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| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT: Immunotherapy may provide an alternative treatment for cancer patients, especially when tumors over-express antigens that can be recognized by immune cells. The identification of markers and therapeutic targets that are up regulated in prostate cancer has been important to design new potential treatments for prostate cancer. Among them, the recently identified six-transmembrane epithelial antigen of the prostate (STEAP) is considered an attractive target, due to its over-expression in human prostate cancer tissues. Previously we selected prime/boost vaccination strategy (DNA/GFP-VRP) as the best scheme to induce a specific CD8 T cell response in C57BL/6 mice responsible for tumor delay in complete absence of autoimmunity development but therapeutic vaccination with mSTEAP modestly controlled the growth of well established tumors. In this report we demonstrated that CD4 T cells that produced IFN γ , TNF α and IL-2 play the main role in tumor delay in model as demonstrated by using CD4- and CD8-deficient mice. Prime/boost vaccination was unable to control tumor progression in TRAMP mice with prostatic intraepithelial neoplasia. In a combined treatment of androgen ablation and STEAP vaccination was successful in prostate tumor-bearing TRAMP mice. | | | | | |
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INTRODUCTION

Six-transmembrane epithelial antigen of the prostate (STEAP) was identified in xenografts of advanced human prostate cancer¹, derived from bone and lymph node metastases and propagated in SCID mouse models. Under physiological conditions, low levels of STEAP have been detected in plasma membranes of normal prostate tissues. In contrast, it is highly over-expressed in human prostate cancer, suggesting it could be a potential candidate for immunotherapy. STEAP expression has also been detected in several colon, bladder, ovarian, and pancreatic cancer cell lines, reinforcing the idea that this gene is up regulated in cancer. We recently identified its murine counterpart, expressed in a prostate tumor cell line (TRAMP-C1) derived from the prostate of mice of the transgenic adenocarcinoma mouse prostate model (TRAMP)². The analysis of the nucleotide and amino acid sequence showed that mouse STEAP has 80% homology with human STEAP and it also contains six potential membrane-spanning regions. In TRAMP mice, STEAP is over-expressed in prostate cancer tissues whereas low levels are detected in kidney and testis. Recently, we detected STEAP at low levels in thymus by immunofluorescence, where it could participate in mechanisms of central tolerance induction. This tumor-associated antigen does not have substantial structural changes. For this reason, it could be difficult to induce a good immune response without disturbing the mechanisms of central and peripheral tolerance potentially associated to this antigen. However in a recent report, two human STEAP peptides were identified as excellent inducers of antigen-specific CTLs, which were able to recognize and kill STEAP-expressing tumor cells^{3, 4}. These data suggests that STEAP can be considered as a promising candidate for immunotherapy of prostate cancer.

Body

Previously we demonstrated that mSTEAP-based vaccination, no matter the scheme of treatment, was able to induce a specific CD8 T cell response responsible for tumor delay. There was complete absence of autoimmunity development. In an effort to understand the immune mechanisms involved in tumor growth delay and to improve the prime-boost vaccination schemes, we proposed in the last report (2004-2005) to analyze the populations of inflammatory infiltrating cells and cytokine levels at the tumor site. Task d1.1 was added.

Task 1 d1.1 To determine the participation of CD4 and CD8 T cells in the control of tumor growth in mSTEAP vaccinated mice

First we decided to analyze the role of antigen specific CD8- and CD4 T cells generated during mSTEAP vaccination. We followed tumor growth after challenging vaccinated C57BL/6 and CD4- or CD8-deficient mice with tumor cells. Mice were vaccinated with mSTEAP-pcDNA3, boosted with mSTEAP-VRP and challenged with TRAMP2 cells. Compared to C57BL/6 mice, tumor growth in vaccinated CD8 knockout mice was significantly delayed ($p=0.001$, two tailed), whereas it was significantly accelerated in vaccinated CD4 knockout mice ($p=0.004$, two tailed) (Figure 1A). These findings indicate that CD4 T cells participate in the control of tumor growth. In addition, we observed a marked infiltration of regulatory CD8 T cells at the tumor site (Fig 1B). The difference in the number of regulatory CD4 T cells between C57BL/6- and CD8 KO mice was not significant, but there was a direct relationship between the trend of tumor growth (Fig. 1A) and the total number of infiltrating regulatory CD4 T cells (Fig.1B). Local production of protective cytokines (IL-2, IL-12p70, IFN γ and TNF α) was absent in mice lacking CD4 T cells (Figure 1C). These cytokines are normally linked to the induction of strong cellular tumor immunity and inhibition of angiogenesis and were readily detectable only in C57BL/6 and CD8KO mice (Figure 1C). These mice produced protective cytokines at similar levels, with the exception of IL-2 that was elevated in CD8KO mice. The comparable cytokine production observed in C57BL/6- and CD8KO is consistent with the pattern of tumor growth that was seen in these mice (Figure 1C).

