentation therefore abrogating CD4+ T lymphocyte activation ²⁸. Indirect support that ProstVac VF is a poor stimulator of CD4+ T lymphocytes comes from the finding that patients vaccinated with ProstVac VF rarely produce detectable PSA-specific antibodies ²³. Furthermore, the vaccinia virus is capable of infecting mature DCs that are then capable of directly activating CD8+ T lymphocyte responses ³⁰. Two pathways to tumor-specific CD8+ T lymphocyte activation mediated by ProstVac VF are proposed in **schematic 1** that are not necessarily mutually exclusive. Pox virus, such as the vaccinia virus (VV) used in the ProstVac VF prime, is capable of infecting both somatic cells and DCs via macropinocytosis ³¹. Infected cells then co-express recombinant co-

stimulatory proteins (CD54, CD58 and CD80) and MHC class I/PSA antigen. In *pathway 1* it is proposed that VV infects immature, and possibly mature, DCs which then travel to draining lymph nodes and co-express co-stimulatory proteins and MHC class I/PSA where they trigger activation and proliferation of PSA-specific CD8⁺ T lymphocytes through engagement of the TCR and complementary co-stimulatory ligands (CD11a/CD18, CD2 and CD28). These activated PSA-specific CTLs then circulate to sites of metastases and kill tumor cells through induction of programmed cell death (PCD). Through a second proposed pathway, VV infects somatic cells which then directly activate PSA-specific CTLs, and possibly NK cells, and induce PCD of infected somatic cells. The dying cells are then taken up by DCs that cross-present PSA and stimulate PSA-specific CTLs in draining lymph nodes resulting in further PCD of tumor cells. The role of NK cells in ProstVac VF vaccinations has not been investigated but it is likely that some NK-mediated killing of VV-infected somatic cells does occur, possibly via natural cytotoxicity receptors³², which would then indirectly contribute to PSA-specific CTL-mediated activation receptors as depicted in *schematic 1*. It should be noted that there is as yet no strong evidence to support this second pathway and, unlike adenoviral components, it has been widely reported that poxviral components suppress innate activation pathways in DCs^{33,34}.

The need for combining ProstVac VF with agents that target the suppressive arm of the immune response is recognized as important since the success of CaVacs in general is majorly influenced by the effector:regulatory lymphocyte ratio. It is therefore likely that CaVacs will require adjuvant co-delivery aimed at reducing the levels of regulatory T lymphocytes (Tregs) and promoting effector lymphocyte responses. One way of achieving this is to target Tregs using antibodies to negative immunological checkpoint molecules such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1). Whilst results from phase I trials thus far have recently revealed that the combination of ProstVac VF and ipilimumab, a CTLA-4 antagonist, is safe and tolerable for cancer patients³⁵, a potential drawback is that ipilimumab, or any other anti-CTLA-4 agent, could result in aggressive autoimmune responses³⁶. PD-1 possesses homology to CTLA-4 and is being considered as a potentially safer alternative target. A recent phase I clinical trial on a range of cancer patients, including those with CRPC, demonstrated that the human anti-PD-1 antibody, MDX-1106, was well tolerated, but did result in toxicities that included immune-related adverse effects³⁷. It is still too early to make conclusive comparisons between ipilimumab and MDX-1106, however, it would appear that MDX-1106 results in similar immune-related adverse effects to ipilimumab but that these adverse effects are less frequent and less severe. Should targeting of Tregs prove to be efficacious at extending survival of patients vaccinated with ProstVac VF it may prove worthwhile in future to target tumors and draining lymph nodes (at sites of vaccination) with nanoparticulated Treg antagonists, such as the aforementioned ipilimumab or MDX-1106, in combination with ProstVac VF. This would localize the ablation of Treg activity to relevant microenvironments and minimize autoimmune side-effects.

Combining ProstVac VF with strategically administered chemotherapy could ultimately be of significant benefit to PCa patients, however, clinical trials designed to test this combination are yet to be fully realized. Randomized phase II clinical trials on CRPC

patients were performed with a predecessor to ProstVac VF which involved combining docetaxel with vaccinia encoding PSA, admixed with vaccinia encoding CD80, followed by avipox-PSA boosts³⁸. The main finding was that the chemotherapy did not interfere with, and may have even improved, the PSA-specific response. Docetaxel does not preferentially affect immunosuppressive populations and therefore is likely to be enhancing antitumor immune responses by other means. It has recently been reported in a tolerogenic murine model that the combination of a poxviral vaccine, similar to ProstVac VF (CEA/TRICOM), with docetaxel was therapeutically more beneficial than either treatment alone³⁹. It was found that although docetaxel had no effect on Tregs there was a significant enhancement in production of IFN- γ by CD8+ T lymphocytes.

