Antitumor Immune Response Induced by DNA Vaccine Encoding Human Prostate-specific Membrane Antigen and Mouse 4-1BBL

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OBJECTIVES To determine whether a novel DNA vaccine encoding truncated human prostate-specific membrane antigen (tPSMA) can be enhanced by a genetically enhanced adjuvant (4-1BB ligand [4-1BBL]).

METHODS A eukaryotic expression plasmid pDC316-tPSMA-internal ribosome entry site-mouse 4-1BBL (pDC316-tPSMA-IRES-m4-1BBL) was constructed. The efficacy of vaccination using pDC316-tPSMA-IRES-m4-1BBL was compared with pDC316-tPSMA in terms of the antigen-specific cytotoxic T lymphocyte activity and antitumor immunity to RM-1-tPSMA in a murine tumor model.

RESULTS pDC316-tPSMA-IRES-m4-1BBL induced potent cytotoxicity against RM-1-tPSMA cells expressing tPSMA (42.6% specific killing) compared with pDC316-tPSMA vaccinated mice (24.8% killing) and mice not vaccinated (10.8% killing; $P < .01$). Moreover, the vaccination of mice with pDC316-tPSMA-IRES-m4-1BBL induced a potent protective antitumor immunity to RM-1-tPSMA in a subcutaneous tumor model.

CONCLUSIONS These results suggest that a specific antitumor immune response is enhanced by DNA vaccines expressing PSMA and 4-1BBL. This approach could offer a new strategy for treating carcinoma of the prostate after standard therapy. UROLOGY 76: 510.e1–510.e6, 2010. © 2010 Elsevier Inc.

Prostate cancer is the most frequently diagnosed cancer in older men and the second-leading cause of male cancer death in Western countries.[1] The incidence and mortality of prostate cancer have been increasing in China. Although radical prostatectomy and radiotherapy remain the primary choice for localized prostate cancer, no effective treatment is available for patients who develop recurrence or hormone-refractory prostate cancer, or who have metastatic disease at diagnosis. Therefore, effective novel therapeutic approaches are needed.

DNA vaccination provides an alternative cancer immunotherapy.[2] Compared with live viral or bacterial vectors, plasmid DNA is relatively safe, can be easily administered, is easy to prepare on a large scale with high purity and high stability, and can be engineered to express antigenic peptides or proteins.[3] However, a major drawback of the DNA vaccine is its limited potency to induce an immune response. Also, optimal strategies to enhance the efficiency of DNA immunization remain to be established. Alternative strategies to improve DNA vaccine potency include the use of co-stimulatory molecules related to T cell proliferation, such as 4-1BB ligand (4-1BBL). 4-1BBL (CD137L), a type II surface glycoprotein belonging to the tumor necrosis factor superfamily,[4] which is expressed by antigen present cell, including B cells, macrophages, and dendritic cells (DCs).[5] 4-1BBL, with its receptor 4-1BB expressed on the T cell, has profound effects on T cells, including activation of both CD4+ and CD8+ T cells, enhanced expansion,[6] increased long-term survival,[7] and antiapoptosis of activation-induced CD8+ T cells.[8] Co-stimulation through 4-1BB can also enhance the production of cytokines, such as interleukin-2, interleukin-4, and interferon-γ.[9] Taken together, a DNA vaccine encoding 4-1BBL combined with an antigen could potentially improve the antigen-specific immune responses.

In the present study, we investigated a novel DNA vaccine encoding mouse 4-1BBL and truncated human prostate-specific membrane antigen (tPSMA). The PSMA is an overexpressed membrane-bound cell surface protein on prostate cancer cells and retains most of the known features of prostate cancer.[10] It has been an ideal target for a variety of therapeutic approaches, including

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the delivery of immunoconjugates, immunotherapy, and prodrugs.[11-13] The objective of the present study was to determine whether the potency of the DNA vaccine encoding tPSMA and mouse 4-1BBL (m4-1BBL) could be enhanced by 4-1BBL.

