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14. ABSTRACT

Androgen Ablation (AA) constitutes the most common therapy for the treatment of advanced prostate cancer. While initially effective at reducing tumor burden, most patients recur with androgen insensitive disease. AA affects the immune system both systemically as well as at the prostate. Androgens have immunosuppressive effects and it is therefore of interest to investigate if immunotherapy can benefit from androgen ablation when immunosuppression is reduced. AA results in infiltration of activated T cells, dendritic cells (DCs) and macrophages into the prostate. DCs are the only antigen-presenting cells that can activate naïve T cells to a novel antigen and are the key players of successful vaccination regimes. Many DC-directed prostate cancer immunotherapy strategies are in clinical trials but there is little information with regards to the effects of androgen ablation on DC function. We have set forth to investigate the effects of androgen ablation on DC costimulation. The ultimate goal of studying the effects of AA on the immune system is to determine if a prostate-directed immunotherapy strategy can benefit from androgen ablation and we have directly tested this in an antigen-specific manner using a prostate-specific antigen, six transmembrane epithelial antigen of the prostate (STEAP).

15. SUBJECT TERMS
Prostate Cancer, Immunotherapy, Androgen Ablation, Mouse model, Clinical trial.

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Introduction

Androgens are required for the normal growth, development and function of the prostate gland and also support the growth of prostate neoplasms. Since the early 1940s, androgen ablation (AA) therapy has been a standard treatment for advanced prostate cancer after Huggins and Hodges[1] made the observation that prostate tumors are partially responsive to androgen withdrawal. AA remains a palliative approach as it does not totally eliminate all the prostate cancer cells. While initially effective at reducing tumor burden, most patients eventually develop disease refractory to androgen withdrawal. The hormone refractory stage of prostate cancer represents the terminal stage of the disease as treatment options are limited and the median survival for such patients is approximately 1 year.

Androgens exert potent immunosuppressive effects. Testosterone has been found to increase production of the immunosuppressive interleukin 10 (IL-10) by $CD4^+$T-helper (T_h) cells[2]. In addition, testosterone treatment of transgenic T cells lines during their initial antigen encounter led to the development T-helper 2 (Th_2) cells[3]. Medical castration has also been shown to reduce the percentage of $CD4^+$ $CD25^+$ T regulatory cells[4]. It is possible that when the immunosuppressive effects of testosterone are reduced during AA, anti-tumor immunity mediated by the Th_1 response will improve. Castration of mice stimulates B and T lymphopoiesis, thymic and bone marrow hyperplasia[5]. A recent report demonstrated that AA leads to the activation of thymic regeneration in both mice and men[6]. The thymic regeneration led to increased thymic emigration of T cells that restored the defects in peripheral T cell function observed in aged mice[6]. The splenic enlargement observed post AA was largely due to expansion of the B-cell population but contained more activated T cells than intact controls[7]. This finding was corroborated by Roden et al. who found that T cell proliferation following non-specific stimulation using anti-CD28 and anti-CD3 antibody treatment were augmented following AA[8]. Such findings have spurred investigators in the field of prostate cancer to investigate the possibility that AA could have a synergistic effect on prostate cancer immunotherapy. In the AA setting where testosterone-mediated immune-suppression is reduced and prostate antigens are released due to the apoptosis of androgen-dependent prostate cells or prostate cancer cells, the naïve thymic emigrants can potentially be activated through vaccination to induce auto-immune attack of prostate antigen-expressing cells.

Dendritic cells are specialized antigen presenting cells that migrate through the periphery to capture antigens, process them into polypeptides, migrate into lymphoid organs and present these antigens to lymphocytes. Depending on the level of inflammatory stimulation during antigen encounter, the DCs can prime or tolerize lymphocytes to the antigen. Inflammatory stimuli cause the DCs to mature and upregulate expression of costimulatory molecules, making them the most potent antigen presenting cells (APCs) in the immune system[9, 10]. DCs are also the only APCs that

can induce primary responses in naïve T cells[9]. DC-based vaccines, alone or in combination with other therapies are being widely tested in clinical trials for prostate cancer immunotherapy[11-16]. AA leads to infiltration of T cells, macrophages and DCs into prostate tissues in prostate cancer patients[17]. While it is known that AA augments T cells levels and responses[8], its effects on DCs has not been investigated. The aim of this project is to determine the effects of AA on DC costimulation and if AA can augment immune responses in combination with prostate cancer immunotherapy.

Body

This final report describes the conclusion of the full period of research that started in September 2002 and concentrates on the data obtained since September 2005 to August 2006. Efforts in this project have deviated slightly from the original approved Statement of work in the grant proposal. In the original Statement of work, Specific Aim 2 dealt with trying to ascertain if the combined effect of AA and CTLA-4 blockade would lead to a clonotypic expansion in the T cells that infiltrate the prostate. A report by Roden et al.[8] demonstrated that AA did not lead to a clonotypic expansion of the T cells based on TCR spectratyping similar to the tasks outlined in Specific Aim 2. In addition, obtaining TCR spectratyping data from T cells infiltrates post AA will only provide inferential evidence of an antigen-specific response within the prostate. We therefore proposed in the last progress report, to get to the ultimate aim of this proposal, which is to determine if AA will enhance a prostate-directed immunotherapy. This new direction of the proposal has been approved by the DOD as well as the removal of Aim3 of the original grant. Both Aim 1 and aim2 have now been fully executed.

In the past year, we have tested out three vaccination strategies following AA to answer the question if AA can work in synergy with prostate cancer immunotherapy to augment immune responses. In our laboratory, we have developed several prostate-antigen specific vaccines using different vaccination approaches that we intend to use to test if castration leads to an increase in the response to prostate cancer-directed vaccines. We have used the Venezuelan equine encephalitis virus replicon particle (VRP) as an RNA vector[18, 19] and the gene gun that delivers DNA intradermally in homologous and heterologous vaccination protocols to determine the optimal mode of vaccination. Our laboratory has previously identified several prostate-specific antigens such as the murine six-transmembrane epithelial antigen of the prostate (mSTEAP), murine prostate stem cell antigen (mPSCA), and murine prostate-specific membrane antigen (mPSMA)[20]to be expressed in TRAMP-C2 prostate cancer cells. These antigens can be used as potential targets for prostate cancer immunotherapy. Unlike the popular prostate–specific antigen (PSA) that is found in humans but has no known orthologue in mice, STEAP and PSCA and PSMA are found in both mice and humans. In addition, mSTEAP and mPSCA are expressed on the surface of normal and prostate cancer cells and not secreted, making them even more attractive as potential immunotherapy targets. We have used mSTEAP as the target antigen in our vaccination scheme.

Also, a manuscript describing the effects of AA on DC costimulatory function has been drafted and is awaiting the results of a final experiment before it is completed. This manuscript is attached in the appendix section and contains the data accumulated from Specific Aim 1. Items underlined within the drafted manuscript are sections that are dependent on the results of our ongoing experiments and will be finalized as outlined in the draft.

Specific Aim 1 : Task 12 Complete data analysis and manuscript reporting findings of DC studies

We have previously demonstrated in the last report (2004-2005) that DCs isolated from castrated or sham-castrated mice did not differ in their ability to support an allogeneic mixed leukocyte reaction (allo-MLR). Further analysis of the DCs isolated from the spleens and lymph nodes of castrated and sham-castrated mice was carried out in order to write a thorough report on the effect of AA on DC costimulation.

Eight to ten weeks old C57BL/6 mice were castrated or sham-castrated and their spleens and lymph nodes harvested three weeks later. DCs were isolated using a density gradient and FACs analysis carried out to compare the DCs isolated from castrated or sham-castrated mice.

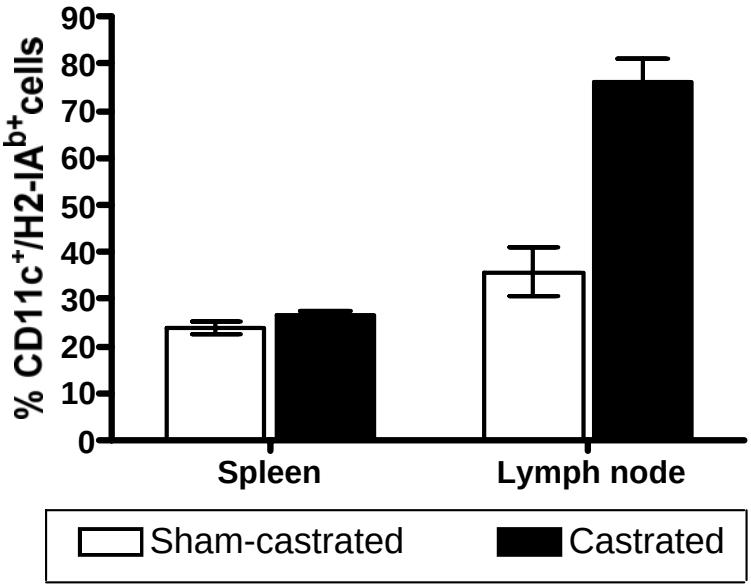


Figure 1. FACs analysis of DCs isolated from spleens and lymph nodes of sham-castrated or castrated mice.

The data show that there is no significant change in the percentage of DCs isolated from the spleens of sham-castrated or castrated mice but there was a significant increase in percentage DCs isolated from the lymph nodes ($p<0.0001$). Next, an analysis of costimulatory molecules expressed such as CD80, CD86 and CD40 was carried out on the CD11c and H2-IA^b double positive DCs.