Future treatments designed to maximize the efficacy of the ProstVac VF are required. Passive or active tumor targeting with nanoparticulated chemotherapeutic drugs, or simply using lower doses of chemotherapy, would substantially alleviate side-effects and decrease the detrimental effect that high dose chemotherapy generally has on the antitumor immune response. Additionally, the choice of the chemotherapeutic agent is crucial as there are certain chemotherapeutic drugs that, aside from their direct tumoricidal properties can also promote antitumor immune responses. For instance, cyclophosphamide and 5-fluorouracil are capable of selectively depleting immunosuppressive cellular populations such as Tregs and myeloid derived suppressor cells (MDSCs) respectively^{40,41}. Also, anthracyclines, such as doxorubicin, are known to induce an immunogenic tumor cell death⁴². Support for potentially combining a cocktail of these agents with PCa vaccines in general come from preclinical studies that have noted the synergistic antitumor benefits of combining doxorubicin and cyclophosphamide⁴³.

Ad5-PSA

Although ProstVac VF has progressed furthest in clinical trials, there is mounting evidence that adenoviruses can also act as suitable TAA vectors. Adenoviruses comprise one of the most efficient methods for gene delivery *in vivo* for vaccine applications. Replication-deficient recombinant adenovirus type 5 vectors (Ad5) are strong candidates for the delivery of TAAs since they are efficient at transducing genes to a range of cell types⁴⁴. More significantly, Ad5 and other Ad strains (e.g. Ad35) are also useful adjuvants for recombinant TAA genes due to their inherent ability to potently infect and stimulate DCs^{18,19}.

Administration of Ad5-PSA admixed with the collagen matrix, Gelfoam®, results in both stronger immune responses and protection from high titer anti-adenovirus antibodies, when compared to Ad5-PSA administered alone⁴⁵. A phase I clinical trial was established using PCa patients with evidence of metastatic disease where a correlation was seen between T lymphocyte responses and survival^{46,47}. Should Gelfoam® prove to consistently protect against neutralization by host Ad5 antibodies, Ad5 vectors may be worth further clinical investigation since they have been suggested to be more immunogenic than vaccinia or canarypox vectors⁴⁶. It is suggestive of the immunopotency of Ad5 that the results described above were performed with patients who only received one administration of Ad5-PSA⁴⁷.

Given the tropism for DCs that Ad5 possess, combined with their capacity to induce, maturation of DCs it is likely that the mechanism of PSA-specific CTL activation is through direct stimulation by infected DCs, and probably involving CD4⁺ T lymphocyte help. As outlined in *schematic 2*, Ad5-PSA directly infects immature DCs via a receptor-mediated process¹⁸ resulting in DC maturation¹⁹ and presentation of MHC class I/PSA epitopes. In addition, Ad5-PSA is capable of infecting somatic cells and can be subsequently recognized and killed by NK cells⁴⁸ through an E1A-independent pathway involving NKG2D/stress-induced protein interaction⁴⁹. NK-mediated killing can result in apoptotic bodies being taken up by immature DCs. These apoptotic bodies would comprise viral components capable of promoting DC maturation, as well as tumor antigens to be presented as MHC class II/PSA epitopes. In the lymph node, activated DCs could then trigger the activation and proliferation of PSA-specific CD8⁺ T lymphocytes possibly with the aid of activated CD4⁺ T lymphocytes supplying additional IL-2⁴⁸. PSA-specific CTLs then circulate to tumor sites and kill tumor cells through PCD (4).

In terms of future combinatorial treatments, Ad5-PSA vaccination may be an even stronger candidate for co-administration with antibodies to negative immunological checkpoint proteins than ProstVac VF due to the potentially stronger promotion of CD4⁺ T lymphocyte activation and IL-2 production. In addition, adenovirus cancer vaccines in general are amenable to co-treatment with Toll-like receptor (TLR) agonists unlike pox viruses which tend to block TLR-triggered DC maturation signals^{33,50}. Preclinical studies in a non-prostate cancer model have shown that combining Ad5-TAA with intratumoral doses of the TLR-9 agonist CpG (class B) result in a synergistic therapeutic benefit⁵¹. In addition, co-administration of Ad5-PSA or Ad5-OVA vaccines with a class B CpG produced enhanced destruction of tumors^{52,53}.