MATERIAL AND METHODS

Animals and Cell Lines

Female C57BL/6 (H-2kb) mice, 6-8 weeks old, were obtained from Shanghai SLAC Laboratory Animal (Shanghai, China). The mice were maintained at the Central Animal Facility of Wuhan University according to standard guidelines, and the experiments were conducted according to an approved protocol following the guidelines of the China Council for Animal Care. RM-1,[14] a murine prostate cancer cell line, was obtained from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 medium with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Expression Vector Construction

The cDNA of m4-1BBL was derived from pcDNA3-m4-1BBL plasmid provided by T. Watts (Department of Immunology, University of Toronto, Toronto, ON, Canada) and inserted in the pDC316-capsid protein VP3 (VP3)-internal ribosome entry site (IRES)-endothelial cell growth factor (sEndo) in the *Not*I/*Nhe*I-restriction sites, creating pDC316-VP3-IRES-m4-1BBL. The cDNA for the extracellular domain of tPSMA was taken from pCR3.1-University-hPSMA plasmid (from Xiangzhong Yu, Department of Biological Sciences, Clemson University, Clemson, SC) and inserted in the plasmid pDC316-IRES-enhanced green fluorescent protein (EGFP) in the *Age*I/*Sac*I-restriction sites, creating pDC316-tPSMA-IRES-EGFP. Next, the fragment tPSMA was digested from pDC316-tPSMA-IRES-EGFP through restriction enzyme and subrate cloned into pDC316-VP3-IRES-m4-1BBL, resulting in pDC316-tPSMA-IRES-m4-1BBL. The cDNA of tPSMA was subrate cloned into pDC316 in the *Bgl*II/*Hind*III, resulting in pDC316-tPSMA.

Transfection

The plasmid pDC316-tPSMA-IRES-m4-1BBL, pDC316-tPSMA, and pDC316 were extracted with an endotoxin-free extraction kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RM-1 cells were transfected with pDC316-tPSMA-IRES-m4-1BBL, pDC316-tPSMA, or an empty (mock) vector by the mediation of Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. After 2 days of incubation at 37°C in 5% carbon dioxide, the cells were collected for Western blot assay.

Western Blot Analysis

The cells were lysed by 200-μL lysis buffer (50 mM NaCl, 0.01 M Tris-Cl [pH 8.0], 5 mM ethylenediaminetetraacetic acid, 0.5% NP-40, 1 mM phenyl-methylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin). The supernatants were collected and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Next, the protein was transferred to a nitrocellulose membrane (GE Healthcare [formerly Amersham Biosciences], Piscataway, NJ). The transferred membrane was probed with polyclonal goat polyclonal anti-PSMA antibody or goat polyclonal anti-4-1BBL antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a horseradish peroxidase-conjugated antigoat IgG secondary antibody (Santa Cruz Biotechnology). The antibodies on membrane were visualized by chemiluminescence (Pierce Biotechnology, Rockford, IL). Western blot for β-actin was used as an internal sample.

Stable Transfection of RM-1 Cells With tPSMA Plasmid

The RM-1 cells were transfected with 2 μg of pCDNA3.1-tPSMA or empty vector by the mediation of 6 μL Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 2 days of culture, the cells were reseeded into a 10-cm dish and cultured for another 2 days. Complete Roswell Park Memorial Institute-1640 medium containing 1000 μg/mL G418 (Sigma, St. Louis, MO) was added to the culture. After 20 days of selection, all nontransfected cells had died, and discrete clones were visible in the transfected cells. These clones were expanded in the presence of 200 μg/mL G418, and positive cells expressing tPSMA were named RM-1-tPSMA. Western blot analysis was performed as detailed in the "Western Blot Analysis" section.

DNA Vaccination of C57BL/6 Mice

Plasmids were purified using an EndoFree plasmid Maxi Kit (Qiagen, Valencia, CA). Three groups, each including 6 C57BL/6 mice, were immunized: a pDC316-tPSMA-IRES-m4-1BBL group, a pDC316-tPSMA group, and a control group that received empty plasmid. All mice were injected with 0.25% lidocaine in the quadriceps femoris muscle 3 days before vaccination to enhance the uptake of the plasmids by the muscles. The mice then received a bilateral intramuscular injection of 50 μg of plasmid in the regenerating muscles. All mice received a boost every 4 weeks during a 12-week interval.

