Published in final edited form as:

J Immunother. 2011 October ; 34(8): 569–580. doi:10.1097/CJI.0b013e31822b5b1d.

Vaccines Targeting the Cancer Testis Antigen SSX-2 Elicit HLA-A2 Epitope-Specific Cytolytic T Cells

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Abstract

The cancer-testis antigen SSX-2 is a potentially attractive target for tumor immunotherapy based upon its tissue-restricted expression to germline cells and its frequent expression in malignancies. The goal of the current study was to evaluate a genetic vaccine encoding SSX-2 to prioritize HLA-A2-specific epitopes and determine if a DNA vaccine can elicit SSX-2-specific cytolytic T lymphocytes (CTL) capable of lysing prostate cancer cells. HLA-A2-restricted epitopes were identified based on their *in vitro* binding affinity for HLA-A2 and by the ability of a genetic vaccine to elicit peptide-specific CTL in A2/DR1 (HLA-A2.1+/HLA-DR1+/H-2 class I-/class II-knockout) transgenic mice. We found that SSX-2 peptides p41-49 (KASEKIFYV) and p103-111 (RLQGISPKI) had high affinity for HLA-A2 and were immunogenic *in vivo*, however peptide p103-111 was immunodominant with robust peptide-specific immune responses elicited in mice vaccinated with a plasmid DNA vaccine encoding SSX-2. Furthermore, p103-111-specific CTL were able to lyse an HLA-A2+ prostate cancer cell line. The immunodominance of this epitope was found not to be due to a putative HLA-DR1 epitope (p98-112) flanking p103-111. Finally, we demonstrated that SSX-2 epitope-specific CTL could be detected and cultured from the peripheral blood of HLA-A2+ prostate cancer patients, notably patients with advanced prostate cancer. Overall, we conclude that SSX-2 peptide p103-111 is an immunodominant HLA-A2-restricted epitope, and epitope-specific CD8+ T cells can be detected in patients with prostate cancer, suggesting that tolerance to SSX-2 can be circumvented *in vivo*. Together, these findings suggest that SSX-2 may be a relevant target antigen for prostate cancer vaccine approaches.

Keywords

SSX-2; CTA; DNA vaccine; Prostate Cancer; CTL; Epitopes; HLA-A2

Introduction

As the most commonly diagnosed and second leading cause of cancer-related death among American men, prostate cancer is a significant health concern with limited treatment options available for advanced stages of disease [1]. Approximately one-third of men diagnosed will ultimately develop recurrent disease after primary surgery or radiation therapy [1, 2]. Tumor immunotherapy has become an increasingly active area of research for recurrent or metastatic prostate cancer [2, 3], and genetic vaccines encoding cDNAs for tumor antigens represent one type of immunotherapeutic vaccine delivery system being evaluated in clinical trials and showing promise [4-9].

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Disclosures: All authors have declared there are no financial conflicts of interest in regards to this work.

Our group is particularly interested in identifying appropriate antigen targets for anti-prostate tumor vaccines, and vaccine strategies that are simple and capable of eliciting antigen-specific cytotoxic T lymphocytes (CTL). DNA plasmid vaccines offer several advantages over other genetic vaccine strategies by providing a safe immunization platform, simple preparation, and inexpensive manufacture, while also specifically targeting only a select antigen(s) or antigenic epitope(s) [10, 11]. Several DNA vaccines have been shown to elicit anti-tumor immune responses in patients with prostate cancer, however, the majority of antigens targeted to date have been tissue-specific and not tumor-specific [5-7, 12]. An important consideration for the development of future vaccines is to identify target antigens that are specific to tumor tissue, thereby circumventing potential cytotoxicity against normal tissues.

Cancer-testis antigens (CTA) are one class of tumor antigen not previously explored to a great extent for prostate cancer immunotherapy. These proteins are defined based upon their tissue-restricted expression to germline cells and their frequent ectopic expression in a variety of tumors [13-16]. The nearly exclusive expression of CTA in germline tissue makes them attractive therapeutic targets because germ cells do not express class I MHC molecules. Moreover, because CTA are expressed in many types of cancer, and especially advanced-stage disease, immunotherapeutic vaccines targeting these antigens may have efficacy for multiple types of malignancy in both a prophylactic and therapeutic setting.

One of the first CTA identified was HOM-MEL-40, or SSX-2, which is known to be expressed in advanced-stage tumors of different histological types [17, 18]. Moreover, SSX-2 mRNA expression has been associated with a worse prognosis in several types of cancer [19]. We have identified this antigen as a potential target for prostate cancer based upon its ectopic expression in advanced prostate cancer, and because IgG responses specific for SSX-2 can be detected in some patients [18]. Additionally, we have shown that SSX-2 expression can be selectively induced in prostate cancer cells upon treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dc) [18]. This may have clinical application because these epigenetic modifying agents are being evaluated as cancer treatments [20]; increased antigen expression with epigenetic modifiers may increase antigen processing and epitope presentation on prostate cancer cells, leading to enhanced recognition by antigen-specific CTL.

To aid in the evaluation of SSX-2 as an immunotherapeutic target, several groups have sought to identify MHC class I-restricted SSX-2 epitopes. The majority of the investigations have utilized reverse immunology approaches culturing T cells from healthy donors or cancer patients with pools of SSX-2 peptides [19, 21-25]. While these studies have successfully identified several HLA-A2-binding peptides, it is not known whether SSX-2 epitopes are elicited as a result of *in vivo* vaccination or whether dominant epitopes exist for this antigen. The current study was conducted to evaluate the potential of a genetic vaccine encoding SSX-2 to elicit epitope-specific immune responses in an *in vivo* mouse model, to more comprehensively compare SSX-2 HLA-A2-restricted T-cell epitopes, determine whether epitope-specific CTL can lyse prostate tumor cells, and evaluate whether these epitope-specific CTL exist within the T-cell repertoire of prostate cancer patients. To identify and characterize SSX-2 epitopes, we used two approaches. The first was a standard approach to determine the HLA-A2 affinity of SSX-2-derived peptides *in vitro*, and secondly we used a genetic vaccine to determine which peptides are processed and presented as epitopes by antigen-presenting cells (APCs). For these vaccine studies we immunized transgenic A2/DR1 mice, which are engineered to express human HLA-A2 and HLA-DR1, but not murine MHC class I and II complexes [26]. We found that a plasmid vaccine encoding SSX-2 could elicit robust epitope-specific CTL capable of lysing prostate cancer cells. Additionally, these same epitope-specific CTL could be detected in patients with

prostate cancer, particularly in patients with advanced disease, suggesting that this antigen is naturally immunogenic. These findings suggest that vaccines targeting SSX-2 might be developed to augment existing immunity, or perhaps in a prophylactic setting to prevent the development of SSX-2+ prostate cancer tumors.