Cell-based PCa vaccines

Cell-based CaVacs, in general, can be of at least 2 types (reviewed by⁵⁴). The first of these involves the use of the patient's own antigen presenting cells or DCs that have been transfected with an appropriate TAA, and is a generally more labor intensive procedure. The second treatment strategy involves the use of irradiated allogeneic or autologous cancer cells, or cancer cell lines, that have been transfected with an immunostimulatory cytokine. Here we present examples of each type of cell-based vaccine that have reached clinical trials for PCa patients.

Sipuleucil-T

The first FDA-approved PCa vaccine, sipuleucel-T (Provenge[®], Dendreon), is available for treatment of patients with metastatic CRPC. Sipuleucel-T involves the patient's own peripheral blood mononuclear cells (PBMCs) that have undergone leukapheresis, are enriched for monocytic cells and then transduced with PA2024, a recombinant fusion protein of human PAP and GM-CSF. As treatment, patients receive three infusions of the prepared cells at biweekly intervals. Seminal integrated phase III trials, known as IMPACT, were performed that demonstrated significant but marginal (approximately 4 months) increases in survival^{5,55}. Interestingly, no change in time to disease progression was observed after sipuleucel-T treatment despite the increases in survival time. The lack of

evidence of any measurable tumor regression or reduced tumor burden has left clinicians puzzling over how sipuleucel-T has impacted positively on OS ⁵⁶. It is interesting that the mechanism of action of sipuleucel-T is unknown. A correlation was seen between patients with increased survival benefit and those expressing high titers of anti-PAP antibodies, however an antibody-mediated form of tumor destruction is unlikely considering PAP is not a cell surface protein. It is therefore logical to assume that PAP-specific CTLs are the primary mediators of immune-mediated tumor killing, however, there are no published clinical or preclinical data that affirms sipuleucel-T is capable of this. It would appear that sipuleucel-T is more efficient at generating antibody and T lymphocyte responses to the fusion product PA2024 than to PAP epitopes ⁵⁷. This is not unexpected given that the fusion protein is non-self. Indirect evidence using a rat model, where vaccination with a rat equivalent of sipuleucel-T resulted in the generation of prostatitis, suggests that this vaccine can break immune tolerance to normal self antigen ⁵⁸. Controversially, a recent report has presented previously unpublished data analyses from the IMPACT trials ⁵⁹. This report suggests that the increased survival in the vaccinated group may have been due to the lower survival in the older versus younger patients in the placebo groups. This observed age-dependent adverse impact on OS may be a result of patients > 65 years of age being less capable of replenishing the repeated depletions of circulating mononuclear cells.

Never-the-less, *inschematic 3*, we have provided a proposed mechanism of action for sipuleucel-T by which it may promote it's yet to be proven antitumor effect in PCa patients. Vaccination with sipuleucel-T involves an initial infusion with the transduced antigen presenting cells which are likely to travel to the spleen ⁶⁰ where they secrete PAP-GM-CSF fusion protein. The secreted GM-CSF portion may chemotactically attract splenic DCs and stimulate DC maturation (through autocrine and paracrine GM-CSF function) and presentation of MHC class II/PAP epitopes. These splenic DCs and/or sipuleucel-T APCs may then activate CD4+ T lymphocytes, as suggested from clinical data ⁵⁷. IL-2 from the activated CD4+ T lymphocytes could then promote proliferation of PAP-specific CD8+ T lymphocytes that have been stimulated directly by sipuleucel-T, or by splenic DCs that have been induced to cross-present PAP epitopes by GM-CSF (not pictured). These activated CD8+ T lymphocytes would then be capable of tumor cell killing. In order to enhance the immunopotency of sipuleucel-T in the future it may be required to enhance the numbers or ratio of DCs that are ultimately transfused back into the patients. In addition, a recent systematic review and meta-analysis of 17 clinical trials using DCs as vaccine vectors for prostate cancer suggest that the current route of sipuleucel-T vaccine delivery may not be optimal, and therefore the targeting of lymph nodes as opposed to the spleen may be worth investigating ⁶¹. Finally, combinatorial therapy that down-regulates Treg numbers or function (as described above for ProstVac VF) and/or eliminates tumor suppressive MDSCs would also be worthy of investigation.

