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Manipulating T-Cell Receptor Signaling in Order to Alter Peripheral
Regulatory T-Cell Activity

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14. ABSTRACT Immunotherapeutic strategies are a novel treatment option for incurable late-stage and metastatic prostate cancer. Several prostate-related antigens have been identified and even used clinically in therapeutic vaccine strategies, but the results have been disappointing. The activity of CD4 ⁺ CD25 ⁺ Foxp3 ⁺ regulatory T cells (Tregs) is a mechanism of peripheral tolerance that regulates immune responses, including those induced by therapeutic vaccination against cancer-associated antigens. PEST-domain enriched tyrosine phosphatase (PEP) is a critical negative regulator of the strength of TCR signaling in the thymus, which in turn has a central role in the development of regulatory T cells. Knockout of PEP leads to an increased number of peripheral Tregs. Thus, it was expected that transgenic mice harboring a gain-of-function variant of the human ortholog of PEP (called LYP) would have fewer peripheral Tregs, but this study has revealed that this is not the case. In response to this unexpected finding, an alternative breeding and research strategy has been developed and the necessary transgenic mice have been procured. In addition, it has been demonstrated that the efficacy of therapeutic immunotherapy is significantly reduced as prostate cancer advances, and that regulatory T cells are likely to have a role in limiting vaccine effectiveness. These data form the basis of a manuscript that has been submitted for publication.					
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INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and is the third most common cause of cancer death in the Western world. Patients with primary prostate cancer may be treated with radiation therapy and prostatectomy. However, if prostate cancer recurs the only treatment option is androgen ablation therapy which is effective for less than eighteen months on average. Androgen-ablation therapy is essentially palliative because most patients go on to develop hormone-refractory prostate cancer and have a median survival time of twelve months.

Immunotherapeutic strategies have been proposed as a novel treatment option for late-stage and metastatic prostate cancer. These strategies entail treatment of the patient with a vaccine directed against a prostate cancer-associated antigen. This will induce an immune response against that antigen that should result in the killing of the prostate cancer cells that express it, thereby eradicating the tumor. We are currently exploring the Venezuelan Equine Encephalitis (VEE) virus replicon system as vector for vaccination against prostate cancer associated antigens. VEE virus and other enveloped, positive-stranded RNA alpha viruses (AV) such as Sindbis and Semliki Forest Virus have been engineered as replication-incompetent viral-delivery vectors or replicons (RP) [1]. The replicon vectors are engineered to express the antigen of interest in lieu of viral structural genes. The replicons retain AV replicase genes, the protein product of which mediates RNA replication and high-level protein (antigen) expression. However, without the viral structural genes no progeny viruses are generated. The replicon-recombinant RNA encoding the antigen of interest must be packaged into VEE replicons (VRP) *in vitro* on provision of the structural RNA in *trans* [2].

STEAP (six membrane epithelial antigen of the prostate) was identified as a possible target antigen for prostate cancer immunotherapy. Under physiological conditions, low levels of STEAP have been detected in plasma membranes of normal prostate tissues but it is highly over-expressed in human prostate cancer tissue. STEAP has also been detected in several colon, bladder, ovarian, and pancreatic cancer cell lines, reinforcing the idea that this gene may be generally upregulated in transformed cells [3]. We identified the murine counterpart of human STEAP, expressed in a prostate tumor cell line (TRAMP-C2) derived from the prostate of mice of the transgenic adenocarcinoma mouse prostate (TRAMP) model [4]. Analysis of the nucleotide and amino acid sequences of mouse STEAP (mSTEAP) showed 80% homology with human STEAP (hSTEAP) and that it also contains six potential membrane-spanning regions. In TRAMP mice, mSTEAP is expressed at high levels in malignant prostate tissue. Recently, hSTEAP peptides were identified as excellent inducers of antigen-specific CTL that were able to recognize and kill STEAP-expressing tumor cells [5] and stimulate specific CD8⁺ T cells from HLA-A*0201 healthy donors [6]. We have demonstrated unequivocally that STEAP is a suitable antigen for prostate cancer immunotherapy when we induced very significant protection against challenge with TRAMP-C2 cells in mice by vaccinating them against STEAP [7]. Based on our extensive experience with cancer vaccines in a variety of cancers, we have deliberately chosen to prime with a DNA based vaccine that encodes the STEAP antigen and boost with an alphavirus-based vaccine that contains the same antigen. Our preliminary data show that a heterologous DNA prime, VRP boost vaccination strategy against STEAP can dramatically improve the survival of TRAMP mice that spontaneously develop prostate cancer. Vaccination against STEAP induced an antigen-specific immune response to the tumor in all mice. Nevertheless, long-term survival in vaccinated mice was only 64% at 420 days post-vaccination (Figure 1).

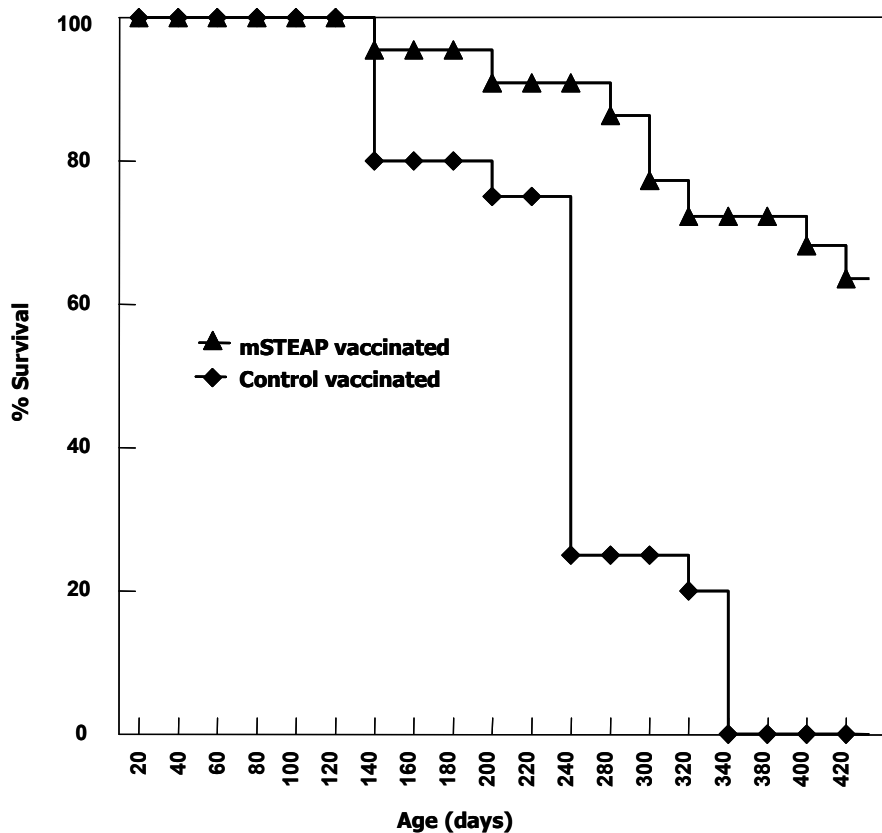


Figure 1: STEAP vaccination of TRAMP mice with prostate tumors.

A group of 22 male TRAMP mice were vaccinated with STEAP DNA at 16 weeks of age, when prostate tumors have developed, and boosted once 2 weeks later with STEAP VRP. A group of 20 control mice were vaccinated with control DNA and boosted with control VRP. No other treatment was given and mice were followed over time for survival. At one year post-vaccination, all control-vaccinated mice had died of aggressive prostate cancer, whereas 64% of the STEAP-vaccinated mice had survived. Survival of vaccinated mice was statistically significantly better than control mice ($p < 0.001$)

We hypothesize that the limited efficacy of our prostate cancer immunotherapy strategy is due to the activity of multiple peripheral mechanisms of tolerance that suppress immune responses to autoantigens in order to prevent autoimmune disease. These peripheral mechanisms of tolerance exist because a large number of potentially self-reactive T cell clones escape negative selection in the thymus and are readily detectable in the periphery. We observed no evidence of autoimmune responses in vaccinated mice, even in organs that normally express STEAP [7].

The activity of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) has been accepted as a mechanism of peripheral tolerance that has a role in regulating autoimmune responses [8]. It has been proposed recently that Tregs also act to inhibit anti-tumor immune responses, both natural and induced by therapeutic vaccination against cancer-associated antigens [9]. We observe that approximately 16% of the $CD4^+$ tumor infiltrating lymphocyte population in the prostate tumors of unvaccinated TRAMP mice are $CD4^+CD25^+Foxp3^+$ regulatory T cells (Figure 2). This raises the possibility that the limited efficacy of our vaccination strategies to date may be due to the presence and activity of these cells within the prostate tumor.

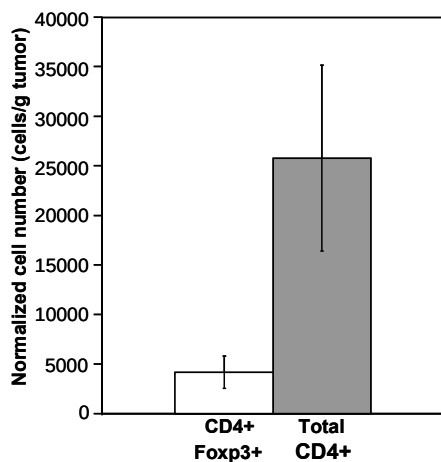


Figure 2: Identification of $CD4^+Foxp3^+$ regulatory T cells within the prostate tumors of TRAMP mice.

A group of 6 male TRAMP mice were sacrificed when they developed palpable prostate tumors. Tumor infiltrating lymphocytes were isolated and analyzed by flow cytometry. Bars represent mean number of cells per gram of tumor tissue analyzed. Approximately 16 percent of the total $CD4^+$ tumor infiltrating lymphocyte population was identified as $CD4^+Foxp3^+$ regulatory T cells.

In recent years it has become increasingly apparent that an immunosuppressive milieu exists within the tumor microenvironment that consists of regulatory cells and secreted factors [10]. Several groups have attempted to enhance the anti-tumor immune response by disrupting the intratumoral suppressive network using various mechanisms. These efforts have met with limited success, most likely because multiple redundant mechanisms of suppression develop within the tumor over the course of tumorigenesis. Furthermore, the methods of abrogating the function of regulatory T cells that are currently available have their limitations. For example, depletion of Tregs using an anti-CD25 monoclonal antibody (PC61) is effective but has several drawbacks. These include the necessity for repeated PC61 treatments for long-term depletion of Tregs and the concomitant depletion of recently-activated effector cells that also express CD25. To investigate the role of Tregs in the anti-tumor immune response, it is important to develop a model system in which Treg activity is modulated prior to tumorigenesis in order to prevent the development of the intratumoral suppressive milieu. The development and characterization of such a system is a primary focus of this proposal.

The strength of TCR signaling in the thymus has a critical role in the development of regulatory T cells. During thymic negative selection, T cells with high affinity TCRs are normally deleted to prevent autoimmunity [11]. Regulatory T cells have TCRs with high affinities that are just below the upper limit to avoid negative selection. Lymphoid tyrosine phosphatase (LYP) and its murine homolog PEST-enriched phosphatase (PEP) are critical negative regulators of TCR signaling [11]. One of our collaborators at USC was the first to identify a single-nucleotide polymorphism in the gene encoding LYP (*PTPN22*) that results in arginine 620 (R620) being changed to tryptophan (W620) in the coding region. The R620W polymorphism produces a gain-of-function version of the phosphatase that predisposes individuals carrying it to autoimmunity [12]. We hypothesize that the increased activity of the gain-of-function LYP/PEP proteins leads to weaker TCR signaling and therefore insufficient generation and/or activity of Tregs. We have obtained PEP knockout (KO) mice [B6.Cg-*Ptpn8*^{tm1Gne}] [13] which have increased numbers of peripheral Tregs (Andrew Chan, personal communication). Mice that over-express LYP-W620 on a PEP KO background (PEP KO(LYP-W620)) in the thymus are in the final stages of development at USC to investigate whether they display reduced numbers of Tregs in the periphery. The availability of PEP KO and PEP gain-of-function mutant mice gives us an opportunity to study the effect of increased and decreased numbers of Tregs on the efficacy of our prostate cancer vaccines.

