Evaluating the SSX Family of Cancer-Testis Antigens as Immunological Targets for the Treatment of Prostate Cancer

by

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Abstract

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Prostate cancer remains a significant health concern worldwide, and continues to be the most commonly diagnosed and second-leading cause of cancer-related death among American men. While several therapies have been developed showing clinical efficacy in patients with early-stages of prostate cancer, there remains a lack of curative treatments for advanced or metastatic disease. Tumor immunotherapy is one area of research currently being evaluated for the treatment of prostate cancer and gaining increased attention with the recent FDA approval of the first cancer vaccine for castrate-resistant prostate cancer. These types of active immunotherapies, or “cancer vaccines,” are designed to harness the host immune system to recognize and attack cancer cells. While several antigen-specific immunotherapeutic vaccines have been evaluated in clinical trials for the treatment of prostate cancer, relatively few robust clinical responses have been demonstrated, highlighting the importance of identifying new and

more relevant target antigens for the development of anti-cancer vaccines. The SSX family of proteins represents an attractive group of target antigens for the immunotherapeutic treatment of prostate cancer, due to their normal expression in immune privileged tissue and their frequent expression in prostate cancer. We have identified that some prostate cancer patients can develop spontaneous immune responses to one dominantly expressed SSX family member, SSX2. We have also shown that different SSX family members can be expressed in prostate cancer cell lines and tissues, particularly SSX2, and that this expression appears to be restricted to metastatic prostate cancer. Utilizing the HHDII-DR1 transgenic mouse model, we have shown that a DNA plasmid vaccine encoding SSX2 can elicit robust immune responses to two HLA-A2-restricted SSX2 epitopes, p41-49 and p103-111, in immunized mice. CTL from these mice were found to lyse peptide-pulsed target cells and SSX2+ prostate cancer cells *ex vivo* in an HLA-A2-restricted manner, indicating that SSX2 proteins encode epitopes presented by prostate cancer cells. It was also found that SSX DNA plasmid vaccines could elicit cross-reactive T cells recognizing a dominant epitope shared among multiple SSX proteins, which suggests that these vaccines could potentially be used to target more than one SSX family member simultaneously. We have also demonstrated that SSX vaccine efficacy can be enhanced using an altered peptide ligand strategy to increase epitope affinity for MHC class I. Finally, we found that both native and modified SSX2 vaccines can elicit anti-tumor immune responses in terms of a decrease in SSX2-expressing tumor

deveopment in HHDII-DR1 mice. These data verify that SSX proteins are attractive targets for the treatment of prostate cancer, and that a DNA vaccine encoding this antigen should be evaluated in clinical trials of patients with advanced prostate cancer or at risk for metastatic disease.

Dedication

This thesis was possible only due to the never-ending support of my family and friends. In particular those who instilled within me a desire to learn, provided the nudges and reassurance I needed during both the good times and the difficult, and helped me in any possible way that they could,

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List of Abbreviations

Abbreviation	Name
ADT	Androgen deprivation therapy
APC	Antigen-presenting cell
APL	Altered peptide ligand
AR	Androgen receptor
ARLBD	Androgen receptor ligand binding domain
5-aza-dc	5-Aza-2’-deoxycytidine
BSA	Bovine serum albumin
CFA	Complete Freund’s adjuvant
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
ConA	Concanavalin A
CpG	Cytosine-guanine
CTA	Cancer-testis antigen
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DT	Doubling time
EMA	Epigenetic modifying agents
EMT	Epithelial-to-mesenchymal transition
E:T	Effector-to-Target cell ratio

ELISPOT	Enzyme-linked immunospot assay
FDA	Food and Drug Administration
FCS	Fetal calf serum
GM-CSF	Granulocyte-macrophage colony stimulating factor
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
MFI	Mean fluorescent index
MHC	Major histocompatibility complex
NK	Natural killer
NLS	Nuclear localization sequence
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PSA	Prostate specific antigen
PSCA	Prostate stem cell antigen
PSMA	Prostate specific membrane antigen
RNA	Ribonucleic acid
SFU	Spot-forming unit
SS	Synovial sarcoma

SSX	Synovial sarcoma X chromosome breakpoint
STEAP	Six-transmembrane epithelial antigen of prostate
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TIL	Tumor infiltrating lymphocytes
TSA	Trichostatin A
USDA	U.S. Department of Agriculture

Chapter 1

Introduction to Prostate Cancer, Tumor Immunotherapy, and the Synovial Sarcoma X Chromosome Breakpoint Family of Cancer-Testis Antigens

Some of this work was published in 'Smith, H.A. and D.G. McNeel, *The SSX Family of Cancer-Testis Antigens as Target Proteins for Tumor Therapy*. Clinical and Developmental Immunology, 2010. Article ID 150591.'

Prostate cancer pathology and current therapies

Prostate cancer is a significant health concern in the aging male population, and remains the most frequently diagnosed and second leading cause of cancer-related death among American men (1). In 2012 approximately 240,000 men will be diagnosed with prostate cancer and another 28,000 individuals will succumb to this disease (1). For the majority of patients, prostate tumors are diagnosed as organ-confined, androgen-dependent malignancies, for which surgery or radiation therapy are standard first-line treatments. Tumor resection or radiation therapy are typically curative, however, one-third of these patients relapse within five years after surgery with biochemically-recurrent disease, as measured by an increase in the prostate cancer serum marker, prostate specific antigen (PSA) (2-4). Recurrent tumors are usually androgen-dependent and patients will often have metastatic disease wherein tumor cells have spread to other organ sites, most often to the bone and lymph nodes (2). At this stage, individuals will have a median survival of 3-7 years, and will usually undergo androgen-deprivation therapy (ADT), which is a targeted therapy used to ablate or eliminate the patient's sources of the hormone testosterone or its chemical precursor forms (2, 4). ADT is a process that can be carried out either by chemical means, such as administering leutinizing hormone releasing hormone (LHRH) agonists, anti-androgens, or these agents simultaneously) or through surgical castration of the testes where androgens are primarily produced (2, 4). While androgen ablation

will provide tumor regression in approximately 80-90% of these patients, the response is short lived, and within 30 months most of these individuals relapse, developing a more aggressive “castrate-resistant” disease usually characterized by androgen-independent tumors and a metastatic phenotype (4-6). Currently, for this stage of disease there are very few therapies that provide considerable improvement in overall survival. Certain chemotherapies such as docetaxel are standard-of-care for this patient population (median of only 2.9 month survival benefit), but the side effects are quite severe, and at the present there is a dire need to develop new therapeutics and treatment modalities to combat this stage of disease (7, 8).

Tumor immunology, immunotherapy, and prostate cancer vaccines

Tumor immunotherapy is one area of prostate cancer research that has been the focus of increased investigation and heightened attention in recent years. The overall goal of this type of tumor therapy is to develop strategies to harness or augment a patient’s own immune defenses to recognize cancer cells and eliminate tumor tissue. Tumor immunotherapy takes advantage of certain molecules such as proteins expressed on the surface of tumor cells that can be recognized by the host immune system. In most instances these proteins are expressed on the tumor cell surface where they can be recognized by cells of the immune system such as T cells. The human immune system is comprised of

numerous cell types and molecules that function to detect and eliminate pathogens, molecules, and altered cells that are recognized as “foreign” or “non-self,” which can include tumor cells.

The immune system is divided into two subsystems; the innate immune system and the adaptive immune system. The innate immune system is comprised of cells such as macrophages, dendritic cells (DCs) and natural killer (NK) cells, and other myeloid cells that can immediately function in a fairly non-specific way to eliminate parasites, infected cells, or cells displaying foreign antigens that mark them as abnormal. Innate immunity is the host’s first-line defense against tumor cells displaying aberrantly expressed proteins; however, the adaptive immune system employs a more sophisticated or fine-tuned strategy to detect cancer cells. The adaptive immune system is divided into two branches, humoral (B cell) and cellular (T cell) immunity. Humoral immunity is mediated primarily by the antibody production of mature, bone marrow-derived B cells called plasma cells that have become activated after encountering their specific extracellular antigen and start to release immunoglobulin (Ig). These antibodies can potentially bind to proteins on the surface of tumor cells and label them for destruction. Alternatively, cellular immunity is mediated by CD4+ T-helper cells (Th cells) and CD8+ cytotoxic T lymphocytes (CTL). These cells originate from progenitor cells in the bone marrow that traffic to the thymus and undergo a complex process of differentiation and maturation. These cells develop the

capacity to detect a very unique protein peptide bound to major histocompatability complexes (MHC, also known as human leukocyte antigens (HLA) in humans) on the surface of cells via their T cell receptor (TCR). The TCR of a Th cell can recognize peptides 15 amino acids long (15-mers) presented on the surface of professional APCs bound to MHC class II molecules, while the TCR of a CD8+ T cell recognizes and binds to 9-mer peptides presented in the context of MHC class I molecules, which present peptides from endogenous antigens. MHC class I molecules are expressed on the surface of all nucleated, non-germ cells including tumor cells and present peptides from random intracellular proteins, in essence showing a peptide pool from proteins expressed in the cell to T cells of the immune system for detection. This snapshot of the proteins inside the cell provides for immune surveillance of any infections or irregularities. When CD8+ T cells encounter a cell or APC displaying the unique antigen/MHC complex for which it is specific, along with costimulatory signals from APCs, it can become activated and proliferate into a larger pool of effector T cells (Teff) with the same antigen specificity of the original cell. These CTL can then go back into circulation searching for other cells displaying the same peptide, which they can then bind to and lyse through a variety of effector mechanisms. The ability of CD8+ effector cells to directly lyse tumor cells has led to increased focus on this subset of T cells for tumor immunotherapy.

It has been known since the seminal work of Paul Ehrlich in 1909 that the host immune system can recognize and kill cancer cells (9). Since this time there has been ample evidence that the host immune system can play a critical role in tumor surveillance and can protect individuals from the outgrowth of tumors (10, 11). Case studies have shown instances of immune-mediated and spontaneous tumor regression, particularly in patients with melanoma and neuroblastoma (12). Additionally, it's been observed that many tumor types (including prostate cancer) show associations between tumor infiltrating lymphocytes (TIL) or immune activity and overall survival or good prognosis (13, 14). This includes the presence of T cells and antibodies that recognize specific TAA in several tumor types, including prostate cancer (15-20). Other evidence of immune involvement with cancer development is demonstrated by increased tumor burden or susceptibility to cancer in patients with immunosuppression (e.g. transplant patients), or individuals with compromised immune systems such as the elderly or AIDS patients (21-24). These examples of immune involvement with cancer progression provide evidence that the host immune system is capable of recognizing and/or destroying tumor tissue.

Prostate cancer has been found to be a particularly immunogenic type of malignancy, and is often prone to inflammation and lymphocyte infiltration (25, 26). ADT has been used as a therapy that enhances the inflammatory state of the prostate, leading to tissue involution and infiltration of the organ by

macrophages which can phagocytize tumor cells and present tumor antigens to other immune cells for enhanced and specific cell-mediated immune responses (27-30). It has been shown that increased TIL in prostate tumor tissue correlates with decreased cancer recurrence (14). Other work has demonstrated that prostate cancer patients frequently have pre-existing immune responses to prostate cancer antigens, such as PSA (20), prostatic acid phosphatase (PAP – (20, 31, 32), prostate specific membrane antigen (PSMA – (32), androgen receptor (AR – (33)), and a number of others (16, 33, 34). An additional feature of prostate cancer that makes it amenable to immunotherapy is the long natural history of the disease. Many patients will have more than a decade between initial diagnosis and when they finally succumb to their disease, which provides a relatively long window of time to intervene with immune-based therapies.

With this knowledge, the broad goal of tumor immunotherapy is to augment or harness the host's own immune system to elicit a more enhanced, robust, and/or specific immune response against the tumor tissue. As such, cancer immunotherapy in general can be divided into two main categories: passive and active immunotherapy. Passive immunotherapy involves the transfer of certain immune agents to the host such as tumor-specific antibodies or whole activated immune cells (autologous T cells or other cells of the immune system) that are engineered to directly recognize and eliminate tumor cells without re-directing or augmenting the host's own immune system. An alternative approach is active

immunotherapy, which is designed to boost or augment the host immune system through delivery of antigen-specific or antigen-non-specific vaccines. An antigen non-specific vaccine is designed to elicit an anti-tumor immune response to whatever antigens the host tumor cells might be expressing. For example the prostate cancer GVAX vaccine delivers irradiated prostate tumor cells to the host (35, 36). In theory these cells are recognized by T cells and B cells of the host immune system as “foreign antigens” or antigens that are not frequently encountered in normal cells. T cells and B cells can recognize protein motifs of altered or over-expressed proteins on the cell surface of the delivered cells, or the cell can be engulfed and broken down by phagocytosing cells such as macrophages, which can present epitopes of degraded proteins on their cell surface to host immune cells.

Unlike antigen non-specific vaccines, antigen-specific vaccines are engineered to elicit an immune response to an individual antigen expressed and presented by tumor cells, and in some cases, even specific peptides derived from the target antigen. The critical first step and a major consideration in designing antigen-specific vaccines is to first identify which tumor antigens might make the best immune targets, and several factors come into play in making this decision. Immunogenicity, tumor specificity, expression level, stem cell expression, presence of epitopes, cellular localization, frequency in antigen-expressing cancer, and the potential role of the antigen in tumorigenicity have all been

identified as important factors to consider when identifying target antigens for vaccine design (17). For prostate cancer, most vaccine target antigens have been chosen on the basis of their tissue-restricted expression to the prostate, including such antigens as PSA, PAP, PSMA, and prostate stem cell antigen (PSCA). While the expression of these antigens is restricted to the prostate, which would help to avoid cytotoxicity against other normal tissue during immune targeting, these antigens lack other, perhaps more important, features of ideal target antigens. For instance, choosing target antigens whose expression is more critical for tumor cell survival, such as antigens driving tumorigenicity, might help to avoid antigen loss variants or "escape variants" during immune targeting. For this reason, new, antigen-specific vaccine targets need to be identified for prostate cancer immunotherapy.

Not only does the vaccine target need to be carefully selected during antigen-specific vaccine design, but the modality of target antigen delivery must be considered as several options are currently being extensively used in both pre-clinical models and in clinical trials, including:

- Viral vectors
- Peptide or protein
- Antigen-pulsed or loaded APCs
- Bacterial delivery (e.g. *Listeria monocytogenes*)
- DNA plasmid vectors

Each delivery strategy has its own advantages and disadvantages as demonstrated by a large body of evidence in both pre-clinical rodent models and in clinical trials. Direct immunization with purified peptides or protein antigens has the benefit of being highly specific without the risk of introducing or eliciting an immune response to other extraneous antigens that might occur with antigen-loaded DCs, viral based vaccines, or other whole cell vaccines including irradiated tumor cell vaccines. A number of peptide vaccines targeting certain prostate tumor antigens have been, or are being, evaluated in clinical trials in patients with cancer including PSA and HER2/neu (37, 38). Although protein or peptides may be taken up by APCs and cross-presented to T cells, as extracellular antigens these vaccines elicit predominantly humoral immune responses, which are less effective in eliciting immune responses to intracellular tumor antigens, which make up a large majority of TAA (39, 40). In addition to this significant disadvantage, another limitation of peptide vaccines is their restricted presentation by a defined subset of MHC haplotypes. Individual peptides will have preferential expression on certain HLA alleles and so these vaccines may only benefit a subset of the patient population that happens to have the correct MHC haplotype. Alternatively antigen-pulsed or loaded APC vaccines have the benefit of delivering autologous, antigen-primed APCs into the same patient from whom they were taken. Sipuleucel-T (Provenge ® Dendreon Corp., Seattle, WA) is one such vaccine, which is designed to target PAP, and is currently the only immunotherapy currently approved by the FDA for the

treatment of prostate cancer (41-45). With this vaccine, DCs taken from an individual prostate cancer patient are loaded with a PAP:GM-CSF fusion protein, activated and expanded *ex vivo*, and re-introduced back into the autologous patient to augment the patient's own intrinsic immune system by activating peptide-specific T cells. This vaccine has shown a very modest clinical benefit in overall survival and is currently approved for patients with castrate-resistant prostate cancer (42). However, this vaccine and this methodology suffer from some large drawbacks, including a very long and labor-intensive production, storage and delivery issues, and expensive cost per treatment for the patient.

An alternative strategy for antigen delivery is administration of a genetic vaccine, which includes viral-based vaccines, genetically altered bacterial based vaccines, and DNA plasmid vaccines. These delivery systems are designed to carry cDNA molecules encoding a unique antigen or antigens that can be taken up by host APCs (46, 47). The APCs can then potentially transcribe the encoded cDNA message into mRNA that can then be translated into protein by the APC's own intracellular machinery. The translated protein can then be broken down into peptide fragments by the proteosome into 9-mer and 15-mer peptides to be presented on the APC cell surface bound to MHC class I and class II molecules to circulating T cells (46, 47) Thus, genetic vaccines are able to elicit both humoral and cellular antigen-specific immunity to a given encoded protein simultaneously, which sets these vaccines apart from many other methods of

antigen delivery (46, 47). With viral-based vectors the encoded cDNA is carried by the virus and is usually able to enter host APCs directly at the injection site (48, 49). An example of a viral-based vaccine that has been evaluated in clinical trials to treat prostate cancer is PROSTVAC (37). This is a vaccinia- and fowlpox-based vaccine system that encodes PSA, and has been shown to elicit modest immune responses in patients with prostate cancer (37, 50). Likewise, bacterial based vectors like *Listeria* that have been transfected to encode TAA are also being evaluated for their ability to elicit antigen-specific immune responses in cancer patients (51). A major drawback of both viral and bacterial-based vaccines is that the host immune system can mount significant immune responses to viral or bacterial antigens that overwhelm and mask any immune response generated to the encoded tumor antigen. This is a significant limitation of this vaccine system. One way around this problem is to immunize directly with a bacterial plasmid DNA vaccine that encodes the tumor antigen cDNA on a DNA plasmid backbone. There are several advantages to plasmid DNA vaccines including safety, relatively inexpensive production cost, ease of manufacture, storage, shipping, and pliability of the DNA backbone if changes need to be made to the DNA sequence. While these vaccines may have reduced APC uptake compared to viral-based vectors, the immune response generated is far more specific and the threat of immune responses to extraneous antigens is eliminated. These vaccines have been shown to elicit potent, long-term immune responses in hosts with multiple types of malignancy in both pre-clinical rodent

models as well in clinical trials (52-58). In addition to these advantages it should be noted that up until PROVENGE was approved for prostate cancer treatment there had been no FDA (Food and Drug Administration) approved vaccine for any type cancer. In fact, the only immunotherapy for clinical cancer treatment was a DNA plasmid vaccine encoding human tyrosinase that was approved by the US Department of Agriculture (USDA) for the treatment of canine melanoma, which provided evidence for feasibility and potential efficacy of DNA plasmid vaccines in future human trials (59).

DNA vaccines and their utilization in prostate cancer

The work that led to the first DNA plasmid vaccine was pioneered at the University of Wisconsin-Madison by Wolff and colleagues who found that plasmid DNA, when injected into recipient mice, is taken up by host cells and the encoded antigen is expressed (60). The first application of DNA plasmids as vaccines was conducted in immunization studies of BALB/c mice by Ulmer and colleagues, in which a DNA plasmid encoding a nucleoprotein derived from the influenza virus was found to elicit protective immunity in mice against influenza infection (58). Since this time, plasmid DNA vaccines have been highly investigated in a multitude of different types of cancers including melanoma, cervical cancer, and prostate cancer, which have demonstrated that this immunization platform is capable of eliciting potent antigen-specific immune

responses (61-65). These studies have formed the basis of DNA plasmid vaccines as an effective antigen-delivery modality by proof of principle.

DNA plasmids are designed with multiple components to generate enhanced immune responses *in vivo* and maximize expression levels of the encoded antigen. Cytosine-guanine (CpG) repeats are frequently included in the DNA plasmid backbone. The CpGs remain unmethylated in the bacteria in which they are generated but have been found to be recognized by toll-like receptors in the immunized host, initiating an innate immune response to the vaccine (66). This innate immune response can help localize immune cells to the site of vaccination that can secrete chemokines and other molecules that can also stimulate an adaptive immune response. In addition to these motifs, many plasmid vaccines also encode viral promoters, such as the human cytomegalovirus (CMV) immediate-early promoter to stimulate strong transcription of the encoded antigen. The pliability of plasmid vaccines lends itself to easy manipulation of the sequence and incorporation of additional antigens, peptide epitopes, adjuvants, and other factors (67, 68). Sequence tags could be added such as PEST sequences which will target the encoded antigen for ubiquination and degradation by the host proteosome which might enhance antigen peptide presentation (69, 70). Other means of altering peptide antigen presentation might include encoding mutations to increase binding of the peptide to the MHC

complex or mutations resulting in heteroclitic peptides that bind more strongly to the TCR on T cells for enhanced T cell signaling and activation (71).

Several plasmid DNA vaccines have been developed and tested in pre-clinical models for anti-tumor efficacy. A few of these are now making their way into clinical trials for prostate cancer, specifically targeting PAP and PSA. pTVG-HP, a vaccine encoding the PAP protein has been tested extensively in pre-clinical models and has been found to elicit antigen-specific immunity in prostate cancer patients that has been associated with a decrease in PSA doubling-time (DT) (61, 72). PSA DT is a measurement that can be evaluated as a clinical endpoint where the rise in PSA, shown to be correlated with the growth of tumor burden, is decreased relative to control patients or what could have been expected without therapeutic intervention. Likewise, a vaccine targeting PSA has been evaluated in similar trials where it was found to elicit both cellular and humoral antigen-specific immune response (73, 74). In addition to these antigens other investigators have evaluated plasmid vaccines targeting such prostate tumor antigens as PSMA, PSCA, six-transmembrane epithelial antigen of prostate-3 (STEAP), as well as a vaccine that targets a group of epitopes from these antigens (75-79). More recently, our group has completed pre-clinical rodent tumor studies evaluating the AR ligand binding domain (ARLBD) as a plasmid vaccine target with demonstrated efficacy, and clinical trials are anticipated in the near future (80). While these vaccines have demonstrated the ability of DNA

plasmid vaccines to augment anti-tumor immune responses to select antigens, they have yet to elicit strong anti-tumor immune responses that lead to objective clinical responses. For this reason, the identification of new, highly immunogenic prostate tumor antigens that are important for tumor survival and growth is a priority for prostate cancer immunotherapy research. Based upon certain features of the cancer-testis antigen (CTA) family of proteins, these antigens may be the ideal therapeutic targets for prostate cancer that have been lacking thus far.

Cancer-Testis Antigens

The class of proteins known as cancer-testis antigens (CTAs) are a subgroup of tumor proteins with normal expression found almost exclusively in testis germline tissues and aberrant expression in many types of cancer (81). More than 110 CTA genes have been identified to date, with approximately 30 members encoded by genes located on the X chromosome, frequently in multigene families (82). These CTA genes located on the X chromosome, called *CT-X* genes, are predicted to comprise ~10% of the genes encoded by X chromosomal DNA, and many of these *CT-X* genes have homologues in mice that are also located on the X chromosome and restricted in expression to testis tissues (82-84). Typically, the *CT-X* genes cluster in two chromosomal regions, at a telomeric region between Xq24 to Xq28 and a centromeric region from Xp11.2 to 11.4. In addition to their tissue-restricted expression patterns, CTA proteins

share a number of other common characteristics. For instance, many are encoded by multigene families, can be epigenetically regulated in expression level with drugs such as 5-aza-2'-deoxycytidine (5-aza-dc), and many have unknown functionality yet appear to play some role in tumorigenesis. Additionally, most CTAs have heterogeneous expression in cancer tissues and are frequently expressed in high-grade or late tumor stages, with expression often correlated with a worse prognosis. Tumors expressing one CTA are also often found to express multiple CTAs, and several have been found to be targets of spontaneous humoral or cell-mediated immune responses (82). The majority of CT-X antigens are expressed in the testis at the spermatogonia stage of spermatogenesis (85), whereas other CTAs appear to be restricted in expression to haploid cells (83). Most non-*CT-X* genes are single-copy genes with no chromosomal clustering, and expression patterns are often not entirely restricted to MHC-deficient germ cells with some low-level expression in other normal tissues. While non-CT-X proteins represent potential therapeutic targets for cancer based upon their predominant expression selectively in tumor tissues, they are particularly attractive as targets for tumor immunotherapy, specifically due to their preferential expression in immune-privileged testis tissue. Several physical and molecular mechanisms contribute to the immunoprivileged nature of testis tissue including localized cytokine-mediated immune suppression, antigen-specific immunoregulation, the presence of the blood-testis barrier, and an absence of MHC class I molecules on testis germ cells (86, 87). The lack of

MHC class I on the surface of germline cells means these cells are unable to present endogenous peptides to the host CD8+ T cells, suggesting that testis-specific proteins should be recognized as neo-antigens when expressed ectopically in tumor tissue elsewhere in the body. While the immune-privileged nature of testis tissue will likely result in decreased peripheral immune tolerance to CTAs expressed in tumors, it should be noted that some central tolerance to these antigens may be present since CTA proteins could be expressed in lymphoid tissues such as medullary cells of the thymus during T-cell selection (88, 89). However, CTAs encoded by the *CT-X* genes appear to be the most promising CTAs as therapeutic vaccine targets since their expression is most highly restricted to only testis and cancer tissues compared to other non-CT-X CTAs.

To date, only a few CTAs have been shown to elicit both humoral and cell-mediated immune responses in humans, including SSX, MAGE-A1, MAGE-A3, and NY-ESO-1 (82). These proteins are currently the most promising CTA targets for tumor immunotherapy, and several clinical trials are underway to evaluate the efficacy of MAGE-A3 and NYESO-1 as tumor targets (90, 91). The SSX proteins represent one family of therapeutic targets that may be potentially applicable to many types of cancer instead of tumors of a restricted histological type. While SSX proteins appear to be very promising targets for tumor therapy, the work evaluating this family as targets for cancer is less advanced compared

to the MAGE or NY-ESO-1 proteins. This body of work will focus on the SSX family of cancer testis antigens. Recently these proteins have been identified as high-priority targets for cancer therapy based upon certain predefined criteria of ideal target antigens such as tissue specificity, oncogenicity, expression level, and number of identified epitopes (17). Some members of this family can become fused to the SS18 protein in synovial sarcoma (SS) through gene translocations, which is how these proteins were first identified. Interestingly, the SS18-SSX fusion genes are expressed in >95% of SS and appear to directly contribute to the cancer phenotype (92). Much work has been conducted in the last decade to characterize this family of proteins, and although a great deal of information has been elucidated regarding their expression patterns, functionality, cellular localization, and immunogenicity, their role in cancer is still incompletely understood. The information obtained from these investigations has only emphasized the importance of SSX proteins as tumor targets.

SSX Family of Cancer Testis Antigens as Therapeutic Targets

SSX Identification

The SSX gene family was first identified through cytogenetic studies of synovial sarcoma (SS) in which approximately 70% of both biphasic and monophasic SS tumor types were found to contain the same characteristic chromosomal translocation event *t*(X;18)(p11.2;q11.2) (93-96). By screening a cDNA library

derived from a SS cell line with a yeast artificial chromosome (YAC) probe spanning the *t*(X;18) chromosome breakpoint, Clark, et. al. identified two novel genomic fragments by Southern blot analysis. Both of these transcripts, when sequenced, failed to exhibit strong homology to any published genomic sequences and were thus designated SYT for "Synovial Sarcoma Translocation" (now known as SS18 for Synovial Sarcoma gene of chromosome 18) and SSX for "Synovial Sarcoma X chromosome breakpoint" (97). It was further found that the C-terminus of SSX was fused with the N-terminus of SS18, which indicated that the translocation would result in an SS18-SSX fusion protein. Because of the presence of this fusion event in such a high percentage of SS, it was predicted that this fusion protein must have inherent transforming activity independent of the normal function of the SS18 and SSX proteins. Sequencing additional SS cDNA clones of this fusion site, Crew et al. found that the C-terminal regions of two distinct genes, designated SSX1 and SSX2, can become fused to the N-terminus of SS18 (98). In the case of both genes, it was observed that the last 78 C-terminal amino acids of SSX1 and SSX2 replaced the last 8 amino acids of the C-terminus of SS18 in most tumors, however, alternate fusions were also observed less frequently for some tumor specimens. Based upon their predicted open-reading frames, these two genes were expected to encode proteins of 188 amino acids, exhibit ~81% protein sequence identity, contain consensus sequences for both N-glycosylation and tyrosine phosphorylation, be rich in charged amino acids (40%-41%), and in particular

both proteins were found to have acidic C-terminal tails that are sometimes hallmarks of nuclear proteins. Using a nonspecific SSX probe, they also found by Northern blot that SSX1 and SSX2 expression was restricted to thyroid and testis tissues. Türeci et al. later identified SSX2 as the cancer testis antigen (CTA) HOM-Mel-40 when it was found that some melanoma patients have IgG antibody immune responses specific for SSX2 using the serological analysis of recombinant cDNA expression libraries (SEREX) methodology (99). By screening a cDNA library derived from melanoma tissue with sera from multiple melanoma patients it was found that high-titer IgG antibodies to HOM-Mel-40/SSX2 were found in approximately 10% of patients with melanoma (10/89), whereas SSX2 responses were absent in sera from healthy controls (0/41). In the same report, it was found that out of 32 tissues evaluated for SSX2 expression by RTPCR and Northern Blot, SSX2 expression was restricted to testis tissue, and contrary to the report by Crew et al. expression in the thyroid was barely detectable by RTPCR, whereas SSX2 expression was found in the testis by both Northern blot and RT-PCR. These results confirmed that SSX2 is essentially testis-specific among normal body tissues. Türeci also reported in the same paper that SSX2 was expressed in about 50% of melanoma tumor samples, 30% of hepatocellular carcinoma tumor samples, 25% of colon and prostate cancer samples, 20% of breast cancers, and several other tumor samples of different histological origin examined by RT-PCR. These findings firmly established the inclusion of SSX2 as a member of the CTA class of

proteins. The presence of SSX2 expression in so many tumor types suggested that this antigen is upregulated in cancer independently from fusion events with SS18, perhaps by alterations in methylation status or by the overexpression of SSX2 transcriptional activators. In fact, it has been shown cytogenetically by several groups that the fusion member derived from the X chromosome ("der(X)" or SSX2) rather than the derivative member from chromosome 18 ("der(18)" or SS18) is important for maintaining proliferation and that der18 can be lost during the course of SS progression suggesting that the expression of the C-terminal portion of SSX may be the dominant event leading to tumor progression (93, 94, 96, 100, 101).

A Family of Proteins

A short time after the identification of the two SSX proteins involved in the SSX-SS18 fusion event it became clear that SSX1 and SSX2 were part of a larger family of homologous proteins, numbered sequentially based on their discovery. A study by Chand et al. using a pulse-field analysis of digested YACs spanning the X chromosome (OATL1 loci), revealed that perhaps as many as five copies of the SSX gene were present in this region. The analysis indicated that SSX or this genetic locus had undergone a series of duplication events (102). Following on these observations, de Leeuw and colleagues identified a third SSX family member designated SSX3 by screening a testis cDNA library (103). This gene had 90% identity to SSX1 and 95% identity to SSX2 at the nucleic acid level.

Additionally, somatic cell mapping studies indicated that this third family member was located in the same region of the X chromosome (Xp11.2 → p11.1) as SSX1/2. However, in contrast to SSX1/2, RT-PCR data from 44 SS tumor samples revealed that SSX3 was not a fusion partner with SS18. SSX family members SSX4 and SSX5 were subsequently identified by Southern blot analysis using PCR and restriction map analysis (104). Human genomic DNA was probed using a ^{32}P-labeled SSX cDNA and the genomic DNA pulled out was sequenced for identification. The two new members brought the total membership of the SSX protein family to five, including a shorter SSX4 transcript representing an alternative splice. These family members shared 88–95% nucleotide homology and 77–91% amino acid homology. Cloning and sequencing of SSX2 as a prototypic SSX gene revealed a coding region containing six exons spanning a total genomic distance of 8 kb. RT-PCR showed that all five members were expressed in the testis. Further PCR analysis of melanoma cell lines indicated common expression of SSX1 and SSX2 (3/12 lines), while SSX4 and SSX5 detection was more rare (1/12), and SSX3 expression was not detected (104). Not long after the first five homologues were described, a sixth family member, SSX6, was identified through database searches by dos Santos et al. (105), and three additional unique members were identified by Güre et al. through the screening and sequencing of a placenta genomic library with an SSX probe and extensive database analysis (106). This brought the SSX gene family to a total of 9 members. In these database queries,

ten additional SSX pseudogenes were identified, all of which mapped to chromosome X with the exception of *ψ*SSX10 found on chromosome 6. The protein homology of these family members ranges from 73%–92%, and the cDNA homology was found to be between 87%–96%. Each SSX homologue was found to encode the KRAB-A domain on two exons (4 and 5) and the SSX repression domain (encoded by exon 9), described below, and each complete gene occupied 8–10 kb with the distance between genes on the X chromosome ranging from 10–50 kb. However, in contrast to their previous study, which found that SSX members have 6 coding exons, further analysis of expressed sequence tags (ESTs) in GenBank revealed additional 5' and 3' exons in untranslated regions. This finding brought the total number of SSX exons to 10; however, it was found that only 8 of the 10 exons are utilized by all SSX members. From these sequencing studies, the authors confirmed that all members share conserved exon/intron junctions, exon sizes, and most introns, with a few exceptions for alternative splice isoforms (i.e., SSX2, SSX4, SSX5 and SSX7). SSX1–9 were all predicted to encode proteins of 188 amino acids except for SSX8 which has a premature termination in the 7th exon and is predicted to encode a shorter protein of 142 amino acids. Protein expression of SSX8 as well as the alternative splice variants for SSX2, 4, 5, and 7 have not been shown. With the exception of *ψ*SSX10 on chromosome 6, all SSX genes and pseudogenes were found to cluster within two loci on the X chromosome 2mb apart. Based on the observed clustering of SSX family members within the

contigs available in GenBank, Güre and colleagues suggested that the two main SSX gene clusters arose from a large duplication spanning an approximately 100 kb region (106). A tenth SSX family member, designated SSX10 and predicted to encode a protein product, has been annotated in GenBank (GeneID: 100128582), but to date no information about its expression in normal or malignant tissues has been described. The discovery that the SSX family contains several protein members is very characteristic of other CT-X antigens described thus far. For instance, it has been shown that the MAGE-A family consists of 12 members, GAGE 16 members, NY-ESO 3members, and the SPANX family has 12 members, just to name a few of the more than 110 CTAs identified to date (82). Not only have many SSX homologues been defined in humans, but several orthologues have been annotated for other species as well. The known SSX orthologues appear to be restricted to mammalian genomes with several SSX orthologues filed in the NCBI GenBank database including *Homo sapian* (human; accession XP 001128182), *Pan troglodytes* (chimpanzee; accession XP 001137291), *Macaca mulatta* (Macaque; accession XP 001103483), *Callithrix jacchus* (Marmoset; accession XP 002762880), *Canis lupus familiaris* (domesticated dog; accession XP 855346), *Equus caballus* (horse; accession XP 001917891), *Rattus norvegicus* (Norway rat; accession XP 002727549), and *Mus musculus* (common house mouse; accession EDL33981.1). All of these orthologues share the SSX repression domain (SSXRD), described below, and KRAB domains that are common among human

SSX (hSSX) family members, with the SSXRD being the most highly conserved (Figure 1). Several of the identified SSX orthologues, like human SSX (hSSX), appear to have homologous members within a species. For instance, a family of as many as 13 murine homologues have been well characterized on the mouse X chromosome (107). These genes were first identified by Chen et al. by sequencing cDNA clones from mouse testis and tumor tissues. The mouse homologues were divided into two distinct classes, “Ssxa” and “Ssxb,” based on their sequence homologies. Ssxa consists of one member (Ssxa1), while Ssxb was found to have 12 members (Ssxb1–12). Ssxb family members were highly homologous overall with 75–98% nucleotide homology and 67–99% protein homology, however Ssxa and Ssxb were only approximately 30% homologous to each other, showing relatively large diversity between these two classes. These orthologues were located on chromosome X:cM position 5.9, and interestingly, this region is syntenic with human chromosome Xp11.3–p11.23 where the hSSX family members cluster. Again, like hSSX, the conserved SSXRD and KRAB domains are encoded by one and two exons, respectively. All intron sizes are similar between human and mice SSX genes, and all SSX genes span genomic regions of 8 kb. Also, like hSSX family members, some mouse (mSSX) homologues were expressed in murine tumors (predominantly Ssxb1 and Ssxb2), whereas others were not, and Ssxa and Ssxb mRNA expression was restricted to testis tissue among other normal tissues. Overall, mSSX genes are less than 35% homologous to hSSX, however, the KRAB and SSXRD domains

were found to be as much as 49–66% homologous depending on the hSSX/mSSX members being compared (107). The distinct similarities in expression patterns and other characteristics shared between mSSX and hSSX suggest that these orthologues may serve as useful models systems for preclinical tumor therapy studies.

