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T CELLS LOCALIZED TO THE ANDROGEN-DEPRIVED PROSTATE ARE T_H1 AND T_H17 BIASED

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Abstract

BACKGROUND—T cells infiltrate the prostates of prostate cancer patients undergoing neoadjuvant androgen deprivation. These prostate-infiltrating T cells have an oligoclonal phenotype, suggesting the development of an antigen-specific T-cell response. We hypothesized that androgen deprivation might elicit a prostate tissue-specific T-cell response that could potentially be combined with other immune-active therapies, and consequently sought to investigate the nature and timing of this T-cell response following castration.

METHODS—We investigated the phenotype and cytokine expression of T cells at various time points in the prostates of Lewis rats following surgical castration, and used adoptive transfer of prostate-infiltrating lymphocytes to determine whether the infiltration by T cells was mediated by effects of castration on the prostate or lymphocytes.

RESULTS—Prostate T-cell infiltration shortly after castration was T_H1 biased up to approximately 30 days, followed by a predominance of T_H17-type cells, which persisted until at least 90 days post castration. Prostate-infiltrating lymphocytes from sham-treated or castrate rats localized to the prostates of castrate animals.

CONCLUSIONS—These observations suggest castration elicits a time-dependent prostate-specific T-cell infiltration, and this infiltration is likely mediated by effects of castration on prostate tissue rather than T cells. These findings have implications for the timing of immunotherapies combined with androgen deprivation as treatments for prostate cancer.

Keywords

Castration; inflammation; cytokine; prostate; rat

INTRODUCTION

Prostate cancer remains the most commonly diagnosed malignancy, and the second leading cause of cancer-related death in American men (1). Androgen deprivation therapy (ADT) is an established standard treatment for recurrent metastatic prostate cancer as it provides a survival benefit to patients (2). ADT induces apoptosis of the prostate secretory epithelium in part by upregulating expression of TGF- β mRNA along with increasing expression of TGF- β receptor II on secretory epithelial cells post castration (3–6). Administration of TGF- β can induce apoptosis and glandular regression in the ventral prostate of rats, further supporting this mechanism of prostate regression (7). Nevertheless, despite the initial

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efficacy of ADT-induced tissue regression, prostate cancer typically recurs as a castrate-resistant disease, thus there is a need for further therapeutic interventions.

Following treatment of humans or rodents with ADT there is an infiltration of lymphocytes into human and rodent prostates, potentially mediated by direct effects on prostate tissue or effects of ADT on adaptive immune cells leading to an increase in the pool of prostate-reactive T cells (8–11). In prostate cancer patients ADT induces an increase in circulating naïve T cells, likely by reversing thymic involution (12,13). The reversal of age-related thymic involution by ADT involves an increase in CCL25 expression on thymic epithelial cells, a ligand for early thymic progenitor entry (14). There is also evidence suggesting that androgens influence T_H bias. T cells specific for myelin basic protein from male mice, or female mice implanted with dihydrotestosterone, secrete increased levels of IL-10 compared with females implanted with a placebo (15). T cells of castrated male mice immunized with a vaccine encoding a prostate antigen were shown to upregulate IFN γ expression when restimulated *ex vivo* with the vaccine antigen (16). These findings suggest male androgens might shift the T_H bias from a T_H1-type immunity.

Similar to androgen deprivation, treatment with 17 β -estradiol has also been shown to induce lymphocytic infiltration of the prostate (17). Treatment of male rats with 17 β -estradiol increased the incidence and severity of non-bacterial prostatitis (18,19). Male Wistar rats implanted with 17 β -estradiol and DHT were shown to increase transcription of IL-1B, IL-6, MIP-2, and iNOS genes 4 days post implant, and increased transcription of IL-4, IL-5, IL-6, MIP-2, eotaxin, and iNOS by 4 weeks of hormone implant (20). Further, naïve rats receiving an adoptive transfer of lymphocytes from the prostates of rats treated with 17 β -estradiol and stimulated with concanavalin-A or anti-CD3 were shown to develop inflammation of the prostate, demonstrating that the lymphocytic inflammation observed in the naïve rats was an autoimmune response elicited against prostate tissue (21).