GVAX-PCa

GVAX (Cell Genesys) is a generic name encompassing a range of CaVacs that involve whole cell tumor cells transfected with GM-CSF ⁵⁴. Although the patient's own tumor cells can be used, this is considered, as with sipuleucel-T, to be both labor-intensive and limited by cell numbers. Alternatively, established allogeneic cell lines have been in clinical

development in a range of cancers that include colorectal, breast, pancreatic, and PCa. GVAX for PCa (GVAX-PCa) comprises a mixture of two irradiated human PCa cell lines, LNCaP and PC-3, which constitutively express GM-CSF. Phase I/II clinical trials with patients with metastatic CRPC demonstrated safety, immunogenicity and increased survival when patients were vaccinated with GVAX-PCa (intradermally, prime plus multiple boosts) in the presence and absence of radiotherapy ^{62,63}. The principle of GVAX-PCa is that the two tumor cell lines potentiate a wide range of tumor-targeting T lymphocytes, as well as antibodies, with varying specificities. As outlined in *schematic 4* the irradiated allogeneic tumor cells (LNCaP + PC-3) secrete recombinant GM-CSF which potentially has the triple role of chemotactically attracting immature host DCs, inducing DC maturation, and promoting cross-presentation of proteins derived from apoptotic GVAX-PCa cells. Apoptotic bodies from irradiated tumor cells can be taken up by DCs, as well as by B cells. Due to the potential for radiotherapy to induce immunogenic cell death, these apoptotic bodies may promote DC-mediated uptake and DC maturation through the expression of calreticulin and HMGB1 respectively (not illustrated) ^{64,65}. Mature DCs expressing exogenously acquired tumor antigens could then activate both TAA-specific CD4+ T lymphocytes and CD8+ T lymphocytes. The activated CD4+ T lymphocytes may stimulate TAA-specific B lymphocytes and also provide IL-2 to TAA-specific CD8+ T lymphocytes. The TAA-specific antibodies to cell-surface proteins, produced by the activated B lymphocytes, could then induce tumor cell death through ADCC or a complement-mediated pathway. Activated CD8+ T lymphocytes would kill tumor cells by PCD through recognition of MHC class I/TAA epitopes.

As has been reported with the ProstVac VF CaVac, the combination of GVAX-PCa with ipilimumab has been investigated in an open-labelled, non-randomized clinical trial ⁶⁶. The phase I study performed with GVAX-PCa and escalating doses of ipilimumab was partially prompted by results from murine pre-clinical studies in mice that showed synergy between ipilimumab and GM-CSF-secreting melanoma cells ⁶⁷. Additionally, ipilimumab has been shown in two randomized phase III trials to improve survival of patients with metastatic melanoma ^{68,69}. It was discovered that immune-related toxicities, such as hypophysitis (inflammation of pituitary gland) and/or sarcoid alveolitis, were diagnosed in patients receiving high doses of ipilimumab (5 mg/kg). The remaining 16 patients therefore received ipilimumab at 3 mg/kg in combination with GVAX-PCa and it was concluded that this combination was safe and well tolerated by patients. In murine studies using GVAX for melanoma it was shown that co-administration of the TLR-4 agonist, LPS, resulted in enhanced therapeutic outcomes ⁷⁰. Therefore, future vaccinations with GVAX-PCa may benefit from the addition of this or other “danger signals” capable of triggering TLRs.

A significant setback for GVAX-PCa emerged from the events of two phase III trials (VITAL-1 and VITAL-2) which were prematurely terminated due to the findings from a futility analysis for VITAL-1 and due to an imbalance of patient deaths in VITAL-2. In the VITAL-2 study a comparison was made of patients treated with a combination of GVAX-PCa and docetaxel with patients treated with docetaxel and prednisone. Inexplicably, in a routine safety review, the GVAX-PCa/docetaxel arm had recorded 67 deaths compared to 47 deaths for the docetaxel/prednisone arm. It has been argued that these two trials were

entered into too rashly and the FDA has recently lifted a hold it had placed on GVAX-PCa⁷¹.

DNA Vaccines

DNA vaccination has been shown to be effective at promoting cellular immune responses in murine models⁷². Consequently, there have been human clinical trials using prostate TAA-encoding plasmids. Two of the DNA vaccines that have reached clinical trials in PCa patients are described in detail below since these have not been described in recent reviews.

DNA-PAP

In an open label, single institution phase I/II clinical trial, 22 PCa patients with biochemical recurrence but no evidence of metastatic disease were vaccinated intradermally with a plasmid (pTVG-HP) encoding human PAP⁷³. Patients were vaccinated 6 times at fortnightly intervals and, each time, GM-CSF (200 µg) was co-administered. No adverse effects were recorded for all doses of DNA plasmid (max. 1500 µg). Of the 22 patients vaccinated, 7 of them had at least a doubling of PSA doubling time (PSADT). PAP-specific T lymphocyte responses were detected in 10/22 patients. At least a 3-fold increase in PAP-specific CD8+ proliferative T lymphocytes was detected in 3/22 patients. A follow-up of this phase I trial was performed to gain more immunologically-based information that could be of potential use when optimizing vaccination protocols in future clinical trials⁷⁴. It was determined that the more vaccinations given to patients, the greater the likelihood of generating PAP-specific T lymphocyte responses. In addition, it was shown that 6/8 patients with long-term PAP-specific interferon- γ T lymphocyte responses had > 200% increase in PSADT whilst only 1/14 that had no change in PSADT developed detectable responses ($p = 0.001$).

