T-Cell Infiltration and Adaptive Treg Resistance in Response to Androgen Deprivation With or Without Vaccination in Localized Prostate Cancer

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ABSTRACT

Purpose: Previous studies suggest that androgen deprivation therapy (ADT) promotes antitumor immunity in prostate cancer. Whether a vaccine-based approach can augment this effect remains unknown.

Patients and Methods: We conducted a neoadjuvant, randomized study to quantify the immunologic effects of a GM-CSF–secreting allogeneic cellular vaccine in combination with low-dose cyclophosphamide (Cy/GVAX) followed by degarelix versus degarelix alone in patients with high-risk localized prostate adenocarcinoma who were planned for radical prostatectomy.

Results: Both Cy/GVAX plus degarelix and degarelix alone led to significant increases in intratumoral $CD8^+$ T-cell infiltration and PD-L1 expression as compared with a cohort of untreated, matched controls. However, the $CD8^+$ T-cell infiltrate was accompanied by a proportional increase in regulatory T cells (Treg), suggesting that adaptive Treg resistance may dampen the immunogenicity of ADT. Although Cy/GVAX followed by degarelix was associated with a modest improvement in time-to-PSA progression and time-to-next treatment, as well as an increase in PD-L1, there was no difference in the $CD8^+$ T-cell infiltrate as compared with degarelix alone. Gene expression profiling demonstrated that *CHIT1*, a macrophage marker, was differentially upregulated with Cy/GVAX plus degarelix compared with degarelix alone.

Conclusions: Our results highlight that ADT with or without Cy/GVAX induces a complex immune response within the prostate tumor microenvironment. These data have important implications for combining ADT with immunotherapy. In particular, our finding that ADT increases both $CD8^+$ T cells and Tregs supports the development of regimens combining ADT with Treg-depleting agents in the treatment of prostate cancer.

Introduction

Prostate cancer remains the second most common cause of cancer-related mortality in men and definitive local therapy represents the only treatment modality with the potential for cure (1). Despite advances in surgical approaches, patients with high-risk localized prostate cancer continue to have a high likelihood of disease recurrence following definitive local therapy (2, 3). To date, no neoadjuvant therapy preceding prostatectomy has demonstrated sufficient efficacy to warrant FDA approval.

In contrast to traditional therapies, which decrease tumor bulk prior to surgery, immunotherapy has the potential to reengage systemic antitumor immune responses, thereby eradicating distant micro-metastases. Although the development of sipuleucel-T for castration-resistant prostate cancer (CRPC) demonstrated the potential for immunotherapy in prostate cancer, immune checkpoint inhibitors have not yielded significant responses, except perhaps when used in combination (4–10). One significant challenge to inducing antitumor immunity in prostate cancer is the noninflamed tumor microenvironment (TME; ref. 11). Prostate tumors also generally have a low mutational burden and low PD-L1 expression; these factors predict response to immunotherapy in other tumor types (12, 13). In addition, prostate tumors demonstrate multiple mechanisms of immune escape including defective antigen processing, decreased MHC class I expression, and infiltration with regulatory T cells (Treg), myeloid-derived suppressor cells, and M2 macrophages (14–17).

Prostate GVAX is an allogeneic cell-based prostate cancer vaccine composed of two irradiated cell lines (PC3 and LNCaP) that have been genetically modified to secrete GM-CSF (14). The release of GM-CSF by these modified tumor cells promotes the recruitment of dendritic cells and subsequent presentation of tumor antigens to T cells with associated activation of antitumor immune responses. Prior randomized

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Translational Relevance

In preclinical models of prostate cancer, androgen deprivation therapy (ADT) promotes immunogenic cell death, transiently mitigates T-cell tolerance to tumors, and augments vaccine-induced antigen-specific $CD8^+$ T-cell responses. However, there are limited data on the immunologic effects of ADT on the tumor microenvironment in patients. In a neoadjuvant trial, we treated men with high-risk localized prostate cancer with either ADT or ADT plus low-dose cyclophosphamide and a cell-based vaccine (Cy/GVAX), prior to radical prostatectomy. ADT induced a complex immune cell infiltrate and increased intratumoral cytolytic $CD8^+$ T cells. However, this $CD8^+$ T cell increase was accompanied by a proportional increase in $FoxP3^+$ regulatory T cells (Treg), proving strong evidence for adaptive Treg resistance. When given prior to surgery, Cy/GVAX modestly augmented the immunologic effects of ADT and decreased disease recurrence compared with ADT alone. These data support the observation that ADT has proinflammatory effects. However, these antitumor effects appear to be counterbalanced by a proportional increase in local immunosuppression.

controlled trials of GVAX as monotherapy or in combination with docetaxel in metastatic CRPC (mCRPC) failed to show a survival benefit over chemotherapy, suggesting that allogeneic cell-based immunotherapy may be insufficient on its own to generate a robust T-cell response against prostate cancer (14). This may be particularly relevant in advanced mCRPC, wherein a more immunosuppressive TME predominates (15). However, preclinical studies demonstrate that administering low-dose cyclophosphamide prior to a cell-based GM-CSF–secreting vaccine can increase $CD8^+$ T-cell infiltration in the prostate and transiently deplete Tregs (16, 17). These preclinical data are supported by clinical trials combining GVAX with low-dose cyclophosphamide in breast cancer, colorectal cancer, and pancreatic cancer (18, 19).

In addition, prior studies in murine models show that castration results in *de novo* presentation of prostate-restricted antigens in tumor-draining lymph nodes, with transient mitigation of T-cell tolerance (18). Androgen deprivation therapy (ADT) can also induce a proinflammatory immune cell infiltrate, supporting the hypothesis that androgen ablation may augment vaccine-induced effector T-cell responses, particularly during the peri-castration period (18). Whether similar immune modulation occurs in patients remains poorly understood.

To address these questions, we conducted a randomized neoadjuvant study to test the hypothesis that the combination of low-dose cyclophosphamide plus GVAX (Cy/GVAX) could augment the ADT-induced immune response in men with localized high-risk prostate cancer. The luteinizing hormone-releasing hormone (LHRH) antagonist degarelix acetate was selected as ADT for this study based on its rapid onset-of-action allowing shorter time-to-surgery, lack of transient increase in testosterone reducing risk of tumor flare, and the observation that degarelix leads to a robust immune cell infiltrate in preclinical models, peaking around 2 weeks after administration (18). A secondary endpoint of the study was to test whether ADT plus Cy/GVAX prolongs time-to-PSA recurrence as compared with ADT alone. We also sought to more deeply profile the immunologic changes in the prostate TME mediated by ADT with or without Cy/GVAX.