It has been well-documented that vaccines are less efficacious in aged mice and elderly humans due to progressive involution and loss of function of the thymus in both species [14]. This results in a reduction of the number of IFN-gamma secreting CD8+ T cells [15] and CD4+ T helper cells [16] that are produced in response to vaccination. Androgen ablation has been proposed to enhance the efficacy of prostate cancer immunotherapy [17]. In aged mice, this may occur due to the regeneration of the thymus and the concomitant restoration of immune cells in the periphery [18]. To date, no studies have been published that show a beneficial effect of androgen ablation on vaccine efficacy in aged mice. Our own studies in young mice show no improvement in vaccine efficacy in castrated mice (Figure 3). In addition, we have demonstrated that the protection induced by immunotherapy against prostate cancer in young TRAMP mice is limited (Figure 1). **We hypothesize that the immune response elicited by prostate cancer immunotherapy is suppressed by the action of Tregs that develop with and are released from the thymus.**

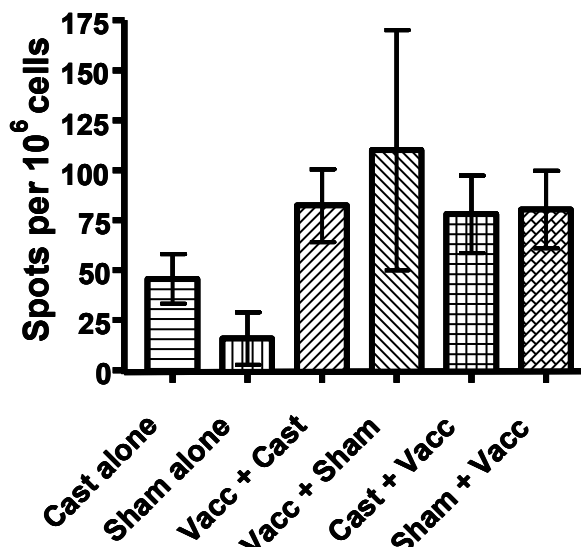


Figure 3: Castration does not improve the efficacy of vaccination.

Groups of 6-8 week old male TRAMP mice were either castrated (cast) and implanted with a flutamide pellet (25 mg/ml) or sham castrated (sham) and given a placebo. Three weeks post-surgery, groups of mice were vaccinated (vacc) with DNA and boosted once 2 weeks later with VRP. Control groups were vaccinated with control DNA and boosted with control VRP. Group sizes were as follows: Cast alone, n = 3; Sham alone, n = 3; Vacc + Cast, n = 3; Vacc + Sham, n = 2; Cast + Vacc, n = 7; Sham + Vacc, n = 5. Mice were sacrificed two weeks after boosting, splenocytes isolated and ELISpot for IFN-gamma performed. Bars represent mean number of IFN-gamma producing cells per 10⁶ cells analyzed. Castration did not enhance the response elicited by vaccination.

After androgen ablation-mediated thymic regeneration, the reconstituted organ functions normally and releases functional CD4 and CD8 T cells in the same manner as the thymus of a young mouse [18]. Though it was not studied specifically, there is no evidence to suggest that Treg development in the regenerated thymus is abnormal. **Therefore, we hypothesize that thymic regeneration in aged mice due to androgen ablation will be insufficient to enhance the immune response to vaccination because that response will be inhibited by Tregs that are produced in and released by the regenerated thymus. We further hypothesize that vaccine efficacy will be improved if thymic Treg production is disrupted during androgen ablation-mediated thymic regeneration.** Most men that have been involved in clinical trials of prostate cancer vaccines to date have already failed to respond to all other treatment options, including androgen ablation therapy. Androgen ablation is predicted to improve thymic function in these men, yet clinical trials involving prostate cancer immunotherapies have been strikingly ineffective. If the latter hypothesis proves to be correct, it may partially explain this as being the result of increased output of Tregs from the thymus which go on to inhibit any anti-tumor immune response induced by immunotherapy. The PEP KO and PEP gain-of-function mutant mice allow us to study the effect of disrupting Treg production during androgen-ablation mediated thymic regeneration on the efficacy of our prostate cancer vaccination strategy.

Hypothesis: The development of regulatory T cells in the thymus can be inhibited by modifying the strength of TCR signaling in the thymus, thereby preventing the suppression of antitumor immune responses elicited by vaccines directed against prostate cancer-associated antigens and improving their efficacy.

Objective: Develop strains of mice which spontaneously develop prostate cancer and which have altered numbers of regulatory T cells in order to assess their effects on the anti-tumor response induced by vaccination against a prostate cancer-associated antigen.

Specific Aims:

- 1.) Generate mice that spontaneously develop prostate cancer and which generate in the thymus and release into the periphery altered numbers of regulatory T cells.
- 2.) Determine whether vaccine efficacy is affected by the number of Tregs present in the periphery of STEAP-vaccinated mice that have normal, increased and decreased numbers of thymic Tregs.
- 3.) Establish whether altered production of thymic Tregs changes the combined efficacy of androgen ablation mediated thymic regeneration and prostate cancer immunotherapy in aged mice.

BODY

TRAINING AIMS

Aim 1: Broaden knowledge of cancer biology, immunology and molecular biology by the regular attendance of classes, workshops, laboratory group discussions, journal clubs and seminars at USC.

Status: Completed in months 1-12. Ongoing for months 13-36.

Aim 2: Formally present data and a detailed progress report to all members of Thesis Committee annually.

Status: Completed in months 1-12. Ongoing for months 13-36.

Aim 3: Improve presentation skills by regularly presenting data to faculty and peers at weekly seminars and laboratory group discussions.

Status: Completed in months 1-12. Ongoing for months 13-36.

Aim 4: Learn practical skills through interaction with faculty, postdoctoral fellows and peers.

Status: Completed in months 1-12. Ongoing for months 13-36.

Aim 5: Enhance knowledge of tumor immunology and develop professional contacts by attending at least one pertinent conference per year.

Status: Completed in months 1-12 by attending the International Society for Biological Therapy of Cancer Annual Meeting 2008 in San Diego, California. The awardee presented a poster based on data obtained from the execution of the experiments proposed as part of this award. Ongoing for months 13-36.

Aim 6: Analyze data and write up results for publication in peer-reviewed journals.

Status: Completed in months 1-12. Since the award was made, the awardee has published two manuscripts as the second author [19, 20] and has submitted another manuscript as the first author to the journal *Vaccine* (see below for details). Ongoing for months 13-36.

Aim 7: Formally defend thesis work.

Status: Cannot be completed until month 36.

RESEARCH AIMS

Specific Aim 1

Predicted deliverable: Generation of mice that spontaneously develop prostate cancer and which generate in the thymus and release into the periphery altered numbers of regulatory T cells.

Task 1.1: Cross TRAMP mice with PEP KO mice.

Task 1.2: Cross TRAMP mice with PEP KO(LYP-W620) mice.

Task 1.3: Verify by flow cytometry that the desired Treg phenotype has been obtained in each of the mouse lines generated in aim 1, tasks 1 and 2.

Progress: To begin work on Specific Aim 1, we first obtained PEP KO(LYP-W620) mice from our collaborator, Dr. Nunzio Bottini. As discussed above, these mice harbor a gain-of function variant of LYP, the human ortholog of the PEP protein. Given that PEP knockout mice have increased numbers of peripheral Treg, we predicted that PEP KO(LYP-W620) mice would have decreased numbers of peripheral Treg. Task 1.2 was to cross PEP KO(LYP-W620) mice with TRAMP mice, thus generating an animal model that should spontaneously develop prostate cancer and have reduced numbers of peripheral Treg. Prior to beginning this time-consuming process, it was deemed wise to check whether PEP KO(LYP-W620) mice did indeed have reduced numbers of Treg as was predicted. To determine this, we analyzed the numbers of Treg in the thymuses

and spleens of PEP KO(LYP-W620) mice and wild type littermate controls by flow cytometry. There was a difference in the number of thymic $CD4^{+}FoxP3^{-}$ T cells but not $CD4^{+}FoxP3^{+}$ Treg in PEP KO(LYP-W620) mice. There was no difference in the numbers of peripheral $CD4^{+}FoxP3^{-}$ T cells or $CD4^{+}FoxP3^{+}$ Treg in the spleens of PEP KO(LYP-W620) mice compared to those of their wild type littermates (Figure 4).

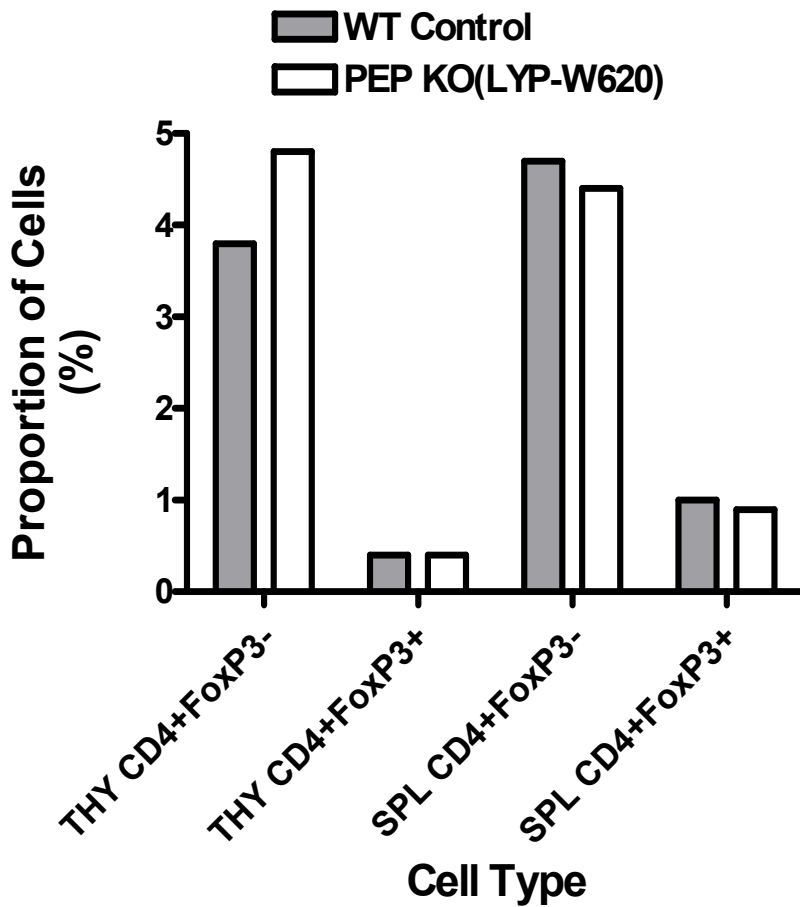


Figure 4: There is no difference in the numbers of peripheral Treg in PEP KO(LYP-W620) mice compared to wild type littermate controls. Splenocytes and thymocytes isolated from four week old PEP KO(LYP-W620) mice and wild type littermate controls were washed and stained with anti-mouse $CD8$ -PE/Cy5 and anti-mouse $CD4$ -PE/Cy7. Cells were fixed and permeabilized overnight, then stained with anti-mouse FOXP3-PE and analysed by flow cytometry. Events were collected gated on either live $CD4^{+}$ cells (spleens) or single-positive $CD4^{+}CD8^{-}$ cells (thymuses) and then further gated on the $CD4^{+}FOXP3^{-}$ fraction and the $CD4^{+}FOXP3^{+}$ fraction. THY = thymus. SPL = spleen.

