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Research Article

# Potential Immunotherapy for Prostate Cancer: Recombinant Bacille Calmette-Guerin Expressing Prostate Specific Molecules

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## Abstract

**Purpose:** The emergence of prostate cancer as a major health issue and the absence of curative treatment for metastatic disease requires the development of new treatment modalities. Prostate specific antigen (PSA) and prostate-specific membrane antigen (PSMA) are possible targets for prostate cancer immunotherapy. We have previously shown that PSA and PSMA can be expressed in recombinant bacille Calmette-Guérin (BCG) strains.

**Methods:** The in vivo immunogenicity of the prostate-specific proteins produced by this recombinant BCG strain were examined by detection of specific antibody responses and delayed-type hypersensitivity (DTH) responses in mice vaccinated with these strains. These immune responses were compared with those of control mice vaccinated with phosphate buffered saline diluent or soluble PSA or PSMA.

**Results:** Mice vaccinated with rBCG-PSA developed low levels of anti-PSA antibodies and strong DTH to PSA. Mice vaccinated with rBCG-PSMA developed strong DTH to PSMA and no anti-PSMA antibodies.

**Conclusions:** We conclude that recombinant BCG expressing PSA or PSMA induce strong cellular immune responses to these antigens. We propose that the innate adjuvant capacity of BCG could help stimulate a specific immune response against prostate-specific proteins produced by the bacteria, which in turn, could lead to the eradication of undetected metastatic prostate cancer cells in post-surgical patients.

**Keywords:** prostate cancer; BCG; bacille Calmette-Guerin; PSA; prostate specific antigen; PSMA; prostate-specific membrane antigen; immunotherapy

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**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

The emergence of prostate cancer (CaP) as a major health issue and the absence of curative treatment for metastatic disease necessitate the development of new treatment modalities. In 1990, CaP surpassed lung cancer as the most commonly diagnosed cancer among American men[1]. Approximately 233,000 cases, or twenty-seven percent of all newly diagnosed cancers in American men in 2014 will be CaP[2]. One in six American men will be diagnosed with CaP in his lifetime[1], and this cancer is the second leading cause of cancer deaths in American men with approximately 29,480 deaths estimated for the year 2014[1].

Ninety percent of organ-confined CaP cases can be cured with surgery if discovered early, but because there is no effective systemic therapy for this disease, the prognosis is poor once the tumor has spread beyond the gland itself and about half of the patients with CaP have clinically advanced (extraprostatic/extracapsular) disease at the time of initial diagnosis[3]. Even in those patients initially determined to have organ-confined disease, one-third actually have undetected micrometastatic disease, as determined by subsequent pathological staging or disease progression[4]. In all, more than 65% of patients with CaP develop metastatic disease.

Due to the high failure rate of conventional therapies (surgery, radiation, hormonal), current research efforts are focused on alternative approaches for the treatment of metastatic prostate cancer such as immunotherapy [4, 5]. Immunotherapy for the treatment of metastatic prostate cancer is based on the activation of the host's immune response against tumor-associated antigens (TAA) present on tumor cells that distinguish them from normal cells. TAA may be normal, tissue-specific cellular proteins that are upregulated on cancer cells, mutated proteins, oncofetal antigens, growth factor receptors, oncogene and tumor-suppressor gene products, among others [6].

Prostate cancer is an ideal candidate for immunotherapy for many reasons. There is a substantial failure rate of current therapies for the primary tumor and a lack of effective chemotherapy for metastatic disease. The prostate contains organ-specific TAA that can serve as targets of an immune response. The prostate is not essential and its removal or destruction in many patients with CaP eliminates the concern for potential autoimmune disease [7].

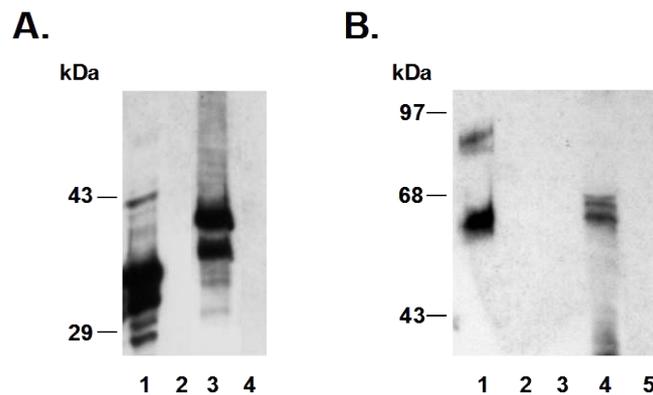
Prostate-specific antigen (PSA) and prostate specific membrane antigen (PSMA) are two prostate-specific TAA. PSA is expressed almost exclusively in normal, benign, and malignant prostate cells. Circulating PSA levels are frequently elevated with primary, locally recurrent or metastatic prostate cancer [8] PSMA is predominantly found in the prostate and is upregulated in primary and metastatic prostate cancer. Moreover, PSMA has been observed in endothelial cells of capillary beds in certain tumors including those of the prostate. Therefore, PSMA may be targeted in tumor neovasculature as well as in carcinoma cells[9].

