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ORIGINAL ARTICLE

# FOXO3, estrogen receptor alpha, and androgen receptor impact tumor growth rate and infiltration of dendritic cell subsets differentially between male and female mice

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**Abstract** Tumors evade immune recognition and destruction in many ways including the creation of an immune-suppressive tumor microenvironment (TME). Dendritic cells (DC) that infiltrate the TME are tolerogenic, and suppress effector T cells and anti-tumor activity. Previous reports demonstrated that a key regulator of tolerance in DC is the transcription factor FOXO3. Gender disparity has been studied in cancer in relation to incidence, aggressiveness, and prognosis. Few studies have touched on the importance in relation to impact on the immune system. In the current study, we show that there are significant differences in tumor growth between males and females. Additionally, frequencies and the function of FOXO3 expressed by DC subsets that infiltrate tumors vary between genders. Our results show for the first time that DC FOXO3 expression and function is altered in females. In vitro results indicate that these differences may be the result of exposure to estrogen. These differences may be critical considerations for the enhancement of immunotherapy for cancer.

**Keywords** Tumor · Immunity · Gender · Dendritic cells · T cells · Hormone receptors

**Abbreviations**

| | |
|---|---|
| AR | Androgen receptor |
| BMDC | Bone marrow-derived DC |
| ChIP | Chromatin immunoprecipitation |
| DHT | Dihydrotestosterone |
| E2 | 17-β-estradiol, |
| ERα | Estrogen receptor alpha |
| ERβ | Estrogen receptor beta |
| HCC | Hepatocellular carcinoma |
| Het | Heterozygous |
| MDSC | Myeloid-derived suppressor cell(s) |
| pDC | Plasmacytoid DC |
| TADC | Tumor-associated dendritic cell(s) |
| Tfm | Testicular feminized mouse |
| TME | Tumor microenvironment, |
| Treg | T regulatory cell(s) |

## Introduction

Distinct sex differences exist in immunological responses to many diseases including infections, autoimmune disorders, and cancer [1–8]. Generally female mount increased immune responses to bacterial and viral infections but also are more susceptible to autoimmune disorders [1, 3, 5]. Conversely, males have a tendency toward lower immune activity against infection and self-antigens and have a greater incidence of cancer [8, 9]. Sex hormone receptors are expressed on all cells, including cells of the immune system. These nuclear hormone receptors, including estrogen receptors alpha and beta (ERα/β) and androgen receptor (AR), are functional receptors that impact development and cell signaling. In fact, hormone signaling plays a critical role in the development of antigen presenting cells [10–13]. Hormone regulation of the immune system is likely involved in fine-tuning the balance of immune cell development and function. This critical balancing act can

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be disrupted in tumor microenvironments (TME) where hormone ligands are present at concentrations much greater than in the serum [14–16]. However, the effects of hormone signaling in mature immune cells, especially at elevated concentrations found in the TME, are less clear. Androgen receptor (AR) signaling was shown to regulate recruitment and the production of pro-inflammatory cytokines in macrophages and neutrophils, and overall control or dampen immune responses [10, 17, 18]. Conversely, there are mixed reports regarding the effects of ER signaling in immunity and the outcome of immune function is much more complex [19]. While estrogen is clearly required for immune cell development, especially in antigen presenting cells [20], depending on the stimulus (bacterial, viral, tumor, etc.) upon ER ligation, it may also result in induction of immune tolerance [21]. Effects may be observed depending on the concentration of 17-β-estradiol (E2).

In this study, we show for the first time that tumor-associated dendritic cells (TADC) up-regulate nuclear hormone receptors and that stimulation by their cognizant ligands, dihydrotestosterone (DHT) or E2, results in increased expression or phosphorylation of FOXO3. We and others have previously shown FOXO3 to be an important factor in regulating DC function [22, 23]. Furthermore, both hormone receptors can induce tolerogenic DCs; they do so by impacting FOXO3 in differing manners. The mechanisms suggest that the sex of the patient should be considered when targeting immune responses in cancer.

## Materials and methods

### Experimental mice

Balb/C, C57Bl/6, Testicular feminized mouse (Tfm) (AR mutant mice), and ERα$^{-/-}$ male and female mice aged 6–8 weeks were obtained from Jackson laboratories. For B16 melanoma models, C57Bl/6, Tfm, and ERα$^{-/-}$ mice were shaved and injected subcutaneously with $1\times10^5$ B16 cells in the right hindquarter. Tumors were allowed to grow and measured every 2–3 days until they reached a size of 150 mm$^3$, at which point the mice were euthanized and tumors were harvested. For the 4T-1 breast tumor model, $1\times10^5$ 4T1 cells were injected into the mammary fat pads of Balb/C mice and allowed to grow until they reached a volume of 40 mm$^3$ at which point the mice were sacrificed and tumors were harvested. For in vitro studies, C57Bl/6 mice were used as a source of bone marrow-derived DC (BMDC) and primary DC were also purified by magnetic beads from splenocytes. Mice were housed under specific pathogen-free conditions and were treated in accordance with NIH guidelines under protocols approved by the animal care and use committee (IACUC) of Loyola University Chicago (Maywood, IL).

### Cell isolations

DC were isolated from single cell suspensions of harvested tumors or spleens using the Miltenyi Biotec MACS cell separation system and Pan-DC magnetic beads as previously described [23]. T-cell isolation was performed on single cell suspension of tumors that had been subjected to a percoll gradient; briefly, cell suspensions were prepared in 10 ml cell culture media and underplayed with 35% and subsequently 70% percoll to separate out leukocytes from tumor cells. Cells were collected at the 35/70 interface for further purification. Leukocytes from the percoll gradient separation were separated using the Miltenyi Biotec T-cell negative selection kit and the MACS cell separation system.

### Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out on TADC purified from tumors from male or female mice. Splenocytes from aged matched control mice were used as a positive control. Additionally, DC from spleen were purified and compared to TADC. ChIP was preformed according to the *SimpleChIP Plus* (cell signaling) kit instructions outlined briefly below. $4\times10^6$ cells were cross-linked in a 1% formaldehyde solution of RPMI for 10 min at room temperature. Cells were washed twice in ice cold PBS and resuspended in lysis buffer at 4 °C with rotation for 10 min. The nuclei were then pelleted by centrifugation and the supernatant was removed and used as the cytoplasmic fraction. The nuclear fraction was incubated with micrococcal nuclease to aid DNA fragmentation and place at 37 °C for 20 min. After micrococcal digestion, the nuclear fraction was sonicated to break nuclear membranes and release chromatin fragments. A small aliquot of chromatin fragments was RNase and proteinase k treated and final chromatin concentration was determined. The remainder was then utilized in the ChIP assays. 10ug of cross-linked chromatin was incubated with anti-FOXO3 ab (cell signaling #75D8) or rabbit IgG as a control and incubated at 4° overnight with rotation. ChIP grade agarose beads were added to the chromatin solution and incubated for 2 h at 4 °C with rotation. Beads were washed three times in a low-salt wash and once in a high-salt wash to remove nonspecific binding. After washing, chromatin was eluted from the beads and protein was removed via proteinase K treatment at 65 °C overnight. DNA was then purified using spin columns and was quantified via PCR using primers to the ERα promoter (F 5′-CACCAGATTAAAGCCCCAAA-3′, R 5′-TCGCTC AGCAGTTCTTGTCA-3′) and the AR promoter (F 5′-TCT

CCCTTCTGCTTGTCCTGGT-3′, R 5′-TAGGCTCCA AAGCAGAAGCGAT-3′) that contained the consensus sequence for FOXO3.

### Western Blots and immunoprecipitations

Whole cell lysates were generated from BMDCs using lauryl-maltoside (Sigma Aldrich) immunoprecipitation buffer supplemented with protease inhibitor (Roche). Proteins were run by electrophoresis on a 4–15% gradient polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blotted for FOXO3 using anti-FOXO3 antibody (Cell Signaling) or for β-Actin (Sigma–Aldrich) for housekeeping control.

### Flow cytometry

Cell suspensions were blocked with anti-CD16/32 antibody and then incubated with the indicated antibodies; CD45, PDCA-1 (CD317), GR-1, CD4, CD8, F4/80, CD11c, CD11b, B220, CD80, CD86 and MHC II (eBioscience). For internal staining, cells were incubated in 90% cold methanol on ice for 30 min before incubation with FOXO3, ER, and AR antibodies. Stained cells were stored in fix/perm solution before cells were run on a BD Fortessa flow cytometer. Data were then analyzed using the flowjo software package.

### Statistics

Statistical analysis for differences between group means was performed using unpaired students *t* test. Data are presented as mean ± SD and values of $p < 0.05$ were considered statistically significant.

## Results

### Gender influence tumor growth rate in implantable mouse tumors

In initial experiments, equal numbers of tumor cells were injected into both sexes; however, upon observations that tumors were growing faster in female mice compared to the male mice, it was hypothesized that this could be due to female mice being smaller than male mice. Therefore, in a second set of experiments, the injections were controlled for by weight of the animal. To determine the effects of sex on tumor growth, we injected $1 \times 10^5$ B16 melanoma tumor cells per 22 g of weight into male and female C57BL/6 mice and similarly 4T-1 into Balb/C mice that were 5–6 weeks of age. The tumor cells in both models are not directly responsive to hormonal stimulus and do not express either ER or AR thus providing a good background to study the function of immune cells expressing these receptors in the tumor microenvironment (TME). Examination of both B16 melanoma and 4T-1 triple negative breast cancer shows that tumor growth proceeded significantly faster in female mice compared to male mice. Differences in growth rate were detected at early stage by day 12 in melanoma and by day 8 in the breast cancer (Fig. 1a, b). By day 19 for melanoma and day 10 for breast cancer, there were significant differences in the tumor growth between the male and female mice (Fig. 1a, b). To further test tumor growth rate and rule out possible variables due to origin of tumor cell line, we tested a third tumor model of hepatocellular carcinoma (HCC). In this model, constitutively active c-MET and beta catenin oncogenes are delivered via hydrodynamic injection into the tail vein. Hepatocytes take up the oncogenes along with sleeping beauty transposons. Tumors arise spontaneously due to constitutive expression of these oncogenes [24]. Again it was found that upon initiation,

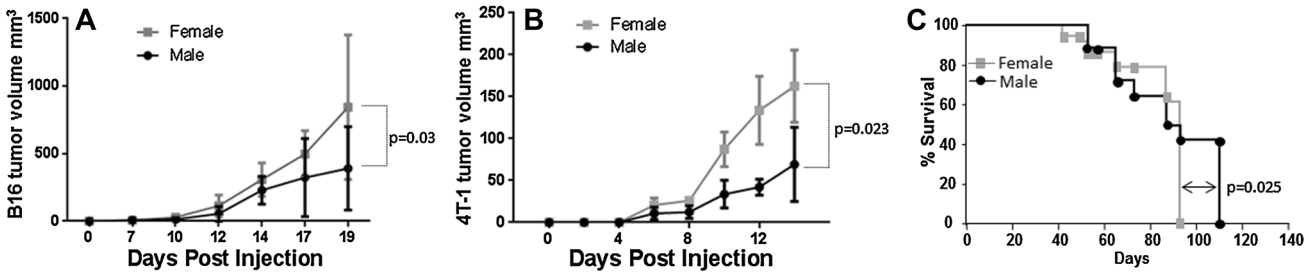


**Fig. 1** Tumors grow more rapidly in female compared to male mice. Male and female C57Bl/6 and Balb/c mice were injected subcutaneously with **a** B16 melanoma or **b** intra-breast fat pad with 4T-1 tumor lines, respectively. Tumors cells were injected at a concentration of $1 \times 10^5$ per 22 g of weight per mouse to ensure that the size of the mouse did not impact the rate of tumor growth. **c** Plasmid DNA and transposons were injected via hydrodynamic injection into the tail vein of male and female mice. Data points represent the average +/− SD tumor volume of five mice per group. *p* values were calculated by student's *t* test at each point. At end stage, these values were statistically significant. This data is representative of five separate experiments

tumors grew more rapidly and female mice expired due to tumor burden more rapidly than male mice (Fig. 1c).

