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PD-1 or PD-L1 Blockade Restores Antitumor Efficacy Following SSX2 Epitope-Modified DNA Vaccine Immunization

Brian T. Rekoske[1], **Heath A. Smith**[2], **Brian M. Olson**[3], **Brett B. Maricque**[3], and **Douglas G. McNeel**[1,3,*]

[1]Department of Medicine, University of Wisconsin-Madison, Madison, WI 53705

[2]Department of Oncology, University of Wisconsin-Madison, Madison, WI 53705

[3]Carbone Cancer Center, University of Wisconsin-Madison, Madison, WI 53705

Abstract

DNA vaccines have demonstrated antitumor efficacy in multiple preclinical models, but low immunogenicity has been observed in several human clinical trials. This has led to many approaches seeking to improve the immunogenicity of DNA vaccines. We previously reported that a DNA vaccine encoding the cancer-testis antigen SSX2, modified to encode altered epitopes with increased MHC class I affinity, elicited a greater frequency of cytolytic, multifunctional $CD8^+$ T cells in non-tumor-bearing mice. In this report we sought to test if this optimized vaccine resulted in increased antitumor activity in mice bearing an HLA-A2-expressing tumor engineered to express SSX2. We found that immunization of tumor-bearing mice with the optimized vaccine elicited a surprisingly inferior antitumor effect relative to the native vaccine. Both native and optimized vaccines led to increased expression of PD-L1 on tumor cells, but antigen-specific $CD8^+$ T cells from mice immunized with the optimized construct expressed higher PD-1. Splenocytes from immunized animals induced PD-L1 expression on tumor cells *in vitro*. Antitumor activity of the optimized vaccine could be increased when combined with antibodies blocking PD-1 or PD-L1, or by targeting a tumor line not expressing PD-L1. These findings suggest that vaccines aimed at eliciting effector $CD8^+$ T cells, and DNA vaccines in particular, might best be combined with PD-1 pathway inhibitors in clinical trials. This may be particularly advantageous for vaccines targeting prostate cancer, a disease for which antitumor vaccines have demonstrated clinical benefit and yet PD-1 pathway inhibitors alone have shown little efficacy to date.

Keywords

DNA vaccine; prostate cancer; PD-1; PD-L1; SSX2

*To whom correspondence should be addressed: 7007 Wisconsin Institutes for Medical Research, 1111 Highland Avenue, Madison, WI 53705. Tel: (608) 265-8131 Fax: (608) 265-0614 dm3@medicine.wisc.edu.

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Introduction

Prostate cancer is the most commonly diagnosed cancer in the United States and the second leading cause of cancer-related death in American men (1). Despite primary therapy with prostatectomy and/or radiation therapy, approximately 1/3 of tumors will recur and can ultimately develop into castration-resistant, metastatic disease, the lethal form of prostate cancer (2,3). In 2010, sipuleucel-T (Provenge®, Dendreon Corp.), an autologous cellular vaccine targeting prostatic acid phosphatase (PAP), was approved by FDA for the treatment of patients with metastatic prostate cancer based on trials demonstrating an improved overall survival following treatment, underscoring the potential for antigen-specific vaccines to impact the clinical care of patients with advanced prostate cancer (4). Similarly, encouraging results observed in randomized phase II trials using PROSTVAC® (rilimogene galvacirepvec/glafolivec, Bavarian Nordic), a viral based vaccine targeting prostate-specific antigen (PSA), has renewed interest in the development of other antigen-specific immunotherapies for the treatment of prostate cancer and other malignancies (5). In fact, due to these and many other recent successes in the cancer immunotherapy field, including clinical results observed from T-cell checkpoint molecule blockade (PD-1, CTLA-4, etc.), the journal *Science* named cancer immunotherapy as its “Breakthrough of the Year” for 2013 (6).

We have focused on DNA vaccines as an approach for the treatment of patients with recurrent prostate cancer. We have completed clinical trials evaluating the safety and administration schedule of a DNA vaccine encoding PAP, and a randomized phase II trial is currently ongoing (7,8). However, despite being shown to be safe across many phase I clinical trials, and despite demonstrable efficacy as a treatment for diseases in other animals (including dogs, horses, and fish), no other human DNA vaccines for the treatment of cancer have progressed beyond phase I trials (9–12). As such, much effort has been devoted to better understanding of the mechanisms of action of DNA vaccines and exploring methods to enhance their immunogenicity and possible clinical effectiveness.

One such method that has been extensively studied is the encoding of altered peptide ligands (APL) with point mutations in the presented epitopes to enhance their binding affinity for the major histocompatibility complex (MHC) and/or the T-cell receptor (TCR) (13,14). These types of modifications have been shown to increase the immunogenicity of both peptide and DNA vaccines targeting different viral and tumor antigens that were otherwise weakly immunogenic (15–18). One vaccine encoding an APL currently in clinical trials is PROSTVAC®, the vaccinia- and fowlpox-based vaccine encoding PSA described above (19). In preclinical development of this vaccine it was observed that a native HLA-A2-restricted PSA epitope was weakly immunogenic and that its immunogenicity could be enhanced when encoding an APL with enhanced MHC binding affinity (20,21). We have studied synovial sarcoma X breakpoint 2 (SSX2) as a prostate tumor antigen, and have demonstrated that a DNA vaccine encoding SSX2 was able to elicit HLA-A2-restricted $CD8^+$ T cells with cytolytic activity (22,23). Recently, we identified that point mutations made to these epitopes could be used to immunize HLA-A2-expressing mice to elicit higher frequency of $CD8^+$ T cells that recognized the native epitopes (24). Furthermore, a DNA vaccine encoding these optimized epitopes (pTVG-$SSX2^{opt}$) was able to elicit a greater

frequency of antigen-specific multifunctional $CD8^+$ T cells that were better able to lyse both peptide-pulsed target cells and the SSX2-expressing LNCaP prostate cancer cell line *in vitro*.

