AD_______________

Award Number: W81XWH-€Ì ËFË€FGG

TITLE: Ò} @æ} &ã * Á@^ÁÒ~ã&æ&^Á[-ÁU¦[•cæe^ÁÔæ} &^¦ÁQ { ˘ } [c@^¦æ} ^ Áa^ÁT æ} ã ˘ |æcã * Á
VËÔ^||ÁÜ^&^] q[¦ÁÙã } æ|ã * Áã ÁU¦å^¦Á[ÁŒ|c^¦ÁÚ^¦ã] @^¦æ|ÁÜ^* ˘ |æq[¦^ÁVËÔ^||ÁŒ&cãçãc

PRINCIPAL INVESTIGATOR: ŒÌ å¦^¸ ÁÕ¦æ

CONTRACTING ORGANIZATION: University of Ù[˘ c@^¦} ÁÔæ|ã[¦} ãæ
Š[• ÁŒ * ^|^• ÊÁÔŒÁJ€€Ì JÁ

REPORT DATE: Ju|^ ÁG€FF

TYPE OF REPORT: Annual Ù˘ { { æ|^

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*
01-07-2011

2. REPORT TYPE
Annual Summary

3. DATES COVERED *(From - To)*
1 JUL 2008 - 30 JUN 2011

4. TITLE AND SUBTITLE
Enhancing the Efficacy of Prostate Cancer Immunotherapy by Manipulating T-Cell Receptor Signaling in Order to Alter Peripheral Regulatory T-Cell Activity

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0122

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Andrew Gray

E-Mail: graya@usc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Southern California
Los Angeles, CA 90089

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 was executed involving the novel depletion of Treg (DEREG) mouse model, completing Specific Aim 1. Using these mice, it has been demonstrated that the efficacy of therapeutic immunotherapy is significantly reduced as prostate cancer advances, and that regulatory T cells have a role in limiting vaccine effectiveness. Overall, these data indicate the crucial role of Tregs in limiting the efficacy of therapeutic cancer vaccines, and highlight the urgent need to develop specific pharmacological means by which to inhibit these cells in humans.

15. SUBJECT TERMS
Regulatory T cells, prostate cancer, mouse models, therapeutic cancer vaccine, immunotherapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

	Page
Introduction…………………………………………………………….………..…..	4
Body………………………………………………………………………………..	8
Key Research Accomplishments……………………………………….……..	18
Reportable Outcomes…………………………………………………………	18
Conclusion……………………………………………………………………	18
References…………………………………………………………………….	19

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).

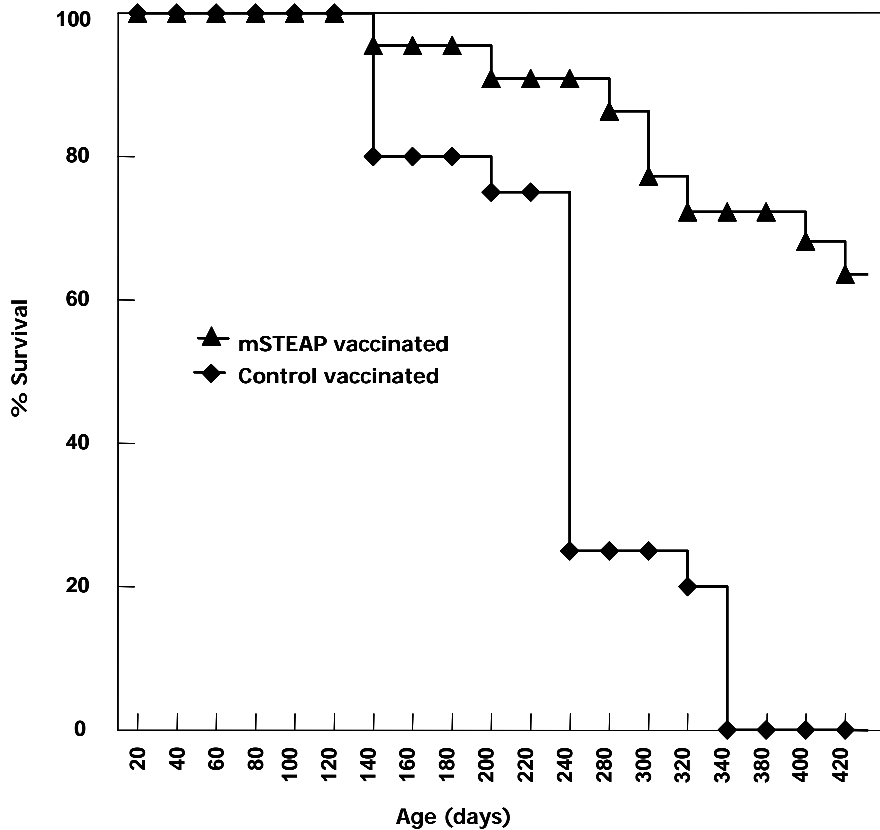


Figure 1: STEAP vaccination of TRAMP mice with prostate tumors.
A group of 22 male TRAMP mice were vaccinated with STEAP DNA at 16 weeks of age, when prostate tumors have developed, and boosted once 2 weeks later with STEAP VRP. A group of 20 control mice were vaccinated with control DNA and boosted 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.

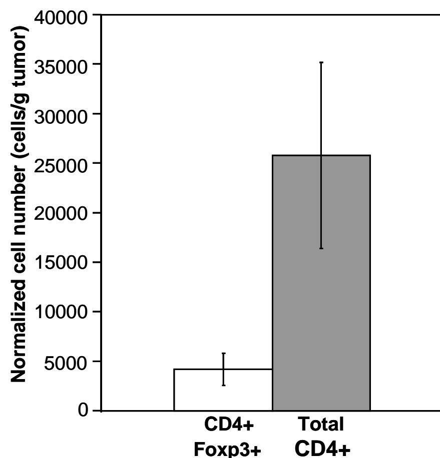
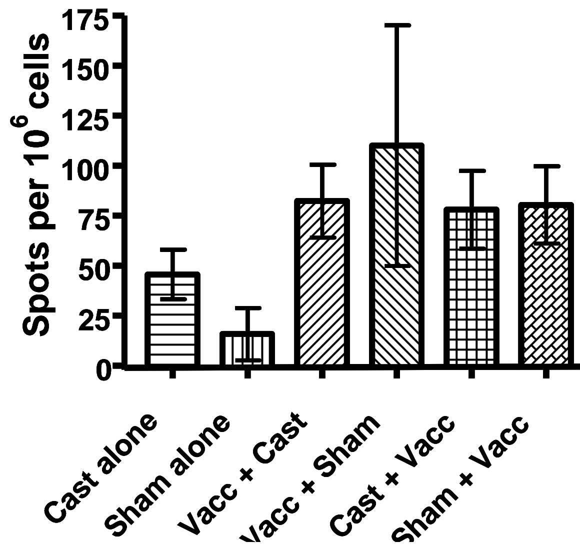


