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The androgen receptor: a biologically relevant vaccine target for the treatment of prostate cancer

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Abstract

The androgen receptor (AR) plays an essential role in the development and progression of prostate cancer. However, while it has long been the primary molecular target of metastatic prostate cancer therapies, it has not been explored as an immunotherapeutic target. In particular, the AR ligand-binding domain (LBD) is a potentially attractive target, as it has an identical sequence among humans as well as among multiple species, providing a logical candidate for preclinical evaluation. In this report, we evaluated the immune and anti-tumor efficacy of a DNA vaccine targeting the AR LBD (pTVG-AR) in relevant rodent preclinical models. We found immunization of HHDII-DR1 mice, which express human HLA-A2 and HLA-DR1, with pTVG-AR augmented AR LBD HLA-A2-restricted peptide-specific, cytotoxic immune responses in vivo that could lyse human prostate cancer cells. Using an HLA-A2-expressing autochthonous model of prostate cancer, immunization with pTVG-AR augmented HLA-A2-restricted immune responses that could lyse syngeneic prostate tumor cells and led to a decrease in tumor burden and an increase in overall survival of tumor-bearing animals. Finally, immunization decreased prostate tumor growth in Copenhagen rats that was associated with a Th1-type immune response. These data show that the AR is as a prostate cancer immunological target antigen and that a DNA vaccine targeting the AR LBD is an attractive candidate for clinical evaluation.

Keywords

Androgen receptor ligand-binding domain; Prostate cancer vaccine; HHDII-DR1; TRAMP; T-cell immunity

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Introduction

Prostate cancer is the most common non-cutaneous malignancy and second leading cause of cancer-related death in American men [1]. While the long natural history of this disease usually allows for several levels of therapeutic intervention, most patients who have recurrent disease following initial definitive treatment (~1/3 of all patients diagnosed) will ultimately develop castrate-resistant disease, the lethal form of the disease. One treatment approach being investigated is active immunotherapy, or anti-cancer vaccination, a strategy designed to elicit and/or augment anti-tumor immune responses. Prostate cancer has proven to be particularly amenable to immunotherapeutic intervention, with sipuleucel-T [an antigen-presenting cell-based therapy targeting prostatic acid phosphatase (PAP)] and PROSTVAC [a viral-based vaccine targeting prostate-specific antigen (PSA)] each having shown survival advantages in large, randomized clinical trials [2, 3]. However, while these vaccines may provide attractive therapeutic options for patients with advanced disease, they both target antigens that may not be critical to the growth and development of prostate tumor cells [4].

Given the possibility of antigen-loss variants as a means of tumors to evade immune detection, the selection of biologically relevant vaccine target antigens remains important. Other qualities of ideal antigens, as highlighted by a recent National Cancer Institute consensus panel, include therapeutic efficacy in vivo, immunogenicity, the role of the antigen in oncogenicity, specificity, expression level and percent of antigen-positive cells, stem cell expression, number of patients expressing the antigen, number of antigenic epitopes, and the cellular location of antigen expression [4]. One antigen that fits most of these criteria is the androgen receptor (AR), a steroid hormone receptor whose functional importance in prostate cancer oncogenicity (as well as frequency and amplitude of expression in prostate cancer patients) has long been established [5–10]. Indeed, the importance of the AR in the development and progression of cancer has led it to be the central molecular target for patients with recurrent disease for more than half a century. In fact, therapies targeting the AR signaling axis continue to be actively pursued, with the FDA approval of abiraterone (an androgen biosynthesis inhibitor; [11]) and enzalutamide (an androgen signaling pathway inhibitor), and several others currently being developed. However, as one of the central means of resistance to many of these therapies is increased AR expression [12, 13], the pharmacological targeting of the AR paradoxically can cause the AR to remain a target in patients with advanced, castrate-resistant disease.

We have shown that the AR is also a potentially attractive immunological target, showing that prostate cancer patients can have preexisting AR-specific antibody and T-cell responses [14] and identifying several HLA-A2-restricted antigenic peptide epitopes derived from the AR that are recognized by T cells in prostate cancer patients and which can lyse prostate cancer cells [15]. These characteristics, plus the observation that many common treatments for prostate cancer can upregulate AR expression, suggest that the AR may be an ideal immunological target antigen for prostate cancer. In fact, the AR was included among a short list of prioritized potential cancer vaccine target antigens by the recent NCI study group [4].