In the current study we examined the *in vivo* antitumor efficacy of this optimized DNA vaccine using a novel murine syngeneic tumor cell line model developed in HLA-A2-expressing mice. We found that the optimized DNA vaccine elicited an *inferior* antitumor response relative to the native vaccine not encoding the APLs. We demonstrated that this inferior response was associated with increased expression of the immune regulatory molecule Programmed Cell Death-1 (PD-1) on antigen-specific $CD8^+$ T cells elicited by the optimized vaccine, and that the inferior antitumor response could be rescued by using PD-1 or PD-L1 blocking antibodies in combination with vaccination, or by targeting a PD-L1-deficient tumor. These findings demonstrate that efforts to improve vaccine efficacy by encoding altered peptide ligands might actually have a deleterious effect by leading to higher PD-1 expression. Moreover, these findings suggest that combining PD-1 pathway blockade with vaccines, and DNA vaccines in particular to augment antigen-specific $CD8^+$ T cells, is a rational approach for future human clinical trials.

Materials and Methods

Mice and Cell Lines

HLA-A2.01/HLA-DR1-expressing, murine MHC class I/II knockout mice (HHDII-DR1) on a C57Bl/6 background were obtained from Charles River Labs (France) courtesy of Dr. François Lemonnier (25). Mice were maintained under aseptic conditions and all experiments were conducted under an IACUC-approved protocol.

To generate the A2/Sarcoma cell line, HHDII-DR1 mice were injected subcutaneously with 0.5 mg 3-methylcholanthrene (MCA, Sigma-Aldrich, St. Louis, MO). Once tumors became palpable, tumors were collected, minced, and cultured in DMEM/High Glucose medium (Mediatech, Manassas, VA). A2/Sarcoma cells were then stably transfected with lentiviral constructs encoding either green fluorescent protein (GFP) or SSX2. Expression was confirmed by immunoblotting using an SSX2-specific monoclonal antibody (Clone 1A4, Abnova, Walnut, CA) or for GFP and MHC expression by flow cytometry.

To generate the A2/Sarcoma-SSX2-ΔPD-L1 line, we utilized the CRISPR-Cas genomic editing system by transfecting the A2/Sarcoma-SSX2 cells with a CRISPR U6-gRNA/CMB-Cas9-GFP plasmid (gRNA Sequence: TTTACTATCACGGCTCCAA, Sigma, St. Louis, MO), and cells were sorted using a FACS Aria sorter for live/singlet/GFP^+ cells. A line incapable of expressing PD-L1 in response to IFNγ was isolated, and genomic DNA from this line was collected and sequenced to confirm the PD-L1 deletion mutation (data not shown).

DNA Constructs

DNA vaccines encoding native or modified SSX2 were purified and used as previously described (22,24). pTVG-$SSX2^{opt}$ was previously described as pTVG-SSX2 p41-AL/p103-RF, and pTVG-$SSX2^{KO}$ was previously described as pTVG-SSX2 p41-VP/p103-IP.

PD-1/PD-L1 Antibodies

Antibodies for mouse PD-1 (G4) and PD-L1 (10B5), both gracious gifts from Dr. Lieping Chen, were purified from Armenian Hamster hybridoma lines using a HiTrap Protein G column (GE Healthcare, United Kingdom) following previously published methods (26–28).

DNA and Peptide Immunization Studies

6–8 week old HHDII-DR1 mice were immunized with plasmid DNA or peptides as we have previously reported (22,24). For tumor protection studies, HHDII-DR1 mice were immunized intradermally six times biweekly with 100 μg of native or modified SSX2 vaccines followed two weeks later by subcutaneous inoculation with 2×10^4 SSX2- or GFP-expressing sarcoma cells in contralateral flanks. Tumor-cell suspensions were prepared in 50% High Concentration, LDEV-Free Matrigel (BD Biosciences, San Jose, CA). Tumor volume was measured in cubic centimeters according to the following formula: $(\pi/6)(\text{long axis})(\text{short axis})^2$. For tumor therapy studies, animals were first inoculated with tumor cells followed by weekly vaccination beginning one day after tumor implantation. In tumor studies using PD-1/PD-L1 blocking antibodies, 100 μg of antibody (or IgG isotype control, BioLegend, San Diego, CA) was injected intraperitoneally on the day following each vaccination.

Flow Cytometry Analyses

Tumors obtained at necropsy were digested in media containing 1 mg/mL collagenase and 20 μg/mL DNAse I (Sigma) for 2 hours at 37°C, and passed through a 100 μm screen to obtain a single-cell suspension. $CD8^+$ T cells were isolated (STEMCELL Technologies, Vancouver, BC Canada) and then stained with anti-CD3 (17A2, eBioscience), anti-CD8 (53-6.7, eBioscience) and anti-CD69 (H1.2F3, eBioscience) antibodies and DAPI. For PD-L1 quantification, tumor suspensions were stained with anti-CD45 (30-F11, BD Bioscience), anti-CD11b (M1/70, eBioscience), anti-GR1 (1A8, BD Bioscience), anti-F4/80 (BM8.1, Tonbo Biosciences), anti-PD-L1 (MIH5, eBioscience) and DAPI.

