Am J Clin Exp Urol 2022;10(2):98-110
www.ajceu.us /ISSN:2330-1910/AJCEU0140469

Original Article

Sex steroid modulation of macrophages within the prostate tumor microenvironment

Zohra Berrehail[1,2], Clovis Boibessot[1,2], Typhaine Gris[1,2], France-Hélène Joncas[1,2], Fanny Gaignier[1,2], Chantal Guillemette[1,2,3], Louis Lacombe[1,2,4], Yves Fradet[1,2,4], Paul Toren[1,2,4]

[1]CHU de Québec-Université Laval Research Center, Quebec City, Quebec, Canada; [2]Cancer Research Center, Université Laval, Quebec City, Quebec, Canada; [3]Faculty of Pharmacy, Université Laval, CHU de Québec Research Center, Quebec City, Quebec, Canada; [4]Department of Surgery, Université Laval, Quebec City, Quebec, Canada

Received November 10, 2021; Accepted March 10, 2022; Epub April 15, 2022; Published April 30, 2022

Abstract: Background: The role of androgens and other sex steroids is known to influence the prognosis and progression of prostate cancer through different disease states. While androgens are generally regarded as immunosuppressive and estrogens as inflammatory, the specific influence of sex steroids on the immune microenvironment of prostate tumors remains incompletely understood. Material and methods: In this study, we evaluate the link between sex steroids and prostate cancer immune cells, particularly macrophages. Using *in vitro* and *in vivo* models, as well as *ex vivo* culture of patient prostate tissue, we evaluated the influence of androgen, estrogen, and progesterone on immune cells of the prostate microenvironment. Results: *In vitro*, we observed sex steroids induced indirect changes on prostate cancer cell proliferation via THP-1 derived macrophages, but no clear changes were induced using human monocyte derived macrophages. Comparing immunohistochemistry for immunosuppressive macrophage marker CD163 with concomitant circulating sex steroids from the same patients, we observed a correlation with higher dehydroepiandrosterone (DHEA)-sulfate and estrone-sulfate levels associated with higher prostate CD163 expression. Similar relationships between DHEA and CD163 levels were observed in *ex vivo* cultured prostate biopsies. Finally, in a murine prostate cancer model of long-term sex steroids we observed significant differences in tumor growth in mice implanted with estrogen and DHEA diffusion tubes. Conclusions: Our results highlight the complex influence of sex steroids on the immune cell composition of prostate tumors. Understanding this biology may help to further personalized therapy and improve patient outcomes.

Keywords: Prostate cancer, sex steroids, macrophages, tumor microenvironment, immunosuppressive phenotype

Introduction

Prostate cancer (PCa) is very common and contributes to approximately 10% of cancer-related deaths [1]. Since the discoveries of Huggins and Hodges over 65 years ago, it is well known that androgens and the androgen receptor (AR) remain central to prostate cancer cell growth [2]. AR antagonists are now widely used for treatment of both treatment-naïve and treatment-resistant PCa. Then as now, an understanding of the hormonal biology of PCa is important in order to optimize treatment approaches and patient outcomes.

The tumor microenvironment (TME) is the context for tumor cell growth as well as the development of resistance. In addition to tumor cells, it includes endothelial cells, fibroblasts, innate and adaptive immune cells as well as locally secreted soluble factors. The cell-to-cell interactions in the TME plays a crucial role in cancer progression and response to treatments like immunotherapy and androgen deprivation therapy (ADT). The TME of PCa is characterized by an immunosuppressive phenotype which decreases anti-tumoral T-cell activity [3]. Accumulation of innate immune cells of myeloid origin, such as myeloid derived suppressor cells (MDSCs), and immunosuppressive macrophages impede the immune response against the cancer cells and promote disease progression and metastasis [4, 5]. Accordingly, immune checkpoint inhibitors focused on the cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed cell death-1

(PD-1)/programmed death-ligands 1 (PD-L1) axis have had limited success in PCa to date [6].

Macrophages are cells with high plasticity in response to different environmental stimuli. The spectrum of activation within macrophage populations may be simplified into a dichotomy of M1 and M2 phenotypes, also referred to as classically activated macrophages and alternatively activated macrophages, respectively. While pro-inflammatory M1 is involved in host defense against pathogens and tumor cells, M2 is anti-inflammatory and promotes tumor growth by releasing anti-inflammatory cytokines, enhance angiogenesis and tumor escape. M1 and M2 tumor associated macrophages (TAMs) coexist within tumors but the abundance of M2 phenotype corresponds to a poorer prognosis in most solid tumors [7]. Similarly, a high density of $CD206^+$ cells (M2 marker) is common in metastatic castration-resistant prostate cancer (CRPC) [8].

Sex steroid receptors are expressed by innate immune cells which can be regulated by intrinsic and extrinsic factors. Androgen, estrogens and progesterone interact with AR, estrogen receptor (ER) and progesterone receptor (PR) respectively which are act as nuclear transcription factors when bound to their ligand. This binding induces the activation or repression of the expression of genes involved in proliferation, differentiation cells, inflammation and/or epigenetic regulation. Further, sex steroids can also act in the cell by non-genomic pathways [9].

It is generally accepted that hormonal differences between sexes contribute to immune differences [10, 11]. Testosterone or its more potent metabolite dihydrotestosterone (DHT) are thought to be immunosuppressive, though testosterone may also be aromatized to estrogen [12]. Estrogens are through to exert dose-dependent effects which may be pro-inflammatory [11]. In PCa, we previously reported that the relative level of testosterone in the blood serum of patients who received ADT predict the long-term development of resistance in patient with minimal volumes of cancer, suggesting an immune-mediated mechanism [13]. Others have also demonstrated that the levels of different steroids as well as the genetic variations related to steroidogenesis can influence PCa patient outcomes [14]. While the mechanisms of how androgens alter tumor cell AR activation have been previously explored in CRPC patients [15, 16], relatively few studies have evaluated the effect of sex steroid differences on the immune system and the anti-tumor response in PCa.

The aim of this study is to investigate the influence of different steroids on macrophage phenotype and their involvement in PCa progression and metastasis. To this end, we use both human THP-1 cell line-derived macrophages and human monocyte-derived macrophages (MDM) to evaluate the direct effects of sex steroids on macrophage phenotype and coculture experiments to evaluate the effect of sex steroid treated macrophages on PCa cell function. Further, we evaluate the effect of sex steroids using *ex vivo* cultured human PCa tissue which better reflects the complexity of the TME. Finally, we use PCa murine models with steroids implants to assess longer-term effects of sex steroids on the immune composition and tumor progression.

Material and methods

Cell culture

Human monocytic THP-1 cells (from American type culture collection, ATCC Manassas, VA) were maintained in culture in Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen, Carlsbad, CA) containing 10% of heat-inactivated fetal bovine serum (FBS, Wisent, Saint-Bruno-de-Montarville, QC, Canada) supplemented with 2.5 g/l D-glucose (BDH CHEMICALS, Montréal, QC, Canada #10117), 10 mM Hepes (Sigma-Aldrich, Oakville, ON, Canada), 1 mM pyruvate and 50 pM β-mercaptoethanol (Sigma-Aldrich). LNCaP cells (kind gift from Martin Gleave, Vancouver Prostate Centre) were maintained in RMPI (Invitrogen) supplemented with 10% of heat-inactivated FBS (Wisent). MR49C cells (kind gift from Amina Zoubeidi, Vancouver Prostate Centre) were maintained in Dubelcco's modified Eagle's minimal essential medium (DMEM, 1 g glucose/l, Wisent) supplemented with 10% FBS. PC3 cells (kind gift from Martin Gleave, Vancouver Prostate Centre) was maintained in DMEM supplemented with 10% FBS. LNCaP and PC3 cell lines were authenticated using short tandem repeat (STR) analysis in 2020 (Promega).

TRAMP-C2 cells were obtained from ATCC. Cells were cultured in DMEM media (Wisent), 5% FBS (Wisent) added with 5% NUserum Growth Medium Supplement (Corning, NY), 0.03% Insulin (Insulin solution from bovine pancreas, Sigma, St. Louis, MO) and 0.01 nM DHT (Toronto Research Chemicals, North York, ON, Canada).

