Prostate Stem Cell Antigen Vaccination Induces a Long-term Protective Immune Response against Prostate Cancer in the Absence of Autoimmunity

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Abstract

Prostate stem cell antigen (PSCA) is an attractive antigen to target using therapeutic vaccines because of its overexpression in prostate cancer, especially in metastatic tissues, and its limited expression in other organs. Our studies offer the first evidence that a PSCA-based vaccine can induce long-term protection against prostate cancer development in prostate cancer–prone transgenic adenocarcinoma mouse prostate (TRAMP) mice. Eight-week-old TRAMP mice displaying prostate intraepithelial neoplasia were vaccinated with a heterologous prime/boost strategy consisting of gene gun–delivered PSCA-cDNA followed by Venezuelan equine encephalitis virus replicons encoding PSCA. Our results show the induction of an immune response against a newly defined PSCA epitope that is mediated primarily by CD8 T cells. The prostates of PSCA-vaccinated mice were infiltrated by CD4-positive, CD8-positive, CD11b-positive, and CD11c-positive cells. Vaccination induced MHC class I expression and cytokine production [IFN-γ, tumor necrosis factor-α, interleukin 2 (IL-2), IL-4, and IL-5] within prostate tumors. This tumor microenvironment correlated with low Gleason scores and weak PSCA staining on tumor cells present in hyperplastic zones and in areas that contained focal and well-differentiated adenocarcinomas. PSCA-vaccinated TRAMP mice had a 90% survival rate at 12 months of age. In contrast, all control mice had succumbed to prostate cancer or had heavy tumor loads. Crucially, this long-term protective immune response was not associated with any measurable induction of autoimmunity. The possibility of inducing long-term protection against prostate cancer by vaccination at the earliest signs of its development has the potential to cause a dramatic paradigm shift in the treatment of this disease. [Cancer Res 2008;68(3):861–9]

Introduction

Prostate cancer is the most commonly diagnosed malignancy in the western world and is the second leading cause of cancer-related death in American men (1). Therapeutic options for advanced and metastatic prostate cancer are limited (2–4). Immunotherapy is a promising prospective new treatment for this disease.

Prostate cancer immunotherapy involves the use of antigen-presenting cells that present prostate cancer–associated antigenic peptides. To date, the targeted antigens include prostate-specific antigen (PSA; ref. 5), six-transmembrane epithelial antigen of the prostate (6), prostate stem cell antigen (PSCA; ref. 7), prostate-specific membrane antigen (PSMA; ref. 8), and prostatic acid phosphatase (9).

PSCA, a 123–amino acid protein, was selected as the target antigen for this study. Analysis of the amino acid sequence of PSCA predicts that it is a membrane-bound protein related to the Ly-6/Thy-1 gene family (10). Human PSCA is expressed in esophagus, stomach, bladder, and prostate basal cells (11). It is overexpressed in 33% of primary prostate tumors and always present in samples from patients with bone metastasis. Higher levels of PSCA correlate with increasing tumor grade, stage, and the progression to androgen independence (12). Recently, we detected a murine homologue (mPSCA) with 65% similarity to human PSCA at the nucleotide and amino acid levels. Mouse PSCA is expressed in the kidneys, colon, and testes, as well as in the normal prostate and in prostate tumor cells in the transgenic adenocarcinoma mouse prostate (TRAMP) cancer model (13). TRAMP mice develop *in situ* and invasive carcinomas of the prostate as a result of prostate-specific expression of the SV40 T antigen driven by the minimal probasin promoter. The whole spectrum of human prostate cancer progression ranging from prostate intraepithelial neoplasia (PIN) to androgen-independent disease is recapitulated in these mice (14). Therefore, it is a relevant model for analyzing vaccine-induced immune responses and disease progression before translating the findings to the clinic.

One of the latest advances in vaccinology is the development of heterologous prime-boost vaccination regimens that involve sequential vaccination with different antigen delivery systems encoding the same antigen (15). For example, Goldberg et al. showed that priming first with DNA and boosting with recombinant alphavirus replicon particles expressing the melanoma antigen tyrosinase resulted in a better immune response and tumor protection than vaccinating with plasmid DNA alone (16). One effective alphavirus replicon is the Venezuelan Equine Encephalitis replicon particle (VRP) system that has been used to break immune tolerance in rats vaccinated against the tumor-associated self antigen neu (17) and induce immunity against human papillomavirus–induced cervical cancer (18, 19). In addition, VRP induces protective immune responses in primates (20), indicating that they may be an excellent tool for vaccine delivery in humans.

In this study, we tested a heterologous prime-boost vaccination strategy directed against mPSCA. Our results showed that priming

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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with mPSCA cDNA, followed by boosting with mPSCA-VRP, was an effective strategy to induce long-term protective tumor immunity in TRAMP mice, apparently without inducing autoimmune disease.

Materials and Methods

Plasmid DNA constructs and peptide synthesis. A DNA fragment encoding mouse PSCA was obtained from pCR-mPSCA (13). For the generation of PSCA-expressing plasmid (pcDNA3-PSCA), mPSCA DNA was amplified using two specific primers. Primer sequences were designed to include either a *Hin*dIII or a *Bam*HI site: 5′-CCCAAGCTTATGACTCACAGG-3′ and 5′-TGTGAGGAGTGCACA-3′. Amplification was performed for 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. An additional extension step was performed for 10 min at 72°C. The PCR product was then cloned into the pcDNA3 expression vector (Invitrogen). DNA sequencing was performed to confirm that the pcDNA3-PSCA construct had the desired sequence and open reading frame. pcDNA3-PSCA or empty vector (pcDNA3) were transformed into TOP10 competent *Escherichia coli* (Stratagene). Plasmid DNA copies were amplified in liquid culture and purified using a plasmid maxi kit (Qiagen Sciences). DNA used for vaccination had an A_{260}/A_{280} ratio of 1.9.

For VRP production, mPSCA DNA was amplified by using specific primers 5′-GACTCACAGGACTACTACGTGGGCAAGAA-3′ and 5′-TTAATTAAGGCGAGC-TCCTACAACC-3. A Pac-1 site was added to the antisense primer. Amplification was performed for 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C. An additional extension step was performed for 10 min at 72°C. The PCR product was digested with the appropriate 3′ enzyme and ligated into pERK (AlphaVax replicon vector plasmid) that had been digested with EcoRV and the matching 3′ enzyme. mPSCA presence in the construct was verified by DNA sequencing. VRP was made using a two-helper system (21).

Peptides. Peptides were synthesized according to standard methods used at the Norris Comprehensive Cancer Center facility at University of Southern California. Peptides were dissolved at 50 mg/mL in DMSO (Sigma) and stored at −80°C until further use.

Peptide binding assay. An MHC class I stabilization assay was performed. Briefly, RMA-S cells were incubated at 30°C overnight. Peptide at various concentrations was added for 1 h at 30°C, and cells were incubated overnight at 37°C. Cells were stained on ice with FITC-antimouse H-2K^b (clone AF6-88.5) or H-2D^b (clone KH95; PharMingen) for 30 min at 4°C. Peptide binding was determined by flow cytometry using a Beckman Coulter Cytomic FC 500 flow cytometer equipped with CXP software (Beckman Coulter, Inc.). The fluorescence index (FI) was calculated as follows: FI = [(fluorescence after peptide stimulation / fluorescence without peptide stimulation) − 1].