We have previously reported development of recombinant BCG (rBCG) strains that express PSA and PSMA with the goal of using such recombinant BCG as cancer vaccines in treatment of CaP. The current report describes the stimulation of cellular and humoral immune responses against these prostate specific molecules in mice vaccinated with these recombinant BCG. We propose that recombinant BCG may elicit a specific immune response against the prostate-specific antigens it produces which in turn may lead to the eradication of undetected metastatic prostate cancer cells that express these proteins.

## Materials and methods

### Mice

Six to ten week old (C57BL/6 x BALB/c) F1 (CBF1) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The animals were housed 5 per cage in the Department of Comparative Medicine Animal Facility of New York Medical College that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).



**Figure 1** Expression of recombinant PSA and PSMA in BCG. (A) Expression of PSA in BCG. Lane 1, LNCaP prostate cancer cell lysate (positive control); Lane 2, recombinant BCG expressing RAS protein (negative control); Lane 3, BCG-PSA lysate; Lane 4, BCG-PSMA1300 lysate. Recombinant full length PSA consistently appears as a doublet of approximately 33 kDa (Figure 1A, lane 3). The larger of the two molecules may be full length PSA still covalently associated with the antigen 85B signal sequence encoded in the pMM7 expression vector. (B) Expression of PSMA in BCG. Lane 1, influenza virus lysate (HA serotype, X-47E4940) (positive control); Lane 2, influenza virus lysate (HA serotype PR8 E5201) (negative control); Lane 3, BCG vector lysate control; Lane 4, BCG-PSMA1300 lysate, BCG-PSMA 1300 appears as a doublet of approximately 50-55 kDa. As with PSA protein, the larger of the two molecules may be PSMA covalently associated with the antigen 85B signal sequence. Lane 5, BCG-PSA lysate. Molecular weight determination was made using Life Technologies high molecular weight protein markers.

### Recombinant Bacteria

BCG were engineered to express PSA or PSMA as previously described [10]. Briefly, the entire coding region of PSA was cloned into the pMM7 vector. Following electroporation into BCG and antibiotic resistance selection, clones expressing PSA protein were identified by western blot analysis (Figure 1a) [10].

The prostate cancer cell line, LNCaP, grown in RPMI 1640 medium, supplemented with 10% FCS (Mediatech, Cellgro, Herndon, VA) was used as a positive control for PSA protein expression. The LNCaP cells were lysed in lysis buffer (10 mM tris, 1 mM EDTA, 15 mM NaCl, 10% NP-40) containing protease inhibitors (1 microgram/ml of aprotinin, leupeptin, and 17 micrograms/ml PMSF). PSA protein expression was quantified using known amounts of purified human PSA as standards on western blot, followed by densitometry (Table 1).

**Table 1** Levels of protein expression in rBCG lysates. Amounts of protein expression were quantified using serial dilutions of standards on western blots, followed by densitometry.

<b>Bacterial Clone</b>	<b>Level of Protein Expression</b> ng per microgram of bacterial protein
BCG-PSA	24.00
BCG-PSMA	0.24
BCG-PSMA1300	0.66
BCG-PSMA1500	0.47

The entire coding region of PSMA was cloned into the pMM7-HA vector as well as two overlapping fragments of PSMA [10]. One PSMA fragment contained the 5'- 1314 nucleotides encoding the amino-terminal 437 amino acids of PSMA. The second PSMA fragment contained the 3'- 1483 nucleotides of PSMA encoding the 446 carboxyl-terminal amino acids. The pMM7-HA vector expresses recombinant proteins as a fusion molecule with the hemagglutinin tag epitope, used for detection purposes[10]. Following electroporation and antibiotic resistance selection, clones expressing PSMA protein were identified by western blot analysis. Both PSMA fragments are efficiently expressed in BCG. BCG-PSMA1300, which expresses the amino-terminal fragment of PSMA, was used for vaccination experiments (Figure 1b). Influenza viral lysates were used as positive and negative controls for the HA tag (a gift from Dr. Edwin Kilbourne, NYMC, Valhalla NY). PSMA protein expression in lysates was quantified using serial dilutions of a known 30 kD HA tagged molecular weight markers as standards on western blot, followed by densitometry (Table 1).