For PD-1 quantification on antigen-specific T cells, splenocytes obtained from immunized animals were enriched for $CD8^+$ T cells and stained as above, along with tetramers specific for the SSX2 p41 or p103 epitopes (NIH Tetramer Core Facility, Atlanta GA), anti-PD-1 (J43, BD Bioscience, San Jose CA) and Ghost Dye–780 (Tonbo Bioscience, San Diego, CA).

For *in vitro* culture studies, cells were treated for 18 hours with 1 μg/mL recombinant mIFNγ (Shenandoah Biotechnology, Warwick PA), or cultured for 48 hours with $CD8^+$ splenocytes isolated from immunized animals, and then collected using non-enzymatic cell dissociation solution (Sigma), and stained as above.

Immunofluorescence (IF) Staining

Tumors obtained at necropsy were embedded in FSC 22 Frozen Section Media (Leica Biosystems), frozen, and cut in 10 μm sections. Sections were acetone fixed and stained for PD-L1 (10F.9G2, BioLegend, and eFluor 570 anti-Rat IgG secondary, eBioscience). 20X images were obtained with an Olympus BX51 Fluorescent Microscope. For PD-1 and CD8

IF staining, FFPE tumor sections were stained using standard immunofluorescence methods with 10 μg/mL anti-CD8 (53-6.7, R&D Systems) and 5 μg/mL anti-PD-1 (ab117420, Abcam), followed by AlexaFluor-488 anti-rat IgG and AlexaFluor-647 anti-goat IgG (Life Technologies). Images were taken on a Nikon Ti microscope and analyzed using ImageJ software (NIH). Images of whole tumor sections were collected and assembled using the Nikon Elements large-image stitching function.

Results

Immunization of SSX2 tumor-bearing mice with an optimized DNA vaccine elicits inferior antitumor response than the native vaccine

We previously reported that animals immunized with a DNA vaccine encoding SSX2 with APL optimized for MHC-I binding (pTVG-SSX2^{opt}) had a greater number of Th1-biased multifunctional, SSX2-specific CD8$^+$ T cells than animals immunized with the native construct (24). Therefore, we hypothesized that pTVG-SSX2^{opt} would be able to elicit a greater antitumor response *in vivo* than the native vaccine. Consequently, we generated a sarcoma cell line from HHDII-DR1 mice that was stably transduced to express either SSX2 or GFP. Expression of SSX2 or GFP, and HLA-A2 (and lack of murine H-2K^b expression), were confirmed by western blot and flow cytometry analysis (Supplementary Figure 1). HHDII-DR1 mice were then immunized six times biweekly with either pTVG4 (vector control), pTVG-SSX2 (encoding native SSX2), pTVG-SSX2^{opt} (encoding two amino acid substitutions to increase the binding of each epitope to HLA-A2), or pTVG-SSX2^{KO} (encoding two amino acid substitutions to ablate the binding of each epitope to HLA-A2). Two weeks after the final vaccination, animals were implanted with tumors expressing either SSX2 or GFP in contralateral flanks. As shown, animals immunized with the native or optimized plasmids had SSX2-expressing tumors that were significantly smaller than those that received the control or knockout plasmids (Figure 1A).

In the next studies, animals were first inoculated with the sarcoma cells followed by weekly vaccination. Again in these studies, tumors in animals immunized with the native and optimized plasmids were significantly smaller than tumors in animals immunized with the control or epitope knockout constructs. These studies confirmed that the antitumor response was mediated by HLA-A2-restricted CD8$^+$ T cells, as the ablation of the HLA-A2 epitopes abolished the antitumor effect observed. However, in these studies the tumors from animals receiving the optimized vaccine were significantly larger than tumors from animals receiving the native vaccine (Figure 1B). Similar results were observed in five independent experiments. In all of these studies, no significant differences were observed for growth of GFP-expressing tumors among the different vaccine constructs, confirming that the antitumor responses observed were antigen-specific.

To assess if differences in tumor-infiltrating T cells (TIL) were responsible for the observed inferior antitumor response elicited by the optimized vaccine, tumor-infiltrating cells were analyzed for evidence of activation. As shown in Figure 1C, while CD69 expression was generally higher on TILs from tumors expressing SSX2, CD8$^+$ TILs from animals that received the native vaccine had higher levels of CD69 expression compared to animals receiving either control or optimized vaccines.

Immunization elicited CD8+ T cells that increased PD-L1 expression on murine tumor cells

The observed inferior *in vivo* antitumor activity, and lower number of activated infiltrating $CD8^+$ T cells, elicited from the APL-optimized vaccine was unexpected given our previous report in which the optimized vaccine was able to elicit T cells with greater Th1 cytokine release and greater cytolytic activity against HLA-$A2^+$ tumor cells *in vitro* (24). Others have shown that IFNγ can lead to an upregulation of PD-L1 on tumor cells (29,30), so we hypothesized that immunization with pTVG-$SSX2^{opt}$ might have elicited higher PD-L1 expression on tumors due to an increased number of antigen-specific IFNγ-secreting $CD8^+$ T cells. As shown in Figure 2, tumors from animals receiving either the native or optimized SSX2 vaccine had higher expression of PD-L1 than tumors from animals receiving the control vaccine, as demonstrated by flow cytometry (Figures 2A and B) or immunofluorescence staining (Figure 2C). However, there was no statistically significant difference in tumor PD-L1 expression between tumors from native and optimized vaccine-treated animals. This upregulation of PD-L1 was likely a result of IFNγ secretion by vaccine-elicited T cells, given that A2/Sarcoma cells were found to have increased expression of PD-L1 following *in vitro* culture with recombinant IFNγ (Figure 2D). Moreover, this was demonstrated to be mediated by antigen-specific cells, given that culture of $CD8^+$ T cells isolated from splenocytes from HHDII-DR1 mice immunized with pTVG-SSX2 versus control-immunized mice led to increased PD-L1 expression on SSX2-expressing tumor cells *in vitro*, but not on control tumor cells not expressing SSX2 (Figure 2E).