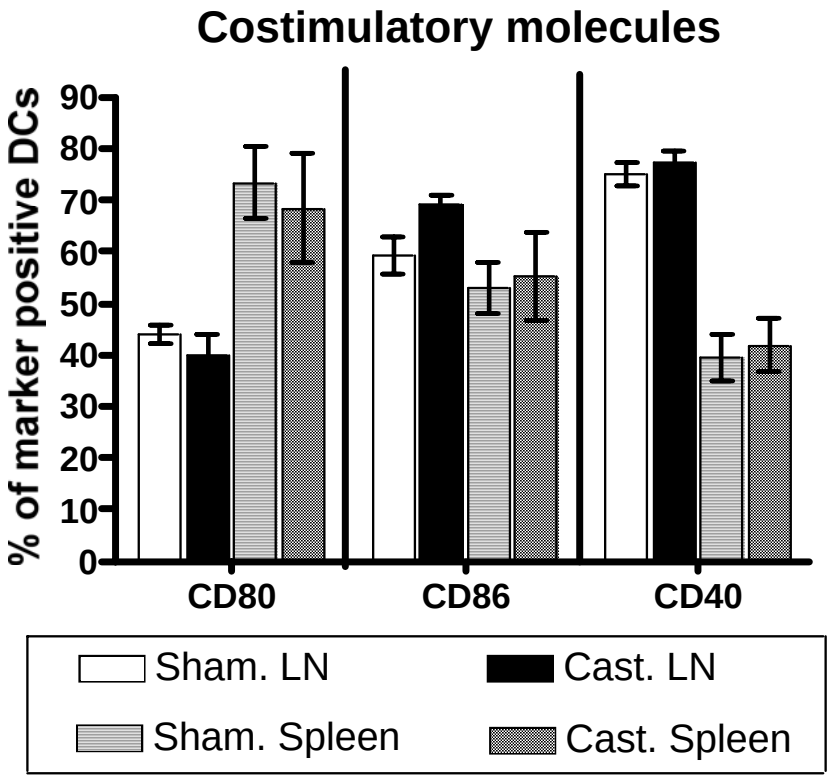


Figure 2. Analysis of costimulatory molecules expression on DCs isolated from the spleens and lymph nodes of sham-castrated and castrated mice.

There were no significant differences expression levels of CD80, CD86 or CD40 in the percentage of CD11c and H2-IA^b double positive DCs from the sham-castrated or castrated mice.

Specific Aim 2 : To determine if AA augments the immune response in a prostate-directed vaccination protocol.

Effect of AA on different vaccination protocols

Three different vaccination protocols directed at STEAP were carried out on sham-castrated or castrated mice. Two of the protocols were homologous prime-boost and were carried out with gene-gun (Gene-Gene) and VRP (VRP). The third vaccination protocol was a heterologous prime-boost with gene-gun first then VRP (Gene-VRP).

Castrated or sham-castrated C57BL/6 mice were vaccinated and then boosted with gene-gun alone, VRP alone or gene-gun then VRP. Three weeks after the boost, the mice were sacrificed and their spleens were harvested. The splenocytes were incubated with $mSTEAP_{25-35}$ (DSYSTKDSGET) or with an irrelevant peptide, PSA5(VTWIGAAPL). An ELISPOT assay was carried out to determine the numbers of mSTEAP-specific IFNγ-producing cells after subtracting the background IFNγ-producing cells present in the irrelevant peptide control wells. Control mice were vaccinated with the empty pcDNA3 vector using the gene-gun or GFP-expressing VRP.

Figure 3. ELISPOT analysis of mSTEAP-specific IFNγ-producing cells

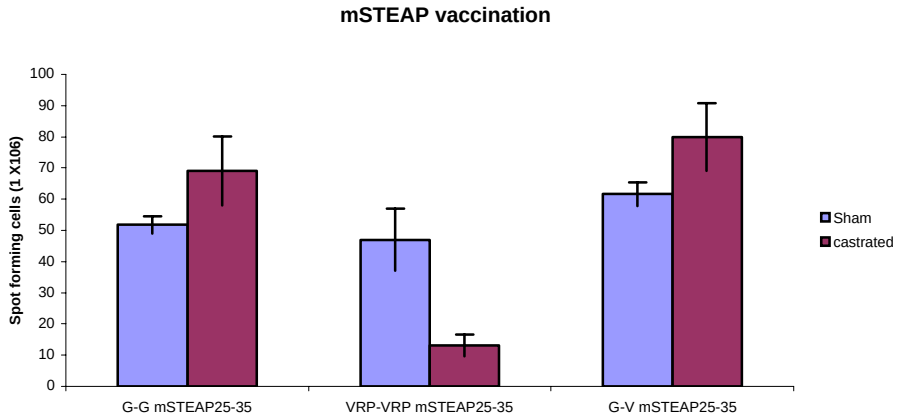


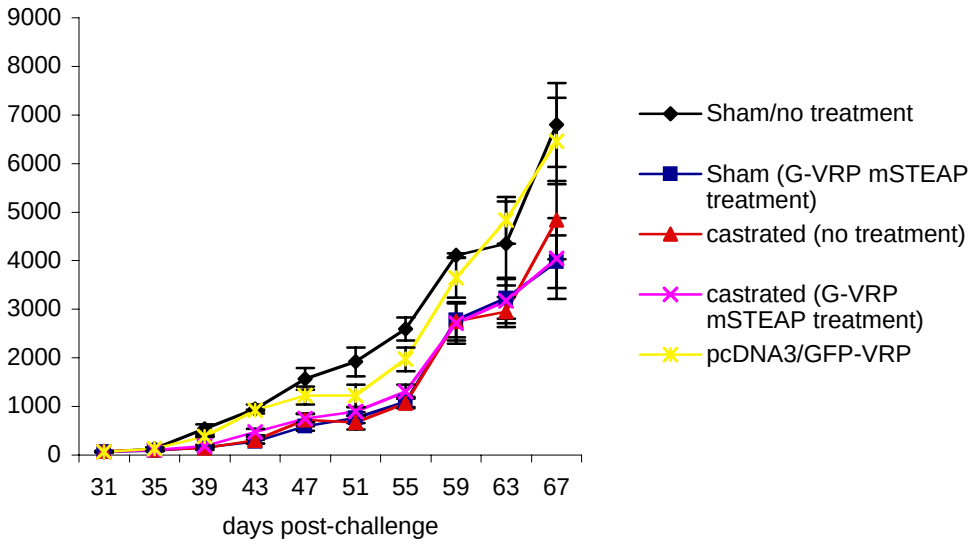
Figure 3. Effect of AA on STEAP-directed vaccination protocols

Based on the results obtained, we were able to generate an immune response against mSTEAP. Castration resulted in a slight but not statistically significant increase in the numbers of mSTEAP-specific IFNγ-producing cells for the homologous prime-boost with gene-gun alone or with the heterologous prime-boost with gene-gun and VRP. However, castration led to a reduction in the numbers of mSTEAP-specific IFNγ-producing cells in the homologous prime-boost vaccination protocol with VRP alone.

Effect of AA on a mSTEAP-targeted vaccination protocol in a prophylactic setting

Castrated or sham-castrated C57BL/6 mice were vaccinated with gene-gun and then boosted with VRP. The mice were then challenged with $5x10^5$ TRAMP-C2 prostate cancer cells and tumor growth measured over time. Control mice were vaccinated with the empty pcDNA3 vector using the gene-gun or GFP-expressing VRP.

Figure 4. Effect of AA ablation on mSTEAP-targeted heterologous prime boost vaccination with gene-gun then VRP



The data shows that castration alone without vaccination was able to delay tumor growth as well as vaccinated mice. Vaccinating the mice with mSTEAP after castration did not reduce the growth of the tumors. This shows that AA does not enhance antitumor immunity following prostate-directed immunotherapy.

Key research accomplishments

1. Tested out homologous and heterologous prime-boost vaccination strategies for mSTEAP.
2. Completed tumor challenge experiments to test out effects of AA on mSTEAP-targeted vaccinations.
3. Drafted manuscript entitled “Androgen ablation does not improve costimulatory function of dendritic cells”

Reportable outcomes

Manuscripts, abstracts, presentation:

Manuscript entitled, "Androgen ablation does not improve dendritic cell costimulatory function" drafted

Patents and licenses applied for and/or issued:

None

Degrees obtained that are supported by this award:

PhD (Molecular Microbiology and Immunology) Yi Ting Koh to be awarded in May, 2007

Development of cell line, tissue or serum repositories:

None

Informatics such as databases and animal models, etc:

None

Funding applied for based on work supported by this award:

Dr. García-Hernández was awarded a fellowship from the Department of Defense #PC041078

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

Senior postdoctoral position for Dr. Maria de la Luz García-Hernández at Trudeau Institute, NY, starting in December 2006.

Conclusions

The main hypothesis of this grant proposal was that AA promotes antitumor immunity by augmenting the antigen-presenting functions of DCs. To test this hypothesis, we have isolated DCs from the spleens and lymph nodes of castrated and sham-castrated mice and analyzed them in several ways: quantitative, costimulatory molecules expression and functionally in an allo-MLR. Although there were higher percentages of DCs found in the lymph nodes of castrated mice (Figure 1) there was no difference in the levels of costimulatory molecules expressed. In addition, the allo-MLR data shown in the September 2004-August 2005 report demonstrated no functional differences in the ability of DCs from castrated or sham-castrated mice to support an allogeneic reaction. We have since drafted a manuscript entitled "Androgen ablation does not improve DC costimulation". The manuscript at present is almost complete but we are still in the process of obtaining the results for a final experiment to determine if AA leads to a polarization of T-helper cells into a Th2 phenotype that favors humoral responses over cell-mediated immunity.