The AR is a large, multi-domain protein with known sequence variability among human populations. However, the carboxy-terminal ligand-binding domain (LBD), the final 260 amino acids of the AR, has a completely identical protein sequence not only among various human populations, but also among species, including mice, rats, and humans. This suggests that there may be some evolutionary importance to the sequence of this domain making it less amenable to mutation or allelic differences among different individuals. Furthermore, the sequence identity among species permits the evaluation of vaccines in preclinical models

where it is also a “self-antigen” expressed under natural conditions (i.e. by both normal and malignant prostate tissue), making the results more directly translatable to human studies with less concern for xenogeneic differences.

In the current report, we sought to directly determine whether the AR could be an immunological target antigen, and in particular whether a vaccine targeting the AR LBD could treat existing prostate cancer. We used several relevant rodent models, including HHDII-DR1 transgenic mice engineered to express human HLA-A2 and HLA-DR1, an autochthonous TRAMP murine prostate cancer model, and a Copenhagen rat prostate cancer cell line model. Our results demonstrate that a DNA vaccine targeting the AR LBD has immune and anti-tumor efficacy in multiple preclinical models in vivo, and the AR therefore represents a rational, biologically relevant immunological target for the treatment for prostate cancer, an approach which could be pursued in human trials alone or in combination with other AR-targeted therapies.

Materials and methods

Animals

HHDII-DR1 transgenic mice, expressing the $\alpha 1$ and $\alpha 2$ chains of human HLA-A2*01 chimeric with the intracellular $\alpha 3$ chain of the H-2D^b allele, and expressing HLA-DR1, with mouse MHC class I (H-2^b) and II (I-A^b) knocked out, were graciously provided by Dr. François Lemonnier (Institut Pasteur, Paris, France, and Charles Rivers Laboratories, Paris, France) [16–18]. HHDII-DR1^{+/-} mice were generated by crossing homozygous C57Bl/6 (Charles Rivers Laboratories, Wilmington, MA) and HHDII-DR1 mice. A2/TRAMP mice were generated by crossing homozygous TRAMP mice and HHDII-DR1 mice. Male Copenhagen rats were obtained commercially (Harlan, Indianapolis, IN, USA). Animals were housed in a facility maintained by the Laboratory Animals Resources of the University of Wisconsin Medical School, and all treatments were conducted under an institutional animal care and use committee (IACUC)-approved protocol.

A2/TRAMP prostate tumor cell line

Tumor cell lines were cultured from prostate tumors arising in 26-week-old A2/TRAMP male mice in DMEM high glucose media (Mediatech, Manassas, VA, USA) supplemented with L-glutamine and 5 % Nu-Serum IV (BD Biosciences, Franklin Lakes, NJ, USA), 5 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 5 μ g/mL insulin (Sigma-Aldrich, St. Louis, MO, USA), 10⁻⁸ M R-1881 (Sigma-Aldrich), and 25 IU/mL penicillin–streptomycin (Mediatech) [19].

Plasmid vaccine construction

The cDNA sequence of the AR LBD (comprising base pairs 1989–2763 of the full-length AR sequence, NCBI reference sequence NM_000044.2), along with a Kozak sequence and ATG start codon added to the 5' end of the gene, was inserted into the pTVG4 vaccine vector (previously described [20]) to generate pTVG-AR. RNA and protein expression was confirmed by transient transfection assays of reporter cell lines. Plasmid DNA was purified using a Qiagen endonuclease-free kit (Qiagen, Valencia, CA, USA).

Peptides

Peptides used for immunization or analysis were previously described [15, 21].