Figure 2: Identification of CD4+FOXP3+ regulatory T cells within the prostate tumors of TRAMP mice.
A group of 6 male TRAMP mice were sacrificed when they developed palpable prostate tumors. Tumor infiltrating lymphocytes were isolated and analyzed by flow cytometry. Bars represent mean number of cells 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.**



<u>Figure 3:</u> 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 10^6 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 trails 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 1-36

Aim 2: Formally present data and a detailed progress report to all members of Thesis Committee annually.
Status: Completed in months 1-36.

Aim 3: Improve presentation skills by regularly presenting data to faculty and peers at weekly seminars and laboratory group discussions.
Status: Completed in months 1-36.

Aim 4: Learn practical skills through interaction with faculty, postdoctoral fellows and peers.
Status: Completed in months 1-36.

Aim 5: Enhance knowledge of tumor immunology and develop professional contacts by attending at least one pertinent conference per year.
Status: Completed in months 1-36 by attending the International Society for Biological Therapy of Cancer 23rd Annual Meeting, San Diego, CA (2008), the La Jolla Immunology Conference at the Salk Institute, La Jolla, California (2009) and the Department of Defense Prostate Cancer Research Program Innovative Minds in Prostate Cancer Today (IMPaCT) Meeting, Orlando, FL (2011).

Aim 6: Analyze data and write up results for publication in peer-reviewed journals.
Status: Completed in months 1-36. During the period of funding, the PI has published a primary research paper in the journal *Vaccine* as the first author (19), two primary research papers as the second author (20, 21) and two reviews (22, 23). In addition, the PI is the first author of a Viewpoint article in the journal *Molecular Interventions* (24). The editors of this journal requested that the PI write this Viewpoint article based on the expertise demonstrated in the *Vaccine* paper that was supported by this award. The data presented here from the TRAMP DEREG mouse studies will be written up and submitted for publication as soon as the long term survival studies are completed (anticipated before the end of 2011).

Aim 7: Formally defend thesis work.
Status: Thesis defense date set for August 19th, 2011.

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.
Task 1.4: Added as an alternative research strategy. See below. Cross TRAMP mice with DEREG mice.
Task 1.5: Verify by flow cytometry that the desired Treg phenotype has been obtained in the mouse line generated in aim 1, task 4.

Progress: Specific Aim 1 has been completed. To begin work on Specific Aim 1, PEP KO(LYP-W620) mice were first obtained from Dr. Nunzio Bottini, a close collaborator. 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).

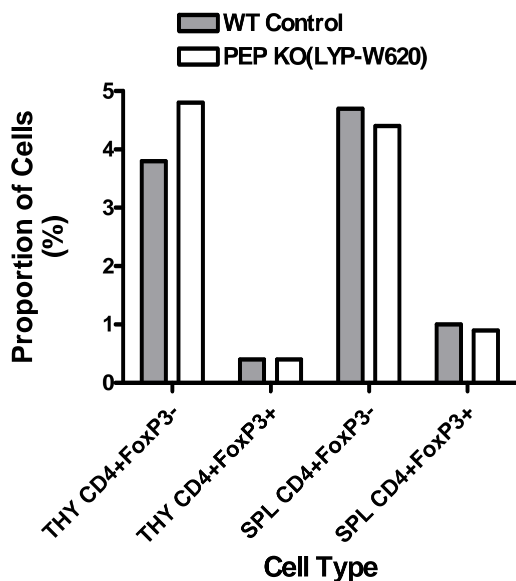


Figure 4: There is no difference in the numbers of peripheral Treg in PEP KO(LYP-W620) mice compared to wild type littermate controls. Splenocytes and thymocytes isolated from four week old PEP KO(LYP-W620) mice and wild type littermate controls were were washed and stained with anti-mouse CD8-PE/Cy5 and anti-mouse CD4-PE/Cy7. Cells were fixed and permeablized 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 was devised. 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 DEREG mouse (25). 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 (DT) to the mice.

Though it was not part of the original statement of work, the use of the DEREG 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 DEREG 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 to cross DEREG mice to TRAMP mice was embarked upon, 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 TRAMP DEREG (TD) mouse line replaced Task 1.2. The TRAMP DEREG mouse replaced the PEP KO(LYP-W620) X TRAMP mouse in all relevant subsequent experiments. To complete **Task 1.4**, TRAMP DEREG mice were successfully bred and were demonstrated to have the desired genotype (Figure 5).

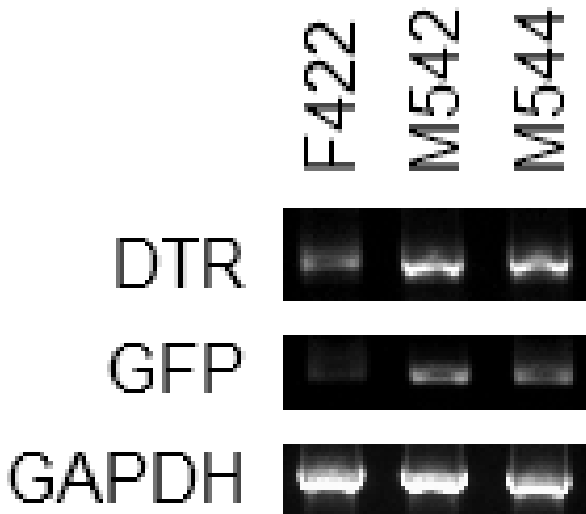


Figure 5: TRAMP DEREG mice have the DTR-GFP transgene. Representative genotyping PCR data from three TRAMP DEREG mice, demonstrating that they are positive for both diphtheria toxin receptor (DTR) and green fluorescent protein (GFP) genomic DNA; therefore they have the desired DTR-GFP transgene construct and their Tregs can be depleted by administration of diphtheria toxin.