Materials and Methods

Mice

HLA-A2.01/HLA-DR1-expressing, murine MHC class I/II knock-out transgenic (A2/DR1) mice on C57Bl/6 background were obtained from Charles River Labs (France) courtesy of Dr. Francois Lemonnier [27]. Mice were maintained in microisolator cages under aseptic conditions and all experimental procedures were conducted under an IACUC-approved protocol.

Construction, purification, and expression confirmation of SSX-2 DNA vaccine

SSX-2 cDNA was cloned from a testis cDNA library into the pTVG4 immunization vector [28] using standard molecular biology techniques. pTVG4 and pTVG-SSX-2 plasmids were purified using the Endo-free Plasmid Giga Kit for animal studies (Qiagen, Valencia, CA). Expression of SSX-2 was confirmed using transient transfection assays of Cos-7 cells (Effectene transfection reagent, Qiagen). SSX-2 mRNA expression was confirmed by reverse-transcription PCR (One-Step RT-PCR kit, Qiagen), and SSX-2 protein expression from cell lysates was confirmed by Western blot using an SSX-2-specific monoclonal antibody (clone 1A4, Abnova, Walnut, CA).

HLA-A2 T2 Binding Affinity Assays

Seven nonamer peptides derived from the amino acid sequence of SSX-2 and predicted to have affinity for HLA-A2 in the SYFPEITHI and BIMAS prediction algorithms [29, 30] were synthesized, and the purity and identity of each peptide was confirmed by mass spectrometry and gas chromatography (United Biochemical Research, Seattle, WA). T2 binding assays were conducted as previously described [31]. Peptide-HLA-A2 binding was measured as relative mean fluorescent intensity (MFI), normalized to vehicle-pulsed T2 MFI, and all measurements were conducted in triplicate.

Peptide and DNA immunization of A2/DR1 mice

For peptide vaccination experiments, 4-6 week-old A2/DR1 mice were immunized subcutaneously with 100µg peptide in complete Freund's adjuvant (Sigma, St. Louis, MO). Mice were euthanized seven days later and spleens were collected, processed through a mesh screen, and splenocytes were isolated by centrifugation after red blood cell osmotic lysis with ACK lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM EDTA). For DNA vaccination studies, 4-6 week-old A2/DR1 mice were immunized intradermally in the ear pinna with 100µg of either pTVG4 or pTVG-SSX-2 vaccines. Mice were immunized every 14 days. Two weeks after the sixth immunization, mice were euthanized and spleens were collected and processed as described above.

IFN γ ELISPOT Assay

IFN γ ELISPOT was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN), and as previously described [6, 31]. Dried plates were counted with an automated plate reader (Autoimmun Diagnostika). The number of spots was corrected for media alone negative control, and reported as the number of IFN γ spot-forming units (SFU) per 10⁶ splenocytes.

Lactate Dehydrogenase Cytotoxicity Assay

Splenocytes were stimulated for seven days with 2 µg/ml peptide in RPMI 1640 media supplemented with 10% FCS, 50mM HEPES, 1 mM sodium pyruvate, 0.1 µM β-mercaptoethanol. 10U/mL murine interleukin-2 (IL-2) (R&D Systems) was added 24 hours later. Following culture, splenocytes were collected and incubated for four hours with target cells (peptide-pulsed T2 cells, DU145 or LNCaP prostate cancer cells transfected to constitutively express HLA-A2 (gift of Dr. Lawrence Fong), or LNCaP cells previously incubated for 15 minutes with 5 µg/mL anti-human HLA-A2 blocking antibody (BD Biosciences)) at various effector to target ratios. This LNCaP cell line was demonstrated to express HLA-A2 and SSX-2. After incubation, supernatants were collected and levels of lactate dehydrogenase were measured using the CytoTox 96 Non-Radioactive Assay (Promega, Madison, WI). The percentage of peptide-specific lysis was quantified using the following equation:

$$\% \text{Cytotoxicity} = \frac{((\text{Experimental Media Alone}) - (\text{Effector Spontaneous} - \text{Media Alone}) - (\text{Target Spontaneous} - \text{Media Alone}))}{((\text{Target Maximum} - \text{Media Alone}) - (\text{Target Spontaneous} - \text{Media Alone}))}$$

Human T-Cell Culture

Blood was obtained from HLA-A2+ male volunteer blood donors, and from patients with prostate cancer, with IRB-approved, written consent. Peripheral blood mononuclear cells (PBMCs) were prepared by density centrifugation, and cryopreserved in liquid nitrogen until use. CD8+ T cells were enriched from PBMCs by magnetic bead negative selection and were cultured *in vitro* with peptide-loaded and irradiated autologous dendritic cells (DCs) each week for up to 5 weeks in RPMI 1640 media supplemented with 10% human AB serum, 1 mM sodium pyruvate, 0.1 µM β-mercaptoethanol, and 10U/mL human interleukin-2 (IL-2) as described previously [31]. Cells were evaluated for target cell lysis weekly by cytotoxicity assays as described above.