SSX Function

Domain Functions and Cellular Localization

Like most CTAs, the function of SSX proteins, as well as the fusion partner SS18, is still not fully understood. It was determined from the initial identification of SSX that these proteins have Kruppel-associated box (KRAB)-like domains and consensual sequences for N-glycosylation and tyrosine phosphorylation (98, 103). Since SSX proteins also have an acidic tail (98) and a KRAB domain shown to have transcriptional repressor activity in a subgroup of zinc finger proteins, it was surmised that SSX may be involved in transcriptional regulation (108, 109). Some of the first work characterizing the function of SSX proteins was carried out by dos Santos et al. evaluating the subcellular localization of FLAG- or VSV-tagged SS18, SSX, and the SS18-SSX2 fusion constructs using specifically-designed polyclonal antibodies (110). They found that all three proteins were expressed in the nucleus of COS-1 cells transfected with constructs encoding cDNAs for these proteins. SSX2 had a diffuse pattern of expression in the nucleus, while SS18 and SS18-SSX2 had more punctate

nuclear expression patterns (110), with no expression found in nucleoli. Staining of SS cells or HeLa cells with these antibodies revealed a punctate pattern of expression similar to that found for SS18- and SS18-SSX-transfected COS-1 cells. Double labeling of SS18 or SS18-SSX proteins with other known nuclear domains in COS-1 cells revealed no significant colocalizations or associations. These results suggested that SS18 may be directing SS18-SSX fusion proteins to SS18-associated regions in the nucleus where the fusion protein may exert abnormal effects. This was later found to be incorrect, with the *SSX* domain directing the fusion constructs to subcellular locations, as described below. In addition to these findings, dos Santos and colleagues also identified three putative bipartite nuclear localization signals (NLSs) that had not been previously reported. These NLSs, rich in lysine and arginine residues, were distributed along the protein sequence (aa9–23, aa52–64, aa158–172) with NLS3 (aa158–172) completely conserved among the known SSX family members. Following on this work, it was found shortly thereafter that the SS18 domain of SS18-SSX fusion constructs can act as a transcriptional coactivator (70-fold activation) and SSX1 could act as a transcriptional repressor (50-fold repression) when these constructs were coupled to a GAL4 DNA-binding domain in reporter assays (111). These investigators theorized that the C-terminal SSX domain may actually direct the SS18 activation domain to new or different target promoters. Lim and colleagues later confirmed that the SS18-SSX1/2 proteins are transactivators, while the KRAB domains of SSX genes function as low-level

transcriptional repressors (112). In GAL4 repression assays the KRAB domains alone from SSX1 and SSX2 downregulated transactivation of a reporter gene by 3.5- and 3.3-fold, respectively, compared to >83-fold repression for a control KOX1-KRAB domain that has been shown to have potent repression activity (113). This result further suggested that the SSX-KRAB domain may not function the same way as in other KRAB-containing proteins. Interestingly, the full-length SSX1 protein was able to repress transactivation in the GAL4 assay by 74-fold. They therefore postulated that another repression domain must exist in SSX that is distinct from the KRAB domain. Assaying deletion constructs, they narrowed down the area of the protein responsible for this repression to the last 33 C-terminal amino acids of SSX2. They called this domain the SSX repression domain (SSXRD), which was predicted to fold into an alpha helix. This domain had greater than 95% homology among the first five SSX family members identified at the amino acid sequence level and no homology was found for this domain among any proteins in the ProDom database within SwissProt. Of note, this domain is retained in SS18-SSX fusion proteins and therefore may contribute to the functional activity of this fusion construct. Since the full-length SSX protein was found to have greater repression activity in reporter assays than the SSXRD itself, this suggested that the KRAB domain may augment the activity of the SSXRD. The domains of SS18 and SSX responsible for nuclear and spatial targeting were later delineated by dos Santos et al. (114). Creating a series of FLAG- or VSV-tagged deletion mutants, they showed that the N-terminal amino

acids 51 to 90 of SS18 are responsible for its nuclear localization, even though this region does not contain an NLS, whereas SSX nuclear targeting was dependent on the last 34 C-terminal amino acids (SSXRD). Since this domain is conserved between family members in humans and other species, it suggests that this region is used in nuclear targeting for all SSX proteins. The putative NLS3, which was predicted earlier by dos Santos et al. to be the dominant SSX NLS (110), was confirmed by amino acid substitutions to play a role in nuclear targeting. However, knockout of NLS3 resulted in both cytoplasmic and nuclear staining suggesting that SSX may use additional, non-basic amino acid residues for nuclear targeting. Additionally, it was observed from truncated SS18-SSX constructs that the SSXRD is required for sub-nuclear localization for the fusion protein, producing the diffuse, even staining and fine punctate dots observed by others (110, 111, 115). Furthermore, these truncation experiments showed that the SSXRD is responsible for the association of SSX with mitotic chromosomes and localization to sites of Polycomb group silencing complex bodies (PcG bodies) in both transfected cells and 518A melanoma cells, suggesting that these functions of the SSXRD are endogenous. Another SSX domain was also identified by dos Santos et al., which is immediately upstream of the SSXRD and shows relatively high divergence in sequence between SSX family members (114). They called this sequence the SSX divergent domain (SSXDD), and its function remains unknown.

Interaction with the Polycomb Group Silencing Complex

To further elucidate SSX protein function, Soulez et al. carried out immunolabeling experiments with hemagglutinin (HA)-tagged SS18, SS18-SSX1, SS18-SSX2, SSX1, and SSX2 proteins expressed exogenously in transfected COS-7 cells, the SS cell line CME-1, and in the human fibrosarcoma cell line 2C4 (115). As observed by dos Santos et al. (110), the expression patterns of SSX1 and SSX2 were diffuse, whereas SS18 constructs were punctate. However, in the Soulez paper, toroidal-like structures were observed in 2C4 cells transfected with SS18 or SS18-SSX1/2, and two prominent foci were observed in the cells transfected with SS18-SSX1/2 or SSX1/2. To determine if these proteins colocalize with any known nuclear compartments, these investigators carried out double immunofluorescence studies with antibodies against SS18, SSX, and other characterized nuclear antigens. Soulez and colleagues confirmed that neither SSX, SS18, nor the fusion constructs localize at sites of active transcription, however, they did find that SS18-SSX1 and SS18-SSX2 expression colocalized with members of the polycomb group (PcG) complex (116). Specifically, SS18-SSX2 was found to localize to sites of RING1 expression in all cells examined, and SS18-SSX1 and SS18-SSX2 colocalized to sites of endogenous BMI-1 expression (both members of the PcG complex). Further labeling experiments showed no association between SS18 and BMI-1, yet SSX1 and SSX2 were both shown to colocalize with BMI-1. This result suggested that the SSX portion of SS18-SSX directs localization of the fusion constructs within

the nucleus to sites of PcG body accumulation, contrary to what was previously theorized by dos Santos et al. and Brett et al. (110, 111). Specifically, it has been shown that the SSXRD is responsible for association of SSX with PcG proteins and core histones (114, 117). Although much is still not known about the function of PcG complexes in mammals, it has been demonstrated that these protein complexes have some role in the regulation of cell cycle progression and differentiation (118). PcG knockout experiments and overexpression studies have shown that PcG genes are associated with proliferation of progenitor cells in the murine haematopoietic system, with BMI-1 overexpression leading to lymphoma development (119, 120). It has been shown in *Drosophila* that multimeric PcG complexes function to maintain homeotic gene silencing, serving as a stable repressor system to ensure the proper timing of developmental programs (121, 122). Like SSX proteins, many PcG complex members lack DNA-binding domains and are believed to exert their repressive functions through epigenetic regulation of chromatin structure via protein-protein interactions (123). Co-immunoprecipitation studies of tagged SSX or SS18-SSX using antibodies for RING-1 and BMI-1, however, showed no *direct* association between these proteins. This result suggested that SSX may interact with other components of the large PcG complexes, perhaps proteins not yet identified (Figure 2). Since it had been previously reported that PcG complexes interact with chromatin throughout mitosis (116), Soulez et al. carried out immunolabeling experiments of transfected cells with propidium iodide-stained chromatin to

determine if SSX associates with condensed chromatin (115). No association was found between SS18 and chromatin distribution, however both SSX1 and SSX2 were found to be distributed evenly over condensed chromosomes during mitosis with especially dense staining during prometaphase and metaphase. Unlike SSX1 or SSX2, SS18-SSX2 associated with prometaphase/metaphase chromosomes in punctate patterns similar to those described for RING-1 and BMI-1 (115), perhaps suggesting a functional difference between wild type SSX and the fusion protein that could potentially lead to SS.

Interaction with RAB3IP and SSX2IP

Using yeast two-hybrid assays and glutathione-S-transferase (GST) pulldown assays, de Bruijn et al. identified the first two proteins that directly associated with SSX2 (124). Three clones were identified from the yeast two-hybrid screens, encoding sequences 1.1, 1.5, and 1.7 kb long. The 1.7- and 1.5-kb clones pulled out were sequenced and identified as RAB3IP, a human homologue of the *Rattus norvegicus Rabin3* gene, which shared 84% cDNA and 88% protein identity, respectively. The two different-sized clones were found to be alternative splices of *RAB3IP*, and were designated *RAB3IPα* and *RAB3IPγ*, with *RAB3IPα* encoding a predicted 51 kDa protein 460 amino acids long, which is analogous to Rabin3 in rats. The 1.7- and 1.5-kb inserts also showed homology to the *Pat-12* gene that has been characterized in mice (125). Both *Rabin3* and *Pat-12* genes are thought to potentially encode Ras-like GTPase-

binding proteins, but these proteins are not fully characterized (126). The 1.1-kb clones identified as SSX2IP were predicted to encode a 71 kDa protein 614 amino acids long, which had no homology to any known sequences. RAB3IP did not interact with SSX1, SSX3, or SSX4, whereas SSX2IP interacted with SSX3 but not SSX1 or SSX4, which may suggest that the SSX members have different functions. By carrying out deletion mutant studies, they also determined that these two proteins interact with the N-terminal moiety of SSX2, with RAB3IP interacting with SSX2 aa 25 to 80 and SSX2IP interacting with SSX2 aa 1 to 80 (Figure 2). Interestingly, the region of SSX2 that interacts with RAB3IP almost exactly overlaps with the SSX2 KRAB domain, which spans aa 20 to 83. Since RAB3IP was found to be expressed in many different tissues by Northern blot analysis and has homology to proteins in species as diverse as *C. elegans* and yeast, it was suggested that RAB3IP may be a house-keeping gene. Murine homologues to SSX2IP were identified from blast analysis and ubiquitous expression was found for this gene as well. The presence of four coiled-coil domains, and its homology to known structural proteins, suggests that SSX2IP may function in nuclear architecture, while the many motifs identified in RAB3IP, including coiled-coiled and ER-retention motifs, indicate that this protein may carry out diverse functions. Interestingly, RAB3IP is found on chromosome 12q13-14, a region known to be frequently rearranged or amplified in human cancers (127, 128), and SSX2IP is on 1p22 also in a region commonly deleted, amplified, or translocated in human tumors (129-131). Immunofluorescence

studies showed that RAB3IP normally localized to the cytoplasm (like Rabin3), but interestingly, when cells were transfected with both RAB3IP and SSX2, 10% of RAB3IP protein translocated to the nucleus, whereas SSX2IP was always shown to colocalize with SSX2 in the nucleus (124). Further analysis of SSX2IP was recently reported by Breslin and colleagues in a review on SSX2IP and its emerging role in cancer (132). They report that SSX2IP was identified as a leukemia antigen through a SEREX screen, specifically recognized in the sera of patients with acute myeloid leukemia (AML) as compared to sera from healthy donors, and that SSX2IP expression was found in 33% of leukemic cells from AML patients but not in normal donor hematopoietic cells tested by RT-PCR (133). SSX2IP has also been shown to be highly expressed on the cell surface of myeloid leukemia cells and AML tissue samples using immunohistochemistry, flow analysis, and confocal microscopy (134). This surface expression on myeloid leukemia cell lines appeared to peak during mitosis. SSX2IP is also known as human afadin DIL domain-interacting protein (ADIP) for its homology to the mouse and rat ADIP genes, which are 88% and 87% identical to SSX2IP, respectively. Microarray data has demonstrated lower expression of SSX2IP in AML patients with *t*(8;21) translocation, while higher expression of SSX2IP was associated with the *t*(15;17) translocation (135). Of note, other genes elevated in expression in patients with the *t*(15;17) translocation include proteins involved in cell cycle regulation (e.g., p57Kip2, cdk7, cyclins D2, D3, E2 and B2), replication

(Cdc6), and mitosis (survivin and CENPJ). In human leukemias, SSX2IP appeared to be expressed in cell cycle-regulated patterns.

Interaction with LHX4

In an attempt to comprehensively identify proteins that associate with SSX, de Bruijn and colleagues conducted yeast two-hybrid studies and found that one of the clones pulled out encoded the LIM homeobox protein LHX4 (136). This protein was previously shown to be deregulated or translocated in multiple forms of leukemia (137-141). The C-terminal SSXRD domains of SSX1, SSX2, and SSX4 all interacted with LHX4 in yeast two-hybrid assays, suggesting that this protein interaction is common to SSX proteins. LHX4 is a 390 amino-acid protein containing two LIM domains (LIM1 and LIM2), a homeobox domain (HOX), two zinc fingers, and was found to have a C-terminal tail with a novel transcriptional activation domain. Utilizing immunofluorescence, de Bruijn and colleagues demonstrated that SSX1 and SS18-SSX colocalized with LHX4 in transfected COS1 and HeLa cells; however, in a small percentage of cells the overlap was not entirely complete suggesting that these interactions between SSX1 and LHX4 are dynamic. The interaction of SSX1 and SS18-SSX with LHX4 was subsequently verified through co-immunoprecipitation analysis. To evaluate whether SSX or SS18-SSX can alter the expression of LHX4 target genes, de Bruijn et al. conducted ChIP and transactivation assays. The glycoprotein hormone-*α* (*CGA*) gene, encoding CGA, is a known target of LHX4 binding and

transcriptional regulation (142). LHX4 was shown to bind directly to the CGA promoter, with increased CGA transactivation in conjunction with SS18-SSX2 in luciferase reporter assays of transfected SYO1 SS cells. Interestingly, in similar assays it was found that SSX2 alone actually had an inhibitory effect on CGA activation, which indicates that SS18-SSX2 and SSX2 have opposite activities of transcriptional regulation in SS. It was proposed in this study that the interaction between SSXRD and LHX4 may be dynamic, with LHX4 competing with PcG proteins for the SSXRD, perhaps altering the transcriptional corepression activities of SSX (Figure 2).

Expression in Mesenchymal Stem Cells

The expression patterns of CTA proteins in testis germline cells that are constantly undergoing proliferation prompted Cronwright and colleagues to evaluate SSX expression in normal somatic cells that are also capable of self-renewal and endless proliferation (143). Specifically, this group investigated the expression of members of CTA gene families including NY-ESO-1, MAGE-A, GAGE, RAGE, and SSX in human postnatal bone marrow and fetal liver mesenchymal stem cells (MSCs). SSX was found to be expressed in melanoma cells, fetal MSCs, adult MSCs, and bone marrow by RT-PCR. Surprisingly, immunofluorescence analysis of MSCs showed both fine and granulated patterns of SSX expression in the cytoplasm of these cells. Bone marrow smears stained for SSX expression showed a few SSX-positive cells that were also CD34+

(hematopoietic stem cells). Immunostaining the MSCs for CTAs after the cells were allowed to partially differentiate into adipocytes or osteocytes, Cronwright's group observed that the CTA expression was markedly decreased, especially for SSX (143). This result was confirmed by RT-PCR analysis as well. Decreased levels of SSX expression were also observed in human fetal tissues allowed to differentiate. Double-immunolabeling of SSX with known proteins associated with migration, invasion, and metastasis were also carried out. The specific proteins evaluated were matrix metalloproteinase 2 (MMP2), which cleaves a number of cell matrix proteins and is associated with invasion and metastasis, vimentin, a component of cytoskeleton intermediate filaments, and fibronectin and laminin, involved with cell adhesion. SSX colocalized with both MMP2 and vimentin. SSX expression was observed to overlap with laminin but no direct colocalization was observed, whereas no overlap was observed with fibronectin. Direct associations between SSX and these proteins were not observed from coimmunoprecipitation studies, however it was found that when the melanoma cell line DFW (known to express SSX) was knocked out for SSX expression, DFW cells had 40% decreased migration in soft agar compared to SSX+ DFW cells, and this reduction was accompanied by a decrease in MMP2 expression. Vimentin and E-cadherin levels were not influenced by lack of SSX expression in these cells. Also, it was observed that SSX expression is ablated during MSC differentiation, while MMP2 expression decreases and vimentin levels remain unchanged. Overall, even though SSX was not found to directly interact with

MMP2, it does appear to indirectly influence its expression patterns, implicating a link between SSX expression and the processes of self-renewal and tumorigenicity. This study provided the first potential evidence of a link between SSX expression and the processes of cell migration and metastasis. It was also proposed in this study that if CTAs are expressed in stem cells with limitless proliferation potential then their ectopic expression in tumor tissue may be a hallmark of dedifferentiation back into a stem-cell like state, perhaps through an altered regeneration program (143).

SSX Expression in Testis and Tumor Tissues

The normal expression of SSX mRNA has been shown by multiple investigators to be predominantly restricted to testis germline tissue (98, 99, 104, 144). SSX protein expression in the testis is predominantly localized to the nucleus of spermatogonia cells and occasionally in spermatocytes as demonstrated by IHC (145). These expression patterns were heterogeneous, with only a subset of spermatogonia found to have SSX nuclear staining. Constitutive genome-wide demethylation has been demonstrated in spermatogonia cells where SSX proteins are normally expressed (146, 147), and interestingly, these same global demethylation patterns have been observed in tumor tissue and tumor cell lines and are associated with the reactivation of silenced genes (148, 149). This finding indicates that the same physiological mechanisms that lead to SSX expression in the testis may be ectopically reactivated in tumor tissues. Except

for SSX6 and SSX10, expression of all SSX family members has been demonstrated in testis tissue (Table 1) (106, 150-152).

The expression of SSX family members has also been demonstrated ectopically in many types of cancer (Table 2). When Türeci et al. initially identified SSX2 as the tumor antigen HOM-Mel-40, they also reported by RT-PCR and Northern blot that SSX2 mRNA was expressed in the following tumor tissues: melanoma (8/16), colorectal carcinoma (9/35), prostate cancer (5/25), breast cancer (7/36), hepatocellular carcinoma (3/6), glioma (2/23), lymphoma or leukemia (1/9), gastric carcinoma (1/12), and thyroid carcinoma tissue samples (2/4) (Table 2) (99). In 1996 Türeci et al. carried out a more comprehensive study evaluating SSX family member mRNA expression in cancers of different histological types. In this analysis they found that SSX1, 2, and 4 were expressed in 8%, 15%, and 15% of tumors, respectively, with no detection of SSX3 in any samples and detection of SSX5 in only 7 out of the total 325 tumor specimens evaluated (153). Specifically, SSX mRNA was expressed in 75% of synovial sarcoma, 57% of head and neck cancer, 55% of bladder cancer, 50% of ovarian cancer, 43% of malignant melanoma, 40% of prostate cancer, 36% of non-Hodgkin's lymphoma, 33% of stomach cancer, 27% of colorectal cancer, 21% of breast cancer, 21% of lung, 16% of glioma, 13% of endometrial, and 4% of renal cell carcinoma tissues (Table 2). Expression of more than one SSX family member was found in

several tumor types, and altogether 27% of tumor tissues expressed at least one SSX family member.

In other reports of SSX family member tumor expression, SSX5 expression has been found in multiple myeloma (154), osteosarcoma (155), and hepatocellular carcinoma (156), while SSX1 expression has been found in these tumors as well as nonsmall cell lung cancer, Hodgkin's lymphoma, nonHodgkin's lymphoma, leukemia, bladder cancer, breast cancer, colorectal cancer, endometrial cancer, head and neck cancer, and malignant melanoma (153-162). SSX2 and SSX4 expression have been found in all of the cancer types listed above as well as neuroblastoma, gastric cancer, ovarian cancer, synovial sarcoma, mesothelioma (SSX2), pancreatic cancer (SSX4), and intrahepatic cholangiocarcinoma (SSX4) (Table 2) (99, 153-169). SSX expression has also been evaluated by RT-PCR in brain tumors in which SSX1 was only expressed in astrocytomas, while SSX2 was expressed in astrocytomas and oligoastrocytomas, and SSX4 was expressed in both of these as well as oligodendrogliomas (169).

Using a novel sequence-based amplification assay to determine SSX mRNA quantities in 211 bone and soft tissue tumor specimens, Naka et al. found that SSX expression levels varied greatly among tumor samples, and they found that malignant tumors showed much higher SSX expression levels than benign tumors ($P < .0001$) (170). Additionally, stage III tumors had significantly higher

SSX mRNA expression levels as compared to stage I or stage II tumors (P < .005). These results demonstrated an association of SSX expression with more advanced stage of disease in cancer patients. In another study by Taylor et al. it was found that SSX1, SSX2, SSX4, and SSX5 were all coexpressed in 20% of patients with multiple myeloma (MM), and this coexpression was found to correlate with adverse prognosis and reduced survival (P = .0006) (154). Of these four SSX family members, SSX2 was found to be the most strongly associated with worse prognosis (P =.0001). Other MM patients not expressing all four of these SSX family members were often found to express one or more SSX proteins. In addition to MM, SSX2 expression in prostate cancer has been reported by our group to be associated with advanced-stage prostate cancer (151).

Like normal testis cells, the overall expression of SSX protein in human tumor tissue samples has been shown to be quite heterogeneous in expression (145). SSX nuclear staining was found in one report to be present in 24% of melanoma locoregional metastases and 40% of primary melanoma tissue. Within the majority of all lesions less than 25% of the tumor cells were found to be SSX positive. Staining was observed in four main patterns, (a) widespread, with >75% of cells expressing, (b) focal, with clustered positive cells in a limited tumor area, (c) scattered, with a few positive cells localized throughout the lesion, or (d) isolated, with just a few positive cells in several tumor areas. This

heterogeneous expression has been a major concern for tumor immunotherapy since the outgrowth of antigen-negative cells might develop with targeted antigen-specific therapy.

Since SSX expression has been shown in mesenchymal stem cells (143), melanoma stem cells (171), and only heterogeneously in many tumor types, it may be necessary to use agents to increase SSX antigen expression in tumor tissue to therapeutically target these antigens. Within tumor tissue it may be that cells expressing SSX proteins are stem cells, dividing cells, or those that have malignant potential, and perhaps this is why SSX expression appears so heterogeneous in tumor lesions. These may be precisely the cells of most interest for therapeutic targeting. Conversely, if not all malignant cells express the SSX antigen, then outgrowth of SSX-negative escape variants could potentially develop. Recent work with epigenetic modifying agents may present one possible solution to this problem. In a report by dos Santos et al. it was found that SSX2 mRNA and protein expression could be induced with 5-aza-2-deoxycytidine (5-aza-dc) treatment in cultured BLM melanoma cells (SSX-negative) and K562 erythroleukemia cells (SSX-positive) (145). Specifically, RT-PCR showed that SSX mRNA expression was clearly induced in BLM cells and increased in K562 cells with 5-aza-dc treatment, while SSX protein expression was found by immunofluorescence staining in 9–13% of all treated cells. No protein expression was found in the absence of 5-aza-dc treatment. SSX

expression has also been shown to be upregulated with 5-aza-dc in bladder cancer cell lines that are SSX-negative prior to treatment (172), and SSX2 has been shown to be inducible with 5-aza-dc in mesothelioma (165). We have also reported that SSX2 expression can be upregulated selectively in prostate cancer cell lines, but not normal prostate epithelial cells, by treatment with 5-aza-dc (151). In a report by Güre et al. it was found that SSX4 was inducible with the histone deacetylase inhibitor Trichostatin A (TSA) in melanoma cell lines, with minor effects for SSX2 and SSX6, whereas 5-aza-dc was able to more effectively induce expression of SSX1, 2, 4, and 5 in melanoma cell lines (106). SSX6 expression was only slightly inducible with 5 azadc in these studies, and overall, SSX4 was the most frequently induced family member in these particular cells, followed by SSX2, SSX5, SSX1, and SSX6 (Table 1). Finally, in another report by Sigalotti et al. it was shown that upregulation of SSX expression in melanoma cell clones with 5-aza-dc treatment was directly correlated with promoter demethylation patterns (171). This finding provided a clear mechanism of action for upregulating CTA expression in cancer cells with DNA methyltransferase inhibitors, which was thought to explain the intratumoral heterogeneity of CTA expression patterns, and provided further support for the use of pharmacologic agents to upregulate antigen expression on tumor cells for targeted tumor immunotherapy (171).

SSX Immunology

The interest in SSX proteins as immunotherapeutic targets began with the identification of SSX2 as the tumor antigen HOM-Mel-40 (99), which had been shown to be the target of humoral immune responses in as many as 10% of patients with melanoma (99, 173, 174). In other studies humoral SSX2 immune responses were observed in 2 out of 74 colon cancer patient sera samples (175), 1 out of 100 prostate cancer patient sera (151), and antibody responses to SSX4, but not SSX2, have been identified in 2 out of 131 patients with gynecological cancers but not in healthy controls (176). SSX common antigen antibody responses have also been detected in sera from pancreatic, lung, breast, colon, and ovarian cancer patients but not in healthy control individuals (177). Additionally, antibodies specific for SSX1, 2, 3, and 4 have been found in the sera of cancer patients with melanoma, colon cancer, and/or breast cancer (104, 175, 178). In another study by Valmori et al. analysis of SSX4 IgG immune responses in epithelial ovarian cancer (EOC) patients showed that two patients out of 109 had both SSX2 and SSX4 IgG antibody responses (179). These responses were confirmed by ELISA, and both patients with dual antibody responses were alive and showed antibody responses >5 years after initial therapy. Observing that cancer patients can have preexisting SSX2 IgG-specific immune responses, other investigators sought to identify if cancer patients can have cell-mediated immune responses to SSX proteins. Ayyoub et al. carried out the first investigation to identify SSX2 T-cell epitopes by utilizing an altered reverse immunology strategy (180). Purified standard proteasome complexes

from human erythrocytes were incubated *in vitro* with a library of overlapping SSX2 peptides covering the entire 188 aa protein sequence. The digested products were analyzed by mass spectrometry, which identified 12 peptides that were processed from the proteasome: SSX2p5–13, p7–15, p15–24, p16–24, p40–49, p41–49, p50–59, p53–61, p57–65, p58–67, p59–67, and p103–111. These peptides were subsequently cultured with cells from the tumor-infiltrated lymph node (TILN) of an SSX2-positive metastatic melanoma lesion (LAU 50), and an IFN*γ*-ELISPOT was performed to detect SSX2 peptide-specific T-cell immune responses. SSX2 peptide p41–49 elicited the greatest T-cell immune response from the TILN, which led the group to synthesize HLA-A2 multimers containing SSX2 peptide p41–49 (Table 3). These multimers were used to stain the TILN cells and showed clearly positive SSX2 p41–49-specific CD8+ T cells. It was also shown that SSX2p41–49 CD8+ T cells (CTL clone LAU 50 E2.4) could lyse peptide-pulsed T2 cells and HLA-A2+ melanoma cell lines. Interestingly, when this clone was incubated with COS7 cells transfected with plasmids encoding HLA-A2 and SSX2 or SSX4, high levels of IFN*γ* secretion and lysis were observed by the clone with SSX2-transfected cells but not SSX4-transfected cells. Since SSX2 and SSX4 only differ by 2 aa within the antigenic epitope region, this result suggested high specificity of the clone for SSX2 p41-49. None of the other predicted SSX2 epitopes were recognized by TILN from this patient. Following on this work, Ayyoub et al. utilized fluorescent HLA-A2/SSX2p41–49 multimers to determine the relative frequencies of SSX2 p41-49

CD8+ T cells in melanoma patients with SSX2-positive or negative tumors and in healthy donors (181). They found that SSX2 p41–49-specific CTLs were identifiable in both melanoma patients and healthy donors, although at lower frequency in healthy donors or patients with SSX2-negative tumors. They showed that p41–49-specific CTLs from the TILN and peripheral blood mononuclear cells (PBMCs) of patients with SSX2-expressing tumors were better able to lyse tumor cells compared to CTLs from the TILN and PBMCs from patients with SSX2-negative tumors or PBMCs from healthy donors. It was also shown that SSX2-specific CTL frequency increased with disease progression in at least one melanoma patient. SSX2 p41–49 T cells have also been identified in patients with hepatocellular carcinoma (HCC) (182). These CD8+ T cells were identified using SSX2 p41–49/HLA-A2 multimers in one out of six patients with HCC, both in the TILN and PBMCs. A polyclonal SSX2 p41–49 T-cell line generated from this patient lysed p41–49-pulsed T2 cells, melanoma cell line MEL 275, and a p41–49 peptide-pulsed HCC cell line. This was the second solid human malignancy with evidence of recognition by SSX2 p41–49-specific CTL. The immunogenicity of SSX2 p41–49 was later highlighted when Rubio-Godoy and colleagues identified this peptide through an approach called positional scanning of synthetic combinatorial peptide library analysis (183). In this report, 3.1×10^{11} nonamer peptides, arranged in a positional scanning format, were screened for melanoma-reactive CTL of unknown specificity and assessed for their ability to elicit peptide-specific CTL. The identified optimal peptide

sequence (AAAPKIFYA) was very similar to SSX2 peptide p41–49 (KASEKIFYV). Recognition of this epitope by melanoma-reactive CTL clone LAU 50/4D7 was confirmed by cytotoxicity assay, and interestingly, the native SSX2 peptide p41–49 had recognition that was in the same range as the optimal peptide. Attempts to modify the anchor residues of the optimally identified peptide or the native p41–49 epitope did not result in enhanced peptide recognition. Additionally, no cross-reactivity of p41 from SSX1, SSX3, SSX4, or SSX5 was observed. Further, a biometrical analysis of the screening data was used to generate a scoring matrix of all predicted peptides in public protein databases that could potentially be recognized by clone LAU 50/4D7, and, amazingly, this approach ranked SSX2 p41–49 27th out of approximately 16 million nonamer peptides (184). If this analysis was restricted to known tumor antigens in humans, this rank was 2nd out of 400,271 peptides. Ranked scores for other SSX peptides were much lower, however. It was noted that this method of scoring does not omit other SSX peptides as potentially immunogenic, but it does emphasize the immunogenicity of SSX2 p41–49. In an effort to identify additional class I MHC-restricted SSX2 epitopes, Wagner et al. used a reverse immunology approach to identify SSX2 epitopes that were predicted to have affinity for the human HLA-A2 MHC class I complex using the SYFPEITHI algorithm (185, 186). PBMCs from seven breast cancer patients and eleven healthy donors were evaluated for reactivity to peptides p41–49 and p103–111, and p167–175, which had all been predicted from the peptide-binding algorithm

to have affinity for HLAA2. They found that 5/7 (71%) of the HLA-A2+ breast cancer patients and 6/11 (55%) of the HLAA2+ healthy controls had T cells that were reactive to SSX2 peptide p103–111 by IFN*γ*-ELISPOT assay. HLA-A2 restriction of the responses was verified using specific HLA blocking antibodies. SSX2 p103–111 was also shown to be a naturally presented SSX2 epitope by the recognition of SSX2+ SKMEL-37 melanoma cell line and transfected COS7 cells by p103-111 peptide-specific CD8+ T cells (Table 3). Also, SSX2 p103-111-specific T cells could lyse peptide-pulsed target cells in cytotoxicity assays. Interestingly, no association was found between SSX2 antibody titer in breast cancer patient serum and p103–111-specific T-cell responses, indicating that the humoral and cell-mediated responses to this antigen are regulated independently. Rentzsch and colleagues found that PBMCs from one out of ten primary breast cancer patients exposed to SSX2-p103–111 significantly increased their mRNA expression of IFN*γ* by QT-RTPCR analysis (187). This immune response was also detected in one of eleven healthy control individuals. Not only has the endogenous processing and presentation of SSX2 peptide p103–111 been demonstrated by reactivity of p103–111-specific CTL for SSX2-expressing tumor cells, it has also recently been shown that this peptide epitope is directly presented on the surface of cancer cells (150). By utilizing a human phage library screening technique Held et al. generated SSX2 p103-111-specific Fab antibodies that specifically recognized and bound to this peptide in the context of HLA-A2. These antibodies were used to stain melanoma cell lines in

fluorescence microscopy studies, and it was observed that a majority of SK-Mel-37 cells expressed p103-111/HLA-A2, whereas Me275 cells expressed very little (<1% of cells). These cells were also used to stimulate SSX1 p103-111-specific T cell clones, and it was determined that the expression of p103-111/HLA-A2 on the cell surface, and not the total SSX2 protein expression levels of the cells, was correlated with enhanced CTL recognition and activation. Ayyoub et al. also identified the first CD4 class II MHC SSX2 epitope (188). This epitope was mapped to aa 19-34 of SSX2 using truncated peptide assays and was recognized by CD4+ T cells from an SSX2-expressing melanoma patient (Table 3). No responses to this epitope were found in healthy controls, and class II blocking antibody experiments verified that this epitope is recognized in the context of HLADPB1*0101. Many studies have documented the atypical expression of class II MHC molecules on the surface of colon carcinomas, breast cancer cells, sarcomas, and melanoma cells (189-193). This expression prompted the investigators in the current study to evaluate whether SSX2 p19-34 is processed endogenously and presented on the surface of tumor cells. Interestingly, SSX2 CD4+ T cells failed to recognize IFN*γ*-treated melanoma cells expressing SSX2 and class II MHC molecules (HLA-DP); however, they were stimulated by antigen-loaded dendritic cells. This result suggests that p19-34 may not be endogenously presented on tumor cells but *can* be loaded exogenously and presented by APCs. Ayyoub et al. also identified an HLA-DRB*1101-restricted SSX2 class II peptide p45-59 (194). CD4+ T cells specific

for this epitope were detected among PBMCs and in the TILN from melanoma patients but not in healthy control individuals. Using the SYFPEITHI algorithm, p45-59 was ranked as the 2nd highest predicted HLA-DR-binding peptide for SSX2, whereas this peptide ranked 3rd, 13th, and 2nd for SSX1, SSX4, and SSX5, respectively. Titrated concentrations of peptides incubated with p45-59-specific CD4+ T cells showed cross-reactivity to SSX5 p45-59 and low-level reactivity to SSX1 p45-59 in IFN*γ* ELISA assays. Eleven of nineteen melanoma patients had CD4+ T cells responses to this epitope region (i.e., aa 37-58). Contrary to what was found for the class II peptide p19-34, T cells recognized epitope p45-59 on both antigen-loaded DCs as well as HLA-DR+ tumor cells. This peptide was also described by Neumann and colleagues in which p45-59 was found to be restricted to HLA-DRB1 subtypes *0701, *1101, *1302 and B3*0301, and it was demonstrated that p45-59 CD4+ T cell responses could be induced in 3/6 of breast cancer patients and 1/5 of healthy controls (195). However, no correlation was found between SSX2 antibody titer and CD4+ T cell responses. After the identification of the first two class II SSX2 epitopes Ayyoub and colleagues identified yet another CD4 peptide that overlapped with these epitopes (196). This epitope region was narrowed down to amino acids p37-51 of SSX2 by truncation experiments and was shown to be restricted to HLA-DRB*0301 by class II MHC blocking antibody assays. Since this was the third immunogenic peptide identified in the KRAB domain region of SSX2, it was theorized that this region may be a “hot spot” for T-cell recognition. Again, wild

type melanoma cell lines expressing MHC class II molecules or tumor cells transfected with plasmid encoding SSX2 were not recognized by epitope-specific CD4+ T cells directly, however, p37-51 peptide-pulsed tumor cells *were* recognized by both SSX2 p37-51-specific CD4+ T cells and SSX2 p41-49 peptide-specific CD8+ T cells. Of note, the transfected cells were recognized by the p41-49-specific T cells but not the p37-51-specific CD4+ T cells, confirming that the p41-49 peptide can be endogenously processed and presented by tumor cells. Cross reactivity of SSX2 p37-51-specific CD4+ T cells was observed for SSX4 and SSX5 but not SSX1 or SSX3 p37-51 peptides in an IFN*γ* peptide titration ELISA assay. Since it was previously observed that SSX4 was expressed in ~20% of malignant melanomas in a report by Türeci et al. (153), Ayyoub and colleagues also evaluated the presence of CD4+ T cells specific to this antigen in melanoma patients by stimulating CD4+ T cells isolated from the PBMCs of four melanoma patients with overlapping peptides derived from the amino acid sequence of SSX4 in the presence of APCs (197). Intracellular IFN*γ* cytokine staining revealed that all four patients had CD4+ T cells that were activated in the presence of at least one of the SSX4 peptides (Table 3). Again, as was found for the SSX2 class II peptides, CD4+ T cells could not recognize melanoma cells directly but were activated by APCs loaded with SSX4 antigen, which suggested that this peptide may not be presented through the endogenous pathway in tumor cells. Interestingly, five out of the seven peptides identified in this study mapped to the KRAB domain of SSX4. Four previously identified