Mice and cell lines. C57BL/6 and MLR/lpr mice were obtained from Taconic Farms. CD8 and CD4 knockout mice were purchased from Jackson. RAG knockout mice were kindly donated by Chaim Jacob (University of Southern California). TRAMP mice (22) on the C57BL6 background were bred at University of Southern California. Research was conducted in compliance with the institutional animal use guidelines. TRAMP-C2 and EL-4 cells were cultured as previously described (6).

Immunization and tumor challenge. Male C57BL/6 or TRAMP mice (8–10 weeks old) were anesthetized i.p. with 2.4 mg ketamine (Phoenix Pharmaceutical, Inc.) and 480 μg xylazine (Phoenix). DNA gold particles were delivered to a shaved area on the abdomen using a helium-driven gene gun (Bio-Rad) with a discharge pressure of 400 p.s.i. Each mouse received 2 μg of murine PSCA cDNA vaccine. Fifteen days after gene gun vaccination, mice were subcutaneously boosted 1 cm from the tail base with 10^6 infectious units (IU) of mPSCA-VRP. TRAMP mice received an additional dose of 10^6 IU mPSCA-VRP at day 60. As control groups, C57BL/6 or TRAMP mice were vaccinated with pcDNA3 and boosted with 10^6 IU GFP-VRP. Ten days after vaccination, mice were challenged subcutaneously with 5×10^5 TRAMP-C2 cells resuspended in 100 μL HBSS (Sigma). Tumor growth was monitored twice weekly using engineer calipers. Survival was followed until tumors reached volumes of >1,000 mm^3 in accordance with our Institutional Animal Care and Use Committee guidelines.

ELISPOT assay. ELISPOT assay was performed as described (23) with the exception that purified T cells were stimulated with mPSCA$_{83\text{-}91}$ or PSA$_{(-3)\text{-}6}$ peptide (24) in the presence of 20 IU/mL interleukin 2 (IL-2) and spots were developed with 3-amino-9-ethyl-carbazole substrate (Sigma).

CTL assay. EL4 cells were cultured with 10 μg/mL of mPSCA$_{83\text{-}91}$ or PSA$_{(-3)\text{-}6}$ and were labeled overnight with ^{51}Cr. After labeling, the EL4 targets were washed and cultured at 10^4 cells per well. Live T cells from the spleens of vaccinated and control mice were added to the labeled EL4 cells at E/T ratios between 12.5:1 and 100:1 (in triplicate) and cultured for 5 h. Supernatants were collected, and released radioactivity was measured with a Gamma counter. Spontaneous ^{51}Cr release was determined in supernatants of labeled EL4 cells cultured in the absence of effectors. Maximum release was determined in supernatants of EL4 cells lysed with 1% Triton X-100.

Tumor histology and detection of cell populations and immunopathology by H&E stain, immunofluorescence, and immunohistochemistry. Paraffin sections of kidney, colon, prostate, and testis from immunized C57BL/6 and TRAMP mice were made and analyzed as previously described (6). For mPSCA visualization, frozen tissue sections were blocked for endogenous biotin activity and then incubated for 1 h with goat anti-mouse PSCA (Santa Cruz Biotechnology). Slides were then incubated for 30 min with biotin-conjugated rabbit anti-goat antibody (Vector Laboratories). Biotinylated antibodies were visualized with streptavidin-conjugated Alexa 488 or Alexa 594 (Molecular Probes). Tissue-specific autoantibodies directed against self-antigens were detected as previously described (6). Accumulation of IgM and collagen deposition in kidneys was analyzed by immunohistochemistry as described (6).

Isolation of tumor-infiltrating lymphocytes and flow cytometric analysis. Tumor-infiltrating lymphocytes (TIL) were isolated from individual prostate tumors as previously described (25). TIL were analyzed by flow cytometry and the total number of cells per gram of tumor calculated, as previously described (6).

Measurement of intratumoral cytokine expression and PCR. Tumors were collected, weighed, and homogenized in PBS containing 2× Halt Protease Inhibitor Cocktail (Pierce). Bovine serum albumin (1%; Sigma) was added, and the supernatants were collected by centrifugation at 4°C for 20 min. The cytokine levels in the supernatants were quantified using the Bio-Plex mouse cytokine T helper 1 (Th1)/Th2 assay (Bio-Rad) and a Bio-Plex HTF system following the manufacturer's instructions. For quantitative PCR, total RNA was isolated using an RNeasy kit (Qiagen). DNase-treated RNA was reverse transcribed with oligo (dT) and SuperScript III (Invitrogen Life Technologies). Quantitative PCR was performed using TaqMan Universal PCR Master Mix, following the Applied Biosystems protocol. Primers and probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-2, IFN-γ, tumor necrosis factor-α (TNF-α), IL-4, IL-5, and IL-10 were obtained (Applied Biosystems). Quantitative PCRs were performed using a PRISM 7700 instrument from Applied Biosystems. The relative level of mRNA expression for each gene in each tumor was first normalized to the expression of GAPDH RNA in that tumor and then normalized to the level of mRNA expression in tumor from control mice.

Statistical analysis. Tumor growth, cytotoxicity assay, multiplex ELISA, and ELISPOT data were analyzed by a two-tailed, paired Student's t test. Survival rates were analyzed by the log-rank test for survival.

Results

mPSCA vaccination generates CD4 and CD8 T cells that prevent prostate cancer growth. The effectiveness of antigen-specific vaccination against mPSCA in protecting mice against prostate tumor challenge was assessed. Nineteen of 25 mice vaccinated with mPSCA DNA and boosted with mPSCA-VRP remained tumor-free 52 days after challenge with TRAMP-C2 murine prostate tumor cells. In addition, the tumors of the six mPSCA-vaccinated mice that developed tumors were statistically significantly smaller than the tumors of control mice vaccinated with empty-vector DNA and boosted with GFP-VRP ($P < 0.05$,

two-tailed test; Fig. 1*A*). To assess whether vaccination-induced tumor infiltration by lymphocytes, total numbers of intratumoral CD4 and CD8 T cells were quantified by flow cytometry. Our data showed a statistically significant increase in the number of tumor infiltrating CD4 T cells ($P = 0.0244$) in mice vaccinated against mPSCA compared with control mice (Fig. 1*B*). An increase in the number of tumor-infiltrating CD8 T cells was also observed in mPSCA-vaccinated mice, although this was not statistically significant ($P = 0.0841$; Fig. 1*B*).

Multiplex ELISA analysis showed a statistically significant increase in the intratumoral levels of cytokines that are critical for generation of antitumor immunity, such as IL-2, IFN-γ, and granulocyte macrophage colony-stimulating factor (GM-CSF), in vaccinated mice compared with control mice (Fig. 1*C*). To determine the role of CD8 and CD4 T cells in the immunization-induced response, tumor growth was followed in vaccinated CD4-deficient and CD8-deficient mice and vaccinated C57BL/6 mice. Mice were vaccinated with mPSCA DNA, boosted with mPSCA-VRP, and challenged with TRAMP-C2 cells. Tumor growth was detected in only one of five C57BL/6 mice. In contrast, all CD4-deficient and CD8-deficient mice developed tumors. In addition, tumors grew significantly larger in CD4-deficient and CD8-deficient mice than in C57BL/6 mice ($P < 0.05$, two-tailed test; Fig. 1*D*). Tumors in vaccinated CD4-deficient mice were significantly smaller than those in vaccinated CD8-deficient mice ($P < 0.05$, two-tailed test). These findings indicate that both CD4 and CD8 T cells are essential for the regulation of protective tumor immunity.