Though this finding represented a significant hurdle in the progress of this project, an alternative research strategy has been devised and started that is as elegant as it is actionable. This alternative research strategy is based upon a transgenic mouse model in which the diphtheria toxin receptor (DTR) is expressed under the control of the promoter of the Treg-specific transcription factor FoxP3. This animal model is known as the DEREK mouse [21]. In these animals, $FoxP3^{+}$ Treg develop normally, but they all express the DTR at their cell surface. The upshot of this is that the researcher can selectively deplete $FoxP3^{+}$ Treg at their discretion by administering a low dose of the diphtheria toxin to the mice.

Though it was not part of the original statement of work, the use of the DEREK mouse has a number of advantages over the use of the PEP KO(LYP-W620) mouse.

1. The model is fully developed and the ability to selectively deplete Treg has already been shown in published literature.
2. The model is on the C57BL/6 background, like the TRAMP mouse. This will make breeding and immunological analyses much easier.
3. The model allows the selective depletion of Tregs at any time during the development of prostate cancer. If the PEP KO(LYP-W620) mouse had had the desired phenotype, it would have had fewer Treg cells throughout life, possibly leading to unintended consequences (such as long-term autoimmune disease) and potentially confounding the results of this study. Using the DEREK model, Treg can be

specifically depleted immediately prior to and during vaccination. This scenario mimics the treatment protocol envisioned for future human therapy where Treg would be depleted by another means from the patient's system prior to and during therapeutic prostate cancer immunotherapy.

4. There is no manipulation of TCR signaling in the DEREg mouse, and thus no possibility of inadvertently affecting effector T cells that do not express FoxP3.

Thus, the alternative research strategy is now to cross DEREg mice to TRAMP mice, generating a line of animals that spontaneously develop prostate cancer and that can have their FoxP3⁺ Treg population depleted at any time by the administration of diphtheria toxin. The development of the DEREg X TRAMP mouse line will replace Task 1.2. The DEREg X TRAMP mouse will replace the PEP KO(LYP-W620) X TRAMP mouse in all relevant subsequent experiments. An MTA to obtain DEREg mice was recently completed and IACUC approval has been obtained to begin the process of breeding DEREg X TRAMP mice.

Specific Aim 2

Predicted outcome: Determination of whether vaccine efficacy is affected by the number of Tregs present in the periphery of STEAP-vaccinated mice that have normal, increased and decreased numbers of thymic Tregs.

Task 2.1: Vaccinate groups of ten TRAMP mice and groups of ten of each of the mice generated in aim 1, tasks 1 and 2 using STEAP DNA via gene gun. Boost all mice two weeks later using VRPs encoding STEAP. As control groups, mice will be vaccinated with pcDNA3 and VRPs encoding GFP.

Task 2.2: Monitor and compare survival of groups of vaccinated mice over time. The survival endpoint is defined as the development of a palpable prostate tumor.

Task 2.3: Vaccinate groups of five mice as described in aim 2.1. Two weeks after boosting, sacrifice mice and harvest the prostate tumor and spleen.

Task 2.4: Determine and compare the number of Tregs present in the tumors and spleens of vaccinated and control vaccinated mice by flow cytometry.

Task 2.5: Vaccinate groups of five mice as described in aim 2.1. Two weeks after boosting, sacrifice mice and harvest the prostate tumor and lymphoid organs.

Task 2.6: Analyze frozen tumor sections by immunofluorescence for the presence of infiltration with CD4, CD25, Foxp3, GITR, CD8, Granzyme b, CD11b, and CD11c-expressing cells in order to quantify intratumoral T helper cells, IL-2 receptor, T regulatory cells, cytotoxic T lymphocytes, lytic machinery molecules, macrophages and dendritic cells, respectively. Compare tumor infiltration between groups of mice.

Task 2.7: Analyze homogenates of frozen tumor sections for the presence of IL-2, IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, IFN-gamma and TNF-alpha using multiplex cytokine analysis and TGF-beta by ELISA to determine whether vaccination alters the intratumoral expression of any of these cytokines. Compare expression between groups of mice.

Task 2.8: Analyze the lymphoid organs obtained in aim 2, task 5 for T cell responses against an H-2Db restricted mSTEAP peptide (mSTEAP₃₂₆₋₃₃₆, DVSKINRTEM) by chromium release assay and ELISPOT for IFN-gamma. Compare responses between groups of mice.

Task 2.9: Vaccinate groups of five mice as described in aim 2.1. Two weeks after boosting, sacrifice mice and harvest the lymphoid organs.

Task 2.10: Perform MACS separation of lymphoid cells to yield CD4⁺, CD4⁺CD25⁻ and CD4⁺CD25⁺ populations.

Task 2.11: Determine and compare the suppressive capacity of each separated population from each group of mice by Treg suppression assay.

Task 2.12: Determine whether any autoimmunity develops in response to vaccination by measuring auto-antibodies (rheumatoid factor, IgM and anti-ssDNA antibodies, positive control will be serum and tissues from autoimmune MRL/lpr mice) and cell infiltrates and collagen depositions in tissues of all organs including the prostate itself. Note that these organs and tissues will be obtained from the same mice vaccinated in aim 2, task 5.

Progress: Making headway on Specific Aim 2 depends upon the completion of Specific Aim 1. As discussed, technical problems have delayed progress on Specific Aim 1 while an alternative research strategy was developed. Thus, Specific Aim 2 has not yet started. However, the awardee has been conducting experiments that are directly related to this topic, the results of which will have a very significant impact upon how Specific Aim 2 is carried out as described below. These studies have already led to the submission of a manuscript to the journal *Vaccine*. The awardee is the first author on this paper.

The manuscript that has been submitted to *Vaccine*, titled “Prostate cancer immunotherapy yields superior long-term survival in TRAMP mice when administered at an early stage of carcinogenesis prior to the establishment of tumor-associated immunosuppression at later stages” and is of particular relevance to Specific Aim 2. The submitted manuscript including all technical details is included as an appendix to this document. The award number of this grant was stated in the Acknowledgements section of this manuscript.

The major conclusions of this study were twofold. First, therapeutic vaccination using prostate tumor-associated antigens is more efficacious if administered at an earlier stage of carcinogenesis in TRAMP mice (PIN lesions at 8 weeks) than if it is given at a later stage of disease (invasive carcinoma and/or neuroendocrine tumors at 16 weeks). These data are shown in Figure 1 of the manuscript attached as the Appendix. Second, multiple immunosuppressive mechanisms become established in the TRAMP mouse as prostate cancer progresses. Significantly, increasing numbers of CD4⁺CD25⁺FoxP3⁺ Treg were shown to be present in the spleens and prostate tumors of TRAMP mice over the course of prostate cancer progression (Appendix, Figure 2). Overall, these findings suggest that therapeutic vaccination at an early stage of prostate cancer development is more successful because it avoids the tumor-associated immunosuppressive network (including Treg) that develops over the course of disease progression.

The implications of these findings for the completion of Specific Aim 2 are as follows:

1. In the approved Statement of Work, the timepoint at which TRAMP mice and their derivatives are vaccinated was not stated. It is now clear that the timing of therapeutic vaccine administration is vitally important to its efficacy. These studies demonstrate that the efficacy therapeutic prostate cancer vaccination significantly decreases in TRAMP mice between 8 and 16 weeks of age, and that Treg may become involved around 16 weeks of age. Therefore it is essential that mice are vaccinated at 16 weeks of age during the execution of Specific Aim 2 because prior to this time point Treg are unlikely to be involved yet. Thus, their manipulation will most likely make no difference to the already excellent efficacy of therapeutic vaccination at 8 weeks of age.
2. Though there is an association between increased Treg numbers and decreased therapeutic vaccine efficacy, this study does not yet confirm a causative link. The experiments that will be carried out as part of this award are precisely designed to investigate whether this is the case. Thus, the data outlined in the submitted manuscript underline the importance of investigating the impact of Treg on therapeutic prostate cancer immunotherapy and strongly justify continuation of the experiments outlined in this award.

Specific Aim 3

Predicted outcome: Determination of whether altered production of thymic Tregs changes the combined efficacy of androgen ablation mediated thymic regeneration and prostate cancer immunotherapy in aged mice.

Task 3.1: Castrate groups of ten aged (18 month old) male PEP KO mice, PEP KO(LYP-W620) mice and C57BL/6 mice. One week post-castration, challenge the mice subcutaneously with 5×10^4 TRAMP-C2 prostate cancer cells. Two weeks post-castration vaccinate the mice against STEAP and boost two weeks later as described in aim 2, task 1. Negative controls are sham-castrated mice of all three genotypes, and mock vaccinated mice (all genotypes, castrated and sham castrated).

Task 3.2: Monitor and compare survival of groups of vaccinated mice over time. The survival endpoint is defined as the development of a tumor with a volume exceeding 1000 mm³.

Task 3.3: Treat groups of five mice as in aim 3.1. Two weeks after boosting, sacrifice mice and harvest spleens.

Task 3.4: Verify the thymic output of mice treated as in aim 3.1 by flow cytometry. Splenocytes will be analyzed for the presence of CD8⁺, CD4⁺CD25⁻Foxp3⁻ and CD4⁺CD25⁺Foxp3⁺ cells.

Task 3.5: Correlate changes in peripheral T cell populations in each group of mice with improved/worsened survival of the corresponding group in aim 3.1.

Task 3.6: Treat mice as in aim 3.3. Two weeks after boosting, sacrifice mice and harvest spleens.

Task 3.7: Determine the suppressive capacities of Tregs isolated from each group of mice as described in aim 2, tasks 10 and 11.

Task 3.8: Correlate changes in the suppressive capacity of Tregs from each group of mice with improved/worsened survival of the corresponding group in aim 3.1.

Task 3.9: Treat mice as in aim 3.3. Two weeks after boosting, sacrifice mice and harvest spleens.

Task 3.10: Determine the strength of the CTL responses elicited by vaccination in each group of mice as described in aim 2, tasks 6, 7 and 8.

Task 3.11: Correlate changes in the strength of the CTL response of each group of mice with improved/worsened survival of the corresponding group in aim 3.1.

Progress: Work on Specific Aim 3 is not due to start yet. Nevertheless, the awardee has been involved in research directly pertinent to this Specific Aim. This work has led to the publication of a manuscript titled “Androgen Ablation Augments Prostate Cancer Vaccine Immunogenicity Only When Applied After Immunization” in the journal *Prostate* [19]. The awardee is the second author on this paper. It has long been hypothesized that androgen ablation can directly affect the immunogenicity of therapeutic cancer vaccines, and has been proposed as a possible means to augment the efficacy of prostate cancer immunotherapy. The overall conclusion of this manuscript is that castration of mice only affected vaccine efficacy if it took place after the vaccine was administered. Though this does not change how Specific Aim 3 will be approached, it is relevant because we will use castration as a means to increase thymic output of T cells (including Treg) in aged mice prior to vaccinating them. Given that we now know that androgen ablation will not affect the immunogenicity of the vaccine in this case, we can remove consideration of this potential confounding factor from the analysis of the data obtained in the execution of Specific Aim 3.

Revised Timeline and Milestones:

Months 13-16: Complete revised Specific Aim 1. Breed necessary numbers of mice for other experiments and maintain mouse colonies throughout duration of project.

Months 16-34: Complete Specific Aim 2.

Months 16-34: Complete Specific Aim 3.

Months 35-36: Analyze and write up results for publication in peer-reviewed journals.

Month 36: Formally defend thesis.