Antitumor activity of the optimized SSX2 vaccine is recovered when targeting a tumor line not expressing PD-L1

To test if the tumor expression of PD-L1 in response to vaccination was responsible for the observed inferior antitumor activity of the optimized SSX2 vaccine, we used the CRISPR/Cas genomic editing system to generate an A2/Sarcoma-SSX2 cell line incapable of expressing PD-L1 in response to IFNγ (Figure 3A). This ΔPD-L1 tumor cell line was then implanted, as described before, and animals were immunized weekly with either the control, native, or optimized SSX2 vectors. As shown in Figure 3B, immunization with the optimized vaccine elicited an identical, or even slightly better (albeit not statistically significantly), antitumor response compared with the native vaccine. In comparison with the previous findings (Figure 1B), this suggested that tumor PD-L1 expression was at least partially responsible for the inferior antitumor response observed with the optimized vaccine. As shown in Figure 3C, while the tumor cells did not express PD-L1 (as expected), both tumor-infiltrating myeloid-derived suppressor cells (MDSC) and macrophages expressed detectable levels of PD-L1, suggesting that even in these PD-L1-deficient tumors, the PD-1/PD-L1 regulatory axis might still be involved in repressing antitumor responses, as these tumors were not eradicated following immunization.

Antigen-specific CD8+ T cells express higher levels of PD-1 in animals that received the optimized SSX2 vaccine

Given that the expression of PD-L1 did not appear different between treatment groups receiving the native or optimized vaccine (Figure 2), and yet the expression of PD-L1 was

responsible for the decreased antitumor effect observed with the optimized vaccine (Figure 3), we next assessed whether the expression of PD-1 on $CD8^+$ TILs differed with respect to immunization. As shown (Figure 4A), tumors from animals immunized as in Figure 1B had detectable levels of $CD8^+$ TILs, and these cells expressed detectable levels of PD-1. The expression of PD-1 on antigen-specific $CD8^+$ T cells was specifically evaluated on peripheral $CD8^+$ T cells elicited following vaccination of tumor-bearing mice with either the native or optimized plasmids. As shown in Figure 4B, PD-1 expression was significantly higher on p41- and p103-tetramer$^+$ $CD8^+$ T cells following immunization with the optimized construct. This upregulation of PD-1 was found to be independent of the presence of tumor, as non-tumor-bearing HHDII-DR1 mice immunized with either the native or the optimized SSX2 DNA vaccines (or the corresponding native or optimized p41 and p103 peptides alone) demonstrated elevated PD-1 expression on antigen-specific $CD8^+$ T cells (Figure 4C). This finding, taken together with the observation that both native and optimized vaccines induced similar levels of PD-L1 expression on tumors, and the observation of increased antitumor activity of the optimized vaccine when targeting a ΔPD-L1 tumor, suggested that the increased expression of PD-1 was most likely responsible for the decreased antitumor effect following vaccination with the optimized vaccine. Presumably ligation of PD-L1 expressed by tumors led to a decrease in effector function, as has been demonstrated by others (31).

Antibody blockade of PD-1 or PD-L1 increases the antitumor activity of DNA immunization

We had previously demonstrated that antigen-specific $CD8^+$ T cells elicited by immunization with these APLs, or with DNA vaccines encoding these APLs, had greater cytolytic activity *in vitro* (24). However the HLA-$A2^+$ tumor cell target lines used for these analyses (T2 and LNCaP) are human, and do not express PD-L1 following IFNγ stimulation (Supplementary Figure 2). This observation, plus the findings in both Figures 3 and 4, suggested that blocking the ligation of PD-1 on antigen-specific $CD8^+$ T cells might restore or enhance the antitumor efficacy of the APL-encoding DNA vaccine, particularly given the persistence of PD-L1 expression in the tumor microenvironment demonstrated in Figure 3C. To test this, mice were implanted with SSX2-expressing tumor cells, followed by weekly administration of either the control, native or optimized SSX2 vaccines. Mice then received 100 μg of PD-1 or PD-L1 blocking antibody, or isotype control, 24 hours following vaccination. As before, the native vaccine elicited greater antitumor activity compared with pTVG-$SSX2^{opt}$ (Figure 5). However, treatment with the combination of pTVG-$SSX2^{opt}$ and antibodies against either PD-1 or PD-L1 elicited similar antitumor activity to pTVG-SSX2, suggesting that the antitumor activity elicited by pTVG-$SSX2^{opt}$ could be rescued by blockade of either PD-1 or PD-L1. While not statistically different from controls, the frequency of $CD8^+$ TILs was generally higher in animals receiving the native vaccine or optimized vaccine with anti-PD-1, and the expression of PD-1 on activated $CD8^+$ TILs was highest in animals receiving the combined treatment (Supplemental Figure 3). Furthermore, we observed complete tumor eradication in several animals that received pTVG-$SSX2^{opt}$ in combination with anti-PD-1 antibody, suggesting that the combination therapy may be more effective than vaccination alone (Figure 5A).