In addition, **Task 1.5** was completed by demonstrating that the TRAMP DEREG mice have the desired phenotype, with successful depletion of $CD4^+FOXP3^+$ Tregs in these animals upon administration of diphtheria toxin (Figure 6).

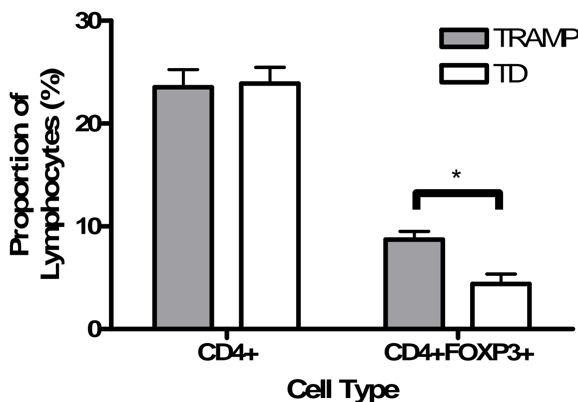


Figure 6: $CD4^+FOXP3^+$ Tregs are depleted in TD mice upon diphtheria toxin treatment. Ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 μg DT daily for six days. Lymphocytes were isolated two days later and analyzed by flow cytometry. The mean proportion of $CD4^+FOXP3^+$ cells was statistically significantly lower in the lymph nodes of TD mice compared to those of TRAMP littermate controls ($p < 0.01$, independent t test). As expected, the overall proportion of $CD4^+$ T cells was unaffected by DT treatment. The * symbol indicates $p < 0.01$.

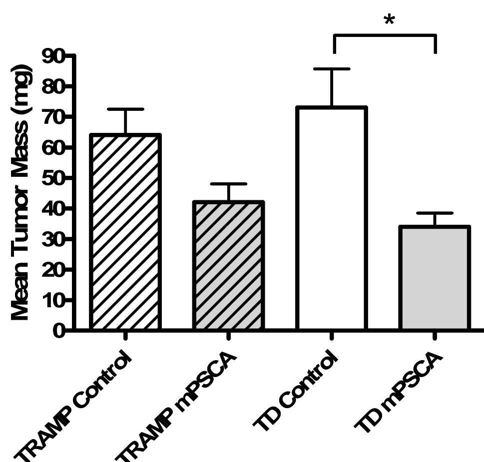
Specific Aim 2

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 using STEAP DNA via gene gun groups of ten TRAMP mice and ten TRAMP DEREG mice after treating all mice with diphtheria toxin in order to deplete Tregs from TRAMP DEREG mice. 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 ($mSTEAP_{326-336}$, 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: Excellent progress has been made on Specific Aim 2, though it is not yet fully complete. One minor variance from the proposed Statement of Work is that mice were vaccinated against murine PSCA (mPSCA) instead of mSTEAP. Our previous studies have shown that vaccination against mSTEAP and mPSCA using our immunization protocol is equally effective (7, 19, 20).

At this time, the survival studies outlined in **Tasks 2.1 and 2.2** are ongoing. The survival of the groups of mice involved will continue until they have all reached the survival endpoint. This is anticipated to occur before the end of 2011. These data will be vital for the manuscript that is being prepared based on the data presented below, so these tasks will certainly be continued even though the period of funding has ended. Though the survival data cannot be presented here, we have data to suggest that therapeutic cancer vaccination combined with depletion of Tregs should yield excellent survival benefits.

The central hypothesis of this study is that that FOXP3$^+$ regulatory T cells inhibit the efficacy of therapeutic prostate cancer vaccines and that their depletion prior to immunization will enhance vaccine efficacy. In order to test this, groups of 8-10 week old TD mice were depleted of Tregs by treating them with 1 μg DT daily for two consecutive days. It had previously been determined that this treatment protocol would efficiently deplete Tregs without the toxicity that was observed when treating the animals for six consecutive days with DT. Treg-depleted TD mice were DNA vaccinated against mPSCA two days after the last DT injection via helium gene gun. A negative control group of Treg-depleted TD mice were DNA vaccinated using the empty vector plasmid. As additional controls, groups of TRAMP littermates (lacking the DEREG transgene and therefore invulnerable to the specific depletion of Tregs by diphtheria toxin) were treated with DT in the same manner and then DNA vaccinated against mPSCA or with empty plasmid vector. Mice that had been DNA primed against mPSCA were boosted fourteen days later using 10^6 VRP encoding mPSCA, whilst those that had received empty vector plasmids were boosted at the same time using 10^6 VRP encoding GFP. In order to assess whether vaccination against mPSCA has functional anti-tumor effects in these animals, the mass of prostate/prostate tumor tissue in each animal was assessed. The mean mass of the prostate/prostate tumor tissue isolated from mPSCA vaccinated TD mice was statistically significantly lower than that of control vaccinated TD mice (Figure 7, p = 0.0157, Student's t test). In addition, there was a reduction in the mean mass of prostate/prostate tumor tissue obtained from mPSCA vaccinated TRAMP mice compared to control vaccinated TRAMP mice, though this did not reach statistical significance. The rapid inhibition of prostate growth in TD and TRAMP mice (53% and 34%, respectively) due to mPSCA vaccination is dramatic but consistent with the spectacular improvement in the survival of TRAMP mice when they are vaccinated against mPSCA at this time point using this immunization protocol (20). Interestingly, even at this early stage of carcinogenesis, depletion of Tregs seems to enhance the inhibition of tumor growth.



<u>Figure 7:</u> **mPSCA vaccination inhibits prostate growth in TD and TRAMP mice.** Eight to ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 μg DT daily for two days. TRAMP and Treg-depleted TD mice were vaccinated two days later by helium-driven gene gun with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at day 14 10^6 IU mPSCA-VRP and 10^6 IU GFP-VRP, respectively. The mice were euthanized fourteen days after boosting and the entire genitourinary (GU) tract harvested. The prostate/prostate tumor tissue was dissected away from the remainder of the GU tract and weighed. Bars represent mean tumor mass ± SEM. The * symbol indicates $p < 0.05$.

