

Combination Therapy with a Second-Generation Androgen Receptor Antagonist and a Metastasis Vaccine Improves Survival in a Spontaneous Prostate Cancer Model

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Abstract

Purpose: Enzalutamide, a second-generation androgen antagonist, was approved by the U.S. Food and Drug Administration (FDA) for castration-resistant prostate cancer (CRPC) treatment. Immunotherapy has been shown to be a promising strategy for prostate cancer. This study was performed to provide data to support the combination of enzalutamide and immunotherapy for CRPC treatment.

Experimental Design: Male C57BL/6 or TRAMP (transgenic adenocarcinoma of the mouse prostate) prostate cancer model mice were exposed to enzalutamide and/or a therapeutic vaccine targeting Twist, an antigen involved in epithelial-to-mesenchymal transition and metastasis. The physiologic and immunologic effects of enzalutamide were characterized. The generation of Twist-specific immunity by Twist-vaccine was assessed. Finally, the combination of enzalutamide and Twist-vaccine to improve TRAMP mice overall survival was evaluated.

Results: Enzalutamide mediated immunogenic modulation in TRAMP-C2 cells. *In vivo*, enzalutamide mediated reduced genitourinary tissue weight, enlargement of the thymus, and increased levels of T-cell excision circles. Because no changes were seen in T-cell function, as determined by CD4⁺ T-cell proliferation and regulatory T cell (Treg) functional assays, enzalutamide was determined to be immune inert. Enzalutamide did not diminish the ability of Twist-vaccine to generate Twist-specific immunity. Twist was confirmed as a valid tumor antigen in TRAMP mice by immunohistochemistry. The combination of enzalutamide and Twist-vaccine resulted in significantly increased overall survival of TRAMP mice compared with other treatment groups (27.5 vs. 10.3 weeks). Notably, the effectiveness of the combination therapy increased with disease stage, i.e., the greatest survival benefit was seen in mice with advanced-stage prostate tumors.

Conclusions: These data support the combination of enzalutamide and immunotherapy as a promising treatment strategy for CRPC. *Clin Cancer Res*; 1–14. ©2013 AACR.

Introduction

Localized prostate cancer is treated with surgery, radiotherapy, or watchful waiting, whereas recurrent disease is further treated with androgen deprivation therapy (ADT; ref. 1). Most patients on ADT eventually develop castration-resistant prostate cancer (CRPC), characterized by an increase in prostate-specific antigen and subsequent pro-

gression of disease despite castrate blood levels of testosterone (2). However, emerging evidence suggests that CRPC remains dependent on androgen receptor signaling for growth. Indeed, CRPC is commonly associated with increased expression of androgen receptor, arising from amplification or mutation of the androgen receptor gene or other mechanisms (3). Enzalutamide is an androgen receptor antagonist that blocks androgens from binding to the androgen receptor and prevents nuclear translocation and coactivator recruitment of the ligand–receptor complex. Enzalutamide has been evaluated in clinical trials (4, 5), including the AFFIRM trial, which demonstrated a 4.8-month advantage in overall survival with enzalutamide compared with placebo (6). Prostate cancer immunotherapy recently achieved significant milestones with the approval of the vaccine sipuleucel-T (7) and the successful clinical trials of the PROSTVAC-VF vaccine (8). Systemic androgen ablation is known to activate thymic regeneration, and androgens are known to regulate a variety of immune responses (9–12). Recently, a phenomenon called

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Translational Relevance

This study examined whether the combination of antiandrogen therapy, enzalutamide, plus a therapeutic vaccine targeting Twist, an antigen involved in epithelial-to-mesenchymal transition and metastasis, could improve survival in TRAMP (transgenic adenocarcinoma of the mouse prostate) mice, a spontaneous prostate cancer model. Enzalutamide mediated immunogenic modulation *in vitro*, reduced genitourinary tissue weight, caused enlargement of the thymus, and increased levels of T-cell excision circles *in vivo*. Because no changes were seen in T-cell function, as determined by CD4⁺ T-cell proliferation and regulatory T cell (Treg) functional assays, enzalutamide was determined to be immune inert. Furthermore, enzalutamide did not diminish the ability of Twist-vaccine to generate Twist-specific immunity. The combination of enzalutamide and Twist-vaccine resulted in significantly increased overall survival of TRAMP mice compared with other treatment groups (27.5 vs. 10.3 weeks). Notably, the effectiveness of the combination therapy increased with disease stage, i.e., the greatest survival benefit was seen in mice with advanced-stage prostate tumors. These findings establish a rationale for the combined use of immunotherapy and antiandrogen therapy enzalutamide as a promising treatment strategy for castration-resistant prostate cancer (CRPC).

immunogenic modulation, exposure of tumor cells to therapies that consequently alter tumor phenotype to render the tumors more susceptible to immune-mediated attack, has been studied and reviewed (13, 14). The immunogenic modulation property of enzalutamide is currently unknown. Our hypothesis is that enzalutamide (i) induces thymic regeneration and (ii) mediates immunogenic modulation in prostate tumor cells, rendering them more sensitive to immune-mediated attack. We further hypothesize that the immunogenic modulation property of enzalutamide could be harnessed in combination with immunotherapy to improve treatment for CRPC.

Twist is a member of a highly conserved BHLH transcription factor that has been implicated in metastasis. Its overexpression is associated with poor prognosis in many cancer types (15–19). In a preclinical study, suppressing Twist expression in highly metastatic murine mammary carcinoma cells prevented the cells from metastasizing from the mammary gland to the lung (20). The same study also showed that activation of Twist led to loss of epithelial cell markers, activation of mesenchymal markers, and induction of cell motility, all of which suggest the important role of Twist in epithelial-to-mesenchymal transition (EMT) and therefore the process of metastasis (20).

In this study, we evaluated the efficacy of combination therapy with enzalutamide plus a yeast-based (*Saccharo-*

myces cerevisiae) vaccine engineered to express Twist antigen in the TRAMP (transgenic adenocarcinoma of the mouse prostate) model of spontaneous prostate cancer, in which tumor development resembles disease progression in humans, from prostatic intraepithelial neoplasia (PIN) to metastatic CRPC (21–23). Immunohistochemistry confirmed Twist as a valid tumor antigen in the TRAMP model, and our data suggest that Twist expression increased as tumors progressed. Here, the combination of enzalutamide and Twist vaccine significantly improved overall survival in TRAMP mice, particularly those with advanced disease, while either modality alone failed.

To our knowledge, this is the first study that demonstrates (i) the immunogenic modulation property of enzalutamide; (ii) the physiologic effect of enzalutamide in C57BL/6 and TRAMP mice, i.e., reduced genitourinary tissue weight, enlarged thymus, and increased levels of T-cell excision circles (TREC); (iii) the minimal effects of enzalutamide on T-cell activity; (iv) the use of Twist, a driver of EMT, as a therapeutic vaccine target, and the ability of Twist-vaccine to generate Twist-specific immunity in C57BL/6 and TRAMP mice; and (v) significantly improved overall survival in TRAMP mice with the combination of enzalutamide and Twist-vaccine. These data support the combination of enzalutamide and immunotherapy as a promising treatment for CRPC.

Materials and Methods

Animals

The National Cancer Institute's Frederick National Laboratory for Cancer Research (Frederick, MD) supplied 8- to 12-week-old male C57BL/6 mice. TRAMP mice on the C57BL/6 background were bred and maintained at the NIH (Bethesda, MD; ref. 24). TRAMP mice were sorted into 4 age groups, which in this model represent different stages of prostate cancer development: 8 to 12 weeks old, PIN; 12 to 20 weeks old, well-differentiated adenocarcinoma; 20 to 28 weeks old, moderately-differentiated adenocarcinoma; and 28 weeks or older, poorly-differentiated adenocarcinoma (23, 24).

Tumor cells

TRAMP-C2 murine prostate adenocarcinoma cells were purchased from American Type Culture Collection and maintained in the recommended medium.

Vaccine constructs

Recombinant *S. cerevisiae* yeast constructs without antigen (control-vaccine) or expressing Twist (Twist-vaccine) were engineered by methods similar to those previously described (GlobeImmune Inc.; ref. 25).

Enzalutamide diet preparation

Enzalutamide (Medivation) was admixed with Open Standard Diet (Research Diets Inc.), which was fed to animals as indicated in each experiment and as previously described (26).

Physiologic and immunologic effects of enzalutamide

C57BL/6 mice ($n = 3/\text{group}$) were not treated or treated with enzalutamide at targeted daily doses of 0, 1, 10, 50, or 100 mg for 14 days. Peripheral blood was collected from the retro-orbital cavity and analyzed for complete blood count (CBC) and enzalutamide concentration in plasma by HPLC. Spleens were harvested and a mixed lymphocyte (H-2^d vs. H-2^b) assay and an anti-CD3 proliferation assay were performed as previously described (27, 28). Genitourinary tissues and thymuses were harvested and weighed. Immune-cell population subsets from splenocytes were analyzed by flow cytometry. In a separate study, TRAMP mice ($n = 2\text{--}9/\text{group}$) were sorted into 4 groups as previously described and randomized to receive no treatment or enzalutamide 10 mg/d for 4 or 12 weeks. Mice were sacrificed and their genitourinary tissues and thymuses were harvested and weighed.