There is interest in vaccines and other immune-based therapies for the treatment of recurrent prostate cancer. In particular, one vaccine has shown a survival benefit in men with castrate-resistant prostate cancer (CRPC), and many other immunotherapies are in clinical trials for the treatment of CRPC (22–24). Preclinical data suggests that the timing of immunotherapies with ADT might be important to improve the efficacy of an immunotherapy for the treatment of prostate cancer. Specifically, mice immunized to prostate-restricted antigens developed antigen-specific IFN γ -secreting responses when immunized prior to castration rather than after castration (16,25). Because ADT is a standard treatment for recurrent prostate cancer, and because there is interest in optimizing the use of immunotherapies for the treatment of prostate cancer, we sought to directly characterize the effect of ADT on prostate-infiltrating lymphocytes (PIL). Thus, in this report we investigated changes to the phenotype of PIL following castration. We then compared the prostate-localized response with T cells in peripheral tissues to determine if the effects were systemic or tissue-specific. Finally, to address whether the prostate T-cell infiltration observed after castration was mediated by direct effects on prostate tissue, or potentially by effects of ADT on adaptive immunity, we conducted adoptive transfer studies using PIL from castrate- and sham-treated rats. Our results demonstrate a T_H1-biased immune response develops initially following castration, and this effect appears to be predominantly mediated by effects on prostate tissue rather than effects on T cells. In addition, castration elicits a chronic T_H17-biased inflammatory response. These findings have implications for the timing of immunotherapies in combination with androgen deprivation.

MATERIALS AND METHODS

Rats

Ten week-old wild type male Lewis type rats were purchased from Charles-River Laboratories (Wilmington, MA). Animals were housed at the University of Wisconsin Hospitals and Clinics vivarium, and at the Wisconsin Institutes for Medical Research vivarium. All experimental protocols were reviewed and approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC).

Surgeries

Rats were anesthetized with 5% isoflurane (Phoenix Pharmaceutical, St. Joseph, MO) and an incision across the scrotum allowed access to the testes. For castration the ductus deferens were then tied off with two opposing surgical knots using size 2-0, nylon monofilament (Johnson and Johnson Somerville, NJ) and the testes were removed. For sham surgery the testes were reinserted into the scrotum, and the incisions were closed with 7mm staples (CellPoint Scientific, Gaithersburg, MD).

T-cell preparation and flow cytometry

Liver and prostate tissues were minced and incubated 2 hours at 37°C with collagenase (2mg/ml; Sigma, St. Louis, MO), and DNase I (20 µg/ml; Sigma) in EHAA medium (Sigma) with 10% fetal calf serum (FCS), L-glutamine (200mM), 2IU/ml penicillin/ 2µg/ml streptomycin (ThermoFisher), 50µM 2-mercaptoethanol. Digested tissues were passed through a wire mesh screen. Splenocytes were washed twice in ACK buffer (0.155M ammonium chloride, 1mM potassium bicarbonate, 0.01mM EDTA) for 30 seconds. Cells were stimulated with 10ng/ml phorbol 12-myristate 13-acetate (Sigma), and 1 mg/mL ionomycin (Thermo Fisher, Waltham, MA), for 5 hours at 37° C with 5% CO₂. Cells were labeled with Fixable blue dead cell stain kit (Invitrogen, Carlsbad, CA), and incubated with antibodies specific for CD3 (1F4; Biolegend, San Diego, CA), CD4 (OX-35; BD, San Jose, CA), and CD8 (OX-8; Biolegend). Cells were fixed and permeabilized with Cytofix/Cytoperm Plus (BD), and stained for intracellular expression of IFN-γ (DB-1; Biolegend) and TNF-α (TN3-19; eBioscience, San Diego, CA), IL-10 (A5-4; BD) and Foxp3 (FJK-16s; eBioscience), IL-4 (OX-81; BD), IL-17 (eBio17B7; eBioscience), or IgG isotype controls (BD). Flow cytometry data was collected on an LSR II flow cytometer (BD), and data was analyzed with FlowJo software (version 8.8.6; Tree Star, Ashland, OR).