In order to determine if AA can augment the immune responses of a prostate-directed vaccine, we vaccinated C57BL/6 mice with mSTEAP using three different vaccination protocols. An immune response was generated against mSTEAP following vaccination (Figure 3). AA did not lead to statistically significant improvement in the vaccination as reflected by the numbers if IFN-γ secreting cells (Figure 3). We decided to carry out a tumor challenge experiment using the heterologous prime-boost vaccination protocol since that was the strategy that gave the highest numbers of IFN-γ secreting cells.

The final figure (Figure 4) included castrated and sham-castrated mice that did not receive mSTEAP vaccination as controls to demonstrate the effects of AA alone on the tumor growth. The results show that AA alone was able to delay tumor growth and AA in combination with vaccination against mSTEAP did not result in an increase in antitumor immunity. This leads us to conclude that AA does not augment the antitumor response in synergy with prostate-directed vaccination and to refute the original hypothesis that androgen ablation would lead to an augmentation of immune responses following vaccination. A manuscript encompassing the data obtained from Aim 1 and Aim2 has been drafted and is attached to this report as an appendix.

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Manuscript Draft

Androgen ablation does not improve dendritic cell costimulatory function

Abstract

Androgens play a critical role in the survival and proliferation of normal and neoplastic prostate cells. Androgen ablation (AA) leads to the apoptosis of androgen-dependent normal and neoplastic prostate cells. Different modalities of AA have been used for the treatment of advanced prostate cancer. However, development of hormone-insensitive disease often ensues within a few years. Immunotherapy is an emerging strategy in the treatment of prostate cancer since the prostate is not essential for survival and can be a targeted by the immune system. Androgens have immunosuppressive effects and it is therefore of interest to investigate if immunotherapy can benefit from androgen ablation when immunosuppression is reduced. Androgen ablation leads to bone marrow hyperplasia, thymic regeneration, T and B cell lymphopoeisis and restoration of age-related peripheral T cell dysfunction. Dendritic cells (DC) are the only antigen-presenting cells that can activate antigen-specific naïve T cells to a novel antigen and are the key players of successful vaccination regimes. Many DC-directed prostate cancer immunotherapy strategies are in clinical trials but there is little information with regards to the effects of androgen ablation on DC function. We have set forth to investigate the effects of androgen ablation on DC costimulation and if a prostate-directed immunotherapy strategy can benefit from androgen ablation. We find that androgen ablation does not improve DC costimulation or augment the response to a prostate-directed vaccination.

Introduction

In 1941, Huggins and Hodges reported that androgen withdrawal led to regression of prostate cancer and the alleviation of patient pain[1, 2]. This demonstrated the androgen dependence of normal prostate and prostate cancer cells for survival. Since then, androgen ablation (AA) therapy has become an important part in managing the disease and is the standard line of treatment for patients with metastatic prostate cancer. AA remains a palliative approach, as it does not totally eliminate all the prostate cancer cells. While initially effective at reducing tumor burden, most patients eventually develop disease refractory to androgen withdrawal. The hormone refractory stage of prostate cancer represents the terminal stage of the disease as treatment options are limited and the median survival for such patients is approximately one year. Prostate-targeted immunotherapy is emerging as an attractive approach compared to other treatments such as chemotherapy. The autoimmune destruction of prostate cells does not eliminate an organ essential to survival. The immune system is subject to suppression by androgens and it is of great interest to see if immunotherapy can be carried out following AA when the immunosuppression is alleviated.

Testosterone-mediated immunosuppression has been documented over the years. Testosterone has been found to increase production of the immunosuppressive interleukin 10 (IL-10) by $CD4^{+}$T-helper (T_h) cells[3]. In addition, testosterone treatment of transgenic T cells lines during their initial antigen encounter led to the development T-

helper 2 (Th_2) cells[4]. Medical castration has also been shown to reduce the percentage of $CD4^+$ $CD25^+$ T regulatory cells[5]. It is possible that when the immunosuppressive effects of testosterone are reduced during AA, anti-tumor immunity mediated by the Th_1 response will improve. Castration of mice stimulates B and T lymphopoiesis and thymic and bone marrow hyperplasia[6]. A recent report demonstrated that AA leads to the activation of thymic regeneration in both mice and men[7]. The thymic regeneration led to increased thymic emigration of T cells that restored the defects in peripheral T cell function observed in aged mice[7]. The splenic enlargement observed post AA was largely due to expansion of the B-cell population but contained more activated T cells than sham-castrated mice[8]. This finding was corroborated by Roden et al. who found that T cell proliferation following non-specific stimulation using anti-CD28 and anti-CD3 antibody treatment were augmented following AA[9]. Such findings have spurred investigators in the field of prostate cancer to investigate the possibility that AA could have a synergistic effect on prostate cancer immunotherapy. In the AA setting where testosterone-mediated immune-suppression is reduced and prostate antigens are released due to the apoptosis of androgen-dependent prostate cells or prostate cancer cells, the naïve thymic emigrants can be potentially activated through vaccination to induce auto-immune attack of prostate antigen-expressing cells.

Dendritic cells are specialized antigen presenting cells that migrate through the periphery to capture antigens, process them into polypeptides, migrate into lymphoid organs and present these antigens to lymphocytes. Depending on the level of inflammatory stimulation during antigen encounter, the DCs can prime or tolerize lymphocytes to the antigen. Inflammatory stimuli cause the DCs to mature and upregulate expression of costimulatory molecules, making them the most potent antigen presenting cells (APCs) in the immune system[10, 11]. DCs are also the only APCs that

can induce primary responses in naïve T cells[10]. DC-based vaccines, alone or in combination with other therapies are being widely tested in clinical trials for prostate cancer immunotherapy[12-17]. AA leads to infiltration of T cells, macrophages and DCs into prostate tissues in prostate cancer patients[18]. While it is known that AA augments T cells level and responses[9], its effects on DCs has not been investigated.

DC infiltration of prostate tissues following AA can facilitate the uptake of prostate antigens released during the apoptosis of androgen-dependent cells. Since DCs are the most potent APCs and are crucial to the success of immunotherapy protocols, this study aims to determine the effects of AA on DC costimulatory function. To this end, we try to answer the following questions. First, is there an immunosuppressive effect of testosterone on DCs *in vitro*? Next, does AA lead to an improvement in DC costimulatory function? Finally we want to determine if AA will augment the immune responses of a prostate-directed DC-based vaccination protocol.

Methods and materials

Mice

Eight to ten week old C57BL/6 and DBA/2 mice were purchased from Taconic (Hudson, NY).

Castration or sham surgery

Surgery was performed on mice anasthetized with intraperitoneal injection of ketamine (50-80mg/kg) and xylazine (5-10mg/kg). Using sterile techniques, an anterior/posterior incision was made longitudinally in the scrotum. The testes were pushed out and hemostatic clamps were used to clamp the vessels and the vas deferens. After a few minutes, the testes were resected and the clamps removed. The wound was closed by compression. Buprenex (0.01-0.05mg/kg) was given as an analgesic subcutaneously. Sham-castrated mice followed the same procedure, except the vessels and vas deferens were not clamped or resected. Mice were sacrificed three weeks post surgery for FACs analysis of dendritic cells or mixed lymphocyte reaction.

Multiplex cytokine assay of serum

Blood was collected from tail snips from C57BL/6 mice a day post surgery in Microtainer (Becton Dickinson, New Jersey) serum gel collection tubes. Blood was allowed to clot for 30min and spun for 2 min at 13 000 x g. Serum is then collected from the top of the tube and stored at –80ºc until use. A 23-plex mouse cytokine assay (Bio-Rad Laboratories Inc., Hercules, Ca) was carried out as per manufacturer's instructions. Briefly, a 96-well Multiscreen Resist Vacuum Manifold filter plate (Millipore, Billerica, Ma) was pre-wetted with Bio-Plex assay buffer, and multiplex beads were added to the wells. Multiplex beads were washed twice with Bio-plex wash buffer and 50µl of reconstituted standards or diluted serum samples were added to the wells. Serum samples were diluted 1:3 with mouse serum diluent (Bio-Rad Laboratories Inc.). The filter plate was incubated with gentle shaking at 300rpm for 30min at room temperature and washed 3 times with Bio-plex wash buffer. Bio-plex detection antibody was then added to the wells and incubated with shaking at 300rpm for 30min room temperature. The filter plate

was washed 3 times with Bio-plex wash buffer and 50µl Streptavidin-PE was added to each well and incubated with shaking at 10min in the dark. The filter plate was washed 3 times with Bio-plex wash buffer and resuspended in 125µl of Bio-plex assay buffer and the beads detected using the Bio-plex HTF system (Bio-Rad Laboratories Inc.) and results analyzed using the Bio-plex Manager software (Bio-Rad Laboratories Inc.).