Patients and Methods

Patients

Men with intermediate- to high-risk localized prostate adenocarcinoma, defined as clinical stage T1c–T3b, N0, M0, and a Gleason sum ≥ 4+3 (grade group ≥3) in at least two cores were considered eligible if they were planning to undergo prostatectomy. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 or 1; and normal kidney, liver, and marrow function. Patients with nodal (N1) or distant (M1) metastases were excluded. Key additional exclusion criteria included prior immunotherapy or vaccine therapy for prostate cancer, prior radiation, hormonal, or chemotherapy, autoimmune disease requiring corticosteroids, or known allergy to cyclophosphamide or G-CSF/GM-CSF. Written informed consent was obtained from all patients, and studies were conducted in accordance with the U.S. Revised Common Rule and approved by institutional review board.

Study design and treatment

Patients were randomized 1:1 to degarelix alone (240 mg subcutaneously) versus cyclophosphamide (200 mg/m^2 i.v.) and GVAX (2.5×10^8 PC3 cells and 1.6×10^8 LNCaP cells) given 2 weeks before degarelix. Randomization was stratified by Gleason sum: 7 versus 8–10. All patients underwent radical prostatectomy 2 weeks after degarelix (**Fig. 1**). Prostatectomy specimens were assessed for Gleason grade, nodal involvement, and pathologic stage using standard methods. Following pathologic review of prostatectomy specimens, a tumor block was selected from the highest grade tumor located in the prostate and microtome sections were prepared for biological analysis of the TME, including IHC staining for CD8, FOXP3, and PD-L1, with additional sections for expression profiling (NanoString). In addition, a contemporaneous cohort of matched-controls (cohort C) who did not receive any neoadjuvant therapy provided untreated radical prostatectomy tumor samples, which were compared with posttreatment prostatectomy samples from study cohort A (degarelix alone) and cohort B (Cy/GVAX plus degarelix) in genetic and IHC analysis. Patients were subsequently followed for biochemical (PSA) and metastatic disease progression.

Outcomes

The coprimary endpoints of the trial were safety and $CD8^+$ T-cell density ($CD8^+$ cells/mm^2) in the prostate tumor tissue following neoadjuvant therapy. Safety was assessed using NCI Common Toxicity Criteria version 4.03. Secondary endpoints included feasibility, Treg density ($FoxP3^+$ cells/mm^2) in the prostate gland, CD8 to Treg ratio, time-to-PSA recurrence, time-to-next anticancer therapy, and time-to-metastatic progression. Time-to-PSA recurrence was defined as the interval from time of prostatectomy to the time when the PSA was ≥0.2 ng/mL for the first of at least two serial rises in PSA (≥2 weeks apart).

IHC

CD8 staining was performed by steaming slides for 45 minutes in Dako Target Retrieval Solution (Agilent Technologies, Inc), followed by incubation with a mouse anti-human monoclonal anti-CD8 antibody for 45 minutes at room temperature (Agilent Technologies, Inc). For FoxP3 staining, slides were steamed for 45 minutes in Dako Target Retrieval Solution (Agilent Technologies, Inc) and then incubated with a mouse monoclonal anti-FoxP3 antibody overnight at 4°C (eBioscience, 1:250 dilution). For CD8, the secondary antibody used was the UltraVision Quanto Detection System HRP DAB (Thermo

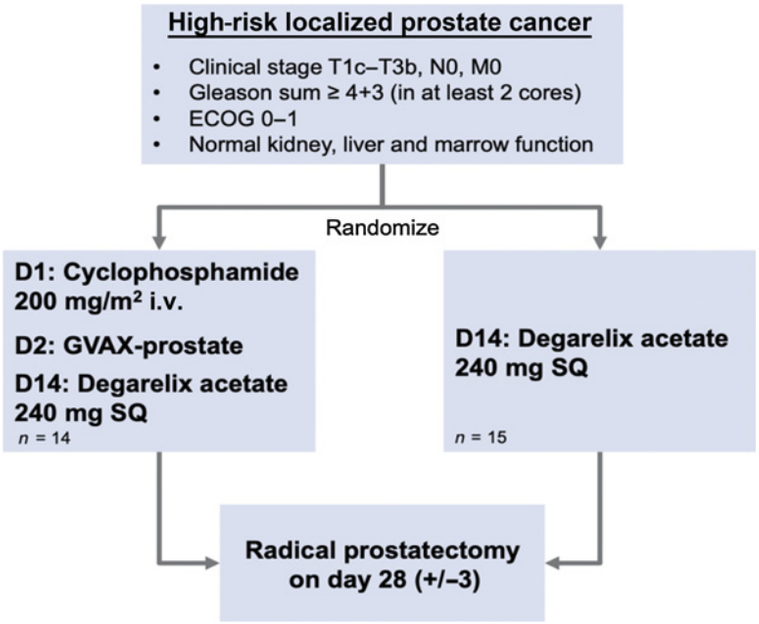


Figure 1.
Clinical trial design and patient disposition diagram. Patients with high-risk localized prostate cancer (T1c–3b, N0, M0, and Gleason 7–10) were randomized 1:1 to degarelix (240 mg subcutaneously) versus cyclophosphamide (200 mg/m^2 i.v.)/GVAX (2.5×10^8 PC3 cells, 1.6×10^8 LNCaP cells) given 2 weeks before degarelix. All patients then underwent radical prostatectomy 2 weeks after degarelix. Abbreviations: ECOG, Eastern Cooperative Oncology Group; D1, day 1; D2, day 2; D14, day 14; SQ, subcutaneously.

Fisher Scientific). For Foxp3, the secondary antibody was the PowerVision+ Kit (Leica Biosystems). Staining was visualized using 3,3′-Diaminobenzidine (Sigma, FAST 3,3′-Diaminobenzidine Tablets) and slides were counterstained with hematoxylin. For CD8 and Foxp3, IHC-stained slides were scanned using an Aperio ScanScope CS. Sections for tumor for image analysis were performed using ImageScope by selecting regions of invasive carcinoma and carefully excluding regions in which inflammatory infiltrates involved benign glands. CD8 and Foxp3 cell data were obtained using positive IHC cell counting algorithms implemented in Aperio Spectrum software by applying hue, saturation, and brightness color space. Cell numbers were normalized to the overall areas/ROI and annotated by a trained pathologist to provide cell density, which was assessed for each patient and compared across study arms. PD-L1 IHC staining and scoring was performed as described previously (13). Although some PD-L1 expression has previously been reported on immune cells in prostate cancer, such cells are morphologically identified as primarily macrophages; here we analyzed and report tumor cell PD-L1 expression.