Next, to determine differences in tumor-infiltrating immune cell populations between male and female mice, tumors were harvested and digested in collagenase and DNAse to form a single cell suspension. Cells were then stained to detect immune cell populations and examined by flow cytometry. Infiltrating populations were expressed as a percentage of the total number of leukocytes (CD45+ cells). There were only slight differences in lymphocyte populations including $CD4^+$ T regulatory cells (Treg), $CD8^+$ T cells, and B cells with the biggest difference being reduced Treg (CD4+/CD25+/FOXP3+)-infiltrating tumors in female mice which was surprising given the increased tumor growth rate (Supplemental Fig. 1A.) In $CD8^+$ T cells, although the relative numbers of infiltrating cells are similar, there still may be critical changes to cellular function of these cells (Supplemental Fig. 1A). Besides T cells, there were statistically significant differences in the frequency of the myeloid cells that infiltrated tumors (Fig. 2). To our surprise, there were actually higher frequencies of myeloid-derived suppressor cells (MDSC) in tumors harvested from male mice compared to female, characterized as CD11b+/Gr-1+/CD11c−. Given the more rapid growth of the tumor in female mice, we anticipated opposite observations especially lower MDSC and Treg in the male population (Fig. 2a, Supplemental Fig. 1).

An equivalent frequency of macrophages characterized as $F4\text{-}80^+/CD11b^+$ was observed (Fig. 2b). On the other hand, there was a significantly higher frequency of plasmacytoid DC (pDC), $CD11c^+$/ $PDCA\text{-}1^+/B220^+$ / $CD11b^-/CD8a^-$, infiltrating female tumors compared to male (Fig. 2c, Supplemental Fig. 1B). The difference in the conventional DC population, $CD11c^+/CD11b^+$ was not significant. DC are critical activators of T cells; therefore, we next sought to determine whether sex-specific signaling contributed to the alteration of an anti-tumor response by $CD8^+$ T cells. Co-stimulatory factors, CD80 and CD86, were slightly modified between male and female TADC, but the most dramatic change was a significantly higher