Generating bone marrow derived DCs

C57BL/6 mice were sacrificed by cervical dislocation and their femurs and tibias removed. Bone marrow was flushed out using a 25-gauge needle and passed over a 70µm cell strainer to remove bone debris. Purified anti-mouse CD4, CD8, H2-IA$^{d/b}$, Gr-1, Ter119 and CD45RA were added to remove hematopoietic cells from other lineages and incubated for 30min on ice. All antibodies were purchased from BD-Pharmingen, San Diego, CA. Cells were washed twice with cold sterile PBS and then incubated with Low-tox rabbit complement (Cedarlane laboratories, Burlington, NC) for 1hr at 37°C. After incubation, the cells were underlaid with Lympholyte-M (Cedarlane laboratories) and spun at 10 000 x g for 10 min without brakes. The cells at the interface were collected, washed with phenol red-free Iscove's modified Dulbecco Medium (IMDM) (Gibco-Invitrogen, Carlsbad, Ca) 10% Charcoal-stripped dextran-treated fetal bovine serum (CS-FBS) (Hyclone, Logan, Utah) and plated at 2x10^5 cells in phenol red-free IMDM 10% FBS (FBS), phenol red-free IMDM 10% CS-FBS (CS-FBS), phenol red-free IMDM 10% CS-FBS 10^{-8}M dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, Mo) or phenol red-free IMDM 10% CS-FBS 10^{-8}M estradiol (Estradiol)(Sigma-Aldrich). All media were supplemented with recombinant murine GM-CSF (500U/ml)(PeproTech, Rocky Hill, NJ)

and recombinant murine IL-4 (500U/ml) (PeproTech). Medium was changed every alternate day and bacterial lipopolysaccharide (LPS) (Sigma-Aldrich) was added after the sixth day for 24 hrs to activate the cells. Cells were then collected for FACs analysis.

Spleen or lymph node cell suspension preparation for DC isolation

The inguinal, brachial, superficial cervical and deep cervical lymph nodes or the spleens were harvested from C57BL/6 mice and dissociated in 0.5mg/ml Collagenase (Sigma-Aldrich) in 5%FBS RPMI supplemented with 10mM HEPES (Gibco-Invitrogen) for 1hr at 37°C, shaking at 25000rpm. The supernatant was collected and cells were harvested by spinning at 12000 rpm, 5min.

Dendritic cell isolation by magnetic cell sorting (MACs)

Cell suspension derived from spleens or lymph nodes were counted resuspended in 400µl of MACs buffer (PBS pH 7.2, 0.5% BSA and 2mM EDTA) per 10^8 cells. 100µl of CD11c (Miltenyi Biotec, Auburn, CA, N418) microbeads were added per 10^8 total cells and incubated on ice for 15min. Cells were washed with 2ml of MACs buffer and collected by centrifugation at 200xg for 10min. The supernatant was removed by pipetting and the cells resuspended in 500µl of MACs buffer per 10^8 cells. Magnetic separation was carried out using the autoMACS separator(Miltenyi Biotec), using the Posseld program.

Dendritic cell isolation using Optiprep

The cell suspension from lymph nodes or spleens were resuspended in 3ml 5% FBS Hanks buffered salt solution (HBSS) with 5μg/ml of DnaseI (Sigma-Aldrich). 1ml of OptiPrep (Axis-shield, Oslo, Norway) was added and the suspension resuspended thoroughly. 5ml of 11.5% OptiPrep (diluted in Solution C) was layered on top of the cell suspension and then another 3ml HBSS was layered on top of the 11.5% OptiPrep. The suspension was spun at 600xg, 15min with no brakes. The DCs floating at the interface of the HBSS and 11.5% OptiPrep was collected and washed with 5%FBS-HBSS before use. Solution C was a diluent made of 0.88% (w/v) NaCl, 1mM EDTA, 0.5%(w/v) BSA, 10mM Hepes-NaOH, pH 7.4.

RNA isolation

Testes from 8-10 weeks old C57BL/6 mice, splenic DCs isolated by MACs and TRAMP-C2 cells were homogenized in TRIZol reagent (Invitrogen) and RNA isolated according to manufacturer's instructions.

Reverse transcription (RT)-PCR

RNA was digested with amplification grade DNase I (Invitrogen) for 15min at room temperature to prevent amplification from genomic DNA. 0.5μg RNA was used for oligo d(T) primed Superscript III first strand synthesis for RT-PCR (Invitrogen) for 50min at 50°C. The reaction was terminated by incubation at 85°C for 5 min and RNase H added for 20min at 37°C to remove RNA-cDNA hybrids. The RT-PCR and PCR reactions were

carried out in a Mastercycler gradient (Eppendorf, Westbury, NY). The primer pair AR-F (5'-GACCTTGGATGGAGAACTACTCCG-3') and AR-R (5'-GGTTGGTTGTTGTCATGTCCGGC-3') spanning 511 nucleotides of the androgen receptor DNA-binding domain was used for the PCR amplification of the androgen receptor. The primer pair GAPDH 5' (5'-TGAAGGTCGGTGTGAACGGATTTGGC-3') and GAPDH 3' (5'-CATGTAGGCCATGAGGTCCACCAC-3') were used to amplify murine GAPDH as a housekeeping gene control. A hot start of 94°C for 1 min was carried out, followed by 35 cycles of 94°C for 1 min, 57°C or 60°C for 1 min 30s for AR or GAPDH, and 72°C for 1 min. A final 10min at 68°C was carried out and the reaction was held at 4°C. PCR fragments were separated in 2% Tris borate-EDTA gels and visualized using a Quantity-One Imager (Bio-Rad laboratories, Inc.).

Splenic lymphocyte isolation by Lympholyte-M gradient

Spleens from the appropriate strain of mice harvested and mashed with the base of a syringe. The cells were passed through a 70μm cell strainer and collected by centrifugation at 12000rpm for 5 min. The cells were treated with ammonium chloride/potassium (ACK) lysing buffer (0.15mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2) to lyse the red blood cells. The cells were suspended in 5ml of 5%FBS-HBSS pH 7.4, layered on top of 5ml of Lympholyte-M (Cedarlane laboratories) and centrifuged at 15000xg for 20min without brakes. The lymphocytes at the interface were collected and washed twice with 5%FBS-HBSS pH 7.4 before use.

Flow cytometry

Cells were stained with P.E-conjugated anti-CD8 (53-6.7), P.E-conjugated anti-CD4 (L3T4), APC-conjugated anti-CD3e (145-2C11), PE-CY7-conjugated anti-CD11c (HL3), PE-conjugated anti-CD40 (3/23), PE-conjugated anti-CD80 (16-10A1), PE-conjugated anti-CD83 (Michel-19), PE-conjugated anti-CD86 (GL1) or FITC-conjugated anti-H2-IA^b (AF6-120.1) and gated based on lymphocyte or dendritic cell population according to the logarithymic forward and side scatter patterns. All antibodies were purchased from BD-Pharmingen. Flow cytometry was carried out using a Cytomics FC500 (Beckman Coulter, Fullerton, Ca) and analyzed with CXP analysis software (Beckman Coulter).

Mixed lymphocyte reaction proliferation assay

DCs isolated from the lymph nodes of castrated or sham-castrated C57BL/6 mice using the OptiPrep discontinuous gradient were irradiated at 3000 rads and cocultured with lymphocytes isolated from DBA/2 mice using Lympholyte-M at a stimulator: responder ratio of 1:10 for 6 days in 20% dextran-treated charcoal-stripped FBS-IMDM in 96-well round-bottom plates. 1μCi of ^{3}H-Thymidine was added to each well on the 5th day and pulsed for 15 hrs. The cells were harvested using the Packard Filtermate harvester (Perkin-Elmer, Boston, Ma) and left to dry overnight. 25μl of Microscint20 (Perkin-Elmer) was added and ^{3}H-Thymidine incorporation measured using the Packard Topcount NXT microplate scintillation and luminescence counter (Perkin-Elmer).

Preparation of DNA plasmid for vaccination

Competent TOP10 *Escherichia coli* cells were transformed with pcDNA3-STEAP or the empty vector, pcDNA3, using heat shock and plated on ampicillin (100μl/ml)/LB

plates at 37^{o}C overnight. Colonies were randomly selected and grown in LB media containing 10% ampicillin. Colonies containing plasmid sequences verified by DNA sequencing were grown in LB media containing 10% ampicillin. DNA was prepared using EndoFree Maxi Prep kits (Qiagen, Valencia, CA).

DNA-gold preparation for gene gun vaccination

DNA-coated gold particles were prepared as follows: 2.5mg of 1.0μm gold microcarrier (Bio-rad, Hercules, Ca) were resuspended in 100μl of 0.05M spermidine (Sigma) and sonicated. 50μg of pcDNA3-STEAP or the empty vector, pcDNA3 were added to the microcarrier and this mixture was precipitated by the addition of 1.0M $CaCl_2$ (100μl) for 10min. The precipitate was washed 3 times with brand new absolute ethanol and resuspended in 3ml of 0.1mg/ml polyvinylpyrrolidone (Bio-rad) dissolved in absolute ethanol. The DNA-coated gold was then loaded into the tubing and allowed to settle for 3 min. Ethanol was gently drawn out and the tubing was dried by flowing nitrogen gas at a rate of 0.3L/min. The tubing was cut into 0.5inch pieces, placed into cartridges and stored at 4°C until use.

VRP preparation

The procedures used for making viral replicon particles (VRP), based on a two-helper system as described previously[19] are described in detail in U.S. Patent No. 7,078,218. In brief, RNA transcripts for the replicon and two helper RNAs, encoding for the capsid and glycoproteins, were transcribed in-vitro from linearized plasmids using a T7 RiboMax kit (Promega, Madison, WI) as per manufacturer's instructions. RNA were purified using RNeasy purification columns (Qiagen) as per manufacturer's instructions.