Discussion

Modifying vaccines to encode altered peptide ligands to enhance epitope binding to the MHC/TCR complex is a method that has been explored as a means of increasing the immunogenicity of vaccines targeting various tumor and viral antigens that are otherwise weakly immunogenic. We recently reported one such epitope-modified vaccine, a DNA vaccine encoding SSX2, that was able to elicit a greater frequency of antigen-specific multifunctional T cells with greater *in vitro* cytolytic activity (24). In this report we sought to identify whether this vaccine encoding modified epitopes was able to elicit a stronger antitumor response against an SSX2-expressing tumor cell line *in vivo*. We demonstrated that this modified vaccine elicited an inferior antitumor response relative to the native vaccine. We found that this was associated with increased PD-1 expression on antigen-specific $CD8^+$ T cells elicited from the optimized vaccine relative to those elicited by the native vaccine, and that immunization with either construct upregulated expression of PD-L1 on antigen-expressing tumors. Finally, we found that the antitumor activity of the optimized vaccine could be increased either by targeting a tumor incapable of expressing PD-L1 or when combined with PD-1 pathway blockade. PD-1 blockade alone had no substantial antitumor activity.

Our results demonstrate that an attempt to enhance the antitumor efficacy of a DNA vaccine by encoding APLs was actually counter-productive, and this was due to increased PD-1 expression on the antigen-specific $CD8^+$ T cells elicited. This is different from reports demonstrating that APLs can be an effective means to increase the efficacy of antitumor and antiviral vaccines, including one DNA vaccine (32,33). However, it is unclear if in these other models PD-1 was similarly upregulated. Moreover, the expression of PD-1 on $CD8^+$ T cells may be of less relevance in circumstances in which the targets of these $CD8^+$ T cells do not express a PD-1 ligand, conceivably this is the case in different tumor and viral antigen systems, and probably the reason we observed an enhanced cytolytic activity *in vitro* from splenocytes obtained from non-tumor-bearing animals immunized with this vaccine (24). Our findings, notably in Figure 4B, are consistent with a recent report demonstrating that T cells stimulated *in vitro* with peptides of varying affinity can lead to different levels of PD-1 expression (34). The precise relationship between epitope-binding affinity and PD-1 expression remains unknown and is a future direction of our research. However, these findings suggest that other methods could be explored to increase the efficacy of anti-cancer DNA vaccines. In a report from Smith and colleagues, the authors demonstrated that epitopes with slightly weakened binding affinity led to lower levels of PD-1 expression, suggesting that modifications to DNA vaccines that decrease epitope-binding affinity, while simultaneously increasing epitope presentation, might be an approach to limit PD-1 expression and increase the antitumor efficacy of DNA vaccines (34).

We also observed in this tumor model that PD-L1 expression levels increased following administration of an antigen-specific DNA vaccine. These findings are similar to those recently reported by Fu and colleagues in which administration of a cellular vaccine was found to upregulate PD-L1 expression on tumors following vaccination (30). In their study, the mechanism for this could not be precisely established due to the absence of a defined antigenic target. We found that this upregulation of PD-L1 was due, at least in part, to

antigen-specific $CD8^+$ T cells elicited with DNA vaccination, as PD-L1 upregulation could be replicated *in vitro* by culturing the tumor cell line expressing the antigen in the presence of antigen-specific $CD8^+$ T cells from immunized animals. These findings have implications for the broader tumor immunology field, suggesting that while antitumor vaccines have potential efficacy in augmenting tumor-specific cytolytic $CD8^+$ T cells, they may concurrently augment counterproductive regulatory ligands present in the tumor microenvironment. This is supported further by our findings that the optimized vaccine had an enhanced antitumor response when targeting tumors engineered to not express PD-L1. At this point, it is not known whether upregulation of the PD-1/PD-L1 axis is specific to genetic vaccines as the method of immunization. This possibility is suggested by the slightly higher, albeit not statistically significantly higher, PD-1 expression observed on $CD8^+$ T cells following DNA immunization compared with direct peptide immunization (Figure 3B). Given that DNA vaccines elicit a Th1-biased response, and $CD8^+$ T-cell responses in particular, it seems likely that this could be a major mechanism of regulation, however more studies are needed to confirm this.

Our findings are also of potential relevance to the broader tumor immunology field given the recent FDA approval of Keytruda® (Pembrolizumab, Merck), and Opdivo® (Nivolumab, BMS), two anti-PD-1 monoclonal antibodies for the treatment of ipilimumab-refractory melanoma, and pembrolizumab for the treatment of non-small cell lung cancer. Several studies have focused on the identification of predictive biomarkers to identify patients likely to respond to anti-PD-1 monotherapy, and the best characterized is tumor expression of PD-L1 (35,36). Our data are consistent with this observed phenomenon – namely that subjects with PD-L1 expression on tumor cells are likely those with pre-existing populations of tumor antigen-specific $CD8^+$ T cells that can secrete IFNγ in the tumor microenvironment, leading to PD-L1 upregulation. Patients without PD-L1 tumor expression, conversely, might not have sufficient populations of tumor antigen-specific $CD8^+$ T cells. Thus, efforts to increase the frequency of tumor antigen-specific $CD8^+$ T cells should be of key importance to increase the efficacy of agents targeting the PD-1/PD-L1 axis, and our results suggest this may be feasible by the use of tumor-targeted antigen-specific vaccines.

Our data are also potentially relevant to the tumor immunology field given a recent surge in attention towards developing personalized tumor antigen-specific vaccines based on the identification of mutated tumor-specific epitopes (37). This method generally proposes to sequence individual tumor exomes to identify tumor-specific, MHC-restricted epitopes based on the presence of mutations leading to novel epitopes. $CD8^+$ T cells specific for such epitopes should escape thymic tolerance, however our results suggest high-affinity T cells might similarly be regulated by the PD-1/PD-ligand pathway. This could, in fact, help explain why checkpoint inhibition has been successful in tumor systems with higher frequencies of tumor-specific mutations (38).