Training Aims 1-5 will be performed throughout the duration of the proposed effort.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that PEP KO(LYP-W620) transgenic mice do not have lower numbers of peripheral regulatory T cells as they were predicted to; developed alternative research strategy to generate a mouse line that spontaneously develops prostate cancer and can have peripheral Treg depleted by crossing the TRAMP mouse to the DEREK mouse.
2. Demonstrated that therapeutic prostate cancer immunotherapy is more effective when administered at the earliest stage of carcinogenesis (the development of PIN lesions) and that Treg are likely to be involved in the relatively poor efficacy of therapeutic vaccination at later stages of disease.

REPORTABLE OUTCOMES

1. Manuscript published in the journal *Prostate*[19].
2. Manuscript submitted for publication to the journal *Vaccine* (see Appendix).
3. Poster presentation given at the 23rd Annual Meeting of the International Society for the Biological Therapy of Cancer, San Diego, California.

CONCLUSION

Though progress has been slowed somewhat by the unexpected finding that PEP KO(LYP-W620) mice do not have lower numbers of peripheral Treg, useful and important data have nevertheless been obtained. An alternative breeding and research strategy has been developed and the necessary transgenic mice have been procured. In addition, it has been demonstrated that the efficacy of therapeutic immunotherapy is significantly reduced as prostate cancer advances, and that regulatory T cells are likely to have a role in limiting vaccine effectiveness. Overall, the progress that has been made over the course of the past twelve months demonstrates the vital importance of this project and provides an excellent basis for its completion over the next two years.

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APPENDIX

Please see below for the original manuscript titled “Prostate cancer immunotherapy yields superior long-term survival in TRAMP mice when administered at an early stage of carcinogenesis prior to the establishment of tumor-associated immunosuppression at later stages” that has been submitted for publication in the peer-reviewed journal *Vaccine*.

Prostate cancer immunotherapy yields superior long-term survival in TRAMP mice when administered at an early stage of carcinogenesis prior to the establishment of tumor-associated immunosuppression at later stages

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Abstract

Prostate cancer immunotherapy clinical trials have been performed, but often in immunocompromised patients with limited clinical success. The study aim was to determine whether the stage of prostate cancer development at which immunization occurs affects vaccine efficacy, and if so which tumor-associated immunosuppressive mechanisms may be involved at later stages. Therapeutic vaccination of TRAMP mice with only precancerous PIN lesions conferred superior protection than immunization after development of invasive carcinoma. The presence of Treg, upregulation of tumor indoleamine-2,3-dioxygenase and TGF β and an immunosuppressive intratumoral cytokine milieu were identified in more advanced prostate cancer. These results indicate that prostate cancer immunotherapy trials will be more successful if conducted in patients with less advanced disease.

Key Words: Prostate cancer; immunotherapy; regulatory T cells

Abbreviated Title: Suppression of prostate cancer immunotherapy efficacy during disease progression

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy in the United States apart from skin cancers, and is the second-leading cause of cancer-related death in American men [22]. There are several therapeutic options available for prostate cancer that is diagnosed early such as prostatectomy, cryotherapy and radiotherapy, but these can have serious side effects including incontinence and impotence [23-25].

There have been myriad attempts to use immunotherapeutic approaches to treat prostate cancer. The overall aim of prostate cancer immunotherapy is to induce a specific immune response against one or more prostate tumor-associated antigens (TAA) in an effort to precisely target and eradicate cancer cells expressing those antigens. Several prostate cancer TAA, including Prostate Specific Antigen (PSA) [26], Six-Transmembrane Epithelial Antigen of the Prostate (STEAP) [7], Prostate Stem Cell Antigen (PSCA) [27], Prostate Specific Membrane Antigen (PSMA) [28] and Prostatic Acid Phosphatase (PAP) [29] have been identified. Though all have been used in clinical trials of prostate cancer immunotherapies, none have been approved by the FDA for use to treat patients [30]. The overwhelming majority of these trials have been conducted in patients with advanced disease. From an ethical standpoint these patients form an ideal group in which to test experimental therapies because they have failed all other therapeutic options and usually have a life expectancy of less than one year. However, these patients are frequently severely immunocompromised due to their advanced cancer and are thus very poor candidates for immunotherapy trials. While generally unsuccessful, there is evidence that the vaccines investigated thus far can elicit protective, specific immune responses against prostate TAAs, but only in the minority of men who are still immunocompetent. This led us to hypothesize that the failure of therapeutic prostate cancer vaccines to date is not necessarily due to a lack of efficacy on the part of those vaccines *per se*, but instead represents either a general inability of the patients' immune systems to respond effectively, or an active inhibition of anti-tumor immunity within the microenvironments of tumors in these patients.

In recent years it has become apparent that there are multiple immunosuppressive mechanisms that can be subverted by tumors in order to blunt patient immune responses mounted against them. The tumor immunology field is currently focused on the activities and functional significance of tumor-associated suppressive immune cells such as regulatory T cells (Treg) [31] and myeloid-derived suppressor cells (MDSC) [32]. In addition, suppression of T cell activity can be mediated by tryptophan depletion due to increased expression of indoleamine-2,3-dioxygenase within the tumor [33], or by increased arginine metabolism due to upregulation of arginase and/or inducible nitrous oxide synthase (iNOS) expression within the tumor [34]. Finally, increased expression of suppressive cytokines such as interleukin(IL)-10 or tumor growth factor β (TGF β) can result in an immunosuppressive tumor microenvironment [35, 36]. It is possible that these tumor-associated immunosuppressive mechanisms become dominant at later stages of prostate cancer, resulting in the relatively poor efficacy of therapeutic vaccines that are administered at those stages. Therefore, we hypothesized that the efficacy of therapeutic vaccines will be improved if they are administered at the earliest stages of disease, thereby circumventing these problems [30].

Prostate cancer is routinely screened for and is frequently diagnosed early in the course of the disease when the patient has only small, non-invasive tumors or even precancerous prostatic intraepithelial neoplastic (PIN) lesions. In these cases, the (pre)cancerous prostate lesions generally cause symptoms in the patients that are less severe than the serious side-effects of the standard prostate cancer treatments. Therefore, the standard of care in these patients is a period of active surveillance that can last for years before the prostate tumor begins to pose a more serious risk to the health of the patient. Prostate cancer immunotherapy may induce a specific immune response that can potentially mediate long-term protection against tumor outgrowth. This may be achieved with significantly fewer side-effects than can occur with conventional treatments for early prostate cancer. The combination of early detection methods, availability of vaccines and a patient population that has received no other clinical interventions means that prostate cancer represents an ideal proving ground for the application of therapeutic cancer vaccines at early stages of disease.

In our previous studies, we have investigated the efficacy of therapeutic cancer vaccines in the TRAMP (transgenic adenocarcinoma mouse prostate) model of spontaneous prostate cancer. Prostate cancer in TRAMP mice closely mimics the course of the human disease, from the development of precancerous PIN lesions to invasive prostate adenocarcinoma or neuroendocrine tumors and then to metastatic disease [37]. Therefore this model is an ideal system in which to investigate the effects of therapeutic vaccination at different stages of prostate cancer progression. We have made extensive use of a heterologous prime-boost vaccination strategy in which mice are immunized with DNA encoding a particular TAA and boosted using Venezuelan Equine Encephalitis virus replicon particles encoding the same antigen. The prostate TAAs STEAP and PSCA are highly upregulated by prostate cancer cells in humans and mice [4, 38]. Our previous studies indicated that vaccination against these antigens using our protocol elicits strong protective immune responses in TRAMP mice and in C57BL/6 mice that have been challenged with TRAMP-C2 prostate cancer cells [7, 20]. In this study, we explored whether superior efficacy of therapeutic vaccination in TRAMP mice can be elicited if it is administered to animals that have only developed PIN lesions compared to when vaccination of these mice occurs after invasive carcinomas or neuroendocrine tumors have developed, and if so what immunosuppressive mechanisms may be involved in hampering therapeutic vaccination effectiveness at the later stages of disease.

MATERIALS AND METHOD

Mice

C57BL/6 and C3H mice were obtained from Taconic farms (Germantown, NY). TRAMP mice [37] on the C57BL6 background were bred at the University of Southern California. Research was conducted in compliance with the institutional animal use guidelines. TRAMP mice were categorized into groups based on their ages: Young, ≤ 8 weeks old; Middle-aged, 16-20 weeks old and Old, ≥ 24 weeks old. These ages reflect different stages of prostate cancer development in the TRAMP model, being prostate intraepithelial neoplasia (PIN), development of prostate adenocarcinoma or neuroendocrine tumors and presence of high-grade or metastatic tumors, respectively.

Immunization

Male TRAMP mice (8 or 16 weeks old) were anesthetized ip with 2.4 mg ketamine (Phoenix Pharmaceutical Inc, St Joseph, MO) and 480 μ g xylazine (Phoenix). DNA-gold particles were delivered to a shaved area on the abdomen using a helium-driven gene gun (BioRad) with a discharge pressure of 400 psi. Each mouse received 2 μ g of either murine PSCA or murine STEAP 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 and mSTEAP, respectively. TRAMP mice received an additional dose of either 10^6 IU mPSCA-VRP or mSTEAP-VRP, as appropriate, at day 60. As control groups, TRAMP mice were vaccinated with empty pcDNA3 plasmid and boosted with 10^6 IU GFP-VRP. Survival was followed until the defined endpoint which was the development of a palpable tumor, in accordance with our IACUC guidelines.

Isolation of tumor infiltrating lymphocytes (TIL) and flow cytometric analysis

TIL were isolated from individual prostate tumors as previously described [39]. TIL were analyzed by flow cytometry and the total number of cells per gram of tumor calculated as previously described [7].

Measurement of suppression of T cell proliferation by regulatory T cells

A single cell suspension of lymphocytes isolated from the tumor-draining lymph nodes of TRAMP mice were purified into CD4⁺CD25⁻ (responder) and CD4⁺CD25⁺ (suppressor) populations by magnetic separation using a CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, except that only half the recommended amount of anti-CD25-PE was used. 5x10⁴ CD4⁺CD25⁺ T cells were cocultured for 72 hours with 5x10⁴ allogenic T cell-depleted splenocytes from C3H mice in complete RPMI supplemented with 1 ug/ml activating anti-mouse CD3 antibody, either alone or with autologous CD4⁺CD25⁻ responder T cells in 1:2, 1:4, 1:8 and 1:16 ratios. As positive controls for maximal proliferation, 5x10⁴ CD4⁺CD25⁻ responder T cells were cocultured with 5x10⁴ allogenic T cell-depleted splenocytes from C3H mice in complete RPMI supplemented with 1 ug/ml activating anti-mouse CD3 antibody. ³H-thymidine (1 µg/well) was added in the last 8 hours of culture. Responder cell proliferation was measured by ³H-thymidine incorporation using a TopCount NXT microplate scintillation counter (Perkin Elmer, Shelton, CT). The relative proliferation index of responder cells for each mouse at each Treg:Tresponder ratio was calculated by dividing the mean Tresponder proliferation at each ratio by the maximal proliferation (Tresponders cultured in the absence of Treg) of Tresponders in that animal.

Quantification of intratumoral cytokine levels

Prostate tumors were harvested from TRAMP mice, weighed and homogenized at 4°C in 10 µl/mg sterile PBS containing 1x Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL) using a PolyTron PT2100 homogenizer (Kinematica AG, Switzerland). Cytokine levels were quantified with a custom 32-plex Milliplex MAP mouse cytokine immunoassay (Millipore, Billerica MA) using the Bio-Plex multiplex system (Bio-Rad, Hercules, CA) following the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction

Prostate tumors were harvested from TRAMP mice, weighed and immediately fixed in RNAlater. After 24 hours, fixed tumors were homogenized at 4°C in 10 µl/mg buffer RLT with β-mercaptoethanol using a

PolyTron PT2100 homogenizer (Kinematica AG, Switzerland). Total RNA was isolated using a QIAGEN RNeasy kit according to the manufacturer's instructions. DNase-treated RNA was reverse transcribed with oligo (dT) and SuperScript III (Invitrogen Life Technologies). Quantitative PCR was performed using Universal PCR Master Mix containing SYBR, following the Applied Biosystems protocol. Primers for GAPDH, TGF β , indoleamine-2,3-dioxygenase and Foxp3 were obtained (USC DNA Core Facility, Los Angeles, CA). 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. The mean relative expression of each mRNA in the tumors of young mice was arbitrarily set as 1.