Tasks 2.3 and 2.4 have been completed. In this case, there was considerable difficulty in obtaining sufficient tumor infiltrating lymphocytes from the mice in order to be able to analyze their phenotypes by flow cytometry. In lieu of this, we instead analyzed the numbers of lymphocytes in the tumor draining lymph nodes of the animals instead. To help determine the immunological mechanisms responsible for the inhibition of tumor growth in mPSCA vaccinated TD mice, the effects of vaccination on the splenic populations of CD4$^+$, CD8$^+$ and CD4$^+$FOXP3$^+$ T cells were first assessed. Splenocytes were isolated from mPSCA vaccinated TD (n = 6), control vaccinated TD (n = 6), mPSCA vaccinated TRAMP (n = 4) and control vaccinated TRAMP (n = 5) mice and analyzed by flow cytometry. There was no statistically significant difference in the mean percentages of splenocytes that were CD3$^+$CD8$^+$ T cells between control vaccinated TD (14.76% ± 5.59%) and control vaccinated TRAMP mice (12.91% ±2.25%), indicating that Treg depletion alone is incapable of boosting peripheral numbers of CD3$^+$CD8$^+$ T cells (Figure 8, Student's t test, p = 0.4961). There was a trend towards an increase in the mean percentage of splenocytes that were CD3$^+$CD8$^+$ T cells in mPSCA vaccinated TD mice

(19.48% ± 3.97%) compared to control vaccinated TD mice (14.76% ± 5.59%) (Figure 8, Student's t test, p = 0.1626) and in mPSCA vaccinated TRAMP mice (16.12% ± 3.28%) compared to control vaccinated TRAMP mice (12.91% ± 2.25%) (Figure 8, Student's t test, p = 0.1236). There was no statistically significant difference in the mean percentages of splenocytes that were $CD3^+CD8^+$ T cells between mPSCA vaccinated TD mice (19.48% ± 3.97%) and TRAMP mice (16.12% ± 3.28%), indicating that Treg depletion does not enhance the numbers of these cells produced in response to vaccination (Figure 8, Student's t test, p = 0.2577). However, there was a statistically significant difference in the mean percentages of splenocytes that were $CD3^+CD8^+$ T cells between mPSCA vaccinated TD mice (19.48% ± 3.97%) and control vaccinated TRAMP mice (12.91% ± 2.25%), indicating that the combination of Treg depletion and vaccination against mPSCA acts additively to increase the numbers of these cells *in vivo* (Figure 8, Student's t test, p = 0.0120).

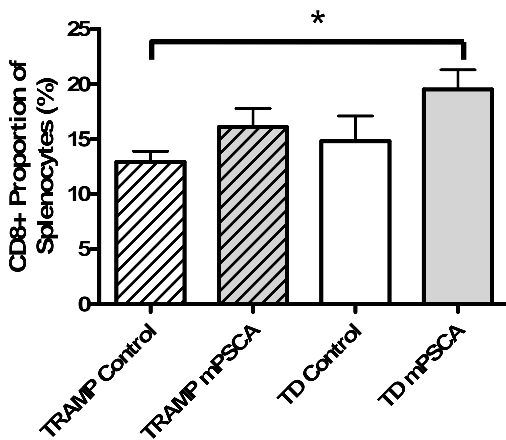


Figure 8: Treg depletion acts additively with mPSCA vaccination to increase peripheral $CD8^+$ T cell numbers. Eight to ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 μg DT daily for two days. TRAMP and Treg-depleted TD mice were vaccinated two days later by helium-driven gene gun with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at day 14 10^6 IU mPSCA-VRP and 10^6 IU GFP-VRP, respectively. The mice were euthanized fourteen days after boosting and the spleens harvested. Single cell suspensions of splenocytes were washed and stained with anti-mouse CD3-FITC and anti-mouse CD8-PE/Cy5, then analysed by flow cytometry. Events were collected gated on live, $CD3^+$ cells and then further gated on the $CD8^+$ fraction. Bars represent mean percentages of cells ± SEM. The * symbol indicates $p < 0.05$.

Next, the mean percentage of splenocytes that were $CD3^+CD4^+FOXP3^-$ non-regulatory T cells was assessed. There was a small but not statistically significant increase in the mean percentages of splenocytes that were $CD3^+CD4^+$ $FOXP3^-$ T cells between control vaccinated TRAMP (8.05% ± 2.22%) and control vaccinated TD mice (10.13% ± 6.02%) (Figure 9, Student's t test, p = 0.5006). There was a statistically significant increase in the mean percentage of splenocytes that were $CD3^+CD4^+$ $FOXP3^-$ T cells in mPSCA vaccinated TRAMP mice (11.82% ± 1.47%) compared to control vaccinated TRAMP mice (8.05% ± 2.22%) (Figure 9, Student's t test, p = 0.0245). Though there was an increase in the mean percentage of splenocytes that were $CD3^+CD4^+$ $FOXP3^-$ T cells in mPSCA vaccinated TD mice (13.91% ± 4.15%) compared to control vaccinated TD mice (10.13% ± 6.02%), it was not statistically significant (Figure 9, Student's t test, p = 0.2618). There was no statistically significant difference in the mean percentages of splenocytes that were $CD3^+CD4^+$ $FOXP3^-$ T cells between mPSCA vaccinated TRAMP and TD mice, indicating that Treg depletion does not enhance the numbers of these cells produced in response to vaccination (Figure 9, Student's t test, p = 0.3677). Despite this, there was a statistically significant difference in the mean percentages of splenocytes that were $CD3^+CD4^+$ $FOXP3^-$ T cells between mPSCA vaccinated TD mice (13.91% ± 4.15%) compared to control vaccinated TRAMP mice (8.05% ± 2.22%), again indicating that the combination of Treg depletion and vaccination against mPSCA acts additively to increase the numbers of these cells *in vivo* (Figure 9, Student's t test, p = 0.0237).