Tetramer Analysis

Patient PBMCs or murine splenocytes were evaluated directly or stimulated *in vitro* for one week with 1 µg/ml of peptides p41-49 or p103-111 prior to staining with APC-labeled tetramers or APC-labeled IgG κ isotype control (BD Pharmingen, San Diego, CA). Lymphocytes were gated on CD3+/CD8+ populations and tetramer-positive staining was defined as the percentage of CD8+/APC+ events among CD3+CD8+ T cells by comparison with a corresponding IgG-APC isotype control gate set to include ≤ 0.05% of CD3+CD8+APC+ events.

RESULTS

Four SSX-2 peptides have affinity for HLA-A2 *in vitro*

To evaluate potential HLA-A2-restricted SSX-2 epitopes, two peptide-binding prediction algorithms were used to scan the 188-amino acid SSX-2 protein sequence for possible HLA-A2*0201-binding peptides [29, 30]. Among the ten highest predicted HLA-A2 binding peptides identified by each algorithm, seven nonamer peptides were shared and chosen for further analysis. T2 binding assays were then carried out to rank the binding of each peptide to HLA-A2, the results of which are shown in Figure 1A. Four peptides (p41-49, p57-65, p99-107, and p103-111) were identified with binding scores comparable to a known influenza A matrix protein HLA-A2 epitope (GILGFVFTL). As shown in Figure 1B, peptides p41-49 and p103-111 were found to have the greatest avidity, stabilizing the surface expression of HLA-A2 at lower concentrations than peptides p57-65 and p99-107.

The identified human peptides were also compared to the corresponding amino acids from two murine SSX orthologues (Ssxa1 and Ssxb1) to determine whether these peptides might represent highly homologous “self” antigens in a murine system and have similar HLA-A2 affinity. As shown in Table 1, the murine homologues were not highly homologous to the human peptides.

HLA-A2 SSX-2 peptide p103-111 represents an epitope presented by prostate cancer cells

To determine whether the four identified HLA-A2-binding peptides can be presented by APCs *in vivo*, immunization studies were carried out in HLA-A2 transgenic A2/DR1 mice [27]. Animals immunized once with one of the four predicted HLA-A2 binding peptides or an influenza A matrix protein peptide were tested for the frequency of peptide-specific immune responses by IFN γ ELISPOT. As shown in Figure 2, approximately half of the p103-111-immunized animals developed high frequency p103-111-specific immune responses ($p = 0.041$), while lower frequency SSX-2 p41-49-specific responses were observed in mice immunized with p41-49 ($p = 0.039$). In multiple vaccination experiments no peptide-specific immune responses to peptides p57-65 or p99-107 were detected. Animals immunized with the influenza peptide developed robust responses that were specific to this peptide but not to the p103-111 or p41-49 peptide. These results demonstrate that SSX-2 peptides p41-49 and p103-111 are immunogenic in this transgenic mouse model.

To evaluate whether these SSX-2 peptides are epitopes presented by prostate cancer cells, splenocytes from peptide-immunized animals were evaluated for lysis of an HLA-A2- and SSX-2-expressing LNCaP cell line. Splenocytes from animals immunized with peptide p41-49 or flu peptide lysed peptide-pulsed target cells but not LNCaP cells (panels A and E), while splenocytes from animals immunized with p57-65 (panel B) or p99-107 (panel C) were not lytic (Figure 3). Peptide p103-111-specific CTL were also capable of lysing LNCaP cells (panel D), and this lysis was abrogated in the presence of an HLA-A2 blocking antibody (data not shown).

As a complementary approach to characterize HLA-A2-restricted epitopes, we evaluated a genetic vaccine encoding SSX-2. We reasoned that a DNA vaccine should elicit both MHC class I and class II restricted responses by direct antigen processing and presentation by host antigen-presenting cells. SSX-2 cDNA was cloned into the pTVG4 immunization vector [28], and transcription and translation from the pTVG-SSX-2 construct was confirmed (Supplementary Figure 1). A2/DR1 mice immunized with pTVG-SSX-2 were found to develop robust ($p=0.0012$) p103-111-specific immune responses detectable directly *ex vivo* (Figure 4A). Additionally, significant p41-49-specific immune responses ($p = 0.022$) could be detected as well; these responses were of lower frequency as *in vitro* stimulation was necessary to detect these responses (Figure 4B). T cells specific for p103-111 could similarly be detected directly by tetramer staining; few p41-49-specific T cells could be detected without *in vitro* stimulation. Epitope-specific T cells were not detected in pTVG4-immunized animals (Figure 4C), and responses to other SSX-2-derived peptides were not detected in either group even following peptide stimulation *in vitro*. These results indicate that peptides p41-49 and p103-111 are naturally-processed, immunogenic SSX-2 epitopes. Splenocytes from DNA-immunized mice were also evaluated for SSX-2 peptide-specific CTL (Figure 5). Mice vaccinated with pTVG-SSX-2 developed p41-49 and p103-111 peptide-specific CTL (Figure 5A) that could also lyse the SSX-2+ LNCaP prostate cancer cell line, but not an HLA-A2-expressing prostate cancer cell line not expressing SSX-2 (DU-145); the CTL lysis was not detectable in the presence of an HLA-A2 blocking antibody (Figure 5B). Splenocytes from animals immunized with the pTVG4 control did not generate SSX-2-specific CTL (5C and 5D).

Identification of an HLA-DR1 15-mer peptide encompassing SSX-2 p103-111

We next questioned why the responses elicited to peptide p103-111 were more robust than peptide p41-49, even though both had similar HLA-A2 avidity *in vitro* and both were being presented from the same DNA construct. We suspected this may be due to the amino acid context surrounding these epitopes. Using a peptide prediction algorithm, we identified a putative class II peptide (p98-112) that encompasses p103-111 and among all potential 15-mer peptides derived from the amino acid sequence of SSX-2 was predicted to have the highest affinity for HLA-DR1 [30]. To evaluate this further, mice were immunized with SSX-2 peptide p98-112 or peptide p103-111 and assessed for peptide-specific immune responses. Animals immunized with peptide p98-112 developed p98-112-specific IFN γ -secreting immune responses that could be blocked with an HLA-DR blocking antibody, but interestingly, no HLA-A2-restricted response was generated. In contrast, all animals immunized with p103-111 developed potent p103-111-specific immune responses that could be blocked with an HLA-A2 blocking antibody (Figure 6A). Similarly, A2/DR1 mice (n=8) immunized with pTVG-SSX-2 were tested for p98-112 peptide-specific immune responses. While the DNA vaccine elicited robust p103-111-specific immune responses that could be blocked with an HLA-A2 blocking antibody, no significant p98-112-specific immune responses were detectable (Figure 6B). These results indicate that while p98-112 is an immunogenic HLA-DR1 peptide, it may not necessarily serve as an epitope processed and presented by APCs, and did not facilitate recognition of the p103-111 epitope.