Macrophage polarization

THP-1 cells: M0 macrophages were differentiated from THP-1 cells during 24 h of incubation with 300 nM of phorbol 12-myristate 13-acetate (PMA) followed by 48 h incubation in RPMI (Wisent). Then, M1 macrophages were cultured with 20 ng/ml of interferon (IFN)-γ (Cedarlane, Burlington, ON, Canada, #CL-101-06) and 10 ng/ml of LPS (Sigma-Aldrich) for 6 h. Macrophage M2 polarization was obtained by incubation with 20 ng/ml of interleukin (IL)-4 (Cedarlane, #CL-101-04) and 20 ng/ml of IL-3 (Cedarlane, #CL-101-13) for 6 h (RT-qPCR) or 24 h (proliferation assay). For each conditioned media 100 nM DHEA, 10 nM estradiol, or 100 nM progesterone were added during incubation (Figure S1A).

Monocyte-derived macrophages (MDM): Blood from healthy male donors was collected in ethylenediaminetetraeacetic acid (EDTA). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-PAQUE Plus density gradient centrifugation (GE Healthcare Pharmacia, endotoxin tested #17-1440-02, Amersham, United Kingdoms). Monocytes were purified from PBMCs using positive selection with magnetic-activated cell sorting (MACS) in LS Columns (Miltenyi Biotec, #130-042-401). CD14 positive monocytes were seeded in 12-well plates (1×10^6 cells/well) with 10 ng/ml of Monocyte-Colony Stimulating Factor (M-CSF) for 5 days to differentiate into M0 macrophages. At Day 6, 50% of volume of fresh medium supplemented with 10 ng/ml of M-CSF was added. At Day 7, M0 macrophages were polarized into M1 macrophages using IFN-γ (20 ng/ml, Cedarlane, Burlington, ON, Canada, #CL-101-06) and LPS (10 ng/ml, Sigma-Aldrich) for 4 additional days of culture (flow cytometry) or 24 h (proliferation assay). Macrophage M2 polarization was induced using IL-4 (20 ng/ml, Cedarlane, #CL-101-04) and IL-13 (20 ng/ml, Cedarlane, #CL-101-13). For steroid treatments 100 nM DHEA, 10 nM DHT, 10 nM estradiol, or 100 nM progesterone were added during incubation as detailed in Figure S1B.

RNA isolation and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from THP-1 macrophages using Trizol/chlorophorm phase extraction. TURBO DNase (2 units, Ambion, AM2239, Austin, TX) was used to cleave non-specific DNA, followed by reverse transcription of 2 µg of mRNA into cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, #4368814, Waltham, MA) according to the manufacturer's instructions. Amplification was performed using SYBRGreen PCR Master Mix (Applied Biosystems, #A25741) and primers (IDT, 300 nM). RSP9 (40S ribosomal protein S9) was used as the reference gene for normalization and mRNA abundance was quantified using the threshold cycle method. mRNA integrity was verified by RNA Integrity Number (RIN) (RNA with a RIN>8 was selected).

Proliferation assay

LNCaP or MR49C cells were seeded in a 24-well plate (150000 cells/well) 24 h before coculture experiments to let the cells become adherent. Coculture without sex steroids starts by changing media and adding a bilayer insert on the bottom of the well containing PCa cells (Figure S2). After 72 h, the supernatant was removed and cells were fixed with 10% glutaraldehyde for 10 min. Each well was rinsed with distilled water and 100 µl of crystal violet solution (0.5% w/v) was added into each well for 5 min. Wells were rinsed 3 times with distilled water and air dried. 200 µl of Sorensen's solution was added into each well for 5 min and optical density (OD) was read at 560 nm.

Cytokine measurements

IL-12 levels in the supernatant of cultured THP-1 derived macrophages or MDM from healthy donors were measured as part of a 9-plex human cytokine kit (Bio-Plex Pro Human Cytokine Th1/Th2 Assay, Bio-Rad, Mississauga, ON, Canada, #M5000005L3). Medium alone was used as blank control and untreated samples were compared with treated samples. Assays were run on Bio-Plex® 200 Systems and data analyzed using Bio-Plex Manager™ Software 6.1.

Wound healing assay

PC3 cells were first cultured to confluence (>90%) in 12-well dishes. A scratch was made with a 1000 µl plastic pipette tip. Thereafter, the detached cells were removed and new media with 2% FBS was added. Coculture started by adding the insert at day 0 for 48 h, with M1 or M2 macrophages pre-treated or not with different sex steroids (100 nM DHEA, 10 nM estradiol, 100 nM progesterone). Cells were observed microscopically over time (0 h, 24 h and 48 h). The remaining scratch area was measured using ImageJ software (v 1.6.0) in 3 different places in each well. The median distance was calculated for 3 independent experiments.

Murine studies

The protocol was approved by the research ethics committee of Centre Hospitalier Universitaire (CHU) de Québec-Université Laval (#2016-2835). C57BL/6 male mice, 6-8 weeks old, were randomized to receive one of four 15 mm silastic diffusion tube implants (interior diameter 1.55 mm, exterior diameter 2.38 mm) at time of surgical castration, with 12 mice per group. Tubes were filled with cholesterol (control), progesterone (1:10 progesterone:cholesterol), β-estradiol (1:10 estradiol:cholesterol), or DHEA (1:5 DHEA:cholesterol). Pilot studies indicated respective elevations in the blood at 7-, 24- and 56-days post-surgery as measured by mass spectroscopy. Mice were surgically castrated 1 week following inoculation with 1×10^6 TRAMP-C2 cells subcutaneously on both flanks (Figure S3). After appearance of a tumor, tumor size was measured twice weekly and tumor volume was calculated using the formula: $Volume = \frac{\text{shortest diameter}^2 \times \text{longest diameter}}{2}$. Mice were sacrificed according to pre-specified tumor volume (2 cm^3) and overall condition endpoints.

Flow cytometry

Tumors and spleen from sacrificed mice were minced into 2-4 mm^2 fragments, mechanically dissociated using a GentleMACs dissociator (Miltenyi Biotec, San Diego, USA). The dissociated tumor cells/splenocytes were placed in RPMI media containing 1500 collagenase digestive units (CDU) of collagenase I (Sigma-Aldrich, Ontario, CA) and 5 mU/mL of dispase II (Roche Diagnostics, Meylan, France) at 37°C and agitated for 20 min. The resulting cell suspension was filtered through a 70 µm cell strainer to remove clusters. Samples were centrifuged and the red blood cells were lysed with ammonium chloride potassium (ACK) lysis buffer. After washing with Hank's balanced salt solution (HBSS, Wisent), the tumor cells/splenocytes were processed for flow cytometry staining using the BD LSRFortessa cytometer (BD Biosciences). Compensation controls were performed. Cells were incubated with anti-CD16/CD32 Fc Block (BD Pharmingen, San Jose, CA) for 15 min. After washing, cells were incubated with fixable viability stain 450 (FVS450, BD Biosciences, #562247) for 30 min. After another wash, cells were incubated with one of two antibody panels (Table S1) before passage in the cytometer. Results were analyzed with FlowJo (v10.7.1).

Ex vivo culture

Ethics approval for human studies was obtained from the research ethics committee of the CHU de Québec-Université Laval (#2019-4181). Briefly five 18-gage PCa biopsies from 23 human radical prostatectomy specimens were divided in half and cultured for 72 h according to a previously described protocol [17] in the presence of indicated steroids (100 nM DHEA, 10 nM estradiol, 100 nM progesterone). Subsequently, biopsies were fixed and stained with monoclonal antibodies against CD163 (clone 2G12, dilution 1:2000, Abcam, Toronto, ON, Canada) or CD68 (clone KP1, dilution 1:400, Abcam) accordingly to previously described methods [18]. CD163 and CD68 expression throughout the biopsy halves were evaluated independently by two trained readers with mean scores used for analyses. The number of $CD68^+$ and $CD163^+$ cells in each of the biopsy halves were counted and those with no $CD68^+$ cells or $CD163^+$ cells were excluded as were slides judged to be of poor quality for interpretation due to artifacts, tissue folding or significantly different biopsy halves' architecture. In total, this resulted in 50% of the biopsies being included. Paired biopsy halves (individually divided between control vs treatment) allowed comparison of treatment effect between patients. To compare results between treated biopsies, the ratio of $CD163^+/CD68^+$ cells was used.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0, with Student's T-test to compare means and two-way ANOVA for difference between treatment groups. A linear regression model was used to compare slopes to assess for correlation analyses.