Task 1 d.2) To determine the therapeutic effect of mSTEAP vaccination in tumor bearing C57BL/6 mice by using a prime-boost vaccination scheme.

Given that therapeutic vaccination with mSTEAP only controlled modestly the growth of well-established tumors, we suggested in the last report that it could be better to evaluate whether combined schemes, involving androgen ablation and mSTEAP therapy, could induce a synergistic effect that will be translated in tumor rejection or at least a delay in tumor growth. Sham-castrated mice, with well-implanted tumor were immunized with mSTEAP-DNA at day 31 after challenge with TRAMP-C2 cells, followed by a subcutaneous injection of mSTEAP-VRP at day 46 post-challenge. This therapeutic vaccination scheme induced a small but statistically significant delay in tumor growth ($p=0.0022$, two tailed) compared with a group of unvaccinated mice or a group of mice vaccinated with pcDNA3 and GFP-VRP (Fig 2).

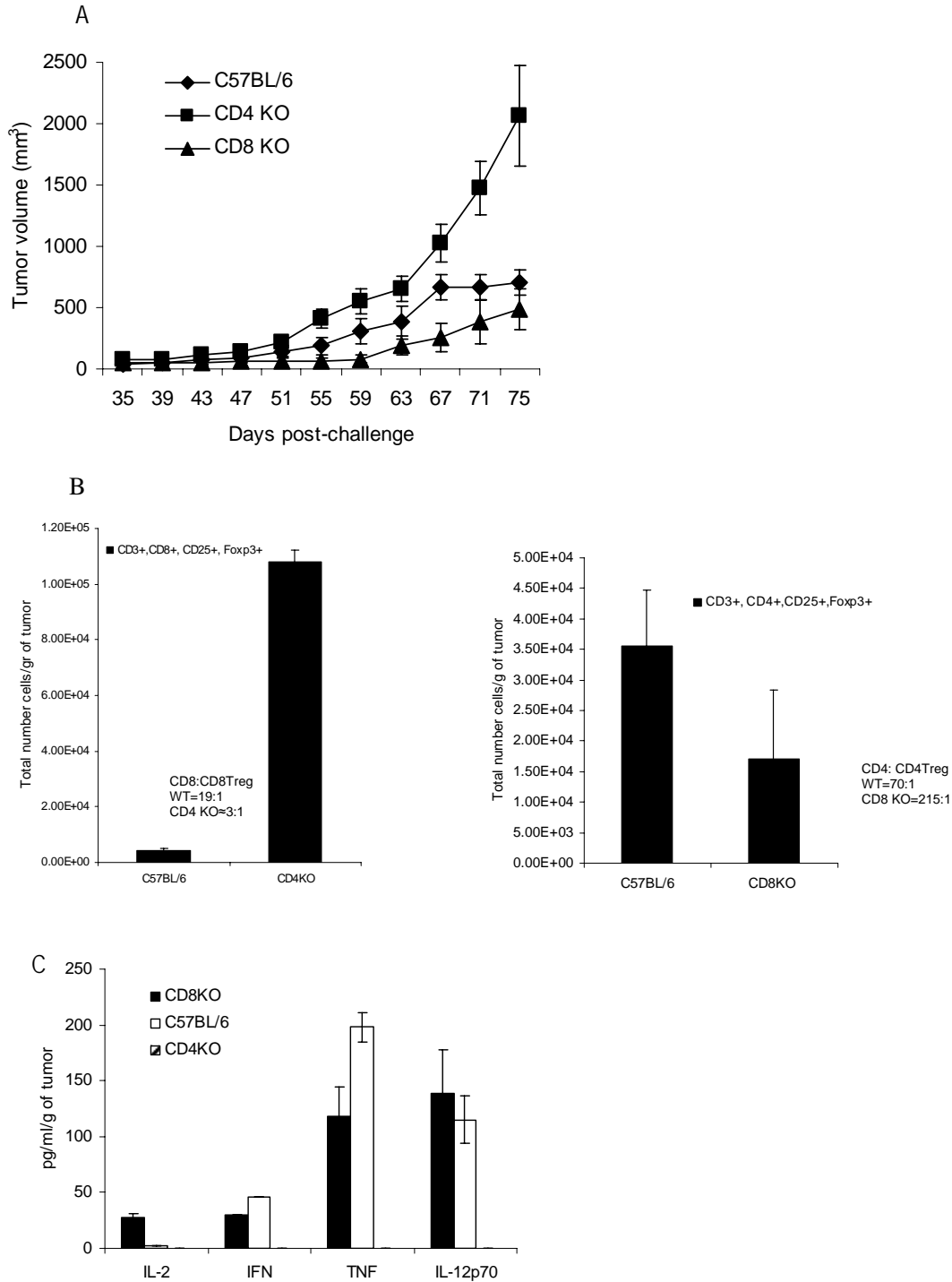


Fig 1 Role of CD4 and CD8 T cells in the control of growth of prostate tumor cells implanted subcutaneously. (A) CD4 T cells play a main role in the control of tumor growth. Groups of 10 wild-type C57BL/6, CD8, and CD4 knockout mice were vaccinated with mSTEAP-pcDNA, boosted with mSTEAP-VRP and challenged with TRAMPC2 cells. Tumor growth was monitored two times per week. Results represent the mean of ten mice \pm SE. (B) mSTEAP vaccination induces regulatory T cells. Tumors were collected, weighed and dissociated with 0.1% dispase. TILs from vaccinated C57BL/6 mice, CD4KO were

analyzed by flow cytometry to calculate the number of infiltrating CD3+, CD25+, Foxp3+ CD4 and CD8-T cells. Data are shown as total number cells/g of tumor and represent the mean of 8 mice \pm SE. C) mSTEAP vaccination induces Th1 cytokines. Tumors were collected, weighed and homogenized with a polytron. The amount of cytokines in the supernatants from tumor of vaccinated C57BL/6 mice CD8 KO and CD4KO were quantified Using a Bio-Plex HTF system, equipped with Bio-Plex manager software 4.0 Data are shown as total number cells/g of tumor and represent the mean of 8 mice \pm SE.

It seems androgens could influence tumor growth in our experimental model, because androgen ablation by castration at day 31 of unvaccinated mice caused a moderated decrease in tumor size ($p=0.041$ two tailed). In the combined scheme based on androgen ablation followed by vaccination with mSTEAP-containing DNA and VRPs, there was no statistically significant reduction in tumor growth compared with mice that only received mSTEAP vaccination ($p=0.99$, two tailed) and there was no significant increase of Th1 cytokines in tumors from sham-castrated and castrated mice (Table I). These findings suggest that the effectiveness of mSTEAP therapeutic vaccination is not augmented after androgen ablation by castration.