Statistical analysis

Quantitative real-time PCR, flow cytometry, suppression of T cell proliferation and multiplex immunoassay were analyzed by a two tailed, paired Student's t test. Survival rates were analyzed by the log rank test for survival. Delta survival was calculated by subtracting the cumulative percentage survival of the relevant negative control mouse group from that of the age-matched vaccinated mouse group at each time point at which at least one animal died in either group. Changes in delta survival over time were analyzed by linear regression. Slopes of linear regressions were compared by F-test.

RESULTS

To evaluate whether the stage of prostate cancer progression at which therapeutic vaccination is applied affects the efficacy of the vaccine, we compared the long-term survival rates of TRAMP mice that were vaccinated at two distinct stages of prostate cancer carcinogenesis. TRAMP mice were vaccinated at 8 weeks of age, at which point they have developed precancerous prostate intraepithelial neoplastic (PIN) lesions, or at 16 weeks of age when prostate neuroendocrine carcinomas or adenocarcinomas have developed. The groups of mice were vaccinated using an identical heterologous DNA prime/VRP boost scheme, though the antigens targeted were different (mPSCA at 8 weeks and mSTEAP at 16 weeks). However, we have previously demonstrated that vaccination against both mPSCA [20] and mSTEAP [7] using this immunization scheme induce strong and comparable immune responses in mice. Survival in mice vaccinated at 8 weeks and at 16 weeks was statistically significantly improved compared to age matched controls ($p < 0.0001$ and $p = 0.0001$, respectively), indicating that vaccination at both time points yielded excellent protection from prostate cancer development (Figure 1a). In the case of TRAMP mice vaccinated at 8 weeks, there was a dramatic difference in tumor burden between mPSCA-vaccinated mice and negative controls euthanized at 340 days (Figure 1b).

The survival of mice vaccinated at 8 weeks and at 16 weeks could not be directly compared because the mice vaccinated at 16 weeks were castrated prior to immunization. The effect of androgen ablation on long-term TRAMP mouse survival is seen in the statistically significant difference in survival between the non-castrated 8 week negative control group and the castrated 16 week negative control group ($p = 0.0074$). Both of these groups were immunized with an empty DNA vector and boosted with a GFP-VRP, so the difference in survival between the two groups is due to castration in the 16 week group. We have demonstrated in a previous study that castration only affects the results of prostate cancer immunotherapy when it is carried out three weeks after vaccination [19]. Therefore, the effect of castration on survival in TRAMP mice that were vaccinated at 16 weeks is most likely due to the retardation of androgen-dependent prostate tumor growth rather than any immunological effect. To remove this confounding factor from our analysis, we calculated the difference between the cumulative survival of the vaccinated groups from the cumulative survival of their age- and

castration-matched control groups (which we termed “delta survival”) at each time point. Scatter plots of delta survival versus survival time were plotted, and linear regression analysis performed (Figure 1c). There was a very strong correlation between delta survival and survival time in groups of mice vaccinated at 8 weeks ($R^2 = 0.856$) and a weaker correlation at 16 weeks ($R^2 = 0.695$). This indicated that, as expected, the difference in survival probability between vaccinated mice and negative controls steadily increases over time. To determine whether the improvement in survival in mice vaccinated at 8 weeks was better than that of mice vaccinated at 16 weeks, the slopes of the regressions were compared via F-test. The linear regression slope of the 8 week vaccination groups was statistically significantly higher than that of the 16 week groups ($F = 5.398$, $p = 0.0346$). This demonstrates that the survival of the group vaccinated at 8 weeks improved statistically significantly more than did the survival of the group vaccinated at 16 weeks when each was compared to their age- and castration-matched control groups.

In an effort to understand the improvement of survival in mice vaccinated earlier in carcinogenesis, we hypothesized that one or more prostate tumor-mediated immunosuppressive mechanisms become established at later stages of carcinogenesis which are responsible for the reduction in therapeutic vaccine efficacy at later stages of disease. Several immunosuppressive mechanisms have been identified as having a possible role in prostate cancer. Given the significantly worse response to therapeutic vaccination at later stages of prostate cancer progression, we investigated whether any of these mechanisms either become active or are more prevalent at later stages of disease.

We first assessed whether regulatory T cells (Treg) are more prevalent in either the periphery or prostate tumors of TRAMP mice with more advanced disease. Splenocytes and tumor infiltrating lymphocytes (TIL) were isolated from young (≤ 8 weeks old, $n = 3$), middle-aged (16-20 weeks old, $n = 4$) and old (≥ 24 weeks old, $n = 3$) TRAMP mice and analyzed by flow cytometry. The mean percentage of splenocytes that were $CD3^+CD4^+FOXP3^-$ non-regulatory T cells in young TRAMP mice ($9.57 \pm 0.28\%$) was statistically significantly higher than in middle-aged ($7.65 \pm 0.28\%$, $p < 0.01$) and in old ($5.47 \pm 0.26\%$, $p < 0.01$) TRAMP mice (Figure

2a). In addition, the mean percentage of splenic CD3⁺CD4⁺FOXP3⁻ T cells was statistically significantly decreased in old compared to middle-aged TRAMP mice ($p < 0.01$) (Figure 2a). The mean percentage of splenocytes that were CD3⁺CD4⁺FOXP3⁺ Tregs also decreased with age, but not significantly. As a result, the ratio of mean CD3⁺CD4⁺FOXP3⁺ splenocyte percentage to mean CD3⁺CD4⁺ splenocyte percentage in TRAMP mice statistically significantly increased with age ($p = 0.021$, ANOVA), indicating an increased accumulation of Treg in the spleens of TRAMP mice the course of prostate cancer progression. Flow cytometric analysis of tumor infiltrating lymphocytes revealed a statistically significant increase in the mean number of tumor infiltrating CD3⁺CD4⁺FOXP3⁻ cells in old (35844 cells/g tumor) compared to middle-aged (7182 cells/g tumor) TRAMP mice (Figure 2b, $p = 0.001$). In addition, the mean number of tumor infiltrating CD3⁺CD4⁺FOXP3⁺ Treg cells in old (5962 cells/g tumor) increased compared to middle-aged (1008 cells/g tumor) TRAMP mice (Figure 2b). In contrast to the situation in the periphery, the proportion of tumor infiltrating CD3⁺CD4⁺FOXP3⁻ cells that were CD3⁺CD4⁺FOXP3⁺ Treg cells remained constant (15.6% versus 15.4% in middle-aged and old mice, respectively). However, the absolute number of Treg per gram of tumor increased over the course of disease progression. No data were available from young TRAMP mice because insufficient TIL for flow cytometry could be isolated from the prostates of these animals.

To assess the suppressive capacity of Tregs in early prostate tumor development, lymphocytes were isolated from the prostate tumor draining lymph nodes of young TRAMP mice and divided into CD4⁺CD25⁻ effector and CD4⁺CD25⁺ regulatory T cell populations. The proliferation of the CD4⁺CD25⁻ effector steadily decreased when cocultured with increasing numbers of CD4⁺CD25⁺ regulatory T cells, indicating that this population is functionally suppressive (Figure 3). Taken together, these data indicate an increase in functionally suppressive Treg during prostate cancer progression, which may interfere with the efficacy of therapeutic vaccination.

To determine whether the cytokine profile of the tumor microenvironment becomes immunosuppressive or inhibitory over the course of prostate cancer development, expression of a panel of cytokines and chemokines was measured by multiplex immunoassay (Figure 4). The expression of several cytokines and chemokines,

including IL-1a, IL-2, IL-4, IL-5, IL-9, IL-10, IL-12(p40 and p70), IL-13, IL-15, IFN γ , G-CSF, GM-CSF, LIX, MIP-1a, MIP-2 and TNF α was reduced in the prostate tumors of middle-aged TRAMP mice compared to the prostates of young TRAMP mice. Conversely, there were increases in the expression of M-CSF, MIG, RANTES and VEGF. In the case of mean MIP-1a expression, the reduction was statistically significant ($p = 0.023$). In contrast, mean KC and MCP1 expression was statistically significantly increased in middle aged mice compared to young mice ($p = 0.027$ and $p = 0.028$, respectively). Overall, there was a trend towards a reduction in both Th1 and Th2 function in middle-aged mice compared to young mice, while expression of angiogenic factors (KC and RANTES) increased with age.

Expression of several immunosuppressive factors, including TGF β and indoleamine 2,3-dioxygenase has been shown to be increased in multiple tumor types. To assess whether their expression increases with prostate tumor progression, we quantified their expression in the prostate tumors of TRAMP mice by quantitative real-time PCR. Relative expression of both TGF β and indoleamine 2,3-dioxygenase mRNA normalized to GAPDH were increased in middle-aged mice compared to young mice (Figure 5). The relative expression of FOXP3 mRNA normalized to GAPDH was also increased in the prostates of middle-aged mice compared to young mice, as was expected given the increase in the number of FOXP3-expressing Treg per gram of prostate tumor with increasing age (Figure 2b). Taken together, these data suggest a reduction in pro-inflammatory cytokines combined with an increase in the expression of immunosuppressive factors during disease progression may lead to an inhibition of prostate cancer-specific immunity and reduced efficacy of vaccination in the later stages of prostate cancer. These data support our hypothesis that immunosuppressive mechanisms become active over the course of prostate cancer progression, and that immunization at early stages of disease may yield superior vaccine efficacy by avoiding their effects.

DISCUSSION

Here we demonstrate that a heterologous DNA prime/VRP boost prostate cancer therapeutic vaccination strategy yields superior long term protection when it is applied at a precancerous stage of disease compared to when it is administered after prostate cancer has developed in the TRAMP mouse model of human prostate cancer. In addition, we have identified several immunosuppressive mechanisms that may be involved in the differential efficacy of a therapeutic vaccine at different stages of prostate carcinogenesis in TRAMP mice.

A wide variety of immunotherapeutic strategies for treating prostate cancer have undergone clinical trials, but none have yet been FDA approved for use in patients. A fundamental problem with these clinical trials is that they have been carried out almost exclusively in terminally ill patients who have failed all other therapeutic options. These patients are frequently immunocompromised, and are therefore poor candidates in whom to test immunotherapies. With this in mind, we have proposed that therapeutic prostate cancer vaccines should be applied in the preventive setting [30]. Prior to seeking approval for clinical trials that involve immunizing early-stage prostate cancer patients, it is vital to demonstrate that application of a therapeutic cancer vaccine at the early stages of disease is likely to yield superior results than when it is administered in the advanced stages of disease. Therefore, we decided to evaluate whether therapeutic vaccination confers superior protection in the TRAMP mouse model of human prostate cancer when it is applied at a precancerous stage of cancer development (PIN lesions at 8 weeks) compared to when the mice have more advanced disease (adenocarcinomas or neuroendocrine tumors at 16 weeks) [40]. To evaluate whether the stage of prostate cancer progression at which therapeutic vaccination is applied affects the efficacy of the vaccine, we compared the long-term survival rates of TRAMP mice that were vaccinated at 16 weeks of age to the survival rates of TRAMP mice that had been vaccinated at 8 weeks of age as part of a previous study [20].