DNA immunization studies

Mice were immunized at biweekly intervals, or as otherwise described, intradermally (in ear pinna) with 100 µg plasmid DNA. Two weeks following the final immunization, spleens were collected and splenocytes were prepared by osmotic lysis. Prostates dissected from these animals were fixed in 10 % buffered formalin for paraffin sectioning and IHC analysis. For survival studies, immunization was continued until time of natural death or veterinarian-directed compassionate euthanasia. For Copenhagen rat studies, 6- to 8-week-old male rats were immunized with 100 µg plasmid DNA. Where indicated, rats were challenged with 10⁴ Mat-LyLu syngeneic prostate tumor cells subcutaneously and followed for tumor growth until tumors reached 10 cm³ [Volume = ($\pi/6$)*(diameter_{long})*(diameter_{short})²].

Enzyme-linked immunosorbent spot (ELISPOT) assay

Frequencies of peptide-specific IFN γ -secreting immune responses were measured after 48 h of antigen challenge using a murine IFN γ ELISPOT kit (R&D Systems, Minneapolis, MN) as previously described [15]. Experimental results are shown as the number of IFN γ spot-forming units (SFU) per 10⁶ splenocytes normalized against media alone control wells. Comparisons between treatment groups were made using a Student's *t* test, with *p* < 0.05 defined as a significant T-cell response.

Cytotoxicity assays

Splenocytes were restimulated with 1 µg/mL purified AR LBD protein (Invitrogen, Grand Island, NY, USA), supplemented on day two with 20 U/mL recombinant IL-2 (Millipore), and cytolytic activity was measured by LDH release, as previously described [15]. Targets used were peptide-pulsed human T2 target cells, the LNCaP human prostate cancer cell line, or the A2/TRAMP prostate tumor cell line.

Immunofluorescence, immunohistochemical staining, and histological analysis

Genitourinary complexes from A2/TRAMP mice were analyzed by investigators blinded to the treatment regimen for the presence of various grades of prostate tumor development based on the metrics of Kaplan and Lefko [22] and in consultation with pathologists (Dr. Ruth Sullivan and Dr. Weixiong Zhong). For analysis, sections were immunofluorescently stained using rabbit anti-laminin (Sigma) and mouse anti-SV40 (BD Biosciences) primary antibodies and goat anti-rabbit Texas Red and horse anti-mouse-FITC secondary antibodies (VECTOR laboratories, Burlingame, CA, USA), and mounted with DAPI VECTOR mounting medium. Slides were imaged using an Olympus BX51 fluorescent microscope (Olympus, Center Valley, PA, USA) in combination with SPOT RT analysis software (SPOT Imaging Solutions, Sterling Heights, MI, USA). At least forty glands were analyzed in a blinded fashion, and the highest pathological score per gland was scored.

For AR and CD8+ immunohistochemical staining, sections were stained with primary antibodies (AR: clone sc-7305, Santa Cruz Biotechnology, Santa Cruz, CA, USA; CD8: clone 53-6.7, R&D Systems), developed using the LSAB+ System-HRP (Dako, Carpinteria, CA, USA) and Metal Enhanced DAB Substrate Kit DAB metal concentration (Thermo Fisher Scientific, Rockford, IL, USA), and imaged as above.

Computer-assisted histological scoring analysis

Dissected anterior, dorsolateral, and ventral prostate lobes from A2/TRAMP mice were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Slides were scanned to digital images using the Vectra™ imaging system (Caliper Life Sciences, Hopkinton, MA, USA). Using inForm software (Caliper Life Sciences), an analysis program was trained to distinguish histological grades of disease based on morphological patterns based on the

metrics of Kaplan-Lefko et al. [22] and in consultation with pathologists. This program was then used to analyze prostate sections for regions of normal, prostatic intraepithelial neoplasia, well-differentiated carcinoma, or non-tissue areas. All identifiable prostate glands (minimum of twenty) were scored for the worst histological grade of disease based on computer analysis and reviewed for accuracy in a blinded fashion. Number of glands (median/range) counted for each lobe of pTVG4-immunized animals: anterior (36/28–47), dorsolateral (50/43–115), and ventral (61/41–86). Number of glands (median/range) counted for each lobe of pTVG-AR-immunized animals: anterior (40.5/28–47), dorsolateral (54/25–75), and ventral (64/43–85).

Enzyme-linked immunosorbent assays

AR LBD-specific IFN γ secretion was measured by ELISA, as previously described [20].