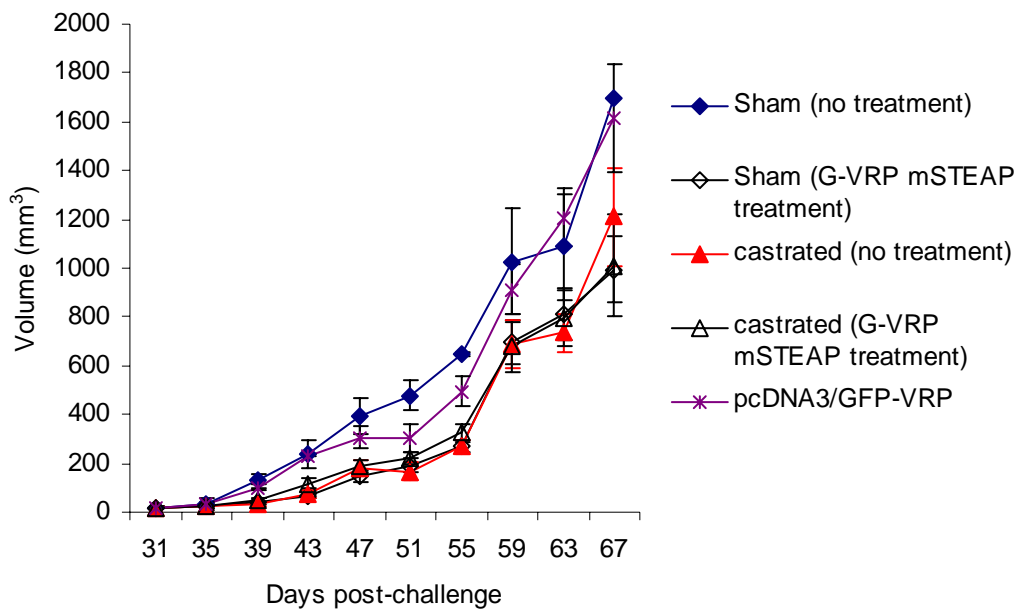


Fig 2 Androgen ablation did not enhance the effectiveness of therapeutic vaccination with mSTEAP of pre-existing tumors. Five different groups of male C57BL/6 mice were inoculated with TRAMPC2 cells. At day 31, all the mice had a palpable tumor. In one group, mice were sham-castrated and vaccinated pcDNA-mSTEAP and mSTEAP-VRPs. In control group, mice were sham-castrated and received a shot of empty vector, followed by a boost with GFP-VRP. A third group mice were sham castrated and did not receive any treatment. A four group was castrated and vaccinates with mSTEAP. A five group was castrated and vaccinated with empty vector and GFP-VRP. Tumor growth was monitored twice a week. Results represent the mean of ten mice \pm SE. One of two separated experiments with similar results is shown.

Table I

Cytokine detection in tumor from sham-castrated and castrated C57BL/6 mice vaccinated with mSTEAP

| Cytokine pg/ml/g of tumor | mSTEAP-PcDNA3/mSTEAP-VRP vaccinated sham-castrated group | mSTEAP-PcDNA3/mSTEAP-VRP vaccinated and castrated group |
|------------------------------|---|--|
| IL-2 | 0.28±0.2 | 9.8±7.3 |
| IFN | 3.9±2.54 | 5.43±5.0 |
| GM-CSF | 185±97.5 | 42.5±14 |
| TNF | 247±181.7 | 272±84 |
| IL-5 | 17.5±3.67 | 259±6.7 |
| IL-4 | 2.1±2.0 | 2.0±0.21 |
| IL-10 | 663±367 | 482±114 |
| IL-12 | 522±100 | 929±370 |

Tumors from mSTEAP vaccinated mice were homogenized and cytokine levels were quantified using the Bio-Plex mouse cytokine Th1/Th2 assay. Data represents the mean of 16 STEAP vaccinated and 4 control mice ± SE

Task 1e) To determine the effectiveness of mSTEAP vaccination in male TRAMP mice.

Based on data obtained from C57BL/6 mice, we selected as the most adequate scheme for vaccination the prime/boost (DNA/VRP) strategy for TRAMP mice. Groups of 19 young male, TRAMP mice that had developed prostatic intraepithelial neoplasia (8 weeks) were vaccinated with mSTEAP using DNA and boosted at days 15 and 60 with 10^6 IU of virus replicon particles (VRPs) containing mSTEAP mRNA. A control group was vaccinated with empty vector and boosted with GFP-VRP. We evaluated the efficacy of antigen-specific vaccination by monitoring survival. Mouse vaccinated with mSTEAP or empty vector/GFP-VRP had the same survival rate and 100% of mice developed large prostate tumor (Fig 3). In the previous report we detected mSTEAP expression in thymus, suggesting that T cells that express high or low-avidity TCRs could undergo positive and negative selection during thymic differentiation. However, there was still release of self-reactive CD8 T cells to the periphery, cells that were still able to recognize STEAP-expressing tumor cells, indicating that negative selection is not completely effective. These autoreactive CD8 T cells are controlled in the periphery by several

regulatory mechanisms performed by regulatory T cell populations. We detected CD4- and CD8 regulatory T cells in 8 weeks old, unvaccinated TRAMP mice (fig 3B). This suggests that the prime/boost vaccination scheme needs additional immunological intervention to overcome the mechanisms of central and peripheral tolerance imposed by regulatory T cells. Initially, we proposed to test mSTEAP vaccination in 8 and 12 weeks old TRAMP mice, however we did not detect any effect on survival rate after vaccination in 8 weeks old mice. Therefore, we decided to test a combined therapy based on mSTEAP vaccination and androgen ablation 16 weeks old mice. At this age, mice have developed prostate tumor. Two groups of TRAMP mice were castrated one day before vaccination.