Results

Impact of sex steroids on macrophage phenotype and function

We first evaluated the impact of sex steroids on the phenotype and function of polarized macrophages derived from the human monocytic THP-1 cell line. After incubation with different sex steroids throughout macrophage differentiation and polarization, we evaluated mRNA expression by using qRT-PCR. As expected, CD206, CD163 and IL-10 were strongly expressed in M2 phenotype, while the mRNA level of CCR7 is absent in this population and found only in M1 phenotype. For all evaluated sex steroids, we observed a relatively small increase in M2 marker (CD163, CD206) in M1 polarized macrophages (**Figure 1A**). The relative expression of genes was normalized to M0 macrophages.

We next evaluated proliferation of various PCa cell lines cocultured with macrophages similarly incubated with sex steroids throughout their differentiation and polarization as above. PCa cell lines sensitive to enzalutamide (LNCaP and $VCaP^{Ctl}$) or resistant to enzalutamide (MR49C and $VCaP^{ER}$) were selected to reflect a diversity of AR-positive human PCa cell lines. As expected, coculture of PCa cells with macrophages decreased PCa cell proliferation, particularly with M1 macrophages (**Figure 1B**). We observed that macrophages previously incubated with sex steroids generally slightly enhanced the anti-proliferative effect of both M1 and M2 macrophages among LNCaP and $VCaP^{Ctl}$ cell lines. DHEA was an exception, where M1 macrophages cultured with this sex steroid exerted less-antiproliferative effects on both enzalutamide-sensitive cell lines (LNCaP and $VCaP^{Ctl}$). In contrast, enzalutamide-resistant (MR49C and $VCaP^{ER}$) cell lines had greater proliferation when cocultured with macrophages previously incubated with sex steroids.

To evaluate directly human macrophages, we utilized MDM from healthy donors for similar experiments with androgens (R1881, DHEA and DHT), progesterone and estrogens. Flow cytometry analysis demonstrated no change in the expression profile of $CD163^{high}$, $CD206^{high}$ and CCR7 markers, with the expected differences between M1 and M2 controls observed (**Figure 2A**). Further experiments assessed whether sex steroids altered the proliferation of LNCaP or MR49C cell lines. First, we observed M2 MDMs had no negative effect on PCa cell proliferation (**Figure 2B**). In contrast to results with THP-1 macrophages, we observed no differences induced by sex steroids. Similarly, no differences in cell migration of PC3 cell line following treatment with the sex steroids were observed (**Figure 2C**). Only untreated M1 MDMs resulted in a significant reduction in PCa cell migration (**Figure 2C**). Finally, we evaluated whether sex steroids altered IL-12 secretion from M1 polarized MDMs. When M1 MDMs were cocultured with MR49C cells, we observed a decrease of IL-12 secretion in the media which was more pronounced in estrogen-treated M1 MDMs (**Figure 2D**). The analogous experiment with macrophages derived from THP-1 display no changes in IL-12 production (**Figure 2E**).

Evaluation of the impact of sex steroids on human prostate macrophages

With our *in vitro* results demonstrating only a few suggestions that sex steroids may alter the macrophage phenotype or function, we next sought to investigate whether any effect of sex steroids on prostate macrophages could be detected in a complex cellular environment. Using a tissue microarray (TMA) from 240 patients with concomitant measurement of circulating steroids previously performed, we first evaluated the relationship between circulating sex steroids and CD163 expression. To account for variation in the immune infiltrate, results were normalized to CD45 expression. DHEA-S and E1-S were selected since sulfated sex steroids are more representative of longer-term sex steroid levels. Both DHEA-S and E1-S levels were positively associated with the CD163/CD45 ratio on linear regression analysis (coefficient 0.52, P=0.017; 0.64, P=0.043, respectively) (**Figure 3A**). When age was included as a covariate (given the expected correla-

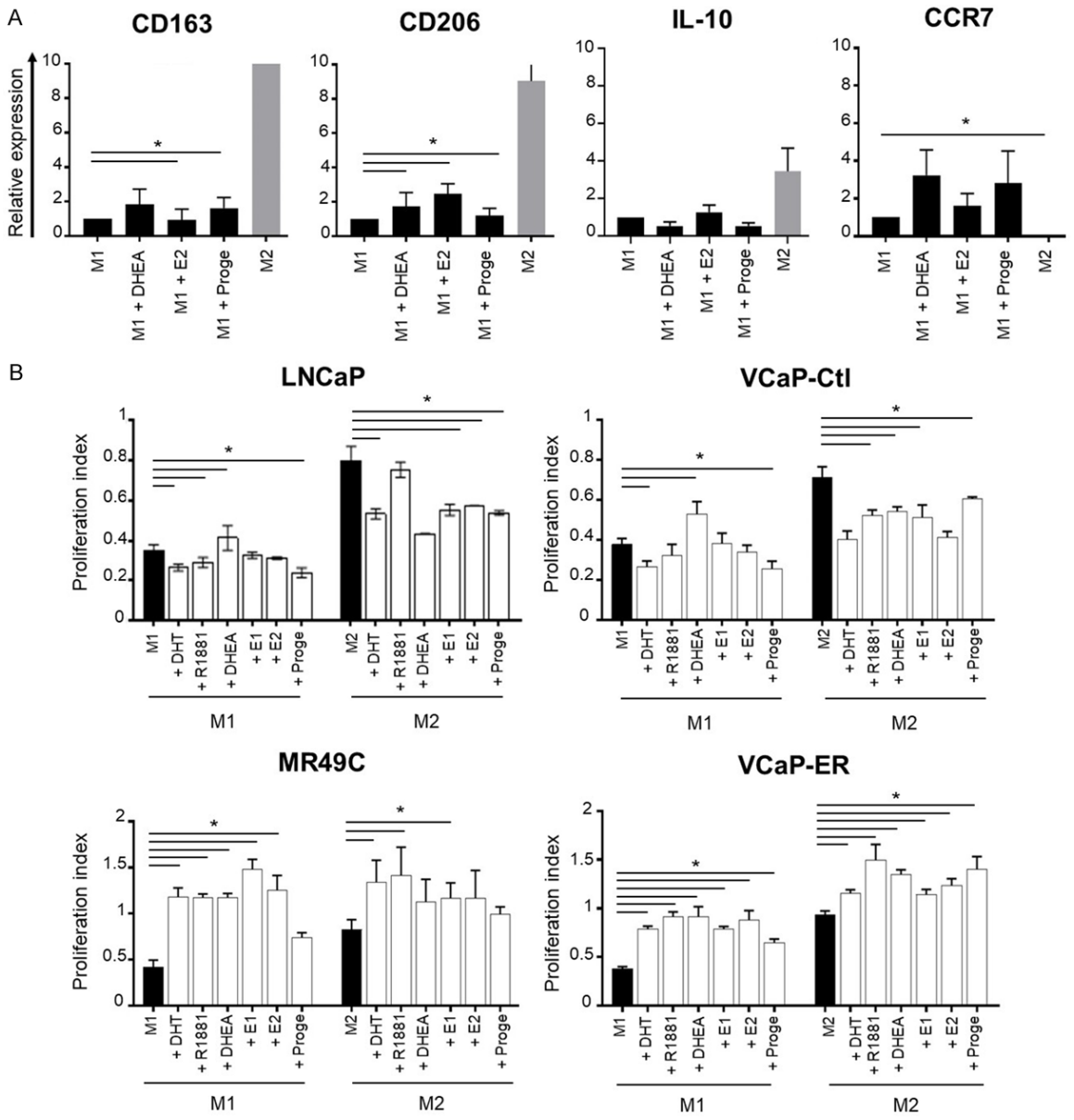

Figure 1. Impact of sex steroids on THP-1 differentiated macrophages. Macrophages were cultured during 5 days with or without sex steroids, then polarized into M1 or M2 macrophages for 6 h. Note that sex steroids were not present during subsequent coculture with indicated PCa cell lines. A. Expression of indicated markers was assessed by qRT-PCR following exposure to sex steroids. B. Proliferation of PCa cell line cocultured with M1 and M2 polarized macrophages after 24 h during 72 h. DHEA = dehydroepiandrosterone; DHT = dihydrotestosterone; R1881 = synthetic androgen methyltrienolone; E1 = estrone; E2 = estradiol; Proge = progesterone. *P<0.05.

tion between DHEA-S and age), similar significant results were obtained.