SSX-2-specific CD8⁺ T cells can be detected and cultured from the PBMCs of prostate cancer patients

We next wished to determine whether SSX-2 epitope-specific T cells are detectable in the peripheral blood of patients with prostate cancer, as evidence of whether these cells are present within the repertoire of HLA-A2⁺ individuals and thus might be able to be augmented with immunization. PBMC were obtained from volunteer male HLA-A2⁺ blood donors without prostate cancer (n=6), HLA-A2⁺ (n=7) or HLA-A2⁻ (n=6) men with biochemically recurrent prostate cancer, and HLA-A2⁺ men (n=8) with advanced, castrate-resistant prostate cancer. These PBMC were stimulated for 1 week *in vitro* with peptides p41-49 and p103-111 and evaluated for the presence of peptide-specific T cells by tetramer staining. As shown in Figures 7A and 7B, SSX-2-specific CD8⁺ T cells were detectable at a significantly higher frequency in patients with prostate cancer compared with volunteer HLA-A2⁺ male blood donors. Moreover, the frequency of SSX-2-specific CD8⁺ T cells was generally higher in patients with more advanced disease. CD8⁺ T cells were also isolated from the peripheral blood of eleven HLA-A2⁺ patients with prostate cancer and tested for their ability to lyse SSX-2 p103-111 peptide-pulsed target cells. CTL specific for peptide-pulsed target cells were detectable from one of seven HLA-A2⁺ patients with biochemically recurrent prostate cancer after five *in vitro* stimulations, and were similarly detectable from three of four HLA-A2⁺ patients with castrate-resistant prostate cancer following two-to-three stimulations *in vitro* (Figure 7B and 7C). All patients with T cells showing demonstrable lysis were found to have demonstrable p103-111 tetramer-specific T cells (data not shown). In one patient with a high frequency of p103-111 peptide-specific T cells after *in vitro* stimulation found by tetramer staining (0.85% of CD3⁺/CD8⁺ splenocytes), peptide-specific CTL could be detected after three *in vitro* stimulations with peptide and could also lyse the LNCaP prostate cancer cell line (Figure 7C).

Discussion

Several vaccines designed to elicit antigen-specific immune responses are currently being evaluated in clinical trials as specific, targeted treatments for patients with prostate cancer. Because the prostate is an expendable organ, most immunotherapy studies have targeted

tissue-specific antigens. For example, vaccines targeting prostate tumor-associated antigens such as prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) have been shown to elicit antigen-specific immune responses in patients with prostate cancer [5, 6, 12, 32-35]. However, PSA and PAP are not tumor-specific proteins; the identification of tumor-specific targets remains important to the development of immunotherapeutic treatments for prostate cancer, as well as other types of malignancy. Recently, Cheever *et al.* reported that SSX-2 and other sarcoma translocation breakpoint antigens may be higher priority targets for cancer therapy than PSA or PAP based upon certain pre-defined criteria for the prioritization of tumor antigens, such as specificity, oncogenicity, expression in multiple tumor types or advanced disease, and number of identified epitopes [36]. Since the majority of prostate cancer antigens targeted therapeutically to date have been tissue-specific and not necessarily tumor-specific, and because the evaluation of targets associated with more advanced disease may have relevance to prostate cancer, we sought to further characterize the immunogenicity of SSX-2. In addition, because this cancer-testis antigen is not normally expressed in MHC class I-expressing cells, unlike the tissue-restricted antigens currently being investigated for prostate cancer, we reasoned that it might be possible to evaluate SSX-2 in the future as a prophylactic cancer vaccine antigen, ideally permitting immunization in a setting prior to expression of the antigen in tumor cells and thereby potentially avoiding immune tolerance.

In the present study we used a genetic vaccine to identify and compare SSX-2 HLA-A2-restricted epitopes relevant to prostate cancer. Specifically, we used peptide prediction algorithms and HLA-A2 affinity assays to identify and prioritize potentially immunogenic peptides, followed by *in vivo* vaccination with SSX-2 peptides or a DNA plasmid encoding SSX-2 to evaluate the immunogenicity of predicted peptides and their ability to elicit anti-tumor CTL. With this approach we identified four SSX-2 peptides that have significant affinity for the HLA-A2 complex *in vitro*. We subsequently carried out peptide vaccination studies in A2/DR1 mice and found that p41-49 and p103-111 could elicit peptide-specific immune responses in this transgenic model. Using a genetic vaccine encoding SSX-2 we found that significant responses to p41-49 and p103-111 were elicited, demonstrating that both peptides are HLA-A2-restricted epitopes. However, peptide p103-111 appeared to be clearly dominant with robust responses elicited by either direct peptide or DNA vaccination. p103-111 immunization elicited CTL in animals that could lyse both p103-111 peptide-pulsed target cells and LNCaP prostate cancer cells. The dominance of the p103-111 epitope was found to not be due to a putative MHC class II epitope encompassing it. Finally, we also found that HLA-A2+ patients with prostate cancer can have p41-49 and p103-111-specific T cells in their peripheral blood, demonstrating that these cells are within the repertoire of patients with advanced disease in particular.

