

## Enhancement of the T-cell Armamentarium as a Cell-Based Therapy for Prostate Cancer

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### Abstract

Prostate cancer is frequently characterized by a large inflammatory infiltrate that includes T cells. Although T cells traffic to cancer lesions in large numbers, they are unable to generate a therapeutic response because of the immunosuppressive microenvironment. Therefore, arming T cells with a cytotoxic agent that is capable of killing cancer cells independent of these immunosuppressive signals is a rational approach to enhance their potency. Essentially, the T cells would serve as a cell-based vector, or "Trojan Horse," to selectively deliver a protoxin to disseminated prostate cancer lesions. The selective delivery of a protoxin using T cells represents an ideal method to maximize their therapeutic potency through a "field effect." Because systemically infused T cells are expected to traffic to sites of inflammation other than cancer, an additional level of specificity may be needed to prevent toxicity to nontarget tissues. Toward this goal, genetic engineering can be used to make protoxin expression dependent upon T-cell recognition of the prostate-specific membrane antigen by a chimeric antigen receptor. Furthermore, selective activation of the protoxin using a tissue- or tumor-specific protease, such as PSA, can promote further specificity. Thus, T-cell potency can be enhanced by targeted protoxin secretion and greater specificity achieved using combinatorial antigen recognition and protoxin activation. *Cancer Res*; 74(13): 3390–5. ©2014 AACR.

### Introduction

Prostate cancer represents the largest number of new cancer diagnoses in men each year. Despite recently approved therapies, such as abiraterone and sipuleucel-T, more than 30,000 men will succumb to cancer-related morbidities associated with prostate cancer metastasis this year in the United States alone. This clearly justifies the need for innovative therapeutic strategies capable of treating advanced metastatic disease if we hope to make long-term patient survival the norm.

After decades of research, cancer immunotherapy has emerged as a promising anticancer platform. A steady stream of immunotherapies has made their way into the clinic over the past few years with many more percolating through the pipeline. Sipuleucel-T, an autologous cell-based vaccine incorporating a prostatic acid phosphatase (PAP)-granulocyte macrophage colony-stimulating factor (GM-CSF) fusion protein, was approved by the FDA in 2010 for the treatment of asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (mCRPC). Numerous other cancer vaccines, including ProstVac VF and GVAX, show promise and are in active clinical development for prostate

and other cancers (1). In 2011, the CTLA-4 immune checkpoint inhibitor, ipilimumab, received FDA approval for the treatment of metastatic melanoma following the demonstration of a 3.5-month survival benefit (2). Antibodies against the PD-1 immunoinhibitory receptor have also shown remarkable clinical responses in select cancer types, most notably non-small cell lung cancer (NSCLC), a disease previously thought not to be amenable to immunotherapy (2, 3). Chimeric antigen receptors (CAR) represent another promising strategy. Adoptive transfer of T cells expressing anti-CD19 CARs have led to striking tumor responses in patients with relapsed, chemotherapy-refractory acute, and chronic lymphocytic leukemia (ALL and CLL, respectively; refs. 4 and 5).

Although dramatic therapeutic responses have been achieved, they are generally limited to a subset of patients (3). Furthermore, despite increased overall survival, many of these patients still ultimately succumb to the disease. Although it is unclear why some patients respond to these immunotherapies and others do not, the latter observation clearly indicates that complete eradication of the tumor has not been achieved, ultimately leading to disease relapse and death. Evidence indicates that smaller tumors may be more susceptible to immunotherapy (1, 6), suggesting that large tumors may overwhelm the therapeutic response. Initiation of therapy at earlier stages of disease or combination therapy of various flavors may overcome this limitation and extend responses into a greater number of patients (7). However, enhancing the potency of the immune system through synthetic methods represents an intriguing alternative to boost the performance of immunotherapeutic strategies and generate sustained clinical responses.