Results

Immunization with a DNA vaccine targeting the AR LBD augments multiple peptide-specific immune responses that can lyse prostate tumor cells

We have previously identified HLA-A2-restricted AR LBD-specific epitopes, T cells specific for which could lyse prostate tumor cells. Moreover, we detected AR epitope-specific T cells in HLA-A2+ prostate cancer patients and HHDII-DR1 transgenic mice [15]. To determine whether AR LBD epitope-specific cytolytic immune responses can be augmented through the use of a genetic vaccine, we cloned the cDNA sequence of the AR LBD into the pTVG4 plasmid DNA immunization construct (pTVG-AR). HHDII-DR1 transgenic mice were then immunized six times biweekly with pTVG-AR or the empty pTVG4 vector (six biweekly vaccinations being a standard immunization schema we have previously identified for autologous antigens in both rat [20] and human [23] studies). Two weeks following the final immunization, animals were evaluated for peptide-specific immune responses against ten HLA-A2-binding peptides derived from the AR LBD [15] or the full-length AR LBD protein. Mice immunized with pTVG-AR developed significantly higher levels of peptide-specific immune responses against peptides AR761, AR805, and AR811, as well as responses generated against the full-length AR LBD (Fig. 1a). Moreover, splenocytes from pTVG-AR-immunized animals (but not control animals) were able to lyse the human HLA-A2+ LNCaP prostate cancer cell line (Fig. 1b).

To more closely model a MHC diverse human population, we crossed HHDII-DR1 mice with wild-type C57BL/6 animals to generate F1 mice heterozygous for human and mouse MHC (HHDII-DR1^{+/-}). HHDII-DR1^{+/-} mice similarly immunized with pTVG-AR had significantly augmented responses to AR761, AR811, and the full-length AR LBD protein, as well as statistically higher responses specific for AR814 and AR859 (Fig. 1c), showing that even in a MHC diverse model in which the AR LBD is a self-antigen, immunization with pTVG-AR can augment epitope-specific immune responses.

Immunization of A2/TRAMP mice with pTVG-AR augments multiple peptide-specific immune responses that can lyse prostate tumor cells

To determine whether immune responses elicited to the AR LBD might have direct anti-tumor efficacy in tumor-bearing animals, we crossed HHDII-DR1 and TRAMP mice, generating a F1 strain of heterozygous A2/TRAMP mice. These animals were identified as being heterozygous for human and mouse MHC, as well as for the SV40 large T antigen, and were also found to develop prostate adenocarcinoma following similar kinetics as reported for wild-type C57Bl/6 TRAMP heterozygous mice (Supplementary Figure S1) [22]. Furthermore, prostate tumors from these animals were harvested to generate a syngeneic A2/TRAMP tumor cell line (Supplementary Figure S2). Six-week-old A2/

TRAMP animals were immunized biweekly with either pTVG-AR or the empty vector, and at 16 weeks of age, immune responses and tumor development were assessed. This provided a time point where we could effectively detect histological differences in tumor development while potentially avoiding the development of large, bulky tumors [24–26]. A2/TRAMP animals immunized with pTVG-AR were found to have peptide-specific immune responses against multiple peptides derived from the AR LBD (Fig. 2a) and could lyse syngeneic A2/TRAMP tumor cells (Fig. 2b, c). Lysis could be inhibited by an HLA-A2 blocking antibody and could also be partially inhibited by blocking a murine MHC class I, suggesting that cytotoxic T lymphocytes (CTL) were elicited to murine epitopes as well as HLA-A2-restricted epitopes. Of note, immune responses were also detected to other AR-derived peptides that were not previously identified as HLA-A2-restricted epitopes, as well as the AR LBD protein, in tumor-bearing, control-immunized animals that were absent in non-tumor-bearing control animals (Fig. 2a compared to Fig. 1a, c).