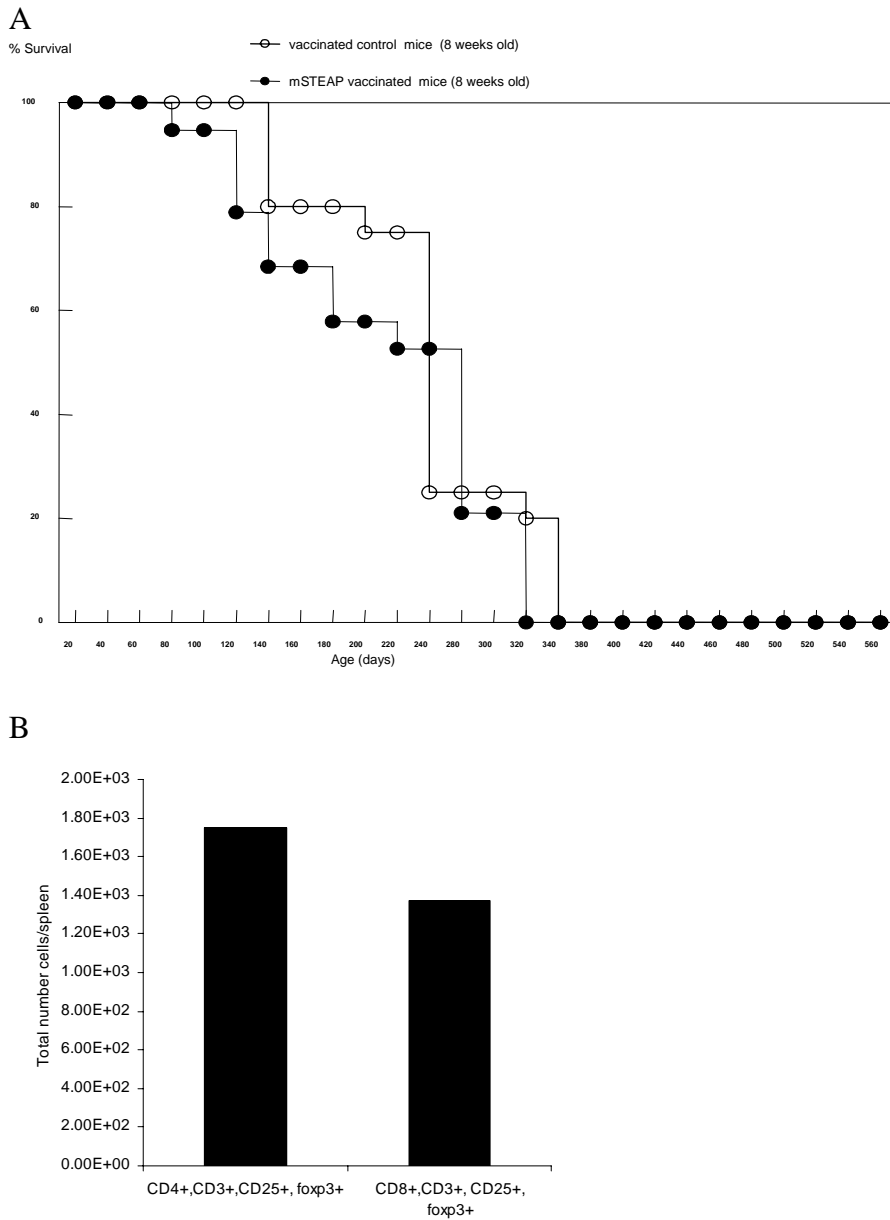


Figure 3. **mSTEAP vaccination did not control tumor protection in TRAMP mice** Male TRAMP (8 weeks old) mice were vaccinated with pcDNA-mSTEAP and subcutaneously boosted with 10^6 IU of mSTEAP-VRP. In control group mice were vaccinated and boosted with empty vector and GFP-VRPs. Tumor control was evaluated by following survival of 19 vaccinated mice. B) TRAMP

mice have regulatory T cells. Spleen cells from unvaccinated TRAMP mice were analyzed by flow cytometry to calculate the number of infiltrating CD3+, CD25+, Foxp3+ CD4- and CD8-T cells. Data are shown as total number cells/g of tumor and represent the mean of 3 mice \pm SE.