The two long-term survival studies evaluated here were not originally designed to be compared to each other. Most notably, the antigens targeted were different (mPSCA at 8 weeks and mSTEAP at 16 weeks). However, we have previously demonstrated that vaccination elicits strong and comparable immune responses against both

mPSCA [20] and mSTEAP [7] using this immunization scheme. Here we show that vaccination of TRAMP mice against either mPSCA and mSTEAP yields very strongly statistically significant improvements in survival compared to age-matched negative controls (Figure 1a). Another difference in the design of the two studies is that the mice vaccinated at 16 weeks were castrated prior to vaccination. In the time since this study was initiated, we have demonstrated that castration only elicits an immunological effect on prostate cancer vaccine efficacy when it is carried out three weeks after vaccination [19]. Therefore, we concluded that the apparent improvement in long-term TRAMP mouse survival due to castration (Figure 1a, compare 8 weeks control curve to 16 weeks control curve) is a direct result of androgen ablation on prostate tumor growth rather than an immunological effect. In order to analyze the effect on survival of vaccination at 8 weeks and at 16 weeks without the confounding factor of castration, the difference between the survival of the vaccinated groups and their age- and castration-matched control groups (which we termed delta survival) was calculated for each time point. Linear regression analysis revealed that the improvement in survival of mice vaccinated at 8 weeks compared to their age-matched controls was statistically significantly better than that of mice vaccinated at 16 weeks compared to their age- and castration-matched controls (Figure 1c). It should also be noted that of the mice vaccinated at 8 weeks with mPSCA, 90% had not yet reached the survival endpoint at 340 days post-vaccination compared to 0% in age-matched controls. Unfortunately, despite not reaching their survival endpoint the mPSCA vaccinated mice were euthanized at 340 days, according to the study design that was then being followed. At the time of their euthanasia, the mPSCA-vaccinated mice were in outstanding condition. They looked outwardly healthy, were eating and behaving exactly as would be expected of one year old wild type C57BL/6 mice and showed no signs of pain or distress. Upon necropsy, the prostates of these animals were outwardly normal. This was in stark contrast with the few animals of the control group that reached the survival endpoint on the same day and were euthanized. These mice were weak, obviously unhealthy and had very large, palpable tumors compared to the mPSCA vaccinated mice. Figure 1b shows the dramatic difference in tumor burden in mPSCA-vaccinated TRAMP mice and controls euthanized at 340 days. Overall, we conclude that therapeutic vaccination against prostate cancer TAAs confers superior survival benefits if it is applied when PIN lesions are present but invasive adenocarcinomas or neuroendocrine tumors have not yet developed.

In an effort to explain the difference in survival between TRAMP mice vaccinated at different stages of disease, we investigated whether several different tumor-mediated immunosuppressive mechanisms become present or are more active at later points of prostate cancer development. There are conflicting data regarding the role of CD4⁺CD25⁺FOXP3⁺ regulatory T cells in prostate cancer. It has been concluded that these cells are more prevalent in the blood of prostate cancer patients [41] and more active [42], while a conflicting study asserted that peripheral tolerance in prostate cancer was not mediated by Treg [43]. Consistent with the first two of these studies, our data indicate a relative increase in the numbers of Tregs in periphery of TRAMP mice with increasing age (Figure 2a). The percentage of splenic CD4⁺FOXP3⁺ T cells decreased with increasing TRAMP mouse age, but the percentage of splenic CD4⁺FOXP3⁺ Tregs stays relatively constant, suggesting progressively increasing systemic immunosuppression. We next assessed whether Treg also accumulate within the prostate tumors of TRAMP mice. We found that though the number of Tregs per gram of prostate tumor did indeed increase over time, it was part of a general accumulation of CD4⁺ T cells within the prostate tumor as it grew (Figure 2b). The constant presence of Treg within the tumor infiltrating lymphocyte population coupled with the fact that the prostate tumors of unvaccinated TRAMP mice will grow rapidly and inevitably kill the animal suggests that despite increased immune infiltration of the prostate, the constant presence of Treg impedes the ability of the attempted immune response to control tumor growth. What is not clear from these data is whether the Treg that infiltrate the tumor do so as part of a general accumulation of lymphocytes within the tumor, whether they are attracted there specifically and independently of the mechanism attracting effector T cells, or whether they are induced *in situ* from tumor infiltrating effector T cells. Resolving this question will be crucial in the development of mechanisms to prevent Treg infiltration to or induction within the prostate tumor, and is an area of active research for our group.

Though Treg are capable of infiltrating prostate tumors, it was unknown whether they were functionally active. Given the difficulty in isolating sufficient numbers of live Treg from prostate tumors, we investigated the suppressive capacities of CD4⁺CD25⁺ Treg isolated from the tumor-draining lymph nodes of TRAMP mice

(Figure 3). The data show that there are functionally suppressive Treg in the draining lymph nodes of prostates of young mice.

If the microenvironment within prostate tumors becomes increasingly immunosuppressive as they advance, it is expected that this would be reflected in the intratumoral cytokine milieu. To investigate this, we quantified the expression of a panel of cytokines and chemokines within TRAMP prostate tumors by multiplex immunoassay (Figure 4). Our data indicate a general reduction of Th1 and Th2 type cytokines in more advanced tumors, suggesting that the tumor microenvironment is indeed more immunosuppressive in these tumors. There were decreases in the expression of G-CSF, GM-CSF, IFN γ , IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-15 and TNF α in the prostate/prostate tumor tissues of young mice versus those of old mice. Conversely, there were increases in the expression of M-CSF, MIG, RANTES and VEGF. Though these differences were not statistically significant, they were large and indicated a trend towards differential expression of these cytokines and chemokines at different stages of prostate cancer development. Of particular interest was the somewhat counterintuitive reduction in IL-10 expression in more advanced tumors. Though it is generally considered an anti-inflammatory cytokine, several studies have indicated that IL-10 has strong anti-tumor effects [44]. Thus, its downregulation in more advanced tumors is not unexpected. Interestingly, there was a statistically significant decrease in the expression of macrophage inflammatory protein-1 α (MIP-1 α) but not MIP-1 β . A previous study has demonstrated that MIP-1 α stimulated the release of IL-1 and TNF α by the peripheral blood monocytes of women with breast cancer but not those of healthy women [45]. In contrast, the same study showed that MIP-1 β could stimulate release of these cytokines only healthy women and not in breast cancer patients. These findings are consistent with our own results, and suggest that downregulation of MIP-1 α that we observed is a mechanism of prostate tumor immune escape that may be responsible for the decreases in IL-1 α and TNF α that occur in more advanced prostate tumors. A statistically significant increase in the expression of monocyte chemoattractant protein 1 (MCP1) was observed in more advanced prostate tumors, which given that this chemokine normally drives Th2 responses was initially puzzling [46]. However, it has been shown that vaccine-induced eradication of r-p185 carcinoma was dramatically increased in MCP1 knockout mice [47]. This result

would be consistent with our own, and suggests an important role for this protein in tumor progression. Finally, we observed a statistically significant increase in keratinocyte-derived cytokine (KC, CXCL1). This cytokine is driven by prostaglandin E2 and promotes angiogenesis [48]. The upregulation of KC coupled with that of VEGF may result in an increase in prostate tumor vasculature, which might explain the observed general increase in tumor infiltrating lymphocytes in mice with more advanced disease.

Finally, we sought to determine whether other immunosuppressive mechanisms thought to be involved in tumor immune evasion are upregulated in more advanced prostate tumors. Quantitative real-time PCR data revealed increases in TGF β and indoleamine-2,3-dioxygenase in the prostate tumors of older TRAMP mice. The increase in TGF β suggests that the increase in the numbers of regulatory T cells observed within advanced prostate tumors is at least partially due to *in situ* Treg induction, but this requires further study. The increase in indoleamine-2,3-dioxygenase indicates that advanced prostate tumors may be directly capable of downregulating the activity of tumor infiltrating lymphocytes. In addition, indoleamine-2,3-dioxygenase has a role in the generation of inducible Treg [49, 50] and in preventing their further conversion in to proinflammatory Th17 cells [51]. These data suggest that increased expression of immunosuppressive molecules involved in the induction and maintenance of inducible Treg is an important event in the establishment of an immunosuppressive prostate tumor microenvironment in TRAMP mice. It is not clear whether the tumor cells upregulate expression of these molecules themselves, or whether they are produced by infiltrating immune cells. We are currently conducting studies to establish this. We saw no change in arginase 2 expression in the prostate tumors of older compared to younger mice, suggesting that the loss of vaccine efficacy at this stage of prostate tumor development is not a result of loss of T cell function due to decreased availability of arginine.

Overall, our data indicate that therapeutic vaccination against prostate TAA confers superior protection on TRAMP mice in terms of survival when they are vaccinated at the earliest possible stage of prostate cancer development. In addition, we have identified for future study multiple prostate tumor mediated

immunosuppressive mechanisms that may be responsible for the reduction in efficacy of therapeutic prostate cancer immunotherapy at the later stages of disease progression, the neutralization of which would assist in the successful use of therapeutic vaccination at those later stages.

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CAPTIONS TO ILLUSTRATIONS

Figure 1a: Therapeutic vaccination against prostate tumor-associated antigens results in long term survival in TRAMP mice. Groups of 20 male 8 week old TRAMP mice were vaccinated by helium-driven gene gun at day 0 with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at days 15 and 60 with 10^6 IU mPSCA-VRP and 10^6 IU GFP-VRP, respectively. Groups of 20 male 16 week old mice were vaccinated by helium-driven gene gun with either 2 ug mSTEAP-pcDNA or 2 ug empty vector and boosted at days 15 and 60 with 10^6 IU mSTEAP-VRP and 10^6 IU GFP-VRP, respectively.

Figure 1b: Therapeutic vaccination against mPSCA results in reduced prostate tumor burden in TRAMP mice. All surviving mPSCA-vaccinated TRAMP mice and their age-matched controls were euthanized at day 340 and necropsy performed. Representative images of a prostate tumor isolated from a negative control animal (left, “Control”) and that of an mPSCA-vaccinated mouse (right, “mPSCA”) are shown.

Figure 1c: Therapeutic vaccination against prostate tumor-associated antigens results in superior survival when administered to TRAMP mice with PIN lesions. The difference between the cumulative survival of each group of vaccinated mice and the cumulative survival of their age-matched controls was calculated at each time point at which one or more mice died. This value was termed “delta survival” and was plotted against survival time. Linear regression analysis was performed on each data set, and the slopes of the regression lines compared.

Figure 2a: Relatively increased numbers of Tregs in periphery of TRAMP mice with increasing age. Splenocytes isolated from groups of young (≤ 8 weeks old, $n = 3$), middle-aged (16-20 weeks old, $n = 4$) and old (≥ 24 weeks old, $n = 3$) TRAMP mice were washed and stained with anti-mouse CD3-FITC and anti-mouse CD4-PE/Cy7. Cells were fixed and permeabilised overnight, then stained with anti-mouse FOXP3-PE and

analysed by flow cytometry. Events were collected gated on live, CD3⁺ cells and then further gated on the CD4⁺FOXP3⁻ fraction and the CD4⁺FOXP3⁺ fraction. The * symbol indicates $p < 0.05$.

Figure 2b: Increased infiltration of CD4⁺ T cells and Treg into the prostate tumor with increasing age.

Tumor infiltrating lymphocytes isolated from groups of young (≤ 8 weeks old, $n = 3$), middle-aged (16-20 weeks old, $n = 4$) and old (≥ 24 weeks old, $n = 3$) TRAMP mice were washed and stained with anti-mouse CD3-FITC, anti-mouse CD8-PE/Cy5 and anti-mouse CD4-PE/Cy7. Cells were fixed and permeabilised overnight, then stained with anti-mouse FOXP3-PE and analysed by flow cytometry. Events were collected gated on live, CD3⁺CD8⁻ cells and then further gated on the total CD4⁺FOXP3⁻ fraction and the CD4⁺FOXP3⁺ fraction. The ‡ symbol indicates $p < 0.001$.