Several other investigators have also sought to identify MHC class I-restricted epitopes specific for SSX-2. Ayyoub *et al.* previously evaluated SSX-2 peptides by incubating an overlapping library of SSX-2 peptides with standard proteasome complexes *in vitro* [25]. They demonstrated that peptide p41-49 is an HLA-A2-restricted epitope recognized by CTL from melanoma patients [25]. Peptide p41-49 was also shown to be an epitope presented in hepatocellular carcinoma, and p41-49-specific CTL were found to lyse melanoma cells and the sarcoma cell line SW 872 [25, 37-39]. Wagner *et al.* independently demonstrated that SSX-2 peptide p103-111 is an HLA-A2-restricted epitope recognized by CTL from breast cancer patients and presented by melanoma cell lines [21]. Held *et al.* further demonstrated that this epitope is directly presented on melanoma cells using peptide/HLA-A2-specific Fab antibodies [40]. He *et al.* suggested that SSX-2 p57-65 was also an HLA-A2-restricted epitope by demonstrating that peptide-specific CTL cultured from healthy donors are capable of lysing peptide-pulsed T2 cells [41], however, they did not demonstrate that this peptide is presented as an MHC class I-restricted epitope by tumor cells. Together, these

prior studies highlighted these three SSX-2 peptides as possible HLA-A2-restricted epitopes. Prior to our study, a comparison of HLA-A2-restricted epitopes had not been carried out, and it had not been determined whether SSX-2 vaccines can elicit epitope-specific CTL to these peptides. Our studies confirm the work of others that p41-49 and p103-111 are HLA-A2-restricted epitopes. However, while p57-65 can bind HLA-A2, it does not appear to be a presented HLA-A2-restricted epitope, and it does not appear to be immunogenic in our transgenic model.

We found that *in vivo* immunization of transgenic mice with peptide p103-111 or a DNA vaccine encoding SSX-2 generated robust p103-111-specific immune responses that could be detected directly *ex vivo*. Peptide p41-49-specific immune responses generated by peptide or genetic immunization were only rarely detected unless the splenocytes were expanded by *in vitro* peptide stimulation (Figure 4B and 4C). These results were not expected from our *in vitro* analysis, which showed p41-49 to have the greatest affinity for HLA-A2. It could be that p41-49 is not efficiently processed by the murine proteasome. Interestingly, the Ssx1 murine peptide corresponding to human peptide p41-49 shares the greatest homology with the human peptide, while the region corresponding to p103-111 is absent in the mouse protein sequence (Table 1). Consequently, it is possible that there is some tolerance to the p41-49 epitope in the mouse that contributed to the reduced immunogenicity observed. Peptide p41-49-specific CTL from DNA-immunized mice were able to lyse p41-49-pulsed target cells, suggesting that p41-49 is an HLA-A2-restricted epitope, yet in our studies p41-49-specific CTL failed to lyse SSX-2+ LNCaP cells (Figure 3A). These results were also unexpected but may indicate that LNCaP cells either do not endogenously process and present this peptide, present low levels of this peptide, or perhaps more likely, p41-49-specific CTL generated from vaccination are too low in frequency or avidity to detect this epitope on LNCaP cells. In any case, our results suggest that p103-111 is the dominant HLA-A2-restricted SSX-2 epitope, at least in this transgenic mouse model.

We found that genetic vaccination offered several advantages over direct peptide immunization for epitope identification, including the induction of stronger immune responses and simultaneous recognition of multiple epitopes. Immunization of mice with pTVG-SSX-2 elicited p103-111-specific immune responses in every immunized animal and with higher frequency than p41-49-specific cells as determined by both IFN γ ELISPOT and tetramer staining of splenocytes directly *ex vivo*. Interestingly, the best *in vitro* lysis of LNCaP cells and peptide-pulsed T2 cells (figure 5A and 5B) was found by splenocytes from an animal that had a robust immune response to both peptides p41-49 and p103-111 by direct *ex vivo* IFN γ ELISPOT assay (data not shown). This finding may indicate that superior lysis of prostate tumor cells can be achieved by eliciting CTL specific for multiple SSX-2 epitopes. A possible future direction may be to develop the SSX-2 DNA vaccine as an immunotherapeutic tool by optimizing its ability to elicit responses to multiple epitopes simultaneously.

In addition to identifying SSX-2 peptide p103-111 as an immunodominant epitope, we were able to detect T cells specific to this peptide in the peripheral blood of patients with prostate cancer using tetramer staining, with particularly high frequencies of T cells specific to either peptide p41-49 or p103-111 in patients with advanced disease. In concordance with these tetramer results we found that one of seven patients with early-stage prostate cancer had CTL that could specifically lyse p103-111-pulsed target cells, while three of four patients with advanced-stage disease had higher frequencies of tetramer positive CTL that could lyse these target cells or the LNCaP prostate cancer cell line after fewer rounds of *in vitro* peptide stimulation. These findings suggest that advanced prostate tumors might have greater SSX-2 expression than earlier stage disease, as has been suggested by findings of increased SSX-2 mRNA expression in metastatic prostate cancer and other tumor types [18,

42], which could lead to increased cross-presentation of SSX-2 and generation of SSX-2-specific CD8⁺ T cells. If true, this might permit an opportunity to consider immunization early in the course of disease, more analogous to the prophylactic setting, to ideally prevent the growth of SSX-2-expressing tumors. However, to date the expression and function of SSX-2 in prostate tumors of various stages remains largely unknown. These will be areas of future research.