Next, to better recapitulate the biological complexity of tumors we evaluated the expression of these markers using our *ex vivo* PCa culture model treated or not with sex steroids (**Figure 3B**). Five biopsies of tumor areas were obtained from radical prostatectomy within 1 hour after surgical removal. Each biopsy was cut in half, with one half treated with one type of sex steroid and the other with the vehicle. After treatment, embedded biopsies were stained for the pan-macrophage marker CD68 and the M2-polarized macrophage marker CD163. The mean of $CD68^+$ and $CD163^+$ positive cells per biopsy was determined and a ratio of $CD163^+$/$CD68^+$ cells calculated for each patient. Compar-

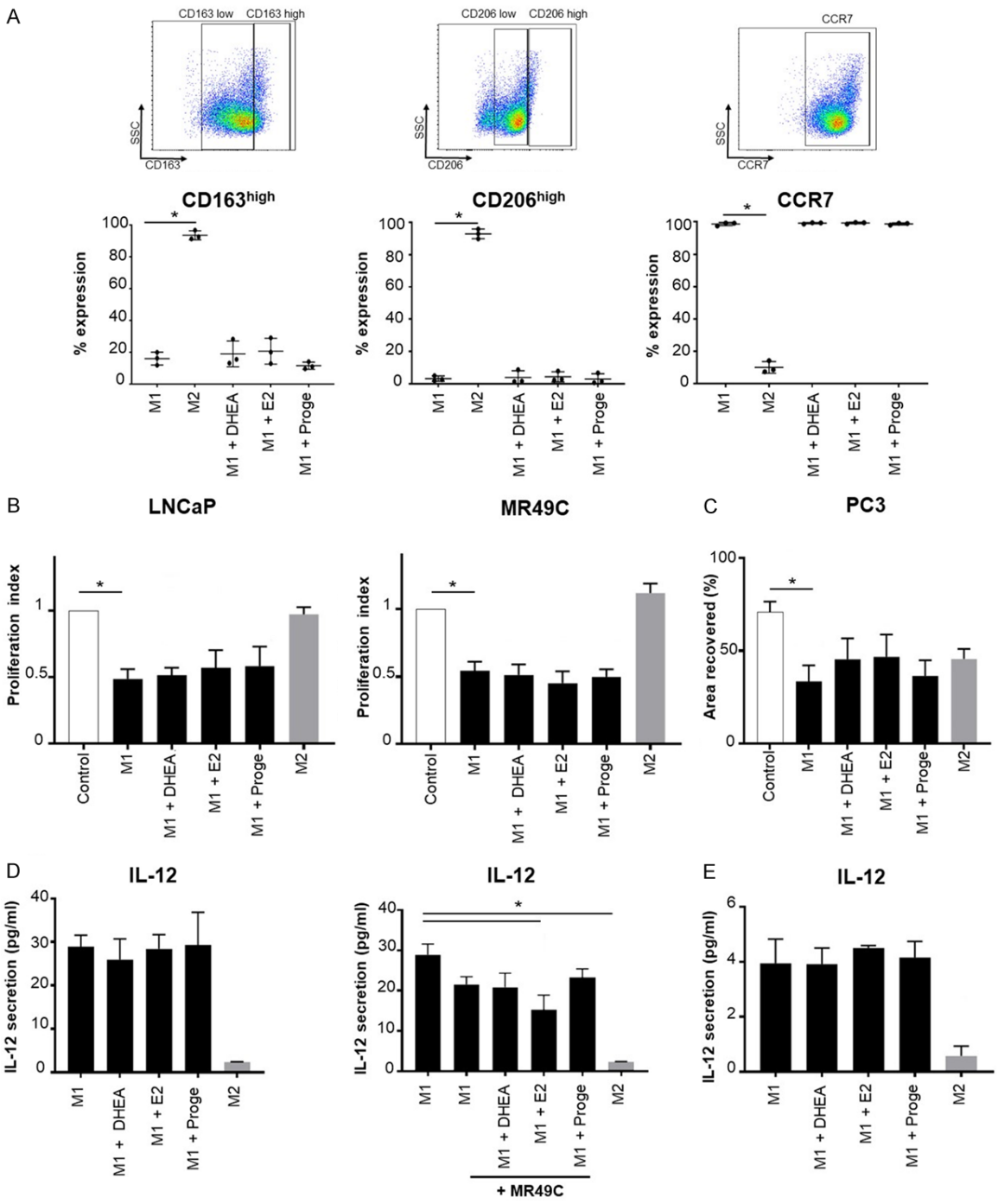

Figure 2. Impact of sex steroids on peripheral blood mononuclear cell (PBMC) differentiated macrophages. Differentiated macrophages were cultured during 5 days with or without sex steroids, then polarized into M1 or M2 macrophages for 96 h. (A) Flow cytometry analysis of macrophage phenotypic polarization markers from PBMC of healthy donors after exposure to sex steroids. (B) Proliferation of indicated PCa cell lines cocultured with M1 and M2 polarized macrophages after 24 h following indicate steroid treatment. (C) Migration of PC3 cocultured with M1 and M2 macrophages for 48 h. (D, E) Impact of sex steroids on IL-12 production from MDM (with or without coculture with MR49C PCa cells) (D) and from THP-1 macrophages (E). *P<0.05.

ing treatment to control for each paired biopsy demonstrated a significant increase of $CD163^+$/$CD68^+$ cells ratio with DHEA versus estradiol or progesterone treatment (**Figure 3C**).

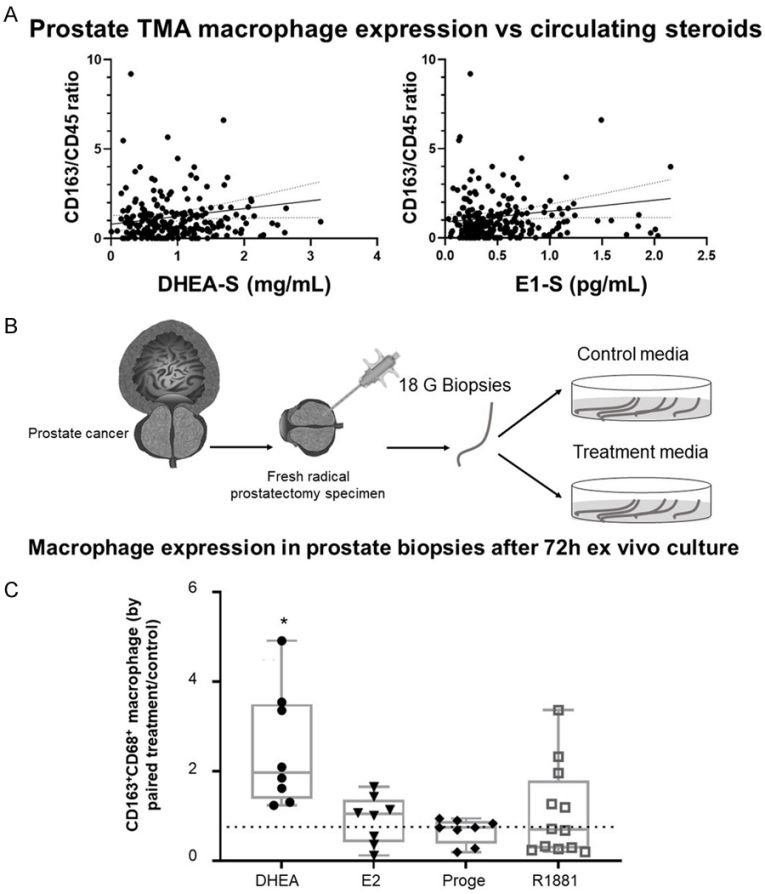

Figure 3. Sex steroids effect on human prostate tissue and correlation with immune cells infiltration. A. Relationship of CD163 expression with DHEA-S (left) and E1-S (right). IHC expression of CD163 in radical prostatectomy specimens was normalized for CD45 expression and plotted according to circulating steroids measured by mass spectroscopy from pre-operative plasma of the same patient. B. Schema of experiments using ex vivo culture of biopsies from fresh radical prostatectomy specimens. C. Ratio of $CD163^+$/$CD68^+$ expression from *ex vivo* culture of biopsies of fresh radical prostatectomy specimens in indicated steroids for 72 h prior to fixation and IHC staining. *P<0.05.