Immunization decreases the prevalence of prostate adenocarcinoma and increases the frequency of tumor-infiltrating lymphocytes, in A2/TRAMP mice in vivo

Sixteen-week-old A2/TRAMP animals immunized with pTV G4 or pTVG-AR were evaluated for prostate adenocarcinoma development using previously established histological parameters [22]. To enhance this histological analysis and distinguish between well- and moderately differentiated adenocarcinoma, prostate tissue sections were analyzed in blinded fashion using immunofluorescent staining (examples shown in Fig. 3a–d, with individual color images in Supplemental Figure 3). As demonstrated in Fig. 3, immunization with pTVG-AR was found to result in a significant decrease in the prevalence of well- and moderately differentiated carcinoma than control-immunized animals at sixteen weeks of age (Fig. 3e).

Similar studies were conducted assessing for tumor development in individual lobes of the prostate at 18 weeks of age, an age which displays the full range of adenocarcinoma development, including poorly differentiated tumors not detectable at 16 weeks of age. Prostate sections from immunized animals were scored for histological evidence of disease using computer-assisted image analysis, analyzed in blinded fashion (example shown in Fig. 4a). As before, animals immunized with pTVG-AR had a decrease in the prevalence of adenocarcinoma in all lobes of the prostate (Fig. 4b–d, left panels). Animals immunized with pTVG-AR were also found to have a higher frequency of infiltrating CD8⁺ T cells when compared to control-immunized animals (Fig. 4e). In addition, animals immunized with pTVG-AR had fewer poorly differentiated carcinomas than control-immunized animals (Fig. 4b–d, right panels); however, these were of low frequency and the difference did not meet statistical significance. These poorly differentiated tumors (but not autologous glands with PIN or WDC) were found to lack expression of the AR (Supplementary Figure S4A). Moreover, compared to tumors with earlier histological grades of disease in which lymphocytes were found infiltrating the tumors, pTVG-AR-immunized animals with poorly differentiated tumors were found to have CD8⁺ T cells at the tumor margins but not infiltrating the tumors themselves (Supplementary Figure S4B).

Immunization with pTVG-AR delays tumor growth in a rat model of prostate cancer

Given that the sequence of the AR LBD is identical among multiple species and that immunization with pTVG-AR was found to decrease the prevalence of adenocarcinoma, we conducted additional tumor studies in a well-characterized rat model of prostate cancer. Male Copenhagen rats were immunized six times biweekly with either pTVG-AR or an empty vaccine control, and 2 weeks following, the final immunization were challenged with syngeneic Mat-LyLu prostate tumor cells. Immunization with pTVG-AR resulted in a significant delay in tumor growth, with pTVG-AR-immunized animals having a median

time to 10 cm³ of 51 days, compared to 43 days for pTVG4-immunized animals (Fig. 5a; $p = 0.016$). Similarly, in studies in which animals were first challenged with tumor cells prior to weekly vaccinations, immunization with pTVG-AR was found to provide a trend toward a delay in tumor growth, with pTVG-AR-immunized animals having a median time to 10 cm³ of 47 days, compared to 42 days for pTVG4-immunized animals (Fig. 5b, $p = 0.076$). Animals that did not develop tumors were found to generate long-term, durable AR LBD-specific IFN γ -secreting immune responses (Fig. 5c), responses not detectable in tumor-bearing animals or animals immunized with pTVG4 (data not shown).

Immunization with pTVG-AR increases survival of A2/TRAMP mice

We next sought to determine whether immunization would prolong the survival of tumor-bearing vaccinated animals. Six-week-old A2/TRAMP mice were immunized twice biweekly, with this immunization cycle repeated every 10 weeks, until the animals succumbed to their disease. Immunization with pTVG-AR was found to provide a significant increase in survival compared to control-immunized animals (Fig. 6), with pTVG-AR-immunized animals having a median survival of 226 days (range 183–370 days) compared to the median survival of pTVG4 animals of 199 days (range 168–232 days; $p = 0.0024$).