Physiologic effects of enzalutamide versus castration

C57BL/6 mice ($n = 4/\text{group}$) were not treated or subjected to castration 14 days before sacrifice. Genitourinary tissues and thymuses were harvested and weighed.

Phenotypic analysis by flow cytometry

Cells were stained and fixed as previously described (26). Multicolor cytometric analyses were performed using a Becton Dickinson LSRII and analyzed using FACSDiva software or using a FACScan flow cytometer using CellQuest software (BD Biosciences).

TREC RT-PCR

C57BL/6 mice ($n = 7/\text{group}$) were not treated or treated with enzalutamide 10 mg/d for 14 days. Blood was collected and DNA was purified using the QIAamp-DNA Mini Kit (Qiagen). PCR was performed using the previously described sjTREC primers and probe for C57BL/6 mice (29). In a separate study, TRAMP mice ($n = 2\text{--}8/\text{group}$) were not treated or treated with enzalutamide for 4 weeks, after which TREC levels were analyzed as described.

Immunological assays

C57BL/6 mice ($n = 6/\text{group}$) were not treated or were vaccinated with control-vaccine or Twist-vaccine at 4 yeast units (YU) per animal ($1\text{ YU} = 10^7$ yeast particles) on days 0, 7, 14, and 21. On day 35, mice were sacrificed and splenocytes were analyzed for Twist-specific CD4⁺ T-cell proliferation using Twist peptide (FSVWRMEGAWSMSAS; CPC Scientific), as previously described (30). LCMV peptide (RPQASGVYMGNLTAQ) and ConA were used as negative and positive control of CD4⁺ T-cell proliferation, respectively. In a subsequent study, C57BL/6 mice ($n = 6/\text{group}$) were not treated, vaccinated 4 times with Twist-vaccine at 4 YU per animal on days 0, 7, 14, and 21, and/or treated with enzalutamide 10 mg/d starting on day zero. On day 35, spleens were harvested and analyzed for Twist-specific CD4⁺ T-cell proliferation as described above. In another independent study ($n = 5/\text{group}$), C57BL/6 and TRAMP mice harboring well-differentiated tumors were not treated

or vaccinated three times with Twist-vaccine at 4 YU per animal weekly. On day 28, spleens were harvested and analyzed for Twist-specific CD4⁺ T-cell proliferation and IFN- γ production. In a separate study, TRAMP mice at various stages of tumor development were vaccinated with Twist-vaccine at 4 YU per animal and treated with enzalutamide 10 mg/d for 12 months. Three mice that received the combination of enzalutamide and Twist-vaccine survived and were analyzed for immunologic responses. Pooled splenic T cells from these mice were analyzed for Twist-specific CD4⁺ T-cell proliferation, as described above, and for peptide-specific IFN- γ and TNF- α production. To evaluate CD8⁺ T-cell responses, spleens were harvested and cocultured for 7 days with Twist peptide (1 $\mu\text{g}/\text{mL}$; TQSLNEAFL), prostate stem-cell antigen (PSCA) peptide (1 $\mu\text{g}/\text{mL}$; NITCCYSDL), survivin peptide (1 $\mu\text{g}/\text{mL}$; CFFCFKEL), and p15E peptide (1 $\mu\text{g}/\text{mL}$; KSPWFITL, referred to as gp70 peptide). Supernatants from these cultures were collected and analyzed for murine IFN- γ and TNF- α by cytometric bead array according to the manufacturer's instructions (BD Biosciences).

RNA interference (siRNA)

siRNA duplexes targeting Twist sequences and control were purchased from Santa Cruz Biotechnology. TRAMP-C2 cells were transfected with Twist siRNA and control siRNA according to the manufacturer's instructions. The interference of Twist expression was confirmed by real-time PCR (RT-PCR) analysis using TaqMan probes for Twist (Mm00442036_m1; Applied Biosystems). All values are expressed as a ratio to the endogenous control GAPDH (glyceraldehyde-3-phosphate dehydrogenase), as previously described (31). A migration assay was performed as previously described (32).

Cytotoxicity T-cell assays

The H-2Kb-restricted gp70-specific CD8⁺-cytotoxic T cells that recognize the peptide p15_{e604} have been previously described (33). The CTL assays were performed as previously described (34).

Twist immunohistochemistry

At each stage of prostate cancer development in the TRAMP mice, Twist expression was detected using rabbit polyclonal antibody to Twist (Abcam) according to the manufacturer's instructions. Entire slides were digitally scanned by an Aperio ScanScope CS scanning system and analyzed by Aperio ImageScope Viewer software (Aperio Technologies Inc.). Twist-positive tumor regions were measured using the Positive Pixel Count v9 algorithm.

Treg functional assay

TRAMP mice ($n = 2\text{--}8/\text{group}$) were not treated or treated with enzalutamide for 4 weeks. Spleens were harvested from sacrificed mice, and CD4⁺CD25⁺FoxP3⁺ Tregs were purified using a Treg isolation kit according to the manufacturer's instructions (Stem Cell Technologies). Tregs were found to be more than 90% pure by flow cytometry. Treg

functional assays were performed as previously described (27, 35). CD8⁺ T cell from naïve untreated C57BL/6 mice were used as responders.

Survival study

TRAMP mice ($n = 25$ –27/group) were not treated or randomized to receive enzalutamide 10 mg/d, Twist-vaccine 4 YU per animal, or a combination of enzalutamide and Twist-vaccine. At initiation of treatments, average mouse ages were 11 weeks (PIN), 17 weeks (well-differentiated adenocarcinoma), 25 weeks (moderately-differentiated adenocarcinoma), and 34 weeks (poorly-differentiated adenocarcinoma). Vaccinations were given weekly for 3 months, then twice a month for 3 months, and monthly thereafter.

Statistical analysis

GraphPad Prism 5 statistical software (GraphPad Software) was used to measure two-tailed unpaired Student *t* tests for differences between groups, one-way ANOVA for differences among groups with a Bonferroni multiple comparison test and Wilcoxon tests of survival.