Sex steroids induced alteration in the immune microenvironment of prostate tumor in vivo

Using a TRAMP-C2 murine subcutaneous model, 48 male mice were divided into 4 groups of 12 mice each treated with control, estradiol, DHEA or progesterone. Tumor growth was higher in all treatment groups, but significantly higher among mice treated with estradiol (**Figure 4A**). Furthermore, median survival in the estradiol group (22 days) was significantly lower than the control group (27 days, P=0.0002) (**Figure 4B**) and ulcerations were observed at a greater extent among the estradiol treatment group.

Flow cytometry analysis of dissociated tumors and spleens evaluated panels of lymphocyte T-cells, natural killer (NK) and myeloid markers (Table S1). Sex steroid exposure did not induce changes in tumoral $CD4^+$ lymphocytes ($CD45^+$ $CD3^+CD4^+$ cells) nor NK cells ($CD45^+CD49b^+$ cells) between different treatments (**Figure 4D**, **4E**). However, tumoral $CD8^+$ lymphocytes ($CD45^+$ $CD3^+CD8^+$) were lower in all treatment groups compared to control (**Figure 4E**). While results from the myeloid panel in tumors were unevaluable due to technical difficulties, evaluation of this panel in splenic cells demonstrated a lower fraction of both M1 ($CD45^+Ly6C^-CMHII^+F4/80^+$ $CD11c^-CD11b^+$ cells) and M2 ($CD45^+Ly6C^-CMHII^+F4/80^+$ $CD11c^+CD11b^+$ cells) macrophages among estradiol treatment conditions (**Figure 4C**). Similar to tumor immune cells, there was no differences in CD4 lymphocytes or NK cell fractions in splenic samples.

Discussion

Understanding PCa biology is critical to advance personalized treatment approaches. Sex hormones have different effects on the immune system via signaling pathways, cytokines and chemokines production [19]. Our experiments leverage *in vitro* immune cell coculture studies, examination of human prostate tissue and murine studies to better understand the effect of sex steroids on PCa associated macrophages. A better understanding of these interactions may contribute to developing strategies to optimize treatments for patients with advanced PCa.

In our study, we focused on the effect of sex steroids on M1 macrophages. Our results suggest that sex steroids exert an impact mostly via transcriptional changes with protein phenotype changes usually undetectable. The androgen, estrogen and progesterone receptors are generally reported in sequencing studies to be expressed in limited quantities on human ma-

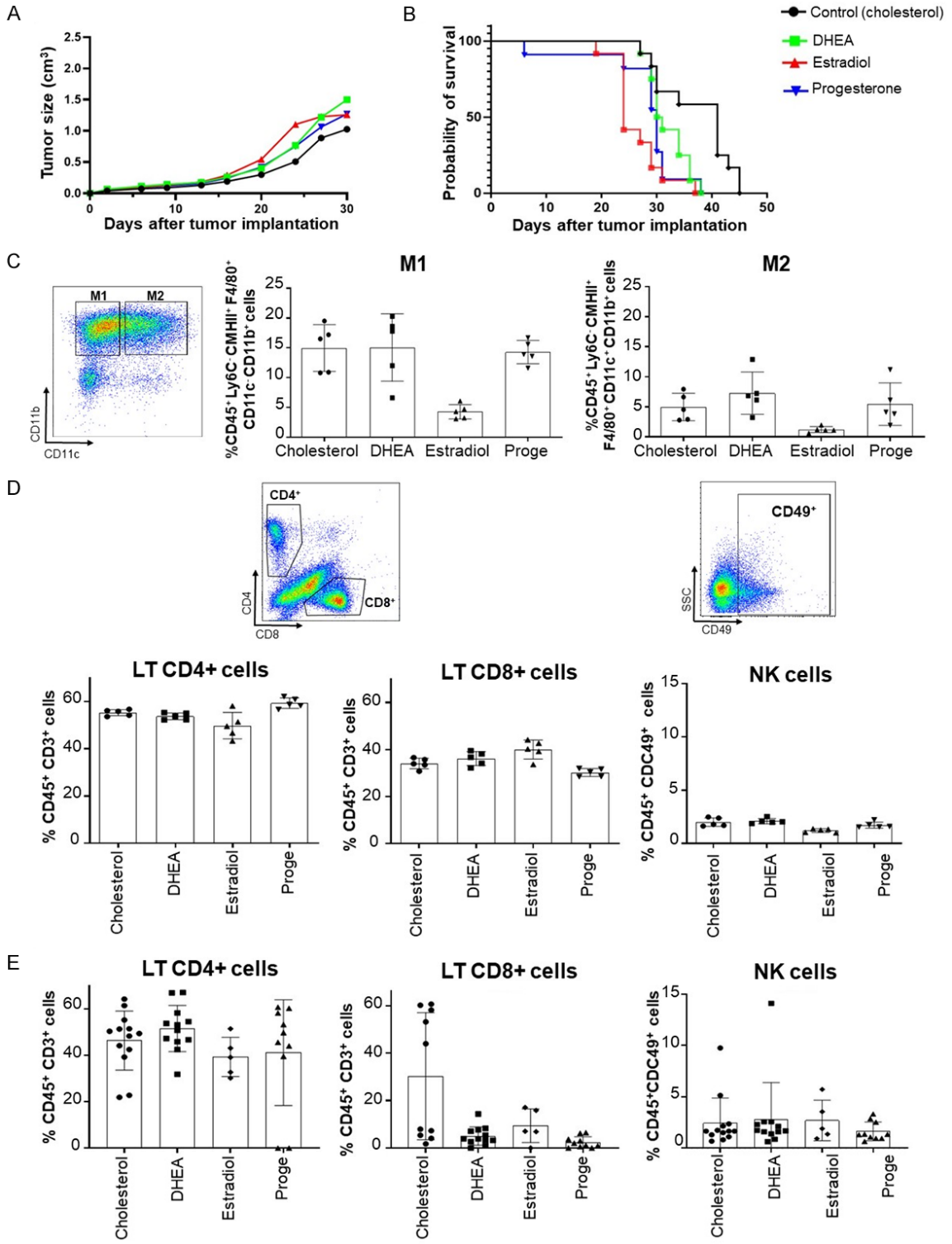

Figure 4. Effect of sex steroids on tumor growth and immune infiltrate in TRAMP-C2 murine prostate cancer model. (A, B) Tumor growth (A) and survival (B) analysis of mice bearing TRAMP-C2 prostate cancer. Two-way ANOVA was used for statistical analysis. (C) Flow cytometry analysis of M1 ($CD45^+Ly6C^-CMHII^+F4/80^+CD11c^-CD11b^+$ cells) and M2 ($CD45^+$ $Ly6C^-CMHII^+F4/80^+CD11c^+CD11b^+$ cells) macrophages from dissociated spleens. (D, E) Flow cytometry analysis of immune cells ($CD45^+$ cells) infiltration and evaluation of lymphocyte T $CD3^+CD4^+$ cells, $CD3^+CD8^+$ cells, and NK cells ($CD49^+$ cells) from dissociated tumors (D) and spleens (E).

crophages and is reported to be functionally active in THP-1 macrophages [20]. Differences between models may explain in part some of our disparate results, with greater responsiveness to estrogens notably more evident in our human and murine studies.