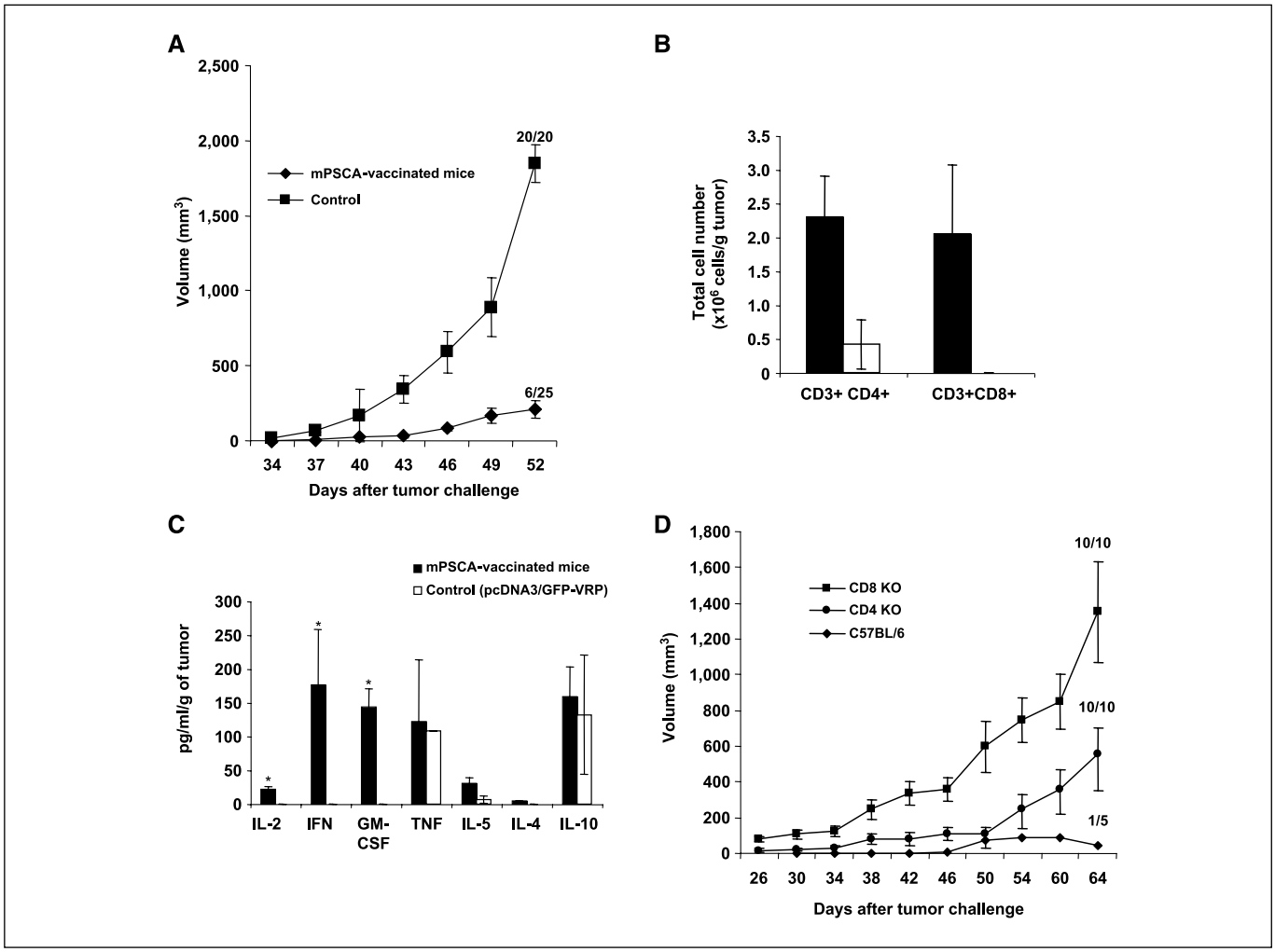

Figure 1. mPSCA vaccination protects mice from TRAMP-C2 tumor challenge in a process involving CD4 and CD8 T cells. *A,* male C57BL/6 mice were immunized at day 0 with 2 μg pcDNA-PSCA by gene gun and boosted 15 d later with 10^6 IU mPSCA-VRP. Ten days after boosting, mice were subcutaneoulsy challenged with 5×10^5 TRAMP-C2 cells. Tumor size was measured twice per week using engineer calipers. *Points,* mean tumor volume of 25 mPSCA-vaccinated and 20 control-vaccinated mice in three separate experiments; *bars,* SE. The fraction of mice that developed tumors in each group is indicated. In cases where some mice developed tumors and others did not, results indicate the mean tumor volume (±SE) only of those mice that developed tumors. *B,* mPSCA vaccination induces increased tumor infiltration by T cells and increased local production of cytokines associated with tumor control. Tumors from mPSCA-vaccinated and control mice were collected, weighed, and enzyme-digested as described in Materials and Methods. TIL were stained with antimouse CD3-FITC and either CD8-PE or CD4-PE antibodies, then analyzed by flow cytometry. Absolute numbers of positive cell per single tumor were calculated, and the cell numbers were normalized per gram of tumor. *C,* cytokine levels were quantified using the Bio-Plex mouse cytokine Th1/Th2 assay as previously described. *, $P \leq 0.05$ mPSCA-vaccinated mice versus control group. *D,* groups of wild-type C57BL/6, CD8 knockout, and CD4 knockout mice were vaccinated with mPSCA and challenged with 5×10^5 TRAMP-C2 cells as described above. Tumor size was measured twice per week using engineer calipers. *Points,* mean tumor volume of 10 CD4 knockout, 10 CD8 knockout, and 5 C57BL/6 wild-type mice; *bars,* SE. The fraction of mice that developed tumors in each group is indicated. In cases where some mice developed tumors and others did not, results indicate the mean tumor volume (±SE) only of those mice that developed tumors.

mPSCA vaccination induces antigen-specific CD8 T cells. Under normal culture conditions, unstimulated TRAMP-C2 cells express low levels of MHC class I (Fig. 2*A*) and they are not able to activate purified T cells *in vitro* (data not shown). However, TRAMP-C2 cells stimulated *in vitro* with IFN-γ expressed high levels of MHC class I as determined by flow cytometry (Fig. 2*A*), making them attractive targets for cytolytic CD8 T cells. Most importantly, to determine whether the increase in IFN-γ expression induced by vaccination can elicit tumor MHC class I expression *in vivo*, we analyzed the tumors of vaccinated mice by immunofluorescence. The number of tumor cells expressing MHC class I was increased in mPSCA-vaccinated mice compared with controls (Fig. 2*B*), suggesting that antigen-specific CD8 T cells induced by vaccination will be able to mediate the killing of tumor cells *in vivo*. To verify that CD8 T cells from mPSCA-vaccinated mice are specific for mPSCA, *in vitro* ELISPOT and CTL assays were carried out. We identified mPSCA peptide candidates that could potentially bind to MHC class I molecules H-2D^b or H-2K^b and selected two peptides based on the highest score of peptide/MHC class I half life of dissociation generated using prediction programs (26, 27). We also evaluated their capacity to stabilize MHC class I on RMA-S cells (Table 1). The peptide mPSCA$_{83\text{-}91}$ had the highest predicted binding affinity of 24 and a measured mean fluorescence index of 4. This peptide was loaded onto spleen cells to evaluate MHC class I–restricted CD8 T-cell responses by ELISPOT and CTL assay. Significantly, more IFN-γ–producing splenocytes were detected