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doi: 10.1158/0008-5472.CAN-14-0249

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## T Cells as a Therapeutic Platform

T cells represent an especially interesting candidate for developing a cell-based platform with enhanced potency for cancer therapy. The association between inflammation and cancer has been well known for over a century. Although the concept of using tumor-infiltrating lymphocytes (TIL) for therapy is not new, the strategies to accomplish this goal have evolved. Rosenberg first championed the idea of TIL adoptive transfer for cancer immunotherapy in the 1970s and 1980s. Although initially pursued with enthusiasm, the isolation and expansion of TILs from many solid tumors proved challenging, time consuming, and cost prohibitive (8). Various strategies have since emerged to activate naïve T cells *ex vivo* or prime them against a tumor-associated antigen using vaccine-based approaches, including those described above (3). In addition, the use of viral transduction to overexpress various proinflammatory cytokines, chemokines, and their receptors, including IL2, IL12, CCL5, and CXCR2, represents another promising approach in various stages of development (9). Time will tell whether the targeted delivery of IL12 and other immune modulators will prove successful; however, there remain concerns over driving an uncontrolled expansion of activated T cells and potentially significant immune-related adverse events (IRAE). Significantly, however, these and similar studies have demonstrated that insertional mutagenesis seems to be an extremely rare event in T cells. Numerous clinical trials using retrovirally transduced T cells have been performed over the past decade with no reports of transformation (9), thereby demonstrating the overall safety of T cells for gene delivery.

## Prostate Cancer as a Model System for Immunotherapy

Prostate cancer represents an especially relevant model for evaluating T-cell therapeutic platforms (10). An inflammatory infiltrate can be identified in >80% of biopsied prostates and chronic inflammation has been suggested as an initiating event in prostate cancer etiology (11). Immunohistochemical analyses indicate that CD3<sup>+</sup> T cells represent the predominant cell type (60%–70%) within this infiltrate (12). In addition, androgen-deprivation therapy (ADT), which all men diagnosed with advanced prostate cancer receive upon disease progression, has been shown to further increase the prostate-infiltrating T-cell population by >6-fold (10). Although T cells are highly prevalent within primary and metastatic prostate cancer lesions, this infiltrate is characterized by a "suppressor" or "exhausted" phenotype (i.e., CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs, and PD-1 expression), and, therefore, are unable to perform their effector functions (13–15). These collective observations suggest that T cells efficiently traffic to prostate tumors in large numbers; however, they are unable to effectively control tumor growth because of the immunosuppressive microenvironment. In addition, the successful preclinical development of the FDA-approved anti-CTLA-4 monoclonal antibody, ipilimumab, using a murine autochthonous prostate cancer model provides

further validation for prostate cancer as a clinically relevant model for immunotherapy development (16).

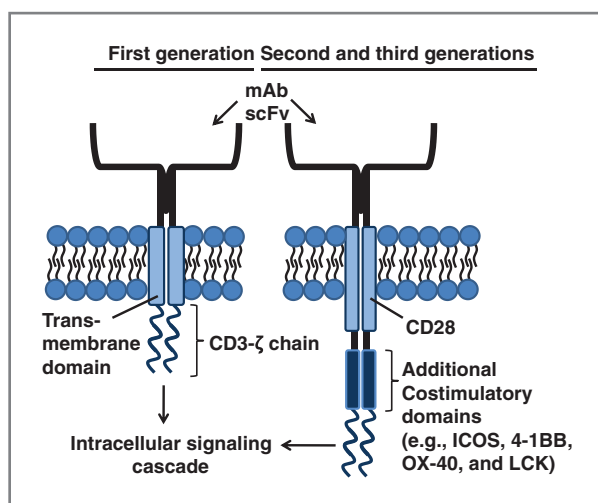
## Overcoming the Immunosuppressive Microenvironment