SSX2 epitopes also mapped to this region, further suggesting that this may be a "hot spot" for T-cell recognition. Valmori et al. found that epithelial ovarian cancer (EOC) patients also had SSX4 CD4+ T cells that recognized some of these epitopes (179). Several of the SSX4 class II peptides that were identified previously were later reported by Godefroy et al. to also be epitopes encoded from the SSX1 protein (198). In this study an overlapping pool of peptides spanning the entire SSX1 protein sequence was incubated with PBMCs from cancer-free individuals; SSX1-peptide-specific CD4+ T cells were identified in 5/5 of the donors analyzed. These T cell populations almost exclusively recognized epitopes from three defined regions of the protein sequence, including the KRAB domain and two C-terminal regions of the protein that are retained in the SS18-SSX fusion construct (Table 3) (197). Evaluating the cross-reactivity of these peptide-specific clones for other SSX family members, however, revealed only limited recognition of p41-60 for SSX5. This lack of cross-reactivity was explained by the presence of several amino acid differences in this epitope region between family members. As before, no endogenous processing and presentation of SSX1 epitopes by tumor cells was demonstrated. Since it had been shown that tumors can express more than one SSX family member at the same time and that T-cell responses can be found to multiple SSX family members in cancer patients, He et al. assessed the utility of an altered peptide ligand strategy to identify class I epitopes that could be used to target multiple SSX family members (199). Using peptide prediction algorithms He and

colleagues identified SSX p57-65 and p99-107 as shared epitopes between family members SSX1-9. Since SSX p57-65 had the higher binding score for all nine family members, this peptide was selected for further analysis. Using T2 binding assays with four altered p57-65 peptides specific to SSX1-9, it was shown that altered peptide P4 (AMTKLGFNV), which is encoded by SSX6 and SSX8, had the greatest *in vitro* binding affinity for HLA-A2, and this affinity was stable at low peptide concentrations. All four altered p57-65 peptides were shown to elicit peptide-specific CTL from the PBMCs of healthy HLA-A*0201 individuals, however P4-specific CTL showed the greatest lysis and IFN*γ* secretion when incubated with peptide-pulsed target cells. Additionally, P4 showed the greatest cross-reactivity to other altered p57-65 peptides presented by target cells in cytotoxicity assays. HLA-A2.1/Kb mice immunized with P4 generated CTL that could be cultured *ex vivo*, and were shown to also lyse T2 cells displaying the immunizing peptide or the three other altered p57-65 peptides, which suggests that this altered peptide strategy might be successfully utilized to target multiple SSX family members (Table 3).

Concluding Remarks and Future Directions

The SSX proteins are a highly homologous group of CTX antigens with demonstrated immunogenicity in patients with cancer, and a number of characteristics that make them attractive targets for tumor therapy. Cheever et al. recently reported that SSX2 in particular may be a high-priority target for

cancer therapy based upon certain predefined criteria for prioritization of tumor antigens, such as tumor specificity, oncogenicity, expression level, and number of identified epitopes (17). As essentially tumor-specific antigens from the immunological perspective, SSX proteins may therefore represent ideal targets for tumor immunotherapy.

As described previously, several groups have demonstrated that SSX mRNA or protein expression in tumors is correlated with more advanced stages of disease and a worse patient prognosis. In the work that will be presented here we seek to identify which SSX family members are expressed in prostate cancer, both in cell lines and prostate cancer tissue biopsies. Additional work needs to be done to evaluate the function of SSX proteins in cancer to determine if the expression of these proteins is a bystander effect of epigenetic reprogramming or whether the proteins themselves have inherent transforming activity and contribute to the cancer phenotype. For instance, it has been shown that SSX proteins in mesenchymal stem cells colocalize with MMP2, and in knock-down studies it appears that SSX expression is correlated with an invasive phenotype (143). Additionally, the presence of SSX in the SS18-SSX fusion construct that is expressed in >95% of SS appears to contribute to transformation as well (92), and may be a reasonable target for drug or vaccine-based therapy. Although this fusion protein is expected to have a different function than native SSX proteins,

the frequent association of SSX proteins with a cancer phenotype suggests a possible functional role leading to tumor formation.

Due to their high degree of homology and the identification of conserved “hot spots” of epitope recognition, it might be feasible to target multiple SSX family member proteins simultaneously in tumors co-expressing these SSX family members. In the studies presented here, we seek to identify immunogenic SSX2 epitopes and evaluate whether a DNA vaccine encoding a single SSX family member can elicit cross-reactive immune responses to multiple SSX family members in pre-clinical rodent studies. It may be possible to elicit immune responses to these proteins by immunizing patients with vaccines encoding native SSX peptides or protein, or modified vaccines designed to target several SSX family members. Thus, we also sought to determine if SSX2 DNA vaccine efficacy could be enhanced by encoding altered peptides with increased binding to MHC class I, thereby potentially eliciting more robust epitope-specific immune responses *in vivo*. Moreover, we evaluate the ability of both native and modified SSX vaccines to elicit anti-tumor immune responses in an antigen-controlled tumor model.

Doctoral Thesis Objectives

For my doctoral thesis, I propose to evaluate the SSX family of cancer-testis antigens as immunological targets for the treatment of prostate cancer, testing the hypothesis that immunotherapeutic vaccines targeting SSX proteins can elicit anti-tumor immune responses *in vivo*. To test this hypothesis, I will evaluate three specific aims:

1. To determine which SSX family members are potential target antigens for prostate cancer.
2. To identify HLA-A2-restricted SSX epitopes and determine if SSX vaccines can elicit peptide-specific CTL capable of lysing prostate cancer cells.
3. To determine if SSX DNA vaccine efficacy can be enhanced by encoding mutations designed to increase HLA-A2 binding of specific peptides, and if vaccination can induce anti-tumor immune responses in A2/DR1 mice bearing SSX-expressing tumors.

Figures and Tables

Figure 1. Protein homology and conserved domains among SSX orthologues.

Shown is a sequence alignment of species-specific SSX protein sequences from canine, horse, human, macaque, chimpanzee, marmoset, mouse, and rat sources. Blue highlighting represents >75% sequence identity with consensus sequence shown on the bottom. The KRAB and SSXRD domain regions denoted by solid green bars are shown below the sequence alignment.

Figure 1. Protein homology and conserved domains among SSX orthologues.

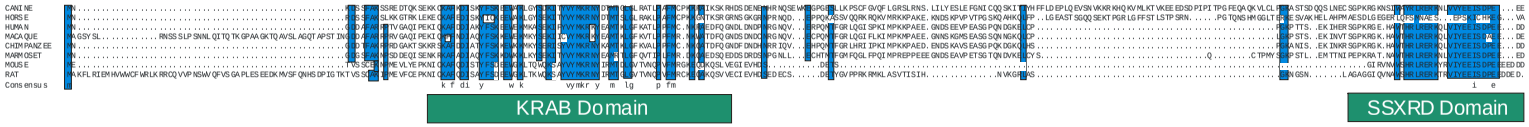

Figure 2: Functional interactions of the SSX proteins.

The RAB3IP and SSX2IP protein interaction with the SSX KRAB domain and LHX4 and PcG protein interactions with the SSXRD domain are depicted. Dynamic intracellular and intranuclear interactions are highlighted.

Figure 2: Functional interactions of the SSX proteins.

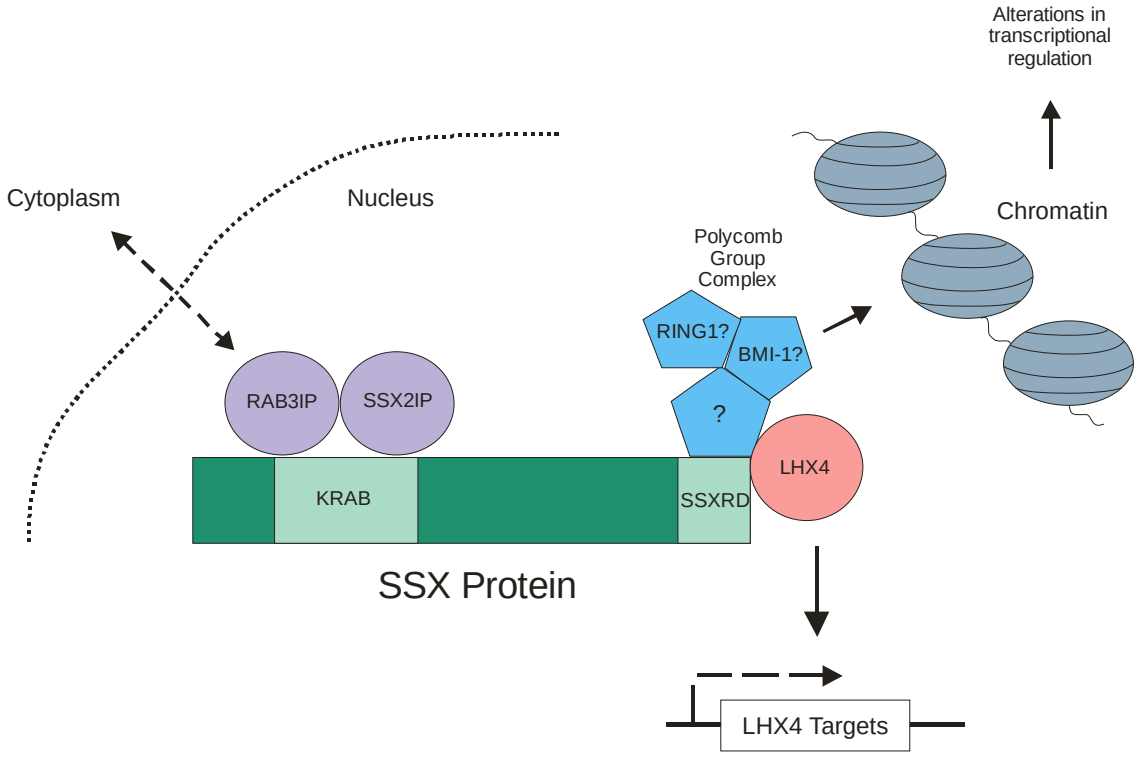

Table 1: SSX family members: expression, fusion constructs, inducibility, and isoforms.

The mRNA expression patterns of the ten known SSX family members are shown for testis and tumor tissues in columns 2, 3, and 4, while SSX family members known to be involved in the SS18-SSX fusion event are shown in column 5. SSX members shown to be inducible with epigenetic modifying agents or known to have alternative splice isoforms are shown in columns 6 and 7, respectively.

Table 1: SSX family members: expression, fusion constructs, inducibility, and isoforms.

	Transcribed	Expressed in testis	Expressed in cancer	Fusion partner with SS18	Inducible	Alternative splices
SSX1	+	+	+	+	+	
SSX2	+	+	+	+	+	+
SSX3	+	+	−*		−	
SSX4	+	+	+	+**	+	+
SSX5	+	+	+/−		+	+
SSX6	+	−	−*		+/−	
SSX7	+	+	−*		−	+
SSX8	+	+	−		−	
SSX9	+	+	−		−	
SSX10	+	n.d	n.d	n.d.	n.d.	n.d.

+ = positive/strong, +/− = positive/weak, − = negative/undetectable.
*very seldom.
**Only one case observed [75].
n.d. = not demonstrated.

Table 2: Expression of SSX family members in cancers of different histological types.

The expression of SSX family members 1-10 are shown for 24 cancers of varying histological origin and in normal testis tissue. “+” indicates that the presence of SSX mRNA or protein has been detected in these tumor tissues.

Table 2: Expression of SSX family members in cancers of different histological types.

	SSX1	SSX2	SSX3	SSX4	SSX5	SSX6	SSX7	SSX8	SSX9	SSX10
Bladder cancer	+	+		+						
Breast cancer	+	+		+						
Cholangiocarcinoma				+						
Colorectal carcinoma	+	+		+	+					
Endometrial cancer	+	+		+	+					
Gastric carcinoma		+		+						
Glioma		+		+						
Head and neck cancer	+	+		+	+					
Hepatocellular carcinoma	+	+		+	+					
Hodgkin's lymphoma	+	+		+						
Leiomyosarcoma										
Lymphoma/Leukemia	+	+		+						
Lung cancer	+	+		+	+					
Melanoma	+	+		+	+	+*	+*			
Multiple myeloma	+				+					
Neuroblastoma		+		+						
Non-Hodgkin's lymphoma	+	+		+						
Osteosarcoma	+	+		+	+					
Ovarian cancer				+						
Pancreatic cancer				+						
Prostate cancer		+								
Renal cell carcinoma		+								
Synovial sarcoma		+		+	+					
Thyroid cancer		+								
Normal testis tissue	+	+	+	+	+		+	+	+	

* Only observed in cell lines.

Table 3: SSX epitopes: sequences, haplotype restrictions, and recognition.

The known SSX immunogenic class I and class II MHC peptides or epitopes are shown. Amino acid sequence, haplotype restriction, tumor presentation, family member recognition, and references are outlined for each peptide/epitope. Symbol designations include + = SSX4 sequence, n.d. = not demonstrated. † = p41–49 epitope shown to be recognized on Me 275, SK-MEL-37, T343B, and T567A melanoma cells and SW 872 liposarcoma cells; p103–111 shown to be recognized on SK-MEL-37 melanoma cells and LNCaP prostate cancer cells; and p45–59 shown to be recognized on Me 275 cells. * = Recognized on antigen-loaded dendritic cells but not endogenously presented by tumor cells.

Table 3: SSX epitopes: sequences, haplotype restrictions, and recognition.

Class	Name	Native peptide sequence	Haplotype	Naturally presented by SSX+ tumor cells	Known family member recognition
I	p41–49	KASEKIFYV	HLA-A*0201	Y†	SSX2
	p57–65	AMTKLGFKA	HLA-A*0201	n.d.	SSX1–9
	p103–111	RLQGISPKI	HLA-A*0201	Y†	SSX2
II	p19–34	EKIQKAFDDIAKYFS	HLA-DPB1*0101	n.d.*	SSX2
	p37–51	WEKMKASEKIFYVYM	HLA-DR3*0301	n.d.*	SSX2, SSX4, SSX5
	p45–59	KIFYVYMKRKYEAMT	HLA-DRB1*1101, *0701, *1101, *1302, and B3*0301	Y†	SSX2
	p21–40	RSKAFDDIATYFSKKEWKKM	HLA-DRB1*1501	n.d.*	SSX1
	p31–50	YFSKKEWEKMKSSEKIVYVY+	HLA-DRB*0301 and *1101 (SSX4)	n.d.*	SSX1, SSX4
	p41–60	KSSEKIVYVYMKLNYEVMTK+	HLA-DRB1*1501 or DRB5*0101 (SSX4) and HLA-DR1*1601 (SSX1)	n.d.*	SSX1, SSX4
	p51–70	MKLNYEVMTKLGFKVTLPPFM+	HLA-DRB1*0701 (SSX4)	n.d.*	SSX1, SSX4
	p61–80	LGFKVTLPPFMRSKRAADFH	HLA-DRB1*1101	n.d.*	SSX4
	p101–120	FGSLQRIFPKIMPKKPAEEE+	HLA-DRB1*1101 (SSX4)	n.d.*	SSX1, SSX4
	p141–160	PPGKANISEKINKRSGPKRG	HLA-DR1*1601	n.d.*	SSX1
	p151–170	INKTSGPKRGKHAWTHRLRE	HLA-DPB1*1001	n.d.*	SSX4
	p161–180	KHAWTHRLRERKQLVVYEEI	HLA-DRB1*08 and HLA-DRB3*0202	n.d.	SSX4

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Chapter 2

Expression and Immunotherapeutic Targeting of the SSX Family of Cancer-Testis Antigens in Prostate Cancer

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Abstract

Recent U.S. Food and Drug Administration approval of the first immunotherapy for advanced prostate cancer has encouraged efforts to evaluate new target antigens for the immunotherapeutic treatment of prostate cancer. The synovial sarcoma X chromosome breakpoint (SSX) proteins comprise a set of cancer–testis antigens that are upregulated in MHC class I–deficient germline cells and in various types of advanced cancers with a poor prognosis. Humoral and cell-mediated immune responses to the SSX family member SSX2 can arise spontaneously in prostate cancer patients. Thus, SSX2 and other proteins of the SSX family may offer useful targets for tumor immunotherapy. In this study, we evaluated the expression of SSX family members in prostate cancer cell lines and tumor biopsies to identify which members might be most appropriate for immune targeting. We found that SSX2 was expressed most frequently in prostate cell lines, but that SSX1 and SSX5 were also expressed after treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine. Immunohistochemical analysis of microarrayed tissue biopsies confirmed a differential level of SSX protein expression in human prostate cancers. Notably, SSX expression in patient tumor samples was restricted to metastatic lesions (5/22; 23%) and no expression was detected in primary prostate tumors examined (0/73; $P < 0.001$). We determined that cross-reactive immune responses to a dominant HLA-A2–specific SSX epitope (p103-111) could be elicited by immunization of A2/DR1 transgenic mice with SSX vaccines. Our findings suggest that multiple SSX

family members are expressed in metastatic prostate cancers which are amenable to simultaneous targeting.

Introduction

Prostate cancer is the most frequently diagnosed and second leading cause of cancer-related death among American men, and a significant health concern worldwide (1). Organ-confined prostate cancer is initially treated by surgery or radiation therapy; however, approximately one-third of patients relapse, and another one-third of these patients will ultimately develop life-threatening, castrate-resistant tumors (1-3). Sipuleucel-T was recently U.S. Food and Drug Administration approved as the first immunotherapeutic vaccine shown to improve overall survival in patients with castrateresistant prostate cancer (4). The success of this approach suggests that other simpler immunotherapies could be investigated targeting additional antigens, potentially with the goal of preventing the development of castrate-resistant metastatic disease.

Over the course of the last decade, several prostate cancer antigens have been evaluated in clinical trials as immunotherapeutic targets for prostate cancer therapy, including prostate-specific antigen (5, 6), prostatic acid phosphatase (PAP; (7, 8)), prostate specific membrane antigen (PSMA) (9), and prostate stem cell antigen (PSCA; (10, 11)). Although these protein targets are frequently expressed by prostate cancer cells, they are also expressed by normal prostate tissue and may not be critical for the survival of the tumor; hence, their expression might be down-regulated by cancer cells during the course of immune targeting or disease progression (12-16). Thus, there remains a need to

identify immune targets of prostate cancer that are highly expressed in metastatic disease and/or critical to the progression of the disease, as simultaneous antigen targeting may be important to prevent the outgrowth of escape variants arising during the course of targeted therapy.

Cancer–testis antigens (CTA) are one class of tumor associated antigen upregulated in tumors of different histologic origin, including prostate cancer, and especially prevalent in advanced disease (17-19). Shown in some cases to be spontaneously immunogenic in cancer patients, these proteins are normally only expressed in germ cells of the testis (20-25). Because of the blood–testis barrier, a paucity of antigen-presenting cells, and a lack of MHC molecules on their surface, proteins exclusively expressed in germline tissue are considered immune privileged (26, 27). Thus, the ectopic expression of CTA in cancer tissue makes these proteins ideal immunotherapeutic targets. NY-ESO-1 and the MAGE family of antigens have been the most extensively studied CTAs. Proteins of these families have been found to be preferentially expressed in metastases over primary tumor of various histologic origin and are currently being evaluated as target antigens in clinical trials (28, 29).

Another superfamily of CTA thought to be attractive targets for cancer immunotherapy are the synovial sarcoma X chromosome breakpoint (SSX) proteins (30-32). Although the precise function of these proteins remains

unknown, SSX expression has been associated with stem cell migration, suggesting a potential biologically important role to the metastatic phenotype (33). The most investigated member SSX2 has been shown to be expressed in prostate cancer lesions at the mRNA level (34), whereas expression of other SSX family members has been shown to be inducible with epigenetic modifying agents (EMA) in colon carcinoma cell lines (35). We have shown that SSX2 expression can be upregulated in prostate cancer cell lines upon treatment with 5-aza-20-deoxycytidine (22). We have also shown that SSX2 antibodies and SSX2 peptide-specific T cells can be found in the peripheral blood of some patients with prostate cancer, indicating that patients can have preexisting immune responses to SSX2 (22, 36). Because the SSX antigens are highly immunogenic and essentially tumor specific, these proteins might be more ideal as targets for immunotherapy than other prostate-associated antigens. However, the relative expression of specific SSX family members in prostate cancer cell lines and prostate tumor tissues remains unknown. In this study, we sought to identify which SSX proteins are expressed in prostate cancer cell lines and tissues and, therefore, potentially relevant target antigens for prostate cancer immunotherapy. We then next sought to determine whether it is possible to simultaneously target these SSX family members using immunotherapeutic vaccines.

Specific Aims

I aim to identify which SSX family members are potential therapeutic targets expressed in prostate cancer cells and tissues, and determine whether these proteins can be simultaneously targeted using therapeutic vaccines.

In this chapter, I will provide evidence demonstrating:

1) SSX1, SSX2, and SSX5 mRNA are frequently expressed in prostate cancer cell lines and inducible with epigenetic modifying agents.
2) SSX antibodies can recognize different sets of SSX proteins, showing that family members are differentially expressed in prostate cancer lesions.
3) SSX proteins are primarily expressed in metastatic prostate cancer lesions (5/22; 23%) and not in primary prostate carcinoma (0/73; $p<0.001$, chi square).
4) Cross-reactive immune responses to a dominant HLA-A2-restircted SSX epitope (p103-111) can be elicited in A2/DR1 transgenic mice by SSX peptide or DNA vaccination.

Results

Identification of gene-specific primers for ten SSX family members

To evaluate mRNA expression of SSX family members, primers and annealing temperatures were first determined that would result in specific product amplification for each known family member. Using the published SSX gene-specific primers and annealing temperatures for PCR (30, 35), we observed cross-amplification of products from image clone DNA plasmids encoding different SSX family member cDNAs, particularly for the SSX4, 6, 7, and 9 primer pairs (data not shown). On the basis of these results, we designed new primers and conducted gradient PCR to establish conditions that would result in complete primer specificity. The primers and annealing temperatures used for these experiments are shown in Table 1, and the location of the published and redesigned primers within the aligned SSX sequences are shown in Figure 1. Specific amplification was observed for all SSX primers (Fig. 2). Image clones encoding SSX8 and SSX10 were not available; however, SSX8 and SSX10 primer sets were tested independently for cross-amplification from the available image clones, with no amplification observed (Fig. 2B). These primers were also tested for PCR amplification from a human testis cDNA library. Products of the appropriate size were found for SSX8 but not SSX10 (Fig. 2C). Amplified products of the predicted size for SSX8 were sequenced, confirming primer specificity and SSX8 expression in the testis.

SSX family members 1, 2, 3, 5, and 8 are expressed or inducible in prostate cell lines

Previous work by our group and others has shown that certain CTA, including SSX2, can be induced in cancer cell lines with EMA (22, 35). To determine which SSX members could be potential targets for prostate cancer immunotherapy, three human androgen-dependent prostate cancer cell lines (LAPC4, LNCaP, and VCaP), three androgen-independent cell lines (22-RV1, PC3, and DU-145), and two immortalized prostate epithelial cell lines (RWPE-1 and PREC-E6) were cultured in vitro and treated with or without two different EMA. Specifically, cells were treated with a DNA methyltransferase inhibitor, 10 mmol/L 5-aza-dc, a histone deacetylase inhibitor, 100 nmol/L TSA, or both agents simultaneously, whereas untreated controls were treated with vehicle alone. RNA was collected 72 hours after treatment for RT-PCR analysis (Fig. 3). SSX1 was expressed at baseline only in untreated PC3 cells but was frequently inducible following 5-aza-dc treatment. SSX2 was expressed at baseline in most of the cell lines and was inducible to varying degrees in the androgen-independent cells and the normal prostate epithelial cell lines. In addition, in some instances SSX3, 5, and 8 were inducible with EMA treatment. In the case of SSX8, amplified products were gel purified and sequenced, confirming SSX8 amplification in DU-145, LNCaP, and PREC-E6 cells, which to our knowledge is the first identification of SSX8 expression in any cancer cell line or tissue.

qRT-PCR was conducted with the same RNA samples in all cases in which amplification was observed by RT-PCR. The expression patterns found by RT-PCR for SSX1, 2, 3, 5, and 8 were highly similar by qRT-PCR (Fig. 4; SSX3, SSX8 not shown). TSA did not appear to significantly induce expression either alone or in combination with 5-aza-dc, suggesting that regulation of SSX gene expression is predominantly mediated, directly or indirectly, by changes in DNA methylation. Overall, these results show that SSX2 is the most commonly expressed family member in prostate cancer cell lines, with higher (and more frequent) expression detected relative to other SSX family members. However, SSX1 and SSX5 expression were frequently induced following treatment with a methylation inhibitor. Individual cell lines could also express multiple SSX family members.

SSX proteins are expressed in human prostate cancer tissues

We next sought to determine whether the SSX family member proteins are expressed in human prostate tumors, and whether this expression is associated with stage of disease. Because the SSX proteins have highly similar amino acid sequences, the cross-reactivity to SSX1-5 GST-tagged recombinant proteins was evaluated by Western blot. Recombinant proteins for SSX family members 6 to 10 were not available and were thus not evaluated. We found that a mAb, 1A4, recognized SSX2 and SSX3, whereas a murine polyclonal antibody, B01P, recognized SSX1, 3, 4, and 5, but not SSX2 (Fig. 5A). These antibodies could

also detect SSX protein expression in paraffin-embedded human testis tissue (Fig. 5B). Using a paraffin-embedded tissue microarray, which included biopsy samples from 95 patients with varying stages of prostate cancer, 25 patients with high-grade prostatic intraepithelial neoplasia (HGPIN), or benign cores from 72 control patients, we evaluated SSX expression in cancer tissues by immunohistochemistry (IHC). SSX expression was detected in some of these tissue specimens with differential staining observed depending on which SSX antibody was used (Fig. 5C and Fig. 6). Given the cross-reactivity of these antibodies, the identification of specific SSX family members was not possible, but the presence of staining with 1A4 and not B01P in certain lesions, together with our findings in cell lines, suggested that some tissues may express only SSX2 (among SSX1–SSX5). Staining with B01P, but not 1A4, suggested that other tissues may express one or more of the other SSX family members. In addition, the nuclear SSX staining patterns varied among tissues; in some cases SSX expression was homogenous, whereas in other tissues the nuclear staining was punctate, with expression detected in some cells but not adjacent tumor cells. Interestingly, SSX protein expression among tumors was found exclusively in metastatic lesions (5/22, 23%), and not in primary tumors (0/73, $P < 0.001$, χ^2; Table 2). A single benign tissue sample was scored positive; however, given predominantly cytoplasmic staining, this might have been a staining artifact (Figure 6).

T cells specific for epitope p103-111 from SSX1 and SSX2 differentially recognize peptide p103-111 from other SSX proteins

Ultimately, these studies were conducted to identify which SSX family members might be attractive targets for prostate cancer immunotherapy. The results above suggested that SSX1, 2, and 5, and possibly SSX3 and 8, may be the most relevant members expressed in prostate tumors. Because these proteins are highly homologous, we next wanted to determine whether they could be immunologically targeted following immunization with one SSX family member. To test this, we focused on SSX2 as the dominant SSX family member, with highest expression among prostate cancer cell lines, and restricted our analysis to a single HLA-A2–restricted epitope, SSX2 p103-111 (36). We previously identified that T cells specific for this immunodominant epitope can be detected in HLA-A2+ patients with advanced prostate cancer and can lyse HLA-A2+ SSX2+ prostate cells (36). We therefore wanted to assess whether SSX2 p103-111-specific T cells could recognize the corresponding peptide derived from other relevant SSX family members. Using peptide-binding algorithms to predict HLA-A2 affinity, we found that most of the corresponding p103-111 peptides from the different family members were predicted to have moderate HLA-A2 affinity (Fig. 7A). These peptides were then synthesized and evaluated for their *in vitro* HLA-A2 affinity (Fig. 7B). We found that each SSX p103-111 peptide had affinity for HLA-A2, and this binding corresponded with the predicted binding algorithm

scores. As a negative control, peptide p103-111 from SSX2 was modified (SSX2-IP) to abolish HLA-A2 binding.

To evaluate whether T cells specific for SSX2 p103-111 can recognize peptides derived from different SSX family members, we immunized HLA-A2 transgenic (A2/DR1) mice with an SSX2-encoding DNA plasmid vaccine (36). T cells specific for SSX2 p103-111 recognized p103-111 from SSX3/5/9, but no other family members (Fig. 7C), suggesting that an SSX2 vaccine that elicits SSX2 p103-111 peptide-specific T cells could also be used to target SSX3, 5, or 9. Similarly, T cells from A2/DR1 mice immunized directly with peptide p103-111 from SSX1 or SSX2 were evaluated for cross-reactive immune responses by IFNγ ELISPOT (Fig. 7D). Again we found that T cells specific for SSX2 p103-111 could recognize peptide p103-111 from SSX3/5/9, whereas T cells elicited by immunization with SSX1 p103-111 cross-recognized peptides p103- 111 from SSX4, SSX6/8, and SSX7. Together, these results show that multiple SSX family members can be expressed in prostate cancer and could potentially be targeted simultaneously using immunotherapeutic vaccines.

Discussion

SSX2 mRNA expression has previously been reported in human prostate tumor tissues (22, 34, 37). Initially, Türeci and colleagues observed SSX2 expression in 5 of 25 prostate cancer lesions by RT-PCR (37) and later found SSX1, 2, 4, and 5 expression in a number of different cancer tissues, however, SSX2 was the only member found in prostate cancer lesions (34). When SSX6-9 were subsequently identified, it was found that SSX1, 2, 4, 5, and 6 could be induced with EMA treatment in colon cancer cell lines (35). Prior to our study, a comprehensive study evaluating SSX family member mRNA and protein expression had not been conducted for prostate cancer cell lines or tumor tissues.

In this study, we have identified primers that specifically amplify individual SSX family members. These primers were used to evaluate SSX expression in prostate cancer cell lines by RT-PCR and qRT-PCR. We found that SSX2 mRNA transcripts were commonly expressed in prostate cancer cell lines at baseline, whereas SSX1 and SSX5 were frequently inducible with EMA. To a lesser extent, SSX3 and SSX8 were also induced (4/8 and 3/8 cell lines, respectively). Using SSX antibodies that recognize different SSX family members, we found that tumors can have differential SSX protein expression, and expression is essentially restricted to metastatic lesions. We also found that T cells specific for an HLA-A2–restricted SSX2 immunodominant epitope can

recognize the corresponding p103-111 peptide derived from other SSX family members. Cross-reactivity of SSX2 p103-specific T cells was observed for the corresponding p103-111 peptide from SSX3/5/9, whereas SSX1 p103-111–specific T cells seem to recognize p103 from SSX6/8 and perhaps SSX4 and SSX7.