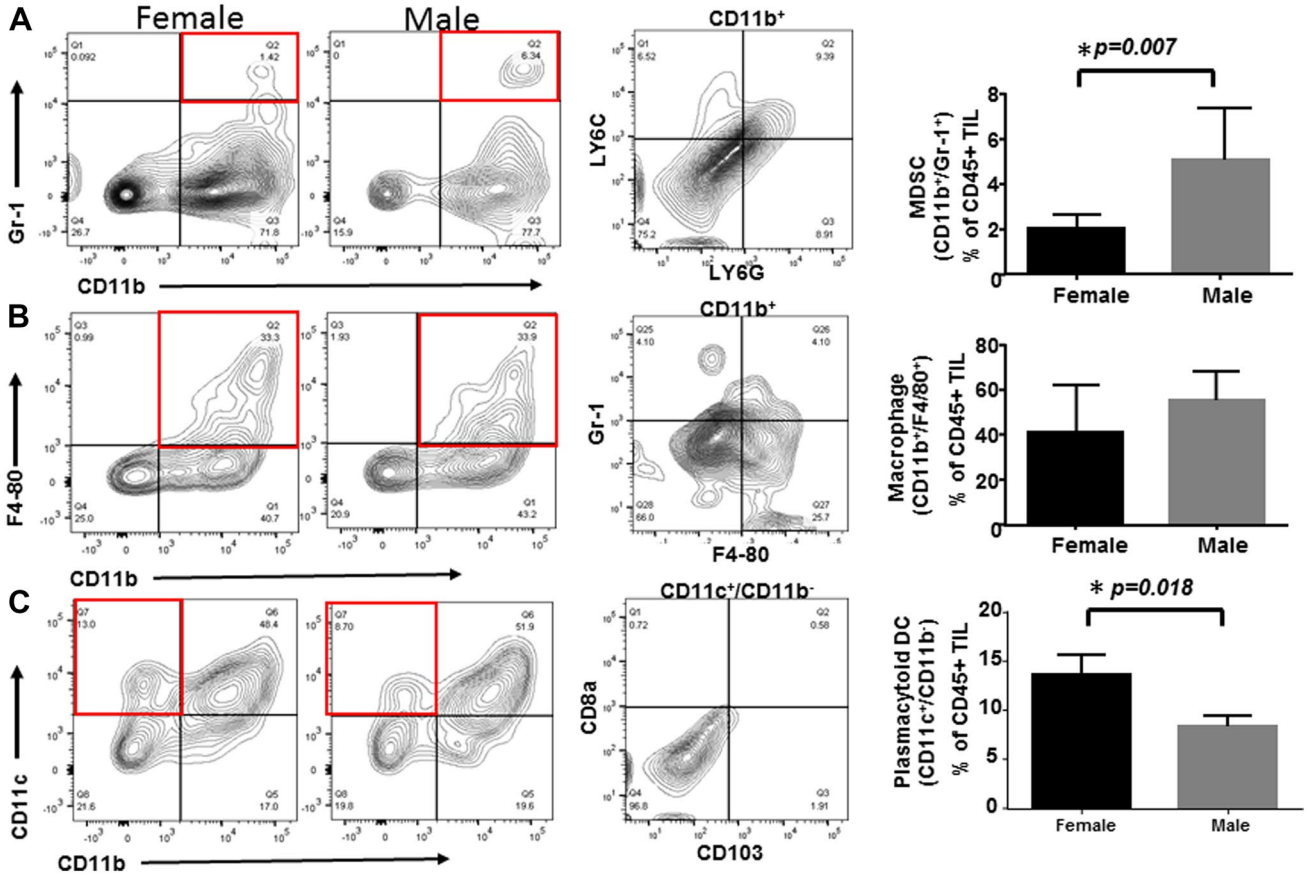


**Fig. 2** Sex impacts myeloid cell tumor infiltration. B16 tumors were excised at $150mm^3$ and assessed for immune cell infiltration by flow cytometry. Gates were set to first assess live cells (excluding dead) and single cells (doublets excluded). A gate for CD45+ cells was then set to further assess cell population frequencies for **a** MDSC, **b** macrophages, and **c** DC. Data are representative of six individual mice from four separate experiments

level of MHC II expression in male TADC compared to female (Supplemental Fig. 1C). These data led us to question whether hormone receptor expression would affect DC function including activation of T cells and if there was cross-talk between the hormone receptors and FOXO3 that may influence the ability to stimulate or inhibit immune responses to tumors.

### Hormone treatment of BMDCs in vitro with DHT or E2 reduces pro-inflammatory cytokine production while increasing anti-inflammatory cytokines

We next sought to determine the direct effects of hormone stimulation on DC function through expression of co-stimulatory molecules and cytokine production. However, sufficient quantities and purification of TADC were not able to be acquired from these tumors for a vast array of cytokine analysis. Therefore, bone marrow precursor cells from C57Bl/6 mice were cultured in complete cell culture medium which does contain trace amounts of estrogen, important for cellular development with GM-CSF for 9 days to induce differentiation of DCs (BMDC). On day 9, cells were placed in hormone-free media and treated with 100 ng/ml LPS with varying concentrations of DHT or E2 for 24 h. Treatment of BMDC with DHT and E2 did not significantly alter costimulatory molecule or MHC II expression (Supplemental Fig. 1D). After 24 h cytokine concentrations were measured by ELISA for IL-6 and TNF-α or via multiplex array ELISA for IL-6, IL-10 and IL-4 measurements. BMDC treated with increasing concentration of DHT, but not E2 resulted in decreased IL-6 production as hormone dosage increased (Supplemental Fig. 2). IL-6 is required for DCs to become fully mature and has been shown to inhibit DC-induced tolerance of $CD8^+$ T cells. A reduction in IL-6 levels may then reduce autocrine DC signaling that leads to immune activation. In contrast to IL-6, BMDCs treated with hormones had an initial decrease in TNFα under physiological concentrations; then as the concentration of hormone was elevated to supra physiological levels, there was a sharp increase in TNF-α (Supplemental Fig. 2).

Prior work has shown that in lymphoid cells E2 treatment reduced the expression of inflammatory cytokines while increasing the expression of anti-inflammatory cytokines such as IL-4 [25, 26]. These observations coinside with the pattern of cytokine expression by tolerogenic DCs. Tolerogenic DC also produce increased levels of anti-inflammatory cytokines while reducing the amount of pro-inflammatory cytokines they secrete [27]. BMDC production of anti-inflammatory cytokine IL-4 was increased at low to medium exposure levels of DHT or E2 (Supplemental Fig. 2). The increase was more pronounced in E2-treated cells. IL-10 levels initially decreased upon BMDC exposure to low levels of hormone stimulation (<50 nM) but as DHT and E2 concentrations increased IL-10 production increased (Supplemental Fig. 2). These data support the hypothesis that increase in hormone receptor ligation favors generation of DC that support immune tolerance in favor of immune activation.

### FOXO3 protein expression is impacted by E2 and DHT stimulation but differ in quantity and persistence over time

To first confirm FOXO3 expression is increased in TADC from males and females, a western blot was used to detect phosphorylated or total FOXO3. FOXO3 was previously found upregulated in TADC and undetectable by this method in wild type, normal splenic DC (Fig. 3a). To further explore the extent of tolerogenic phenotype and function of DC treated with increased concentrations of hormones, BMDCs were treated with DHT or E2 at various concentrations for exposure times of 60 or 240 min. After exposure cells were lysed and protein levels of FOXO3 were quantified by western blot and normalized to untreated controls, ER stimulation through E2 increased FOXO3 protein levels up to twofold over untreated cells at the 60 min time point but this increase in FOXO3 expression was much more transitory as demonstrated at 240 min FOXO3 levels had dropped below the untreated controls (Fig. 3b). This indicates that FOXO3 is either more actively degraded in ER stimulated cells or that initial ER stimulation triggers an increase in FOXO3 but this increase is self-limiting. The appearance of a double band indicates phosphorylation. The upper band is phosphorylated protein, while the lower band is unphosphorylated. Conversely, DHT treatment only generated a modest increase in FOXO3 protein levels at all concentrations of DHT at both the 60 and 240 min time points, but did induce a change in phosphorylation at the 240 min time frame (Fig. 3c). These data suggest that there is either continued FOXO3 expression or more likely based upon our previous findings in prostate cancer, FOXO3 accumulates in the cytoplasm and is stable due to interactions with other proteins such as NF-κB [23]. Such interactions could promote a reduced ability to degrade FOXO3 proteins in AR-stimulated BMDCs.