Vero cells ($1x10^8$) suspended in PBS were combined with 30μg of replicon and each helper RNA in 0.4cm electroporation cuvettes and electroporated using a Bio-Rad Gene Pulser (Bio-Rad). The cells and RNA were pulsed four times with the electroporator set at 590 volts and 25μFarads. Electroporated cell suspensions were seeded into individual roller bottles containing 150ml of OptiPro medium (Invitrogen) supplemented with antibiotics and incubated at 37°C in 5%CO_2 for 16-24hr. VRP were harvested and the titers of the VRP determined by immunofluorescence assay using goat anti-V22 nsP2 specific polyclonal antiserum as the primary antibody and donkey anti-goat Alex Fluor 488 (Invitrogen) as the secondary antibody on methanol fixed cells using a Nikon Eclipse TE300 fluorescence microscope. The VRP were tested for the presence of contaminating replication competent venezuelian equine encephalitis virus (VEE) using two blind passages of Vero cells. Briefly, $1x10^8$ VRP were used to infect Vero cells in $75cm^2$ flasks (MOI=0.5) for 1hr. The VRP inoculum was removed, the cell monolayers were washed with PBS and 35ml of fresh media was added to each flask. The flasks were then incubated at 37°C for 24hr. After incubation, the first pass media was collected and used to inoculate fresh $75cm^2$ flasks of Vero cells for 1hr. The first pass media was then removed, 35ml of fresh media added to each flask and the flasks incubated for an additional 72hr at 37°C. After this second pass of media, the flasks were inspected for the presence of cytopathic effects. The absence of cytopathic effects in second pass flasks was deemed to indicate the absence of replication competent VEE. VRP were purified by affinity chromatography using HiTrap Heparin HP columns (Amersham Biosciences), resuspended in an isotonic PBS with 1% mouse serum (formulation buffer) and stored at -80°C until use.

Immunization and tumor challenge

Groups of ten male C57BL/6 mice received gene gun vaccination on the same day they underwent orchiectomy or sham-surgery during the anesthesia period. DNA-gold particles were delivered to a shaved are of the abdomen using a helium-driven gene gun (Bio-Rad) with a discharge pressure of 400psi. Each mouse received 2ug of pcDNA3-mSTEAP DNA vaccine or the pcDNA3 empty vector as control. Fifteen days after gene gun vaccination, the mice were subcutaneously boosted at approximately 1cm from the base of the their tails with 10^6 infectious units (IU) of mSTEAP-VRPs or GFP-VRP as controls. Ten days after the boost, the mice that only underwent orchiectomy or sham-surgery, and the mice that underwent orchiectomy or sham-surgery that were also vaccinated with mSTEAP or control vaccines were challenged with $5x10^5$ TRAMP-C2 cells, resuspended in HBSS (Sigma). Tumor volume was measured twice a week using an engineer caliper.

Statistical analysis

Graphs and statistical analysis was carried out using the Prism software (Graphpad). A one-tailed paired t-test at 95% confidence interval was used.

Results

Dendritic cells express androgen receptor

The expression of the androgen receptor by DCs was investigated in order to determine if AA could directly or indirectly affect DCs. Unlike human DCs that vary in their

expression of CD11c, all subtypes of mouse DCs express CD11c[20, 21], facilitating their purification based on CD11c. Using MACs-purified dendritic cells from spleens of C57BL/6 mice, androgen receptor transcript expression was detected by RT-PCR (Figure 1). Protein expression has to be confirmed by immunofluorescent staining of the lymph nodes to show co-staining of AR with DEC205^{+} cells (Going to be Figure 2).

Effect of dihydrotestosterone on bone marrow-derived DCs

In order to determine if testosterone has any direct immunosuppressive effects on the growth or activation of DCs, bone-marrow (BM) derived DCs were generated *in vitro* in the absence or presence of testosterone. Phenol-red free media was used because phenol-red has mild estrogenic effects *in vitro*[22] and estrogens promote the differentiation of DCs from bone marrow precursors[23]. BM-derived DCs were cultured in phenol-red free medium with normal fetal calf serum (FBS), hormone-deficient charcoal dextran treated FBS (CS-FBS), CS-FBS with dihydrotestosterone (DHT) or CS-FBS with estradiol. Culturing the BM-derived DCs in hormone-deficient medium enables the delineation of the effects of DHT, an androgen or estradiol, an estrogen, when compared to FBS that contains both estrogens and androgens. After six days, the BM-derived DCs cultured in the different media were stimulated with bacterial lipopolysaccharide (LPS) for 24hrs and FACs analysis carried out on the percentage of DCs obtained from the BM precursors. From the data obtained (Figure 3), growing and activating BM-derived DCs in a hormone-deficient medium using the CS-FBS resulted in a reduction in the percentage of CD11c and H2-IA^{b} double positive DCs compared to medium containing normal FBS. Addition of either DHT or estradiol to the CS-FBS-containing medium

restored the percentage of CD11c and H2-IA^b double positive cells to that observed in medium containing normal FBS. Overall these data show that testosterone does not suppress the growth or activation-induced proliferation of BM-derived DCs.

In addition to the growth of DCs, expression of costimulatory and maturation markers on the DCs was also assessed before (Figure 4a) and after (Figure 4b) LPS stimulation in order to determine if testosterone affected the differentiation of DCs. CD80, CD86 and CD40 are costimulatory molecules that are upregulated upon DC activation and CD83 is a DC maturation marker that enhances it costimulatory activity[24]. When the BM DCs were cultured in hormone-deficient conditions (CS-FBS), there was a trend of slightly lower expression levels of CD80, CD83, CD86 and CD40. DCs express the estrogen receptor and estrogens have been shown to promote the differentiation of DCs from BM precursors[23]. The levels of CD80, CD83, CD86 and CD40 were increased in the presence of estradiol before and after LPS stimulation the cells cultured in normal FBS. In the presence of DHT, the expression levels of the costimulatory molecules were usually close to that of the BM DCs cultured in normal FBS, with the exception of lower CD83 expression post LPS stimulation ($p=0.0015$). Since lower CD83 levels can lead to impaired costimulation, we decided to analyze DCs *ex vivo* following AA to determine the effects of AA on the costimulatory marker expression levels.

Ex vivo analysis of AA on DCs

Spleen and lymph node cell suspensions from castrated or sham-castrated eight to ten weeks old C57BL/6 mice were analyzed for DC numbers and levels of costimulatory

molecule expression three weeks post surgery. The cells were gated based on CD11c expression and assessed for H2-IA^b and CD80, CD86 or CD40 staining. We observed an obvious increase in the size of the spleen and lymph nodes three weeks after androgen ablation. The percentage of DCs in the spleens of castrated and sham-castrated mice were not significantly different (Figure 5a) but there was a significant increase in the percentage of DCs in the lymph nodes. Next, the DCs were stained for costimulatory markers such as CD80, CD86 and CD40. There was no difference in the percentages of marker positive DCs in castrated or sham-castrated mice (Figure 5b). (Have to stain for CD83 to check if there is a difference) Therefore the *ex vivo* analysis of the DCs from castrated or sham-castrated mice showed that although there were higher percentages of DCs in the lymph nodes of castrated mice, there were no differences in the levels of costimulatory molecules expressed. This demonstrates that AA leads to an increase of DCs homing to the lymph nodes but does not change their level of costimulatory molecule expression. (If there is an increase in CD83 staining in the castrated mice due to AA, then we can suggest that androgens can suppress DC function through CD83 downregulation and AA results in alleviation of this suppression and can possibly improve DC costimulatory function. And then we test this functionally through the MLR, which says that it doesn't. And if there isn't an increase in CD83 staining, then we can say that although we saw the effect in vitro but we couldn't find it in vivo.)

Functional analysis of DCs post AA

In order to functionally demonstrate the differences in the costimulatory capacities of DCs from castrated or sham-castrated mice, a mixed lymphocyte reaction (MLR) was

carried out. The allo-MLR measures the proliferation of T cells in response to allogeneic antigens encoded by the major histocompatibility complex and the proliferation is enhanced by the costimulatory capacities of allogeneic stimulator cells. It was shown (Figure 6a) that irradiated DCs from castrated or sham-castrated mice did not differ in their ability to support an alloreactive T cell response. To increase the probability of detecting age-related differences in the immune response, the allo-MLR was also carried out using older, seven to nine months old C57BL/6 mice (Figure 6b). Again, there was no difference in the proliferation response of the allo-reactive T cells. These data show that there was no functional improvement in the costimulatory ability of the DCs post AA in young or middle-aged mice (despite a possible increase in CD83 levels post AA).

Effects of AA on serum cytokine/chemokine levels

A multiplex cytokine assay was carried out on the serum from castrated and sham-castrated mice a day post surgery in order to determine the changes in cytokine or chemokine milieu resulting from AA. (Further analysis of serum cytokine levels at other time-points needs to be repeated, but has so far shown no differences) There was no skewing in the Th1 versus Th2 (Figure 7a) cytokines between the castrated or sham-castrated mice. Inflammatory cytokines such as IL-1a, IL-1b and IL-6 were slightly higher in the castrated mice (Figure 7b). However, there was a four-fold increase in the neutrophil attractant, keratinocyte chemoattractant (KC)-chemokine and granulocyte colony-stimulating factor (G-CSF) levels in castrated mice (Figure 7c).
G-CSF can mobilze DCs that polarize T cells to a Th2 response. To test this, I will co-incubate DCs from castrated and sham-castrated mice with CD4+ T cells and collect the

supernatants and test them using a Th1/Th2 multiplex cytokine assay kit to see if there was a skewing in the T-helper response towards the Th_2 phenotype.