Together, our findings provide a clear rationale for pursuing clinical trials combining cancer vaccines, and DNA vaccines in particular, with PD-1 blockade. In our model, we found that PD-1 blockade alone had little antitumor effect, but we observed a marked increase in antitumor activity when combined with a DNA vaccine that is able to elicit tumor antigen-specific $CD8^+$ T cells. Interestingly we also observed that the optimized vaccine elicited

complete responses when combined with PD-1 blockade, but not when targeting ΔPD-L1 tumors, suggesting that PD-L1 expression by both tumor cells and infiltrating immune cells play a role in regulating antitumor immune responses. The role of these PD-L1-expressing hematopoietic cells in regulating antitumor immunity in the tumor microenvironment will be another area of future research. However, these findings are of particular relevance to the treatment of advanced, metastatic prostate cancer given that anti-PD-1 monotherapy has shown relatively little success in early phase clinical trials (35,39), whereas vaccines have already demonstrated clinical benefit (4,5). This may be an ideal clinical setting in which to evaluate treatments combining antitumor vaccination to elicit tumor-specific $CD8^+$ T cells in combination with PD-1 blockade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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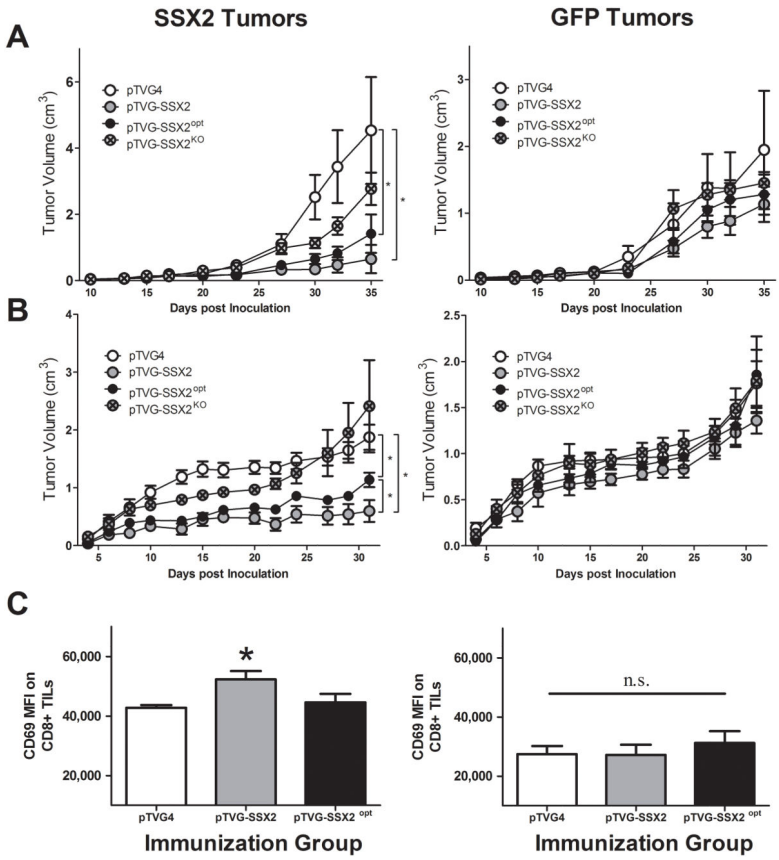

Figure 1. Immunization of SSX2 tumor-bearing mice with an optimized DNA vaccine elicits inferior antitumor response than the native vaccine

HHDII-DR1 mice (n=6 per group) were immunized six times biweekly (panel A) or weekly (panel B) with pTVG4, pTVG-SSX2, pTVG-SSX2^{opt}, or pTVG-SSX2^{KO} either prior to (panel A) or one day after (panel B) subcutaneous inoculation with SSX2- or GFP-expressing sarcoma cells in contralateral flanks. Tumor growth was measured as indicated. Results are representative of two (panel A) or five (panel B) independent studies. Panel C: Tumor-infiltrating CD8$^+$ T cells were assessed for expression of CD69. Shown is the mean fluorescence intensity (MFI) for CD69 on CD8$^+$ TIL cells from SSX2-expressing (left panel) or GFP-expressing tumors (right panel, n=6 animals per group). Error bars denote standard error. * indicates a p value < 0.05 using a Mann-Whitney test for Panels A and B, and a one-way ANOVA followed by a *post hoc* protected Fischer's LSD test for Panel C.