Discussion

After more than half a century, the androgen receptor remains the primary pharmacological target for prostate cancer. Recent data have shown that the AR remains a target even in “castrate-resistant” disease, with a majority of these patients continuing to express (or oftentimes overexpress) the AR. Here, we report the first investigation into the AR as a vaccine target antigen, reasoning that such an approach would target a protein critical to the oncogenicity of the tumor, thus potentially avoiding escape variants that lose antigen expression. In addition, such an approach has the potential of complementing other AR-directed therapies, as AR overexpression has been shown to be a means of resistance to a variety of these treatments [12, 13]. Targeting functionally important antigens, while previously not pursued in the development of prostate cancer immunotherapies, is an approach taken in other solid malignancies. Here, using a variety of relevant pre-clinical models, we show that a vaccine targeting the AR LBD can safely elicit immune and anti-tumor responses, suggesting that in addition to being a pharmacological target, the AR is also an immunological target and that vaccines targeting the AR, particularly the AR LBD, are attractive approaches for clinical development.

The development of antigen-specific vaccines targeting proteins whose function is critical to the oncogenicity of tumor cells is an approach that has been taken in several other malignancies, with vaccines targeting functionally important antigens such as HER-2/Neu [27–29], CEA [30–32], MUC1 [32–34], and EGFR vIII [35–37] for a variety of solid tumors. However, it has been conspicuously absent in the development of vaccines for prostate cancer, despite ‘oncogenicity’ being one of the most important characteristics of an ideal tumor antigen as determined by an NCI study group [4]. In addition, the AR also has many of the other qualities of a potential ideal target antigen, including its intracellular expression, the high frequency of patients with elevated AR expression, the high homogenous expression within individual tumors, and its frequent overexpression in prostate cancer. These are all factors which are lacking for the commonly targeted antigens PSA and PAP, which are both secreted proteins that likely do not play a central role in oncogenicity and are not necessarily overexpressed in prostate cancer. These antigens have been targeted in large part due to their expression being largely restricted to tumor cells. While AR is expressed in normal prostate tissue as well as some other tissues, and this might a priori be considered a disadvantage, it should be noted that the AR has been safely targeted

for over 50 years using a wide variety of pharmacological therapies. Moreover, in our studies, we did not observe any obvious toxicity (e.g. muscle wasting, changes in animal behavior). On the contrary, tumor-bearing immunized animals lived longer than control-immunized animals, further suggesting a lack of significant toxicity. Additionally, while the AR is expressed by other normal tissues, it is not as widely expressed as antigens which have been safely targeted using a variety of approaches in other solid malignancies, such as HER-2/neu and EGFR.

To evaluate whether a DNA vaccine targeting the AR was a rational approach for human testing, we utilized a variety of animal models relevant to our previous human in vitro studies evaluating immune responses to the AR as well as the highest relevance for future potential clinical evaluation. Foremost, in all four of the models evaluated, the AR is a true 'self-antigen' (i.e. naturally expressed by the normal prostate and thus undergoes the natural process of central and peripheral tolerance). Additionally, the protein sequence of the AR LBD in these models is not only identical, but also identical to the sequence targeted in humans, meaning the AR LBD encoded by the pTVG-AR DNA vaccine could be studied in rodents and humans without concerns for xenoantigen-type responses. Using HHDII-DR1 and TRAMP transgenic mice, we established murine prostate tumor models expressing HLA-A2, allowing us to use these models to demonstrate that a DNA vaccine targeting the AR LBD can augment HLA-A2 epitope-specific immune responses that we have previously identified as having the ability to lyse prostate cancer cells [15]. More importantly, it provided us an autochthonous model of prostate cancer demonstrating that immunization leads to a decrease in tumor prevalence and increased survival (the "gold standard" for clinical trials). Furthermore, while older TRAMP mice undergo a neuroendocrine switch resulting in a loss of AR expression (Figure S4A), we found that animals with poorly differentiated tumors immunized to the AR had CD8⁺ T cells at the tumor margins. While the relevance of these CD8⁺ T cells remains to be established, it is conceivable that immunization results in antigen spread to other tumor-associated antigens. This will be an important area of future research, notably because the development of AR-loss variants could otherwise be a means of tumor evasion as has been suggested in pharmacological studies [38, 39].