We focused on multi-day incubation of macrophages with steroids, attempting to mimic the physiological situation where monocyte cells are constantly exposed to circulating steroids before recruitment and differentiation into tissular macrophages. However, this study design does not capture acute sex steroid induced changes. With the prostate known for intraprostatic synthesis of androgens [21], this constant exposition of sex steroids may be more pertinent to the study of prostate macrophage biology.

To our knowledge, our observation of a correlation between circulating DHEA-S or E1-S levels and prostate CD163 expression is the first data directly linking circulating hormone levels to the prostate macrophage phenotype. Sulfated steroids are relatively stable and thus DHEA-S is more representative of long-term androgen levels. In a prospective cohort of 1,766 localized PCa patients, the association between circulating level of sex steroids and disease-free survival suggests that higher levels of DHEA-S corresponds to a higher risk of progression following surgery for localized PCa [38]. With prior studies also implicating a higher risk of progression for similar patients with higher immunosuppressive macrophage infiltration [39-41], our data suggest the first possible biological link between these observations in circulating steroids and the macrophage phenotype.

Similar to our observation of low level changes in M2 macrophage markers following exposure to sex steroids, others have observed that steroids may increase the expression of M2 markers (e.g.: CD206 and CD163) in THP-1 derived macrophages and also favor anti-inflammatory cytokine production [22]. Testosterone has been reported to suppress T cell function by suppressing pro-inflammatory IFN-γ and IL-12 while increasing immunosuppressive IL-10 and the abundance of Treg $CD25^+$ $Foxp3^+$ cells [23-27]. Estrogen appears to exert dose- and receptor-dependent effects on immune cells [10, 11, 28, 29], while the estrogen receptor also plays a role in modulating inflammatory signalling [30]. Limited data suggest progesterone may have anti-inflammatory effects [31]. Similar to our results, other report that progesterone does not influence the phenotype and function of macrophages *in vitro* [32-34]. Two studies evaluating the effect of DHEA on THP-1 macrophages found that it decreased LPS-stimulated TNF-α [35, 36]. Overall, our results in both THP-1 and human *in vitro* models add to the relatively sparse literature evaluating hormonally induced macrophage phenotypic alterations.

Interestingly, our *in vivo* results concur with others who report that estrogens may promote an anti-inflammatory response by decreasing the Th1 response by increasing Treg FoxP3 recruitment and the production of IL-10 [37]. The changes in the splenic immune populations we observed suggest changes in systemic immune cell circulation induced by estrogens could ultimately impact the immune infiltration and tumor growth. However, no clear changes in the tumor immune infiltrate were observed in our study. Interestingly, all sex steroids reduced T $CD8^+$ cells infiltration within the TME compared to control group, suggesting the possibility these differences explained the altered tumor growth we observed. Nonetheless, the diffusion increased sex steroids through implants does not recapitulate physiologic hormonal variations. In addition, the absence of hormones in gonadectomised mice may alter the number and phenotype of immune cells after tumor inoculation.

Our study has several limitations. First, *in vitro* experiments assessed only one time point, with differences potentially more detectable at different treatment intervals not assessed in these studies. While the use of DHEA may avoid some direct stimulation of the AR in PCa cells *in vivo*, we cannot fully compensate for the steroid metabolism and activation of downstream androgen or estrogens receptors with this and other sex steroids evaluated [42, 43]. The influence of steroids and their respective metabolites may have a direct or indirect effects, including epigenetic or metabolic changes not evaluated in these studies. Finally, while *ex vivo* culture of patient biopsies under different hormonal media preserves cell architecture and most TME cell types, there remains significant heterogeneity which can

affect results. Indeed, PCa is known to be highly heterogeneous and controlling for the variability of immune infiltration within each biopsy was not feasible.

Conclusion

Our studies in multiple PCa models demonstrate the complexities of understanding the specific role of sex steroids on the immune system phenotype. While we observed minimal changes in many experiments, there was a consistency of changes with evaluated steroids favoring an immunosuppressive macrophage. This was mirrored in our correlative studies with patient prostate samples. Further research is needed to explore the epigenetic pathways which may contribute to undetected phenotypic changes as well as to further defined changes in other immune cell phenotypes.

Acknowledgements

This work was supported by Cancer Research Society (#23483) and the Fonds de Recherche de Québec - Santé (#32774).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Paul Toren, Centre de Recherche du CHU de Québec-Université Laval and Centre de Recherche Sur Le Cancer, Université Laval, Uro-Oncologist, CHU de Québec-Université Laval, 10 McMahon, Rm 0877 Québec, Quebec G1R 3S1, Canada. Tel: 418-525-4444 ext. 17064; Fax: 418-691-5562; E-mail: paul.toren@crchudequebec.ulaval.ca

References

[1] Paschalis A and de Bono JS. Prostate cancer 2020: "the times they are a'changing". Cancer Cell 202; 38: 25-27.
[2] Huggins C and Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin 1972; 22: 232-40.
[3] Bilusic M, Madan RA and Gulley JL. Immunotherapy of prostate cancer: facts and hopes. Clin Cancer Res 2017; 23: 6764-70.
[4] Gajewski TF, Schreiber H and Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 2013; 14: 1014-22.
[5] Hanahan D and Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012; 21: 309-22.
[6] Ribas A and Wolchok JD. Cancer immunotherapy using checkpoint blockade. Science 2018; 359: 1350-5.
[7] Yang M, McKay D, Pollard JW and Lewis CE. Diverse functions of macrophages in different tumor microenvironments. Cancer Res 2018; 78: 5492-503.
[8] Zarif JC, Baena-Del Valle JA, Hicks JL, Heaphy CM, Vidal I, Luo J, Lotan TL, Hooper JE, Isaacs WB, Pienta KJ and De Marzo AM. Mannose receptor-positive macrophage infiltration correlates with prostate cancer onset and metastatic castration-resistant disease. Eur Urol Oncol 2019; 2: 429-36.
[9] Shepherd R, Cheung AS, Pang K, Saffery R and Novakovic B. Sexual dimorphism in innate immunity: the role of sex hormones and epigenetics. Front Immunol 2020; 11: 604000.
[10] Klein SL and Flanagan KL. Sex differences in immune responses. Nat Rev Immunol 2016; 16: 626-38.
[11] Boibessot C and Toren P. Sex steroids in the tumor microenvironment and prostate cancer progression. Endocr Relat Cancer 2018; 25: R179-96.
[12] Gamat M and McNeel DG. Androgen deprivation and immunotherapy for the treatment of prostate cancer. Endocr Relat Cancer 2017; 24: T297-310.
[13] Klotz L, O'Callaghan C, Ding K, Toren P, Dearnaley D, Higano CS, Horwitz E, Malone S, Goldenberg L, Gospodarowicz M and Crook JM. Nadir testosterone within first year of androgen-deprivation therapy (ADT) predicts for time to castration-resistant progression: a secondary analysis of the PR-7 trial of intermittent versus continuous ADT. J Clin Oncol 2015; 33: 1151-6.
[14] Lévesque É, Huang SP, Audet-Walsh É, Lacombe L, Bao BY, Fradet Y, Laverdière I, Rouleau M, Huang CY, Yu CC, Caron P and Guillemette C. Molecular markers in key steroidogenic pathways, circulating steroid levels, and prostate cancer progression. Clin Cancer Res 2013; 19: 699-709.
[15] Chang KH, Li R, Kuri B, Lotan Y, Roehrborn CG, Liu J, Vessella R, Nelson PS, Kapur P, Guo X, Mirzaei H, Auchus RJ and Sharifi N. A gain-of-function mutation in DHT synthesis in castration-resistant prostate cancer. Cell 2013; 154: 1074-84.
[16] Dai C, Chung YM, Kovac E, Zhu Z, Li J, Magi-Galluzzi C, Stephenson AJ, Klein EA and Sharifi N. Direct metabolic interrogation of dihydrotestosterone biosynthesis from adrenal precursors in primary prostatectomy tissues. Clin Cancer Res 2017; 23: 6351-6362.