Figure 3: CD4⁺CD25⁺ T cells isolated from prostate tumor draining lymph nodes are functionally suppressive.

T cells from tumor-draining lymph nodes isolated from young (≤ 8 weeks old, $n = 3$) TRAMP mice were purified into CD4⁺CD25⁻ (responder) and CD4⁺CD25⁺ populations by magnetic separation. 5×10^4 CD4⁺CD25⁺ T cells were cocultured for 72 hours with 5×10^4 allogenic T cell-depleted splenocytes from C3H mice in complete RPMI supplemented with 1 $\mu\text{g/ml}$ activating anti-mouse CD3 antibody, either alone or with autologous CD4⁺CD25⁻ responder T cells in 1:2, 1:4, 1:8 and 1:16 ratios. As positive controls for maximal proliferation, 5×10^4 CD4⁺CD25⁻ responder T cells were cocultured with 5×10^4 allogenic T cell-depleted splenocytes from C3H mice in complete RPMI supplemented with 1 $\mu\text{g/ml}$ activating anti-mouse CD3 antibody. ³H-thymidine (1 $\mu\text{g/well}$) was added in the last 8 hours of culture. Responder cell proliferation was measured by ³H-thymidine incorporation. The relative proliferation index of responder cells for each mouse at each Treg:Tresponder ratio was calculated by dividing the mean Tresponder proliferation at each ratio by the maximal proliferation (Tresponders cultured in the absence of Treg) of Tresponders in that animal.

Figure 4: The cytokine/chemokine expression profile of spontaneous TRAMP prostate tumors changes over time.

Spontaneously arising prostate tumors harvested from groups of young (≤ 8 weeks old, $n = 5$) and

old (≥ 24 weeks old, $n = 4$) were homogenized at 4°C in $10\text{ }\mu\text{l/mg}$ sterile PBS containing $1\times$ protease inhibitors. Cytokine levels were quantified with a custom 32-plex Milliplex MAP mouse cytokine immunoassay (Millipore, Billerica MA) using the Bio-Plex multiplex system (Bio-Rad, Hercules, CA). The * symbol indicates $p < 0.05$.

Figure 5: Increased expression of immunosuppressive molecules in prostate cancer. Spontaneously arising prostate tumors harvested from groups of young (≤ 8 weeks old, $n = 3$) and old (≥ 24 weeks old, $n = 3$) mice, fixed in RNAlater and were homogenized at 4°C in $10\text{ }\mu\text{l/mg}$ buffer RLT with β -mercaptoethanol. Total RNA was isolated using a QIAGEN RNeasy kit according to the manufacturer's instructions. Complementary DNA was generated using this RNA as a template, then quantitative real-time PCR performed.