Cytotoxic T Lymphocyte Assay

At 2 weeks after the last immunization, the mice were killed. The splenocytes were harvested, homogenized, and red blood cell lysed with lysis buffer. Purified T cells subsequently generated using nylon wool column were used as effector cells. These T cells, then 2×10^7, were restimulated in 6-well culture plates with 4% paraformaldehyde prefixed 2×10^6 RM-1-tPSMA cells in the presence of 10 U/mL of murine interleukin-2 at 37°C. After 6 days of incubation, the restimulated T cells were harvested and separated from the dead cells. The target cells (RM-1 cells or RM-1-tPSMA cells) were placed in 96-well tissue culture plates at 1×10^4 cells per well and co-cultured with effector cells (cytotoxic T lymphocytes [CTLs]) at a ratio of 1:10, 1:20, 1:40, and 1:80 for 48 hours at 37°C in 5% carbon dioxide. The target cells and effector cells incubated in medium alone served as the target control and effector control, respectively. The cytotoxic activities were determined using the Cell Counting Kit-8 assay (Dojindo, Japan). Cell Counting Kit-8 resolution (10 μL) was added to each well of 100 μL medium. Absorbance was measured at 450 nm using an automatic enzyme-linked immunosorbent assay reader (Triturus, Grifols, Barcelona, Spain). All determinations were performed in triplicate and repeated 3 times. The percentage of specific cytotoxicity was calculated as [target control − (experimental − effector control)/target control] × 100%.

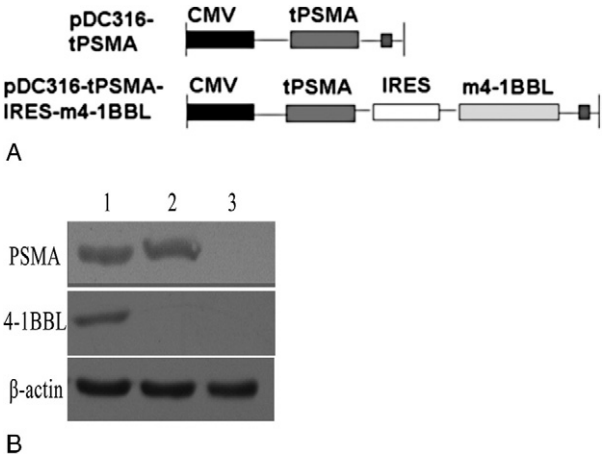

Figure 1. Construction of DNA vaccine. **(A)** Schematic overview of recombinant plasmid pDC316-tPSMA-IRES-m4-1BBL and pDC316-tPSMA. **(B)** Western blot using anti-PSMA pAb or anti-4-1BBL pAb of cell lysates of pDC316-tPSMA-IRES-m4-1BBL-transfected, pDC316-tPSMA-transfected, or mock-transfected RM-1 cells separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis, showing specific band corresponding to molecular mass of tPSMA and 4-1BBL. β-Actin used as reference.

In Vivo Antitumor Immunity

For the evaluation of tumor prevention, the C57BL/6 mice were divided into 3 groups and immunized as detailed in the previous sections. RM-1-tPSMA cells were trypsinized and resuspended in phosphate-buffered saline; 2×10^5 cells/100 mL were subcutaneously injected into the left flank of the mice. Tumor establishment was determined by palpation. The tumor volume was measured 2-dimensionally with a caliper along the longest axis (x) and the axis (y) perpendicular to the longest axis. The tumor volume was estimated using the following formula: volume $= 1/2 \times x \times y$.[2] The treatment groups consisted of 5 mice/group. When the tumor volume reached 3000 mm^3, the mice with tumors were killed. The experiments were independently repeated 3 times. The combined data from these experiments are presented.

Statistical Analysis

The data are presented as the mean ± standard deviation. Statistical differences were considered significant at $P < .05$, as determined by analysis of variance or Student's t test using the Statistical Package for Social Sciences, version 13.0 (SPSS, Chicago, IL).

RESULTS

Construction of DNA Vaccine

We constructed the DNA vaccines carrying human tPSMA gene driven by the cytomegalovirus promoter and mouse 4-1BBL gene driven by an IRES (Fig. 1A). To check the expression of tPSMA protein and m4-1BBL protein, the total protein of the cells transfected with DNA vaccine was collected and analyzed using Western blotting. The results showed that tPSMA protein and m4-1BBL protein was only expressed in the cells transfected with pDC316-tPSMA-IRES-m4-BBL compared with the control cells (Fig. 1B). β-Actin protein was used as an internal control.