[17] Neveu B, Jain P, Tetu B, Wu L, Fradet Y and Pouliot F. A PCA3 gene-based transcriptional amplification system targeting primary prostate cancer. Oncotarget 2016; 7: 1300-10.
[18] Boibessot C, Joncas FH, Park A, Berrehail Z, Pelletier JF, Gris T, Bergeron A and Toren P. Using ex vivo culture to assess dynamic phenotype change in human prostate macrophages following exposure to therapeutic drugs. Sci Rep 2021; 11: 19299.
[19] Cutolo M, Accardo S, Villaggio B, Barone A, Sulli A, Coviello DA, Carabbio C, Felli L, Miceli D, Farruggio R, Carruba G and Castagnetta L. Androgen and estrogen receptors are present in primary cultures of human synovial macrophages. J Clin Endocrinol Metab 1996; 81: 820-7.
[20] Cioni B, Zaalberg A, van Beijnum JR, Melis MHM, van Burgsteden J, Muraro MJ, Hooijberg E, Peters D, Hofland I, Lubeck Y, de Jong J, Sanders J, Vivié J, van der Poel HG, de Boer JP, Griffioen AW, Zwart W and Bergman AM. Androgen receptor signalling in macrophages promotes TREM-1-mediated prostate cancer cell line migration and invasion. Nat Commun 2020; 11: 4498.
[21] Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME and Nelson PS. Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. Cancer Res 2007; 67: 5033-41.
[22] Mendoza-Cabrera MI, Navarro-Hernández RE, Santerre A, Ortiz-Lazareno PC, Pereira-Suárez AL and Estrada-Chávez C. Effect of pregnancy hormone mixtures on cytokine production and surface marker expression in naïve and LPS-activated THP-1 differentiated monocytes/macrophages. Innate Immun 2020; 26: 84-96.
[23] Lin AA, Wojciechowski SE and Hildeman DA. Androgens suppress antigen-specific T cell responses and IFN-γ production during intracranial LCMV infection. J Neuroimmunol 2010; 226: 8-19.
[24] Kissick HT, Sanda MG, Dunn LK, Pellegrini KL, On ST, Noel JK and Arredouani MS. Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation. Proc Natl Acad Sci U S A 2014; 111: 9887-92.
[25] Liva SM and Voskuhl RR. Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production. J Immunol 2001; 167: 2060-7.
[26] Fijak M, Damm LJ, Wenzel JP, Aslani F, Walecki M, Wahle E, Eisel F, Bhushan S, Hackstein H, Baal N, Schuler G, Konrad L, Rafiq A, O'Hara L, Smith LB and Meinhardt A. Influence of testosterone on inflammatory response in testicular cells and expression of transcription factor Foxp3 in T cells. Am J Reprod Immunol 2015; 74: 12-25.
[27] Fijak M, Schneider E, Klug J, Bhushan S, Hackstein H, Schuler G, Wygrecka M, Gromoll J and Meinhardt A. Testosterone replacement effectively inhibits the development of experimental autoimmune orchitis in rats: evidence for a direct role of testosterone on regulatory T cell expansion. J Immunol 2011; 186: 5162-72.
[28] Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. Cell Immunol 2015; 294: 63-9.
[29] Galván-Ramírez ML, Ramírez De Arellano A, Rodríguez-Pérez LR, Lopez-Pulido EI, Muñoz-Valle JF and Pereira-Suárez AL. Hormonal modulation of Toxoplasma gondii infection: regulation of hormonal receptors and cytokine production in THP-1 cells. Exp Parasitol 2019; 204: 107721.
[30] Calippe B, Douin-Echinard V, Delpy L, Laffargue M, Lélu K, Krust A, Pipy B, Bayard F, Arnal JF, Guéry JC and Gourdy P. 17Beta-estradiol promotes TLR4-triggered proinflammatory mediator production through direct estrogen receptor alpha signaling in macrophages in vivo. J Immunol 2010; 185: 1169-76.
[31] Matias ML, Romao-Veiga M, Ribeiro VR, Nunes PR, Gomes VJ, Devides AC, Borges VT, Romagnoli GG, Peracoli JC and Peracoli MT. Progesterone and vitamin D downregulate the activation of the NLRP1/NLRP3 inflammasomes and TLR4-MyD88-NF-κB pathway in monocytes from pregnant women with preeclampsia. J Reprod Immunol 2021; 144: 103286.
[32] Tsai YC, Tseng JT, Wang CY, Su MT, Huang JY and Kuo PL. Medroxyprogesterone acetate drives M2 macrophage differentiation toward a phenotype of decidual macrophage. Mol Cell Endocrinol 2017; 452: 74-83.
[33] Polikarpova AV, Levina IS, Sigai NV, Zavarzin IV, Morozov IA, Rubtsov PM, Guseva AA, Smirnova OV and Shchelkunova TA. Immunomodulatory effects of progesterone and selective ligands of membrane progesterone receptors. Steroids 2019; 145: 5-18.
[34] Peltier MR, Tee SC and Smulian JC. Effect of progesterone on proinflammatory cytokine production by monocytes stimulated with pathogens associated with preterm birth. Am J Reprod Immunol 2008; 60: 346-53.
[35] Corsini E, Galbiati V, Papale A, Kummer E, Pinto A, Serafini MM, Guaita A, Spezzano R, Caruso D, Marinovich M and Racchi M. Role of androgens in dhea-induced rack1 expression and cytokine modulation in monocytes. Immun Ageing 2016; 13: 20.

[36] Di Santo E, Foddi MC, Ricciardi-Castagnoli P, Mennini T and Ghezzi P. DHEAS inhibits TNF production in monocytes, astrocytes and microglial cells. Neuroimmunomodulation 1996; 3: 285-8.
[37] Tai P, Wang J, Jin H, Song X, Yan J, Kang Y, Zhao L, An X, Du X, Chen X, Wang S, Xia G and Wang B. Induction of regulatory T cells by physiological level estrogen. J Cell Physiol 2008; 214: 456-64.
[38] Lévesque E, Caron P, Lacombe L, Turcotte V, Simonyan D, Fradet Y, Aprikian A, Saad F, Carmel M, Chevalier S and Guillemette C. A comprehensive analysis of steroid hormones and progression of localized high-risk prostate cancer. Cancer Epidemiol Biomarkers Prev 2019; 28: 701-6.
[39] Boibessot C, Molina O, Lachance G, Tav C, Champagne A, Neveu B, et al. Macrophage reeducation in prostate cancer: subversion of inflammatory macrophages to a mixed immunosuppressive tumor associated-macrophage phenotype. Clin Transl Med 2021.
[40] Zarif JC, Baena-Del Valle JA, Hicks JL, Heaphy CM, Vidal I, Luo J, Lotan TL, Hooper JE, Isaacs WB, Pienta KJ and De Marzo AM. Mannose receptor-positive macrophage infiltration correlates with prostate cancer onset and metastatic castration-resistant disease. Eur Urol Oncol 2019; 2: 429-436.
[41] Erlandsson A, Carlsson J, Lundholm M, Fält A, Andersson SO, Andrén O and Davidsson S. M2 macrophages and regulatory T cells in lethal prostate cancer. Prostate 2019; 79: 363-9.
[42] Webb SJ, Geoghegan TE, Prough RA and Michael Miller KK. The biological actions of dehydroepiandrosterone involves multiple receptors. Drug Metab Rev 2006; 38: 89-116.
[43] Labrie F, Luu-The V, Labrie C and Simard J. DHEA and its transformation into androgens and estrogens in peripheral target tissues: intracrinology. Front Neuroendocrinol 2001; 22: 185-212.