HHDII-DR1 and other human MHC transgenic mice have been valuable tools for identifying and characterizing T-cell epitopes in a variety of disease settings. However, our results highlight the importance of complementary in vitro studies with human cells. For example, in this report, we identified an HLA-A2-specific epitope (AR761) presented by murine HLA-A2⁺ prostate tumor cells, but which we have previously shown is not naturally presented by human HLA-A2⁺ tumor cells [15]. In studies not shown, we found that T cells specific for this peptide could lyse murine prostate tumor cells in an HLA-A2-restricted fashion, but not human prostate tumor cells. This suggests that there may be antigen processing and presentation differences between the human and murine immunoproteasomes and that this may result in the presentation of alternative peptides by HLA-A2.

While these studies did not extensively evaluate safety, we did not observe any overt signs of toxicity. This, combined with compiled evidence showing the safety of DNA immunization in a variety of malignancies, suggests that this approach could be feasibly translated to clinical evaluation in patients with prostate cancer. Our data suggest logical avenues for such investigation in early-phase clinical trials. For example, many have suggested that earlier stages of disease, such as in individuals experiencing biochemical recurrence of prostate cancer following primary therapy, might be an optimal population for active immunotherapy [40, 41]. In addition to lower tumor burden, these patients might also have fewer of the suppressive mechanisms that are associated with advanced tumor growth, as has been documented in genetic vaccine models of prostate cancer in TRAMP mice [42].

Alternatively, as described above, a common mechanism of resistance to several AR-directed therapies is overexpression of the AR [12, 13]. This suggests that a logical direction is to combine AR-directed pharmacological and immunotherapeutic approaches to specifically target tumors with increased AR expression. This is a future direction we will explore using the preclinical models developed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AR	Androgen receptor
LBD	Ligand-binding domain
MDC	Moderately differentiated carcinoma
PDC	Poorly differentiated carcinoma
PIN	Prostatic intraepithelial neoplasia
TRAMP	Transgenic adenocarcinoma of the mouse prostate
WDC	Well-differentiated carcinoma

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Fig. 1.

Immunization of HHDII-DR1 mice with pTVG-AR augments AR LBD HLA-A2-restricted, peptide-specific immune responses that can lyse prostate cancer cells. Splenocytes from HHDII-DR1 mice immunized with either pTVG4 or pTVG-AR were analyzed for AR LBD-specific immune responses by IFN γ ELISPOT. **a** Splenocytes from animals immunized with either pTVG4 (*open symbol*, $n = 11$) or pTVG-AR (*closed symbol*, $n = 15$) were stimulated with AR LBD HLA-A2-restricted peptides, AR LBD protein, or ConA-positive control. Shown is the frequency of peptide-specific T cells (SFU, spot-forming units) for individual animals (average of triplicate samples corrected for media alone, group mean shown by the solid bar). $*p < 0.05$ as calculated by a Student's t test. **b** Splenocytes from four individual animals immunized with pTVG4 (*open symbol*) or pTVG-AR (*closed symbol*) were tested for their ability to lyse HLA-A2+ LNCaP human prostate cancer cells. Shown is the mean percent specific lysis and standard error, determined by triplicate samples at four effector/target ratios. **c** HHDII-DR1^{+/-} mice were immunized six times biweekly with either pTVG4 (*open symbol*, $n = 4$) or pTVG-AR (*closed symbol*, $n = 6$) and analyzed for the frequency of peptide-specific immune responses by IFN γ ELISPOT as in **a**

Fig. 2.

Immunization of A2/TRAMP mice with pTVG-AR augments AR LBD peptide-specific immune responses and HLA-A2-restricted CTL. **a** A2/TRAMP mice were immunized five times biweekly. Cells from individual animals immunized with pTVG4 (*open symbol*, $n = 6$) or pTVG-AR (*closed symbol*, $n = 9$) were analyzed for the frequency of IFN γ SFU to the listed antigens by ELISPOT. * $p < 0.05$ as calculated by a Student's t test. Pooled splenocytes from animals immunized with pTVG4 (**b**) or pTVG-AR (**c**) were tested for their ability to lyse untreated syngeneic A2/TRAMP prostate cancer cells (*black*) or cells pre-treated with human MHC class I (*solid grey*) or murine MHC class I (*dashed grey*) blocking antibodies. Shown is the mean percent specific lysis and standard error, determined by triplicate samples, at three effector/target cell ratios

Fig. 3.