Effect of AA on prostate cancer immunotherapy

In order to definitively demonstrate that AA does not augment prostate cancer immunotherapy when carried prior to vaccination, sham-castrated and castrated mice were vaccinated using a heterologous prime-boost vaccination strategy targeted at the six transmembrane epithelial antigen of the prostate (mSTEAP) (Figure 8). The data shows that castration alone without vaccination was able to delay tumor growth as well as vaccinated mice. Vaccinating the mice with mSTEAP after castration did not reduce the growth of the tumors and may actually aborogate the delay in tumor growth induced by vaccination. This shows that AA does not enhance antitumor immunity following prostate-directed immunotherapy. We intend to vaccinate before or after AA in order answer the ultimate question, which is, when should vaccination be carried out, relative to AA.

Discussion

DCs are the most potent activators of the immune system. Many current immunotherapy clinical trials for prostate cancer involve the re-infusion of autologous DCs either transfected or loaded with prostate cancer associated antigens[13]. In prostate cancer patients, AA resulted in T cell, macrophages and DC infiltration of the prostate

tissue[18]. AA is the standard line of treatment for patients with advanced prostate cancer but the effects of AA on DCs or vaccine approaches have not been reported.

The gender dimorphism in susceptibility to autoimmune diseases has been attributed to the dampening effects of testosterone on the immune system and the ability of estrogens to enhance immune reactivity[25]. Androgens have been shown to directly stimulate the secretion of IL-10 from $CD4^+$ T lymphocytes[3]. IL-10 inhibits the proliferation of T-helper 1 (Th_1) cells and enhances humoral responses[26]. IL-10 can also dampen the global immune response by reducing the levels of costimulatory molecules expression on DCs and the production of proinflammatory cytokines[27]. Androgens accelerate thymocyte apoptosis[28] and AA leads to thymic regeneration in mice and humans[7, 29]. Chemical castration leads to a reduction on $CD4^+CD25^+$ T regulatory cells that suppress the immune response and an increase in the percentage of natural killer cells[5] that can mediate anti-tumor immunity. AA also leads to an increase in B and T cell lymphopoiesis [6, 8, 30]. Therefore, androgens have potent immunosuppressive effects and AA results in an alleviation of the immunosuppression and the induction of naïve T cells into the periphery. The finding that AA also activates thymic regeneration in mice and humans and restored the defects in peripheral T cell function observed in aged mice[7] has prompted many to hypothesize that AA might be able to improve prostate cancer immunotherapy. This report deals with the effects of AA on DCs and if prostate cancer immunotherapy could benefit from the reduction of the immunosuppressive effects of testosterone following AA.

We show that the androgen receptor is expressed by DCs and therefore, DCs can be a target of testosterone-mediated immunosuppression (Figures 1 and 2). BM-derived DCs generated in medium containing CS-FBS with DHT had slightly lower levels of CD83 expression when activated LPS (Figure 4b). Lower expression of CD83 was reportedly correlated with an impaired MLR stimulatory activity in herpes simplex virus type 1-infected DCs even though they express similarly high levels of costimulatory markers such as CD80 and CD86 as uninfected cells[31]. This shows that DHT was capable of mediating an immunosuppressive effect on BM-derived DCs. *Ex vivo* analysis of freshly isolated DCs from the spleens and lymph nodes of castrated and sham-castrated mice showed that AA led to an increase in DC numbers in the lymph node (Figure 5a). ??? Is there going to be a change in the CD83/ maturation status of the DCs???

However, DCs from castrated mice did not have enhanced stimulatory capacity compared to sham-castrated mice (Figure 6a). Peripheral T cell function declines with age due to thymic atrophy due to a reduction in the export of naïve T cells, known as recent thymic emigrants[32, 33]. Consequently, homeostatic proliferation compensatory mechanisms lead to an increase in the memory T cells population[34, 35] resulting in a narrowing of TCR repertoire[36] and diminished responses to new or previously encountered antigens[37]. Following AA, thymic regeneration leads to export of naïve T cells that restore the defects in peripheral T cell function. Little information is available with regards to the impact of ageing on DC function. In order to detect possible age-related differences in the costimulatory capacity of DCs, we repeated the MLR using middle-aged mice. Again, there were no significant differences in the ability of the DCs

to stimulate allo-reactive T cells in a MLR (Figure 6b). This shows that AA does not lead to a functional improvement in DC costimulation. In addition, DCs derived from peripheral blood mononuclear cells from old (>65 years) were just as able to stimulate proliferation and cytokine production of T cells as younger donors (<30 years)[38]. It could be that DC function does not decline with age and may not be as affected by the alleviation of testosterone-mediated immunosuppression as the T cells.

Serum levels of KC-chemokine and G-CSF increased four-fold in castrated mice a day post AA. KC-chemokine, a member of the C-X-C family of chemokines is a potent neutrophil attractant[39] and G-CSF is a hematopoietic growth factor that stimulates the growth of granulocytes[40]. G-CSF also promotes neutrophil survival, proliferation and differentiation[41, 42]. G-CSF has been used to mobilize peripheral blood monocytes to derive DCs for the purpose of cancer vaccine therapy[43] but these G-CSF-mobilized DCs were shown to polarize naïve T cells to differentiate to a Th_2 phenotype[44-46]. Anti-tumor immunity is mediated by Th_1 cells that help drive the CTL response. Thus, we decided to investigate if AA leads to polarization of $CD4^+$ T helper cells into Th_2 cells. If we do see a skewing then, this would explain why AA did not improve a Th1-dependent anti-tumor response. If the T cell response has been skewed towards a Th_2 phenotype, then it would account for the inability of AA to enhance the effects of a Th_1-dependent vaccine.

Despite the lack of differences in DC costimulatory capacities between castrated and sham-castrated mice, we decided to carry out a prostate antigen-targeted vaccination

strategy post AA to determine if the thymic regenerative effects of AA could improve the immunotherapy (Figure 8). From the data obtained, vaccinated mice had smaller tumors than the control mice but there was no significant improvement in the survival of mice that were castrated then vaccinated over the mice that were sham-castrated and vaccinated. This shows that AA does not improve prostate cancer immunotherapy.

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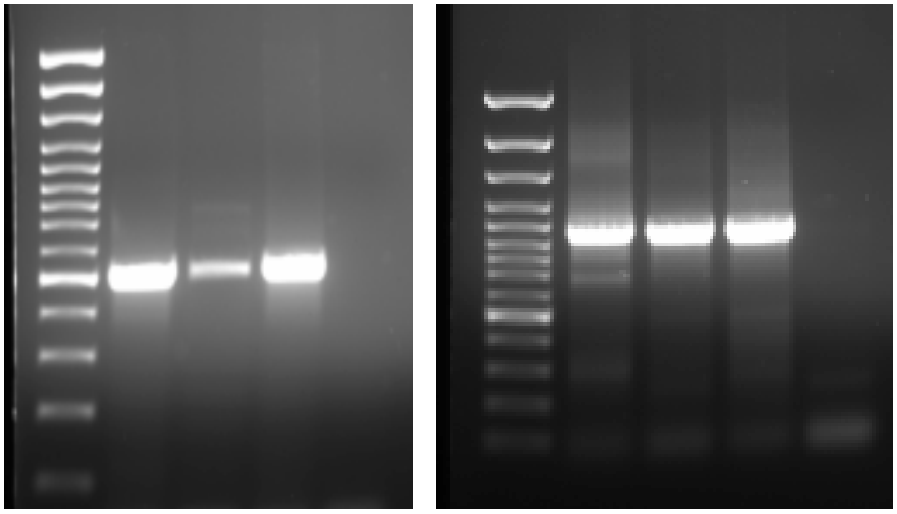
Figures

Figure 1. RT-PCR analysis of androgen receptor (Fig. 1a) and GAPDH (Fig. 1b) expression



Lane 1 : Testes
Lane 2 : Splenic DCs
Lane 3 : TRAMP-C2 prostate cancer cell line
Lane 4 : Water control

Figure 2. Immunofluorescent staining of LN DCs (Incomplete)

Figure 3. Effects of hormones on DC growth
Bone marrow-derived DCs were cultured in phenol red-free IMDM 10% with 10% FBS (FBS), CS-FBS (CS-FBS), 10% CS-FBS 10^{-8}M DHT (DHT) or 10% CS-FBS 10^{-8}M estradiol (Estradiol). LPS was added six days later for 24 hrs to activate the DCs and cells were collected for FACS analysis. Control cells were not treated with LPS. DHT did not affect the growth or activation-induced proliferation of bone marrow-derived DCs.