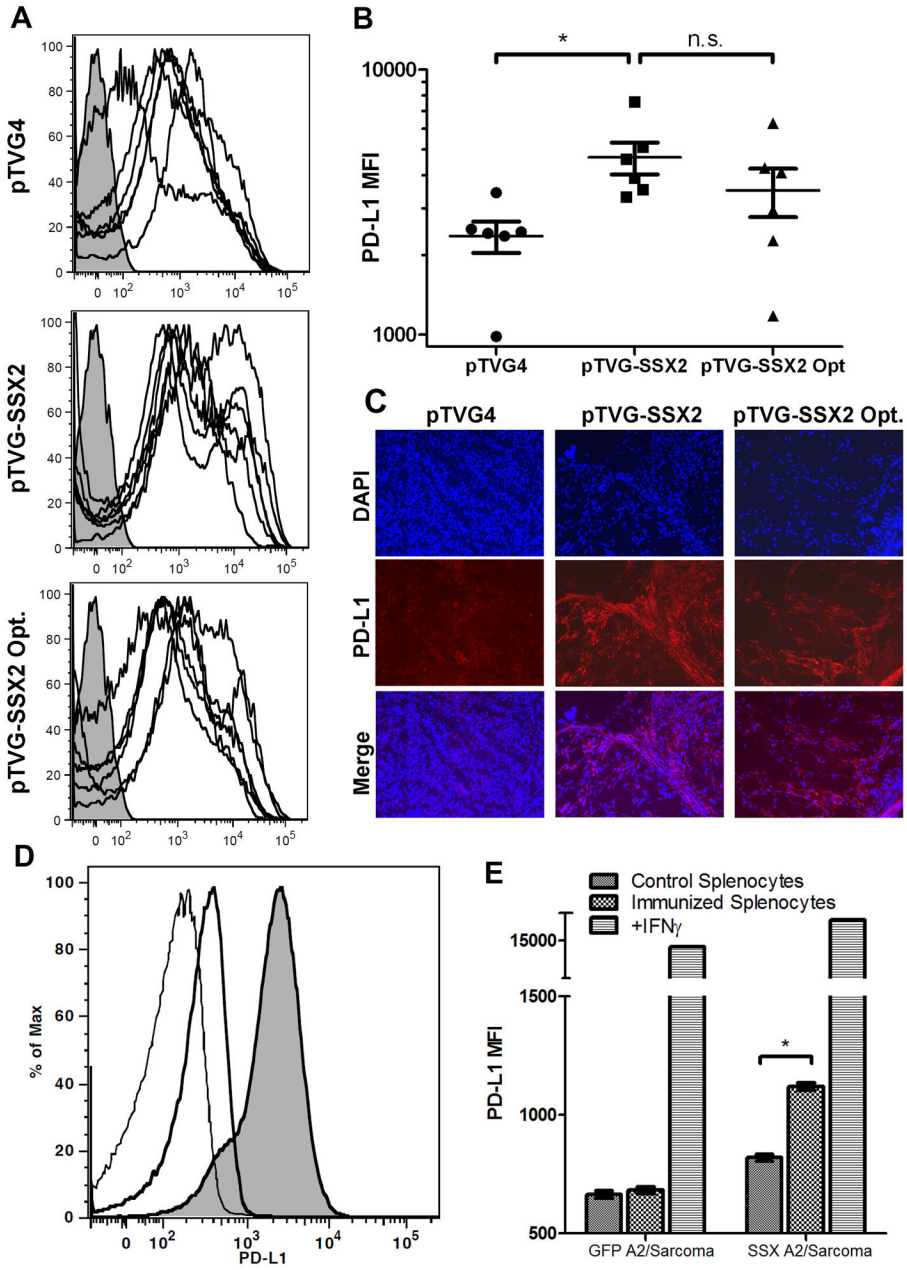

Figure 2. Immunization elicited CD8$^+$ T cells that increased PD-L1 expression on murine tumor cells

Panels A–C: SSX2-expressing tumors were collected at necropsy from HHDII-DR1 mice (n=6 per group), and analyzed for PD-L1 expression by flow cytometry (panel A) or immunofluorescence imaging (panel C). Panel B shows average PD-L1 MFI values from histograms in Panel A.

Panel D: A2/Sarcoma cells were cultured for 18 hours in the presence of 1 μg/mL IFNγ and stained for PD-L1 expression. Shown are histograms for PD-L1 expression on live stimulated (shaded) and unstimulated (dark line) cells, or isotype control (light line).

Panel E: SSX2- or GFP-expressing A2/Sarcoma cells were cultured for 36 hours in the presence of $CD8^+$ cells isolated from splenocytes from HHDII-DR1 mice previously immunized with either pTVG4 or pTVG-SSX2, or cultured in the presence of IFNγ, and evaluated for PD-L1 expression by flow cytometry. Results are representative of two independent experiments. For all panels, * indicates a p value < 0.05 using a Mann-Whitney test.

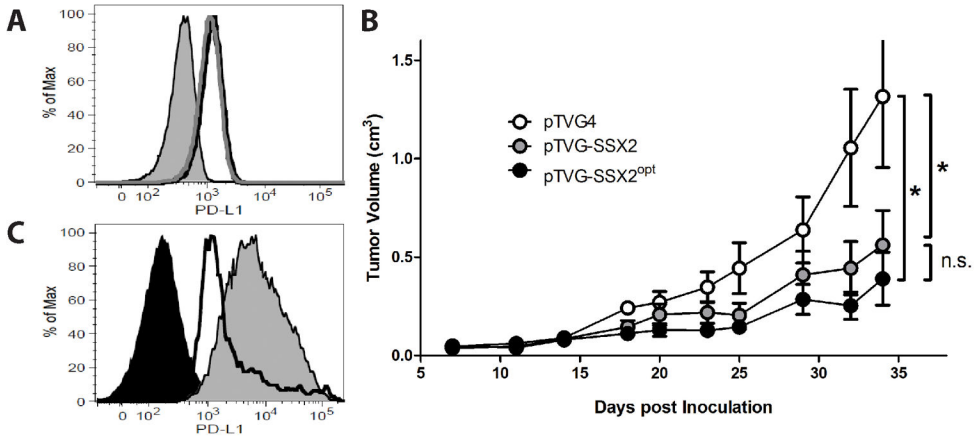

Figure 3. Antitumor activity of the optimized SSX2 vaccine is recovered when targeting a tumor line not expressing PD-L1

Panel A: SSX2-expressing sarcoma cells modified to delete the PD-L1 gene were untreated (gray line), or cultured in the presence of recombinant IFNγ (black line), and evaluated for PD-L1 expression (or IgG isotype control, solid gray) by flow cytometry. Panel B: HHDII-DR1 mice (n=6 per group) were implanted with ΔPD-L1 A2/Sarcoma-SSX2 tumors, followed by weekly vaccinations with either pTVG4, pTVG-SSX2, or pTVG-SSX2^{opt}. Groups were compared using a one-way ANOVA followed by a *post hoc* protected Fisher's LSD test (* indicates a p value < 0.05). Panel C: Tumors from panel B were collected at necropsy, digested, and analyzed for the expression of PD-L1 on various cell subsets. Shown are histograms for tumor cells (solid black, CD45^{-}), MDSCs (black line, CD45^{+}/CD11b^{+}/GR1^{+}), and macrophages (solid gray, CD45^{+}/CD11b^{+}/F4/80^{+}) from a representative animal.