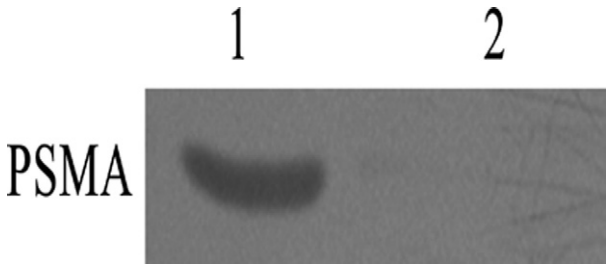

Figure 2. Detection of stable transfection of RM-1-tPSMA cells by Western blotting. Total cell lysates were harvested and presence of PSMA protein was detected by anti-PSMA polyclonal antibody. Specific band identified in positive clone but not in RM-1 cells transfected with empty vector.

Detection of Stable Transfection of RM-1-tPSMA Cells

To detect the transfection efficiency, the lysates from positive cells were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting. A band with a similar size to 95 kDa was identified in the positive clones but not in mock-transfected RM-1 cells (Fig. 2). These positive clones were designated RM-1-tPSMA and were used as target cells for the CTL assay.

Tumor-Specific CTL Activity

To define the tumor-specific CTL activity, T cells from C57BL/6 mice were restimulated for 6 days with 4% paraformaldehyde-prefixed RM-1-tPSMA cells. Cytotoxicity was measured by Cell Counting Kit-8 resolution from attacked RM-1-tPSMA cells or RM-1 cells. Splenocytes from mice in the pDC316-tPSMA-IRES-m4-1BBL group and pDC316-tPSMA group exhibited specific lysis against RM-1-tPSMA. In contrast, those from the mice in the control group did not acquire killing activity. The differences between the empty vector group and the other 2 groups were statistically significant ($P < .01$). CTL reactivity was significantly enhanced in the mice treated with pDC316-tPSMA-IRES-m4-1BBL compared with pDC316-tPSMA ($P < .01$; Fig. 3A). Specific lysis was not observed in any group when RM-1 cells were used as target cells (Fig. 3B). This result showed that pDC316-tPSMA-IRES-m4-1BBL could enhance tumor-specific CTL activity.

Antitumor Immunity In Vivo

To examine the antitumor immunity in vivo, C57BL/6 mice (n = 5/group) were vaccinated with pDC316-tPSMA-IRES-m4-1BBL, pDC316-tPSMA, and empty vector separately that had been challenged with RM-1-tPSMA cells. Protection was observed in the subcutaneous tumor model. After 19 days, the mice vaccinated with pDC316-tPSMA-IRES-m4-1BBL had an obviously