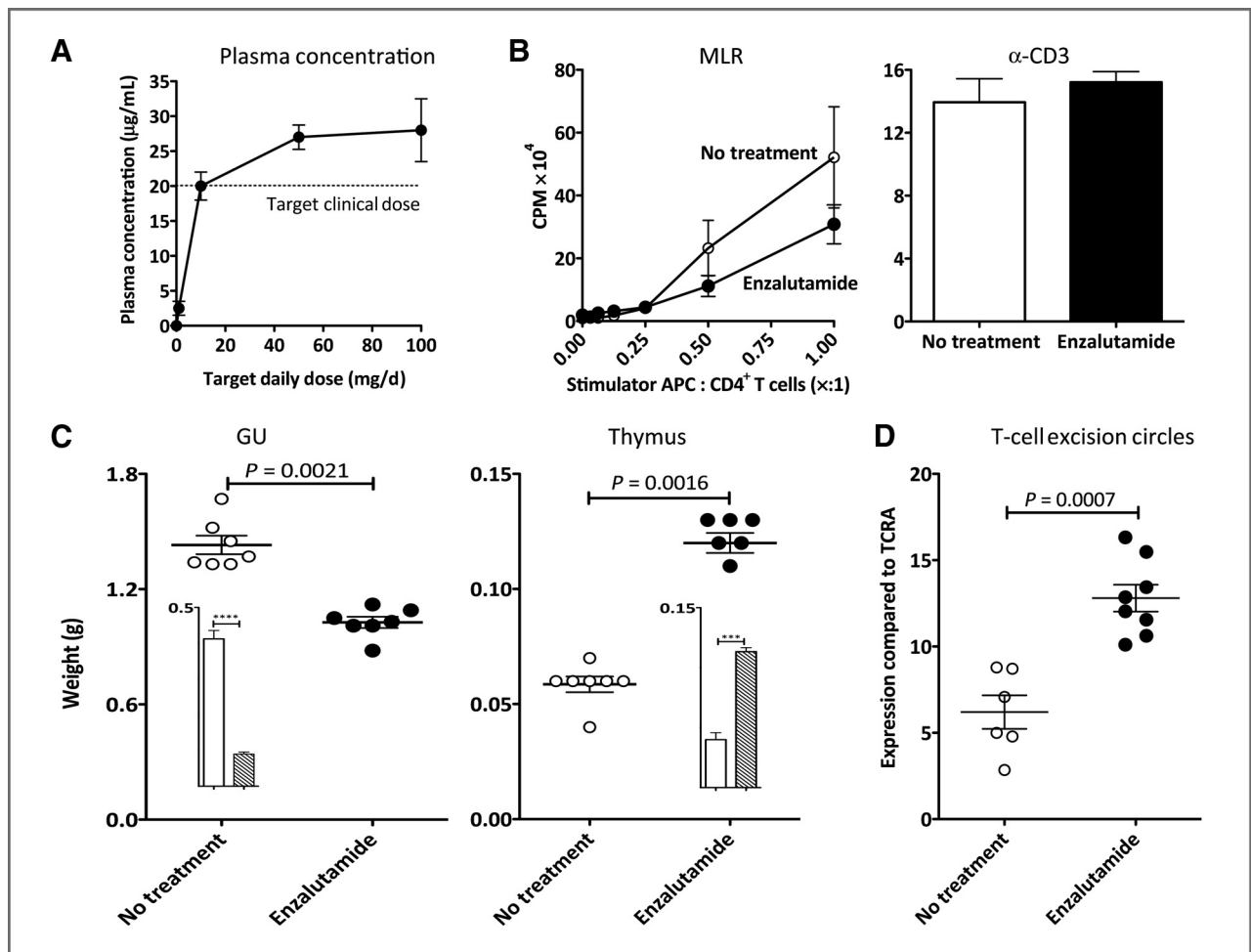


Figure 1. Enzalutamide does not affect T-cell activity in male C57BL/6 mice, but does mediate a reduction in genitourinary tissue weight, enlargement of the thymus, and increased TREC levels. A, enzalutamide concentration in plasma of mice on daily enzalutamide diet. Mice ($n = 3$) were treated with enzalutamide at target daily doses of 0, 1, 10, 50, and 100 mg for 14 days. Collected blood was tested for enzalutamide concentration in plasma. B, effect of enzalutamide on CD4⁺ T-cell activity. Mice (H-2^b; $n = 3$) were not treated (open circles) or treated with enzalutamide (closed circles) for 14 days. CD4⁺ T cells were collected and cocultured with allogeneic splenocytes (stimulator; H-2^d) for 5 days (left) at different stimulator to CD4⁺ T cells responder ratios. Effect of enzalutamide on anti-CD3-induced proliferation of CD4⁺ T cells was evaluated by culturing purified CD4⁺ T cells with 2 µg/mL plate-bound anti-CD3 (right). Proliferation in response to stimuli was measured by incorporation of ³H-thymidine, which was added during the final 18 hours. C, exposure to enzalutamide reduced genitourinary tissue weight and enlarged thymus in mice ($n = 7$) not treated (open circles) or treated with enzalutamide (closed circles) for 14 days. Mice ($n = 4$) were left untreated (open bar) and subjected to castration 2 weeks (hashed bar) prior to sacrifice. Mice subjected to castration showed significant reduction of genitourinary tissues ($P = 0.0001$) and enlarged thymus ($P = 0.0004$) when compared with control mice (inset). D, enzalutamide significantly increased TREC levels in mice ($n = 7$) not treated (open circles) or treated with enzalutamide (closed circles) for 14 days. One hundred nanograms of DNA from blood was collected and TREC levels were quantified by RT-PCR. Results were normalized against the constant gene segment of TCRA, which serves as an endogenous reference gene. All experiments were carried out three times with similar results. Statistical analyses were conducted by a Student *t* test. Error bars, mean \pm SEM from triplicate measurements. ****, $P = 0.0001$. ***, $P = 0.0004$.

Results

Enzalutamide has minimal effects on T-cell activity

To facilitate preclinical animal modeling, enzalutamide, which is administered orally to humans, was formulated into rodent diet at different concentrations to achieve target daily doses of 0, 1, 10, 50, and 100 mg. After 14 days of treatment, dose-dependent levels of enzalutamide were detected in mouse plasma (Fig. 1A). The dose level in animals that achieved the equivalent therapeutic levels observed in humans (4) was 10 mg/d, and thus was the dose used in all subsequent studies. Untreated and enzalutamide-treated mice had comparable food intake, body weight, and CBCs. Flow cytometry analysis of splenocytes revealed no differences between untreated and enzalutamide-treated mice in the number of immune-cell population subsets (CD4⁺ and CD8⁺ T cells, Tregs, and myeloid-derived suppressor cells [MDSC]; $P > 0.05$). The functional activity of CD4⁺ T cell was determined by mixed lymphocyte reaction (MLR) and anti-CD3 proliferation. By MLR, no differences were observed in allogeneic CD4⁺ T-cell proliferative responses between untreated and enzalutamide-treated mice ($P > 0.05$; Fig. 1B). In addition, CD4⁺ T-cell proliferation, stimulated with plate-bound anti-CD3, was similar between untreated and enzalutamide-treated mice ($P = 0.48$; Fig. 1B). These findings indicate that enzalutamide has minimal effects on T-cell activity and is immune inert.

Enzalutamide reduces genitourinary tissue weight, enlarges the thymus, and increases TREC levels

Clinically, ADT reduces prostate size and weight and increases thymus weight (10). In this study, male mice were castrated or sham-castrated, as previously described (24), then sacrificed 2 weeks later. Harvested genitourinary tissue and thymuses from untreated and sham-castrated mice were similar in weight, but castrated mice showed significantly reduced genitourinary tissue weight ($P = 0.0001$) and increased thymus size ($P = 0.0004$) compared with untreated mice (Fig. 1C insets). To determine whether enzalutamide creates similar effects, mice were not treated or treated with enzalutamide for 14 days. Enzalutamide-treated mice had significant reductions ($P = 0.0021$) in genitourinary tissue weight and significant increases ($P = 0.0016$) in thymus weight (Fig. 1C) compared with untreated mice. To determine whether increased thymus size corresponded with increased thymic function, we evaluated changes in levels of TREC, an episomal DNA by-product generated when gene segments encoding the T-cell receptor are rearranged (29). We collected blood from enzalutamide-treated and untreated mice and performed RT-PCR to detect the presence of TREC. We observed a significant increase ($P = 0.0007$) in TREC levels in peripheral blood from enzalutamide-treated mice compared with untreated mice (Fig. 1D). These data indicate that enzalutamide mediates significant physiologic changes in mice, including reduction in genitourinary tissue weight, enlargement of the thymus, and increased levels of TREC in peripheral blood.

Vaccination with Twist-vaccine generates Twist-specific immune responses

Twist, a highly conserved transcription factor, plays an essential role in metastatic processes and is highly expressed in prostate cancer tissue, making it a potential target for vaccine immunotherapy (15, 20). To determine whether a Twist-vaccine could elicit Twist-specific immune responses, groups of mice were not treated or were vaccinated with 1 YU of control-vaccine or Twist-vaccine on days 0, 7, 14, and 21 and sacrificed 14 days later. Untreated mice and mice vaccinated with control-vaccine showed negligible CD4⁺ T-cell proliferation (Fig. 2A). In contrast, vaccination with Twist resulted in a 5-fold increase in antigen-specific CD4⁺ T-cell proliferation compared with untreated ($P = 0.0003$) or control-vaccine ($P = 0.0001$). To determine whether the addition of enzalutamide beneficially affected the generation of Twist-specific immunity, mice were not treated, or were given Twist-vaccine alone, enzalutamide alone, or enzalutamide and Twist-vaccine on days 0, 7, 14, and 21, and sacrificed 14 days later. Untreated mice generated minimal Twist-specific CD4⁺ T-cell proliferation (Fig. 2B). Enzalutamide treatment resulted in a 2-fold increase in CD4⁺ Twist proliferation ($P = 0.001$) compared with untreated mice. Vaccination with Twist resulted in a 3.6-fold and 1.8-fold increase in CD4⁺ Twist proliferation compared with untreated ($P = 0.005$) and enzalutamide-treated mice ($P = 0.02$), respectively. Combination treatment with Twist-vaccine and enzalutamide resulted in a 3-fold and 1.5-fold increase in CD4⁺ Twist proliferation compared with untreated ($P = 0.002$) and enzalutamide-treated mice ($P = 0.03$), respectively. There was no significant difference in CD4⁺ Twist proliferation between mice receiving Twist-vaccine and mice receiving the combination treatment. All groups demonstrated similar CD4⁺ T-cell proliferation against LCMV peptide (negative control; Fig. 2) and ConA (positive control; Fig. 2 insets). These data demonstrate that the combination of enzalutamide and Twist-vaccine does not negatively affect the production of Twist-specific immunity.