A first group of castrated mice was vaccinated with mSTEAP-pcDNA3 and boosted with mSTEAP-VRP at day 15 and 60 after castration. The control group was vaccinated with empty vector and boosted with GFP-VRP. Survival of TRAMP mice, castrated and vaccinated with mSTEAP was prolonged significantly compared with the control group ($p=0.038$) (Fig 4A). Over 440 days, only 14% of mice from control group survive, but they developed a large prostate tumor (figure 4B). Analysis of tissue section showed poorly differentiated adenocarcinoma. 86% of control mice died with large prostate tumor. In radical contrast 13 out of 21 mice vaccinated with mSTEAP had very small prostate with focal well-differentiated adenocarcinoma and large hyperplasia zones. 8 mice died but they did not have apparent prostate tumor. Tumor size and survival rate correlated with a tumor environment dominated by type 1 cytokines (table II), which play a central role in the generation of protective- and memory tumor immunity.

Given that, the final experiment to analyze whether mSTEAP vaccination induced an autoimmune disease in castrated mice and to characterize the infiltrating cells responsible for tumor regression was initiated a few weeks ago, we are unable to show these results. However we have obtained enough data to start new vaccination protocols including antibody treatments to block regulatory T cell function and suppressive functions of CTLA-4 in 8- and 12 weeks old, TRAMP mice. In addition, we are analyzing the mechanisms involved in tumor regression in older mice that will help us to design new strategies to control early neoplastic growth in our experimental model. These data will be submitted to a peer-reviewed journal as soon as we complete the experiments.

Finally it is important to mention that the data obtained and included in the progress and final report showed clearly mouse STEAP can be used as tumor rejection antigen as we proposed in Task I and part of our results have been submitted to a peer-reviewed journal as we mentioned in task II

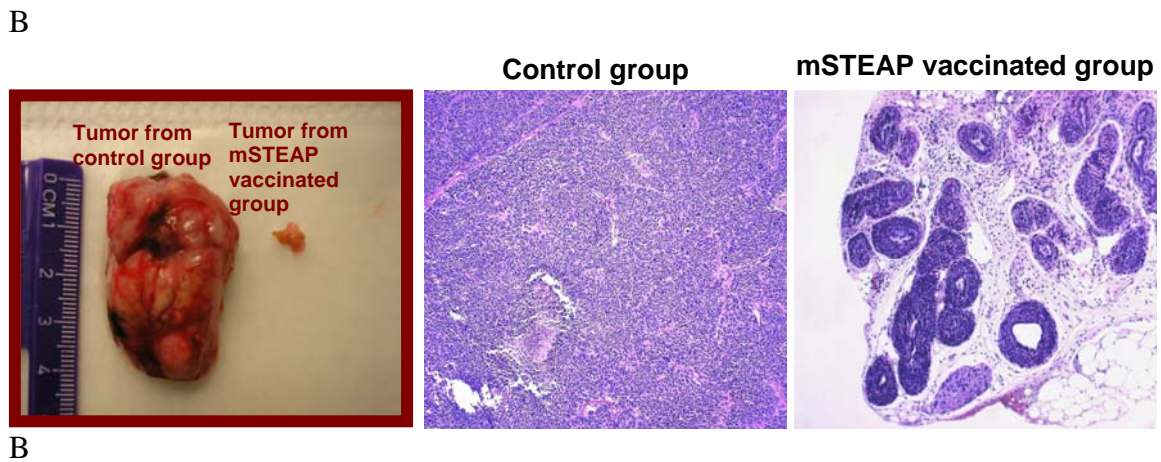
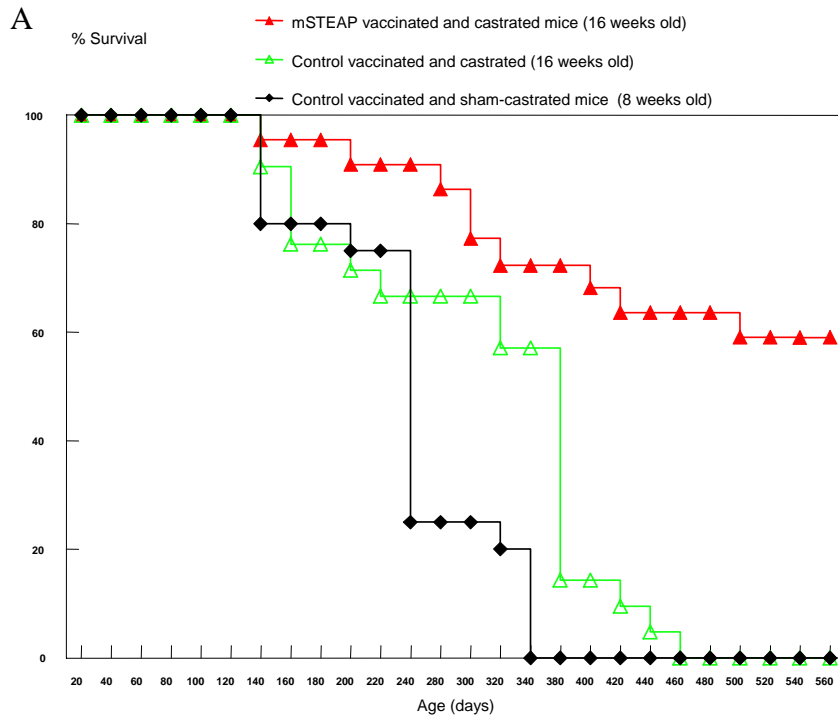