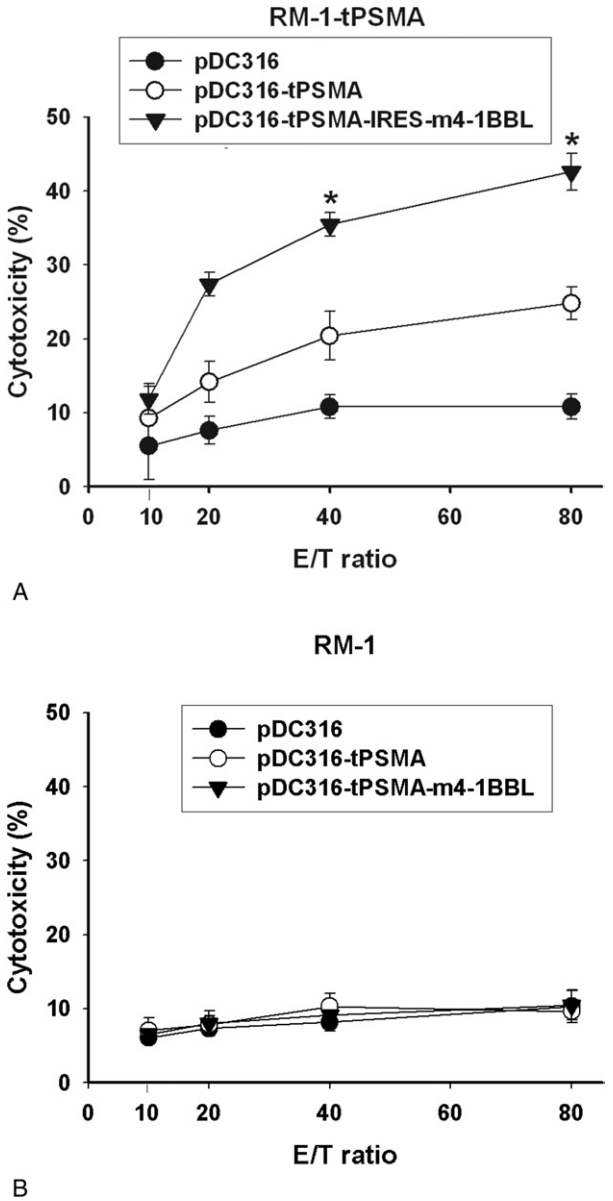

Figure 3. Cytotoxicity assay. Spleen lymphocytes harvested from mice vaccinated with pDC316-tPSMA-IRES-m4-1BBL, pDC316-tPSMA, and pDC316 separately. T cells were subsequently generated by co-cultivation of these spleen lymphocytes with 4% paraformaldehyde prefixed RM-1-tPSMA cells for 6 days. Cytotoxicity was measured using Cell Counting Kit-8 resolution from attacked RM-1-tPSMA cells or RM-1 cells. Splenocytes from both pDC316-tPSMA-IRES-m4-1BBL and pDC316-tPSMA immunized mice specifically recognized and lysed **(A)** RM-1-tPSMA ($P < .01$), but not **(B)** RM-1 cells. Level of CTL activity induced by pDC316-tPSMA-IRES-m4-1BBL was greater than that of pDC316-tPSMA ($P < .01$). Each point represents mean of triplicate experiments; 1 of 3 representative experiments shown.

lower tumor growth than the mice vaccinated with pDC316-tPSMA or empty vector ($P < .01$; Fig. 4). This result suggested that vaccination with pDC316-tPSMA-IRES-m4-1BBL significantly enhanced the antitumor effects.

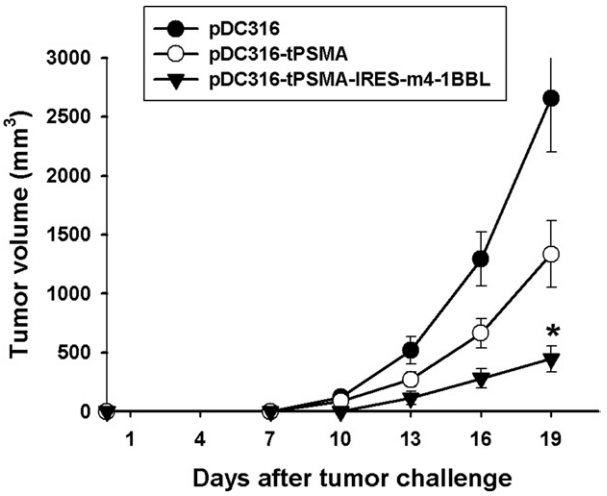

Figure 4. Antitumor immunity in vivo. C57BL/6 mice (n = 5/group) vaccinated with pDC316-tPSMA-IRES-m4-1BBL, pDC316-tPSMA, or empty vector separately challenged with RM-1-tPSMA cells. Tumor size in each group of mice was monitored. After 19 days, mice vaccinated with pDC316-tPSMA-IRES-m4-1BBL and pDC316-tPSMA resulted in significant inhibition of tumor growth, and mice vaccinated with pDC316-tPSMA-IRES-m4-1BBL had obviously lower tumor growth than mice vaccinated with pDC316-tPSMA or empty vector ($P < .01$). Each point represents mean of triplicate experiments; 1 of 3 representative experiments shown.