Expression profiling

Immune gene expression in the prostate TME was profiled using the NanoString IO360 Immune Panel (19). Sufficient tissue for analysis was available from 13 patients from arm A (degarelix) and 12 patients from arm B (degarelix + Cy/GVAX), as well as 18 untreated matched control patients. NanoString count data were normalized by first thresholding to exceed mean + 1 SD of negative controls, then scaling each sample by a positive control normalization factor to correct for total counts, and additionally, scaling with a set of predefined housekeeping genes, as described in the NanoString documentation (20). Three housekeeping genes (*FCF1, POLR2A*, and *TUBB*) were excluded from the normalization process due to high cross-sample variance, and two additional genes (*CC2D1B* and *GUSB*) were excluded due to poor correlation with other housekeeping genes. This scaling corrected for background noise and differences in total gene count across samples, allowing for differential gene expression between groups to be calculated by unpaired *t* test. For each pairwise comparison, we performed Benjamini–Hochberg multiple-testing correction and reported the number of differentially upregulated and downregulated genes with a corrected $P < 0.05$.

NanoString data were used to computationally infer an absolute abundance of immune cell types in each sample to compare the two study arms with each other and with the untreated group. These analyses were performed using the CIBERSORT algorithm, which deconvolutes gene expression matrices to a mixture of known immune cell types by fitting to a validated reference matrix of 22 immune cell subtypes, where each cell subtype has a defined set of differentially expressed genes (21). This approach was limited by the fact that NanoString profiles a limited set of targeted genes rather than the whole transcriptome, so not all differentially expressed genes in the CIBERSORT reference matrix were captured. However, NanoString specifically targets immune-related genes and there are a significant number of differentially expressed genes captured for each immune cell subtype by the NanoString panel. These are reported in Supplementary Table S1. CIBERSORT was able to deconvolute immune cell composition from these genes with a $P < 0.05$ for 13 treatment arm A samples, 10 treatment arm B samples, and 12 untreated control samples.

Statistical analysis

Our primary hypothesis was that men receiving Cy/GVAX followed by ADT would have a twofold (100%) increase in CD8$^+$ T-cell infiltration as compared with men receiving ADT alone. With 16 patients per arm, and assuming an 86% coefficient of variation for the average CD8$^+$ T-cell density, a one-sided 0.05 α-level *t* test of the logarithms of these ratios would provide 82% power to detect a twofold (100%) increase in CD8$^+$ T-cell density between treatment groups. Thus, the trial was powered to recruit 32 patients, with a total of 29 patients ultimately recruited. The primary statistical endpoint of this study was CD8$^+$ T-cell density quantified by the number of nuclei staining positive for CD8 per mm^2. Following a log transformation, the mean CD8$^+$ T-cell densities were compared between treatment arms using a two-way ANOVA with the stratification variable, Gleason score, treated as a block factor. Event time distributions for time-to-PSA progression, time-to-metastasis, and time-to-next cancer treatment were

estimated with the method of Kaplan and Meier and compared using a stratified Cox proportional hazards model. For all comparisons of differential gene expression, *t* tests were applied to the normalized NanoString counts matrix, and *P* values corrected for multiple testing by the Benjamini–Hochberg method. Similarly, *t* tests with Benjamini–Hochberg correction were applied to the inferred CIBERSORT immune cell abundance matrices, and to the IHC density values for CD8 and FOXP3. In a secondary analysis, hypothesis testing for unbiased association of clinical variables with time-to-PSA recurrence and time-to-next treatment was performed using multiple Cox regression with backward feature selection using the Akaike information criterion (AIC; refs. 22–24), and visualized using hazard ratio forest plots and Kaplan–Meier survival curves. The same multiple Cox regression with backward feature selection was performed to test for association of clinical variables with metastasis and time to testosterone recovery (Supplementary Fig. S1). Pearson correlation was also calculated between all clinical, gene expression, and IHC variables as well as correlation of each variable with disease recurrence, visualized in Supplementary Fig. S2. Statistical analyses were performed using R version 3.5.3 and SAS version 9.2.

Results

Thirty-two patients were recruited to the study with 16 randomized to each arm. One patient randomized to degarelix alone and two patients randomized to degarelix plus Cy/GVAX withdrew consent before study drug initiation. Therefore, 29 patients received study treatment. Fifteen patients received degarelix alone and 14 received degarelix plus Cy/GVAX (one patient in this group withdrew following cerebrovascular ischemia, and was subsequently lost to follow-up). Clinical characteristics of the two treatment groups were similar with respect to age, risk status, Gleason sum, tumor stage, regional nodal involvement, and surgical margins (**Table 1**). Sixty-four percent of patients had Gleason ≥8 disease, 56% had pathologic stage T3b, and 18% were found to have N1 disease at the time of surgery.

Table 1. Patient baseline demographics and disease characteristics.

Clinical variable	Degarelix (*N* = 15)	Degarelix + Cy/GVAX (*N* = 13)
Median age (interquartile range), years	58 (55–64.5)	61 (54–63)
Very high risk (%)	6 (40%)	8 (61%)
Gleason sum[a], *n* (%)		
7	6 (40%)	4 (31%)
8	1 (7%)	2 (15%)
9	8 (53%)	6 (46%)
10	0 (0%)	1 (8%)
T stage, *n* (%)		
pT2	4 (27%)	3 (23%)
pT3a	5 (33%)	6 (46%)
pT3b	6 (40%)	4 (31%)
ECOG status, *n* (%)		
0	15 (100%)	13 (100%)
Regional lymph node involvement, *n* (%)	2 (13%)	3 (23%)
Positive margin, *n* (%)	7 (47%)	5 (38%)
Recurred, *n* (%)	9 (60%)	4 (31%)
Developed metastasis, *n* (%)	2 (13%)	3 (23%)

Note: Clinical variables for patients treated with degarelix alone versus degarelix plus Cy/GVAX.
Abbreviation: ECOG, Eastern Cooperative Oncology Group.
[a]Gleason sums for the histologic pattern of carcinoma range from 7–10 with higher scores indicating a higher-grade tumor.

Safety

Both degarelix alone and degarelix plus Cy/GVAX were well-tolerated. A single grade 3 alanine aminotransferase (ALT) elevation was reported in the degarelix plus Cy/GVAX group, with no other treatment-related grade 3 or 4 adverse events reported (**Table 2**). All enrolled patients successfully underwent radical prostatectomy, with no significant unexpected surgical complications or toxicities reported. Significant surgical complications were defined as blood loss in excess of 2,500 mL, operative time in excess of 3.5 hours, hospital stay in excess of 4 days, or systemic symptoms including fever, rash, or myelosuppression.