Figure 4. mSTEAP vaccination control tumor growth in TRAMP mice. Male TRAMP (16 weeks old) mice were vaccinated with pcDNA-mSTEAP and subcutaneously boosted with 10^6 IU of mSTEAP-VRP. In control group mice were vaccinated and boosted with empty vector and GFP-VRPs. Tumor protection was evaluated by following survival of 21 vaccinated mice. B) Tumor section from TRAMP mice immunized with mSTEAP or empty vector and GFP were embedded in paraffin. Sections of 5 μ m in thickness were stained with hematoxylin and eosin and the histopathological changes features were compared with tumor from control mice.

Table II. Cytokines detection in tumor from castrated TRAMP mice vaccinated with mSTEAP

| Cytokine pg/ml/g of tumor | mSTEAP-PcDNA3/mSTEAP-VRP sham-castrated vaccinated group | mSTEAP-PcDNA3/mSTEAP-VRP castrated and vaccinated group |
|------------------------------|---|--|
| IL-2 | 5066±1573 | 0 |
| IFN | 1519±1025 | 0 |
| GM-CSF | 210±91 | 0 |
| TNF | 58330±24755 | 108±2 |
| IL-5 | 78044±63507 | 6.57±5 |
| IL-4 | 9746±3564 | 0 |
| IL-10 | 7710±4212 | 132.78±8 |
| IL-12 | 8463±2346 | 76±32 |

Tumors from mSTEAP vaccinated mice were homogenized and cytokine levels were quantified using the Bio-Plex mouse cytokine Th1/Th2 assay. Data represents the mean of 16 PSCA vaccinated and 4 control mice ± SE

Key research accomplishments

- 1) Analysis of cell populations involved in prostate tumor outgrowth delay using C57BL6 mice
- 2) Effect of androgen ablation on tumor growth in C57BL/6 mice vaccinated with mSTEAP.
- 3) Successful treatment based on androgen ablation therapy and mSTEAP vaccination in prostate tumor bearing TRAMP mice.

Reportable outcomes

Manuscripts, abstracts, presentations:

Poster presentation at AAI meeting 2005, San Diego Convention Center, April 2-6 2005
Garcia Hernandez ML, Koh YT, Hubby B and Kast WM. PSCA vaccination induces a prostate cancer protective immune response in the absence of autoimmunity

Chapter book:

Koh Y, **Garcia Hernandez ML** and Kast WM. Chapter Title: Tumor Immune Escape Mechanisms. Book Title: "Cancer Drug Resistance," edited by Beverly Teicher, PhD.