The recognition that T cells in the tumor microenvironment of many cancers, not just the prostate, are characterized by an exhausted or tolerized phenotype has instigated numerous investigations into immune checkpoint blockade to reactivate endogenous T-cell activity (2). Although many immune checkpoint inhibitors are currently in development, monoclonal antibodies against CTLA-4 and PD-1 have progressed the farthest. Although promising results have been achieved using both CTLA-4 and PD-1 inhibitors in select cancer subtypes, including melanoma, renal cell carcinoma, and NSCLC, their benefit as single agents is limited to a subset of patients (2, 3). This is at least partially because of the upregulation of compensatory checkpoints (2, 7). Preclinical and clinical evidence supports the use of immunotherapy in combination with chemotherapy, radiotherapy, or other immunotherapies to increase response rates (7). Unfortunately, these combined approaches may not be sufficient to drive endogenous T-cell cytolytic functions in the context of the highly immunosuppressive tumor microenvironment either. Adoptively transferred tumor-specific CD8<sup>+</sup> T cells quickly lose their cytotoxic functions following tumor infiltration in an autochthonous prostate cancer model (17). This occurred despite antibody blockade of the PD-1 checkpoint and knockout of TGFβ inhibitory signals (17).

A second strategy to circumvent the immunosuppressive tumor microenvironment is the introduction of CARs, which are akin to synthetic T-cell receptors (TcR). As a result, T cells that target specific tumor-associated antigens in an HLA-independent manner can be generated. In their simplest form, CARs are recombinant receptors composed of an antigen recognition domain, most frequently a single-chain variable fragment (scFv), fused to the CD3-ζ chain intracellular signaling domain (Fig. 1), although several variations exist. These recombinant proteins are then capable of stimulating T-cell activation upon antigen recognition by initiating an intracellular signaling cascade (18). These first-generation CARs were improved upon by incorporating various combinations of costimulatory domains (i.e., CD28, 4-1BB, ICOS, OX-40, or Lck), which are required for full T-cell activation (Fig. 1; ref. 18). Recent reports from the June and Sadelain/Brentjen groups have documented remarkable clinical responses using second-generation CD19-targeted CAR-expressing T cells (CART) in patients with relapsed chemotherapy-refractory chronic and acute lymphoblastic leukemia (CLL and ALL, respectively; refs. 5 and 19).

## Combinatorial Antigen Recognition for Enhanced Specificity

A particularly interesting approach is the dual-targeting CAR recently described by Sadelain and colleagues, which requires combinatorial antigen recognition [PSCA and prostate-specific membrane antigen (PSMA)] to drive endogenous T-cell functions (20). The rationale behind this strategy is that



**Figure 1.** Chimeric antigen receptors (CAR). CARs are essentially HLA-independent synthetic TcRs that can be engineered to recognize a specific antigen. First-generation CARs consist of an antigen recognition domain, such as an scFv, fused to the CD3- $\zeta$  chain intracellular signaling domain. Antigen binding by the CAR activates the CD3- $\zeta$  chain intracellular signaling cascade that leads to T-cell activation. Second-generation CARs include a single costimulatory domain, such as CD28, ICOS, 4-1BB, OX-40, or LCK, to enhance CAR signaling. Further promotion of T-cell activity has been accomplished by incorporating multiple costimulatory domains into third-generation constructs. It should also be noted that there are several variations on this general platform.

enhanced specificity can be achieved by titrating the signal strength of the anti-PSCA CAR to be ineffective in the absence of simultaneous costimulation by chimeric costimulatory receptor recognition of a distinct antigen, that is, PSMA. This combinatorial recognition requires that both antigens be present on the same cell, and thus, so-called "on-target, off-tumor" effects targeting single-positive cells in nontumor tissue can be reduced. The need for enhanced specificity is highlighted by the death of a patient treated with a single high dose of erBB2-targeted CARTs (21). Low-level expression of the target in normal lung tissue and a massive systemic release of cytokines (i.e., a cytokine storm) are thought to be responsible for this fatal toxicity. Because very few targets are truly cancer-specific and T cells are capable of recognizing extremely low levels of antigen, careful consideration of target expression in nontumor tissue is needed to prevent similar patient responses.