Silencing Twist reduces the migratory capacity of TRAMP-C2 cells

Twist plays a critical role in the metastasis of murine mammary carcinoma, as previously demonstrated (20). To investigate whether Twist also plays a critical role in the migratory capacity of TRAMP-C2 cells, we used Twist-siRNA to transiently downregulate the expression of Twist. When levels of Twist mRNA expression were analyzed by RT-PCR (Fig. 3A), we observed a 45% reduction in the levels of Twist mRNA ($P = 0.001$). An *in vitro* migration assay demonstrated that the partial reduction of Twist expression reduced the migratory capacity of TRAMP-C2 cells by 60% ($P = 0.008$) compared with control cells (Fig. 3B).

Enzalutamide mediates immunogenic modulation in TRAMP-C2 cells

Immunogenic modulation is exposure of tumor cells to therapies that sensitizes them to immune-mediated attack (14). To determine whether enzalutamide mediates

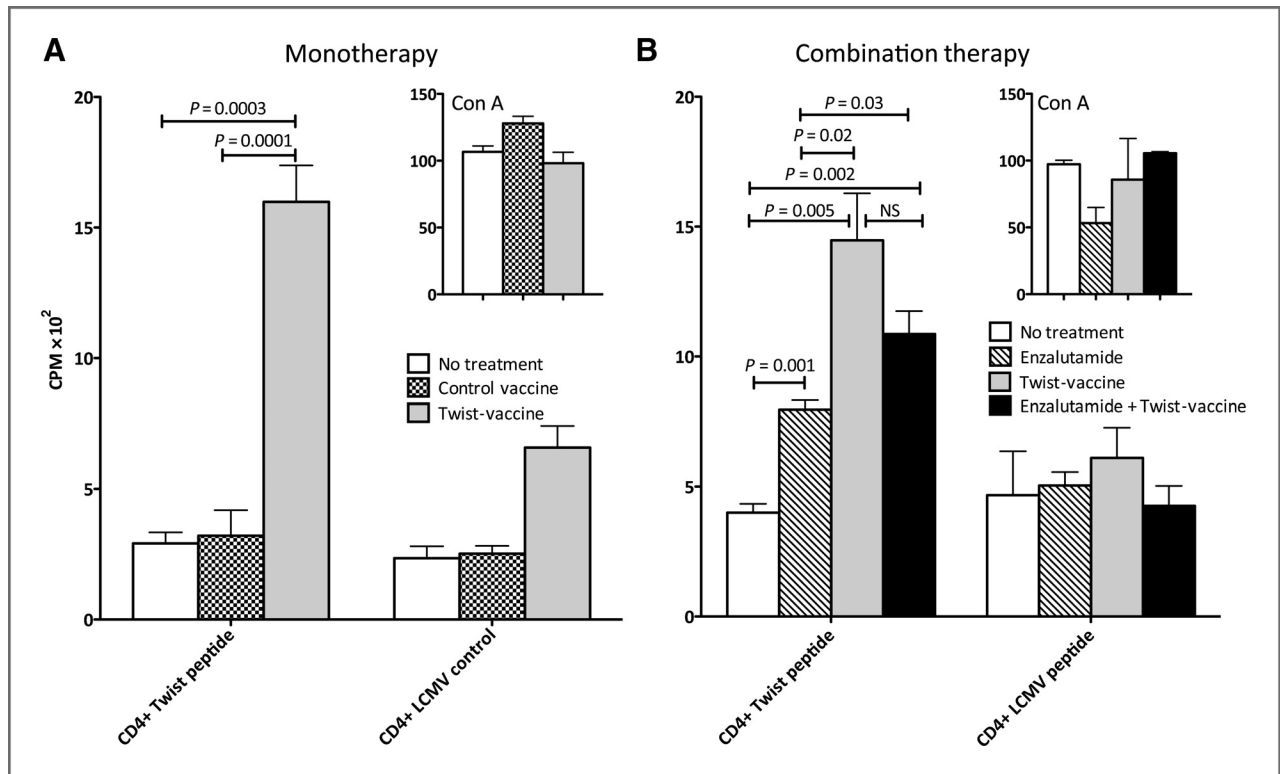


Figure 2. Vaccination with Twist induces antigen-specific T-cell responses in male C57BL/6 mice. **A**, mice ($n = 6$) were not treated or were vaccinated with 1 YU of control-vaccine or Twist-vaccine on days 0, 7, 14, and 21. On day 35, mice were sacrificed, spleens were harvested, and CD4⁺ T cells were purified and tested for proliferation by culturing with irradiated APCs and Twist peptide (5 $\mu\text{g}/\text{mL}$) or LCMV control peptide (5 $\mu\text{g}/\text{mL}$) or ConA positive control (10 $\mu\text{g}/\text{mL}$) for 5 days. Proliferation in response to stimuli was measured by incorporation of ³H-thymidine, which was added during the final 18 hours. **B**, mice ($n = 6$) were not treated, or were treated with enzalutamide alone, 1 YU of Twist-vaccine alone, or a combination of enzalutamide and Twist-vaccine. Mice were vaccinated on days 0, 7, 14, and 21 and sacrificed on day 35. Spleens were harvested and CD4⁺ T cells were purified and tested for proliferation as described above. All experiments were done twice with similar results. Statistical analyses were carried out by a Student *t* test. Error bars, mean \pm SEM from triplicate measurements.

immunogenic modulation, TRAMP-C2 cells were treated with enzalutamide *in vitro* and used as target cells in gp70-mediated killing, which is a specific CTL line that recognizes the endogenous retrovirus *env* peptide p15_{e604}. Exposing TRAMP-C2 cells to enzalutamide significantly enhanced ($P = 0.008$) p15_{e604}-specific CTL-mediated lysis relative to tumor cells exposed to vehicle (Fig. 3C). Increased expression of cell surface expression of Fas and MHC class I molecules has been implicated in enhancing antitumor T-cell responses through diverse mechanisms (36). Flow cytometry analyses demonstrated that enzalutamide exposure increased the expression of Fas and MHC class I molecules on the surface of TRAMP-C2 cells (Fig. 3D).

Twist expression increases along with tumor progression in TRAMP mice

TRAMP is an ideal animal model for prostate cancer because tumor progression in TRAMP mice closely mimics prostate cancer progression in humans (22, 23). When TRAMP mice of different ages (representing different stages of disease) were sacrificed and their prostates were harvested, we observed increases in Twist expression as mice aged (i.e., tumors progressed from PIN to poorly-differen-

tiated adenocarcinomas) by immunohistochemistry (Fig. 4A). Digital quantification of tissue slides revealed the following percentages of Twist⁺ cells: 47% in mice with PIN; 57% in mice with well-differentiated adenocarcinoma; 64% in mice with moderately-differentiated adenocarcinoma; and 74% in mice with poorly-differentiated adenocarcinoma (Fig. 4B–D). This accords with findings in human prostate cancer, in which elevated Twist protein levels are positively associated with Gleason score, suggesting that increased Twist expression is associated with poor prognosis in prostate cancer (15).

Vaccination with Twist-vaccine generates Twist-specific immune responses in TRAMP mice

To determine whether the Twist-vaccine could elicit Twist-specific immune responses in TRAMP mice, groups of C57BL/6 and TRAMP mice were not treated or vaccinated three times weekly with 1 YU of Twist-vaccine. Untreated C57BL/6 and TRAMP mice showed similar negligible CD4⁺ T-cell proliferation (Fig. 5A) and Twist-specific IFN- γ production (Fig. 5B). In contrast, vaccination with Twist resulted in an 8-fold and 2.5-fold increase in Twist-specific CD4⁺ T-cell proliferation compared with no treatment