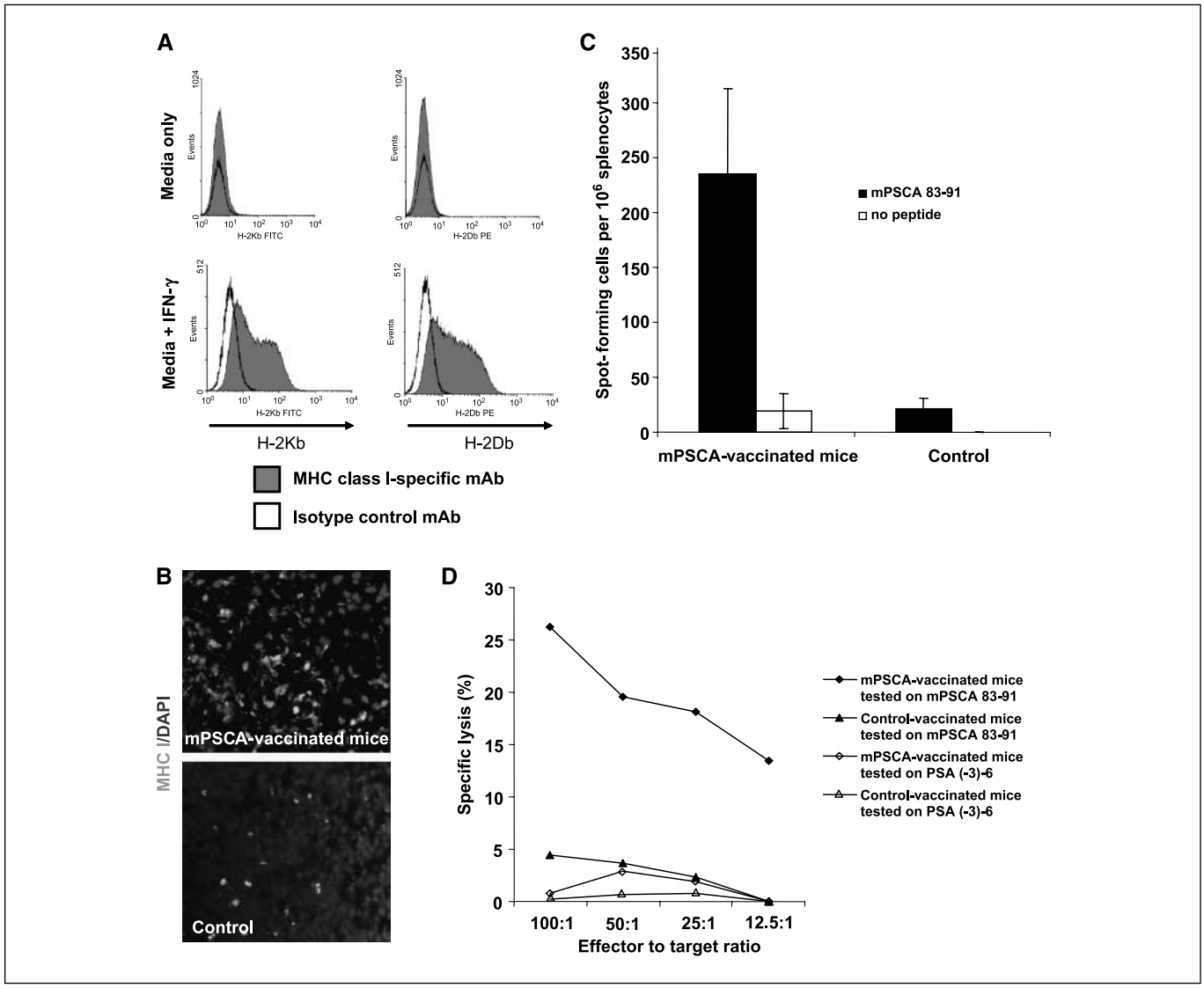

Figure 2. MHC class I expression by TRAMP-C2 cells increases in the presence of IFN-γ. *A,* TRAMP-C2 cells were cultured for 48 h in standard media or in media supplemented with IFN-γ (500 units/mL). Cells were then washed and stained with antimouse H-2Kb-FITC and H-2Db-PE antibodies, then analyzed by flow cytometry. *B,* frozen sections of tumors obtained from mPSCA-vaccinated and control mice were incubated with biotinilated antimouse H-2Kb antibody at room temperature for 12 h, then incubated with streptavidin Alexa Fluor 488 and counterstained with mounting solution with 4′,6-diamidino-2-phenylindole (*DAPI*). Slides were imaged at 250× magnification. A specific cellular immune response against mPSCA is induced in vaccinated mice. *C*, ELISPOT. Male C57BL/6 mice were vaccinated once with 2 μg mPSCA-pcDNA3 or empty vector and boosted with 10^6 IU mPSCA-VRP or GFP-VRP, respectively. T cells from the spleens of mPSCA-vaccinated mice were activated with mPSCA$_{83\text{-}91}$ or PSA$_{(-3)\text{-}6}$ peptide in the presence of 5 IU IL-2 for 48 h. The results are presented as spot-forming cells per 10^6 splenocytes. Spot numbers represent the mean of four mice in each vaccinated group; *bars,* SE. *D,* chromium-release assay. Lymph node cells from mPSCA-vaccinated mice or controls were stimulated for 5 d with 20 U IL-2 and 10 μg mPSCA$_{83\text{-}91}$ peptide. Cells were cultured with ^{51}Cr-labeled EL4 targets loaded with mPSCA$_{83\text{-}91}$ (♦, ▲) or PSA$_{(-3)\text{-}6}$ (◇, △) for 5 h at the indicated effector-to-target ratios, and the specific lysis calculated. The data shown represent one of two separate experiments with similar results.

Table 1. Stabilization of cell surface MHC by PSCA peptides

Peptide	Sequence	Score (estimate of half time of dissociation)	H2 molecule selected	Fluorescence index
$PSCA_{83-91}$	NITCCYSDL	24	K^b	4
$PSCA_{23-31}$	AQMNNRDCL	220	D^b	0.2
$E7_{49-57}$	RAHYNIVTF	3	D^b	7
$PSA_{(-3)-6}$	VTWIGAAPL	4	K^b	4

NOTE: MHC binding assays were performed by loading RMAS cells with the peptides indicated and assessing the level of MHC-I by flow cytometry. The fluorescence index was calculated as described in Materials and Methods. Scores were obtained using an online HLA peptide binding prediction program (http://bimas.cit.nih.gov/).

by ELISPOT after incubation with the $mPSCA_{83-91}$ peptide (Fig. 2*C*). In addition, significantly more peptide-specific IFNγ-producing splenocytes were present in mPSCA-vaccinated mice compared with control mice ($P < 0.05$, two-tailed test), confirming specificity of our peptide. The peptide-specific CTL response generated against $mPSCA_{83-91}$ was also significantly higher in mPSCA-vaccinated mice than in controls ($P < 0.05$, two-tailed test; Fig. 2*D*).