In contrast to previous reports, we found that published primers for SSX4, 6, 7, and 9 led to amplification of other family member transcripts. In our hands, SSX4 primers previously reported amplified products from SSX2, 3, and 4 cDNA templates at the annealing temperatures published for SSX4, whereas SSX6, 7, and 9 primers cross-amplified products from the SSX7 and SSX9 cDNA templates (data not shown). We also observed cross-amplification of SSX7 and SSX9 transcripts using the published SSX5 primers. We report here a set of primers and annealing temperatures specific to each family member that eliminated cross-amplification (Table 1; Fig. 1). We believe that cross-amplification with the original SSX4 primers is a significant finding due to the reported expression of this family member in cell lines and tumor tissues in numerous studies. Because we found these primers to cross-amplify SSX2, which is the most frequently reported family member in cancer cell lines and tissues, it could be that some reports of the frequency of SSX4 expression in various tumors are inaccurate.

The observed patterns of SSX family member expression and induction from our studies suggest underlying epigenetic marks in different cell lines that mediate responsiveness to EMA agents. Differences in promoter methylation of CpG islands, methylation of intragene cysteines, or alterations in chromatin folding because of acetylation or methylation of histone residues could all contribute to variations in SSX family member expression. From our studies, the largest induction of transcription was found after DNA methyltransferase inhibition with 5-aza-dc treatment, which is not surprising in light of previous findings that hypomethylation events arise early in prostate cancer tumorigenesis (38-40). Although the methylation status of CpG islands have not been reported for the SSX gene family members, whole genome bisulfite sequencing data from cancer cell lines is available online through the UCSC Genome browser (41). There seem to be no documented methylation sites or CpG islands in the promoter region of SSX2, 3, 4, 6, 7, 8, or 9, even far upstream of the start site or within intragene regions. Methylated sites are found in the promoter region of SSX1, and in an intragene region of SSX5 that could account for differences in baseline expression or inducibility. Other regulation sites or mechanisms may be present to account for the increased expression of SSX2, 3, and 8 in some cell lines after EMA treatment. In two of the three cell lines showing SSX8 inducibility, expression is only present with combined 5-aza-dc and TSA treatment, suggesting that chromatin remodeling may play a role in transcriptional regulation

of this family member. Future studies will assess methylation patterns found in SSX genes at the DNA level, before and after EMA treatment.

We also show for the first time SSX protein expression in human prostate cancer tissues. We observed different patterns of SSX family member protein expression in prostate tumors in terms of member and homogeneity of staining. SSX expression was found almost exclusively in metastatic prostate cancer lesions as compared with primary prostate cancer lesions ($P < 0.001$, χ^2). Expression identified here in bone, lymph node, and brain metastases suggest that SSX protein expression is inherent to metastatic disease, independent of tissue site. Interestingly, primary tumor biopsies from patients with metastases (stage IV) also were SSX negative, whereas metastatic tissues from 5 of 22 patients were SSX positive ($P = 0.027$, χ^2), further associating SSX expression specifically with metastatic disease. These results correspond to our previous studies looking at the frequencies of SSX2 p103-111 peptide-specific T cells in the peripheral blood mononuclear cells (PBMC) of patients with prostate cancer by tetramer analysis (36). We found that patients with advanced disease had significantly higher frequencies of SSX-specific T cells compared with healthy donors and patients with early-stage disease, further suggesting that these antigens may be associated with advanced prostate cancer. These findings may or may not suggest a biological importance of SSX expression in metastatic disease progression.

The expression of multiple SSX family members in some metastatic prostate cancer lesions suggests that SSX immunotherapy may need to consider targeting several SSX proteins simultaneously. We have previously identified an immunodominant SSX2 epitope (p103-111) that can elicit epitope-specific T cells in transgenic A2/DR1 mice immunized with a plasmid vaccine encoding SSX2 (36). Cross-reactivity of SSX2 p103-specific T cells from DNA or peptide-immunized mice was observed for the corresponding p103-111 peptide from SSX3/5/9, whereas SSX1 p103-111–specific T cells recognized p103 from SSX6/8, SSX4, and SSX7. Interestingly, whereas SSX antibodies were unable to fully distinguish between family members, our T-cell cross-reactivity results show that subtle differences in amino acids in the TCR-interacting portion of the epitopes conferred specificity for some members and cross-reactivity to others. These studies do not rule out the possibility that other SSX epitopes could be recognized among family members. Future work with T-cell-directed therapies needs to consider whether SSX1, 2, and 5, as the most expressed SSX family members, will specifically be targeted for prostate cancer. Immune targeting could potentially be accomplished by immunizing with multiple plasmids encoding these members, or polytope vaccines could be designed encoding cross-reactive epitopes that will elicit T cells that can specifically recognize peptides from all SSX family members relevant to prostate cancer.

Although SSX proteins may be expressed in advanced prostate tumors, it should also be noted that this expression may be heterogenous, as has been described for other CTA in various tumor types. This could potentially allow for antigen loss variants during immune targeting with a vaccine. Antigen heterogeneity might be overcome with EMA treatment to upregulate antigen expression in tumor tissue, however, it should be noted that these EMA might have deleterious effects on immune function or may upregulate antigen expression in normal tissues. Future work will need to assess the feasibility of combining these two treatments simultaneously to optimize tumor immunotherapy.

Figures and Tables

Table 1. SSX family member sequence sources and primer design

Shown are the GenBank accession numbers for each SSX family member evaluated, the primer sequences and annealing temperatures used to amplify specific SSX products in PCR and RT-PCR studies, and the expected product size of each amplified family member transcript.

Table 1. SSX family member sequence sources and primer design

Family member	Accession number	5′-Primer sequence 3′Primer sequence	Product size (bp)	Annealing temperature (°C)
SSX1	NM_005635.2	5′-CTAAAGCATCAGAGAAGAGAAGC-3′ 5′-AGATCTCTTATTAATCTTCTCAGAAA-3′	422	60
SSX2	NM_175698.1	5′-GTGCTCAAATACCAGAGAAGATC-3′ 5′-TTTTGGGTCCAGATCTCTCGTG-3′	435	60
SSX3	NM_021014.2	5′-GGAAGAGTGGGAAAAGATGAAAGT-3′ 5′-CCCCTTTTGGGTCCAGATATCA-3′	378	65
SSX4	NM_001034832.2	5′-ATGCTCAAATATCAGAGAAGTTACGA-3′ 5′-GCAAGAAAATACAGCGGAAACGCCA-3′	590	69
SSX5	NM_175723.1	5′-GTTCTCAAATACCAGAGAAGATG-3′ 5′-CTCTGCTGGCTTCTCGGGCG-3′	437	60
SSX6	NT_079573.4	5′-CTAAAGCATCAGAGAAGAGAAGC-3′ 5′-TCTGAGAGTCTGTGGCCCGTT-3′	198	67
SSX7	NM_173358.2	5′-TTTGCAAGGAGACCTAGGGC-3′ 5′-CTTGGTTTTCCTGGAGGGCACAGG-3′	419	65
SSX8	NR_027250	5′-AAAGAGACCCAGGGATGATGA-3′ 5′-CTCTTCATAAATCACCAGCTGG-3′	515	65
SSX9	NT_079573.4	5′-TTTGCAAGGAGACCTAGGGC-3′ 5′-GTTGGTTTTCCCGGGGGGCACAGC-3′	419	73
SSX10	NT_079573.4	5′-TTTGCAAGGAGACCCAGGGT-3′ 5′-TCAGTCCTGGAGCAGGATTCTGCAG-3′	438	60

Figure 1. Sequence alignment and primer design for SSX1-10.

SSX1-10 mRNA sequence alignment with the location of primers used in these studies in red and published priers not used in these experiments in green. Start and stop codons are shown in bold lettering.

Figure 1. Sequence alignment and primer design for SSX1-10.

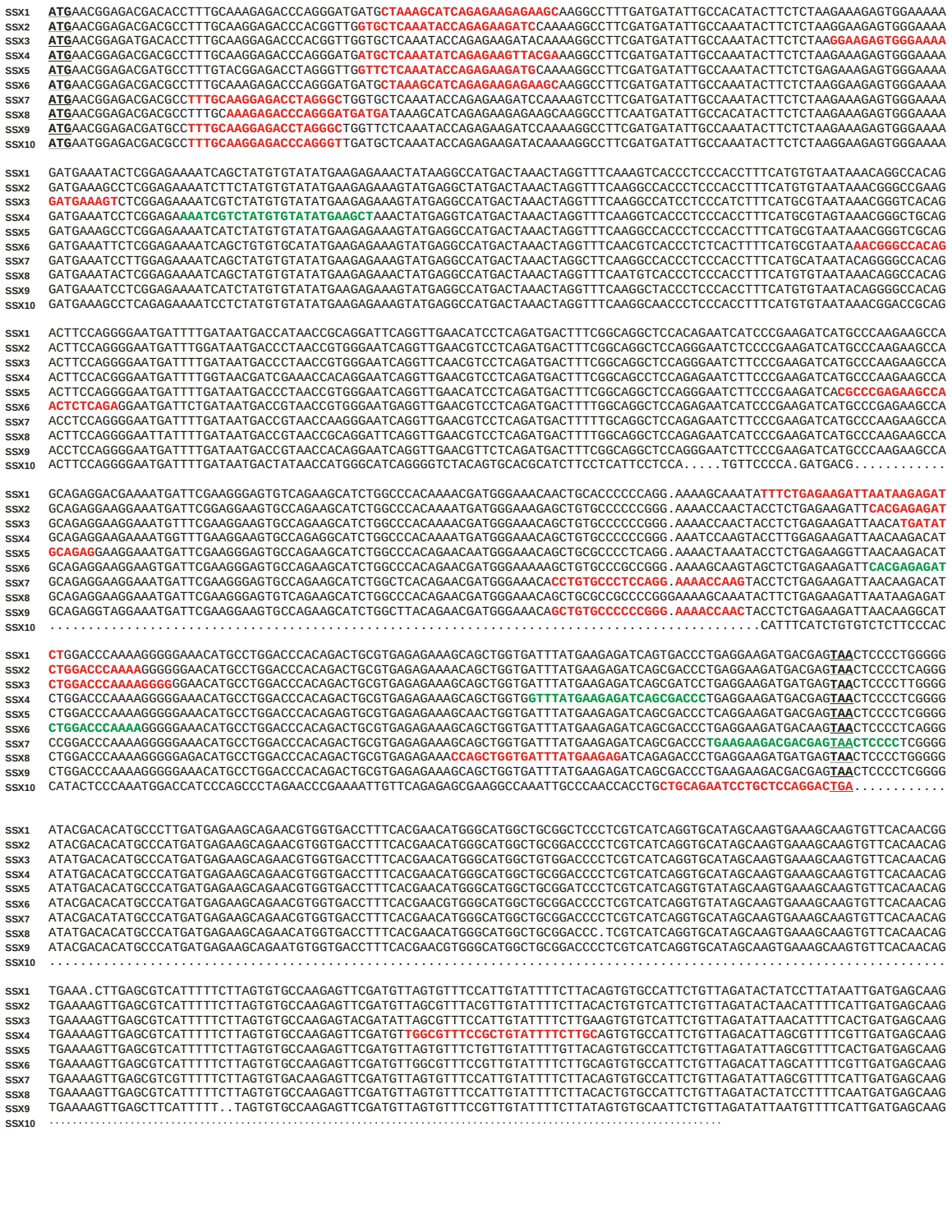


Figure 2. PCR amplification of SSX gene products from plasmids expressing specific SSX family members

Panel A. Primer sets specific fo each SSX family member were tested for PCR amplification using available image clone plasmids encoding different SSX family members as template DNA. SSX8 and SSX10 image clones were unavailable.

Panel B. SSX8 and SSX10 primer sets were tested against the available SSX image clones.

Panel C. SSX2, 8, and 10 primer sets were evaluated by PCR amplification from human testis cDNA to verify specificity of SSX8 and SSX10 primers. Data in each panel is representative of 2 independent experiments.

Figure 2. PCR amplification of SSX gene products from plasmids expressing specific SSX family members

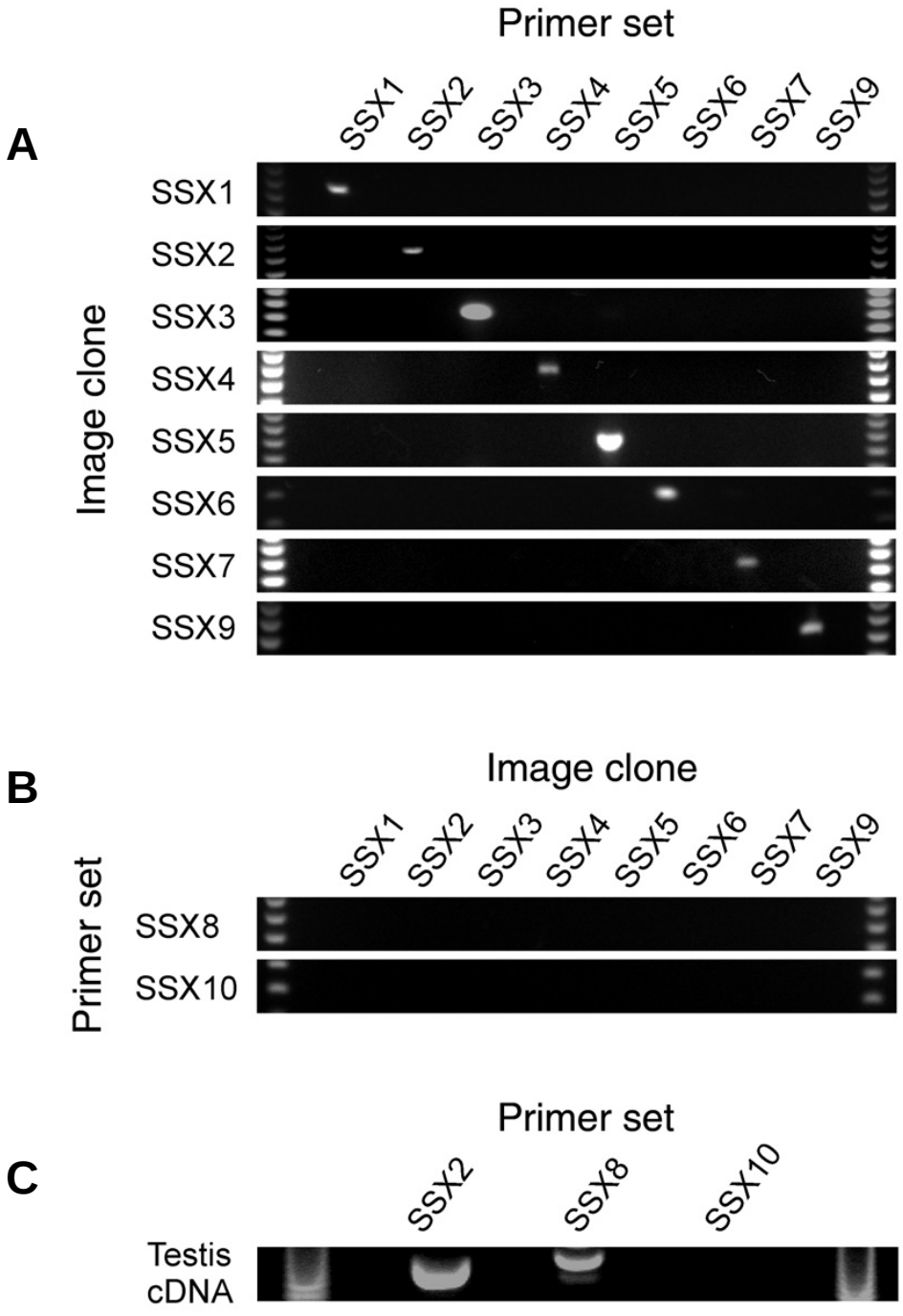


Figure 3. RT-PCR analysis reveals SSX CTA expression in prostate cancer cell lines and inducible expression of SSX mRNA transcripts with epigenetic modifying agents

Three androgen-dependent and three androgen-independent prostate cancer cell lines and two immortalized prostate epithelial cell lines were evaluated for expression of SSX1-10 mRNA either in media alone without treatment (NT) or after treatment with 10 mmol/L 5-aza-dc (AZA), 100 nmol/L TSA (TSA), or treatment with both agents simultaneously (A+T). PCa = prostate cancer. These results are representative of two independent studies.

Figure 3. RT-PCR analysis reveals SSX CTA expression in prostate cancer cell lines and inducible expression of SSX mRNA transcripts with epigenetic modifying agents

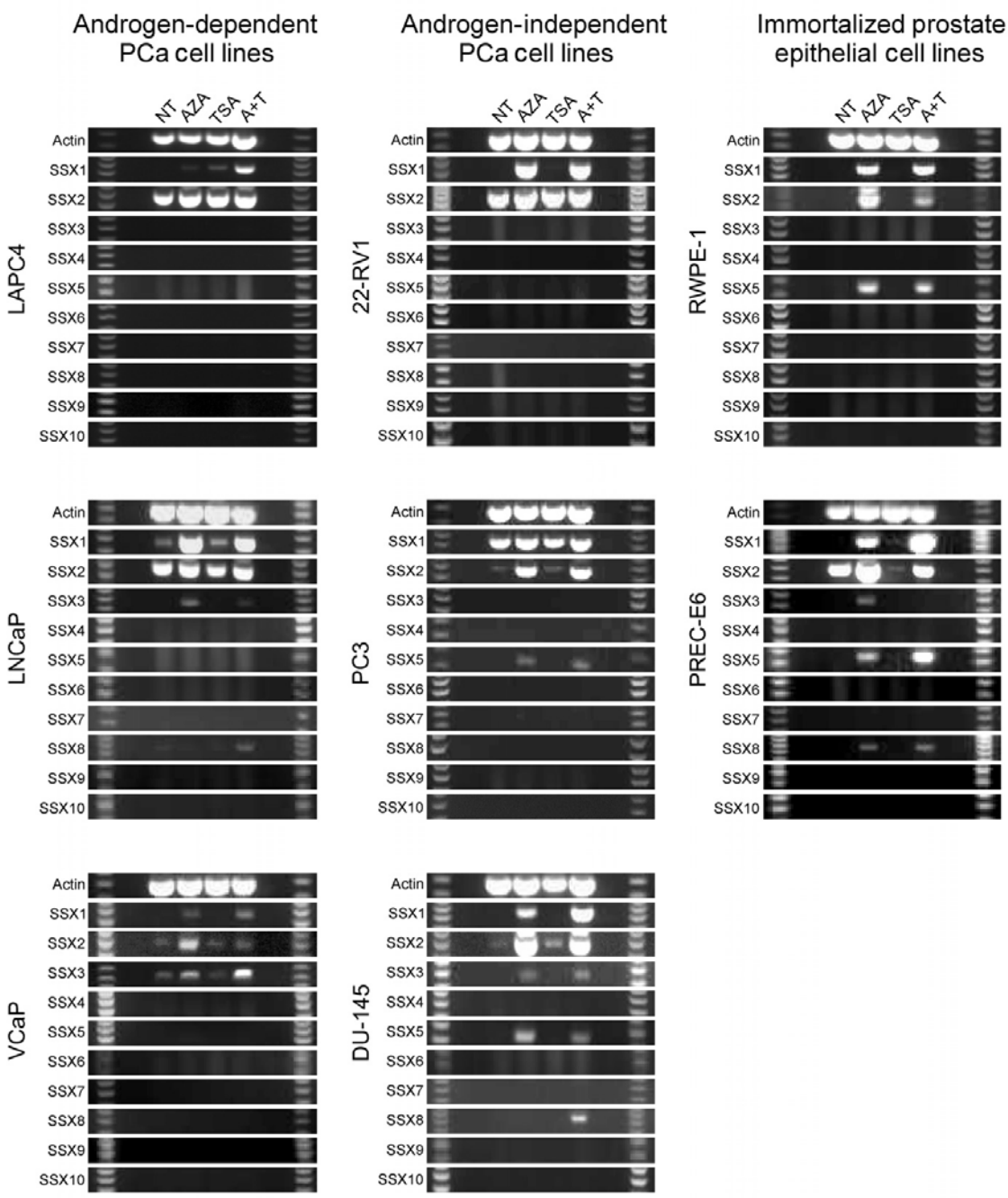


Figure 4. Quantitative analysis of relative expression and fold induction of SSX 1, 2, and 5 mRNA in prostate cancer cell lines treated with epigenetic modifying agents

RNA isolated from prostate cancer cell lines showing SSX expression by RT-PCR was evaluated by qRT-PCR for expression relative to an internal control transcript (P0; *Panel A*) and specific fold induction (*Panel B*) of SSX transcripts following EMA treatment. qRT-PCR was done with primers specific for SSX1, 2, and 5, conducted in triplicate, and repeated in an independent experiment. Error bars represent the mean and SD of 6 wells from these two experiments. Comparison between groups was made with a 1-way ANOVA followed by post hoc analysis with the Tukey test. *, $P < 0.05$ compared with vehicle treatment (A) or with TSA treatment (B).

Figure 4. Quantitative analysis of relative expression and fold induction of SSX 1, 2, and 5 mRNA in prostate cancer cell lines treated with epigenetic modifying agents

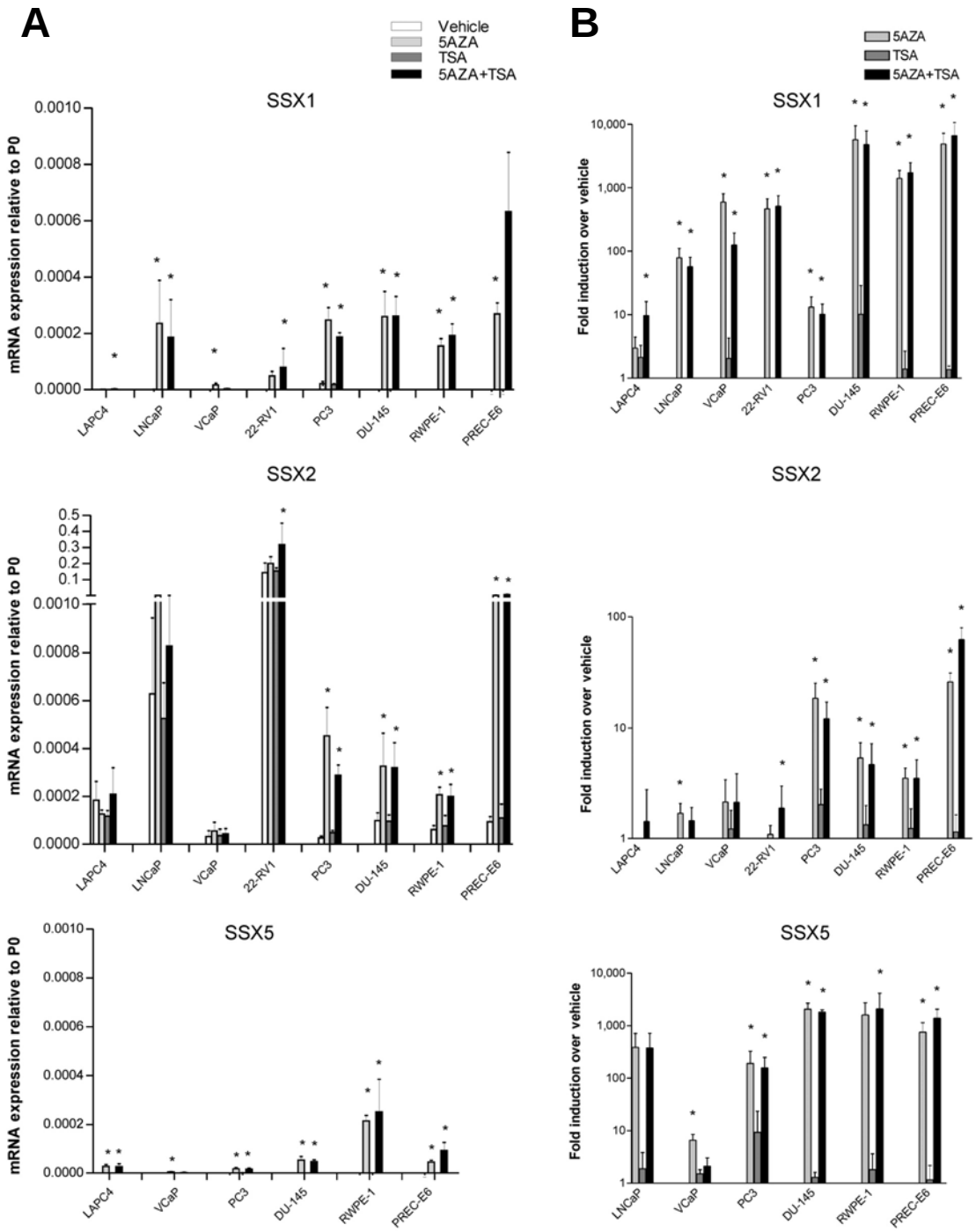


Figure 5. SSX proteins can be detected in tumor biopsy specimens from prostate cancer patients

Panel A. 200 ng of GST-tagged recombinant SSX proteins (SSX1-5) or PAP protein (negative control) were resolved by SDS-PAGE. Cross-recognition of SSX proteins was determined by staining with mAb (1A4) or pAb (B01P) in Western blot.

Panel B. These antibodies were also evaluated for their recognition of SSX proteins in paraffin-embedded testis tissue by IHC. An IgG isotype control was used as a primary antibody negative control for staining specificity.

Panel C. Tissue microarray slides were stained using mAb 1A4 mAb, pAb B01P, or an IgG isotype control. Specimens were considered positive for SSX expression if 2 blinded observers independently scored the cores positive. Metastatic biopsy samples from 3 patients with differential SSX expression are shown (Patient 1: bone metastasis; patient 2: brain metastasis; and patient 3: lymph node metastasis). Positive and negative staining are denoted by "+" and "-", respectively.

Figure 5. SSX proteins can be detected in tumor biopsy specimens from prostate cancer patients

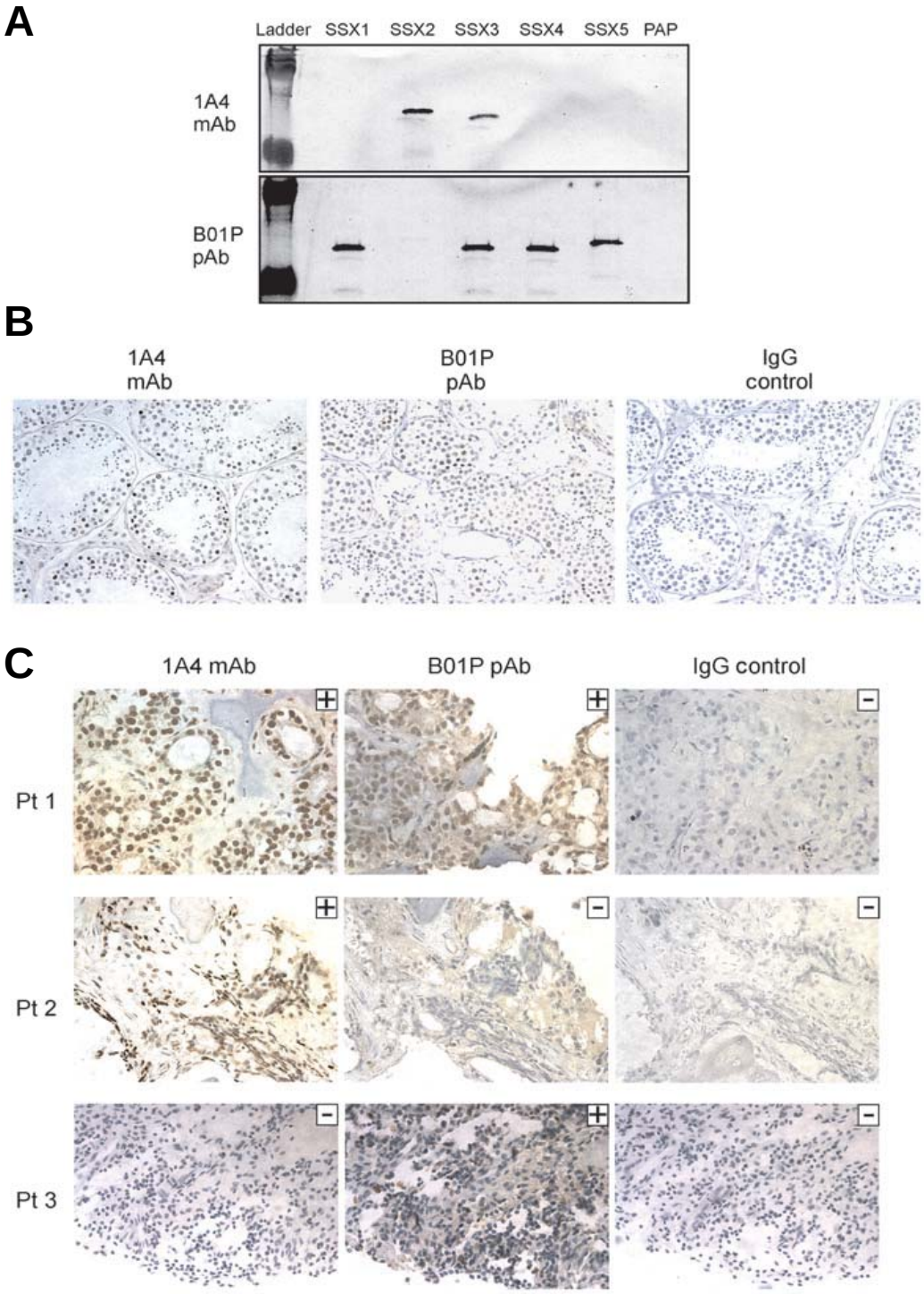


Figure 6. Additional prostate cancer biopsy cores from microarray showing punctate SSX staining.

Panel A: Benign core stained with 1A4 mAb, B01P pAb, or IgG isotype control showing punctate B01P cytoplasmic staining.

Panel B: Metastatic tissue core showing punctate B01P (nuclear staining).

Figure 6. Additional prostate cancer biopsy cores from microarray showing punctate SSX staining.

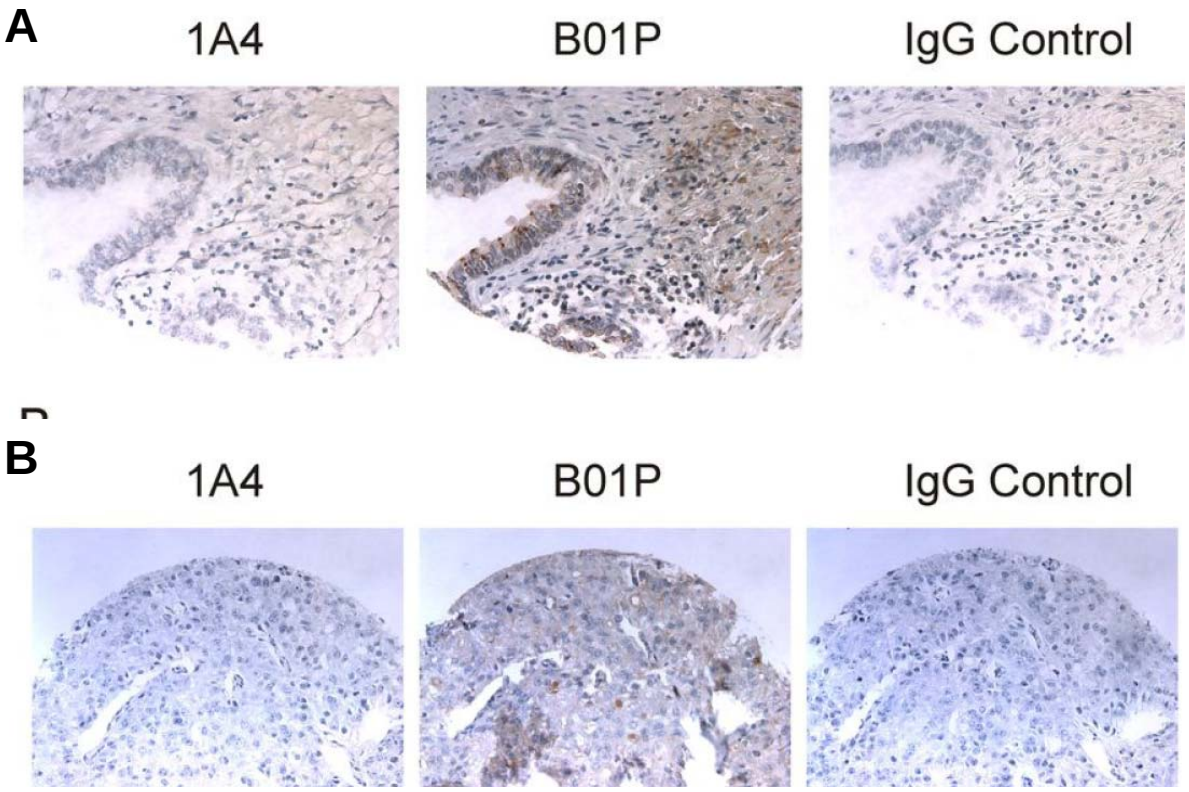


Table 2. SSX proteins are preferentially expressed in metastatic prostate cancer lesions

Shown are the numbers of patients in each disease stage with biopsy cores staining positive with either of the 2 SSX antibodies, or both antibodies simultaneously (total SSX) by tissue microarray IHC. *Staining shown in Supplementary Fig. S2).

Table 2. SSX proteins are preferentially expressed in metastatic prostate cancer lesions

	SSX expression				
Disease stage	**1A4+ B01P+**	**1A4+ B01P−**	**1A4− B01P+**	**Total SSX**	**Total tissues**
Benign prostate tissue	—	—	1[b]	1[b]	48
Benign prostatic hyperplasia	—	—	—	—	24
HGPIN	—	—	—	—	25
1° Prostate tumor–stage 2	—	—	—	—	42
1° Prostate tumor–stage 3	—	—	—	—	12
1° Prostate tumor–stage 4	—	—	—	—	19
Metastasis	1	2	2	5	22

Figure 7. SSX1 and SSX2 p103-111–specific T cells exhibit cross-recognition for other SSX proteins.

Panel A. Shown are the homologous amino acid sequences for SSX1-9 corresponding to the p103-111 epitope described previously for SSX2 (36). Highlighted amino acids represent nonidentical residues compared with SSX2. Columns 3 and 4 show the predicted HLA-A2 affinity scores for each peptide using the SYFPEITHI (42) and BIMAS (43) prediction algorithms.

Panel B. *In vitro* HLA-A2 affinity of p103-111 peptide from each family member at titrated peptide concentrations by T2 binding assay mean fluorescence intensity (MFI). These results are representative of 2 independent experiments.

Panel C. Splenocytes from individual A2/DR1 mice immunized with either DNA vaccine encoding SSX2 (pTVG-SSX2, n = 9, ■) or vector control (pTVG4, n = 3, ○) were analyzed to determine the frequency of peptide-specific T cells by IFNγ ELISPOT. Splenocytes were stimulated with one of each of the six p103-111 peptides from the different SSX family members, SSX2-IP, influenza matrix A peptide, or a ConA positive control. Each dot represents the frequency of peptide-specific IFNg SFU from an individual mouse, measured in triplicate with background subtracted.