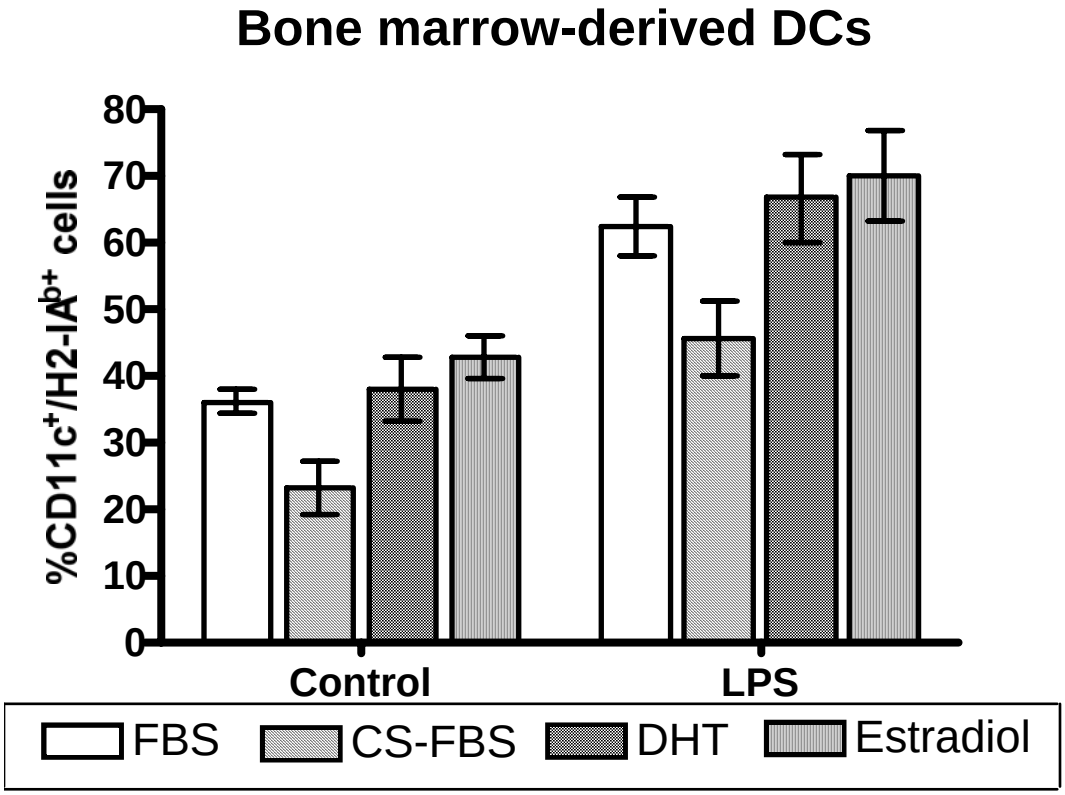


Figure 4. Effect of hormones on costimulatory marker expression
Bone marrow-derived DCs were cultured in phenol red-free IMDM with10% FBS (FBS), 10% CS-FBS (CS-FBS), 10% CS-FBS 10^{-8}M DHT (DHT) or 10% CS-FBS 10^{-8}M estradiol (Estradiol). LPS was added six days later for 24 hrs to activate the DCs (Figure 4b) and cells were collected for FACS analysis. Control cells were not treated with LPS (Figure 4a). DHT led to slightly lower (p=0.0015, one tailed t-test) expression of CD83 after LPS stimulation compared to cells grown in FBS (Fig. 4c).

Figure 4a.

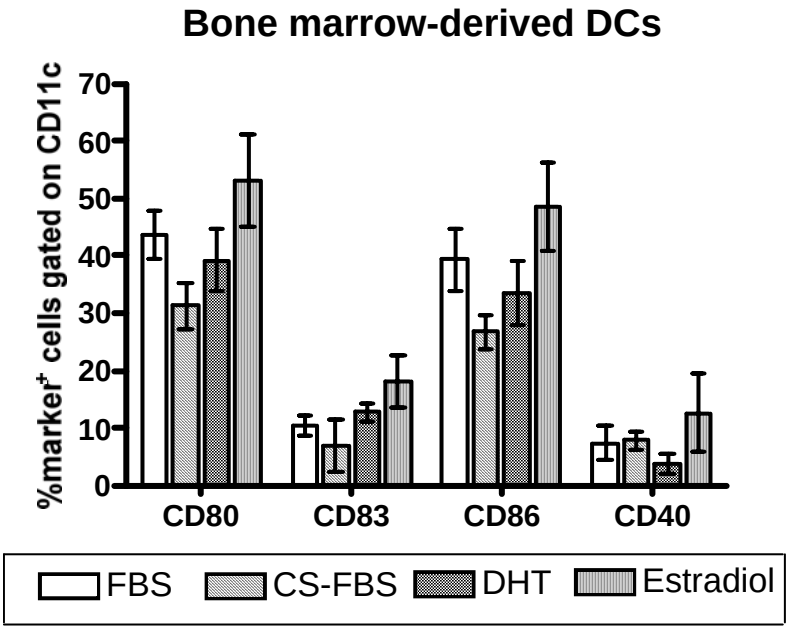


Figure 4b.

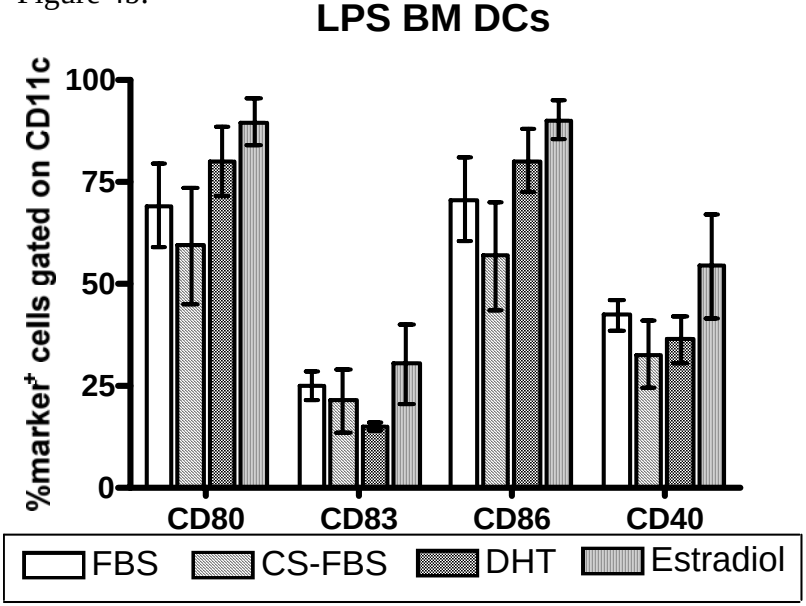


Figure 4c.

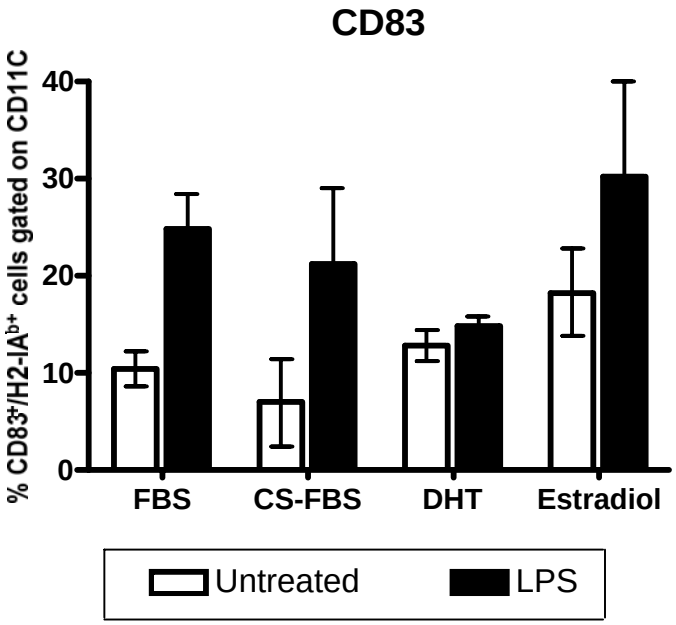


Figure 5. *Ex vivo* analysis of DCs post AA
DCs were isolated from the spleen and lymph nodes from castrated or sham-castrated C57BL/6 mice three weeks post surgery and analyzed using flow cytometry. There was an increase in CD11c and H2-IA^b double positive cells in castrated mice (Figure 5a). However, there were no differences in the levels of CD80, CD86 or CD40 expression (Figure 5b).

Figure 5a.

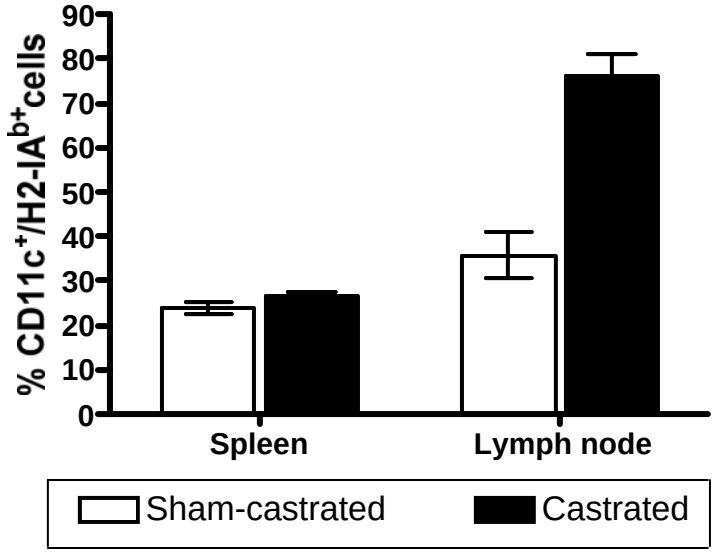


Figure 5b.