### FOXO3 binding to AR and ER promoter regions is greater in TADC isolated from male compared to female tumors

Given that FOXO3 is a transcription factor, we next sought to determine whether upregulation of FOXO3 in the TME may be involved in the regulation of ERα and AR expression in TADC. There are known FOXO3-binding sites in both the ERα and AR promoter regions

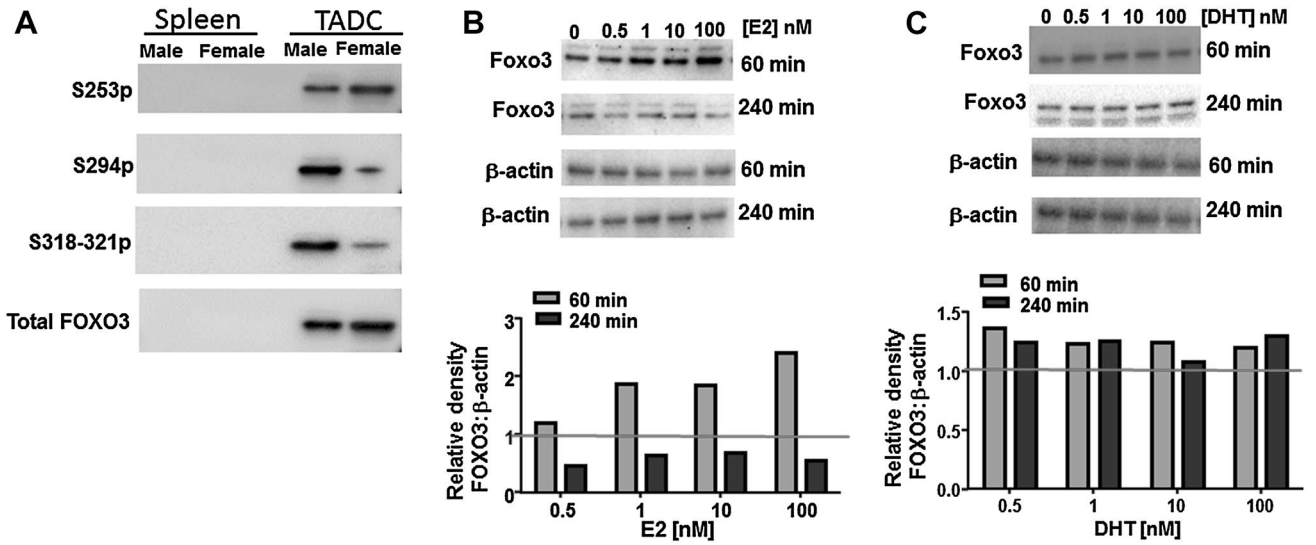


**Fig. 3** Estrogen and Androgen receptor stimulation differentially impacts FOXO3 expression in dendritic cells. **a** FOXO3 expression is upregulated in TADC from males and females. FOXO3 expression in splenic DC is below level of detection by western blot. **b** Bone marrow-derived DC were stimulated with physiological and super physiological doses for E2 or DHT for 60 to 240 min. Upon ER stimulation, FOXO3 expression rapidly increased but also rapidly degraded. **c** Upon AR stimulation, FOXO3 expression only slightly increases but remains elevated for an extended period of time. The red line on the bar graphs represents the value of the 0 nM stimulation and is equal to 1. Values are representative of $1\times10^6$ DC tested by western blot in two separate experiments. FOXO3 expression is displayed as relative to b-actin

[28, 29]. ChIP experiments were conducted on DCs purified from B16 tumors using an anti-FOXO3 ab to determine if FOXO3 was able to bind to the promoter regions of AR and ERα. Increased FOXO3 localization was observed in TADC from male tumors at both the ERα and AR promoter regions while only minimal binding was detected in TADC female samples (Fig. 4a). This result indicates that FOXO3 expressed in male TADC is capable to yield transcriptional activity such as induction or repression of AR and ERα. In opposition, FOXO3 isolated from female TADC were unable or very minimally able to bind either of the known sequences. To determine whether these results were consistent in all male and female DC populations or whether they were TME induced, FOXO3 was taken from TADC and splenic DC from male and female mice. Given that FOXO3 isolated from splenic DC from the same female mice did in fact bind these consensus sequences (Fig. 4b), these data suggest that FOXO3 in female TADC may either be rapidly degraded or may have undergone extensive post-translational modifications that render it inactive in the form of a transcription factor. In either case, FOXO3 expressed by female TADC is significantly different than FOXO3 expressed by male TADC.

## The removal of AR but not androgen reduced tumor growth and decreased infiltration of DC

To test the role of androgen in regulating immune cell responses to tumors, male wild-type C57BL/6 mice were anesthetized and castrated or given a sham operation at 8 weeks of age and allowed to recuperate for 10 days. After surgery, mice were injected with $1\times10^5$ B16 melanoma cells/22 g mouse in the right flank. Tumor growth was monitored and tumors were harvested upon control groups reaching 1500mm$^3$. Tumor-infiltrating immune cells were stained and assessed by flow cytometry. Depletion of androgen had no significant impact on tumor growth compared to sham operations (Supplemental Fig. 3A). Additionally, the only differences detected in infiltrating immune cell populations were a slight increase in pDC in the androgen-depleted mice compared to control tumors (Supplemental Fig. 3B). Because castration does not fully eliminate androgen from the environment, Tfm mice which lack a functional AR were obtained. Similar to our previous experiments, tumors grew more rapidly in female control and female Tfm mice compared to wild-type male mice. However, tumors grew most rapidly in male Tfm mice compared to all other groups (Fig. 5a). Interestingly, we