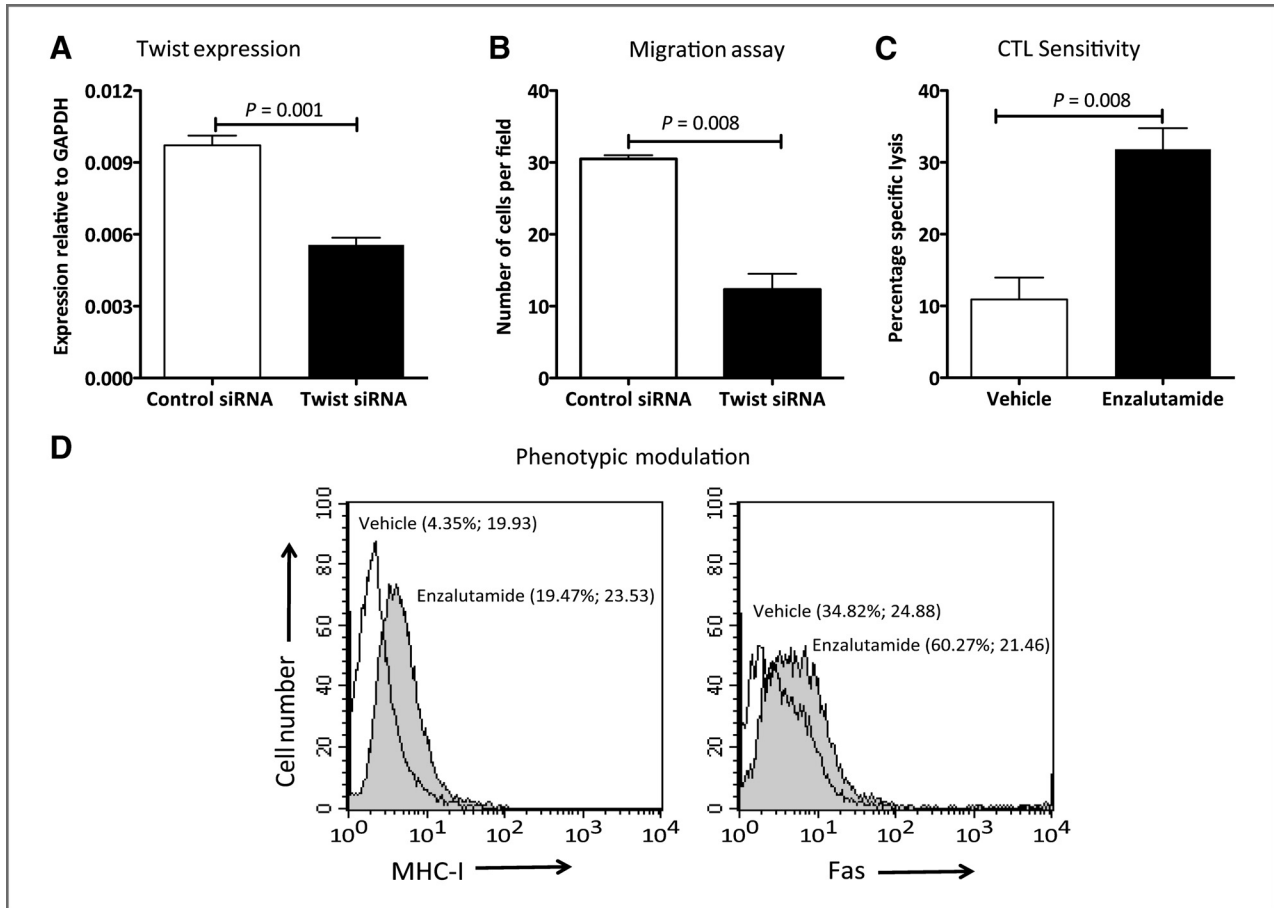


Figure 3. Twist plays an important role in the migratory function of TRAMP-C2 cells and enzalutamide mediates immunogenic modulation. **A**, expression of Twist was analyzed by RT-PCR in TRAMP-C2 cells transiently transfected with Twist or control siRNAs. **B**, *in vitro* cell migration assay was performed using the transiently transfected cells. **C**, enzalutamide improved TRAMP-C2 cells' sensitivity to gp70-specific CD8⁺-cytotoxic T-cell lysis. TRAMP-C2 cells were exposed *in vitro* to either vehicle (dimethyl sulfoxide, DMSO) or 10 μ M enzalutamide for 48 hours. Cells were harvested, washed, and labeled with ¹¹¹In. The sensitivity of TRAMP-C2 target cells to gp70-specific killing was determined after cells were incubated at an effector:target ratio of 50:1. **D**, enzalutamide mediated increased expression of cell surface Fas and MHC-I molecules on TRAMP-C2 cells. TRAMP-C2 tumor cells were treated *in vitro* for 48 hours with vehicle (open histogram) or 10 μ M enzalutamide (shaded histogram) and were analyzed for surface expression Fas and MHC-I by flow cytometry. Numbers in parentheses indicate the percentage of positive cells and mean fluorescence intensity. Data are representative of two independent experiments. Statistical analyses were carried out by a Student *t* test. Error bars, mean \pm SEM from triplicate measurements.

group in C57BL/6 ($P = 0.0002$) and in TRAMP mice ($P = 0.001$), respectively (Fig. 5A). All groups showed similar insignificant proliferation toward LCMV-negative control (Fig. 5A, inset). In addition, vaccination with Twist generated a 14-fold and 3.5-fold increase in Twist-specific IFN- γ production compared with no treatment group in C57BL/6 and TRAMP mice, respectively (Fig. 5B).

In TRAMP mice, enzalutamide mediates reduction of genitourinary tissue weight and thymic enlargement while remaining immune inert

TRAMP mice of different ages (representing different tumor stages) were randomized to receive no treatment or enzalutamide for 4 or 12 weeks. Mice were sacrificed at the end of treatment and their genitourinary tissues and thymuses were harvested and weighed. Treatment with enzalutamide mediated reduced genitourinary tissue weight in all tumor stages (Fig. 6A). Significantly reduced genitouri-

nary tissue weight was observed in mice with PIN ($P = 0.002$), well-differentiated adenocarcinomas ($P = 0.02$), and poorly-differentiated adenocarcinomas ($P = 0.023$). In enzalutamide-treated mice, significant increases in thymus weight were observed in all tumor stages (PIN, $P = 0.007$; well-differentiated, $P = 0.02$; moderately-differentiated, $P = 0.007$; and poorly-differentiated, $P = 0.05$) compared with untreated mice (Fig. 6B). Enzalutamide-treated mice also showed increases in TREC levels in all tumor stages (PIN, $P = 0.01$; moderately-differentiated, $P = 0.006$; and poorly-differentiated, $P = 0.03$) compared with untreated mice (Fig. 6C). Immunohistochemistry analyses of tumor tissues demonstrated that there was a 4-fold increase in CD3⁺ tumor-infiltrating lymphocytes in testis ($P = 0.006$) of enzalutamide-treated mice when compared with untreated mice. Flow cytometry analyses of splenocytes and thymocytes, however, revealed no significant differences in the number of subsets of immune-cell populations (CD4⁺ and