### Prostate Specific Molecules

Human seminal PSA was obtained from Scripps Laboratories (San Diego, CA). Recombinant PSMA was prepared using the MaxBac2.0 Baculovirus Expression system. (Invitrogen, Carlsbad, CA).

### Immunization of mice

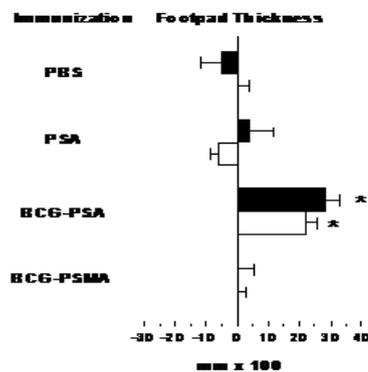
CBF1 mice were subcutaneously injected with one million colony-forming units of BCG-PSA, BCG-PSMA1300 (expressing the 5'-1314 nucleotides encoding the amino terminal 438 amino acids of PSMA) [10]BCG (with vector only), 5 micrograms of PSA protein, 5 micrograms of PSMA protein or PBS (100 microliters total volume).

### Detection of DTH

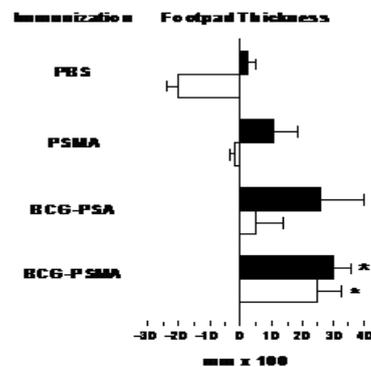
Animals were challenged 12 weeks after vaccination with 10 micrograms PSA or 5 micrograms PSMA in 10 microliters of saline into the footpad using 100ul Hamilton syringe fitted with a 30 or 26 gauge needle. Footpad thickness was measured by a vernier caliper, prior to and 24 and 48 hours after challenge [11]Statistical analysis was performed using one-way analysis of variance

(ANOVA) analysis with a Tukey-Kramer multiple comparison post-test.(Figure 2).

### A. PSA Challenge



### B. PSMA Challenge



**Figure 2** Analysis of DTH response. Mice were challenged with either soluble PSA (Panel A) or PSMA (Panel B) and the response was quantified by increased footpad thickness (black bars, 24hours;white hatched bars, 48 hours) as well as histologic evaluation of infiltrating cells (48 hours).

### Detection of an Antibody Response

Individual samples of serum were collected from each mouse by retro-orbital bleeding prior to, and 5 and 10 weeks after immunization. Equal amounts of sample from animals in each group were pooled. ELISA was used to assay for anti-PSA and PSMA antibodies. Ninety six-well Immunlon-2 plates were coated with 100 ng/well of PSA in 100 microliters of coating buffer (0.1 M NaHCO<sub>3</sub> pH 9.6). The plates were incubated overnight at 37<sup>0</sup>C after which the excess liquid was decanted. One hundred micrograms of PSMA was solubilized in 8M urea, then coated onto wells and incubated overnight at 4<sup>0</sup>C after which the excess liquid was decanted, the plate was rinsed once with PBS. The plates were blocked with 200 microliters of 5% BSA in PBS per well for 1 hour at room temperature. Samples in PBS containing 1% BSA and 0.05% Tween-20 were then added to the wells (total volume, 100 microliters per well) and incubated for 1 hour at room temperature. Preimmune sera were diluted 1:20 and the post-immune sera were 2-fold serially diluted (1:20 to 1:2560). The plate was washed 5 times with 0.05% Tween-20 in PBS and once with PBS. HRP-labeled, secondary antibody, rabbit anti-mouse IgG (H+L) (Jackson

ImmunoResearch Laboratories, West Grove, PA) was added at a 1:1000 dilution at 100 microliters per well in 1% BSA, 0.05% Tween-20 in PBS. The plate was incubated for 1 hour at room temperature and washed as above. Colorimetric analysis was performed using TMB Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD)(Figure 4). Endpoints for antibody titers were defined as the highest dilution at which the values of absorbance at 450nm ( $A_{450}$ ) were greater than the pre-immune sera, diluted 1:20, plus 2 standard deviations [12].