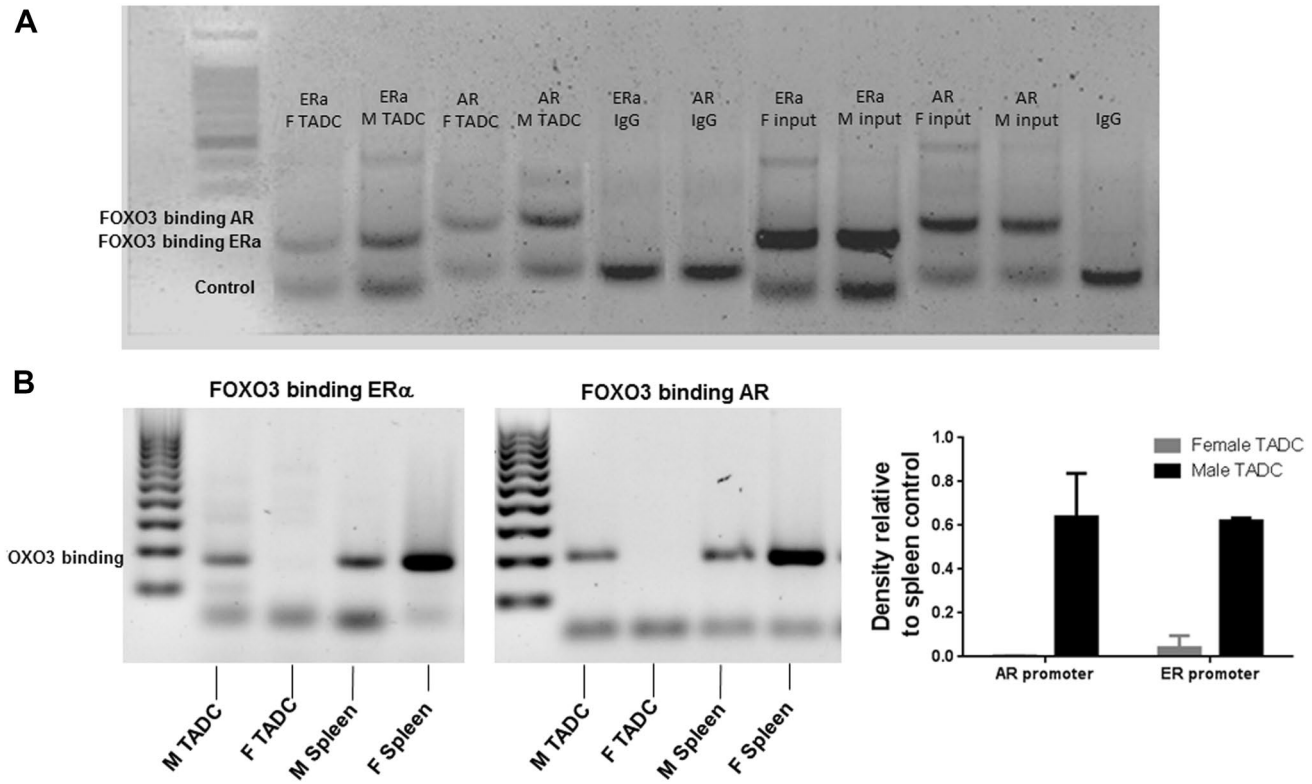


**Fig. 4** FOXO3 expressed in male but not female TADC retains ability to bind DNA consensus sequences. **a** TADC were isolated from B16 tumors in male and female mice. DC were homogenized and FOXO3 was extracted and tested by ChIP for FOXO3 binding at the ERα and AR promoter regions which are known consensus sequences. **b** To confirm FOXO3 DNA-binding comparisons were made between male and female TADC and splenic DC. Data are representative of four independent trials of three mice per group ($n = 12$)

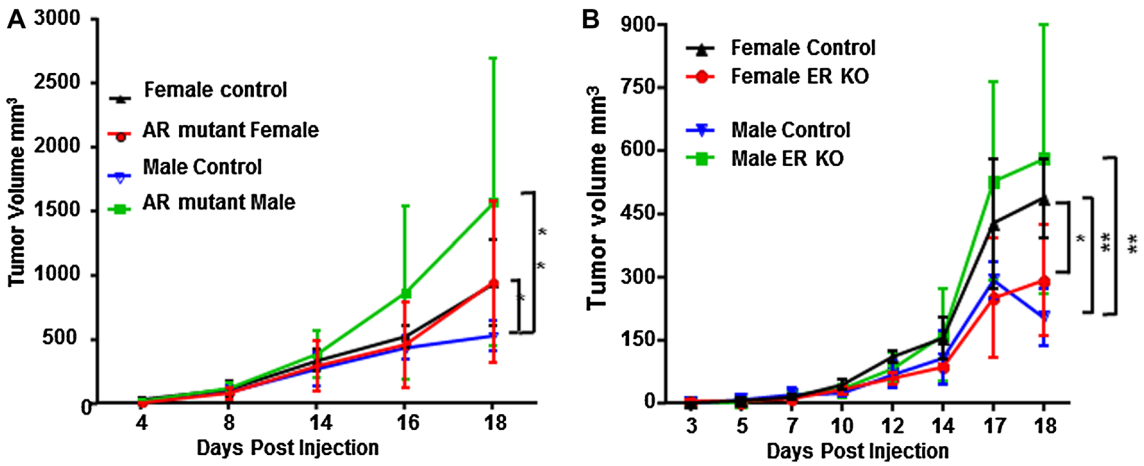


**Fig. 5** The absence of sex hormone receptor signaling differentially impacts tumor growth in males and females. **a** B16 tumors were injected in male and female Tfm mice which lack functional androgen receptors. Tumors grew much quicker in male Tfm mice compared to WT male similar to the rate of growth observed in both female mouse groups. $*p < 0.05$. Data are representative of 6 individual mice from 2 experiments $*p < 0.05$. Data are representative of 6 individual mice from 2 experiments. **b** B16 were injected in C56Bl/6 wild type (Control) or estrogen receptor alpha knockout mice. Tumors grew slower in female mice absent of ERα compared to wild-type male mice. Unexpectedly, tumors grew more rapidly in male mice deficient in ERα. $*p < 0.05$, $**p < 0.01$. Data are representative of nine individual mice

saw a similar trend in the fluctuation of DC and MDSC in these tumor groups. Wild-type male mice had more MDSC than female wild-type mice; but upon mutating, the AR more MDSC infiltrated these female tumors (Supplemental Fig. 3C). Similarly, mutation of the AR resulted in fewer DC, both conventional and pDC phenotypes infiltrating tumors in both the male and female groups. Although it should be recognized that the decrease may be partially due to an overall lower frequency of DC in Tfm mice compared to wild-type mice.