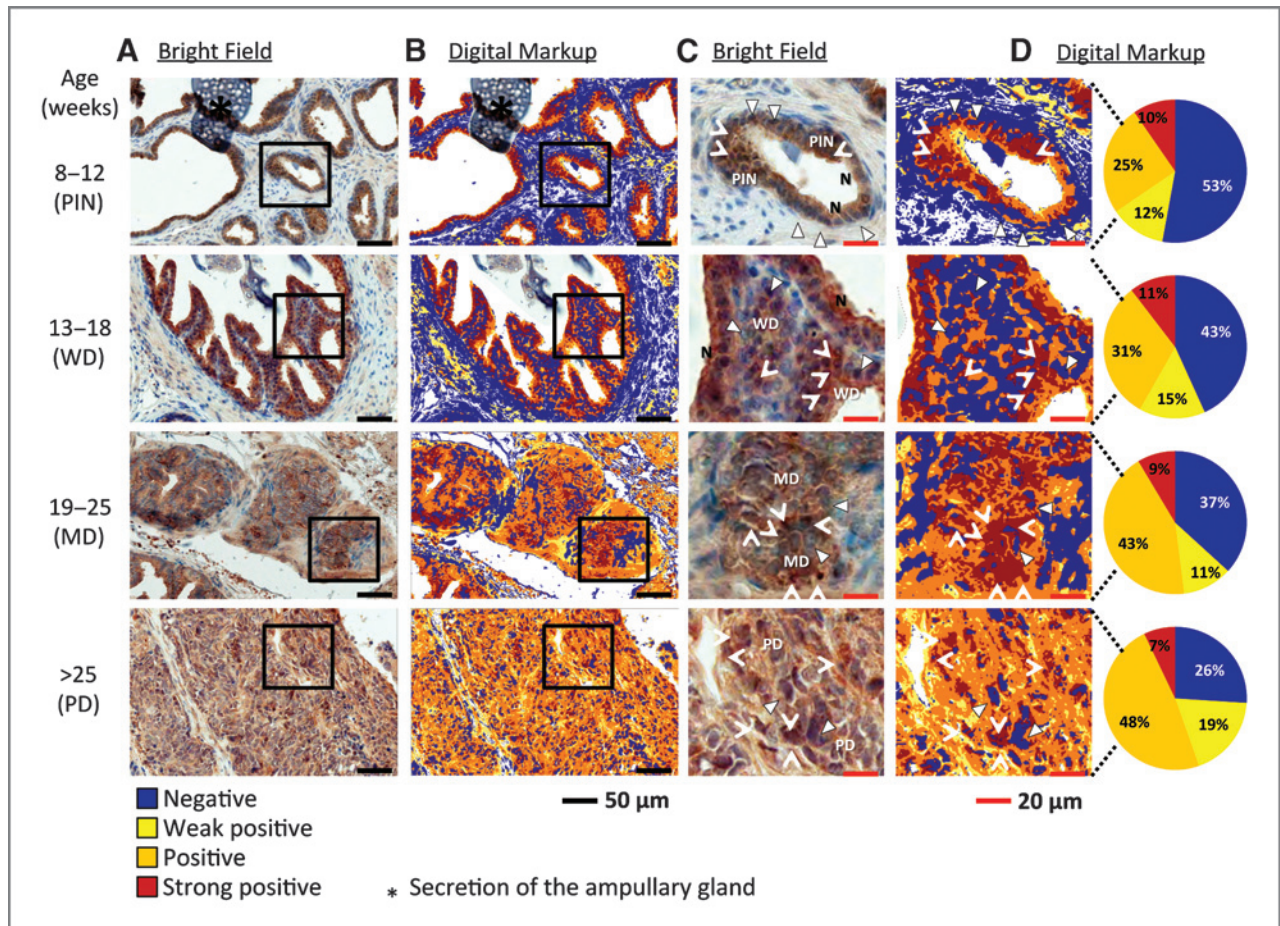


Figure 4. Twist expression in prostate tissues from TRAMP mice varies at different stages of tumor development. A, immunohistochemical analysis of prostate sections from TRAMP mice ($n = 3/\text{group}$) of different ages (representing different stages of tumor development). Sections were stained with Twist-DAB and counterstained with hematoxylin. B, digital markup of sections depicted in column A, elaborated by Positive Pixel Count v9 algorithm using Aperio ImageScope image analysis software. C, magnification of tissues within squares in column A. D, magnification of tissues within squares in column B and digital quantification of pixels indicating Twist expression: blue, negative; yellow, weak positive; orange, positive; brown, strong positive; triangles, blue nuclei/negative for Twist; arrowheads, brown nuclei, positive for Twist. N, normal epithelium; WD, well-differentiated adenocarcinoma; MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma. Triangles, note the absence of Twist expression in nuclei of normal epithelia compared with neoplasias. *, secretion of the ampullary gland.

CD8⁺ T cells, Tregs, and MDSCs) between untreated and enzalutamide-treated mice ($P > 0.05$). There was no difference in Treg suppressive function between enzalutamide-treated and untreated mice. In both groups, Tregs were able to suppress CD8⁺ T-cell proliferation by 50% (Fig. 6D). By MLR, no differences were observed in allogeneic CD4⁺ T-cell proliferative responses between untreated and enzalutamide-treated mice (data not shown).

Combination treatment with enzalutamide and Twist-vaccine significantly improved overall survival in TRAMP mice

To determine whether the combination of enzalutamide and Twist-vaccine would beneficially affect overall survival in TRAMP mice, mice were randomized to receive no treatment, enzalutamide, Twist-vaccine, or the combination of enzalutamide and Twist-vaccine. No differences were observed among untreated mice and mice receiving

Twist-vaccine or enzalutamide alone (Fig. 7A). However, mice receiving the combination treatment showed a significant increase in overall survival (28 vs. 14.5 weeks after treatment) compared with untreated mice ($P = 0.0001$) or mice receiving Twist-vaccine ($P = 0.0003$) or enzalutamide ($P = 0.008$) alone. This represents a 75% reduction in death rate (HR, 3.9) for mice receiving combination therapy compared with untreated mice.

Similar results were observed in mice with all tumor stages except PIN (Fig. 7B). Mice receiving Twist-vaccine or enzalutamide alone showed no improvement in overall survival over untreated mice. In contrast, mice receiving the combination treatment showed a statistically significant increase in overall survival (27.5 vs. 10.3 weeks after treatment) compared with untreated mice ($P \leq 0.0001$) or mice receiving Twist-vaccine ($P = 0.0003$) or enzalutamide ($P = 0.0009$) alone. Subset analyses based on different ages of mice (representing different stages of tumor development)

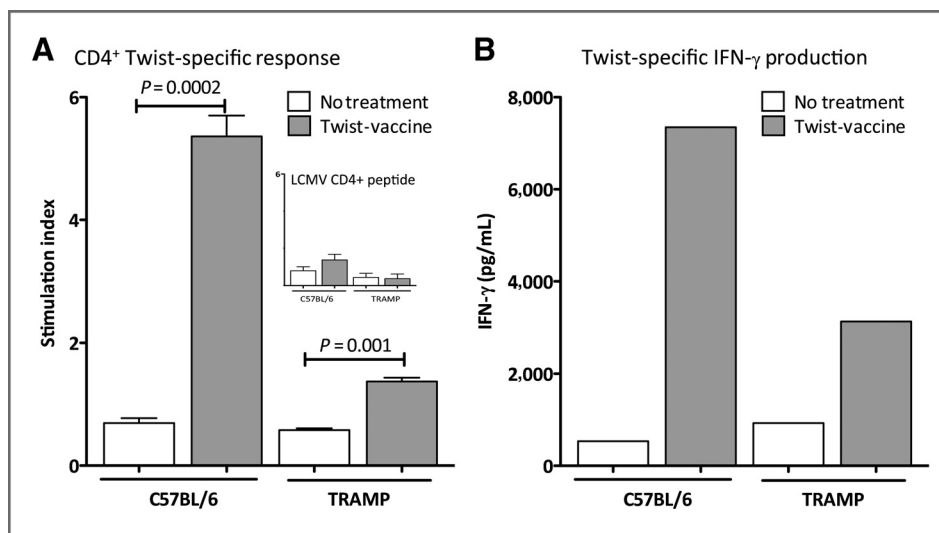


Figure 5. Vaccination with Twist induces antigen-specific T-cell responses in TRAMP mice. **A**, C57BL/6 and TRAMP mice ($n = 5$) harboring moderately differentiated adenocarcinomas at average age of 17 weeks were not treated or were vaccinated with 1 YU of Twist-vaccine on days 0, 7, and 14. On day 28, mice were sacrificed, spleens were harvested, and CD4⁺ T cells were purified and tested for proliferation by culturing with irradiated APCs and Twist peptide (5 $\mu\text{g}/\text{mL}$) or LCMV CD4⁺ peptide (5 $\mu\text{g}/\text{mL}$) or ConA positive control (10 $\mu\text{g}/\text{mL}$) for 5 days. Proliferation in response to stimuli was measured by incorporation of ³H-thymidine, which was added during the final 18 hours. Statistical analyses were done by a Student *t* test. Error bars, mean \pm SEM from triplicate measurements. **B**, to evaluate CD8⁺ T-cell responses, spleens were harvested and coincubated for 7 days with Twist peptide (1 $\mu\text{g}/\text{mL}$). Lymphocytes were restimulated with fresh irradiated naive splenocytes and each peptide: 1 $\mu\text{g}/\text{mL}$ of Twist peptide or 1 $\mu\text{g}/\text{mL}$ of HIV-gag peptide. Twenty-four hours later, the supernatant fluid was collected and analyzed for murine IFN- γ using the Cytometric Bead Array Kit. Nonspecific IFN- γ production in response to HIV-gag control peptide was subtracted from that induced by Twist peptide. Data are from two separate independent experiments.

showed no significant differences in mice with PIN among all treatment groups (Fig. 7C). Mice with well-differentiated adenocarcinomas treated with the combination therapy had improved survival compared with untreated mice ($P = 0.0026$) or mice receiving Twist-vaccine alone ($P = 0.0031$). Mice with moderately-differentiated adenocarcinomas treated with the combination therapy had significantly improved survival compared with untreated mice ($P = 0.01$) or mice receiving enzalutamide alone ($P = 0.01$). Mice with poorly differentiated adenocarcinomas treated with the combination therapy had improved survival compared with untreated mice ($P = 0.02$) or mice receiving enzalutamide alone ($P = 0.04$). Data from these subset analyses suggest that tumor-bearing mice treated with the combination of enzalutamide and Twist-vaccine received the greatest survival benefit.

Evidence of antigen cascade and tumor-infiltrating lymphocytes in TRAMP mice receiving combination therapy

To detect the presence of Twist-specific immune responses and antigen cascade to other tumor antigens, the 3 mice that survived and received combination therapy from the aforementioned survival study were sacrificed and their spleens were analyzed. Mice receiving the combination therapy generated a 4-fold increase in Twist-specific CD4⁺ T-cell proliferation compared with controls (Supplementary Table S1). CD8⁺ T cells from mice receiving the combination therapy generated 4- to 20-fold increases in Twist-specific IFN- γ and TNF- α production compared with age-matched controls. Combination-treated mice also

showed 4- to 32-fold increases in CD8⁺ T-cell responses against PSCA, survivin, and gp70 antigens compared with age-matched controls (Supplementary Table S1). Analyses of CD3⁺ tumor-infiltrating lymphocytes of tumor tissues from these mice revealed similar number in CD3⁺ infiltrates compared with controls (data not shown).