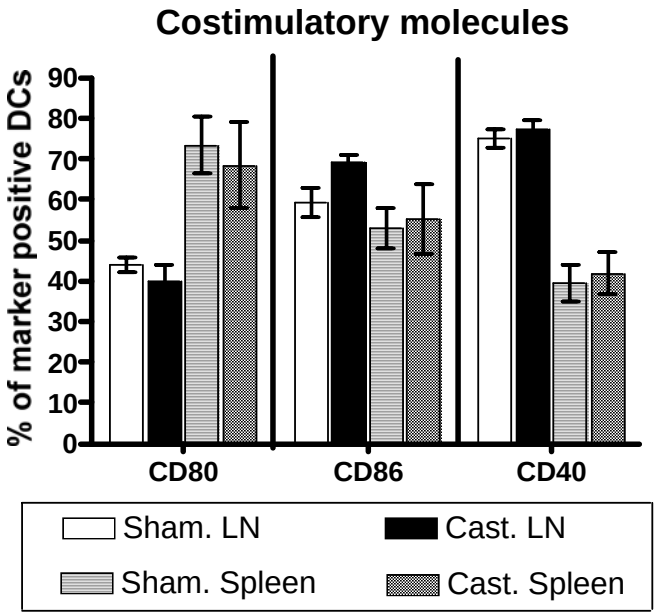


Figure 6. AA does not improve costimulatory function of DCs.
DCs were isolated from the lymph nodes of sham-castrated or castrated eight to ten weeks (Figure 6a) or seven to nine months old C57BL/6 mice three weeks post surgery. The DCs were irradiated and used as stimulators in a mixed leukocyte reaction with the lymphocytes isolated from eight to ten weeks old DBA/2 mice at a 1:10 stimulator : responder ratio. Scatter dot plots show the proliferation for each individual mouse. The mean of each group is shown as a bar with standard error of mean.

Figure 6a.

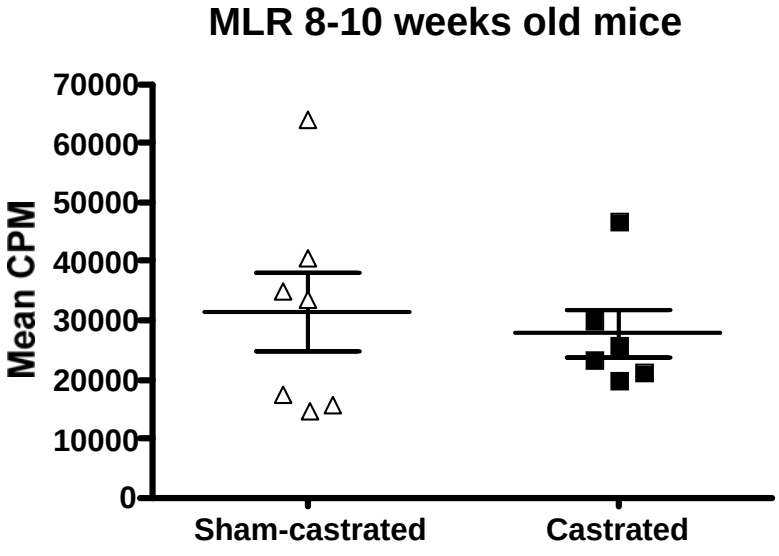


Figure 6b.

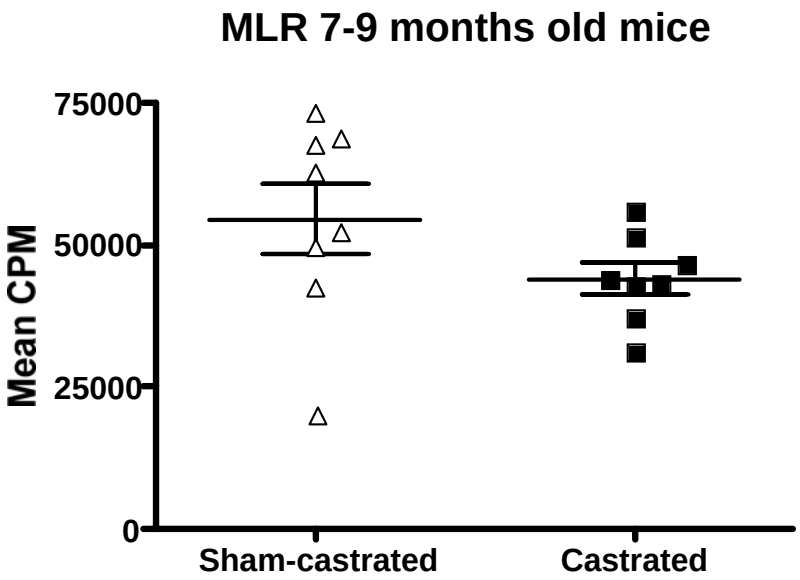


Figure 7. Effects of AA on serum cytokine/chemokine levels
Serum was collected from sham-castrated and castrated C57BL/6 mice a day post surgery and a multiplex-cytokine assay was carried out to determine if there were changes in cytokine/chemokine levels between sham-castrated and castrated mice. There were no significant differences in the Th1 versus Th2 cytokines (Figure 7a). Proinflammatory cytokines such as IL-6, IL-1a and IL-1b were slightly elevated in castrated mice (Figure 7b). There was a four-fold increase in serum levels of KC chemokine (Figure 7c) and G-CSF (Figure 7d) in castrated mice.

Figure 7a

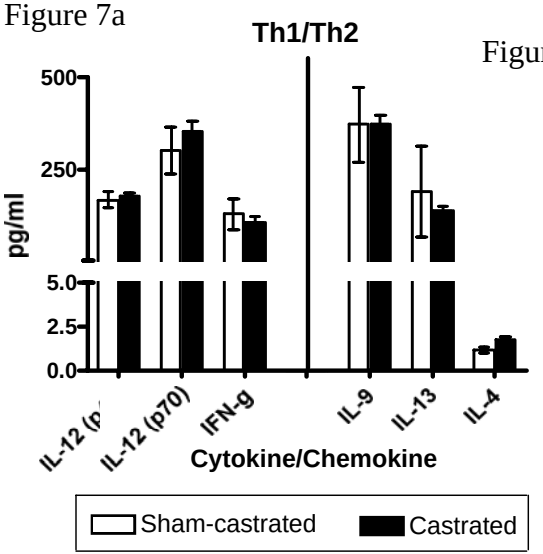


Figure 7b.

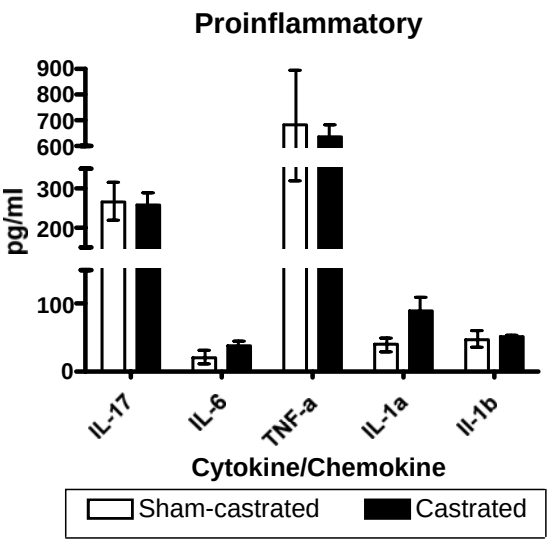


Figure 7c

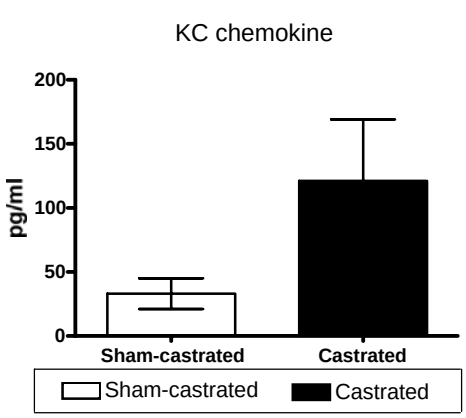


Figure 7d

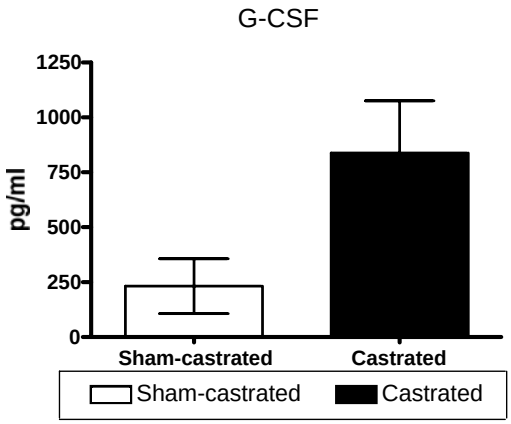


Figure 8. Eight to ten weeks old C57BL/6 mice were sham-castrated or castrated and then vaccinated with pcDNA3-mSTEAP using gene-gun and boosted two weeks later with viral relicon particles encoding for mSTEAP. Control mice were vaccinated with the empty pcDNA3 vector using the gene-gun or GFP-expressing VRP. Two groups of mice that were sham-castrated or castrated but did not receive vaccination were included to control for the effects of AA on tumor growth. The mice were then challenged with $5x10^5$ TRAMP-C2 prostate cancer cells and tumor growth measured over time.