Degarelix (ADT) induces CD8 T-cell infiltration with a proportional increase in Tregs

Prostatectomy samples from both treatment arms, degarelix and degarelix + Cy/GVAX, showed significantly increased intratumoral $CD8^+$ T-cell density by IHC as compared with untreated matched controls (**Fig. 2C**). However, this CD8 infiltration was balanced by a proportional increase in Treg infiltration, such that the CD8/Treg ratio remained consistent across all treatment groups (**Fig. 2D** and **E**). While there was a significant increase in both $CD8^+$ T-cell and Treg infiltrate with degarelix versus controls and degarelix + Cy/GVAX versus controls, there was no statistically significant difference between the degarelix and degarelix + Cy/GVAX treatment groups (**Fig. 2**), suggesting that the GVAX vaccine did not induce additional CD8 infiltration in this setting as compared with degarelix alone. Because FOXP3 can potentially be expressed in other T-cell populations, we also analyzed our transcriptomic data to identify whether treatment led to increased expression of other Treg markers including GITR (*TNFRSF18*), CTLA-4, and CD25 (*IL2RA*). We observed increased expression of GITR, CTLA-4, and CD25 with both degarelix alone and degarelix plus Cy/GVAX compared with untreated controls (Supplementary Fig. S3). However, there was no difference in expression of these markers between degarelix and degarelix plus Cy/GVAX.

Increased PD-L1 expression after GVAX vaccination

Consistent with prior reports, tumor cell PD-L1 expression was minimal in untreated patients (**Fig. 3**). Degarelix alone appeared to modestly increase PD-L1 expression, consistent with the notion that cytokine secretion from infiltrating $CD8^+$ T cells may drive upregulation of immune checkpoints. Tumor samples from patients treated with degarelix + Cy/GVAX were found to have increased PD-L1 staining compared with patients treated with degarelix alone, with a higher proportion of samples exceeding 5% PD-L1 positivity (**Fig. 3**); this trend was not statistically significant. Although there appeared to be some areas of PD-L1 staining in inflammatory cells in the stroma, the majority of cells staining positive for PD-L1 were tumor cells. Taken together, these data suggest that while the GVAX vaccine does not significantly increase $CD8^+$ T-cell density, the infiltrating immune cells induced by GVAX may be capable of promoting PD-L1 upregulation.

Degarelix and degarelix plus Cy/GVAX induce complex changes in immune gene expression

Pairwise differential gene expression was performed on normalized NanoString data from prostatectomy samples, comparing

Table 2. Adverse events reported by treatment group.

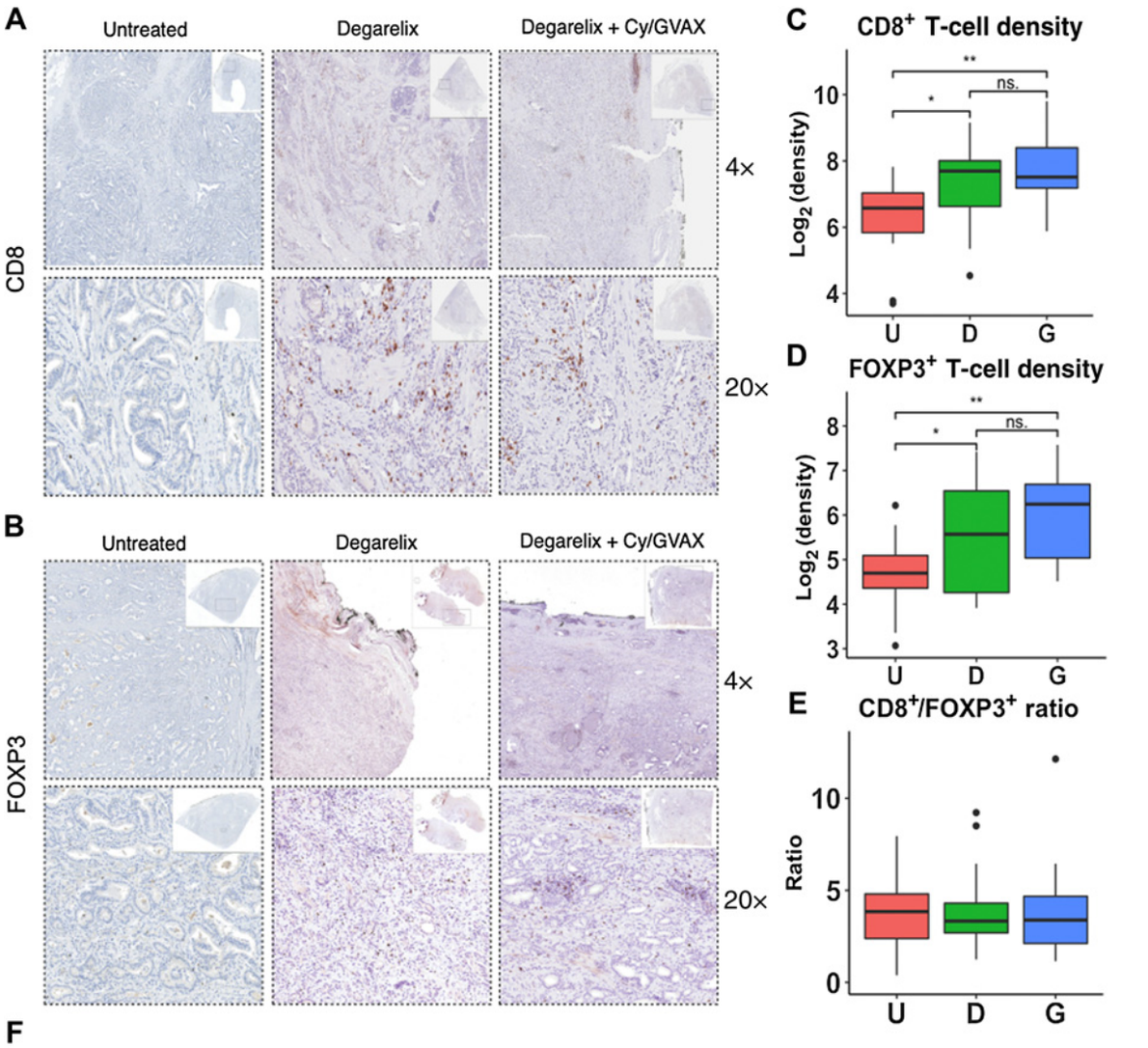
	Degarelix ($N = 15$)			Degarelix + Cy/GVAX ($N = 14$)		
Adverse events	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 1**	**Grade 2**	**Grade3**
General disorders						
Injection site reaction	10 (66%)	0	0	11 (79%)	2 (14%)	0
Fatigue	4 (27%)	0	0	3 (21%)	0	0
Chills	2 (13%)			1 (7%)		
Fever	0	0	0	2 (14%)	0	0
Flu-like symptoms	0	0	0	0	1 (7%)	0
Malaise	0	0	0	1 (7%)	0	0
Edema, limbs	1 (7%)	0	0	0	0	0
Localized edema	0	0	0	1 (7%)	0	0
Gastrointestinal disorders						
Abdominal pain	2 (13%)	0	0	1 (7%)	0	0
Nausea	0	0	0	1 (7%)	0	0
Vascular disorders						
Hot flashes	6 (40%)	0	0	8 (57%)	0	0
Reproductive system disorders						
Erectile dysfunction	1 (7%)	0	0	2 (14%)	0	0
Testicular disorder	0	0	0	1 (7%)	0	0
Urinary disorders	0	0	0	0	0	0
Urinary incontinence	1 (7%)	0	0	1 (7%)	0	0
Laboratory abnormalities						
Elevated ALT	1 (7%)	0	0	0	0	1 (7%)
Elevated AST	2 (13%)	0	0	0	1 (7%)	0
Skin disorders						
Rash (systemic)	0	0	0	3 (21%)	2 (14%)	0
Dizziness	0	0	0	1 (7%)	0	0
Musculoskeletal disorders						
Arthralgias	1 (7%)	0	0	2 (14%)	0	0
Myalgias	1 (7%)	0	0	1 (7%)	0	0
Nervous system disorders						
Lethargy	0	0	0	1 (7%)	0	0
Dizziness	0	0	0	1 (7%)	0	0
Headache	0	0	0	2 (14%)	0	0
Ischemia, cerebrovascular	0	0	0	1 (7%)	0	0
Surgical complications						
Postop hematoma (pelvic)	0	0	0	0	1 (7%)	0