In conclusion, this work provides direct evidence that SSX-2 is a relevant vaccine target antigen for prostate cancer. In particular these findings highlight the importance of SSX-2 peptide p103-111 as a dominant HLA-A2-restricted immunogenic epitope. To date the majority of the work evaluating SSX-2 has focused on SSX-2 peptide p41-49 as an HLA-A2-restricted epitope in patients with different types of malignancy. Future studies will explore the expression and function of SSX-2, and other SSX family members, in prostate tumors. Other studies will explore modified DNA vaccines as a means to enhance peptide-specific immune responses to this antigen and will assess strategies to augment the anti-tumor potential of these SSX-2-specific vaccines *in vivo*, such as using these vaccines in combination with epigenetic modifying agents that might increase SSX-2 expression in tumor cells (18).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported for HAS by NIH (T32CA009135-33) and for HAS and DGM by the US Army Medical Research and Materiel Command Prostate Cancer Research Program (W81XWH-08-1-0341 and W81XWH-10-1-0495). We also thank Dr. Francois Lemonnier for his provision of the A2/DR1 mice, Dr. Brian Olson for providing technical support and reviewing the manuscript, and the NIH Tetramer facility (Atlanta, GA) for providing the HLA-A2 tetramers.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. *CA Cancer J Clin.* 2009; 59:225–249. [PubMed: 19474385]
2. Vieweg J. Immunotherapy for advanced prostate cancer. *Rev Urol.* 2007; 9(Suppl 1):S29–38. [PubMed: 17387370]
3. McNeel DG. Prostate cancer antigens and vaccines, preclinical developments. *Cancer Chemother Biol Response Modif.* 2005; 22:247–261. [PubMed: 16110615]
4. Wolchok JD, Gregor PD, Nordquist LT, et al. DNA vaccines: an active immunization strategy for prostate cancer. *Semin Oncol.* 2003; 30:659–666. [PubMed: 14571413]
5. Miller AM, Ozenci V, Kiessling R, et al. Immune monitoring in a phase 1 trial of a PSA DNA vaccine in patients with hormone-refractory prostate cancer. *J Immunother.* 2005; 28:389–395. [PubMed: 16000958]
6. McNeel DG, Dunphy EJ, Davies JG, et al. Safety and immunological efficacy of a DNA vaccine encoding prostatic acid phosphatase in patients with stage D0 prostate cancer. *J Clin Oncol.* 2009; 27:4047–4054. [PubMed: 19636017]
7. Mincheff M, Tchakarov S, Zoubak S, et al. Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: a phase I/II clinical trial. *Eur Urol.* 2000; 38:208–217. [PubMed: 10895014]
8. Kantoff PW, Schuetz TJ, Blumenstein BA, et al. Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol.* 28:1099–1105. [PubMed: 20100959]
9. Madan RA, Arlen PM, Mohebtash M, et al. Prostavac-VF: a vector-based vaccine targeting PSA in prostate cancer. *Expert Opin Investig Drugs.* 2009; 18:1001–1011.

10. Monie A, Tsen SW, Hung CF, et al. Therapeutic HPV DNA vaccines. *Expert Rev Vaccines*. 2009; 8:1221–1235. [PubMed: 19722895]
11. Carvalho JA, Prazeres DM, Monteiro GA. Bringing DNA vaccines closer to commercial use. *IDrugs*. 2009; 12:642–647. [PubMed: 19790015]
12. Pavlenko M, Roos AK, Lundqvist A, et al. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. *Br J Cancer*. 2004; 91:688–694. [PubMed: 15280930]
13. Caballero OL, Chen YT. Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci*. 2009; 100:2014–2021. [PubMed: 19719775]
14. Simpson AJ, Caballero OL, Jungbluth A, et al. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer*. 2005; 5:615–625. [PubMed: 16034368]
15. Costa FF, Le Blanc K, Brodin B. Concise review: cancer/testis antigens, stem cells, and cancer. *Stem Cells*. 2007; 25:707–711. [PubMed: 17138959]
16. Ayyoub M, Taub RN, Keohan ML, et al. The frequent expression of cancer/testis antigens provides opportunities for immunotherapeutic targeting of sarcoma. *Cancer Immun*. 2004; 4:7. [PubMed: 15298487]
17. Tureci O, Sahin U, Schobert I, et al. The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. *Cancer Res*. 1996; 56:4766–4772. [PubMed: 8840996]
18. Dubovsky JA, McNeel DG. Inducible expression of a prostate cancer-testis antigen, SSX-2, following treatment with a DNA methylation inhibitor. *Prostate*. 2007; 67:1781–1790. [PubMed: 17929270]
19. Ayyoub M, Hesdorffer CS, Mettetz G, et al. Identification of an SSX-2 epitope presented by dendritic cells to circulating autologous CD4+ T cells. *J Immunol*. 2004; 172:7206–7211. [PubMed: 15153546]
20. dos Santos NR, Torensma R, de Vries TJ, et al. Heterogeneous expression of the SSX cancer/testis antigens in human melanoma lesions and cell lines. *Cancer Res*. 2000; 60:1654–1662. [PubMed: 10749136]
21. Wagner C, Neumann F, Kubuschok B, et al. Identification of an HLA-A*02 restricted immunogenic peptide derived from the cancer testis antigen HOM-MEL-40/SSX2. *Cancer Immun*. 2003; 3:18. [PubMed: 14677925]
22. Ayyoub M, Hesdorffer CS, Montes M, et al. An immunodominant SSX-2-derived epitope recognized by CD4+ T cells in association with HLA-DR. *J Clin Invest*. 2004; 113:1225–1233. [PubMed: 15085202]
23. Ayyoub M, Merlo A, Hesdorffer CS, et al. Distinct but overlapping T helper epitopes in the 37–58 region of SSX-2. *Clin Immunol*. 2005; 114:70–78. [PubMed: 15596411]
24. Viatte S, Alves PM, Romero P. Reverse immunology approach for the identification of CD8 T-cell-defined antigens: advantages and hurdles. *Immunol Cell Biol*. 2006; 84:318–330. [PubMed: 16681829]
25. Ayyoub M, Stevanovic S, Sahin U, et al. Proteasome-assisted identification of a SSX-2-derived epitope recognized by tumor-reactive CTL infiltrating metastatic melanoma. *J Immunol*. 2002; 168:1717–1722. [PubMed: 11823502]
26. Pajot A, Michel ML, Fazilleau N, et al. A mouse model of human adaptive immune functions: HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout mice. *Eur J Immunol*. 2004; 34:3060–3069. [PubMed: 15468058]
27. Perarnau B, Saron MF, San Martin BR, et al. Single H2Kb, H2Db and double H2KbDb knockout mice: peripheral CD8+ T cell repertoire and anti-lymphocytic choriomeningitis virus cytolytic responses. *Eur J Immunol*. 1999; 29:1243–1252. [PubMed: 10229092]
28. Johnson LE, Frye TP, Arnot AR, et al. Safety and immunological efficacy of a prostate cancer plasmid DNA vaccine encoding prostatic acid phosphatase (PAP). *Vaccine*. 2006; 24:293–303. [PubMed: 16115700]
29. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*. 1994; 152:163–175. [PubMed: 8254189]