mPSCA vaccination delays tumor progression and induces long-term tumor protection. The effectiveness of vaccination in protecting TRAMP mice from developing prostate cancer was tested. TRAMP mice were vaccinated at 8 to 10 weeks of age after they had developed PIN (22). TRAMP mice were immunized with mPSCA DNA or empty vector at day 0 and boosted with mPSCA-VRP or GFP-VRP, respectively, at days 16 and 60. Survival of TRAMP mice vaccinated against mPSCA was prolonged significantly compared with the control group ($P = 0.035$; Fig. 3*A*). Over 320 days, 16 of 20 mice from the control group (pcDNA3/GFP-VRP) died due to the presence of large and poorly differentiated prostate adenocarcinomas. In stark contrast, only 4 of 20 mPSCA-vaccinated mice died, and of these, only two mice developed a large prostate tumor. When surviving mice (4 control and 16 mPSCA vaccinated) were sacrificed, tumors from mPSCA-vaccinated mice had few or no cells staining for mPSCA when compared with the strong staining observed in sections of tumors taken from the control group (Fig. 3*B*). In addition, the majority of the mPSCA-vaccinated group showed small well-differentiated focal adenocarcinomas with extensive hyperplasia and multiple apoptotic zones (Fig. 3*C*). Overall, tumors from mPSCA-vaccinated mice had lower Gleason scores than those of control-vaccinated TRAMP mice, which all had a very large tumor burden at the time of sacrifice (Fig. 3*C*). The tumors of vaccinated TRAMP mice were infiltrated by considerable numbers of immune cells, including macrophages, dendritic cells, and IFNγ-expressing CD4 and CD8 T cells. In contrast, these cell types were present in very low numbers or completely absent in the tumors of control mice (Fig. 3*D*). mPSCA vaccination induced a tumor microenvironment dominated by cytokines that may inhibit tumor growth, including IFNγ, IL-2, GM-CSF, TNF-α, IL-4, and IL-5 (Table 2). These data suggest that vaccination against mPSCA is able to induce a specific immune response that is capable of targeting mPSCA-expressing tumor cells with high specificity, which confers a long-term protective immune response against prostate cancer.

mPSCA vaccination does not induce pathologic autoimmunity. Given that the prime-boost vaccination scheme was able to induce a specific immune response against mPSCA, there was concern that an autoimmune response may be elicited in the organs in which this antigen is expressed or that a systemic autoimmune cascade may be induced. The production of autoantibodies against rheumatoid factor and anti-ssDNA antibodies in serum samples from control and vaccinated groups was assayed. In addition, the presence of inflammatory cells in tissues where mPSCA is expressed was tested. As positive controls, we used serum and tissues from aged MRL/lpr mice. Titers of rheumatoid factor or anti-DNA autoantibodies in most mPSCA-vaccinated mice were equivalent to control levels (Supplementary Fig. S1*A*). Given that mPSCA is also expressed in other organs, such as kidney, testis, colon, and prostate, cellular infiltration of these organs was analyzed by H&E staining of frozen tissues obtained from mPSCA-vaccinated and control mice (Supplementary Fig. S1*B*). No infiltration by inflammatory cells or tissue damage was found in organs in which mPSCA is expressed. Finally, it was determined whether mPSCA vaccination induced autoantibodies that recognized self antigens in the serum of vaccinated mice. Frozen sections of kidney, prostate, testis, and colon from RAG mice, which do not produce endogenous antibodies, were incubated with serum samples from mPSCA-vaccinated and control (pcDNA3/GFP-VRP) mice. Bound antibodies were detected with anti-mouse IgG. Positive reaction on RAG tissues in testis was only detected in 2 of 20 mPSCA-vaccinated TRAMP mice and when the tissues were incubated with serum from MRL/lpr mice (Supplementary Fig. S1*C*), indicating that the vast majority of vaccinated mice do not produce autoantibodies that could be responsible for tissue damage.

Discussion

In this study we have assessed the efficacy of a DNA prime/VRP boost prostate cancer vaccination strategy directed against mPSCA in murine models of prostate cancer. The antigen delivery systems most commonly used to induce protective immunity against transformed cells are based on administration of plasmids by gene gun or inoculation of viral vectors containing the gene of interest. Heterologous prime-boost schemes, where DNA and viruses are inoculated in succession, often induce the best immunity (19, 28).

In several experimental models, T-cell infiltration is considered a good prognostic marker for tumor control (29, 30). In our model, an increased number of tumor-infiltrating CD4 and CD8 T cells was correlated with improved survival and slower tumor growth in vaccinated mice. The increased numbers of TIL likely included a