COMMENT

At present, several tumor vaccines have been entered in clinical trials for patients with prostate cancer. These trials are using a variety of immunization strategies targeting several prostate tumor antigens, including PSMA, six-transmembrane epithelial antigen of the prostate,[15] prostate-specific antigen,[16] prostate stem cell antigen,[17] and prostatic acid phosphatase).[18] DNA-based cancer vaccines provide an attractive approach for cancer immunotherapy. After vaccination, plasmid DNA is taken up by the host tissue, and an active immune response to the target antigen can be elicited through antigen expression and presentation by the resident antigen-presenting cells. However, the clinical outcomes have not been satisfactory, mainly because the tumor-specific CTLs elicited by the vaccines have been insufficient to suppress cancer progression. Other reasons have included indoleamine 2,3-dioxygenase produced within prostate cancer initiates the degradation of tryptophan along the kynurenine pathway, resulting in the production of immunosuppressive catabolites known to inhibit T cell stimulation in vitro and to cause T cell apoptosis.[19] CD8+ CTLs constitute one of the most important arms of the immune system, exhibiting the capacity to recognize and destroy cancerous cells.[20,21] A variety of approaches are under evaluation to activate CD8+ CTLs. To that end, vaccines need to be administered in combination with adjuvants, including cytokines[22] and costimulatory molecules.[23]

In the present study, we generated a novel DNA vaccine encoding tumor-associated antigen (tPSMA) and co-stimulatory molecule 4-1BBL (Fig. 1) and confirmed the expression of these transgenes in infected RM-1 cells (Figs. 2 and 3). PSMA is a well-defined prostate-restricted tumor-associated antigen upregulated in prostate carcinoma, especially in the advanced stages.[16,24] Several PSMA-based vaccines had been developed, and it has been observed in a Phase I- II trial using major histocompatibility complex class I-restricted peptides that PSMA induces immune responses in patients with advanced carcinoma.[25,26] 4-1BBL and its receptor 4-1BB induced on naive T cells after T cell receptor-major histocompatibility complex/peptide and CD80/86/CD28 ligation after interaction of cognate T cells with DCs are a pair of co-stimulatory molecules. Signaling through 4-1BB/4-1BBL enhances T cell expansion, augments T cell effector function (including cytokine release, CD4− mediated "help," and CTL activity),[27] prevents activation-induced apoptosis of CD8+ T cells,[8] overcomes activation-induced nonresponsiveness in CD8+ T cells in vitro,[28] and can break immunologic ignorance (promoting regression of poorly immunogenic tumors in vivo).[29] Therefore, 4-1BBL could be a useful vaccine adjuvant.

Our results have confirmed that splenocytes from both pDC316-tPSMA-IRES-m4-1BBL and pDC316-tPSMA immunized mice specifically recognized and lysed RM-1-tPSMA, but not RM-1 cells (Fig. 3) in vitro. The level of CTL activity induced by pDC316-tPSMA-IRES-m4-1BBL was greater than that of pDC316-tPSMA ($P < .01$). A comparison of the protective antitumor immunity of the vaccination using DNA vaccine in RM-1-tPSMA subcutaneous tumor cells showed that vaccination of mice pDC316-tPSMA-IRES-m4-1BBL resulted in enhanced antitumor immunity in vivo compared with pDC316-tPSMA and pDC316 (Fig. 4). These results were consistent with the CTL activity against RM-1-tPSMA in vitro. The results of the present study suggest that RM-1 cells can affect an immune response in this model. Consistent with previous reports, the results of the previous study have indicated that 4-1BBL enhances cellular immunity.

Our work was done entirely in only 1 cell line, RM-1, which was a limitation of the present study, and clinical prostate cancer or other cell lines might not necessarily result in the same outcomes. Because of an ampicillin resistance gene, the use of pDC316 might be precluded from implementation in human clinical trials. It will important to use an appropriate DNA vector, such as the pNGVL4a vector, which has previously been used in human clinical trials.[30] The pNGVL4a vector lacks the ampicillin resistance gene and thus would be suitable for clinical translation of the current DNA vaccines.[22]

CONCLUSIONS

The antitumor activity of DNA vaccines encoding tPSMA and 4-1BBL have demonstrated significantly enhanced potency against PSMA-expressing tumors by DNA vaccines. The success of the present study supports the notion that additional exploration of other co-stimulatory molecules to optimize T cell function and identify the ideal co-stimulatory molecule to optimize DNA vaccine potency.

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