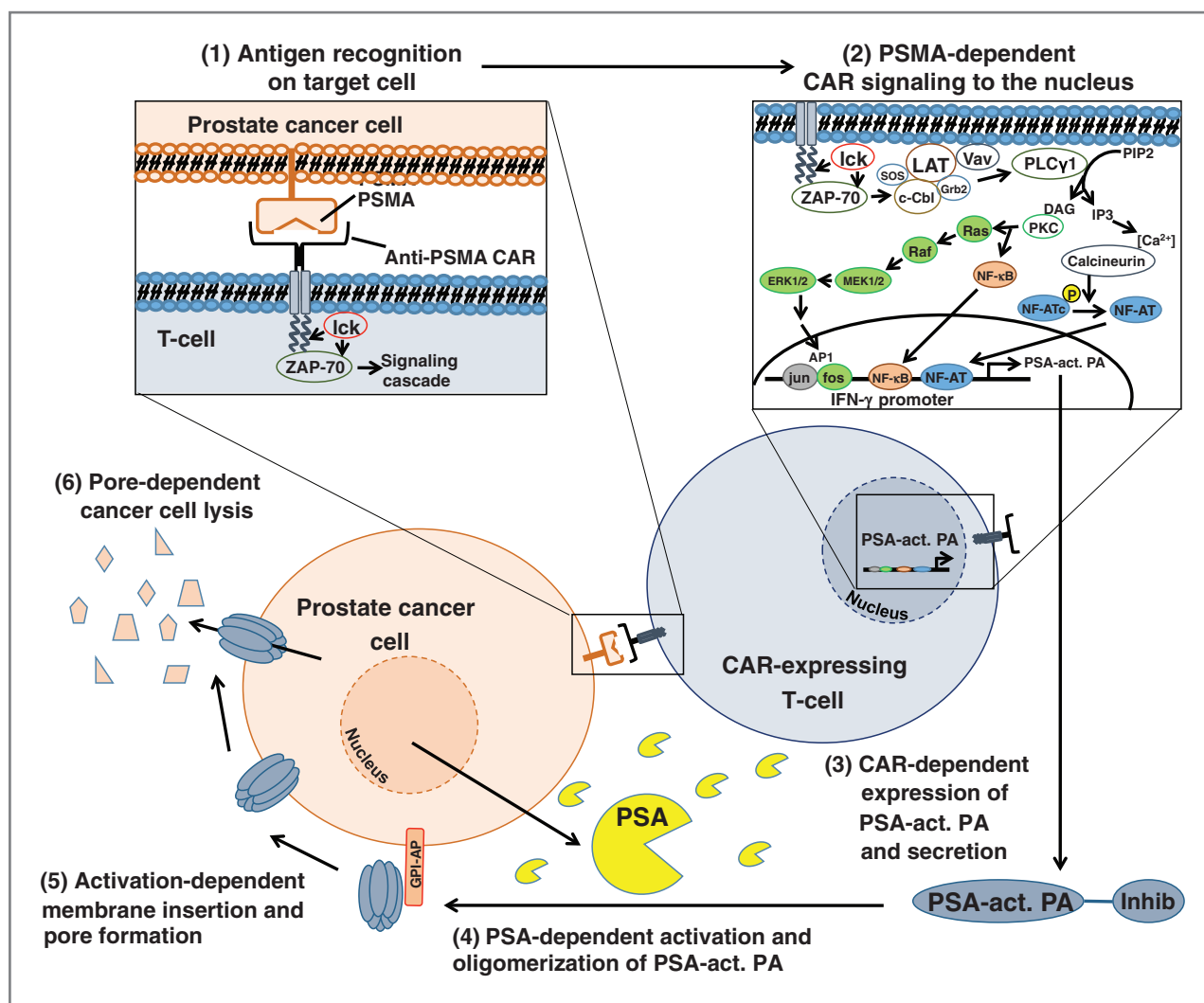
Thus, combinatorial antigen recognition and activation of T cells represents an interesting method of expanding the available repertoire of targets and therefore, tumor types potentially amenable to CART therapy. However, all of these strategies share a dependence upon endogenous T-cell effector functions. Although T cells have been described as "serial killers" and effector-to-target ratios as high as 1:40 have been achieved in preclinical models (22), these ratios do not factor in the proliferation of activated T cells following antigen recognition *in vivo*, which can be quite substantial. Greater than a 1,000-fold amplification of CD19-targeted CARTs has been demonstrated in patients with CLL (19), suggesting that the physi-