Note: Adverse events for patients treated with degarelix alone versus degarelix plus Cy/GVAX were reported for all patients in the study, including one patient in the degarelix plus Cy/GVAX group that subsequently discontinued treatment following cerebrovascular ischemia.
Abbreviation: AST, aspartate aminotransferase.

untreated control patients, degarelix-treated patients, and degarelix + Cy/GVAX–treated patients. This analysis identified 98 genes upregulated in both degarelix and degarelix + Cy/GVAX versus controls (**Fig. 4A**). *CHIT1*, a macrophage activation marker, was the only gene significantly upregulated in degarelix + Cy/GVAX versus degarelix (**Fig. 4B**; ref. 25). The CIBERSORT algorithm was used to deconvolute and infer the abundance of immune cell subtypes in each sample from NanoString gene expression. Fractional contributions of immune cell populations were then compared between treatment groups (**Fig. 4C**). These data show that a complex immune infiltrate was present in prostatectomy samples at time of surgery, with significant populations of B cells, CD4 T cells, M1 macrophages, M2 macrophages, and mast cells. Summing the inferred abundance of each cell type yielded a total immune infiltrate estimate from gene expression data. Those data showed that total immune infiltrate was significantly increased in both degarelix and degarelix + Cy/GVAX compared with control, but not in degarelix + Cy/GVAX as compared with degarelix alone (**Fig. 4D**). CIBERSORT analysis also revealed an increased infiltrate of $CD8^+$ T cells, M2 macrophages, and gamma-delta T cells in both treatment groups as compared with untreated controls, with a raw $P < 0.05$. Although the $CD8^+$ T cell increase is consistent with the IHC data (**Fig. 1**), these differences based on gene-expression analysis were not statistically significant after adjustment for multiple testing (**Fig. 4E**). To further assess whether treatment could increase T-cell activation, we evaluated IFNγ and granzyme B expression levels and demonstrated no significant difference in expression levels between the treatment groups (Supplementary Fig. S3).

Degarelix plus Cy/GVAX is associated with improved clinical outcome

At 24 months postprostatectomy, 69% of patients were free of PSA recurrence in the Cy/GVAX plus degarelix treatment group as compared with 40% in the degarelix-only group (**Table 1**). Initial univariate Cox regression of treatment group against time-to-PSA recurrence stratified by Gleason sum 7 versus Gleason sum greater than 7 yielded a HR of 0.44 [95% confidence interval (CI), 0.13–1.43; $P = 0.17$], with time-to-next-treatment yielding a HR of 0.41 (95% CI, 0.13–1.36; $P = 0.15$). After determining informative clinical variables for prediction of



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	Untreated (U) *N* = 20	Degarelix (D) *N* = 15	Degarelix + Cy/GVAX (G) *N* = 13	*P*
$CD8^+$ T cell density (mean, 95% CI)	96 (72–120)	205 (121–289)	263 (129–397)	<0.01 (G vs U) 0.03 (D vs U) 0.35 (G vs D)
Treg cell density (mean, 95% CI)	29 (21–36)	59 (34–85)	78 (48–107)	<0.01 (G vs U) 0.02 (D vs U) 0.18 (G vs D)
$CD8^+$/Treg ratio (mean, 95% CI)	3.7 (2.9–4.6)	4.0 (2.7–5.3)	3.9 (2.2–5.7)	0.87 (G vs U) 0.68 (D vs U) 0.66 (G vs D)

Figure 2.

Degarelix and degarelix + GVAX increase $CD8^+$ and $FOXP3^+$ T-cell infiltration in prostate tumors. **A,** Representative hematoxylin and eosin (H&E) and IHC for $CD8^+$ T cells, visualized at 4× and 20× magnification. **B,** Representative H&E and IHC for $FOXP3^+$ T cells, visualized at 4× and 20× magnification. **C,** Boxplots of $\log_2$($CD8^+$ T-cell density), quantified from IHC as represented in **A**. **D,** Boxplots of $\log_2$($FOXP3^+$ T-cell density), quantified from IHC as represented in **B**. **E,** Boxplots of the $CD8^+$/$FOXP3^+$ T-cell ratio, quantified from IHC as represented in **A** and **B**. **F,** Table of mean $CD8^+$ T cell density (cells/mm^2), mean Treg density (cells/mm^2), and CD8/Treg ratio for each treatment group and untreated controls, with 95% CIs and *P* values by Gleason stratification–adjusted ANOVA reported for each comparison of groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ns, not significant).

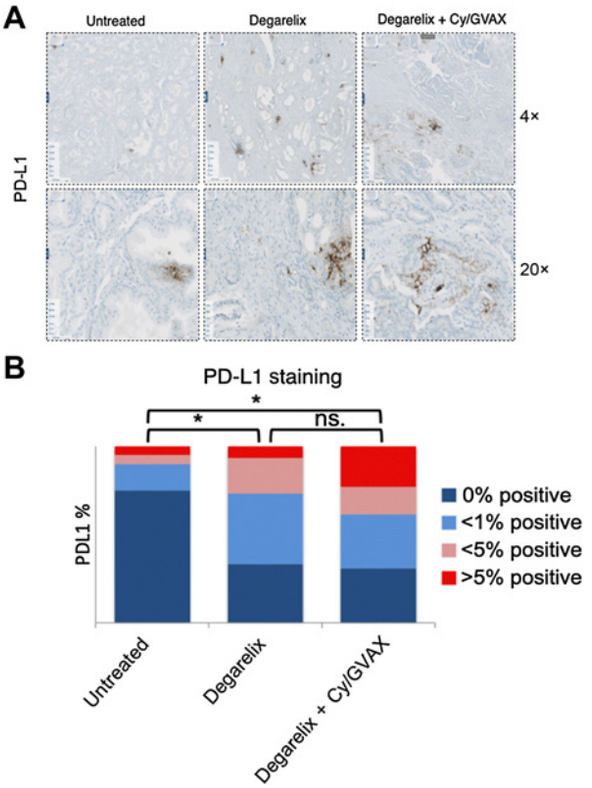

Figure 3.