DNA-PSA

In a phase I trial to evaluate safety and feasibility, 9 patients with advanced PCa were given intradermal administrations of a DNA plasmid encoding PSA (pVAX/PSA) ($5 \times$ at 4 week intervals) as well as subcutaneous administrations of GM-CSF ($40 \mu\text{g/day} \times 3$ days) and IL-2 ($75 \mu\text{g/day} \times 7$ days)⁷⁵. The presence of these adjuvants was shown to be beneficial in a preclinical study⁷⁶. Doses of 100 – 900 µg of pVax/PSA were tested and no dose-limiting toxicities were observed and treatment-related side-effects did not surpass WHO grade 2. Nor was there any evidence of auto-immune disease. At the highest dose of pVAX/PSA (900 µg), 3/3 patients had increased levels of PSA-specific IFN- γ -producing T lymphocytes, although in one patient the increase was not significant. Of the patients receiving the highest dose of pVAX/PSA, 2/3 showed a slowing of their PSADT. A trend was noted that these 2 patients gave the best immune responses and exhibited stabilization of disease.

The mechanism of action of these DNA vaccines is outlined in *schematic 5*. Intradermal administration of plasmid DNA encoding a prostate TAA is probably taken up and expressed primarily by specialized dermal DCs, known as Langerhans cells⁷⁷. GM-CSF, which is co-injected with pDNA/TAA, can attract immature Langerhans cells/DCs and induce their maturation⁷⁸, after which they travel to draining lymph nodes. Transduced and

mature Langerhans cells/DCs, with upregulated CD80 and CD86 (induced by GM-CSF ⁷⁹) and co-expressing MHC class I/TAA epitopes, can stimulate TAA-specific CD8+ T lymphocyte activation by interaction with CD28 and TCR respectively. It is likely that some TAA epitopes are expressed in association with MHC class II resulting in activation of CD4+ T lymphocytes that provide further help (IL-2) for TAA-specific CD8+ T lymphocytes which are then capable of killing tumor cells by PCD.

The main concern of using naked plasmid DNA as vaccines is the potential limitation of inefficient transfections. In an attempt to address this, it has been shown that mice vaccinated with pVAX/PSA had significantly improved PSA-specific CD8+ T lymphocyte responses when the vaccination was combined with *in vivo* electroporation⁸⁰. Interestingly, under varying electroporation conditions, there was not a direct correlation between transfection efficiency and cellular immune responses, suggesting that electroporation parameters must be carefully chosen.

Novel vaccine strategies

Most vaccines discussed thus far, the exception being GVAX-PCa, involve the use of one specific TAA as an immunogen. One possible drawback of such an approach is that it may be too restrictive, given that tumors may lose expression of a crucial epitope, rendering the TAA-specific CTL incapable of target recognition therefore compromising effective antitumor activity. Approaches to circumvent this include the use of the *in situ* tumor cell itself as the vaccine thus generating a wide range of potential target TAAs and making it more difficult for the tumor to avoid immune eradication. Tumors can be induced to undergo an immunogenic cell death. Dying cells that release purine nucleotides, such as ATP, can chemotactically attract DCs, whilst expression of calreticulin on the surface of dying cells can promote their uptake by DCs ²⁰. Here we focus on gene-mediated cytotoxic immunotherapy, however the use of oncolytic viruses is another novel strategy that, although strategically different, is based on a similar principle and has been reviewed in detail elsewhere ^{81,82}.

Gene-mediated cytotoxic immunotherapy

The mode of tumor cell death is of importance if we wish to alert the host's immune system to endogenous TAAs expressed by the tumor cells. Chemotherapy, more often than not, causes a non-immunogenic programmed cell death that does not trigger the innate immune system with "danger signals" and thereby avoids the generation of a potentially effective downstream TAA-specific CTL responses. Gene-mediated cytotoxic immunotherapy (GMCI) involves intratumoral delivery of an adenovirus encoding the Herpes virus gene, thymidine kinase (AdV-tk). Transduced cells in the tumor microenvironment expressing thymidine kinase then convert a systemically administered prodrug such as valcyclovir (VCV) or ganciclovir (GCV) into what eventually becomes a nucleotide analog that is toxic to dividing or DNA-repairing cells ^{83,84}. The activated prodrug can then affect neighboring cells through multiple processes collectively known as the local bystander effect ⁸⁵. Importantly, it was subsequently discovered that GMCI also generated an immune mediated systemic bystander effect that attacked metastases and protected against tumor rechallenge (reviewed by ⁸⁶). **Schematic 6** provides a description of a proposed mechanism by which