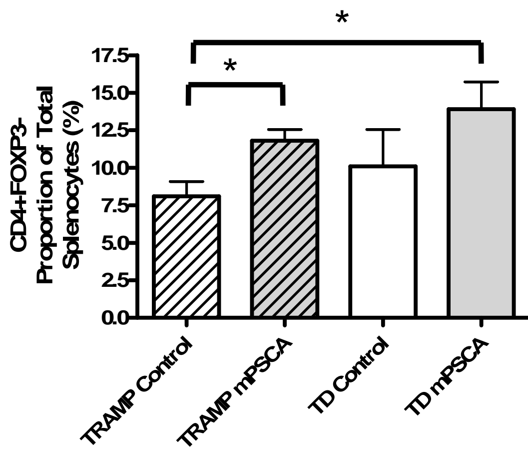


Figure 9: Treg depletion acts additively with mPSCA vaccination to increase peripheral $CD4^{+}Foxp3^{+}$ T cell numbers. Eight to ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 µg DT daily for two days. TRAMP and Treg-depleted TD mice were vaccinated two days later by helium-driven gene gun with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at day 14 10^{6} IU mPSCA-VRP and 10^{6} IU GFP-VRP, respectively. The mice were euthanized fourteen days after boosting and the spleens harvested. Single cell suspensions of splenocytes were washed and stained with anti-mouse CD3-FITC and anti-mouse CD4-PE/Cy7, then analysed by flow cytometry. Events were collected gated on live, $CD3^{+}$ cells and then further gated on the $CD4^{+}$ fraction. Bars represent mean percentages of cells ± SEM. The * symbol indicates $p < 0.05$.

It is known that the FOXP3+ population rebounds in DEREG mice between two and three weeks after depletion (26). Therefore, it was next investigated whether this was the case in TRAMP DEREG mice, and if so whether mPSCA vaccination could prevent or attenuate Treg repopulation in these animals. There was no statistically significant difference in the mean percentages of splenocytes that were $CD3^{+}CD4^{+}$ $FOXP3^{+}$ Tregs between control vaccinated TRAMP (1.59% +/- 1.02%) and control vaccinated TD mice (1.4% +/- 0.46%) (Figure 10, Student's t test, p = 0.5006). This indicates that the Treg population of TD mice had completely rebounded to pre-depletion levels within the time course of this experiment (approximately four weeks), exactly as would be observed with DEREG mice. Interestingly, vaccination against mPSCA increased the mean percentage of spenocytes that were $CD3^{+}CD4^{+}$ $FOXP3^{+}$ Tregs in both TRAMP and TD mice. This increase was statistically significant in the mean percentage of splenocytes that were $CD3^{+}CD4^{+}$ $FOXP3^{+}$ Tregs in mPSCA vaccinated TRAMP mice compared to control vaccinated TRAMP mice (Figure 10, Student's t test, p = 0.0277), but not in mPSCA vaccinated TD compared to control vaccinated TD mice (Figure 10, Student's t test, p = 0.1314). There was no statistically significant difference in the mean percentages of splenocytes that were $CD3^{+}CD4^{+}$ $FOXP3^{+}$ Tregs between mPSCA vaccinated TRAMP and TD mice, indicating that prior Treg depletion does not change the numbers of these cells produced in response to vaccination (Figure 10, Student's t test, p = 0.8182).

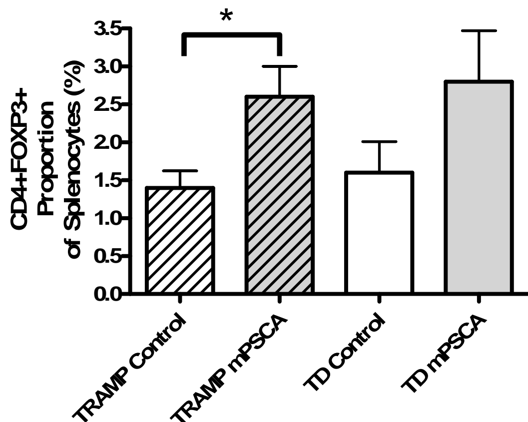


Figure 10: Vaccination against mPSCA increases the proportion of Treg cells in the periphery, regardless of prior Treg depletion. Eight to ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 µg DT daily for two days. TRAMP and Treg-depleted TD mice were vaccinated two days later by helium-driven gene gun with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at day 14 10^{6} IU mPSCA-VRP and 10^{6} IU GFP-VRP, respectively. The mice were euthanized fourteen days after boosting and the spleens harvested. Single cell suspensions of splenocytes were washed and stained with anti-mouse CD3-FITC and anti-mouse CD4-PE/Cy7, fixed and permeablised 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. Bars represent mean percentages of cells ± SEM. The * symbol indicates $p < 0.05$.

Given the critical role of lymph nodes in effector T cell activation and in Treg activity, the effects of vaccination on the populations of $CD8^+$ and $CD4^+FOXP3^+$ T cells were assessed in these organs. Lymphocytes were isolated from mPSCA vaccinated TD (n = 6), control vaccinated TD (n = 6), mPSCA vaccinated TRAMP (n = 4) and control vaccinated TRAMP (n = 5) mice and analyzed by flow cytometry. There was a striking, statistically significant decrease in the mean percentages of lymphocytes that were $CD3^+CD8^+$ T cells between control vaccinated TD mice (26.0% ± 5.3%) and control vaccinated TRAMP mice (38.6% ± 7.7%) (Figure 11, Student's t test, p = 0.106). It is not clear from these data whether this effect of prior Treg depletion is due to a reduction of trafficking of CD8+ T cells to the lymph nodes, or whether it is due to an increase in activation of $CD8^+$ T cells and a subsequent increase in their trafficking out of the lymph nodes and back into the periphery, perhaps to the prostate tumor. Further work is needed to investigate this phenomenon. Frozen tumor tissue is available from these animals which can be used to facilitate this. Interestingly, there was a statistically significant increase in the mean percentage of lymphocytes that were $CD3^+CD8^+$ T cells in mPSCA vaccinated TD mice (36.0% ± 9.3%) compared to control vaccinated TD mice (26.0% ± 5.3%) (Figure 11, Student's t test, p = 0.0247). Though the mean percentages of lymphocytes that were $CD3^+CD8^+$ T cells were generally higher in TRAMP mice than in TD mice, the enhancement in $CD3^+CD8^+$ lymphocytes in TRAMP mice due to mPSCA vaccination was much less pronounced than in their TD littermates. Indeed, the increase in the mean percentage of lymphocytes that were that were $CD3^+CD8^+$ T cells in mPSCA vaccinated TRAMP mice (42.8% ± 9.6%) compared to control vaccinated TRAMP mice (38.6% ± 7.7%) was not statistically significant (Figure 11, Student's t test, p = 0.4886).