### ERα impacts rate of tumor growth in male and female mice

To test the role of ERα in immune regulation of tumor growth, wild type or ERα$^{-/-}$ male and female mice were injected with B16 described above. Given that the tumor line was not genetically deficient in ERα, any observable differences in tumor growth should be due to host response. Tumor growth was monitored and, when the fastest growing tumors reached end stage all groups were harvested. Initiation of tumor growth was consistent among all treatment groups most likely due to injection of fewer tumor cells as ERα$^{-/-}$ are much smaller than wild-type C57BL/6 mice. However, by 2 weeks after tumor cell injection, consistent with all previous results tumors grew faster in wild-type female (black line) compared to wild-type male mice (Fig. 5b). Tumors generated in ERα$^{-/-}$ mice resulted in reduced tumor growth rates in female mice which were comparable to the tumor growth rate in male wild-type mice (Fig. 5b). However, more puzzling was the unexpected increase in tumor growth rate in ERα$^{-/-}$male mice. Examination of infiltrating immune cells demonstrated that there were slight but no significant changes in the frequency of infiltrating DC in ERα$^{-/-}$ mice compared to wild type (Supplemental Fig. 4).

### FOXO3 impacts tumor growth and DC infiltrating in males and females

To directly test the role of FOXO3 in the differential tumor growth observed between males and females, B16 melanoma was inject subcutaneously into the right flank of heterozygous or FOXO3$^{-/-}$ male and female mice. Tumor growth was recorded every 2–3 days for 19 days when the fastest growing group, the female heterozygous mice, had to be euthanized due to tumor burden. As observed previously, tumors grew more rapidly in control female groups compared to male. However, tumors grew at a significantly reduced rate in FOXO3$^{-/-}$ mice in both genders. In this reduced growth rate, there were no detectable differences between the male and female groups in terms of tumor growth (Fig. 6a). There were no detectable differences in conventional DC frequencies; however, there was a significant reduction in pDC infiltration in female FOXO3$^{-/-}$ mice compared to control which was increased over all other groups (Fig. 6b). Interestingly, there was no change in the frequency of infiltrating MDSC in either the female or male control or FOXO3$^{-/-}$ group, but MDSC frequency remained slightly higher in males compared to females (Fig. 6c).

## Discussion

In this work, we sought to examine the contribution of sex and hormone receptor activation regulating an immune response to a growing tumor. To our surprise, upon adjusting for animal weight, a higher tumor growth rate of both B16 melanoma and 4T-1 breast tumors was detected in female mice compared to male mice. Although, it may be argued that these tumor lines were originally derived in female mice, we and our collaborators have observed similar phenomena in a model of hepatocellular carcinoma (HCC) where oncogenes are delivered via the tail vein and tumors develop in the liver upon plasmid incorporation. These data argue against the origin of the tumor line. In our studies to identify potential mechanisms regulating these differences, we chose to focus on two key suspects' estrogen and androgen which are regulated differently in males and females. Examination of the infiltrating immune cells in B16 melanoma tumors revealed that the proportion of macrophages were equivalent in male and female mice (Fig. 2). It was unexpected, however, that the frequency of MDSC (CD11b$^{+}$/Gr-1$^{+}$ cells), which are typically referred to as myeloid derived suppressor cells, would be significantly higher in the male mice given that the tumors grew slower in male mice compared to the female. It was also unexpected that there would be a lower frequency of Treg infiltrating female tumors. While that observation had been previously reported by other groups [30], it is counter intuitive to the fact these tumors grew more rapidly. When we examined DC infiltration, we saw that there was a slight increase in DC infiltration in female mouse tumors. However, there were no detectable differences in the frequency of T cells, which suggests that although the numbers are there, there is likely a difference in function.

DC-induced tolerance is well known in the tumor especially by mechanisms involving IDO, β-catenin, and FOXO3 [23, 31], but the effects of hormone signaling on TADC, to our knowledge, has not been described. Based on our previous work, a key mediator of DC tolerance is the transcription factor FOXO3 [23]. This factor is part of a large family of DNA binding proteins that generally have been associated with regulation of cell cycle progression and cell death. In tumor cells, the presence of FOXO factors