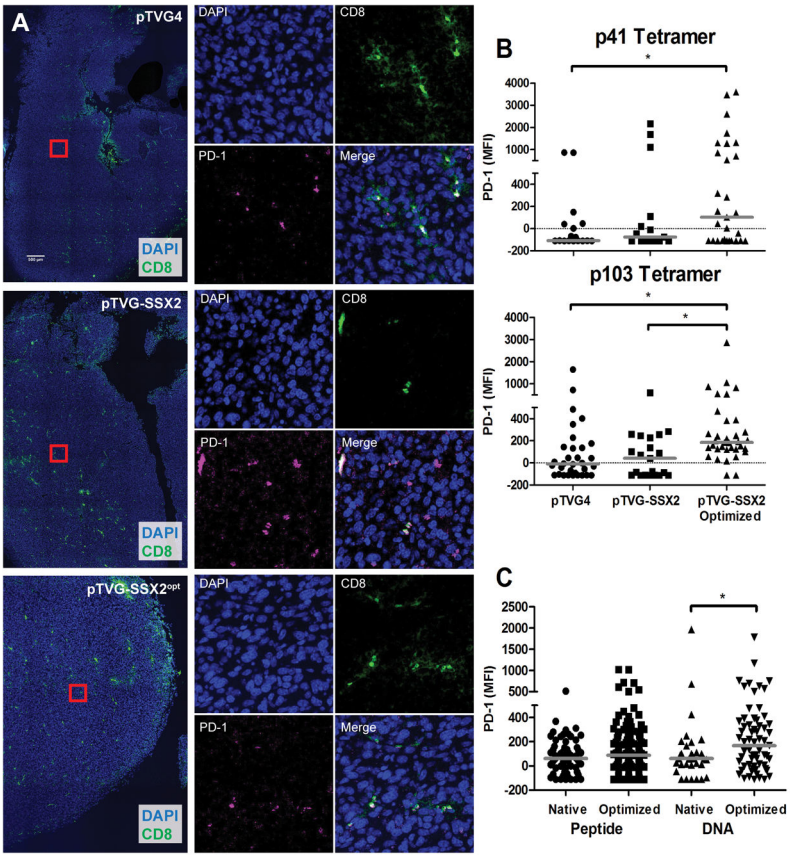

Figure 4. Antigen-specific CD8$^+$ T cells express higher levels of PD-1 in animals that received the optimized SSX2 vaccine

Panel A: FFPE tumor sections obtained at necropsy were stained for CD8 and PD-1 expression. Shown are both the whole tumor sections (10x, assembled using Nikon Elements software) and higher-powered images (20x) from representative animals from each treatment group. For the assembled tumor sections, the scale bar in the upper-left image indicates a length of 500 μm, and the red boxes indicate the regions where the higher-power images were taken. Panels B and C: CD8$^+$ T cells were isolated from splenocytes from tumor-bearing (panel B) or tumor-free (panel C) HHDII-DR1 mice that had received the indicated peptide or DNA vaccine treatment, and stained for SSX2 p41 or p103 with tetramers (separated in panel B, pooled in panel C) and for PD-1 expression. Shown are the MFI values for PD-1 expression on individual live/CD8$^+$/tetramer$^+$ events, with the median represented by the solid gray line. Each test represents splenocytes from at least three animals per group pooled together, and * indicates a p value < 0.05 using a Mann-Whitney test.

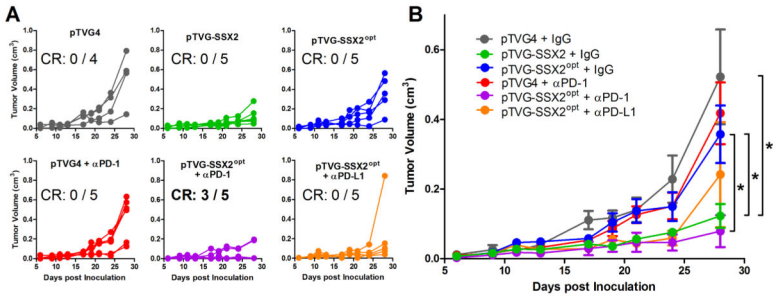

Figure 5. Antibody blockade of PD-1 or PD-L1 increases the antitumor activity of DNA immunization

HHDII-DR1 animals (n=4–6 per group) were subcutaneously implanted with SSX2-expressing sarcoma cells followed by weekly immunizations with pTVG4, pTVG-SSX2, pTVG-SSX2^{opt}. On the day after each immunization, all animals were treated with 100 μg of antibodies blocking PD-1, PD-L1, or with IgG isotype control (as indicated by the graphs). Animals were subsequently monitored for tumor growth. Shown are individual animal tumor growth curves (panel A) and group means with standard error (panel B). Results are representative of three independent studies. On graphs in panel A, the number of animals with a complete tumor response (CR) is noted. Error bars denote standard error and * indicates a p value < 0.05 by Mann-Whitney test.