GMCI promotes antitumor immunity. Tumor cells are transduced with AdV-tk and convert a pro-drug to a toxic drug that induces an immunogenic cell death. Apoptotic tumor cells, possibly through cell-surface expression of calreticulin (not pictured) are taken up by immature DCs and B lymphocytes and then certain tumor components, such as HMGB1, or as yet undefined viral components, contribute to DC maturation. Mature DCs then directly activate tumor antigen-specific CD4⁺ T lymphocytes and CD8⁺ T lymphocytes. The activated CD4⁺ T lymphocytes may further stimulate B lymphocyte activation as well as provide IL-2 for the activated CD8⁺ T lymphocytes. B lymphocytes could produce TAA-specific antibodies to cell-surface proteins that mediate ADCC or complement-mediated tumor cell death (specifics not shown). Activated CD8⁺ T lymphocytes then kill tumor cells through recognition of TAA/MHC class I epitopes.

A phase I/II clinical trial on 23 PCa patients with locally advanced disease was performed prior to prostatectomy⁸⁷. Analysis of the resected prostates of treated patients revealed a highly significant influx of CD8⁺ T lymphocytes into the tumor compared to untreated control patients. Levels of activated CD8⁺ T lymphocytes also rose significantly in the blood. This was in contrast to the changes in levels of CD4⁺ T lymphocytes, NK cells and B lymphocytes. Although there was no significant increase in prognosis for the treated patients, as measured through biochemical recurrence of PSA, valuable information was obtained. This included the finding that GMCI preferentially demonstrated cytopathological effects in cancerous, over benign, tissue. However, the lack of improved prognoses prompted the investigation into therapies that combine standard therapies such as chemotherapy or radiotherapy with GMCI. Radiation and some chemotherapeutic drugs cause DNA damage, increase DNA repair activity and, resultantly, can make cancer cells more sensitive to GMCI. Although the combination of chemotherapy and AdV-tk/prodrug has not yet been tested in clinical trials there has been a phase I/II clinical trial completed where the effect of combining AdV-tk/prodrug with radiotherapy was tested in patients with varying stages of PCa⁸⁸. The patients were grouped into 3 categories/arms which included: 29 patients at low-risk (stage T1-T2a, Gleason score < 7), 26 patients at high-risk (stage T2b-T3, Gleason score > 6), and finally, 4 patients with stage D1 PCa. Encouraging results were found with biopsy data demonstrating good locoregional control in low-risk and high-risk patients. With median follow-ups at greater than 13 months, biochemical control of PSA levels was also noted for all low-risk and high-risk patients at last follow-up. However, 3 out of 4 patients with stage D1 had biochemical failure at 3, 3 and 7.7 months.

Future directions: Strategic approaches to PCa vaccine therapy

It is becoming evident that PCa patients with less advanced disease maybe more susceptible to the benefits of PCa vaccines²⁴. If one is to seriously consider PCa vaccines as a treatment modality then it is required that, at some stage, these vaccines are administered under conditions that maximize their potential. To date this has not been done, understandably, since patient safety concerns initially need to be met. With the advent of sensitive screening methods, the majority (> 90%) of PCa patients are diagnosed without detectable metastatic disease. Since a substantial percentage (> 30%) of these patients will inevitably succumb to metastatic disease, a strong argument can be made for vaccinating immediately. Further encouragement for such an approach comes from the crucial findings that, in virtually all

PCa vaccine trials, these CaVacs are safe and well tolerated by PCa patients. Additionally, these treatments appear to be slowing disease progress or, at the very worst, they do not speed it up. In essence, PCa patients can be seen as having minimal risk of serious side-effects and potentially experience life extending benefits by undergoing a CaVac therapy as early as possible. Skepticism as to whether PCa TAAs, such as PSA and PAP, are viable targets for CTLs has been largely quashed through the findings that substantial percentages of PCa patients can be induced to generate PCa TAA-specific CD8⁺ T lymphocyte responses^{47,73}. Such findings discount the possibility that prostate-specific T lymphocytes have been deleted through central tolerance mechanisms.

It may be highly edifying, in the future, if PCa vaccines were administered prior to surgical resection whenever possible, particularly when one is assessing the efficacy of a new PCa vaccination therapy. This is because the tumor milieu, with respect to ratios of tumor-specific lymphocytes to suppressor populations, such as Tregs and myeloid derived suppressor cells (MDSCs) can be ascertained subsequent to vaccination and correlations could then be made to resulting OS. Such an approach could provide valuable information as to what future changes to the vaccine protocol might be required.