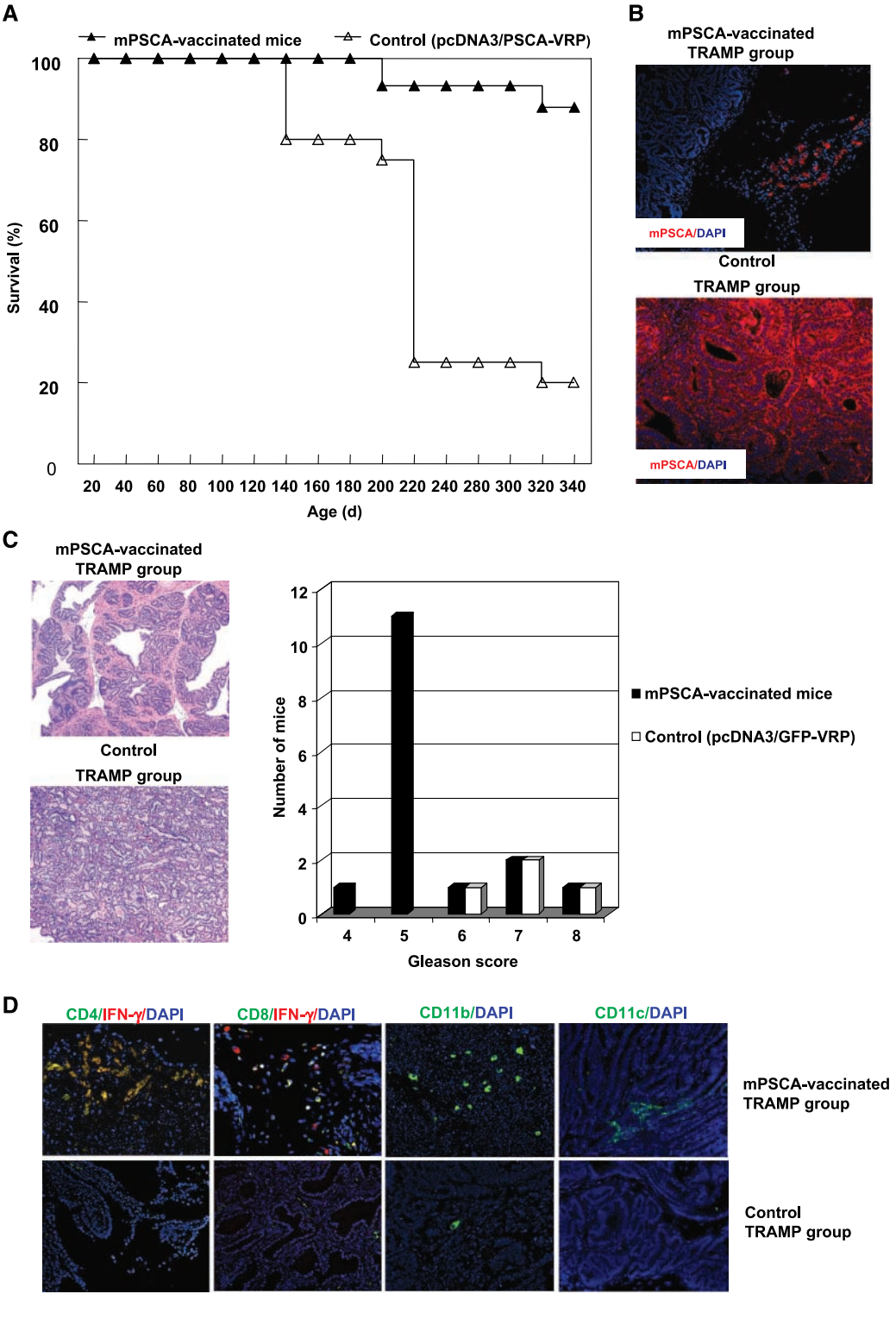

Figure 3. mPSCA vaccination improves survival in TRAMP mice. *A,* groups of 20 TRAMP mice were vaccinated at day 0 with either 2 μg mPSCA-pcDNA or 2 μg empty vector and boosted at days 15 and 60 with 10^6 IU mPSCA-VRP and 10^6 IU GFP-VRP, respectively. *B,* tumors from mPSCA-vaccinated TRAMP mice have fewer mPSCA-expressing cells than control-vaccinated TRAMP mice. Frozen tumor section from mPSCA-vaccinated or control mice were incubated with biotin antimouse PSCA at room temperature for 1 h then incubated with streptavidin conjugated to Alexa 594. *C,* tumors from mPSCA-vaccinated TRAMP mice have lower grade adenocarcinoma than control-vaccinated TRAMP mice. Paraffin-embedded sections of tumors obtained from mPSCA-vaccinated or control-vaccinated mice were stained with H&E and their Gleason scores calculated by a certified pathologist. *D,* the tumors of mPSCA-vaccinated TRAMP mice are infiltrated by greater numbers of immune cells than those of control-vaccinated TRAMP mice. Frozen tumor sections from mPSCA-vaccinated and control-vaccinated TRAMP mice were incubated as indicated with IFN-γ-PE and biotin antimouse CD4, CD8a, CD11c, and CD11b at room temperature for 1 h then incubated with streptavidin conjugated to Alexa 488. Representative sections from 16 mPSCA-vaccinated and four control-vaccinated TRAMP mice with similar results. Slides were imaged at 250× magnification.

Table 2. Quantitation of cytokines and mRNA levels in TRAMP mouse prostate tumors

Cytokine	Cytokine level (pg/mL/g tumor)		Fold change in mRNA expression/gram of tumor	
	PSCA-PcDNA3/ PSCA-VRP–vaccinated group	pcDNA3/GFP-VRP control–vaccinated group	PSCA-PcDNA3/ PSCA-VRP–vaccinated group	pcDNA3/GFP-VRP control–vaccinated group
IL-2	1.2 ± 0.5	0.6 ± 0.3	7.45 ± 2.81*	1 ± 0.12
IFN-γ	2 ± 0.8*	0	38.7 ± 19*	1 ± 0.148
GM-CSF	210 ± 91	22 ± 9	N/D	N/D
TNF-α	49.45 ± 10.78*	5 ± 2	0.073 ± 0.035	1 ± 0.145
IL-5	100 ± 32*	14 ± 6	34.03 ± 7.85*	1 ± 0.148
IL-4	1 ± 0.2*	0.2 ± 0.04	1.58 ± 0.75	1 ± 0.21
IL-10	402 ± 206	178 ± 98	0.035 ± 0.016	1 ± 0.21

NOTE: Tumors from PSCA-vaccinated TRAMP mice were homogenized and cytokine levels were quantified using the Bio-Plex mouse cytokine Th1/Th2 assay. DNA-free RNA was isolated from the tumors of experimental groups and was reverse transcribed. Quantitative PCR array was performed to determine relative changes in levels of mRNA expression. mRNA levels of each gene in each sample were first normalized to the expression of GAPDH and then normalized to the expression of that gene in the group of control mice. Data represent the mean ± SE cytokine level of 16 mPSCA-vaccinated and four control-vaccinated mice.

Abbreviation: N/D, not determined.

*$P < 0.05$ for mPSCA-vaccinated mice compared with controls.

proportionately larger number of antigen-specific T cells that controlled tumor growth. Vaccinated C57BL/6 mice produced a large population of mPSCA-specific T cells that were able to produce IFN-γ and kill antigen-loaded target cells. The mPSCA-specifc CD8 T cells induced by vaccination were only capable of very limited killing TRAMP-C2 prostate tumor cells that endogenously express mPSCA, 12% lysis at an E/T ratio of 100:1 versus 7% when T cells were used from nonvaccinated animals (data not shown). Nevertheless, the induction of an immune response by prophylactic vaccination was associated with slower tumor growth in vaccinated mice challenged with TRAMP-C2 cells. Although mPSCA vaccination induced strong immunity that prevented growth in most of the tumor-challenged mice, 6 of 25 vaccinated mice still developed tumors. However, tumor growth in these mice was delayed very significantly and was extremely slow compared with tumor growth in unvaccinated mice. Collectively, these data indicate that vaccination against mPSCA induces antigen-specific T cells that are capable of controlling TRAMP-C2 tumor growth *in vivo* although they show relatively poor killing of TRAMP-C2 cells in short-term chromium release assays *in vitro*. Our findings may indicate that small amounts of CD8-mediated killing over the long term is sufficient to control tumor growth or that CD8 T cells do not act alone to control tumor growth by direct killing.

To investigate further the roles of CD4 and CD8 T cells in the control of tumor growth in our orthotopic impantation model of prostate cancer, we made use of CD4-deficient and CD8-deficient mice. We showed that the induction of mPSCA-specific CD8 T cells by our heterologous vaccination strategy is crucial to the efficient control of early tumor growth. In contrast, CD4 T cells seem to be more important in the control of the later stages of tumor growth. Accelerated tumor growth in CD8 knockout mice may be explained by the absence of mPSCA-specific cytotoxic CD8 T cells. A better initial antitumor response was observed in CD4 knockout mice. This was most likely due to increased numbers of CD8 T cells. In addition, negative modulation of tumor immunity by CD4 natural T-regulatory cells was abolished in CD4 knockout mice because this population was completely absent. However, at later stages of tumor growth (after day 50), the presence of CD4 T-helper cells was apparently required to keep tumor outgrowth under control. These data may indicate that in our system CD4 T cells are important in the maintenance and generation of memory cells as previously observed (31). Alternatively, or in addition, the CD4 cells may be required for activation of macrophages and dendritic cells, an event crucial for killing of transformed cells (32). Given that vaccination elicits an increase in intratumoral IL-2 expression, it is also possible that CD4 T cells are required to provide this cytokine to CD8 T cells during the induction of the antitumor immune response. Overall, these data indicate that interactions between CD4 and CD8 T cells are required for proper control of tumor outgrowth. These findings are in keeping with those recently reported by Chaput et al., which showed the importance of CD4 T-helper cell activity in controlling tumor growth (33).

Interestingly, the TRAMP-C2 tumors in CD4 knockout and CD8 knockout mice grow out with very similar growth kinetics, albeit with a delay in tumor growth in CD4 knockout mice. In addition, the growth kinetics of these TRAMP-C2 tumors closely resemble the growth pattern of TRAMP-C2 tumors in unvaccinated wild-type C57BL/6 mice (compare Fig. 1*C* with the control growth curve in Fig. 1*A*). mPSCA-negative tumor variants developed in vaccinated TRAMP mice (Fig. 3*B*) and in vaccinated C57BL/6 mice challenged with TRAMP-C2 tumors (Supplementary Fig. S2, compare PSCA-positive TRAMP-C2 cells from mPSCA-vaccinated C57BL/6 mice in the top left with PSCA-negative TRAMP-C2 cells in the bottom right). mPSCA-negative variants may also develop in vaccinated CD4 knockout and CD8 knockout mice. Tumor immune escape may occur in the knockout mice when TRAMP-C2 mPSCA-negative variants develop, which then grow with normal TRAMP-C2 growth kinetics. Rapid tumor outgrowth is not observed in vaccinated wild-type mice, although mPSCA-negative variants develop in them (Supplementary Fig. S2). This implies that interplay between the CD4 and CD8 T-cell populations is required to facilitate epitope spreading, thereby preventing tumor immune escape by the development of negative variants.