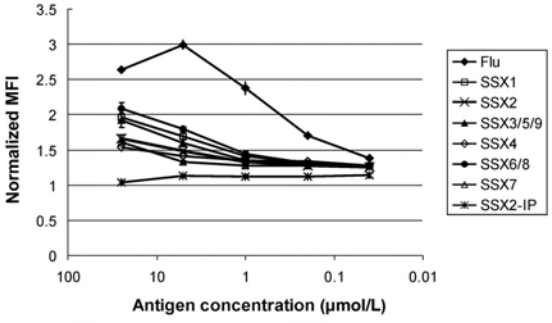
Panel D. Splenocytes from individual A2/DR1 mice immunized with peptide p103-111 from either SSX1 (n = 5, ■) or SSX2 (n = 6,●) or a vehicle control (n = 4, ∆) were analyzed to determine the frequency of peptide-specific T cells by IFNγ ELISPOT as described above.

Figure 7. SSX1 and SSX2 p103-111–specific T cells exhibit cross-recognition for other SSX proteins.

A

Protein	Sequence	SYFPEITHI	BIMAS
SSX1	RLHRIIPKI	25	23.995
SSX2	RLQGISPKI	23	10.433
SSX3/5/9	RLQGIFPKI	23	19.822
SSX4	SLQRIFPKI	23	19.822
SSX6/8	RLQRIIPKI	25	23.995
SSX7	RLQRIFPKI	21	19.822
SSX2-IP	RLQGISPKP	15	0.01
Matrix A (Flu)	GILGFVFTL	30	550.927

B



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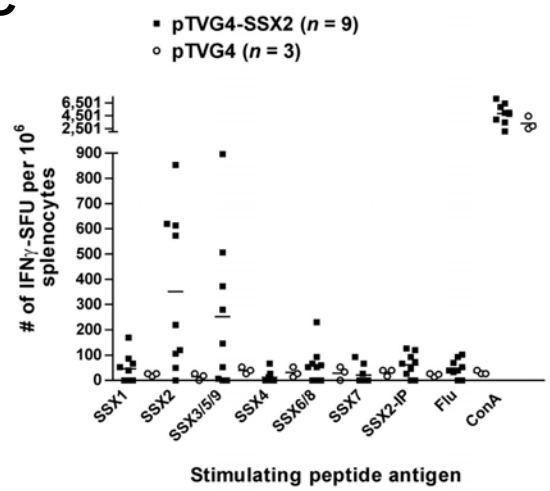

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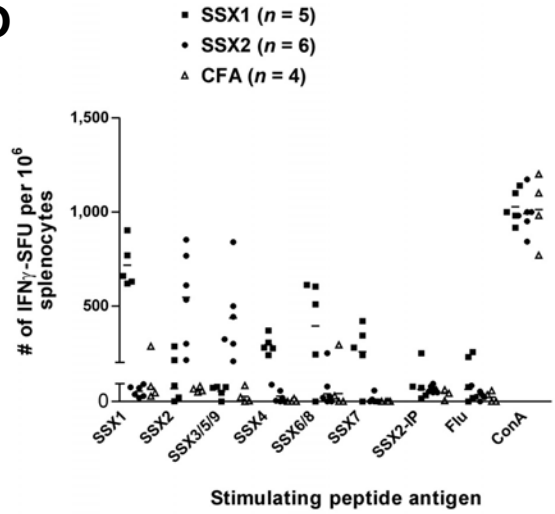

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Chapter 3

Vaccines Targeting the Cancer-Testis Antigen SSX2 Elicit HLA-A2 Epitope-Specific Cytolytic T Cells

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Abstract

The cancer-testis antigen synovial sarcoma X breakpoint-2 (SSX2) 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 this study was to evaluate genetic vaccine encoding SSX2 to prioritize human leukocyte antigen (HLA)-A2-specific epitopes and determine if a DNA vaccine can elicit SSX2-specific cytotoxic T lymphocytes (CTLs) 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+/HLADR1+/H-2 class I-/class II-knockout) transgenic mice. We found that SSX2 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 SSX2. Furthermore, p103-111-specific CTLs were able to lyse an HLAA2+ 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 SSX2 epitope-specific CTLs 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 SSX2 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 SSX2 can be circumvented *in vivo*. Together, these findings suggest that SSX2 may be a relevant target antigen for prostate cancer vaccine approaches

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).

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 (CTLs). DNA plasmid vaccines offer several advantages over other genetic vaccine strategies by providing a safe immunization platform, simple preparation, and inexpensive manufacture, whereas also specifically targeting only a select antigen(s) or antigenic epitope(s) (10, 11). Several DNA vaccines have been shown to elicit antitumor 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 major histocompatibility complex (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.

One of the first CTA identified was HOM-MEL-40, or synovial sarcoma X breakpoint-2 (SSX2), which is known to be expressed in advanced-stage tumors of different histologic types (17, 18). Moreover, SSX2 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 SSX2 can be detected in some patients (18). In addition, we have shown that SSX2 expression can be selectively induced in prostate cancer cells

upon treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (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 CTLs.

To aid in the evaluation of SSX2 as an immunotherapeutic target, several groups have sought to identify MHC class I-restricted SSX2 epitopes. The majority of the investigations have used reverse immunology approaches culturing T cells from healthy donors or cancer patients with pools of SSX2 peptides (19, 21-25). Although these studies have successfully identified several human leukocyte antigen (HLA)-A2-binding peptides, it is not known whether SSX2 epitopes are elicited as a result of *in vivo* vaccination or whether dominant epitopes exist for this antigen. This study was conducted to evaluate the potential of a genetic vaccine encoding SSX2 to elicit epitope-specific immune responses in an *in vivo* mouse model, to more comprehensively compare SSX2 HLA-A2-restricted T-cell epitopes, determine whether epitope-specific CTLs can lyse prostate tumor cells, and evaluate whether these epitope-specific CTLs exist within the T-cell repertoire of prostate cancer patients. To identify and characterize SSX2 epitopes, we used two approaches. The first was a standard approach to determine the HLA-A2 affinity of SSX2-derived peptides *in vitro*, and second, 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 SSX2 could elicit robust epitope-specific CTLs capable of lysing prostate cancer cells. In addition, these same epitope-specific CTLs 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 SSX2 might be developed to augment existing immunity, or perhaps in a prophylactic setting to prevent the development of SSX2+ prostate cancer tumors.

Specific Aims

I aim to identify SSX2-specific epitopes and determine if SSX2 vaccines can elicit peptide-specific CTL capable of lysing prostate cancer cells.

In this chapter, I will provide evidence demonstrating that:

1) Several SSX2-derived peptides can bind to the HLA-A2 complex.
2) A2/DR1 mice have SSX2 p41- and p103-specific T-cells, which can be augmented by vaccination *in vivo*.
3) A genetic vaccine encoding SSX2 can elicit epitope-specific CTL capable of lysing prostate cancer cell lines.
4) SSX2 peptide p103-111 appears to be an immunodominant epitope.
5) SSX2 p98 is an HLA-DR-binding class II peptide, but does not appear to be an epitope presented by APCs.
6) p103-specific CTL can be cultured from the PBMCs of some HLA-A2+ patients with prostate cancer.

Results

Four SSX2 peptides have affinity for HLA-A2 in vitro

To evaluate potential HLA-A2-restricted SSX2 epitopes, two peptide-binding prediction algorithms were used to scan the 188-amino acid SSX2 protein sequence for possible HLA-A2*0201-binding peptides (27, 28). Among the ten highest predicted HLA-A2 binding peptides identified by each algorithm, seven nonamer peptides were shared between the two peptide prediction programs 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 with 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 with 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 HLAA2 affinity. As shown in Table 1, the murine homologs were not highly homologous to the human peptides.

HLA-A2 SSX2 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 (29). 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 IFNγ 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), whereas lower frequency SSX2 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 SSX2 peptides p41-49 and p103-111 are immunogenic in this transgenic mouse model.

To evaluate whether these SSX2 peptides are epitopes presented by prostate cancer cells, splenocytes from peptide-immunized animals were evaluated for lysis of an HLA-A2- and SSX2-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), whereas splenocytes from animals immunized with p57-65 (panel B) or p99-107 (panel C) were not lytic (Fig. 3).

Peptide p103-111-specific CTLs were also capable of lysing LNCaP cells (panel D), and this lysis was abrogated in the presence of an HLA-A2 blocking antibody.

As a complementary approach to characterize HLAA2-restricted epitopes, we evaluated a genetic vaccine encoding SSX2. 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 APCs. SSX2 cDNA was cloned into the pTVG4 immunization vector (30), and transcription and translation from the pTVG-SSX2 construct was confirmed (Figure 4). A2/DR1 mice immunized with pTVG-SSX2 were found to develop robust (P=0.0012) p103-111-specific immune responses detectable directly *ex vivo* (Fig. 5A). In addition, 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 (Fig. 5B). 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 (Fig. 5C), and responses to other SSX2-derived peptides were not detected in either group even after peptide stimulation *in vitro*. These results indicate that peptides p41-49 and p103-111 are naturally processed, immunogenic SSX2 epitopes. Splenocytes from DNA-immunized mice were also evaluated for SSX2 peptide-specific CTLs (Fig. 6). Mice vaccinated with pTVG-SSX2 developed p41-49 and p103-111 peptide-specific

CTLs (Fig. 6A) that could also lyse the SSX2+ LNCaP prostate cancer cell line, but not an HLA-A2-expressing prostate cancer cell line not expressing SSX2 (DU145); the CTL lysis was not detectable in the presence of an HLA-A2 blocking antibody (Fig. 6B). Splenocytes from animals immunized with the pTVG4 control did not generate SSX2-specific CTL (Figs. 6C, D).

Identification of an HLA-DR1 15-mer peptide encompassing SSX2 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 SSX2 was predicted to have the highest affinity for HLA-DR1 (28). To evaluate this further, mice were immunized with SSX2 peptide p98-112 or peptide p103-111 and assessed for peptide-specific immune responses. Animals immunized with peptide p98-112 developed p98-112-specific IFNγ-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 (Fig. 7A). Similarly, A2/DR1 mice (n=8) immunized with pTVG-

SSX2 were tested for p98-112 peptide-specific immune responses. Although 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 (Fig. 7B). These results indicate that although p98-112 is an immunogenic HLA-DR1-binding peptide, it may not necessarily serve as an epitope processed and presented by APCs, and did not facilitate recognition of the p103-111 epitope.

SSX2-specific CD8+ T cells can be detected and cultured from the PBMCs of prostate cancer patients

We next wished to determine whether SSX2 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. PBMCs 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 PBMCs were stimulated for one 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 8A and 8B, SSX2-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 SSX2-specific CD8+ T cells was generally higher in patients with more advanced disease. CD8+ T cells were also isolated from the peripheral blood of 11 HLA-A2+ patients with prostate cancer and tested for their ability to lyse SSX2 p103-111 peptide-pulsed target cells. CTLs 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 after two to three stimulations *in vitro* (Figs. 8B, C). All patients with T cells showing demonstrable lysis were found to have demonstrable p103-111 tetramer-specific T cells. 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 CTLs could be detected after three *in vitro* stimulations with peptide and could also lyse the LNCaP prostate cancer cell line (Fig. 8C)

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. As 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, 31-34). 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, and other types of malignancy. Recently, Cheever et al. (35) reported that SSX2 and other sarcoma translocation breakpoint antigens may be higher priority targets for cancer therapy than PSA or PAP based upon certain predefined criteria for the prioritization of tumor antigens, such as specificity, oncogenicity, expression in multiple tumor types or advanced disease, and number of identified epitopes. As 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 SSX2. In addition, because this CTA 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 SSX2 in the future as a prophylactic cancer vaccine antigen, ideally permitting immunization in a setting before expression of the antigen in tumor cells and thereby potentially avoiding immune tolerance.

In this study, we used a genetic vaccine to identify and compare SSX2 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 SSX2 peptides or a DNA plasmid encoding SSX2 to evaluate the immunogenicity of predicted peptides and their ability to elicit antitumor CTLs. With this approach, we identified four SSX2 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 SSX2, 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 seemed to be clearly dominant with robust responses elicited by either direct peptide or DNA vaccination. Peptide p103-111 immunization elicited CTLs 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 SSX2. Ayyoub et al. (25) previously evaluated SSX2 peptides by incubating an overlapping library of SSX2 peptides with standard proteasome complexes *in vitro*. 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 CTLs were found to lyse melanoma cells and the sarcoma cell line SW 872 (25, 36-38). Wagner et al. (21) independently demonstrated that SSX2 peptide p103-111 is an HLA-A2-restricted epitope recognized by CTLs from breast cancer patients and presented by melanoma cell lines. Held et al. (39) further demonstrated that this epitope is directly presented on melanoma cells using peptide/HLA-A2-specific Fab antibodies. He et al. (40) suggested that SSX2 p57-65 was also an HLA-A2-restricted epitope by demonstrating that peptide-specific CTLs cultured from healthy donors are capable of lysing peptide-pulsed T2 cells; however, they did not demonstrate that this peptide is presented as an MHC class I-restricted epitope by tumor cells. Together, these earlier studies highlighted these three SSX2 peptides as possible HLA-A2-restricted epitopes. Before our study, a comparison of HLA-A2-

restricted epitopes had not been carried out, and it had not been determined whether SSX2 vaccines can elicit epitope-specific CTLs to these peptides. Our studies confirm the work of others that p41-49 and p103-111 are HLA-A2-restricted epitopes. However, although p57-65 can bind HLA-A2, it does not seem to be a naturally presented HLA-A2-restricted epitope, and it does not seem 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 SSX2 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 (Figs. 4B, C). 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. It is interesting to note that the Ssxa1 murine peptide corresponding to human peptide p41-49 shares the greatest homology with the human peptide, whereas 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 CTLs 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 CTLs failed to lyse SSX2+ LNCaP cells (Fig. 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 CTLs 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 SSX2 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-SSX2 elicited p103-111-specific immune responses in every immunized animal and with higher frequency than p41-49-specific cells as determined by both IFNγ ELISPOT and tetramer staining of splenocytes directly *ex vivo*. It is interesting to note that the best *in vitro* lysis of LNCaP cells and peptide-pulsed T2 cells (Figs. 5A, B) 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* IFNγ ELISPOT assay (data not shown). This finding may indicate that superior lysis of prostate tumor cells can be achieved by eliciting CTLs specific for multiple SSX2 epitopes. A possible future direction may be to develop the SSX2 DNA vaccine as an immunotherapeutic tool by optimizing its ability to elicit responses to multiple epitopes simultaneously.

In addition to identifying SSX2 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 CTLs that could specifically lyse p103-111-pulsed target cells, whereas 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 SSX2 expression than earlier stage disease, as has been suggested by findings of increased SSX2 mRNA expression in metastatic prostate cancer and other tumor types (18, 41, 42), which could lead to increased cross-presentation of SSX2 and generation of SSX2-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 SSX2-expressing tumors. However, to date, the expression and function of SSX2 in prostate tumors of various stages remains largely unknown. These will be areas of future research.

In conclusion, this study provides direct evidence that SSX2 is a relevant vaccine target antigen for prostate cancer. In particular, these findings highlight the importance of SSX2 peptide p103-111 as a dominant HLA-A2-restricted immunogenic epitope. To date, the majority of the work evaluating SSX2 has focused on SSX2 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 SSX2, 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 SSX2-specific vaccines *in vivo*, such as using these vaccines in combination with epigenetic modifying agents that might increase SSX2 expression in tumor cells (18).

Figures and Tables

Figure 1. SSX2 nonamer peptides have HLA-A2 affinity in vitro

Panel A. Shown is the relative HLA-A2 binding of each SSX2 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 mean fluorescence intensity (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 mg/mL) in T2 binding assays. Shown are the normalized MFI values, and data are representative of two independent experiments.

Figure 1. SSX2 nonamer peptides have HLA-A2 affinity in vitro

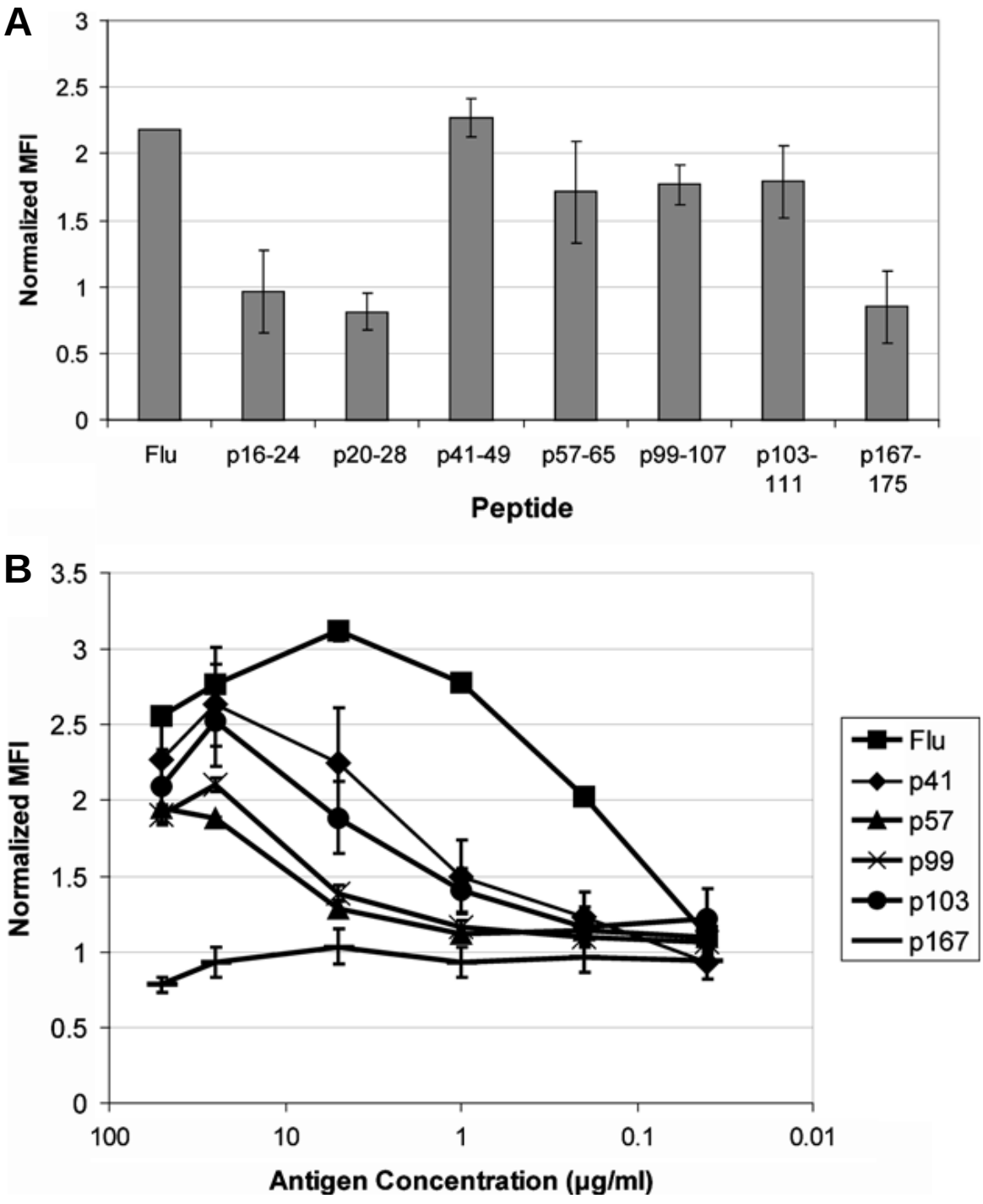


Table 1. Predicted HLA-A2 affinity and homology of human and mouse SSX2 nonamer peptides

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 SSX2, and the respective murine Ssxa1 and Ssxb1 peptide sequences and binding affinities. Predicted HLA-A2 peptide affinity was determined by scanning the SSX2, Ssxa1, and Ssxb1 protein sequence with two different peptide prediction algorithms (27, 28). A known HLA-A2-binding peptide derived from the influenza A matrix protein is shown for comparison. Amino acids within the mouse peptide sequences shown in gray represent identical peptide residues between the human and murine peptides.

Table 1. Predicted HLA-A2 affinity and homology of human and mouse SSX2 nonamer peptides

Human SSX-2 Peptide Name	Peptide Sequence	Predicted Binding Affinity (BIMAS)	Predicted Binding Affinity (SYFPEITHI)	Mouse Peptide Homolog (Ssxa1 Ssxb1)	Predicted Binding Affinity (BIMAS)	Predicted Binding Affinity (SYFPEITHI)
SSX2p16	QIPEKIQKA	6.4	21	HKPEETCQA	0.001	8
				YEPKNICKA	1.022	12
SSX2p20	KIQKAFDDI	4.7	17	ETCQAFEDI	0.010	9
				NICKAFQDI	2.726	16
SSX2p41	KASEKIFYV	1017.1	22	SRSEKITYV	0.501	19
				TQWQKSAYV	321.9	14
SSX2p57	AMTKLGFKA	20.8	16	TMTNLGLRA	3.588	13
				RMTDLGVTV	205.9	23
SSX2p99	MTFGRLQGI	2.1	20	Deleted	—	—
SSX2p103	RLQGISPKI	10.4	23	Deleted	—	—
SSX2p167	RLRERKQLV	21.7	22	QLREKKKPV	21.672	22
				IKRMKLTSV	0.029	19
Influenza	GILGFVFTL	550.9	30	N/A	—	—

Figure 2. Peptide-immunized A2/DR1 mice develop SSX2 peptide-specific immune responses to SSX2 p41-49 and p103-111

A2/DR1 mice were immunized once with 100 mg of each individual SSX2 peptide or an influenza A matrix (Flu) protein peptide (n = 4 to 12 per group) and then assessed for the development of peptide-specific immune responses by IFNγ ELISPOT. Splenocytes were cultured with the specific peptide used for immunization (●), a nonspecific HLA-A2-binding nonamer peptide (○), or Concanavalin A (ConA) positive control (▲). For animals immunized with Flu, the nonspecific peptide used was p103-111. Each dot represents the frequency of IFNγ SFU from an individual animal measured in triplicate with background subtracted. Data are representative of two independent experiments. * indicates a significant response ($P < 0.05$, 2-tailed t test).

Figure 2. Peptide-immunized A2/DR1 mice develop SSX2 peptide-specific immune responses to SSX2 p41-49 and p103-111

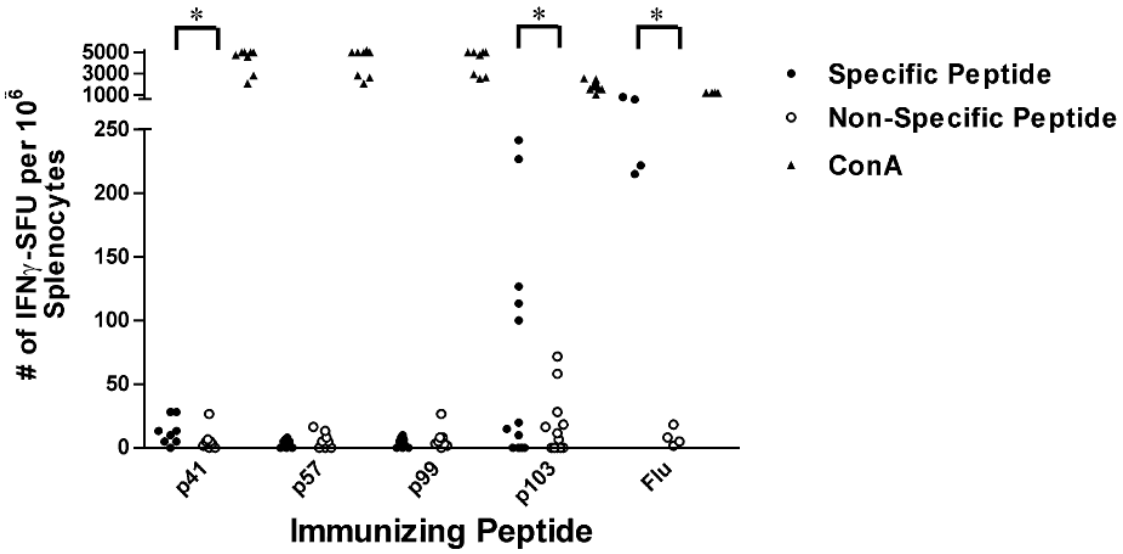


Figure 3. Immunization of A2/DR1 mice with SSX2 peptides can elicit cytotoxic immune responses against peptide-specific target cells and human prostate cancer cells

Splenocytes from A2/DR1 mice vaccinated with SSX2 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 3. Immunization of A2/DR1 mice with SSX2 peptides can elicit cytotoxic immune responses against peptide-specific target cells and human prostate cancer cells

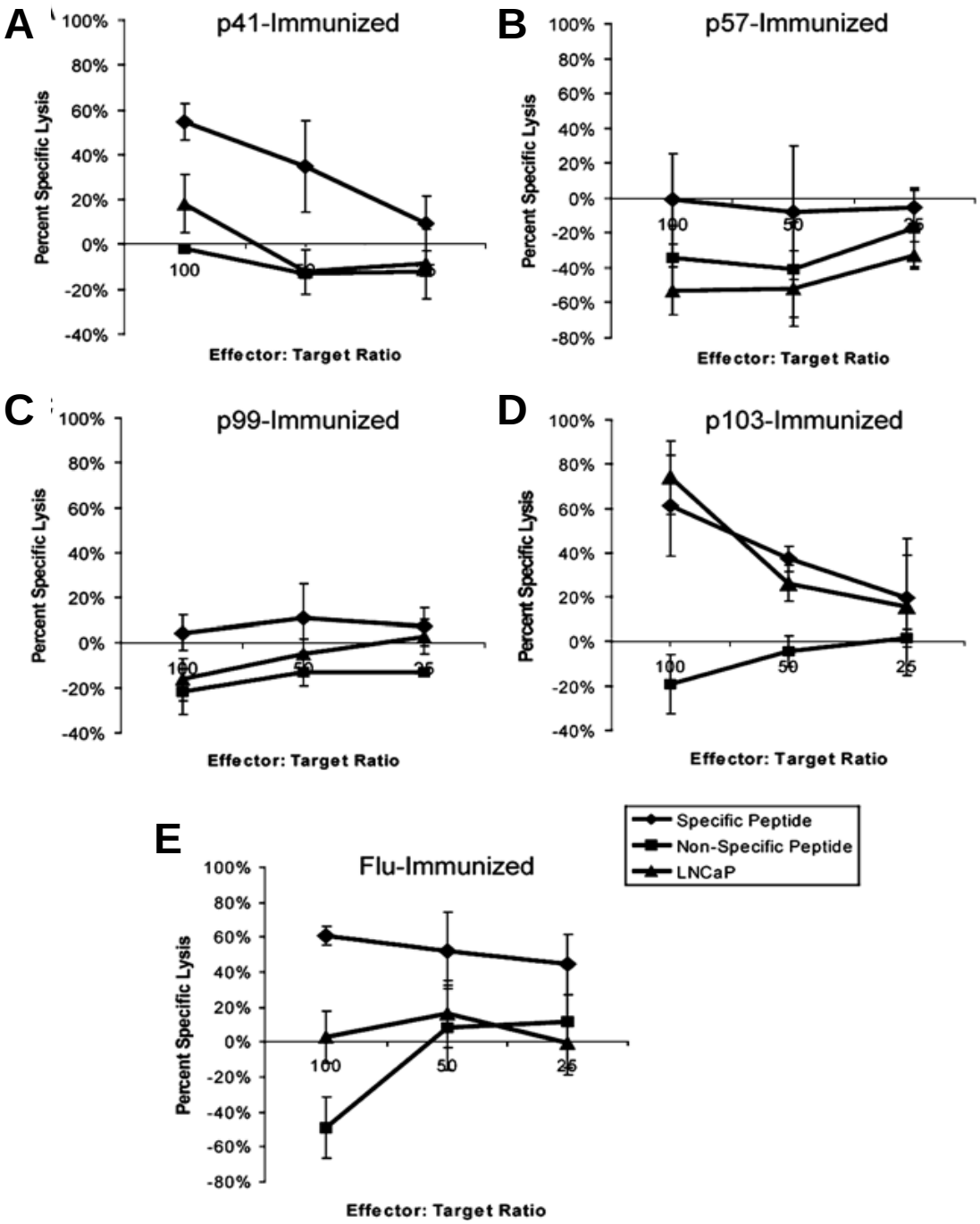


Figure 4. The pTVG-SSX2 DNA vaccine expresses SSX2 mRNA and protein following transient transfection

Full-length SSX2 cDNA was cloned into the pTVG4 immunization vector to construct the DNA vaccine (*Panel A*). To confirm that SSX2 is expressed from the DNA plasmid when taken up by host cells, transient transfection assays were carried out in which Cos-7 cells were transfected with either pTVG-SSX2 or the empty pTVG4 plasmid. RNA and protein samples were collected at 24 and 48 hours post-transfection to test for SSX2 expression via RT-PCR (*Panel B*) and Western blot (*Panel C*). Expected molecular weight of SSX2 protein (approximately 21 kDa) is indicated by arrow.

Figure 4. The pTVG-SSX2 DNA vaccine expresses SSX2 mRNA and protein following transient transfection

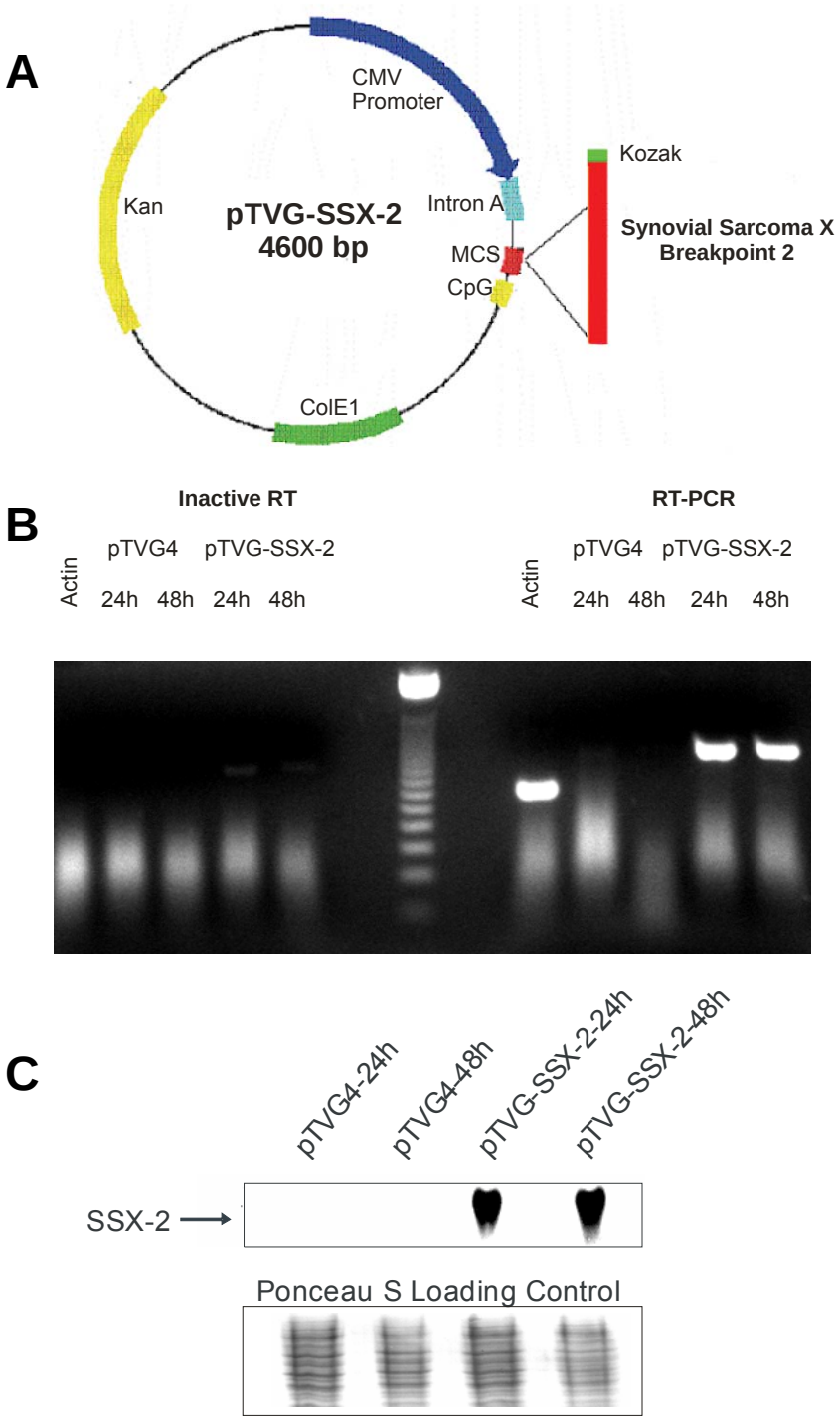


Figure 5. DNA-immunized A2/DR1 mice develop peptide-specific immune responses to SSX2 peptides p41-49 and p103-111

Splenocytes from individual A2/DR1 mice immunized with either pTVG-SSX2 (n = 7, closed circle) or pTVG4 (n = 6, open circle) were analyzed directly *ex vivo* (*Panel A*) or after a 7-day *in vitro* stimulation (*Panel B*) to determine the frequency of peptide-specific T cells by IFNγ ELISPOT. Each dot represents the frequency of peptide-specific IFNγ-SFU from an individual mouse, measured in triplicate with background subtracted. * indicates a significant ($P < 0.05$) difference in the mean number of IFNγ-SFU between the two groups (2-tailed *t* test). Results are representative of two independent experiments. *Panel C*: Splenocytes from representative animals immunized with either pTVG4 or pTVG-SSX2 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. DNA-immunized A2/DR1 mice develop peptide-specific immune responses to SSX2 peptides p41-49 and p103-111

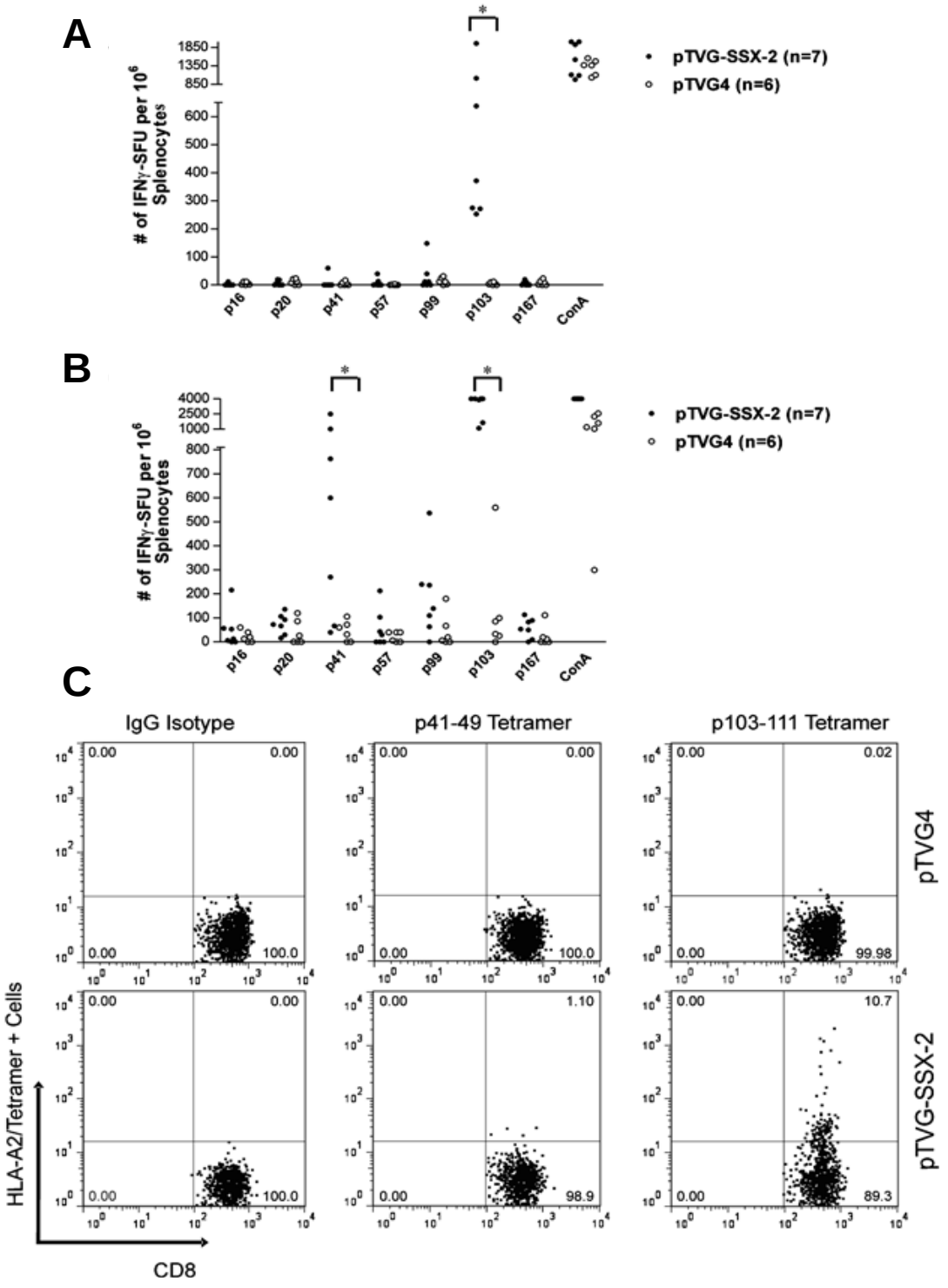


Figure 6. Immunization of A2/DR1 mice with pTVG-SSX2 DNA vaccine elicits cytotoxic immune responses against p41-49 and p103-111 peptide-pulsed target cells and the LNCaP prostate cancer cell line

Splenocytes from animals vaccinated with pTVG-SSX2 (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 $SSX2^-$ DU145 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 (solid triangle) or absence (solid circle, square) of an HLA-A2 blocking antibody.