It is likely that PCa vaccinations in patients with more advanced disease are less effective due to a combination of factors that include; excessive tumor burden, the accumulation of tumor suppressive elements such as Tregs and MDSCs, and the intrinsic suppressive nature of the tumor itself. It is imperative that future therapies involve combinations of an effective CaVac with a safe but potent dampening, or elimination, of suppressive populations. As described above, there are early stage clinical trials using ProstVac VF in combination with antibodies, ipilimumab or MDX-1106, which potentially reduce Treg involvement^{37,89}. It is too early to assess the potential for success of these combinations but they are certainly a step in the right direction. However, if all immunosuppressive elements, including metastatic tumor burden are to be effectively dampened (Tregs) or eliminated (MDSCs and tumor cells) we may be envisaging a multipronged treatment approach that could involve many years of clinical trials. A potential “shortcut” or stop-gap approach could involve strategic low-dose chemotherapy as an accompaniment to a PCa vaccine. The trials reported above involving docetaxel and either ProstVac VF or GVAX-PCa, occurred more by happenstance than through any strategy designed to fully exploit the vaccines’ antitumor potential. Docetaxel has no preferential toxicity for immunosuppressive populations and therefore may not be of great benefit to cancer vaccines, outside of reducing tumor burden and possibly enhancing T cell effector function. Low-doses of the chemotherapeutic drugs, cyclophosphamide (CTX) and 5-fluorouracil (5-FU) are independently capable of dampening Treg function and eliminating MDSCs respectively^{40,41}. Therefore combining PCa vaccines with low-dose CTX and 5-FU has a potential for synergistic immune and therapeutic outcomes in PCa patients.

Conclusions

Over the past decade, results from clinical trials with PCa patients at varying stages of disease using various vaccination strategies have highlighted some important factors. The first of these is that the parameters required for measuring clinical endpoints for therapeutic

CaVacs are likely to be different to those that have been chosen for chemotherapy. OS is ultimately the best parameter at this stage however this involves extensive time-lags between initiation of treatment and assessment of OS. Increased PSADT has a tendency to correlate with decreased time to progression and improvement in OS and is therefore a more favorable parameter for judging CaVac efficacy over measurement of absolute PSA levels. In addition, the T lymphocyte stimulation index may be correlated to CaVac efficacy⁹⁰. In terms of treatment protocol, another important factor to consider is the timing of the CaVac, both with respect to patient disease status as well as in terms of whether the vaccine should be delivered pre- or post-operatively.

The main “take home message” from most clinical trials performed thus far is that CaVac for PCa are safe, feasible and have the capacity to stimulate the production of antitumor T lymphocyte responses. Prognostications as to which of the above approaches will prove most beneficial would be premature at this early stage. Nevertheless, some tentative conclusions can be drawn. Of the vaccines tested in clinical trials, the most advanced is sipuleucel-T. However, the labor and cost involved in generating this individualized vaccine is a significant drawback, particularly when one considers a recent analysis of the IMPACT trial that questions the therapeutic benefit of sipuleucel-T⁵⁹. Changing the route of administration of sipuleucel-T such that the vaccine targets lymph nodes instead of, or as well as, the spleen may prove to be of therapeutic benefit⁶¹. The plasmid DNA/TAA vaccinations are promising but the efficiency of transfection and subsequent strength of T lymphocyte responses remain potential drawbacks. In addition, viral CaVacs seem to perform the same tasks as DNA vaccines only much more efficiently. Although there have been limited clinical studies performed with Ad5-based CaVac in PCa compared to pox viruses (ProstVac VF), the results obtained thus far with Ad5-PSA are promising and warrant further clinical evaluation. Since it is unlikely that any of the PCa vaccines discussed here are going to remain as stand-alone therapies it is important that they have potential for being combined with other therapeutic agents such as TLR agonists or chemotherapy. Poxvirus-based vaccines may not be ideal in that they subvert the innate arm of the immune response and therefore negate the potential for many potential future adjuvant therapies. The clinical trials performed so far have set the stage for what may prove to be great advances in PCa therapy involving strategically designed combinatorial therapies that synergistically promote CaVac-induced antitumor immunity.