Histology & IHC

Tissues were fixed in 10% buffered formalin and embedded in paraffin or frozen in FSC 22 (Surgipath, Richmond, IL) on dry ice. Sections were stained with hematoxylin and eosin. For CD8 staining, tissues were first treated with 0.08% proteinase K (Sigma) (weight/vol) in 1x PBS pH 7.4 and incubated for 20 minutes at 37°C. Slides were blocked in 1x PBS pH 7.4 with 10% (vol/vol) FCS for 30 minutes at room temperature. Slides were incubated overnight at 4°C with anti-rat CD8 at 1:250 (EP1150Y, Lifespan Biosciences, Seattle, WA) in a humidified chamber. Slides were incubated at room temperature with a secondary anti-rabbit IgG Alexa 488 (Invitrogen, Carlsbad, CA) at 1:200, and incubated with DAPI (Vector Labs, Burlingame, CA). Slides were analyzed with a fluorescent microscope using Spot advanced plus software version 4.7 (Diagnostic Instruments, Sterling Heights, MI).

Adoptive Transfer

Liver and prostate were digested as described above and mononuclear cells (lymphocytes and monocytes) were collected by Ficol (GE, Piscataway, NJ) gradient purification. Live cells were counted by hemocytometer, and tissue resident monocytes and lymphocytes from

the prostate were labeled with PKH26 (Sigma), and liver monocytes and lymphocytes were labeled with CellVue Claret (Sigma) according to the manufacturer's protocol. 6×10^5 prostate cells and 8×10^4 liver cells were transferred intraperitoneally, and tissues were collected 24 hours post adoptive transfer. Tissues from transfer recipients were prepared for histological and flow cytometry analysis as described above.

RESULTS

Castration transiently increases prostate-infiltrating CD8+ lymphocytes

T-cell infiltration of both human and rodent prostates following androgen deprivation has been previously reported (8–10). We sought to characterize the kinetics of this infiltration, and to determine if the phenotype of prostate T cells is altered following castration. Wild type Lewis rats received a sham surgery or castration, and the ratio of T cells from the prostates was evaluated from 1 to 90 days post surgery by flow cytometry. Because castration induces atrophy of the prostate, we were unable to meaningfully evaluate the number of lymphocytes per volume of tissue. However, we observed a significant increase in the ratio of CD8+ cells relative to CD4+ cells by 30 days post castration (Fig 1A). Gating specifically on CD3+ cells showed that a significant change in the ratio of CD3+CD8+ to CD3+CD4+ T cells was specifically observed at the 30 day time point (data not shown), demonstrating the changes are specific to the T lymphocytes. We then specifically assessed the localization of CD8+ cells by immunohistochemical tissue staining. We observed a diffuse CD8+ cell infiltration throughout the prostates of castrate animals, greater at 30 days than 90 days following castration; CD8+ cells were predominantly localized to the perivascular areas of sham-treated animals (Fig 1B). These results suggested castration of rats induces a transient CD8+ T-cell enriched infiltration of the prostate by 30 days of castration, which declines by day 90.