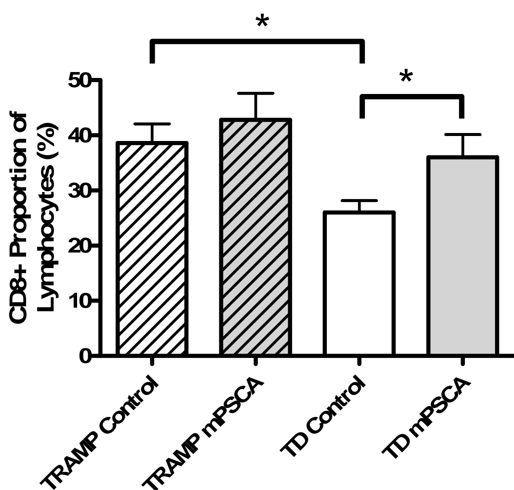


Figure 11: Treg depletion enhances the increase in the $CD8^+$ T cell compartment of lymphocytes in response to mPSCA vaccination. Eight to ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 μg DT daily for two days. TRAMP and Treg-depleted TD mice were vaccinated two days later by helium-driven gene gun with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at day 14 10^6 IU mPSCA-VRP and 10^6 IU GFP-VRP, respectively. The mice were euthanized fourteen days after boosting and the lymph nodes harvested. Single cell suspensions of lymphocytes were washed and stained with anti-mouse CD3-FITC and anti-mouse CD8-PE/Cy5, then analysed by flow cytometry. Events were collected gated on live, $CD3^+$ cells and then further gated on the $CD8^+$ fraction. Bars represent mean percentages of cells ± SEM. The * symbol indicates $p < 0.05$.

Regulatory T cells are known to exert their suppressive function within lymph nodes. Therefore, the effect of prior depletion of $FOXP3^+$ regulatory T cells and concomitant mPSCA vaccination on the Treg populations of TRAMP DEREG lymph nodes was investigated. In stark contrast to the striking increase in the percentage of $CD3^+CD4^+$ $FOXP3^+$ Tregs that was associated with mPSCA vaccination in the spleens of TD and TRAMP mice, there was a only virtually indistinguishable (but consistent) increase in Tregs in the lymph nodes of both TD and TRAMP mice when they were vaccinated against mPSCA. Treg depletion seemed to persist longer in the lymph nodes of TD mice compared to in their spleens. There was a small but non-statistically significant decrease in the mean percentages of lymphocytes that were $CD3^+CD4^+$ $FOXP3^+$ Tregs between control vaccinated TD (1.92% ± 0.35%) and control vaccinated TRAMP (2.28% ± 0.71%) mice (Figure 12, Student's t test, p = 0.2634). Interestingly, the decrease in the mean percentage of lymphocytes that were $CD3^+CD4^+$ $FOXP3^+$ Tregs between mPSCA vaccinated TD (1.99% ± 0.12%) and mPSCA vaccinated TRAMP (2.42% ± 0.30%) mice was statistically significant (Figure 12, Student's t test, p = 0.0147).

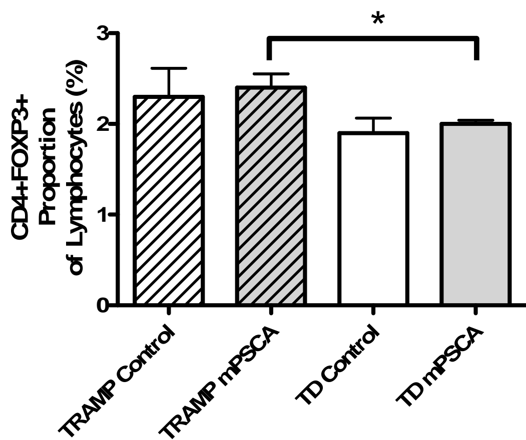


Figure 12: Treg depletion persists in the lymph nodes of TD mice for up to four weeks. Eight to ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 μg DT daily for two days. TRAMP and Treg-depleted TD mice were vaccinated two days later by helium-driven gene gun with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at day 14 10^6 IU mPSCA-VRP and 10^6 IU GFP-VRP, respectively. The mice were euthanized fourteen days after boosting and the lymph nodes harvested. Single cell suspensions of lymphocytes were washed and stained with anti-mouse CD3-FITC and anti-mouse CD4-PE/Cy7, fixed and permeablised 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. Bars represent mean percentages of cells ± SEM. The * symbol indicates $p < 0.05$.

Task 2.5 has been completed, and tissues from the animals involved were cryopreserved so that **Task 2.6, Task 2.7 and Task 2.12** can be completed as soon as possible. In the case of Task 2.7, completion is anticipated by the end of August 2011. The completion of Task 2.6 and Task 2.12 depend on the availability of our resident pathologist, so an estimated time for completion is not currently available. To determine whether vaccination against mPSCA was eliciting a specific anti-tumor immune response and thus complete **Task 2.8**, the ability of $CD8^+$ cytotoxic T cells to produce interferon-gamma (IFNγ) in response to an mPSCA peptide was assessed. Candidate mPSCA peptides that could potentially bind to MHC class I molecules $H\text{-}2D^b$ or $H\text{-}2K^b$ had previously been identified. Two peptides were selected based on the highest score of peptide/MHC class I half life of dissociation generated using prediction programs (27, 28). The capacities of these peptides to stabilize MHC class I on RMA-S cells was also evaluated (20). The peptide $mPSCA_{83\text{-}91}$ had the highest predicted binding affinity and has since been used in order to quantify mPSCA-specific responses elicited by vaccination in TRAMP mice. This peptide was loaded onto spleen cells to evaluate antigen-specific MHC class I-restricted CD8 T-cell responses in terms of IFNγ production by flow cytometry. Splenocytes were isolated from mPSCA vaccinated TD (n = 6), control vaccinated TD (n = 6), mPSCA vaccinated TRAMP (n = 4) and control vaccinated TRAMP (n = 5) mice. In TRAMP mice, there was no statistically change in the mean number of $CD8^+IFN\gamma^+$ T cells per million splenocytes in mPSCA vaccinated mice (86.2 cells per million splenocytes) compared to control vaccinated mice (65.1 cells per million splenocytes) (Figure 13). In contrast, there was a large increase in the mean number of $CD8^+IFN\gamma^+$ T cells per million splenocytes in mPSCA vaccinated TD mice (163.6 cells per million splenocytes) compared to control vaccinated TD mice (436.8 cells per million splenocytes) (Figure 11). This increase was not statistically significant due to large variations in the number of $CD8^+IFN\gamma^+$ T cells in each mouse. Indeed, the splenocytes obtained from approximately half of the mice in each group failed to respond to $mPSCA_{83\text{-}91}$ stimulation at all, which was surprising given that they are all genetically identical at their MHC loci. Overall, there were more IFN-γ producing splenocytes in TD mice than in TRAMP mice, regardless of vaccination status, indicating that prior depletion of Tregs can result in an increase in antigen-specific immune responses even in the absence of vaccination.