ILLUSTRATIONS

Figure 1a

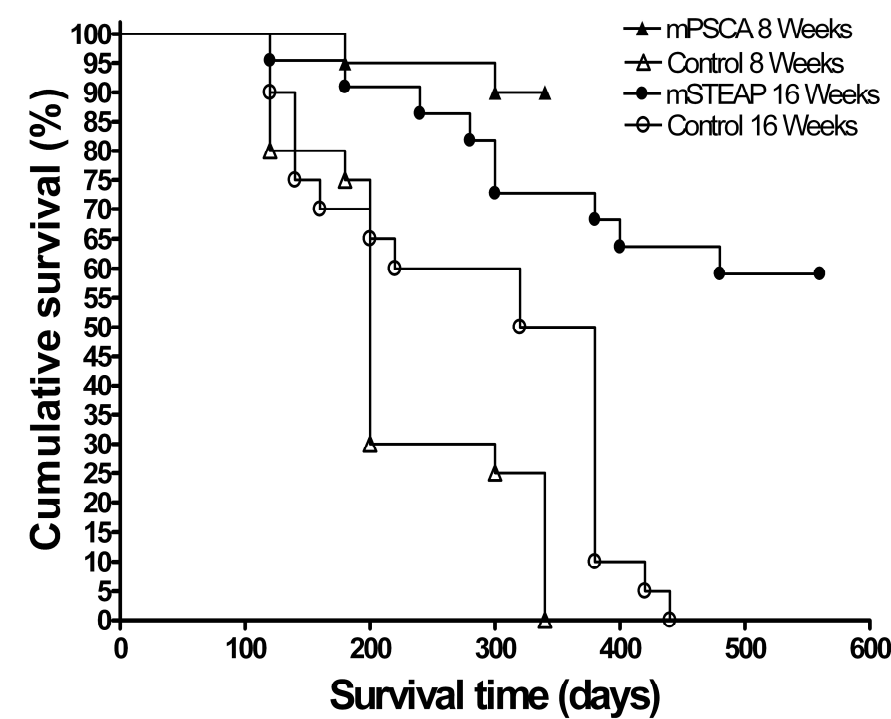


Figure 1b

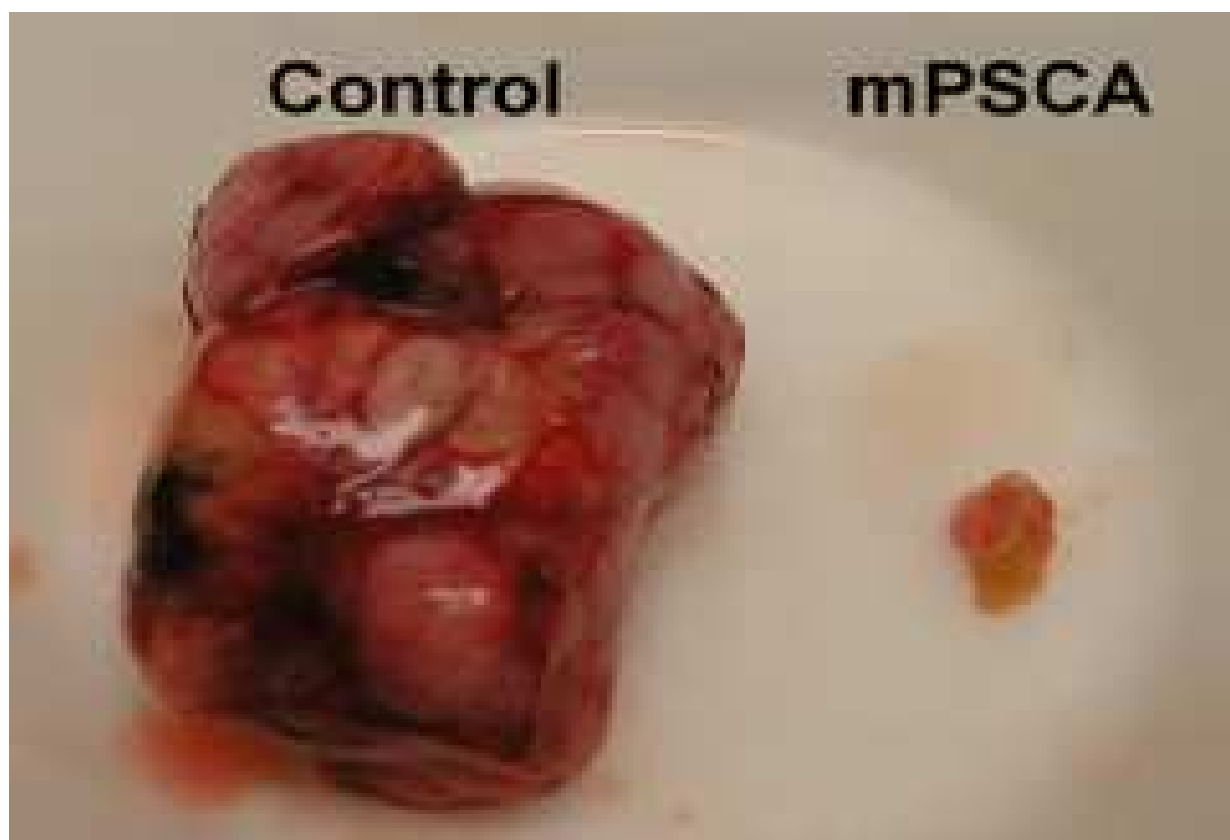


Figure 1c

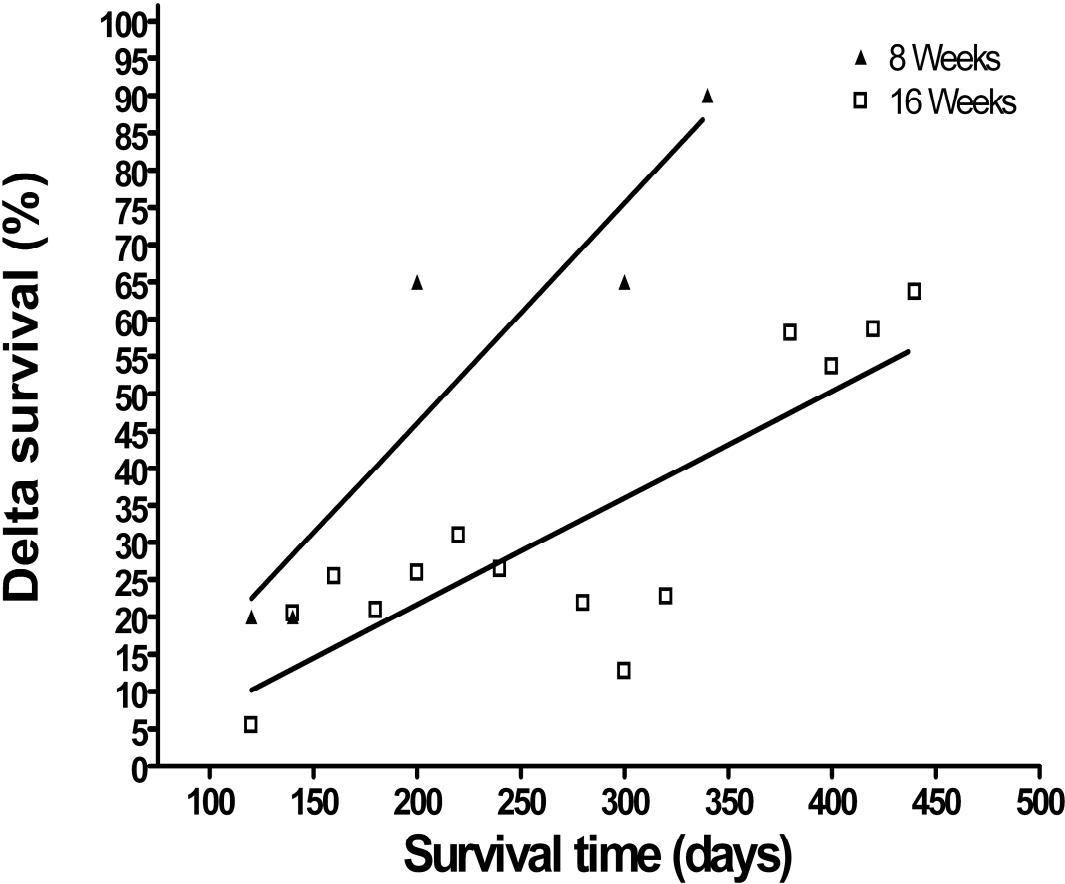


Figure 2a

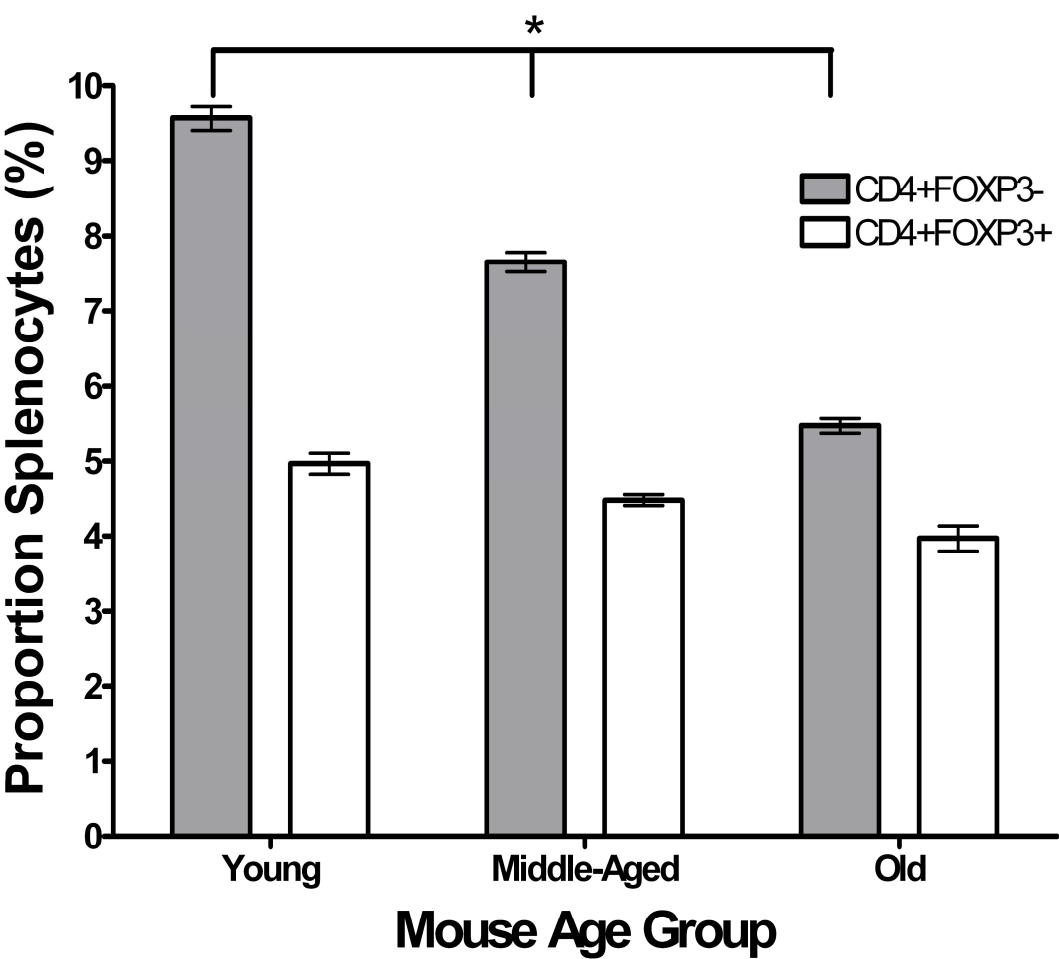


Figure 2b

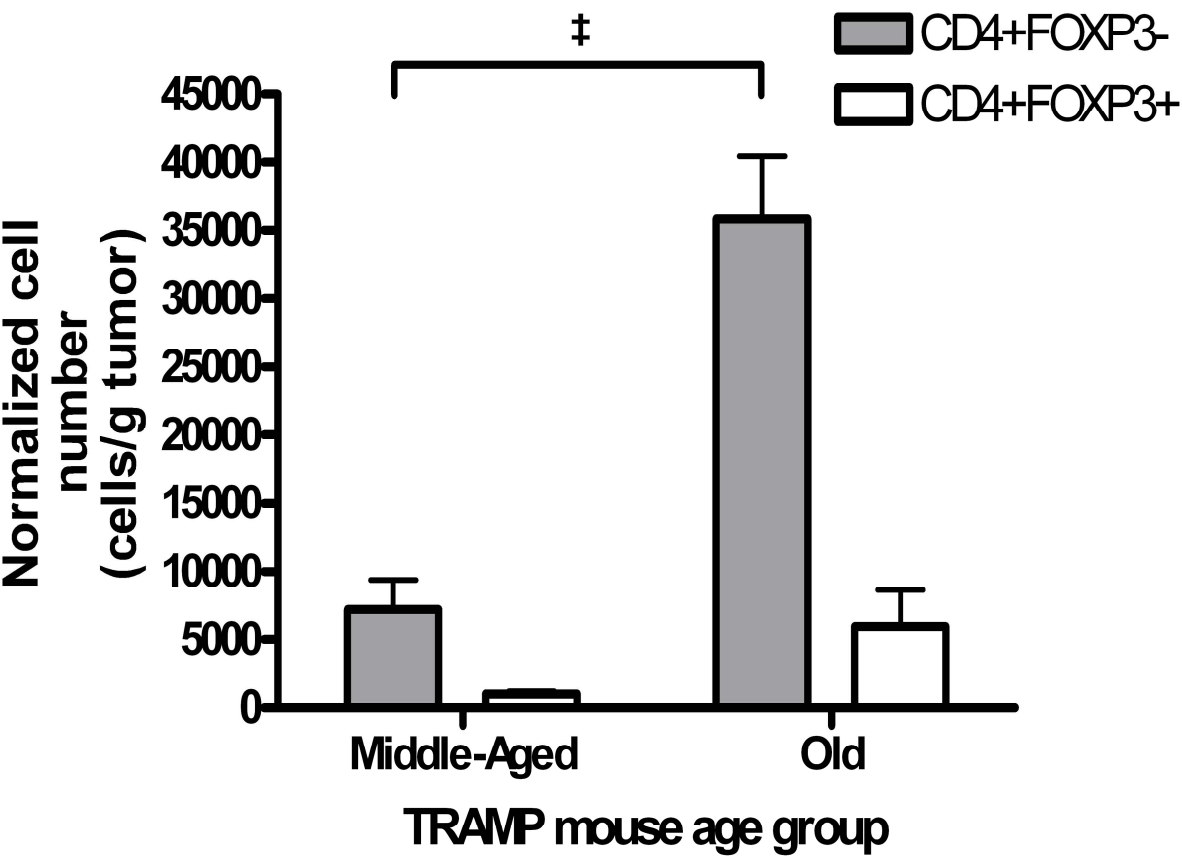


Figure 3

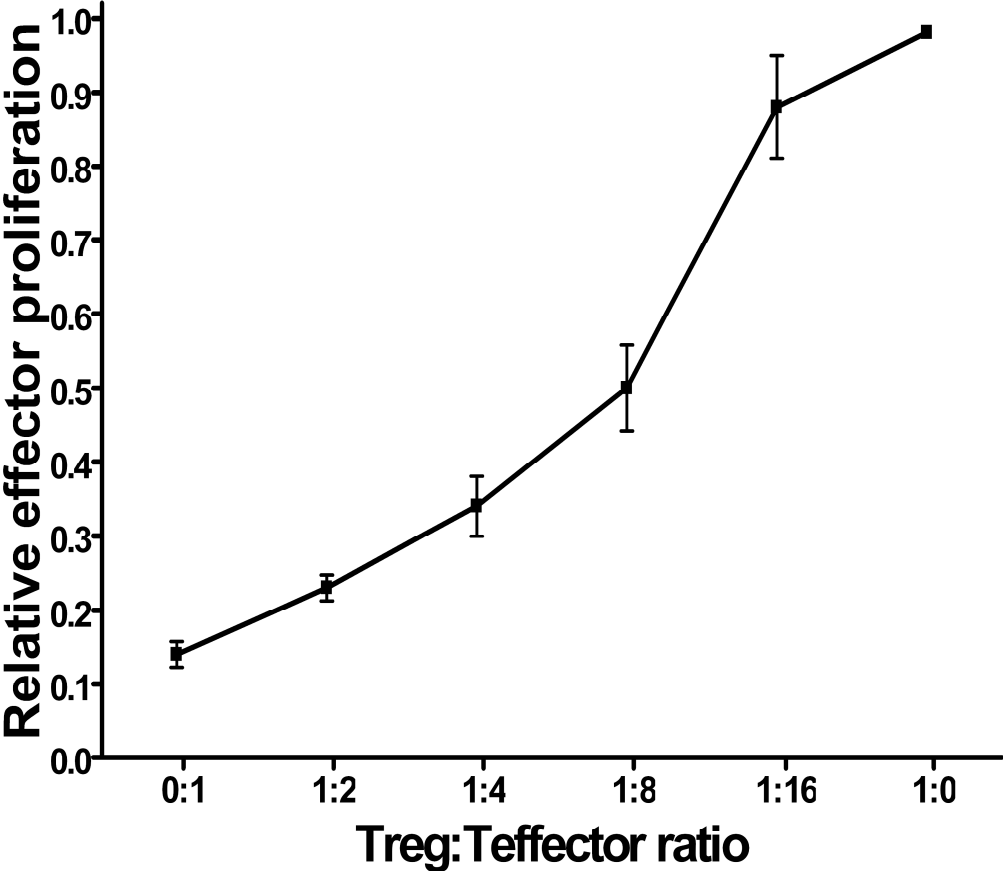


Figure 4

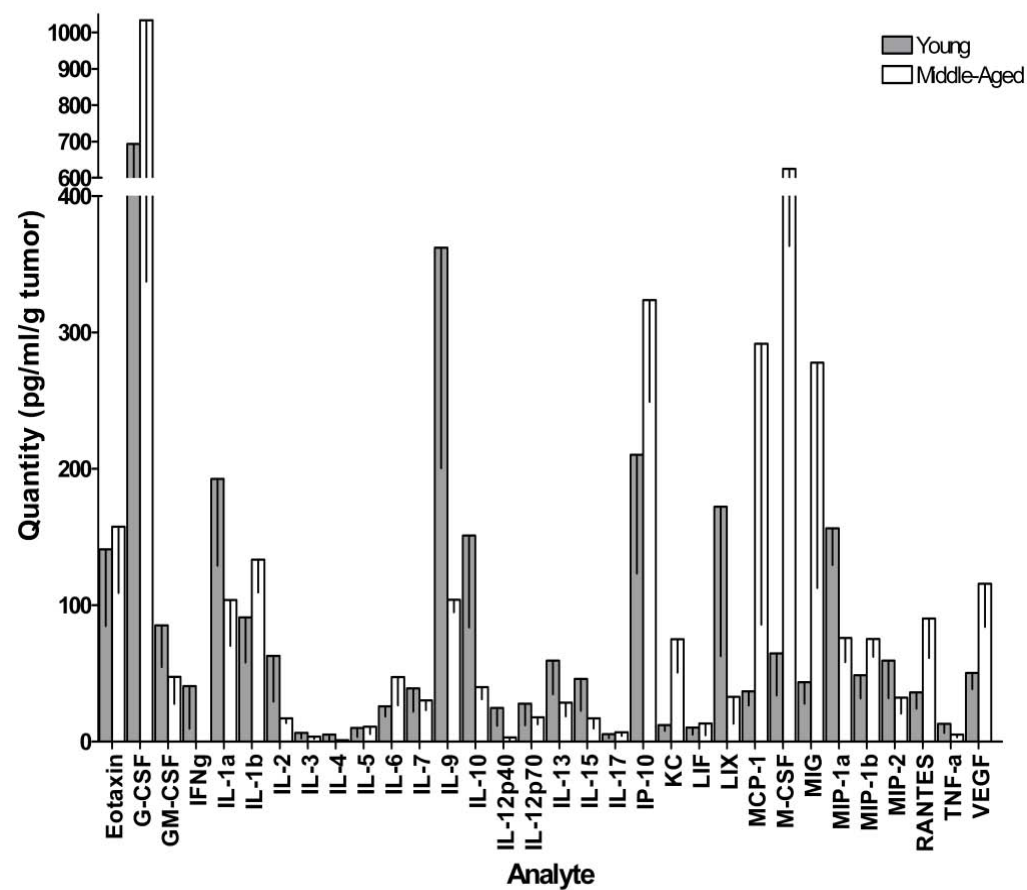


Figure 5