To investigate the efficacy of our vaccination strategy in a more physiologically relevant setting, we immunized TRAMP mice against mPSCA. TRAMP mice vaccinated against mPSCA had vastly better survival rates than unvaccinated control TRAMP mice. This protection was associated with an increased number of infiltrating dendritic cells, macrophages, Th1, and $CD8^+$ $INF\text{-}\gamma^+$ cells in mPSCA-vaccinated mice compared with controls, as detected by immunofluorescence. We also confirmed by flow cytometry that there was a significant increase in the number of CD4 T cells, CD8 T cells, and dendritic cell in the spleens of mPSCA-vaccinated mice compared with control mice (data not shown).

MHC class I expression by tumor cells is essential for their recognition by CD8 cytolytic T cells. Down-regulation of MHC class I is a common tumor escape mechanism (34); therefore, we investigated whether prostate tumors in our model systems expressed MHC class I. IFN-γ produced at the tumor site is important in stimulating the expression of MHC classes I and II molecules by malignant cells, facilitating their clearance (35). We confirmed that this concept is important in our models by demonstrating the induction of MHC class I expression on TRAMP-C2 cells cultured in the presence of IFN-γ. Crucially, vaccination of C57BL/6 mice challenged with TRAMP-C2 tumor cells induced increased expression of IFN-γ in tumors that was associated with increased MHC class I expression *in vivo*. Class I–positive tumor cells, such as these, are excellent targets for tumor-infiltrating CD8 T cells that are activated by IL-2 and IFN-γ in the tumor microenvironment. In addition, stromal cells may pick up mPSCA peptides released by killed cancer cells, rendering them viable targets for killing by tumor-infiltrating CD8 T cells as well. Our vaccination protocol resulted in small but significant increases in expression of IFN-γ, IL-2, IL-4, and IL-5 in the tumors of vaccinated mice. Although the latter two are classic type 2 cytokines that down-regulate cellular immunity, there are reports that point out their relevance in tumor clearance (36–38). The profile of cytokines in our vaccinated mice was similar to that induced by intratracheal administration of dendritic cells transduced with CCL21, which was associated with protection against bronchoalveolar cancer (39). Taken together, these results indicate that our vaccination strategy is capable of inducing immunologic changes in the tumor microenvironment that are very favorable for the killing of transformed cells, thereby allowing tumor clearance.

Killing of transformed cells may release mPSCA peptides that can be captured by immature infiltrating dendritic cells and macrophages. This may cause their activation, maturation, and migration to secondary lymph organs where they may present antigen to T cells, giving rise to the production of a substantial number of mPSCA-specific CD8 T cells. It has been reported that poor macrophage infiltration of tumors is linked to cancer progression (40). This suggests that the increase in the number of tumor-infiltrating macrophages in our vaccinated mice may contribute to the elimination of tumor cells. In addition, we detected considerable local production of GM-CSF and TNF-α, two factors that are required for dendritic cell differentiation and maturation. Consistent with the increased production of IL-2, IFN-γ, GM-CSF, IL-4, IL-5, and MHC class I in the tumors of vaccinated mice discussed above, it is possible that tumor-infiltrating mature dendritic cell help in generating an environment that is favorable for controlling the proliferation of prostate tumor cells (41).

Fewer cells express mPSCA in prostate tumor tissues obtained from vaccinated TRAMP mice than in those taken from unvaccinated mice. This observation suggests that a highly specific anti-mPSCA immune response takes place *in vivo*, which eliminates prostate tumor cells that express high levels of mPSCA. However, mPSCA-expressing cells remain in the prostate tissues of vaccinated mice. It is possible that the strong anti-mPSCA immune response selects tumor cells that express little or no mPSCA, possibly leading to tumor escape. This process is consistent with the tumor immunoediting paradigm proposed by Dunn et al. (42). Tumor immunoediting may explain why tumors were not completely eradicated in vaccinated mice, suggesting that more than one antigen may need to be targeted to completely eliminate tumor cells. Alternatively, the mPSCA-expressing cells observed in vaccinated TRAMP mice may simply be spontaneously arising tumor cells that have yet to be eliminated by the induced anti-mPSCA immune response. The SV40 large T antigen is expressed in the prostate throughout life in TRAMP mice that have not been castrated. Given that we have extended the lives of these mice far beyond their normal life expectancy, it is possible that there is time for multiple primary prostate tumors to develop. The fact that mPSCA-vaccinated TRAMP mice survive and are healthy for so long suggests that if new prostate tumors are continuously developing or if negative variants arise, then they must also be subject to long-term immune control.

Vaccination of mice against mPSCA did not induce significant infiltration by inflammatory cells or cause tissue damage in healthy organs in which mPSCA is expressed nor were any autoantibodies produced in response to vaccination. Taken together, these results suggest that there was no induction of a harmful autoimmune response in vaccinated mice. Our histology data would allow the visualization of tissue damage and cellular infiltration in the context of the structure of the relevant organs, if any occurred, although this approach has the disadvantage that it would not allow the quantification of any cellular infiltration. Nonetheless, our histologic methods were highly sensitive to the autoimmune responses that did occur, as can be seen in our positive MRL/lpr controls and in the rare cases where some autoimmunity was observed in vaccinated mice (Supplementary Fig. S1*C, e*). It is likely that the mPSCA-specific cells induced by vaccination do not respond to tissues that express normal low physiologic levels of mPSCA, including the healthy prostate, simply because there is an insufficient amount of antigen available to stimulate them. In contrast, prostate tumor cells strongly overexpress mPSCA, making them ready targets for the mPSCA-specific T cells induced by our vaccination strategy. Although we have not addressed this issue directly, the T-cell clones generated by our vaccination protocol are apparently of sufficiently high affinity to respond to the very high mPSCA expression levels of tumor cells, but not of such high affinity that they are capable of recognizing and killing healthy cells that express low physiologic mPSCA levels.

Recently a phase I/phase II clinical trial was conducted in which patients with hormone and chemotherapy refractory prostate cancer were vaccinated with dendritic cells loaded with a mPSCA peptide. Although this approach induced high frequencies of peptide-specific T cells *in vitro*, there was limited clinical benefit *in vivo*. Only one patient of 12 had complete regression of a retrovesical lymph node, whereas the others had progressive or stable disease (43). In contrast to this work, our TRAMP mouse studies were carried out in animals that had PIN lesions rather than advanced prostate cancer. The success of this vaccination strategy in inducing long-term protection against prostate cancer

suggests that similar vaccination protocols should also be carried out in humans at the early stages of prostate cancer. For instance, the best strategy may be to vaccinate men after they display rising PSA levels but before they develop overt symptoms of prostate cancer. This would be a major paradigm shift in the treatment of prostate cancer, which is currently centered on watchful waiting for disease progression.

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References

1. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. CA Cancer J Clin 2002;52:23–47.

2. Tannock IF, Osoba D, Stockler MR, et al. Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. J Clin Oncol 1996;14:1756–64.