ologic effector/target ratio is closer to 1 or higher. Furthermore, even with enriched fractions of antigen-specific T cells, much higher ratios are frequently needed (20, 23). Therefore, it is possible that infused T cells will not traffic to target tissues in sufficient numbers to achieve a meaningful clinical response using current strategies. This is especially true for many solid tumors that are often poorly perfused. Furthermore, the immunosuppressive microenvironment in solid tumors is a much harder obstacle to overcome and may limit the proliferation of tumor-targeted T cells *in vivo* (24). These high ratios may also underlie the inability of cytotoxic T cells (CTL) to affect a significant antitumor response for established tumors that are typically seen at clinical presentation (6). Collectively, these observations suggest that T cells with enhanced potency (i.e., a significantly lower effector/target ratio) may be needed to achieve significant long-term therapeutic responses in patients with solid tumors, especially those with a large tumor burden or widespread disseminated disease where the need is most dire.

### Bypassing Endogenous Cytolytic Functions

One strategy to achieve this goal is to bypass endogenous T-cell cytolytic functions and arm the T cells with a highly potent cytotoxic molecule, such as a protoxin. In this scenario, the T-cell would serve as a cell-based vector, or "Trojan Horse" for the tumor microenvironment. This can be accomplished using microparticle cell-loading strategies or through genetic engineering, the latter of which can add specificity through making protoxin expression dependent upon antigen recognition by a tumor-targeted CAR. This would effectively turn the T cells into "biologic microfactories" capable of pumping out large quantities of the protoxin into the tumor microenvironment following tumor-associated antigen recognition. The secreted protoxin would amplify the cytotoxic effect of each T-cell by killing a large number of malignant cells with every infiltrating T-cell (i.e., a "field effect"), thereby significantly enhancing the potency and lowering the effector/target ratio. Importantly, this "field effect" is dependent upon the controlled release of the protoxin within the tumor microenvironment.

As an example, proaerolysin is a bacterial pore-forming protoxin with picomolar toxicity against all cell types because of its nonselective mechanism of action (25). Wild-type proaerolysin is rapidly activated by ubiquitous furin-like proteases, and therefore, does not have a therapeutic index when administered systemically (25). Previous studies have demonstrated that a bolus injection of the protoxin intratumorally also does not produce a therapeutic effect (25). Because proaerolysin is highly lipophilic in its active form, it rapidly and irreversibly inserts into adjacent cell membranes following furin-mediated activation. Consequently, the activated toxin is rapidly sequestered within a finite number of nearby cells within a much localized area following intratumoral injection, thereby preventing sufficient distribution throughout the tumor to create an antitumor effect. Modification of the protoxin using site-directed mutagenesis to replace the wild-type activation domain with a PSA-selective cleavage sequence generated a protoxin that is selectively toxic to prostate cells (25). The