Degarelix and degarelix + Cy/GVAX increase PD-L1 expression in prostate tumors. **A,** Representative IHC for PD-L1, visualized at 4× and 20× magnification. **B,** Stacked bar plot of % PD-L1–positive cells, showing relative proportion of samples with 0% PD-L1 staining, <1% PD-L1 staining, <5% PD-L1 staining, and >5% PD-L1 staining in tumor cells in each treatment group and a cohort of untreated matched controls. Distributions of % PD-L1 categories may be visually compared between groups, such that the degarelix + Cy/GVAX group has the highest proportion of samples with PD-L1 > 5%. Proportions of samples with % PD-L1 > 0 were also compared between groups by Fisher's exact test, with *P* values shown above the plot for each comparison (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ns, not significant).

time-to-PSA recurrence using backwards feature selection by the AIC, multiple Cox regression was performed accounting for interactions between patient age, tumor stage, Gleason sum, and treatment group. Using this regression analysis, treatment with Cy/GVAX plus degarelix showed an increased time-to-PSA recurrence as compared with that observed in patients treated with degarelix alone, with a HR of 0.29 (95% CI, 0.08–1.00; $P = 0.05$; **Fig. 5A** and **B**). Backward feature selection converged to the same set of clinical variables for prediction of time-to-next treatment, where there was a statistically significant treatment effect for degarelix plus Cy/GVAX compared with degarelix alone, with a HR of 0.26 (95% CI, 0.071–0.97; $P = 0.046$; **Fig. 5C** and **D**). There was no significant difference observed between the two treatment groups in prediction of time-to-metastasis, where backward feature selection converged to a null model, and univariate Cox regression with treatment group yielded a *P* value of 0.46 (Supplementary Fig. S1). This may be due to the overall low rate of metastases in this patient population, with only five cases of metastasis observed across the two treatment groups (**Table 1**). There was also no significant difference in time-to-testosterone recovery between the two treatment groups (Supplementary Fig. S1), suggesting that the improved time-to-PSA recurrence cannot be accounted for by differences in the duration of a castrate level of testosterone. Correlation with recurrence is shown in Supplementary Fig. S2 for each variable considered in the first step of the backward feature selection model, such that $CD8^+$ and $FOXP3^+$ density as well as PD-L1 level were each negatively correlated with recurrence, but were not individually predictive of time-to-recurrence and were not additionally informative after accounting for treatment group, age, stage, and Gleason sum.

Discussion

This study demonstrates that neoadjuvant ADT (degarelix acetate) with or without the addition of GVAX immunotherapy and low-dose cyclophosphamide promotes a complex immune response within the prostate TME. Treatment was well-tolerated and did not lead to unexpected surgical complications, providing proof of concept for an immunotherapy-based neoadjuvant approach to prostate cancer treatment. Importantly, we found that ADT significantly increases the intratumoral $CD8^+$ T-cell infiltrate in prostate cancer. However, our comprehensive analyses of the immune TME showed that ADT induces other important immunologic changes, with both proinflammatory and immunosuppressive effects. Perhaps most strikingly, we observed that the $CD8^+$ T-cell infiltrate was accompanied by a proportional increase in Tregs, a key immunosuppressive cell population that mediates immune resistance in multiple tumor types (20). The addition of cyclophosphamide, which has previously been shown to transiently deplete Tregs, did not appear to significantly deplete Tregs in this setting. The addition of Cy/GVAX to ADT did lead to a modest increase in PD-L1 expression, as well as a statistically significant increase in the macrophage marker *CHIT1*, perhaps suggesting increased immunologic activity for the combination therapy. When accounting for patient age, tumor stage, and Gleason sum in a multiple regression model selected by unbiased AIC backward feature selection (22–24), there were significant improvements in time-to-PSA recurrence and time-to-next therapy in patients treated with Cy/GVAX plus degarelix compared with degarelix alone, suggesting the possibility that the combination regimen has some clinical activity.

Prior preclinical and clinical studies showed that androgen deprivation can remodel the immune TME in prostate tumors toward a proinflammatory state. Our group previously demonstrated in the MycCaP murine model that ADT initially leads to a proinflammatory immune cell infiltrate in prostate tumors with increases in $CD8^+$ T cells, Tregs, macrophages, and natural killer cells (18). However, this infiltrate is transient and appears to dissipate with the emergence of castration resistance. Other groups have also shown that androgen ablation can increase B-cell infiltration, which may promote progression to castration resistance through B-cell–derived lymphotoxin production (21). In patients, the androgen receptor blocker flutamide was shown to induce T-cell infiltration and increase expression of proinflammatory immune-related genes (IFNγ, TNFα, and Granzyme A) in prostate cancers when given prior to prostatectomy (25, 26). Several prior studies have also investigated the use of neoadjuvant vaccine-based immunotherapy approaches to enhance antitumor immune responses. For example, the autologous cellular vaccine, sipuleucel-T, was shown to promote lymphocyte recruitment and enhance T_H1 responses when given in the neoadjuvant setting (27, 28).

The findings reported here are largely consistent with these prior observations and suggest that ADT may prime prostate-specific T-cell responses. We observed that ADT led to a robust increase in $CD8^+$ T cells, which was not further enhanced by Cy/GVAX. One possible reason for the lack of further $CD8^+$ infiltration with Cy/GVAX could