CD4+ and CD8+ T cells localized to the prostate following castration are T_H1 and T_H17 biased

We next sought to identify if castration alters the phenotype of T cells in the prostate after castration. Prostate-infiltrating CD3+CD4+ or CD3+CD8+ T cells from Lewis rats were analyzed for intracellular protein expression of IFN γ , TNF α , IL-10, Foxp3, IL-4, or IL-17A at time points from 1 to 90 days post castration. As early as 3 days post castration a significant increase in the frequency of IFN γ -expressing CD4+ T cells was observed compared to the frequency in sham-treated control rats (Fig 2A); by day 30 a significant expansion of CD4+ T cells expressing TNF α and CD4+ T cells expressing IL-17A (Fig 2A and 2C) was observed, with IL-17A-expressing cells persisting through day 90 (Supplementary Figure 1). There were no significant changes in the frequency of CD4+ T cells expressing IL-10, Foxp3, or IL-4 (Fig 2B and D). A significant expansion of CD8+ T cells expressing both IFN γ and TNF α together was observed in the prostates of castrated rats by day 30, however by day 90 less TNF α expression was detected (Fig. 3A and Supplementary Figure 1). CD8+ T cells expressing FoxP3 and/or IL-10 were detected without an obvious trend (Fig 3B). CD8+ T cells expressing IL-17A were observed by day 14 and persisted through day 90, however this change was not significant compared with the sham controls (Fig 3C). No significant changes were observed in the frequency of prostate CD8+ T cells expressing IL-4 (Fig 3D). While a limitation of this analysis is the multiple comparisons made to different parameters at different days, with some transient changes observed at other time points, the general trend arising from these results suggests that the acute prostate-infiltrating T-cell response following castration is predominantly a T_H1 phenotype, whereas, the long-term or chronic T-cell response is T_H17 biased.

The T_H1 response is augmented systemically following castration

We next sought to determine if the accumulation of T_H1 and T_H17 T cells was specific to the prostate or if the T-cell response following castration was systemic. Liver and spleen samples were collected along with the prostates (described above) from sham control and castrate rats. In the liver no significant increase in the frequency of IFN- γ , TNF- α , FoxP3, IL-10, IL-4 or IL-17A expressing CD3+CD4+ or CD3+CD8+ T cells was observed (Fig. 4 and data not shown). In the spleen a significant increase of CD3+CD4+ T cells expressing IFN γ and TNF α were observed on day 3 (Fig. 4A and data not shown). No significant increase of spleen CD4+ T cells expressing Foxp3, IL-4 or IL-17A were observed (Fig. 4A and data not shown). A significant increase in the frequency of spleen CD8+ T cells expressing IFN γ and/or TNF α were observed on day 3 and day 30 (Figure 4B), however no significant increase in CD8+ T cells expressing IL-17A was observed. Once again, while interpretation of the specific statistical analysis at individual days was limited by the multiple comparisons being made, together with the results in Figures 2 and 3, these findings generally suggest the T_H1 response may be augmented systemically following castration, but is specifically localized to the prostate. The T_H17 response occurs subsequently and is specific to the prostate.

Prostate-infiltrating lymphocytes localize to androgen-deprived prostate tissue

The observation that T_H1- and T_H17-biased CD4+ and CD8+ T cells were specifically localized to the prostate following castration suggests the T cells are responding to prostate-associated antigens, possibly presented following castration-induced prostate atrophy, and/or potentially augmented directly as a result of effects of androgen deprivation on T-cell phenotype or function. To evaluate whether the T-cell infiltration was due to effects of castration on the prostate or due to systemic effects on lymphocytes, cells were isolated from sham- and castrate-treated rats 30 days after surgery, fluorescently labeled, and adoptively transferred to syngeneic recipient rats 30 days after sham or castrate treatment. Donor CD4+ and CD8+ T cells from the prostates of both sham and castrate rats localized at an increased frequency to the prostates of castrate recipients compared with sham recipients (Figure 5). CD4+ or CD8+ cells isolated from the liver of a donor sham or castrate rat did not specifically localize to the androgen-deprived prostate at an increased frequency, rather they localized to liver and spleen at frequencies similar to sham recipients (data not shown). Taken together, these findings suggest that castration alters the prostate microenvironment, making it amenable to infiltration by T cells.