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Schematic 1. Proposed mechanism(s) of ProstVac VF action

Pathway 1: VV infects immature and/or mature DCs which travel to draining lymph nodes and express co-stimulatory proteins and MHC class I/PSA (**1a**) where they trigger activation and proliferation of PSA-specific CD8+ T lymphocytes (**1b**), which then circulate to tumor sites and kill tumor cells through programmed cell death (PCD) (**1c**). **Pathway 2:** VV infects somatic cells (**2a**) which then directly activate PSA-specific CTLs (**2b**), and NK cells (**2b'**), which then induce PCD of somatic cells (**2c** and **2c'**) which are then taken up by DCs that are activated by viral components to mature and cross-present PSA (**2d**). These DCs further stimulate PSA-specific CTL activation (**2e**) that cause PCD of tumor cells (**2f**).

Schematic 2. Proposed mechanism of Ad5-PSA action

Ad5-PSA directly infects immature DCs (a receptor-mediated process) (**1a**) resulting in DC maturation and presentation of MHC class I in association with PSA epitopes (**2**). In addition, Ad5-PSA infects somatic cells (**1a'**) which are then recognized (**1b**) and killed (**1c**) by NK cells through an E1A-independent pathway involving NKG2D/stress-induced protein interaction. NK-mediated killing results in apoptotic bodies (**1d**) being taken up by immature DCs (**1e**) and promoting DC maturation and presentation of PSA epitopes in association with MHC class II (**2**). In the lymph node, activated DCs trigger activation and proliferation of PSA-specific CD8⁺ T lymphocytes (**3a**) possibly with the aid of activated CD4⁺ T lymphocytes (**3b**) supplying additional IL-2 (**3c**). PSA-specific CTLs then circulate to tumor sites and kill tumor cells through PCD (**4**).

Schematic 3. Proposed mechanism of action of sipuleucel-T

Infused antigen presenting cells (sipuleucel-T) travel to the spleen where they secrete PAP-GM-CSF fusion protein that attracts splenic DCs and stimulates DC maturation (through autocrine (**1a**) and paracrine (**1b**) GM-CSF function) and presentation of PAP epitopes in association with MHC class II (**1b**). Splenic DCs and/or sipuleucel-T APCs activate CD4+ T lymphocytes (**2**) which then provide IL-2 (**3b**) to PAP-specific CD8+ T lymphocytes (**3a**) which then mediate PCD of tumor cells (**4**).

Schematic 4. Proposed mechanism of action of GVAX-PCa

Irradiated allogeneic tumor cells (LNCAP + PC-3) secrete recombinant GM-CSF which has the triple role of chemotactically attracting immature DCs (**1a**), inducing DC maturation (**2**) and cross-presentation of proteins from apoptotic GVAX-PCa (**1b**, **1c**, **2**). Irradiated tumor cell apoptotic bodies (**1b**) and are taken up by DCs (**1c**) and B cells (**1d**) and also contribute to DC maturation (**2**). Mature DCs activate CD4+ T lymphocytes (**3a**) and CD8+ T lymphocytes (**3b**). Activated CD4+ T lymphocytes further stimulate B lymphocyte activation (**4a**) and provide IL-2 for CD8+ T lymphocytes (**4b**). B lymphocytes produce TAA-specific antibodies to cell-surface proteins that result in ADCC or complement-mediated tumor cell death (specific details not shown) (**5a**). Activated CD8+ T lymphocytes then kill tumor cells through recognition of MHC class I in association with TAA epitopes (**5b**).

Schematic 5. Proposed mechanism of action of DNA cancer vaccines

GM-CSF, which is co-injected with pDNA/TAA, attracts immature DCs (primarily Langerhans cells) and induces their maturation (1a). Simultaneously, pDNA/TAA transduce the DC (1b) and the DC expresses TAA epitopes in association with MHC class I and stimulates TAA-specific CD8+ T lymphocyte activation in the draining lymph node (2a). It is likely that some TAA epitopes are expressed in association with MHC class II resulting in activation of CD4+ T lymphocytes (2b) that provide further help (IL-2) for TAA-specific CD8+ T lymphocytes (3) which are then capable of killing tumor cells by PCD (4).

Schematic 6. Proposed mechanism of action of gene-mediated cytotoxic immunotherapy

Tumor cells are transduced with AdV-tk (1) and convert a pro-drug to a toxic drug that induces an immunogenic cell death (2). Apoptotic tumor cells are taken up by immature DCs (3a) and B lymphocytes (3b) and contribute to DC maturation (4). Mature DCs activate CD4+ T lymphocytes (5a) and CD8+ T lymphocytes (5b). Activated CD4+ T lymphocytes further stimulate B lymphocyte activation (6a) and provide IL-2 for CD8+ T lymphocytes (6b). B lymphocytes produce TAA-specific antibodies to cell-surface proteins that result in ADCC or complement-mediated tumor cell death (specifics not shown) (7a). Activated CD8+ T lymphocytes then kill tumor cells through recognition of MHC class I in association with TAA epitopes (7b).