Figure 6. Immunization of A2/DR1 mice with pTVG-SSX2 DNA vaccine elicits cytotoxic immune responses against p41-49 and p103-111 peptide-pulsed target cells and the LNCaP prostate cancer cell line

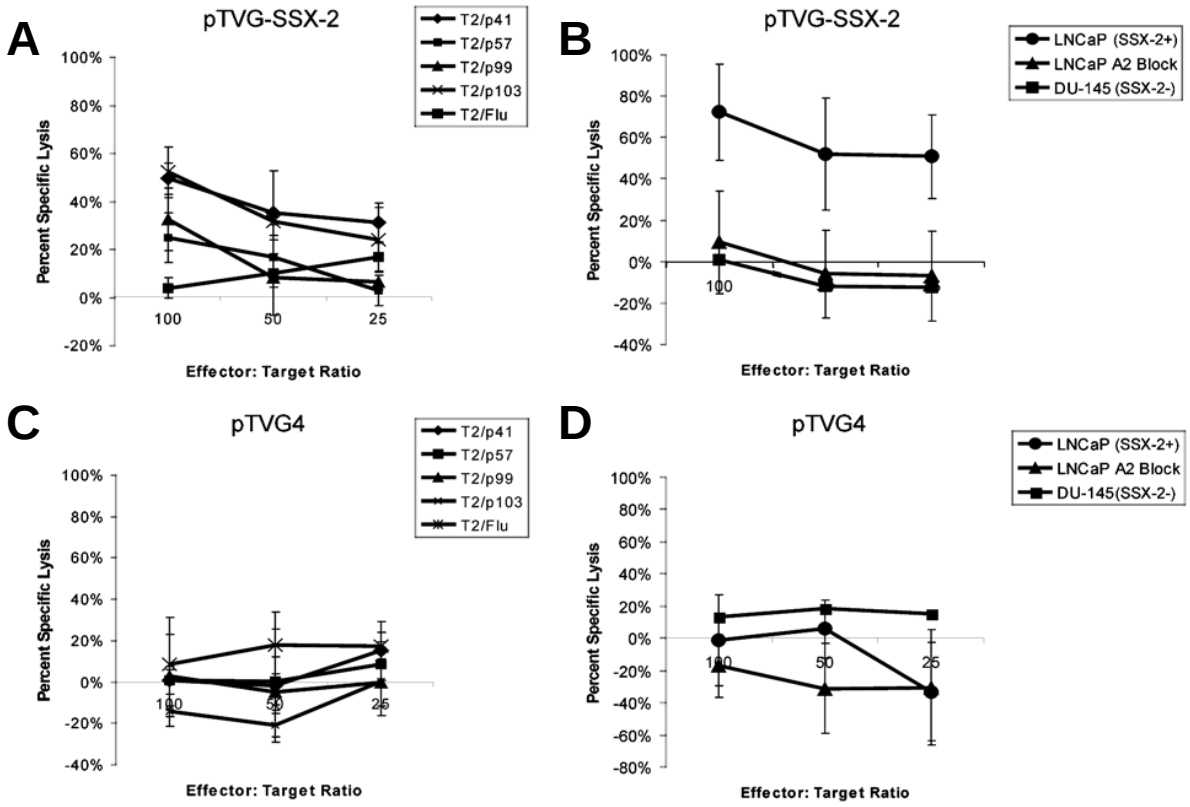


Figure 7. Identification of an SSX2 DR1-binding peptide encompassing SSX2 p103-111

Splenocytes from A2/DR1 mice immunized once with 100 mg of SSX2 15-mer peptide p98-112 (n = 4, solid circle) or nonamer peptide p103-111 (n = 5, solid triangle) were assessed for the development of peptide-specific immune responses by IFNγ 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 mg of pTVG-SSX2 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 IFNγ 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 ($P < 0.05$, 2-tailed *t* test). Data are representative of two independent experiments.

Figure 7. Identification of an SSX2 DR1-binding peptide encompassing SSX2 p103-111

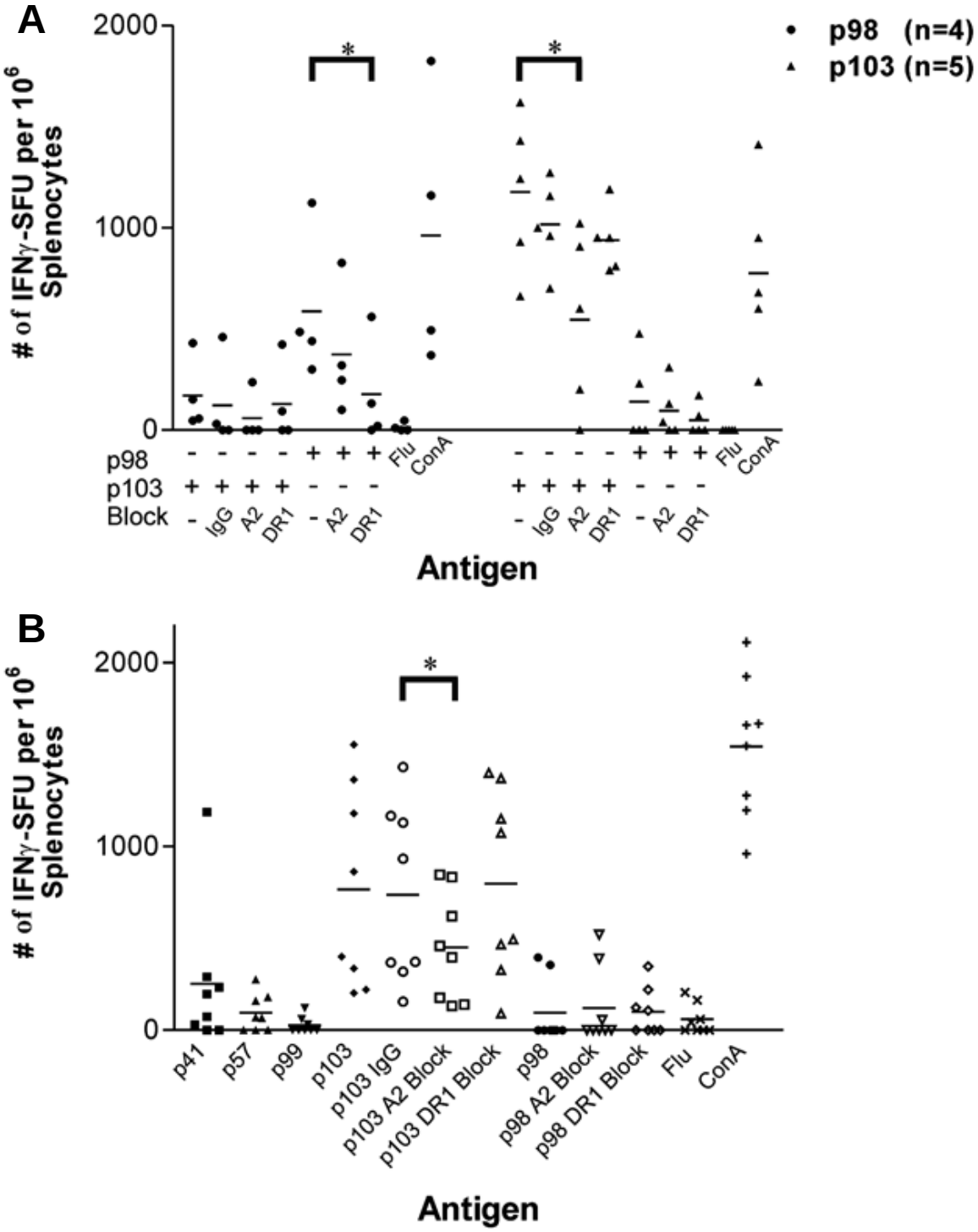


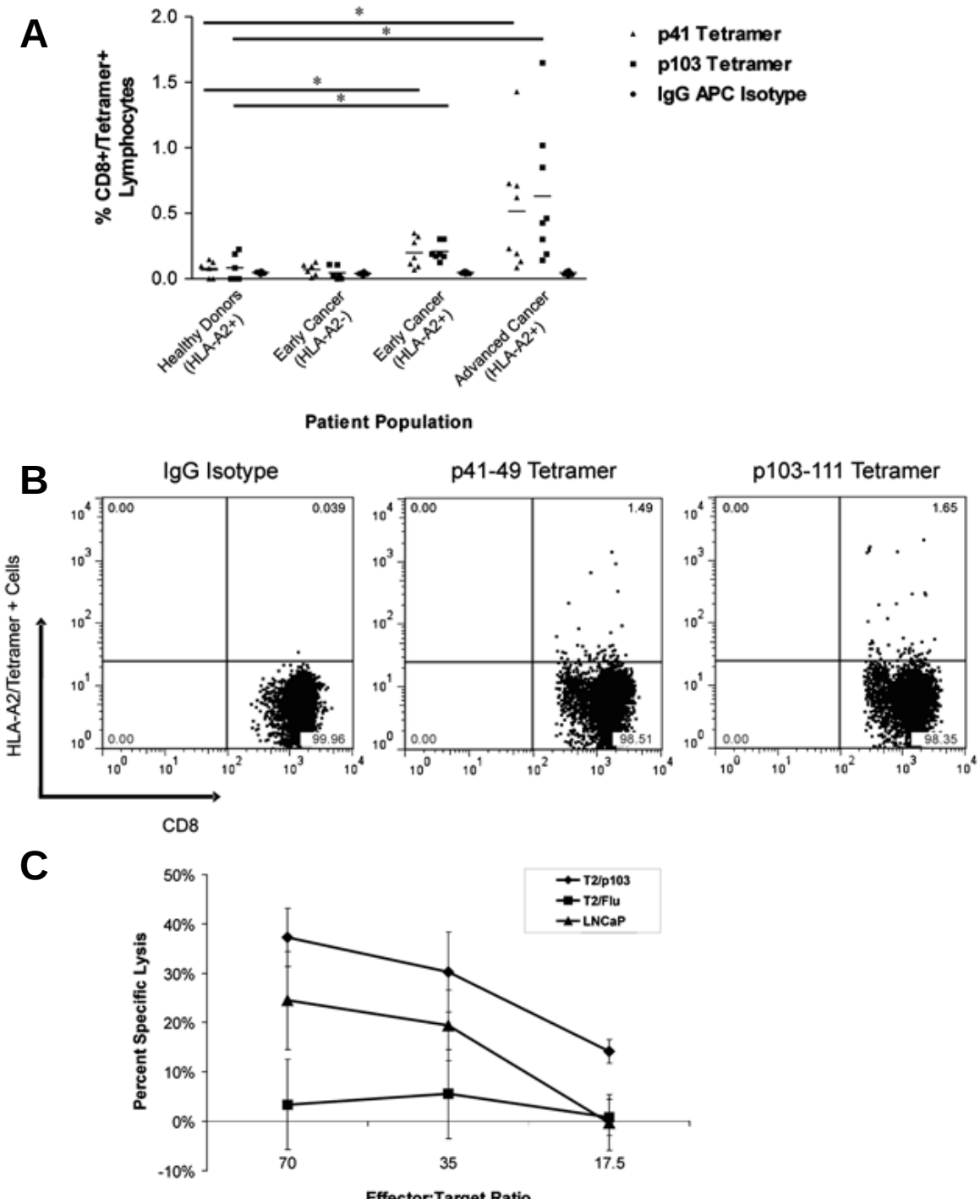
Figure 8. SSX2 epitope-specific CTLs can be detected and cultured from the peripheral blood of prostate cancer patients

Panel A. PBMCs obtained from six HLA-A2+ men without prostate cancer, and from 21 men with prostate cancer, were stimulated *in vitro* for seven 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 ($P < 0.05$, 2-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 SSX2 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.

Figure 8. SSX2 epitope-specific CTLs can be detected and cultured from the peripheral blood of prostate cancer patients



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Chapter 4

Vaccines with Altered Peptide Ligands Targeting the Cancer-Testis Antigen SSX2 Elicit Epitope-Specific Anti-tumor Immune Responses

This work is currently being prepared for submission to the Journal of Immunology.

Abstract

The SSX family of proteins are considered attractive immunotherapeutic target antigens for cancer based upon their normally tissue-restricted expression to immunoprivileged germline cells of the testis and for their ectopic expression in a variety of malignancies. Notably, SSX2 has been identified as the most frequently expressed SSX family member in prostate cancer cell lines and tissues and immune responses to this protein have been found to spontaneously develop in patients with prostate cancer, suggesting that this antigen is naturally immunogenic. We have previously identified two peptides, p41-49 and p103-111, as HLA-A2-restricted prostate cancer epitopes, and we have found that HHDII-DR1 transgenic mice develop peptide-specific immune responses to these epitopes after immunization with a plasmid DNA vaccine encoding SSX2. In the present study we sought to enhance SSX2 vaccine efficacy by utilizing an altered peptide ligand (APL) strategy wherein modifications were made to anchor residues of peptides p41-49 and p103-111 to enhance their binding affinity to HLA-A2. We found that these APL peptides had greater affinity for HLA-A2 *in vitro* and elicited increased frequencies of cross-reactive, peptide-specific T cells in immunized mice compared to animals immunized with the native epitopes. Additionally, CTL from immunized mice could lyse peptide-pulsed target cells and prostate cancer cells. Finally, we designed a SSX2 DNA vaccine encoding APL that elicited robust peptide specific immune responses to epitopes p41-49 and p103-111 simultaneously. Both the native and modified vaccines were found to

elicit anti-tumor immune responses in HHDII-DR1 mice bearing SSX2+ tumors. These results demonstrate that APL can be used to elicit enhanced frequencies of SSX2 peptide-specific T cells specific for multiple epitopes simultaneously, and that these vaccines can elicit anti-tumor immune responses.

Introduction

Advanced prostate cancer is the second leading cause of cancer-related death among American men after lung cancer and remains a significant health concern worldwide (1). Most prostate cancer patients will initially present with organ-confined malignancy, however, one-third of patients who receive primary treatment, such as surgical removal of the prostate or radiation therapy, will relapse with biochemically-recurrent disease (2-4). Ultimately, these relapses are accompanied by metastatic lesions in the lymph nodes and/or bone (2). While androgen ablation therapy can provide a brief tumor remission, approximately 80-90% of these patients will ultimately relapse again developing a more aggressive disease, and for these individuals there are few therapies providing considerable improvement in overall survival (4, 5).

Tumor immunotherapy is one type of cancer treatment that has been gaining increased attention in the last decade for prostate cancer. Recently the Food and Drug Administration (FDA) approved the first tumor immunotherapy vaccine for castrate-resistant prostate cancer, Sipuleucel-T (Provenge®, Dendreon Corp.) (6). This antigen-presenting cell (APC) vaccine loaded with prostatic acid phosphatase (PAP) fused to granulocyte-macrophage colony stimulating factor (GM-CSF) was found to confer a modest survival benefit in patients with castrate-resistant metastatic disease over placebo. However, the high cost and cumbersome production of this vaccine have limited its widespread implementation (7). Several other immunotherapy techniques for tumor antigen

delivery are being actively pursued in pre-clinical and clinical studies to target specific tumor associated antigens (TAA) expressed by prostate cancers. These include peptide or protein vaccines, viral based vectors, and DNA plasmid vaccines (8-13). Of these, plasmid DNA vaccines offer several advantages over many other types of antigen delivery systems including safety; relative ease and inexpensive manufacturing; shipping and storage; pliability of the DNA backbone for alteration of the encoded antigen and/or encoding multiple antigens or immune-modulation factors. One of the key challenges in designing genetic vaccines is the choice of ideal target antigens.

Recently, a tumor vaccine consortium panel outlined a number of specific antigen characteristics that appear to be hallmarks of strong tumor targets (14). We have been evaluating a family of cancer-testis antigens (CTA), the synovial sarcoma chromosome X breakpoint (SSX) proteins, found to possess many of these features of an attractive immunotherapeutic target antigen including immunogenicity, specificity, expression level and percent of antigen-positive cells, stem cell expression, and number of antigenic epitopes (15-20). We have specifically focused on SSX2, which we have previously shown to be expressed in ~25% of metastatic prostate cancer lesions (17). As a CTA this protein likely has limited central tolerance since its expression is restricted to immune-privileged testis tissue (21). This could contribute to the previous observations that prostate cancer patients can have pre-existing humoral and cell-mediated immune responses specific for SSX2 (16, 18). We have also identified two HLA-

A2-restricted SSX2 epitopes (p41-49 and p103-111); T cells specific for which can can lyse SSX2-expressing prostate cancer cells. Moreover, we have shown that HLA-A2 transgenic mice immunized with a DNA vaccine encoding SSX2 develop p41-49 and p103-111-specific T cells (16, 17).

In this study we sought to determine whether modifications to a plasmid DNA vaccine encoding SSX2 could be designed to augment the frequency and efficacy of HLA-A2-restriced CTL and enhance peptide-specific anti-tumor activity. Specifically, we wanted to first identify altered peptide ligands (APL) for SSX2 with greater or reduced HLA-A2 binding. We then sought to establish whether immunization with these peptides resulted in cross-reactive CTL specific for the native epitope and investigate whether a DNA vaccine encoding altered peptides could be used to elicit these responses. Finally we wanted to determine which modifications could be made to an SSX2 DNA vaccine that would result in broad epitope-specific CTL responses and anti-tumor efficacy. Specifically, we hypothesized that eliminating dominant epitopes or enhancing the binding of subdominant epitopes could boost the immune responses generated to SSX2 or elicit immune responses to the greatest number of epitopes.

Specific Aims

I aim to evaluate whether SSX2 DNA vaccine efficacy can be enhanced using an altered peptide ligand strategy to increase HLA-A2 binding of specific SSX2 peptides, and determine if SSX DNA vaccination can induce peptide-specific anti-tumor immune responses in A2/DR1 mice.

In this chapter, I will provide evidence demonstrating that:

1) SSX2 peptides designed to have enhanced or reduced affinity for HLA-A2 have increased or decreased binding to HLA-A2 *in vitro* as predicted.
2) Modified SSX2 peptides and APL DNA vaccines can elict enhanced frequencies of peptide-specific T cells that are cross-reactive with the native epitopes.
3) APL SSX2 vaccines can elicit CTL capable of lysing prostate cancer cell lines *ex vivo*.
4) pTVG-SSX2 p41-49-AL/p103-111-RF, modified to increase binding of both a dominant and subdominant epitope, appears to elicit the highest frequency of peptide p41-49 and p103-111 peptide-specific T cells in the greatest number of immunized A2/DR1 mice.
5) Native and modified SSX2 DNA vaccines can elicit antigen-specific anti-tumor immune responses in A2/DR1 mice bearing SSX2-expressing tumors.

Results:

Modified SSX2 peptides designed to have enhanced affinity for the HLA-A2 complex bind more strongly to HLA-A2 compared to native peptides

We have reported previously that SSX2 peptides p41-49, p57-65, p99-107, and p103-111 have affinity for the HLA-A2 complex (16). While all four of these peptides were found to bind HLA-A2 *in vitro*, peptide immunization studies in the HHDII-DR1 transgenic mouse model revealed that only p41-49 and p103-111 were able to elicit peptide-specific immune responses. Vaccination with these peptides directly or a plasmid DNA vaccine encoding SSX2 elicited peptide p41-49- or p103-111-specific T cells and CTL capable of lysing peptide-pulsed target cells or the LNCaP prostate cancer cell line (16). In this study we evaluated whether the T-cell responses to all four of the previously identified HLA-A2-binding SSX2 peptides could be enhanced by modifying the anchor residues of the peptides for the HLA-A2 complex, while maintaining recognition of the native peptide residues by T cells.

Using two MHC-peptide binding algorithims, BIMAS (22), and SYFPEITHI (23), we designed homologous peptides for p41-49, p57-65, p99-107, and p103-111 with predicted increased or decreased HLA-A2 binding affinity (Table 1). T2 binding assays were then carried out to determine the *in vitro* binding affinity of each peptide for the HLA-A2 complex. We found that all peptides designed to have enhanced binding to HLA-A2 were able to stabilize the surface expression of HLA-A2 on T2 cells to a higher degree than the native peptides (Figure 1),

while peptides designed to ablate HLA-A2 affinity showed no HLA-A2 stabilization above vehicle control. These studies confirmed that the designed peptides could bind to HLA-A2 with the affinities predicted.

HHDII-DR1 Mice Immunized with Modified SSX2 Peptides Develop Enhanced Peptide-Specific Immune Responses and CTL Capable of Lysing Prostate Cancer Cells

To determine whether modified SSX2 peptides can elicit enhanced, cross-reactive peptide-specific immune responses, immunization studies were carried out in HLA-A2 transgenic (HHDII-DR1) mice. Animals were immunized once with either a native peptide (p41-49, p57-65, p99-107, or p103-111) or a modified peptide. Splenocytes were collected from these animals and tested for the frequency of peptide-specific immune responses by IFNγ ELISPOT. As shown in Figure 2, animals immunized with the enhanced HLA-A2 binding peptides designed for p41-49 and p103-111 developed higher frequencies of peptide p41-49 or p103-111-specific T cells compared to animals immunized with the native peptides. These T cells not only recognized the modified immunizing peptide, but could also cross-recognize the native epitopes. Animals immunized with modified p57-65 or p99-107 peptides did not develop increased frequencies of peptide-specific T cells (Figure 2), suggesting that these peptides can not be modified to enhance their immunogenicity through enhanced HLA-A2 binding.

Splenocytes isolated from peptide-immunized animals were tested for their ability to recognize and lyse peptide-pulsed target cells and prostate cancer cells. We found that animals immunized with p41-49 were unable to lyse prostate cancer cell lines as we have reported before, however, splenocytes from animals immunized with the modified peptide p41-49-AL or p41-49-AV (not shown) were able to lyse peptide-pulsed T2 target cells as well as the LNCaP prostate cancer cell line (Figure 3). Lysis was abrogated in the presence of an HLA-A2 blocking antibody, demonstrating that peptide p41-49 is an HLA-A2-restricted epitope presented by LNCaP cells. Interestingly, it appears that immunization with the native p41-49 peptide did not elicit peptide-specific cytotoxic T lymphocytes (CTL) in high enough frequency to detect lysis of target cells *in vitro*. Splenocytes from animals immunized with the native p103-111 peptide or the modified p103-111-RF peptide were able to lyse both peptide-pulsed target cells and the LNCaP cell line, but again this lysis was HLA-A2 restricted as no lysis was observed in the presence of an HLA-A2 blocking antibody. No CTL lysis was observed by splenocytes isolated from animals immunized with native or enhanced-binding p57-99 or p99-107 peptides. These results demonstrate that peptides p41-49 and p103-111 were the only HLA-A2-restricted epitopes, and CTL elicited by these peptides are capable of lysing a prostate cancer cell line in an HLA-A2 restricted manner. Additionally, in the case of peptide p41-49, it was advantageous to immunize with the APL peptide to allow for the highest frequency of peptide p41-49-specific T cells.

HHDII-DR1 mice immunized with enhanced, modified SSX2 plasmid vaccines develop p41-49 and p103-111 peptide-immune responses at higher frequency than animals immunized with the native vaccine

Single peptide immunizations demonstrate the ability of an APL strategy to enhance peptide-specific immune responses to native SSX2 epitopes. However, we ultimately wanted to identify the best immunization strategy to develop enhanced immune responses to multiple SSX2 epitopes simultaneously by DNA plasmid vaccination. With direct peptide vaccination there is no internal competition between epitopes for presentation or T cell recognition, yet other groups have shown that epitope competition can result in the development of dominant and subdominant immune responses to multiple encoded peptides (24-26). We hypothesized that dominant epitope-specific immune responses generated by a DNA vaccine encoding SSX2 may mask subdominant epitopes by competition for MHC presentation and/or T cell activation. Therefore, we tested vaccines that both ablated HLA-A2 binding of dominant epitopes as well as vaccines that were designed to enhance binding of subdominant epitopes in an effort to identify an optimal vaccine to elicit simultaneous immune responses to multiple SSX2 epitopes. Modified plasmid vaccines were generated by utilizing site-directed mutagenesis to alter the native pTVG-SSX2 vaccine. Our first immunization studies were carried out to confirm that the only robust HLA-A2-specific immune responses are conferred by the dominant (p103-111) and

subdominant (p41-49) SSX2 epitopes and evaluate whether these responses were masking other potential weaker epitopes (e.g. p57-65 and p99-107). Animals were immunized six times biweekly with the native SSX2 vaccine or a modified SSX2 vaccine encoding peptides p41-49 and p103-111 with proline mutations to ablate HLA-A2 binding. As expected, ablation of peptides p41-49 and p103-111 by introduction of proline mutations abrogated immune responses to both, however, animals also failed to elicit immune responses to other HLA-A2-binding peptides p57-65 or p99-107 (Figure 4A). These results also confirm that peptides p57-65 and p99-107 are not epitopes in this model and may not be amenable to further enhancement in modified DNA constructs, while p41-49 and p103-111 are the sole HLA-A2-restricted immunogenic epitopes.

We next evaluated whether immunization with a DNA vaccine encoding modifications designed to increase HLA-A2 binding of the dominant epitopes could enhance peptide-specific immune responses, and whether ablating binding of peptide p103-111 to HLA-A2 could increase immune responses to the subdominant p41-49 epitope. Alterations to the DNA plasmid were based on the results from the direct peptide vaccination studies, which indicated that p41-AL and p103-RF were more immunogenic than p41-AV and p103-IV, respectively. We immunized mice with a vaccine that enhanced peptide p41-49 binding to HLA-A2 (pTVG-SSX2 p41-AL), a vaccine that ablated peptide p103-111 binding to HLA-A2 (pTVG-SSX2 p103-IP), or a vaccine that both enhanced peptide p41-49 HLA-A2 binding and ablated peptide p103-111 binding (pTVG-SSX2 p41-

AL/p103-IP). Interestingly, we found that ablating binding of peptide p103-111 to HLA-A2 did not increase peptide p41-49 peptide-specific immune responses as we had predicted (Figure 4B), however both constructs encoding peptide p41-49-AL elicited statistically significantly higher frequencies of p41-49 peptide-specific T cells in the immunized animals.

We next immunized mice with the native vaccine, a vaccine encoding peptide p103-111 with enhanced HLA-A2 affinity (pTVG-SSX2 p103-RF), and a vaccine that ablated peptide p103-111 binding to HLA-A2 (pTVG-SSX2 p103-IP). As expected, we found that a greater number of animals had higher frequencies of peptide p103-111-specific immune responses when immunized with p103-111-RF compared to the native vaccine (Figure 4C). While few responses to peptide p41-49 were detected, this frequency was not significantly affected by the vaccine encoding p103-111-RF.

Finally, we conducted an immunization study to determine the optimal vaccine to elicit robust p41-49 and p103-111 immune responses. We immunized mice with the native vaccine or vaccines enhancing p41-49 alone (pTVG-SSX2 p41-49-AL) or enhancing p41-49 and p103-111 simultaneously (pTVG-SSX2 p41-49-AL/p103-111-RF). We found that the latter vaccine was able to elicit the highest frequency of peptide specific immune responses to both of the native p41-49 and p103-111 epitopes (Figure 4D).

Immunization of SSX2 and GFP tumor-bearing mice with native and enhanced, modified SSX2 vaccines induces antigen-specific tumor regression

To evaluate whether the native or modified SSX2 DNA vaccines have anti-tumor activity we generated an HHDII-DR1 syngeneic sarcoma cell line line that was stably transduced with a lentiviral vector encoding either SSX2 or GFP for subcutaneous tumor studies. Sarcoma cell expression of SSX2 and GFP was confirmed by Western blot and flow cytometry analysis, respectively (Figure 5). To evaluate anti-tumor vaccine efficacy we carried out both prophylactic and therapeutic immunization studies. In prophylactic tumor studies HHDII-DR1 mice were immunized six times biweekly with pTVG4 vehicle, native SSX2 vaccine (pTVG-SSX2), optimized SSX2 vaccine (pTVG-SSX2-p41-49-AL/p103-11-RF), or a vaccine that ablated HLA-A2 binding of both dominant epitopes (pTVG-SSX2-p41-49-VP/p103-111-IP). Two weeks after the last vaccination the animals were inoculated with 10^4 SSX2-expressing sarcoma cells or 10^4 GFP-expressing sarcoma cells in the contra lateral flank. We found that animals immunized with the native SSX2 and optimal modified vaccine had SSX2-expressing tumors that were significantly smaller than SSX2-expressing tumors in animals immunized with the control plasmid or the vaccine ablating HLA-A2 binding of peptides p41-49 and p103-111 (Figure 6 panel A). Likewise, we observed similar results in the tumor therapy study (Figure 6 panel B), with SSX2-expressing tumors in animals immunized with the native SSX2 and optimal

modified vaccine significantly smaller than tumors in mice immunized with the other constructs. There was not a statistically significant difference in GFP-expressing sarcoma tumor size among any of the groups, which suggests that the SSX2 vaccines elicit anti-tumor immune responses in an antigen-dependent manner.

Discussion

Since the concept of using APL to augment T-cell immune responses was first introduced two decades ago, this strategy has been used to investigate multiple aspects of antigen-specific immunity (27, 28). APL have been utilized by many groups to both down-regulate and up-regulate immune responses to peptide antigens expressed by pathogens, tumor cells, and somatic cells to circumvent infectious disease, cancer, and autoimmune diseases (28). In many cases APL serve as antagonists to dampen the immune response or inactivate T cell effector functions induced by the native epitope in autoimmune or infectious diseases (29). The goal for tumor immunology, however, is to augment epitope-specific immune responses against peptides expressed on tumor cells to increase the frequency and/or cytolytic activity of anti-tumor CTL. Tumor-associated antigens (TAA) are usually weakly immunogenic, containing epitopes that have low binding affinity for HLA molecules, yet strong immune responses have been generated to several TAA in both pre-clinical mouse studies and human clinical trials using APL strategies (29). For example, Lazoura, et. al., demonstrated that immune responses to mucin 1 could be enhanced in mice after *in vivo* vaccination with MUC1-8, an altered peptide with 100-fold increased binding to H-2Kb due to an anchor residue modification (30). Strong immune responses have also been generated in both animals and in human clinical trials after APL immunization with a peptide from the melanocyte differentiation antigen TRP-2 ($TRP_{180\text{-}188}$) (31). To date, APL have demonstrated efficacy in eliciting

enhanced immune responses to many different tumor and viral antigens that are otherwise weakly immunogenic or tolerized (24-26, 32-34).

In the present study we sought to enhance epitope-specific immune responses to the SSX2 tumor antigen using an APL strategy to boost responses to multiple SSX2 epitopes simultaneously. We evaluated whether APL vaccines could elicit enhanced frequencies of SSX2 peptide-specific T cells, and determine if native and APL vaccines possess anti-tumor efficacy in an SSX2-expressing subcutaneous tumor model. Unlike previous studies, we specifically used a DNA vaccine to deliver two altered ligands, which allowed for the delivery of multiple APL to the same APC for presentation and activation of T cell immunity. It should be noted that we specifically made amino acid substitutions to peptide anchor residues to enhance HLA-A2 binding while potentially maintaining interaction with the TCR of lymphocytes specific to these peptide/MHC complexes. We did not design these peptides to enhance TCR recognition in a heteroclitic peptide strategy.

We evaluated two distinct and well-defined SSX2 MHC class I epitopes, peptides p41-49 and p103-111, and our analysis was entirely restricted to one MHC haplotype (HLA-A2). Peptides p41-49 and p103-111 were first identified as HLA-A2-restricted SSX2 epitopes recognized by T cells in melanoma and breast cancer patients, respectively (35, 36). Since this time, we have demonstrated that HHDII-DR1 mice immunized with these SSX2 peptides develop CTL that can lyse HLA-A2+ prostate cancer cell lines (16). Results from immunization studies

with a plasmid encoding SSX2 indicated that peptide p103-111 is the immunodominant SSX2 epitope based on the frequency of peptide-specific T cells from immunized animals (16). Furthermore, we demonstrated that HHDII-DR1 mice immunized with a vaccine encoding SSX2 develop cross-reactive immune responses to multiple p103-111 peptides from homologous SSX family members. Interestingly, some prostate cancer patients also have peptide p103-111-specific T cells, which are detectable at higher frequency with more advanced disease (16, 17). These previous findings highlight the potential importance of peptides p41-49 and p103-111 as immunotherapeutic target epitopes for the treatment of SSX+ prostate cancer.

In the current study we found that APL for peptides p41-49 and p103-111, modified at MHC/peptide anchor residues, had enhanced binding affinity to HLA-A2 *in vitro*, and we also demonstrated that these APL could elicit greater numbers of peptide-specific T cells in HHDII-DR1 mice after peptide immunization. T cells from these animals were not only able to recognize the APL peptide, but more importantly, were also cross-reactive to the native epitopes. In addition these T cells could lyse peptide-pulsed target cells and the LNCaP (HLA-A2+ and SSX2+) prostate cancer cell line in an HLA-A2-restricted manner. Thus, we were able to augment immune responses to both p41-49 and p103-111 with altered peptides.