DISCUSSION

We sought to investigate the nature and timing of the prostate-infiltrating T-cell response that has been demonstrated by many investigator groups to occur following castration. We found that in the Lewis rat there is a relative increase of CD8+ T cells into the prostate by 30 days post castration, and this response decreases over time. Similarly, CD4+ and CD8+ infiltrating T cells at this time demonstrated a T_H1 phenotype. A T_H17 response was also observed by day 30, and this persisted for at least 90 days post castration, when fewer CD8+ T cells and T_H1-associated responses were observed. While T_H1 responses were observed systemically following castration, the T_H17 response was only observed in the prostate. We then demonstrated that PIL from rats localized to androgen-deprived prostate tissue at an increased frequency relative to PIL transferred to sham control rats, suggesting that castration alters prostate tissue making it more amenable to T-cell infiltration. Our data are consequently the first to demonstrate that castration promotes a time-dependent prostate-localized T_H1 response and a chronic T_H17 response.

We observed an increase in the frequency of infiltrating CD8+ cells relative to CD4+ cells by 30 days post castration, and further identified that the prostate T cells upregulated expression of T_H1 cytokines (IFN γ and TNF α) at this same time, consistent with an inflammatory, cytolytic-type immune response. The frequency, localization, and T_H bias of tumor-infiltrating lymphocytes (TILs), in patients with colon and lung cancer, have been reported to be predictive of prognosis (26). Similarly, primary prostate tumors with gene expression profiles demonstrating increased T_H1-biased genes are associated with a better prognosis (27). However, in our study the CD8+ T-cell infiltration and T_H1 bias we observed was diminished by 90 days post castration. While we don't understand the mechanism by which these cells declined in frequency, and similar time-dependent studies have not been conducted in patients with prostate cancer following treatment with androgen deprivation to our knowledge, it is interesting to speculate that strategies to prolong the duration of T_H1-type cytolytic T-cell responses following castration might be exploited as a prostate cancer treatment.

The role of T_H17 cells in tumor development and progression remains controversial (28). Correlations linking a T_H17 bias both with tumor promotion and suppression have been reported in prostate cancer patients. In one study, an increased frequency of T_H17 cells in patient PBMC was significantly associated with a shorter time to metastatic progression (29). In a separate study, a high frequency of T_H17-expressing PIL in prostate cancer patients was associated with a lower pathologic Gleason score (30). It should be noted that both of these studies were relatively small, and larger studies may be needed to address the association of T_H17 bias with disease outcome, and whether the difference is due to the local or systemic response. T_H17 PILs may be responding specifically to tumor tissue, whereas the T_H17 cells in the periphery may be more indicative of global inflammation. Interestingly, STAT3 activation is necessary for the development of the T_H17 phenotype, and has been shown to be active following castration (31,32). Inhibition of activated STAT3 significantly slowed prostate tumor growth following castration in mice (32). Functional analysis of the T_H17 cells in normal and tumor-bearing prostates will be needed to demonstrate their role affecting other immune cells and their association with prostate cancer regression or recurrence. Similarly, tumor-bearing animal models may be useful to determine whether T_H17 immunity persists or wanes with the emergence of castrate-resistant disease.

Finally, we show in our study that PILs from Lewis rats localized to androgen-deprived prostates at an increased frequency compared with sham controls. These results suggest that castration alters the prostate tissue environment to enable T-cell localization. This may be due to destruction of prostate cells with increased presentation of T-cell antigens, due to disruption of vasculature enabling T-cell trafficking, due to effects on chemokine expression, or potentially due to effects on other prostate tissue populations permitting T-cell localization and retention. Specifically, it has been demonstrated in human tissues that androgen deprivation increases prostate epithelial gene expression of the chemoattractant IP-10/CXCL10, suggesting this might increase T-cell trafficking and retention (33). While these observations do not exclude the possibility that castration may directly affect the phenotype and repertoire of T cells able to infiltrate the prostate, this does not appear to be the predominant reason, since PIL from either castrate- or sham-treated animals were able to similarly infiltrate castrate-treated prostate tissues. In any case, our findings show that castration temporarily leads to a T_H1-type T-cell infiltration of the prostate. This response, and the timing of this response, might be combined with other immune-based therapies to prolong or increase the presence and activity of cytolytic T cells within prostate tumors as a treatment for prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Castration transiently increases prostate-infiltrating CD8+ lymphocytes