30. Rammensee H, Bachmann J, Emmerich NP, et al. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 1999; 50:213–219. [PubMed: 10602881]
31. Olson BM, Frye TP, Johnson LE, et al. HLA-A2-restricted T-cell epitopes specific for prostatic acid phosphatase. *Cancer Immunol Immunother*. 59:943–953. [PubMed: 20140431]
32. Burch PA, Breen JK, Buckner JC, et al. Priming tissue-specific cellular immunity in a phase I trial of autologous dendritic cells for prostate cancer. *Clin Cancer Res*. 2000; 6:2175–2182. [PubMed: 10873066]
33. Burch PA, Croghan GA, Gastineau DA, et al. Immunotherapy (APC8015, Provenge) targeting prostatic acid phosphatase can induce durable remission of metastatic androgen-independent prostate cancer: a Phase 2 trial. *Prostate*. 2004; 60:197–204. [PubMed: 15176049]
34. Small EJ, Schellhammer PF, Higano CS, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. *J Clin Oncol*. 2006; 24:3089–3094. [PubMed: 16809734]
35. Higano CS, Schellhammer PF, Small EJ, et al. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. *Cancer*. 2009; 115:3670–3679. [PubMed: 19536890]
36. Cheever MA, Allison JP, Ferris AS, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res*. 2009; 15:5323–5337. [PubMed: 19723653]
37. Ayyoub M, Rimoldi D, Guillaume P, et al. Tumor-reactive, SSX-2-specific CD8+ T cells are selectively expanded during immune responses to antigen-expressing tumors in melanoma patients. *Cancer Res*. 2003; 63:5601–5606. [PubMed: 14500401]
38. Bricard G, Bouzourene H, Martinet O, et al. Naturally acquired MAGE-A10- and SSX-2-specific CD8+ T cell responses in patients with hepatocellular carcinoma. *J Immunol*. 2005; 174:1709–1716. [PubMed: 15661935]
39. Ayyoub M, Brehm M, Mettez G, et al. SSX antigens as tumor vaccine targets in human sarcoma. *Cancer Immun*. 2003; 3:13. [PubMed: 14533943]
40. Held G, Neumann F, Sturm C, et al. Differential presentation of tumor antigen-derived epitopes by MHC-class I and antigen-positive tumor cells. *Int J Cancer*. 2008; 123:1841–1847. [PubMed: 18688854]
41. He Y, Mao L, Lin Z, et al. Identification of a common HLA-A*0201-restricted epitope among SSX family members by mimicking altered peptide ligands strategy. *Mol Immunol*. 2008; 45:2455–2464. [PubMed: 18295890]
42. Naka N, Joyama S, Tsukamoto Y, et al. Quantification of SSX mRNA expression in human bone and soft tissue tumors using nucleic acid sequence-based amplification. *J Mol Diagn*. 2005; 7:187–197. [PubMed: 15858142]
43. Sauma SY, Gammon MC, Bednarek MA, et al. Recognition by HLA-A2-restricted cytotoxic T lymphocytes of endogenously generated and exogenously provided synthetic peptide analogues of the influenza A virus matrix protein. *Hum Immunol*. 1993; 37:252–258. [PubMed: 8300410]

Abbreviations

SSX-2	synovial sarcoma X breakpoint-2
CTA	cancer testis antigen
CTL	cytotoxic T lymphocyte
PBMC	peripheral blood mononuclear cell
TAA	tumor associated antigen
MHC	major histocompatibility complex
LDH	lactate dehydrogenase
ELISPOT	enzyme-linked immunosorbent spot

HLA	human leukocyte antigen
5-aza-dc	5-Aza-2'-deoxycytidine

Figure 1. SSX-2 nonamer peptides have HLA-A2 affinity *in vitro*

Panel A: Shown is the relative HLA-A2 binding of each SSX-2 peptide by T2 binding assay. Values were measured in triplicate for each peptide and normalized against a vehicle control. Results are representative of three independent experiments. A known HLA-A2-binding peptide derived from the influenza A matrix protein was used as a positive control for comparative MFI value [43]. **Panel B:** The affinities of peptides p41-49, p57-65, p99-107, and p103-111 for HLA-A2 were evaluated at multiple peptide concentrations (50, 25, 5, 1, 0.2, and 0.04 μg/ml) in T2 binding assays. Shown are the normalized MFI values, and data are representative of two independent experiments.

Figure 2. Peptide-immunized A2/DR1 mice develop SSX-2 peptide-specific immune responses to SSX-2 p41-49 and p103-111

A2/DR1 mice were immunized once with 100 μ g of each individual SSX-2 peptide or an influenza A matrix (Flu) protein peptide (n=4-12 per group) and then assessed for the development of peptide-specific immune responses by IFN γ ELISPOT. Splenocytes were cultured with the specific peptide used for immunization (●), a non-specific HLA-A2-binding nonamer peptide (○), or Concanavalin A (ConA) positive control (▲). For animals immunized with Flu, the non-specific peptide used was p103-111. Each dot represents the frequency of IFN γ spot-forming units (SFU) from an individual animal measured in triplicate with background subtracted. * indicates a significant response ($p < 0.05$, two-tailed T test). Data are representative of two independent experiments.

Figure 3. Immunization of A2/DR1 mice with SSX-2 peptides can elicit cytotoxic immune responses against peptide-specific target cells and human prostate cancer cells

Splenocytes from A2/DR1 mice vaccinated with SSX-2 peptides p41-49 (panel A), p57-65 (panel B), p99-107 (panel C), p103-111 (panel D) or influenza A matrix peptide (panel E) were stimulated with the immunizing peptide for five days and tested for specific lysis of peptide-pulsed T2 target cells (specific peptide with which mice were immunized (◆), or non-specific peptide (■)) or against the LNCaP cell line (▲). Shown is the mean and standard deviation of percent specific lysis at three different effector-to-target ratios. Data is from individual mice and is representative of two mice per group.