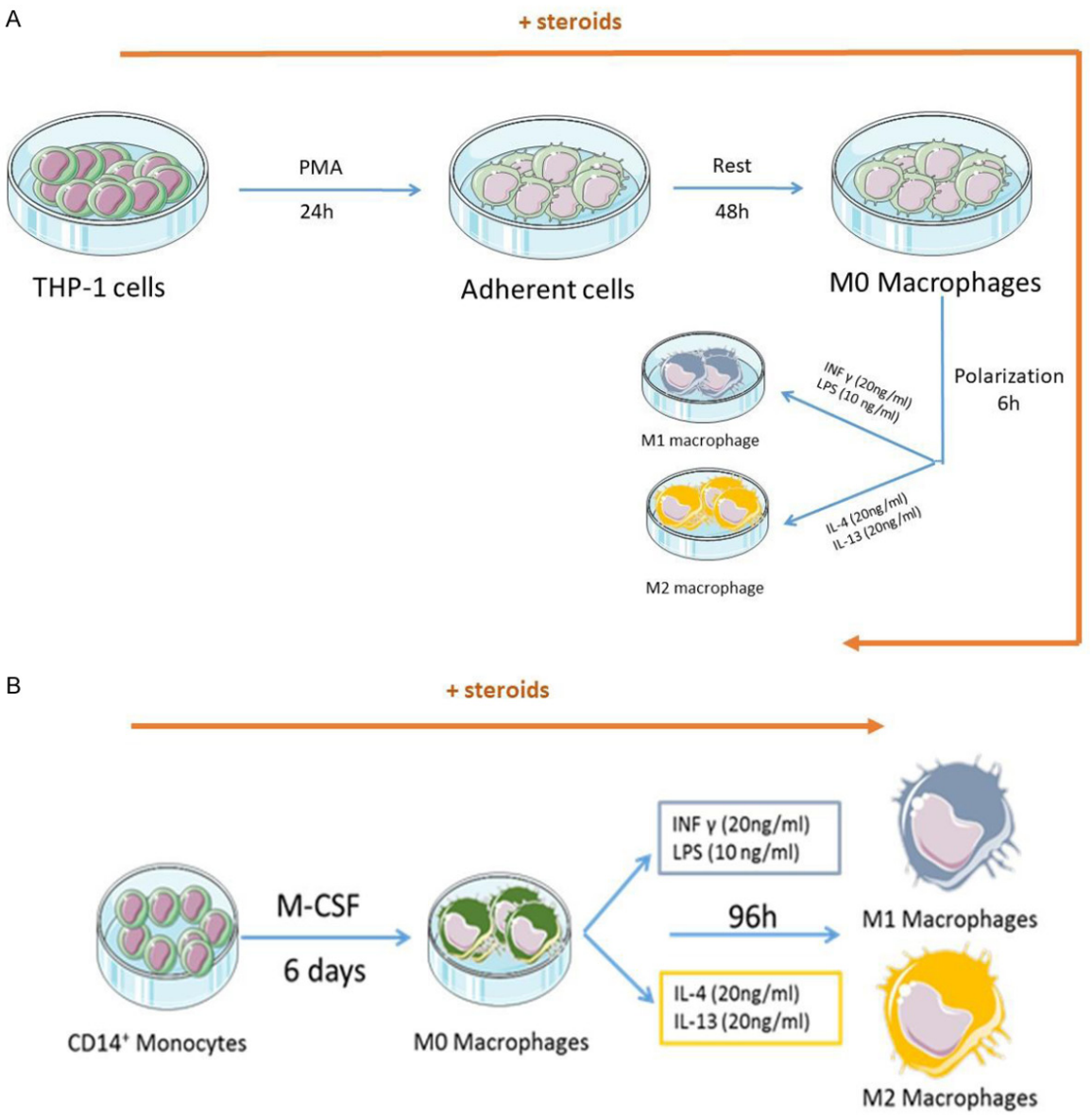

Figure S1. Schematic representation of polarization of macrophages. A. THP-1 macrophages were cultured with or without sex hormones during 24 h with PMA followed by 48 h of incubation in RPMI medium, then polarized into M1 or M2 for 6 h. B. $CD14^+$ monocytes were magnetically isolated from male donor blood and cultured for 6 days with M-CSF, then polarized into M1 or M2 for 96 h with or without sex steroids.

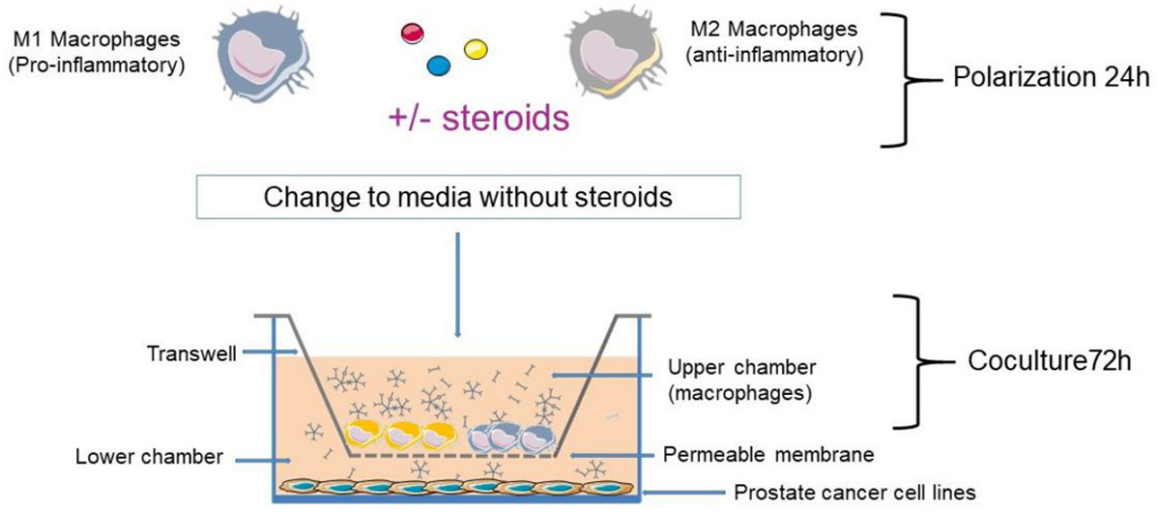

Figure S2. Schematic representation of insert coculture system. The upper chamber contains sex hormones induced macrophages polarized after 24 h and the lower chamber holds indicated PCa cell line, which were seeded in 24-well plate 24 h before coculture. The assessment of proliferation was performed after 72 h of coculture without sex steroids as described in material and methods.

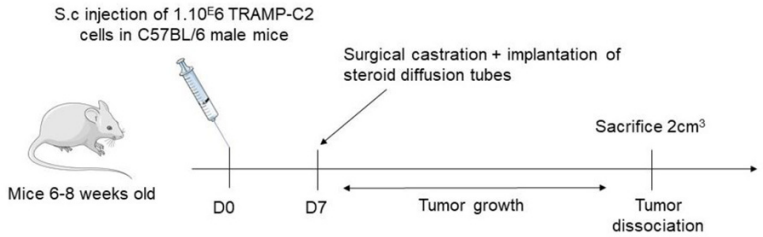

Figure S3. Schematic representation of murine experiment. 48 6-8 weeks old C57BL/6 male mice were used. 12 mice per group of each sex steroid with implantation of steroid diffusion tubes at surgical castration one week following subcutaneous inoculation of TRAMP-C2 cells.

Table S1. Murine antibodies used in multi-parametric flow cytometry analyses

Panel 1: Lymphoid cell line			
Antibodies	Fluorochrome	Clone	Supplier
CD45	V500	30-F11	BD Biosciences
CD3e	APC-Cy7	145-2C11	BD Biosciences
CD4	FITC	GK1.5	BD Biosciences
CD8a	BV605	53-6.7	BD Biosciences
CD49b	PE-Cy7	DX5	Biolegend
Panel 2: Myeloid cell line			
Antibodies	Fluorochrome	Clone	Supplier
CD45	V500	30-F11	BD Biosciences
I-A/I-E (CMHII)	BV605	M5/114.15.2	BD Biosciences
CD11c	APC-Cy7	HL3	BD Biosciences
CD11b	PerCPCy5.5	M1/70	BD Biosciences
F4/80	FITC	BM8	Biolegend
Ly-6G	PeCF594	1A8	BD Biosciences
GR1	AF700	RB6-8C5	BD Biosciences
Ly6C	PE-Cy7	AL-21	BD Biosciences