A) Prostate cell suspensions were evaluated for CD4+ and CD8+ cells by flow cytometry from individual prostates of sham or castrated Lewis rats. Shown is the ratio of CD8+ to CD4+ cells in the wild type Lewis rat prostates from 1 to 90 days post surgery. Data are compiled from multiple experiments for the different time points. p values are the result of a two-tailed unpaired T test. B) Representative H&E and CD8+ (Alexa 488) with DAPI staining of prostate tissues from 30- and 90-day sham control and castrate rats. Serial sections are shown for the CD8+ stains, H&E, and negative controls. Original magnification 200 $\times$.

Figure 2. CD4⁺ T cells localized to the prostate following castration are T_H1 and T_H17 biased
 Intracellular staining was performed with T cells collected from prostates obtained 1 to 90 days post castration or sham control. Cells were stimulated 5 hours with PMA and ionomycin prior to cytokine detection. Flow plots were gated on live, CD3⁺CD4⁺ T cells. A) Mean frequency of IFN γ - and/or TNF α -expressing cells; B) IL-10 and/or FoxP3; C) IL-17A; D) IL-4. Cytokine positive gates were determined for each rat by setting the gate of the fluorochrome IgG isotype control at 0.5%. * indicates a *p* value < 0.05 determined by two-tailed unpaired *T* test.

Figure 3. CD8+ T cells localized to the prostate following castration are TC1 biased

Intracellular staining of T cells was performed with cells collected from prostates obtained 1 to 90 days post castration or sham control. Cells were stimulated and analyzed as described in Figure 2. Shown is the mean % of CD3+ CD8+ T cells expressing cytokines and/or FoxP3. A) IFN γ and/or TNF α ; B) IL-10 and/or FoxP3; C) IL-17A; D) IL-4 expression. Gating was performed as described in Figure 2. Comparisons between groups were performed as in Figure 2, and * indicates a p value ≤ 0.05 .

Figure 4. The T_H1 response is augmented systemically following castration

Cells from the liver or spleen of castrated or sham control rats, collected from 1 to 90 days post surgery, were stimulated 5 hours with PMA and ionomycin prior to cytokine detection. A) The mean % of CD3+CD4+ T cells expressing IFN γ and/or TNF α or IL-17A. B) The mean % of CD3+CD8+ T cells expressing IFN γ and/or TNF α or IL-17A. * indicates a p value < 0.05 determined by two-tailed unpaired T test.

Figure 5. Prostate-infiltrating lymphocytes localize to androgen-deprived prostate tissue
 Total lymphocyte populations were obtained 30 days after castration or sham control surgery from prostate tissue and labeled with a membrane intercalating dye. Labeled prostate (PKH26) lymphocytes from donor rats were adoptively transferred (6×10^5 prostate lymphocytes) to recipient 30-day sham or castrate rats. A) Donor cells were recovered from recipient rat prostates 24 hours post adoptive transfer and analyzed by flow cytometry. Shown are the % of live, CD3+CD4+ or CD3+CD8+ T cells per total prostate events analyzed ($n=3$ per group). Comparison between groups was made with two-tailed unpaired *T* tests, and the *p* values are shown. B) Immunohistochemistry of representative recipient rat prostates showing donor (PKH26) and CD4+ (ALEXA 488) cells under 200 $\times$ magnification.