Figure 4. DNA-immunized A2/DR1 mice develop peptide-specific immune responses to SSX-2 peptides p41-49 and p103-111

Splenocytes from individual A2/DR1 mice immunized with either pTVG-SSX-2 (n=7, ●) or pTVG4 (n=6, ○) were analyzed directly *ex vivo* (panel A) or following a seven-day *in vitro* stimulation (panel B) to determine the frequency of peptide-specific T cells by IFN γ ELISPOT. Each dot represents the frequency of peptide-specific IFN γ -SFU from an individual mouse, measured in triplicate with background subtracted. * indicates a significant (p<0.05) difference in the mean number of IFN γ -SFU between the two groups (two tailed t-test). Results are representative of two independent experiments. Panel C: Splenocytes from representative animals immunized with either pTVG4 or pTVG-SSX-2

were stained, without prior *in vitro* stimulation, with HLA-A2 tetramers specific for p41-49 or p103-111. Shown are the events gated on CD3+CD8+ T cells. The numbers represent % of events in each gated quadrant.

Figure 5. Immunization of A2/DR1 mice with pTVG-SSX-2 DNA vaccine elicits cytotoxic immune responses against p41-49 and p103-111 peptide-pulsed target cells and the LNCaP prostate cancer cell line

Splenocytes from animals vaccinated with pTVG-SSX-2 (panels A and B) or pTVG4 (panels C and D) were tested for their ability to lyse peptide-pulsed T2 target cells, the SSX2+ LNCaP cell line or the SSX2- DU-145 prostate cancer cell line transfected to express HLA-A2. Shown in panels A and C are the percent specific lysis at three different effector to target ratios of splenocytes incubated with peptide-pulsed T2 target cells. In panels B and D, splenocytes were evaluated for lysis of one of the prostate cancer cell lines in the presence (▲) or absence (●, ■) of an HLA-A2 blocking antibody.

A **B**

Figure 6. Identification of an SSX-2 DR1-binding peptide encompassing SSX-2 p103-111
 Splenocytes from A2/DR1 mice immunized once with 100 μ g of SSX-2 15-mer peptide p98-112 (n=4, ●) or nonamer peptide p103-111 (n=5, ▲) were assessed for the development of peptide-specific immune responses by IFN γ ELISPOT (panel A), with or without the addition of an HLA-A2 or HLA-DR blocking antibody. In panel B, splenocytes from mice immunized twice with 100 μ g of pTVG-SSX-2 were assessed for peptide-specific immune responses. Responses to p98-112 and p103-111 were also assessed in the presence of HLA-A2 or HLA-DR blocking antibodies or an IgG control. Each dot represents the frequency of IFN γ SFU from an individual animal measured in triplicate with background subtracted, and lines show the means for experimental groups. * indicates a significant difference between compared groups (p < 0.05, two-tailed t-test). Data are representative of two independent experiments.

Figure 7. SSX-2 epitope-specific CTL can be detected and cultured from the peripheral blood of prostate cancer patients

Panel A: PBMC obtained from 6 HLA-A2+ men without prostate cancer, and from 21 men with prostate cancer, were stimulated *in vitro* for 7 days with p41-49 and p103-111 and evaluated for the frequency of peptide-specific CD8+ T cells by tetramer staining similar to that shown in Figure 4C. Shown is the frequency of tetramer+ cells among CD3+CD8+ cells. * indicates a significant difference between compared groups ($p < 0.05$, two-tailed t-test). Panel B: Representative tetramer staining for a patient from Panel A with advanced disease that had both p41-49 and p103-111 tetramer positive events. Panel C: T cells from a representative HLA-A2+ patient with prostate cancer and high frequencies of p103-111

tetramer-positive cells were cultured with SSX-2 peptide p103-111-loaded DCs and tested for their ability to lyse p103-111 peptide-pulsed T2 target cells or LNCaP cells. Shown is the percent specific lysis at three different effector to target ratios after three *in vitro* stimulations with peptide.

Predicted HLA-A2 affinity and homology of Human and Mouse SSX-2 nonamer peptides

Table 1

Shown are the peptide names, amino acid sequences, and predicted HLA-A2 affinity scores of potential HLA-A2 peptides derived from the amino acid sequence of human SSX-2, as well as the respective murine Ssx1 and Ssxb1 peptide sequences and binding affinities. Predicted HLA-A2 peptide affinity was determined by scanning the SSX-2, Ssx1, and Ssxb1 protein sequence with two different peptide prediction algorithms [29, 30]. A known HLA-A2-binding peptide derived from the influenza A matrix protein is shown for comparison [39]. Amino acids within the mouse peptide sequences shown in gray represent identical peptide residues between the human and murine peptides.

Human SSX-2 Peptide Name	Peptide Sequence	Predicted Binding Affinity [BIMAS]	Predicted Binding Affinity [SYFPEITHI]	Mouse Peptide Homologue (Ssx1 Ssxb1)	Predicted Binding Affinity [BIMAS]	Predicted Binding Affinity [SYFPEITHI]
SSX2p16	QIPEKIQKA	6.4	21	HKPEETCQA YEPKNJCKA	0.001 1.022	8 12
SSX2p20	KIQKAFDDI	4.7	17	ETCQAFEDI NICKAFQDI	0.010 2.726	9 16
SSX2p41	KASEKIFYV	1017.1	22	SRSEKITVY TQWQKSAYV	0.501 321.9	19 14
SSX2p57	AMTKLGFKA	20.8	16	TMTNIGLRA RMTDLGTV	3.588 205.9	13 23
SSX2p99	MTFGRQLGI	2.1	20	Deleted	-	-
SSX2p103	RLQGISPKI	10.4	23	Deleted	-	-
SSX2p167	RLRERKQLV	21.7	22	QLREKKKPV IKRMKLTSV	21.672 0.029	22 19
Influenza	GILGFVFTL	550.9	30	N/A	-	-