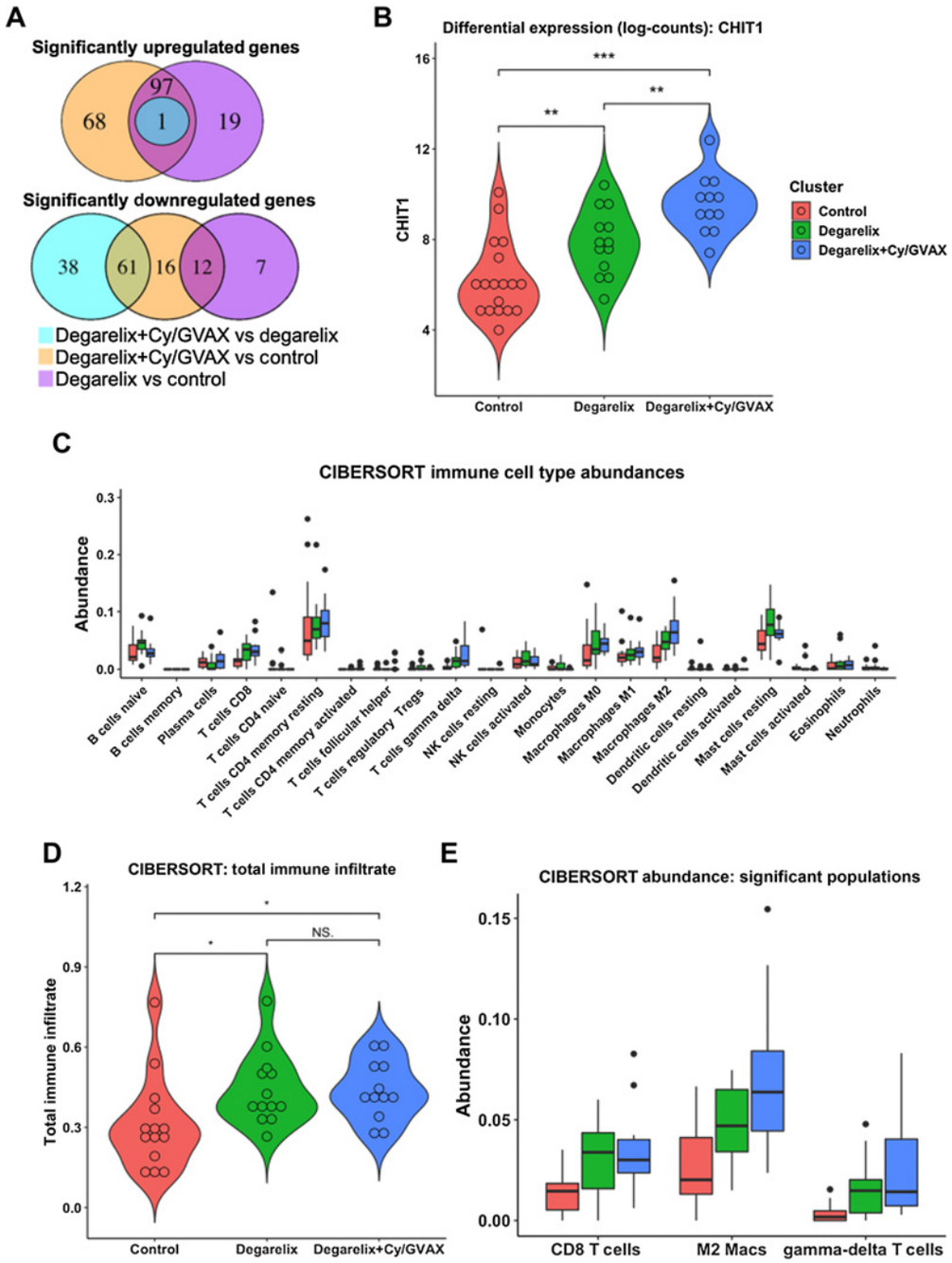

Figure 4.

Degarelix and degarelix + Cy/GVAX induce complex changes in immune gene signatures in primary prostate tumors. **A,** Differential expression of immune-related genes by NanoString immune profiling panel in primary prostate tumors after degarelix, degarelix + Cy/GVAX, and untreated matched controls. Euler plots showing number of genes with Benjamini–Hochberg corrected *t* test $P < 0.01$ for each pairwise comparison of groups, such that "upregulated genes" refers to genes that have higher mean frequency in the degarelix + Cy/GVAX group than in the degarelix group (cyan), higher mean frequency in the degarelix group than the untreated control group (purple), and higher mean frequency in the degarelix + Cy/GVAX group than the untreated control group (orange), and "downregulated genes" refers to genes that have lower mean frequency in degarelix + Cy/GVAX versus degarelix (cyan), degarelix versus controls (purple), and degarelix + Cy/GVAX versus controls (orange), respectively. **B,** Violin plot of log-scaled postnormalization NanoString gene counts for *CHIT1* in each treatment group and untreated controls. In **A**, *CHIT1* is the sole gene significantly upregulated in each comparison. **C,** Boxplot of immune cell type absolute abundances as inferred by CIBERSORT, colored by treatment group, and reported for all samples with CIBERSORT $P < 0.05$. **D,** Violin plot of total immune cell infiltrate for each sample by treatment group, such that total immune cell infiltrate represents the sum of CIBERSORT immune cell abundances as shown in **C**. **E,** Boxplot of immune cell populations for which *t* test comparing abundance between groups showed an uncorrected $P < 0.05$. *P* values were obtained by unpaired *t* test with Benjamini–Hochberg multiple-testing correction, and shown in **B** and **D** with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; NS, not significant.

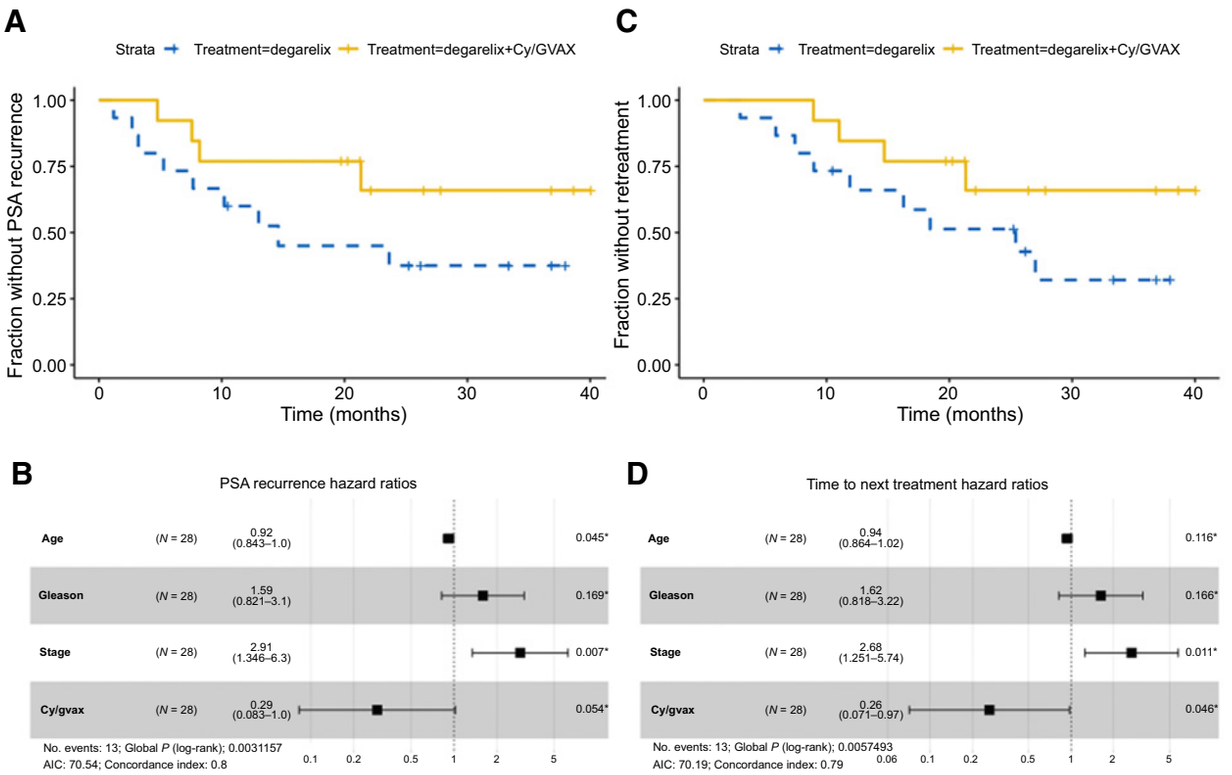

Figure 5.