## Results

### Recombinant BCG express high levels of recombinant proteins

Of the four recombinant prostate molecule constructs, PSA was synthesized at the highest level, perhaps due to its smaller size and higher hydrophilicity than PSMA (Table 1). Perhaps for the same reasons, both fragments of PSMA were synthesized at higher levels than the entire molecule (Table 1). Recombinant full length PSA consistently appears as a doublet of approximately 33 kDa (Figure 1A). The larger of the two molecules may be full length PSA still covalently associated with the antigen 85B signal sequence encoded in the pMM7 expression vector. BCG-PSMA 1300 appears as a doublet of approximately 50-55 kDa (Figure 1B). As with PSA, the larger of the two molecules may be PSMA still covalently associated with the antigen 85B signal sequence. BCG-PSA and BCG-PSMA 1300 (because of its higher expression) were used for the immunization of mice.

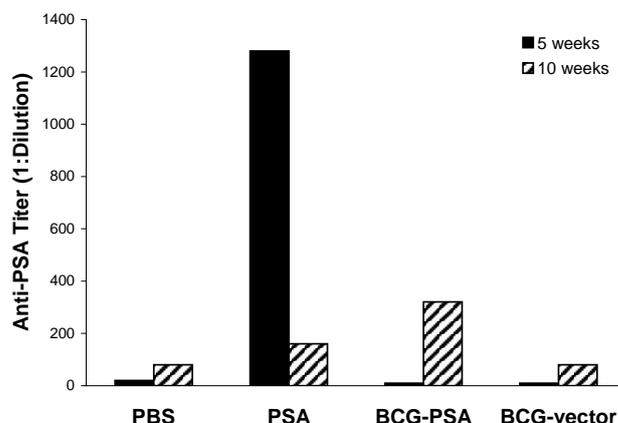
### Mice immunized with BCG-PSA or BCG-PSMA1300 develop a DTH response against the respective prostate specific molecule

To determine if vaccination with recombinant BCG can stimulate a DTH response in animals challenged with a target protein, groups of 5 immunized and control mice were injected with 10 micrograms of PSA or 5 micrograms PSMA protein into the hind footpad twelve weeks after vaccination. Footpad thickness was measured 24 and 48 hours later.

BCG-PSA and BCG-PMSA vaccination induced specific DTH as measured by increases in footpad thickness that were maximal at 24 h that could only be elicited by the homologous protein antigen. Thus, mice immunized with BCG-PSA only exhibited a significant increase in footpad thickness to PSA protein (Figure 2A), while mice immunized with BCG-PMSA only exhibited a significant increase in footpad thickness to PMSA protein (Figure 2B). These data suggest that immunization with recombinant BCG that express prostate specific molecules can stimulate cellular immune responses against the recombinant proteins.

### Mice immunized with BCG-PSA generate a weak antibody response to PSA

Serum was prepared from mice 5 and 10 weeks after immunization and assayed for the presence of anti-PSA or anti-PSMA antibodies. Mice immunized with human PSA had a titer of 1:1250 antibody at 5 weeks, which decreased to 1:160 by week 10 (Figure 3). Mice immunized with BCG-PSA also had an anti-PSA antibody response, but this response was not detectable until week 10 after vaccination and had a lower titer, 1:320. BCG with vector alone was unable to stimulate an anti-PSA antibody response and showed a profile similar to vaccination with PBS. Similar results were obtained in a second experiment (data not shown). There was no detectable antibody response against PSMA in any animals immunized with PSMA or BCG-PSMA1300.



**Figure 3** Antibody response to PSA by mice immunized with BCG-PSA. Mice were immunized with PBS, PSA, BCG-PSA or BCG-PSMA1300 and serum was tested for the presence of anti-PSA antibodies by ELISA. Sera not showing a response at the lowest dilution tested, 1/10, were assigned a titer of 10.