Discussion

The ability of ADT to initially reduce tumor burden makes it a cornerstone of prostate cancer treatment (1, 37). However, most patients eventually become refractory to ADT and develop CRPC. Although it was initially thought that CRPC was completely resistant to ADT, it has since been demonstrated that CRPC remains dependent on androgen signaling for growth and that CRPC is sensitive to further manipulation of androgen signaling (38). ADT continues to play a major role in the management of CRPC. Androgens regulate a variety of immune responses, and the effects of ADT on the immune system are well documented (10, 11, 39). In aged male mice, androgen ablation regenerates the thymus, enhances the T-cell repertoire, restores peripheral T-cell phenotype, and abrogates immune tolerance of prostate tumor cells (9, 10). In humans, ADT also induces T-cell infiltration of the prostate (40). Immunogenic modulation is exposure of tumor cells to conventional therapies that consequently alters tumor phenotype to render the tumors more susceptible to immune-mediated attack (13, 36). The immunomodulatory effects of the conventional therapies include upregulation of tumor antigens, costimulatory molecules, Fas, and MHC moieties. The

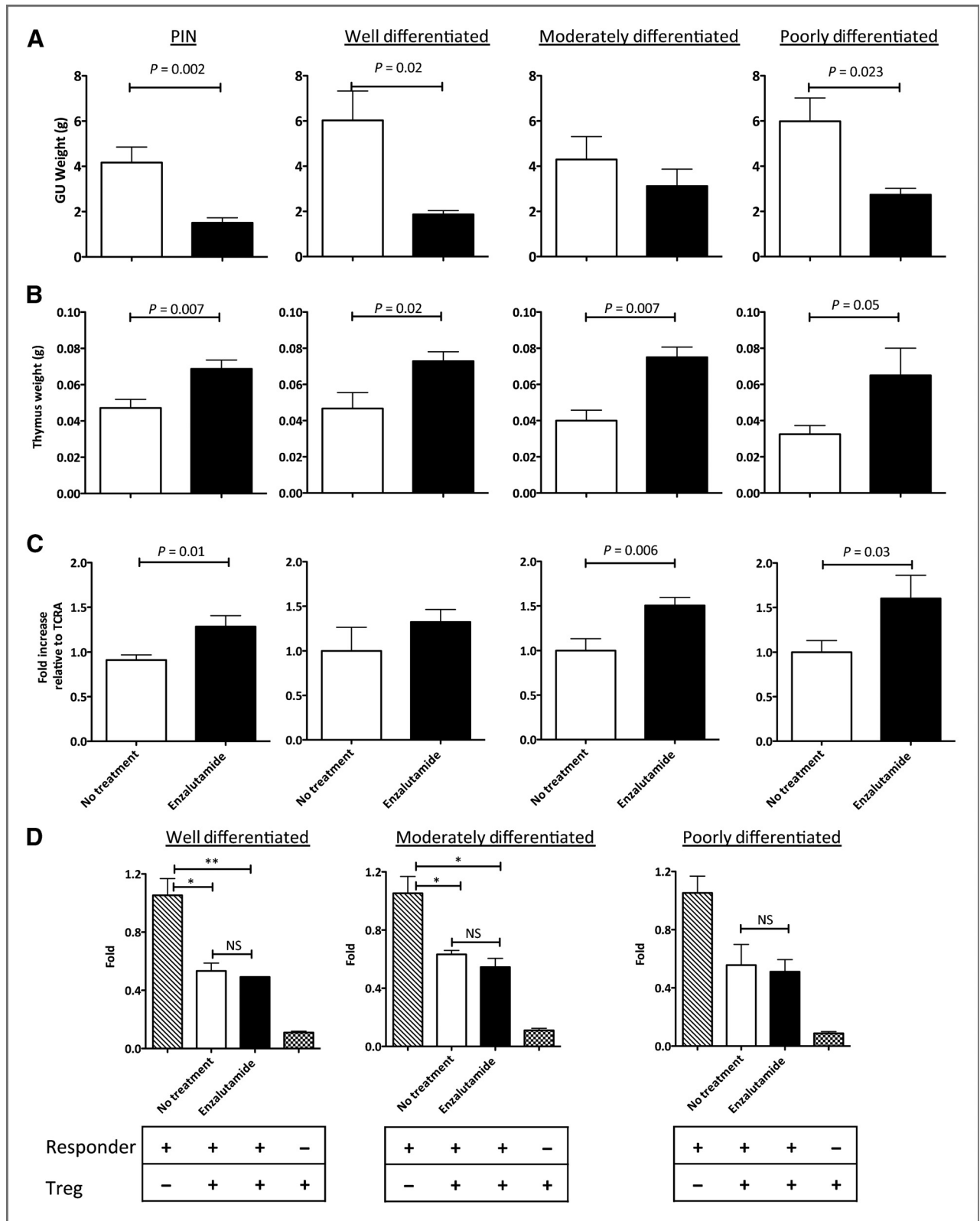


Figure 6. In TRAMP mice, enzalutamide reduces genitourinary tissue weight, enlarges the thymus, and is immune inert. A, enzalutamide mediates reduction of genitourinary tissue weight. TRAMP mice ($n = 4-9$) of different ages (representing different stages of tumor development) were not treated or treated with enzalutamide 10 mg/d for 4 weeks. (Continued on the following page.)

immunogenic modulation property of ADT, enzalutamide in particular, has not been described. We thus hypothesized that enzalutamide induces thymic regeneration and mediates immunogenic modulation and therefore renders prostate tumor cells' sensitivity to immune-mediated attack. Because of the immunomodulatory properties, we further hypothesized that enzalutamide could be exploited in combination with immunotherapy in the form of a therapeutic vaccine to create a more robust treatment option for CRPC.

This study demonstrated that enzalutamide mediates a reduction in genitourinary tissue weight and enlargement of the thymus in both C57BL/6 (Fig. 1) and TRAMP mice (Fig. 6). In TRAMP mice, enzalutamide treatment also significantly increased the numbers of CD3⁺ tumor-infiltrating lymphocytes in tumor tissues when compared with untreated mice. Quantification of TREC levels in peripheral blood provides an estimate of recent thymic emigrant levels and thus indirectly indicates the magnitude of thymic function (41). Here, enzalutamide-induced enlargement of the thymus was indeed accompanied by increased TREC levels, indicating a potential improvement in thymic function. However, there were no differences in the phenotype or function of T cells isolated from splenocytes from enzalutamide-treated mice compared with untreated mice, suggesting that enzalutamide can be administered in combination with immunotherapy without affecting immune response.

Twist, a member of a highly conserved BHLH transcription factor, has been implicated in the metastatic process, and its overexpression is associated with poor prognosis in many cancer types (15–19). Twist has been shown to play a major role in metastasis in a 4T1 murine mammary tumor model (20). Here we describe the significant role of Twist in the TRAMP-C2 model. When Twist expression was partially silenced, the migratory potential of TRAMP-C2 cells significantly diminished (Fig. 3B). Twist was confirmed as a valid target antigen in the TRAMP model and its expression increased as tumors progressed (Fig. 4). These data accord with previous studies implicating Twist in EMT and tumor-cell invasion and metastasis and showing that increased Twist protein levels correlate positively with Gleason score (15, 20). Data presented in this study further suggest that the Twist-vaccine is able to elicit Twist-specific immunity in C57BL/6 (Fig. 2) and TRAMP mice (Fig. 5) that persists for an extended period of time. Our first hypothesis was that the increased thymic function induced by enzalutamide generated new pools of naïve T cells, thus creating an ideal environment for vaccination and induction of Twist-specific immunity. Our data, however, suggest that the com-

bination of enzalutamide and Twist-vaccine did not yield any additional benefit in the generation of Twist-specific immunity (Fig. 2). Our second hypothesis was that enzalutamide mediated immunogenic modulation. Indeed, our data demonstrate that treatment of enzalutamide increased cell surface expression of Fas and MHC-I moieties and consequently improved sensitivity of TRAMP-C2 cells to immune-mediated attack (Fig. 3C and D).

Although enzalutamide mediated a reduction in genitourinary tissue weight in TRAMP mice at different ages (representing different stages of tumor development; Fig. 6), the reduction in tumor burden did not translate to improved overall survival when enzalutamide was used as monotherapy (Fig. 7). This observation contrasts with the efficacy of enzalutamide as monotherapy for prostate cancer in humans, in which enzalutamide treatment resulted in a 4.8-month advantage in overall survival compared with placebo (6). When Twist-vaccine was used as monotherapy, there was no improvement in survival compared with untreated mice. When enzalutamide and Twist-vaccine were used in combination, however, increased survival was achieved in mice at various tumor stages (Fig. 7). A subset analysis of survival revealed that older mice with more advanced tumors received the most benefit from the combination therapy. Mice with PIN did not benefit from the combination therapy, perhaps due to negligible Twist expression in this population (Fig. 4). Because Twist is associated with metastasis, Twist may not be highly expressed in precancerous lesions such as PIN (Fig. 4; ref. 15), in which vaccination against other target antigens may be more effective. This observation is supported by Garcia-Hernandez and colleagues, who used PSCA vaccine to treat young TRAMP mice with PIN (42). The PSCA vaccine's successful induction of long-term protection against prostate cancer was due to the fact that the vaccine was delivered in the early stages of tumor development. This highlights the fact that tumor antigen must be expressed at high levels to elicit a robust and effective immune-mediated response. In addition, mice that survived and received combination treatment with enzalutamide and Twist-vaccine for 12 months experienced antigen cascade (Supplementary Table S1), a host response not only to the antigen in the vaccine but also to other tumor antigens associated with a given tumor type (33, 43–45).