Interestingly we did not find that APL strategies could be used to enhance or elicit peptide-specific immune responses to HLA-A2-binding peptides p57-65

or p99-107. He, et. al., had previously demonstrated that APL to peptide p57-65 could elicit CTL capable of lysing peptide-pulsed target cells after *in vitro* stimulation of human PBMC or after immunization of HLA-A2.1/K^b transgenic mice (37). Their ability to generate p57-65-specific CTL could be due to different p57-65 APL and mouse model than we report here, however, the relevance of this is unclear as it was not demonstrated that p57-65-specific T cells could lyse SSX2-expressing HLA-A2+ cells. Hence, peptide p57-65 is not a true epitope in our model system or theirs. Immune responses to peptides p57-65 and p99-107 were not even observed after ablating binding of the dominant p41-49 and p103-111 epitopes after immunization of mice with pTVG-SSX2 p41-VP/p103-IP. In fact, from our tumor studies, mice immunized with a vaccine ablating HLA-A2 binding of peptides p41-49 and p103-111 had average SSX tumor size not significantly different from controls, suggesting that epitopes p41-49 and p103-111 are largely, if not solely, responsible for the significant levels of SSX2+ tumor regression.

By modifying a SSX2 DNA plasmid vaccine to incorporate APL we were able to generate increased frequencies of peptide p41-49- and p103-111-specific immune responses simultaneously. By eliciting multiple, epitope-specific immune responses this APL-encoding DNA vaccine potentially enhances the breadth of immune responses against epitopes that may be presented by prostate cancer cells. Indeed, we previously observed that the most robust lysis of prostate cancer cell lines by cytolytic T cells from pTVG-SSX2-immunized

mice occurred when animals had immune responses to both peptides p41-49 and p103-111 simultaneously as assessed by IFNγ ELISPOT (16). This finding supports the theory that the most efficacious anti-tumor immunity may result from the development of immune responses to multiple epitopes. However, in the tumor protection and therapy studies we observed that anti-tumor responses were not as robust in animals immunized with the enhanced APL vaccine as compared to those immunized with the native plasmid. It may be that the enhanced APL vaccine elicits higher frequencies of IFNγ-secreting T cells, however these cells become regulated in the tumor-bearing mice. This might explain the observed results of the native vaccine having greater anti-tumor efficacy. Other groups have reported similar findings with APL vaccines eliciting higher frequencies of IFNγ-secreting T cells while their CTL activity is diminished (38-40).

Based on previous findings of T cell competition among dominant and subdominant viral epitopes (25, 26), we thought that it may be possible to elicit enhanced immune responses to a subdominant SSX2 epitope using an APL strategy to ablate binding of a dominant epitope encoded by the native SSX2 DNA vaccine. Direct evaluation of T cell competition was possible by vaccination with a DNA plasmid encoding two well-defined HLA-A2 epitopes (p41-49 and p103-111) as direct peptide immunization would not reveal competition between epitopes presented by the same APC. Contrary to our initial hypothesis, we found that ablating HLA-A2 binding of a dominant epitope (p103-111) did not

appear to elicit enhanced immune responses to a subdominant epitope (p41-49). These results indicate that epitope competition is not a major mechanism to "mask" subdominant epitopes, at least in this model system; rather each SSX2 epitope appears to be presented equally. These results are contrary to what other groups have found by ablating HLA binding of dominant HBV antigen epitopes in a DNA plasmid vaccine (26). Riedl, et. al., observed uncovering of immune responses to cryptic epitopes in mice that were immunized with a vaccine that ablated HLA affinity of dominant epitopes, however, this "un-masking" was only observed in a setting of tolerance to the dominant epitope. Thus, this difference in findings may be due to an antigen-dependent effect, the mouse model and HLA haplotype used, or more likely, the presence of regulatory mechanisms already in place to dampen immune responses to the dominant epitopes.

A similar approach to APL that has been investigated by our group and others is utilizing a xenoantigen for immunization to induce robust peptide-specific immune responses to a tumor-expressing protein. In principle, shared epitopes between two species' proteins may be dissimilar by a few amino acids, and in some instances this has been shown to elicit higher frequency epitope-specific immune responses than immunizing with the native protein (41-45). In fact, the only USDA-approved anti-tumor vaccine is a DNA plasmid encoding the human tyrosinase xenoantigen for the treatment of canine melanoma (46). We have similarly evaluated a xenoantigen vaccine by immunizing rats with human

prostatic acid phosphatase (PAP). However, we found that immune responses developed to uniquely human epitopes without cross-reactivity to rat PAP (47). While this strategy has merit, it is equally likely that the xenoantigen may weaken MHC binding of epitopes that are otherwise immunogenic, thereby dampening the epitope/MHC: TCR interaction. Hence, we believe that epitope-specific modifications with defined MHC binding affinity offer a more rational approach for future APL strategies as presented here for SSX2 epitopes.

Figures and Tables

Table 1: Predicted HLA-A2 affinity of modified SSX2 peptides

Shown are the peptide names, amino acid sequences, and predicted HLA-A2 affinity scores of modified SSX2 peptides. Highlighted in red are the amino acid changes to the anchor residues of the native peptide designed to reduce or enhance binding affinity to HLA-A2. Predicted HLA-A2 peptide affinities were determined using two different peptide prediction algorithms (BIMAS and SYFPEITHI)(22, 23).

Table 1: Predicted HLA-A2 affinity of modified SSX2 peptides

Name	Sequence	BIMAS ($t_{½}$ min)	SYFPEITHI (Arbitrary Units)	Purpose
p41	KASEKIFYV	1017	22	Native peptide
p41-AV	K**V**SEKIFYV	6407	22	Enhances
p41-AL	K**L**SEKIFYV	73,228	28	Enhances
p41-VP	KASEKIFY**P**	0.218	12	Reduces
p57	AMTKLGFKA	20	16	Native peptide
p57-AV	AMTKLGFK**V**	291	22	Enhances
p57-AP	AMTKLGFK**P**	0.06	12	Reduces
p99	MTFGRLQGI	2	20	Native peptide
p99-TI	M**I**FGRLQGI	20	24	Enhances
p99-TP	M**P**FGRLQGI	0.9	16	Reduces
p103	RLQGISPKI	10	23	Native peptide
p103-RF	**F**LQGISPKI	48	24	Enhances
p103-IV	RLQGISPK**V**	69	25	Enhances
p103-IP	RLQGISPK**P**	0.01	15	Reduces

Figure 1: Modified SSX2 peptides have enhanced or reduced HLA-A2 affinity in vitro compared to native peptides

Shown are the relative HLA-A2 binding affinities of each modified SSX2 peptide as shown by mean fluorescence intensity (MFI) from T2 binding assay. Values were measured in triplicate for each peptide and normalized to the MFI of a no-antigen vehicle control. Results are representative of two independent experiments. A known HLA-A2-binding peptide derived from the influenza A matrix protein (GILGFVFTL) was used as a positive control for comparable, normalized MFI value.

Figure 1: Modified SSX2 peptides have enhanced and reduced HLA-A2 affinity in vitro compared to native peptides

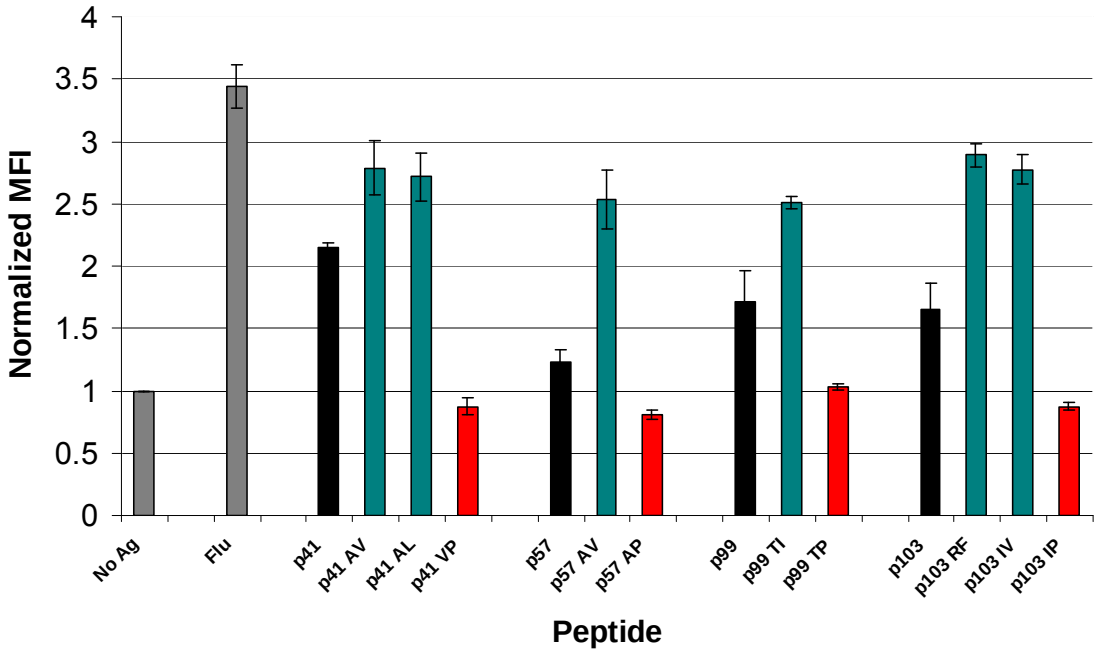


Figure 2: HHDII-DR1 mice immunized with modified peptides p41 and p103 develop enhanced peptide-specific immune responses

Splenocytes from individual mice immunized once with either a native SSX2 peptide, an HLA-A2 enhanced-binding peptide, or an HLA-A2 reduced-binding peptide were analyzed *ex vivo* for the frequency of peptide-specific immune responses by IFNγ ELISPOT. Splenocytes were evaluated for responses to each of the immunizing peptides or concanavalin A (ConA) positive control. Each dot represents the frequency of peptide-specific IFNγ-secreting responses from an individual mouse for the modified p41-49 group (panel A), modified p57-65 group (Panel B), modified p99-107 group (Panel C), and modified p103-111 group (panel D), with n = 4-8 mice per group.

Figure 2: HHDII-DR1 mice immunized with modified peptides p41 and p103 develop enhanced peptide-specific immune responses

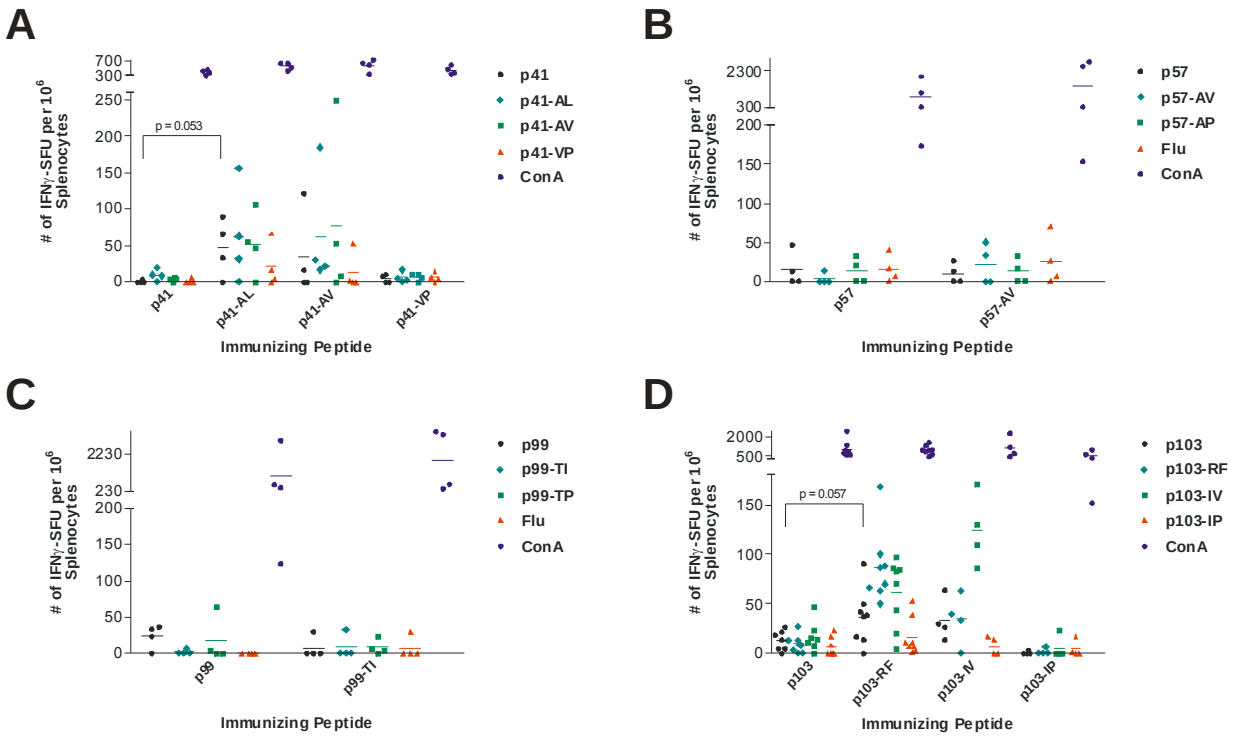


Figure 3: HHDII-DR1 mice immunized with modified SSX2 peptides develop CTL capable of lysing peptide-pulsed target cells and the LNCaP prostate cancer cell line

Splenocytes from HHDII-DR1 mice vaccinated with native SSX2 peptides p41-49 (panel A), p103-111 (panel C) or modified SSX2 peptides p41-AL (panel B) and p103-RF (panel D), were stimulated with the native peptide for five days and tested for specific lysis of peptide-pulsed T2 target cells, the LNCaP prostate cancer cell line, or the LNCaP prostate cancer cell line pre-incubated with an HLA-A2 blocking antibody. Shown are the means and standard deviations of percent specific lysis at three effector-to-target ratios. Data is from individual mice and representative of multiple independent experiments.

Figure 3: HHDII-DR1 mice immunized with modified SSX2 peptides develop CTL capable of lysing peptide-pulsed target cells and the LNCaP prostate cancer cell line

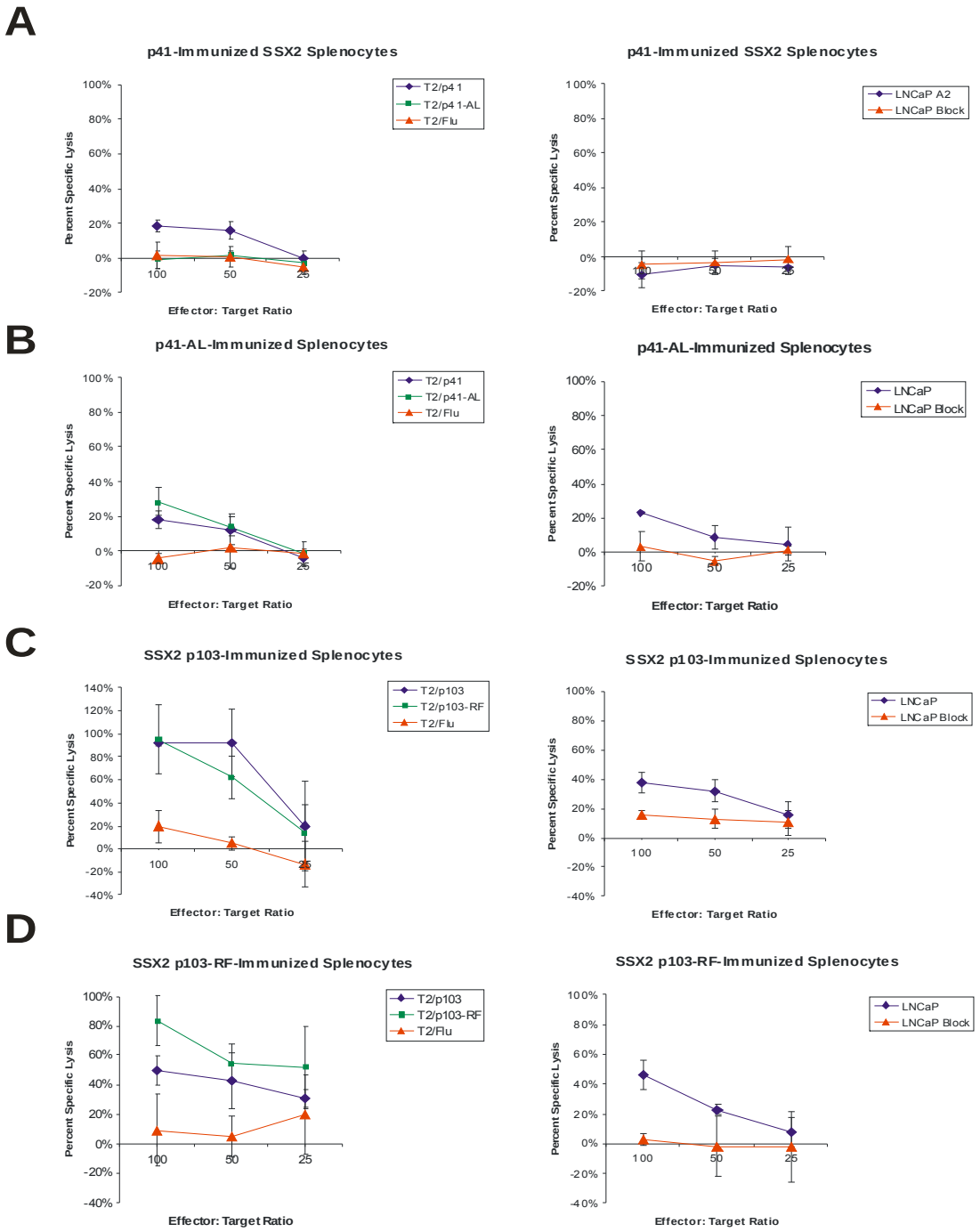


Figure 4: HHDII-DR1 mice immunized with enhanced, modified SSX2 plasmid vaccines develop p41-49 and/or p103-111 peptide-specific immune responses at higher frequency than animals immunized with the native vaccine

Panel A: Splenocytes from individual mice immunized with either native pTVG-SSX2 plasmid vaccine (n=6) or pTVG-SSX2 p41-VP/p103-IP double knockout vaccine (n=6) were analyzed *ex vivo* for the frequency of peptide-specific immune responses by IFNγ ELISPOT. *Panel B:* Splenocytes from individual mice immunized with either native pTVG-SSX2, a plasmid enhancing peptide p41-49 (p41-AL), a plasmid knocking out p103-111 (p103-IP), or a plasmid enhancing p41-49 and knocking out p103-111(p41-AL/p103-IP) were analyzed *ex vivo* for the frequency of peptide-specific immune responses by IFNγ ELISPOT (n=5 for all vaccines). *Panel C:* Splenocytes from individual mice immunized with either native pTVG-SSX2, a plasmid enhancing peptide p103-111 (p103-RF), or a plasmid knocking out p103-111 (p103-IP) were analyzed *ex vivo* for the frequency of peptide-specific immune responses by IFNγ ELISPOT (n=11 for all vaccines). *Panel D:* Splenocytes from individual mice immunized with either native pTVG-SSX2, a plasmid enhancing peptide p41 (p41-AL), or a plasmid enhancing both peptides p41 and p103 (p41-AL/p103-RF) were analyzed *ex vivo* for the frequency of peptide-specific immune responses by IFNγ ELISPOT (n=6 for all vaccines). Splenocytes were stimulated for 48 hours with each of the peptides indicated above or a concanavalin A positive control. Each

dot represents the frequency of peptide-specific IFNγ-secreting responses from an individual mouse. * indicates a significant (P<0.05) difference in the mean number of IFNγ-SFU between the 2 groups (2-tailed t test).

Figure 4: HHDII-DR1 mice immunized with enhanced, modified SSX2 plasmid vaccines develop p41-49 and/or p103-111 peptide-specific immune responses at higher frequency than animals immunized with the native vaccine

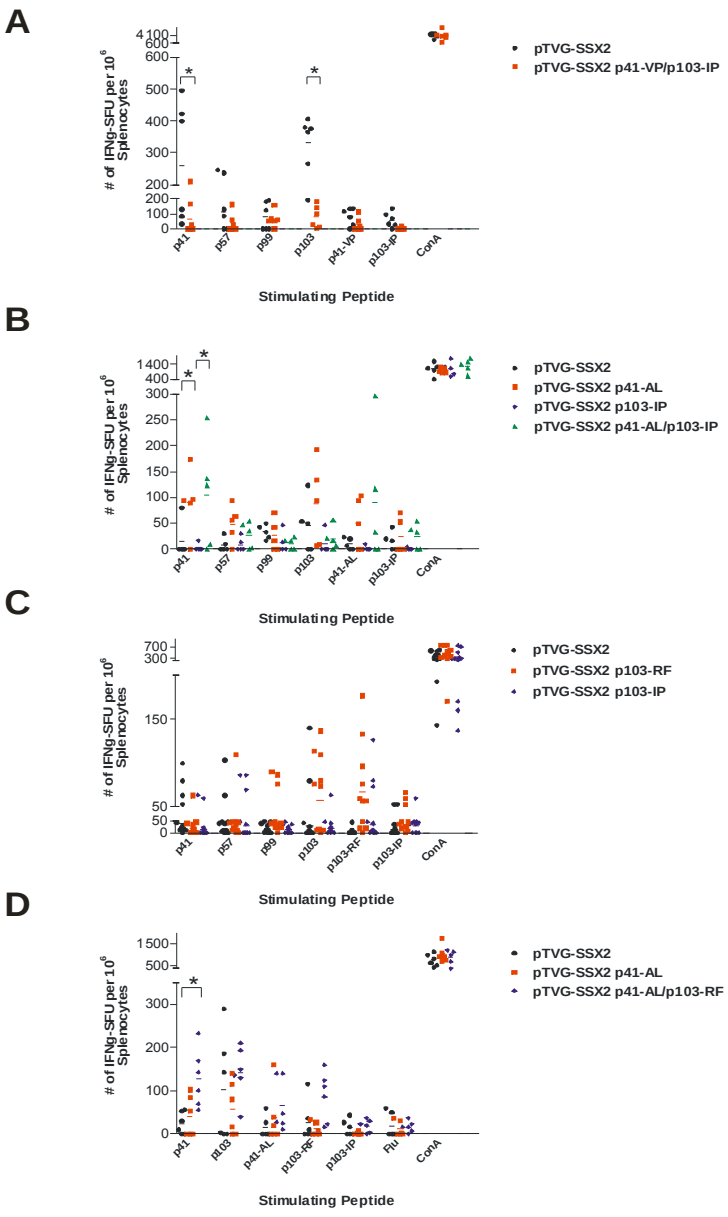


Figure 5: Transduced HHDII-DR1 sarcoma cells express SSX2 protein

Panel A: 100µg of protein from HHDII-DR1 sarcoma cell lysates was resolved on SDS PAGE for Western blot detection with SSX2 mAb. Lane 1, lysate from cos7 cells transiently transfected with pTVG-SSX2 DNA vaccine (+control); Lane 2, lysate from HHDII-DR1 sarcoma cell lines transduced with SSX2 lentiviral vector; Lane 3, lysate from HHDII-DR1 sarcoma cell lines transduced with GFP lentiviral vector. *Panel B:* Flow cytometry analysis of GFP expression in sarcoma tumors after excision from HHDII-DR1 mice. Blue indicates GFP (FITC) level in GFP+ tumors and red indicates GFP level in SSX2+ tumors.

Figure 5: Transduced HHDII-DR1 sarcoma cells express SSX2 protein

A

B

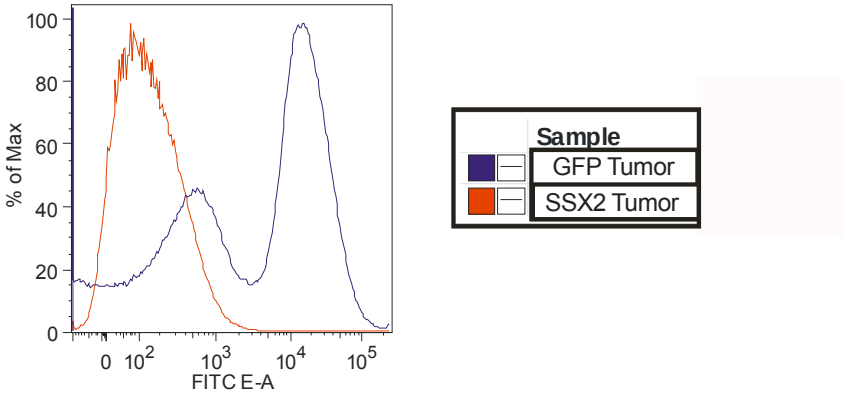
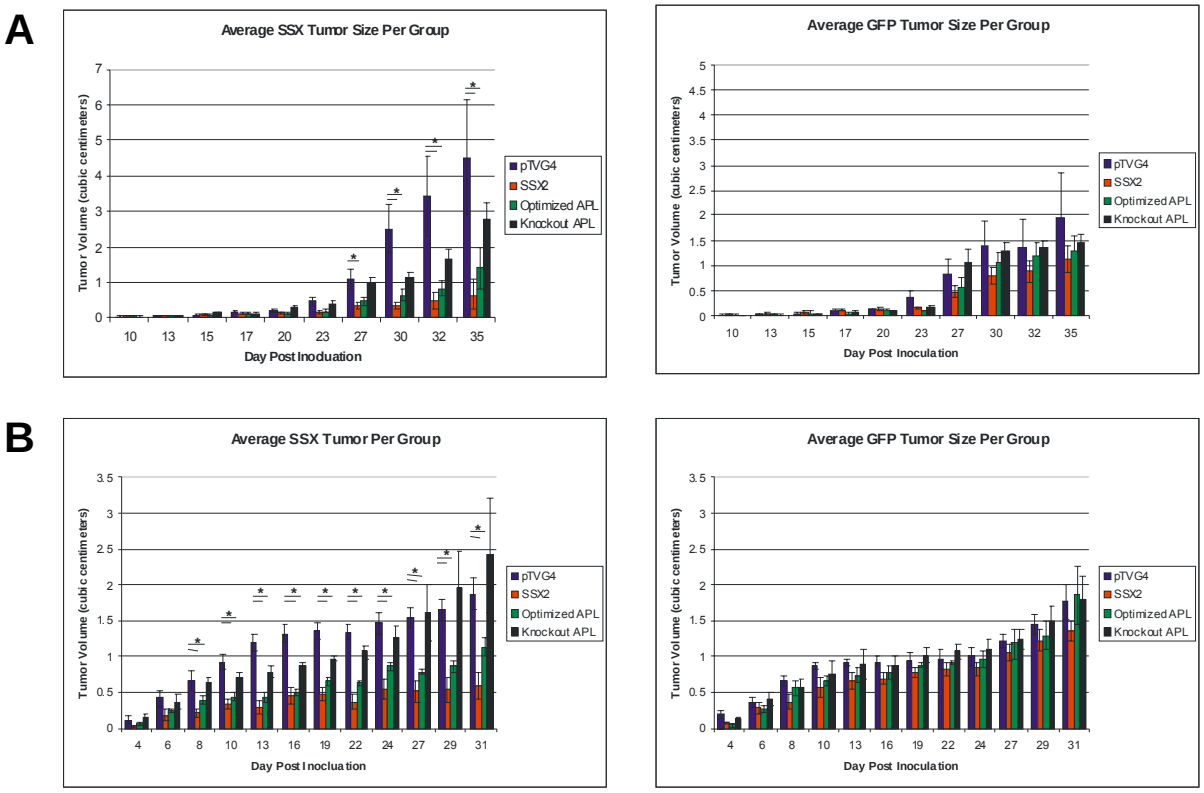


Figure 6: HHDII-DR1 mice immunized with pTVG-SSX2 or pTVG-SSX2-p41-49-AL/p103-111-RF have significantly delayed growth of SSX2-expressing tumors

Panel A: Mice (n=6 per group) immunized six times bi-weekly with pTVG4 (vehicle control), pTVG-SSX2, pTVG-SSX2 p41-49-AL/p103-111-RF (Optimized APL), or pTVG-SSX2 p41-49-VP/p103-111-IP (Knockdown APL) were inoculated with SSX2-expressing or GFP-expressing sarcoma cells after immunization. Animals were subsequently monitored for tumor growth in a protection study.

Panel B: Mice (n=6 per group) were inoculated with SSX2-expressing or GFP-expressing sarcoma cells followed by six weekly immunizations with pTVG4 (vehicle control), pTVG-SSX2, pTVG-SSX2 p41-49-AL/p103-111-RF (Optimized APL), or pTVG-SSX2 p41-49-VP/p103-111-IP (Knockdown APL). Animals were subsequently monitored for tumor growth in a therapy study. Error bars denote standard error. * indicates a significant ($P < 0.05$) difference in the mean tumor volume between the 2 groups (2-tailed *t* test).

Figure 6: HHDII-DR1 mice immunized with pTVG-SSX2 or pTVG-SSX2-p41-49-AL/p103-111-RF have significantly delayed growth of SSX2-expressing tumors



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Chapter 5

Summary, Discussion, and Future Directions

Summary

Over the course of the last decade, most immunotherapeutic vaccines for prostate cancer have targeted antigens expressed almost exclusively by normal prostate tissue, such as PSA, PAP, PSMA, and PSCA. (1-7). While these proteins are indeed also expressed by prostate tumors, the relatively infrequent observation of clear clinical responses in patients highlights the importance of identifying new, more robust anti-tumor targets for prostate cancer immunotherapy. The SSX family of proteins may represent such ideal target antigens due to their normally tissue-restricted expression to immune-privileged germ line cells of the testis and for their aberrant expression in a variety of malignancies including prostate cancer (8). As CTA, these proteins should be predominately recognized as foreign antigens when ectopically expressed in tumor tissue and may thus be more amenable to immune targeting. SSX proteins have been shown to possess several features of ideal immunotherapeutic targets, including immunogenicity, specificity, expression level, percent of antigen-positive cells, stem cell expression, and number of antigenic epitopes (8-14). Based on these observations made by our group and others, we sought to evaluate this family of antigens as potential immunotherapeutic targets for the treatment of prostate cancer. To do so, we first sought to identify which SSX family members were expressed in prostate cancer cell lines and human prostate cancer tissues. We found that SSX1, SSX2, and SSX5 were the most frequently expressed SSX family members at

the mRNA level or inducible with epigenetic modifying agents by RT-PCR and qRT-PCR. We also evaluated SSX protein expression in human prostate cancer biopsy lesions from 192 patients with varying stages of disease. For this analysis we obtained two SSX antibodies, and demonstrated that these antibodies could be used to stain different sets of SSX family member proteins by Western blot. Utilizing these antibodies to stain human biopsies on tissue microarray, we found that SSX protein expression was restricted to metastatic prostate cancer lesions and this expression was not found in primary carcinoma of the prostate. Interestingly, we found differential SSX staining with the two SSX-specific antibodies indicating that different SSX family member proteins could be expressed in metastatic prostate cancer. These results indicated that SSX1, 2, and 5 may be the most relevant SSX family members to target for prostate cancer and clinical trials might best focus on patients with metastatic disease or at high risk for metastatic prostate cancer.

After identifying which SSX family members might be relevant targets for prostate cancer immunotherapy, we sought to identify immunogenic SSX epitopes and evaluate whether DNA plasmid vaccines encoding SSX proteins could elicit peptide-specific immune responses in A2/DR1 transgenic mice. We focused on SSX2 as a model SSX family member due to its predominant mRNA expression in prostate cancer cell lines. We identified several peptides derived from the amino acid sequence of SSX2 that were predicted to bind to the HLA-A2

complex. Of these we found that peptides p41-49, p57-65, p99-107, and p103-111 had demonstrated affinity for HLA-A2 *in vitro*. To test the *in vivo* immunogenicity of these HLA-A2-binding peptides we conducted direct peptide vaccination of A2/DR1 mice and evaluated the development of peptide-specific immune responses from the splenocytes of these immunized animals. We found that only peptides p41-49 and p103-111 were immunogenic by IFNγ ELISPOT assay. In addition we found that animals immunized with a DNA vaccine encoding SSX2 developed these same epitope-specific immune responses, however, peptide p103-111 was clearly immunodominant. Not only did animals develop peptide p103-111-specific T cells, these CTL were also able to lyse the LNCaP prostate cancer cell line and target cells pulsed with peptide p103-111. This lysis was abrogated in the presence of an HLA-A2 blocking antibody demonstrating that this lysis was HLA-A2 restricted. Following on these observations, we evaluated whether prostate cancer patients might have peptide p41-49 and p103-111-specific T cells in their peripheral blood. To do so we tested CD8+ T cells isolated from patient PBMCs for recognition of p41-49 and p103-111 tetramers by flow cytometry. We observed that HLA-A2 prostate cancer patients had significantly higher frequencies of both p41-49- and p103-111-specific T cells compared to T cells from healthy donors or patients with cancer that were HLA-A2 negative. We also observed that there was a substantial increase in the frequencies of these cells in patients with more

advanced diseased, which correlates with our previous findings of SSX expression in metastatic tissues by tissue microarray IHC.

After establishing the immunogenicity of a plasmid DNA vaccine encoding SSX2 and identifying two specific epitopes that conferred robust peptide-specific immune responses, we wanted to determine if it was possible to enhance SSX2 vaccine efficacy by modifying certain peptides to enhance their binding affinity to HLA-A2. Specifically, we made amino acid substitutions to peptide anchor residues in an effort to enhance HLA-A2 binding while potentially maintaining interaction with the TCR of lymphocytes specific to these peptide/MHC complexes. We found that certain modifications to these peptides did enhance their binding to HLA-A2 *in vitro* as demonstrated by T2 binding assay. We also carried out direct peptide vaccinations of A2/DR1 mice to establish any effects on immunogenicity between native and enhanced HLA-A2 binding peptides. We found that animals immunized with peptides designed to increase peptide p41-49 or p103-111 HLA-A2 binding elicited higher frequencies of peptide-specific T cells that were cross-reactive to the native epitopes. CTL from these animals were also able to lyse peptide-pulsed target cells and the LNCaP prostate cancer cell line, but not in the presence of an HLA-A2 blocking antibody. These results demonstrated that peptides p41-49 and p103-111 are both epitopes presented by prostate cancer cell lines and it is possible to elicit higher frequencies of peptide-specific T cells to these epitopes using an altered peptide ligand

strategy. We also demonstrated that DNA vaccines encoding these altered peptides were able to elicit enhanced frequencies of peptide p41-49 and p103-111-specific T cells as well when administered to A2/DR1 mice. Using these modified plasmid vaccines to ablate HLA-A2 binding of dominant epitopes we were also able to demonstrate that there is no T cell competition between epitopes. Using this APL strategy to encode modified peptides, we were able to design an optimal SSX2 plasmid vaccine that was capable of eliciting robust immune responses to peptides p41-49 and p103-111 simultaneously.