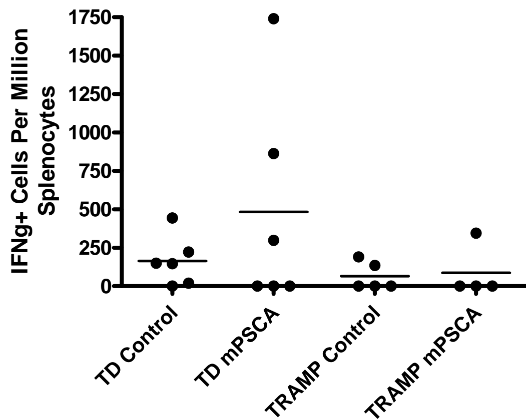


Figure 11: Depletion of Tregs enhances the response of antigen-specific $CD8^+$ T cells. Eight to ten week old TD mice and standard TRAMP littermate controls were treated IP with 1 µg DT daily for two days. TRAMP and Treg-depleted TD mice were vaccinated two days later by helium-driven gene gun with either 2 ug mPSCA-pcDNA or 2 ug empty vector and boosted at day 14 10^6 IU mPSCA-VRP and 10^6 IU GFP-VRP, respectively. The mice were euthanized fourteen days after boosting and the spleens harvested. Splenocytes were treated for twenty four hours with $mPSCA_{83-91}$ and IL-2, with Brefeldin-A being added in the last six hours in order to arrest the Golgi apparatus of the cells, thus allowing detectable levels of IFNγ to build up in the cytoplasm of activated T cells. Splenocytes were washed and stained with anti-mouse CD3-FITC and anti-mouse CD4-PE/Cy7, fixed and permeablised overnight, then stained with anti-mouse IFNγ-PE and analysed by flow cytometry. Events were collected gated on live, $CD3^+$ cells and then further gated on the $CD4^+IFN\gamma^+$ fraction. Dots represent number of $CD8^+IFN\gamma^+$ T cells per million splenocytes in each individual animal. Horizonal bars represent mean number of $CD8^+IFN\gamma^+$ T cells per million splenocytes in each group.

Mice are currently being bred in order to complete **Task 2.9.** When they are available, **Task 2.9, 2.10 and 2.11** will be completed. Estimated time of completion of these tasks is by the end of October 2011.

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 $5x10^4$ 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 mm^3.
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: No progress has been made towards completing Specific Aim 3. There were significant problems in breeding sufficient TRAMP DEREG mice, and in optimizing the diphtheria toxin protocol such that Tregs were successfully depleted in these animals without otherwise harming them. As a result, all available TRAMP DEREG mice were used in the effort to complete Specific Aim 2. Though the experiments outlined for Specific Aim 3 remain interesting and valuable, they could not be completed during the funding period.

KEY RESEARCH ACCOMPLISHMENTS

1. Determined that PEP/LYP gain of function mutations have no effect on peripheral Treg numbers.
2. Developed the TRAMP DEREG mouse line.
3. Characterized the effect of Treg depletion on the immune response induced by therapeutic prostate cancer vaccination.

REPORTABLE OUTCOMES

1. A total of three primary research articles (19-21) and three review articles (22-24) published by the PI over the funding period, all as first or second author.
2. Attended three major conferences over the funding period. At all three, the PI presented data that was generated by completing Specific Aims 1 and 2.
3. As a direct result of the experience and training provided by completing this award, the PI has been offered and has accepted a postdoctoral fellowship position at UCLA under the mentorship of Professor Sanaz Memarzadeh.

CONCLUSION

Specific Aim 1 has been completed, and Specific Aim 2 is on the verge of being completed. It is already apparent from these studies that – as hypothesized – regulatory T cells have a negative impact on the immune responses elicited by therapeutic cancer vaccines. It is known based on the work published by the PI during the funded period that regulatory T cells become more prevalent in this model of prostate cancer as disease progresses (19). Follow up studies are thus being planned to determine whether Treg depletion at later time points in the progression of prostate cancer can enhance the efficacy of therapeutic cancer vaccines. If so, then they may become capable of eradicating tumors that were previously resistant to immunotherapy due to high levels of Treg-mediated immune suppression. The results of these experiments will be extraordinarily exciting, and will be highly beneficial to the field. Though FOXP3-expressing Tregs have long been hypothesized to play a role in limiting the efficacy of prostate cancer vaccines, the lack of a good means by which to ***specifically*** inhibit Tregs has made testing this hypothesis extremely difficult. The work done as part of this award has achieved this for the first time. As a result, there is a much greater impetus for the field to develop specific inhibitors of Tregs – for example, small molecule inhibitors of FOXP3 – thereby developing an entirely new class of pharmacological treatments for prostate cancer, and indeed other cancers. While it is unfortunate that Specific Aim 3 could not be completed during the funding period – ultimately due to the research problems in the first year of the award – the development of the TRAMP DEREG mouse model over the course of this project will at least allow the completion of this aim in the future. Overall, the funding period has been highly productive for the PI. He would like to take this opportunity to thank the Prostate Cancer Research Program for the funding and the extraordinary opportunities that it opened up over the years.