## Discussion

It has been over a century since William Coley's first report of cancer regression induced by immune system activation in response to bacterial toxins[6]. Immunotherapy has always represented an attractive approach to cancer treatment in light of the many shortcomings of conventional treatment such as surgery, radiation and chemotherapy[6]. While many cancer vaccine trials have yielded promising results, active immunotherapy has only recently become an established modality of anticancer therapy[13]. The high incidence of metastatic prostate cancer necessitates the development of new systemic therapies.

One immunotherapeutic approach for cancer involves the use of a patient's tumor cells mixed with various adjuvants, including cytokines, or genetically modified autologous cells that secrete cytokines[3]. Among the drawbacks of whole cell vaccines is that it is labor-intensive and time consuming, especially if the cells are to be genetically modified. The success, or the lack of success, in the expansion of primary cultures for autologous vaccines can limit the courses of vaccinations and, further, an autologous vaccine needs to be specifically made for each patient[14].

Another strategy for generating antigen-specific immunity is the *ex vivo* administration of specific antigen or peptides to antigen-presenting cells (APC). Again, this type of therapy is limited by the need to culture cells from each patient and success in the expansion of primary cultures for autologous vaccines can limit the number of courses of vaccination. Further, the use of peptides to "load" APCs faces the obstacle of finding HLA-restricted peptides for all the different polymorphic HLA molecules[3].

The use of recombinant BCG that express prostate specific molecules for immunotherapy would eliminate the necessity to collect, expand, and preserve autologous tumor cells. Also, BCG engenders a strong, long-lived immune response due to the ability to survive for several weeks in the host's macrophages and this may eliminate the need for numerous vaccine boosts. Live

BCG is currently used as the vaccine for tuberculosis and its safety is already well established. This is beneficial due to the fact that live, antigen-expressing recombinant BCG appear to be critical for providing strong, specific, cell-mediated immunity; dead recombinant BCG and live non-recombinant BCG mixed with antigens are significantly less effective [15].

We have demonstrated that recombinant BCG are capable of eliciting an immune response against PSA and PSMA. Delayed type hypersensitivity was induced against both PSA and PSMA. A delayed antibody response to PSA in animals vaccinated with BCG-PSA was observed compared to vaccination with human PSA. This delay could indicate that live recombinant BCG cells are actively growing and continued growth may be necessary to achieve a critical mass of recombinant bacteria and foreign antigen needed for the stimulation of the immune response [16]. It is noteworthy that BCG has a long doubling time of approximately 20 hrs. It is possible that a longer period of observation may be necessary to obtain an even higher anti-PSA antibody titer from animals immunized with BCG-PSA.

The recombinant BCG vaccine, BCG-PSMA1300 was not capable of eliciting an antibody response. These results suggest that even though cellular responses were detected, the inherent immunogenicity and/or level of antigen expression may be important for engendering a strong humoral response [16]. The BCG-PSA vaccine expressed approximately forty-fold more recombinant protein than BCG-PSMA1300.

The ability of the recombinant BCG vaccines to elicit a cell-mediated immune response is more desirable than its ability to elicit a humoral response. It has been shown that a cell-mediated response is more efficient in eliminating tumor cells. Studies in experimental animals have shown that cellular rather than humoral immune responses are responsible for the rejection of transplanted tumors or allogeneic tissue and that the administration of antibodies has had little impact on inhibiting the growth of tumors [17].

Clinically, it would be anticipated that recombinant BCG vaccines would be administered to patients who have recently undergone a radical prostatectomy, as the low tumor burden may present a window of opportunity for the recombinant BCG vaccine to stimulate an immune response against prostate molecules, and eradicate metastatic prostate cancer cells. It is significant that Wei *et al.* [18] have reported that transgenic BALB/c mice that express human PSA in a tissue-specific and androgen-dependent manner are capable of mounting a cytotoxic immune response against PSA. Thus, the induction of anti-PSA or anti-PSMA immune responses in prostate cancer patients with recombinant BCG immunotherapeutic vaccines appears feasible. Further, other prostate-associated proteins may be used as additional targets for recombinant BCG immunotherapy, as well as the incorporation of recombinant cytokine expression to direct and enhance the immune response against prostate cancer cells.

In summary, we have shown that immunization of mice with recombinant BCG expressing prostate-specific antigens induces readily detectable, specific, cell mediated immune responses to PSA and PSMA, suggesting that these vaccines could be used to stimulate a prostate specific immune response for the eradication of undetected metastatic prostate cancer after radical prostatectomy.

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