**Figure 2.** Proposed combinatorial strategy for enhanced specificity and therapeutic potency of CAR-expressing T cells. In the proposed model, expression and secretion of PSA-activated proaerolysin (PA) is dependent on antigen recognition (PSMA) by genetically engineered T cells. Antigen recognition induces a downstream signaling cascade within the T-cell that leads to activation of an IFN $\gamma$  promoter and protoxin expression. Protoxin activation following secretion into the tumor microenvironment is dependent on the presence of enzymatically active PSA, which is only found in the prostate and at sites of prostate cancer, including primary and metastatic tumors. PSA-dependent cleavage of the inhibitory domain leads to aeryolysin oligomerization and pore formation, which results in rapid cell lysis at nanomolar concentrations following membrane insertion.

engineered protoxin maintained low nanomolar activity against PSA-expressing cells, but the activation kinetics were sufficiently reduced to permit distribution throughout the tumor, thereby enhancing therapeutic efficacy (25). PSA-activated proaerolysin has been described as a "molecular grenade" (26), because once activated in the extracellular fluid by PSA-dependent cleavage of the inhibitory domain, the toxin is completely nonselective and kills any adjacent cell in the microenvironment independent of target expression, which results in a "field effect" or "kill zone." Importantly, the elimination of dormant cells in addition to cancer stem or initiating cells is implicit in this bystander effect.

Systemically administered T cells would also be expected to traffic to sites of inflammation other than cancer, which suggests an additional level of regulation may be necessary

to prevent "on-target, off-tumor" toxicity. For instance, PSMA is expressed on the surface of prostate epithelial cells, including primary and metastatic lesions. It is also expressed on the tumor neovasculature irrespective of disease site, but not on normal endothelial cells. Thus, PSMA potentially represents a nearly universal tumor-associated antigen for CAR-targeting in solid tumors (27). However, because of its expression in the brain and kidney, a combinatorial strategy involving a second regulatory step, such as PSA-mediated protoxin activation, is needed. Importantly, PSA in circulation is inactive because of inhibition by ubiquitous serum protease inhibitors. Using genetic engineering techniques, a PSA-activated proaerolysin construct could be cloned downstream of an NFAT or IFN $\gamma$  promoter, which are activated by CD3- $\zeta$  chain downstream signaling. By engineering the CAR to recognize a distinct

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antigen, such as PSMA, a CAR-dependent inducible expression system with enhanced specificity can be generated. Essentially, this strategy would combine combinatorial antigen recognition with the secretion and activation of a highly potent protoxin to achieve both greater specificity and significantly greater efficacy at lower effector/target ratios (Fig. 2).

### Potential for Rapid Translation

Importantly, each of these components has been independently validated clinically. Retrovirally modified CARTs have been administered to numerous patients in multiple clinical trials with promising results, particularly in ALL (4, 5, 18). Furthermore, these trials have a demonstrated track record of safety extending >10 years with no evidence of integration-mediated toxicity detected in >500 person-years (9). Although CARTs have demonstrated significant efficacy in hematologic malignancies and the concept has been validated in solid tumors, the data suggest that enhanced potency may be needed to achieve clinically significant therapeutic responses in the latter setting. In addition to the high effector/target ratios needed, immunosuppressive pathways may abrogate both endogenous and synthetic T-cell effector functions through inhibition of CD3- $\zeta$  chain downstream signaling pathways. This inhibition may also limit the proliferation of activated CARTs in the tumor microenvironment, thereby providing the rationale for enhancing T-cell potency through a "field effect" by delivering a highly cytotoxic agent. Combination therapy with immune checkpoint inhibitors has been suggested as a means to enhance therapeutic efficacy, and this holds true for synthetic platforms such as the one described as well.

In addition, PSA-activated proaerolysin has been administered to >130 patients and is entering phase III registration trials as a local therapy for symptomatic BPH (28). Importantly, intraprostatic injections into the PSA-producing monkey prostate produced no toxicity in periprostatic tissues, including the lateral pelvic fascia, anal sphincter, urethra, urinary bladder, rectum, or other distant organs, thereby demonstrating that the toxin does not re-enter systemic circulation once activated (25). Unfortunately, although highly effective as a local therapy, its therapeutic index as a systemic agent is limited as result of binding to ubiquitous GPI-anchored proteins present on cells throughout the body. Consequently, a "Trojan Horse" strategy, such as the one described, is needed for protoxin delivery in systemic applications targeting metastatic disease.