REFERENCES

1. Schlesinger, S., and T. W. Dubensky. 1999. Alphavirus vectors for gene expression and vaccines. *Curr Opin Biotechnol* 10:434-439.
2. Pushko, P., M. Parker, G. V. Ludwig, N. L. Davis, R. E. Johnston, and J. F. Smith. 1997. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology* 239:389-401.
3. Hubert, R. S., I. Vivanco, E. Chen, S. Rastegar, K. Leong, S. C. Mitchell, R. Madraswala, Y. Zhou, J. Kuo, A. B. Raitano, A. Jakobovits, D. C. Saffran, and D. E. Afar. 1999. STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors. *Proc Natl Acad Sci U S A* 96:14523-14528.
4. Yang, D., G. E. Holt, M. P. Velders, E. D. Kwon, and W. M. Kast. 2001. 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* 61:5857-5860.
5. Rodeberg, D. A., R. A. Nuss, S. F. Elsawa, and E. Celis. 2005. Recognition of six-transmembrane epithelial antigen of the prostate-expressing tumor cells by peptide antigen-induced cytotoxic T lymphocytes. *Clin Cancer Res* 11:4545-4552.
6. Alves, P. M., O. Faure, S. Graff-Dubois, S. Cornet, I. Bolonakis, D. A. Gross, I. Miconnet, S. Chouaib, K. Fizazi, J. C. Soria, F. A. Lemonnier, and K. Kosmatopoulos. 2006. STEAP, a prostate tumor antigen, is a target of human CD8(+) T cells. *Cancer Immunol Immunother*.
7. Garcia-Hernandez Mde, L., A. Gray, B. Hubby, and W. M. Kast. 2007. In vivo effects of vaccination with six-transmembrane epithelial antigen of the prostate: a candidate antigen for treating prostate cancer. *Cancer Res* 67:1344-1351.
8. Tang, Q., and J. A. Bluestone. 2006. Regulatory T-cell physiology and application to treat autoimmunity. *Immunol Rev* 212:217-237.
9. Lizee, G., L. G. Radvanyi, W. W. Overwijk, and P. Hwu. 2006. Improving antitumor immune responses by circumventing immunoregulatory cells and mechanisms. *Clin Cancer Res* 12:4794-4803.
10. Wang, H. Y., and R. F. Wang. 2007. Regulatory T cells and cancer. *Curr Opin Immunol* 19:217-223.
11. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol* 3:756-763.
12. Vang, T., M. Congia, M. D. Macis, L. Musumeci, V. Orru, P. Zavattari, K. Nika, L. Tautz, K. Tasken, F. Cucca, T. Mustelin, and N. Bottini. 2005. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat Genet* 37:1317-1319.
13. Hasegawa, K., F. Martin, G. Huang, D. Tumas, L. Diehl, and A. C. Chan. 2004. PEST domain-enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells. *Science* 303:685-689.
14. Gruver, A. L., L. L. Hudson, and G. D. Sempowski. 2007. Immunosenescence of ageing. *J Pathol* 211:144-156.
15. Deng, Y., Y. Jing, A. E. Campbell, and S. Gravenstein. 2004. Age-related impaired type 1 T cell responses to influenza: reduced activation ex vivo, decreased expansion in CTL culture in vitro, and blunted response to influenza vaccination in vivo in the elderly. *J Immunol* 172:3437-3446.
16. Eaton, S. M., E. M. Burns, K. Kusser, T. D. Randall, and L. Haynes. 2004. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J Exp Med* 200:1613-1622.
17. Drake, C. G., A. D. Doody, M. A. Mihalyo, C. T. Huang, E. Kelleher, S. Ravi, E. L. Hipkiss, D. B. Flies, E. P. Kennedy, M. Long, P. W. McGary, L. Coryell, W. G. Nelson, D. M. Pardoll, and A. J. Adler. 2005. Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. *Cancer Cell* 7:239-249.
18. Sutherland, J. S., G. L. Goldberg, M. V. Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. R. Blazar, J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. *J Immunol* 175:2741-2753.
19. Gray, A., M. de la Luz Garcia-Hernandez, M. van West, S. Kanodia, B. Hubby, and W. M. Kast. 2009. 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. *Vaccine* 27 Suppl 6:G52-59.

20. Garcia-Hernandez Mde, L., A. Gray, B. Hubby, O. J. Klinger, and W. M. Kast. 2008. Prostate stem cell antigen vaccination induces a long-term protective immune response against prostate cancer in the absence of autoimmunity. *Cancer Res* 68:861-869.
21. Koh, Y. T., A. Gray, S. A. Higgins, B. Hubby, and W. M. Kast. 2009. Androgen ablation augments prostate cancer vaccine immunogenicity only when applied after immunization. *The Prostate* 69:571-584.
22. Gray, A., A. B. Raff, M. Chiriva-Internati, S. Y. Chen, and W. M. Kast. 2008. A paradigm shift in therapeutic vaccination of cancer patients: the need to apply therapeutic vaccination strategies in the preventive setting. *Immunol Rev* 222:316-327.
23. Raff, A. B., A. Gray, and W. M. Kast. 2009. Prostate stem cell antigen: a prospective therapeutic and diagnostic target. *Cancer letters* 277:126-132.
24. Gray, A., L. Yan, and W. M. Kast. Prevention is better than cure: the case for clinical trials of therapeutic cancer vaccines in the prophylactic setting. *Molecular interventions* 10:197-203.
25. Lahl, K., C. Loddenkemper, C. Drouin, J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, and T. Sparwasser. 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* 204:57-63.
26. Lahl, K., and T. Sparwasser. In vivo depletion of FoxP3+ Tregs using the DEREG mouse model. *Methods in molecular biology (Clifton, N.J* 707:157-172.
27. Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol* 152:163-175.
28. D'Amaro, J., J. G. Houbiers, J. W. Drijfhout, R. M. Brandt, R. Schipper, J. N. Bavinck, C. J. Melief, and W. M. Kast. 1995. A computer program for predicting possible cytotoxic T lymphocyte epitopes based on HLA class I peptide-binding motifs. *Hum Immunol* 43:13-18.