Our data demonstrate that enzalutamide is able to reduce tumor burden and mediates immunogenic modulation rendering tumor cells to be sensitive to immune-mediated attack. The antigen-specific immunity generated by a cancer vaccine may add further negative pressure. Tumor-specific

(Continued.) Mice were sacrificed and their genitourinary tissues harvested and weighed. B, enzalutamide induces thymic enlargement. TRAMP mice ($n = 2-8$) were not treated or treated with enzalutamide 10 mg/d for 4 weeks. Mice were sacrificed and their thymic tissues harvested and weighed. C, enzalutamide significantly increases TREC levels in TRAMP mice. TRAMP mice ($n = 4-9$) at different ages were not treated or treated with enzalutamide 10 mg/d for 4 weeks and then sacrificed. Of note, 100 ng of DNA from blood was collected and TREC levels were quantified by RT-PCR. Results were normalized against the constant gene segment of TCRA, which serves as an endogenous reference gene. D, enzalutamide does not alter Treg suppressive function. TRAMP mice ($n = 2-8$) were not treated or treated with enzalutamide 10 mg/d for 4 weeks. Spleens were harvested and CD4⁺ CD25⁺ FoxP3⁺ Tregs were purified and cultured with irradiated APCs, responder (purified CD8⁺ T cells from naïve C57BL/6 mice), and 0.1 µg/mL soluble anti-CD3 cross-linking antibody for 72 hours. Of note, ³H-thymidine was added to the wells for the last 18 hours. Error bars indicate mean ± SEM from triplicate measurements. All experiments were done twice with similar results. Statistical analyses were done by a Student *t* test. *, $P \leq 0.05$. **, $P = 0.005$.

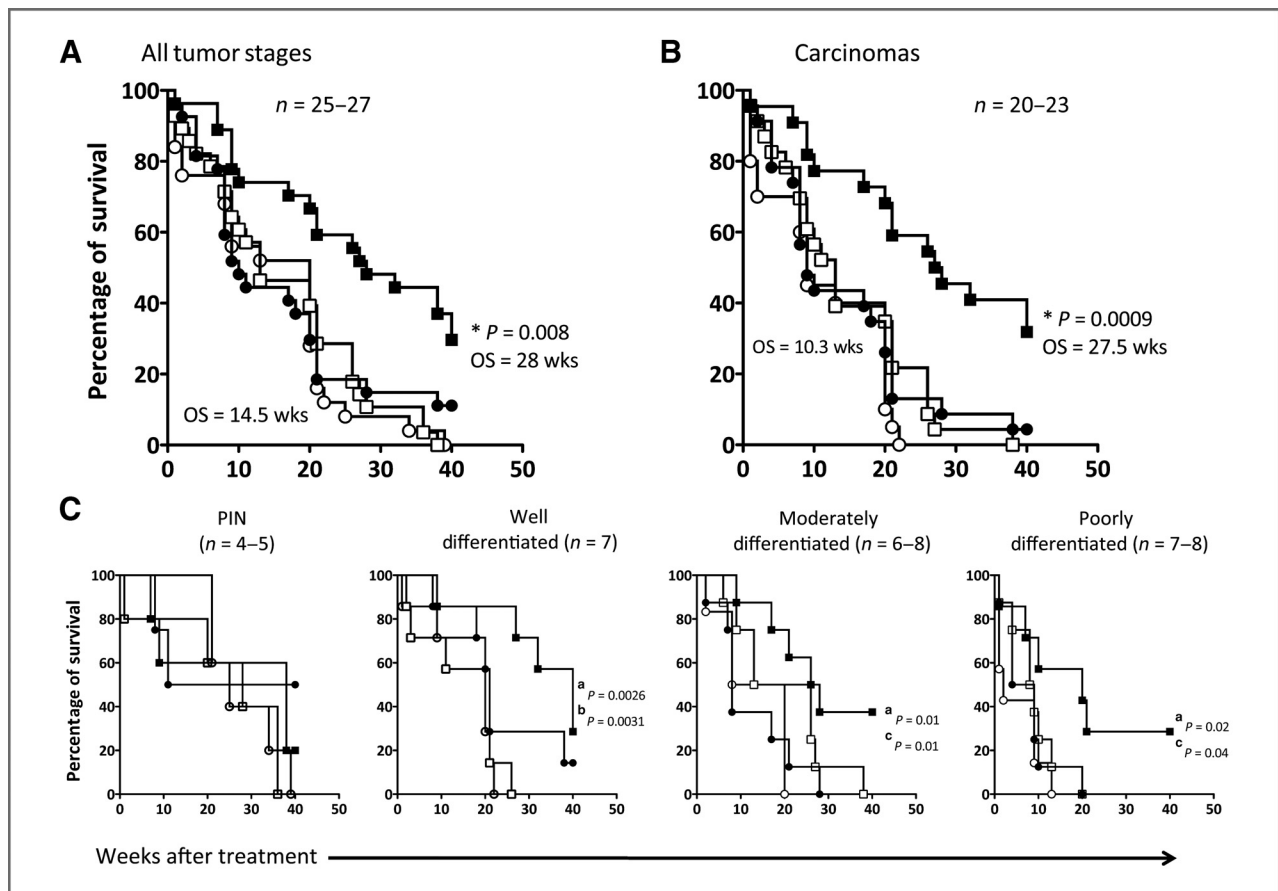


Figure 7. Combination therapy with enzalutamide and Twist-vaccine increases overall survival in TRAMP mice. TRAMP mice ($n = 25–27$) were randomized to receive no treatment (open circles), Twist-vaccine alone (open squares), enzalutamide alone (closed circles), or enzalutamide and Twist-vaccine (closed squares). Vaccine was given weekly for 3 months, then twice a month for 3 months, and monthly thereafter. Survival curve analyses are indicated for: A, mice with all tumor stages; B, mice with all tumor stages except PIN (*, P value of comparison between mice receiving combination treatment and mice receiving enzalutamide alone); C, mice from each stage of tumor progression. a, the P value between untreated mice and mice receiving combination treatment; b, the P value between mice receiving Twist-vaccine and those receiving combination treatment; c, the P value between mice receiving enzalutamide and those receiving combination treatment. OS, overall survival.

immunity induced by a therapeutic vaccine is active, dynamic, and, more importantly, able to persist long after vaccination, potentially conferring protection against tumor recurrence. Findings from this study could provide a rationale for the combination of enzalutamide with a clinically active therapeutic vaccine such as PROSTVAC-VF for the treatment of CRPC. Published data from phase II trials of PROSTVAC-VF in metastatic CRPC ($n = 125$) demonstrate that the vaccine is well tolerated and is associated with a 44% reduction in death rate and an 8.5-month improvement in median overall survival compared with placebo (8). Clinical trials evaluating the efficacy of the combination of PROSTVAC-VF and enzalutamide in patients with CRPC (NCT01867333) and those with nonmetastatic castration-sensitive prostate cancer (NCT01875250) are currently underway (www.clinicaltrials.gov).

To our knowledge, this is the first study that demonstrates (i) the previously unknown immunogenic modulation property of enzalutamide; (ii) the physiologic effect of enzalutamide in the C57BL/6 mice and the TRAMP model,

including reduced genitourinary tissue weight, enlarged thymus, and increased TREC levels; (iii) the minimal effects of enzalutamide on T-cell activity; (iv) the use of Twist, a driver of EMT/metastasis, as a target for therapeutic vaccine; (v) the ability of Twist-vaccine to generate Twist-specific immunity; and (vi) significantly improved overall survival in TRAMP mice treated with the combination of enzalutamide and a therapeutic vaccine, especially in mice with advanced prostate adenocarcinomas. These data support the combination of enzalutamide and immunotherapy as a promising treatment for CRPC.

Disclosure of Potential Conflicts of Interest

A. Protter has stock options in Medivation.

D. Apelian is employed as chief medical officer and has ownership interest in GlobImmune. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Ardiani, B. Farsaci, A. Protter
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Ardiani, B. Farsaci, A. Protter, J.W. Hodge
Writing, review, and/or revision of the manuscript: A. Ardiani, B. Farsaci, J.W. Hodge
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Ardiani, B. Farsaci, C.J. Rogers, A. Protter, J.W. Hodge
Study supervision: J.W. Hodge

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