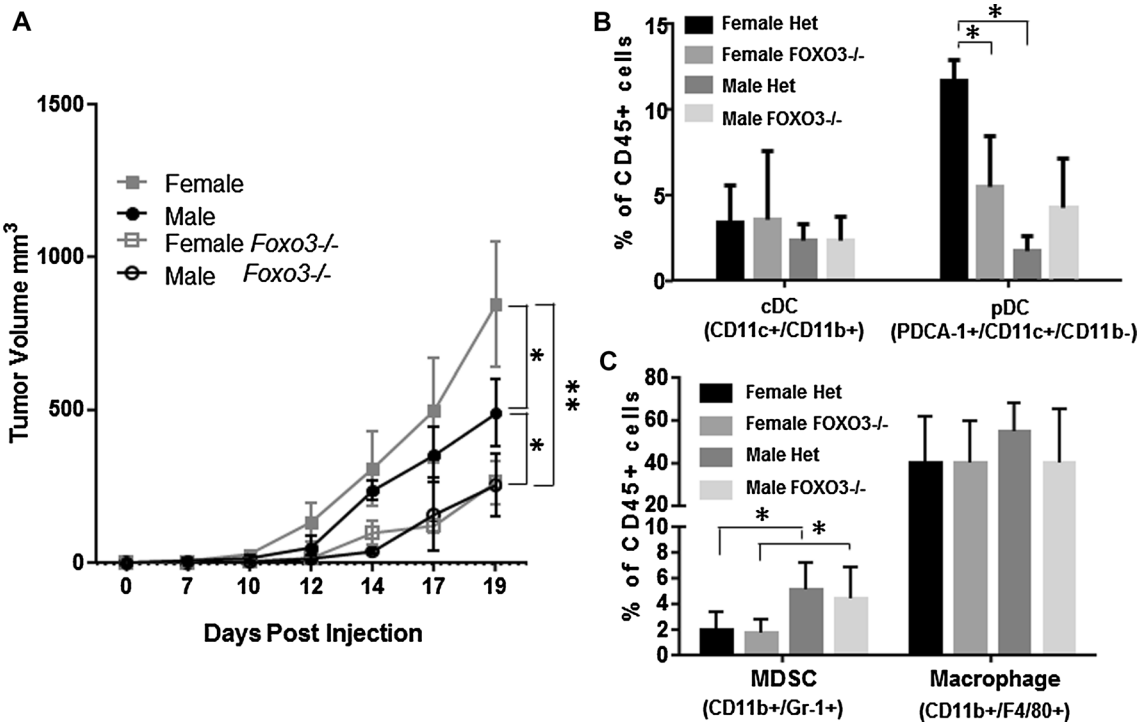


**Fig. 6** FOXO3 impacts tumor growth and pDC infiltration into tumors in female mice. **a** B16 tumors were injected in male and female heterozygous (Het) (as control) and FOXO3$^{-/-}$ mice. Tumor growth was measure every 2–3 days. Tumors grew more rapidly in het mice compared to FOXO3$^{-/-}$ and more rapidly in female het compared to male het mice. Upon deletion of FOXO3$^{-/-}$, differences in tumor growth in males and females disappeared. **b** A higher frequency of pDC infiltrated female tumors in Het mice but were reduced upon deletion of FOXO3. **c** A higher frequency of MDSC remained in the male mice compared to female, but no differences in macrophage populations were detected. *$p < 0.05$. **$p < 0.01$. Results are representative of two experiments with ten individual mice per group

is positive as it is correlated with decreased tumor growth and tumor suppression. However, increasing expression of FOXO, especially FOXO3, results in an increased immune suppression and a reduction in anti-tumor immunity. It may be that the control of anti-tumor immunity is not dependent on the total number of DC present in the tumor but instead on whether or not they have an immune-activating or -tolerizing phenotype. Further analysis of FOXO3 expressed in TADC revealed specific functional differences. FOXO3 from female, TADC was not able to bind known FOXO3-DNA binding sequences (Fig. 4). The possibility that signaling through AR or ER had an effect on DC phenotype including FOXO3 expression was examined. By exposing BMDC to ligands in vitro, we were able to avoid competing effects from other tumor-associated factors that can influence tolerance. From these studies, it was determined that DCs treated with DHT or E2 increase the production of anti-inflammatory cytokines IL-4, with E2 exposure leading to a greater increase in production. Additionally, the pro-inflammatory cytokines IL-6 and TNFα are reduced upon increased hormone exposure. The overall effect of hormone stimulation to supra physiological levels which occur in tumor microenvironments. These changes can be a contributing factor to the tolerogenic phenotype in TADC as tumors aberrantly express aromatase enzymes and hormone precursors which can elevate the local hormone concentration above the physiological level [1, 14–16, 32].

The increase in FOXO3 expression seen upon hormone stimulation is compelling evidence that gender-specific signaling may impact function. Since ER, AR and FOXO3 are all transcription factors that can interact with other proteins to modulate their function, it was examined whether FOXO3 could regulate expression of AR or ER through binding their promoter regions. Co-immunoprecipiation experiments determined that these proteins do not directly interact (data not shown). However, FOXO3 from TADC in males reacted very differently than FOXO3 isolated from female TADC. Male TADC-expressed FOXO3 can regulate expression of AR or ER. In male TADC, FOXO3 was able to bind to the promoter region of both AR and ER much like in splenic DCs. FOXO3 from female TADC, however, was not found at the promoter region of ER or AR. This may explain the differing effects of E2 and DHT signaling on FOXO3 protein levels. If androgen stimulation in male TADC induces FOXO3 expression which in turn promotes AR

expression, it could generate a positive feedback mechanism that keeps FOXO3 elevated for a longer time period. In female TADC, this feedback seems to be absent; so after the initial spike of FOXO3, the protein levels drop off. Determining if this model is correct will require further examination of ER- and AR-deficient DCs to elucidate their contribution to the initial FOXO3 elevation in TADC. Importantly, upon removal of FOXO3 from the host, but not in the tumor cell line, there was a significant reduction in tumor rate. This reduction in tumor growth also lead to a disappearance of differences in tumor growth between males and females. These data suggest, FOXO3 does have a role in the regulation of tumor growth and take together with our data from the ChIP analysis (Fig. 4), FOXO3 may function differentially when expressed in immune cells in males and females.

Overall, our findings indicate that a signaling axis exists between hormone receptors and FOXO3. This could impact DC ability to induce or contribute to anti-tumor immunity but further studies are required. The differential effects seen depend on which hormone is most prevalent or which hormone receptors are upregulated. While many questions remain including our ability to target and alter AR and ER signaling and the impact on generating an anti-tumor immune response, we have for the first time demonstrated that tumor growth, immune recognition and immune cell infiltration of tumors is impacted by sex and the mechanisms involved include signaling through the AR and ER which may occur through either ligand or ligand independent activation of the receptor. Furthermore, the transcription factor FOXO3 is regulated differently in males and females in regards to expression, activation and potential post-translational modifications. Further understanding of all of these factors will lead to enhanced potential to effectively trigger anti-tumor immune responses in patients.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflicts of interest to report.

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