3. Hillman GG, Triest JA, Cher ML, Kocheril SV, Talati BR. Prospects of immunotherapy for the treatment of prostate carcinoma-a review. Cancer Detect Prev 1999; 23:333–42.

4. Crawford ED, Rosenblum M, Ziada AM, Lange PH. Hormone refractory prostate cancer. Urology 1999;54: 1–7.

5. Noguchi M, Kobayashi K, Suetsugu N, et al. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. Prostate 2003;57:80–92.

6. Garcia-Hernandez ML, Gray A, Hubby B, Kast WM. *In vivo* effects of vaccination with six-transmembrane epithelial antigen of the prostate: a candidate antigen for treating prostate cancer. Cancer Res 2007; 67:1344–51.

7. Ross S, Spencer SD, Holcomb I, et al. Prostate stem cell antigen as therapy target: tissue expression and *in vivo* efficacy of an immunoconjugate. Cancer Res 2002;62: 2546–53.

8. Zhu ZY, Zhong CP, Xu WF, et al. PSMA mimotope isolated from phage displayed peptide library can induce PSMA specific immune response. Cell Res 1999; 9:271–80.

9. Wang Y, Harada M, Yano H, et al. Prostatic acid phosphatase as a target molecule in specific immunotherapy for patients with nonprostate adenocarcinoma. J Immunother 2005;28:535–41.

10. Reiter RE, Gu Z, Watabe T, et al. Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. Proc Natl Acad Sci U S A 1998;95:1735–40.

11. Bahrenberg G, Brauers A, Joost HG, Jakse G. Reduced expression of PSCA, a member of the LY-6 family of cell surface antigens, in bladder, esophagus, and stomach tumors. Biochem Biophys Res Commun 2000;275:783–8.

12. Gu Z, Thomas G, Yamashiro J, et al. Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer. Oncogene 2000;19:1288–96.

13. Yang D, Holt GE, Velders MP, Kwon ED, Kast WM. Murine six-transmembrane epithelial antigen of the prostate, prostate stem cell antigen, and prostate-specific membrane antigen: prostate-specific cell-surface antigens highly expressed in prostate cancer of transgenic adenocarcinoma mouse prostate mice. Cancer Res 2001;61:5857–60.

14. Kaplan-Lefko PJ, Chen TM, Ittmann MM, et al. Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. Prostate 2003;55: 219–37.

15. Wang X, Wang JP, Rao XM, et al. Prime-boost vaccination with plasmid and adenovirus gene vaccines control HER2/neu+ metastatic breast cancer in mice. Breast Cancer Res 2005;7:R580–8.

16. Goldberg SM, Bartido SM, Gardner JP, et al. Comparison of two cancer vaccines targeting tyrosinase: plasmid DNA and recombinant αvirus replicon particles. Clin Cancer Res 2005;11:8114–21.

17. Nelson EL, Prieto D, Alexander TG, et al. Venezuelan equine encephalitis replicon immunization overcomes intrinsic tolerance and elicits effective anti-tumor immunity to the 'self' tumor-associated antigen, neu in a rat mammary tumor model. Breast Cancer Res Treat 2003;82:169–83.

18. Velders MP, McElhiney S, Cassetti MC, et al. Eradication of established tumors by vaccination with Venezuelan equine encephalitis virus replicon particles delivering human papillomavirus 16 E7 RNA. Cancer Res 2001;61:7861–7.

19. Cassetti MC, McElhiney SP, Shahabi V, et al. Antitumor efficacy of Venezuelan equine encephalitis virus replicon particles encoding mutated HPV16 E6 and E7 genes. Vaccine 2004;22:520–7.

20. Davis NL, Caley IJ, Brown KW, et al. Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. J Virol 2000;74:371–8.

21. Pushko P, Parker M, Ludwig GV, et al. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes *in vitro* and immunization against heterologous pathogens *in vivo*. Virology 1997;239:389–401.

22. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A 1995;92:3439–43.

23. Wakabayashi MT, Da Silva DM, Potkul RK, Kast WM. Comparison of human papillomavirus type 16 L1 chimeric virus-like particles versus L1/L2 chimeric virus-like particles in tumor prevention. Intervirology 2002;45:300–7.

24. Koh YT, Higgins SA, Weber JS, Kast WM. Immunological consequences of using three different clinical/laboratory techniques of emulsifying peptide based vaccines in incomplete Freund's adjuvant. J Transl Med 2006;4:42.

25. Garcia-Hernandez ML, Hernandez-Pando R, Gariglio P, Berumen J. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology 2002;105:231–43.

26. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163–75.

27. D'Amaro J, Houbiers JG, Drijfhout JW, et al. A computer program for predicting possible cytotoxic T lymphocyte epitopes based on HLA class I peptide-binding motifs. Hum Immunol 1995;43:13–8.

28. Mackova J, Stasikova J, Kutinova L, et al. Prime/boost immunotherapy of HPV16-induced tumors with E7 protein delivered by Bordetella adenylate cyclase and modified vaccinia virus Ankara. Cancer Immunol Immunother 2006;55:39–46.

29. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005;102:18538–43.

30. Piersma SJ, Jordanova ES, van Poelgeest MI, et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer Res 2007;67:354–61.

31. Lin CT, Chang TC, Shaw SW, et al. Maintenance of CD8 effector T cells by CD4 helper T cells eradicates growing tumors and promotes long-term tumor immunity. Vaccine 2006;24:6199–207.

32. Hokey DA, Larregina AT, Erdos G, Watkins SC, Falo LD, Jr. Tumor cell loaded type-1 polarized dendritic cells induce Th1-mediated tumor immunity. Cancer Res 2005;65:10059–67.

33. Chaput N, Darrasse-Jeze G, Bergot AS, et al. Regulatory T cells prevent CD8 T cell maturation by inhibiting CD4 Th cells at tumor sites. J Immunol 2007; 179:4969–78.

34. Koh YT, Garcia-Hernandez ML, Kast WM. Tumor immune escape mechanisms. In: Teicher BA, editor. Totowa: The Humana Press; 2006. p. 577–602.

35. Schmitz-Winnenthal FH, Escobedo LV, Beckhove P, et al. Specific immune recognition of pancreatic carcinoma by patient-derived CD4 and CD8 T cells and its improvement by interferon-γ. Int J Oncol 2006; 28:1419–28.

36. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC, Dutton RW. Effector cell-derived lymphotoxin α and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res 2004;64:406–14.

37. Mattes J, Hulett M, Xie W, et al. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. J Exp Med 2003; 197:387–93.

38. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol 2004;172:1380–90.

39. Yang SC, Batra RK, Hillinger S, et al. Intrapulmonary administration of CCL21 gene-modified dendritic cells reduces tumor burden in spontaneous murine bronchoalveolar cell carcinoma. Cancer Res 2006;66: 3205–13.

40. Yang G, Addai J, Tian WH, et al. Reduced infiltration of class A scavenger receptor positive antigen-presenting cells is associated with prostate cancer progression. Cancer Res 2004;64:2076–82.

41. Zhang S, Zeng G, Wilkes DS, et al. Dendritic cells transfected with interleukin-12 and pulsed with tumor extract inhibit growth of murine prostatic carcinoma *in vivo*. Prostate 2003;55:292–8.

42. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol 2006;6:836–48.

43. Thomas-Kaskel AK, Zeiser R, Jochim R, et al. Vaccination of advanced prostate cancer patients with PSCA and PSA peptide-loaded dendritic cells induces DTH responses that correlate with superior overall survival. Int J Cancer 2006;119:2428–34.