Manuscripts:

Maria de la Luz Garcia-Hernandez, Andrew Gray, Bolyn Hubby and W. Martin Kast. In vivo effects of vaccination with six transmembrane epithelial antigen of the prostate: a candidate antigen for treating prostate cancer. Cancer Research, in revision

Maria de la Luz Garcia-Hernandez, Andrew Gray, Bolyn Hubby Otto J. Klinger and W. Martin Kast. PSCA vaccination induces long lasting tumor immunity in the absence of autoimmunity. Submitted for publication

Patents and licenses applied for and/or issued:

None

Degrees obtained that are supported by this award:

None

Development of cell lines, tissue or serum repositories:

None

Informatics such as databases and animal models, etc:

None

Funding applied for based on work supported by this award:

None

Employment or research opportunities applied for and/or received on experiences/training supported by this award:

Application accepted to continue working in the field of tumor immunology in the laboratory of Richard W. Dutton at the Trudeau Institute.

Conclusions.

The main goal in our project was focused to design a vaccine that augments the host immune response to prostate cancer in order to eradicate tumor cells. The hypothesis was that the presence of mSTEAP in prostate tumor cells will allow the development of a preventive and or immunotherapeutic strategy against prostate cancer. We first detected that mSTEAP is a membrane protein produced in kidney, spleen, thymus, testis and prostate. Thymic STEAP expression suggests there is an active selection of the mature STEAP specific T cells, via interaction with STEAP peptide presented on MHC complex molecules expressed on the surface of medullar epithelial cells or APCs. In order to determine whether mSTEAP can be used as a tumor rejection antigen, we tested two delivery systems and three vaccination strategies in C57BL/6 mice. In the first strategy, mice were primed and boosted with an expression vector that contains mouse STEAP (mSTEAP) cDNA by gene gun. In the second, mice were primed with mSTEAP cDNA by gene gun and boosted with Venezuelan equine encephalitis virus-like replicon particles (VRPs) encoding mSTEAP. In the third vaccination approach, mice were primed and boosted with mSTEAP-VRP. We demonstrated that no matter the scheme of treatment, there was a significant delay in tumor growth, associated with the induction of a STEAP specific CD8 T cell response. However, according to the functional activity of STEAP specific CD8 T cells, measured by a chromium release assay, the gene-VRP scheme was the best in inducing STEAP-specific, cytotoxic CD8 T cells. The data obtained from CD4- and CD8-deficient mice, also demonstrated that CD4 Th1 cells play an active role in the control of transformed cells and that a minimal negative modulation of the immune response by regulatory CD4 T cells is taking place in our model. In addition the dominance of proinflammatory cytokines such as IL-12, IL-2, TNF α and IFN γ in the tumors of C57BL/6 and CD8KO mice suggests that control of tumor growth is CD4 dependent. In therapeutic vaccination, we found a slightly delayed tumor growth that was statistically significant when compared to control mice, but it was not as impressing as to consider STEAP vaccination alone as an efficient therapy to control tumor growth after tumor is implanted and palpable. We considered that the combination of a vaccination protocol with androgen ablation therapy would have a synergistic effect, however tumor volume and cytokine production were similar in sham-castrated and castrated mice. We hypothesized that tumor location in C57BL/6 mice was important to explain why androgen androgen ablation didn't have any enhancing effect on tumor immunity. To test this hypothesis we used the combined treatment in a model of murine prostate cancer (TRAMP) mouse model, where the proliferation of prostate cell depends directly on local production of androgens. In 16 weeks old TRAMP mice, androgen ablation in combination with mSTEAP vaccination had a spectacular enhancing effect on tumor immunity, as was demonstrated by prolonged survival rate, drastic reduction in tumor volume and local production of proinflammatory cytokines, key for creating a favorable environment for tumor eradication. Although, mSTEAP vaccination did not control tumor progression in mice that have developed PIN by itself, there is still opportunity to improve our basic vaccination schemes with additional immunomodulatory tools, in order to achieve complete elimination of prostate tumors, allowing the translation of this immunotherapy into the clinical arena.

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