Combination of Cy/GVAX with degarelix improves time-to-PSA recurrence and increases time-to-next treatment. **A,** Kaplan–Meier curves comparing time-to-PSA recurrence of patients treated with degarelix + Cy/GVAX versus degarelix alone. Informative clinical variables for multivariate analysis were selected by backward feature selection using the AIC. **B,** Forest plot showing time-to-PSA recurrence HRs with 95% CI for multiple Cox regression of progression-free survival against Cy/GVAX status, patient age, tumor stage, and Gleason score. *P* values for each variable are reported, as is the overall log-rank *P* value, AIC value, and concordance index for the regression. **C,** Kaplan–Meier curves comparing time-to-next treatment for patients treated with degarelix + Cy/GVAX versus degarelix alone, with log-rank *P* value reported from multiple Cox regression of time-to-next-treatment against Cy/GVAX status, patient age, tumor stage, and Gleason score. Informative clinical variables were selected as in **A**. **D,** Forest plot showing time-to-next-treatment HRs with 95% CI for multiple Cox regression of time-to-next-treatment against Cy/GVAX status, patient age, tumor stage, and Gleason score. *P* values for each variable are reported, as is the overall log-rank *P* value, AIC value, and concordance index for the regression.

be the allogeneic nature of the GVAX vaccine relative to the patients' tumors. The vaccine cell line PC3 was originally derived from a skull metastasis, and LNCAP is originally derived from a lymph node metastasis, and it is possible that neither consistently shared tissue-specific antigens with the primary prostate tumors in the treated patients. It may also be the case that GM-CSF was insufficiently able to activate dendritic cells, as it has been found that modified versions of GVAX expressing dendritic cell–activating molecules such as STING were far more effective in preclinical models (29). It should also be noted that the prostate cancer microenvironment is particularly immunosuppressive, such that CD8 T cells isolated from the prostate remain refractory to stimulation even in *ex vivo* experiments (30), indicating that improved depletion of Tregs may also improve response to GVAX. Of note, there was also increased PD-L1 expression with ADT, which did appear to be augmented by the addition of Cy/GVAX. The significance of this upregulation of PD-L1 is unclear but could reflect an adaptive response to IFNγ produced by activated T lymphocytes. Future mechanistic work is required to better understand this observation. Furthermore, and consistent with the hypothesis that counter regulatory mechanisms can function to maintain immune evasion, we observed an increase in Treg infiltration with ADT. This process of adaptive Treg resistance has not previously been described in the setting of neoadjuvant ADT, although increases in Treg density have been observed in response to a range of therapies across a number of tumor types, highlighting the notion that adaptive Treg resistance may be a broad-based mechanism that can attenuate maximal responses to immunotherapy in patients with diverse malignancies.

Interestingly, in both treatment groups, differential gene expression analysis showed that degarelix treatment upregulated *CHIT1*, a marker of macrophage activation shown to regulate many inflammatory processes through stimulation of inflammatory mediators such as IL8, MMP9, CCL2, CCL5, and CCL11, and correlated with levels of IL1β and TNFα (31). Given that macrophages are key antigen-presenting cells, this finding corroborates the notion that ADT enhances prostate–antigen presentation and thereby promotes prostate-specific T-cell responses. *CHIT1* expression appeared to be further upregulated by the addition of Cy/GVAX to ADT.

Limitations of this study include the relatively small number of patients in each treatment arm and our inability to capture serial immunologic changes within the prostate TME over time. We hypothesized that 2 weeks of ADT would be optimal to elicit robust immunologic responses, because preclinical data suggest that the immunologic effects of ADT are transient, with the initial immune infiltrate

evolving over time into a more suppressive one, dominated by Tregs (18). The optimal duration of ADT prior to radical prostatectomy remains unknown and it is possible that the single dose of degarelix acetate used in this study was insufficient to sustain a clinically significant immune response. Our study used cyclophosphamide in combination with GVAX based on the hypothesis that low-dose cyclophosphamide would be capable of depleting Tregs and therefore augmenting an antitumor immune response. This approach was supported by preclinical studies which showed significant augmentation of antitumor immunity upon administration of cyclophosphamide approximately 24 hours prior to vaccination with GVAX (32, 33). The dosage of cyclophosphamide used here reflects the dosage in a breast cancer study that also showed augmentation of antitumor immunity with administration of cyclophosphamide prior to a GM-CSF–secreting vaccine (34). However, we observed no difference in Treg density with the addition of Cy/GVAX to degarelix. One possibility is that the dosing regimen of cyclophosphamide used in this study was not optimal for Treg depletion. Since the completion of our study, emerging data showed that oral cyclophosphamide may be more effective for Treg depletion (35, 36). Given these limitations, future studies may be required to fully characterize the evolution of the immune TME over time and to optimize neoadjuvant immunotherapy in patients with prostate cancer.

However, these results do provide important insights into the immunologic effects of ADT, either alone or in combination with an allogeneic cell-based vaccine. Importantly, the complexity of the immune response to ADT suggests that selectively targeting immunosuppressive cell populations may be essential for maximizing the immunogenicity of neoadjuvant ADT. The observation that ADT can induce adaptive Treg resistance provides a strong rationale for novel strategies aimed at depleting Tregs within the prostate TME. Finally, future mechanistic studies aimed at comprehensively understanding how androgen deprivation regulates antitumor immunity in prostate cancer are warranted.

Disclosure of Potential Conflicts of Interest

A.M. De Marzo reports receiving commercial research grants from Janssen Research and Development. C.G. Drake is a paid advisory board member for AstraZeneca, Bristol-Myers Squibb, Compugen, Ferring, F-Star, Genocea, Janssen, Merck, Merck-Serono, Pfizer, Pierre Fabre, Roche/Genentech, Shattuck Labs, Tizona, Urogen, and Werewolf; reports receiving speakers bureau honoraria from Bristol-Myers Squibb; and holds ownership interest (including patents) in Bristol-Myers Squibb and Janssen. No potential conflicts of interest were disclosed by the other authors.

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