Finally, we evaluated whether our native or modified SSX2 vaccines had anti-tumor efficacy. To investigate this we treated A2/DR1 mice with methylcholanthrene (MCA) to induce tumor formation and in this way cultured out a syngeneic A2/DR1 sarcoma cell line that was induced to express GFP or SSX2 using a lentiviral system. Tumor cells were used to challenge mice pre- or post-immunization to establish vaccine anti-tumor efficacy in both a prophylactic and therapeutic setting. We found that animals immunized with SSX2 vaccines (native or modified) had significantly delayed or inhibited growth of SSX2-expressing tumors but not GFP-expressing tumors, while both SSX2 and GFP-expressing tumors continue to grow unabated in animals immunized with empty plasmid or a SSX2-encoding plasmid with peptides p41-49 and p103-111 modified to ablate HLA-A2 binding. These results validated our hypothesis that

immunotherapeutic vaccines targeting SSX2 can elicit anti-tumor immune responses.

Discussion and Future Directions

The last decade has witnessed an increasing interest in immunotherapeutic-directed therapies for the treatment of prostate cancer, including viral-based vaccines, DNA plasmid vaccines, and whole-cell vaccines loaded with tumor antigen. Of particular note is the recently FDA-approved treatment for castrate-resistant metastatic prostate cancer, Sipuleucel-T (Provenge ® Dendreon Corp., Seattle, WA) (15, 16). While the approval of this immunotherapeutic vaccine has been a notable achievement in the field of tumor immunotherapy, the cost of treatment with this therapy is prohibitively expensive and the clinical benefit to patients was found to be very modest compared to patients receiving standard chemotherapy. Thus, there is a dire need to identify new, immunotherapeutic targets and design more efficacious vaccines for the successful treatment of prostate cancer.

Due to several features, prostate cancer is particularly amenable to immunotherapeutic measures. With a long natural progression of disease, prostate tumor development lends itself to immune intervention which typically necessitates longer periods of stimulation and activation of immune cells, such as cytotoxic T cells, during the generation of an adaptive immune response

against tumor antigens. Additionally, prostate tumors may be spontaneously immunogenic, with humoral and cell-mediated immune responses to some antigens already present in patients before any kind of therapeutic intervention (10, 17, 18). Based on these observations and the current lack of curative or highly effective treatments for advanced prostate cancer, tumor immunotherapy is thought to be a very attractive approach for patients with this disease.

While many previous immunotherapies for prostate cancer have met with relatively limited rates of clinical success, it may be possible to identify new target proteins and more efficiently prioritize potential antigens for immune intervention. To date, the majority of prostate cancer vaccines that have been developed target antigens with expression restricted to the prostate (1-7), however, tissue specificity is only one factor of antigen selection that should be considered when designing vaccines. For many other types of solid malignancy, immunotherapeutic vaccines have been designed to target antigens critical for tumor survival such as HER2/neu, MUC1, and CEA (19-23). As these antigens are crucial to tumor growth they may be more relevant targets than those that are only present as bystanders from the tissue from which the tumors arose. In fact, a consortium panel recently outlined a specific set of criteria to consider when prioritizing potential antigens for immunotherapy including: immunogenicity, tumor specificity, expression level, stem cell expression, presence of epitopes, cellular localization, frequency in antigen-expressing cancer, and the potential

role of the antigen in tumorigenicity (9). These characteristics have all been identified as important factors to consider when identifying target antigens for vaccine design, and based upon many studies conducted previously by our group and others we believe that the SSX family of proteins meets many of these criteria.

We have previously reported the natural immunogenicity of SSX2 by the presence of SSX2 epitope-specific T cells in the peripheral blood of prostate cancer patients as assessed by tetramer staining analysis, as well as humoral, antibody-mediated immune responses that develop in a small portion of patients with prostate cancer (10, 12). We have also shown that A2/DR1 transgenic mice immunized with SSX2 peptides or a DNA vaccine encoding SSX2 can develop immune responses to multiple SSX2 epitopes (10). These studies, demonstrate the presence of encoded epitopes and the natural immunogenicity of SSX2, two criteria of an attractive target antigen. Furthermore, the tissue-restricted expression of SSX proteins to immunoprivileged germ-line cells of the testis, and high expression in some prostate cancer tissues, demonstrates two additional characteristics of an ideal immunotherapeutic target; tumor specificity and expression level (11, 12).

Many investigators have conducted studies to investigate the specific function of SSX proteins, particularly SSX1 and SSX2 (8). The function of this family of

proteins has not yet been fully elucidated, however, several studies have clearly demonstrated that SSX proteins possess a transcriptional repression domain (SSXRD) that likely exerts repression of one or more specific target genes, however, these targets have not yet been identified (24). As SSX proteins do not contain a DNA-binding domain, they likely function through direct protein-protein interactions (24). Although a few SSX2 interacting partners have been identified, namely, SSX2IP, RAB3IP, and LHX4, it is still unknown what the significance of these pairings are or what the downstream phenotypic effects of these interactions might have on the cells which express SSX proteins (25, 26). Other studies have shown that SSX proteins co-localize with members of the Polycomb group silencing complex, particularly RING1 and BMI-1, which suggests that SSX protein function may be involved with broad transcriptional regulation or chromatin remodeling (27). Until these downstream effects of SSX interactions and functional partners are elucidated, we will not fully understand the mechanism of SSX in tumor tissue nor any tumor promoting activities that may be conferred by expression of these proteins. We are currently planning studies to more fully evaluate the transforming or oncogenic effects of SSX proteins by overexpressing SSX1 and SSX2 in immortalized prostate epithelial cell lines. Future experiments will determine if overexpression of SSX proteins in otherwise normal prostate epithelial cells confers any invasive, cell migratory, or transforming activity as assessed by cell proliferation, wound healing, and *in vivo* tumor cell inoculation studies. If the SSX proteins do possess tumor promoting

activities, this would confer yet another characteristic of an ideal target antigen to this family of proteins.

Cronright and colleagues previously demonstrated that SSX2 expression was found in mesenchymal stem cells (14). This expression pattern demonstrates yet another characteristic of an ideal target antigen and may hint at a possible function of SSX proteins. As has been found for many oncogenes, SSX expression in cancer may be tumor promoting (driver mutation) and not a passenger effect. Studies indicate that SSX expression is associated with cell migration *in vitro* and co-localization with MMP2, perhaps indicating a more invasive phenotype (14). An invasive or cell migratory effect is a function that has been implicated for other CTA, including a recent report demonstrating enhanced migration of MCF-7 breast cancer cells upon over-expression of SSX2 (28). Additionally, the expression of SSX protein selectively in prostate cancer metastases could indicate that these cells have undergone an epigenetic reprogamming, transcribing genes that were previously expressed during development. As such, SSX expression may be linked to an epithelial-to-mesenchymal transition (EMT), where SSX and other CTA are expressed as cells take on features of mesenchymal cells for extravasation from the primary tumor into the vasculature. This would make sense as SSX expression has already been found in mesenchymal stem cells and the SS18-SSX fusion event is found in >95% of synovial sarcoma, a tumor of mesenchymal origin (8, 29-31).

These features of SSX expression highlight the possibility that these proteins are involved in EMT or cell migration. Future studies in our lab are underway to examine the relationship between SSX expression and markers of proliferation, cell migration, stem cells, and EMT, such as Ki67, E-cadherin, vimentin, Snail, CD133, and MMP-2. This analysis will compare SSX2 expression to these markers in metastatic prostate cancer tissues compared with control tissues by IHC.

Because SSX proteins have previously been shown to possess many of the characteristics of an ideal target antigen as outlined above, we have specifically focused our efforts on the design of DNA plasmid vaccines encoding SSX2 for the immunotherapeutic treatment of prostate cancer. We have fully demonstrated the involvement of peptides p41-49 and p103-111 as immunogenic epitopes recognized by T cells from patients and from transgenic immunized mice. Studies to identify immunogenic SSX2 epitopes are expected to fall short of the true range of epitopes that could potentially be recognized by host T cells. For the studies described here, a single HLA haplotype (HLA-A2) was evaluated for SSX2 peptide presentation. Many different HLA haplotypes exist in the human population that could potentially present epitopes to T cells that were not identified in these studies. Not only class I HLA epitopes, but class II epitopes could also be presented to T helper cells, which could also significantly affect the outcome of cytotoxic immune response generated against this tumor antigen.

Thus, the scope of these studies is expected to severely underestimate the range of potential epitopes presented on different MHC molecules. Additionally, we only evaluated HLA-A2 affinity of peptides that were predicted to bind to HLA-A2 based on certain *in silico* peptide binding prediction algorithims. As only two different algorithms were utilized to narrow down the possible list of peptides to evaluate for HLA-A2 affinity *in vitro*, we expected this method to underestimate the number of epitopes that may have HLA-A2 affiinity. This may not be the case as tumors in animals immunized with an SSX2 vaccine ablating HLA-A2 binding of peptides p41-49 and p103-111 did not grow significantly different than control-immunized animals. Although peptides p41-49 and p103-111 appear to be the dominant HLA-A2 epitopes for SSX2 recognition, we still believe that the data provided here does not represent an exhaustive evaluation of potential SSX2 epitopes that could be utilized for tumor immunotherapy. Rather, these studies provide a proof-of-principle that the SSX proteins encode immunogic epitopes and vaccines can augment the immune response to these epitopes *in vivo*.

One of the critical resources utilized for this work was the HHDII-DR1 or A2/DR1 transgenic mouse. It should be noted that the transgenic mouse was chosen based upon expression of human HLA-A2 and HLA-DR1 molecules, while the expression of murine class I and class II MHC molecules had been ablated (32). In this way, epitopes could be presented on mouse APCs that would be similarly presented in the same context by human APCs, essentially serving as a model of

the presentation of human epitopes in a murine host. For SSX2 immunization studies this mouse model was vaccinated with a DNA plasmid encoding a foreign antigen, not normally encountered by the murine immune system. Thus, the immune responses elicited to this antigen may be more robust than what might be observed in a human host. Since human medullary cells in the thymus normally express a variety of host proteins to T cells during negative selection, we might expect some central tolerance to this antigen. Due to the restricted expression of SSX proteins in immunoprivileged cells of the testis, there is still expected to be limited tolerance to this antigen in the human host as this expression should not confer peripheral tolerance. Therefore, the mouse model is likely to be quite representative of the immune responses that might be elicited by SSX2 DNA plasmid vaccines delivered to a human host. During the course of evaluating the SSX2 vaccines for immune efficacy we did observe that immune responses were elicited *in vivo*, and we did not observe any overt signs of toxicity from the vaccine. However, the cytotoxic effects of vaccines still need to be evaluated more precisely by histological analysis to verify that no adverse affects are likely to occur as a result of autoimmunity elicited against healthy tissues. This possibility is not likely since SSX proteins have expression restricted to the testis, however this analysis would be required before any clinical trial could be initiated. A similar plasmid DNA vaccine targeting the androgen receptor ligand binding domain (AR-LBD) was not observed to result in any significant cytotoxicity in healthy tissues of immunized A2/DR1 mice, even though AR

expression can be found in several normal murine tissues including skeletal muscle and normal prostate tissue [submitted].

It is clear from tetramer analysis of T cells isolated from the PBMCs of patients with prostate cancer that SSX2 epitope-specific T cells can arise spontaneously in these individuals (10). It is also apparent that patients with more advanced disease have higher frequencies of epitope p41-49 and p103-111-specific T cells, which may be a direct result of higher SSX protein expression in metastatic prostate tumor lesions as we previously found by tissue microarray IHC (11). Yet these patients still succumb to their disease. Why? Perhaps SSX2 epitope-specific T cells are encountering peptides presented on the surface of SSX2-positive tumor cells, but are not proliferating and expanding to frequencies that can lead to tumor regression. More likely, the SSX2-positive tumors secrete chemokines or factors to circumvent immune detection, or regulate the immune response in the local tumor microenvironment. This may also account for why we observed less anti-tumor activity in mice immunized with the optimized vaccine in the tumor study. Perhaps the enhanced frequencies of T cells are activating a regulatory response that dampens the cytolytic activity of the peptide-specific effector T cells. Many previous studies have demonstrated a myriad of ways in which tumors can recruit regulatory T cells (Tregs) such as Fox3p+/CD25+/CD4+ Tregs or myloid-derived suppressor cells (MDSCs) to tumor tissue thereby dampening the immune response (33-37). Using

therapeutic vaccines designed to elicit SSX2 epitope-specific CTL we hope to augment the immune response in patients to levels that can achieve clinical benefit. It has been previously shown that immunization of non-small cell lung cancer or esophageal carcinoma patients with a DNA plasmid vaccine encoding the CTA NY-ESO-1 resulted in the development of significant levels of Tregs that suppressed the immune responses elicited by the DNA vaccine (38). These results demonstrate directly that vaccination of cancer patients with a DNA plasmid vaccine encoding a CTA can result in immune regulation. However, currently it is unknown whether additional agents will be necessary to bolster the immune response or prevent regulatory immune responses to an SSX2-encoding DNA vaccine. It is feasible to administer agents such as anti-CTLA4 or anti-PD-1 (key proteins expressed by suppressive cells mediating immune regulation) as adjuvant therapy to prevent immune regulation of epitope-specific T cells. These antibodies have been used by themselves or in conjunction with other immunotherapies to induce a more robust immune response to tumor antigens (33, 39-44). Future clinical trials will establish the degree of immune regulation after immunization of patients with SSX DNA vaccines, and determine whether these additional agents in combination with vaccination would be beneficial to patient therapy.

While direct immunization with a plasmid DNA vaccine encoding SSX2 was found to elicit robust immune responses to epitope p103-111, we also sought to

enhance vaccine efficacy and elicit more robust immune responses to subdominant epitopes such as peptide p41-49. Using an altered peptide ligand (APL) strategy we designed peptides that were modified at MHC-binding anchor residues to increase their affinity to HLA-A2. We found that these peptides did, indeed, elicit more robust peptide-specific immune responses in immunized A2/DR1 mice after direct peptide vaccination. Similarly, we sought to enhance SSX2 plasmid DNA efficacy by incorporating these same APL in the encoded plasmid by site-directed mutagenesis. Using this strategy we were able to elicit higher frequencies of epitope-specific T cells to multiple SSX2 peptides simultaneously, however, in anti-tumor studies both the native and enhanced vaccines were observed to possess similar levels of anti-tumor activity. It may be that both vaccines are equally robust in eliciting anti-tumor immune responses, however, tumor tissue in responding mice was not completely destroyed. How then can we enhance SSX2 vaccines to further enhance their anti-tumor efficacy? Multiple strategies have been employed by others to elicit more robust immune responses to vaccines either through increased antigen expression, processing, and presentation of the antigen, or to include immunostimulatory sequences to the plasmid backbone. Various delivery strategies have also been utilized to increase plasmid DNA uptake by APCs, such as using electroporation or gene guns, which have been previously demonstrated to result in higher levels of DNA transfection efficiency and resulting antigen presentation despite lower concentrations of DNA (45-49). Antigen expression itself can be increased by

including sequences to designed to enhance antigen transcription (e.g. using alternative promoters) or translation (optimizing codon usage of the encoded antigen) (50-52). Groups have also sought to enhance the processing of antigen epitopes by incorporating sequences to bolster the degradation of the translated antigen, such as encoding PEST sequences, which target the antigen for ubiquination and subsequent proteasomal degradation (53-55). Alternatively, presentation of antigen-derived peptides can also be enhanced by co-expressing heat-shock proteins (Hsp), which have been shown to increase the presentation of certain peptides on MHC class I complexes (56-58). Finally, it may also be possible to enhance vaccine efficacy by incorporating multiple tumor antigens or immunostimulatory sequences into a single vaccine. Encoding multiple tumor antigens, or epitopes from multiple TAA, could provide a means of preventing escape variants that downregulate the expression of a single antigen or dominant epitope that is being targeted therapeutically, as has been described for other antigens such as the CTA NY-ESO-1 (59, 60). In addition to tumor antigens, other immunostimulatory sequences could be incorporated into a plasmid vaccine such as inflammatory cytokines or costimulatory molecules, which has been found to enhance anti-tumor immune response *in vivo* (61-64).

The experiments discussed here demonstrate that multiple SSX family members can be expressed in prostate cancer and that SSX expression appears restricted to metastatic disease. As it is still unknown whether different SSX family

members have redundant function, it may be prudent to design vaccines that will target multiple SSX proteins simultaneously to prevent the possible development of escape variants during targeted therapy. We found that SSX1, SSX2, and SSX5 were the most frequently expressed or epigenetically inducible in prostate cancer cell lines, and likely the members that may be expressed in prostate cancer lesions (11). We also found that it was possible to target SSX2 and SSX5 simultaneously by immunizing mice with a DNA vaccine encoding SSX2. As these two family members share dominant epitopes, the cross-reactive immune response recognized peptide p103-111 from both family members, however, peptide p103-111 encoded by SSX1 was not recognized (11). Since immune responses to SSX1 peptide p103-111 were elicited in A2/DR1 mice after direct peptide vaccination, it does appear that this family member could be targeted using an immunotherapeutic vaccine. Future studies will address the optimal means to target SSX1, 2, and 5 simultaneously. We will specifically evaluate whether two separate vaccines encoding SSX1 and SSX2 will be necessary, or whether a single polytope vaccine with epitopes from both family members would be optimal.

If SSX vaccines should be moved into human clinical trials it would be best to determine which patient populations are most likely to benefit from this therapy. DNA vaccines may be most efficacious for patients with early stage disease when tumors have not accumulated overwhelming amounts of genetic alterations

and variability that could circumvent immune recognition. At earlier stages of disease there should also be less tumor burden by mass and more opportunity for peptide-specific T cells to infiltrate the tumor. However, as it appears that SSX proteins are perhaps exclusively expressed in metastatic disease it may be more beneficial to select patients with late-stage tumors or metastases for clinical trials. Alternatively, the SSX vaccines could be beneficial as a prophylactic strategy to elicit immune response to an antigen expressed on metastatic cells to prevent the outgrowth of SSX+ metastatic prostate cancer lesions. Our current thought is that SSX proteins may be involved in epithelial-to-mesenchymal transition where SSX expression goes up during extravasation and circulation from the primary tumor into the vasculature, while SSX expression might be turned off epigenetically during mesenchymal-to-epithelial transitions at the site of metastatic seeding. Preliminary results indicate that SSX mRNA message can be found in a large percentage of PBMC from patients with advanced stage disease (data not shown). This could indicate that SSX expression is elevated in circulating prostate tumor cells, and could represent an optimal stage of disease to target these cells using immunotherapeutic vaccines; before large tumor burden and the development of distant metastases and while relatively few cells express SSX proteins. Additionally, SSX vaccination could be administered as an adjuvant therapy after surgical removal of prostate cancer tissue or radiation therapy. Future studies are underway to evaluate the specific timing of SSX

expression during prostate cancer development so a patient population can be optimally chosen for clinical trials.

Overall, the compiled results from these studies illuminate the potential importance of the SSX family of antigens for prostate cancer therapy. The studies described specifically demonstrate which SSX proteins are expressed in prostate cancer, and that this expression appears to be restricted to metastatic lesions. SSX2 was the family member most frequently expressed in prostate cancer cell lines and tissues. Other experiments identified which peptide epitopes encoded by SSX2 had affinity for HLA-A2 *in vitro* and were immunogenic *in vivo*. Using a plasmid DNA vaccine, it was possible to elicit immune responses to multiple SSX epitopes simultaneously, and the frequency of these immune responses could be amplified using an APL strategy to enhance peptide affinity for the HLA-A2 complex. We also found that both native and modified SSX2 vaccines were capable of eliciting anit-tumor immune responses in A2/DR1 mice with tumors expressing SSX2. Overall, these results demonstrate the feasibility of using DNA plasmid vaccines to elicit antigen-specific immune responses to a protein expressed in metastatic prostate cancer. These pre-clinical studies lend further evidence that the SSX family of proteins could be valuable clinical targets for the immunotherapeutic treatment of prostate cancer.

Refernces

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Chapter 6

Materials and Methods

Some of this work was published in 'Smith, H.A., *et al*. *Expression and Immunotherapeutic Targeting of the SSX Family of Cancer-Testis Antigens in Prostate Cancer*. Cancer Research, 2011. **71** (21): p. 6785-6795.' and in 'Smith, H.A. and McNeel, D.G. *Vaccines Targeting the Cancer-Testis Antigen SSX2 Elicit HLA-A2 Epitope-Specific Cytolytic T cells*. The Journal of Immunotherapy, 2011. **34** (8): p. 569-580.' Some is currently being prepared for publication.

Materials and Methods – Chapter 2

Cell culture

Prostate cancer cell lines (LNCaP, 22-RV1, PC3, and DU145) and immortalized prostate epithelial cell lines (RWPE-1 and PREC-6) were grown in RPMI 1640 media supplemented with 10% fetal calf serum (FCS; Invitrogen), 200 U/mL penicillin/streptomycin, 1 mmol/L sodium pyruvate, and 0.1 μmol/L β-mercaptoethanol. VCaP and LAPC4 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% glucose, 10% FCS, 200 U/mL penicillin/streptomycin, 1 mmol/L sodium pyruvate, and 0.1 μmol/L β-mercaptoethanol. Where indicated, cells were additionally cultured with 10 μmol/L 5-Aza-2'-deoxycytidine (5-aza-dc; Sigma), 100 nmol/L Trichostatin A (TSA; Sigma), or both agents simultaneously for 72 hours. RNA was collected after treatment using the RNeasy Mini kit (Qiagen). Cell lines were obtained from American Type Culture Collection (ATCC), verified using polyphasic (genotypic and phenotypic) testing to confirm identity, and passaged in our laboratory for less than 6 months.

PCR, RT-PCR, and quantitative RT-PCR analysis

Gradient PCR was carried out to amplify DNA products from SSX image clones encoding SSX1, 2, 3, 4, 5, 6, 7, or 9 cDNAs (ATCC), without cross-amplification of other SSX family members. Image clones exclusively encoding SSX8 and SSX10 were not commercially available. Amplification of products with SSX8

and SSX10 primers in cell lines was consequently verified by direct DNA sequencing. SSX primers used for PCR analysis were designed directly, or from published sequences (1, 2), and were commercially synthesized (Integrated DNA Technologies). PCR conditions were as follows: 95°C for 1 minute followed by 30 cycles of 95°C for 1 minute, the specific annealing temperature for 1 minute, and 72°C for 3 minutes. A final extension time was 10 minutes at 72°C. Products were then separated and evaluated by agarose gel electrophoresis. Reverse-transcriptase PCR (RT-PCR) using the One-Step RT-PCR kit (Qiagen) was carried out using RNA isolated from cell lines with the primers and annealing temperatures listed in Table 1, as well as β-actin control primers

(actinA:50-TCATGAAGTGTGACGTTGACATCCGT-30,

actinB: 50-CTTAGAAGCATTTGCGGTGCACGATG-30)

under the following PCR conditions: 50°C for 30 minutes, 95°C for 15 minutes, 35 cycles at 95°C for 1 minute, specific annealing temperature for 1 minute, and 72°C for 1 minute; final extension for 10 minutes at 72°C. For quantitative RT-PCR (qRT-PCR), 1 μg RNA was collected and reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. qRT-PCR was done using PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Inc.) according to the manufacturer's instructions with 1 μL of the cDNA synthesis reaction mixed with primers specific for each family member and the SuperMix. Samples were analyzed using a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad) with annealing temperatures as

shown in Table 1. All results were analyzed by the $2^{-\Delta Ct}$ method (3) relative to the ribosomal protein P0 as a control gene (4):

P0 forward: 50-ACAATGGCAGCATCTACAAC-30;

P0 reverse: 50-GCAGACAGACACTGGCAAC-30).

Fold induction over vehicle treatment was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously published (3).

Western blot

Recombinant SSX GST-tagged proteins (SSX1-5; Abnova) or PAP protein (Fitzgerald) were resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes using standard procedures. Membranes were probed with an SSX2 monoclonal antibody (mAb; clone 1A4; Abnova) or an SSX5 polyclonal antibody (B01P; Abnova).

Immunohistochemistry

Paraffin-embedded tissue sections were washed in xylene and rehydrated in ethanol, followed by antigen retrieval using Tris-EDTA buffer supplemented with 5 mmol/L $CaCl_2$, 0.5% Triton X-100 (pH 8.0), and 20 μg/mL Proteinase K. After washing in PBS/0.1% Tween-20 and blocking in PBS/10% BSA, slides were stained with primary antibody overnight at 4°C. Samples were then stained with goat anti-mouse IgG secondary antibody (Sigma), washed, and stained using the LSAB System HRP kit (Dako) and DAB metal concentrate (Pierce). Slides were

then counterstained with hematoxylin, mounted with coverslips, and imaged using an Olympus BX51 microscope (Olympus) and SPOT RT analysis software (Diagnostic Instruments).

HLA-A2 T2 binding affinity assays

SSX nonamer peptides, derived from the amino acid sequences of SSX1-9 and corresponding to SSX2 epitope p103-111, were evaluated for their predicted HLA-A2 affinity using the SYFPEITHI and BIMAS prediction algorithms (5, 6). The purity and identity of synthesized peptides was confirmed by mass spectrometry and gas chromatography (United Biochemical Research, Seattle, WA). *In vitro* HLA-A2 affinity for each peptide was determined by stabilization of HLA-A2 on TAP-deficient T2 cells as previously described (2, 7). Peptide-HLA-A2 stabilization was measured as relative mean fluorescent intensity (MFI), normalized to vehiclepulsed T2 MFI, and all measurements were conducted in triplicate.

Mice

HLA-A2.01/HLA-DR1 expressing, murine MHC class I/II knock-out, transgenic (HHDII-DR1) mice on C57Bl/6 background were provided by Dr. François Lemonnier (8). Mice were maintained in microisolater cages under aseptic conditions, and all experimental procedures were conducted under an I Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Immunization studies

Four- to 6-week-old HHDII-DR1 mice were immunized subcutaneously with 100 µg of an SSX peptide or vehicle control in complete Freund's adjuvant (Sigma). For DNA vaccination studies, mice were immunized every 14 days intradermally in the ear pinna with 100 µg of either plasmid DNA vector control (pTVG4) or a plasmid DNA vaccine encoding SSX2 (pTVG-SSX2). Mice were euthanized seven (peptide immunization) or 14 (DNA immunization) days after the last immunization, and splenocytes were isolated by centrifugation after red blood cell osmotic lysis.

IFNγ enzyme-linked immunosorbent (ELISPOT) assay

A 48-hour IFNγ ELISPOT was used to determine frequencies of peptide-specific T lymphocytes using a murine IFNγ ELISPOT kit according to the manufacturer's instructions (R&D Systems) as previously described (9, 10). Briefly, 96-well nitrocellulose plates (NC-plates, Millipore Corporation, Bedford, MA) were coated with 50µl of an anti-murine IFNγ capture antibody diluted 1:100 overnight at 4°C. The following day, plates were blocked in PBS/10%BSA for one hour and washed in murine T-cell media. Splenocytes were added ($2X10^5$ cells/well) to plates, followed by antigens: specific and non-specific peptides at concentrations of 2µg/mL or 10 µg/mL concanavalin A (Calbiochem), or media alone. Plates were allowed to incubate at 37°C for 48 hours. After incubation, plates were

washed with PBS/0.05% Tween-20, followed by incubation with biotinylated anti-IFNγ antibody (BioRad) for 2 hours at room temperature in PBS/1%BSA. Plates were again washed and developed with 100µl/well 5-bromo-4-chloro-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) colorimetric substrate (BioRad) for 15-30 minutes. Colorimetric reaction was stopped by rinsing the plates under cool tap water, and wells were allowed to completely dry before spots were enumerated. Spots were counted with an automated plate reader (Autoimmun Diagnostika). The number of spots was corrected for the media alone negative control, and reported as the mean number of antigen-specific IFNγ spot-forming units (SFU) per 10^6 splenocytes from triplicate samples.

Materials and Methods – Chapter 3

Mice

HLA-A2.01/HLA-DR1-expressing, murine MHC class I/II knockout transgenic (HHDII-DR1) mice on C57Bl/6 background were obtained from Charles River Labs (France) courtesy of Dr François Lemonnier (11). Mice were maintained in microisolater cages under aseptic conditions and all experimental procedures were conducted under an IACUC -approved protocol.

Construction, purification, and expression confirmation of SSX2 DNA vaccine

SSX2 cDNA was cloned from a testis cDNA library into the pTVG4 immunization vector (12) using standard molecular biology techniques. pTVG4 and pTVG-SSX2 plasmids were purified using the Endo-free Plasmid Giga Kit for animal studies (Qiagen, Valencia, CA). Expression of SSX2 was confirmed using transient transfection assays of Cos-7 cells (Effectene transfection reagent, Qiagen). SSX2 mRNA expression was confirmed by reverse-transcription polymerase chain reaction (One-Step RTPCR kit, Qiagen), and SSX2 protein expression from cell lysates was confirmed by Western blot using an SSX2-specific monoclonal antibody (clone 1A4, Abnova, Walnut, CA).

HLA-A2 T2 binding affinity assays

Seven nonamer peptides derived from the amino acid sequence of SSX2 and predicted to have affinity for HLAA2 in the SYFPEITHI and BIMAS prediction algorithms (5, 6) 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 (10). 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 HHDII-DR1 mice

For peptide vaccination experiments, 4 to 6-week-old HHDII-DR1 mice were immunized subcutaneously with 100 µg peptide in complete Freund 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 ammonium chloride / potassium chloride lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). For DNA vaccination studies, 4 to 6-week-old HHDII-DR1 mice were immunized intradermally in the ear pinna with 100 µg of either pTVG4 or pTVG-SSX2 vaccines. Mice were immunized every 14 days. Two weeks after the sixth immunization, mice were euthanized and spleens were collected and processed as previously described.

IFNγ enzyme-linked immunosorbent (ELISPOT) assay

Interferon (IFN)γ enzyme-linked immunosorbent spot (ELISPOT) was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN), and as previously described (10, 12). 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 IFNγ spot-forming units (SFU) per 10^6 splenocytes.

Lactate dehydrogenase cytotoxicity assay

Splenocytes were stimulated for seven days with 2 μg/mL peptide in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal calf serum, 50mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1mM sodium pyruvate, 0.1 μM β-mercaptoethanol. 10 U/mL murine interleukin-2 (R&D Systems) was added 24 hours later. After 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 SSX2. 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:

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

Human T-cell culture

Blood was obtained from HLA-A2+ male volunteer blood donors, and from patients with prostate cancer, with Institutional Review Board-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 each week for up to five weeks in RPMI 1640 media supplemented with 10% human AB serum, 1mM sodium pyruvate, 0.1 μM β-mercaptoethanol, and 10U/mL human interleukin-2 as described previously (10). Cells were evaluated for target cell lysis weekly by cytotoxicity assays as previously described.

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 before staining

with APC-labeled tetramers (NIH Tetramer Core Facility, Emory University, Atlanta, GA) 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.

Materials and Methods – Chapter 4

Mice

HLA-A2.01/HLA-DR1-expressing, murine MHC class I/II knock-out transgenic mice (HHDII-DR1) on C57Bl/6 background were obtained from Charles River Labs (France) courtesy of Dr. François Lemonnier (8). Mice were maintained in microisolater cages under asceptic conditions and all experimental procedures were conducted under an Institutional Animal Care and Use Committee-approved protocol.

HLA-A2 T2 binding affinity assays

Modified peptides were designed from the amino acid sequence of SSX2 to have either reduced or enhanced affinity for HLA-A2 in the SYFPEITHI and BIMAS prediction algorithms (5, 6). These peptides were synthesized, and the purity and identity of each peptide was confirmed by mass spectrometry and gas chromatography (United Biochemical Research, Seattle, WA) (10). Peptides were incubated with TAP-deficient/HLA-A2+ T2 cells as described previously in T2 binding assays (10, 13). Peptide-HLA-A2 binding was measured as relative mean fluorescent intensity (MFI) of peptide-stabilized HLA-A2 surface staining, normalized to MFI for vehicle-pulsed T2 cells, and all measurements were conducted in triplicate.

Plasmid DNA vaccine constructs

A DNA vaccine encoding SSX2 (pTVG-SSX2) was described previously (13). Modifications to the DNA vaccine were made by site-directed mutagenesis using the Phusion™ Site-Directed Mutagenesis Kit (New England BioLabs (Ipswich, MA, USA)). Amplified products were ligated, cloned, and sequence verified using standard molecular biology techniques. pTVG4, pTVG-SSX2, and modified pTVG-SSX2 plasmids were purified from *E. coli* using the Endo-free Plasmid Giga Kit (Qiagen, Valencia, CA).

Peptide and DNA immunization of HHDII-DR1 mice

For peptide vaccination experiments, 4-6 week-old HHDII-DR1 mice were immunized subcutaneously with 100µg of an individual 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 ammonium chloride/potassium chloride lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) (13). For DNA vaccination studies, 4-6 week-old HHDII-DR1 mice were immunized intradermally in the ear pinna with 100µg of DNA plasmid. Mice were immunized at 14-day intervals. Two weeks after the last immunization, mice were euthanized and spleens were collected and processed as described above.

IFNγ enzyme-linked immunosorbent (ELISPOT) assay

Interferon (IFN)γ enzyme-linked immunosorbent spot (ELISPOT) was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) as previously described (9, 10). 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 mean number of peptide-specific IFNγ spot-forming units (SFU) per 10^6 splenocytes. All assays were conducted in triplicate.

Lactate dehydrogenase cytotoxicity assay

Splenocytes were stimulated for seven days with 2μg/ml peptide in Roswell Park Memorial Institute 1640 media supplemented with 10% fetal calf serum, 50mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM sodium pyruvate, 0.1μM β-mercaptoethanol. 10U/mL murine interleukin-2 (R&D Systems) was added 24 hours later. After culture, splenocytes were collected and incubated for four hours with target cells (peptide-pulsed T2 cells, 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. 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:

% Cytotoxicity =

((Experimental - Media Alone) – (Effector Spontaneous – Media Alone) – (Target Spontaneous – Media Alone)) / ((Target Maximum – Media Alone) – (Target Spontaneous – Media Alone))

Generation of SSX2 and GFP sarcoma cell lines

HHDII-DR1 mice were treated with methylcholanthrene (MCA) to generate a syngeneic sarcoma cell line. Cells were transduced with a lentivirus encoding either SSX2 or the green fluorescent protein (GFP). Expression of SSX2 was verified by Western blot analysis with an SSX2 mAb (Abnova, clone 1A4), while expression of GFP was verified microscopically.

Tumor studies

For tumor protection studies, HHDII-DR1 mice were immunized six times biweekly with native or modified SSX2 vaccines followed two weeks later by inoculation with 10^4 SSX2-expressing sarcoma cells in the right flank or 10^4 GFP-expressing sarcoma cells in the left flank. Tumor cell suspensions were prepared in 50% Matrigel solution (BD Biosciences, San Jose, CA). Tumor growth was monitored every three days after inoculation by palpitation and measurement with calipers. The volume was measured in cubic centimeters

according to the following formula: (3.14/6)(long measurement)(short measurement^2). For tumor therapy studies animals were first inoculated with tumor cells as described above, and followed by weekly vaccination with the native pTVG-SSX2 vaccine or modified SSX2 vaccines.

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