Immunization with pTVG-AR decreases the prevalence of prostate adenocarcinoma at 16 weeks. Sixteen-week-old A2/TRAMP animals immunized with pTVG4 (*open symbol*, $n = 7$) or pTVG-AR (*closed symbol*, $n = 8$) were analyzed for tumor development by histological analysis combined with immunofluorescence using antibodies specific for SV40 T antigen (*green*), laminin (*red*), and DAPI (*blue*) based on the metrics of Kaplan-Lefko et al. [22]. Examples in **a–d** show the spectrum of disease observed (10× magnification: *scale bar* 100 μm): normal prostate glands (**a**), prostatic intraepithelial neoplasia (**b**), well-differentiated carcinoma (**c**), or moderately differentiated carcinoma (**d**: invasive cells indicated by *arrows* in the 20× magnification inset: *scale bar* 50 μm). The results are compiled for the most advanced histological stage in each gland in **e**. *Each dot* represents the frequency of a particular histological grade in an individual animal, with *solid bars* indicating the group mean. * $p < 0.05$ as calculated by a Student's *t* test

Fig. 4.

Immunization with pTVG-AR decreases the prevalence of prostate adenocarcinoma in all prostate lobes and increases the frequency of tumor-infiltrating CD8⁺ T cells at 18 weeks. Prostates were collected from 18-week-old A2/TRAMP-immunized animals. Individual anterior, dorsolateral, and ventral lobes were dissected and analyzed for tumor development. **a** An example of this analysis, with regions that were identified as normal (*red*), prostatic intraepithelial neoplasia (*green*), well-differentiated carcinoma (*blue*), or non-tissue area (*yellow*). At least twenty glands from individual animals were then scored for the worst histological grade of disease based on this analysis and corrected when necessary based on tissue or reading artifacts (e.g. *black arrow*) in blinded fashion from anterior lobes (**b**), dorsolateral lobes (**c**), and ventral lobes (**d**). Dot plots in the *left panels* show the frequency of histological grades in individual animals immunized with pTVG4 (*open symbols*) or

pTVG-AR (*closed symbols*), with solid bars indicating the group mean. $*p < 0.05$ as calculated by a Student's *t* test. *Right panels* show the frequency of animals per immunization group with poorly differentiated carcinoma. **e** Prostates from 18-week-old A2/TRAMP animals were analyzed for the frequency of infiltrating CD8⁺ T cells. **e** A representative immunohistochemical staining (40× magnification *scale bar* 50 μm), and the *right panel* shows the normalized frequency of infiltrating CD8⁺ T cells in anterior, dorsolateral, and ventral lobes of the prostate in animals immunized with pTVG4 (*open symbol*) or pTVG-AR (*closed symbol*). $*p < 0.05$ as calculated by a Student's *t* test

Fig. 5.

Immunization with pTVG-AR delays tumor growth in a subcutaneous rat model of prostate cancer. **a** Copenhagen rats immunized six times biweekly with either pTVG4 (*dashed line*, $n = 10$) or pTVG-AR (*solid line*, $n = 11$) were challenged with 10^4 May-LyLu syngeneic prostate tumor cells subcutaneously and followed for tumor growth. **b** Copenhagen rats were implanted with 10^4 Mat-LyLu syngeneic prostate tumor cells and then immunized weekly with either pTVG4 (*dashed line*, $n = 20$) or pTVG-AR (*solid line*, $n = 22$) and followed for tumor growth until tumors reached 10cm^3 . Statistical comparisons were made by log-rank test. **c** Splenocytes from pTVG-AR-immunized animals lacking tumor at day 245 (**a**) were analyzed for AR LBD-specific immune responses by IFN γ ELISA. Group averages are shown by solid bar, and $*p < 0.05$ as calculated by a Student's t test

Fig. 6.

Immunization with pTVG-AR increases overall survival of A2/TRAMP mice. A2/TRAMP mice were immunized with either pTVG4 ($n = 13$, *dashed line*) or pTVG-AR ($n = 14$, *solid line*) (immunizations indicated by *black arrows* along *x-axis*) until the time of natural death or veterinarian-directed compassionate euthanasia. Shown is a Kaplan–Meier plot of survival, with statistical comparison made by log-rank test