Although the strategy has been designed to minimize systemic toxicity and off-target effects, IRAEs implicated in other forms of immunotherapy may be of concern here as well, particularly if used in combination with checkpoint inhibitors. One strategy to further abrogate toxicity to single-positive, nontargeted tissue may be to use a suboptimal first-generation CAR to titrate intracellular signaling strength and prevent activation of endogenous T-cell activity following antigen recognition. "Leakiness" at any of the regulatory steps, including protoxin secretion and activation, would also be of potentially significant concern. In addition, although it is unlikely that cells can become resistant to proaerolysin because of its

mechanism of action (i.e., pore formation), it is possible for the tumor to evade cytotoxicity by downregulating the activating protease or CAR-targeted antigen. PSA- and PSMA-negative cells would also be killed via the "field effect"; however, it is possible to select for a resistant tumor through clonal outgrowth of these negative cells.

Because of the high costs and risks currently associated with adoptive T-cell therapy, advanced mCRPC represents the only suitable setting for clinical evaluation at this time. Furthermore, immunotherapy represents a particularly attractive treatment option in these patients because of its ability to potentially recognize a broad array of novel tumor antigens, thereby reducing the risk of resistance. Combination therapy with second-generation anti-androgens, such as enzalutamide, in late-stage disease also represents a potentially interesting option. Recent preclinical data have demonstrated an additive antitumor effect using MUC1-targeted immunotherapy in combination with anti-androgen therapy (29). An androgen receptor-negative neuroendocrine or "small cell" phenotype has been observed in a subset of patients with very aggressive disease (30), the prevalence of which is predicted to increase in patients failing second-generation anti-androgen therapies. However, it should be noted that nearly half of small cell patients with prostate cancer in one study still demonstrated evidence of androgen receptor activity as indicated by elevated PSA levels (30). This suggests that the proposed strategy may still be of benefit in a significant fraction of patients with highly aggressive mCRPC, and provides further support for the use of immunotherapeutic strategies in this patient population to circumvent tumor heterogeneity.

### Summary

Thus, the proposed strategy has the potential to enhance T-cell potency within the highly immunosuppressive prostate cancer microenvironment and bypasses tumor heterogeneity through the use of a "molecular grenade." Exploiting the intrinsic tumor tropism of T cells as a cell-based vector to deliver a PSA-activated protoxin following stimulation of a PSMA-targeted CAR represents a novel therapeutic platform, one that potentially offers significantly greater specificity and potency than current immunotherapies through bypassing endogenous cytolytic functions. Furthermore, this platform can easily be adapted to other tumor types through engineering alternative combinations of tumor- or tissue-specific targets into the system.

### Disclosure of Potential Conflicts of Interest

C.G. Drake has commercial research grant from BMS. C.G. Drake is a consultant/advisory board member of BMS, Compugen, Roche/Genentech, and Dendreon. No potential conflicts of interest were disclosed by the other authors.

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**Development of methodology:** W.N. Brennen

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.T. Isaacs

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.T. Isaacs

**Writing, review, and/or revision of the manuscript:** W.N. Brennen, C.G. Drake, J.T. Isaacs

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** W.N. Brennen  
**Study supervision:** W.N. Brennen

### Acknowledgments

The authors thank the Prostate Cancer Foundation (W.N. Brennen, PCF Young Investigator Award) and the Department of Defense (W.N. Brennen, Post-

Doctoral Fellowship, W81XWH-12-1-0049). These studies were supported by NIH-Prostate SPORC Grant (P50 CA058236).

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Received January 27, 2014; revised March 10, 2014; accepted March 12, 2014; published OnlineFirst April 18, 2014.

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